STRUCTURAL INVESTIGATIONS INTO CELL WALL BIOMASS IN THE ULVA SEAWEED GENUS

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STRUCTURAL INVESTIGATIONS INTO CELL WALL BIOMASS IN THE ULVA SEAWEED GENUS

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Abstract

Climate change is a notorious and serious problem that needs to be attended with no delays. Mitigation of fossil fuel emissions are mandatory to reduce the production of green-house gases, however, as population increases, the global energy demand increases as well, with fossil fuels acting as the main energy source, globally. Worldwide, different strategies towards the development of sustainable societies applying circular economy principles and decarbonization of the industrial infrastructures are among the most important goals to achieve for the coming years. The use of biomass to accomplish such goals by producing bio-derived commodities and biofuels have gained track on the last two decades. One important source of biomass that has been highly studied in the recent years is the one coming from the aquatic feedstock called macro algae, which is normally used for food and cosmetic applications. However, the application of the macro algae to produce the so-called third generation biofuels or other chemical commodities, although having some drawbacks, seem to lead to promising results for the coming years with more and more mature technologies for such tasks. Belonging to the genus *Ulva,* the sea lettuce, a group of green algae have been identified as a potential candidate to be used to produce bio-derived products. In this work, through a literature review, the effects of the use of carbon and oil worldwide were appraised, as well as some of the most studied alternatives to mitigate the negative effects caused to the environment using fossil fuels. The possible use of macro algae as a promising candidate to produce bio-derived products was also highlighted. Using an *Ulva* sp. collected from the coast at county Durham, qualitative analysis of the cellulose and ulvan biochemical fractions were obtained. Through Fourier-Transformed Infrared Spectroscopy, the possible existence of acetylated cellulose in algae was explored. Mass-spectrometry analysis was performed to clarify the obtained results from the Fourier-Transformed Infrared analysis and finally,

Thin-Layer chromatography analysis was performed to assess whether the presence of the endotransglycosylase enzymes could catalyze the linking between wall polysaccharides from the sample and some oligosaccharides. In this work, a better understanding of the composition from the *Ulva* sp. collected from Durham coast was gained, developing a foundation for more in-depth focused research of ulvan biomass as a resource to produce high quality biofuels in the coming future.

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1. Acknowledgments

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2. Introduction

Recent years have seen increased awareness of the fact that pollution worldwide has increased alarmingly, generating a diverse range of problems and threats to the environment and to humanity [1, 2]. In a well-cited study, Grossman & Krueger [3] explain that the generation of pollutants is an inevitable result of human industrialization, supporting work by the American Chemical Society (ACS) [4] that shows that the concentrations of CO_2, NO_x and CH_4 in the atmosphere have been increasing exponentially since 1750 (Figure 1).

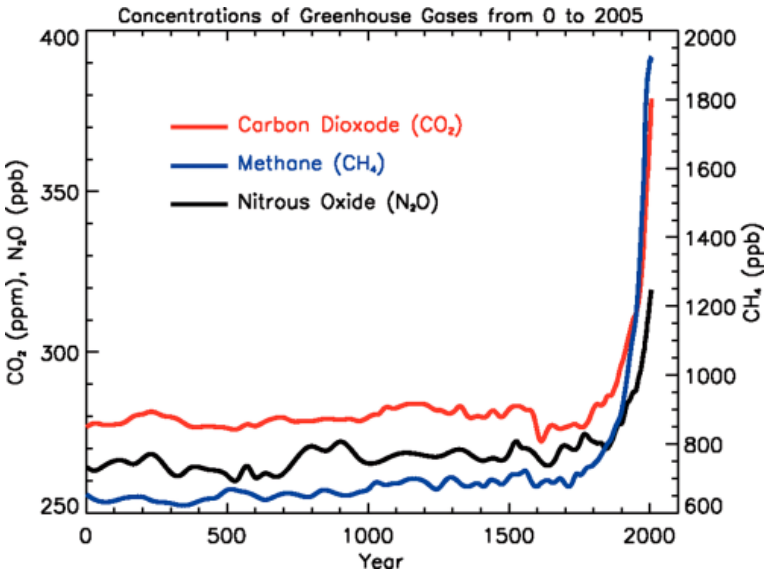

Figure 1. Greenhouse gases emissions raising since industrial revolution to the new millennia. The x axis shows the years up to the year 2000 whereas the y axis refers to the greenhouse gases emissions raising exponentially the closer the latter approach the year 2000 [4].

The sharp increase in atmospheric CO_2, NO_x and CH_4 is commonly associated with the beginning of the industrial revolution. Starting around 1800 and rising ever since, the consumption of oil, gas and coal have also increased dramatically, as shown in Figure 2 [5].

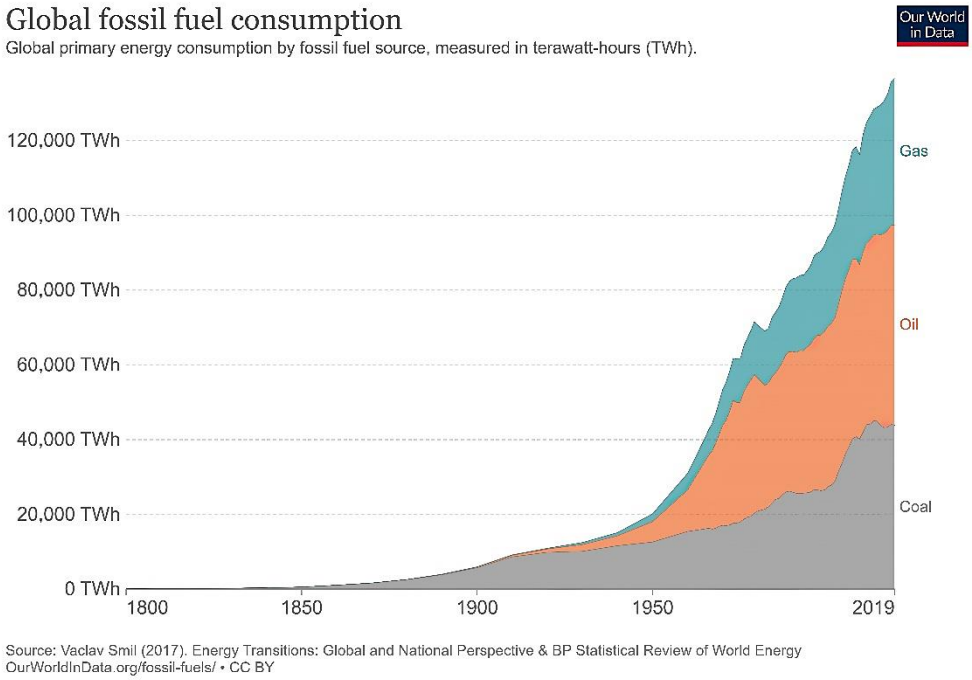

Figure 2. Consumption of fuel raising from the 1800 until 2019 (x axis). The y axis refers to the increase in the terawatt-hour production due to the use of fossil fuels since the 1800's. The latter increase of the fossil fuel consumption correlates well with the increase of the presence of the green-house gases [5].

These data show clearly that the exploitation of fossil fuels and their derivatives is directly proportional to the production of greenhouse gases (GHG), which is the typical name given to the CO_2, NO_x and CH_4 gases.

A recent study [6] estimates that two-thirds of the GHG emissions total are due to the combustion of fuels by petroleum-based vehicles and although gigantic efforts are underway to reduce the production of GHG by using battery-powered alternatives, the electricity produced by renewable sources is not enough to convert aircrafts, ships and other large craft in the transportation sector, all of which will keep needing high-energy density fuels. It is, therefore, increasingly urgent to find sustainable alternatives that can help to mitigate the adverse effects of the excessive consumption of gasoline and diesel.

In fact, according to Chu and Majumdar [7], nothing less than another industrial revolution is required, in which energy can be obtained in a sustainable and affordable manner. This search for cleaner ways to harvest energy and

produce commodities while transforming societies into actual "sustainable societies" is not a new research topic; however, since 1990, more and more efforts have been and continue to be focused into the matter [8].

Concepts such as sustainability and the circular economy have gained traction within many research communities, especially over the past decade. In an attempt to define exactly what these terms mean, Geissdoerfer *et al.* [9] compiled usages by more than 100 researchers to settle on a definition in which the circular economy was "an economic system in which resource input and waste, emission, and energy leakages are minimised by cycling, extending, intensifying, and dematerialising material and energy loops. This can be achieved through digitalisation, servitisation, sharing solutions, long-lasting product design, maintenance, repair, reuse, remanufacturing, refurbishing, and recycling". We can see, then, that both concepts focus heavily on the search for an economy and society that can feasibly coexist without compromising the environment either for our generation or for the ones to come [10, 11].

However, reaching such laudable goals will not be an easy task. The sustainability approach is gigantic and involves a daunting range of research fields. Nevertheless, and as stressed by Figure 2, decarbonizing our energy infrastructures will be one of the most important problems, if not the most important problem, for humanity to tackle to counteract our indiscriminate use of oil and coal over the past 200 years, and its subsequent impacts on climate change (i.e., warmer oceans, melting glaciers, extreme weather conditions, rising the sea levels , etc) [12].

2.1 Biomass

One of the most studied directions towards a more sustainable economy is the biomass-led approach, which aims to valorise biomass and the potential sustainable value-added products that can be obtained from it [13-16]. Biomass is abundant, low-cost, and sustainable [6, 16-18]. Among the wide range of potential biomass feedstocks are agricultural and forest residues, vegetable oils and algae [17, 19]. All of these are renewable and relatively easy to obtain and process, with many of them being considered undesirable waste materials [16, 17, 19]. These characteristics have made biomass a promising subject of study in the search for sustainable and feasible alternatives to the current fossil fuel-based economy and infrastructure.

According to Shylesh *et al*. [6], an exemplar approach towards a more sustainable biomass-based future could be based on a scheme that combines renewable electricity, biofuels and bio-derived feedstocks for the production of added-value products [6]. Some authors named the latter the biorefinery approach [20-22].In this proposal, the carbohydrates coming from biomass (such as cellulose and the hemicelluloses) are converted into chemical feedstocks that may then be used to produce a diverse range of products such as fuel and lubricants (Figure 3) through thermochemical (e.g. pyrolysis, which refers to the degradation of a material in an oxygen-free atmosphere) or biochemical processes (i.e., enzymatic hydrolysis) [6, 22].

