Bio-refining of seaweed: A sustainable source of chemicals and fuel?

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Abstract

The research presented in this thesis evaluated a range of UK native seaweeds as potential feedstocks for both bioethanol and speciality chemical production, with the objective of developing a putative bio-refining process.

The gross chemical compositions of nine different UK species of seaweed were determined (Chapter 3) to evaluate their potential for bioethanol production. The carbohydrate contents were within the range 16.8 – 41.8 % (d/w). Three species were selected for further work targeted at bioethanol production. Methodologies involved using thermo-chemical pre-treatments followed by enzymatic hydrolysis. Optimisation experiments concluded that water based (hydrothermal) pre-treatments were most suitable for both *D. carnosa* and *U. lactuca* spp (121°C, 24 – 30 min, 15% and 10% (w/v) solids, respectively) to maximise glucose liberation. In contrast dilute acid hydrothermal pre-treatment (1.5 N H_2SO_4, 121°C, 24 min, 25% (w/v) solids) was most suitable for *L. digitata*. Feedstocks were produced using optimised conditions for trial fermentations (using *S. cerevisiae* NCYC2592). These produced yields of 78.4 - 94.5% theoretical ethanol, which equated to 15.4-20.4 kg of pure ethanol per metric tonne of native seaweed.

The thermo-chemical pre-treatment of seaweed generated a liquid fraction rich in an array of different monosaccharides inherent to seaweed which was used to identify microorganisms capable of fermenting seaweed monosaccharides into ethanol using a novel phenotypic microarray approach (Chapter 5). Whilst *S. cerevisiae* spp were suggested to be the most suitable (and efficient) for

fermentation of C6 sugars, the yeast stain *P. anomala* TP12 was able to utilize a mixture of sugars inherent to seaweed.

Attempts were made to link both bioethanol production and fucoidan extraction as part of a novel bio-process using *L. digitata* (Chapter 6). At pilot plant scale, fucoidan was extracted with a purity of 65% and an overall extraction efficiency of 17% was attained. Pre-treatment and enzymatic hydrolysis of the remaining waste residue generated a feedstock that achieved 93.8% theoretical glucose, which when fermented yielded 94.4% of theoretical ethanol. Furthermore, extracts from waste streams of the putative process exhibited both antioxidant and antimicrobial activities.

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This thesis is dedicated to my Grandfather,

Costantinos Papacostas

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1 Introduction

Undoubtedly, our reliance on fossil fuels and their impacts on the environment and climate are causing major constraints on our planet, and the search to find alternative, sustainable and cleaner burning sources of energy has become an extensive area of research. The concentration of CO_2 in the atmosphere will continue to rise unless major changes are finally made in the way fossil fuels are utilised (Wise *et al.*, 2009). CO_2 emissions are expected to increase to approximately 37 Gt by 2035 (Change, 2013) and this has facilitated the drive behind the policies of many countries to encourage the growth of renewable energy. Importantly, the development of renewable bioenergy is a stepping stone that will lead the way to a more sustainable and efficient way of life for future generations.

Bioethanol, which can be derived from sugar-based biomass through yeast-based fermentation processes, can be used as an energy-rich transportation fuel (Karakashev *et al.*, 2007). Indeed traditional feedstock materials such as corn, wheat and sugarcane have been and are still widely exploited to generate bioethanol (Bothast and Schlicher, 2005). Controversy however has arisen over the utilization of such feedstocks and attention has been diverted to lignocellulosic biomass (2^{nd} generation biomass), which comprises mainly of agricultural waste products (Lu *et al.*, 2009).

Macroalgae, commonly known as seaweed, are third generation biomasses that have great potential in bioenergy production. Additionally, macroalgae contain a multitude of novel and valuable chemicals compounds with bioactivity such as polyunsaturated fatty acids (PUFAs), sterols and

antioxidants which are of great interest to the pharmaceutical and cosmetics industries (Sun *et al.*, 2007). Polysaccharides from various species have also been found to have antiviral activity (Plaza *et al.*, 2008) and so the screening of more species of seaweed to identify useful bioactivities that have clinical applications should be researched. This highlights the potential for utilising seaweed feedstocks in bio-refinery based processes, as the biomass can be fractionated into multiple process streams for either biofuel/chemical production, as well as extracting compounds with bioactive properties.

This introductory Chapter will focus on the recent advances made in biofuel generation, and in particular bioethanol production from seaweed, including the identification of the most suitable microorganisms utilized during fermentation and pre-treatment techniques applied to generate desirable ethanol yields. Furthermore, the concept of seaweed bio-refineries will also be explored and the significant factors which are involved in the successful bio-conversion of biomass substrates into useful products will be discussed. Additionally, a variety of valuable speciality compounds and chemicals which can be extracted from UK species of seaweed will be considered.

1.1 A review of biofuel production

Major research effort was made for the development of refinery processes based on the fractionation of petroleum, coal and natural gas from the readily, yet cheaply available fossil fuel feedstocks back in the twentieth century. However, questions were soon after raised about the availability, sustainability and economic status of fossil fuel resources. It has been made apparent that the future supply of fossil fuels is finite, and reliance on oil has had a negative social and environmental impact. This was highlighted for example in 2010,

when approximately 5 million barrels (mb) of oil leaked from the BP Deepwater oil platform in the Gulf of Mexico, which resulted in a global backlash against BP due to the adverse publicity created. The burning of fossil fuels has contributed to the increase in carbon dioxide levels in the atmosphere which has ultimately been directly associated with the global warming and climate changes observed in recent decades (www.shell.com). Therefore, the quest to find more sustainable and environmentally friendly sources of energy for the planet's industrial economies and consumer societies has increased (Naik *et al.*, 2010). Biofuels, produced from bio-materials such as plants or organic wastes, are an important source of renewable energy and a plausible alternative to fossil fuel resources as they have the potential to help reduce the global dependence on oil and carbon dioxide emissions (Naik *et al.*, 2010). Ideally biofuels need to be produced from cheap, non-food feedstocks that are readily available throughout the year and are as energy dense as petrol or diesel (Savage, 2011). Such fuels are already making a contribution in the sustainable transport sector, where they are being mixed with gasoline to create a 10-25% (E10-E25) bioethanol blend. Ideally, a shift from utilising fossil fuels to an alternative industrial feedstock and the development of green processes to produce fuels and product chemicals from renewable biomass resources is key (Stevens and Verhé, 2004).

1.1.1 1st generation bioethanol

The term 'first generation bioethanol' refers to ethanol that is derived from agricultural biomass such as sugar cane, corn, oilseeds, grains and wheat. Such biofuels are commonly produced through yeast based fermentation of sugars liberated from these biomass feedstocks. Interestingly the current commercial

success of biofuels (bioethanol and biodiesel) has been based on first generation biofuel technologies which account for approximately 5% of total world ethanol production (Berg, 2004).

Typically, starch (derived mainly from corn and maize) and sugar (sugar beet and cane) are the main carbohydrate sources that are fermented to produce bioethanol. Sucrose (glucose-α-1,2-fructose), the main building block found in sugar containing feedstocks such as sugar cane and sugar beet. The polysaccharide can be directly fermented by the yeast *Saccharomyces cerevisiae* as the enzyme 'invertase' that is responsible for the breakdown of sucrose into it hexose monomers (fructose and glucose) is inherent to the yeast strain (Figure 1.1). The yeast can then subsequently ferment the two hexose monosaccharides directly to bioethanol (Schiener, 2013).

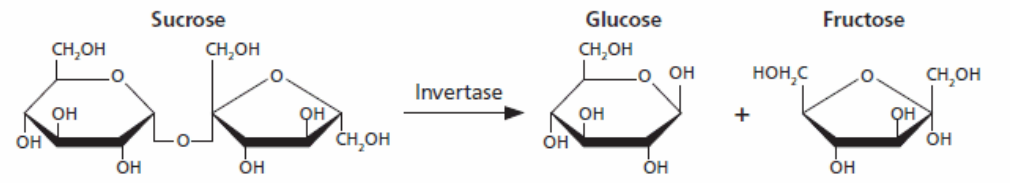


Figure 1.1 Hydrolysis of sucrose by invertase enzyme (from www.sigmaalrich.com)

In order to produce bioethanol from starch containing feedstocks (which contains both α-1,6 and α-1,4-glycosidic linkages within the polysaccharide), some form of a pre-treatment step is required prior to fermentation to liberate the sugars within. This is typically because fermentative microorganisms such as *S. cerevisiae* do not have the capability to utilise starch directly. Simple sugars are firstly extracted from the plant either by milling or by steeping in

hot water. Wort is the product that is produced from this form of pre-treatment to which enzymes such as amylases are added (if not already present in the raw material) during the steeping process for activation. α-Amylases break down the starch complex into soluble sugars which additionally decreases the overall viscosity of the wort. A 'saccharification' step normally follows, in which glucoamylases are added that can then convert the soluble dextrins into glucose monomers (Figure 1.2) which can then be fermented by yeast to bioethanol.

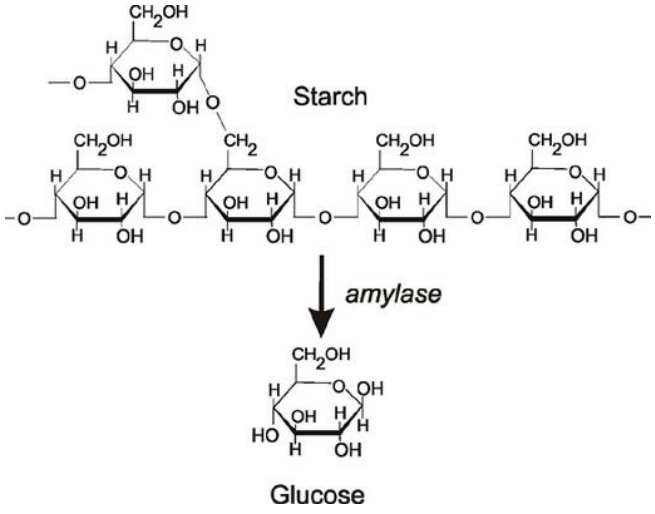


Figure 1.2 Saccharification of starch to glucose from the addition of amylase enzymes (www.biotek.com)

The future competitiveness of first generation fuels is however looking somewhat limited because of issues related to their social and environmental sustainability. There are concerns about the sourcing of feedstocks and the impact they may have on biodiversity, land use and most importantly, competition with agricultural production.

1.1.2 2nd generation bioethanol

To alleviate the concerns highlighted above there has been increased focus on second generation bio-fuel production in recent years. Crops such as wheat straw, corn stalks, wood chips and sugarcane, are sources of plant biomass which are derived from the cheap and abundant non-edible portions of the plant (Gomez *et al.*, 2008).

Currently, the full potential of second generation crops cannot be exploited because of the lignocellulosic complexes within which the carbohydrate polymers are bound to. The lignocellulosic material is found in the cell walls and is normally composed of three distinct layers; the middle lamella, the primary cell wall and the secondary cell wall (Chundawat *et al.*, 2011).

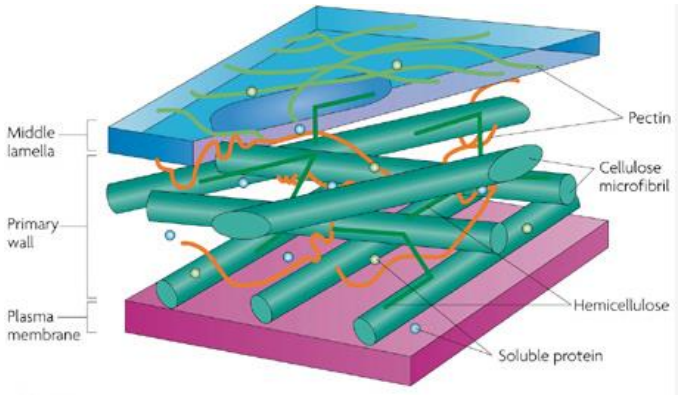


Figure 1.3 Schematic overview of lignocellulosic structure (Sticklen, 2008)

Three main components form the basis of the lignocellulosic material; cellulose, hemicellulose and lignin. A high degree of structural complexity exists within lignocellulosic biomass. Its general structure is highly amorphous due to the random interactions between the three sub-components (also including the random polymerisation of lignin). However, cellulose, one of the

principal components of the material, is often itself highly crystalline in form. This high level of crystallinity confers a significant degree of the recalcitrance of lignocellulosic biomass and is resistant to deconstruction. The lignocellulosic material itself is considered to have been produced as a direct response to the coevolution of plants with animals and other potential predators such as certain microbial species which may attempt to use the plant directly as a source of food (Dodd and Cann, 2009). Even though approximately 75% of the cell walls of these plants are polysaccharides (Pauly and Keegstra, 2008), the presence of lignin presents a physco-chemical barrier that needs to be disrupted in order to access the carbohydrate component. This currently makes the production of biofuels from second generation crops less energy and cost-efficient than first generation technologies.

To produce liquid biofuels from 2nd generation biomass crops, two main routes are typically involved; thermochemical processing and biochemical processing (Naik *et al.*, 2010). Thermochemical processing is the conversion of biomass into a range of different products by thermal decay and chemical reformation which generally involves heating the biomass in the presence of a chemical reactant (Gomez *et al.*, 2008). However, this form of pre-treatment required for the removal of lignin is an energy intensive step which can be relatively expensive and time consuming. Although dilute and concentrated acid processes are both close to commercialisation (Yang *et al.*, 2008), improvements in physico-chemical pre-treatment are still required in order to maximise efficiency and best open up the cellular structure of the feedstock for subsequent hydrolysis (Chandra *et al.*, 2007). Biochemical conversion is the application of enzymes to breakdown the polysaccharide fractions of the

biomass into monomeric submits for fermentation to bioethanol. The production of new and improved enzymes and enzyme mixes continue to be developed in order to effectively increase the hydrolysis of the inter-connected matrix of cellulose, hemicellulose and lignin (Sims *et al.*, 2010). This however, requires the employment of a number of different aggressive cellulases, hemicellulases and β-glucosidases. The enzymes which are commonly used are derived from wood-rotting fungi such as *Trichoderma*, *Penicillium* and *Aspergillus*; however production expense continues to be the main concern as costs are still relatively high. It is therefore important to find a suitable feedstock material for the production of biofuels, which does not require as much energy to pre-treat; making it feasible to generate biofuels as inexpensively as possible.

1.1.3 3rd/Advanced generation bioethanol

Seaweeds are a promising feedstock for the production of so-called third generation biofuels. There are several main advantages that seaweeds have over the traditional terrestrial feedstocks, such as corn, wheat straw and wood chippings which have been used for the production of biofuels:

1) The production of seaweeds does not require arable land, avoiding the current land-use issues and the 'food v fuel' debate associated with 1st generation bio-fuel production.

2) The majority of seaweed species are not currently exploited as major food commodities, in contrast to feedstocks such as corn or sugarcane.

3) Fresh water is not a requirement for seaweed cultivation (and many regions globally face increasing pressure on the availability of fresh water for agriculture).

4) Seaweeds do not have lignin crosslinking molecules in their cellulosic structures as are found in terrestrial plants, implying that less severe pre-treatments can be employed.

4) Seaweeds contain relatively high amounts of carbohydrate (ranging from 23 to 79.4% d/w (Mohammadi *et al.*, 2013, Murphy *et al.*, 2013) which makes them a potentially attractive resource to be exploited for biofuel production.

5) Seaweed productivity through aquaculture can be higher than for land based plants such as corn and soybean, where on average yields of around 1-4 dry t/ha/yr are common, compared to yields of *Saccharina japonica* that can reach up to 20-30 dry t/ha/yr (Burton *et al.*, 2009).

6) Seaweeds may offer the possibility of multiple harvesting within a year. Furthermore, different species might have maximum carbohydrate contents at differing times of the year. This may render seaweeds suitable for biofuel production due to year-round availability of feedstocks.

7) Seaweeds do not require additional nutrients during cultivation, therefore eliminating fertiliser costs.

1.2 An introduction to seaweed

There are a vast variety of seaweed species with the potential to be used as biomass in a bio-refinery process. From an evolutionary perspective, seaweeds colonised our planet some 2 billion years ago, reducing the atmospheric nitrogen and carbon dioxide levels and most importantly increasing the oxygen levels to the current level of 21% (Mouradov and Stevenson, 2012). Approximately 50% of biomass on the planet is thought to be derived from a marine environment (Carlsson *et al.*, 2007) and more than 10,000 different species of seaweed are distributed in diverse coastal environments worldwide (Phillips, 2001), primarily in near-shore coastal waters where an ideal substrate for attachment can be found (Roesijadi *et al.*, 2010).

Algae are a diverse group that exist in varying forms from unicellular microscopic (microalgae) to multicellular organisms of great size (Plaza *et al.*, 2008), and may be either autotrophic/mixotrophic or heterotrophic (Mata *et al.*, 2010). The autotrophic algae use sunlight and fix the inorganic carbon from atmospheric CO_2 to generate carbohydrates as a food reverse; a process known as photosynthesis (John *et al.*, 2011). On the contrary, heterotrophic algal species take up small organic molecules from the environment they inhabit, and convert them into the necessary building blocks they require to survive, mainly protein and fat or oils (John *et al.*, 2011). Either way, over a short amount of time algae are able to produce a sufficient amount of endogenous lipids, proteins or carbohydrates, which, from a human perspective, have the potential to be extracted or processed to generate biofuels and other useful compounds.

Traditionally in Asian countries, many species of seaweed have been utilized as a source of food and as herbal drug remedies (Jiao *et al.*, 2012). China lead the way in macroalgal production with an estimated 7.9 million wet metric tons being produced annually (FAO., 2008). In Western countries however, macroalgae have been used industrially as sources of phycocolloids and gelling agents which can be used in the chemical and food industries (Mabeau and Fleurence, 1993). Edible macroalgal species such as *Undaria pinnatifida*, commonly known as 'Wakame' in Japan or 'Gim' in Korea and *Saccharina japonica* (Japanese kelp), are widely eaten with rice or used in soups to create broths. These species are not only rich in soluble dietary fibre, proteins and minerals with low calorific value, but also contain a range of polyunsaturated fatty acids, phytochemicals and antioxidants (Mohamed *et al.*, 2012). Such functional products, which are defined as those having 'beneficial effects beyond nutrition when consumed or applied' (Hasler, 2000), are widely desirable and the global market for such products has been steadily growing (Table 1.1).

Table 1.1 Examples of human commercial exploitation of macroalgae (Lukovitz., 2008)

Product	**Example**
Human Food	Nori, wakame, aonori, kombu
Algal Hydrocolloids	Agar (Food ingredient, biological/microbiological, pharmaceutical) Alginate (Textile printing, food additive, pharmaceutical, medicinal) Carrageenan (Food additive, pet food, toothpaste)
Bioactive Compounds	Sulphated polysaccharides, phlorotannins, carotenoids (pharmaceutical/medicinal)
Other products	Fertilizers, animal feed

The presence of bio-active compounds such as sulphated polysaccharides and carotenoids in some species of seaweed have potential medicinal applications and could be used as complementary and/or alternative therapies (Lukovitz., 2008).

1.2.1 Species of UK seaweed

Seaweed are classified into three groups based on the nature of chlorophyll, cell wall chemistry and flagella, however the main criterion that is applied to classify algal species is primarily based on the composition of photosynthetic pigments; *Phaeophyta* (brown algae), *Rhodophyta* (red algae) and *Chlorophyta* (green algae; (Roleda *et al.,* 1997). The presence of these different photo-pigments can also be related to the marine habitat a particular species resides in, as different species require different intensities of light in order to photosynthesize (Lordan *et al.*, 2011). For example, green macroalgae can be found in coastal waters due to the fact that they are able to absorb large amounts of light energy, as opposed to brown and red seaweeds which succeed at greater depths where minimum light penetrates (Bocanegra *et al.*, 2009). Approximately 6000 macroalgal species are divided between these three groups, with the brown algae being the largest group (59%), followed by red algae (40%) and finally green algae (<1%) (D'Orazio *et al.*, 2012). A diverse range of macroalgal species are found around the UK and Ireland shoreline, inhabiting approximately 644 different species (Brodie *et al.*, 2015). Table 1.2 depicts some of examples of UK native species of seaweed.

Table 1.2 Examples of seaweed species found around the UK shoreline (www.nhm.ac.uk)

	Latin Name	Common Name	Comments	Image
Brown *(Phaeophyta)*	*Ascophyllum nosodum*	Egg Wrack	Single large egg-shaped air bladders where each bladder represents one year of growth	
	Bifurcaria bifurcata	N/A	Rounded branches that fork in at the end. Seen in rock pools	
	Fucus serratus	Toothed Wrack	Fronds flattened with toothed edges and a mid-rib	
	Himanthalia elongata	Thongweed	No airbladders, starts growing as a small button	
	Laminaria hyperborea	Tangle/Cuvie	Found in the sub littoral zone of the Northern Atlantic	
	Saccharina latissima	Sugar Kelp	A large crinkly frond with wavy edges	
Red *(Rhodophyta)*	*Corallina* sp	N/A	Stiffed, finely branched coral-like pink seaweed made up of tiny segments	
	Chondrus crispus	Irish Moss	Grows abundantly along rocky parts of Atlantic cost Europe	
	Palmaria palmata	Dulse	Found along the Northern coast of Atlantic and Pacific oceans	
Green *(Chlorophyta)*	*Ulva lactuca*	Sea lettuce	Also has a world-wide distribution	

1.3 Seaweed composition

The chemical composition of seaweeds varies according to species, time of harvest and geographical habitat in addition to many external factors such as water temperature, light intensity and the nutrient content present in the water (Mabeau and Fleurence, 1993, Marinho-Soriano *et al.*, 2006, Marsham *et al.*, 2007). Figure 1.4 shows the changeable composition of seaweed and in general, there are substantial differences in the concentrations of nutritional factors such as proteins, minerals, lipids and carbohydrate with time of harvest.

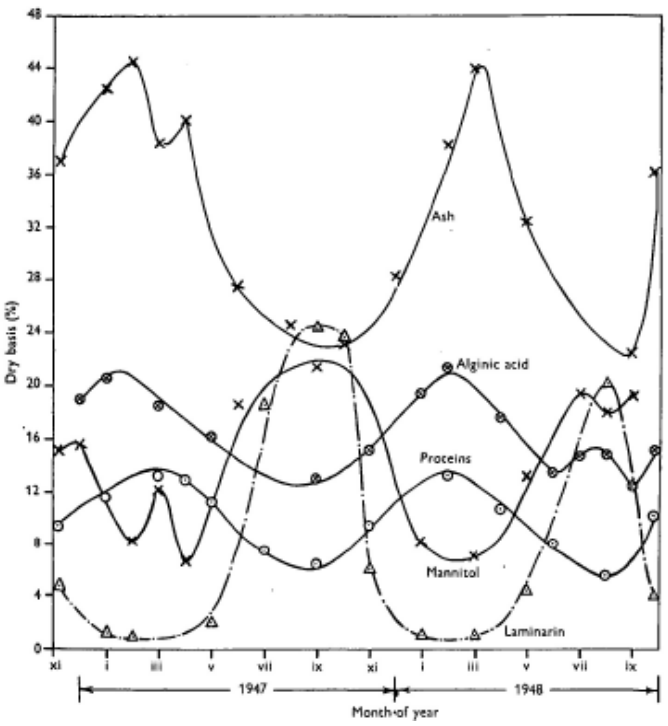


Figure 1.4 Seasonal variation of chemical components in *Saccharina latissima* taken from (Black, 1950).

Interestingly, mannitol and laminarin contents (found in brown seaweeds) are highly variable throughout the year (Adams *et al.*, 2011a, Schiener *et al.*, 2015b). The sugar alcohol mannitol is one of the main primary photosynthetic products, and serves as a storage compound along with the polysaccharide

laminarin in brown algae (Bartsch *et al.*, 2008). Based on experiments with radioactive carbon, a study concluded that both mannitol and laminarin are biochemically interchangeable storage molecules in brown seaweed and there is a shift in these major storage compounds from mannitol in spring to laminarin in autumn (Dethier and Williams, 2009). Kelps remobilise stored carbon (such as mannitol) for the development of new algal tissue during the autumn season, in order to be available both morphologically and physiologically for the summer months. New carbon is fixed and stored during the summer months without further growth of the marine plant (Gómez and Huovinen, 2012). Naturally there is also great diversity in composition between the different taxonomic groups of seaweed and studies have investigated the seasonal patterns of the different carbohydrates in greater depth (Schiener *et al.*, 2015b, Mabeau and Fleurence, 1993, Marsham *et al.*, 2007).

1.3.1 Ash

Seaweeds are known to be a significant source of minerals due to their ability to absorb inorganic substances from the environment they inhabit. This ability is due to the presence of certain polysaccharides in the cell walls which act as a 'storage unit' (Hashim and Chu, 2004). The presence of different polysaccharides in the seaweed cell wall (discussed in more detail in Section 1.3.5) is the reason why the different groups of seaweeds have varying abilities to uptake minerals (this topic is discussed in more detail in Section 3.3.1.1).

1.3.2 Protein

Seaweeds are known to contain significant amounts of nitrogenous compounds that include essential amino acids in the form of protein/peptides and additionally contain a small amount of non-protein nitrogen such as free amino acids, chlorophyll, nitrate, ammonium ions and nucleic acids (Lourenço *et al.,* 2002). It has been widely reported that seaweeds have relatively high protein contents with typical values varying between 10-30% (on a dry weight basis) (Ramos *et al.*, 2000). However, in certain species of red seaweed, such as *Palmaria palmata* and *Porphyra tenera* (commonly known as Dulse and Nori), the protein contents can be as high as 35-45% dry matter (Marsham *et al.*, 2007). On the contrary, in brown seaweed the protein content is generally lower, between 5-15% (Dawczynski *et al.*, 2007). The fact that seaweeds are a more than adequate source of protein suggests that they could be a complementary source of food proteins for human and animal nutrition. Furthermore, studies dating back to the late 1980s revealed the beneficial effects of incorporating the red species *Porphyra* into the diet of fish (Mustafa *et al.*, 1995, Mustafa and Nakagawa, 1995).

1.3.3 Lipid

The lipid content of seaweeds is typically less than 5% of the total dry weight (McDermid and Stuercke, 2003). This value is relatively low and indicates that macroalgae are not a suitable feedstock material for conversion to biodiesel, although concentrations as high as 20% have been reported in a study by McDermid and Stuercke (2003) comparing the nutritional composition of edible Hawaiian species. It is evident therefore that the production of biofuels from seaweeds predominantly depends on the

conversion of carbohydrates rather than the extraction of energy rich lipids or oils which can then be processed to biodiesel or hydrocarbons.

Despite their low lipid content, seaweed are a good source of long chain ω-3 and ω-6 polyunsaturated fatty acids (PUFA), as compared to land based vegetables (Kumari *et al.*, 2010). Green algae such as *Codium* spp have been shown to contain a higher C:18 PUFA content, whereas red algae, including *Palmaria* and *Porphyria* spp have a greater C:20 PUFA content (Khotimchenko *et al.*, 2002, Sánchez-Machado *et al.*, 2004, Kumari *et al.*, 2010). Brown seaweeds, especially species that are harvested in cold water, contain high levels of ω-3 PUFA such as stearidonic and eicosapentanoic acid (Terasaki *et al.*, 2009). Seaweeds are an attractive alternative source of PUFA to fish and fish oils and the consumption of these seaweeds may be beneficial for the prevention of cardiovascular diseases, hypertension and diabetes (Dawczynski *et al.*, 2007, Colombo *et al.*, 2006).

1.3.4 Lignin

As noted in section 1.1.3, one attractive attribute of seaweeds for biofuel production, is the lignin-free cell walls, in comparison to terrestrial crops which have matrices comprised of lignin bound to cellulose microfibrils and other cell wall components (Ralph *et al.*, 2004). The buoyancy of seaweed negates the requirement of structural biopolymers such as lignin and hemicellulose, therefore seaweed did not require the evolution of biosynthesis of these components (unlike terrestrial biomass). However, recently, low levels of lignin were discovered in the intertidal red alga *Calliarthron cheilosporioides*, raising questions about the evolution of cell walls, particularly algal cell walls (Martone *et al.*, 2009). Evolutionary scenarios

have been speculated in an attempt to explain the occurrence of lignin in red algae and vascular plants including (1) the transfer of lignin biosynthetic genes between organisms (Niklas, 2004); (2) the evolution of lignin in both independent pathways (Popper and Tuohy, 2010); and (3) the removal of ancient algal lignin biosynthetic genes during the evolution to vascular plants (Niklas and Kutschera, 2009).

1.3.5 Polysaccharides

High levels of polysaccharides exist in each of the three taxonomic groups of seaweed, which thus are potential biochemical feedstocks for the production of biofuels. However the seaweed taxonomic groups have substantially different carbohydrate compositions and accumulate characteristic polysaccharides which differ between the brown, red and green algae (Table 1.3).

Table 1.3 Main carbohydrate compositions of the three macroalgae taxa (based on dry weight).

(Adams *et al.*, 2011a, Davis *et al.*, 2003, Gómez-Ordóñez *et al.*, 2010, Marinho-Soriano and Bourret, 2005, Percival and McDowell, 1967, Martone *et al.*, 2009, Sebaaly *et al.*, 2012, Souza *et al.*, 2012, Veroy *et al.*, 1980, Yaich *et al.*, 2015, Yaich *et al.*, 2011)

Brown Algae ***(Phaeophyta)***	**Green Algae** ***(Chlorophyta)***	**Red Algae** ***(Rhodophyta)***
Laminarin (0.8-24%)	Starch (17%)	Carrageenan (7-11.5%)
Mannitol (5-20%)	Hemicellulose (20%)	Agar (6-39%)
Alginate (10-30%)	Cellulose (~9%)	Cellulose (7-10%)
Fucoidan (1-10%)		Lignin (only identified in one intertidal species)
Cellulose (2-7%)		

It is evident that green seaweeds contain similar carbohydrate complexes to those found in higher plants, albeit with lower levels of cellulose. For many years, red algae have been exploited for the production of carrageenan, agar and alginates which have great commercial value and represent potential co-products for existing markets (Roesijadi *et al.*, 2010). The total market for these three hydrocolloids reached 1,018 million US$ in 2009 (Bixler and Porse, 2011). The polysaccharides can be divided into three functionally different groups; structural cell wall polysaccharides, intracellular mucilage polysaccharides and storage polysaccharides (Bocanegra *et al.*, 2009).

1.3.6 Structural polysaccharides

The structural cell wall components found in seaweeds provide mechanical strength and flexibility to the plant in order to help it withstand water and tidal fluctuations and to avoid tearing. Seaweeds are able to synthesise a number of different structural polysaccharides that comprise their cell wall structures:

1.3.6.1.1 Alginate

Alginate, which is also known as algin or alginic acid, is a polysaccharide that is found in brown species of seaweed, and is the most abundant structural polysaccharide (Davis *et al.*, 2003). It is typically an unbranched polymer consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) rich regions with alternative G- and M-rich regions (Draget *et al.*, 2005). A regular repeating pattern is absent in this polysaccharide as the linear alginate polymer is composed of homopolymorphic M or G region, interspersed with alternate M and G regions (Figure 1.5).

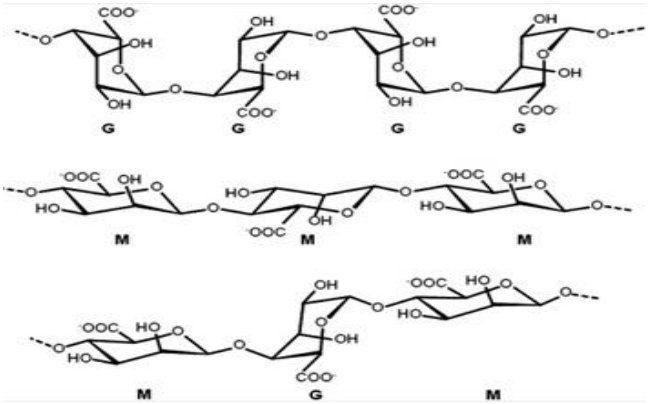


Figure 1.5 Chemical structures of alginate.

Alginates are a family of linear co-polymers containing blocks of (1-4)-linked β-D-mannuronate (M) and α-L-guluronate (G). Depending on source, alginates differ in M and G content in addition to the length of each block (Lee *et al.*, 2012)

1.3.6.1.2 Carrageenan

Carrageenans, the main component in the cell wall of red species of seaweed, are sulphated linear polysaccharides of D-galactose and 3,6-anhydro- D-galactose (Campo *et al.*, 2009). Three different structures of carrageenan exist in nature (Figure 1.6) depending on the algal species they have been sourced from, growth conditions, process of extraction and the amount and position of sulphate groups (Stanley, 1987, Préchoux *et al.*, 2013).

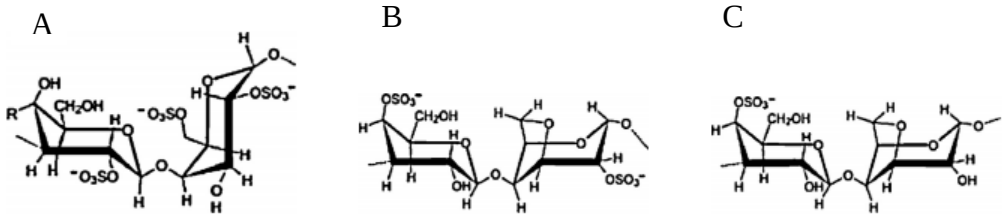


Figure 1.6 Diversity of the different carrageenan structures dependent on red algal species, growth conditions and extraction process A) λ (lambda) carrageenan B) ι (iota) carrageenan and C) κ (kappa) carrageenan (Prajapati *et al.*, 2014).

Each form has its own unique properties and application in the industry. For example, κ-carrageenan (sourced from *Kappaphycus alvarezzi*) makes an extremely strong gelling agent, whereas ι-carrageenan (from *Eucheuma denticulatum*) can be used to create elastic gels (Préchoux *et al.*, 2013).

In addition to their application in the food industry as thickening, gelling and stabilizing agents, carrageenans have more recently been used in various non-food industries such as cosmetics, printing, pharmaceutical and textiles (Weiping *et al.*, 2000). Studies have also revealed that carrageenan is in fact a very potent infection inhibitor for a number of viruses such as the human papillomaviruses (Buck *et al.*, 2006) and may also be able to act as a microbicide to block HIV infection (Council, 2008). However, *in vitro* assays have concluded that carrageenan was active against HIV but only at concentrations 1000 times higher than those required to inhibit HPV (Council, 2008). It is evident that carrageenans can be used as a model to tailor the design of novel anti-HIV or other anti-viral drugs.

1.3.6.1.3 Agar

Similarly to carrageenan, agar is extracted from certain species belonging to the red group of seaweeds. It is a linear polymer with alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-galactopyranosyl units (O'sullivan *et al.*, 2011). The structure of the monomers that make up this linear polysaccharide can be seen in Figure 1.7.

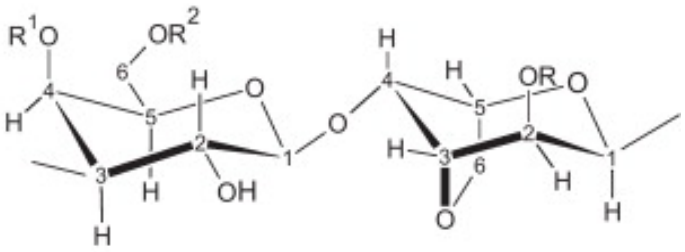


Figure 1.7 Molecular structure of agar.

(Francavilla *et al.*, 2013)

Interestingly, agar is the most expensive industrially produced hydrocolloid extracted from red seaweeds belonging to the *Gracilaria* and *Gelidium* genera (Cregut and Rondags, 2013) and is commonly used as a gelling agent in industry, especially for the food and pharmaceutical domains. It is also widely used by biologists as a microbial growth medium (agar plates). At the time of writing there is a global shortage in supply of agar and wholesale prices have increased to all-time highs of US$35-45 per kilogram (Callaway, 2015).

1.3.6.1.4 Ulvan

Ulvan, found in the cell walls of green species of seaweed, is a complex sulphated hetero-polysaccharide (O'Sullivan *et al.*, 2010). It is built on repeating disaccharide unit sequences composed of sulphated rhamnose and glucuronic acid, iduronic acid or xylose (Percival and McDowell, 1967, Quemener *et al.*, 1997). The two major repeating disaccharides are aldobiurinic acids that are designated as type A: ulvanobiuronic acid 3-sulphate (A_{3S}) and type B: ulvanobiuronic acid 3-sulphate (B_{3S}) (Figure 1.8)

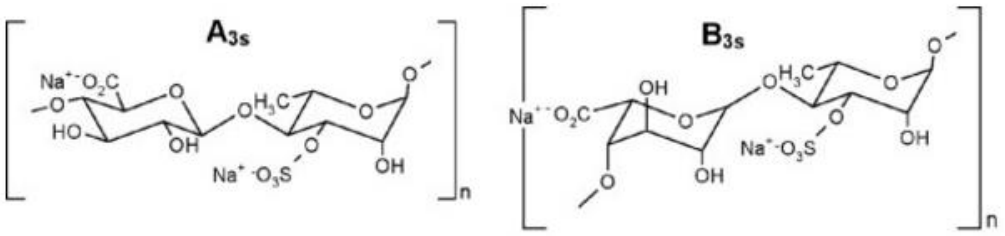


Figure 1.8 Structure of the main repeating disaccharide units in ulvan

(Robic *et al.*, 2009)

1.3.6.1.5 Cellulose

Cellulose is a naturally occurring polysaccharide and is the most abundant organic substance on this planet (Klemm *et al.*, 2005), consisting of a chain of β-1-4-linked glucose units (Figure 1.9).

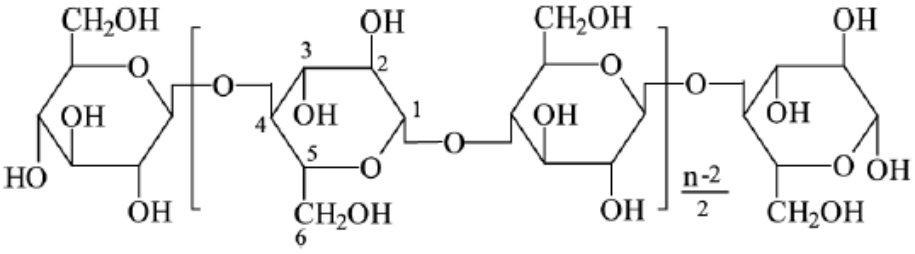


Figure 1.9 The chemical structure of cellulose (Gao *et al.*, 2005)

Cellulose is crystalline in nature and it exists as a mixture of two crystalline forms; α and β (Siddhanta *et al.*, 2009). The glucan chains in α-Cellulose are oriented in parallel to form the nanostructure that is known as a microfibril. With β-Cellulose, the molecules are organised in an antiparallel orientation and a more thermodynamically stable structure exists as hydrogen bonds hold the molecules together (Brown, 2004).

In comparison to terrestrial plants where the cellulose fractions occur in abundance and comprise 40-45% of the cell wall structure (Sjöström and Alén, 2013), the cellulose content in the cell walls of seaweed are generally lower at between 1-20% (dry weight). The actual content of cellulose in seaweeds depends on seaweed group, as studies have found that green species of seaweed usually contain more cellulose than red species of seaweed (Koyama *et al.*, 1997).

1.3.6.1.6 Fucoidan

Fucoidans are heterogeneous, sulphated water soluble polysaccharides that are inherent to brown species of seaweed. Little is known about the exact role this polysaccharide plays in marine plants, however studies have suggested that fucoidan may provide protection against dehydration, be involved in algal cell wall organisation and assist in the cross-linkage of alginate and cellulose (Mabeau *et al.*, 1990, Bisgrove and Kropf, 2001, Kloareg and Quatrano, 1988). Fucoidans essentially exist in two distinct forms of glucosaminoglycans, either in F- or U- form. F-fucoidan, which accounts for more than 95% of the fucoidans found in brown seaweeds, is composed of sulphated esters of L-fucose, and U-fucoidan is approximately 20% glucuronic acid and 80% L-fucose (Morya *et al.*, 2012). The polysaccharide may also contain other monosaccharides such as xylose, galactose and mannose in addition to the L-fucose units depending of seaweed species, along with proteins (Venugopal, 2009). Fucoidan in general comprises less than 10% (dry weight) of the seaweed's composition (Pomin *et al.*, 2005).

Interestingly, the chemical structures of fucoidans vary from species to species. However, all fucoidans have a 1-3-linked α-L-fucopyranosyl

prevalent core backbone and an alternating α-1-3 and α-1-4-linked L-fucopyranosyl structure, with various extents of sulphate substitutions (Morya *et al.*, 2012). The variations in the structural properties of fucoidans from different brown seaweed species clearly confirms the natural biodiversity of fucoidans and Figure 1.10 illustrates some of the structures found in different species of brown seaweed.

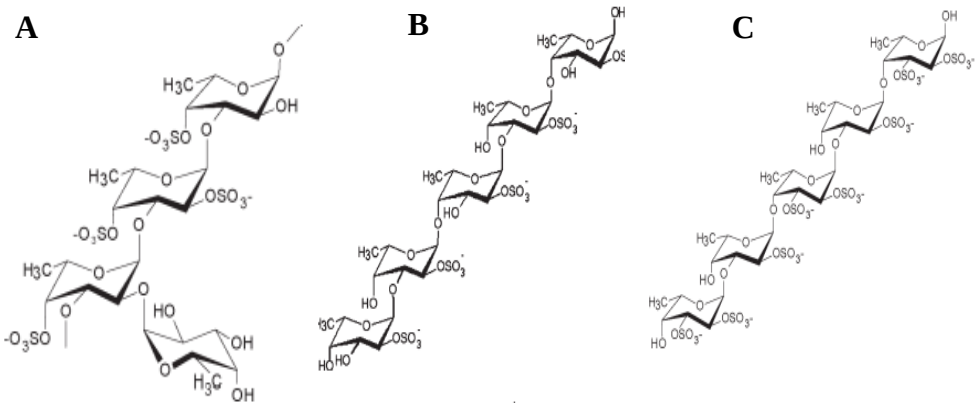


Figure 1.10 Chemical structures of fucoidans, isolated from different members of the order Fucales

(A) *Saccharina latissima* (B) *Fucus serratus* (C) *Ascophylum nodosum* (http://www.staff.dtu.dk/mata/Fucoidan/Structures)

In recent years, the interest in fucoidan, its structure and biological applications has increased. This is mainly because the sulphated polysaccharide has been shown to exhibit a number of pharmaceutically

interesting biological activities that are directly related to structure, composition, sulphate content, position of sulphate ester group and molecular weight (Ale and Meyer, 2013). Table 1.4 lists the different biological activities that have been previously ascribed to fucoidans.

Table 1.4 Examples of the different biological activities elicited by extracts of fucoidan, including species source and article reference.

Biological Activity	Species Source	Reference
Anticoagulant/antithrombotic	*E. cava*	(Athukorala *et al.*, 2006)
	E. cava	(Jung *et al.*, 2007)
	A. nodosum	(Chevolot *et al.*, 1999)
	S. fulvellum	(De Zoysa *et al.*, 2008)
Immunomodulation	*F. vesiculosus*	(Kim and Joo, 2008)
	U. pinnatifida	(Yoo *et al.*, 2007)
Anti-inflammation	*L. japonica*	(Li *et al.*, 2011)
Antitumor/anti-proliferation/anticancer	*U. pinnatifida*	(Synytsya *et al.*, 2010)
	E. cava	(Athukorala *et al.*, 2009)
	F. evanescens	(Alekseyenko *et al.*, 2007)
Angiogenesis	*A. nodosum*	(Matou *et al.*, 2002)
Antiviral	*U. pinnatifida*	(Hemmingson *et al.*, 2006)
Cardio protection	*C. okamuranas*	(Thomes *et al.*, 2010)

Given that the bioactivity of the polysaccharide is related to particular structural features, it is important that the isolation procedure which is employed maintains the structural integrity of the fucoidan and relevant features for each specific biological activity (Ale and Meyer, 2013). Therefore, a consistent extraction process is a prerequisite for determining the

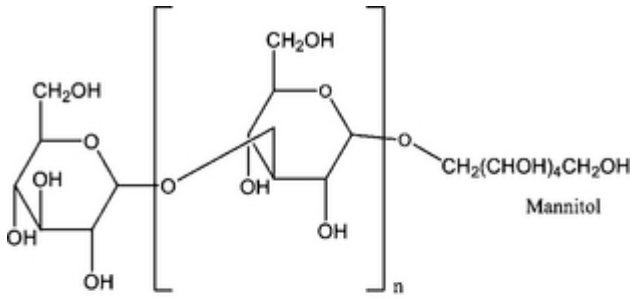
structure-function relationship that promotes the different biological activities listed in Table 1.4. As a result of there not currently being a standardised extraction methodology, the developments of any pharmaceutical, dermatological, nutraceutical and other potential applications have yet to be approved (Ale and Meyer 2013). Fucoidan is discussed in more detail in Chapter 6 where its extraction from *L. digitata* as part of a seaweed biorefinery concept is explored.

1.3.7 Storage polysaccharides

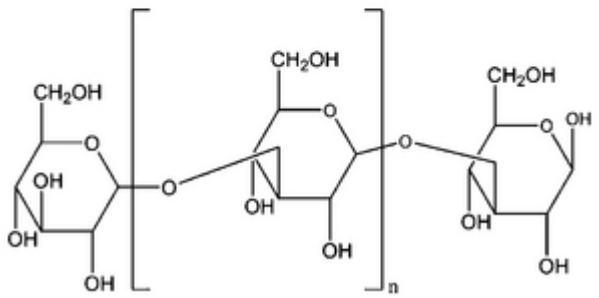
The main storage polysaccharides found in the vast majority of living organisms are α-glucans such as starch and glycogen which are provisional sources of energy. In a similar way to terrestrial starches, seaweeds contain storage polysaccharides that are also almost glucose (α-glucan) based, but differ according to seaweed taxonomic group. Starch is in fact the main storage polymer found in green species of seaweed and is most similar in structure to the starch found in terrestrial plants. In green seaweeds it is deposited in the chloroplasts and in higher plants as semi-crystalline granules (Viola *et al.*, 2001). In red seaweeds however, floridean starch is the main storage polysaccharide which is similar in structure to terrestrial plant starch, yet does not possess any amylose (Shimonaga *et al.*, 2008). Floridean starch is deposited outside the plastids of the seaweed (Shimonaga *et al.*, 2007). The storage polysaccharides of brown seaweeds on the contrary have the least similarity to the starches of land plants and these are discussed in more detail in the next section.

1.3.7.1.1 Laminarin

Laminarin (found in cell vacuoles) is a linear polysaccharide of β-(1-3)-D-glucose with terminating chains of D-mannitol and occasional β-(1-6)-glycosidic linkages (Horn *et al.*, 2000a). M and G are two types of laminarin chains that depend upon the reducing ends; for example M chains end with 1-O-substituted D-mannitol, whereas G chains end with glucose as the reducing end (Figure 1.11). As this molecule is a storage glucan, it is used as a food reserve in seaweeds. The structure and the ratio of the two types of laminarin vary according to a number of different environmental factors, including nutritive salt and the age of the seaweed (Chizhov *et al.*, 1998).



(a) M chain Laminarin



(b) G chain Laminarin

Figure 1.11 The molecular structure of laminarin

(Kadam *et al.*, 2015)

The structural features of laminarin vary according to species of seaweed with respect to the M:G ratio and this is believed to influence the biological activities of laminarin (Rioux *et al.*, 2010) which include antitumour and wound-healing activities (Lee *et al.*, 2012). Glucanases which are found in a number of microorganisms are able to easily hydrolyse laminarin to its glucose monomers which could subsequently be fermented to ethanol.

1.3.7.1.2 Mannitol

Mannitol, a sugar derived from mannose has numerous pharmaceutical and cosmetic applications because of its hydrating and antioxidant activities (Iwamoto and Shiraiwa, 2005)(Figure 1.12).

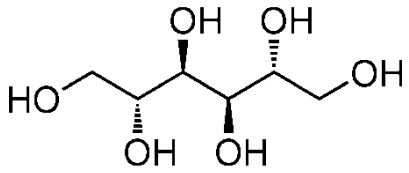


Figure 1.12 The molecular structure of mannitol

Mannitol is commonly found in brown seaweeds, however it is not readily available (Fasahati and Liu, 2012). As many microorganisms are not able to carry out the anaerobic fermentation of mannitol, it must first be oxidised to fructose by mannitol dehydrogenase; a reaction generating NADH (Horn *et al.*, 2000a, Horn *et al.*, 2000b).

1.4 Bioethanol production

It has been estimated that the potential ethanol yield which could be produced from seaweeds is approximately 23,400 L ha^{-1} y^{-1} (Adams *et al.*, 2009); much

superior to many other terrestrial crops which have been used for bioethanol production (Table 1.5).

Table 1.5 Comparison of yield and potential bioethanol volume between major terrestrial bioethanol crops and macroalgae.

(Adams *et al*., 2009)

	Wheat (grain)	**Corn (kernel)**	**Sugarcane**	**Macroalgae**
Average world yield (kg ha^{-1} year^{-1})	2800	4815	68,260	730,000
Potential volume of bioethanol (L ha^{-1} year^{-1})	1010	2010	6756	23,400

In general, there are four steps for the production of bioethanol from any type of biomass: pre-treatment, enzymatic hydrolysis, fermentation and distillation. The following sections will review in turn the current state of knowledge relating to: i) various pre-treatments that have been reported in recent studies ii) microorganisms which have been involved in either the enzymatic hydrolysis step or for the fermentation of sugars into fuel and iii) the various species of seaweed which have been utilized and the reported ethanol yields.

1.4.1 Pre-treatment and Saccharification

The objective of applying pre-treatment technologies is to alter the chemical structure of the biomass of interest, in order to make polysaccharides more accessible to hydrolytic enzymes which can then generate fermentable sugars. However, before a pre-treatment method can be applied, the desired biofuel product should be considered, and in order to do this it is important to firstly identify the main polysaccharide constituents of the seaweed of interest.

Adams *et al* (2009) tested a range of pre-treatments to improve the conversion of laminarin from *Saccharina latissima* into bioethanol using the yeast *Saccharomyces cerevisiae*. Interestingly, the results from this study suggested that a pre-treatment method prior to the fermentation process was not required. Higher ethanol yields were in fact generated from the untreated biomass that was fermented, yielding 0.45% (v/v) of ethanol, compared to those which had altered pH or temperature pre-treatments where 0.13% (v/v) ethanol was produced. This might suggest that the cost of bioethanol production from macroalgal species can be dramatically reduced as there is for the potential to dispense with expensive pre-treatment of biomass. This is in contrast to the findings of other pre-treatment research performed on seaweeds. For example, Wi *et al* (2009) carried out work on the red algal species *Gelidium amansii* (commonly known as Ceylon moss) by comparing different fermentation pre-treatments. By treating *G. amansii* with sodium chlorite before enzymatic saccharification, maximum glucose yields as high as 70% were obtained, while only 5% of glucose yields were achieved if no pre-treatment was applied. This work also revealed that the sodium chlorite pre-treatment increased the efficiency of the enzymatic hydrolysis.

A study by Jang *et al* (2012) utilized a simultaneous saccharification and fermentation (SSF) method for ethanol production from *Saccharina japonica* slurry. The group performed thermal H_2SO_4 hydrolysis (optimum conditions: 40 mM H_2SO_4 at 121˚C for 60 minutes) with the addition of 1g dw/L of an isolated marine bacterium in situ (*Bacillus* sp. JS-1) for the saccharification treatment of *S. Japonica*, yielding 45.6 ± 5.0 g/L of reducing sugars and a 69.1% yield of the total carbohydrates of the macroalgae. A theoretical yield

of 33.3% with an ethanol concentration of 7.7 g/L was achieved after using the newly isolated *Bacillus* sp. JS-1 strain for the saccharification and the yeast *Pichia angophorae* for the production of ethanol. This work indicated that the SSF method can generate higher ethanol yields in comparison to the traditional separate hydrolysis and fermentation procedure; reducing the number of unit operations needed and hence the final total cost.

Alternatively, Wang *et al* (2011) demonstrated that the combination of a two-stage hydrolysis is capable of producing sufficient saccharification of biomass, as opposed to dilute acid hydrolysis on its own. Dilute H_2SO_4 hydrolysis (under optimum conditions: 120˚C and 2% sulphuric acid for 30 minutes) along with enzymatic hydrolysis (industrial cellulases) managed to produce 13.8g glucose/kg of *Gracilaria salicornia*, a red macroalgal species, whereas only 4.3g glucose/kg was obtained for just dilute H_2SO_4 hydrolysis. The genetically engineered *E. coli* KO11 strain which can ferment a variety of C_6 and C_5 sugars, was able to produce 79.1g ethanol/kg during the fermentation of the monosaccharides present in the agal hydrolysate after the two-step pre-treatment hydrolysis. However, the ethanol yield achieved from this study is still somewhat low compared to previously reported values using extracts from *Laminaria hyperborea* (Horn *et al*., 2000a). The group suggest reasons behind this including (1) the presence of inhibitory compounds in the algal hydrolysate generated from the *E. coli* KO11 strain, (2) the monosaccharides which had been produced after the two-stage hydrolysis are not easily fermentable to ethanol by the *E. coli* KO11 strain and (3) the ethanologenic microbial strains utilised in previous studies might be better at fermenting the spectrum of sugars produced to ethanol than *E. coli* KO11.

Jeong *et al* (2011) tested the feasibility of non-enzymatic biomass hydrolysis using a two-stage acid hydrolysis of the red species *Gelidium amansii*. Although enzymatic saccharification is an environmentally friendly approach to hydrolyse carbohydrates of biomass into fermentable monosaccharide units which requires minimum energy input, the cost of such desirable enzymes are relatively expensive and the process is a major bottleneck, needing longer reaction times for the process to take place. This study showed that 68.58% of the cellulose derived from *G. amansii* could be converted into glucose using a two-stage acid hydrolysis approach; however, significant amounts of products known to inhibit fermentative microorganisms, such as 5-HMF, formic acid and levulinic acid (Jeong *et al* (2011), were generated in each stage of acid hydrolysis.

For the first time, a group in China analysed macroalgal processing residues for their potential as biofuel feedstocks. Ge *et al* (2011) explored the use of *Laminaria japonica* floating residue wastes from the alginate industry for the production of ethanol. Dilute H_2SO_4 hydrolysis (optimum conditions: 0.1% w/v, 121˚C, 1 h) was employed in the pre-treatment step along with enzymatic hydrolysis (60˚C, pH 4.8, 48 h) utilizing cellulase and cellobiase. The findings of this research revealed that the floating residues contained high cellulose and low hemicellulose contents and virtually no lignin.

The generation of fermentation inhibitory factors from any type of biomass material is a potential hindrance for the conversion of valuable sugar monomers into bioethanol. It is believed that toxic compounds can be divided into four groups: (1) compounds derived from lignocellulose structures, (2) sugar degradation products, (3) lignin degradation products and (4) heavy

metal ions (Olsson and Hahn-Hägerdal, 1996). The route through which inhibitory compounds can be generated in macroalgae is primarily through sugar degradation as lignin is generally absent from the materials. Park *et al* (2012) believe that their development of a dilute acid continuous hydrolysis process using a continuous Plug and Flow reactor system design, is an efficient way of generating a hydrolysate rich in fermentable sugars (glucose and galactose) and low in inhibitory factors. A comparison was made between the concentrations of fermentable sugars and inhibitor concentrations in hydrolysates of *G. amansii* from batch and continuous reactors; 26 g/L of fermentable sugars were produced from the batch reactor as opposed to 30 g/L from the continuous reactor, and 10.4 g/L of inhibitory products were yielded from the batch reactor whilst only 3.1 g/L was generated from the continuous reactor. Additionally, the hydrolysates from each reactor were fermented using the yeast *Brettanomyces custersii* KCTC 18154P; a total of 11.8 g/L (yield: 0.13) of ethanol was achieved from the batch reactor, in comparison to 27.6 g/L (yield: 0.38) from the continuous reactor. The authors state that this difference in yield might be attributed to the amount of inhibitors present in the batch reactor hydrolysate.

In contrast, ye Lee *et al* (2013) demonstrated that 0.06% H_2SO_4 at 170˚C for 15 minutes is more than adequate for the pre-treatment of the brown alga species *Laminaria japonica*. A maximum glucan content of 29.09%, which is four fold higher than that of raw *L. japonica,* was achieved after this pre-treatment. The advantages of applying a low acid concentration in the pre-treatment step are that the subsequent processes are a lot easier and cost effective. For example, there is no need for a neutralization step as well as

minimal waste treatment which essentially leads to a more sustainable and environmentally friendly process.

Meinita *et al*, (2012a) worked with the red macroalgae species *Kappaphycus alvarezii* harvested from Indonesian waters. This species is considered one of the most promising algal candidates for bioethanol production based on its high carbohydrate content, particularly galactose. The group reported optimal dilute acid hydrolysis using 0.2 M H_2SO_4 at 130˚C for 15 minutes with 100 g/L of powdered *K. alvarzii* and fermentation of the acid hydrolysate with 3.3 g/L of *Saccharomyces cerevisiae* (wet weight). An ethanol yield of 0.21 g/g of the sum of galactose and glucose (present in the starting hydrolysate) was produced, corresponding to a 41% theoretical yield. In order to remove inhibitory compounds present in the acid hydrolysate, activated charcoal, a form of carbon which has been processed to be extremely porous thus creating a large surface area available for absorption, was applied. Although the ethanol yield increased slightly after charcoal treatment, the treatment was not selective in its removal of hydrolysate inhibitors 5-HMF and levulinic acid, and also removed some galactose and glucose. Therefore, this study could not demonstrate a suitable method for the removal of inhibitors, suggesting further research is required in the area of inhibitor removal if solely acid-based hydrolysis protocols are to be successfully developed.

1.4.2 Fermentation

The production of ethanol is a biological process performed by many different species of yeast and bacteria, and is one of the oldest fermentation technologies discovered by humans. Simple sugars are the favourable sources of carbon and energy for yeast and the metabolic pathways by which they are

utilised have been well studied (Chu and Lee, 2007). C6 monosaccharides are taken up by yeast cells through relevant hexose transporters such as specific permeases like Hxt1, whereas complex carbohydrates like di- and tri-saccharides need to undergo hydrolysis into their monomeric units prior to entry into any metabolic pathway. Yeast such as *Saccharomyces cerevisiae* and *Brettanomyces custerii* are able to convert simple sugars such as glucose to ethanol and CO_2 under anaerobic conditions by way of the Embden-Meyerhof (glycolysis) pathway. The pathway is described by the following overall reaction:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + CO_2$$

In theory, one molecule of a hexose sugar can yield two ethanol molecules, and in terms of mass, around 0.51 kg of ethanol and 0.49 kg of CO_2 can be produced from 1 kg of hexose sugar (Bai *et al.*, 2008). Figure 1.13 depicts the pathways, including glycolysis and the hexose monophosphate shunt, that are involved in the fermentation of glucose to ethanol. During glycolysis adenosine triphosphate (ATP) is generated via substrate level phosphorylation. The product of glycolysis, pyruvate, is then further reduced to ethanol with the release of CO_2 via acetaldehyde during the TCA cycle (Boulton and Quain, 2008). Two ATP molecules generated during the glycolysis pathway are also used to drive the biosynthesis of yeast cells; indicating that ethanol production is tightly coupled with yeast cell growth and yeast biomass is a co-product of the process (Bai *et al*, 2008). Furthermore, a proportion of the carbon flow devolving from hexose sugars is dissimilated via the hexose monophosphate shunt and generates nicotinamide adenine dinucleotide phosphate$^+$ (NADPH)

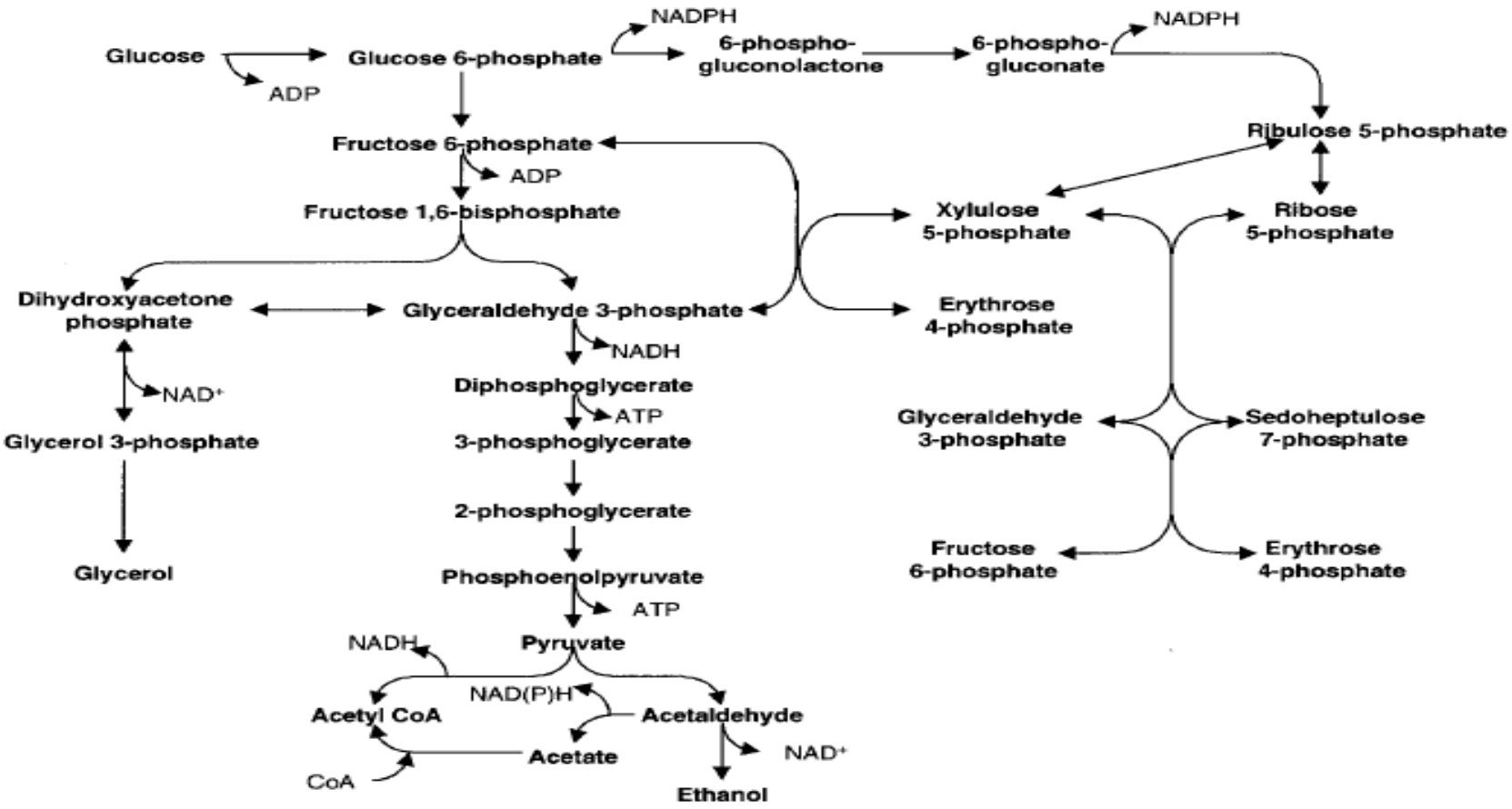


Figure 1.13 Principal pathways involved in the dissimilation of glucose to ethanol in yeast

(Taken and adapted from Boulton and Quain, 2008)

which is then subsequently used in anabolic pathways such as lipid synthesis (Boulton and Quain, 2008). It is important that the reducing power of the systems involved in the balance of redox potential within the yeast cell are maintained at all times. A 100% theoretical conversion of sugars to ethanol is never really achievable as various by-products are also produced during ethanol fermentation (Briggs *et al.,* 2004). Glycerol is the main product and can be produced at a level of about 1% (w/v) for most ethanol fermentations (Bai et al, 2008). It is produced in response to osmotic stress via the reduction of intermediates of the glycolysis pathway by the glycerol-3-phosphate dehydrogenase enzyme and also generates excess nicotinamide adenine dinucleotide (NAD^+) to maintain redox balance.

Other by-products such as organic acids and higher alcohols can also be produced as carbon end-points but at much lower levels. Hexose monosaccharides such as glucose, fructose, galactose and mannose have a higher affinity for uptake into yeast cells than pentose sugars such as xylose. Despite the fact that yeasts such as *S. cerevisiae* possess all of the relevant C5 pentose sugar transport and metabolism genes, for unknown reasons yeasts do not express these genes (Jeffries, 2006, Gírio *et al.*, 2010). Interestingly, yeasts can transport pentose sugars such as xylose using its hexose transporter system, however they will only transfer xylose into their cells in the absence of glucose (or any other 'preferred' C6 sugar) (Girio *et al.,* 2010). Pentose sugars that are taken up by the yeast cell are channelled through the Pentose Phosphate Pathway (PPP), in which this pathway interfaces with the glycolytic pathway at either1) Glucose-6-phosphate, 2) Glyceraldehyde-3- phosphate, 3) Dihydroxyacetone phosphate or 4) Fructose-3- phosphate (Flores *et al.,* 2000).

1.4.3 Fermentation of seaweed

It is evident that the full potential of ethanol production from seaweeds cannot be fully achieved because a suitable industrial microorganism that can efficiently ferment the principal sugars and polysaccharides (alginate, mannitol and fucoidan), is not available. When selecting a suitable microorganism to carry out the fermentation step for the production of bioethanol, it is important to firstly identify the main polysaccharides in specific macroalgae biomasses and then check the efficiencies of different microorganisms to ferment these sugars to bioethanol. The fermentation step during bioethanol production from renewable feedstocks is usually undertaken by yeast, but other microorganisms such as bacteria and other genetically engineered strains are also being considered for industrial usage. Here we will review both natural microorganisms (either yeast or bacteria) that have been able to produce bioethanol from the sugars released from seaweed, as well as the development of genetically engineered microbes to produce bioethanol.

Horn *et al* (2000a) revealed the possibility of simultaneously fermenting both laminarin and mannitol extracts from *Laminaria hyperborea* to ethanol using *Pichia angophorea*. A maximum yield of 0.43g ethanol/g of substrate was recovered. Further research by Horn *et al* (2000b) demonstrated that the bacterium *Zymobacter palmae* was able to ferment synthetic mannitol under different oxygen regimes. *Z. palmae* was able to grow successfully and generate bioethanol in the synthetic mannitol media under oxygen-limiting conditions, yielding 0.38g ethanol per g of mannitol. Additionally, in the same work, Horn *et al* (2000c) also tested the ability of this bacterium to ferment mannitol from *L. hyperborea* extracts in a mixed substrate medium consisting

of glucose and mannitol. The final yields were 0.53g of ethanol per g of mannitol after 11.7 hours, and 0.61g ethanol per g of mannitol at 21.9 hours. This implies that the bacterium is able to ferment both sugars simultaneously and produce a greater yield of ethanol. Interestingly however, to date no mannitol pathways have been identified in *Z. palmae* and in order for mannitol to be converted to ethanol, a number of catabolic reactions need to take place. Mannitol is catabolically converted to fructose-6-phosphate by one out of two possible pathways (Figure 1.14).

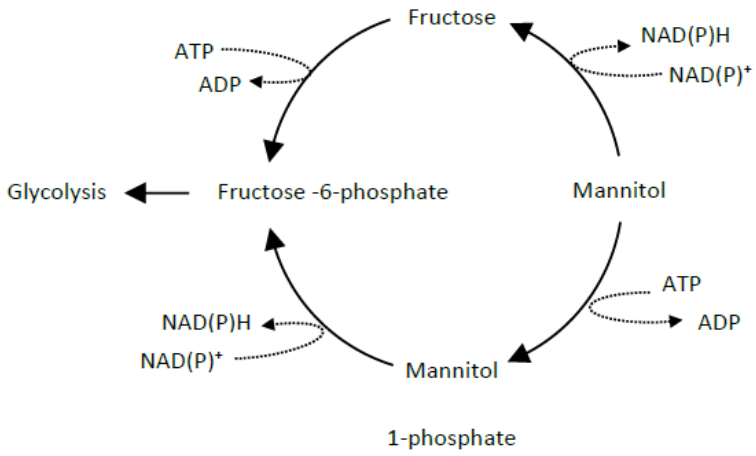


Figure 1.14 Mannitol utilisation pathways identified in yeast

Mannitol could firstly be oxidised to fructose via a hexokinase enzyme, and then mannitol dehydrogenase can phosphorylate the fructose to fructose-6-phosphate. Alternatively, the enzyme mannitol kinase can convert mannitol to mannitol-1-phosphate which would then be oxidised to fructose-6-phosphate by action of mannitol-1-phosphate dehydrogenase. At the end of both pathways, the same product (fructose-6-phosphate) can subsequently be fed

into the glycolysis pathway in the yeast cell where it will then be converted to pyruvate and lastly to ethanol (Quain and Boulton, 1987).

As mentioned in the previous section, traditional fermenting yeast strains such as *S. cerevisiae* can easily convert glucose into ethanol via the glycolysis pathway whereas galactose is metabolised by the more complex Leloir pathway (Figure 1.15). In this pathway, galactose is taken up into the cell by galactose permease 2 and is then converted to glucose-6-phosphate by galactosekinase (Galp1), galactose-1-phosphate uridyltransferase (Gal7p), UDP-galactose-4-epimerase (Gal10p) and phosphoglucomutase (two isozymes: Pgm1p and Pgm2p) (Lee *et al.*, 2015).

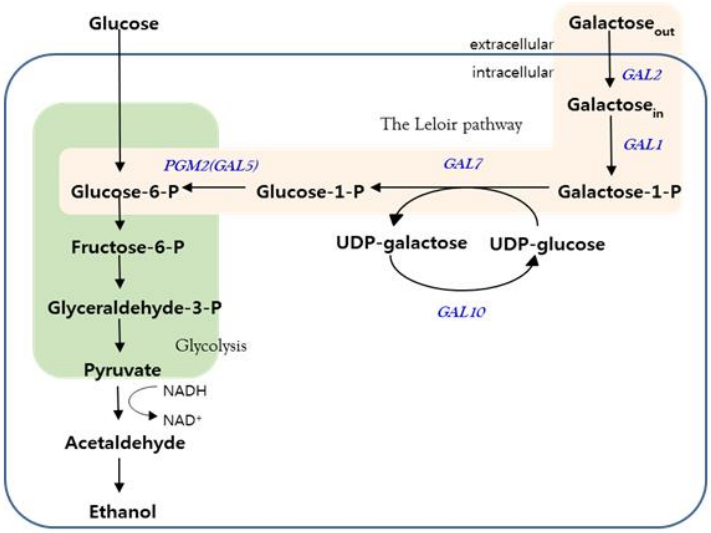


Figure 1.15 Galactose metabolism in *S. cerevisiae*

(Taken from Lee et al, 2015)

However due to the complexity of galactose metabolism, the sugar is consumed more slowly than glucose and it's uptake may be repressed in the presence of glucose (Ostergaard *et al.*, 2000). Kim *et al* (2015) evolutionarily

engineered and constructed an evolved *S. cerevisiae* strain that was able to rapidly consume galactose in both batch and repeated-batch cultures of galactose alone or a mixture of galactose and glucose. This research was targeted at the complete conversion of the major sugars found in red seaweeds, which are predominately glucose and galactose. The newly evolved strain was able to metabolise 5-fold more galactose and produce ethanol 2.1-fold faster than its parental strain. This study demonstrated the potential applications of evolutionary enginnering to develop yeast strains that can rapidly ferment sugars other than glucose in a seaweed hydrolysate.

Khambhaty *et al* (2012) isolated a marine yeast from the warm waters surrounding India, and subsequently utilized it for the production of macroalgal-based ethanol. Successfully, the marine yeast which was characterized as *Candida* sp. was able to ferment a *Kappaphycus alvarezzi* (*Rhodophyta*), hydrolysate to ethanol. What made this study interesting was the fact that the algal hydrolysate contained a high salt concentration and that galactose is the main polysaccharide present in this species as opposed to glucose, therefore experiments were conducted using galactose in the fermenting media containing different salt concentrations. The *Candida sp*. was able to yield 1.23-1.76% ethanol despite the hydrolysate containing varying levels of galactose and salt between 2.7-5.5% and from 6.25-11.25%, respectively. This marine yeast proved that it had tolerance to a range of salt concentrations and by using such a halotolerant microorganism the step of desalting prior to fermentation could be skipped and potentially make the fermentation process more economically viable.

The depolymerisation of alginate into monomeric sugars was researched by Kim *et al* (2012), who scrutinized alginate lyase genes from various bacterial microorganisms. They found that *Saccharophagus degradans* 2-40 possessed many genes that had been shown to encode for alginate lyases, and the group managed to successfully clone, express and characterize a particular gene from this bacterium 'Alg17C'. This gene was identified to be an exo-type oligoalginate lyase that has decomposing activities against alginate oligomers, hydrolyzing them into monomeric sugars. More research however is required to examine this gene's full hydrolysing potential as there are no studies to date which have used Alg17C in enzymatic hydrolysing assays of alginate prior to the main fermentation process for bioethanol production.

It is evident that the selection of suitable industrial microbial strains, which have been either isolated from or adapted towards a marine environment, would be a key step towards achieving commercial yields of ethanol from the fermentation of seaweed. One hurdle which researchers are currently faced with is that present commercial enzymes such as amylases, cellulases and proteases are usually more efficient at depolymerising terrestrial biomass than marine biomass.

Wargacki *et al* (2012) made a breakthrough in 2012 in the field of genetic engineering, after having successfully created a microbial platform of *Escherichia coli* that had the ability to convert a large amount of brown seaweed sugars to bioethanol. In order for this *E. coli* strain to be able to metabolise alginate, over 20 new genes from the species *Vibrio splendidus* 12B01, *Pseudoalteromonas* sp. A1 and *Agrobacterium tumefaciens*, were expressed in *E. coli*. Furthermore seven endogenous genes were deleted in

order to prevent the conversion of sugars into inhibitor co-products. The authors managed to achieve an ethanol titre of almost 5% (v/v) and reported yields of over 80% of the maximum theoretical yield based on the sugars found in the *Saccharina japonica* substrate used for fermentation. What was also interesting about this research was the fact that no chemical, thermal or biological pre-treatment was applied to the algal biomass before fermentation; indicating the true value of this novel engineered strain which may abolish the need to apply any costly pre-treatment methods to macroalgae before the vital fermentation step.

Similarly, alginate and mannitol degradation, transportation and metabolism genes were also introduced into *S. cerevisiae* to produce ethanol from these substrates (Enquist-Newman *et al.*, 2014). Re-engineered modules for alginate and mannitol fermentation were developed and the alginate monomer 4-deoxy-L-erythro-5-hexoseulose uronate (DEHU) transporter that is encoded by the Ac_DHT1 gene from *Astermyces cruciatus* was additionally introduced into the yeast. A final ethanol titer of 4.6% (v/v) was produced and up to 83% of the maximum theoretical yield from the consumed sugars.

1.5 Seaweed bioactives

For many years the bioactivities of macroalgae as complementary medicines has been studied, with all three taxa of macroalgae showing therapeutic properties for the management of health and certain diseases (Table 1.6). It has been proven that bioactive food components are able to alter the genetic expression of a number of cellular events. This could have an influence on an individual's susceptibility to certain diseases or heath defects, or alternatively provide a number of beneficial antioxidant (Milner, 2004) or enzyme-

inhibitory activities (Huang *et al.*, 1994). Macroalgae are rich in soluble dietary fibres, minerals, vitamins, proteins, antioxidants, phytochemicals and polyunsaturated fatty acids (PUFAs).

Table 1.6 Examples of macroalgal species from the three taxonomic groups with examples of their known bioactive compounds and possible effects on human health

(Plaza *et al*., 2008; de Quiros *et al*, 2010; Ferraces-Casais *et al*., 2012; Jiao *et al*., 2012; *Ji et al*., 2012; Mohamed *et al*., 2012; O'Sullivan *et al*., 2011; Smit *et al*., 2004; Yamasaki-Miyamoto *et al*., 2009).

Seaweed species	**Functional Ingredients**	**Possible effect on health**
Fucus serratus	Polyphenols	Antioxidant activities DNA protective effects
	PUFAs	Reduced risk of certain heart diseases
Laminaria hyperborea	Laminarin	Anti-tumor properties
	Fucoidans	Anti-tumor properties
	Mannitol	Antioxidant activity
	Sulphated polysaccharides	Apoptotic activities Reduced risk of certain heart diseases
	PUFAs	
Laminaria digitata	Laminarin	Anti-tumor properties
	Mannitol	Antioxidant activity
	Sulphated polysaccharides	Reduced total and LDL cholesterol
	PUFAs	Apoptotic activities
Ulva lactuca	Sterols	Reduced total and LDL cholesterol
Palmaria palmata	PUFAs	Reduced risk of certain heart diseases
Plumaria elegans	Carrageenan	Anti-viral activities
	PUFAs	Reduced risk of certain heart diseases
Chondrus crispus	PUFAs	Reduced risk of certain heart diseases
	Sterols	Reduced total and LDL cholesterol
	Soluble fibre	Reduced total and LDL cholesterol
	Sulphated polysaccharides	Apoptotic activities

1.5.1 Fibre

Macroalgae contain high amounts of dietary fibre which could assist in the control of calorie absorption in the small intestine (Mohamed *et al*, 2012). The main carotenoid found in brown algae, fucoxanthin, was recently shown to have possible antiobesity effects from a study trial where 151 non-diabetic, obese pre-menopausal women were given a standardised botanical food supplement containing xanthigen, which contains 300mg pomegranate seed oil and 300mg brown seaweed extract containing 2.4 mg of fucoxanthin (Abidov *et al.*, 2010). The results revealed a significant reduction of body weight, fat and systolic/diastolic blood pressure. Fucoxanthin may help reduce the bodyweight of patients by upregulating the expression of the mitochondrial uncoupling protein 1 (UCP1) gene in white adipose tissue (Maeda *et al.*, 2005).

Hypertension is a very serious risk factor that can increase one's chances of developing heart disease or inducing strokes. The brown macroalgal species *Undaria pinnatifida* (Wakame), which is consumed as part of a daily diet in Japan and South Korea, has been shown to reduce the cholesterol, systolic and diastolic blood pressure in a case controlled study using hypersensitive human patients (Hata *et al.*, 2001).

1.5.2 Polysaccharides

A link has been established between macroalgae and anticancer properties. Daily macroalgae intake has been shown to reduce colorectal (Kato *et al.*, 1990) and breast cancers (Yang *et al.*, 2010), in addition to benign and neoplasis cancer risks (Aceves *et al.*, 2005).

Water-soluble polysaccharides such as laminarin found in brown macroalgae, sulphated polysaccharides and fucoidans are known for their anti-tumour (Yamasaki-Miyamoto *et al*., 2009) and immunological regulation effects which are closely related to cell apoptosis. A study by Ji *et al* (2012) recognized that laminarin extracted from *Laminaria japonica* can induce apoptosis in human cancer colon LOVO cells through a novel mitochondrial pathway, suggesting that laminarin can be used as an effective drug for tumour prevention and treatment.

The structural features of some polysaccharides found in macroalgae, such as sulphated and uronic acid groups differentiate them from polysaccharides from terrestrial plants, yet they are similar to mammalian glycosaminoglycans. Through an increase in research on algal polysaccharides, anti-viral properties have been identified and activities have been reported against a wide range of viruses; human immunodeficiency virus (Baba *et al.*, 1988), herpes simplex virus (Hasui *et al.*, 1995) and influenza A virus (Leibbrandt *et al.*, 2010). A study by Jiao *et al.* (2012) identified that polysaccharides extracted from brown macroalgae exhibited higher anti-influenza (H1N1) activities compared to those from red or green algae. The group speculate that because brown algae have sulphate, fucose and uronic acid groups along their polysaccharide backbones, a certain degree of ionization is provided which may have the ability to inhibit viral reverse transcriptase activity. Brown algae have also been shown to have the highest antimicrobial activities, followed by the red and green algae (Nightingale *et al*., 2009).

1.5.3 Polyunsaturated fatty acids

Although the lipid content of macroalgae is generally low, the fatty acid (FA) distribution in algae showed higher levels of ω3 FA where eicosapentaenoic acid reached levels as high as 50% of the total FA content (Dawczynski *et al.*, 2007). The lipid and fatty acid content however varies between algal species, habitat condition, location and season (Mohamed *et al*., 2012). Macroalgae can be utilized as sources of ω3 fatty acids and such dietary ω3 fatty acids which have been shown to reduce heart disease risk, decrease low density lipoprotein (LDP) cholesterol, but cannot reduce high density lipoprotein (HDL) cholesterol (Mohamed *et al*., 2012). By incorporating macroalgae into a slimming food regimen, the management of weight can be controlled due to their low lipid, high dietary fibre and protein contents (Matanjun *et al.*, 2009). For example, marine oils have been used to supply substantial amounts of ω3 PUFAs into meat products, such as frankfurters enriched with ~5% *Himanthalia elongata* extracts (López-López *et al.*, 2009).

1.5.4 Antioxidants

The harsh marine environment macroalgae inhabit exposes them to a combination of different light and oxygen concentrations, which essentially leads to the formation of free radicals and other strong oxidizing agents. The fact that macroalgae seldom suffer from any photodynamic damage during metabolism, implies that over millennia, these aquatic organisms have developed potent protective antioxidative mechanisms in their cells (Matsukawa *et al.*, 1997) to deal with such conditions. The accumulation of reactive oxygen species in the human body can lead to oxidative damage and cause age-related degenerative diseases and even cancer. Potential antioxidant

compounds have been identified, such as various pigments (fucoxanthin, carotenoid etc) and polyphenols (phenolic acids, tannins) which avert free radical formation (Al-Azzawie and Alhamdani, 2006). Such compounds have been reported in macroalgae (Duan *et al.*, 2006) and the identification of natural sources of antioxidants has increased over the years, in order to avoid the use of synthetic forms such as butylated hydroxyanisole (Cox *et al.*, 2012). Algae have long been investigated as a potential source for the isolation of natural antioxidants because their polyphenols especially may have the potential to be more powerful than analogous phenols from terrestrial biomass; due to the presence of up to 8 interconnected phenol rings (Hemat., 2007). Antioxidant activities vary between macroalgal species, green algae have been recognized to have the highest 'free-radical scavenging' properties, followed by brown algae and lastly the red algae (Mohamed *et al*., 2012). Numerous studies have been undertaken on the antioxidant properties of different macroalgae and a recent study by O'Sullivan *et al* (2011) compared the antioxidant activities of macroalgae located around the Irish shoreline. The brown algae *Fucus serratus* exhibited the highest phenolic levels in its extract and *Fucus vesiculosus* also showed potent antioxidant activities.

1.6 Seaweed bio-refineries

A bio-refinery is a conceptual model in which both fuels and high value co-product materials are commercially produced (Demirbas, 2009). This type of concept attempts to convert various biological non-food feedstocks, using similar methods that have been applied to refine petroleum, in an economical way to generate biofuels, bio-chemicals, heat and power (Figure 1.16). The key concept is that biomaterials are fractionated into product streams, each of

which can be valorised to fully exploit the resource. Some of the main challenges however, are to make this process as 'green' and sustainable as possible and to ensure that maximum conversion efficiencies are achieved and minimal waste is generated (Clark *et al.*, 2012).

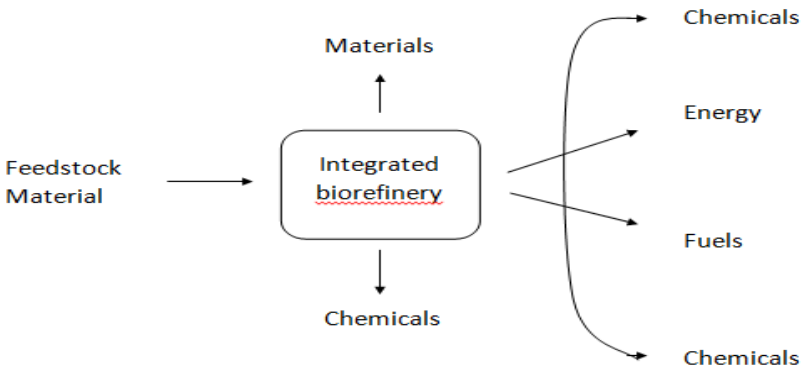


Figure 1.16 The integrated bio-refinery approach
(Clark *et al*, 2012)

Many production processes of chemicals from seaweeds have focussed on a single product, for example alginic acid, carrageenan or even colourants, and the rest of the seaweed is treated as waste material. Furthermore, many current seaweed research programmes also focus on a single product objective, with much current research focus on biofuels (John *et al.*, 2011, Langlois *et al.*, 2012). As an alternative to developing processes based on single products from seaweeds, cascading bio-refineries are being sought in order to maximise the inherent value of all components present in the biomass (van Hal *et al.*, 2014, Bikker *et al.*, 2013, Schiener *et al.*, 2015a, Yuan and Macquarrie, 2015c). Seaweeds are excellent feedstocks for such bio-refineries as they contain both high value components (such as speciality sugars and bioactive molecules) and what are considered to be the platform chemicals for the bio-

based economy such as glucose and xylose (van Hall *et al*, 2014). Additionally, various nutrients, pigments (Kumar *et al.*, 2013) and even seaweed salts (Magnusson *et al.*, 2016) could also be extracted from seaweeds, thus increasing the potential value of bio-refining processes.

An overview of the seaweed bio-refinery concept is portrayed in Figure 1.17.

Such a concept has the potential to produce a number of highly valuable bio-based products and fuels in a cost efficient way, by employing different processes to valorise the many substrates and co-products isolated from native seaweed species.

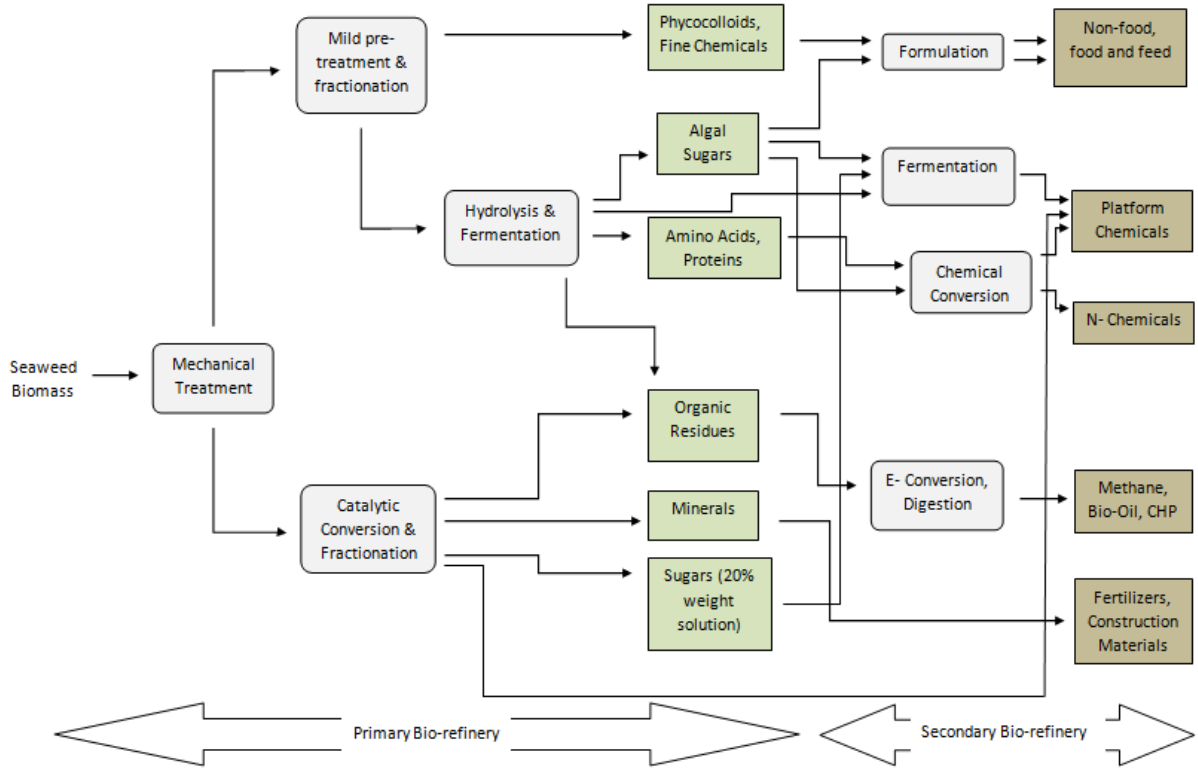


Figure 1.17 A schematic representation of the seaweed bio-refinery concept

(Kraan, 2013)

The bio-refinery can be divided into two main sections. The primary bio-refinery is where the seaweed is fractionated into its main chemical constituents, such as sugars, proteins and minerals through a number of approaches including mechanical, chemical and thermal pre-treatments, hydrolysis and fermentation. In the secondary bio-refinery, the main components extracted from the first bio-refinery are then converted to into bulk chemicals and fuels (www.seaweed.biorefinery.nl/home/). Researchers are investigating ways that can efficiently convert seaweed substrates into such bio-based products and finely-tune the bio-refinery concept for underexploited species. Large scale projects are currently/have been underway in Europe aiming to achieve this precise goal as well as projects to support large scale aquaculture research, including:

•**BioMara** - EU Programme. Production of sustainable fuel from marine sources;

•**MacroFuels** - Horizons 2020. Project aimed at producing advanced biofuels from seaweed;

•**MISTRA** - Sweden. Development of sustainable and large scale cultivation methods of seaweed

•**BIOFEED** - Norway. Research and development to produce feed from non-food biomass including seaweed

•**SINTEF** - Norway. Large scale seaweed cultivation project for biofuels;

•**Supergen II –** EPSRC, UK. Potential of Marine Biomass for Energy, Fuels and Chemicals;

•**EOS LT** – SenterNovem, The Netherlands. Seaweed Biorefinery Project;

•**Biorefinery and Biofuel Competence Centre** – Enterprise Ireland

Interestingly, bio-refinery concepts for various brown species have already been developed primarily for the extraction of such desirable polymers and minerals which can be subsequently converted into valuable commercial products (Figure 1.18).

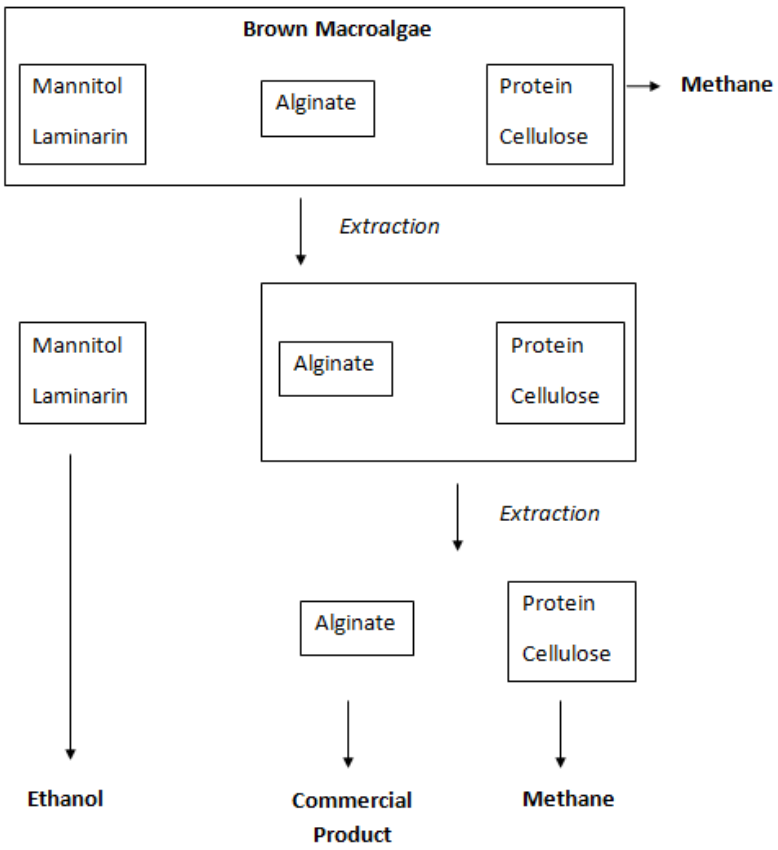


Figure 1.18 The bio-refinery concept for brown macroalgae
(Taken from Horn *et al*, 2000)

Bioethanol, biomethane and biobutanol are of great importance and can be considered to be target products (Kraan, 2013), as they have the potential to replace the current commercialised fuels derived from petroleum, and subsequently reduce CO_2 emissions. The potential for fractionation of seaweed feedstocks into carbohydrate, protein, or lipid-rich process streams and subsequently biofuels looks promising. However this research is still in its infancy and has not proceeded to scale-up. The concept of seaweed based bio-refining will be discussed in more detail in Chapter 6.

1.7 Challenges to the widespread cultivation and industrial utilisation of macroalgae

The production of fuel and co-products from seaweeds is faced with many challenges that need to be overcome, in order for the production of advanced generation biofuels to prevail. Firstly, the fact that there are seasonal differences in the availability and composition of macroalgae needs to be taken into consideration when designing plants which will successfully integrate species with high carbohydrate contents into an energy platform (Lewis *et al*., 2011). Seaweed farming will require careful consideration, as significant volumes of the biomass will be required for the production of biofuel and chemicals on an industrial scale (Burton *et al.*, 2009). This means that mass production of seaweed will require special cultivation in a suitable aquatic environment and site location, with appropriate water temperatures, light, nutrients, salt content and even water movement (Rocca *et al.*, 2015). Ultimately, the most suitable seaweed farming system will need to be developed for the species of interest, and the environmental impacts of seaweed farming will need investigating to help attain the balance between the

development of seaweed bio-refineries and their subsequent environmental costs (Wei *et al.,* 2013). Additionally, at present, there is no acceptable method for storing large tonnages of seaweed which have been harvested during the months which generate high amounts of fermentable components. In order for an all year round operation of a seaweed fermentation plant to produce biofuels and other platform chemicals, a suitable storage system must be conceived.

Although seaweeds in general contain a low concentration of easily fermentable components (mainly mannitol and laminarin) in comparison to other bio-crops, various compounds such as polyphenols in *Ulva* spp for example which are relatively high, may decrease the efficiency of bioconversion by binding to other proteins and inhibiting yeast or bacteria during the production of biofuels (Moen and Ostgaard, 1997). The high concentrations of salt present in seaweed could inhibit enzymes during hydrolysis steps and yeast based fermentations for the production of bioethanol Furthermore, high salt concentrations in generated hydrolysates may corrode the stainless steel fermentation vessels (van Hal et al, 2014). Research into inhibitory compounds present in macroalgae and similar compounds generated during the pre-treatment stage of seaweed is paramount because such factors could be a costly hindrance during the vital stage for the production of biofuels.

1.8 Research aims and objectives

In the research described in this Thesis, species of seaweed which occur in abundance in UK waters were evaluated for their potential use as feedstocks for bioethanol and speciality chemical production. British seaweeds have

seldom been exploited and furthermore, accessed for their potential to be applied in seaweed bio-refineries, in comparison to other species sourced from the waters of the Far East or surrounding Scandinavia. This research is important to develop scientific understanding of the potential for exploitation of UK seaweeds in bio-refinery processes. This would include elucidating whether UK seaweeds contain any novel compounds or a high accumulation of storage and structural polysaccharides which could be of benefit to the pharmaceutical or nutraceutical industries, and possess biofuel potential.

The primary focus of the research was to determine the feasibility of producing bioethanol from different species of UK native seaweeds. This would ultimately require the deconstruction of the seaweed biomass into simpler monomeric fractions, thus liberating smaller sugars from larger complex polysaccharides which would then be used as a feedstock for fermentation to ethanol. However, as noted (Section 1.3.5), the carbohydrate composition of seaweeds is different to that of terrestrial biomass and differs further between seaweed taxonomical groups. Therefore, the modification of existing or new procedures will be required to underpin this research.

Preliminary work (first section of Chapter 3 Section 3.1) determined the chemical composition of nine native UK seaweeds; four of which belonged to the brown seaweed group, a further four belonging to red, and finally one green species. Particular attention was paid to the carbohydrate contents and monosaccharide profiles of each of the nine seaweeds, in order to examine which species were most suitable for bioethanol production. This involved identifying candidate species with the potential to produce higher yields of

ethanol based on their C6 composition (since hexose sugars are preferred by industrial fermenting yeast strains such as *S. cerevisiae*).

The second part of Chapter 3 deals with the evaluation and screening of thermo-chemical pre-treatment systems for three selected species of seaweed. One species from each taxonomical group was selected and attempts were made to optimise the pre-treatment parameters for maximum sugar solubilisation from each of the three seaweeds; with the assumption that the optimum pre-treatments would differ between the 3 biomasses as their carbohydrate compositions vary. This research compared various dilute sulphuric acid pre-treatments and water based pre-treatments (autohydrolysis) and reaction times at a set temperature of 121°C. Furthermore, the solids loading content (seaweed biomass to reactant ratio) was also investigated in order to determine the impact on sugar yields following enzymatic hydrolysis. The ability to operate efficiently at high solids-loading would both increase throughput and reduce the amount of energy and water usage per tonne of biomass. It would also assist in generating concentrated feedstocks for fermentation. The efficacy of the pre-treatment was measured using glucose yields achieved following enzymatic saccharification, as this would likely be the primary carbon source used for ethanol production using a wild type yeast strain. Furthermore, it is the most common monosaccharide present across the three different seaweed groups. Finally, laboratory scale mini-fermentations were conducted using feedstocks generated from the optimised processes on each seaweed biomass to evaluate their potential for bioethanol production.

Due to issues which became apparent through the ongoing research, Chapter 4 considers the optimisation of quantification of carbohydrates in seaweed

biomass. Technical difficulties were encountered when attempting to accurately quantify the carbohydrate content (in particular glucose) of pre-treated seaweed. As such, a more accurate carbohydrate quantification protocol was required and a more suitable assay was developed.

The project then moved on to the identification of suitable microorganisms able to metabolise and ferment the sugars which originate from seaweed biomass (Chapter 5). Results obtained from Chapter 3 revealed that hydrolysates generated from acid pre-treatment contained a mixed array of the sugars inherent to seaweeds; not only C6 sugars such as glucose and galactose. Fermentative strains such as *S. cerevisiae* however do not contain the relevant metabolic pathways to uptake and convert the monosaccharides found in seaweed, such as fucose and uronic acids; meaning that the full potential of ethanol production from seaweeds cannot be achieved. Efforts were made to identify natural yeast strains that ferment media containing seaweed monomers. A novel phenotypic microarray (PM) approach was used to screen 24 different strains of yeast cultured on synthetic minimal media containing one sugar inherent to seaweed, or cultured on seaweed hydrolysates. Further investigation of promising strains highlighted by the PM assays were examined in laboratory-scale fermentations for ethanol production utilising different seaweed hydrolysates.

Finally, attempts were made to develop a novel bio-refinery process for an abundant brown UK seaweed, *Laminaria digitata* (Chapter 6). The process was based around the extraction of the highly valuable, sulphated polysaccharide fucoidan. Fucoidan was targeted as the key compound for extraction due to its natural bioactivities that are of potential value to the

pharmaceutical and food industries. Attempts were then made to scale up the process at CRODA's (industrial co-sponsors) pilot plant site in Leek, UK in order to obtain sufficient quantities of the polysaccharide. The extracted fucoidan and certain waste streams and products from the process were subsequently assessed for antimicrobial and antioxidant activity, and efforts were made to produce bioethanol from the seaweed solids which remained at the end of the extraction process.

2 Materials and Methods

2.1 Reagents

All reagents were of AnalaR grade and obtained from Sigma-Aldrich (UK) and Fisher Scientific (UK) unless otherwise specified. All water used was subjected to deionised reverse osmosis and of ≥18 mega-ohm purity.

2.2 Seaweed collection

The seaweeds used in this thesis (*L. digitata*, *F. serratus*, *L. hyperborea*, *S. latissima*, *P. elegans*, *C. crispus*, *P. palmata*, *D. carnosa* and *U. lactuca*) were collected at spring low tides in May 2013) near Downderry in Cornwall (OS grid coordinates: SX 315 540) in 2013 (Figure 2.1)

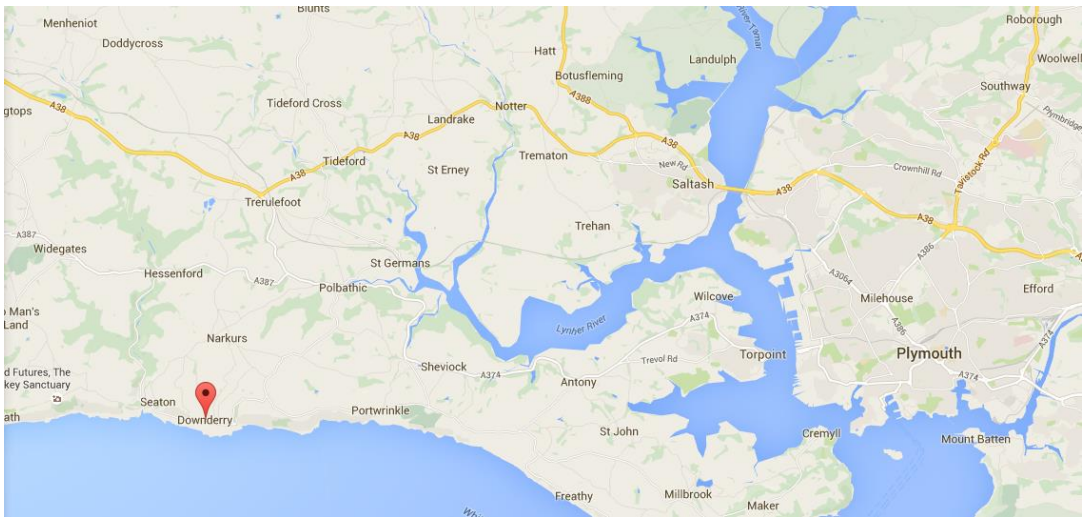


Figure 2.1 Harvesting site for seaweeds at Downderry

(Google Maps)

Downderry is a shingle environment with rocky reefs exposed at low tides. The live seaweeds were harvested (use of cutting tools where necessary) and identified on site using taxonomic references/guides. The seaweeds were

separated into separate storage bags based on species before being brought back to Plymouth Marine Laboratory, Plymouth UK, for further processing (see below).

2.3 Preparation of the seaweed

The seaweed samples were rinsed in distilled water to remove salt and debris, and then dried in a fan oven at 80 °C for a minimum of 48 h until perceived to be dry. The seaweed samples were then milled using a ball mill (Fritsch, Germany) to obtain a fine homogeneous powder and stored in a desiccator away from direct sunlight and moisture until further analyses (see below).

2.4 Compositional analysis techniques

2.4.1 Determination of moisture content in seaweed

Moisture content was measured by drying ca. 5.0 g of each seaweed species in a convection oven at 105 °C for 90 min (Santos *et al.*, 2003). After heating, the samples were placed in a desiccator for 30 min to prevent rehydration before being weighed again. All experiments were conducted in triplicate. Results were then used to calculate the compositional data on a dry weight basis.

2.4.2 Determination of ash content

Seaweed (ca. 1.0 g) was weighed into crucibles of known weight which were then ashed in a muffle furnace at 580°C for 24 hours. After ashing the samples were placed directly into a desiccator for 30 minutes. The sample was then accurately re-weighed. All experiments were conducted in triplicate.

2.4.3 Determination of protein content

A Thermo Flash Nitrogen Analyser (ThermoFischer Scientific, USA) was used to determine the protein content of each species. Sample (50 mg) was sealed in a tin capsule and combusted at approximately 1800 °C. Combustion gases were passed into a reduction reactor (at 680 °C and containing reduced copper) where nitrogen oxides are converted to elemental nitrogen. Carbon dioxide, sulphur dioxide and water were removed via filters of soda lime, magnesium perchlorate and a molecular sieve. The effluent stream was passed through a nitrogen separation column (50 °C) and into a thermal conductivity detector. Quantitation was achieved with Eager 300 software using an L-aspartic acid standard. Protein was determined using the N×6.25 conversion factor. All experiments were conducted in triplicate.

2.4.4 Determination of lipid content

Lipid content was determined using an adaption of the Folch method (Folch *et al.*, 1957). Powdered sample (ca. 400 mg) was added to a 50mL glass centrifuge tube. To each sample, 12 mL of Dichloromethane:methanol (2:1, v/v) was added and left for 2 h with occasional agitation. The glass tubes were then centrifuged at 1000 rpm for 5 min to pellet the sample. Using a glass syringe, the upper organic phase was removed and transferred into a clean 50-mL centrifuge glass tube to which 2.5 mL KCl (0.88 %, w/v) was added before being inverted, vortexed and centrifuged at 1000 rpm for 5 min. The lower organic phase (lipid extract) was removed using a glass syringe and transferred into a pre-weighed glass tube. The lipid extract was then dried with nitrogen gas and then weighed. All experiments were conducted in triplicate.

2.4.5 Determination of carbohydrate content

Carbohydrate content was measured following a modified version of the Dubois assay (Dubois *et al.*, 1956) where 1 mL of 12 M H_2SO_4 was added to 30 mg of seaweed in a heat resistant screw cap glass tube and incubated at 37 °C for 1 h. Water (11 mL) was then added to the sample to dilute the acid strength to 1 M. Samples were then incubated at 100 °C for 2 h. Resulting liberated monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) were analysed by HPAED-PAD (see section 2.7.1.). All experiments were conducted in triplicate.

2.4.6 Multi element analysis of seaweed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

2.4.6.1 Sample preparation

For the quantification of elements (Ag, Al, As, B, Ba, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, S, Se, Sr, Ti, U, V and Zn) in seaweed, samples were prepared as follows:

Seaweed biomass (200 mg) was accurately weighed into digestion vessels to which 6 mL of HNO_3 (concentrated) was added. The digestion vessels were then placed into a microwave rotor (Anton Paar Multiwave Pro 24HVT50) where they were heated to 140°C for 20 min and then cooled at 55°C for 15 min. Once the digestion was complete, Milli-Q H_2O was added to make a final volume of 20 mL. Samples were then transferred to a universal storage bottle and stored at 4°C until analysis. Experiments were conducted in triplicate.

For the quantification of iodine, samples were prepared according to the method of (Watts and Mitchell, 2009). Seaweed samples (250 mg) were

accurately weighed into Pyrex tubes, to which 5 mL of 5% (v/v) Tetramethylammonium hydroxide (TMAH) was added. Samples were shaken before being placed into a convection oven at 70°C for 3 h, with bottles shaken at 1.5 h. DI water (5 mL) was added to the samples after the 3 h incubation period, and the samples were transferred to 50 mL centrifuge tubes and centrifuged at 2500 for 25 min. The supernatant was transferred to a clean universal storage bottle and diluted to a final concentration of 1% (v/v). This was repeated in triplicate for each species of seaweed.

2.4.7 Analysis

All trace multi-element analysis was performed on an ICP-MS (Thermo-Fisher iCAP-Q) equipped with a 'Flatopole collision cell' (typically charged with helium gas) upstream of the analytical quadrupole to reduce polyatomic interferences. Internal standards were introduced to the sample stream via a T-piece and typically included Sc (50 µg/L), Ge (20 µg/L), Rh (10 µg/L) and Ir (5 µg/L) in the preferred matrix of 2% HNO_3. External calibration standards were all in the range 0 – 100 µg/L. Samples were introduced via a covered autosampler (Cetac ASX-520) through a concentric glass venturi nebuliser (Thermo-Fisher Scientific) or a PEEK Burgener Miramist nebuliser. Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific). Results were reported as gravimetric concentrations (µg/L or mg/L).

2.5 Novozymes Cellic® Ctec2 enzyme hydrolysis

2.5.1 Enzyme buffer preparation for Novozymes Cellic® Ctec2

All Novozymes Cellic® Ctec2 enzyme assays were conducted in a 50 mM sodium citrate buffer that was adjusted to pH 5.0 ± 0.2. Method details of additional enzyme assays are described in the materials and methods sections in the relevant Chapters.

2.5.2 Determination of cellulase activity of enzyme preparations used for subsequent saccharification (FPU)

Filter paper discs (cellulase material; Whatman No 1; 90 mm diameter; Whatman Ltd., Kent, UK) were cut into 1 × 10 cm rectangular strips of 50 mg weight. The rectangular filter paper strips were placed into test tubes that contained 1.0 mL of 50 mM sodium citrate buffer (pH 4.8). A 0.5 mL aliquot of various diluted Novozymes Cellic® Ctec2 enzyme mixtures were added separately to the tubes. A range of dilutions, 1:10, 1:100, and 1:1000, were made to determine the appropriate dilutions which would be required to recover the goal target of 2.0 mg of glucose (as stated by the protocol). Standards of glucose were prepared (concentration range: 1 – 3.35 mg/0.5 mL) with 50 mM sodium citrate buffer. All reaction vessels were incubated for 1 h at 50°C in a water bath (Grant Instruments, UK), to which 3 mL of 3, 5-dinitrosalicylic acid (DNS) reagent was added to each test tube (prepared as described in Adney and Baker; 1996). Reaction vessels were incubated again at 100°C for 5 min until a change in colour developed. After the boiling step the absorbance (540 nm) of samples was measured using a Jenway 7315 UV–Vis spectrophotometer (Bibby Scientific Limited, Staffordshire, UK). A colour change is observed if the DNS reagent is reduced to 3-amino-5-

nitrosalicylic acid in the presence of reducing sugars; the carbonyl group on the sugar becomes oxidised to form a carboxyl group. Glucose concentrations in the unknown samples were then calculated from the standard curve prepared using known glucose concentrations. The following equations were then used to calculate FPU:

$$\frac{(2.0\ mg\ glucose\ /mg\ glucose\ /\mu mol)}{0.5\ mL\ diluted\ enzyme\ \times 60\ mL} = 0.37\ \mu mol\ per\ minute\ mL$$

$$= \frac{filter\ paper\ activity}{(enzyme\ dilution) releasing\ 2.0\ mg\ glucose}\ units\ mL$$

2.6 Fermentations

2.6.1 Preparation of yeast cultures

Yeast strains were taken from glycerol stocks stored at −80 °C and inoculated into a liquid medium containing 10 g/L yeast extract (Oxoid, UK), 20 g/L peptone, and 20 g/L glucose (YPD) and placed in an orbital shaker (180 rpm; CERTOMAT BS-1 incubator, Germany) at 30 °C under aerobic conditions for 48 h. A sample (100 µL) of these of these cells was then spread on YPD slopes and incubated at 30 °C under aerobic conditions for a further 48 h. A sequential 3-cycle propagation system was used in order to culture a sufficient cell density that was required for the fermentation vessels. This involved firstly transferring a loop-full of the yeast cells grown on the YPD slopes into 5 ml of 4% YPD media containing 4% glucose, 2% peptone and 1% yeast extract in a sterile 30 mL plastic vessel (Sterillin, UK). Cultures were then placed in an orbital shaker (180 rpm; CERTOMAT BS-1 incubator, Germany)

at 30 °C under aerobic conditions for 48 h. The 5 mL culture was transferred into 50 mL of sterile 4% YPD (in a 250 mL glass conical flask) for a further incubation period of 48 hours (180 rpm agitation). Finally, the 50 mL culture was propagated into 500 mL of sterile 4% YPD (in a 1 L glass conical flask) and incubated for the last 48 hours at 30 °C (180 rpm agitation). Cultures were then subjected to three washing cycles to ensure all the residual glucose and YPD nutrients had been removed prior to inoculation, which consisted of the addition of 100 mL water (into a sterile 500 mL Nalgene centrifuge tube) and centrifugation at 5000 rpm for 5 min (Heraeus Megafuge 16, Thermo Scientific, UK) after which the supernatant was discarded. After the final wash the supernatant was discarded and the yeast pellets re-suspended in 50 mL of sterile water. Cell counts were conducted on the washed yeast suspension using a haemocytometer to determine the concentration of cells present (cells/mL) and methylene blue was used to indicate cell viability.

2.6.2 Laboratory-scaled fermentations

Fermentations (25 mL total volume) were conducted in glass serum bottles (30 mL; Wheaton, USA) using a method adapted from Quain *et al*. (1985) and (Powell *et al.*, 2003). The setup of the laboratory-scaled fermentation system can be seen in Figure 2.2. The bottles were made anaerobic by sealing the vessels with rubber septa and a one-way valve was used in order to facilitate the expulsion of CO_2 produced during the fermentation process as sugars are converted to ethanol. The fermentation vessels containing the fermentation feedstock were inoculated with the selected yeast strain at a pitching rate of ca 10^7 cells/ mL. The vessels were incubated at 30 °C (MIR-253 incubator, Sanyo Electric Co., Japan) with magnetic stirring set at 120 rpm, and the

progression of the fermentation was monitored by tracking the weight loss of the vessels at frequent intervals resulting from the expulsion of CO_2. The end of the fermentation was indicated by the vessels reaching constant mass. Samples were taken at the end of the fermentation for sugar and ethanol analysis via HPLC (see section 2.7.3.). All fermentations were carried out in triplicate.

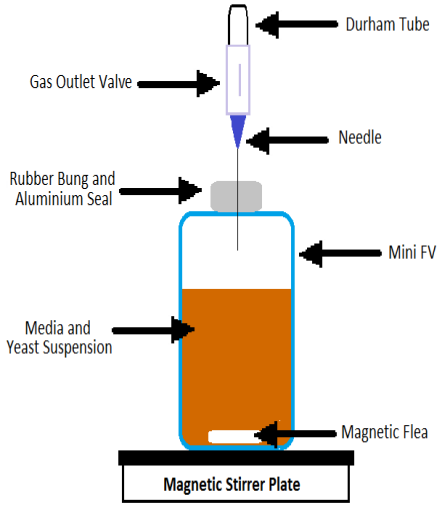


Figure 2.2 Schematic diagram of the miniature fermentation vessel used for laboratory-scaled fermentations

2.7 High Performance Liquid Chromatography

2.7.1 Quantification of monosaccharides using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

The monosaccharide concentrations were quantified using Dionex ICS-3000 Reagent-Free Ion Chromatography, electrochemical detection using ED 40 and computer controller. A CarboPacTM PA 20 column (3×150 mm) was used, with a mobile phase of 10 mM NaOH at an isocratic a flow rate of 0.5

mL/min. The injection volume was 10 μL and the column temperature was maintained at 30 °C. Authentic standards of monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) were used to generate calibration curves (0.0625-1 g/L) for monosaccharide quantification in seaweed hydrolysates.

2.7.2 Quantification of sugar degradation products using reverse phase high performance liquid chromatography (RP-HPLC) with photodiode array (PDA) detection

The analysis of sugar degradation products: (5-hydroxy-methyl-furfural (HMF), furoic acid, furfural, vanillic acid, vanillin, ferulic acid and p-coumaric acid) were quantified by HPLC using UV detection at 270 nm (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA) and a Techsphere ODS C18 column (5 μm, 4.6 mm×250 mm; HPLC Technologies, UK) was used at room temperature. The sample volume was 10 μL, and the mobile phase was a gradient of methanol in 1 % acetic acid at an overall flow rate of 1.0 mL/min. The methanol concentration was increased from 20 to 50 % over 30 min with a 100 % methanol column cleaning phase for 1 min and a 9-min re-equilibration period. Data were recorded using Millennium Chromatography software (Waters, USA).

2.7.3 Quantification of ethanol concentrations using reverse phase high performance liquid chromatography (RP-HPLC) with refractive index (RI) detection

Ethanol yields produced during fermentation were quantified by HPLC using an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC Pump (Jasco, Japan). The Rezex ROA Organic Acid H^+ organic acid column (5 μm,

7.8 mm × 300 mm; Phenomenex, UK) was operated at ambient temperature with a mobile phase of 0.005 N H_2SO_4 at a flow rate of 0.5 mL/min. A Refractive Index cell (RI-2031 Intelligent Refractive Index detector, Jasco, Japan) was used for detection, and the injection volume was 10 μL. Data were acquired using the Azur software package v. 4.6.0.0 (Datalys, France). Prior to HPLC analysis, all samples and standards were filtered using Whatman GD/X syringe filters (GF/C 25 mm filter diameter/1.2 μm pore size; Whatman, UK).

3 Screening of UK seaweeds as potential feedstocks for bioethanol production

3.1 Introduction

Seaweeds have historically been used globally as fertilizers, animal feed, for their hydrocolloid content and for human consumption (Indergaard and Ostgaard, 1991, Norziah and Ching, 2000). Interestingly, the concept of using seaweed as a potential feedstock for the production of renewable energy sources is not as novel as one might think. The idea was first conceived in the late 1960s by Howard Wilcox and throughout the 1970s and 1980s extensive research in the USA was carried out to investigate whether seaweeds could be an alternative source of natural gas via the process of anaerobic digestion. This research was however suspended and subsequently frozen due to the lack of available funding and political deposition. In the last decade, Japan, Europe and the USA have started to re-investigate the use of seaweeds as a source of biofuels, mainly due to concerns associated with the long-term sustainability of first and second generation biofuels. Seaweeds have several advantages over terrestrial plants as potential feedstocks for the production of renewable fuels (section 1.1.3). The annual global production of seaweed has been estimated to be approximately 26 million tons (wet weight) with China, being the largest producer, generating almost 13.5 million tonnes (FAO, 2014). The fact that seaweed cultivation does not compete for arable land, has high productivity and no freshwater and/or fertilizer requirements makes it an attractive feedstock for the biofuel industry.

The development of seaweed as a feedstock for biofuel production is currently a focus of global research. Efforts are being made to develop the technological innovations involved in both seaweed cultivation technologies and biomass conversion process technologies in order to generate bioethanol from seaweed at the required scale (Roesijadi *et al.*, 2010). Establishing a strategy that will successfully and efficiently achieve the complete saccharification of seaweed polysaccharides is a complex task. This is mainly because the chemical structure and composition of seaweeds differ greatly as compared to land-based plants. Studies on bioethanol production from seaweed resources have typically followed conventional methods of biomass hydrolysis, which were primarily developed for terrestrial plants for first and second generation bioethanol production. This has been accomplished through the use of a single stage chemical hydrolytic pre-treatment of the biomass, by employing an acid or an alkali as a catalyst to release fermentable sugars which can then be fermented to bioethanol. The uses of a range of acids and concentrations have been extensively explored to optimise the saccharification of seaweed polysaccharides (Ge *et al.*, 2011, Fasahati *et al.*, 2015). This has often been investigated with different combinations of reaction times, temperatures and pressures as these parameters have been shown to influence the maximum final yields of sugar that are liberated from the seaweed biomass (Meinita *et al.*, 2012b, Jang *et al.*, 2012). Fermentable sugars such as galactose and glucose which form the carrageenan and agar polymers of red seaweeds, have been readily hydrolysed by applying dilute acid pre-treatments (Kim *et al.*, 2012, Park *et al.*, 2012, Khambhaty *et al.*, 2012). Microwave assisted hydrolysis using dilute acid yielded 127 mg/g monosaccharides from the

brown seaweed *Ascophylum nodosum* in which 60.7% of the liberated sugars were successfully converted to bioethanol via fermentation using *S. cerevisiae* (Yuan and Macquarrie, 2015b). Although the use of dilute acid pre-treatment for the hydrolysis of the naturally recalcitrant polymers found in seaweeds is favourable due its low cost, such chemical pre-treatments are often coupled with higher temperatures and/or longer reaction times to maximise the efficiency of this hydrolytic step. However, higher process temperatures can lead to the production of degradation products such as HMF and furfural, and the generation of organic acids (formic and levulinic acids). Such products may have inhibitory effects on downstream processes, such as enzymatic reactions and fermentations (Jönsson *et al.*, 2013). With regards to terrestrial plant biomass, the generation of degradation products with inhibitory effects (such as ferulic acid, *p*-coumaric, vanillin and syringaldehyde), arises as a result of the degradation of lignin, to derivatives of its monomeric subunits (Wilkinson *et al.*, 2014). Seaweeds contain little to no lignin, with the exception of a handful of Rhodophyte species that contain slightly higher levels of the polymer (Martone *et al.*, 2009). This implies that the production of such degradation products during seaweed thermo-chemical pre-treatment could result from the thermal degradation of liberated pentose and hexose sugars (Larsson *et al.*, 1999, Boopathy *et al.*, 1993). Inevitably, this reduces sugar recovery efficiencies which is not desirable for bioethanol production. Additionally, these degradation products can inhibit the subsequent fermentation process by inhibiting the microorganisms used in the process. The enzymatic and biological activities performed by fermentative yeast

strains are damaged and protein and RNA synthesis become inhibited (Modig *et al.*, 2002, Sanchez and Bautista, 1988).

In order to improve the sugar recovery yield from seaweed biomass and to avoid the production of undesirable by-products, enzymatic hydrolysis has been applied to residues which have already been subjected to a mild/dilute form of thermo-chemical pre-treatment. Specialised enzymes or enzymatic blends (a combination of two or more enzymes) have been applied to directly target active sites which may still be present in the residue of the seaweed; often in the form of un-hydrolysed polysaccharides which can further be depolymerised to their monomeric subunits. This implies that higher saccharification yields can be obtained, which would then generate higher yields of ethanol post fermentation. Various studies have shown that a two stage process (dilute acid chemical pre-treatment followed by enzyme saccharification) can increase the yields of fermentable sugars by at least two-fold (Yazdani *et al.*, 2011, Jang *et al.*, 2012). Ra et al (2015) added a blend of enzymes to saccharify the dilute thermal acid hydrolysis slurry of red algal species *G. verrucosa*, achieving an 84.2% conversion of the species' total carbohydrate content. However this method of conducting enzymatic saccharification on chemically pre-treated slurry (no separation of seaweed solids from the acid generated hydrolysate) has often proven to be somewhat challenging, as sugar degradation products produced from the previous pre-treatment step can impede enzymatic hydrolysis. To overcome this, materials with high adsorption properties, such as activated charcoal, have been used as a means to detoxify slurries of undesirable products (Ra *et al.*, 2015a, Meinita *et al.*, 2012a). 100% removal of undesirable products has been achieved with

short adsorption times; however attaining high removal rates is often costly as monosaccharides can also be removed from hydrolysates due to the non-specific binding to activated charcoal (Ra *et al.*, 2015b). Establishing a pre-treatment method that would make the glucan content more accessible to enzyme hydrolysis is a key aspect for efficient fermentable sugar liberation. An important pre-requisite for bioethanol production from any feedstock material is an effective biomass pre-treatment that either directly solubilises polysaccharides (into a pre-treatment hydrolysate) or enables subsequent hydrolysis with the aid of enzymes, and without sugar degradation. However it is also important to consider the input of energy that would be required, and hence the overall costs of the process. Before the production of biofuels from any feedstock is to be undertaken, comprehensive chemical analysis of the raw feedstock composition needs to be conducted (this is later discussed in more detail in Chapter 4). As previously discussed in Chapter 1, the biochemical composition of seaweeds varies considerably between species but other factors may also influence composition; seasonal variations, environmental conditions, seaweed maturity and gender (Ito and Hori, 1989, Murakami *et al.*, 2011). Studies have been conducted on species of brown seaweeds to monitor the changes in chemical profile which may arise over long periods of time. This would aid in identifying suitable harvest times which would result in the species being an adequate substrate for bioethanol production (Schiener *et al.*, 2016, Adams *et al.*, 2011a, Adams *et al.*, 2011b). The work described in this Chapter firstly focusses on the compositional analysis of 9 UK native seaweeds, four Phaeophytes, four Rhodophytes and one Chlorophyte, with the aim of evaluating their potential as feedstocks for bioethanol production.

These species were selected based upon their abundance and availability in the waters near Plymouth Marine Laboratory. The second part of this Chapter describes process development for bioethanol production from seaweeds belonging to three different taxonomical groups. Pre-treatment reaction conditions (at 121°C) were varied using a range of different dilute sulphuric acid concentrations, reaction times and also a range of solid loadings (biomass to liquid ratio). Both direct attempts at enzyme saccharification (without any prior pre-treatment) and also combinations of both acid pre-treatment and subsequent enzyme hydrolysis were examined in order to identify the most suitable conditions for maximal sugar liberation from each of the three shortlisted seaweeds. The aim here was to compare and identify pre-treatment conditions and/or combinations that would liberate the maximum yield of fermentable sugars (mainly glucose), with the assumption that the optimum conditions would most likely differ between the three species under investigation. Finally, fermentations were conducted using *S. cerevisiae* to produce bioethanol from each of the three seaweed hydrolysates.

3.2 Materials and Methods

3.2.1 Composition analysis of seaweed

3.2.1.1 Proximate Analysis

The moisture, ash and protein contents of the 9 native seaweeds were evaluated according to the methods outlined in Sections 2.4.1, 2.4.2 and 2.4.3. The total carbohydrate and solvent-extractable lipid contents were quantified using the methods described in Sections 2.4.4 and 2.4.5. As there are no standard methods for determining the total carbohydrate content of seaweeds,

a total acid hydrolysis protocol established by the National Renewable Energy Laboratory (NREL) was followed in order to achieve complete hydrolysis and liberation of monosaccharides from the seaweed feedstocks. Individual monosaccharides (mannitol, fucose, galactose, glucose, arabinose and xylose) were subsequently quantified by HPAEC-PAD as described in section 2.7.1. Carbohydrate was estimated as the sum of the analysed monosaccharides, and it is assumed that the unaccounted for dry matter is principally polysaccharide material which was either not broken down under the hydrolysis conditions employed or was not quantified against authentic standards during HPAED-PAC analysis.

3.2.1.2 Trace metal analysis

Identification and quantification of all trace multi-elements were determined on an ICP-MS (Thermo-Fisher) following the method outlined in Section 2.4.6.

3.2.2 Thermo-chemical and auto-hydrolytical pre-treatment of seaweed biomass

Three species of seaweed (one from each taxonomical group) were selected from the 9 seaweeds that had been analysed for their chemical composition. Seaweeds containing the highest concentrations of quantified carbohydrate were selected for further trials, as this suggested that a higher yield of ethanol might be achievable.

A range of dilute sulphuric acid concentrations and also DI water were selected as the catalysts for investigation of pre-treatment of the three seaweed species used in this work. Previous research into seaweed bioethanol

production has examined the use of different concentrations of sulphuric acid as a pre-treatment, however the formation of sugar degradation products has often been a challenge (Park *et al*, 2012). Water based treatments have been traditionally employed during the first stages of phycocolloid extraction from seaweeds (for their applications in the food and pharmaceutical industries) as these mild treatments enable the target polysaccharides of interest to remain intact (Jiménez-Escrig *et al.*, 2015, Kadam *et al.*, 2015, Souza *et al.*, 2012). A dilute sulphuric acid thermal hydrolysis was selected as the pre-treatment. This is because prior studies have suggested that a higher yield of fermentable monosaccharides can be achieved through the application of dilute acid followed by enzymatic saccharification of seaweed feedstocks (Yanagisawa *et al.*, 2011, Trivedi *et al.*, 2013, Choi *et al.*, 2012). An aqueous auto-hydrolytical (water) pre-treatment was also performed in order ascertain whether this treatment was better suited for seaweed biomass prior to enzyme saccharification and to aid in the liberation of fermentable monosaccharides.

3.2.2.1 Experimental Design and Statistical Analysis

Experimental designs were generated using Design Expert v 7.0 (Stat-Ease Inc., Minneapolis, USA). The *D-optimal* designs used an algorithm which chose the treatment combinations (the exact parameter settings; e.g. acid concentrations and reaction times) to include in the design from a specific range for that parameter set by the user. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided statistically valid data. Additional experimental runs were also conducted to improve the statistical validity of the derived models. The experimental matrix is shown in Table 3.1. Alongside the dilute acid pre-

treatment, auto-hydrolytical treatments for each seaweed species were also run in parallel with reaction times of 5-30 min at 121°C.

Table 3.1 Experimental design matrix for the optimisation of the dilute acid hydrothermal pre-treatment of seaweed biomass in a bench-top autoclave.

Optimisation was conducted through screening different concentrations of sulphuric acid (0.25-5N) and reaction times (5-30 min) in a bench-top autoclave set at 121°C, according to a *D-optimal* design space.

Experimental Run Order	**Factor A** H_2SO_4 Acid (N)	**Factor B** Reaction Time (min)
1	0.25	5
2	1.5	11
3	2.5	5
4	2.5	18
5	3.5	11
6	0.25	30
7	0.25	30
8	5	18
9	0.25	18
10	2.5	30
11	2.5	18
12	5	5
13	5	30
14	5	30
15	2.5	18
16	5	5
17	3.5	30
18	0.25	18
19	1.5	5
20	1.5	18
21	1.5	30
22	3.5	5
23	0.25	24
24	3.5	24
25	5	24
26	5	11
27	1.5	24
28	2.5	24

3.2.2.2 Dilute Thermo-chemical and Auto-hydrolytical Pre-treatment of Seaweed Biomass in a Bench-Top Autoclave

Homogenised powdered samples of *L. digitata, D. carnosa* and *U. lactuca* (description of sample preparation as outlined in section 2.3) were separately loaded into glass Pyrex reaction tubes for heating in a benchtop autoclave. The Pyrex reaction tubes were individually filled with 1 g of each seaweed sample and mixed with 10 mL of either dilute acid (0.25-5 N) or DI water to prepare a solids loading of 10% (w/v). The reaction tubes were placed in a benchtop autoclave set to heat at 121°C and heated for 5-30 min. Experimental conditions were performed in accordance with the designed experiment outlined in Table 3.1 (above).

3.2.2.3 Recovery of Treated Seaweed Residues

Thermo-chemically and auto-hydrolytically treated biomasses were left to cool to ambient temperature before being centrifuged at 5000 rpm for 25 min in order to separate the solid residues from the pre-treated hydrolysates. The supernatants were then passed through a 0.45 μm filter (Whatman Ltd, UK) and stored until further analysis. The recovered seaweed residues were exhaustively washed with DI water to remove any degradation products that could have been generated during the pre-treatment step, and left to dry in a convection oven at 80°C for a minimum of 48 h until dry. Dried pre-treated seaweed residues were compared to the mass initially loaded into the Pyrex reaction tube in order to determine mass loss before being stored in an air-tight environment away from direct sunlight until further analysis.

3.2.2.4 Carbohydrate Analysis of the Treated Residues

The amounts of carbohydrate in the insoluble pre-treated residues (section 3.2.2.3) were quantified by the two-stage acid hydrolysis method (section 2.4.5) and liberated monosaccharides were determined by HPAEC-PAD (section 2.7.1).

3.2.2.5 Analysis of the Hydrolysates Separated from the Treated Seaweed Residues

Hydrolysates generated as described in sections 3.2.2.2 - 3.2.1.3 were analysed for monosaccharide and degradation products by HPAEC-PAD (section 2.7.1) and RP-HPLC (section 2.7.2). Amounts of monosaccharides and sugar degradation products in the recovered hydrolysates were calculated as a percentage of the original weight of the untreated seaweed.

3.2.2.6 Examining the Efficacy of the Pre-treatment Conditions on the Seaweed Feedstocks

Subsamples (0.1 g) of dry pre-treated seaweed residues (section 3.2.2.3) were mixed with 20 mL of 50 mM sodium citrate buffer (pH 5) (prepared by following the method in section 2.5) and dosed with an excess of Novozymes Cellic® CTec2 (ca. 50 FPU/g biomass). The samples were then incubated at 50°C for 48 h in a shaking incubator set at 120 rpm. Amounts of glucose present in the enzyme hydrolysate were quantified by HPAEC-PAD (Section 2.7.1) and calculated as the amount (mg) liberated from 1 g of pre-treated seaweed residue. Higher glucose yields obtained from the enzymatic saccharification were indicative of a more effective pre-treatment. All experiments were conducted in triplicate.

3.2.2.7 Examining the Effect of Variable Solids Loading (Biomass: Liquid) Using the Optimal Pre-treatment Conditions for Each Seaweed

Once the optimal pre-treatment conditions for each species of seaweed under investigation were identified, a small study was conducted in order to determine whether varying the biomass: liquid ratio during the thermal pre-treatment step would have any influence on the release of glucose from the seaweed residues, post enzyme saccharification with Novozymes Cellic® CTec2. Dried (native) seaweed samples (section 2.3) were accurately weighed into Pyrex reaction tubes and mixed with the optimised reaction catalyst (either dilute H_2SO_4 or water) to give a final solids loading rate of 5, 10, 15, 20 and 25% (w/v). Reactions were set up in triplicate. The samples were then heated in a benchtop autoclave at 121°C for the optimum reaction time determined from the experiments described in section 3.2.2.2. After the samples cooled to ambient room temperature, the generated hydrolysates were separated from the seaweed residues by centrifugation as outlined in section 3.2.2.3. Treated residues were washed, dried and weighed for mass loss and quantified for carbohydrate (mainly glucose) concentration. The hydrolysates were analysed for their concentration of liberated monosaccharides from the seaweed biomass (section 3.2.2.5). Dried seaweed samples were then subjected to cellulolytic saccharification using Novozymes Cellic® CTec2 (section 3.2.2.6) and glucose liberation from the treated seaweeds was quantified.