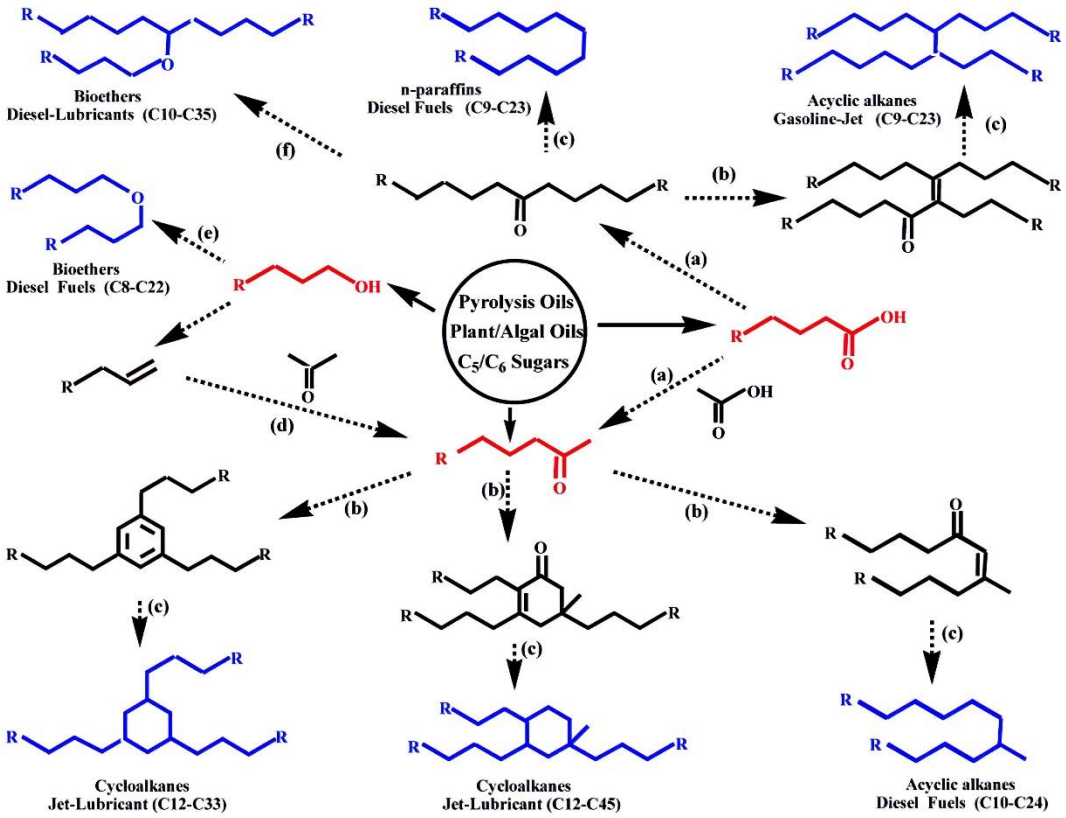

Figure 3. Taken from Shylesh et al.,[6]. A proposal of different routes for the obtention of bio-derived synthons, which can further be converted into valuable added-value products. As observed, the base biomass blocks can be further transformed into three main molecules (carboxylic acids, esters and alcohols) to be finally processed to obtain different diesel-like composition molecules.

These feedstocks, which are sometimes called bio-synthons, are the building blocks from which the final added-value products formed. These are mostly ketones, alcohols, and carboxylic acids. The ketones arise from dehydration of the C_5 and C_6 sugars in the biomass followed by hydrogenolysis of the resultant furfural species, whereas the alcohols result from fermentation of the aforementioned sugars, followed by the Guerbet reaction, in which a primary alcohol is converted into a β-alkylated dimer alcohol. The carboxylic acids, in contrast, are obtained by hydrolysis of triglycerides from animal or plant oils [6].

Once these building blocks have been formed through the chemical reactions such as ketonization and aldol-condensation and subsequent

tranformations, among others, valuable alkanes are obtained which in many cases meet the same specifications, in terms of energy and physical properties, as petrochemical-derived hydrocarbons [6, 16]. However, on their own, the production of "biosynthons" is not usually enough to make biomass processing economically viable.

Accordingly, attention is turning to biorefineries as a way to extract maximum value from all elements of the biomass. Biorefinery approaches follow similar principles, which is to obtain as many added-value products from biomass as possible, giving them use inside the biorefinery scheme. For example, Navarro-Pineda *et al*. [21] describe a conceptual design of a biorefinery to process *Jatropha curcas:* an inedible crop species that has been extensively studied as a strong candidate to produce biofuels due to its resistance to drought and ability to grow in marginal land conditions [23]. Through chemical processes such as transesterification of the fatty acids coming from *Jatropha curcas* and thermochemical processes such as pyrolysis of the pressed-cake, fruit shell and seed husk, biodiesel and bio-oil with bio-char may be obtained [21, 23].

Other studies go further, with Sacramento-Rivero, *et al*. [22] proposing a biomass-based production system in which all the commodities that are usually obtained through conventional petroleum-based methods may be obtained through the correct exploitation of biomass resources. These researchers evaluated a conceptual design of a biorefinery to transform switchgrass into added-value commodities, including bioethanol, phenols, methane, and energy production in the form of heat and electricity. With this approach, the cellulose and hemicellulose fractions of the biomass undergo hydrolysis to ethanol and water through saccharification and fermentation of the C_5 and C_6 sugars. The waste liquid streams undergo additional anaerobic digestion to biogas (largely methane) and any lignin is passed through pyrolysis to produce bio-oil and valuable phenolics. Similarly ambitious pipelines have been proposed for other biomass sources, such Cuevas-Castillo *et al.*'s [20] overview of microalgal

biomass as a feasible source of potential added-value products such as fuels and chemical commodities, in which the microalgae are used to produce lipids for biodiesel to blend in mixtures with the current fuel infrastructure. Moreover, the residues from the lipid extraction processes could potentially be used as raw materials for bio-char production or polyunsaturated fatty acids that have pharmaceutical and food applications.

Nonetheless, given current infrastructure costs, sustainable biorefinery schemes for the production of energy, chemicals and biofuels remain only theoretically feasible [2, 14, 20, 24]. However, research into current bottlenecks is offering increasing support for biomass as a potential route to decarbonize the current energy infrastructure systems.

2.1.1. The limitations of Biofuels

Biomass-derived fuels, colloquially called biofuels, can be subcategorized into four different generations, depending on they are produced.

The so-called first-generation biofuels are made from edible materials such as starch or corn, which are usually converted into biodiesel or bioethanol [25, 26]. Biodiesel is made from vegetable oil or animal fat and is composed mainly of methyl esters that have similar characteristics to conventional diesel fuels [16, 27]. Bioethanol, on the other hand, is mainly produced by fermenting sugar-rich crops [16]. The technology for the production of first generation biofuels is mature and accessible, and biodiesel or bioethanol can be used as drop-in fuels with today's petroleum-based infrastructure or in blends with petrol and diesel fuels [28].

Bioethanol, for example, can be used in mixtures like E85 which consist of 85 % bioethanol and 15 % gasoline and specialized engines can run E100 (100 % bioethanol) [29]. Biodiesel, on the other hand, is usually mixed with regular diesel

up to a concentration of 20 % biodiesel to 80 % diesel, due to the physical and chemical characteristics of the fuel [30].

The strongest drawback to first-generation biofuels is the use of edible crops and their associated resources such as water or land , which places them in direct competition with food production. This has raised real controversies over the past few years, with communities being asked to sacrifice food security for fuel security. Biodiesel, in addition, has limitations to its use as a diesel substitute. Biodiesel has higher viscosity than regular diesel, a lower heating value and a higher density. Its production of No_x is also higher, and the damage to a typical car engine by the use of a biodiesel blend higher than 20% with such characteristics can be severe [16, 30].

The second-generation biofuels, by contrast, are produced using non-edible lignocellulosic material, generally using crop residues from agriculture or waste[31, 32]. The latter helps to overcome the food vs fuel dilemma caused by the first generation biofuels; however, the capital and operative cost of a second-generation facility is still significant [13, 25, 33] and needs to mature into more feasible infrastructure.

When subjected to pyrolysis (thermochemical decomposition in an oxygen-free atmosphere), lignocellulosic biomass may be transformed into bio-oil [34, 35], which is a highly oxygenated liquid (product of the quenching from the liberated gases when biomass goes under oxygen-free cracking reactions) that, with further treatments, is a promising fossil fuel substitute; and into bio-char, which is the solid residue of the reaction that can be used as a soil fertilizer or to produce activated charcoal [23, 36].

The use of algal biomass to produce biofuels is considered as the third-generation production route, specifically the use of microalgae[25, 32, 33]. The biggest advantage of third generation biofuels is the high production of lipids that can be delivered; however, other advantages also include the rapid growth of the microalgal biomass, as well as relatively low cultivation costs under the

right conditions [25, 37]. As with second generation biofuels, the technology is not fully mature, with much of the microalgal data coming from laboratory studies. Scaling up these processes, along with their development implications, is a challenge that remains to be fully addressed [20].

Nevertheless, the major disadvantage when considering algae as a source of energy is, according to Dasan *et al.* [38] and Cuevas-Castillo *et al.* [20] the significant energy inputs required into the production system. Using a specialized environment for cultivation of microalgae, such as bubble column photoreactors rather than cheaper open pond systems, is invariably an energy intensive process [38, 39].

The last and final generation of biofuels is the fourth one, which is defined as the use of genetically modified microorganisms to produce fuels through a photosynthetic process. These microorganisms include yeast, cyanobacteria, and microalgae, among others[25, 32, 40]. Some of the advantages obtained from the fourth generation route include the CO_2 sequestration produced by such modified microorganism as well as a high lipid production and the reduced environmental impact compared with the other generations in terms of land use [25, 31, 32]. However, the technology for such tasks is still very much in the early stages of development [25, 32].

2.2 Macroalgae biofuels and *Ulva* spp. as a feedstock

Despite the drawbacks of using algal biomass to produce biofuels, their potential means that extensive research has been performed in the past decade to overcome bottlenecks [41-43]. In particular, considerable attention has been given to the multicellular macroscopic relatives of the microalgae, the macroalgae, or seaweeds that are typical of coastal environments. The macroalgae retain many of the advantages of their unicellular relatives: they grow fast, efficiently

assimilating the required nutrients and resources needed for their development and are generally considered to be third generation biofuels [20, 44]. In fact, macroalgae have higher yields than even the faster growing land crops, producing more biomass per hectare than their terrestrial counterparts [45].

Milledge *et al.* [46] present a summary of all the possible alternatives (Table 1) to harvest energy from algae, which include different processes, highlighting one more time the vast versatility of such algal biomass as a sustainable source of renewable raw material for energy and bio-derived added-value products.

Table 1. Methods of energy extraction of macroalgal biomass, adapted by Milledge et al., [46]

Method	**Use of all organic biomass**	**Drying of biomass after harvesting**	**Principal derived energy product**
Direct combustion	Yes	Yes	Heat
Pyrolysis	Yes	Yes	Primarily pyrolysis oil
Gasification	Yes	Yes[b] (conventional)	Primarily gas
Biodiesel production	No	Yes[c]	Liquid
Hydrothermal treatment	Yes	No	Primarily liquid
Bioethanol production	No[a]	No	Liquid
Biobutanol production	No[a]	No	Liquid
Anaerobic digestion	Yes	No	Gas

[a]Polysaccharides require hydrolysis to fermentable sugars. Some of the sugars produced from the breakdown of seaweed polysaccharides are not readily fermented; [b]Supercritical water gasification (SCWG) an alternative gasification technology can convert high moisture biomass; [c]No current commercial process for the wet trans-esterification of wet macroalgal biomass.

However, macroalgae are a broad group and are more evolutionarily and biochemically diverse than the land plants. The most familiar classification of macroalgae is based on the presence or absence of accessory phytopigments (ie non chlorophyll pigments), which divide them [47, 48] into three different classes (Figure 4): brown algae (Phaeophyceae), red algae (Rhodophyceae) and green algae (Chlorophyceae).