3.2.3 Enzymatic saccharification optimisation (seaweed residue: buffer ratio)

This experiment was conducted to ascertain whether varying the biomass to buffer ratio during enzymatic saccharification of the thermally treated seaweed residues would have any effect on:

i. The release of glucose liberated from the treated seaweed (mg glucose per g pre-treated seaweed)
ii. The concentration of glucose in the enzyme hydrolysate (g glucose per L enzyme hydrolysate)
iii. The recovered volume of enzyme hydrolysate (mL)

Dried (pre-treated) seaweed samples (section 3.2.2.7) were accurately weighed into falcon tubes and mixed with 50 mM sodium citrate buffer (pH 5) to make final solid loadings rates of 2, 4, 8 and 16% (w/v). An excess of Novozymes Cellic® CTec2 was dosed into the reactions and tubes gently inverted before being incubated at 50°C for 48 h at 120 rpm. Trials were conducted in triplicate. Levels of glucose were determined by taking samples of the hydrolysate at the end of the incubation period and quantified by HPAED-PAD. Hydrolysates were separated from the seaweed residue by centrifugation at 5000 rpm for 25 min, filtered and stored.

3.2.4 Fermentation of seaweed hydrolysates

Hydrolysates (section 3.2.3) were fermented using *S. cerevisiae* strain NCYC 2592 following the method outlined in section 2.6. Final glucose and ethanol yields were quantified by HPAED-PAD and HPLC respectively (section 2.7).

3.3 Results and discussion

3.3.1 Gross chemical composition of seaweed species

The gross chemical compositions of the species of seaweed investigated in this study are summarised in Table 3.2. It should be noted that achieving an accurate and complete mass balance is difficult when using the combination of analytical techniques applied to the different seaweeds in this work. Unfortunately, there are no specific agreed methodologies for determining the chemical composition of seaweed biomass and assays which have been developed for terrestrial plants are frequently employed. Additionally, as the composition and the chemical make-up vary greatly between the different genera of seaweed, one set methodology may not provide accurate results across all species. The compositional data presented here are representative of the proportions of each fraction present in each species and were acquired using the protocols described in section 2.4, with several methods requiring minor modification in order to be successfully applied to seaweed biomass. All compositional assays provided only proximate values for each component, and the technical difficulties associated with accurate quantification of the total carbohydrate content (see Chapter 4) were considered to account for the lack of complete mass closure (mass balances do not equate to 100%). However, overall results were considered adequate (58.8 – 91.3 % mass accounted for) and still provided a suitable measure of the composition of each different seaweed biomass type.

Table 3.2 Proximate composition of seaweed species used in this study

	Composition % (dry weight basis)					
Seaweed Species	**Ash**	**Protein**	**Lipid**	***Carbohydrate**	**Moisture**	**Unaccounted (%)**
L. digitata	24.3 ± 0.38	26.8 ± 0.19	1.9 ± 0.09	21.7 ± 0.68	12.1 ± 0.39	25.3
F. serratus	18.8 ± 0.58	9.6 ± 0.72	2.8 ± 0.38	26.4 ± 0.75	10.6 ± 0.06	42.4
L. hyperborea	28.1 ± 0.12	20.0 ± 0.10	2.6 ± 0.59	15.9 ± 0.63	10.5 ± 0.10	33.4
S. latissima	26.3 ± 0.74	14.8 ± 1.30	0.9 ± 0.47	16.8 ± 1.30	1.9 ± 0.16	41.2
P. elegans	32.7 ± 0.13	24.3 ± 0.68	0.4 ± 0.01	17.6 ± 1.50	3.9 ± 0.35	25
C. crispus	19 ± 1.02	19.9 ±0.27	0.5 ± 0.25	21.8 ± 1.57	12.6 ± 0.21	38.8
P. palmata	25.7 ± 0.31	22.9 ± 0.16	3.3 ± 0.60	39.4 ± 1.00	5.2 ± 0.01	8.7
D. carnosa	15.4 ± 025	22.2 ± 0.64	1.3 ± 0.70	41.8 ± 0.64	0.8 ± 0.49	19.3
U. lactuca	21.5 ± 0.29	16.4 ± 0.14	1.0 ± 0.23	23.8 ± 0.80	10.0 ± 0.01	37.3

*Carbohydrate was estimated as the sum of monosaccharides arabinose, galactose, glucose, xylose, fucose and mannitol. It is assumed that the unaccounted for dry matter is principally polysaccharide material either nor broken down under the hydrolysis conditions employed or not quantified against authentic standards during HPLC analysis. Data are the mean ±SD of three measurements

3.3.1.1 Ash

Remarkably high ash contents (Table 3.2) were observed across all species ranging from 18.8-32.7% dry weight (d/w), with the red seaweed *P. elegans* having the highest ash content of 37%. Interestingly, the other three Rhodophytes included in this study had significantly lower ash contents compared to that of *P. elegans*, within the ranges of 15.4 to 25.7 % (d/w). The ash content within the Phaeophyte group of seaweeds ranged from 18.8-28.1 % (d/w) and the content of the only Chlorophyte in this study *U. lactuca*, was 21.5 % (d/w). In general, ash content in seaweeds is substantially higher than the amounts present in most terrestrial plants (Ghadiryanfar *et al.*, 2016). Seaweeds have the ability to easily absorb inorganic substances from their environment (Yanik *et al.*, 2013) and the high ash content in their chemical composition is a result of their capacity to accumulate high levels of metal ions, mostly potassium and sodium. The ash content of seaweed biomass mainly consists of Sodium, Magnesium, Potassium and Calcium (Schiener *et al.*, 2015b). In this research, the major elements found at high concentrations across the 9 seaweeds under investigation can be seen in Figure 3.1. The concentrations of Potassium were the highest out of all the elements analysed across the 9 species of seaweed and were identified to be within the range of 13902.21 to 5017.54 mg kg^{-1} of seaweed. The next most significant elements found in the 9 seaweeds tested were (in descending order of concentration): Sulphur, (Sodium), Calcium, (Iodine) and Magnesium, followed by Strontium, Iron, Aluminium, Manganese, Nickel and Zinc. However, the levels of the latter group of ions (Strontium - Zinc) were significantly lower than the six

most abundant elements and varied considerable between each different seaweed species.

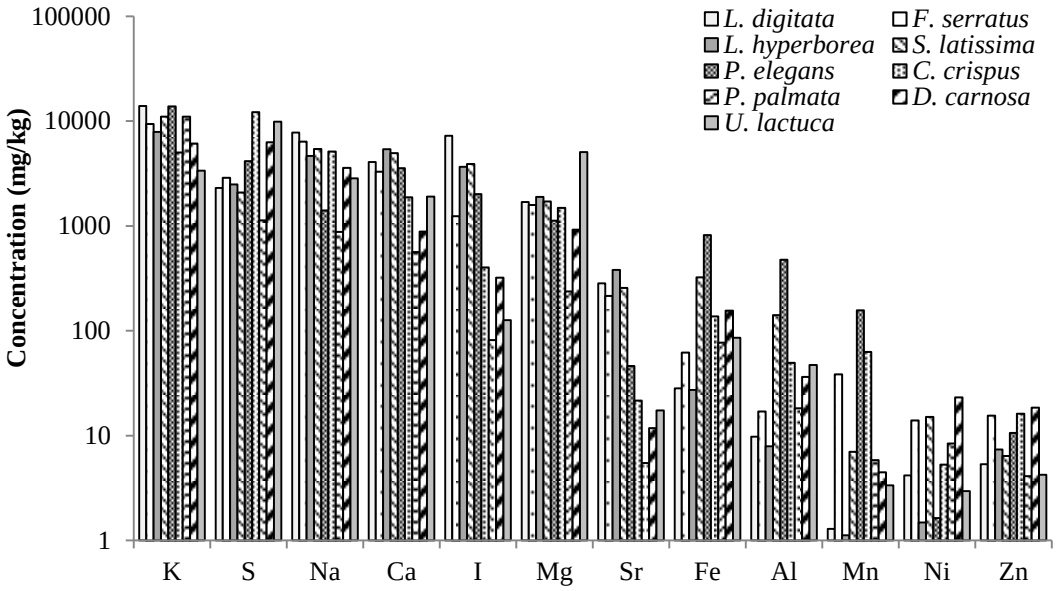


Figure 3.1 Concentrations of the most prevalent elements identified across all 9 species of seaweed

Elements arranged according to abundance (mg/kg) across the 9 seaweeds. Analysis via inductively coupled plasma spectrometry (ICP) with mass spectrometry detection. Data are the mean ±SD of three measurements.

A similar trend in elemental composition was also observed by Schiener et al (2014). The total elemental analysis of the 9 seaweed species, (including trace elements) is shown in Table-9.1 and 9.2 (Appendix A). High levels of Iodine were observed in the brown seaweeds (7267.9-1241.1 mg kg^{-1}). Concentrations were lower within the red group of seaweeds (402.1-82.0 mg kg^{-1}) and in *U. lactuca* (126.3 mg kg^{-1}). However, interestingly, red seaweed *P. elegans* was found to contain surprisingly high levels of Iodine (2023.2 mg kg^{-1}) which has not been reported previously. It has been documented that brown seaweeds, especially species belonging to the genus *Laminaria,* are significant sources of iodine and have been used for the prevention of goitre

(Verhaeghe *et al.*, 2008). Published studies have proposed that the presence of different polysaccharides in the cell walls of seaweed is the main contributing factor to the differential uptake of mineral ions (Davis *et al.*, 2003, Bocanegra *et al.*, 2009). Brown seaweed species possess a higher metal absorption capacity over red and green seaweeds due to the presence of the polysaccharide alginate and salts of alginic acids. Sulphated galactan polysaccharides which are found in red species such as carrageenan and agar play a role in metal binding and the degree of sulphation on these polysaccharides along with the presence of hydroxyl and carboxyl groups may also contribute to binding activity (Vasconcelos and Leal, 2001). Such polysaccharides have strong ion-exchanging properties mostly for elements found in high abundance such as Calcium, Magnesium, Sodium and Potassium (Antunes *et al.*, 2003). The average concentrations of Calcium, Magnesium, Sodium and Potassium detected in the four brown and four red species investigated in this study (Figure 3.2) were compared. As there was only one species belonging to the green taxonomic genus in this study, it was not included in this comparison as the data from 1 species would be insufficiently representative for this purpose. Results from this study found that the average concentrations of Calcium, Magnesium, Sodium and Potassium were higher in the group of Brown seaweeds than the group of Red seaweeds under investigation, which is in agreement with the findings of Antunes *et al* (2003). High levels of such ions in seaweeds make them a significant source of minerals which are essential in the human diet.

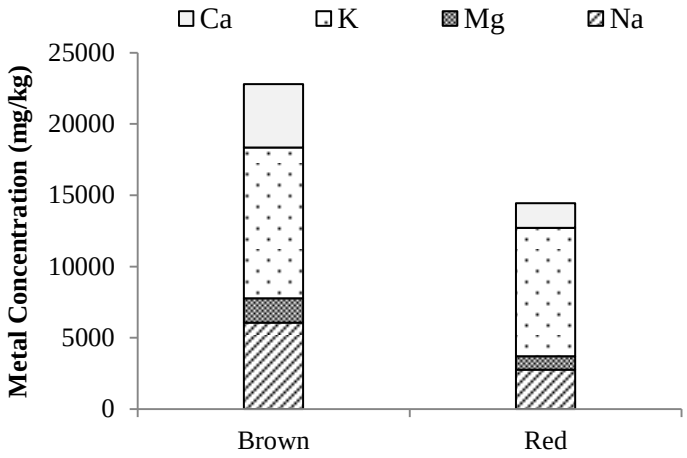


Figure 3.2 A comparison of the average concentrations of Calcium, Potassium, Magnesium and Sodium (in mg kg^{-1}) found in the Brown and Red seaweed groups in this study

Brown seaweed group is represented by *L. digitata*, *F. serratus*, *L. hyperborea* and *S. latissima* and the red seaweed group by *P. elegans*, *C. crispus*, *P. palmata* and *D.carnosa*. Metals were identified by ICP-MS and expressed in concentrations of mg kg^{-1}.

The high metal sorption also enables seaweeds to be used as bio-indicators of the environmental pollution in oceans (Conti *et al.*, 2007, Markert *et al.*, 2003, Bellinger and Sigee, 2015). However, the presence of such high levels of metals may cause processing difficulties for bioethanol production. For example, the high calcium and sodium levels may inhibit the activity and growth of *Saccharomyces spp* (Bautista-Gallego *et al.*, 2008).

3.3.1.2 Protein

Protein contents were within the range 9.6-26.8% (d/w) with *F. serratus* having the lowest content and *L. digitata* the highest content of protein. It has generally been reported that red and green species of seaweed have relatively high protein concentrations with averages reaching around 10-30% (d/w) (Ramos *et al.*, 2000, Mabeau and Fleurence, 1993). This finding is in agreement with the results of the present study. On the contrary, brown

seaweeds have been reported to have lower protein contents, of the order of 5-15% (d/w) (Burtin, 2003, Dawczynski *et al.*, 2007). In the present study only *F. serratus* and *S. latissima* fell within this range (9.6% and 14.8% (d/w), respectively), whilst *L. digitata* and *L. hyperborea* had higher protein contents (26.8% and 20.0% (d/w) respectively). Some other studies have reported high protein contents in brown seaweeds, for example, Marsham *et al.* (2007) reported 44% (d/w) protein in *F. serratus* and the content in *Undaria pinatifida* was recorded to be 21.3% (d/w) (MišurCoVá *et al.*, 2010). Is it clear that protein contents are variable according to species within the same taxonomic group and also vary according to environmental conditions. Additionally, studies have shown that there is a seasonal influence on the levels of protein in seaweeds, with contents higher in winter and early spring months from February to May, whilst the lowest values were determined to be in the summer and autumn from July to October (Denis *et al.*, 2010, Fleurence, 1999, Schiener *et al.*, 2015b) High protein contents could be deemed advantageous when screening for feedstocks for bioethanol production. Organic nitrogen in the form of peptides and amino acids are important nutrients for fermentative microorganisms. It would have been of interest to have also screened the protein amino acid profiles in each species of seaweed, particularly for *L. digitata* and *L. hyperborea* as they had relatively high protein contents for brown species. However due to time constraints and priorities this was not undertaken. After the production of bioethanol from the carbohydrate fraction of seaweed has been achieved, a protein enriched residue may remain which could then be used for animal feed manufacture. This suggests a preference for feedstocks that are high in both carbohydrate

and protein fractions, as it may then be possible to generate multiple value added products in a bio-refinery process.

3.3.1.3 Lipid

The lipid content of seaweed species in this study was between 0.48 - 3.3% (d/w). This is in agreement with published literature typically ranging from 1-5% of dry seaweed material (Kebelmann *et al.*, 2013, Burtin, 2003). However, the majority of the lipids found in seaweeds are rich in polyunsaturated fatty acids (PUFAs) which are beneficial to human health (Norziah and Ching, 2000). Brown seaweeds such as *Undaria sp* and *Laminaria sp* contain high levels of oleic acid (C18:1, n-9), alpha linolenic acid (C18:3, N-3) and eicosapentaenoic acid (EPA) (C20) and are currently of great commercial interest for their n-3 PUFAs and to the fact that they can be cultivated on a large scale at sea (Kumari *et al.*, 2010).

3.3.1.4 Carbohydrate

Due to the complexity and the diversity of carbohydrates which are inherent to seaweed biomass, the carbohydrate content was measured as the total of the monosaccharides glucose, galactose, mannitol, fucose, xylose and arabinose, quantified by Ion Chromatography (HPAEC-PAD) following a two stage acid hydrolysis. Amongst the five seaweed species screened in this study, *D. carnosa* had the highest carbohydrate content, amounting to 41.8% of the dry material, which likely reflects the hydrolysis of agar, a major component located in the extracellular matrix of red seaweed (Noseda *et al.*, 1999). *P. palmata* was next highest in carbohydrate content (39.4%). Interestingly, the brown kelps *L. hyperborea* and *S. latissima* had the lowest measured

carbohydrate contents of 15.9% and 16.8%, respectively. This finding was somewhat surprising as levels of carbohydrates in the same species of seaweed have been reported at higher concentrations, as high as 65.5% (d/w) for *L. hyperborea* and 63.1% (d/w) for *S. latissima* (Schiener *et al*, 2015).The low levels of phaeophycean carbohydrates recorded in this analysis may be due to the fact that seaweeds were harvested in May for this study. It has been well documented that the composition of seaweed carbohydrates are variable and dependent on the season (Marinho-Soriano *et al.*, 2006, Schiener *et al.*, 2015b, Chapman and Craigie, 1978). Furthermore, a study by Adams *et al*. (2011) revealed that the most suitable month to harvest *L. digitata* for bioethanol production was July, as this is when carbohydrate yields (mainly mannitol and laminarin) were found to be highest. Unfortunately due to practical limitations it was not feasible to harvest the seaweeds for this project during the summer months. Undoubtedly, this influenced the final yields of bioethanol that could be generated. It is also possible that some monosaccharides (e.g. mannuronic and guluronic acids from the polysaccharide alginate) were released but were not quantified using the analytical method applied. Figure 3.3 shows the breakdown of monosaccharides (mannitol, fucose, arabinose, glucose, galactose and xylose) analysed following the 2 stage acid hydrolysis protocol. The monosaccharide data for the different seaweed species are in accordance with the major polysaccharides they are known to accumulate (see Table 1.3). The main cell wall constituents that make up brown seaweeds are typically cellulose microfibrils which are embedded in an amorphous matrix of alginate and fucoidan polysaccharides (Kloareg *et al.*, 1986).

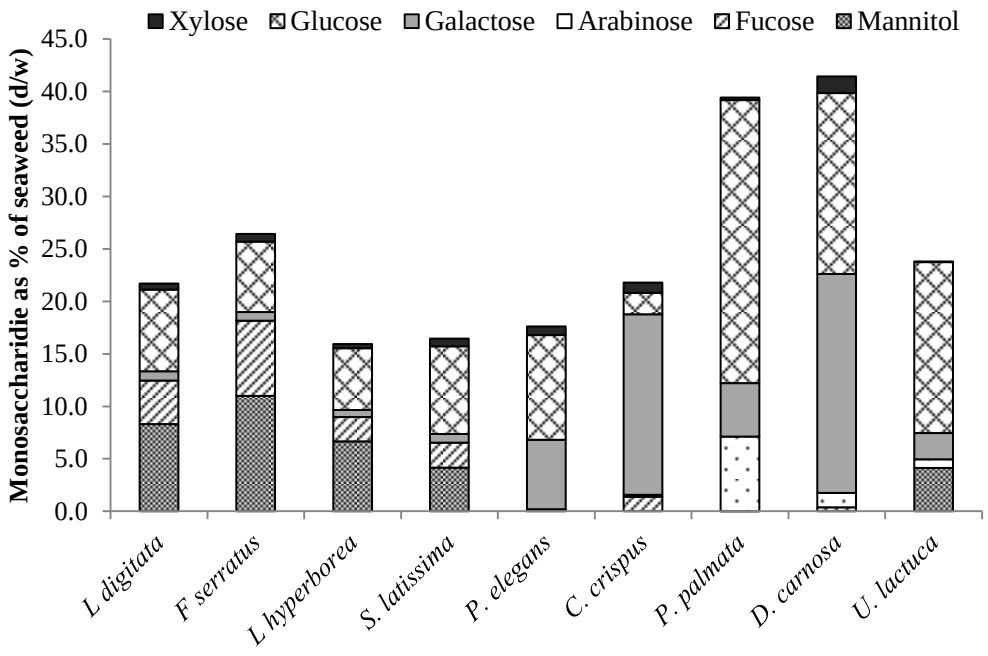


Figure 3.3 Analysis of the monosaccharides released from the 9 seaweeds after the two stage acid hydrolysis

Carbohydrate was estimated as the sum of monosaccharides arabinose, galactose, glucose, xylose, fucose and mannitol. Data are the mean ±SD of three measurements

Mannitol is often found as a terminating side chain on laminarin, a polymer of β-(1-3)-D-glucose that can be easily extracted from brown seaweed (Horn et al. 2000a). The concentrations of mannitol across the four brown seaweeds in this study were within the range of 4-11 %. Fucose monomers have only been detected in the brown seaweeds, as a result of the hydrolysis of the sulphated polysaccharide fucoidan (Rodriguez-Jasso *et al.*, 2011). The varying levels of fucose which was released (between 0.8-7.0%), doubtless reflects the different concentrations of fucoidan within the seaweed. It is not possible, based on the present data, to conclude whether the relatively low levels of glucose (5.9-8.4 %) analysed resulted from the hydrolysis of laminarin or from the cellulose component in brown seaweeds. Studies have reported that laminarin is an

easily hydrolysable polymer which can dissociate from the seaweed complex under relatively mild conditions (Horn *et al*, 2000b). Various researchers have used laminarinase enzymes in order to quantify the concentration of laminarin in different species of seaweed, through the direct release of D-glucose (Adams *et al.*, 2011b). However studies that have applied this methodology have solely focused on the laminarin content, and have not conducted subsequent assays on the remaining carbohydrate content within the seaweed. It remains possible that some of the glucose which was liberated as a result of the two stage acid hydrolysis protocol was from the more recalcitrant polymer cellulose. The harsh conditions of the two stage acid hydrolysis could have converted laminarin glucose into a degradation product. Such processes would lead to an underestimation of the true glucose content of the brown seaweeds in this study. Similarly the monomeric sugars of which alginate is comprised could not be quantified using the established HPLC method applied in this work, which could also explain the overall lower carbohydrate contents reported for the brown seaweeds. The profile of monosaccharide sugars from the hydrolysis of red algal species (*C. crispus*, *D. carnosa*, *P. elegans* and *P. palmata*) revealed a different composition of carbohydrates. Significant amounts of glucose and in particular galactose are noticeable, which are products of the hydrolysis of agarose or carrageenan and cellulose. Agarose, a galactose based polysaccharide of L or D-galactose and 3,6-anhydro-galactose subunits (Knutsen *et al.*, 1994) is comprised of β-1-4 glucosidic bonds. Carrageenans are sulphated linear galactan polysaccharides with a basic structure comprised of alternating β-1-3- and α-1,4-linked galactose residues (Chapman and Chapman, 1980). Cellulose has β-1-4 glucosidic bonds

between two D-glucose monomers. *P. palmata* had the lowest content of galactose (5.1%), however this species yielded the highest content of glucose (27.8%) compared to the other three red seaweeds. This may imply that this particular species had a higher content of cellulose in its structure than carrageenan or agarose. Surprisingly, an arabinose content of 7.1% was identified in *P. palmata* whereas only 1.4% was found in *D. carnosa* and no traces of arabinose were detected in *P. elegans* or *C. crispus*. The monosaccharide profile of *D. carnosa* was noteworthy as it had high concentrations of both glucose (17.2%) and galactose (20.9%). The fact that over 37% of dry matter was fermentable hexose monosaccharides makes this red seaweed a potential candidate for bioethanol production. Xylose was detected in all red seaweeds albeit at relatively low concentrations (0.2-1.6%). Findings from other research groups have revealed that red seaweeds belonging to the orders *Nemaliales* and *Palmariales* contain an inner fibrillar network of xylan instead of cellulose in the algal cell wall, which consists of mixed β-D-(1-4) and β-D-(1-3) linkages (Usov, 2011, Viana *et al.*, 2011). Interestingly, the major polysaccharide of *P. palmata* is reported to be xylan (Galland-Irmouli *et al.*, 1999) however the xylose content of the *P. palmata* investigated in this work was extremely low and only contributed to 0.2% of the carbohydrate content, which contradicts the previous finding. This low yield could reflect the incomplete hydrolysis of xylan. It is not feasible to determine, based on the current data, where specifically the galactose originated from in the red seaweeds. Fucose was quantified at 1.4% in *C. crispus*. This monosaccharide is typically only found in phaeophytes, not rhodophytes and this finding calls into question the correct assignment of the

HPLC peak to fucose in this instance. It may be concluded that *C. crispus* contained a monosaccharide which demonstrated chromatographic similarity to the fucose standard under experimental conditions. Likewise it eluted within the same retention window as the peak assigned to fucose identified in the brown seaweeds. However, LC-MS studies would be required to further confirm this identification. *Ulva lactuca*, the only green species of seaweed examined in this study had a glucose content of 16.3%. The cell walls of green algae are extremely similar in structure and composition to those of terrestrial plants (Domozych *et al.*, 2012), therefore one can speculate that the glucose monomers released were most likely the product of cellulose hydrolysis. Xyloglucans are another minor polysaccharide that can be located within the cell walls of green algae, in addition to arabinogalactans (Domozych *et al*., 2012), explaining the presence, albeit at low levels, of arabinose (0.8%), galactose (2.5%) and xylose (0.2%).

3.3.2 Optimisation of pre-treatment conditions for bioethanol production from selected seaweed species

L. digitata, *D. carnosa* and *U. lactuca* were selected (from the original nine species subjected to compositional analysis) to evaluate their potential as feedstocks for bioethanol production. Although the kelp *L. digitata* only had the second highest carbohydrate content of 21.7% in the brown seaweed group, it was selected due to its high abundance in UK waters. Abundance is an important factor to consider when evaluating any potential biomaterial (biomass type) as a feedstock for bioethanol production, especially if a bioethanol plant is to be developed in the UK for seaweed processing and fuel generation. *D. carnosa*, was shown to have the highest carbohydrate content

within the Rhodophyte group (41.8%) and had a desirable monosaccharide profile consisting principally of easily fermentable glucose and galactose. Finally, *U. lactuca* was included in this trial as an example of a green seaweed species. It was thought desirable to include a species from each taxonomic group in this study, to provide an indication of whether optimum pre-treatment conditions varied across them. This is the first study which has explored the potential use of purely water (with reactions proceeding entirely via 'auto-hydrolysis') as the catalyst during the pre-treatment step for seaweed bioethanol production. Relatively short experimental run times (up to a maximum of 30 mins) were chosen in an attempt to make the pre-treatment process as short and economical as possible. Additionally, reaction temperature (121 °C) was not a variable. This was because the bench-top autoclave (utilised as the bio-reactor to conduct these trials) had a maximum operating temperature of 121°C. In addition, there is also a wealth of published research that confirms the use of 121°C reaction temperatures as being suitable for pre-treatment of lignocellulosic biomass (Cao *et al.*, 2012, Wilkinson *et al.*, 2014, Pereira *et al.*, 2015). Optimum pre-treatment conditions were identified based on the maximum liberation of glucose from the treated residue after enzymatic saccharification. An overview of the pre-treatment and enzymatic saccharification stages involved in the investigations with seaweed biomass is outlined in Figure 3.4.

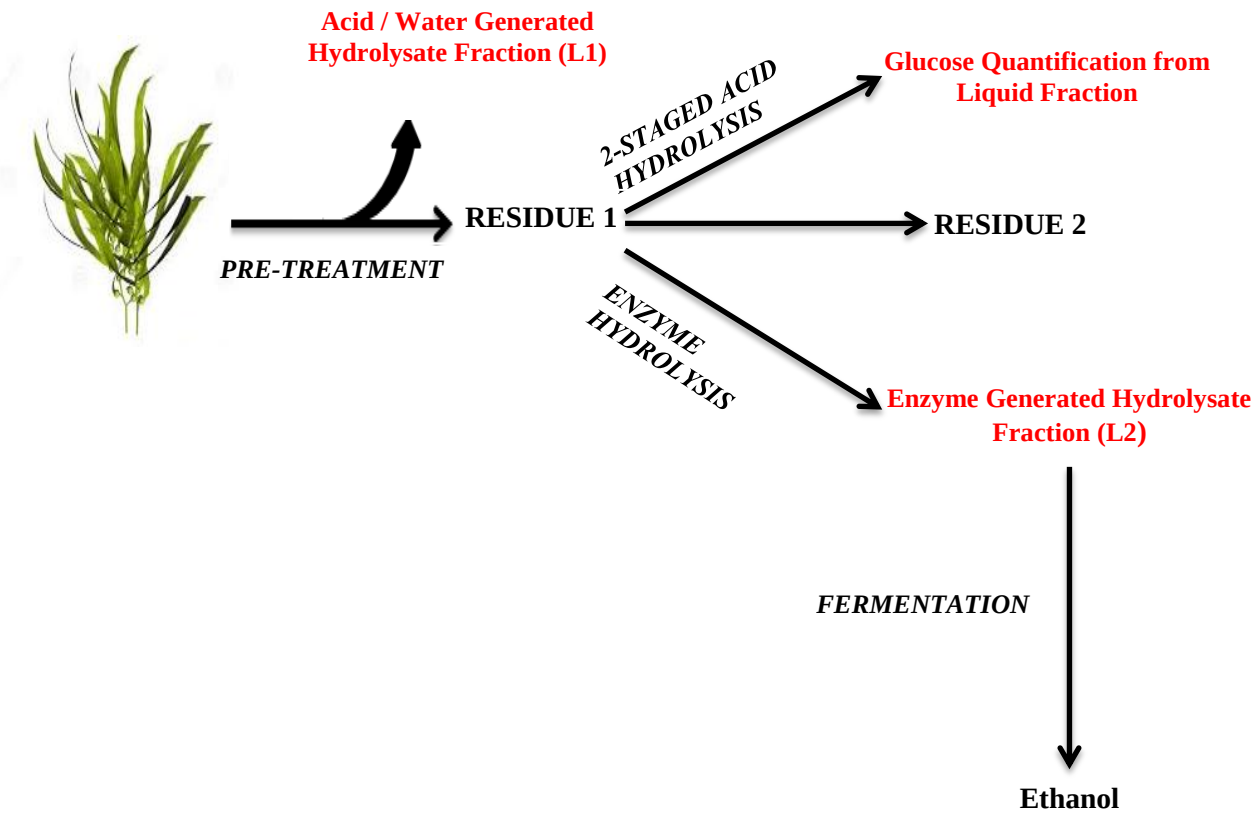


Figure 3.4 Schematic overview of the pre-treatment, enzymatic saccharification and the fermentation stages of the optimisation process for bioethanol production from selected seaweeds

Generated liquid fractions quantified for monosaccharide content are highlighted in red.

3.3.2.1 The effects of dilute acid hydrothermal and water based auto-hydrolytical pre-treatment on: concentrations of monosaccharides and degradation products in the generated hydrolysate (L1 fraction)

Thermo-chemical pre-treatments of seaweed biomass usually result in the direct liberation of monosaccharides into the pre-treatment generated hydrolysate (from easily hydrolysable polysaccharides with low recalcitrance). Identification and quantification of monosaccharides and other products released from the seaweed during a pre-treatment provides useful information on the efficacy of the treatment, and may identify other waste streams with potential value. Typically such waste streams are discarded, however analysis

of the products contained in the liquid fractions (L1 in particular) which are generated is essential for establishing a bio-refining approach for bioethanol production from seaweed. The concentrations of monosaccharides released into the L1 liquid fraction from the kelp *L. digitata* can be seen in Figures 3.5 A-E. The total yields of liberated monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) were all within the range of 30 – 48 mg/g (initial seaweed biomass used) for all experimental permutations investigated. The highest yield of total sugar (48.5 mg/g) was liberated by treating the *L. digitata* with 1.5 N sulphuric for 5 min at 121°C (Figure 3.6 A). As the strength of the sulphuric acid increased from 0.25N to 5N at 121°C for 5 and 11 min (Figures 3.5 A and B), the overall total sugar yields decreased. Interestingly, similar concentrations of total sugar were released during the treatment at reaction times 18, 24 and 30 min across all investigated concentrations of sulphuric acid. It appeared that reaction time was not a rate significant factor (p=0.22) in relation to the liberation of total sugars from the *L. digitata* biomass (a flat experimental response was seen as no significant change in yields of sugar were detected as the reaction time increased from 5 to 30 min). This may suggest that these values represent the maximum amounts of total sugar which could be liberated from the *L. digitata* into the acid hydrolysate from the permutations under investigation. Alternatively, reaction temperature could have possibly been a more significant parameter that may contribute to the liberation of sugars from the biomass. However this was not varied for the reasons previously discussed. Due to the nature of the experimental design and how the *D-optimal* algorithm selects the treatment combinations, not all permutations were evaluated.

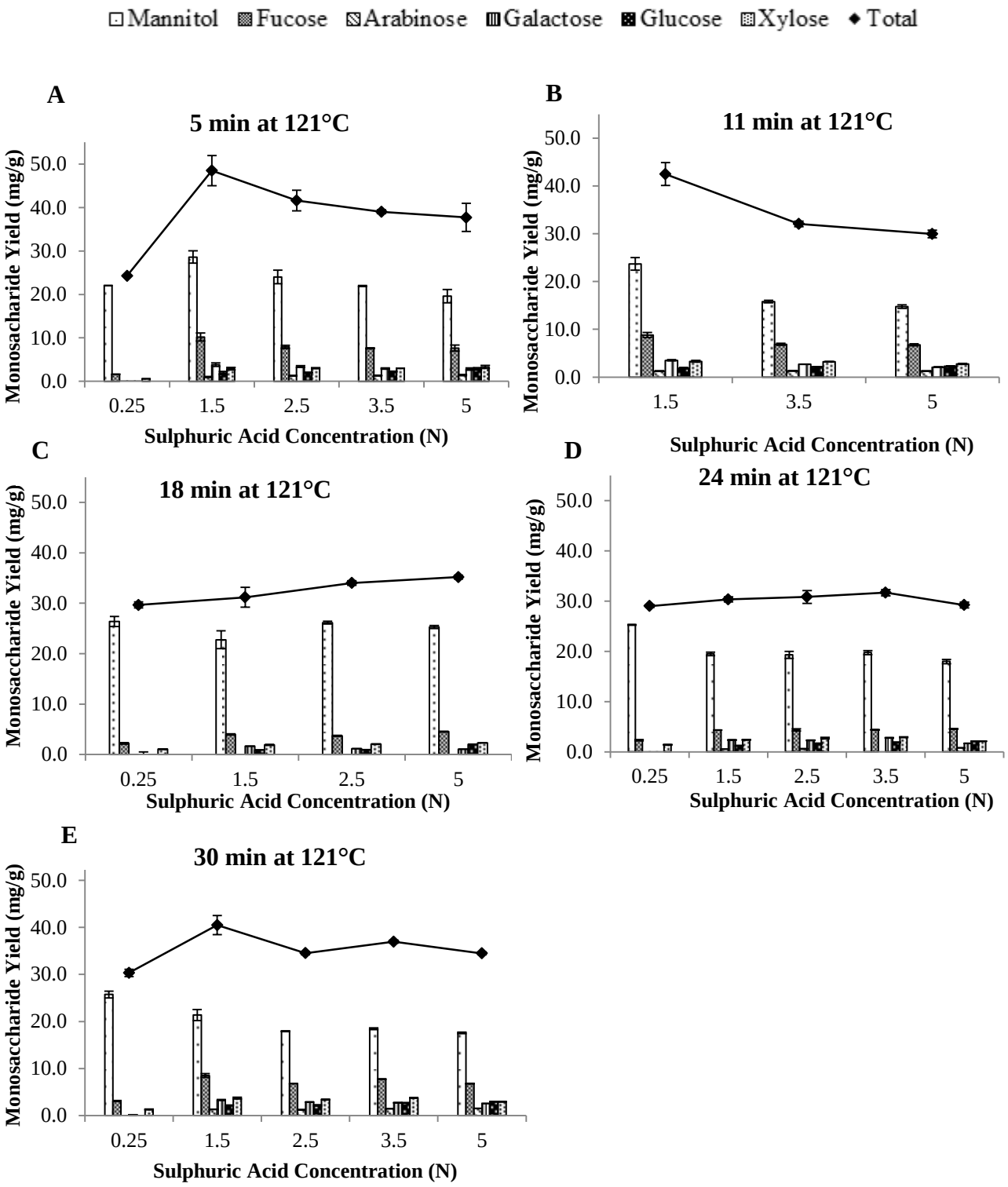


Figure 3.5 Analysis of monosaccharides released from *L. digitata* into the L1 liquid fraction (hydrolysate) after pre-treatment at 121°C, comparing various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E

The experimental design matrix (produced from Design Expert) outlined in sections 3.2.2.1 was followed. The acid generated hydrolysates were analysed for monosaccharide composition using HPAEC-PAD. Total (sugars) corresponds to the sum of monomers. All experimental permutations were conducted in triplicate.

This is why sulphuric acid concentrations of 1.5, 3 and 5 N were only investigated for 11 min at 121°C. It may be possible that if 0.25 N was included in the 11 min reaction time, a similar trend to that displayed in Figure 3.6 A might have been observed. As brown seaweeds do not contain lignin in their biochemical composition, such harsh parameters are not typically required. It may be possible that as the reaction times increased from 11 to 30 min, the monosaccharides liberated from *L. digitata* degraded into other products over the prolonged reaction time. The formation of selected degradation products was assessed in the acid hydrolysates by HPLC and yields are shown in Figure 3.6 along with mass loss (seaweed mass loss) values that resulted from the thermo-chemical pre-treatment of the brown seaweed biomass. 5-Hydroxymethyfurfural (HMF) was the only degradation product that was quantified in the acid generated hydrolysates. In general, the overall yields of HMF were relatively low with the highest concentration reaching ca 7 mg/g. For some reaction condition permutations, HMF yields continue to rise in the absence of any further increase in liberated sugar yield. With an increase in acid strength at reaction times 5 and 11 min, the concentrations of total sugars decreased as the yields of HMF increased (from 0 – 5 mg/g). This agrees with the suggestion that solubilised monosaccharides had been degraded to HMF in stronger acidic environments. The concentrations of total sugars were more stable across reaction times 18, 24 and 30 min (Figures 3.6 C-F) averaging ca 30-35 mg/g. However the yields of HMF were slightly higher (ranging from 3 – 15 mg/g) compared to the yields generated during the 5 and 11 min (≤10 mg/g) reaction times pre-treatments.

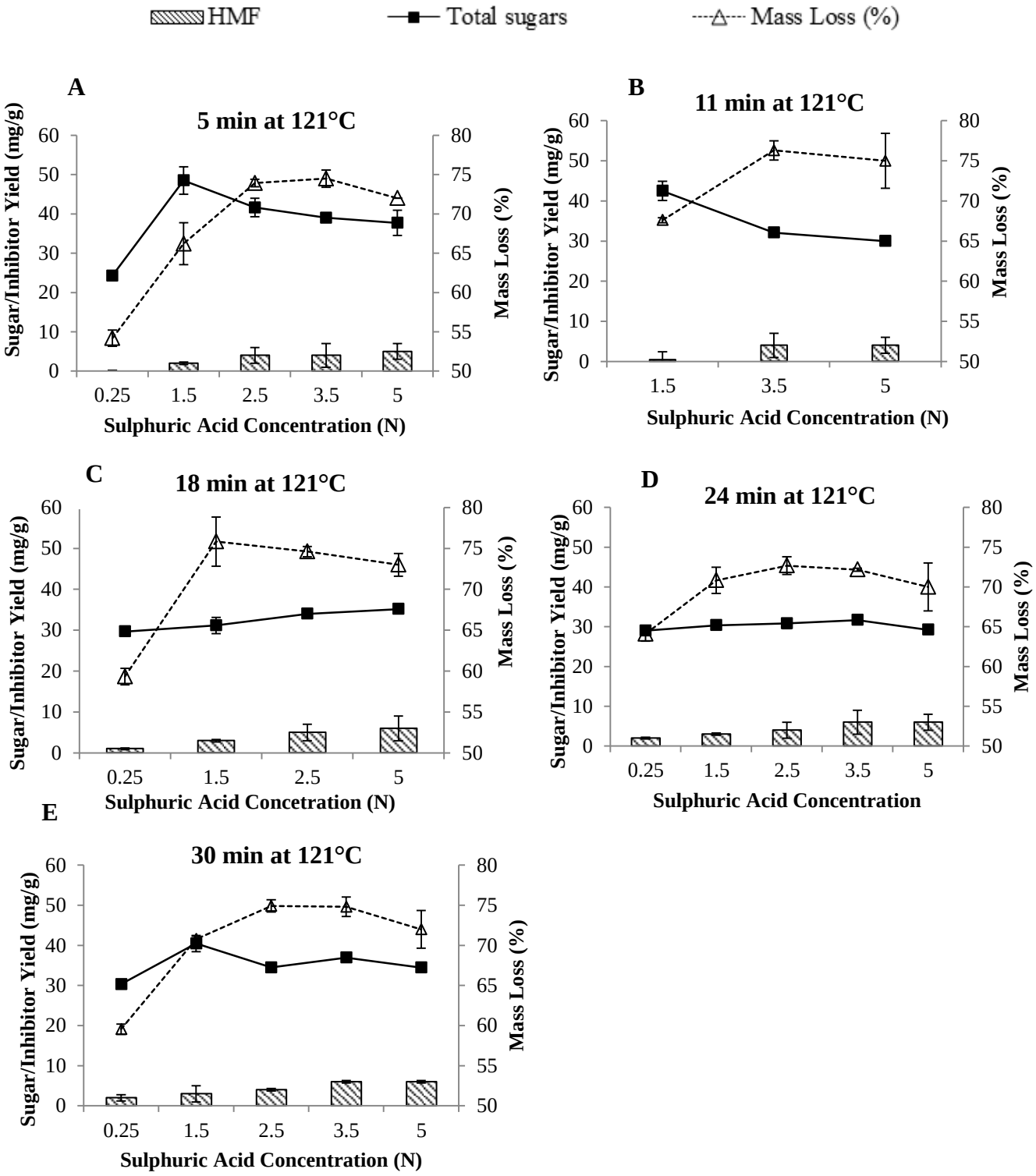


Figure 3.6 Comparison of total sugar yields, degradation product (HMF) concentration and mass loss (%) generated from pre-treatment *L. digitata*

Pre-treatment at 121°C in a benchtop autoclave using various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E.

This might mean that the concentrations of total sugars and HMF levels are the maximum yields that could be obtained from the different pre-treatment permutations investigated in the study. The same general trend in mass-loss was seen in Figures 3.7 A-E as the acid concentration was increased at 121°C; mass loss increased simultaneously with an increase in total sugar yields, however levels eventually plateaued or decreased as acid strength increased beyond 1.5 – 2.5N. A decrease in the total sugar yields achieved was seen as the strength of the acid was increased and this also correlated with an increase in mass loss. Simultaneously, HMF formation increased when the degree of mass loss intensified. This supports the hypothesis that liberated monosaccharides from the seaweed biomass were beginning to be thermally degraded to HMF. The acid generated hydrolysates contained a mixture of monosaccharides that correspond to the chemical building blocks of the different polysaccharides inherent to brown seaweeds. This cocktail of sugars is a reservoir of different carbon sources which could be used for the production of bioethanol or other platform chemicals; however the presence of HMF may be a problem to fermentative microorganisms. This finding is discussed in more detail in Chapter 5.

With regards to *D. carnosa*, increased concentrations of monosaccharides were released from the seaweed as the strength of the sulphuric acid increased from 0.25 to 5 N (Figures 3.7 A-E). However, throughout all the pre-treatment conditions, galactose was present at a much higher concentration than any other monosaccharide in the acid hydrolysates, followed by glucose. This is indicative of the hydrolysis of carrageenan and agar which are mainly comprised of alternating galactose units. The lower levels of glucose may be a

result of the partial hydrolysis of the cellulose component found in red seaweeds. Negligible levels of fucose and xylose were released from the *D. carnosa* biomass across all pre-treatment permutations. The highest concentration of total monosaccharides liberated from *D. carnosa* used an acid concentration of 1.5 N with a reaction time of 5 min at 121°C. This resulted in a total monosaccharide yield of ca 104.2 mg per g of seaweed biomass. As acid strength increased from 2.5 to 5 N for 5 min at 121°C, total monosaccharide yields decreased within the range 89.2 to 81.3 mg/g (Figure 3.7 A). Monosaccharide profiles generated by pre-treating the red seaweed for either 24 or 30 mins (Figures 3.7 D and E) showed that the extended reaction time (30 min) yielded higher glucose concentrations (26.3 mg/g) than those achieved when using 24 min (15.2 mg/g). In contrast galactose yields were similar (averaging ca 32.2-36.8 mg/g) irrespective of the variation in the reaction time. This suggests that a longer reaction time enabled limited breakdown of the cellulose fraction of the red seaweed, hence liberating more of the glucose monomer. Additionally, it may be possible that another intrinsic glucose based polymer that is of greater recalcitrance becomes exposed after 18 min of the reaction at 121°C and is subsequently broken down in to glucose units. This may explain the difference in glucose yields which reached their highest concentration during a 5 min reaction time (between 30.2 - 36.4 mg/g). Surprisingly, the levels gradually decreased as the reaction time increased to 24 min. However yields increased yet again during the 30 min reaction treatment.

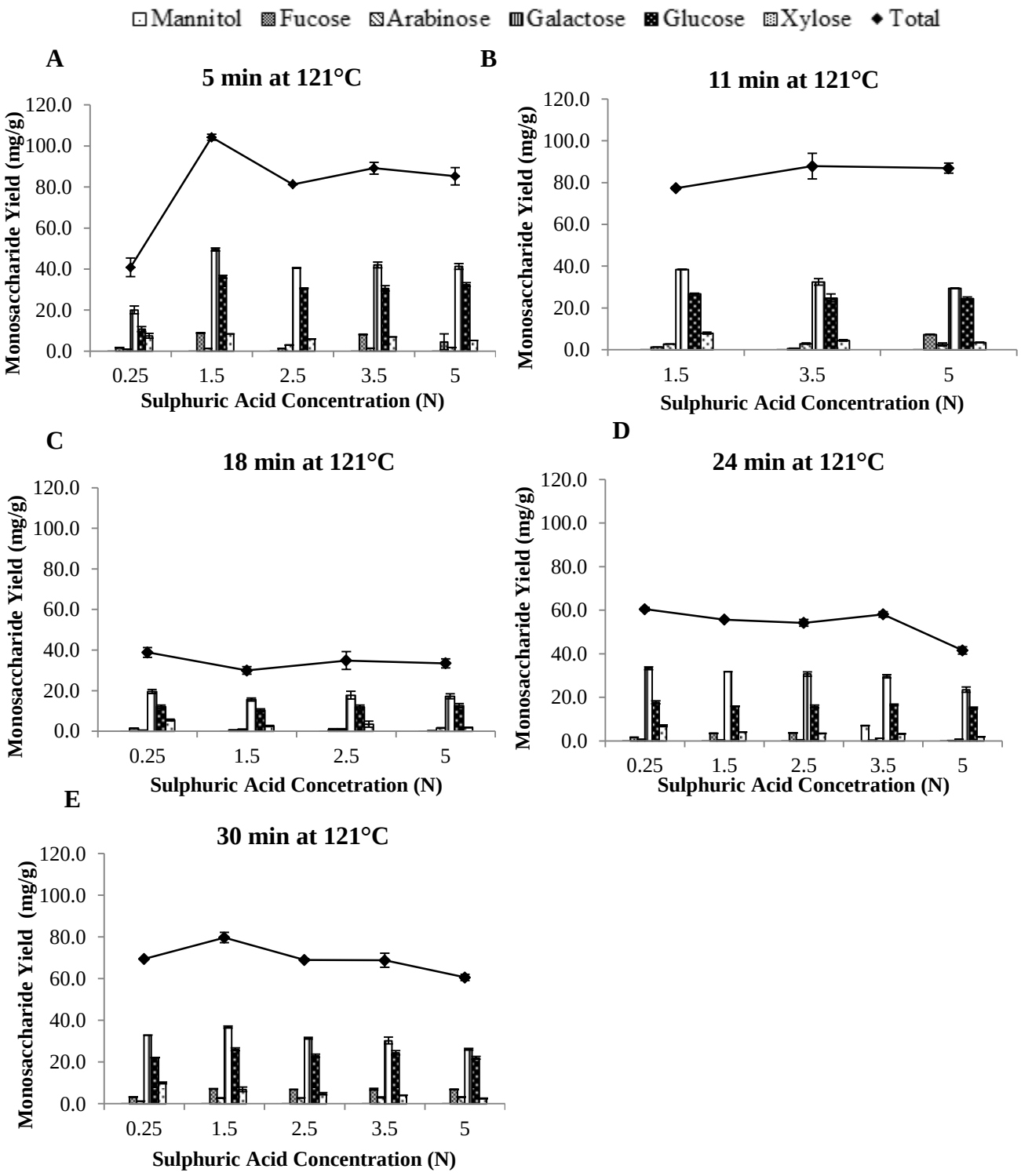


Figure 3.7 Analysis of monosaccharides released from *D. carnosa* after pre-treatment at 121°C, comparing various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E

The experimental design matrix (produced from Design Expert) outlined in sections 3.2.2.1 was followed. The acid generated hydrolysates were analysed for monosaccharide composition using HPAEC-PAD. Total corresponds to the sum of monomers: mannitol, fucose, arabinose, galactose, glucose and xylose. All experimental permutations were conducted in triplicate.

Mass loss data, depicted in Figures 3.8 A-E, may assist in understanding this behaviour.

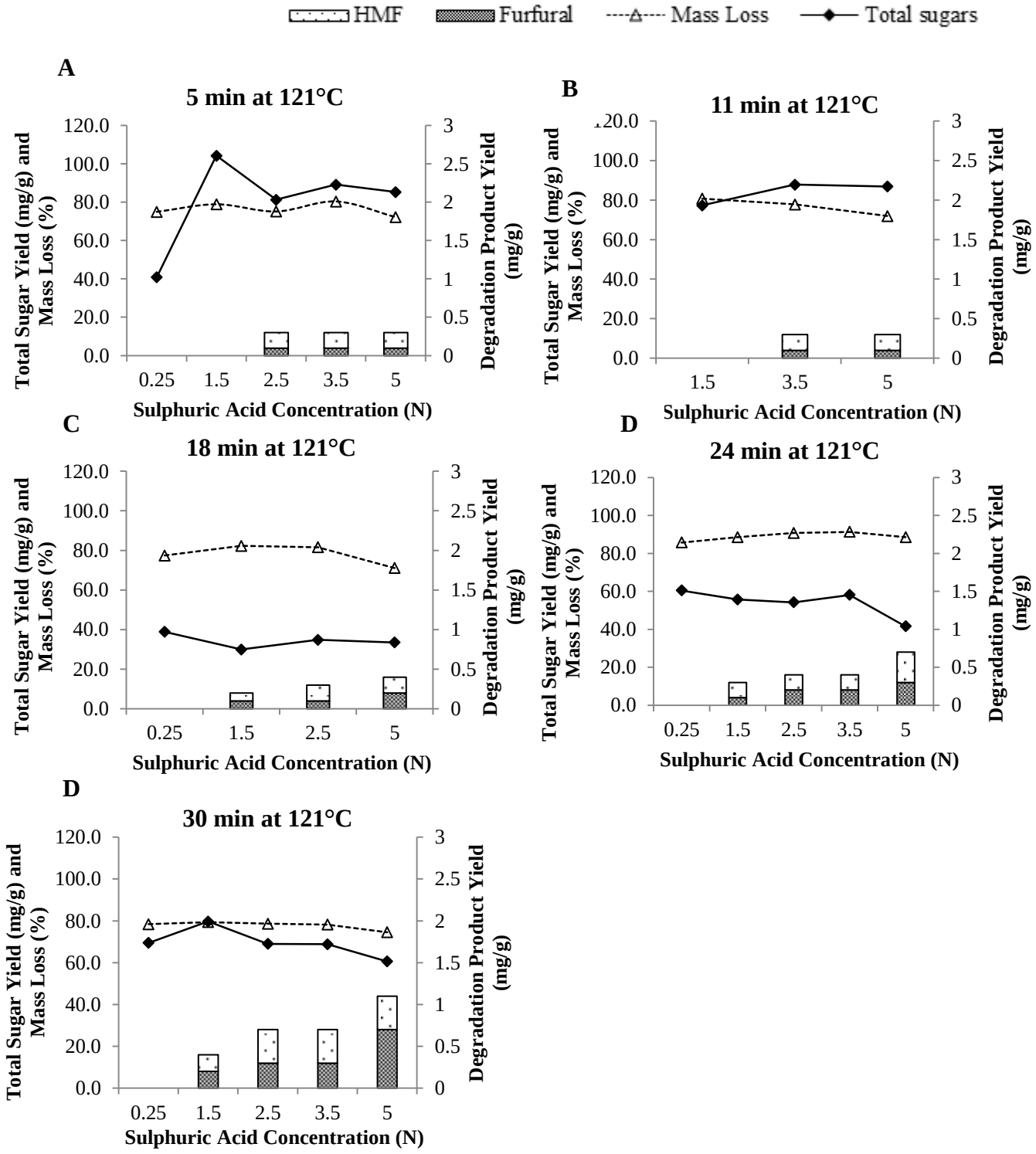


Figure 3.8 Comparison of total sugar yields, degradation product (HMF) concentration and mass loss (%) generated from pre-treatment of *D. carnosa*

Pre-treatment at 121°C in a benchtop autoclave using various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E

Furfural and HMF degradation products were both identified in the acid hydrolysates produced from the thermo-chemical pre-treatment of *D. carnosa* biomass. It is evident that both product levels varied according to pre-treatment severity. For example, an increase in the concentrations of furfural was seen when the seaweed was treated with stronger concentrations of acid for longer reaction times (Figures 3.8 D-E). This increase in the production of both degradation products during 24 and 30 min of acid pre-treatment may be a result of glucose thermal degradation as seen and predicted from the change in glucose yields depicted in Figures 3.8 D and E. The most severe pre-treatment in this investigation (5N sulphuric acid, 30 min, 121°C) yielded a total degradation product value of ca 1.1 mg per g of *D. carnosa*. Although this was the highest yield achieved from the investigated pre-treatment permutations, the concentrations were significantly lower than the levels generated by pre-treating lignocellulosic biomasses with dilute acid based pre-treatments. Furfural levels have reached yields as high as 20 mg/g of lignocellulosic biomass and HMF yields up to 7.5 mg/g (Silva-Fernandes *et al.*, 2015). This supports the desirability of using seaweed as a feedstock material for the production of bioethanol, as relatively lower levels of degradation products reduce the possibilities of downstream processing problems occurring, such as yeast intolerance to such products during fermentation. For *S. cerevisiae* fermentations, ethanol production can be inhibited by up to 79% in hydrolysates containing ca 4 g/L of furfural (Palmqvist *et al.*, 1999), and up to 50% in the presence of 8 g/L HMF (Clark and Mackie, 1984). As the current levels obtained in this investigation do not pose a direct threat to bioethanol production, the need to include a

detoxification step to ‘clean up’ hydrolysates is not required. Interestingly, various studies had reported that HMF (which had been generated from severe pre-treatment on lignocellulosic feedstocks) was actually being converted to another compound during the fermentation of such hydrolysates by yeast, and the compound had been interpreted as an HMF alcohol (Taherzadeh *et al.*, 2000, Palmqvist *et al.*, 1999). However, (Liu *et al.*, 2004) later identified this HMF conversion product as 2, 5-*bis*-hydroxymethylfuran and this bio-transformation utilises aldehyde as an electron accepter which can simply convert HMF to 2, 5-*bis*-hydroxymethylfuran by $2e^-$ reduction (Belay *et al.*, 1997). later reported that *Scheffersomyces stipitis* KCTC 7228 fermentation of the red seaweed *G. amansii* hydrolysate resulted in the decrease of HMF and the increase of 2, 5-*bis*-hydroxymethylfuran during ethanol production.

Regarding *U. lactuca*, the highest concentration of liberated sugar was achieved by treating the seaweed biomass with 2.5 N sulphuric acid for 5 min at 121°C (Figure 3.9 A). This resulted in a total sugar yield of 160.0 mg per g of seaweed. From all three species of seaweeds shortlisted for this investigation, the highest concentrations of total sugars liberated into the acid hydrolysates were recovered from a 5 min reaction time at 121°C, albeit with different strengths of sulphuric acid. The monosaccharide profile revealed that glucose was the predominant monomeric sugar identified in the generated acid hydrolysates (L1). Glucose liberation resulted from the direct hydrolysis of cellulose, and the lower levels of xylose and arabinose detected in the hydrolysates arose from their catalytical removal from cellulose backbone (as they are located there as terminating side chains on the polysaccharide) (Percival 1979).

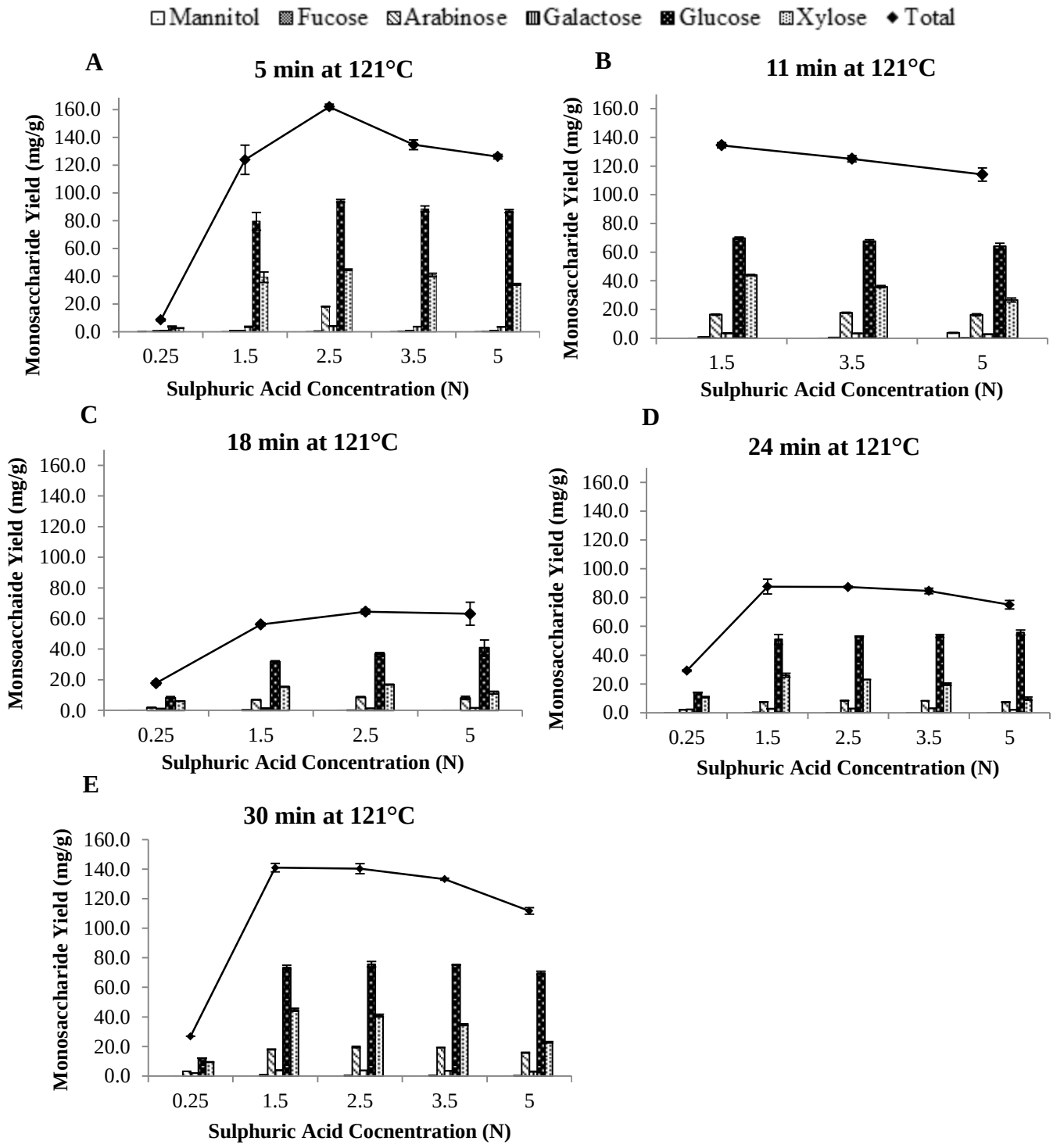


Figure 3.9 Analysis of monosaccharides released from *U. lactuca* after pre-treatment at 121°C, comparing various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E

The experimental design matrix (produced from Design Expert) outlined in sections 3.2.2.1 was followed. The acid generated hydrolysates were analysed for monosaccharide composition using HPAEC-PAD. Total corresponds to the sum of monomers: mannitol, fucose, arabinose, galactose, glucose and xylose. All experimental permutations were conducted in triplicate

Levels of glucose varied according to pre-treatment severity, however it was noticed again with this green seaweed that the yields were greater after short reaction time exposures at 121°C (5 and 11 min). Furfural and HMF were formed from reactions conducted on *U. lactuca* and higher concentrations were acquired when the reaction time increased beyond 18 min, particularly for HMF (Figure 3.10 A-E). Thermochemical degradation of any liberated hexose sugars may lead to significant concentrations of HMF. Analysis of the acid hydrolysates revealed that glucose was the predominant sugar, and this can explain the increase in HMF concentrations as the thermochemical pre-treatments increased in severity. Once again, a flat response was seen with mass loss as there was no significant increase in mass loss as the reaction severity (acid concentration and reaction times) increased. This correlated with similar findings for the red seaweed *D. carnosa*, as minimal changes in mass loss were also observed.

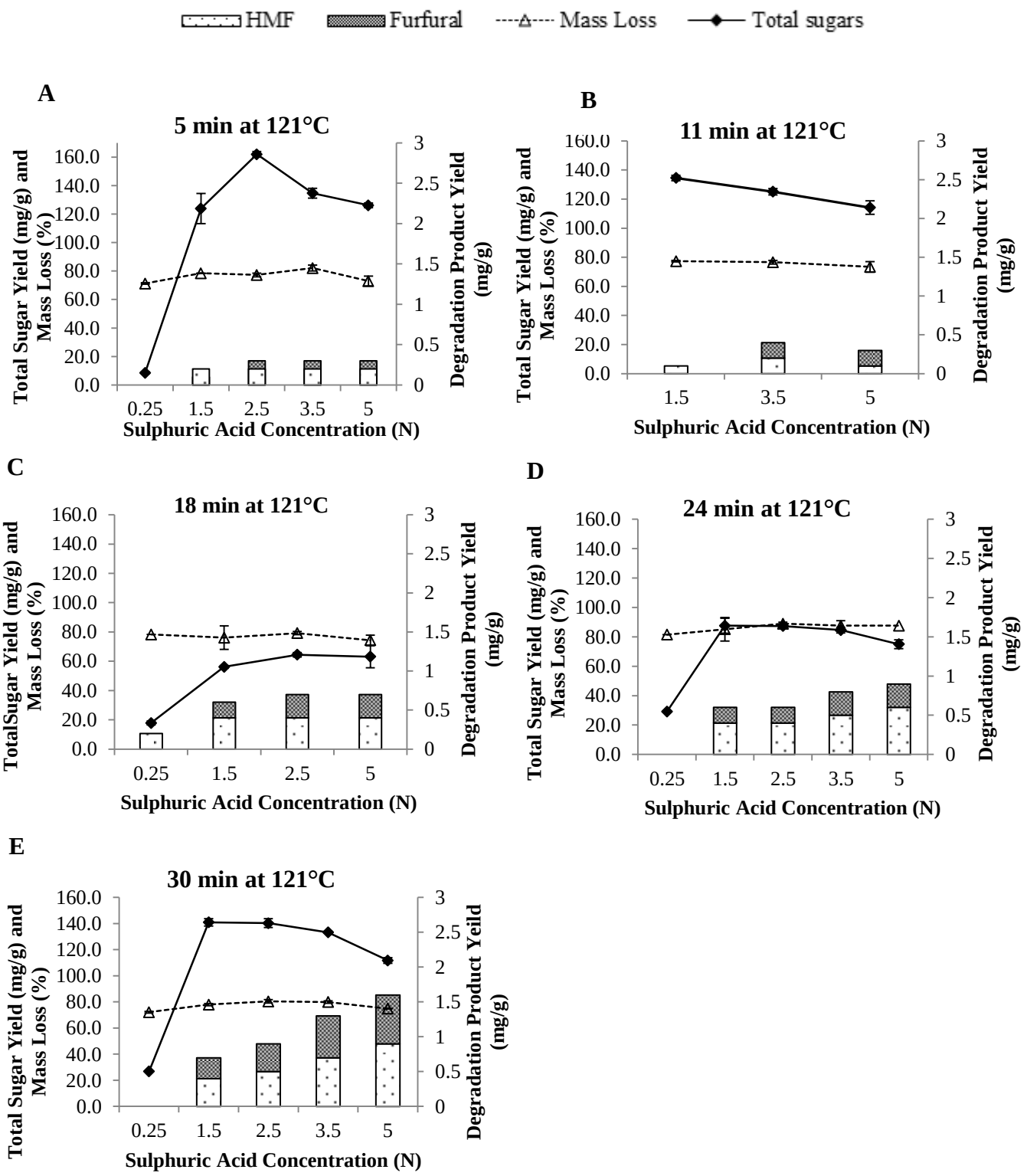


Figure 3.10 Comparison of total sugar yields, degradation product (HMF) concentration and mass loss (%) generated from pre-treatment of *U. lactuca*

Pre-treatment at 121°C in a benchtop autoclave using various concentrations of dilute sulphuric acid (0.25-5 N) and reaction times (5-30 min) A-E.

Sugar based compound levels detected in the L1 fraction and associated mass loss as a result of water based auto-hydrolytical pre-treatment can be seen in Tables 3.3 A and B.

Table 3.3 Comparison of the water based (auto-hydrolytical) pre-treatment generated hydrolysates from the 3 species of seaweed

A

Water pre-treatment hydrolysate mannitol concentrations (mg/g) and mass loss (%)						
Time	*Mannitol			Mass loss (%)		
(min)	*L. digitata*	*D. carnosa*	*U. lactuca*	*L. digitata*	*D. carnosa*	*U. lactuca*
5	0.0 ± 0.0	N/A	N/A	30.2± 2.6	36.1±1.2	N/A
11	4.3 ± 0.3	N/A	N/A	32.6± 1.7	38.9± 6.7	31.9± 4.7
18	2.8 ± 0.1	N/A	N/A	31.5± 1.2	37.5± 4.3	33.2± 1.9
24	4.1 ± 0.5	N/A	N/A	33.6± 3.2	38.8± 5.2	34.2± 4.2
30	4.4 ± 0.5	N/A	N/A	36.2± 4.1	40.0± 3.5	37.6± 3.1

B

Water pre-treatment monosaccharide concentrations (mg/g) in *U. lactuca***							
Time (min)	Mannitol	Fucose	Arabinose	Galactose	Glucose	Xylose	Total Sugar***
30	0.0 ± 0.0	4.1 ± 0.2	1.7 ± 0.8	21.6 ± 2.3	16.2 ± 1.7	4.1 ± 0.5	47.9 ± 5.4

*Mannitol was the only monosaccharide detected in significant quantities across all three species of seaweed

***U. lactuca* was the only seaweed that was found to contain significant concentrations of any additional monosaccharides other than mannitol.

***Total sugar corresponds to the average of the sum of monomers mannitol, fucose, arabinose, galactose, glucose and xylose.

Data are the mean±SD of three measurements

Negligible levels of sugar degradation products were detected from any of the L1 hydrolysate fractions across the three seaweeds. For *L. digitata*, mannitol was the only sugar based compound detected in any significant quantity. However, no trace of mannitol was detected from samples obtained from *D. carnosa* and *U. lactuca* liquid fractions. In the specific case of *D. carnosa*, no

sugars were detected at all. In contrast, the *U. lactuca* L1 fraction contained a range of different monosaccharide sugars which ultimately yielded ca 48.0 mg/g of total sugars (Table 3.3 B). Mass loss was within 30-40% (w/w) regardless of reaction time and species of seaweed. A general trend was seen of increasing mass loss with increasing reaction time. This was as predicted as increased reaction times likely resulted in increased hydrolysis of components from the seaweed biomass. Reactions that involved heating the *D. carnosa* and *U. lactuca* biomass for 5 min at 121°C did not yield any recoverable volumes of hydrolysate for analysis. These seaweeds had absorbed all water present in the reaction tube and an extremely viscous slurry was generated. Seaweeds are extremely hygroscopic due to the presence of certain polysaccharides in their algal cell walls. In particular, red and green seaweeds (which contain polysaccharides such as carrageenan, agar and ulvan) are often extracted for their gelling and thickening properties and are used in various food industries (Souza *et al* 2012). This formation of a viscous slurry (and no recovery of hydrolysate), is indicative of such gelling properties inherent to seaweed polysaccharides. This viscosity-related issue would make subsequent downstream bio-refinery processes extremely difficult to manage.

3.3.3 Quantification of glucose levels in pre-treated residues (R1) using the two-stage acid hydrolysis protocol

As the primary aim of the work presented in this Chapter was to produce bioethanol from UK seaweeds, it was important to evaluate the levels of glucose that could be released from the remaining seaweed residues post thermo-chemical and auto-hydrolytical pre-treatment. Although the seaweeds investigated in this study contain other C6 monosaccharides, such as galactose

that are readily fermented by yeast, it was decided to focus solely on the presence and liberation of glucose. This was because commercial enzyme blends are not currently available for seaweed hydrolysis, and as an alternative a commercial enzyme blend tailored for lignocellulosic biomass had to be used instead. Glucose is the main hydrolysis product that is released using this enzyme blend. Therefore, the remainder of the work that was conducted concentrated on the liberation and the availability of glucose in the pre-treated seaweed residues. However, a representative sub sample of the total number of experimental runs from the design space (Table 3.1), were chosen for the quantification of glucose in the residues generated after pre-treatment (thermo-chemical and auto-hydrolytical pre-treatments). This was determined for all three species of seaweed under by applying the two stage acid hydrolysis method (Section 2.4.5) and ultimately quantifying the liberated glucose via HPAEC-PAD. The glucose contents of selected pre-treated seaweed residues (determined by applying the 2 stage acid hydrolysis method for carbohydrate quantification) are shown in Figure 3.11. These numbers constitute the overall content of glucose present in each of the residues that remained after the application of different pre-treatments; and theoretically the maximum amount of glucose that could be released from each of the residues (e.g. through enzyme saccharification). It is evident that there is a clear difference in the amounts of glucose that could be released from the pre-treated residues across the three different seaweeds.

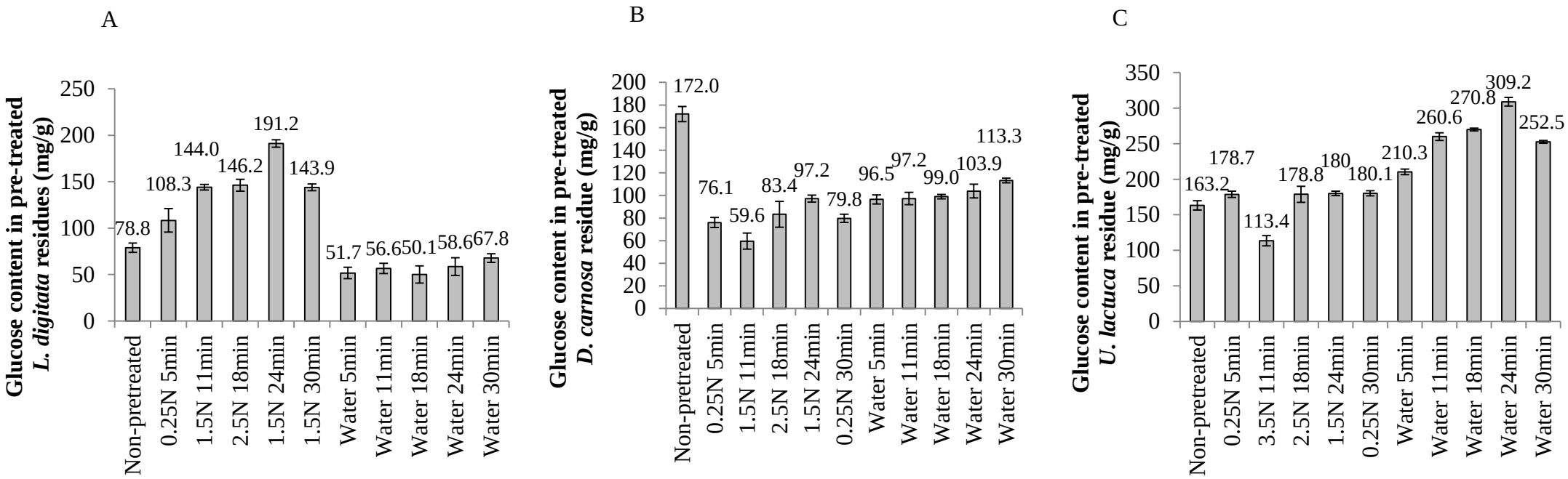


Figure 3.11 Contents of glucose in sub-samples of pre-treated seaweed residues determined by applying the 2 staged acid hydrolysis protocol. A) *L. digitata* B) *D. carnosa* C) *U. lactuca*

Data are the mean±SD of three measurements

It appeared that a mild form of acid pre-treatment was required in order to produce a *L. digitata* residue with a rich glucose content. Residues that were treated with dilute sulphuric acid contained a higher proportion of glucose than the auto-hydrolytical (water) based pre-treatments. The residue which contained the highest content of glucose (191.2 mg/g pre-treated seaweed) was produced from pre-treating the seaweed biomass with 1.5N sulphuric acid for 24 min at 121°C. This value is higher than the content of glucose present in the non-pre-treated (native) *L. digitata* seaweed (78.8 mg/g). This confirms a significant degree of enrichment of the carbohydrate through fractionation of other components due to the high degree of mass loss (c.a. 66.4%) as seen in Figure 3.6 A. The pre-treatment hydrolysate (L1 fraction) contained only a small quantity of total sugar (ca. 30 mg/g; of which the majority of carbohydrate consisted of liberated mannitol). The pre-treatment hydrolysate also only contained a very low proportion of glucose (1.9 mg/g) and only a low quantity of thermal degradation products (HMF) were detected (ca.2.0 mg/g). Overall this suggested that the vast majority of the carbohydrate remained in the insoluble residue generated after pre-treatment which therefore represents the main carbohydrate fraction.

In the case of *D. carnosa*, there was not much of a dramatic difference in the amounts of glucose that were present in the pre-treated residues between either of the pre-treatments (both acid and water based; Figure 3.11 B). However, the residue which contained the highest content of glucose (113.3 mg/g) was generated from pre-treating the *D. carnosa* biomass with water for 30 min at 121°C. This value is lower than the content of glucose which was present in the non-pre-treated (native) *D. carnosa* biomass (172.0 mg/g). Considering

that no glucose or degradation products were detected in the L1 hydrolysate fraction and a relatively low degree of mass loss was seen (Table 3.3 A; 40% mass loss), it is difficult to account for the loss of glucose after the water based pre-treated had been performed. Looking at *U. lactuca*, there is a clear difference in the amount of glucose present in the auto-hydrolytically pre-treated residues (210.3-309.2 mg/g) and the thermo-chemically treated residues (113.4-180.1 mg/g). Auto-hydrolytical pre-treatment of the green seaweed for 24 min at 121°C generated a residue that contained ca 309.2 mg of glucose per g of pre-treated *U. lactuca.* Yet again, as seen with *L. digitata* seaweed, this value was higher than the content of glucose present in non-pre-treated *U. lactuca* (163.2 mg/g). There was ca 34.2% mass loss however no glucose or degradation products were detected in the L1 hydrolysate fraction; suggesting that other components were fractionated from the green seaweed during this pre-treatment and left behind an enriched carbohydrate fraction of predominately glucose polymers. Based on these results, the best pre-treatment conditions for maximum enrichment of what would be presumed to be the cellulose fraction (as glucose was the measure) were: *L. digitata* 1.5N sulphuric acid for 24 min at 121°C; *D. carnosa* auto-hydrolytical for 30 min at 121°C; *U. lactuca* auto-hydrolytical for 24 min at 121°C.

3.3.4 Enzyme hydrolysis:

3.3.4.1 Effect of variation of pre-treatment reaction conditions on liberated glucose yields from pre-treated seaweed residues, after enzymatic hydrolysis

Due to practical time limitations, screening a range of different commercial enzymes with greater specificity towards each type of seaweed was not

feasible. Therefore, pre-treated residues were subjected to enzyme hydrolysis with Novozymes Cellic® CTec2. This commercial enzymatic mixture was originally developed for lignocellulosic feedstocks; however as no specific product was available for seaweeds at the time of this research this enzymatic mixture was used instead. Novozymes Cellic® CTec2 was chosen as a benchmark commercial enzyme preparation in this investigation as the highly specific enzymes present in the mixture (a blend of cellulases, hemicellulases and β-glucosidases) are likely to be compatible with the glucose based insoluble polysaccharide complexes which remain in the pre-treated seaweed residues. Cellulases and hemicellulases target the larger cellulose and hemicellulose polymers (respectively), whereas β-glucosidases hydrolyse cellobiose. In order to truly evaluate the efficacy of all of the pre-treatments screened, glucose liberation after enzyme hydrolysis was chosen as the most suitable response factor. The same sub-set of residues chosen in section 3.3.3 (for glucose determination by the two stage acid hydrolysis protocol) were also used to determine pre-treatment efficacy and the results can be seen in Figure 3.12. For *L. digitata,* dilute sulphuric acid based pre-treatments liberated c.a. 110.0 – 218.9 mg of glucose per g of pre-treated residue. The highest amount of glucose was liberated from a residue that was produced after treatment with 1.5 N sulphuric acid for 24 min at 121°C. This agrees with data obtained by applying the 2 stage acid hydrolysis method for carbohydrate quantification (Figure 3.11 A).

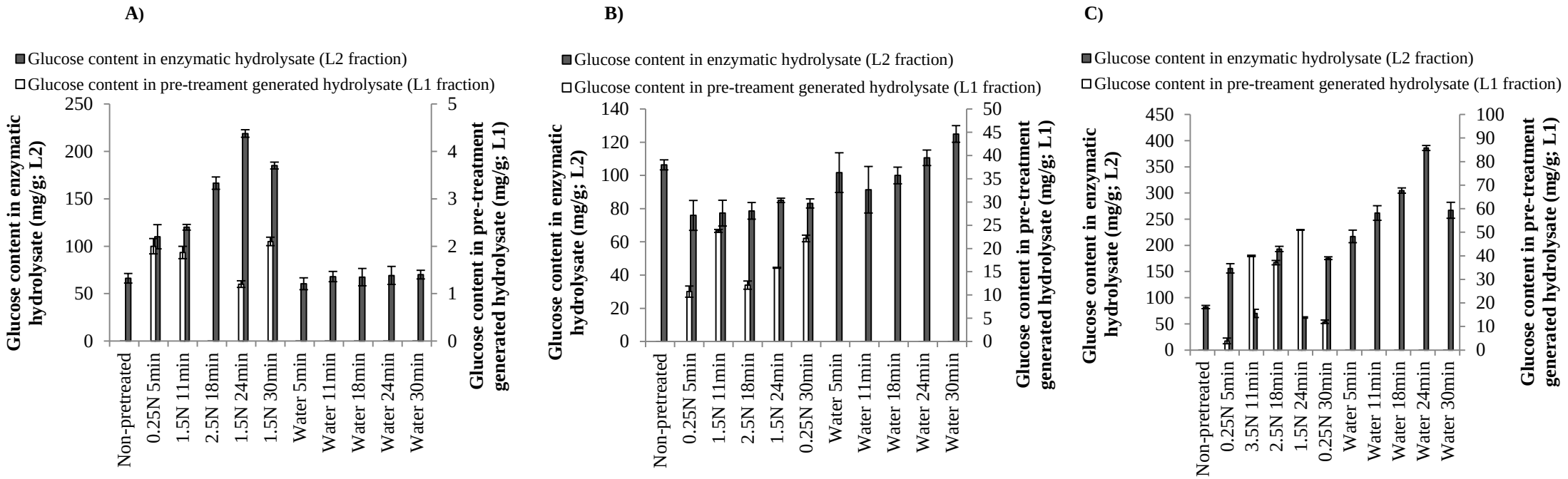


Figure 3.12 Glucose content in hydrolysates generated after enzyme saccharification (L2 fraction) of pre-treated residues and glucose content in pre-treatment hydrolysates (L1 faction). A) *L. digitata.* B) *D. carnosa*. C) *U. lactuca*

Data are the mean±SD of three measurements

In contrast, auto-hydrolytical based pre-treatments liberated a lower content of glucose within the ranges of 60.5 – 70.1 mg/g. The enzyme treatment clearly worked equally as well for all pre-treatments suggesting that the best pre-treatment was one that enriched the glucose containing fraction of the *L. digitata* biomass the most.

Interestingly, a high amount of glucose (106.4 mg/g) was released from non-pre-treated (native) *D. carnosa* species. This calls into question the actual requirement for any form of pre-treatment on this seaweed. Furthermore, it appeared that auto-hydrolytical based pre-treatments were better suited to this species of seaweed, as they achieved higher glucose yields (91.4-125.0 mg/g) than those released from thermo-chemically pre-treated residues (73.3-83.6 mg/g). Treatment for 30 min solubilised the greatest amount of glucose, (125.0 mg/g); only 18.6 mg/g more than non- pre-treated (native) *D. carnosa*. It is possible that the thermo-chemical treatment altered the structural integrity of the *D. carnosa* cell wall to such an extent, that the availability of binding sites that are compatible with the enzymes in Novozymes Cellic®CTec2 diminishes. Additionally, *D. carnosa* may consist of structural complexes that are less recalcitrant than the other two seaweeds and milder pre-treatments (if any are needed at all) are better suited to this species. At the time of writing this Thesis, no published literature on bioethanol production from *D. carnosa* was available. It is evident that bio-processing methodologies which have already been developed for other species of seaweed cannot be applied to this red seaweed. Auto-hydrolytical treatment for 30 min at 121°C was found to be the best treatment for *D. carnosa* prior to enzymatic saccharification. This of course would make the production of bioethanol from *D. carnosa* relatively

cost effective as no acid treatment was required. Nevertheless, the fact that direct CTec2 saccharification (on dried, non-pre-treated *D. carnosa*) yielded only 18.6 mg/g less glucose than the auto-hydrolytical pre-treatment is a major finding. Not only is this the first study that investigated the suitability of *D. carnosa* for bioethanol production, but also identifying a feedstock which requires no form of thermo-chemical modification to its structure prior to enzymatic saccharification is novel.

Conversely, it appeared that some form of pre-treatment was essential in order to solubilise greater yields of glucose from *U. lactuca*, as only 82.2 mg/g glucose was released from direct Novozymes Cellic®CTec2 hydrolysis of non- pre-treated seaweed. Auto-hydrolytical treatment for 24 min at 121°C followed by Novozymes Cellic®CTec2 saccharification then ultimately solubilised 386.0 mg/g glucose from the pre-treated *U. lactuca* residue. This pre-treatment on the green seaweed appeared to be the optimum for total glucose liberation post enzymatic saccharification. The overall yields of glucose liberated following enzymatic hydrolysis on acid catalysed pre-treatment residues were not as high (62.0-193.1 mg/g) as for those that underwent auto-hydrolytical pre-treatment (217.0-386.0 mg/g). Although glucose was detected in the liquid fraction generated during the thermo-chemical pre-treatment step (L1), the glucose yields from the solid residue following pre-treatment were lower in all cases for acid pre-treatment relative to auto-hydrolysis.

However, percentage theoretical glucose yields across all 3 species of seaweed (the amount of glucose liberated from the enzyme hydrolysis step relative to the starting amount of glucose in the pre-treated biomass) were calculated to

be higher than 100%. Whilst the yields were only slightly higher than 100%, these values were still not possible and thus highlighted a possible inaccuracy of the 2 staged carbohydrate assay and the subsequent underestimation of glucose present in pre-treated seaweed biomass. The assay, which was originally developed for carbohydrate quantification in terrestrial plants, may not be compatible with pre-treated seaweed residues. Accurate quantification of glucose in both the starting seaweed material and the newly generated pre-treated residues is a fundamental factor for correct process and pre-treatment efficiency calculations. This warranted further investigation and potential modification of the protocol in order to achieve more accurate quantification and this is discussed in more detail in Chapter 4. The remainder of this Chapter which investigates pre-treatment, enzymatic saccharification and bioethanol production for the three UK species of seaweed can be found in Appendix B.

3.4 Conclusions

Work conducted in this Chapter revealed the biochemical composition of nine native UK species of seaweed belonging to three different taxonomical groups. Amongst the nine that were investigated in this preliminary screen, *L. digitata*, *D. carnosa* and *U. lactuca* were shortlisted for further experiments aimed at bioethanol production. The red seaweed *D. carnosa* and the green seaweed *U. lactuca* were selected based on the fact that they both had the highest relative carbohydrate contents within their respective taxonomic groups (at 41.8% and 23.8% (d/w), respectively). Although the brown seaweed *L. digitata,* contained just 21.7 % (d/w) of carbohydrate, it was selected due to its high abundance around the coastal regions of the UK. Such

a species is highly suitable for mass cultivation and can be grown in both exposed and sheltered areas (Hill 2013). Two different pre-treatment methods were investigated for each of the three selected seaweeds (acid catalysed and aqueous auto-hydrolytical pre-treatment) prior to enzymatic saccharification with Novozymes Cellic® CTec2. This was performed in order to identify optimum pre-treatment conditions for each species of seaweed for maximum glucose liberation, which could then be fermented to bioethanol. However during the optimisation experiments, it became apparent that greater than 100% yield of theoretical glucose appeared to be obtained. This clearly is not possible therefore further development of the carbohydrate assay was required.

4 Optimisation of an assay for determining carbohydrates in seaweed

4.1 Introduction

In the previous Chapter (Chapter 3), the two-stage acid hydrolysis (NREL) protocol that was employed to determine the total glucose content of thermo-chemically and auto-hydrolytically pre-treated seaweed residues produced discrepancies with yields in excess of 100% theoretical.

The rationale of work described here was to optimise the NREL protocol (primarily designed for lignocellulosic biomass) to increase suitability for seaweed biomass and accurate carbohydrate quantification. Indeed, to develop an effective bio-refinery process for any feedstock, it is essential to fully characterise the composition of the starting material, utilizing both rapid and reliable methodologies. This would facilitate the working of mass balance and overall process-efficiency calculations. In the specific case of bioethanol production, particular attention must be paid to the carbohydrate quantification because carbohydrate content is directly proportional to ethanol yields in biochemical conversion processes (Montague, *et al*. 2002).

Standardised methods for proximate composition e.g. lipid and protein analyses of biomass *per se*, have been previously developed and are well established (Kjeldahl, 1883, Folch *et al.*, 1957). At the time of writing this thesis, there were no standardised methods for the total quantification of carbohydrates specifically in seaweeds. In order to quantify the carbohydrate content in terrestrial plants, modified versions of the NREL two-staged acid

hydrolysis protocol for determining carbohydrates (Sluiter *et al.*, 2008) are typically performed. This involves carrying out a two-stage acid hydrolysis on the biomass followed by the determination of liberated monosaccharides by high performance liquid chromatography or gas chromatography mass spectrometry (HPLC or GC-MS, respectively) or colourimetry (Dubois *et al*. 1956). A number of different methods have additionally been applied to quantify carbohydrates in seaweeds (see Table 4.1). These range from variations in the type of chemical based deconstructive treatments applied to the seaweed (which are often modifications of the NREL method), to the use of specific enzymes which cleave off specific polysaccharides (such as laminarin and fucoidan) and are then measured either spectrophotometrically or chromatographically. Targeting one specific polysaccharide within seaweed for quantification through the use of enzymes could be the most suitable approach if the interest of the work solely relies on the use of that one particular polysaccharide for its conversion to bioethanol or additional high value products/carbon end points. However, this is based on the assumption that a 100% conversion efficiency is achieved by the enzyme. In practise, achieving 100% conversion (converting 100% of a substrate into its monomeric units) is not theoretically possible due to mass transfer losses, especially if an enzyme is added to a system containing complex matrices (which is often the case with seaweeds that contain various different polysaccharides), as opposed to a pure substrate. The use of specialised enzymatic assays for carbohydrate quantification would likely be relatively expensive in comparison to simpler chemical reagent based protocols, and hence may not be suitable for high throughput rapid screening purposes.

Table 4.1 Summary of the different methodologies used previously to determine the total carbohydrate content in seaweed.

Method	Description	Reference
Colorimetric	Total and reducing sugars of *K. alvarezii* measured spectrophotometrically by phenol-sulphuric acid method (Dubois et al. 1956) and Nelson method (Nelson 1944). Galactose was analyzed by HPLC.	Khambhaty *et al.* (2012)
	Total carbohydrate species determined by Dubois *et al* (1956).	Manivannan *et al.* (2008), Trivedi *et al.* (2013),Mutripah *et al.* (2014)
	U. rigida was treated with 1 mL of 2N H_2SO_4 and boiled for 1 h. Carbohydrates determined by phenol-sulphuric acid method (Dubois *et al*. 1956).	(Korzen *et al.* (2015)
Chemical	*U. lactuca* treated with 72% H_2SO_4 at 30°C for 1 h, followed by dilution of H_2SO_4 to 1M and hydrolysis for 3h at 100°C. Glucose, glucose, xylose, rhamnose and mannitol in the hydrolysate detected by HPLC. Uronic acids detected spectrophotometrically using *m*-hydroxydiphenyl assay using galactouronic acid as a standard (Blumenkrantz and Asboe-Hansen 1973).	(van der Wal et al. 2013)
	Cellulose and hemicellulose content from floating residues (*L. japonica*) determined by modified NREL method (Sluiter, Hames *et al*. 2008) Sample was treated with 3 mL 72% H_2SO_4 at 40°C for 120 min, followed by addition of 84 mL deionized water and hydrolysis at 121°C for 60 min. Monosaccharide composition determined by gas chromatography.	(Ge et al. 2011)
	Various Thai seaweed species were hydrolysed with 0.5 mL of 5N HCl at 100°C for 15 h. Hydrolysates were washed with water to remove acid and then rotary evaporated. HPLC analysis was used to determined rhamnose, xylose, fucose, arabinose, mannose, glucose and galactose.	(Chirapart *et al.*, 2014)
Enzymatic	Laminarin content of *L. digitata* was calculated by the use of laminarinase to hydrolyse soluble laminarin to glucose. Glucose was measured using the Megazyme GOPOD enzyme assay.	Adams et al. (2009), Adams et al. (2011), Adams et al. (2014)
Total by difference	Carbohydrate content calculated by subtracting the sum of moisture, ash, crude fat, crude fibre, and crude protein from 100.	Borines *et al.* (2013), Wu *et al.* (2014)

Other methods of carbohydrate quantification include a 'total by difference method', which is a summation of all other non-carbohydrate based compounds (moisture, ash, protein and lipid) that is then subtracted from 100%. This indirect 'estimation' of the carbohydrate content, is clearly not an accurate way of determining carbohydrate content, nor can it be considered a high-throughput approach. Furthermore, the values obtained may be inaccurate and lead to the miscalculation of achieved theoretical yields (and subsequently inaccurate process efficiency yields).

There are distinct differences in both the subunits (monomers) and also the specific linkages within seaweed polysaccharides when compared to polysaccharides in terrestrial plants. Furthermore, differences in polysaccharides content exist between seaweed taxonomic groups. Previous results (Chapter 3) suggested that the acid digestion based carbohydrate assays could possibly be too thermo-chemically harsh for seaweeds, since they were principally designed for lignin-based biomass. As such there does not appear to be a standardised and validated protocol for carbohydrate determination in seaweed.

The NREL carbohydrate assay is composed of 2 distinct stages. The first stage is a low temperature concentrated acid phase for initial biomass swelling and fragmentation of the larger polysaccharides. The second stage involves a less concentrated acid but high temperature phase which hydrolyses the larger sugar units into monomeric units that can then be quantified. The objective of this work was to optimise each part for maximum carbohydrate liberation. *L. digitata* was chosen as a benchmark species for the initial method development and the formulation of the newly optimised protocol. Although

erroneous percentage theoretical yields were detected from pre-treated seaweed residues (the solid insoluble residue remaining after pre-treatment) in the previous Chapter, not enough seaweed samples were available to produce sufficient pre-treated biomass for this optimisation. Therefore, the optimisation was conducted on 'native' seaweed biomass (without any prior pre-treatment); and this would also reveal whether higher yields could also be obtained from the starting material. The optimised methodology was then tested on other seaweeds, namely *C. crispus* and *U. lactuca*. Although *D. carnosa* was shortlisted for bioethanol production optimisation in Chapter 3, (and the unsuitability of the standard two-staged acid hydrolysis protocol was additionally made apparent with this species) it was not included in the work described herein due to insufficient amounts of material that remained (after pre-treatment optimisation and fermentations). Therefore, *C. crispus* was selected as a substitute for the red alga in its absence.

The degree of sugar liberation from seaweed polysaccharides post acid hydrolysis treatment (see materials and methods below) were quantified using both a colourimetric based assay for total sugars (Dubois 1956) and a chromatography based analytical system (HPAEC-PAD) for the quantification of individual sugars (as originally used for carbohydrate content quantification in Chapter 3).This was because despite the fact that the colourimetric assay is a fast and simple quantification method, it provides no information about the specific profile of monosaccharides.

4.2 Materials and methods

4.2.1 Biomass preparation

Seaweeds *Laminaria digitata*, *Chondrus crispus*, *Ulva lactuca* were prepared as described in the method outlined in section 2.3. Wheat straw (Orbit, harvested from Sutton Bonington) was used as a control substrate. The wheat straw was milled to ca. 2 mm particle size prior to use using a knife mill (Fritsch, Idar-Oberstein, Germany).

4.2.2 Standardised control protocol (SCP) for quantification of total carbohydrate content in biomass

A modified version of the NREL protocol for the quantification of carbohydrates was used as the standard bench mark (control) protocol (as described in section 2.4.5). This protocol involved 2 distinct stages and then a subsequent quantification step afterwards (Figure 4.1) as follows: Stage 1 utilises concentrated sulphuric acid (12 M) at a relatively low temperature (37˚C) for 1 h and then stage 2 involves diluting the acid strength to 1 M and boiling the reaction vessels for 2 h. Conditions within these two stages were optimised for the analysis of sugars in seaweed. Post acid digestion (both stage 1 and 2), sugars were then quantified using one of two methodologies. Firstly, a colourimetric based assay measuring total reducing sugar content (Dubois, 1956). Here 100 μL of acid digested sample was transferred into a test tube and mixed with 1 mL 2.5% (v/v) phenol and 2.5 mL concentrated H_2SO_4 at room temperature for 5 min. The absorbance (490nm) of the resulting yellow/orange solution was recorded spectrophotometrically (Jenway7315, Staffordshire, UK).

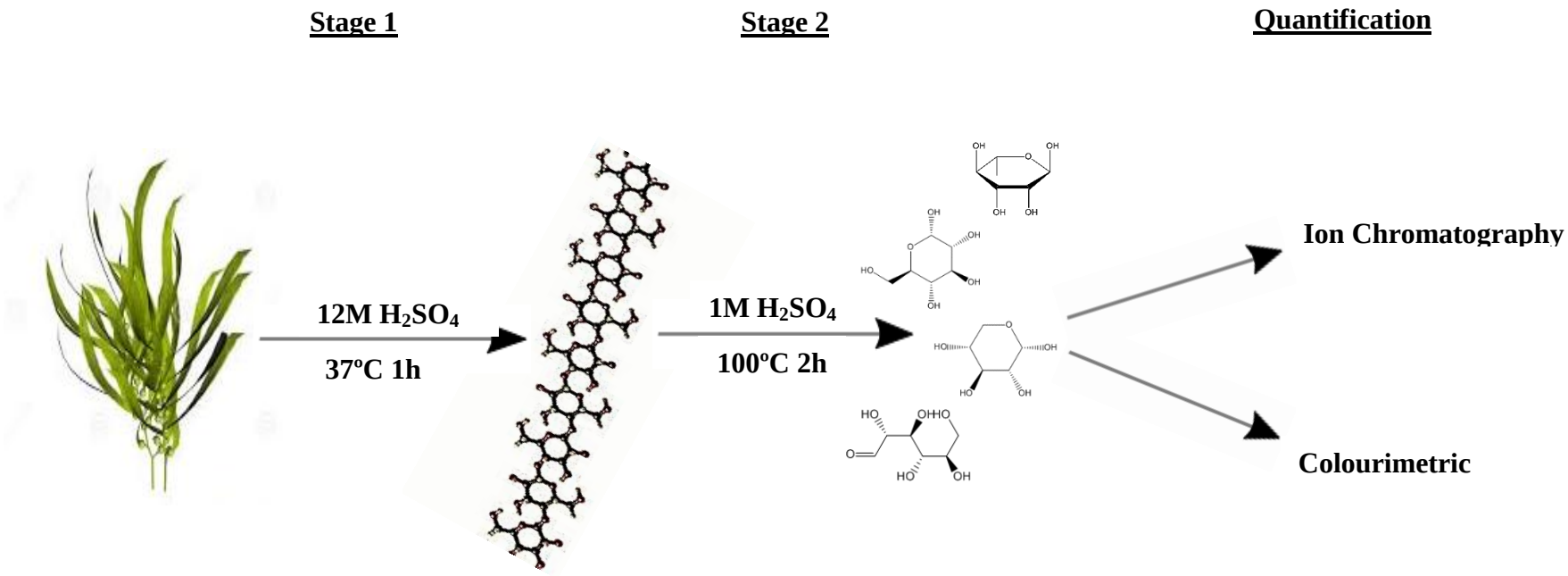


Figure 4.1 Overview of the SCP for determining carbohydrate analysis of lignocellulosic biomass.

Stage 1: 1 mL of 12M H_2SO_4 is added to biomass and incubated at 37 °C for 1h, liberating the larger polysaccharides from the biomass. Stage 2: acid strength diluted with distilled water to 1 M and incubated at 100 °C for 2 h which hydrolyses the polysaccharides into their monomeric constituents. Quantification then achieved either using HPAEC-PAD or colourimetric method.

Total sugar concentrations were determined by comparison to a glucose standard curve (prepared by using 0, 200, 400, 600, 800 and 1000 μg/mL concentrations).

Secondly, Ion Chromatography (HPAEC-PAD; outlined in section 2.7.1) was used to quantify individual monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) in the acid digested samples. Furthermore, sugar degradation products (in particular furfural and 5-hydroxymethylfurfural) were also quantified using HPLC (section 2.7.2).

4.2.3 Evaluation of acid digestion stages 1 and 2 of the standard control protocol (SCP)

A hydrolysis trial was conducted with *L. digitata* and *C. crispus* (n=3) using only the low temperature concentrated acid step (stage 1) to establish if this alone was sufficient for accurate quantification of total carbohydrates in seaweed. Wheat straw was used alongside as a control substrate. These results were then compared to the same substrates subjected to both acid digestion stages 1 and 2 combined. The 2 different sugar quantification methods (colourimetric and HPAEC-PAD) were used to determine the total carbohydrate contents.

4.2.4 Stage 1 optimisation

The first stage of the two stage hydrolysis method was investigated to determine whether heating for 1 h with 12M H_2SO_4 concentration at 37°C was too extreme for *L. digitata* biomass

Stage 1 optimisation involved using a range of sulphuric acid concentrations (3-12 M) and reaction times (15-60 min), whilst maintaining the original 37°C

incubation temperature of the benchmark (SCP) protocol. To test and explore the interaction of these acid and reaction time parameters, a *D*-optimal experimental design approach was utilised (see section 4.2.7 on experimental design methodology) which simultaneously varied the 2 experimental variables. Whilst reducing the total number of experimental runs from that of a full factorial experimental design, the design space still provided statistically significant data. Optimal parameters would then be modelled using liberated sugar concentration as the response factor. Both the acid concentration and reaction time conditions found in the SCP were included in the design of this experiment and replicates were included in the design (n=3). The experimental design matrix can be seen in Table 4.2.

Table 4.2 Experimental design used to optimise stage 1 (37°C) of the total acid hydrolysis methodology for quantifying carbohydrates in seaweed species *L. digitata*.

Optimisation conducted through screening different sulphuric acid concentrations (3-12M) and reaction times (15-60 min) at 37°C, according to a *D-optimal* design space.

Run	**Factor 1** A: H_2SO_4 Acid Conc (M)	**Factor 2** B: Time at 37°C (min)
1	12	15
2	3	15
3	3	15
4	7	15
5	7	35
6	3	60
7	5	45
8	12	35
9	7	35
10	5	25
11	12	60
12	10	25
13	3	35
14	3	60
15	12	15
16	7	60
17	12	60
18	7	35
19	7	35
20	10	45
21	5	60
22	10	60
23	10	15
24	12	60
25	12	60
26	12	15
27	12	15
28	8	15
29	8	15
30	8	60
31	12	35
32	8	35
33	10	15
34	10	25
35	10	25
36	10	45

37	10	45
38	10	60
39	9	20
40	9	50
41	11	20
42	11	50

L. digitata (30 mg) was mixed with 1 mL of H_2SO_4 (3M-12M) and incubated at 37°C at the different temperatures (15-60 min). RO water was added to dilute the strength of the acid to 1M and then the samples were heated at 100°C for 2 h. Reducing sugars were measured following the method outlined by Dubois *et al* (1956) and individual monosaccharides and sugar degradation products were quantified by HPAEC-PAD analysis.

4.2.5 Stage 2 optimisation

After the optimisation of stage 1, these optimal conditions were used as a basis for the subsequent optimisation of the second step. Optimisation of stage 2 again involved the evaluation of a range of acid concentrations (0.5-2 M) and a range of different reaction temperatures (70-100°C), whilst maintaining the 2 h reaction time from the original protocol. As with the stage 1 optimisation, a *D*-optimal experimental design was utilised (Table 4.3). Both colourimetric and HPAEC-PAD based quantification methods were again used as with the stage 1 optimisation experiments.

Table 4.3 Experimental design used to optimize stage 2 of the total acid hydrolysis methodology for quantifying carbohydrates in *L. digitata*.

Optimisation conducted by screening different sulphuric acid concentrations (0.5-2 M) and temperatures (20-100°C) for 2 h, according to a *D-optimal* design space. All stage 2 optimisation attempts were conducted using the previously optimised stage 1 parameters prior (11 M H_2SO_4 at 37°C for 1 h).

Run	**Factor 1** A: H_2SO_4 Conc (M)	**Factor 2** B: Time at 100°C (min)
1	0.5	20
2	1	60
3	0.5	80
4	2	60
5	1.25	80
6	1.25	60
7	2	20
8	1	60
9	0.5	60
10	1.25	20
11	0.5	100
12	2	40
13	0.5	100
14	0.5	100
15	2	100
16	1.25	100
17	2	80
18	2	100
19	1	40
20	1	80
21	1.25	60
22	2	100
23	0.5	80
24	1.25	60
25	0.5	60
26	1.5	40
27	1.5	90
28	0.75	90
29	0.75	30
30	1.8	80
31	1.8	30
32	1.8	90
33	1.8	60

4.2.6 Validation of the fully optimised method (combined optimisations of both stage 1 and 2) on other seaweed biomass types

Derived optimal conditions for sugar determination in *L. digitata* were further evaluated on two other seaweed species; *U. lactuca* and *C. crispus* and compared to the non-optimised SCP. Liberated monosaccharides and inhibitory compounds were then analysed via HPAED-PAD and HPLC, respectively.

4.2.7 Experimental design and statistical analysis

Experimental design, response surface modeling and ANOVA were all performed using Design Expert v 7.0 (Stat-Ease Inc., Minneapolis, USA). The experimental designs and response surface modelling (RSM) used a *D-optimal* format from which the *D-optimal* algorithm chose the treatment combinations (the exact parameter settings; e.g. acid concentrations, reaction times and temperatures) to include in the design from a specific range for that parameter set by the user. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided statistically valid data. Additional experimental runs were also conducted to improve the statistical validity of the derived models. Additional statistical analyses were conducted using a student's *t-test.*

4.3 Results and Discussion

The aim of the work conducted in this Chapter was to optimise an assay for carbohydrate determination for a range of seaweeds. Previously, the glucose yields from pre-treated seaweed residues (SCP; NREL reference) were questionable and the assay may have underestimated the content of glucose in the pre-treated seaweed residues. When the same pre-treated residues were

subjected to cellulolytic saccharification with Novozymes Cellic® CTec2, slightly higher yields of glucose were quantified compared to the NREL acid hydrolysis assay. This led to the suggestion that the acid hydrolysis assay may be too extreme for seaweed biomass and therefore thermally degrade measureable reducing sugars; the protocol should be optimised for seaweed feedstocks.

All experimental data within this section are presented in terms of achieved total sugars from two different methods of quantification in order to compare their reliability. Quantification method 1 was the colourimetric based assay outlined in the protocol followed by Dubois *et al* (1956) which enables the calculation of the reducing sugar yields (mg/g) liberated from the hydrolysis of biomass (through comparison to glucose standards). This assay is a well-established quantification method that has been extensively used in many fields of research, for carbohydrate quantification on a number of different feedstocks including Brewer's spent grains (Wilkinson *et al*, 2014), sorghum grains (Holmes *et al*, 2013), and *Arabidopsis thalania* (van Acker *et al*, 2013). Individual monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) liberated from the hydrolysis and their sum (total of each sugar for comparison with the Dubois assay), in addition sugar degradation products were analysed using HPAEC-PAD and HPLC, respectively. Sugar yields (determined via both quantification methods) and degradation product yields were expressed on a dry weight basis per g of starting seaweed biomass.

4.3.1 Comparison of sugar yields from stage 1 and stage 2 of the SCP carbohydrate assay on *L. digitata*, *C. crispus* and wheat straw

The differences in the release of reducing sugars (quantified using the Dubois based colourimetric assay) and individual monosaccharides (HPAEC-PAD) between the two stages of the standardised benchmark control protocol, from the three types of biomass, can be seen in Figures 4.2 A-D.

When quantifying sugar yields from both acid digestion phases using the colourimetric assay (Dubois *et al.* 1956) there appeared to be no difference in the yields of reducing sugars between stage 1 and stage 1+2 (Figure 4.2 A) for both *L. digitata* and wheat straw substrates. However, there was a 108.5 mg/g difference in sugar yields observed between stage 1 and stage 1+2 for the red seaweed *C. crispus*. Nevertheless, in contrast, the chromatography based data revealed a clear and significant difference in the release of monomeric sugars between both stages of the SCP for all three biomasses (Figures 4.2B-D).

Stage 1: 12M H_2SO_4 at 37°C for 1 h

Stage 1+2: 1M H_2SO_4 at 100°C for 2 h

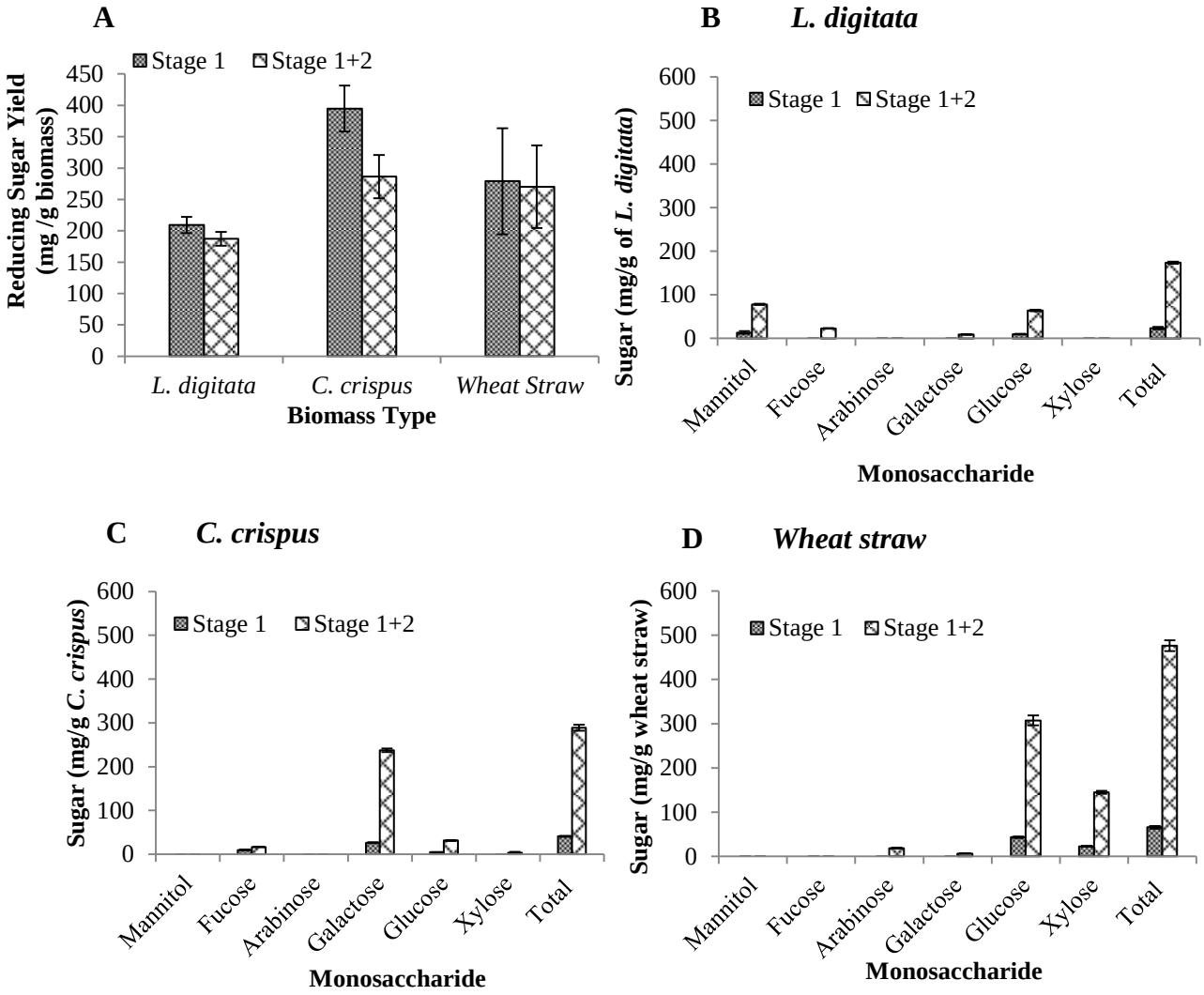


Figure 4.2 Comparison of reducing sugar yields (mg/g dry biomass) and monosaccharide yields (mg/g dry biomass) released from various different biomass types during either just stage 1 or stage 1+2 of the control protocol.

A Reducing sugar yields (mg/g) released from *L. digitata*, *C. crispus* and wheat straw (control substrate) during the 2 separate stages of control method, with quantification via the phenol-sulphuric acid colourimetric assay (Dubois). Data are the mean ± SD of three replicate measurements. **B-D** HPAEC-PAD data showing yields of individual monosaccharides released during stages 1 and 2 of the original control method. Data are the mean ± SD of three replicate measurements. **B**: *L. digitata*. **C**: *C. crispus*. **D**: Wheat straw. Total sugar yields refer to the sum of the six different monosaccharides.

Despite these differences in yield between stages 1 and 1+2, the total sum of monosaccharides (quantified by HPAEC-PAD) and reducing sugar yields (calculated from the Dubois colourimetric assay) were similar for both *L. digitata* (173.7 ± 9.5 mg/g and 187.2 ± 15.3 mg/g, respectively) and *C. crispus* (288.6 ± 10.8 mg/g and 286.3 ± 25.6 mg/g, respectively). On the contrary, the total sum of monosaccharides quantified by HPAEC-PAD from wheat straw biomass was 56.7 % higher than the Dubois colourimetric assay. This suggested that the Dubois assay many not be the most suitable method for quantifying the sugar content of certain biomass feedstocks, as it appeared to not be sensitive enough to detect all present reducing sugars from hydrolysed wheat straw. Additionally, this data suggested that the Dubois colourimetric reagent may possibly be reacting with other compounds that may be present (or generated) in the stage 1 phase, which have resulted in achieving higher yields for stage 1 alone compared to stage 1+2 (Figure 4.2 A) for all three feedstocks. The Dubois assay is based upon a phenol-sulphuric acid reaction which depends on the dehydration of hydrolysed sugars to furfural derivatives during their reaction with concentrated sulphuric acid (Dubois *et al*. 1956, Bicker *et al*. 2003, Lima *et al*. 2010). When phenol is added, the furfural derivatives react further and a coloured complex is formed which can then be read using a spectrophotometer at a maximum absorbance wave-length of 490 nm (Dubois *et al*. 1956). Although this method is relatively easy to follow and gives rapid results, there are a significant number of limitations mainly related to the actual quantification conducted by comparison to the absorbances of the purely glucose standards; and the results from this initial screen prove this. The method of quantification, that uses

glucose as a reference standard compound, may lead to inaccuracies in the calculation of the carbohydrate content of biomass types that are composed of carbohydrate polymers which are not entirely glucose based, as it is the case with many species of seaweed (Albalasmeh *et al.*, 2013). Additionally, differences which exist in the chemical reactivity of various carbohydrates with derivatisation reagents (sulphuric acid in this case) resulting from the carbohydrate being either neutral or anionic, could influence their molar absorption co-efficiencies (Mecozzi, 2005). Interestingly, a study by Manns *et al.* (2014) additionally found that the Filisetti-Cozzi and Carpita (1991) colourimetric assay which is employed to determine total uronic acid content in biomass, greatly underestimated the yields found in brown seaweeds. This again validates the fact the rudimentary colourimetric based quantification assays do have certain limitations with their use.

HPAEC-PAD analysis (which revealed the monosaccharide profiles between the two stages for *L. digitata*, *C. crispus* and the wheat straw control substrate), showed that higher yields (mg/g of biomass) of the specific monosaccharides, particularly glucose, galactose and xylose, were released during the second stage of the protocol. This ultimately increased the total yields of monomeric sugars. This demonstrated that the second step (stage 2) was still crucial for accurate quantification of the total carbohydrate content in seaweed.

The increase in galactose sugar yields that were detected using the HPAEC-PAD based quantification method (liberated from *C. crispus*) using both stage 1 and stage 2 of the protocol (compared to when only stage 1 was used) are as a result of carrageenan hydrolysis. This confirmed the requirement of the

second stage of the protocol in order to completely hydrolyse carrageenan. In a similar fashion, the increases in glucose yields from wheat straw were as a result from the complete hydrolysis of cellulose.

4.3.2 Optimisation of stage 1 of the control benchmark protocol using *L. digitata*

Figure 4.3 shows the simultaneous interaction of time (min) and sulphuric acid concentration (M) at set incubation temperature (37 ˚C) on the yield of reducing sugars (mg/g) using the (A) Dubois assay for quantification and (B) the release of monosaccharides (mg/g) using HPAEC-PAD analysis from the first stage of the protocol. The derived response surface model (RSM; Figure 4.3 A) suggested that time was not a significant factor that had any influence on the final yield of reducing sugars liberated from *L. digitata* (p=0.17). However in contrast, the initial concentration of sulphuric acid used appeared to be highly significant (p<0.0001). It was evident that there was an increase in the final yield of reducing sugars as the concentration of acid increased however overall yields then started to decrease when the acid concentration exceeded 11 M sulphuric acid. From the HPAEC-PAD data it appeared that using an 11M sulphuric acid concentration released 209 mg of total sugars (mg/g of *L. digitata*) compared to only 166.4 mg/g when using 12M acid (Figure 4.3 B).

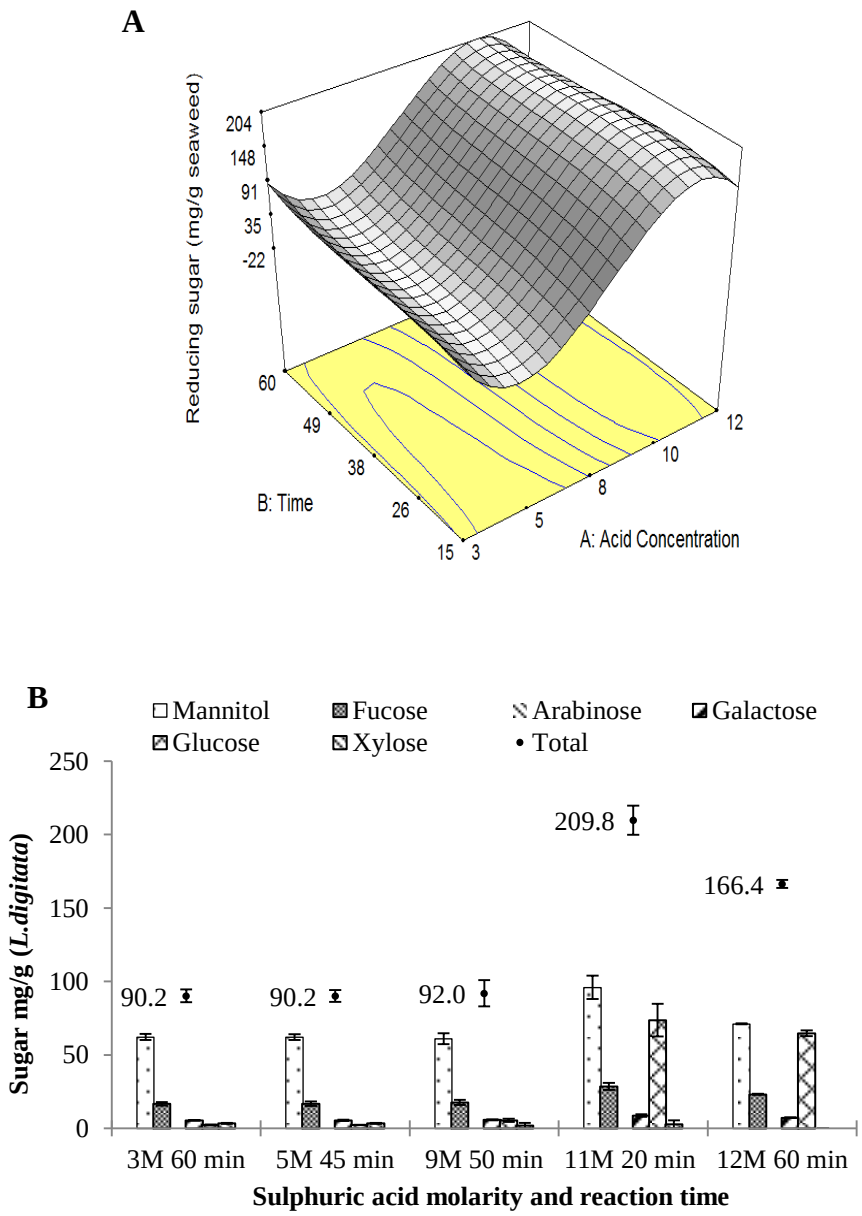


Figure 4.3 The effect of sulphuric acid concentration and reaction time on the release of sugars from *L. digitata* during stage 1 (at 37°C) of the benchmark protocol.

A 3D response surface model showing the impact of different sulphuric acid concentrations (M) and reaction times (min) on the release of reducing sugars (mg/g). Quantification using the phenol-sulphuric acid (Dubois) colourimetric assay. Model R^2: 0.56. **B** HPAED-PAD based quantification of the individual monosaccharides yields (mg/g biomass) liberated from seaweed using a range of H_2SO_4 concentrations (3-12 M) and a range of reaction times (15-60 min). Data are the mean ± SD of three replicate measurements.

In addition, the higher acid concentrations (11M-12M H_2SO_4) liberated ca. 65-75 mg/g (glucose per g *L. digitata*) compared to only ca. 2-5 mg/g for the lower concentrations (3-9 M H_2SO_4; $p<0.05$). This large difference confirmed seaweed biomass, similar to that of lignocellulosic biomass, did require strong acid concentrations, although with an optimum concentration possibly slightly lower than 12M as used for lignocellulosic biomass. It may be possible that treatment with 12M H_2SO_4 could have actually liberated more sugars from the seaweed but may have converted a greater proportion of liberated sugars into degradation products as observed with lignocellulosic biomass (Wilkinson *et al.*, 2015). Figure 4.4 shows example HPLC chromatograms of degradation products that were detected in the hydrolysate generated during the stage 1 optimisation of the assay and an example chromatogram of sugar degradation product standards used for comparison and quantification. Furfural was the only compound identified that could be verified against an authentic standard (Figure 4.4 B i).

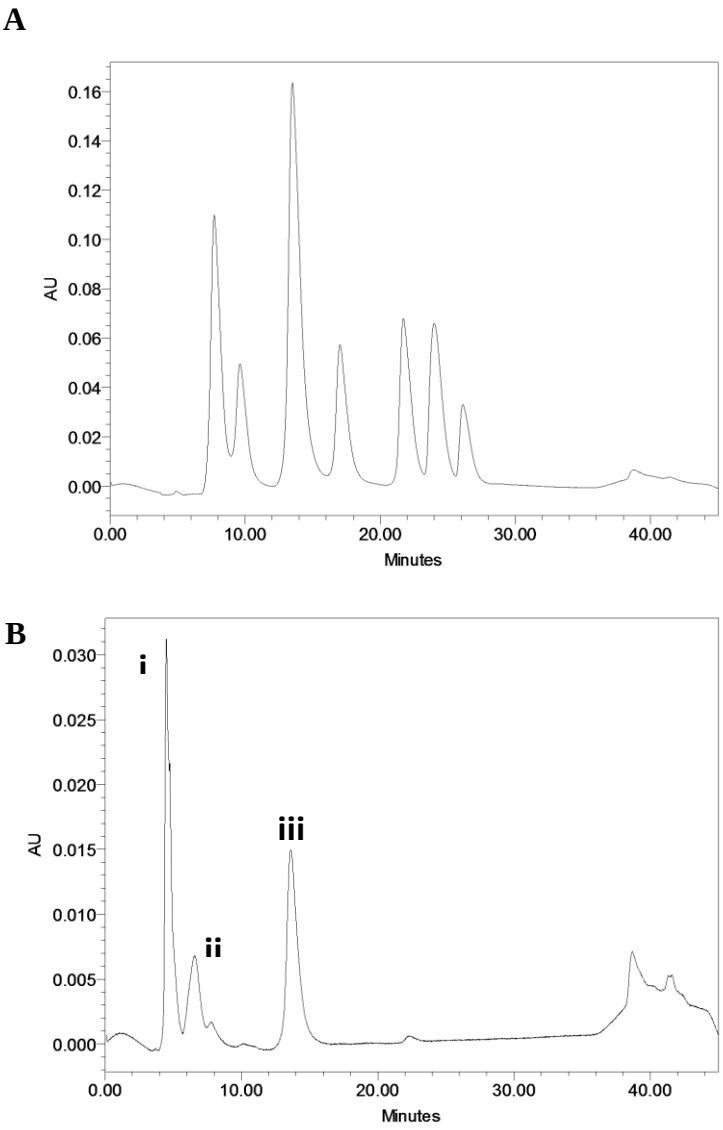


Figure 4.4 HPLC chromatograms of sugar degradation products generated during stage 1 optimization of the SCP.

A HMF, furoic acid, furfural, vanillic acid, vanillin, ferulic acid and *p*-coumaric acid (Left to right). **B** Degradation products released from *L. digitata* after exposure to 12M H_2SO_4 for 60 min at 37°C. **i**. Unknown peak at 4.5 min **ii**. Unknown peak at 6.5 min **iii**. Furfural.

There was an increase in the production of furfural when using higher concentrations of sulphuric acid (ca 1.3 ± 0.2 mg/g furfural increases to ca 2.6 ± 0.4 mg/g) and a slight increase with furfural concentrations (1.2 ± 0.1 mg/g

rising to 1.8 ± 0.1 mg/g) with increasing reaction time (R^2 = 0.50) (Figure 4.5 A).

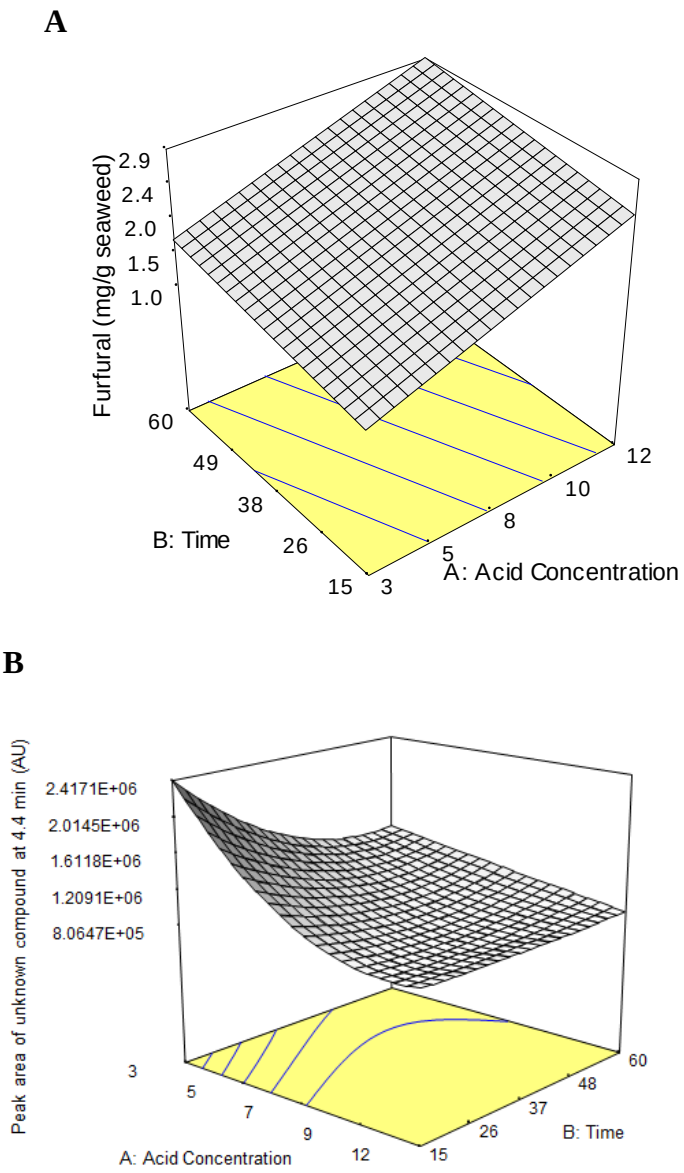


Figure 4.5 The impact of different sulphuric acid concentrations (M) and reaction times (min) on A) the generation of furfural from *L. digitata* during stage 1 (at 37°C) of the total acid hydrolysis and B) the degradation of unknown compound with elution time of 4.4 min from *L. digitata* during stage 1 (at 37°C) of the total acid hydrolysis.

3D response surface models showing the influence of simultaneous variation of the sulphuric acid concentration and reaction time on the release of A) furfural (mg/g) (model R^2: 0.50) and B) the production of an unknown degradation product at 4.4 min (model R^2: 0.90). Data are the mean ± SD of three replicate measurements.

Regarding furfural production, reaction time was suggested to be somewhat significant (p=0.0113). Furthermore, the strength of the sulphuric acid played a much more significant role with regards to furfural generation (p<0.0001).

Interestingly, two additional unknown compounds were detected in the hydrolysate with elution times at 4.4 min (Figure 4.4 B i) and at 6.5 min (Figure 4.4 B ii). It was not possible to identify or accurately quantify these unknown compounds without a reference standard to compare against, therefore peak area sizes were evaluated in response to variation parameters. Figure 4.6 A illustrates how the unknown compound that eluted at 4.4 min decreased in size (reduction in peak area) as acid concentration and reaction time increased. However the model suggested that reaction time was not likely to be a significant factor (p=0.56). Although the specific identification of this unknown compound (that eluted at 4.4 min) was not achieved, it was speculated to potentially be an additional sugar degradation product that is produced in increasing quantities with increasing acid strength. Alternatively, the unknown compound may have been a further degradation product of one of the sugar degradation products previously produced under less severe reaction conditions.

It was important to incorporate the original parameters of the carbohydrate assay into the design space (the SCP protocol), as this would provide a direct comparison of the original assay to the less severe permutations. This enabled the identification of a second unknown compound (with a retention time of 6.6 min) in samples treated by this original control protocol (12 M H_2SO_4 for 60 mins) and by treatment with 12 M H_2SO_4 for 35 min (Figure 4.6).

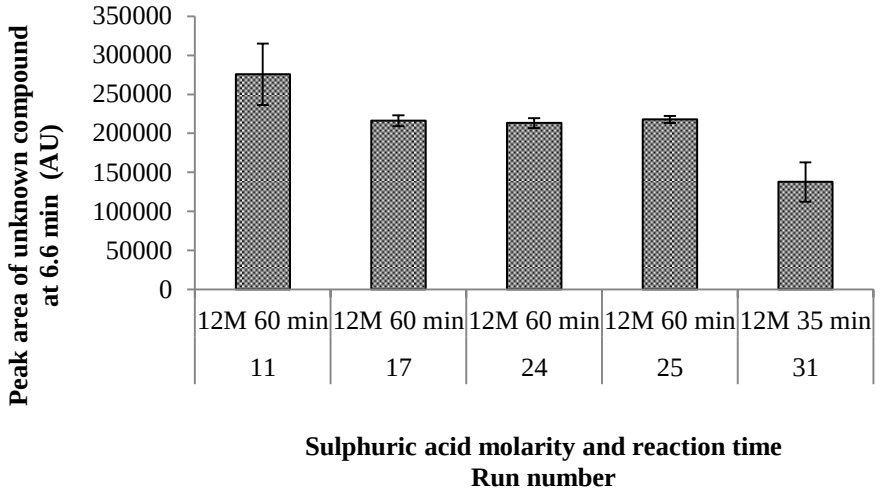


Figure 4.6 Peak area values (AU) of the unknown compound (with a retention time of 6.5 min) found only in samples exposed to 12M H_2SO_4.

Data are the mean ± SD of three replicate measurements.

As this compound was also not identifiable without a reference standard, it was not possible to determine its identity; however it may additionally be a sugar degradation product as this would explain the lower sugars yields that were achieved from using the SCP on *L. digitata* biomass.

Overall, using 11 M sulphuric acid was suggested to be the optimum acid concentration to use for the complete hydrolysis of *L. digitata* as (regardless of which of the 2 different quantification methods were used) as it liberated more sugars (as indicated by both higher monosaccharide concentrations from HPAEC-PAD and also more reducing sugars from the Dubois assay) and also fewer sugar degradation products than the SCP. In addition, reaction time was not suggested to be a significant factor for any of the responses measured. Using the optimal sulphuric acid concentration (11 M), an additional experiment was conducted to determine whether reduced reaction times (<

60min) could be used in the assay to increase throughput.Results in Figure 4.7 confirmed that reduced reaction times (<60min) *digitata* were not optimal.

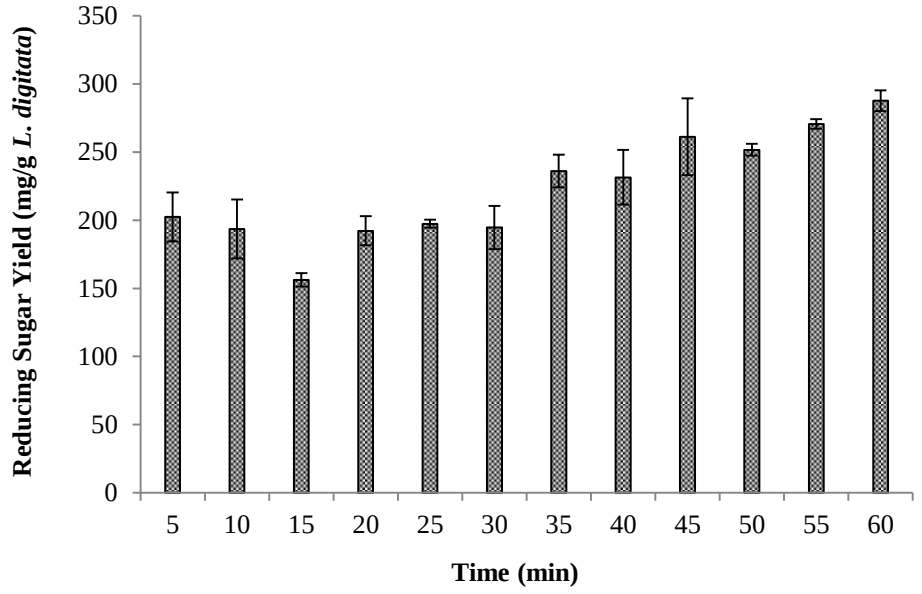


Figure 4.7 A comparison of reaction time (5-60 min) on the release of reducing sugars from *L. digitata* at 37°C using 11M sulphuric acid concentration.