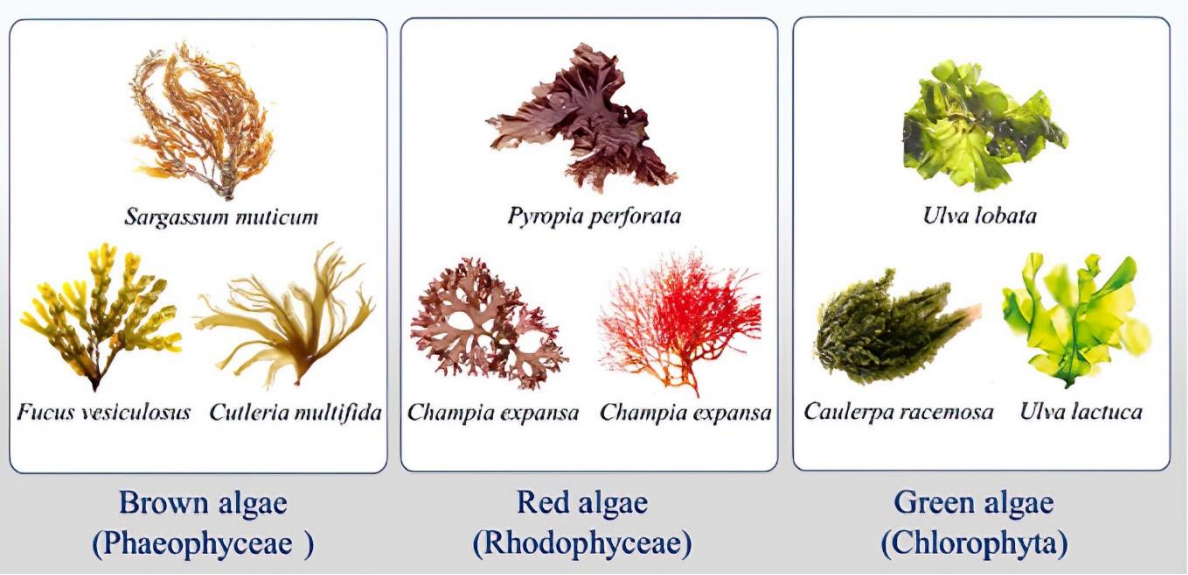

Figure 4. Classification of macroalgae according to the lack of phytopigments others than chlorophyll. Adapted from Thompson *et al.*,[47]

Within these three groups, most aquaculture-based macroalgal biomass production comes from five genera [46]: *Laminaria, Undaria, Porphyra, Euchema* and *Gracilaria* [48]. Indeed, around 100 times more cultivated macroalgal than microalgal biomass is currently produced worldwide and farming and harvesting infrastructures are well established in many countries. Nevertheless, information remains scarce concerning efficient methodologies for growing, harvesting and preserving seaweed in scaled up commercial systems [46].

As mentioned above, the green, red and brown macroalgae have distinct biochemical properties. Table 2 shows the diversity in carbohydrates, lipids, and proteins among all three classes [45].

Table 2. Composition of macroalgae in terms of carbohydrates, lipids, proteins and sugars released by hydrolysis of some common seaweeds. Adapted from Murphy *et al.* [45].

Seaweed	**Class**	**Carbohydrate composition**	**Carbohydrates (%, total)**	**Lipids (%)**	**Protein (%)**	**Ash (%)**	**Sugar released by hydrolysis (%)**	**Sugar composition**
Gelidium amansii	Red	Agar, Carrageenan, Cellulose	75.2 - 83.6	0.6–1.1	12.2–18.5	3.3–5.7	34.6–67.5	Glucose, Galactose
Laminaria japonica	Brown	Laminarin, Mannitol, Alginate, Fucoidan, Cellulose	51.9–59.5	1.5–1.8	8.1–14.8	30.9–31.5	9.6–37.6	Glucose, Mannitol
Sargassum fulvellum	Brown	Laminarin, Mannitol, Alginate, Fucoidan, Cellulose	39.6	1.4	13	46	9.6	Glucose, Mannitol
Ulva lactuca	Green	Starch, Cellulose	54.3	6.2	20.6	18.9	19.4	Glucose
Ulva pertusa	Green	Starch, Cellulose	65.2	2.6	7	25.2	59.6	Glucose

The composition is important because, to date, bioenergy approaches have focussed on the brown seaweeds, which are more common off the coasts of Europe and North America [44]. The brown seaweeds, especially those in the genera Laminaria, *Saccharina* and *Undaria*, are also farmed in North-East Asia for commercial purposes, mainly for food. The major constituents of the brown seaweeds are alginate, fucoidan, mannitol and laminarin, all of of which have potential uses in the cosmetic and food industries [49]. Nevertheless, when used

for biofuel production, the production costs of brown seaweeds remain high enough to discourage their use as raw materials for biofuel production [46, 49].

Red algae have been also studied, using a biorefinery approach, to assess whether these seaweeds have the potential to produce added value products [44]. Because of their biochemical differences to the brown seaweeds (Table 2), red algae are mainly used to obtain agar and carrageenan, both carbohydrates extensively used for food and pharmaceutical purposes. Several studies have built on this to consider red algae within a circular economy framework. For example, Alvarez-Vinas *et al.* [44] proposed a cascade biorefinery scheme in which not only biofuels, but also fertilizer and peptides may be obtained from red seaweeds, although other authors have highlighted that the feasibility of red algae biorefineries is critically dependent on infrastructure and may often not be currently viable [25, 26, 46].

The green seaweeds have also been explored for their potential use as a feedstock to obtain added-value products, such as chemicals or fuels, using a production chain based on the use of green seaweed as raw material [41, 50]. Particularly promising green seaweeds are those in the *Ulva* genus, which include the species responsible for 'green tide'. Ulva spp. are among the most studied Atlantic seaweed species, with high protein contents and significant amounts of polysaccharides (ulvan, uronic acid, xylose and starch), which make up over 50 % of their dry weight. The *Ulva* genus comprises many different species, with some of the better known being the sea lettuce *Ulva lactuca,* the gutweed *Ulva intestinalis*, and the green-tide forming species *Ulva prolifera.* Most Ulva species are believed to be close relatives whose physical and chemical characteristics do not differ greatly between species [41, 51].

These characteristics make *Ulva* spp. strong candidates for exploitation as suitable feedstocks to produce value-added products such as biofuels and bio-derived chemical commodities [41, 52]. For example, Bikker *et al*. [41] highlighted that after 24 h enzymatic hydrolysis with a cellulase cocktail at 50 °C with

continuous stirring, *Ulva lactuca* gave good yields of fermentable sugars. It has, however, been argued [51] that although Ulva offers high biomass yields, the difficulty of processing its biomass to bioethanol and biogas is too low yield to make their use economically viable and further feasibility analyses of cascade biorefineries need to be performed in order to assess these arguments.

However, if high value products (such as proteins) were to be extracted prior to conversion of the biomass into energy, the economic feasibility of a production process based on *Ulva* could well be increased. This focusses attention on the exact composition of Ulva biomass, which is relatively high in soluble uronic-acid rich polymers (called 'ulvan') and relatively low in cellulose, compared to land plant biomass. The exact composition of *Ulva* spp. can be affected by the growth conditions and hydrolysis conditions, so a better understanding of the chemistry of these cell wall components is desirable when considering *Ulva* as a biomass source.

2.2.1 Ulvans

Ulvans are pectin-like sulphated polysaccharides and one of the most important components of *Ulva* cell walls, being their major soluble polysaccharides and constituting about 30% of the dry weight of Ulva fronds [53].

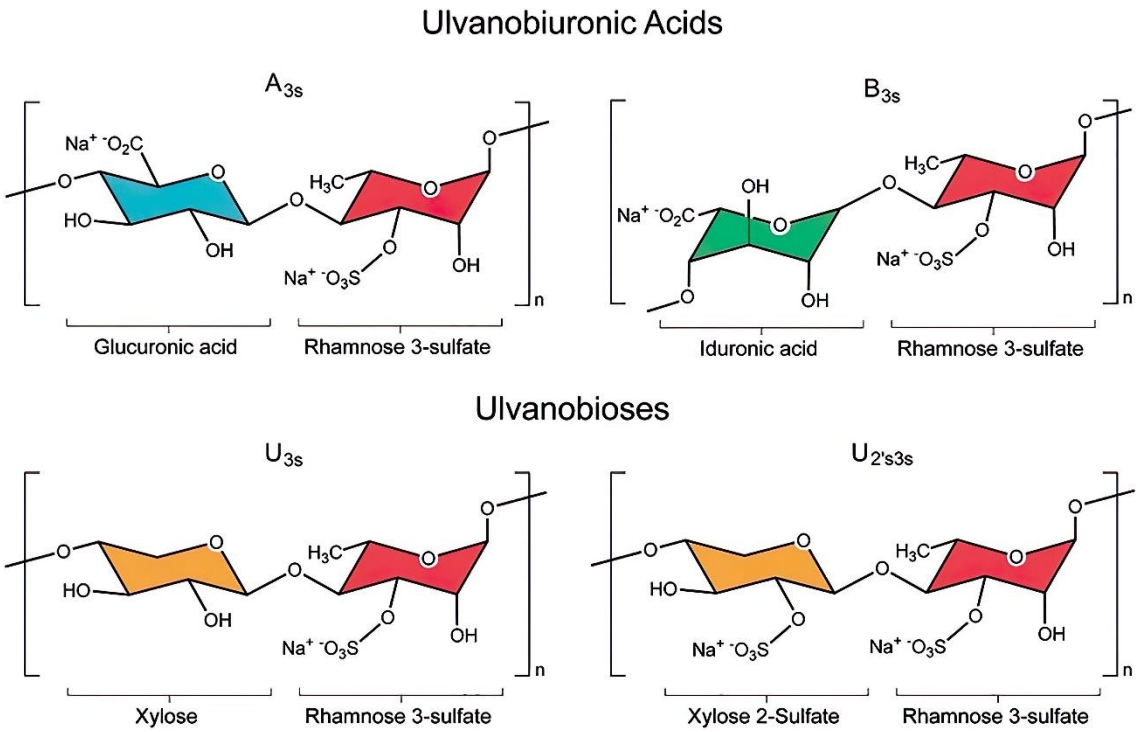

Figure 5. Ulvan most characteristic disaccharides. The latter disaccharides refer to the major repeating units that can be found in Ulvan's composition. Taken from Kidgell *et al.* [54].

Ulvan is mainly comprised of five monosaccharides: Glucose, Glucuronic acid, Iduronic acid, L-rhamnose and Xylose. A scheme of ulvan composition is shown in Figure 5 [54].

Ulvan is extensively used in the food industry as an additive but its use has been extended to other areas, such as a biological ingredient used for therapeutic preparation because of its ability to form thermoreversible gels in the presence of borate and divalent cations [55, 56]. In a recent survey, Cindana Mo'o *et al.* [55] reviewed the diverse uses for extracted ulvan, describing its use as a preventative nutraceutical agent that, when used to produce food, biomedical additives or dietary supplements, shows a wide variety of benefits that show it acting as an anti-inflammatory, antioxidant, anti-bacterial and anticancer agent. Moreover, in some studies regarding its biomedical applications, it has been proposed as a strong candidate for tissue engineering. For example, when combining loaded dexamethasone ulvan particles within a matrix of poly (D-

lactic acid) (PDLLA), an efficient localized drug delivery agent can be produced [55, 57]. Because of this, ulvan is of great interest for its potential utilization in the food, agricultural, pharmaceutical, and chemical sectors [58].