Reducing sugars quantified by phenol-sulphuric acid colourimetric assay. Data are the mean ± SD of three replicate measurements.

The highest sugar yield was still achieved using the original incubation time of 60 min (29%; as with the SCP protocol) so this was selected as the incubation time for further experiments. Therefore, the optimised conditions chosen for stage 1 of the control protocol for *L. digitata* were **11M sulphuric acid at 37°C for 60 min.** These conditions were then subsequently used for further optimization studies with stage 2 of the assay.

4.3.3 Optimisation of stage 2 of the control protocol for *L. digitata*

This series of experiments were designed to determine the optimum acid concentrations and incubation temperatures for maximum sugar release. The

effect of these experimental parameters was then determined through quantification of reducing sugars (Dubois 1956). From the results, the derived response surface model (Figure 4.8) had a poor R^2 value of 0.17 (Figure 4.8) suggesting that neither variations in acid concentration (0.5-2M) nor incubation time (5-60 min) improved sugar release compared to conditions in the original protocol Therefore, the original protocol for stage 2 was not altered.

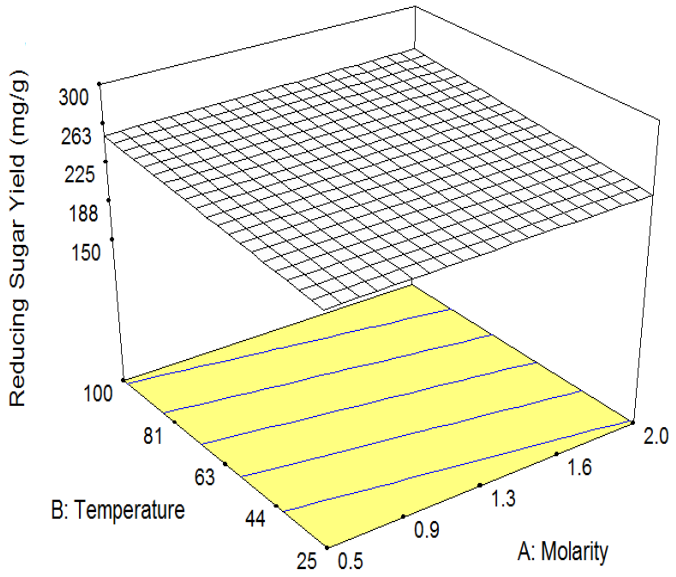


Figure 4.8 3D response surface model showing the effect of simultaneous variation of acid concentration (M) and reaction temperature (°C) on the release of reducing sugars (%) from *L. digitata* during stage 2 of the total acid hydrolysis protocol.

Stage 2 incubation time: 2 h. Reducing sugars quantified by phenol-sulphuric colourimetric assay. SCP involved using previously optimised stage 1 parameters: 11 M H_2SO_4, 37°C, 1 h. Model R^2: 0.17.

In conclusion it suggested that optimisation of stage 1 of the process was more crucial than stage 2 in the specific case of *L. digitata* species. It was concluded that initially treating the seaweed with a sufficient concentration of acid (i.e. stage 1) was required to successfully liberate seaweed polysaccharide

fragments from the biomass. These fragments were then subsequently fully hydrolysed to monosaccharides during stage 2. However, experimental data concluded the original SCP stage 2 conditions were optimal. As such, stage 2 of the process remained unchanged as part of the new optimised process used subsequently for testing other species of seaweed.

4.3.4 Evaluation of the new optimised protocol for quantification of the total carbohydrate content in alternative species of seaweed

The newly optimized method for determining the carbohydrate content of *L. digitata* and also the original SCP protocol were compared using two other species of seaweed belonging to different taxonomical groups. *C. crispus* (a rhodophyte) and *U. lactuca* (a chlorophyte) were selected because the carbohydrate structures and composition differ significantly between the groups (Percival and McDowell, 1981, Adams *et al.*, 2011b). Any significant difference in carbohydrate composition between species would imply that an altered chemical structure and different bond linkages will additionally exist. As such different severities of treatment may be required to effectively hydrolyse the carbohydrates for accurate quantification. Figure 4.9 shows the difference in the liberation of total sugars (the sum of mannitol, fucose, arabinose, galactose, glucose and xylose concentrations) and sugar degradation product furfural for each *C. crispus* and *U. lactuca*.

Original: 12M H_2SO_4 37°C 1 h/ 1M 100°C 2 h

New: 11M H_2SO_4 37°C 1 h/ 1M 100°C 2 h

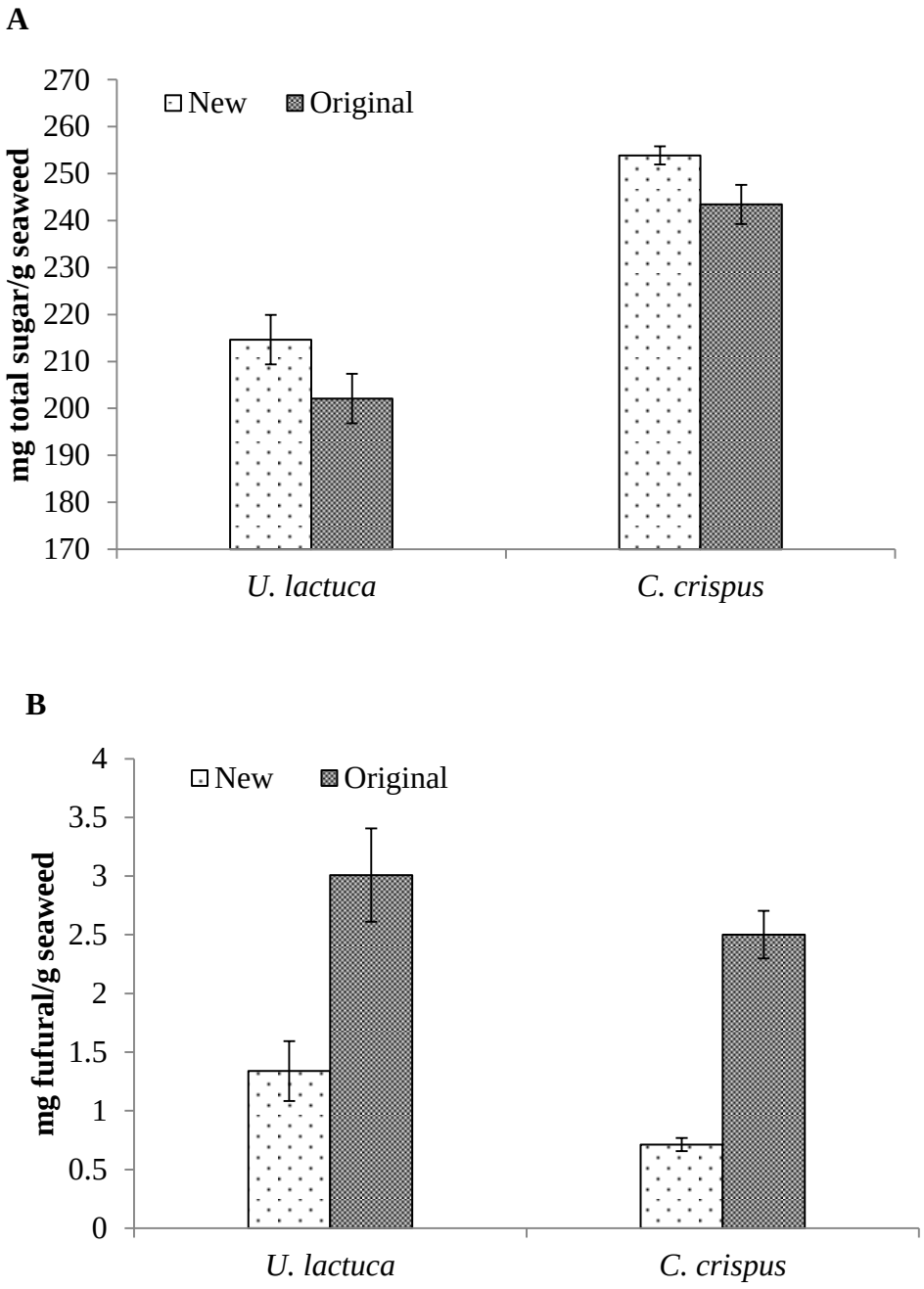


Figure 4.9 Comparison between control (SCP) and optimised carbohydrate quantification assay on the seaweed species *U. lactuca* and *C. crispus*

A Total sugar yields (sum of mannitol, fucose, arabinose, galactose, glucose and xylose; quantified by HPAEC-PAD) from both *U. lactuca* and *C. crispus*. **B** Furfural concentrations generated from both *U. lactuca* and *C. crispus* from both the original control protocol and the newly optimised protocol.

The newly optimized quantification protocol liberated significantly (p <0.05) higher total sugar yields for both the *C. crispus* and *U. lactuca* species when compared to the original SCP protocol (Figures 4.9 A+B). Total sugar yields of 215 and 254 mg/g were achieved using the new protocol (for *C. crispus* and *U. lactuca*, respectively) compared to only 202 and 244 mg/g respectively from the SCP. Additionally, there was a significant difference (p<0.05) in the production of furfural (which was the only sugar degradation product detected in the samples), between the newly optimized protocol and SCP applied to both *C. crispus* and *U. lactuca* (p<0.005). Higher concentrations of furfural were generated from both species of seaweed when the SCP was applied (2.5 and 3 mg/g for *C. crispus* and *U. lactuca*, respectively) when compared to the newly optimised protocol (NOP) which generated 0.7 and 1.3 mg/g, respectively. This suggested that the SCP would provide an under estimation of the true total carbohydrate content in other seaweed species. Furthermore, findings from this study confirm the importance to optimise protocols for each different species of seaweed in order to accurately determine the content of carbohydrates. Whilst a fully optimised protocol was developed for *L. digitata* this was not done for other seaweed species (a comparison of the yields from the NOP was merely included as a comparison to confirm the specific optimisation requirements).

Other research groups have attempted to develop novel and highly specific quantification methods for seaweed monomeric sugars and carbohydrates. Manns *et al.* (2014) compared the monosaccharide compositions of samples of *L. digitata* and *S. latissima* by using different HPAEC methods after different acid hydrolysis methods (similar to the work within this Chapter) or after

cellulase enzyme treatment. The group identified that HPAEC-PAD analysis (also the same method used in this study for total monosaccharide quantification) of seaweeds after treatment with their own optimised version on the NREL two-stage acid hydrolysis (12M H_2SO_4 for 1 h at 30°C and then 0.75 M at 120°C for 40 min). This protocol yielded 51% w/w glucose (510 mg/g) from *L. digitata* and also appeared to be the best methodology to apply for the hydrolysis and quantification of carbohydrate in brown seaweeds. The study additionally revealed that enzyme decomposition of the brown seaweeds (an alternative to thermo-chemical hydrolysis) from the use of commercial enzyme mixes revealed higher glucose yields compared to the acid treatments screened in their study. This was also observed in results obtained from the work described in Chapter 3 (section 3.3.4.1), where pre-treated residues had slightly higher yields of glucose solubilized into the liquid fraction post enzymatic saccharification than subjection to the NREL two-stage acid hydrolysis protocol. Although enzymatic hydrolysis may be a more suitable alternative that can provide more reliable values for certain monosaccharides, the main disadvantage of employing enzymes for composition purposes is that only specific polysaccharides are targeted for depolymerisation Therefore the overall decomposition of the biomaterial into its monomeric constituents remains incomplete and in addition there is the high cost of enzymes compared to the relatively low cost of concentrated acids.

4.4 Conclusions

A comprehensive optimisation of the total acid hydrolysis based protocol for the quantification of the carbohydrate content of seaweeds has been presented in this Chapter. It can be concluded that the most commonly followed

protocol, which is typically a modification of the NREL based assay (specifically designed for carbohydrate analysis from lignocellulosic biomass), was excessively thermo-chemically harsh for seaweed biomass. This can result in erroneously low total sugar quantification as the reaction severity can degrade a proportion of the liberated sugars to decomposition products.

In addition the first stage of the hydrolysis protocol was shown to be the most crucial for maximising sugar yields.

This work also revealed that different seaweed species most likely required specific individual optimisations of the protocol for accurate total sugar quantification. This was concluded by applying the newly optimised method (for *L. digitata*) on samples of *C. crispus* and *U. lactuca*. Not only were higher total sugar yields obtained by applying the new protocol to the 2 species of seaweed (when compared to the yields achieved using the SCP), but significantly lower levels of the sugar degradation product furfural were produced. Additionally complete optimisation of the assay (for the 2 additional seaweed species) may also liberate even higher sugar concentrations from the biomass.

5 Novel application of a phenotypic microarray approach to identify yeast strains with metabolic capability to ferment seaweed hydrolysates

5.1 Introduction

Thermo-chemical pre-treatment on biomass, to release polysaccharides, is usually an important primary stage for bioethanol production. The modified feedstock structure can then usually be targeted by enzymes which can then hydrolyse the larger polysaccharides into their monomeric constituents; ready for microbial conversion to ethanol. Previously, work in Chapter 3 (section 3.3.2.1) identified that a relatively mild acid pre-treatment using sulphuric acid was required on the brown seaweed *L. digitata*, and the optimum conditions for the pre-treatment were deduced. The thermo-chemical pre-treatment of *L. digitata* generated a liquid fraction that was rich in an array of monomeric sugars specific to the seaweed. This liquid fraction (hydrolysate) is normally discarded, yet its contents could be used as a direct source via fermentation to produce metabolic carbon end points such as fuels and/or platform based chemicals. However, the monosaccharide types found in the hydrolysate, such as fucose and mannitol, may not be easily utilised by traditional yeast strains (such as *Saccharomyces cerevisiae*) as these yeasts lack the relevant metabolic pathways for the conversion of such sugars into ethanol (Roesijadi *et al*., 2010). This suggests that the maximum conversion of available

monosaccharides in the hydrolysates into bioethanol may not be fully achieved.

As the interest in bioethanol production from seaweeds has increased, the search to find wild type microorganisms with abilities to ferment sugars derived from the breakdown of seaweed biomass has proven to a major obstacle (Wargacki *et al*, 2012, Enquist-Newman *et al*, 2013). Attempts have been made to generate genetically engineered microorganisms with the ability to ferment sugars inherent to seaweed into ethanol (as discussed in Chapter 1), or to identify novel natural (wild type) strains. Phenotypic screening of yeast strains may be employed for this purpose, and identify strains with desirable traits for bioethanol production (Greetham *et al.*, 2014). This was demonstrated by (Wimalasena *et al.*, 2014), where candidate yeast strains (*Saccharomyces* spp) for efficient second generation bioethanol production were identified by employing a phenotypic microarray (PM) analysis to measure tolerance to stresses associated with the fermentation process e.g. inhibitory sugar degradation products and tolerance to ethanol itself. The PM technique gives an insight into the yeast cell's metabolic activity against various media (e.g. individual monosaccharide carbon sources). Although not directly indicative of fermentation activity, the approach can identify microorganisms with abilities to metabolise different carbon sources. Even when an organism does not produce ethanol as a major metabolic product, it remains useful to identify organisms which can assimilate and metabolise the sugars liberated from macroalgae. In the PM method, changes in colour of a redox sensitive dye are monitored as yeast cells respire on selected media arranged in a 96 well plate format (Bochner *et al.*, 2001). The colour

development is a measure of the yeast's metabolic activity, and essentially indicates its ability to metabolise the sugar (or compound under investigation) present in the assay. When a yeast cell is able to grow and metabolise on a particular compound present in the microarray plate well, cellular respiration generates NADH. As a consequence, the tetrazolium dye that is used in the assay is reduced to formazan, and this results in a colour change from colourless to purple. The OmniLog (Biolog) machine records an image every 15 minutes and the visual image is then converted into a number; reflecting metabolic output. The metabolic activity of the yeast can be plotted against time computationally. There are many advantages of utilizing a PM approach, which include high throughput nature and avoidance of traditional yeast fermentation methodologies which are comparatively laborious and expensive (Greetham, 2014). Furthermore, because end-products of metabolic activity e.g. bioethanol are not the goal using this approach, strains that can metabolise seaweed sugars *per se* can be identified, raising the potential for the exploration of other novel end-products e.g. xylitol and succinic acid, in future studies (Wimalasena *et al*, 2014).

In this Chapter, the novel application of PM analysis to the screening and selection of yeast strains able to metabolise monosaccharides inherent to seaweeds was evaluated. This was conducted by generating synthetic minimal media containing monosaccharides unique to seaweeds. Furthermore, promising strains highlighted by the PM assays were further screened for ability to metabolize hydrolysates prepared from the acid hydrolysis of 5 native UK seaweeds. Post this PM screening approach, selected yeast strains

were further examined in laboratory-scale fermentations specifically for ethanol production utilising different seaweed hydrolysates.

5.2 Materials and Methods

5.2.1 Seaweed collection and preparation

Laminaria digitata and *Fucus serratus*, *Chondrus crispus* and *Palmaria palmata*, and *Ulva lactuca* were collected and prepared as described in the method outlined in Chapter 2 Sections 2.2 and 2.3.

5.2.2 Preparation of seaweed hydrolysates (acid hydrolysis)

For the production of seaweed hydrolysates, 200 g of seaweed powder was dispersed in 2 L of 1.5-1.7N H_2SO_4 in 2 L Duran bottles and heated in a benchtop autoclave at 121 ˚C for 15 min. After hydrolysis, the liquid fraction was separated from the residue by filtration using a Büchner funnel and flask connected to a pump. The filtrate (seaweed hydrolysates) were adjusted to pH 7 using NaOH (5M) and filter sterilised (GF/C 25 mm filter diameter/0.2 μm pore size; Whatman International Ltd., Banbury, UK) before being analysed for monosaccharides and concentrations of sugar degradation products (see below).

5.2.2.1 Composition analysis of hydrolysates

Free amino nitrogen (FAN) was determined by following the European Brewing Convention ninhydrin spectrophotometric method, which utilises glycine as a standard (Analytica – EBC, 2000). Monosaccharide concentrations were determined by HPAEC-PAD (Chapter 2 Section 2.7.1) and sugar degradation products were quantified by HPLC (Chapter 2 Section 2.7.2).

5.2.3 Yeast strain screening using the PM method (Biolog Omnilog)

5.2.3.1 Yeast strains and growth conditions

All 24 yeast strains which were screened in this investigation listed in Table 5.1 were taken from glycerol stocks stored at -80 ˚C and inoculated into a yeast extract peptone (YPD) liquid medium containing 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose and placed in an orbital shaker (180 rpm; CERTOMAT BS-1 incubator, Sartorius, Germany) at 30ºC under aerobic conditions for 48 h. 100µL of these cultures were then spread on YPD slopes and incubated at 30ºC under aerobic conditions for a further 48 h.

Table 5.1 List of yeast strains investigated in this study.

*TP – courtesy of Trevor Phister, *NCYC – National Collection of Yeast Cultures, *BSYC – Brewing Science Yeast Culture, *EL – courtesy of Ed. Loui

Microorganism	Source	Equivalent strain destignations/Ref	ID in this study
Kluyveromyces marxianus	*TP	ATCC 22296, CBS 5671, NRRL Y8287, Phaff 71-15	TP1
Candida veronae	TP	-	TP2
Pichia Mexicana	TP	ATCC 28874, CBS 5815, NRRL Y17672, Phaff 69-32	TP3
Candida utilitis var thermophila	TP	CBS 1517, NRRL Y-1082, Phaff 74-62, DBVPG 7306	TP4
Candida mogii	TP	-	TP5
Candida sorboxylosa	TP	Phaff 91-491.3	TP6
Candida stellate	TP	Phaff 72-1034	TP7
Candida utilis	TP	ATCC 9950, CBS 5609, Phaff 74-61	TP8
Candida santjackobensis	TP	ATCC 58898, CBS 8183	TP9
Pichia stipites	TP	ATCC 58785, CBS 6054, NRRL Y11545	TP10
Candida valida	TP	Phaff 94-150.3	TP11
Pichia anomala	TP	CBS 5759, NRRL Y-366, Phaff 76-71T	TP12
Saccharomyces cerevisiae	*NCYC	Melbourne No. 1 strain, NCTC 4919	NCYC 192
Hanseniaspora valbyensis	NCYC	CBS 479, NRRL Y-1626, ATCC 10631, IFO 0670, NCTC 478	NCYC 17
Candida tenuis	NCYC	ATCC 58782, CBS 4285, CCRC 21772, NRRL Y-17106	NCYC 2545
Candida succiphila	NCYC	ATCC 46049, CBS 8003, CCRC 21410, IAM 12489, IFO 1911, JCM 9445, KL30, NRRL Y-11998	NCYC 1403
Saccharomyces cerevisiae	NCYC	CBS 1200, ATCC 4126, CCRC 21494, MUCL 39497, CLIB 409	NCYC 2592
Candida lyxosophila	NCYC	CBS 8194, CCY 29-173-1, JCM 7532	NCYC 2379
Pichia guilliermondii	NCYC	ATCC 46036, CCRC 21697, CCRC 22093, DBVPG 6571, IFO 10106, NRRL Y-2075	NCYC 443
Candida shehatae var lignosa	NCYC	CBS 4705, ATCC 58779, CCRC 21774, IGC 3590, JCM 9837, NRRL Y-12856, NRRL Y-17027	NCYC 2389
Candida arabinofermentans	NCYC	CBS 8468, NRRL YB-2248	NCYC 2916
Candida shehatae	*BSYC	-	BSYC 1
Saccharomyces cerevisiae	*EL	Liti *et al*., (2009)	YPS128
Saccharomyces cerevisiae	EL	Liti *et al*., (2009)	Y12

5.2.4 Phenotypic microarray analysis (Biolog-Omnilog) using synthetic minimal media and seaweed hydrolysates

5.2.4.1 Preparation of customised phenotypic microarray Biolog plates

The Biolog-Omnilog phenotypic microarray (PM) system (Biolog, Hayward, CA, USA) was used according to the manufacturer's instructions with minor modifications according to Greetham *et al*. (2014a) to screen for yeast strains that can metabolize different carbon sources, in particular monosaccharides liberated from the acid hydrolysis of seaweed species. Synthetic minimal media containing different individual carbon sources (arabinose, fucose, galactose, glucose, mannitol, rhamnose and xylose) at 6% w/v were prepared in order to ensure a sufficient concentration of sugar was provided to the yeast strains under investigation. An aliquot (120 µL) of the synthetic minimal media was supplemented into a solution containing 0.67% (w/v) of yeast nitrogen base (YNB; without amino acids and ammonium sulphate), 2.6 µL of yeast nutrient supplement mixture (NSx48- 24 mM Adenine-HCl, 4.8 mM L-histidine HCl monohydrate, 48 mM L-leucine, 24 mM L-lysine-HCl, 12 mM L-methionine, 12 mM L-tryptophan and 14.4 mM uracil), and 0.2 µL of dye D. Reverse osmosis (RO) sterile distilled water was added to make a final volume of 60 µL, which was then aliquoted into individual wells of the PM array plates. Seaweed hydrolysate (56 µL) which was generated in Section 5.2.2, was supplemented with 0.4 µL of dye D (instead of 0.2 µL used with the synthetic media) due to the hydrolysates' darker colour in order to see any potential colour change and then aliquoted into the wells of the PM plates. 60 µL of hydrolysate without dye D was also aliquoted into specific wells in order to act as a control.

5.2.4.2 Preparation of yeast strains for inoculation onto PM Biolog plates

Anaerobic conditions were achieved by vacuum packing the PM plates in PM gas bags (Biolog) before being incubated in the OmniLog reader at 30 ºC for 95 h. The Omnilog reader was programmed to photograph the PM plates every 15 min for 95 h, converting the pixel density of each well into a signal data value that represents cell metabolic output and dye colour change. The reduction of the dye, producing a colour change from colourless to violet, results from the cells' metabolic activity when in contact with the different media in each well; this has been defined here as redox signal intensity. The redox signal intensity data was assembled in the Biolog software and exported using Microsoft® Excel. All assays were conducted in triplicate and the mean values along with standard deviations are presented.

5.3 Fermentations of seaweed hydrolysates using selected yeast strains

Yeast strains which scored a redox signal intensity value (RSI) of ≥ 20 in the PM assay when utilising seaweed specific sugars and/or seaweed hydrolysates as carbon substrates were used in subsequent bioethanol fermentations. The hydrolysates were fermented by following the method outlined in section 2.6.2. At the end of the fermentations, samples were taken for monosaccharide and ethanol analysis via HPAED-PAD and HPLC, respectively (section 2.7). All fermentations were carried out in triplicate.

5.4 Results and Discussion

5.4.1 Analysis of the hydrolysates generated from acid pre-treatment

The composition of acid hydrolysates derived from the seaweed can be seen in Table 5.2. Red seaweed species *C. crispus* and *P. palmata* (with carbohydrate

contents of 21.8% and 39.4%) and the green seaweed *U. lactuca* (23.8% carbohydrate content) yielded the highest concentrations of glucose; 2.02, 2.99 and 5.30 g/L, respectively, which most likely resulted from the hydrolysis of cellulose which is abundant in these species (Griffiths *et al.*, 2016, Yaich *et al.*, 2015). *C. crispus* and *P. palmata* produced the highest yields of galactose (4.24 and 6.84 g/L respectively) which would most likely be a product of carrageenan hydrolysis (Prajapati *et al.*, 2014). Brown seaweed species *L. digitata* and *F. serratus* (carbohydrate contents of 21.7% and 26.4%) only released 1.51 and 0.95 g/L of glucose respectively, but yielded 2.99 and 3.77 g/L of fucose and 3.49 and 4.38 g/L of mannitol, respectively. Interestingly, *C. crispus* yielded 3.04 g/L fucose. Although this sugar has not previously been reported or identified in this particular red species before, its presence has been continuously detected in the current project. According to the compositional analysis undertaken in Chapter 3, *P. palmata* had the highest carbohydrate content (39.4%) and actually yielded the highest concentration of total sugars in its hydrolysate. Although it appears that this particular species could be an ideal candidate for bioethanol production, its abundance in UK waters is not as high as that of certain brown kelp species, such as *L. hyperborea* and *A. nodosum* (Wilkinson, 1995).

Table 5.2 Analysis of monosaccharides, yeast inhibitory compounds and **Free Amino Nitrogen (FAN) present in seaweed hydrolysates

Data are the mean ±SD of three measurements

		Seaweed Hydrolysate				
		L. digitata	***F. serratus***	***C. crispus***	***P. palmata***	***U. lactuca***
	***Carbohydrate % (d/w)**	21.7	26.4	21.8	39.4	23.8
Monosaccharide (g/L)	**Arabinose**	0.07 ± 0.01	0.08 ± 0.00	0.14 ± 0.01	0.05 ± 0.01	2.15 ± 0.00
	Fucose	2.29 ± 0.11	3.77 ± 0.10	3.04 ± 0.15	0.08 ± 0.00	0.11 ± 0.02
	Galactose	0.57 ± 0.03	0.91 ± 0.03	6.84 ± 0.17	4.24 ± 0.21	0.78 ± 0.00
	Glucose	1.51 ± 0.08	0.95 ± 0.03	2.02 ± 0.07	2.99 ± 0.15	5.30 ± 0.03
	Mannitol	3.49 ± 0.08	4.38 ± 0.09	0.01 ± 0.02	0.04 ± 0.00	0.06 ± 0.00
	Xylose	0.55 ± 0.03	1.00 ± 0.03	1.31 ± 0.06	8.71 ± 0.42	3.52 ± 0.02
	Total	8.48 ± 0.34	11.09 ± 0.30	13.36 ± 0.47	16.13 ± 0.79	11.91 ± 0.08
Inhibitors (g/L)	**Furfural**	-	-	-	0.25 ± 0.01	0.05 ± 0.00
	HMF	0.02 ± 0.02	-	2.13 ± 0.16	0.02 ± 0.00	0.09 ± 0.00
Other (g/L)	****FAN**	0.24 ± 0.00	0.38 ± 0.00	0.36 ± 0.01	0.38 ± 0.01	0.16 ± 0.00

*Carbohydrate was estimated as the sum of monosaccharides arabinose, galactose, glucose, xylose, fucose and mannitol. It is assumed that the unaccounted for dry matter is principally polysaccharide material either nor broken down under the hydrolysis conditions employed or not quantified against authentic standards during HPLC analysis.

L. digitata had the lowest measured carbohydrate content of 21.7% (d/w) in this study. However, this may be due to the fact that the *Laminaria* biomass in this study was harvested in spring (May) when levels of endogenous phaeophycal polysaccharides (in particular laminarin and fucoidan) may not be particularly high. A study by Adams *et al* (2011) revealed that the most suitable month to harvest *L. digitata* for bioethanol production was July, as this is when carbohydrate yields are highest. Further, some specialised monosaccharides such as mannuronic and guluronic acids from the polysaccharide alginate, which is prevalent in this species, were not quantified using the analytical chromatography method applied. Alginate is extremely difficult to hydrolyse and under the conditions utilised in this study is unlikely to have liberated significant quantities of these particular monosaccharides (Benvegnu and Sassi, 2010). The main cell wall constituents that make up brown seaweeds are typically cellulose microfibrils which are embedded in an amorphous matrix of alginate and fucoidan polysaccharides (Kloareg *et al*., 1986). Mannitol is often found as a terminating side chain on laminarin, a polymer of β-(1-3)-D-glucose that can be easily extracted from brown seaweed (Horn *et al*. 2000a) and the fucose yields have resulted from the breakdown of fucoidan in the brown seaweed hydrolysates.

The hydrolysates derived from seaweed were also analysed for the presence of known yeast-inhibitory compounds (a range of phenolic compounds, organic acids and furan compounds), which can be formed in the pre-treatment of lignocellulosic biomass (Wilkinson *et al*, 2014). Such compounds are generated during the acid hydrolysis of biomass at high temperatures and can negatively impact on ethanol production during fermentation (Meinita et al.,

2012). HMF and furfural were the only compounds present in the hydrolysates, but were present at low levels compared to similar hydrolysates from other biomass types (Wilkinson *et al,* 2015) and were not detectable in the hydrolysate of *F. serratus*. HMF derives from the breakdown of hexose sugars and furfural from the breakdown of pentose sugars. *C. crispus* generated the highest amount of HMF (2.13 g/L) which is consistent with its high glucose and galactose contents (C-6 sugars).

Levels of free amino nitrogen (FAN) are also reported for each of the seaweed hydrolysates. Amino acids represent a significant nitrogen source required for yeast growth and metabolism during fermentation. FAN levels were within the range 0.16 to 0.38 g/L. The minimum FAN content normally specified for brewing fermentations is 0.15 g/L, so the seaweed hydrolysates appeared to contain sufficient quantities of amino acids to avoid the need for supplementary nitrogen sources to aid fermentation.

There had been no previous reports on the utilisation of hydrolysates produced from the acid pre-treatment of seaweeds for bioethanol production, until Mutripah *et al.* (2014) fermented the hydrolysate of the rhodophyte *Palmaria palmata* with *Saccharomyces cerevisiae*, where 17.3 mg of ethanol per gram of seaweed was produced. Typically, the objective of applying an acid (or in some cases alkaline) pre-treatment step is to modify the chemical structure of the biomass by removing the more recalcitrant complexes. Hydrolysis of polysaccharides into their monosaccharide constituents is typically a further step that is sequential to the acid pre-treatment, by the addition of selective enzymes (Meinita *et al.*, 2012b). However, addition of enzyme is expensive and it is necessary to apply the correct enzyme blend to suit the particular

species of seaweed that is being processed. The liquid fraction generated from the acid pre-treatment step is usually discarded. Moreover, analysis of the hydrolysates derived from the pre-treatment of seaweeds in this study clearly indicated that they contained a cocktail of available monosaccharides which could possibly be fermented to ethanol, without the need to apply an enzyme hydrolysis step, and contained low levels of yeast-inhibitory compounds which should not unduly influence yeast performance during fermentation.

5.4.2 Phenotypic Microarray Analysis of yeast strains

The yeast strains used in this study (mainly *Candida* and *Pichia* strains) for the initial screen were selected based upon their ability to ferment C5 sugars, such as xylose (Hahn-Hägerdal *et al.*, 2007), which were present in the seaweeds studied here. Therefore, it was of interest to examine whether these yeast could utilize the sugars which are only found in seaweeds and whether they would produce ethanol as a result.

5.4.2.1 Metabolic output of yeast strains grown on synthetic minimal media with different carbon sources

The metabolic outputs (defined here as redox signal intensity) of the 22 yeast strains on minimal media containing individual carbon sources present in seaweeds (glucose, galactose, mannitol, xylose, fucose and rhamnose; all at 6% w/v) were measured (Figure 5.1 A - F).

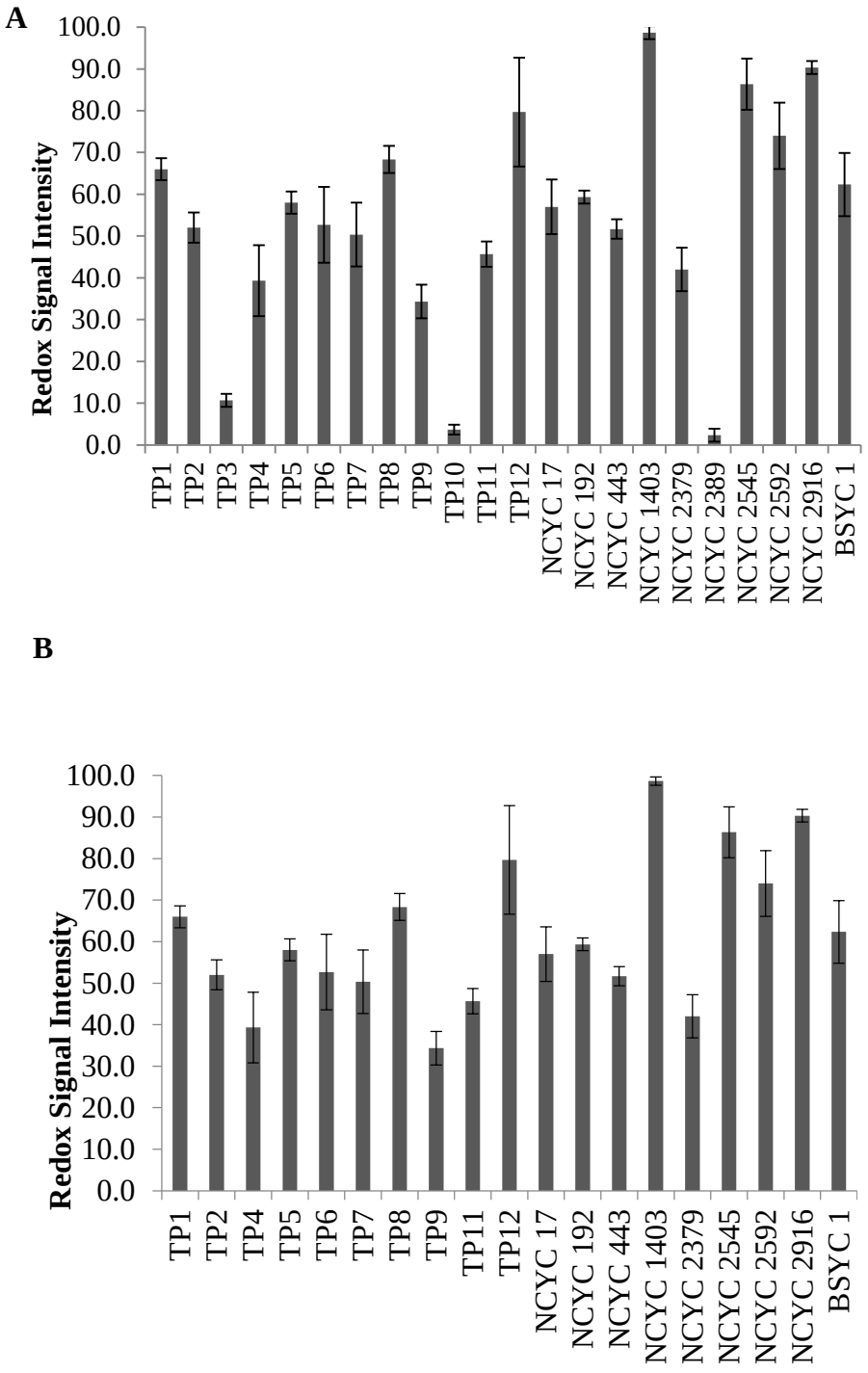


Figure 5.1 Phenotypic microarray analysis (redox signal intensity for yeast strains grown in minimal media supplemented with A) Glucose; B) Mannitol at 6% (w/v) inclusion.

Data are the mean ± SD of three replicates.

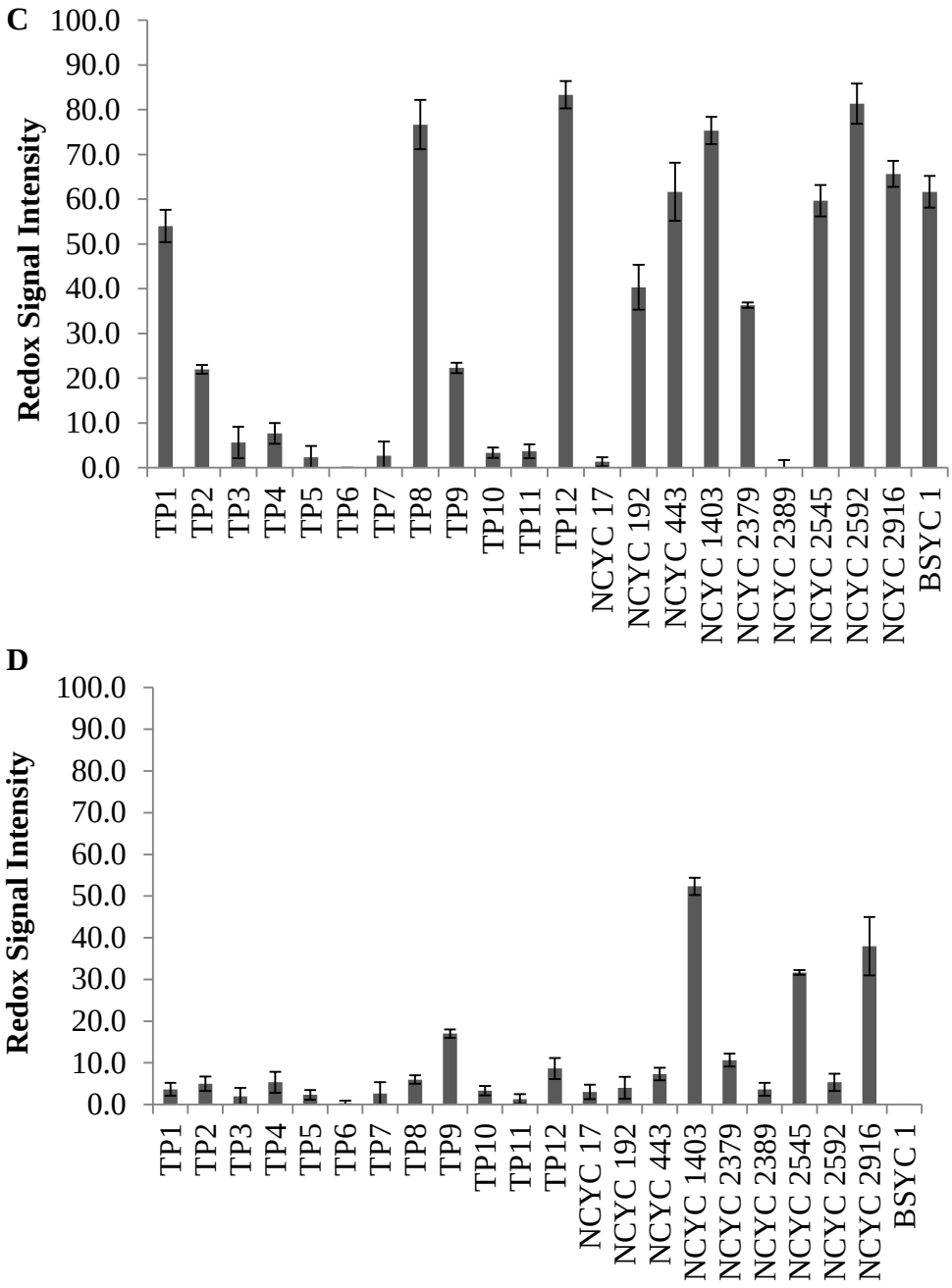


Figure 5.1 Phenotypic microarray analysis (redox signal intensity for yeast strains grown in minimal media supplemented with C) Galactose and D) Fucose at 6% (w/v) inclusion.

Data are the mean ± SD of three replicates.

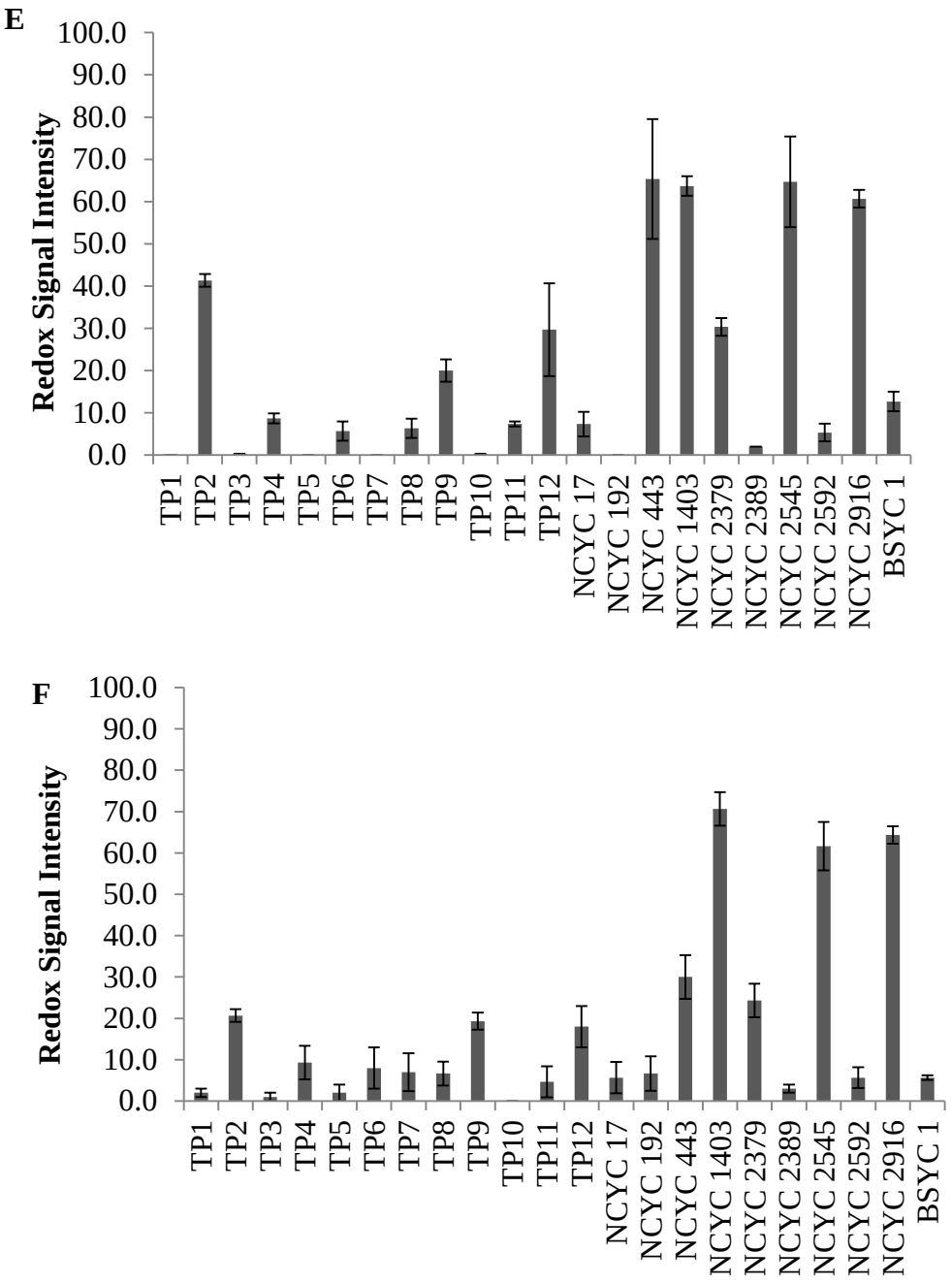


Figure 5.1 Phenotypic microarray analysis (redox signal intensity for yeast strains grown in minimal media supplemented with E) Xylose and F) Rhamnose at 6% (w/v) inclusion.

Data are the mean ± SD of three replicates.

There was measurable metabolic output for all strains utilizing glucose (Figure 5.1 A) and the RSI of the strains cultured on this monosaccharide ranged from 2.3 to 98.7 RSI. Similarly, the majority of the strains displayed a high (>20 RSI) metabolic output (34.3 to 98.7 RSI) when utilising galactose (Figure 5.1 C). This finding was as expected since such monosaccharides are favourable carbon sources for yeast (Flores *et al*., 2000). Nevertheless, some strains exhibited extremely low RSI levels when utilising glucose as a sole carbon source, namely *Candida shehatae var lignosa* NCYC 2389 and *Pichia stipites* TP10, and were removed from further study. The metabolic outputs of all strains screened here appeared to be similar in response when utilising mannitol, xylose and rhamnose (Figure 5.1 B, E and F). When the yeast strains were evaluated for similarities of sugar metabolism through a statistical comparison of the data using R-script (Figure 5.2), it became clear that there was a high degree of shared similarities for all strains in the utilization of mannitol, xylose and rhamnose (Xylose and Mannitol: R=0.94, Xylose and Rhamnose: R=0.92, Mannitol and Rhamnose: R=0.90). Interestingly, the *Candida* and *Pichia* spp examined in this study displayed an increase in metabolic activity, albeit at varying intensities, when utilising mannitol, xylose or rhamnose as sole carbon sources (Figure 5.1 B, E and F) compared to *Saccharomyces*, *Kluveromyces* and *Hanseniaspora* spp in this study. *Candida* spp and *Pichia* spp have been previously reported to be able to metabolise and ferment pentose sugars (Kordowska-Wiater *et al.*, 2008, Dien *et al.*, 1996), however only 1% of all known xylose utilizing yeast are known to display the potential of fermenting xylose to ethanol (McMillan and Boynton, 1994).

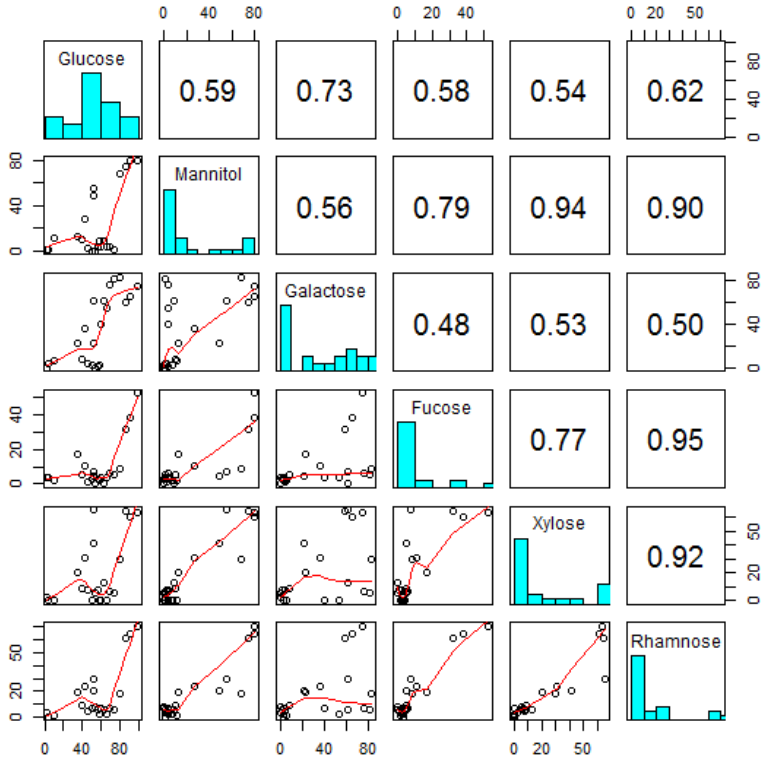


Figure 5.2 Statistical comparison (using R) of sugar metabolism between the 22 yeast strains.

Yeast strains were grown on minimal media containing different carbon sources (glucose, mannitol, galactose, fucose, xylose and rhamnose at 6% (v/w) inclusion) at 30°C for 95h. Shared phenotypic responses to each carbon source at 50 h are shown here. Histograms represent a spread of the data for all yeast strains across all sugars. Additionally, the histrograms indicate how each of the investigated yeast strains utilise each sugar; i.e. can all yeast strains use one sugar or are the majority for the yeast strains poor users of that particular sugar. The closer the numbers are to 1, the similar the phenotype (use of sugar).

Pichia angiophorae has already been shown to be able to ferment both mannitol and laminarin in seaweed extracts to ethanol (Horn *et al*. 2000a). The strains exhibited very poor metabolic output when utilizing fucose as the sole carbon source (Figure 5.1 D), which was as expected since to date no yeast strain has been identified to have fucose fermenting abilities.

In order to have a better understanding of each yeast strains' ability to metabolize unusual carbon sources, the PM assay results were adjusted to show each strain's metabolic output for these sugars in relation to their utilisation of glucose (Table 5.3).

Table 5.3 Phenotypic microarray results (RSI at 50h) for each yeast strain investigated in this study on minimal media containing: galactose, mannitol, fucose, xylose and arabinose (6% v/w) relative to the corresponding glucose value (RSI)

Data are the mean ± SD of three replicates

		Monosaccharide (% RSI in comparison to glucose)				
Yeast ID	**Glucose (RSI)**	**Galactose**	**Mannitol**	**Fucose**	**Xylose**	**Rhamnose**
TP1	66.0	81.8	6.1	5.6	0.0	3.0
TP2	52.0	42.3	93.6	9.6	79.5	39.7
*TP3	10.7	53.1	112.5	18.8	3.1	9.4
TP4	39.3	19.5	26.3	13.6	22.0	23.7
TP5	58.0	4.0	14.4	4.0	0.0	3.4
TP6	52.7	0.0	0.6	0.6	10.8	15.2
TP7	50.3	5.3	0.0	5.3	0.0	13.9
TP8	68.3	112.2	5.4	8.8	9.3	9.8
TP9	34.3	65.0	36.9	49.5	58.3	56.3
*TP10	3.7	90.9	36.4	90.9	9.1	0.0
TP11	45.7	8.0	5.1	2.9	16.1	10.2
TP12	79.7	104.6	84.9	10.9	37.2	22.6
NCYC 17	57.0	2.3	5.8	5.3	12.9	9.9
NCYC 192	59.3	68.0	6.2	6.7	0.0	11.2
NCYC 443	51.7	119.4	106.5	14.2	126.5	58.1
NCYC 1403	98.7	76.4	80.7	53.0	64.5	71.6
NCYC 2379	42.0	86.5	65.9	25.4	72.2	57.9
*NCYC 2389	2.3	0.0	71.4	157.1	85.7	128.6
NCYC 2545	86.3	69.1	86.5	36.7	74.9	71.4
NCYC 2592	74.0	109.9	2.3	7.2	7.2	7.7
NCYC 2916	90.3	72.7	88.6	42.1	67.2	71.2
BSYC 1	62.3	98.9	14.4	0.0	20.3	9.1

*** Removed from study due to low metabolic output on glucose**

Interestingly, strains which had relatively high utilisation of glucose (≥79.7) also displayed high metabolic outputs when utilizing galactose, mannitol, fucose, xylose and rhamnose. These were: *C. succiphila* NCYC 1403, *P. anomala* TP12, *C. tenuis* NCYC 2545. *C. tenuis* NCYC 2545, *C. succiphila* NCYC 1403 and *P. anomala* TP12 exhibited high RSI levels on all monosaccharides analysed in relation to their corresponding glucose values, which implies that they are able to metabolize these monomeric units. All screened sugars are somewhat similar in their chemical structures, however it is interesting that only a selected few strains of yeast displayed high metabolic activity when cultured on the synthetic media containing only one carbon source. It is a possibility that these yeast strains could have a higher degree of sensitivity towards the monosaccharides and uptake ability, compared to the other strains that were investigated in this Chapter; ultimately making them ideal candidates for further research. Additionally, these yeast species appeared to be metabolically active on synthetic media containing fucose, with an RSI ≥ 20 (Figure 5.1 D). Fucose is a hexose sugar and is the fundamental subunit of the fucoidan polysaccharide found in brown seaweeds (Ale *et al.*, 2011). This suggested that these strains may possibly contain a fucose utilization pathway or surface transporter, enabling the uptake of fucose into the cell. *C. albicans* contains the, C2_09280C_B gene, which is predicted to be either a membrane transporter, member of the fucose: proton symporter (FHS) family or part of a major facilitator superfamily (MFS), and orthologous genes have been identified in other *Candida*, *Aspergillus* and *Saccharomyces* species (Gaur *et al*, 2008, Lohse *et al*, 2010; www.candidagenome.org;). Although beyond the scope of this thesis, it would

be of interest to confirm whether the same or orthologous genes associated with fucose uptake or metabolism can also be identified in the *Candida spp* examined in the present study. This could ultimately lead to the cloning and transferring of such genes into other yeast strains to produce a genetically engineered capable of fermenting fucose containing hydrolysates from seaweeds.

5.4.3 Metabolic output of yeast strains grown on seaweed hydrolysates

Three yeast strains, *C. tenuis* NCYC 2545, *C. succiphila* NCYC 1403 and *P. anomala* TP12 were able to metabolise the majority of the individual sugars as evaluated using PM analysis. They were thus selected for further experiments to assess their abilities to metabolise the acid hydrolysates generated from five seaweed species (Figure 5.3 A-E). In addition, *S. cerevisiae* NCYC 2592 was also included in the trial as a reference distiller's production strain (www.ncyc.co.uk), expected to provide efficient conversion of glucose and galactose to bioethanol. Additionally, *S. cerevisiae* Y12 and YPS128 (Liti *et al.*, 2009), were also as yeast strains that have been derived from clean lineages that have had no alternations to their genomes from human influences or domestication events (Liti *et al*., 2009). It was important to include such strains as alternatives to brewing yeast strains, due to their different genomes which may contain the necessary metabolism genes suited more towards monosaccharides that are inherent to seaweeds.

Assays revealed that all strains cultured on *L. digitata*, *F. serratus* and *U. lactuca* hydrolysates were metabolically active (Figure 5.3 A, B and E). However, assays with *C. tenuis* NCYC 2545 revealed a decrease in RSI after

20 h from ca 50-60 RSI to ca 10 RSI in both brown seaweed hydrolysates (Figure 5.3 A and B).

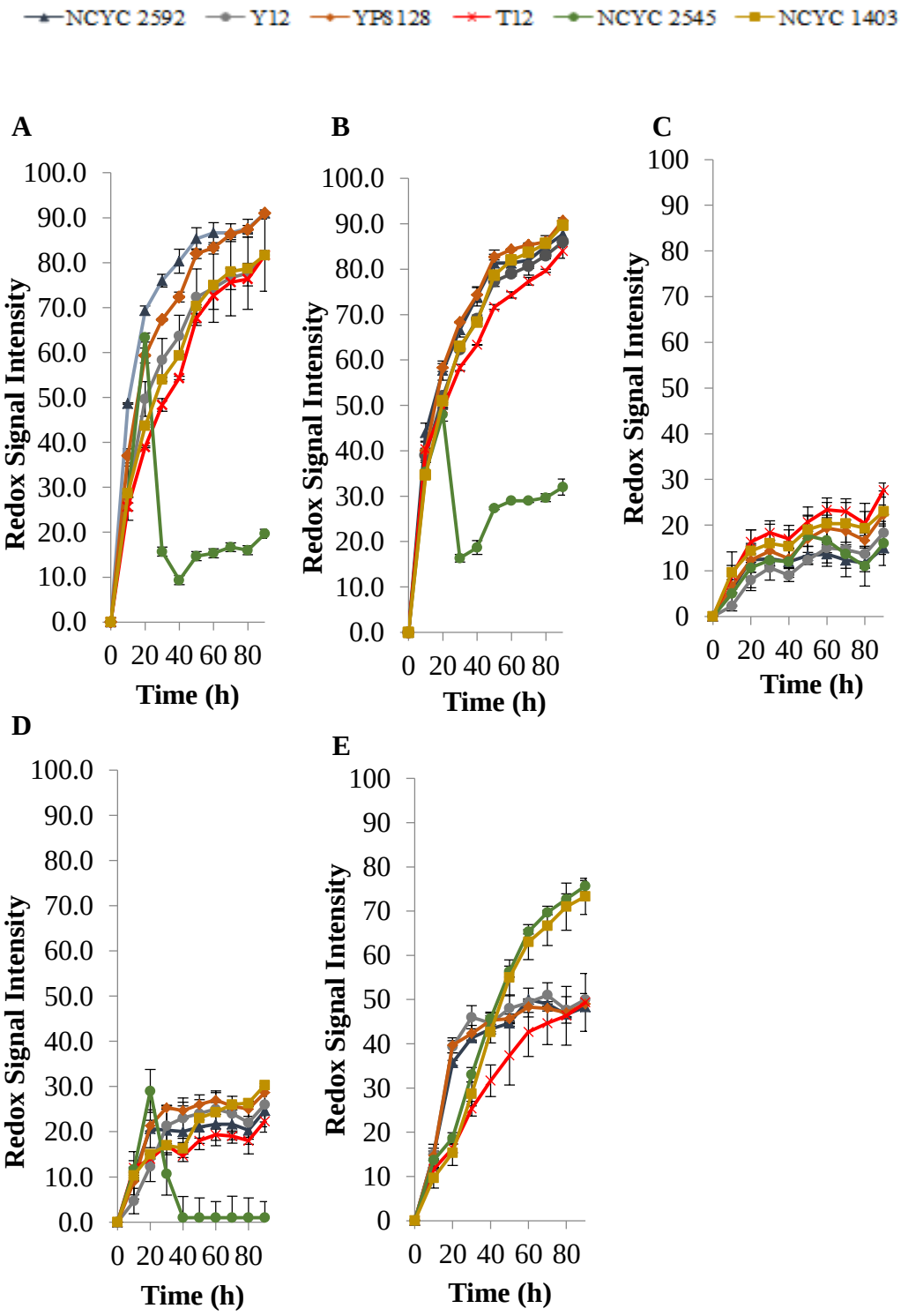


Figure 5.3 Phenotypic microarray analysis (redox signal intensity) for yeast on A. *L. digitata* hydrolysate B. *F. serratus* hydrolysate C. *C. crispus* hydrolysate D. *P. palmata* hydrolysate E. *U. lactuca* hydrolysate.

Data are the mean ± SD of three replicates.

Although this phenomenon has been observed in other microarray assays before, it is unknown why it occurs but could be down to cell lysis and loss of the irreversibly reduced dye (Darren Greetham, personal communication). Due to this result, *C. tenuis* NCYC 2545 was not included in future work. The metabolic activities of the shortlisted strains *C. succiphila* NCYC 1403, *P. anomala* TP12, *C. tenuis* NCYC 2545 and *S. cerevisiae* NCYC 2592 when cultured on red seaweed hydrolysates could not be reliably interpreted using the present methodology. This is because the colour of the hydrolysates interfered with detection of the tetrazolium dye (Figures 5.3 C and D). This is undoubtedly a limitation on the current application of the PM. If this approach is to be employed in future work, it would be necessary to experiment with the use of other reporter dyes such as DAPI, dihydroethidium or GFP.

5.4.4 Screening yeast strains grown on seaweed hydrolysates for bioethanol production

Trial fermentations were subsequently conducted with the yeast strains *S. cerevisiae* NCYC 2592, *C. succiphila* NCYC 1403, *P. anomala* TP12, and *S. cerevisiae* strains YPS128 and Y12 to determine whether they could produce bioethanol. Acid hydrolysates derived from the five seaweed species were neutralized and fermented using each yeast strain. Fermentation progression (Figures 5.4 A-E) was monitored as weight-loss due to the evolution of carbon dioxide a metabolic product of the fermentation of any monosaccharide and can be described from the following reaction:

$$C_6H_{12}0_6 \rightarrow 2C_2H_5OH + 2CO_2$$

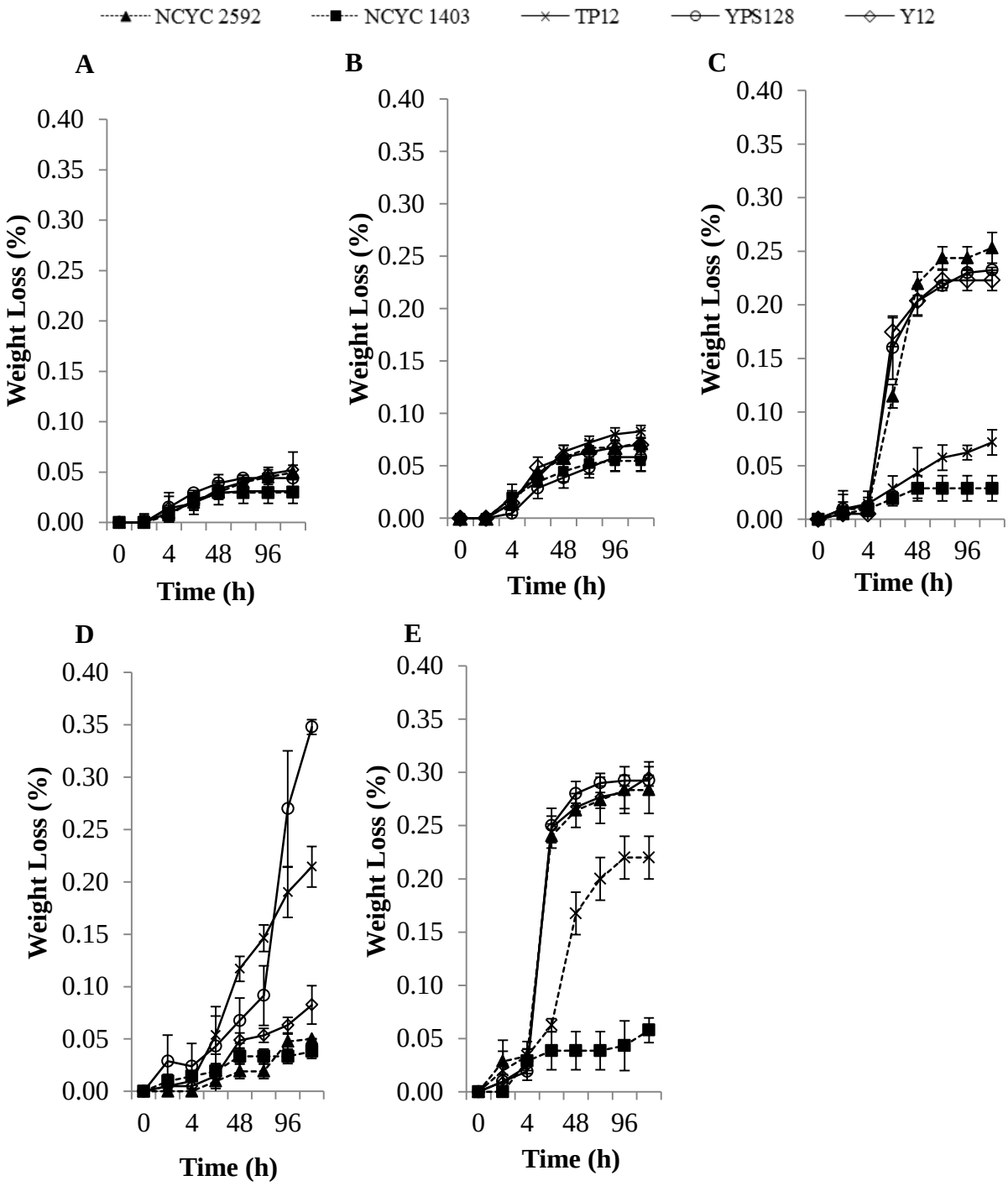


Figure 5.4 Fermentation progression of yeast strains on A *F. serratus* hydrolysate; B *L. digitata* hydrolysate; C *P. palmata* hydrolysate; D *C. crispus* hydrolysate; E *U. lactuca* hydrolysate by yeast strains, (indicated by vessel weight loss resulting from the release of carbon dioxide)

Data are the mean ± SD of three replicates

There were clear differences in the fermentation progression for each hydrolysate using the strains employed in this trial. Overall, fermentation of the *L. digitata* and *F. serratus* hydrolysates by all strains did not perform well as there were only a maximum of 0.08% and 0.05% weight loss after 120 h (Figures 5.4 A and B). On the contrary, fermentations using *P. palmata* and *C. crispus* hydrolysates progressed better, with *P. anomala* TP12 performing better on the *C. crispus* hydrolysate (0.21% weight loss), albeit with a longer lag-phase (up to 72 h) and longer attenuation time (48 h) (Figures 5.4 C and D). *S. cerevisiae* YPS128 displayed a similar fermentation profile with *P. anomala* TP12 when grown on the *C. crispus* hydrolysate, yet *S. cerevisiae* Y12 did not readily ferment this substrate (maximum weight loss of 0.08%). All three *S. cerevisiae* strains demonstrated significant weight loss (0.28 to 0.30 %) on the *U. lactuca* hydrolysate, whereas it took up to 24h for *P. anomala* TP12 to start to ferment this hydrolysate (Figures 5.4 E).

Saccharomyces cerevisiae spp are traditionally used in fermentations for the production of commercial bioethanol, as these strains can adequately consume hexose sugars and are resistant to osmotic and ethanol toxicity stresses, occur during the fermentation (Zhao and Bai, 2009). However, their inability to utilize pentose sugars, despite having the relevant pathways in their genomes (Hahn-Hägerdal *et al*., 2007), limits the full potential of their use in the production of bioethanol. Utilisation of monosaccharides pre and post fermentation and the mean bioethanol yields achieved from each strain on the five hydrolysates at the end of the fermentations, can be seen in Table 5.4.

Table 5.4 Utilisation of monosaccharides in hydrolysates of *L. digitata*, *F. serratus*, *C. crispus*, *P. palmata* and *U. lactuca* and production of bioethanol at the end of fermentation by different yeast strains post fermentation

Data are the mean ± SD of three replicate.