2.3 Conclusions

The importance of moving towards a more sustainable infrastructure to mitigate and reduce the effect of pollution and climate change worldwide should be one of the priorities of mankind if irreversible changes to the environment are to be avoided. Sustainability could be accomplished by the gradual production and use of bio-derived products from biomass resources, such as algae, among others. The research focused on algae has increased in the last decade, as they are promising sources of sustainable products such as biofuels and bio-derived added-value products, with potential use in several industries such as the chemical, textile, pharmaceutical and agricultural sectors.

3. Scope and aims of this project

The aim of this project was to assess the carbohydrate composition of *Ulva* spp. biomass to help understand how these algae can be used as an alternative feedstock for biofuel production.

3.1 Specific goals

- Qualitative analysis of the cell wall carbohydrate composition of *Ulva sp.* through Thin Layer Chromatography.
- Separation of the soluble and insoluble fractions of the *Ulva* sp. cell wall (ulvan and cellulose, respectively)
- To determine whether acetate species are present in either cell wall fraction.

4. Materials and methods

The chemicals used for this project were purchased from Sigma-Aldrich, Megazyme or Thermo Fisher Scientific unless stated and are listed below:

- Chloroform, Fisher chemical, 99%
- Methanol, Fisher chemical, 99.5%
- Proanol-2-ol, Fisher chemical, 99.7%
- Sodium Hydroxide pellets, Fisher chemical
- Citric acid, Aldrich, 99.5%
- K-ACETAK Kit, Megazyme
- Mannose, Aldrich, 99%
- D-Glucose, Aldrich, 99%
- Galactose, Aldrich, 99%
- Inositol, Aldrich, 99%
- TFA, Fisher, 99%

- Methanolic HCl, Aldrich, for GC derivatization
- Hexane, Fisher chemical, 98.09%
- TMSI pyridine. Fisher chemicals, 98 %
- All the chemicals used for the Thin-Layer chromatography experiments were supplied by Dr Lenka Frankova from Newcastle University.

4.1 Extraction of ulvan and cellulose fractions from Ulva spp.

Ulva spp. samples were collected at Seaham Harbour in county Durham. Thalli were taken to the laboratory in containers with seawater for cleaning and maintained in an Thermo Scientific MaxQ 6000 Incubator with agitation at 8ºC, maintained in artificial seawater that was changed once a week. For the extraction of the fractions, a modified version of a methodology described elsewhere was followed [59]. *Ulva* spp. biomass was weighed, chopped and ground under liquid nitrogen. The resulting powder was put into falcon tubes with distilled water and left to agitate overnight in a 4ºC room on a Stuart Roller Mixer SRT1. Afterwards, the samples were centrifuged using a Beckman Allegra X-22R centrifuge at 4,000 g for 30 minutes; the obtained supernatant was discarded. The remaining pellets were resuspended with a solution of 2:1 methanol:chloroform, agitated for 2h at 4ºC and then centrifuged at 4,000 g for 30 minutes. The methanol:chloroform washes were performed 4 times. After the last wash, the tubes were left open in a fume hood for the solvents to evaporate overnight. Pellets were then resuspended in distilled water and put in a water bath (Grant GD100) at 80ºC for 2h. Immediately after that, the suspension was filtered through a muslin cloth; the liquid filtration product contained the dissolved ulvan and the solid sample remaining on the muslin cloth was cellulose fiber. The obtained cellulose fiber was washed with distilled water in falcon tubes

to eliminate any traces of ulvan. A room temperature procedure was used rather than high temperature one trying to avoid any possible degradation of the cellulose fraction.

The dissolved ulvan was put into falcon tubes and was mixed with a 3:1 isopropanol solution and those samples were left to freeze at -80 ºC overnight to favor the precipitation of ulvan; the samples were defrosted, centrifuged, and left in the fume hood to dry.

4.2 Hydrolysis of the ulvan and cellulose fractions from *Ulva* sp.

Samples of ulvan and cellulose were freeze-dried and placed into Eppendorf tubes with 1M NaOH; each tube was put into a water bath for 8 h at 80ºC. After the first hydrolysis, the samples were centrifuged at 3,000 g for 15 minutes and the supernatant was collected, analyzed with a pH meter (Jenway 4330) and subsequently neutralized to pH7.0. The resulting pellet was resuspended with 1 ml of a 1M solution of NaOH and a second hydrolysis was performed. The samples were freeze-dried prior being analyzed using a FTIR PerkinElmer Frontier instrument.

4.3 Fourier-Transformed Infrared Spectroscopy (FTIR) of the ulvan and cellulose fractions from *Ulva* spp.

The fractions of ulvan and cellulose were analysed with a PerkinElmer Frontier IR/NIR spectrometer with an UATR polarization accessory. All the analyses were executed after a background check and with a gauge force of 60N.

4.4 Mass-spectrometry of the cellulose fraction from *Ulva* spp.

The sugar analysis protocol provided by the Durham University proteomics laboratory was used, as follows. 12mg of the cellulose obtained in the extraction process as well as another 12mg of cellulose obtained in the extraction process after hydrolysis were weighed. All the samples were put in glass sample tubes and resuspended in 500µl of distilled water for a subsequent sonicated water bath for 1h.

The standards used for the experiment were glucose, mannose, galactose, fucose and inositol (100µg, aliquoted from 1mg/ml solutions in distilled water), which were dried under N_2 and processed separately. 4M Trifluoroacetic acid and inositol internal standard were added to make 2M final concentration mixture; the hydrolysed cellulose fraction, non-hydrolysed cellulose samples and the standards were placed in a thermoblock Techne dri-block DB-3A at 110ºC for 2h. The samples were transferred to microfuge tubes and centrifuged at 14,000 g for 30 minutes and the resultant supernatant was evaporated to dryness under nitrogen at 40ºC in glass sample tubes.

400µl of 1M MeOH-HCL were added to the glass sample tubes that contained the hydrolysed cellulose fraction, non-hydrolysed cellulose samples and the standards and incubated at 80ºC overnight. After that, 400µl of TMSI-pyridine were added and the samples were incubated at 80ºC for 30 minutes, dried under N_2 and the resultant oil was resuspended in 1ml hexane and with an equal volume of water.

The GC-MS analyses were performed using a single-quadrupole Shimadzu QP-2010-Plus system fitted with a Restek Rxi-5Sil column (30 m, 0.25mm ID). The hydrolysed cellulose fraction, non-hydrolysed cellulose samples and the standards were introduced by split injection and the carrier gas was helium. The injector temperature was 250ºC and the initial oven temperature

was 140ºC, increasing at 2ºC/minute to 180ºC and held at this temperature for 5 minutes before increasing to 275ºC at 10 ºC/minute, held for 10 minutes.

The experiment was carried out in duplicate.

4.5 Thin-layer chromatography (TLC)

The TLC experiments were performed to gain further knowledge about the behavior of the plant cell wall constituents when subjected to specific enzymatic treatments and to ascertain linkage patterns between monosaccahrides in the cell wall of the ulvan.

4.5.1 Preparation of *Ulva* spp. sample.

A buffer was prepared with 11.81g of succinic acid added to a 400ml of distilled water. The pH of the mixture was adjusted to 5.5 with 10M sodium hydroxide, and after that, the mixture was made up to 500ml with distilled water. The resulting mixture was divided into three beakers, to make different buffers. Beaker 1 contained 200ml of the base buffer with nothing added to it; beaker 2 had 200ml of the base buffer plus 11.69g of NaCl; beaker 3 had 100ml of the base buffer plus 96ml of distilled water and 4ml of 1% triton. Each buffer solution (Beaker 1, Beaker 2, and Beaker 3) was split in two, one to work as a control with just the buffer and *Ulva* spp. sample and the other to be a mixed of the buffer, the *Ulva* spp. Sample, and the enzyme (endotransglycosylase).

10g of fresh *Ulva* spp. were chopped and grinded under liquid nitrogen to prepare the fraction. The resultant powder was weighed and divided onto the beakers (control and enzyme).

4.5.2 Thin-layer chromatography experiments

A plate of silica was used, with standard TLC analysis procedures as follow. The origin was at 2 cm from the bottom. 8mm segments were marked for the sample loading with 2 mm gaps between them and leaving about 1cm edge from the sides. This allowed the loading of 18 samples including the markers. We prepared 0.1% marker stock solutions with 16 standards.

Table 3. Standards used as markers for the TLC experiment

1. RG 1-potato	7. Arabinoxylan wheat	13. Manno 5
2. PGA	8. Arabinan S. beet	14. Xylo 4
3. 1-4 Mannan	9. Galactose (Lupin)	15. Arabinan 6
4. Lichenin	10. Xyloglucan	16. Heptasaccharide 6
5. Galactomannan	11. Cello 4	
6. B-Glucan	12. Laminarin 6	

2µl of the samples were loaded onto the origin (i.e., sixteen repeats, one per marker) making sure the band did not overlap. Onto the last two bands we loaded 2µl of the markers, spotting several mixtures of them in the same area. Once dried, 80ml of developing solution (butanol:acetic acid:distilled water 2:1:1) were poured into a glass tank and put the TLCs in the tank with the plastic backs facing the glass wall, the tank was covered with a lid and left overnight. For the staining, the plate was dipped into a solution of 0.5% thymol (w/v) and 5% concentrated H_2SO_4 (v/v) in 96% ethanol (v/v). Then, the plates were left to air dry in a vertical position to later be suspended in an oven at 105ºC for 5-15 minutes or until a pink/purple colour developed.

A diagram representing the methodology and separation of sample beakers (section 4.5.1) is presented in Figure 6.