			Yeast Strain				
			NCYC 1403	**NCYC 2592**	**TP12**	**Y12**	**YPS128**
Hydrolysate	**Monosaccharide/ Ethanol (g/L^{-1})**	***Start***	***End***	***End***	***End***	***End***	***End***
L. digitata	Mannitol	3.49 ± 0.08	3.40 ± 0.07	3.46 ± 0.11	3.44 ± 0.48	3.42 ± 0.48	3.48 ± 0.11
	Fucose	2.29 ± 0.11	2.25 ± 0.07	2.23 ± 0.13	2.28 ± 0.24	2.30 ± 0.24	2.31 ± 0.01
	Arabinose	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.02	0.02 ± 0.05	0.08 ± 0.05	0.07 ± 0.00
	Galactose	0.57 ± 0.03	0.55 ± 0.04	0.02 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.00 ± 0.00
	Glucose	1.51 ± 0.08	1.49 ± 0.08	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
	Xylose	0.55 ± 0.03	0.56 ± 0.02	0.37 ± 0.03	0.14 ± 0.10	0.14 ± 0.10	0.41 ± 0.00
	Ethanol	-	0.09 ± 0.12	1.24 ± 0.12	1.26 ±0.06	1.22 ± 0.05	1.55 ± 0.17
F. serratus	Mannitol	4.38 ± 0.08	4.36 ± 0.55	4.35 ± 0.56	4.32 ± 0.50	4.38 ± 0.11	4.37 ± 0.13
	Fucose	3.77 ± 0.11	3.58 ± 0.40	3.58 ± 0.28	3.75 ± 0.42	3.76 ± 0.05	3.77 ± 0.09
	Arabinose	0.08 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.06 ± 0.02	0.04 ± 0.00	0.04 ± 0.00
	Galactose	0.91 ± 0.03	0.88 ± 0.13	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	0.02 ± 0.00
	Glucose	0.95 ± 0.08	0.89 ± 0.12	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
	Xylose	1.00 ± 0.03	0.94 ± 0.13	0.82 ± 0.12	0.07 ± 0.04	0.80 ± 0.02	0.97 ± 0.09
	Ethanol	-	0.00 ± 0.03	0.58 ± 0.05	0.46 ± 0.04	0.78 ±0.17	1.05 ± 0.07
P. palmata	Mannitol	0.04 ± 0.02	0.02 ± 0.03	0.02 ± 0.00	0.03 ± 0.02	0.01 ± 0.00	0.03 ± 0.08
	Fucose	0.08 ± 0.15	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.15	0.06 ± 0.00	0.07 ± 0.04
	Arabinose	0.05 ± 0.00	0.04 ± 0.02	0.05 ± 0.00	0.03 ± 0.01	0.05 ± 0.00	0.04 ± 0.00
	Galactose	4.24 ± 0.17	4.22 ± 0.04	0.39 ± 0.01	4.23 ± 0.17	0.40 ± 0.01	0.23 ± 0.23
	Glucose	2.99 ± 0.07	2.97 ± 0.02	0.00 ± 0.00	2.96 ± 0.07	0.00 ± 0.00	0.00 ± 0.12
	Xylose	8.71 ± 0.06	8.70 ± 0.02	8.69 ± 0.14	8.70 ± 0.06	8.68 ± 0.25	6.20 ± 0.00
	Ethanol	-	0.00 ± 0.01	5.21 ± 0.27	1.69 ±0.05	6.57 ±1.20	6.08 ±0.40
C. crispus	Mannitol	0.01 ± 0.02	0.00 ± 0.03	0.01 ± 0.00	0.01 ± 0.02	0.00 ± 0.04	0.01 ± 0.01
	Fucose	3.04 ± 0.15	2.35 ± 0.10	1.37 ± 0.00	0.05 ± 0.00	0.25 ± 0.00	0.39 ± 0.00
	Arabinose	0.14 ± 0.01	0.11 ± 0.00	0.13 ± 0.01	0.04 ± 0.00	0.11 ± 0.00	0.15 ± 0.01
	Galactose	6.84 ± 0.17	6.84 ± 0.20	6.63 ± 0.11	0.36 ± 0.01	0.50 ± 0.03	1.32 ± 0.01
	Glucose	2.02 ± 0.07	1.97 ± 0.09	0.00 ± 0.10	0.00 ± 0.00	0.01 ± 0.00	0.03 ± 0.04
	Xylose	1.31 ± 0.06	0.79 ± 0.04	0.84 ± 0.02	0.43 ± 0.13	0.51 ± 0.01	0.43 ± 0.02
	Ethanol	-	0.00 ± 0.02	2.90 ± 0.02	3.26 ±0.01	4.43 ± 0.18	12.84±0.70
U. lactuca	Mannitol	0.06 ± 0.00	0.05 ± 0.05	0.06 ± 0.00	0.04 ± 0.07	0.03 ± 0.01	0.06 ± 0.04
	Fucose	0.11 ± 0.02	0.09 ± 0.00	0.10 ± 0.00	0.11 ± 0.00	0.01 ± 0.01	0.11 ± 0.00
	Arabinose	2.15 ± 0.00	2.15 ± 0.04	1.98 ± 0.08	2.03 ± 0.14	2.15 ± 0.03	1.98 ± 0.02
	Galactose	0.78 ± 0.00	0.71 ± 0.01	0.70 ± 0.03	0.04 ± 0.00	0.01 ± 0.00	0.04 ± 0.00
	Glucose	5.30 ± 0.07	5.28 ± 0.04	0.05 ± 0.00	0.00 ± 0.00	0.03 ± 0.00	0.00 ± 0.00
	Xylose	3.51 ± 0.02	3.49 ± 0.06	2.29 ± 0.04	2.43 ± 0.04	2.71 ± 0.02	2.06 ± 0.01
	Ethanol	-	0.26 ± 0.02	6.14 ± 0.22	3.49 ± 0.06	6.80 ± 0.34	7.31 ± 0.50

Overall, each strain, apart from *C. succiphila* NCYC 1403, managed to produce bioethanol; however the yields varied between strain and hydrolysate. The yeast strains produced more bioethanol on the red seaweed hydrolysates than the hydrolysates derived from both brown and green seaweeds, with the exception of *C. succiphila* NCYC 1403, which failed to produce any ethanol. *Candida* spp are obligate aerobes (need oxygen to respire) and are Crabtree-negative microorganisms. This means that that they respire and produce biomass independent of the sugar concentration as long as oxygen is available (Fredlund *et al.*, 2004). This explains the extremely low levels of ethanol produced in the anaerobic environment encountered during fermentation. Despite the fact that strain *P. anomala* TP12 is also a Crabtree-negative yeast, it demonstrated the ability to produce bioethanol in an anoxic environment from all five seaweed hydrolysates, with the highest ethanol yield of ca 3.5 g/L being produced from the green *U. lactuca* hydrolysate. There are reports that have revealed this strain to be ethanol-producing and potentially resistant to high ethanol concentrations (Passoth *et al.*, 2013, Tao *et al.*, 2011). Interestingly, a study by Ruyters *et al* (2015) revealed that *P. anomala* could be a candidate strain for ethanol production from lignocellulosic hydrolysates, as ethanol yields were similar to those with *S. cerevisiae*, and *P. anomala* displayed tolerance to high levels of ethanol. The group also found that *P. anomala* needed a longer fermentation time, which is in agreement with the results obtained from this study (up to 72 h) when *P. anomala* TP12 was inoculated into the seaweed hydrolysates (Figure 5.4). Although the oxygen-sensitive nature of this strain will undoubtedly limit its fermentation performance, it appeared that *P. anomala* TP12 and possibly other *P. anomala*

strains, are able to successfully ferment seaweed hydrolysates to bioethanol, and should, as a key group, be studied further.

The comparatively poor fermentation performance of the five strains on the brown seaweed hydrolysates resulted in the lowest ethanol yields (< 2 g/L ethanol for all strains). This is likely due to the lower levels of fermentable sugars in the hydrolysate, namely glucose and galactose. These sugars in total amounted to 2.08 and 1.86 g/L in the *L. digitata* and *F. serratus* hydrolysates, respectively; principally as a result of harvesting in May. A recent study by Schiener *et al* (2016) proved that seasonality does have an influence on the yields of glucose that can be hydrolysed from kelps and their subsequent conversion to bioethanol. Brown seaweeds which had been harvested during the autumn months had higher glucose and bioethanol yields, whereas lower yields were obtained from kelps harvesting during the winter and spring months. However studies have shown that brown seaweeds are suitable feedstocks for bioethanol production, as 30 g/L yields of bioethanol have been obtained from the fermentation of an *L. digitata* hydrolysate produced by enzyme hydrolysis (Hou *et al*, 2015). In order to make bioethanol production commercially viable from brown seaweed species, harvesting at the appropriate time period of the year for maximum sugar quantities and potentially further hydrolysis with specific enzymes (for cellulose hydrolysis) will most likely be required, as acid hydrolysis alone was not sufficient to generate an adequate yield of fermentable sugars. Furthermore, the salinity of the generated acid hydrolysates could have been a contributing factor to the yeast strains' poor fermentation performance. As the pH of the generated acid hydrolysates were adjusted to be within the range of pH 7.0 ± 0.5 prior to

fermentation, salinity levels may have increased. Unfortunately, salinity levels were not determined and compared across the five hydrolysates used in this trial.

Wild yeast strains *S. cerevisiae* Y12 and YPS128 were both able to consume the vast majority of monosaccharides (including pentose sugars) that were present in the hydrolysate of *C. crispus*. However *S. cerevisiae* Y12 generated 4.43 g/L of bioethanol, compared to *S. cerevisiae* YPS128 which produced the highest yield of bioethanol in this study of 12.84 g/L from the *C. crispus* hydrolysate. *S. cerevisiae* Y12 has been reported to be extremely sensitive to the presence of inhibitory compounds which arise as a result of the thermo-chemical pre-treatment of biomass (Wimalasena *et al.,* 2014). Hydrolysate from the pre-treatment of *C. crispus* was shown to have the highest ($P<0.05$) yield of HMF at ca 2 g/L, which could explain *S. cerevisiae* Y12's poor fermentation progress in this instance. Conversely, *S. cerevisiae* YPS128 displayed tolerance to inhibitory compounds (Wimalasena *et al*., 2014), and such phenotypes are highly desirable for bioethanol production. *P. anomala* TP12 was also able to consume the majority of the monosaccharides present in the hydrolysate of *C. crispus*, however only 3.26 g/L of ethanol was generated. It is possible that the sugars were converted to other carbon end point products in addition to bioethanol, such as xylitol, glycerol and trehalose (Boulton and Quain, 2008 and Devantier *et al,* 2005). To prove the influence of HMF, it would be necessary to detoxify the hydrolysate from *C. crispus* by selective removal of inhibitory compounds e.g. by activated charcoal, vacuum evaporation or adsorption by ion-exchange resins (see review by Mussatto and Roberto, 2004).

Interestingly, only the fucose which was present in the *C. crispus* hydrolysate (at a concentration of ca 3 g/L) was partially consumed by strains *S. cerevisiae* NCYC 2592, Y12 and *P. anomala* TP12 during fermentation (Table 5.4). This calls into question the correct assignment of the HPLC peak to fucose in this instance. This monosaccharide is typically only found in phaeophytes, not rhodophytes. Overall, the evidence presented here suggests that the *C. crispus* hydrolysate contained a monosaccharide which demonstrated chromatographic similarity to the fucose standard under our analytical conditions, and further investigation is required to determine structure e.g. isolation and NMR and LC-MS^n characterisation..

5.5 Conclusions

In this Chapter, a novel application of the PM assay to evaluate the ability of yeast strains to metabolize individual monosaccharides typically present in seaweed polysaccharides), as well as acid hydrolysates of five UK seaweed species, has been demonstrated. The most suitable yeast strains to utilize sugars derived from macroalgae and convert them into bioethanol were *S. cerevisiae* spp with maximum concentrations of bioethanol (ca 13 g/L) resulting from the fermentation of *C. crispus* hydrolysate by *S. cerevisiae* YPS128. Yeast stain *P. anomala* TP12 was able to utilize the majority of the monosaccharides present in the hydrolysate of *C. crispus* including a 'fucose-like' sugar, generating ca 3.5 g/L of ethanol, which was similar to yields of ethanol produced by *S. cerevisiae* strains NCYC 2592 and Y12 from the same hydrolysate. This revealed that *P. anomala* TP12 could be a possible candidate strain for further research. The present work was focused on yeast strain selection and did not seek to optimize ethanol yields from the fermentation of

the seaweed hydrolysates. This would be required in order to make the industrial production of ethanol from seaweed economically viable. Industrial production of ethanol from seaweeds would require the generation of more efficient strains that can ferment the majority of sugars contained in seaweed polysaccharides. Although ethanol was the only product screened for in this study, other high value, carbon end point products such as xylitol, succinic and acetic acids could also be screened for and sourced from the growth of similar or other microorganisms on seaweed hydrolysates.

6 Development of a novel bio-refinery process for biofuel and chemical production from *L. digitata*

6.1 Introduction

Previous work in this Thesis and from other studies suggested that there is potential to produce bioethanol from seaweeds. The gross chemical composition data from Chapter 3 revealed that an array of substrates are readily available for conversion into products and/or fuels; or could simply be extracted. Although bioethanol was produced from three different species of seaweed in Chapter 3, the production of bioethanol alone from seaweed feedstocks is of questionable viability in terms of sustainability and economics. Cost effective bio-processing routes towards chemicals and fuels need to be developed and despite seaweeds being an attractive feedstock to exploit, seaweed bioethanol is unfortunately a low value commodity. Therefore, the development of seaweed bio-refinery approaches which alongside bioethanol production, also recovers high value commodities and platform based compounds, are essentially a plausible route towards a more sustainable and environmentally friendly chemical and fuel industry.

The ultimate aim of this PhD project was to develop a bio-refinery concept based around the production of bioethanol and speciality chemicals from seaweeds. At the time of writing this thesis, a number of seaweed bio-refinery processes had already been investigated for the production of biofuels and commodity compounds (please refer to Chapter 1 for more detail). This had

been highlighted for example in the work outlined by Kumar *et al* (2013). Post the extraction of agar from red species *Gracilaria verrucosa* the residual pulp was converted into bioethanol; achieving an ethanol yield of 0.43g/g of sugar. Similarly, van der Wal *et al* (2013) generated not only bioethanol but also acetone and butanol from a hydrolysate derived from green seaweed *Ulva lactuca* using clostridial strains *C. beijerinckii* and *C. acetobuticum*. van der Wal *et al* (2013) solubilised over 90% of the sugars found in the green seaweed into solution which was then converted into useful fuels. Despite the success of generating three useful biofuel products, producing biofuels from the solubilisation of all functional polysaccharides inherent to seaweed biomass may in the long term jeopardise the seaweed hydrocolloid industry (Baghel *et al,* 2015). At the time of writing this thesis, the global hydrocolloid market was estimated to be valued at $5.70 billion and is expected to increase to $7.56 billion by 2020 (http://www.marketsandmarkets.com), compared to bioethanol which at the time was currently valued at $23.2 million (Renewable Fuels Association). Therefore the fractionation and selective utilisation of biofuel substrates, such as the cellulose fraction from seaweed biomass (which is a less utilised material) for the production of biofuels, would prevent negative impacts on the present hydrocolloid industry and associated worldwide markets. Additionally, the net worth of a seaweed bio-refinery for fuels and commodity compounds would also increase.

Brown species of seaweeds are amenable to bio-refinery processing as they contain a diverse array of metabolites with existing or potential applications. This includes extracellular matrix polysaccharides such as alginates and fucoidans, storage polysaccharides such as laminarin and mannitol, bio-active

polyphenolic compounds and pigments such as fucoxanthin (Lorbeer *et al*, 2013). The kelp *L. digitata* is one of the most prevalent species of brown seaweed found around the UK and French coastlines. It is by far the largest growing species within the brown taxonomic group (growing up to 4 m in length) and large scale mechanical harvesting of this species takes place in Brittany, France and Iceland (Adams *et al*, 2011). On average, approximately 60,000 tonnes (wet weight) of the seaweed is harvested annually in France (Hill 2008).

Brown seaweeds have been mainly exploited for the anionic polysaccharide alginate that is widely used in the pharmaceutical, food, cosmetic and biotechnology industries due to its favourable gelling properties (refer to Chapter 1). In recent years sulphated polysaccharides have received attention due to their specific biological activities and unique properties; in particular the properties of fucoidans have been of particular interest (Winberg *et al*, 2014). Section 1.3.5.1.6 discusses in detail the unique properties and potential biological activities possessed by fucoidans. Furthermore, a crude extract of fucoidan from brown seaweed *Fucus vesiculosus* was priced at £386.00 for 1 g at the time of writing this thesis (Sigma-Aldrich website accessed 20/02/16).

Results obtained from Chapter 5 identified difficulties in finding a suitable wild type microorganism capable of fermenting fucose, the monomer that forms the basis of the fucoidan backbone. Despite the noted ability of one *Candida* strain to metabolise fucose (although it was unclear whether bioethanol was the metabolic carbon end point product after its uptake into the cell), the sulphated polysaccharide is not considered to be a suitable substrate for bioethanol production. It was therefore decided to attempt to develop a

bio-refinery process based around the extraction of fucoidan from the UK native seaweed *L. digitata*. A bio-refinery process for *L. digitata* was conceived and is depicted in Figure 6.1.

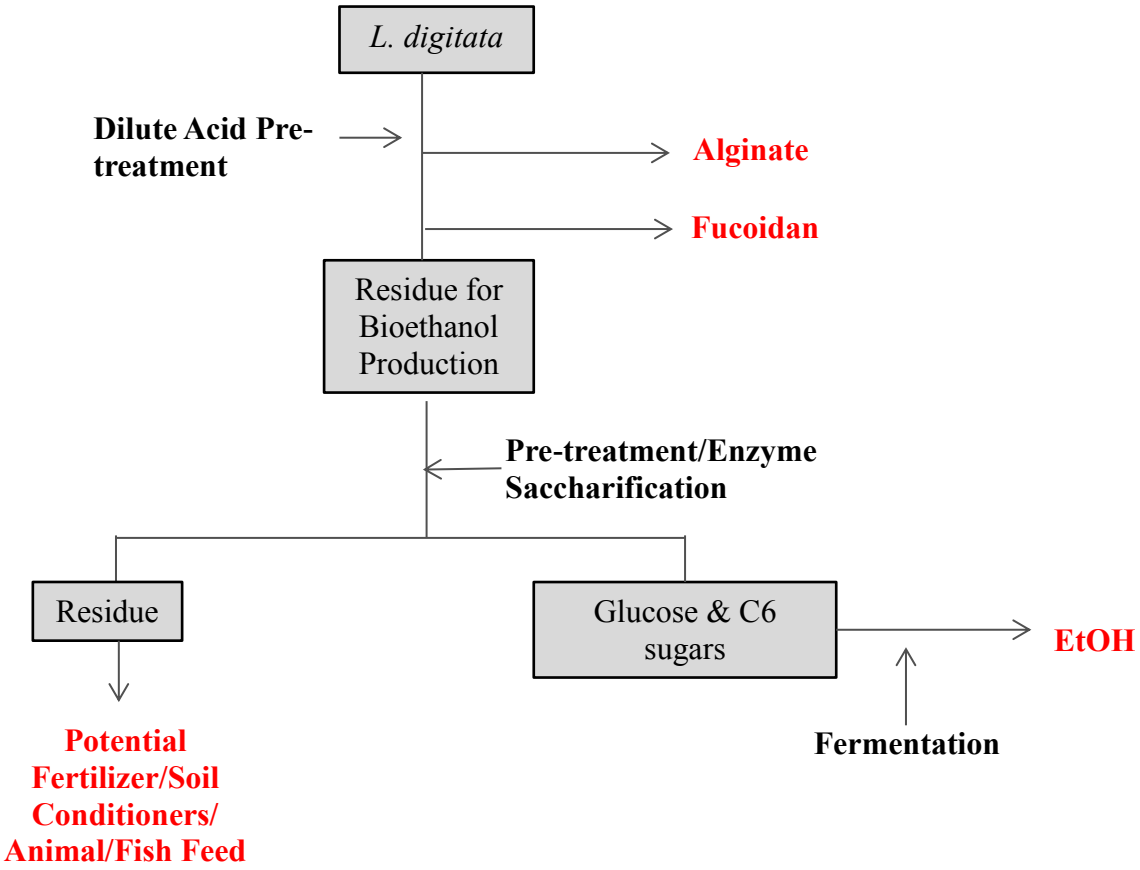


Figure 6.1 Overview of the proposed bio-refinery extraction process for *L. digitata*

A dilute acid pre-treatment was employed for the extraction of the sulphated polysaccharide from *L. digitata* biomass. After successive rounds of modifications to the extraction process were made, the process was scaled up to pilot plant level in order to extract and generate sufficient yields of product/waste material for further analysis. The fucoidan extract was evaluated for biological activities and attempts were then made to produce

bioethanol from the remaining *L. digitata* residue. Waste streams from the developed process were also analysed for potential value.

6.2 Materials and Methods

6.2.1 *L. digitata* collection and preparation

The first batch of *L. digitata* was prepared as described in section 2.3. The second batch of *L. digitata* for the pilot plant trials was sourced from Setalg (France) and arrived as a dried milled powder with a particle size of <250 μm.

6.2.2 *L. digitata* composition analysis

Composition analysis of the first batch of *L. digitata* collected from waters around Plymouth (UK) was evaluated according to the methods described in Chapter 2 (Section 2.4). Composition analysis of the Setalg *L. digitata* sample was conducted externally by Eurofins Food Testing UK Ltd.

6.2.3 Fucoidan extraction

Two methods were evaluated and compared for the extraction of fucoidan from *L. digitata*. The methods were based on prior literature (Black *et al* 1952 and Kim *et al* 2014) and were selected due to their suitability to be conducted in the laboratory and potential for scale-up to pilot plant operation.

6.2.3.1 Black et al (1952) –Extraction Method 1

2 g *L. digitata* (as prepared in section 6.2.1) was mixed with 20 ml of 0.1 M HCl (pH 2-2.5) at 70°C for 1 h. The mixture was stirred at 250 rpm in order to produce a slurry that was then centrifuged at 5000 rpm for 15 min to separate the liquid from the seaweed solids. One volume of 1% (w/v) $CaCl_2$ was added to the recovered liquid, mixed by inversion and kept at 4°C for 72 h. The

precipitate which formed was removed from the liquid phase by centrifugation at 5000 rpm for 20 mins and dried in an oven at 40°C until perceived to be dry. Two volumes of ethanol (absolute) were added to the recovered liquor and the mixture was inverted and kept at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000 rpm for 20 min and the upper liquid phase was discarded. The solid precipitate was dried in an oven at 40°C and analysed for fucoidan purity as described in section 6.2.8.1. This process was repeated in duplicate on the remaining seaweed solids.

6.2.3.2 Kim et al (2014) –Extraction Method 2

2 g *L. digitata* (as prepared in section 6.2.1) was mixed with 60 ml of 1% (w/v) $CaCl_2$ solution and heated to 85°C for 4 h in a water bath. The mixture was stirred at 455 rpm. The liquid phase was then separated from the seaweed solids by centrifugation at 5000 rpm for 15 min before being mixed with two volumes of ethanol (absolute) and one volume of 1% NaCl solution. This mixture was kept at -20°C for 48 h in order to form a precipitate that was ultimately removed by centrifugation at 5000 rpm for 20 min. The precipitate was completely re-suspended in deionised water (100 ml) and the solution was dialysed for 48 h at 4°C using a 15 kDa cut off dialysis membrane. The dialysed fucoidan was then freeze dried until all the water was removed leaving behind the fucoidan powder. The fucoidan was analysed for purity as described in the method outlined in section 6.2.8.1. This process was performed in duplicate on the remaining seaweed solids.

6.2.4 Modifications to Extraction Method 1

The original Black *et al* (1952) extraction method outlined in section 6.2.3.1 was further modified in order to be compatible with the facilities and at

Croda's pilot plant in Leek. The following stages of the extraction process were investigated for further modifications:

6.2.4.1 Consolidation of the triple acid re-extraction to one extraction

To increase the rate of the extraction process and to eliminate the requirement for the triple acid re-extraction from *L. digitata,* the process outlined in Section 6.2.3.1 was modified. The volume of 0.1 M HCl (ca pH 2) was increased by a factor of 3, hence the extraction was conducted at a solids loading rate of 3.3% (w/v) as opposed to the original 10% (w/v). Furthermore, the extraction time increased from 1 h to 3 h yet the extraction temperature remained at 70°C. Figure 6.2 shows the experimental set-up, whereby 100g *L. digitata* was mixed with 3 L of 0.1 M HCl in a 5 L beaker for 3 h, in a water bath at 70°C. A high torque mixer (SS30 overhead stirrer, Stuart Scientific, UK) set at180 rpm was used to ensure the extraction was homogeneous. The viscosity of the slurry post extraction was monitored visually to ensure it was free-flowing in order to prevent the potential for blockages when conducted at pilot scale. The liquor was separated from the *L. digitata* solids by centrifugation at 5000 rpm for 20 min, stored in an airtight container and refrigerated until further use.

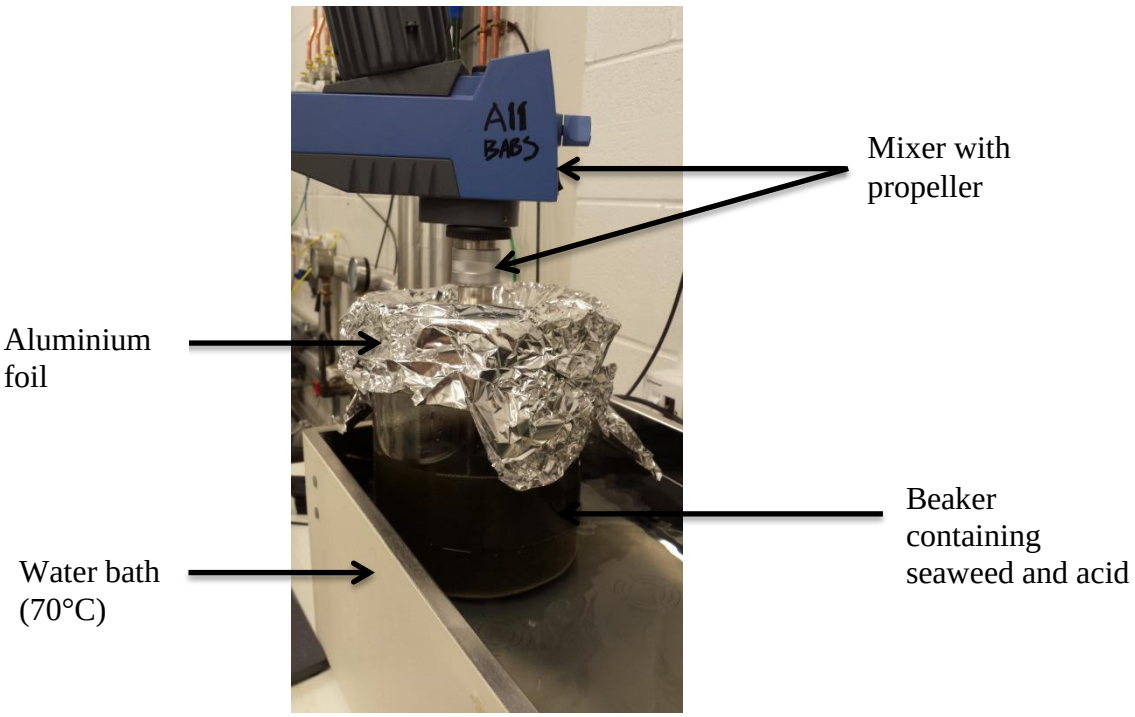


Figure 6.2 Experimental set up for alginate and fucoidan extraction from *L. digitata*; method development using a single batch extraction

6.2.4.2 Evaluation of the efficacy of fucoidan and alginate extractions at ambient temperatures (20°C ± 1°C)

Due to the non-availability of a 4°C refrigeration unit at Croda's pilot plant site, the efficacy of the extraction process was evaluated at room (laboratory) temperature as compared to the original 4°C. The impact of reaction (precipitation) time was also investigated in an attempt to shorten the duration of the extraction. The investigated extraction conditions are listed in Table 6.1.

Table 6.1 Extraction conditions under investigation for the precipitation of alginate and fucoidan

Extraction Temperature (°C)	Extraction Time (h)			
4°C	12	24	48	72
Laboratory Temperature (20°C ± 0.1)	12	24	48	72

Precipitates were generated from the liquor collected in section 6.2.4.1 following extraction using the different conditions outlined in Table 6.1 above. Alginate and fucoidan precipitates were subsequently recovered by centrifugation at 5000 rpm for 20 min and dried in an oven at 40°C prior to mass determination. Fucoidan purity was assessed by the method described in Section 6.2.8.1 and compared against alginate extraction time.

6.2.5 Further method development at Croda Ltd, Leek

6.2.5.1 Extraction set up

Figure 6.3 depicts the apparatus set up in the laboratory at Croda for the extraction of alginate and fucoidan.

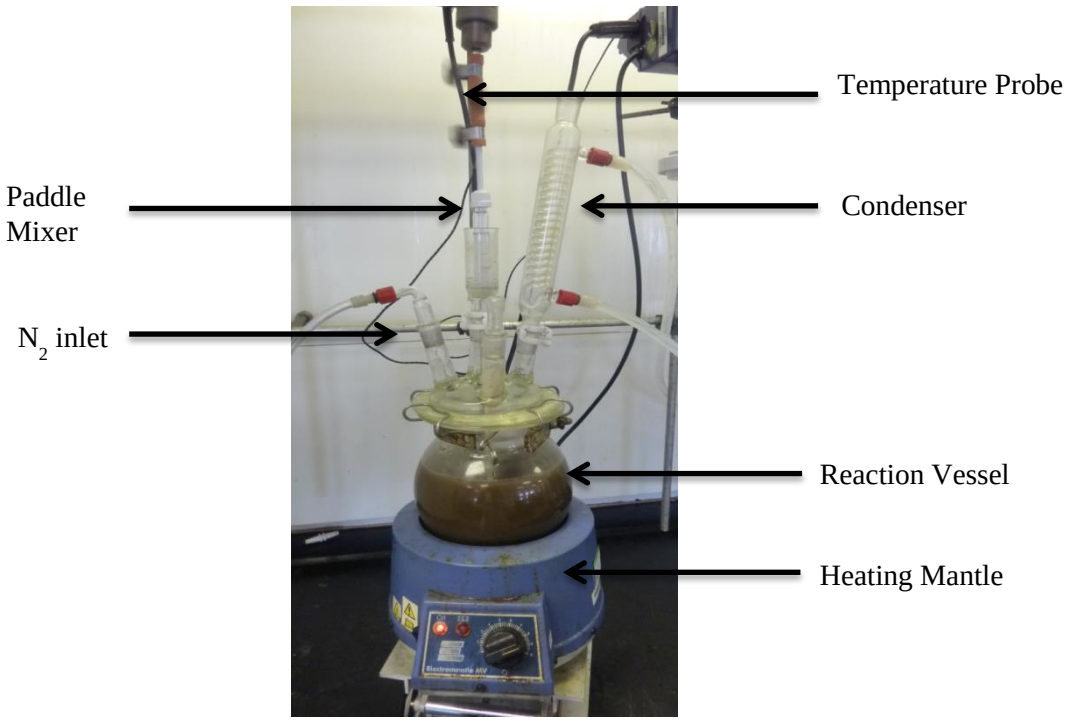


Figure 6.3 Set up of alginate and fucoidan extraction in the laboratory at Croda.

As opposed to using a water bath with a high torque mixer to agitate the reactants (Figure 6.2), smaller scale apparatus which mimic the large scale reactors used in the pilot plant were set up in a fume cabinet. *L. digitata* (50 g; Setalg) was mixed with 1.5 L of 0.1 M HCl in a 5 L reaction vessel for 1 h, with the temperature maintained at a stable 70°C using a heating mantle. The contents of the vessel were stirred with a mixing paddle set at 200 rpm. The condenser prevented the loss of water which could arise as a result of heating the extraction to 70°C and an inflow of nitrogen through an inlet enabled the reaction to remain stable.

6.2.6 Pilot plant scale up extraction

A finalised process schematic conceived from the various rounds of method development at the laboratories at Nottingham and Croda Ltd is outlined in Figure 6.4. This process flow schematic was followed for the pilot scale

extraction of fucoidan and alginate from *L. digitata* (Setalg). 130 L of 0.1 M HCl was charged into the PP1 reactor (max. 150 L capacity) and the pH was adjusted to ca 2.1 with concentrated NaOH (40% w/v) with a slight agitation of 200 rpm. When the pH was adjusted accordingly, 4.73 kg of the powdered *L. digitata* was charged into the reactor. The reactants of stage 1 of the process were heated to 70°C and held at this temperature for 1 h. The slurry was forced under pressure out of the reactor and through a filtration unit containing an enclosed 20 μm General Application Filter (GAF) to capture *L. digitata* solids (Figure 6.5). The filtrate was collected into a pre-weighed drum and the wet seaweed solids were weighed in the laboratory before being dried in an oven at 40°C and brought back for analysis at Nottingham. The mass of the filtrate in the pre-weighed drum was recorded.

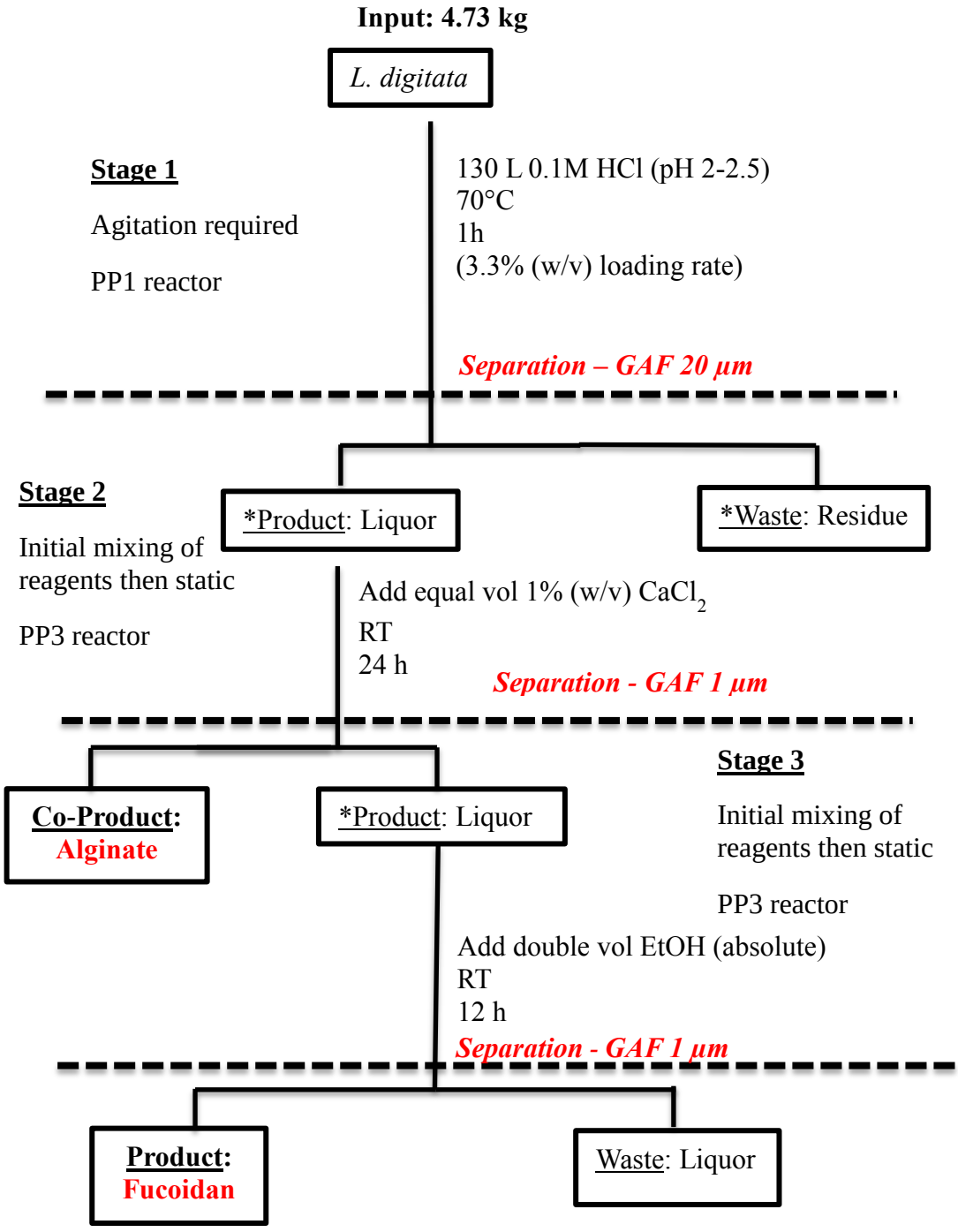


Figure 6.4 Finalised process schematic for the extraction of alginate and fucoidan from *L. digitata* (Setalg) carried out at pilot plant scale

The filtrate collected from stage 1 of the process was subsequently charged into the PP3 reactor (max 1 tonne capacity) and an equal volume of 1% (w/v) $CaCl_2$ was added. The contents of the PP3 reactor were initially mixed at 95

rpm for 15 min before being left static at room temperature for 24 h. The precipitate formed in stage 2 of the process (alginate) was separated from the liquor under pressure out of the reactor and through a filtration unit containing a 1 μm GAF. It soon became apparent that the 1 μm GAF was not capturing the alginate co-product and the entire contents of the PP3 reactor were decanted into the drum. After careful analysis of the GAF, it appeared that the wrong size had been ordered and a 3 μm was being utilised instead of the 1 μm GAF which was actually required. Therefore a 0.45 μm candle filter was bolted onto the filtration system after the 3 μm GAF unit in order to capture the alginate co-product (Figure 6.6). The candle filter had to be opened up in order to recover the captured alginate. The recovered alginate was mixed with DI water and stored in an inert, airtight container to be transported back to Nottingham where the co-product was freeze-dried and weighed for mass balance closures.

Figure 6.5 Stage 1 extraction set up using PP1 reactor at Croda's pilot plant site in Leek

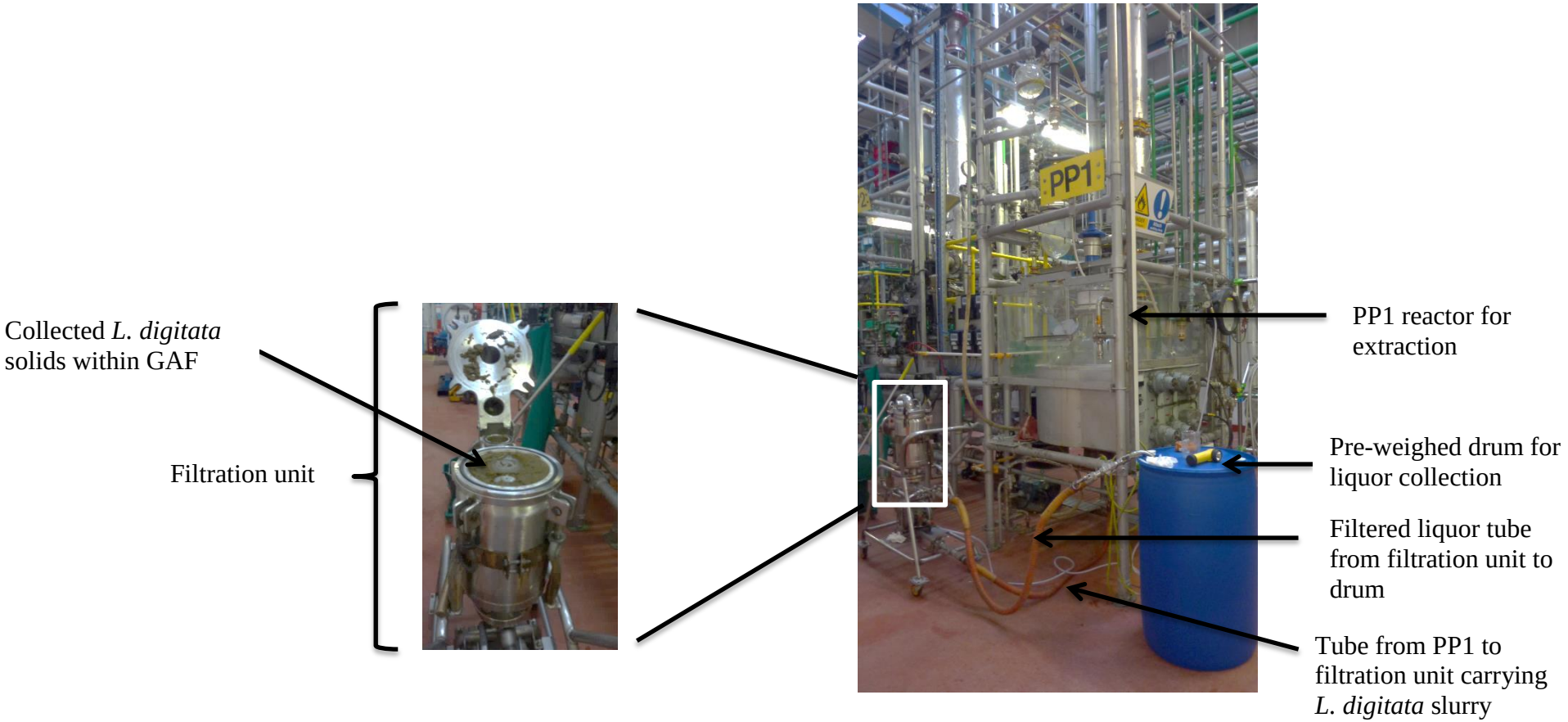
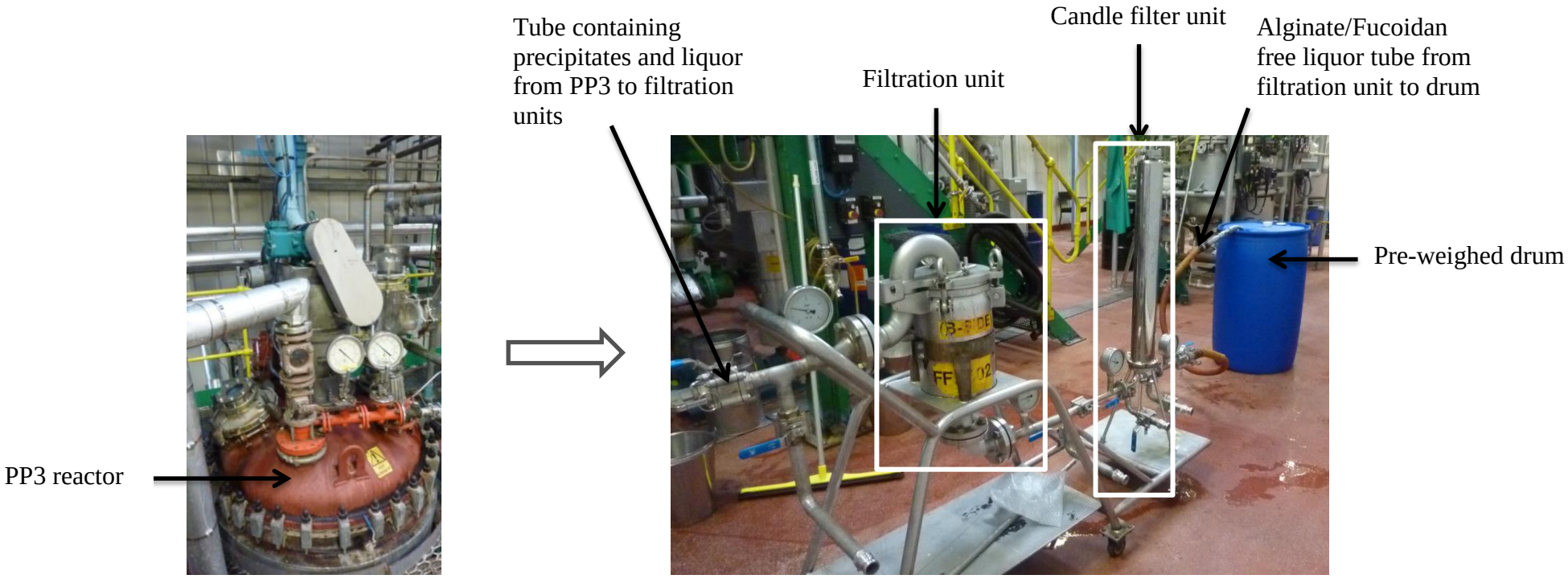


Figure 6.6 Stage 2 and 3 extractions set up using PP3 reactor at Croda's pilot plant site in Leek



After the PP3 reactor had been cleaned with ethanol and water, the alginate-free liquor generated from stage 2 of the process was charged back into the reactor. Double the volume of ethanol (absolute) was added, calculated from the weight of recovered liquor generated during stage 2 of the process. The contents of the reactor were again agitated at 95 rpm for 15 min and then remained static for 12 h at pilot plant room temperature. Separation of the fucoidan extract product from the liquor was achievable under pressure filtration with the 3 μm GAF; therefore the candle filter unit was not required. The GAF was cut open and the product was washed off with DI water. The fucoidan was stored in an inert, airtight container for transportation back to Nottingham where the product was freeze-dried, weighed and stored in an airtight container. The fucoidan extract was stored at -80°C until further analysis.

6.2.7 Residue composition analysis

L. digitata (Setalg) residue generated from the process outlined in section 6.2.3.5 was analysed for moisture, ash, protein, lipid, crude fibre and carbohydrate contents. This was conducted externally by Eurofins Food Testing UK Ltd.

6.2.8 Characterisation of fucoidan extract

6.2.8.1 Fucoidan extract quantification and determination of purity

Fucoidan extracts were prepared for quantification and purity by making a 10 g/L solution with DI water. The samples were run on a HPLC using an AS-2055 Intelligent Auto-sampler and a PU-1580 Intelligent HPLC Pump (Jasco, Japan). The Rezex ROA Organic Acid H+ organic acid column (5 μm, 7.8

mm×300 mm; Phenomenex, UK) was operated at ambient temperature with a mobile phase of 0.005 N H_2SO_4 at a flow rate of 0.5 mL min^{-1}. A Refractive Index cell (RI-2031 Intelligent Refractive Index detector, Jasco, Japan) was used for detection, and the injection volume was 10 µL. Data were acquired using the Azur software package v. 4.6.0.0 (Datalys, France). Prior to HPLC analysis, all samples and standards were filtered using Whatman GD/X syringe filters (GF/C 25 mm filter diameter/1.2 µm pore size; Whatman, UK). All experiments were conducted in triplicate. Authentic standards of fucoidan with concentrations within range of 10 g/L^{-1} to 0.5 g/L^{-1} were used for quantification. Fucoidan purity was calculated according to the following equation.

$$\%\ Purity\ = \frac{Peak\ Area\ of\ Extract}{Peak\ Area\ of\ Standard} \times 100$$

6.2.8.2 Monosaccharide profile

The monosaccharide profile of the extracted fucoidan was obtained by following the method of Rodriguez-Jasso *et al* (2011). Fucoidan extract (10 ± 0.2 mg) was hydrolysed with 2 M Trifluoroacetic acid (0.5 mL) at 121°C for 2 h in N_2 sealed heat resistant screw cap glass tubes. The tubes were then cooled in an iced water bath before being centrifuged at 5000 rpm for 5 min. Samples were then prepared for monosaccharide quantification using the HPAED-PAD method described in Chapter 2 Section 2.7.1.

6.2.8.3 Sulphate content

The sulphate group content of the extracted fucoidan was determined using a sulphate assay kit (Sigma-Aldrich®, USA) according to manufacturer's

instructions. Proprietary reagents were mixed to induce a concentration dependant colour change which was read at 600 nm using a Jenway Spectrophotometer. Quantification was performed by comparison to proprietary standards of barium sulphate (Sigma Aldrich; USA) over a range of concentrations (0, 0.5, 1.0, 2.0 mM).

6.2.8.4 FT-IR

The IR spectra of the extracted fucoidan and of a standard of fucoidan (Sigma-Aldrich®) were determined using a Fourier transform infrared spectrophotometer (FTIR) (Bruker VERTEX 70) and ATR probe with Golden Gate attachment. The software used for this analysis was OPUS 3.0 (Bruker). Spectra were recorded in the wavenumber range of 600 to 4000 cm^{-1} using 132 scans.

6.2.9 Bioactivity screening on selected fractions from the bio-refinery process

6.2.9.1 Extract preparation

Native seaweed (*L. digitata*), the waste residue following extraction (*L. digitata* residue), waste liquor from the process, the fucoidan extract and a standard of fucoidan were investigated for selected bioactivity analysis. The extracts were prepared in either methanol and/ or water in order to evaluate which solvent gave the best extraction. The fucoidan extracts however, were only prepared using water due to their insolubility in methanol. Table 6.2 provides of the sample codes used to identify the prepared extracts.

Table 6.2 Sample codes for the extracts of fractions from the *L. digitata* biorefinery process which were analysed for selected bioactivities.

Sample	Water Extraction	Methanol Extraction
L. digitata	LD-W	LD-M
Waste Residue	WR-W	WR-M
Waste Liquor	WL-W	WL-M
Fucoidan Extract	FE-W	n.a
Fucoidan Sigma	FS-W	n.a

Glass beads (100 mg, 1.0 mm) were added to ca 20 mg of biomass/residue in an Eppendorf tube followed by the addition of 1 mL of cold methanol (100%) or water. The samples were placed in a tissue lyser (Qiagen TissueLyser II, USA) for 10 min at 30 Hz and then centrifuged at 14,000 rpm for 1 min. The supernatant was decanted into an evaporation tube and stored on ice. A further 1 mL of methanol/water was added to the Eppendorf tube and the process was repeated one more time, pooling together the relevant supernatants. In order to concentrate the extract, the solvents were evaporated and then the residue re-suspended in a reduced volume (1 mL) of either methanol and/or water. Extracts were then stored in amber vials at -80°C until further use.

6.2.9.2 Determination of antioxidant activity

6.2.9.2.1 DPPH$^{\bullet}$ scavenging capacity assay

A 200 μM solution of DPPH was prepared in methanol (100%) in an amber conical flask to avoid contact with light. For the assays, 100 μL of DPPH (200 μM) solution was mixed with 100 μL of extract and incubated in the dark at 30°C for 30 min. The reduction of the DPPH$^{\bullet}$ radical was measured by continuous monitoring of decolourisation at 518 nm. The control solutions

contained 100 µL of distilled water or 100 µL methanol (100%). $DPPH^{\bullet}$ percent of inhibition was calculated according to the following equation:

$$Inhibition\ (\%) = [(1 - A_{\text{sample_518}}/\text{A}_{\text{control_518}})] * 100$$

EC_{50} values were also calculated which indicates the concentration of sample required to scavenge 50% DPPH radicals, according to the following equation:

$$\text{EC}_{50} = \left[\frac{\text{sample concentration (mg/ml)}}{\text{DPPH inhibition (\%)}}\right] \times 50$$

6.2.9.2.2 $ABTS^{\bullet+}$ scavenging capacity assay

The $ABTS^{\bullet+}$ assay was carried out according to the protocol outlined in the work of Martinez-Avila *et al* (2012). In order to generate the radical ($ABTS^{\bullet+}$), 12.5 mL of potassium persulfate (2.45 mM) was mixed with 25 mL of ABTS (7 mM). The mixture was maintained in the dark at room temperature for 12-16 h. The absorbance was measured at 734 nm and the $ABTS^{\bullet+}$ solution was then diluted with ethanol until an absorbance value of 0.7 ± 0.01 was achieved. For the assays, 950 µL of $ABTS^{\bullet+}$ solution was added to 50 µL of extract and the absorbance was measured after 1 min of the reaction. The control solutions contained 50 µL of distilled water or 50 µL methanol (100%). $ABTS^{\bullet+}$ percent of inhibition was calculated according to the following equation:

$$Inhibition\ (\%) = [(1 - A_{\text{sample_734}}/\text{A}_{\text{control_734}})] * 100$$

The radical-scavenging capacity of each sample was calculated according to a Trolox standard curve (0 to 50 µM in 5 µM increments) and expressed as Trolox equivalent antioxidant capacity (TEAC) by the extrapolation of

$ABTS^{\bullet+}$ percent inhibition of each tested sample. Assays were conducted in triplicate.

6.2.9.3 Determination of antimicrobial activity

6.2.9.3.1 Preparation of bacterial broths, agar plates and culturing of bacterial strains

Eight human pathogenic strains and 3 marine pathogenic strains were investigated in this study (Table 6.3). The strains were harvested from local waters around the Plymouth shoreline. Two types of bacterial broth and agar plates were made depending upon bacterial strain. LB broth (for human pathogenic strains): 5 g bactopeptone, 1 g yeast extract made up to 1L with DI water. Marine LB broth (for marine pathogens): 5 g bactopeptone, 1 g yeast extract made up to 1L with filtered sea water. YPD plates were made following the same recipe as mentioned above for both pathogen types, however contained 2 g agar. The bacterial strains were taken from glycerol stocks stored at -80°C and spread onto appropriate YPD plates and kept at 26°C for 24 h. A loop-full of cells were then inoculated into 3 mL of LB/marine LB broth. Cultures were placed in a shaking incubator at 26°C for 24 min at 130 rpm.

Table 6.3 List of human and marine pathogens included in this study with their corresponding strain IDs.

*Marine pathogens.

Strains	Strain ID
Staphylococcus aureus	1
Salmonella enterica	2
Burkholderia cenocepacia	3
Burkholderia multivorans	4
Bacillus cereus	5
Streptococcus pyogenes	6
Acinetobacter iwofii	7
Listeria monocytogenes	8
**Yersinia ruckerii*	9
**Vibrio anguillerium*	10
**Aeromonas hydrolphila*	11

6.2.9.3.2 Agar disc diffusion assay

Cells (taken from section 6.2.7.3.1) were subsequently diluted 1/100 into 25 mL of LB/marine LB agar and the molten agar was poured into a sterile petri dish. Sterile Whatmann discs (10 mm diameter) were gently placed onto the dish and numbered (Figure 6.7). Each number corresponded to an extract generated from section 6.2.7.1 (see Table 6.3 for extract number codes). 20 μL of extract was inoculated onto the disc and plates were then incubated for 24 h at 30°C for bacterial growth to occur. Zones of clearance around each disc were indicative of antimicrobial activity and were examined by eye. Each extract was tested in triplicate and water and methanol were included in this trial as controls.

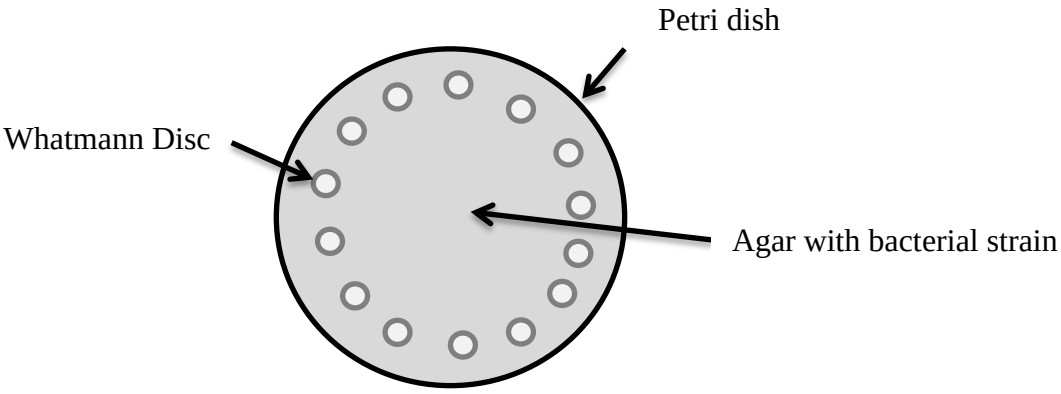


Figure 6.7 Schematic representation of the spot plate set up to evaluate the antimicrobial activity of extracts generated from bio-refinery process against 11 pathogens

6.2.9.3.3 Validation test of antimicrobial activity

Results generated from the method described in section 6.2.7.3.2 were highly subjective as any observed zones of clearance were assessed visually. Therefore an assay was set up in order to validate the findings from section 6.2.7.3.2. To confirm the agar disc diffusion assay results, antimicrobial activity was also evaluated in liquid media. Bacterial strains (from 6.2.7.3.1) were diluted 1/50 in LB/marine LB broth and 180 µL of the broth and cell mix were subsequently pipetted into a well on a 96 well plate. Extract (20 µL) was added and the plates were incubated for 24 h at 30°C. Absorbance was measured at 600 nm using a plate reader and absorbance values were compared against the absorbance of wells containing only LB/marine LB broth.

6.2.10 Bioethanol production from the bio-refinery process residue

6.2.10.1 Preliminary screening of enzymes for saccharification of the residue

A range of different enzymes (Table 6.4) were investigated in attempts to identify the most suitable enzyme or enzyme mixture for saccharification of the *L. digitata* residue for maximum glucose liberation. All enzymes were dosed in substantial excess for proof of principle, unless otherwise stated.

Table 6.4 List of enzymes investigated on the *L. digitata* residue generated from the bio-process

Enzyme	Supplier	Enzyme Specifications
β-Glucanase	Sigma	A mixture of β-1→3/1→4-glucanase, xylanase and cellulase activities
Driselase	Sigma	Crude powder containing laminarinase, xylanase and cellulase
Cellic® CTec 2	Novozymes	Contains a blend of aggressive cellulases, hemicellulases and high levels of β-glucosidases
Laminarinase	Sigma	Digestion of β-1,3-glucan (laminarin)

***β-Glucanase*:** A β-glucanase solution of 10% (w/v) concentration was prepared in 50 mM sodium acetate buffer (pH 5) to which the correct quantity of dry *L. digitata* residue was added to achieve a 1% (w/v) loading rate in 15 mL falcon tubes. The samples were then incubated for 72 h in a shaking incubator at 30°C and with agitation at 120 rpm. This dosing was considered to be greatly in excess of the 0.1% (w/v) dose rate used in the work of Fleita *et al.* (2015). The specific activity of β-glucanase was 1 Unit/mg. One unit was defined as the amount of enzyme required to release one μM of glucose from cellulose per hour at pH 5 at 37°C.

***Driselase*:** A driselase concentration of approximately 20% (w/v) was prepared in 50 mM sodium acetate buffer (pH 5) to which the correct quantity of dry *L. digitata* residue was added to achieve a 1% (w/v) loading rate. The samples were incubated for 72 h in a shaking incubator set at 120 rpm. This was a novel application of this enzyme on seaweed based biomass; therefore enzyme dosing rates were greatly in excess of the 1.5% (w/v) and 2% (w/v) dosing used in Lingard and Bartel (2009) and Pignocchi *et al*. (2009) in which driselase was used to hydrolyse *Arabidopsis* cell walls. The specific cellulase activity was 100 Unit/g; one unit was defined as the amount of enzyme required to release one μM of glucose from cellulose per hr at pH 5.0 at 37°C. The specific laminarinase activity was 10 Units/g; one unit was defined as the amount of enzyme required to release one μg of glucose from laminarin per min at pH 5.0 at 37°C. The specific xylanase activity was 3 Units/g; one unit was defined as the amount of enzyme required to release one μg of reducing sugar from xylan per min at pH 4.5 at 37°C.

***Cellic® CTec 2*:** 0.1 g of dry *L. digitata* residue (section 6.2.3.5) was mixed with 20 mL of 50 mM sodium citrate buffer (pH 5) and dosed with Novozymes Cellic® CTec2 (ca. 50 FPU/g biomass). The samples were then incubated at 50°C for 72 h in a shaking incubator set at 120 rpm.

***Laminarinase*:** The specific activity of laminarinase was 0.2 Unit/mg. One unit was defined as the amount of enzyme required to release one μg of reducing sugar (defined as glucose) from laminarin per min at pH 5 at 37°C. Scullin *et al* (2015) described the use of 0.005U of glucan. However, an excess enzyme dosage of 0.5U/ g *L. digitata* residue was used in this assay. The enzyme was prepared in sodium acetate buffer (pH 5.0) with a biomass

loading rate of 1% (w/v). The samples were then incubated for 72 h in a shaking incubator set at 120 rpm.

All enzyme reactions were conducted in triplicate. The enzymes were all dosed at levels which were considered to be in excess in order to maximise the potential for hydrolysis of the *L. digitata* residue. Enzyme dosing was not optimised in any way in the work conducted in this Chapter. Amounts of liberated glucose in the enzyme hydrolysates generated from each saccharification reaction were then quantified by HPAED-PAD (section 2.7.1) and calculated as the achieved percentage theoretical yield of glucose. This was calculated using the following equation:

$$\frac{Yield\ of\ Glucose\ liberated\ post\ enzyme\ hydrolysis\ (\frac{mg}{g})}{Glucose\ content\ \ of\ L.digitata\ residue\ (\frac{mg}{g})}\ X\ 100$$

Additional samples were taken at the start of each reaction (T_0 controls) in order to quantify any glucose which may already have been present in the enzyme preparation. This could then be subtracted from the achieved yields for accurate quantification of enzyme effectiveness.

6.2.10.2 Pre-treatment screen on residue

The processed residue of *L. digitata* was subjected to three different forms of treatment in order to determine the most compatible pre-treatment with the new feedstock. The optimum pre-treatment conditions for *L. digitata* investigated in Chapter 3 (1.5N H_2SO_4, 121°C, 24 min, 25% (w/v) solids loading) were used as a starting point for bioethanol production. An auto-

hydrolytical treatment was additionally run alongside the acid pre-treatment by way of comparison. This involved treating the process residue with water at a 25% (w/v) solids loading rate for 24 min at 121°C in a bench top autoclave. Residues post pre-treatment were recovered and evaluated for pre-treatment efficacy following the methods outlined in sections 3.2.2.3 and 3.2.2.6. An enzyme hydrolysis was also performed directly on processed *L. digitata* residue without any prior pre-treatment following the method in section 3.2.2.6. Amounts of glucose present in the enzyme hydrolysates were quantified by HPAED-PAD (section 2.7.1) and calculated as mg of glucose released per g of treated *L. digitata* composite. Achieved percentage theoretical yield of glucose were also determined. All trials were conducted in triplicate.

6.2.10.3 Laboratory scale fermentation of residue hydrolysate for bioethanol production

Hydrolysates (section 6.2.8.2) were fermented using *S. cerevisiae* strain NCYC 2592 following the method described in section 2.6. Final glucose and ethanol yields were quantified by HPLC (section 2.7.3). All fermentation experiemnts were conducted in triplicate.

6.3 Results and Discussion

This section is structured in two main parts. The first part outlined in sections 6.3.1 to 6.3.4 focusses on the development of the bio-refinery process based around the extraction of fucoidan from *L. digitata* biomass. The method is successively modified and developed from laboratory scale to pilot plant level. The second part in sections 6.3.5 to 6.3.8 assesses the fucoidan extract and certain process products and waste streams for potentially useful bioactivities.

Lastly, results are reported for bioethanol production from the seaweed residue which remained at the end of the bio-refinery process.

To date, a number of bio-refinery strategies have been devised for brown seaweeds in particular, which involve the use of novel technologies in order to recover sulphated polysaccharides and anionically charged polysaccharides such as alginates. These include supercritical CO_2 extractions, auto-hydrolysis, ultrafiltration and microwave based extractions (Balboa *et al,* 2013, Gonzalez-Lopez *et al*, 2012, Perez-Lopez *et al* 2014, Quitain *et al*, 2013a, Quitain *et al*, 2013b) . Despite these technologies having the potential to recover compounds that are in general more challenging to extract and to utilize less water and chemicals, their adoption at industrial scale may be limited. This is because such techniques require comprehensive and expensive infrastructural upgrades and instalments that various industries may be reluctant to adopt for scale up purposes. Therefore the simplicity of acid based extraction methodologies were preferred in the present work, particularly because of the fit with existing processing capability at Croda's pilot plant (Leek).

6.3.1 Comparison of the 2 extraction methods on *L. digitata*

There is currently no standardised extraction or purification protocol for fucoidan and other fucose containing sulphated polysaccharides (Ale *et al*, 2011), however protocols may involve multiple, aqueous based extractions usually with hot acid (hydrochloric acid). Treatment with acid disrupts the hydrogen bonds between polysaccharides, and this liberates the fucoidan into solution for subsequent recovery (Kim *et al*, 2014). The extractions may further include the addition of a calcium salt to promote alginate precipitation (Chizhov *et al*, 1999, Marais and Joseleau 2001). The maximum yields of

fucoidan that can be extracted from brown seaweeds are usually within the range from 5-7% (on a dry weight basis). Various parameters may influence the final yields of extractable fucoidan from brown species of seaweed, including extraction time duration, extraction temperature and acid concentration/pH. Such parameters may additionally have a direct influence on the sulphated polysaccharide's chemical composition (Li *et al*, 2008). This was highlighted in work conducted by Ale *et al* (2012) who assessed the influences of such parameters on the composition of extracted fucoidan from the seaweed *Sargassum henslowianum*, using a single step extraction procedure. Ultimately, the study revealed that the structural integrity of the compound was best conserved using lower acid treatments. Higher acid treatments with elevated extraction times and temperatures resulted in a lower fucose content, suggesting that the harsher extraction parameters may cause a loosening of the cell wall matrix which then allows local penetration of the acid into the fucoidan polymer. Furthermore, other studies have revealed that a prolonged extraction times led to obtaining an extract with a lower degree of sulphatation and fucose content, but higher proportions of glucuronic acid (Ponce *et al*, 2003). Although extraction parameters should be taken into consideration, especially when attempts are made to extract the polysaccharide from different species of brown seaweed, optimisation of the extraction method was not included in the work conducted in this Chapter. Alternatively, two methodologies were chosen from the literature and compared for final yields and purity of fucoidan obtainable from *L. digitata* biomass. Both methodologies were additionally assessed for their feasibility to be conducted in the laboratory and at pilot scale. Extraction method 1, developed by Black

et al (1952), recommended the use of a three-step hot acid extraction procedure with the addition of $CaCl_2$ for the removal of alginate. Extraction method 2 followed the method outlined by Kim *et al* (2014) does not utilise a hot acid extraction, but includes additional extraction steps such as a dialysing stage. In contrast to extraction method 1, alginate was not directly removed during the process. The yields of extracted fucoidan from both methodologies including fucoidan purity and a comparison of recovered seaweed solids are reported in Table 6.5.