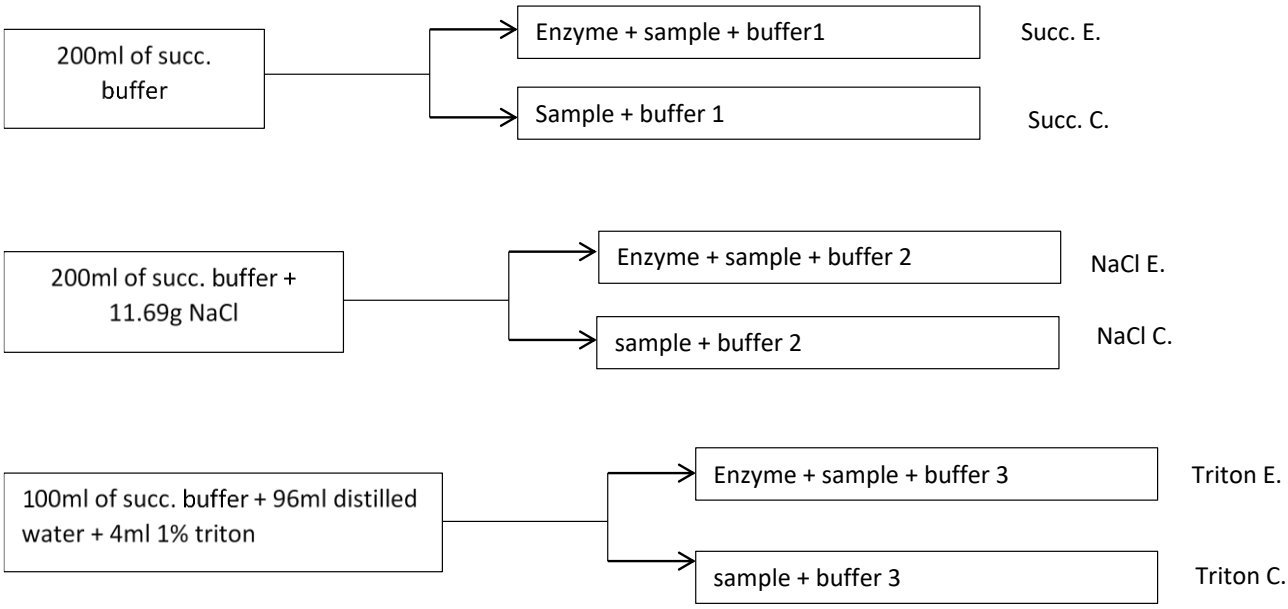

Figure 6. Three different buffers were prepared to be tested with the *Ulva* spp. sample. The first buffer, the second one with the added NaCl and the third one with the added triton. In this figure, it is shown how the three different solutions were prepared.

5. Results and discussions

5.1 Extraction of ulvan and cellulose fractions from *Ulva* spp. and FTIR characterization

Prior the FTIR characterization, ulvan and cellulose fractions were extracted as mentioned in section 4.1. The FTIR experiment was performed first to set a starting point of comparison between the soluble and insoluble fractions from *Ulva* spp. collected and the literature values. The FTIR from the ulvan fraction is shown in Figure 7. When the ulvan fraction was compared with other FTIR spectra published elsewhere [60, 61], the spectra correlated accordingly.

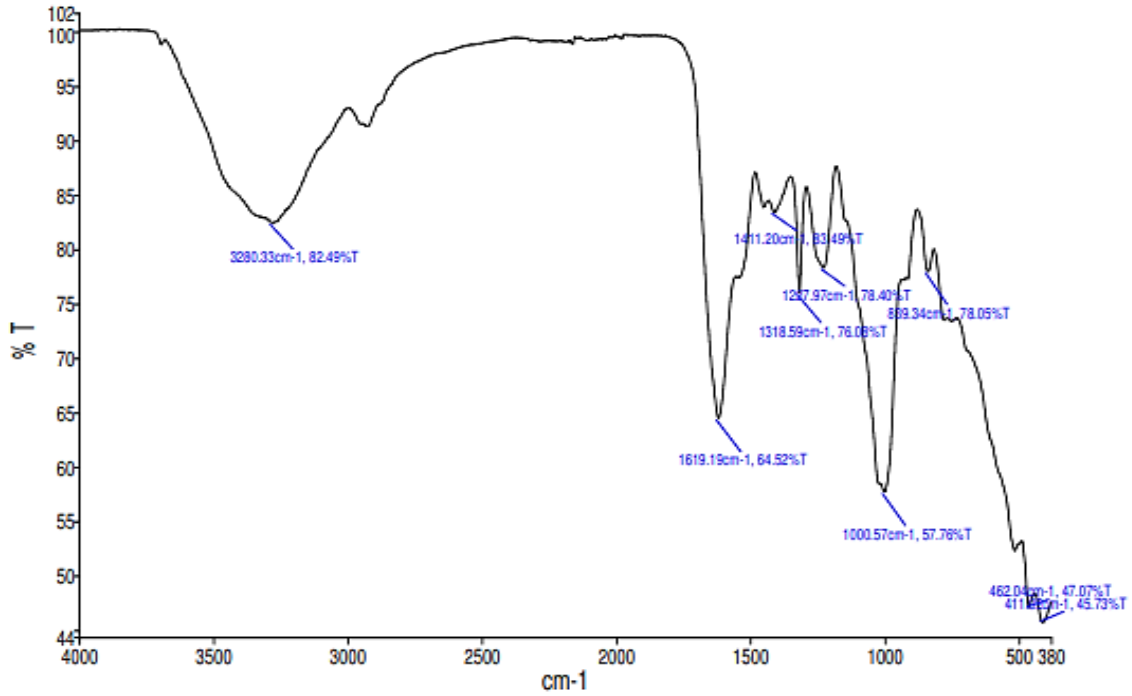

Figure 6. Fourier-Transformed Infrared analysis of the ulvan extracted from the *Ulva* sp. The corresponding peaks to the sulfate esters (1227 cm^{-1}) and peaks related to the uronic acid (1620 cm^{-1}) are observed.

The composition of the ulvan fraction is strongly influenced by the species of seaweed and the extraction process used [54]. Ulvan is mainly composed from polysaccharides [54, 60], therefore FTIR was an excellent technique to give further insight into the ulvan composition. Table 4 includes the assignation to the most relevant peaks and the ones that were able to be identified from the FTIR regarding the ulvan fraction.

Table 4. Main assignations to the identified peaks from the Fourier transform infrared spectroscopy analysis of the ulvan fraction from Ulva sp.

Wavelength (cm^{-1})	**Peak assignation**
1000	Sugar back bone [61]
1227	Sulfate esters [60-62]
1619	Carboxylic acids (e.g., uronic acid) [60, 61]
1411	Carboxylate (e.g., uronic acid derived) [60, 61]
3280	O-H presence (e.g., polysaccharides presence) [60-62]

For Figure 7 the broad peak at 3280cm^{-1} was due to the presence of O-H groups [60-62] in the sample, a clear indication of the aforementioned polysaccharides, particularly disaccharides related to rhamnose and glucuronic acid. The presence of such acids was also suggested by the maximum absorption observed at 1000cm^{-1}, which was assigned to the C-O stretching vibration of the sugars rhamnose and glucuronic acid, as reported elsewhere [61].

The peak observed at 1227cm^{-1} corresponds to the presence of sulfate esters (C-O-S) [60-62]. The strong and sharp peak at 1620cm^{-1} which is usually related to the asymmetric stretching of carboxylate groups, indicates the presence of uronic

acid. The presence of the acid is also confirmed by the weaker symmetric vibration related to the presence of carboxylate groups, presented in a small band a 1411cm^{-1} [60, 61].

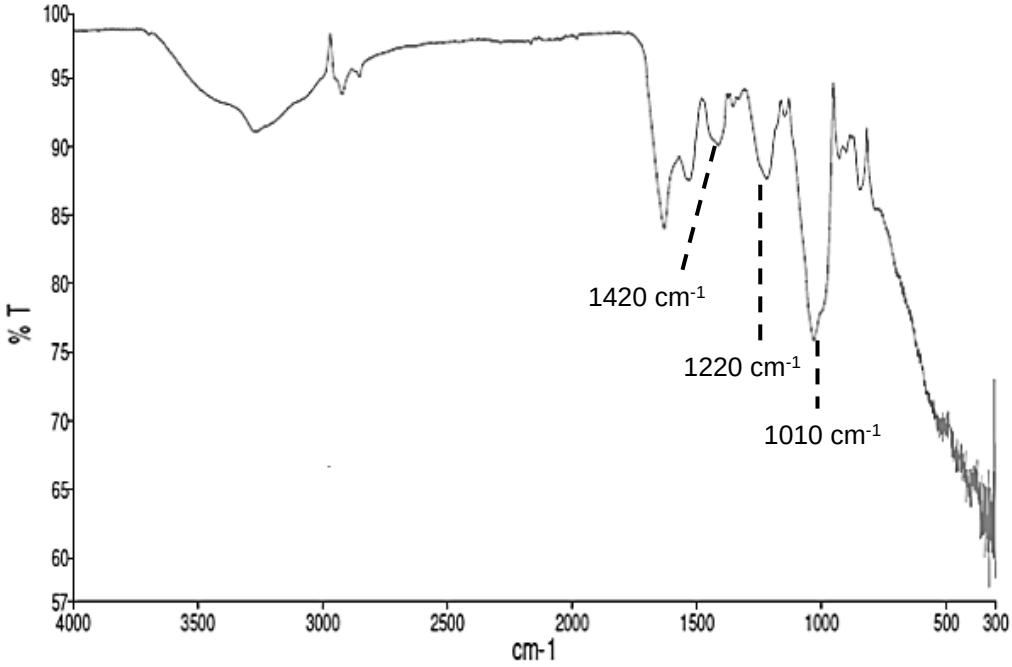

Figure 8. Fourier-Transformed Infrared spectroscopy of the cellulose fraction from the *Ulva* sp. Peaks related to the presence of OH at 3300cm^{-1} and one at 1200cm^{-1} due to possible presence of acetylated cellulose.

The ulvan fraction showed informative differences from the FTIR analysis on the cellulose fraction obtained from *Ulva* spp. as observed in Figure 8.

As with Figure 7, the broad peak at 3300 cm^{-1} is related to the stretching vibration of the O-H groups while the small peaks around 2900 cm^{-1} are due to the stretching vibrations of the C-H bonds [1, 63], both characteristic of cellulose materials. The peak at 1420cm^{-1} was assigned to the H-C-H scissor vibrations [64].

However, the FTIR spectra of the cellulose fraction apparently shared some peaks (ca 1010 cm^{-1} and ca 1200 cm^{-1}) with the ulvan fraction, as observed in Figure 9.

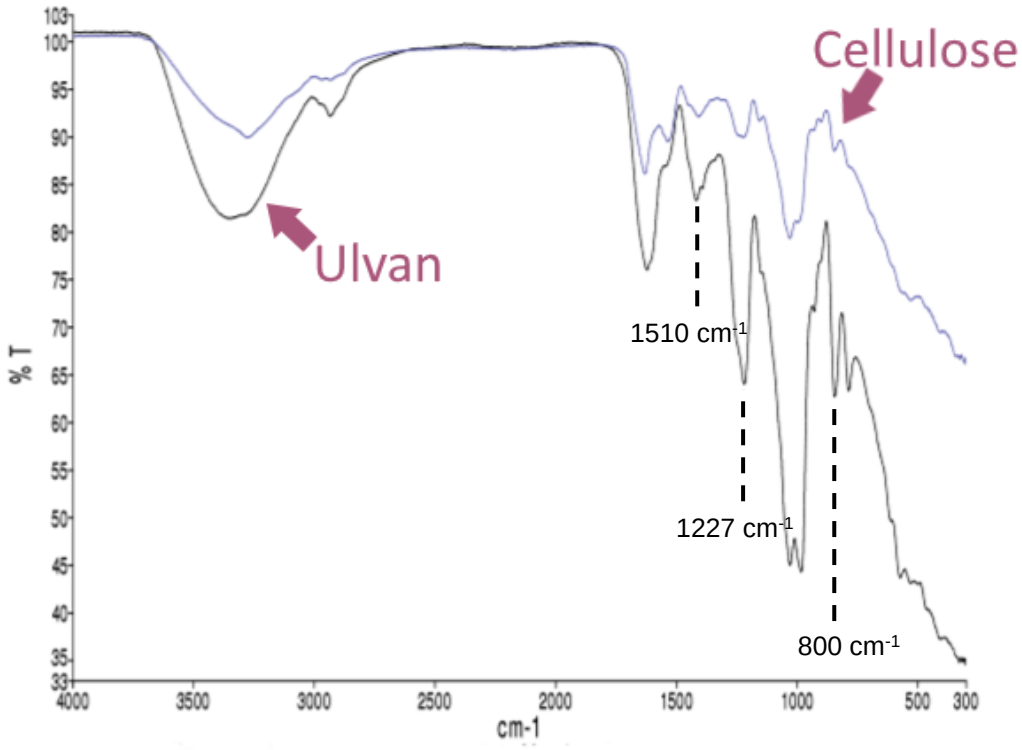

Figure 9. Fourier transform infrared spectra of both, the Ulvan and cellulose fractions together form the *Ulva* spp. Some peaks seem to be shared between both fractions (the peaks *ca* 800 cm^{-1} and the peak *ca* 1220 cm^{-1}) which could be a sign of acetylated cellulose.

From Figure 9 it is clear that the transmittance intensity between both fractions, ulvan and cellulose vary drastically, with the sulfate related peaks *ca* 1227 cm^{-1} and *ca* 800 cm^{-1} being particularly less intense for the cellulose sample when compared with the ulvan sample. Moreover, the small peak observed *ca* 1520 is most likely to be attribute to possible protein contamination. Proteins have a characteristic amide bond (i.e., N-H) that is usually observed *ca* 1500 ~ 1600 cm^{-1}, as described elsewhere [65].

The latter protein assigned peak is less pronounce in ulvan whereas in cellulose is bigger, as expected to be, according to the literature [65]. As observed in Figure 9, the extraction protocol described in Section 4.1 might have influenced the amount of sulfation retained within the ulvan sample, which is of interest as it has been

explored that there exist a strong correlation between the strong antioxidant capabilities of the ulvan with the degree substitution of sulfate groups through the whole polymeric backbone [55, 66]. Moreover, there has been found a correlation between ulvan and anti-inflammatory effects (mainly in an inflamed liver). The latter anti-inflammatory effect has been also attributed to the presence of sulfated polysaccharides [55].

Moreover, in terms of the ulvan fraction, as already mentioned the peak assigned to the wavelength at 1227 cm^{-1} is related to the bound of the C-O-S sulfated groups, some evidence suggests at that same wavelength (*ca* 1200 cm^{-1}), acetylated polymers (i.e., acetylated ulvan) can be observed, specifically referring to the antisymmetric stretching of the C-O-C bonds [67, 68]. Nevertheless, it cannot be assumed that the ulvan is truly acetylated (in algae, it is not expected that the polysaccharides will be acetylated, contrary to land plants [69]) as the peaks might be overlapping and also other techniques such as an acetate assay would be required to assess such claims of the ulvan being acetylated. Nevertheless, if the ulvan was indeed acetylated, in a natural manner or that the acetylation of the latter ulvan could be induced in some manner, that would set the first steps to better understand the behavior of cell walls within the *Ulva* spp.

In a similar manner than with the ulvan FTIR plot in Figure 9 and according to Li *et al.,* [63] when cellulose is acetylated, it can be expected to observe a strong peak around 1200cm^{-1} corresponding to C-O stretching of an acetyl group. As the latter peak was observed *ca 1220* cm^{-1} in the cellulose fraction extracted from *Ulva* spp. an assumption of the cellulose being acetylated was also considered [63]. As with the ulvan case, although not occurring naturally in plants [70], the possibility of the existence of acetylated cellulose in algae, more specifically in *Ulva* spp. was not ruled out, due to the importance of the latter as a widely used raw material with impact in several manufacturing industries such as the fiber, coating and plastic industries

[71]. The cellulose fraction was further washed as per section 4.1 whilst the ulvan fraction was also assessed due to the possibility of the latter ulvan to also be acetylated. Moreover, as proposed in section 4.1, hydrolysis was performed in both samples, as it is expected that during alkaline hydrolysis, removal of acetyl groups will happen [72], leading to a decrease in the intensity of the *ca* 1220 cm^{-1} peak for both, ulvan and cellulose samples.

5.2 Cellulose fraction washes

As per proposed in section 4.1, the cellulose fraction obtained from *Ulva* spp. (Figure 8) was submitted to several washes. It has been reported in the literature that if the anhydro glucose, which is the repeat unit of cellulose, is partially acetylated, it becomes water soluble [73].

If the cellulose fraction was partially acetylated rather than fully acetylated, a change in the peak at 1200 cm^{-1} is expected. However, if the aforementioned fraction is fully acetylated, no decrease at 1200 cm^{-1} will occur. The results are shown in Figure 10.

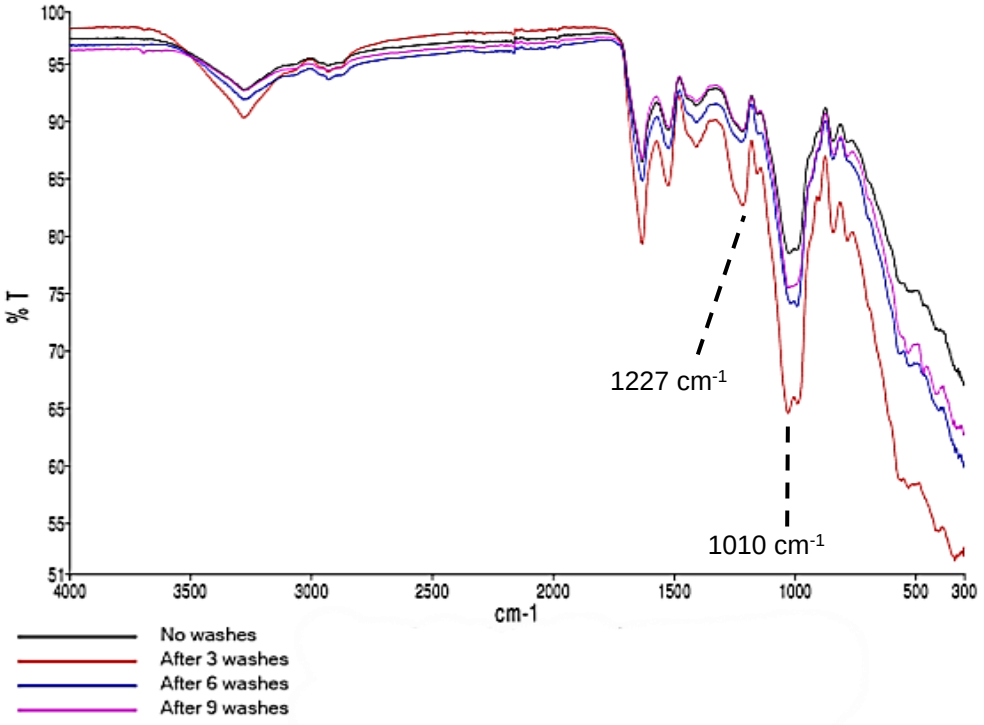

Figure 10. FTIR of the cellulose fraction obtained from the *Ulva* spp. after nine washes with distilled water. No disappearance of the acetylation-related peak can be observed. It should be noted that the intensity of the peaks varied due to the amount of pressure applied to the instrument to read the sample (when placing the sample and using the diamond ATR accessory).

It can be observed than no changes occurred after the washings, with no appreciable disappearance of the relevant peak. The intensity difference between all the peaks could be due to the pressure employed over the sample to run the FTIR analysis, which is commonplace. Based on these observations, further analysis was conducted using FTIR, but with hydrolyzed samples, of both fractions, ulvan and cellulose.

Thus, from the results observed from the washings in Figure 10, as the 1200cm^{-1} did not disappear after the washes, it was possible that the cellulose fraction from the *Ulva* spp. was either contaminated with hemicellulose, that ulvan is heavily bonded to the cellulose fraction or that the cellulose is in fact, fully acetylated [74].

5.3 Hydrolysis of the ulvan and cellulose fractions obtained from the *Ulva* spp.

As noted from the results obtained in section 5.1, one of the main constituents of *Ulva* cell wall biomass are the sulfate esters. These esters can significantly affect the physicochemical properties of *Ulva* spp. and are present in the ulvan. Thus, as described in the literature [75], variations in the ulvan sulfation, for example due to the growing conditions, storage conditions and extraction methodology, could affect cell wall properties. Although no quantitative analysis of the obtained fractions was performed, we assessed whether the sulfate groups on ulvan could be hydrolyzed.

Therefore, as per proposed in section 4.2, an alkaline hydrolysis was performed on the ulvan fraction. Even though there is a lack of references in the literature for the application of alkaline hydrolysis with ulvan, it has been argued [54] that basic conditions for hydrolysis should remove all sulfate esters. The results of the alkaline hydrolysis are shown in Figure 11.

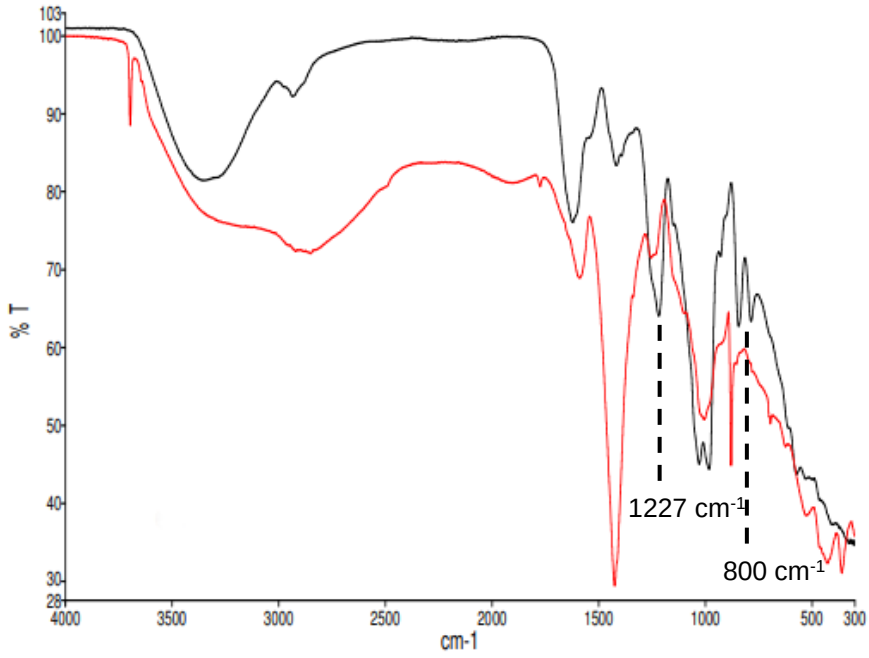

Figure 11. FTIR spectra of as obtained ulvan fraction (black) and ulvan fraction after hydrolysis (red). Drastically reduction of the sulfate ester peak at 1227cm^{-1}.

The intensity of the sharp peak at 1227cm^{-1} and the small one at *ca* 800 cm^{-1}, both related to the presence of the sulfate esters (C-O-S), were drastically reduced (Figure 11). The peak change could be attributed to the fact that the sulfation degree of the ulvan fraction from *Ulva* spp. could be controlled, reducing the sulfate esters through following a basic hydrolysis procedure.