Table 6.5 Comparison of the two methods under investigation for the extraction fucoidan from *L. digitata*

	Extraction Methodologies	
	Black *et al* (1952)	Kim *et al* (2014)
L. digitata IN	2 g	2g
L. digitata OUT	1.54 g ± 0.02	1.07 g ± 0.03
Alginate	98.4 mg ± 0.1	n.a
Fucoidan	130.9 mg ± 1.1	150.6 mg ± 3.1
Fucoidan Purity	65% ± 2.1	67% ± 1.4

It is evident that there was not a substantial difference between the two extraction procedures in terms of yield and the purity of the fucoidan extracts. Extraction method 2 (Kim *et al*, 2014) yielded 1.03% more fucoidan than extraction method 1 (Black *et al*, 1952) and the purity was only 2% higher. This may be due to the dialysis step which is included in the Kim *et al* (2014) method, as any excess salts and other impurities could have been removed from the fucoidan extract during this step. Extraction method 1 was chosen as the most suitable protocol to follow for the extraction of fucoidan from brown seaweeds at applied scale. This was mainly because the protocol was simpler to follow, did not include a dialysis step and would be more compatible with the facilities at Croda's pilot plant. Importantly, the overall yields of the

fucoidan extract and its purity between the two methodologies were comparable with one another.

6.3.2 Modifications to the extraction method at Nottingham

6.3.2.1 Extraction step consolidation

Attempts were made to make the Black *et al* (1952) method for fucoidan extraction more compatible with the facilities at Croda's pilot plant. This included consolidating the extraction into one reaction to eliminate the requirement of re-extracting fucoidan from the already treated *L. digitata* solid residue. To compensate for the loss of re-extraction from the brown seaweed, the acid reactant to seaweed ratio was tripled, resulting in a decrease in solids loading from 10% (w/v) to 3.3% (w/v). The yields of extracted fucoidan from the modified Black *et al* (1952) method can be seen in Table 6.6.

Table 6.6 Yields of fucoidan extract and its' purity, following modifications to the method of Black *et al* (1952) in preparation for pilot scale trials

L. digitata IN	100.27g
L. digitata OUT	58.77g
L. digitata mass loss	41.4%
Alginate	2800mg
Fucoidan	3900mg
Fucoidan purity	54%

Relative to the original extraction protocol, there was a 9% decrease in fucoidan purity (54%) and a drop in ultimate fucoidan yield by modifying the protocol in this way. Overall, 3900 mg of fucoidan was recovered whereas the non-modified extraction method would have yielded approximately 6500 mg of fucoidan from 100 g of *L. digitata*. Although there was a decrease in

fucoidan yield, it was considered that this slight drop did not outweigh the benefits of a simple ‘one-pot’ extraction for the purposes of this pilot trial. Furthermore, it was noted that the modifications to the protocol naturally improved the handling of the extraction and produced a less-viscous slurry which was easier to handle at pilot scale.

6.3.2.2 Investigating the impacts of time and temperature on the yields of alginate and fucoidan

The extraction time and temperature of stages 2 and 3 from the process were investigated as variables in order to identify whether they may influence the final yields of extractable alginate and fucoidan. The impacts of extraction time and temperature on alginate and fucoidan yields can be seen in Figures 6.7 A and B. It appeared that temperature played a small effect in the recovery of both alginate and fucoidan. On average, slightly higher yields of alginate and fucoidan were obtained from the cooler 4°C environment compared to room temperature. Extraction time was not particularly significant for fucoidan yields, with 12 h performing just as well as 72 h (Figure 6.7 B). On the contrary, there was a rather small but gradual increase in alginate yield with additional time beyond 12 h (Figure 6.7 A) and because of this it was important to analyse whether the efficiency of alginate precipitation and its subsequent removal had any impacts on the purity of the fucoidan extract

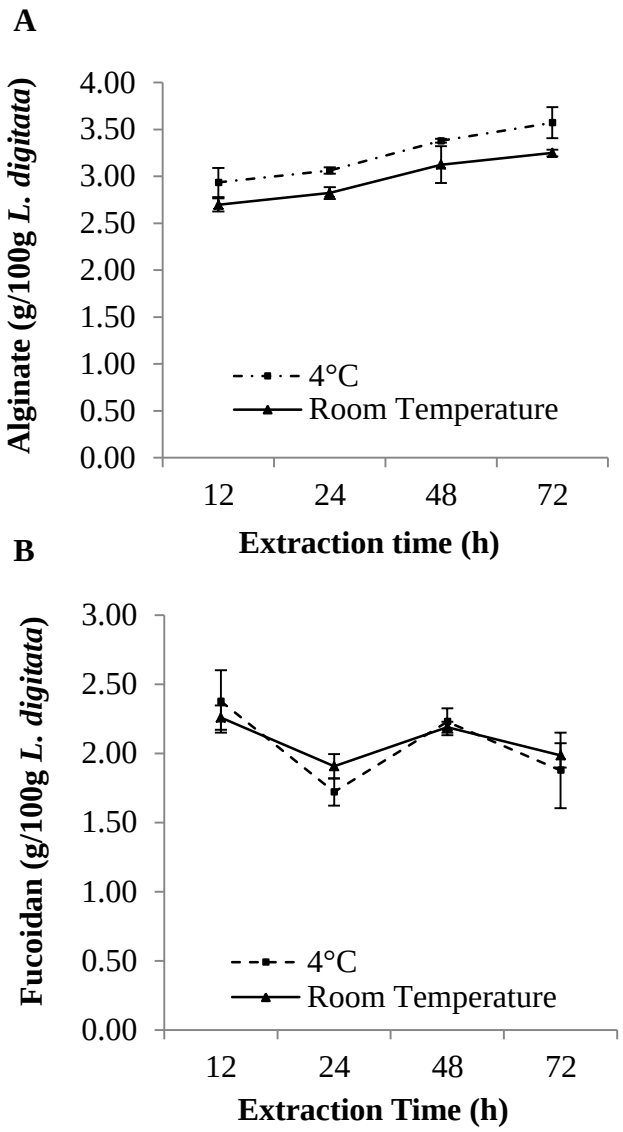


Figure 6.8 The influence of extraction time (12-72 h) and temperature (4°C vs room temperature) on the yields of (A) alginate and (B) fucoidan from *L. digitata*

Figure 6.8 depicts the influence of alginate extraction time on the overall yields and purity of the fucoidan extract.

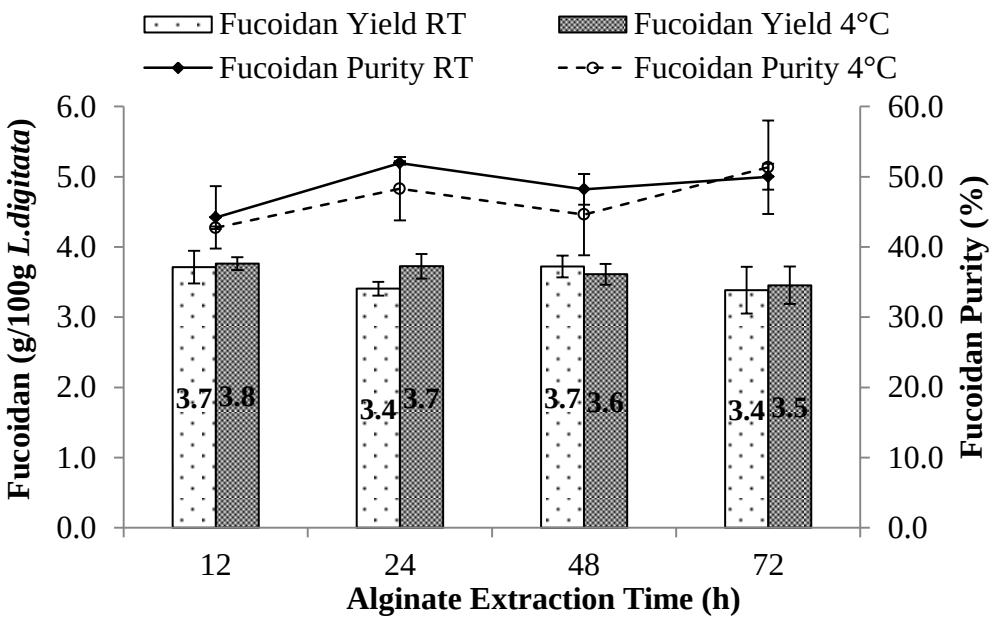


Figure 6.9 The influence of alginate extraction time on the yields and purity of fucoidan

Although there was no direct influence on fucoidan yield with a shorter alginate extraction time, there did appear to be a slight decrease in fucoidan purity. Fucoidan purity after 12 h of alginate extraction was ca 42.7%, however longer alginate extraction times saw an increase in fucoidan purity to around 52.2%. Furthermore, there did not appear to be a significant difference in fucoidan purity between the extractions conducted at room temperature compared to 4°C, although purities were on average slightly higher from extractions at room temperature.

6.3.3 Further modifications to the extraction method at Croda

The aim of this trial was to examine the compatibility of the extraction process developed at Nottingham in Section 6.3.2 with the pilot plant facilities at Croda. This involved collaborating with the process engineers at the pilot plant in order to identify any potential difficulties that could arise in implementing

the developed process at pilot scale. The process could then be adapted accordingly to the suit pilot scale extraction conditions and ultimately develop a finalised protocol. *L. digitata* bulk-purchased from the French marine company Setalg was used in this trial for the first time. It was therefore important to observe how the new material handled under the process conditions prior to scale up and to determine whether similar yields of fucoidan and alginate were attained. Further, to separate solids from liquids in the pilot plant, an oyster press is employed. The oyster press utilises filter paper and the entire system works under pressure. It was important in this trial to investigate the use of different sized filter papers in order to select grades that would successfully separate the main products of interest during processing.

6.3.3.1 Viscosity of the extraction

Stage 1 of the extraction was performed at the same solids loading content (3.3% w/v) used in the extraction in Section 6.3.2. The new batch of *L. digitata* from Setalg appeared to be free flowing and the paddle mixer was able to work freely. The extraction was performed using the apparatus shown in Figure 6.3 (Section 6.2.5.1) in duplicate in order to sequentially examine different pore sized filter papers for successful separation of the main products of interest from the process.

6.3.3.2 Stage 1 separation of seaweed residue from liquor

A filtration system under vacuum pressure was set up for the two extractions (Figure 6.10) in order to remove the seaweed solids from the extraction liquor. Different filter papers (6 vs 11 μm pore size) were evaluated for efficiency of separation. The results are shown in Table 6.8.

A B



Figure 6.10 (A) Filtration of seaweed and liquor under pressure using filter paper. (B) Recovered seaweed solids (wet cake).

Table 6.7 Summary of the main findings from stage 1 separation of seaweed solids from the liquor

Experimental Set Up	Filter Size (µm)	Recovered volume (ml)	Wet weight (g)	Dry weight (g)	Comments
A	6	1340	123.61	23.39	Filtration was very slow and filter was eventually blocked
B	11	1185	147.75	24.62	Tear in filter paper Seaweed cake was not as dry as Extraction A

Due to seaweeds' highly hygroscopic nature, the separation of the solid residue from the liquor proved to be somewhat challenging; raising concerns for when the extraction would be scaled up. Due to the size of the oyster press (which is 110 cm in diameter) only a hand full of available filter papers can accommodate its size. The smallest pore size filter paper available for the oyster press is 1 µm and the largest is 10 µm. Due to this fact and after

observing that the 6 and 11 μm filter papers screened in the laboratory trial were not suitable for the separation of the seaweed solids from the liquor, a 20 μm General Application Filter (GAF) was selected as a more appropriate filter to use, since they are more robust, can filter higher solids and can be easily replaced if a blockage occurs (Figure 611).



Figure 6.11 Example of a General Application Filter (GAF) used in the pilot scale extraction for the separation of stage 1 products

6.3.3.3 Stage 2 - separation of alginate

Two different filter papers (Whatman® Grade GF/F 0.7 μm and Whatman® Grade GF/B1 μm pore size) were compared for the separation of alginate from the liquor: $CaCl_2$ filtrate (Table 6.9).

Table 6.8 Alginate recovery from 50 g (dry weight) Setalg *L. digitata* during the lab-scale trial at Croda

Extraction	Filter Paper Pore Size (μm)	Recovered Alginate (g)
A	1	340 mg
B	0.7	360 mg

Although the filtration process was relatively slow using either filter paper, alginate successfully separated from the liquid and a clear filtrate was

collected. As is evident in Table 6.9, a similar amount of alginate was collected using either filter paper; 0.34 and 0.36 g. However, both filter papers eventually blocked and the rates of filtration decreased; resulting in the need for constant replacement of filter papers. The separation of the alginate from the filter paper however would prove to be technically difficult during the scale up. A 1 μm GAF was considered to be a plausible alternative to use for the scale up extraction due to the practical difficulties of recovering alginate from the filter paper.

6.3.3.4 Stage 3 - separation of fucoidan

Both 1 μm and 10 μm pore size filter papers (Whatman® Grade GF/B and Grade 598) were compared in order to separate fucoidan from the filtrate (Table 6.10) in stage 3 of the process.

Table 6.9 Fucoidan recovery from 50 g (dry weight) of Setalg *L. digitata* during the trial at Croda

Extraction	Filter Paper Pore Size (μm)	Recovered Fucoidan (g)
A	1	1560 mg
B	10	1400 mg

The rate of filtration was slow however slightly faster than the filtration in stage 2 (alginate extraction). A higher yield of fucoidan extract (1560 mg) was recovered using the smaller pore size (1 μm) filter. However, the higher yields may be the result of Extraction A's better yield from stage 1 of the process; a greater volume of liquor was recovered from the seaweed: acid mix, that ultimately produced a drier seaweed cake compared to Extraction B. A 1 μm GAF was recommended for the separation of the fucoidan extract during the scale up. Recovering the fucoidan from the filter paper proved to be somewhat

difficult and the same challenge could also apply when using a GAF at pilot scale. For mass balance calculations it would therefore be necessary to pre-weigh the GAF before use in order to calculate the final yields of fucoidan. Cutting the GAF with scissors to recover fucoidan for analysis might additionally be required. Alternatively, rinsing the GAF with water in order to remove as much fucoidan from the mesh as possible might be required. Subsequent removal of the water from the fucoidan extract by rotary evaporation was discussed as an option. However, freeze drying was considered a more suitable technique to use, in terms of product stability. A freeze drier was not available on site at the pilot plant, therefore freeze drying of fucoidan was conducted at Nottingham.

Due to a technical oversight, the 0.1M HCl was not adjusted to be within the pH range of 2-2.5 in the Croda lab-scale trial as per the protocol of Black *et al* (1952). It is possible that this could have influenced the final yields of fucoidan obtained during the trial, as studies have revealed pH to be an important factor in the extraction of this polysaccharide from brown seaweed (Hahn *et al*. 2012).

6.3.4 Pilot plant extraction at Croda

After the unforeseen technical difficulties which were encountered and overcome during the pilot scale extraction of fucoidan from *L. digitata* (discussed in Section 6.2.6) the final product yields obtained from the process were as detailed in Table 6.11.

Table 6.10 Pilot scale product yields generated from the extraction of fucoidan from *L. digitata* at Croda

L. digitata (starting)	4.7kg
L. digitata (residual weight)	2.3kg
L. digitata mass loss	48.9%
Alginate	60.4g
Fucoidan	80g
Achieved Yield	54.6%
Fucoidan purity	65%

A total of 80 g fucoidan was extracted from 4.7 kg of *L. digitata* at pilot plant scale with a purity of 65%. This yield (1.7% product, 1.1% pure fucoidan on a w/w basis) was lower than expected, based on the values obtained during laboratory extractions (Section 6.3.3.4; 3.1% product yield). Thus the pilot scale process yield was 54.6% of that achieved at laboratory scale. The actual fucoidan content of the *L. digitata* feedstock from Setalg (France) was not determined due to the procedural difficulties in obtaining an accurate fucoidan yield. In this regard, different methods of extraction have been applied to recover fucoidan from different species of brown seaweed and appear to result in different values. These methods include classical fucoidan extraction methods which follow either dilute acid extraction based protocols (similar to the method used in this Chapter), or hot water treatment (Hahn *et al*, 2012). Alternatively enzyme assisted extractions can additionally be employed (Heo *et al*, 2005a, Heo *et al*, 2005b). Subsequently, methods in fucoidan detection and quantification employed by researchers further differ with no agreed quantification guidelines; hence yields of extracted fucoidan are not consistent between studies. As such, it was not possible to determine the complete

overall efficiency of the fucoidan extraction process conducted in this Chapter. Reports have suggested that the fucoidan content of brown seaweeds is typically around 10% (d/w), however this value may vary between species of seaweed, harvesting season and region of isolation (Hahn *et al,* 2012). Based on the assumption that the fucoidan content in brown seaweeds is on average around 10%, it is possible to estimate that the overall extraction efficiency for the pilot process conducted at Croda was around 17%. Despite this value being relatively low, the process was not fully optimised and future method development has the potential to increase yields.

From the 4.7 kg of *L. digitata* used in the process, only 2.3 kg was recovered after the extraction. Therefore approximately 2.4 kg of material was solubilised from the starting material (Figure 6.12). The main products from the extraction process (fucoidan and alginate) together yielded a total of 140.4 g, leaving approximately 2.26 kg of material unaccounted for. This group of unaccounted for material is termed process 'losses' in this work and represents other extracted components of the biomass which had not been precipitated/ recovered. Other extracted materials from the seaweed such as pigments, polysaccharides/monosaccharides, proteins, polyphenols, minerals and salts would have comprised this solubilised matter. Furthermore, products from the extraction process could have been volatilised during stage 1 of the process, in which the contents of the reaction vessel are heated to 70°C.

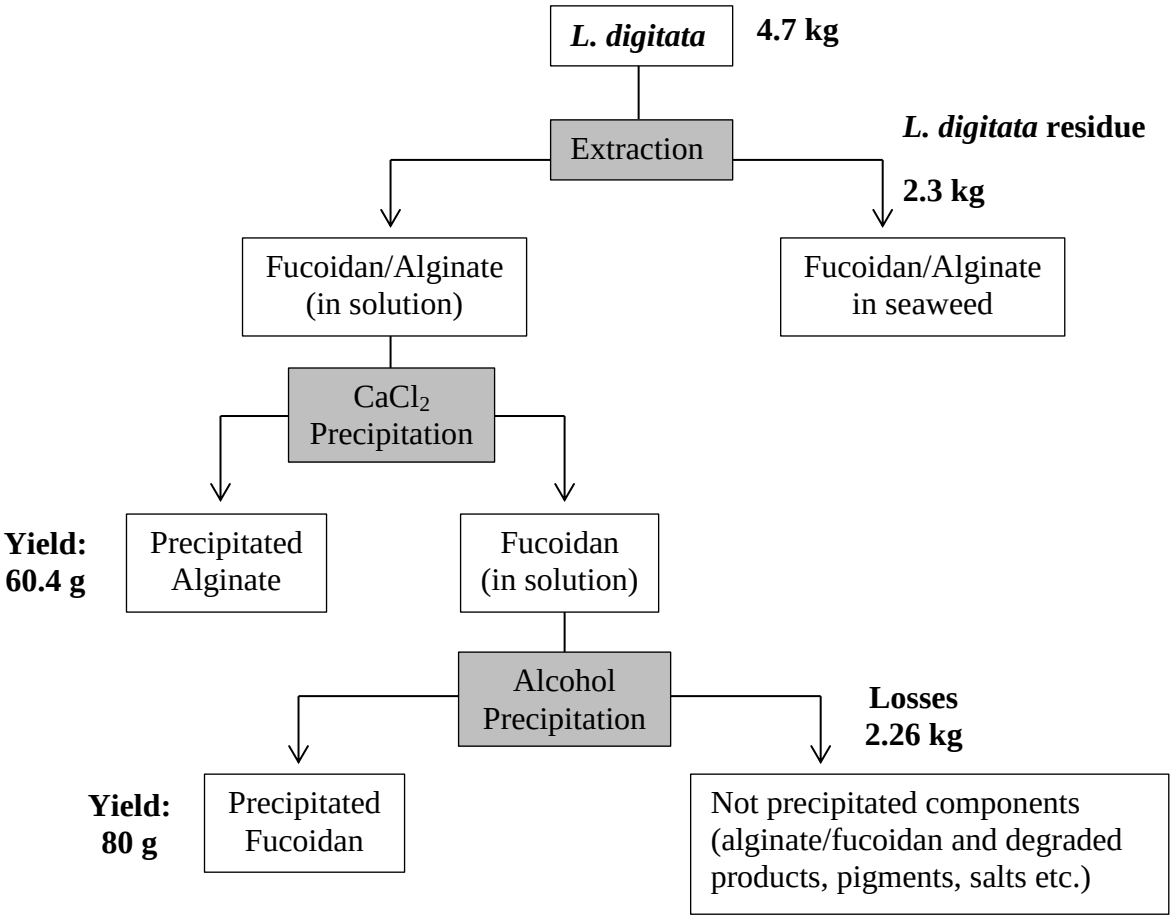


Figure 6.12 General extraction process of fucoidan and alginate from *L. digitata* including product and losses yields

6.3.5 Compositional analysis of *L. digitata* residue

The composition of the seaweed residue following extraction can be seen in Table 6.12 along with the original composition of *L. digitata.* Ash and lipid were present at a lower concentration in the residue, relative to native *L. digitata* which had not undergone the extraction process. The ash decreased from a content of 26.0% (d/w) to 23.4% (d/w). There was hardly any change in lipid content between the two *L. digitata* samples. The protein content increased from 12.9 to 14% (d/w); suggesting a slight enrichment of the protein fraction.

Table 6.11 Proximate composition of Setalg *L. digitata* before and after the extraction of alginate and fucoidan

Composition (d/w %)	Native Seaweed	New Residue
Moisture	8.0	4.4
Protein	12.9	14.1
Ash	26.0	23.4
Lipid	1.0	0.9
Crude Fibre	5.5	15.5
Carbohydrate	46.6	41.4

Although there appeared to be a reduction in the carbohydrate content, from 46.6% to 41.4% (d/w), which most likely resulted from the extraction of alginate and fucoidan, there was an increase in crude fibre content from 5.5% to 15.5% (d/w), respectively. This may be the consequence of an enriched cellulose fraction. The monosaccharide profile of the seaweed residue is shown in Figure 6.13.

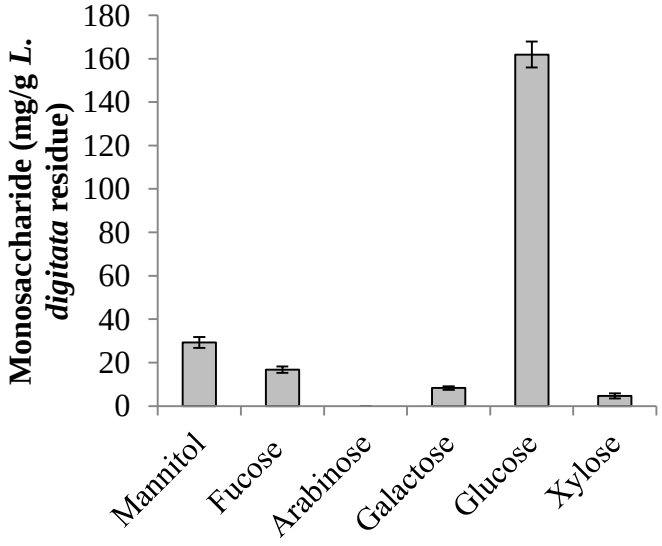


Figure 6.13 Monosaccharide composition of the seaweed residue following extraction of alginate and fucoidan from *L. digitata*

The predominant monosaccharide quantified in the residue was glucose (161.9 mg/g). Minor contents of mannitol, galactose and xylose were present; however a small fraction of fucose (16.7 mg/g) was additionally detected. This suggested that not all of the fucoidan was extracted from the *L. digitata* during the extraction process and adjustments to the process would be required in order to maximise recovery of fucoidan from *L. digitata*. Nevertheless, the presence of a substantial glucose fraction represents a promising substrate for the production of bioethanol.

Figure 6.14 depicts the concentrations of the main elements that were quantified in both the native seaweed and the extracted residue.

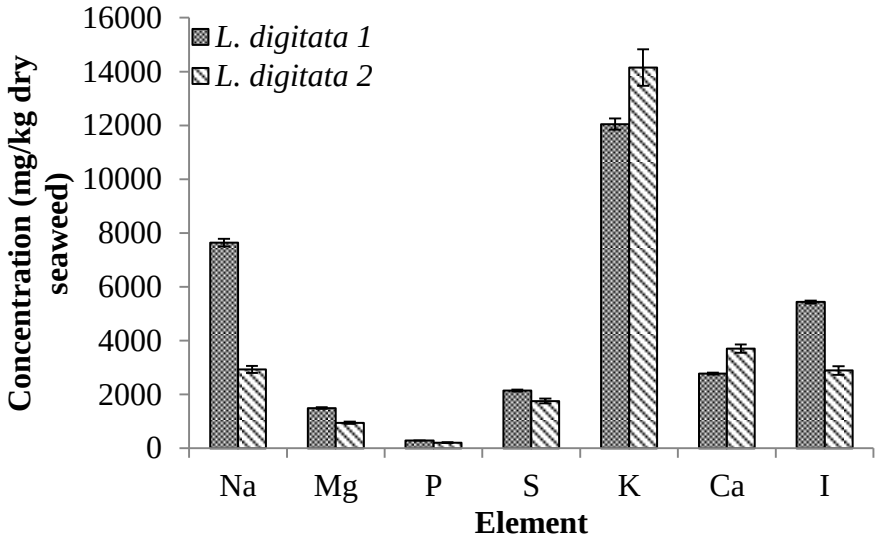


Figure 6.14 Elemental analyses of (1) native *L. digitata* and (2) *L.digitata* residue post the extraction of fucoidan (ICP-MS).

The levels of potassium were enriched in the residue and were the most abundant of the elements quantified (14149.3 ± 679.2 mg/kg). Iodine, which is a major element in seaweeds (especially in the genus *Laminaria)* and is used

for the prevention of goitre, was found at lower levels in the residue (2890.3 ± 156.9 mg/kg). This concentration is much higher than the recommended value for edible seaweed in France where the limit is <0.5 mg/g (dry matter) (Burtin 2003), yet within range of the limit (<5000 mg/g (dry matter)) set for the USA (Mabeau and Florence, 1993). The iodine level present in the *L. digitata* residue may represent an adequate source for subsequent recovery and interestingly, *L. digitata* has been widely used as a health supplement for myxoedema and for the treatment of goitre (Müssig 2009). The total element analysis for both native and new composite *L. digitata* can be seen in Table-9.4 (Appendix C).

6.3.6 Fucoidan characterisation

6.3.6.1 Monosaccharide profile

The fucoidan extract was characterised for its monosaccharide content (Figure 6.15) and sulphate content.

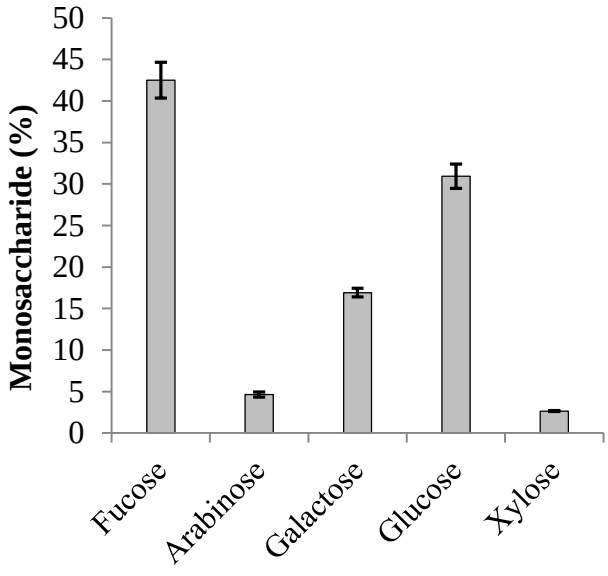


Figure 6.15 Monosaccharide composition of the extracted fucoidan

The fucoidan extract was predominantly composed of fucose followed by a significant proportion of glucose and galactose, and minor amounts of arabinose and xylose. This is in agreement with prior literature which describes fucoidan as being extremely heterogeneous with a branched structure (Marais and Joseleau, 2001). The sulphate content was determined to be 23.8% and is in broad agreement with values reported in the literature (Rodriguez-Jasso et al, 2012).

6.3.6.2 FT-IR analysis

The extracted fucoidan was evaluated by FT-IR in order to determine the specific absorption bands of the present extract. The FT-IR spectrum of the extracted fucoidan is shown in Figure 6.16, as compared to a standard of fucoidan.

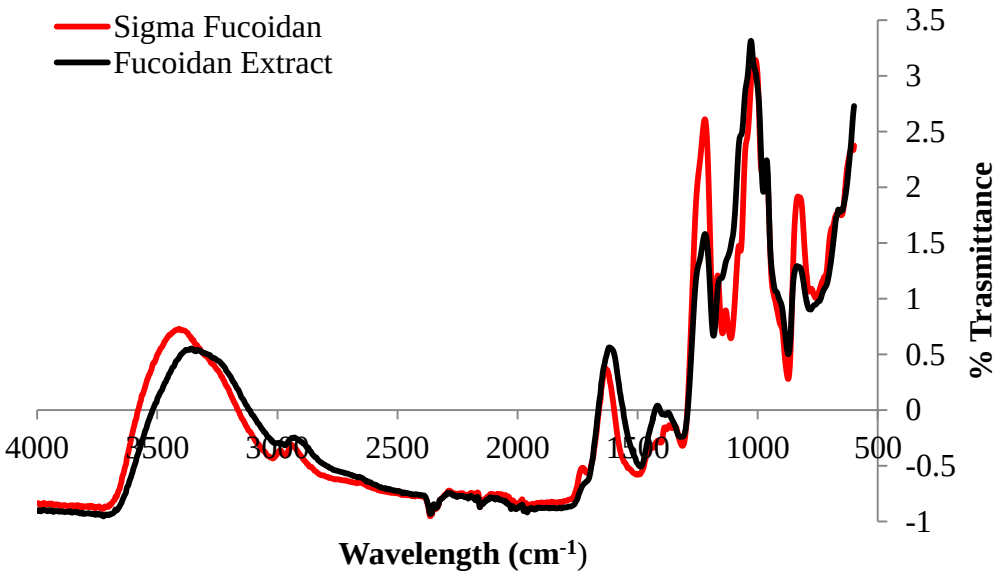


Figure 6.16 FT-IR spectroscopy of fucoidan extract obtained from bio-refining process of *L. digitata* and a standard of fucoidan

The spectra of our fucoidan extract and the standard fucoidan were similar in that they exhibited absorption bands which are typical of the sulphated polysaccharide. The broad band between 3100-3500 cm^{-1} is assigned to hydrogen bonded O-H stretching vibrations and it common to all polysaccharides. The band at 1600 cm^{-1} has been identified to be due to the absorbance of uronic acid (carboxylate O-C-O asymmetric stretching vibrations). However due to the incompatibility of uronic acid with the IC system that was employed to determine the monosaccharide content of the fucoidan extract, it was not possible to quantify the amount of uronic acids present in the fucoidan extract. The absorption band around 1220-1255 cm^{-1} (S=O) confirmed the presence of sulphate in the recovered extract (Mähner , Lechner and Nordmeier, 2001). The sharp band between 820-833 cm^{-1} (C-S-O) suggested a pattern of sulphate substitution, mainly at the C-4 position (axial C-4 substitution of α-linked L-fucopyranose).

6.3.7 Bioactivity analysis of process products/waste streams

Seaweeds have been considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites that can be characterised by a broad spectrum of biological activities (refer to Chapter 1). The interest in new sources of natural bioactives such as antioxidants and antimicrobials has increased in recent years. One reason for this has been a desire to reduce the use of synthetic forms of antioxidants and antimicrobials such as butylated hydrolxytoluene (BHT) and propyl gallate (PG) where strict regulations have been applied due to their potential health hazards. It was therefore of interest to analyse whether any of the products and waste streams

that had been generated from the process had potential antimicrobial and/or antioxidant activities.

6.3.7.1 Antioxidant activity

The antioxidant capacities (free radical scavenging ability) of different products/waste streams from the process were evaluated through the preparation of extracts in both methanol and water. The specific type of solvent used for the extraction may influence the type, yield and the potency of the antioxidant compounds (O'Sullivan *et al*. 2013). Furthermore, the profile of the extracted compounds depends on the polarity of the solvent used, hence the use of both water and methanol. The radical scavenging capacity was determined using both the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. This is because the use of only one antioxidant method to evaluate activity may not be sufficient to determine the true potential of a substance's antioxidant capacity. DPPH is a stable free radical, and the radical scavenging model has been widely used to determine the free radical-scavenging activities of antioxidants (Hu *et al.* 2010). In the assay, absorbance at 518 nm decreases as a result of a colour change as the radical is scavenged by antioxidants through the donation of hydrogen, and the stable DPPH-H molecule is formed. The $ABTS^{\bullet+}$ assay works in a similar way in that the $ABTS^{\bullet+}$ absorbance at 734 nm is measured after 1 min of the reaction and the $ABTS^{\bullet+}$ cation radical becomes reduced in the presence of hydrogen-donating antioxidants. As seen in Figure 6.17, both assays revealed comparable antioxidant activity levels across all extracts investigated. Both of the fucoidan samples (a pure fucoidan standard from Sigma-Aldrich and the extracted fucoidan from *L. digitata*) had the highest scavenging effects in each of the assays.

Sample Key	Water Extraction	Methanol Extraction
L. digitata	LD-W	LD-M
Waste Residue	WR-W	WR-M
Waste Liquor	WL-W	WL-M
Fucoidan Extract	FE-W	n.a
Fucoidan Sigma	FS-W	n.a

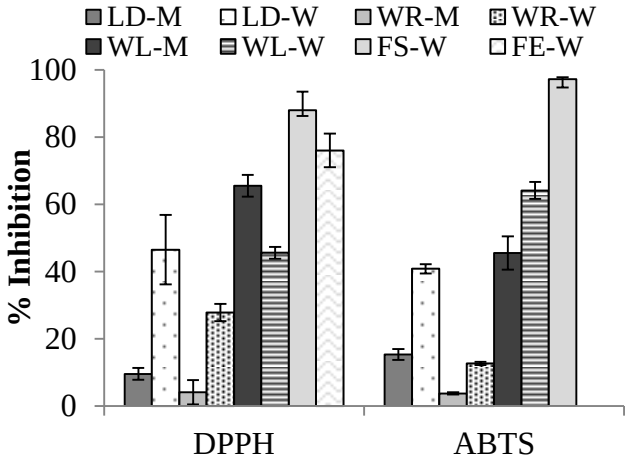


Figure 6.17 Antioxidant activities (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$) of bio-refinery process extracts.

Radical scavenging activity was calculated according to Trolox standards

The highest DPPH$^{\bullet}$ scavenging effect was observed with the pure fucoidan standard, having an inhibition value of 88.0% ± 5.51 at a concentration of approximately 20 mg/mL. The fucoidan extract from the bio-refining process had a 12% lower inhibition value (for the DPPH$^{\bullet}$ assay) than that of the fucoidan standard (76.0% ± 5.0). This was perhaps due to the fact that the fucoidan standard is of a higher purity than the extracted fucoidan. Studies have suggested that the scavenging effect of the fucoidan polysaccharide may result from the presence of the sulphate group positioned at O-2 which is close to the glycosidic bond (Barahona *et al* 2011). In contrast to the DPPH$^{\bullet}$ assay, the extracted fucoidan was not able to interact with the ABTS$^{\bullet+}$ radical and a

colour change was not detected. Therefore, this extract was not included in the ABTS assay$^{\bullet+}$. However, as observed with DPPH$^{\bullet}$, the highest ABTS$^{\bullet+}$ radical values were obtained from the fucoidan standard. Extracts obtained directly from native *L. digitata* exhibited higher inhibition values than extracts from the waste residue, especially from extracts prepared in water as opposed to methanol. The DPPH$^{\bullet}$ scavenging ability of native *L.digitata* (water extract) had an inhibition value of 46.5% ± 10.4, whereas the waste residue extract (water extract) had an inhibition value of 27.9% ± 2.6. The values obtained from the ABTS$^{\bullet+}$ assay for the residue following extraction were even lower, displaying inhibition values of 12.7% ± 0.5. A major cause of these lower values may have been the prior extraction of the fucoidan polysaccharide which itself has been shown to have substantial antioxidant capacity (Rodriguez -Jasso *et al*, 2014). Additionally, other compounds present in the native *L. digitata* that possess antioxidant properties (e.g. phenolic compounds) could have likewise been removed during the extraction process. The DPPH$^{\bullet}$ assay revealed the waste liquor from the process to have an inhibition value of 65.05% ± 3.2 (for the methanol extract). In contrast, the ABTS$^{\bullet+}$ assay had an inhibition value of 45.6% ± 5.0 from the same methanol extract. In order to put the DPPH antioxidant values of the extracts produced from the bio-refining process into perspective, EC_{50} values of the samples were calculated and compared against a known antioxidant (ascorbic acid) and other extracts of fucoidan obtained from the literature (Table 6.12).

Table 6.12 Comparison of DPPH antioxidant inhibition activity (%) of the extracts produced from the bio-refining process against ascorbic acid and extracts of fucoidan from other studies.

EC_{50} values for the $DPPH^{\bullet}$ radical scavenging activity are also included as a means to compare antioxidant capacities.*Concentration of sample required to scavenge 50% DPPH radicals (lower the value, the higher the antioxidant capacity).

Compound/Extract	DPPH Inhibition (%)	Concentration (mg/mL)	DPPH *EC_{50} (mg/mL)	Reference
Ascorbic acid	95.6	3.0	1.6	Yuan *et al*, 2015
Fucoidan extract from *L. digitata*	76.0	20.0	13.2	This work
Native *L. digitata* extract (water)	46.5	20.0	21.5	This work
L. digitata residue (water)	27.9	20.0	35.8	This work
Waste liquor extract (methanol)	65.1	20.0	15.3	This work
Fucoidan from *A. nodosum*	30.4	10.0	16.5	Yuan *et al*, 2015
Fucoidan from *S. vulgare*	22.0	3.0	6.8	Dore *et al*, 2013
Fucoidan from *S. pallidum*	19.1	3.8	10.0	Ale *et al*, 2008

The EC_{50} value is a useful number that enables one to determine the effective concentration (EC) of sample (in this case the extract) required to scavenge 50% of DPPH radicals (Rajauria *et al*, 2011). Generally, the lower the EC_{50} value of the sample, the higher the antioxidant capacity. Comparisons could then be made between the bio-refining process extracts and other known antioxidant samples. Evaluation of these calculated EC_{50} values suggested that none of the extracts produced from the bio-refining process were as powerful (in terms of radical scavenging ability) as pure ascorbic acid (only 1.6 mg/mL of ascorbic acid was required to scavenge at least 50% of DPPH radicals). However, purification of the bio-process extracts may aid in reducing the EC_{50} values of the extracts that were tested here. The fucoidan extract from the bio-refining process (with an EC_{50} value of 13.2 mg/mL) was within the same range of activity as fucoidan extracted from the alternative species of *A. nodosum* (16.5 mg/mL; Yuan et al, 2015) and *S. pallidum* (10.0 mg/mL; Ale *et al*, 2008). In contrast fucoidan extracted from *S. vulgare* exhibited stronger DPPH radical scavenging abilities than the fucoidan extracts from the literature, as only 6.8 mg/mL of the extract is required to scavenge 50% DPPH radicals (Dore *et al*, 2013). Interestingly, the waste liquor extract from the bio-process exhibited an EC_{50} value that was in a similar range with the fucoidan extract (15.3 mg/mL and 13.2 mg/mL, respectively). The fact that relatively high inhibition values were acquired from the waste liquor (and it exhibiting a similar EC_{50} value with that of the extracted fucoidan), suggests the opportunity for another stream of added value from the putative process. It may be possible that algal polyphenols (including tannins and flavonoids) are the principal constituents that are responsible for the antiradical properties of

the waste liquor from the process. However, due to time constraints, it was not possible to characterise the waste liquor and determine its composition. This would provide invaluable information that could potentially identify the specific compound/s that were responsible for the observed antioxidant activities and may also suggest a potential application in either the health and/or nutraceuticals industries.

6.3.7.2 Antimicrobial activity

A total of 11 different bacterial strains, ranging from common food pathogenic, food spoilage and fish pathogenic bacteria were included in this investigation. A crude qualitative assay was conducted initially to screen for antimicrobial activities from the prepared extracts against the selected pathogens. Growth inhibition (defined as clearance zones around an inoculated disc with extract) of the bacteria listed in Table 6.3 were investigated by the standard agar disc diffusion assay. The clearance zones were assessed by eye and recorded. A summary of the pathogen and extract combination results can be seen in Table 6.14, however full result combinations can be seen in Appendix D (Table-Appendices 5-14). Unfortunately not all clearance zones were photographically visible as certain zones were not as prominent as others. However Figure 6.18 shows areas of growth inhibition on agar containing the bacterium *B. cenocepacia*, generated after inoculation of the methanolic extract produced from the process waste liquor.

Table 6.13 Summary of extracts that produced clearance zones (inhibition of pathogen growth) on the standard agar disc diffusion assay

(full table can be seen in Appendix D (Table-9.5-14)).

Extract	Pathogen growth inhibition
Waste liquor (methanol)	*S. enterica*, *B. cenocepacia*, *B. cereus*
Fucoidan extract (water)	*B. cenocepacia*, *S. enterica*
Fucoidan standard (water)	*S. pyogenes*, *L. monocytogenes*, *V. anguillerium*
L. digitata (methanol)	*B. cenocepacia*
Waste Residue (methanol)	*B. cenocepacia*
Waste Residue (water)	*V. anguillerium*, *Y. ruckerii*,
L. digitata (water)	*A. iwofii*, *A. hydrolphila*, *V. anguillerium*

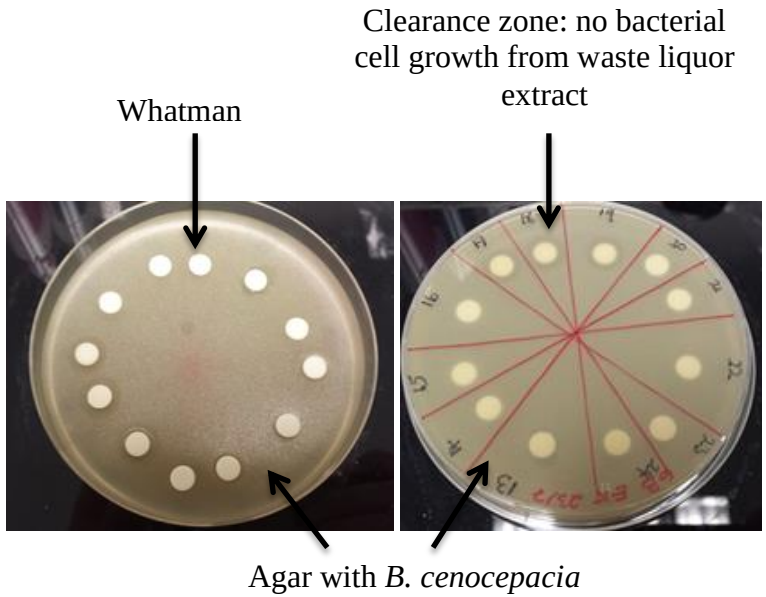


Figure 6.18 Agar disc diffusion assay showing zones of clearance (growth inhibition) on *B. cenocepacia* agar from process waste liquor (methanol extract) at positions 17, 18 and 19 (n=3)

It was important to validate the findings from the agar disc diffusion assay, as the acquired data was highly subjective and qualitative. A quantitative method was hence applied to study selected pathogen and extract combinations in which the pathogen was grown in liquid broth with the extract. Growth inhibition (measured as an absorbance) was determined after a 24 h incubation period. The growth inhibitions of each selected extract against the pathogenic bacteria are presented in Table 6.14. Inhibition of pathogenic growth was confirmed for three out of the 10 shortlisted pathogenic strains. *B. cenocepacia* growth appeared to be inhibited by extracts prepared from the process waste liquor and both native and residue *L. digitata* extracts (methanol extracts). Absorbance readings after 24 h of growth were lower than the reading at 0 h; 0 h absorbance at 600 nm was 1.21±0.14 however after 24 h absorbance values were 1.06±0.19, 1.17±0.59 and 1.10±0.06 for the waste liquor, native *L. digitata* and residue *L. digitata* extracts, respectively. Likewise, *B. multivorans* growth was supressed after 24 h incubation with the *L. digitata* residue extract, as was *B. cereus*; however the waste liquor extract additionally inhibited *B. cereus* growth in the liquid media. It appeared that the inhibition of pathogenic growth was selective to certain strains and extracts in a liquid medium, and not all of the combinations that had been shortlisted from the agar disc diffusion assay displayed inhibitory activity. The reasons behind this are unclear; however the agar disc diffusion assay did serve as a screening tool to shortlist candidate combinations of pathogens and extracts. It additionally appeared that methanol was a good solvent for the extraction of antimicrobials from process products.

Table 6.14 Growth inhibition after 24 h of selected process extracts against selected human pathogenic[(a)], food spoilage[(b)] and fish pathogenic[(c)] bacteria.

Each value is presented as mean±SD (n=3).

	ABS at 600 nm 0 h	Broth & Cells + Extract (ABS at 600nm) 24 h						
Strain Name	Broth&Cells	WL-M	FE-W	WR-W	LD-M	FS-W	LD-W	WR-M
Salmonella enterica[a]	1.13±0.05	1.15±0.29	1.21±0.99	-	-	-	-	-
Burkholderia cenocepacia[a]	1.21±0.14	1.06±0.19	1.24±0.32	-	1.17±0.59	-	-	1.10±0.06
Burkholderia multivorans[a]	1.10±0.52	-	-	1.02±0.06	-	-	-	-
Bacillus cereus[b]	1.08±0.23	1.02±0.32	-	-	1.03±0.09	-	-	-
Streptococcus pyogenes[a]	0.73±0.85	-	-	-	-	0.94±0.36	-	-
Acinetobacter iwofii[a]	1.23±0.03	-	-	-	-	-	1.30±0.36	-
Listeria monocytogenes[a]	1.04±0.56	-	-	-	-	1.21±0.12	-	-
Yersinia ruckerii[c]	0.55±0.36	-	-	0.54±0.09	-	-	-	-
Vibrio anguillerium[c]	0.73±0.25	-	-	0.76±0.19	-	0.79±0.32	0.74±0.09	-
Aeromonas hydrolphila[c]	0.85±0.16	-	-	-	-	-	1.00±0.04	-

The current work inoculated extracts at concentrations around 20 mg/mL and therefore it would be of great interest to further examine the percentage of inhibition from these promising pathogenic strains and extracts and decipher the most effective concentrations at which the extracts can supress bacterial growth.

6.3.8 Bioethanol production from *L. digitata* residue

The residue following extraction of *L. digitata* contained a fraction of fibre which became enriched as a direct result of the bio-refining process. Monosaccharide analysis revealed that ca 161.9 mg/g of the new composite residue was glucose and therefore attempts were made to solubilise the glucose for bioethanol production.

6.3.8.1 Enzyme screen

Four different enzymes were screened to find the most suitable enzyme /enzyme mix for saccharifying the residue. Enzymes with potential specificity towards binding sites that may be present in the brown seaweed residue were selected; Novozymes Cellic® CTec2, *β*-Glucanase, Laminarinase and Driselase (Table 6.4) and dosed in excess. Figure 6.19 shows the theoretical glucose yields (%) that were achieved from application of each of the enzymes to the residue. Two out of the four enzymes and enzyme mixes were compatible with the *L. digitata* residue; Novozymes Cellic® CTec2 and Driselase. A percentage theoretical glucose yield of 66.9% was achieved from Novozymes Cellic® CTec2, indicating that this enzyme mix was most compatible with the residue in terms of glucose solubilisation.

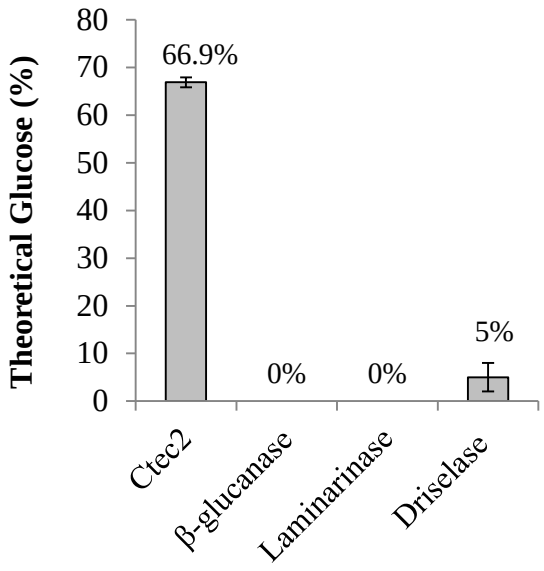


Figure 6.19 Percentage theoretical yields of glucose resulting from treatment of the *L. digitata* extraction residue with the stated enzymes

Data are the mean±SD of three measurements.

Treatment with driselase achieved a 5% theoretical yield of glucose, which was significantly lower than the yields obtained from Novozymes Cellic® CTec2. The fact that Novozymes Cellic® CTec2 was the most suitable enzyme mix additionally provides useful information about the structure of the new composite residue; i.e. the residue contained a dense cellulose fraction that was depolymerised by the mix of aggressive cellulases, hemicellulases and β-glucosidases. Furthermore, the fact that there was no liberation of glucose from a laminarin substrate (by laminarinase) confirms the absence of this polymer in the residue. A study by Adams *et al* (2009) revealed that acid pre-treatment of brown seaweed *Saccharina latissima* prior to enzyme hydrolysis using laminarinase had no effect on ethanol yields. This suggests that the full potential of bioethanol production from brown seaweeds is somewhat limited, as this glucan rich polymer is already removed by any prior pre-treatment of the biomass.

6.3.8.2 Pre-treatment

Although the pre-treatment stage for bioethanol production from the seaweed residue was not optimised, pre-treatments investigated in Chapter 3 were applied as a starting point for the deconstruction of the material. The optimum pre-treatment conditions for *L. digitata* biomass identified in Chapter 3 (1.5 N H_2SO_4, 24 min, 121°C, 25% [w/v] solids loading) in addition to an auto-hydrolytical pre-treatment (24 min, 121°C, 25% [w/v] solids loading) were applied to the seaweed residue from the bio-refining process. The efficacies of these pre-treatments were subsequently evaluated by hydrolysing the pre-treated materials with an excess of Novozymes Cellic® CTec2. The solubilised yields of glucose can be seen in Figure 6.20. A direct Novozymes Cellic® CTec2 saccharification was also conducted directly on the seaweed residue without any prior pre-treatment, in order to evaluate whether a pre-treatment was actually required. Pre-treating the residue with the optimum pre-treatment conditions for *L. digitata* biomass (Chapter 3: 1.5 N H_2SO_4, 24 min, 121°C, 25% (w/v) solids loading) liberated the greatest amount of glucose, achieving 93.8% theoretical yield of glucose following enzyme hydrolysis. The auto-hydrolytically treated residue liberated 118.2 mg/g glucose after enzyme saccharification which corresponded to a 73.2% theoretical yield of glucose. Lastly, almost 50% theoretical yield of glucose resulted from the direct saccharification of the new composite without prior pre-treatment. Overall, it appeared that a dilute form of acid pre-treatment was still required to aid the solubilisation of glucose from this material.

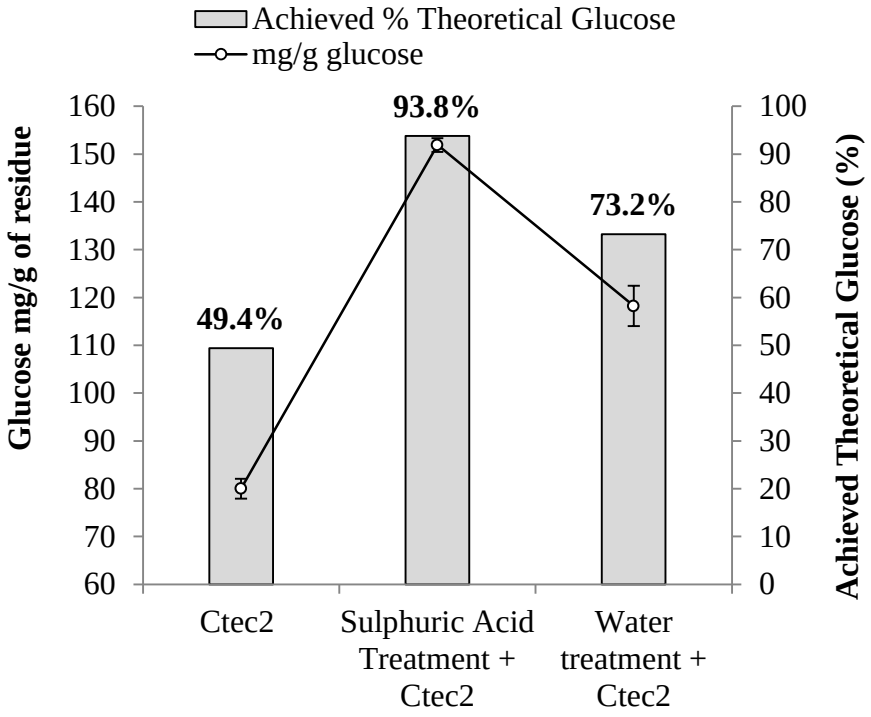


Figure 6.20 Liberation of glucose post enzymatic saccharification of pre-treated *L. digitata* residue.

Data are the mean±SD of three replicates.

6.3.8.3 Bioethanol production from the residue of L.digitata extraction

A trial fermentation with *S. cerevisiae* NCYC2592 was conducted in order to determine final ethanol yields that could be produced from the hydrolysate generated from the residue. No additional nutrients were added into the feedstock prior to fermentation. The fermentation progression and ethanol yield data are shown in Figures 6.21 A and B.

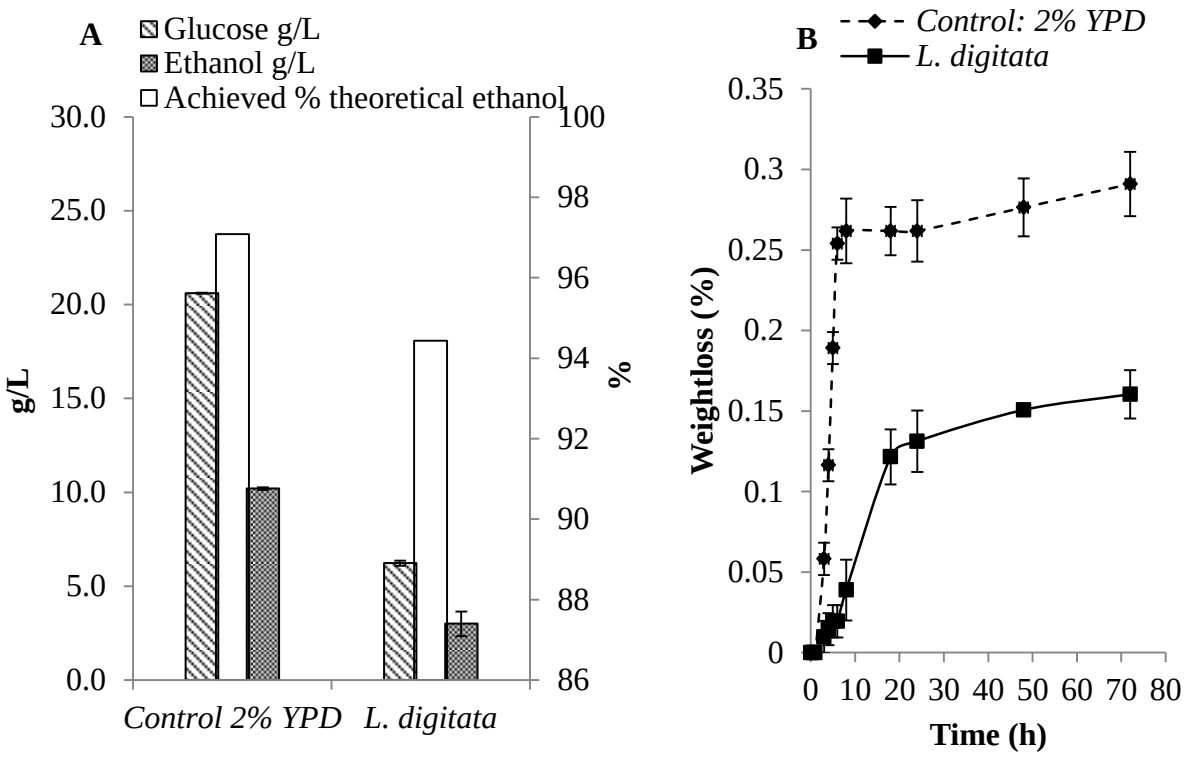


Figure 6.21 Ethanol yield data (A) and fermentation progression (B) for fermentation with *S. cerevisiae* strain NCYC2592 of the saccharified seaweed extraction residue

A: Theoretical ethanol yield based on based on glucose concentration in the three feedstocks.
B: Fermentation progression monitored by weight-loss of vessels due to CO_2 evolution

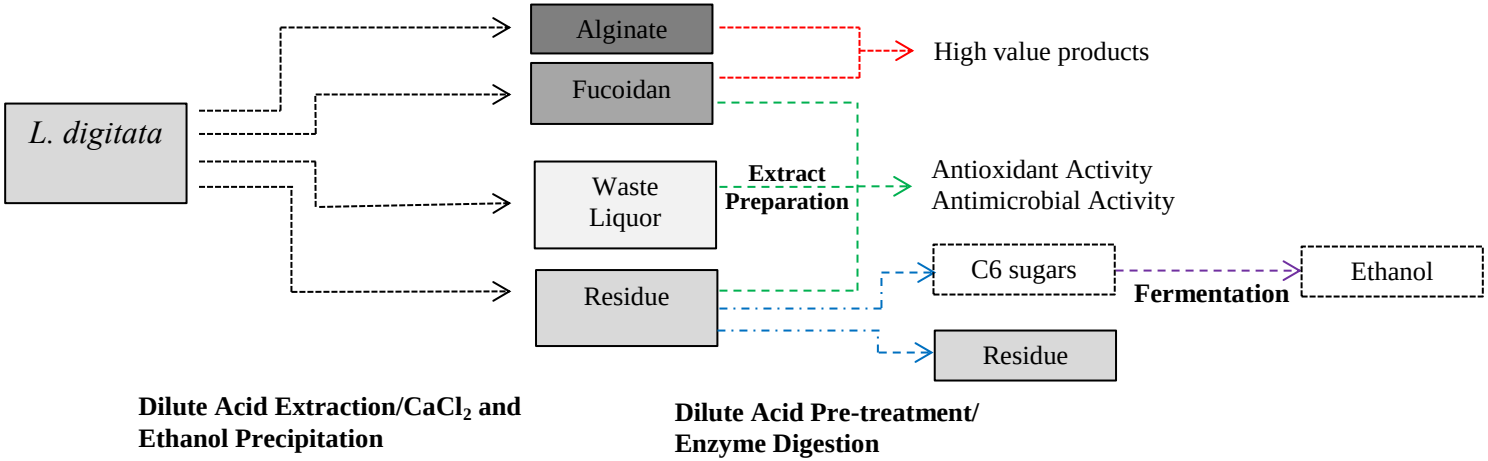
A yield of 3.0 g/L of ethanol was produced from the fermentation of the hydrolysate generated from the new composite. This yield equated to ca 94.4% of theoretical ethanol yield (calculated from the initial content of glucose present in the hydrolysate). Although the volumetric yield of ethanol produced from the hydrolysate was low (0.3% Alcohol By Volume), ethanol production from this composite hydrolysate was not fully optimised for other sugars. However, given that an apparent 94.4% theoretical ethanol yield from glucose was achieved across fermentation it appears that the ethanol titre

following fermentation could only be significantly improved in the present process by increasing the concentration of the hydrolysate prior to fermentation,

6.4 Conclusions

Overall, work conducted in this Chapter has led to the development of a putative bio-refining process for *L. digitata* based around the production of high value products such as fucoidan and alginate (Figure 6.22). Evaluation of co-product streams from the process demonstrated the potential for antimicrobial and antioxidant activity and an insoluble residue with an enriched crude fibre content which was solubilised and converted to bioethanol. The process however still requires additional optimisation in order to enhance the overall process efficiency and ethanol yields, since the latter were too low to be economically viable. Furthermore, the identification for other valuable by-products with interesting bioactivities or screening for potential platform chemicals in waste streams might contribute to the development of a cost efficient bio-refining process for *L. digitata*.

Figure 6.22 Schematic diagram of the bio-refining process developed for *L. digitata* for the extraction of value added compounds, bioethanol and residues/waste streams with potential bioactivity



7 Overall discussion, final conclusions and recommendations for future work

7.1 Overview

This conclusion chapter addresses the main aims and objectives of this Thesis, including the principal findings and associated implications and also recommendations for both the production of ethanol and development of a bio-refinery process for UK seaweeds.

The main outputs from this Thesis include:

- Chemical composition analysis of 9 UK seaweeds
- Optimised pre-treatment and optimised enzymatic saccharification conditions (and also subsequent bioethanol production) for 3 seaweeds native to the UK
- An optimised carbohydrate assay tailored towards each different species of seaweed (including each different pre-treated seaweed residues).
- Identification of a potential fucose assimilating yeast species achieved via screening using a phenotype microarray.
- The development of a preliminary bio-refinery process for *L. digitata* based around the extraction of fucoidan and alginate, and subsequent bioethanol production from seaweed residues

7.2 Overall discussion and final conclusions

Despite the fact that certain species of seaweed are highly abundant in UK waters and have great potential for sustainable harvesting, British seaweeds are an underutilised resource (Chapter *et al*, 2016). At present, there are only a handful of UK research institutes which have investigated the potential of incorporating seaweed biomass into bio-refinery processes and evaluating their potential as precursors for biofuel and biochemical production. This Thesis is one of a few which has attempted to answer and contribute to the exploration of seaweed as a bio-processing feedstock.

Nine species were chemically characterised in this Thesis (Chapter 3; *L. digitata*, *F. serratus*, *L. hyperborea*, *S. latissima*, *P. elegans*, *C. crispus*, *P. palmata*, *D. carnosa* and *U. lactuca*), and this analysis justifies future work in the development of UK based research strategies that could attempt to either fractionate or utilise as many of the components present in the marine organism. In particular the ash (18.8-32.7% [d/w]), protein (9.6-26.8% [d/w]) and carbohydrate (15.9-41.8% [d/w]) fractions found in the listed UK seaweeds could provide potentially higher value uses for the feedstock, such as animal/fish feed or applications in cosmeceuticals (Tiwari and Troy, 2015, Hagino and Masanobu, 2003) from the protein fraction and incorporation of the inorganic elements from the ash into a commercial fertiliser (Philippsen 2013, McHugh, 2003)

The application of a suitable pre-treatment followed by optimised enzymatic saccharification of the feedstock is eminent in releasing fermentable sugars for bioethanol production. Although thermal acid pre-treatment is commonly used

on seaweeds, its application does have the potential disadvantages of formation of sugar degradation products, high energy consumption and also potential for environmental pollution. Therefore, as all these factors need to be taken into consideration, moderate pre-treatment technologies are required in order to improve the economic feasibility and sustainability of the seaweed biofuel industry. Direct enzymatic attempts (without any prior pre-treatment step) were initially trialled in this Thesis in an attempt to liberate a significant quantity of fermentable sugars directly. This was conducted on seaweeds *L. digitata* and *U. lactuca* and for the first time on *D. carnosa*, using Novozymes Cellic® CTec2. However, generally glucose yields were lower than those achieved when also using a prior pre-treatment. Whilst the aim was to attempt to avoid any pre-treatment, the results unfortunately confirmed its requirement. This may be due to the structural modifications to the biomass after pre-treatment application; which possibly enhanced the physical access of the enzymes to the exposed polysaccharides. As such, subsequent optimisations of pre-treatment parameters were conducted; thermo-chemical vs aqueous (water) based pre-treatments, testing of acid concentration (0.25-5 N solids loading content (5-25 % [w/v]; biomass: reactant) and reaction time (5-30 min), to develop effective protocols for each of the 3 seaweed species (Table 3.4, section 3.4). As hypothesised, results from this study revealed that one universal pre-treatment could not be applied to all seaweeds and the optimal pre-treatment conditions were found to be specific to each seaweed species. What was particularly interesting was that the red and green species of seaweeds (*D. carnosa* and *U. lactuca*) were both more suited with an auto-hydrolytical (water-based) pre-treatment (yielding 57.8 and 85.8 mg of

glucose/g of pre-treated seaweed, respectively). Researchers are currently looking into new technologies to overcome the disadvantages of thermo-chemical pre-treatments, and a potential alternative could be the use of hydroxyl radicals and hot water pre-treatment which was used as a pre-treatment on the brown seaweed *Macrocystsis pyrifera* (Gao *et al* 2015). Auto-hydrolytical (water) pre-treatments could be a plausible alternative as they are much more environmentally friendly (than the use of reagents such as acids) and with the conditions used in this work, no sugar degradation products were formed. The use of a water based hydrolysis method for the pre-treatment of seaweeds had not been reported previously until this research. Additionally, this was the first study that evaluated the potential of *D. carnosa* as a feedstock for bioethanol production and results from this work indicate that this red species is an ideal candidate, especially as no form of thermo-chemical pre-treatment is required prior to enzyme saccharification.

On the contrary, the brown kelp *L. digitata* required a mild acid thermo-chemical pre-treatment in order to solubilise more glucose (134.4 mg glucose /g pre-treated seaweed). Although higher yields of ethanol were produced from the red and green seaweed feedstocks, their abundance in UK waters is not as prevalent as other species of seaweeds such as kelps. Kelps (such as *L. digitata*) can be well cultivated in the UK, grown at a range of different scales that would be suitable for commercial production and have a fast growth rate (Wilson *et al*, 2015); suiting the purposes of a bio-refinery. Cultivated biomass also suggests that a sustainable and reproducible source of seaweed would be available for processes that have the potential to be scaled up for commercial production (Schiener *et al*, 2015). Additionally cultivated seaweed eliminates

the need of harvesting wild biomass (such as beech-cast seaweed washed ashore), which may affect the coastal ecosystem (Kirkham and Kendrick, 1997). Beech-cast seaweed provides both nutrients for organisms and also shelter and protection for a range of marine species from predators (Robertson and Hansen, 1982, Robertson and Lenanton, 1984). Seaweed price (production costs) is an important factor that needs to be taken into consideration with regards to the economics of biofuel production (Fasahati *et al*, 2015). Despite the fact that seaweeds can be cultivated both naturally and artificially, there is a significant difference between the production costs of both practices. Cultivation can be performed by offshore and near-shore farming. However even though offshore farming enables the plant to growth to great lengths using the unlimited supply of water in the ocean, dissolved CO_2 in the surface water and nutrients (Chynoweth, 2002), many operational difficulties makes this cultivation process challenging to employ. For example, the economics and feasibility of harvesting and transporting the seaweed from the offshore farming site directly to a bioethanol production plant need to be considered. Near-shore cultivation systems eliminate such hindrances.