Depending on the application or manufacturing process where ulvan can be potentially involved (i.e., biomedical application, anti-inflammatory, anti-cancer, antibacterial, etc.) the possibilities of tuning the sulfation degree of ulvan could be of interest. For instance, when used as a food additive or dietary supplement, it has been documented that the antioxidant properties of the ulvan are strongly related to the sulfate content of the algae [76, 77].

However, taking into account the discussion made in Section 5.1, ulvan could in fact have overlapping FTIR peaks, between the sulfate ester related peaks *ca* 1220 cm^{-1} with the presence of acetyl species within the ulvan (i.e., acetylated ulvan) with are also expected to be observed *ca* 1200 cm^{-1}. If the latter ulvan fraction is in fact acetylated, a diminish in the intensity of the *ca* 1220 cm^{-1} is expected to be observed, due to the fact that basic hydrolysis will lead to removal of acetyl species [72]. As observed in Figure 11, the associated peak (i.e., 1220 cm^{-1}) to the acetylated ulvan indeed suffered a diminish, leading to think that the latter peak could be in fact associated to the presence of acetylated species within the ulvan, which as mentioned in Section 5.1, are not usually presented in algae species, rather just in land plants [69]. Both results, Figure 9 in Section 5.1 and Figure 11 led to think that the possibility of the ulvan fraction being acetylated cannot be ruled out and rather than just being a peak assigned to the vibration of the C-O-S sulfate bond, the peak *ca* 1220 cm^{-1} could indeed indicate the presence of acetylated ulvan.

Returning to the cellulose fraction, another basic hydrolysis was performed, following the procedure of section 4.2. The alkaline hydrolysis was performed as in

Niemela [78] and Sjostrom [79]. Under basic conditions, cellulose can be converted into carboxylic acids, which could have potential uses as bio-derived products. Therefore, exploring the changes in the cellulose fraction under such conditions through a qualitative analysis was relevant. After the hydrolysis, another FTIR spectra was obtained, which is shown in Figure 12.

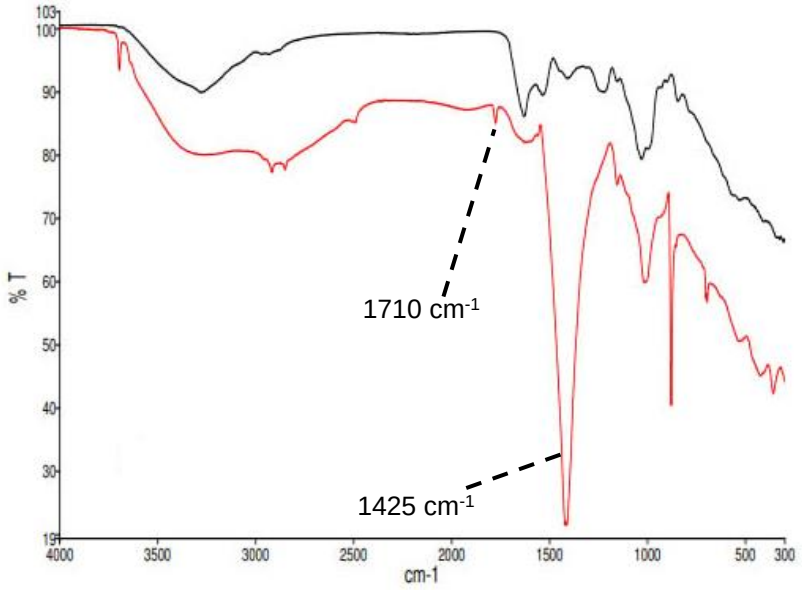

Figure 12. FTIR spectra of the cellulose fraction obtained from *Ulva* spp. before hydrolysis, and after hydrolysis

Although no formal identification and characterization of the derived carboxylic acids was performed, Figure 12 indicates clearly the formation of such acids, because a sharp and small peak at around 1710 cm^{-1} was observed, which correlates well with the C-O bond of the carbonyl group [80]. A clear and sharp carboxylate peak at 1425 cm^{-1} is also observed, supporting the presence of the C-O bond [60, 61].

The results showed that the cellulose fraction from *Ulva* spp. can be processed to generate carboxylic acids. The observation is relevant as one of the main purposes of studying green algae is for their potential use as a raw material for biofuel production, as explained in section 2.2. A qualitative assessment of such uses from

the cellulose fraction of *Ulva* spp. is relevant as a base for future work with this genus.

5.4 Mass-spectrometry of the cellulose fractions

To further assess the possible acetylation of cell wall fractions from *Ulva* and with the options described at the end of section 5.2. (i.e., the existence of acetylated cellulose in the *Ulva* spp., the hemicellulose contamination of the sample or the heavily bonded ulvan to the cellulose) a mass-spectrometry analysis was performed as mentioned in Section 4.4. Since cellulose is a polysaccharide formed by d-glucose units joined by β-1,4-glycosidic linkage [81], during the mass-spectrometry analysis, it was expected to see a single peak of glucose as the main result. The results are shown in Figure 13.

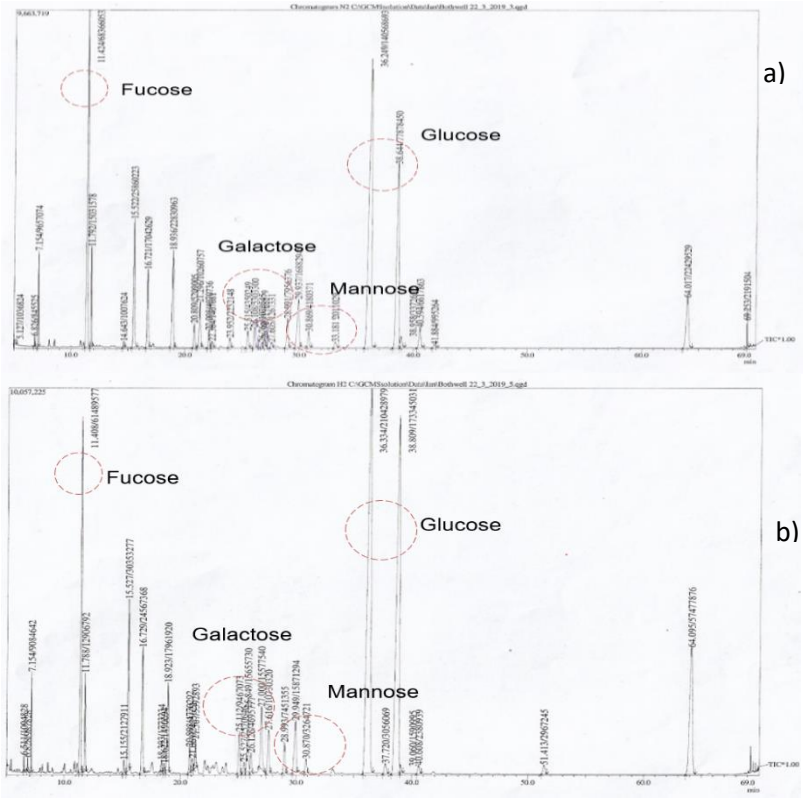

Figure 13. a) shows the mass-spectrometry analysis of the cellulose fraction after being washed, whereas Figure 13 b) refers to the results of the mass-spectrometry analysis of the cellulose sample that was not washed.

From Figure 13 it can be observed that both samples showed signs of contamination by hemicellulose. It can also be observed that the peaks corresponding to glucose, mannose, galactose and fucose are clearly present in both samples. All the aforementioned compounds can be found in hemicellulose, which is a clear sign that the cellulose fractions in fact were contaminated with hemicellulose. Therefore, the presence of acetylated cellulose was not part of the cellulose fraction from *Ulva* spp.

As hemicellulose represents more than 20% of the neutral fibers contained in Ulva algae, the contamination of the cellulose and ulvan fractions likely occurred during extraction procedures.

It should be noted that although the mass-spectrometry experiments were useful in characterising the cellulose obtained from the *Ulva* spp., the lack of access to the facility in the early stages of the project meant that the FTIR procedures were performed before the mass spectrometry experiments.

5.5 TLC analysis of Ulva spp.

The TLC plate analysis is presented in Figure 14, Figure 15 and Figure 16 for the buffer, the buffer plus NaCl and the buffer plus triton, respectively. All experiments w ere performed according to the procedure presented in Figure 6 and as mentioned in Section 4.5.

In the case of the samples with the succinate buffer (Figure 14), bands were assigned to markers five, eleven and twelve, which correspond to galactomannan, cello 4 and laminarin 6, respectively (Figure 14(a))

On the plate with the buffer that had succinate + NaCl (Figure 15) another positive reaction was observed for marker number twelve, which was reproduced for the plate with the buffer containing succinate + triton (Figure 16)

In this section of the study, more detailed experimental is needed, however, due to setbacks on how to approach and perform the TLC methodology, as well as setbacks gathering the information and setbacks analysing the latter information, the data reported in this section was all the information available during the writing of this document.

According to Hoffman [82], the endotransglycosylase enzyme crosslinks charophyte cell walls. In other words, when exogenous oligosaccharides are treated with this enzyme, the wall polysaccharides from charophytes become covalently linked to them. Based on the latter, and according to the methodology presented in Section 4.5.1, the enzyme used for all the TLC experiments is an endotransglucosylase. The latter endotransglucosylase was used to test whether the polysaccharides from the wall would be linked with the some of the oligosaccharides samples [83, 84]. Since *Ulva* belongs to the chlorophyte green algae, we looked at whether such cell wall-modifying enzymes would also link these polymers to chlorophyte cell wall polysaccharides. The latter experimentation were

performed on the basis of obtaining further insights into the. behavior of the *Ulva* spp. polymers within the algae.

As explained before, according to the literature [82, 83] if an oligosaccharide (i.e., the markers used during the TLC experiment such as cello, laminarin or galactomannan) is linked by the enzyme to an Ulva cell wall polysaccharide, a shift in the spot on the TLC plate is expected to be observed as the cell wall polysaccharide increases in size (i.e. a spot further up from the origin where the marker was put).

It can be observed in Figure 14(a) that such reactions did, in fact, occur, with markers five, eleven and twelve (galactomannan, cello-4 and laminarin-6, respectively). This shows that these oligosaccharides were attached to the cell wall polysaccharides by the endotransglycosylases. As with the charophytes [82, 84], the galactomannan reaction was the one that proceeded most strongly (Figure 14(a)).

False negatives may be expected in some columns, if the formed products and reactants were to be indistinguishable [85]. Follow up experiments, with labelling in the form of a radiochemically or fluorescently tagged oligosaccharide, would be needed to confirm the identity of such products. However, due to the unavailability of this technology in the laboratory, only the enzyme reaction was evaluated by TLC, without further identification of the formed products.

As a control and to determine the characteristics of the *Ulva* spp. sample, no reaction was observed on the same markers when the enzyme was not present (i.e., Figure 14 b). Therefore, no linking occurred.

Interestingly, when NaCl was added into the buffer solutions (Figure 15), the activity of the enzyme towards the linking of the galactomannan was hindered. In accordance with the literature, there is the possibility that a high salt concentration solution could affect the activity of the enzyme. The same behavior was observed when triton (Figure 16) was added to the reaction [86].

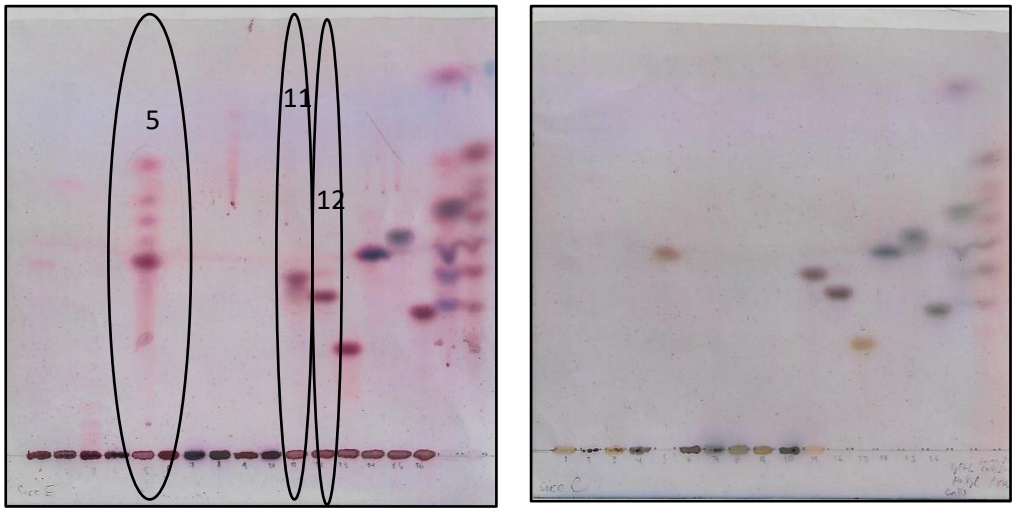

Figure 14. a) Buffer with the *Ulva* sample and the enzyme (i.e., endotransglycosylase) b) Buffer without the enzyme. a) shows the effect the endotransglycosylase is causing when reacting with the markers at position 5, 11 and 12 (galactomannan, cello-4 and laminarin-6, respectively) showing the attachment of the oligosaccharides with the cell wall. b) when the enzyme was not present, no reaction was observed.

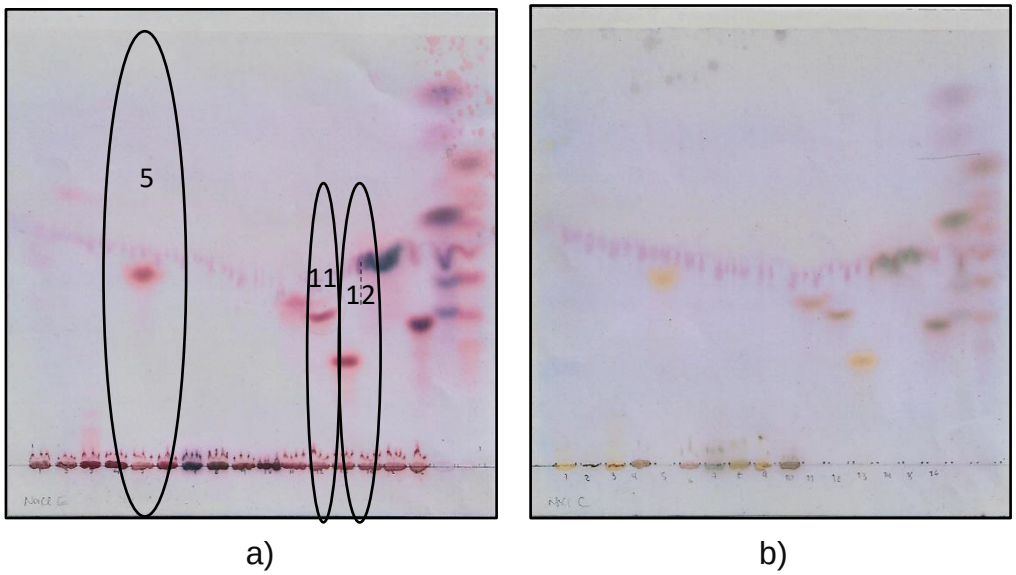

Figure 15. a) Buffer with the *Ulva* sample and the enzyme (i.e., endotransglycosylase) b) Buffer without the enzyme. a) shows the effect the endotransglycosylase is causing when reacting with the markers at position 11 and 12 (cello-4 and laminarin-6, respectively) showing the attachment of the oligosaccharides with the cell wall. The presence of the salt (NaCl) might be hindering further reactions at marker 5, as per the literature mentions. b) when the enzyme was not present, no reaction was observed

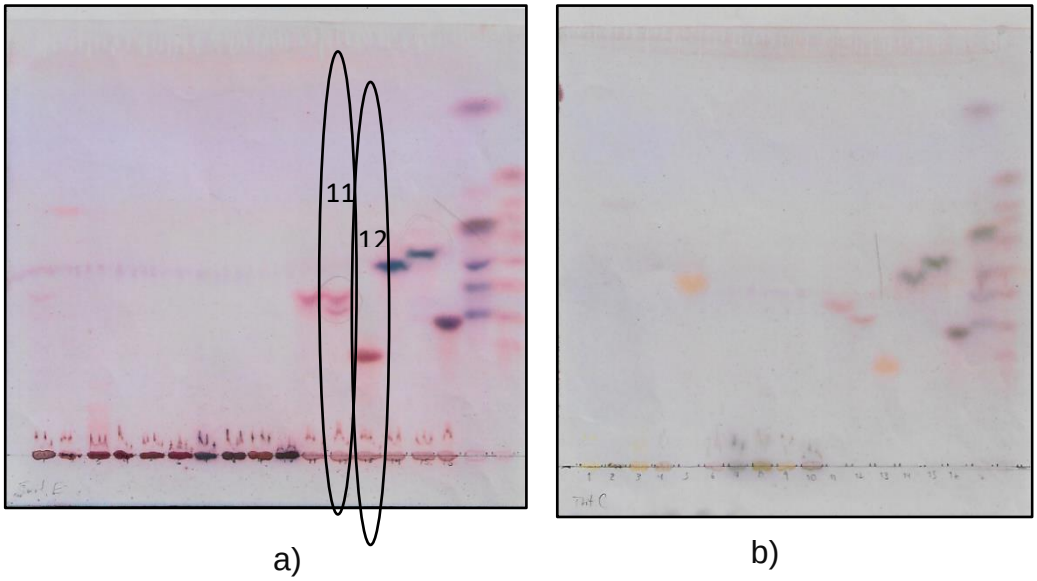

Figure 16. a) Buffer with the *Ulva* sample and the enzyme (i.e., endotransglycosylase) b) Buffer without the enzyme. a) shows the effect the endotransglycosylase is causing when reacting with the markers at position 11 and 12 (cello-4 and laminarin-6, respectively) showing the attachment of the oligosaccharides with the cell wall. The presence of the triton might be hindering further reactions at marker 5, in a similar way as when NaCl was present in the solution. b) when the enzyme was not present, no reaction was observed

The ability of the enzyme to link the wall polysaccharides and the oligosaccharides, however, needs to be further studied with other experimental analysis. For instance, to see whether the endotransglycolase would work in conjunction with xyloglucan as an acceptor substrate, and how these changes occur in vivo during stress [87].

A first approach to better understand some characteristics and capabilities of the *Ulva* spp. cell wall through TLC chromatography and how the latter cell wall to undergo linking of oligosaccharides and polysaccharides could potentially benefit further characterization of the *Ulva* spp. collected from Seaham in county Durham in further studies was performed. Although more studies are needed regarding how the cell wall will react when in presence of enzymes, this first approach results of interest as it lays some foundation to further studies, which could result in

implementing new methods and strategies to improve the mechanical properties and use of the algae.

5.6 The effect of the base hydrolysis and the possibility of the ulvan being naturally acetylated

During the writing of this work no quantitative assessment was performed to identify the amount of possible acetyl molecules being removed when the ulvan fraction was submitted to base hydrolysis. Based on the information presented in Section 5.1 and Section 5.3, the presence of FTIR peaks that could be assigned to the presence of acetylated ulvan (i.e., 1220 cm^{-1}) and the reduction of the latter peaks when base hydrolysis was performed on the ulvan fraction (Figure 11) suggest that the acetylation of the ulvan indeed occurred. As a set of preliminary results, To complete the information presented in the Section 5, followed-up work within the Bothwell group achieved a quantitative measurement of a base-labile acetate liberated when the base hydrolysis on the ulvan was performed. According to the Bothwell group, 50 mg of base-labile acetated was released for 1 g of cell wall.

The release and quantification of acetate species after the base hydrolysis, in conjunction with the information reported in this study in Section 5.1 and Section 5.3 suggests that in fact, the ulvan fraction would be acetylated, with this work laying the foundation of the follow-up work developed in the Bothwell group so far, which indicates that the *Ulvan* spp. cell walls have acetylated conditions such as the ones encountered in land plants [69], which has not been identified before elsewhere.

6. Conclusions

Insights into the composition and cell wall characteristics of the *Ulva* spp. from Seaham harbour in county Durham were acquired. The sulphated polysaccharide fraction, ulvan, was extracted from the *Ulva* spp. and analysed through Fourier-Transformed Infrared Spectroscopy (FTIR) to identify the functional groups present on the sample as well as to act as a point of comparison with other ulvans from other *Ulva* spp. The cellulose fraction was also characterized through FTIR and Mass-spectrometry (MS) to assess whether or not the FTIR analysis of both fractions reflected the presence of acetylation in either the ulvan or cellulose fractions. Finally, a Thin-Layer chromatography (TLC) experiment was performed to see whether the *Ulva* spp. from the county Durham coast could be used as a substrate for enzymes that added oligosaccharides detected in the sample to cell wall components.

My results suggest that acetylation is present on the ulvan fraction, and on hemicelluloses that contaminate the cellulose fraction. Both acetyl and sulfate groups could be removed by basic hydrolysis, which may be a good approach to efficiently processing the ulvan fractions. Moreover, through TLC, it was determined that the presence of endotransglycosylase favoured the covalent linking of the *Ulva* sp. wall polysaccharides to external oligosaccharides, a behaviour previously observed happening with charophytes, the closest relatives to land plants.

7. Future work

More in-depth analysis of the *Ulva* spp. collected from the Seaham coast needs to be done to fully characterize it. Further studies are particularly needed if we are to optimize ulvan extraction from *Ulva* spp. For instance, when extracted, the degree of ulvan sulfation can vary, with some degree of sulfation being removable through basic hydrolysis. This procedure would directly affect the chemical composition of the ulvan. The biological activity of the ulvan is also influenced by its chemical composition, so a study of the biological activity of the ulvan extracted from the Seaham *Ulva* spp. would also be of interest and is being developed by my host group, based on the results that I have presented here. Using a better-defined procedure to extract and isolate ulvan from *Ulva* spp. would result in a product that can be further evaluated for its biological activity, with potential medical or biotechnological applications that may add value to biofuel extraction.

Moreover, further studies into the linking of the polysaccharides with oligosaccharides from the sample are also required, identifying the produced compounds by mass spectrometry to better understand how the mechanical properties of the algae could be exploited.

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