Bioethanol production from seaweed feedstocks can also be potentially limited by the lack of a suitable industrial microorganism that can efficiently ferment the unique sugars and polysaccharides found in seaweed (alginate, mannitol and fucoidan and their corresponding monomeric sugars). However, microorganisms such as bacteria and genetically engineered yeast strains are being developed in order to be able to achieve efficient conversion of seaweed biomass into ethanol (Kim *et al*, 2012, Wargacki *et al*, 2012, Wang *et al*, 2013, Enquist-Newman *et al*, 2014 and Ji *et al*, 2016). The screening of a

range of strains of yeast (which included traditional *Saccharomyces spp*, *Pichia spp* and *Candida spp*) for their ability to metabolise a range of monosaccharides originating from the breakdown of seaweed polysaccharides, as well as the acid hydrolysates of five seaweed species using a phenotypic screening approach, confirmed that *S. cerevisiae* spp were still the most suitable (Chapter 5). Overall, a maximum concentration of 13 g/L ethanol was achieved from the fermentation of a pre-treatment generated hydrolysate from *C. crispus* when using *S. cerevisiae* YPS128. *S. cerevisiae spp* are the most robust and efficient strains of yeast to use for fermentations of C6 sugar-rich feedstocks and are widely used in industry; hence their application in beer production. However for seaweed sugar fermentation, their limitations become more apparent. In this work the yeast stain *P. anomala* TP12 was the only strain able to utilize the majority of the monosaccharides present in the pre-treatment hydrolysate of *C. crispus* including a 'fucose-like' sugar. Despite not knowing whether the fucose itself was converted directly to ethanol (producing 3.5 g/L) or simply just up-taken from the medium, the finding suggests that *P. anomala* TP12 could be a candidate strain for further research and optimisation.

The commercial viability of seaweed bioethanol production on a larger scale would rely on the economic performance of the processes that are involved to produce bioethanol. This includes for example the cost of enzymes used in feedstock saccharification and various energy intensive steps that increase the cost of the overall process significantly; such as pre-treatment of the biomass and distillation of the final product (Vane *et al*, 2008). The costs will additionally differ significantly depending on which species of seaweed is

used for bioethanol production. Based on results presented in this thesis, the kelp *L. digitata* as the feedstock would most likely be more expensive to process compared to *D. carnosa*, as a mild acid thermo-chemical pre-treatment was required for sugar liberation from *L. digitata* instead of aqueous-based pre-treatment which was the most effective for *D. carnosa*. From an industrial perspective, the use of sulphuric acid as a catalyst in the pre-treatment reactors would acquire additional technological steps (Fasahati *et al*, 2015). A neutralisation process would be required following the acid pre-treatment, subsequently suitable for enzymatic treatment and/or microorganisms to function in the subsequent process steps. Furthermore, cladding material would need to be used for the pre-treatment reactors to withstand the corrosive and high temperature environment of the acid pre-treatment (Fasahati *et al*, 2015). The latter would ultimately contribute to the final operation costs.

Bioethanol data generated from the three species of seaweed that were under investigation in Appendix B Chapter 3, despite being somewhat modest (due to the relatively low glucose concentrations liberated during pre-processing), enabled some basic calculations of product yields if operating on a large scale. Theoretical maximum (pure) ethanol yields were calculated based on the processing of 1 metric tonne (1000 kg) of seaweed (using the previously optimised pre-treatment parameters described in Appendix B Chapter 3). These provide a preliminary benchmark for further process improvements (Table 7.1).

Maximal total glucose (kg) and total ethanol yields (kg) that could be attained from processing 1 metric tonne (1000 kg) of seaweed using the previously optimised pre-treatment parameters

(see Appendix B Chapter 3 for details).

Per Tonne Seaweed	Pre-treatment	Enzymatic hydrolysis*	Total glucose** (kg)	Total ethanol*** (kg)
L. digitata	1.5N H_2SO_4, 121°C, 24 min, 25% (w/v) solids loading	2% (w/v) solids loading	40.0	20.4
D. carnosa	Autohydrolytical, 121°C, 30 min, 15% (w/v) solids loading	8% (w/v) solids loading	30.2	15.4
U. lactuca	Autohydrolytical, 121°C, 24 min, 10% (w/v) solids loading	8% (w/v) solids loading	35.9	18.3

*Saccharification conducted at 50°C for 48 h using Novozymes Cellic®CTec2 (50 FPU/g).
**Glucose content calculated from post-enzymatic saccharification hydrolysate.
***Theoretical ethanol concentrations calculated using glucose quantity x 0.51.

Regardless of seaweed species used, final ethanol yields of only ca. 15-20 kg pure ethanol per tonne of seaweed biomass could be attained. These low ethanol yields would not render bioethanol production alone (without any additional bio-refining for extraction of supplementary high value products) to be economically viable, and would unlikely be able to compete with the ethanol produced from other sources (i.e. the ethanol sold today in the marketplace: 1.61 US$/GAL [http://www.tradingeconomics.com/commodity/ethanol]; last accessed 27/05/2016). However, it is important to note that the enzyme hydrolysis step was not fully optimised hence only liberating glucose concentrations that corresponded to ca. 3-4% dry weight of the original seaweed biomass.

In order to enhance the LCA favourably and Techno-Economic Analysis (TEA) of a process, the incorporation of additional high valued commodities and platform based compounds alongside bioethanol production would be required. At present, only a handful of seaweed bio-refinery processes have been investigated for the production of biofuels and commodity compounds (Kumar et al 2013, Trivedi et al 2016, Magnusson et al 2016). However for the first time, a novel bio-refinery process for UK kelp *L. digitata* was developed in Chapter 6. The rationale for choosing *L. digitata* was because of its high prevalence in UK waters and it's suitability of cultivation for commercial production. The preliminary bio-refining process involved a number of stages based around the extraction of the polysaccharides fucoidan and alginate followed by the subsequent production of bioethanol from the remaining residual biomass. To date, fucoidan is not considered to be a suitable substrate for bioethanol production (despite findings obtained in

Chapter 5 suggesting that *P. anomala* TP12 may be able to up-take fucose). However, the polysaccharide has been shown to exhibit specific biological activities and unique properties (such as antioxidant and antimicrobial effects; Ale *et al*, 2013, Li *et al*, 2008, Morya *et al*, 2012). At pilot scale, approximately 80 g of fucoidan was extracted from 4.73 kg *L. digitata* with a purity of 65%. However, the process only had an overall extraction efficiency of 17%. This was based on the amount of extracted fucoidan compared to the amount of fucoidan present in brown seaweeds (ca.10% on a dry weight basis; Hahn *et al*, 2012). Indeed, analysis of the *L. digitata* residue which remained after the extraction still identified a small proportion of fucose in its composition, suggesting that fucoidan extraction yields could be further increased with additional optimisation of the process.

Interestingly, the fucoidan extract and extracts of other waste streams from the *L. digitata* bio-refining process were shown to have both antioxidant and antimicrobial activities. For the first time, extracts generated from the waste liquor (liquid fraction) of the fucoidan extraction process were shown to inhibit the growth of the bacterial pathogen *Burkholderia cenocepacia*, a multi antimicrobial resistant, human pathogen, that patients with cystic fibrosis are particularly susceptible to. The same extracts additionally displayed high levels of antioxidant activity according to the $DPPH^{\bullet}$ assay, in which 76.0% ± 5.0 inhibition activity was recorded with the fucoidan extract and 65.05% ± 3.2 inhibition value from the process waste liquor. Such findings are promising and could potentially enable additional revenue streams to be realised from this bio-refinery process. For example, the identification of novel natural antioxidants (instead of artificial antioxidants such as propyl

gallate, BHA and BHT) and/or novel antimicrobial sources (that have been derived from a renewable source and produced using a low cost process) would clearly be highly advantageous.

An important factor that needs to be taken into consideration if seaweed bio-refinery processes were to be conducted on a commercial scale, is the seasonal variation of the seaweed polysaccharides. Seasonal composition studies have shown that the best times of the year to harvest for maximum carbohydrate content can differ between species, seasons and location (Schiener *et al,* 2015). Therefore, it is highly likely that a seaweed bio-refinery process would not be economically viable if it depended solely on one particular seaweed species. In order to ensure a continuous supply of seaweed was available for a continual bio-refinery process to operate, it would be essential to utilise more than one species.

In conclusion, the work conducted in this Thesis has provided useful information about the composition and thus potential value of nine UK species of seaweed, as well as furthering the knowledge of the key stages in converting seaweed biomass into sugar-rich streams for fermentation to bioethanol. The work has additionally led to the development of a putative bio-refining process for *L. digitata* based around the production of high value products such as fucoidan. Evaluation of co-product streams from the process demonstrated the potential for antimicrobial and antioxidant activity and an insoluble residue with an enriched crude fibre (polysaccharide) content was solubilised and converted to bioethanol. The process however still requires additional optimisation in order to enhance the overall process efficiency and especially ethanol yields, since the latter was too low to be economically

viable on a commercial scale. Furthermore, the identification of other valuable by-products with interesting bioactivities or screening for potential platform chemicals in waste streams might contribute to the development of a cost efficient bio-refining process for *L. digitata*. These experimental findings and the abundance of *L. digitata* (or the ability of aquaculture based methods for its cultivation) may enable a UK based seaweed bio-refinery as a realistic possibility.

7.3 Recommendations for future work

7.3.1 Enzyme saccharification studies

Whilst some moderate degree of investigative experimental work was conducted evaluating different enzyme hydrolysis solids loadings (Chapter 3) and different enzymes enzyme mixtures (Chapter 6) the work was by no means comprehensive. A comprehensive screening of all relevant enzymes in combination may highlight suitable mixture that would be much more compatible with the seaweed feedstock and enhance sugar yields.

In addition, most enzymatic saccharification experiments that were used to evaluate the efficacy of pre-treatment were conducted using excess doses (purely as proof of principle). Also, all reactions were conducted using a rudimentary shaking incubator system for agitation. Consequently this low energy mixing regime may have limited successful enzyme-substrate interactions and limited the final glucose yields achieved. However, due to the complex nature and kinetics of enzyme based reactions there are most likely many additional factors that synergistically interact to reduce enzymatic sugar yields. As such, a comprehensive multi-parameter optimisation of the

enzymatic hydrolysis step could be conducted and 3D response-surface modelling used to look at interactions between different experimental parameters. This could aim, for example, to simultaneously minimise the enzyme dose used, maximise the solids loading used, optimise the reaction time and investigate alternative mixing regimes.

Another area of research that could be explored is the production of seaweed specific enzymes (carbohydrate-degrading for example) through solid-state fermentation (SSF) approaches. The high costs of many enzymes and for the most part their non-specificity towards seaweeds (as the majority of commercial enzyme preparations are tailored towards lignocellulosic feedstocks) renders them unsuitable for large scale usage with seaweeds. As such SSF may be a plausible methodology for the generation of novel and highly specific enzymes which may be able to depolymerise the polysaccharides inherent to seaweed. This is achieved by using biomass as the substrate for the production of enzymes from various fungi such as *Aspergillus niger* and *Trichoderma reesei.* However, as carbohydrate compositions vary significantly between seaweeds, it would be essential to conduct SSF either discreetly on different species of seaweeds individually (for the production of one specific enzyme for one specific seaweed polysaccharide) or alternatively using a mix of 2-3 seaweeds belonging to different taxonomic groups (for the production of a crude mixture of enzymes with broad activity). Such an approach could be incorporated into seaweed bio-refineries and assist in the reduction of processing costs for biofuels or high-value platform chemicals.

7.3.2 Yeast strain investigations and development

To date there are no published studies which have investigated the use of fucose and its uptake by yeast to produce bioethanol. From this work, *Pichia* and *Candida* spp displayed promising abilities to metabolise fucose; the basic backbone monomer of the sulphated polysaccharide fucoidan found in brown seaweeds. However, it was not possible to determine whether the assimilated fucose was converted to ethanol, biomass or any other carbon end-product by the yeast. However, further experiments involving the radioactive labelling of the monosaccharide (with carbon-14 for example or stable isotopes such as C-13) would be one possible way of concluding its metabolic fate. Fermentates should also be screened for other known metabolic carbon end point products, which are considered to be high-value platform chemicals, such as xylitol and succinic acid. This may also assist in explaining the apparent uptake of fucose but low bioethanol titres achieved.

Furthermore, these fucose utilizing yeast strains likely contained a functional membrane transporter protein on the cell surface that enabled fucose transport. An initial point of experimentation would be to identify whether the C2_09280C_B gene (or similar) is present in any of the yeast strains shortlisted in this Thesis. The C2_09280C_B gene is predicted to be either a membrane transporter, member of the fucose: proton symporter (FHS) family or part of a major facilitator superfamily (MFS) and orthologous genes have been identified in other *Candida*, *Aspergillus* and *Saccharomyces* species. This could ultimately lead to the cloning and insertion of these relevant genes into other yeast strains to enable fermentation of the fucose present in hydrolysates derived from seaweeds. Alternatively, accelerated evolution

technology using shortlisted yeast strains in seaweed hydrolysates and/or use of synthetic minimal media containing only fucose as the sole carbon source may produce 'improved' strains. This may enable strains to display both an increase in tolerance to the specific chemical environment (of the seaweed derived hydrolysate), and also potentially an improvement in the ability of the yeast cell to uptake fucose. Improved transport mechanisms may then enable improved fermentation efficiency either to bioethanol or another metabolic end product.

7.4 *L. digitata* bio-process optimisation

Further optimisation of the bio-refinery process developed for the extraction of fucoidan from *L. digitata* (Chapter 6) is required. The overall extraction efficiency was determined to be 17% (which was calculated based on the amount of extracted fucoidan in pilot plant trials compared to the reported quantity 10% (d/w) for brown seaweeds described by Hahn *et al*, 2012). Such a low extraction efficiency would not render this process economically viable on a commercial scale. However, since fucoidan has a current market value of £386 (GBP) per g (www.sigmaaldrich.com), it is certainly worth optimising the extraction protocol to increase the overall yields of extractable fucoidan. Quantifying the actual amount of fucoidan in the native *L. digitata* (used in this study) would prove useful as this would enable accurate calculations of process efficiency to be determined. However, as already highlighted in this Thesis, accurate quantification of carbohydrates (or any component for that matter) is paramount and this would likely entail developing a new, highly specific and precise assay. This would also most likely involve subsequently conducting a multi-parameter optimisation trial of the assay and 3D response-

surface modelling could again be used to explore different experimental parameters for increased extraction.

Unfortunately due to time and practical constraints it was not possible to screen for other groups of potentially high-value products that could have also been co-extracted during the process. For example the waste liquor generated from the process could have been screened for the presence of useful polyphenolic compounds.

A final area of interest would be to evaluate whether the fucoidan extract may be of any potential value to the collaborative company CRODA (plc). CRODA specialise in the chemical derivatisation of many compounds, such as polysaccharides for example. Other areas of interest include extraction of compounds from oil based feedstocks and their incorporation into novel, modified commercial products that can be used for personal care, cosmetics, heath care and crop care. It would have been of significant interest to evaluate the fucoidan extract and determine whether it could be converted into another commercially useful product such as a surfactant. This would involve derivatisation of the extract (e.g. the attachment of hydrophobic moieties such as fatty acids to the sugar backbone) and then assessing whether any potentially useful surfactant-type properties resulted. A group that recently extracted fucoidan from *Sargassum* sp using hot buffer established that the crude extract demonstrated potent emulsion forming and stabilizing capacities (Hifney *et al*, 2016). This discovery provides justification for further research into the properties of fucoidan (and its derivatives) extracted from brown seaweeds and could represent an alternative to the use of synthetic polymers or other natural polysaccharides. These products may have applications in the

food, pharmaceutical and even textile industries. Additionally, it would be important to evaluate the emulsifying properties of fucoidan extracted from different species of seaweeds; as this property may vary between species.

8 Bibliography

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9 Appendices

9.1 Appendix A: Metal Analysis of UK Seaweeds

Table 9.1 Metal Analysis of *L. digitata*, *F. serratus*, *L. hyperborea* and *S. latissima*, expressed as mg per kg^{-1} of seaweed.

	L. digitata	***F. serratus***	***L. hyperborea***	***S. latissima***
B	23.3 ± 0.4	25.1 ± 1.1	22.0 ± 2.1	25.8 ± 0.6
Na	7755.3 ± 212.1	6379.4 ± 297.7	4656.2 ± 420.7	5436.2 ± 74.9
Mg	1693.7 ± 25.6	1589.3 ± 56.7	1892.0 ± 139.2	1709.5 ± 43.7
P	498.9 ± 8.6	649.3 ± 7.1	592.2 ± 50.3	419.3 ± 0.8
S	2302.9 ± 42.0	2889.7 ± 243.1	2486.4 ± 152.5	2082.6 ± 19.1
K	13902.2 ± 287.9	9387.4 ± 460.4	7909.1 ± 703.9	11072.2 ± 217.1
Ca	4075.1 ± 5.6	3306.5 ± 176.3	5386.0 ± 328.2	4968.2 ± 108.1
Ti	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0
Li	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.1
Be	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Al	9.8 ± 0.6	17.0 ± 1.2	7.9 ± 1.1	141.1 ± 33.3
V	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.6 ± 0.1
Cr	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1
Mn	1.3 ± 0.0	38.5 ± 2.2	1.1 ± 0.2	7.0 ± 1.8
Fe	28.4 ± 0.6	62.0 ± 0.8	27.2 ± 2.8	323.9 ± 77.1
Co	0.1 ± 0.0	0.8 ± 0.0	0.0 ± 0.0	0.4 ± 0.0
Ni	4.2 ± 0.8	14.0 ± 0.1	1.5 ± 0.2	15.1 ± 2.5
Cu	1.1 ± 0.1	1.8 ± 0.0	1.6 ± 0.2	0.7 ± 0.1
Zn	5.3 ± 0.2	15.6 ± 0.1	7.4 ± 0.6	6.4 ± 0.1
As	17.8 ± 0.3	12.4 ± 0.4	21.7 ± 1.0	9.2 ± 0.1
Se	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Rb	5.8 ± 0.1	3.2 ± 0.1	3.8 ± 0.3	4.3 ± 0.1
Sr	283.5 ± 0.4	215.5 ± 14.2	381.1 ± 30.2	257.3 ± 3.0
Mo	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
Ag	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cd	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cs	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ba	3.0 ± 0.0	2.6 ± 0.2	3.7 ± 0.2	3.3 ± 0.1
Tl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pb	0.1 ± 0.1	0.7 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
U	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
I	7267.9 ± 148.5	1241.1 ± 0.6	3676.6 ± 73.7	3909.9 ± 129.0

Table 9.2 Metal Analysis of *P. elegans*, *C. crispus*, *P. palmata* and *D. carnosa* and *U. lactuca*, expressed as mg per kg^{-1} of seaweed.

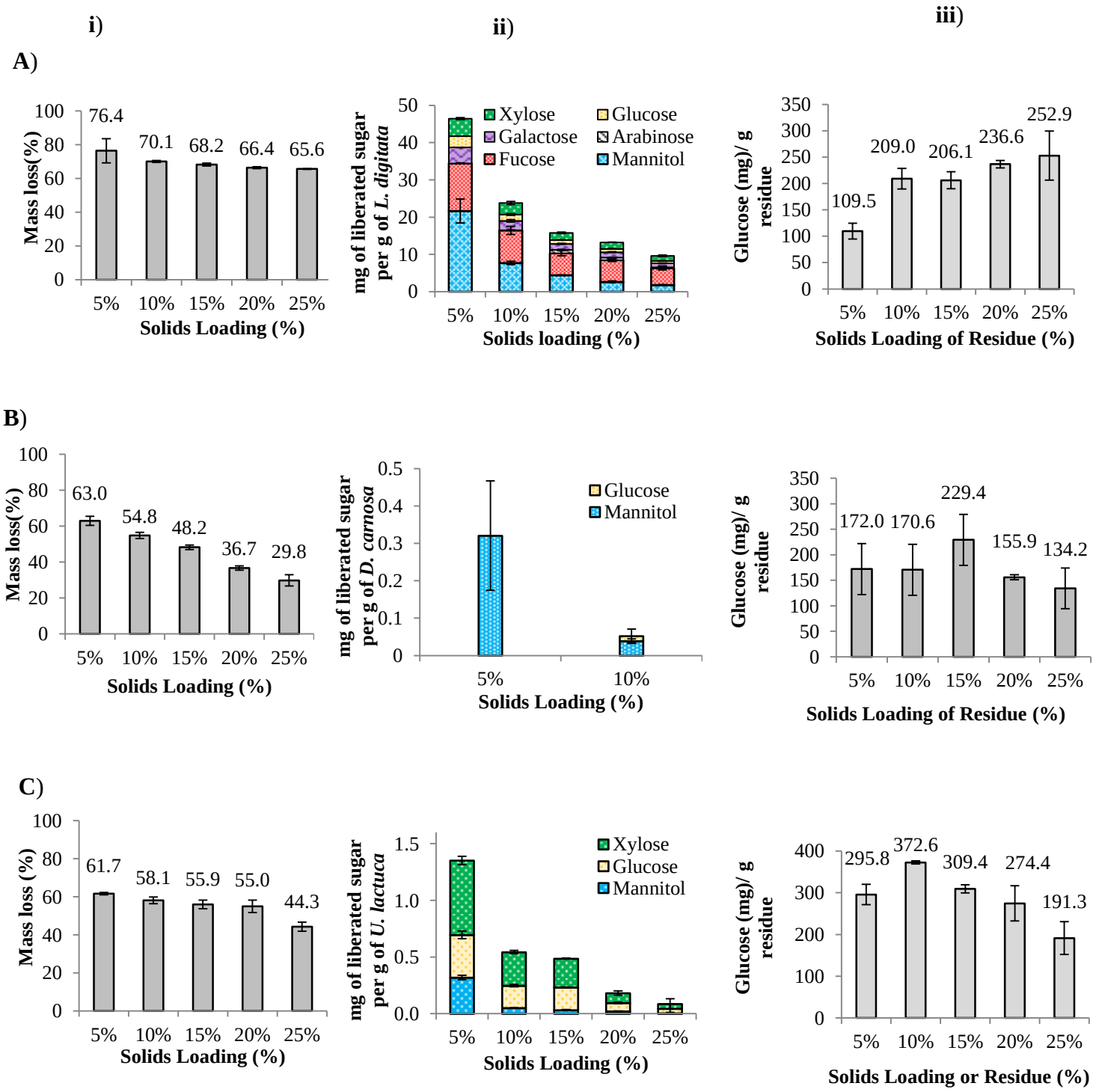
	P. elegans	***C. crispus***	***P. palmata***	***D. carnosa***	***U. lactuca***
B	19.3 ± 1.1	43.7 ± 1.0	28.2 ± 1.1	27.8 ± 1.4	6.6 ± 0.1
Na	1412.4 ± 62.4	5126.5 ± 7.3	882.7 ± 1.4	3584.0 ± 134.5	2844.6 ± 8.9
Mg	1125.1 ± 11.3	1490.5 ± 9.8	237.3 ± 7.2	922.0 ± 35.9	5073.1 ± 43.4
P	456.5 ± 4.7	560.4 ± 14.7	459.7 ± 15.9	613.1 ± 22.9	223.4 ± 3.5
S	4156.8 ± 249.6	12221.2 ± 127.8	1132.6 ± 24.8	6296.2 ± 156.4	9846.9 ± 156.9
K	13868.5 ± 524.6	5017.5 ± 107.6	11053.4 ± 396.6	6131.7 ± 290.9	3363.01 ± 55.5
Ca	3564.8 ± 47.3	1879.8 ± 7.7	563.7 ± 294.3	884.1 ± 22.7	1909.0 ± 11.8
Ti	1.7 ± 0.0	0.6 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.5 ± 0.0
Li	1.4 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Be	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Al	477.3 ± 51.6	49.8 ± 0.8	18.3 ± 0.1	36.3 ± 7.2	47.3 ± 1.1
V	2.0 ± 0.1	1.8 ± 0.0	4.5 ± 0.1	1.3 ± 0.1	0.4 ± 0.0
Cr	1.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Mn	157.7 ± 0.6	62.9 ± 3.2	5.9 ± 0.3	4.5 ± 0.5	3.4 ± 0.2
Fe	820.3 ± 84.8	137.3 ± 3.9	77.2 ± 2.0	155.8 ± 38.9	85.8 ± 2.7
Co	0.9 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.5 ± 0.2	0.1 ± 0.0
Ni	1.6 ± 0.1	5.3 ± 0.7	8.4 ± 0.7	23.2 ± 6.9	3.0 ± 0.4
Cu	3.3 ± 0.1	2.3 ± 0.0	2.6 ± 0.3	2.5 ± 0.1	1.4 ± 0.2
Zn	10.7 ± 0.0	16.2 ± 0.0	4.1 ± 0.1	18.5 ± 0.6	4.2 ± 1.4
As	2.0 ± 0.1	3.4 ± 0.0	1.6 ± 0.0	5.9 ± 0.6	1.1 ± 1.4
Se	0.5 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Rb	4.9 ± 0.1	2.4 ± 0.1	4.4 ± 0.1	2.8 ± 0.1	17 ±0.0
Sr	46.2 ± 0.3	21.6 ± 0.6	5.5 ± 1.9	11.8 ± 0.4	17.5 ± 0.2
Mo	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Ag	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cd	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Cs	0.8 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ba	1.5 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Tl	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pb	6.3 ± 0.2	2.6 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
U	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
I	2023.2 ± 36.1	402.1 ± 9.2	82.0 ± 8.4	322.1 ± 2.6	126.3 ± 1.8

9.2 Appendix B: Continuation of Chapter 3 Bioethanol Production from UK Seaweeds

Theoretical glucose yields are not presented here and the total glucose liberation in all generated liquid fractions (combined L1 and L2 hydrolysate fractions) was used as the indicator for pre-treatment efficacy.

9.2.1 Effect of variation of pre-treatment solids loading on sugar yields liberated into both the pre-treatment hydrolysate and after enzymatic hydrolysis

The next set of experiments investigated the effects of variation of the solids loadings content during the pre-treatment stage. This was investigated in order to evaluate the yields of monosaccharides directly liberated into the pre-treatment liquid fraction (L1) and also, glucose levels post enzymatic saccharification (L2) (with an excess of cellulolytic enzyme mix). Optimised pre-treatment conditions for the seaweeds were applied and results can be seen in Figure 9.1.



9.1 Effects on mass loss, monosaccharide liberation into L1 fraction and the liberation of glucose from treated seaweed residues at various solids loadings

Pre-treatment: *L. digitata* (121°C, 1.5N H_2SO_4, 24 min). *D. carnosa* (121°C, water, 30 min). and *U. lactuca* (121°C, water, 24 min). **A)** *L. digitata* **B)** *D. carnosa* **C)** *U. lactuca* **i)** Mass loss (%) **ii)** Quantification of monosaccharides in L1 fraction **iii)** Glucose liberation post enzymatic saccharification on thermo-chemically pre-treated residues. Data are the mean±SD of three measurements.

For *L. digitata,* previous pre-treatment conditions of 24 min at 121°C with 1.5N sulphuric acid were found to be optimal and maximised glucose solubilisation when conducted at a solids loading content of 10 % (w/v) as described in section 3.3.4.1. A slight decrease in mass loss was observed as the solids loading content increased from 5 to 25 % (Figure 9.1 Ai). At a solids loading of 5% (w/v), 76.4% of the *L. digitata* biomass was solubilised into the hydrolysate. This was the highest degree of mass loss that was observed when *L. digitata* was treated using its optimised pre-treatment conditions. Lower solids loadings typically resulted in higher degrees of mass loss. This may be because the ratio of the surface area to reactant (in this instance 1.5N sulphuric acid) was much higher. Although the concentration of H^+ ions remained the same throughout all reactions (only the solids loading contents varied), a greater proportion of the seaweed's surface was exposed to ionic attack as it was in a more dilute system. This potentially explains the higher concentration of different monosaccharides (inherent to the brown seaweed) that were detected in the liquid fraction (L1) that was produced after the thermo-chemical pre-treatment of *L. digitata* at a loading rate of 5% (w/v) (Figure 9.1 Aii). A total of 48.0 mg/g of the different monosaccharides were quantified in the L1 fraction, with mannitol being the most prominent monomeric sugar (21.6 mg/g seaweed).

The overall concentrations of the inherent brown seaweed monosaccharides decreased as the solids loading content increased beyond 5% (w/v), yet the liquid fractions still contained a mixture of the different monomeric sugars. Typically, processing biomass at elevated solids loading contents results in a greater concentration of monomeric sugars in the pre-treatment liquid fraction.

The decrease in yields seen in this experiment with *L. digitata* seaweed may suggest that the thermo-chemical pre-treatment was not as effective when solids' loading was increased. Efficient heat transfer throughout the sample could be limited when operating at higher solids loadings rates. This fact could be supported by the observation of decreased levels of mannitol in the L1 fraction. Mannitol, which is highly soluble in water and is usually present at quite high levels after pre-treatment (Xia *et al.*, 2015), can be easily extracted from brown seaweeds as it is found as a terminating side chain on the laminarin polymer (Ito and Hori 1989). Additionally, mannitol is easily extractable (together with fucoidan) from the call wall of brown seaweed using hot water (Percival and Ross, 1950, Black, 1954). In 2014 a group found that the sugar alcohol can be efficiently extracted with water from fresh seaweed that had not undergone any form of pre-treatment (Hal and Huijgen, 2014). Due to the high osmotic stress present in seaweed, mannitol would migrate from the cells walls directly to the extraction water phase without the need of physically breaking up the cell and/or cell walls prior to extraction. The decrease in mannitol concentration as the solids loading content increased (seen in Figure 9.1 A ii) suggested that the sugar alcohol could not have been solubilised from the seaweed, as the volume of available reactant decreased; inevitably limiting the flow of heat throughout the biomass. Cellulolytic saccharification of the pre-treated residues following the varying solids loading content experiment provided further information on how the increase in processed biomass affected the availability of cellulolytic binding sites in the pre-treated seaweed residues. The highest yield of glucose was liberated from the experiment using 25% solids loading pre-treatment (252.9 mg

glucose/g pre-treated biomass [at 25% solids loading]; Figure 9.1 Aiii). This may be because at such a high solids loading content the cellulose polymer may not have been exposed by the dilute acid treatment. Instead, the residue was more accessible for hydrolysis by the Novozymes Cellic®CTec2 enzyme mix. Processing at this loading rate is advantageous as more seaweed could be processed with less volume of dilute acid reactant per unit time and per heating cycle at 121°C. Furthermore, solubilised glucose from the enzyme hydrolysis could be directly fermented to bioethanol without the need to detoxify the hydrolysate from degradation products which may hinder the fermentation process. For these reasons, pre-treating *L. digitata* at a solids loading rate of 25% was deemed to be the best loading rate for this species of seaweed. The pre-treatment acid hydrolysate (L1 fraction) contained a mixture of different monosaccharides inherent to *L. digitata* (Figure 9.1 Aii). Due to their presence at low levels (<1% sugars), their fermentation to bioethanol may not be economically feasible. It would be a challenge to find a suitable microorganism to ferment the cocktail of different monosaccharides; especially as mannitol and fucose were the most abundant monomers present in the hydrolysates. However, this aspect is discussed and investigated in more detail in Chapter 5.

On the contrary, there was a gradual decrease in mass loss (63.0 – 29.8%) as the loadings content increased during the pre-treatment of *D. carnosa* (Figure 9.1 Bi). No volume of the water based hydrolysate was recovered from the reactions conducted at solids loading rates of 15, 20 and 25% (w/v). The low volumes of recoverable hydrolysate from the 5 and 10% solids loading reaction contained negligible amounts of monomeric sugars (Figure 9.1 Bii).

Relatively dry slurries remained after pre-treatment at 15, 20 and 25% solids loadings and no volume of hydrolysate (L1) was collected even after centrifugation. Due to the hygroscopic nature of *D. carnosa* biomass (which results from the presence of polysaccharides containing water gelling properties) the formation of no liquid fraction post auto-hydrolytical treatment at 121°C for 30 min was perhaps to be expected. Although auto-hydrolytical pre-treatment at higher solids loading contents did not produce a fermentable liquid fraction, the treatment did not generate any unwanted degradation products. This implies that a washing step between the pre-treatment and enzymatic saccharification stages may not be required and the pre-treated residues could be directly hydrolysed by relevant enzyme mixtures. This would be favourable for bioethanol production from this particular red seaweed on an industrial scale. The enzyme buffer (at an appropriate pH) containing the enzyme could be charged directly into the vessel containing the *D. carnosa* solids for the enzymatic saccharification process to start. Additionally, as the pre-treatment was conducted at 121°C, the residue and the reactor would remain sterile. This reduces the chances of any microbial contamination that could adversely affect the enzyme hydrolysis. A treated residue of *D. carnosa* that underwent auto-hydrolytical treatment at 121°C for 30 min with a 15% (w/v) solids loading liberated ca 229.4 mg glucose/g pre-treated residue post cellulolytic saccharification (Figure 9.1 Biii); the highest yield which appeared to be the optimum loading content for this species of seaweed. The effectiveness of the optimum pre-treatment conditions for *D. carnosa* diminished beyond 15% solids loading. This may be due to the low volume of water present in such reactions which inevitably prevents adequate

heating of the seaweed as the reaction is static. Mixing may need to be employed in order to enable adequate heat transfer throughout the seaweed during its pre-treatment. However this was not feasible with the bench-top autoclave used in this investigation.

As seen with *D. carnosa*, increasing the solids loading content of *U. lactuca* from 5 to 25% during auto-hydrolytical pre-treatment gradually reduced the severity of mass loss (Figure 9.1 Ci). Unlike *D. carnosa* however, liquid fractions (L1) were generated and collected for analysis post auto-hydrolysis at 121°C for 24 min. The liquid fraction produced from the 5% solids loading residue contained the highest content of monosaccharides, including mannitol and glucose but predominately xylose (Figure 9.1 Cii). However, overall yields were extremely low and corresponded to a total value of only 1.35 mg/g. Total yields of liberated monosaccharides decreased as the seaweed loading content increased. Once again this was indicative of a reduced pre-treatment efficacy. It appeared that pre-treatment at 10% solids loading was the optimum loading content that solubilised the maximum amount of glucose from the green seaweed, achieving a yield of 372.6 mg/g (Figure 9.1 Ciii). A yield of 386.0 mg/g glucose was recovered when the pre-treatment conditions were initially examined for efficacy in section 3.3.5.4, all at a solids loading content of 10% (w/v). A 10% solids loading content proved to be most for this particular species of seaweed again under optimum pre-treatment conditions. The efficacy of the pre-treatment decreased as the solids loadings content continued to increase, which ultimately resulted in lower recoverable yields of glucose post enzyme hydrolysis.

9.2.2 Optimisation of the solids loading used during the enzymatic saccharification of pre-treated seaweed residues

The ratio of buffer to seaweed biomass was investigated (2, 4, 8 and 16% [w/v]) in order to examine whether effective enzymatic hydrolysis could still be achieved at higher solids loading contents. It was hypothesised that glucose yields (mg/g pre-treated seaweed biomass) would decrease with increasing solids loading during enzyme hydrolysis. Enzymatic hydrolysates (L2) were recovered and had their volumes recorded. Additionally, hydrolysates were analysed for glucose and the acquired glucose data was expressed in both mg/g glucose and g/L glucose. This was done in order to compare hydrolysates and determine how much of the seaweed residues could be enzymatically solubilised to produce feedstocks suitable for bioethanol fermentation. It is important to quantify glucose in both mg/g and g/L terms. Firstly, mg/g (mg of glucose released per g of pre-treated seaweed) data is indicative of enzyme hydrolysis efficacy whilst g/L indicates the concentration of glucose that is present in the recovered hydrolysate per L (which is important for fermentation). The final volume (mL) of recovered hydrolysate post enzymatic saccharification was also recorded as adequate hydrolysate volumes are needed in order to conduct fermentations and other calculations. Figures 9.2 A-C shows the yields of glucose (mg/g and g/L) and volumes of recovered hydrolysates from the seaweeds post enzyme hydrolysis at different seaweed to enzyme buffer loading rates.

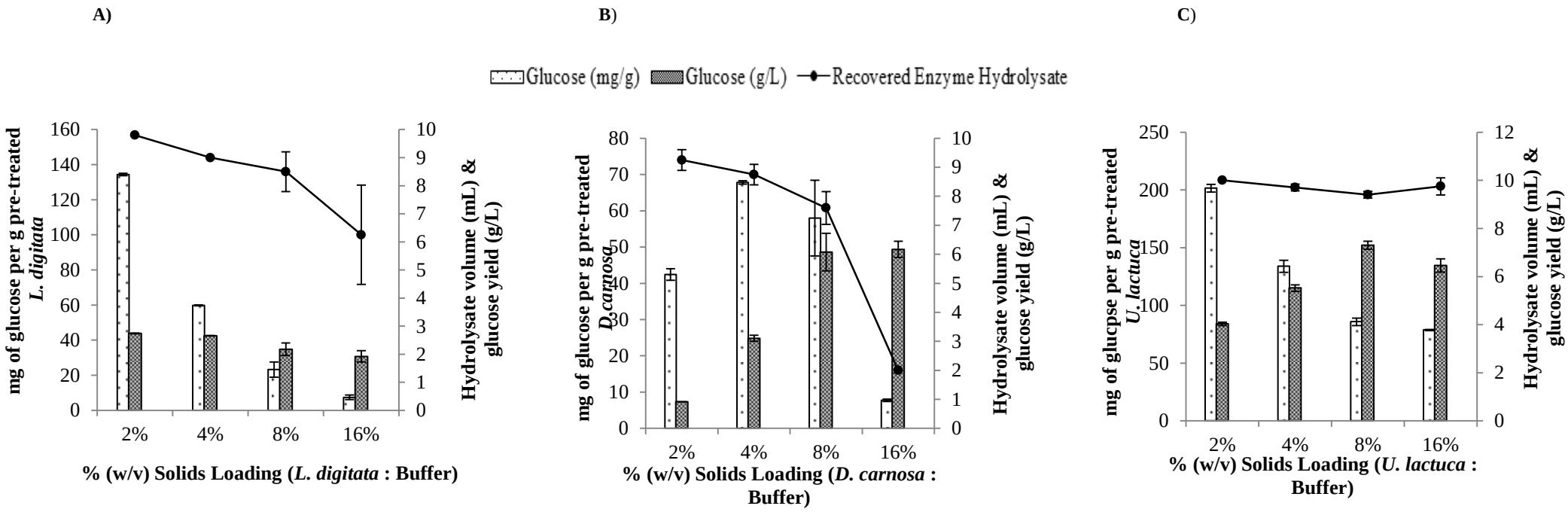


Figure 9.2 Yields of glucose (mg/g and g/L) and volumes of recovered hydrolysates (mL) from seaweeds post enzyme hydrolysis at different seaweed to enzyme buffer loading rates. A) *L. digitata* B) *D. carnosa* C) *U. lactuca*

Pre-treatment: *L. digitata* (121°C, 1.5N H_2SO_4, 24 min, 25% (w/v) solids loading). *D. carnosa* and *U. lactuca* (121°C, autohydrolytical, 30 min and 24 min respectively). Enzyme hydrolysis conducted at different solid loading rates with an excess of CTec2 and final volumes made up to 10 mL. Data are the mean±SD of three measurements.

The ratio of pre-treated *L. digitata* biomass (from optimum pre-treatment conditions) to the volume of enzyme buffer appeared to have a direct influence on the volumes of recoverable hydrolysate and the amounts of glucose released per g of seaweed (Figure 9.2 A). As predicted, the glucose yields obtained from the above enzyme hydrolysis reactions were lower than the yield (252.5 mg/g) obtained from the dilute 0.5% (w/v) loading rate used in section 3.3.3.5. It appeared that the optimum enzyme hydrolysis conditions for pre-treated *L. digitata* were achieved with the lowest loading rate of 2% (w/v) from all those tested. A final enzyme hydrolysate volume of 9.8 mL was recovered post the saccharification (starting volume of enzyme buffer was 10 mL) containing ca 134.4 mg/g glucose. However this corresponded to a glucose concentration of 2.74 g/L. This suggests that although these conditions were the most suitable to liberate glucose from the pre-treated seaweed under enzymatic attack and an adequate volume of hydrolysate was recovered, the overall concentration of glucose (g/L) is not ideally sufficient to produce bioethanol on a commercial scale. Lower volumes of hydrolysate were recovered as the solids loading contents increased (9.8 - 6.6 mL). Additionally, the efficacy of the saccharification reaction with Novozymes Cellic®CTec2 decreased as the loading content increased from 2 to 16% (w/v) (134.3 – 7.4 mg/g glucose). This is possibly due to the interaction of phaeophycal hydrocolloids (such as alginate) with the buffer. As a consequence, when enzymatic hydrolysis was conducted at higher loading rates, particularly at 16% (w/v), more of the exposed hydrocolloid was available for interaction with the buffered liquid. This would ultimately increase the viscosity of the enzymatic reaction and as a result probably

inhibited the ability of the cellulolytic enzymes to reach compatible target sites. Not only did this produce a lower volume of hydrolysate (6.25 mL) but the efficacy of the saccharification reaction was additionally hindered, yielding only ca 7.40 mg/g glucose.

With *D. carnosa*, the glucose yields obtained from the enzyme hydrolysis reactions were lower than the yield (229.4 mg/g) obtained from the dilute 0.5% (w/v) loading rate used in preliminary pre-treatment screening (as predicted). The volume of recoverable hydrolysate (L2) slightly decreased from 9.25 to 7.62 mL as the loading content increased from 2 to 8% (w/v); however the volume dropped dramatically from 7.62 mL to 2.01 mL when the loading content increased from 8 to 16 % (w/v). The same phenomenon seen with *L. digitata* was again observed with *D. carnosa*; the viscosity of the enzyme reaction increased, probably for the same reasons as previously described. The decrease in recoverable hydrolysates gave rise to increased concentrations of glucose (g/L) as solids loading content increased. The yields of glucose solubilised from *D. carnosa* polymers increased from 42.4 to 67.8 mg/g when the loading content doubled from 2 to 4% (w/v). Interestingly glucose yields dropped from 67.8 to 57.9 mg/g as the loading content increased to 8% however a dramatic decline in yields down to 7.7 mg/g was seen during enzyme hydrolysis at a 16% (w/v) loading rate. The most suitable loading content for the enzymatic saccharification of pre-treated *D. carnosa* seaweed was considered to be 8% (w/v). This was concluded based on the fact that it was the only loading content that had yielded the highest concentrations of glucose in both mg/g and g/L values (57.9 mg/g and 6.1 g/L, respectively). Additionally, there was only a 1.65 mL difference between

hydrolysates recovered from the 2% (w/v) reaction that had recovered the highest volume of enzymatic hydrolysate. Ideally, obtaining a hydrolysate containing a high yield of glucose is more desirable for conversion to bioethanol.

A major difference was noticed during the investigation of different loading contents for pre-treated *U. lactuca* biomass during enzyme saccharification compared to *L. digitata* and *D. carnosa* pre-treated residues. A highly viscous slurry was not generated following the reaction, and almost all enzyme generated hydrolysates (L2) were recovered from each reaction. This is may be because *U. lactuca* might not contain polysaccharides with gel forming properties and ulvan in particular (which is found predominantly in green seaweeds) forms weak gels and low viscosity mixtures in the presence of water (Alves *et al.*, 2013). *U. lactuca* hydrolysates post enzyme saccharification were the easiest to handle out of the three species of seaweeds investigated in this Chapter. This is an important factor to take into consideration if bioethanol is to be produced at commercial scale. The formation of free-flowing, non-viscous liquids is ideal in order to prevent blockages in reactors and to enable the easy transfer of the desired liquid fractions into subsequent reaction vessels. It appeared that the efficacy of the enzyme hydrolysis slightly decreased as the solids loading content in the reactions increased. A reaction at 2% loading content yielded 201.5 mg/g glucose whereas 16% liberated only 78.7 mg/g glucose. This did result however in a slight increase in glucose content in the generated hydrolysate, from 4.0 to 6.5 g/L respectively. Despite all reactions producing less viscous, more fluid-like liquid fractions, less glucose was depolymerised from cellulose

like polymers in the biomass at higher solids loading reactions. A higher dose of enzyme may be required to achieve higher yields of glucose from this pre-treated green seaweed. Seeing that at 16% (w/v) no obstacles with regards to viscosity levels during the reaction were encountered, processing more *U. lactuca* biomass at a higher loading rate beyond 16% could be possible. Although processing more biomass is advantageous, a compromise will have to be met as a greater dose of enzyme may then be required; and this will undoubtedly increase the cost of the process. If more time was available, the optimisation of enzyme dosing rates would have been investigated at higher seaweed loading rates during this enzymatic hydrolysis step. A solids loading content of 8% (w/v) was selected as the most optimum condition for enzyme saccharification of *U. lactuca.* This is because the highest concentration of glucose (7.3 g/L) was quantified in the 9.4 mL recovered hydrolysate. Additionally, there was only a 48.1 mg/g glucose difference between the 4% and 8% seaweed loading content reactions, which suggested that there was not a great difference in the efficacy of seaweed hydrolysis reactions between the two.

9.2.3 Trial fermentations of seaweed enzyme hydrolysates using *Saccharomyces cerevisiae*

The three optimised pre-treatment processes (see legend in Figure 9.3) for *L. digitata*, *D. carnosa* and *U. lactuca* were applied to the seaweeds on a 1 L scale. This was in order to generate a sufficient quantity of pre-treated seaweed biomass to enable sufficiently large volume enzymatic hydrolysis reactions to be conducted to produce feedstocks. These feedstocks were then used for trial fermentations in attempts to produce bioethanol. The partially

optimised enzymatic saccharification steps (utilising an excess of Novozymes Cellic® CTec2; ca. 50 FPU per g biomass) were subsequently conducted on each species of seaweed to produce enough hydrolysate to conduct 25 mL fermentations using *S. cerevisiae* strain NCYC2592. Fermentations also using strain NCYC2592 were also performed with 4% YPD, in order to serve as a control. The glucose concentrations of the hydrolysates and achieved ethanol yields from the three seaweeds can be seen in Figure 9.3 A and fermentation progression profiles in Figures 9.3 B. The concentrations of glucose in the enzyme hydrolysates produced from the different seaweeds varied significantly. The *L. digitata* derived enzymatic hydrolysate had a glucose content of 6.6 g/L; the lowest yield achieved from all of the three seaweeds tested. In contrast, *D. carnosa* produced a hydrolysate with a glucose concentration of 13.5 g/L whilst the *U. lactuca* derived hydrolysate had the highest concentration of glucose (17.7 g/L). These yields of glucose are somewhat higher than was previously achieved at smaller lab-scale. The trial fermentations with *S. cerevisiae* NCYC2592 were conducted in order to determine whether either feedstock was able to outperform the other in terms of fermentation rate and final ethanol productivity. No additional nutrients were added into the feedstock hydrolysates prior to fermentation. Different concentrations of ethanol were produced from the three feedstocks with *L. digitata*, *D. carnosa* and *U. lactuca* producing 3.2 g/L, 5.4 g/L and 7.8 g/L of ethanol, respectively. These yields equated to ca 94.5%, 78.4% and 86.5% of theoretical ethanol yield, respectively (calculated based upon the initial content of glucose present in each of the seaweed's hydrolysates and the theoretical maximum ethanol concentration that can be produced).

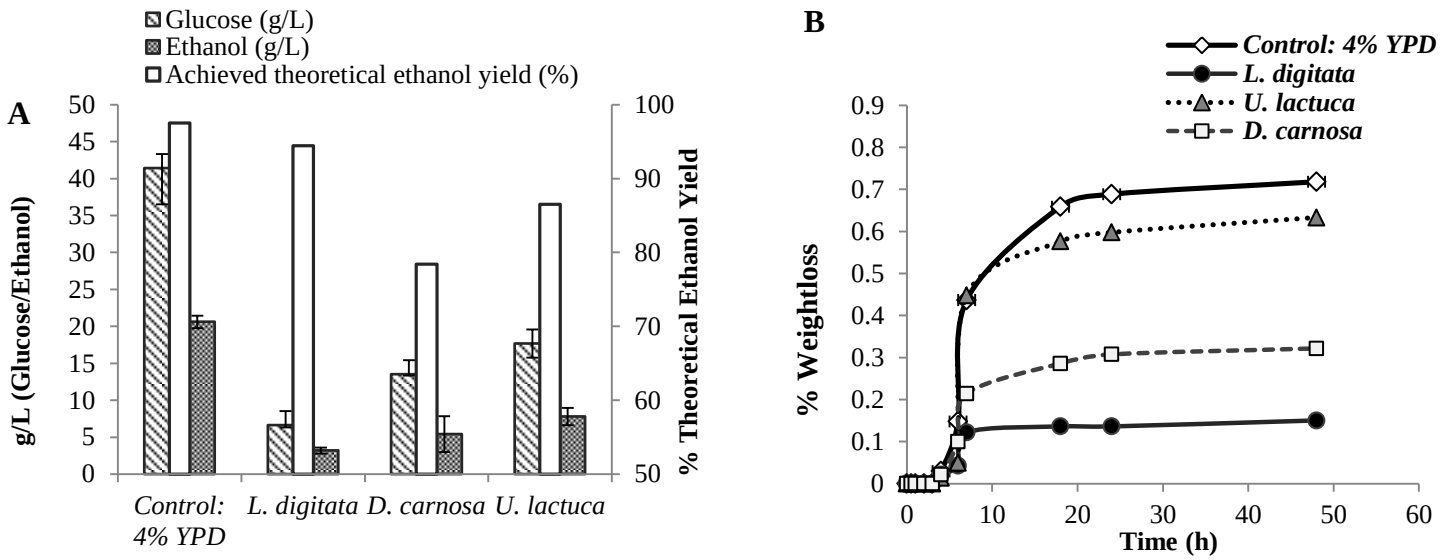


Figure 9.3 Comparison of yield data (A) and fermentation progression (B) from the fermentation trials (with *S. cerevisiae* strain 2592) of enzymatic hydrolysates resulting from either optimised autohydrolytical or dilute acid hydrothermal pre-treatment processes on the three seaweeds. A: Theoretical ethanol yield based on based on glucose concentration in the three feedstocks. B: Fermentation progression monitored by weight-loss of vessels due to CO_2 evolution. Trials were compared against a 4% Yeast Peptone Dextrose (YPD) control.

L. digitata Pre-treatment: 1.5N H_2SO_4 (121°C 24 min) at 25% solids loading (w/v). Enzyme digestion: Novozymes Cellic®CTec2 (dosed in excess ca. 50 FPU per g biomass) 48 h incubation at 50°C at 2% solids loading (w/v).

D. carnosa Pre-treatment: Auto-hydrolytical (121°C 30 min) at 15% solids loading (w/v). Enzyme digestion: Novozymes Cellic®CTec2 (dosed in excess ca. 50 FPU per g biomass) 48 h incubation at 50°C at 8% solids loading (w/v).

U. lactuca Pre-treatment: Auto-hydrolytical (121°C 24 min) at 10% solids loading (w/v). Enzyme digestion: Novozymes Cellic®CTec2 (dosed in excess ca. 50 FPU per g biomass) 48 h incubation at 50°C at 8% solids loading (w/v).

Fermentations conducted at 30°C with stirring (120rpm) using *S. cerevisiae* NCYC2592 at a pitching rate of $1x10^7$ cells/mL in 25 mL of hydrolysate. Data are the mean ± SD of three replicate experiments.

The fermentation progression of each of the three feedstocks can be seen in Figure 9.3 B. All three exhibited rather similar initial lag phase periods; however the times of attenuation differed. It appeared that *L. digitata* feedstock reached attenuation first after ca 6 h, whereas *D. carnosa*, *U. lactuca* and the control feedstocks all entered the attenuation phase after ca 18 h. Due to the lower content of glucose in *L. digitata* feedstock, the fermentation was relatively short (hence faster attenuation) and the vessels had the lowest percentage weight loss over the course of the fermentation. This indicated that the evolution of CO_2 was low and confirmed a less vigorous fermentation due to the lower levels of glucose. Higher percentage weight losses (from the fermentation vessels) were exhibited from the *U. lactuca* and *D. carnosa* feedstocks, which is in agreement with the higher yields of ethanol produced from the increased glucose present in their hydrolysates. The fermentation progression of the *U. lactuca* feedstock was somewhat similar to the YPD control media (which produced the highest yield of ethanol), as adequate concentrations of glucose were present in both feedstocks. *D. carnosa* fermentations progressed a little more slowly than the YPD control and *U. lactuca* feedstock however better than *L. digitata*, possibly due to perhaps the relatively intermediate content of glucose in the feedstock or possibly a deficiency in assimilable nitrogen or some micronutrient (such as an essential trace element like zinc which is essential for the alcohol dehydrogenase enzymes that ultimately produce ethanol).

9.2.4 Conclusions

Results from this work revealed that the application of a pre-treatment did generally enhance enzymatic hydrolysis efficiencies (sugar yields) from

seaweed. Furthermore, one universal pre-treatment could not be applied and the optimal pre-treatment conditions were found to be specific for each individual species (Table 9.3).

Table 9.3 Summary of optimum pre-treatment conditions for each species of seaweed investigated in this Chapter, including achieved ethanol yields following fermentation

Seaweed Species	Optimum Pre-treatment Conditions at 121°C	*Optimum Enzyme Hydrolysis Solids Loading	Achieved Ethanol Yields (g/L)
L. digitata	1.5 N H_2SO_4, 24 min at 25% (w/v) solids loading	2% (w/v)	3.2
D. carnosa	H_2O, 30 min at 15% (w/v) solids loading	8% (w/v)	5.4
U. lactuca	H_2O, 24 min at 10% (w/v) solids loading	8% (w/v)	7.8

* Novozymes Cellic® CTec2 dose was not optimised in this Chapter and as such was dosed in an excess in order to achieve maximum glucose liberation from each seaweed.

The glucose yield after enzyme saccharification of pre-treated *L. digitata* (using the optimised pre-treatment conditions) was ca. 252.9 mg/g seaweed. This value was significantly in excess of the original glucose content quantified in native (non-pre-treated) *L. digitata* biomass (ca. 80 mg/g native seaweed). This highlighted the substantial degree of enrichment of glucose-rich polysaccharides that occurs as a result of the significant mass loss (of non-carbohydrate components) from the pre-treatment reactions (Section 3.3.4.1). A prior study conducted on brown *Sargassum* spp suggested the optimum pre-treatment for this brown seaweed to be between 3.4 - 4.6 % (w/v) sulphuric acid at 115°C for 1.5 h and at 10% solids loadings (Borines *et al* 2013). However, enzyme saccharification with 2 different commercial enzyme mixtures only liberated ca. 45 mg/g glucose from the pre-treated seaweed biomass. Although the concentrations of acid used in the current study and the study conducted by Borines *et al* were relatively similar (1.5 N

sulphuric acid equates to approximately 4.09 % [w/v]) and the reaction temperatures were also similar (115°C vs 121°C) it may be possible that the reaction time was too long and resulted in lower yields of glucose post enzyme hydrolysis. Additionally, Borines *et al* included a washing step between pre-treatment and enzyme saccharification which used hot water, instead of the ambient water used in this work. The use of hot water could have washed away easily soluble monosaccharides, especially glucose and ultimately reducing their final achieved yields of glucose. Although thermal acid pre-treatment is commonly used on seaweeds, its application does have the potential disadvantages of formation of sugar degradation products, high energy consumption and also potential for environmental pollution. Therefore, as all these factors need to be taken into consideration, moderate pre-treatment technologies are required in order to improve the economic feasibility and sustainability of the seaweed biofuel industry. Researchers are currently looking into new technologies to overcome the disadvantages of thermo-chemical pre-treatments, and a potential alternative could be the use of hydroxyl radicals and hot water pre-treatment which was used as a pre-treatment on the brown seaweed *Macrocystsis pyrifera* (Gao *et al* 2015). Auto-hydrolytical pre-treatments could be a plausible alternative as they are much more environmentally friendly (than the use of reagents such as acids) and with the conditions used in this work, no sugar degradation products were formed. Interestingly, results from this work showed that auto-hydrolytical treatment of the red seaweed *D. carnosa* and the green seaweed *U. lactuca* species prior to enzymatic hydrolysis most effectively enhanced glucose liberation. The use of a water based hydrolysis method for the pre-treatment of

seaweeds has not been reported previously. However Trivedi *et al* (2013) investigated whether use of sodium acetate buffer alone (used for enzymatic saccharification at pH 4.8) could enhance the liberation of glucose post enzyme hydrolysis, after *Ulva* spp had been treated with the buffer at 120°C for 60 min at a solids loading content of 5% (w/v). A total of 206 mg glucose per g of the green spp was ultimately released; suggesting that the sodium acetate could have served as a catalyst for the deconstruction of the seaweed cell wall. Nevertheless, auto-hydrolytical pre-treatment without any pH adjustments at 121°C for 24 min at a loading content of 25% (w/v) followed by cellulolytic enzyme hydrolysis on *U. lactuca* in this study liberated 376.2 mg of glucose/g pre-treated seaweed. Additionally, this was the first study that evaluated the potential of *D. carnosa* as a feedstock for bioethanol production and results from this work indicate that this red species is an ideal candidate, especially as no form of thermo-chemical pre-treatment is required prior to enzyme saccharification. Had additional time been available it would have been of interest to have evaluated the differences in the structures of the seaweed surfaces before and after pre-treatment when using either dilute acid or water based treatments. This could have possibly been achieved through SEM imaging and may have provided valuable information about the specific cell wall modifications that resulted from each different pre-treatment. Enzymatic saccharification yields (mg of glucose released per g of pre-treated seaweed biomass) did improve when the solids loading content (seaweed to buffer ratio) was increased. However, the optimal solids loading rate differed between species. The commercial enzymatic mixture which was employed (Novozymes Cellic® CTec2) appeared to be compatible with all three

seaweeds that were shortlisted for further investigation. Other published studies typically utilise a combination of at least 2 commercial enzymes (either a mixture containing a blend of different enzymes) or a single enzyme alone in order to enhance glucose liberation (Ra *et al* 2015, Yanagisawa *et al* 2011, van der Wal 2013). However, the use of different enzymes will make the overall processing costs rather high. In this work enzyme dose was not optimised however it could be speculated that the optimal dose would depend on many different factors. These factors may include the species of seaweed, pre-treatment method and processing conditions. This would mean that enzyme dosage would have to be re-evaluated for each of the different species of seaweed used in this study. One alternative which could reduce process costs and eliminate the need to purchase external sources of enzymes would be to produce crude in-house enzyme mixtures that could be specific to each type of seaweed residue. This could be achieved through solid state fermentations that employ filamentous fungi, as they have the ability to grow and secrete enzymes for plant cell wall deconstruction. Ultimately a crude enzyme mix could then be applied for the hydrolysis step on pre-treated seaweed biomass, and could target the specific polymers present in the seaweed residues for depolymerisation to fermentable monosaccharides. However, this would require further research and optimisation that would have to be tailored to each seaweed residue. In order for bioethanol production to be viable on a commercial scale, ca. 4-5% (ABV) of ethanol has to be produced (Girio *et al*, 2010). As yeast convert glucose to ethanol at a rate of 0.51 g ethanol (per g of glucose), a fermentable hydrolysate containing a minimum of ca. 8% (80 g/L) glucose needs to be generated to make ethanol production from seaweed

economically viable. Although the processes developed in this chapter for the seaweeds investigated have not yet been fully optimised, the highest yield of ethanol was achieved from *U. lactuca* feedstock; reaching almost 1% (ABV). This may be because green species of seaweed contain a greater cellulose fraction in comparison to the other seaweed species. This could explain why Novozymes Cellic® CTec2 worked particularly well with *U. lactuca* and hydrolysed higher yields of glucose per g of biomass (as this enzyme mixture was originally developed for terrestrial plants and their substantial cellulose fractions). However, there is scope for further improvement and optimisation of the process which could ultimately enhance the ethanol production from UK species of seaweed.

9.3 Appendix C: Metal Analysis of *L. digitata* biomass prior to and post bio-refining process

Table 9.4 Metal Analysis of *L. digitata* (Setalg) used in bio-refining process, expressed as mg per kg^{-1} of seaweed. *L. digitata* 1: Native biomass. *L. digitata* 2: new composite residue from bio-process

	L. digitata 1	*L. digitata 2*
B	23.9 ± 0.2	6.7 ± 0.2
Na	7639.9 ± 142.9	2931.1 ± 126.4
Mg	1494.2 ± 29.5	948.6 ± 39.2
P	289.8 ± 0.6	211.9 ± 3.3
S	2145.3 ± 40.0	1758.3 ± 93.0
K	12046.6 ± 208.4	14149.3 ± 679.2
Ca	2779.9 ± 36.8	3703.7 ± 158.8
Ti	1.3 ± 0.0	2.3 ± 0.1
Li	0.2 ± 0.0	0.2 ± 0.0
Be	0.0 ± 0.0	0.0 ± 0.0
Al	63.4 ± 0.9	104.7 ± 3.1
V	0.3 ± 0.0	0.4 ± 0.0
Cr	0.9 ± 0.0	12.6 ± 0.7
Mn	3.3 ± 0.1	4.8 ± 0.4
Fe	215.0 ± 14.0	421.8 ± 34.9
Co	0.1 ± 0.0	0.2 ± 0.0
Ni	0.5 ± 0.0	5.8 ± 0.4
Cu	0.4 ± 0.0	5.7 ± 0.0
Zn	4.9 ± 0.1	5.9 ± 0.3
As	17.2 ± 0.2	6.8 ± 0.3
Se	0.3 ± 0.0	0.4 ± 0.0
Rb	5.8 ± 0.1	3.0 ± 0.1
Sr	178.9 ± 2.8	272.0 ± 11.0
Mo	0.2 ± 0.0	0.5 ± 0.1
Ag	0.0 ± 0.0	0.0 ± 0.0
Cd	0.0 ± 0.0	0.0 ± 0.0
Cs	0.0 ± 0.0	0.0 ± 0.0
Ba	2.2 ± 0.0	4.7 ± 0.1
Tl	0.0 ± 0.0	0.0 ± 0.0
Pb	0.1 ± 0.0	0.3 ± 0.0
U	0.0 ± 0.0	0.0 ± 0.0
I	5437.2 ± 50.8	2890.3 ± 156.9

9.4 Appendix D: Antimicrobial activity of prepared extracts from *L. digitata* bio-refining process

Table 9.5 *Staphylococcus aureus*

Y/N=Yes/No

Extract Code	# on plate	Clearance Zone	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	**Y**	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	**Y**	
WR-M	8	N	
WR-M	9	**Y**	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	N	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.6 *Salmonella enterica*

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	**Y**	
LD-M	2	N	
LD-M	3	N	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	N	
WR-M	8	**Y**	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	**Y**	
WL-M	14	**Y**	
WL-W	15	N	
WL-W	16	N	
FS-W	17	N	
FS-W	18	**Y**	
FS-W	19	N	
FS-W	20	**Y**	
FS-W	21	**Y**	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.7 ***Burkholderia cenocepacia***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	**Y**	
LD-M	3	**Y**	
LD-W	4	**Y**	
LD-W	5	N	
LD-W	6	N	
WR-M	7	**Y**	
WR-M	8	**Y**	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	**Y**	
WL-M	14	**Y**	
WL-W	15	**Y**	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	**Y**	
FS-W	21	**Y**	
FS-W	22	**Y**	
Water	23	N	
Methanol	24	N	

Table 9.8 *Burkholderia multivorans*

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	N	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	**Y**	v. faint
WR-W	11	**Y**	v. faint
WR-W	12	**Y**	v. faint
WL-M	13	**Y**	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	N	
FS-W	21	**Y**	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.9 *Bacillus cereus*

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	**Y**	
LD-M	2	**Y**	
LD-M	3	N	
LD-W	4	N	
LD-W	5	**Y**	
LD-W	6	N	
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	**Y**	
WL-M	13	**Y**	
WL-M	14	**Y**	
WL-W	15	N	
WL-W	16	N	
FS-W	17	**Y**	
FS-W	18	N	
FS-W	19	N	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.10 ***Streptococcus pyogenes***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	**Y**	
LD-M	2	N	
LD-M	3	N	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	**Y**	
WR-M	8	N	
WR-M	9	**Y**	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	**Y**	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	**Y**	
FS-W	18	**Y**	
FS-W	19	N	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.11 ***Acinetobacter iwofii***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	**Y**	
LD-W	4	**Y**	
LD-W	5	**Y**	
LD-W	6	N	
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	**Y**	
WL-M	14	N	
WL-W	15	**Y**	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	**Y**	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.12 ***Listeria monocytogenes***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	N	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	N	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	**Y**	
FS-W	18	N	
FS-W	19	**Y**	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.13 *Yersinia ruckerii*

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	N	
LD-W	4	N	
LD-W	5	N	
LD-W	6	N	
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	**Y**	v. faint
WR-W	11	**Y**	v. faint
WR-W	12	**Y**	v. faint
WL-M	13	N	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.14 ***Vibrio anguillerium***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	N	
LD-W	4	**Y**	v. faint
LD-W	5	**Y**	v. faint
LD-W	6	**Y**	v. faint
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	**Y**	v. faint
WR-W	11	**Y**	v. faint
WR-W	12	**Y**	v. faint
WL-M	13	N	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	**Y**	
FS-W	18	**Y**	
FS-W	19	**Y**	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	

Table 9.15 ***Aeromonas hydrolphila***

Y/N=Yes/No

Extract Code	# on plate	Clearance Zones	Comments
LD-M	1	N	
LD-M	2	N	
LD-M	3	N	
LD-W	4	**Y**	v. faint
LD-W	5	**Y**	v. faint
LD-W	6	**Y**	v. faint
WR-M	7	N	
WR-M	8	N	
WR-M	9	N	
WR-W	10	N	
WR-W	11	N	
WR-W	12	N	
WL-M	13	N	
WL-M	14	N	
WL-W	15	N	
WL-W	16	N	
FS-W	17	N	
FS-W	18	N	
FS-W	19	N	
FS-W	20	N	
FS-W	21	N	
FS-W	22	N	
Water	23	N	
Methanol	24	N	