Enzymatic Hydrolysis of *Ascophyllum nodosum* for the Co-Production of Biofuels and Value-Added Products

by

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Abstract

There has been an increasing interest in the use of biorefining feedstocks for the production of value-added products. Seaweeds are of special interest, due to the fact that they do not compete for land use with food crops, have low to no lignin content, and can be grown in waste water or at sea, thereby using resources that are currently of low value. However, seaweed does have shortcomings, the most important being that seaweed has not been thoroughly studied as is the case for lignocellulosic materials. Canada has a large amount of coastline, and therefore, also has an abundance of algae.

The collaborators at Acadian Seaplants Ltd. produce and sell a plant biostimulant from *Ascophyllum nodosum*, a brown algae. However, their production process results in the generation of large amounts of byproduct seaweed residues that have a highly basic pH and thus have very little current value. The primary objective of this work was to study the incorporation of enzymes to facilitate release of plant biostimulants from *Ascophyllum nodosum*, while simultaneously producing an algal biomass byproduct stream rich in sugars and at a pH that that is more amenable to downstream processing applications.

Characterization of the seaweed sample was performed, and then proceeded with the enzymatic hydrolysis. For these experiments, well-known and commercially relevant cellulase enzyme blends were used, Celluclast® 1.5L, Cellic CTec2, and Viscozyme® L, as well as a commercial alginate lyase. A maximum of 70 ± 9% of the glucose present was released by the most successful enzyme treatment. To test for the presence of plant biostimulant activity in the enzyme hydrolysates, samples were sent for a mung bean rooting assay which was also performed. The rooting assays showed that the liquid hydrolysates had increased rooting activity when compared to the controls. The research presented in this thesis highlights the potential of using

enzyme treatments to simultaneously produce a plant biostimulant and a sugar-rich byproduct stream that could be converted to value-added products through subsequent biorefining conversions.

Preface

Some of the research conducted for this thesis was a business research collaboration, led by Dr. David Bressler's lab at University of Alberta, with Acadian Seaplants Ltd. being the lead collaborator. The ICP-EOS for the elemental analysis in *Sections 3.1.2 and 4.1.2* was done at the Natural Resources Analytical Laboratory (NRAL) in the Department of Renewable Resources, at the University of Alberta. The microscopy equipment used in *Sections 3.4 and 4.4* was operated by a trained technician and done at the Scanning Electron Microscope Lab, in the Department of Earth & Atmospheric Sciences, at the University of Alberta. The mung bean bioassay in *Sections 3.5 and 4.5* was done by a collaboration from Acadian Seaplants Ltd. in Nova Scotia, Canada, and Dr. Balakrishnan Prithiviraj from Dalhousie University in Halifax, Nova Scotia, Canada.

All experimental design, remaining experimental work, and experimentation was performed by myself with the supervision, oversight and guidance of Dr. David Bressler. No part of this thesis has been previously published.

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1. Research Proposal

1.1 Justification

There is an existing commercial market of seaweed extracts for its uses in agriculture. These seaweed extracts, containing plant biostimulants, boost crop productivity and help reduce plant stress. Current processes to extract these plant biostimulants use an alkaline extraction to rupture the cells. This yields a residue stream that is high pH, high salt content, and of little to no value. In order to give value to this residue stream, the process needs to be changed. An alternative for the rupture and further extraction of this plant biostimulants can be enzyme hydrolysis. Using enzyme hydrolysis, we could get both the plant biostimulants, and a sugar rich feedstock. Sugars can further be used with fermentation for the production of biofuels. This project is significant in the following levels:

1.1.1 Energy demand

The world population is increasing rapidly, and with it, there is a global increase in the demand for energy. Biofuels, like ethanol fermented from sugars, is a viable renewable energy source that could supplement the current energy production to meet future energy needs.

1.1.2 Development of value-added products through the use of more environmentally friendly methods

This research involves the use of the brown algae *Ascophyllum nodosum*, a novel feedstock, for the co-production of fermentable sugars and plant biostimulants through a biorefining approach using enzymes. Traditionally, the extraction of plant biostimulants involve the use of caustic chemicals through an alkaline extraction, that then yields a byproduct stream that is of high pH and high salt content and is of very low value. The use of enzymes could eliminate that waste

stream and turn it into a sugar-rich feedstock that, through further fermentation steps, yields bioethanol or other value-added commodities.

1.1.3 Making use of Canada´s rich natural resources while supplementing its strong agriculture industry

Canada has a very large coastline, where seaweed is available naturally. By using seaweed, it can not only help meet energy demands, or use more environmentally friendly methods to produce value-added products, it can also help the agriculture sector. Canada has a strong agriculture economy that can benefit from application of algal-based biostimulants, which can help increase crop production and stress resistance.

1.2 Objectives

This research involves studying the enzymatic hydrolysis of the brown seaweed *Ascophyllum nodosum*. The main objectives of this study are as follows:

- To determine if enzymatic hydrolysis using cellulases used for lignocellulosic material will yield a sugar-rich hydrolysate when used on the algae system.
- To determine if enzyme hydrolysis is capable of extracting plant biostimulants from the seaweed without the use of harsh chemical treatments.

1.3 Research Hypotheses

1. Enzyme hydrolysis of *Ascophyllum nodosum* using industrially-relevant lignocellulosic enzymes will result in the production of a sugar-rich hydrolysate.
2. Enzyme hydrolysis will rupture the cell walls enabling the release of plant biostimulants into the hydrolysate, which will facilitate increased rooting activity.

2. Background

In recent years, there has been an increasing interest on the study and development of newer and more efficient alternative energies, one of them being biofuels. The field of biofuels has progressed over time, becoming more efficient and attempting to compete less for resources that could be otherwise used for other ends such as food. The third generation of biofuels, or biofuels generated using algae, is one of the newest and most promising due to their structural composition of algae as well as their environmental characteristics (Aitken *et al*., 2014). Algae can grow using resources that can be considered waste, like sewage water, and because it grows on water, it doesn't compete for land and resources that could be used for food crops (Borowitzka, 2012). Canada has a large amount of coastline in which algae can be found or farmed, making it an important and readily available resource in the country.

Though the use of algae for the development of biofuels seems promising, the research on it is still ongoing (Tan & Lee, 2014). Most of the research in third generation biofuels has been done on microalgae, though there has been an increasing interest in the use of macroalgae. There are still elements in the process that can be improved, and components and characteristics of algae that have not been studied in depth compared to the knowledge available on the use lignocellulosic materials for biofuels. Elements like which enzymes to use or which pre-treatments could make the process of saccharification more efficient are still being proposed and studied (Martin *et al*., 2016; Tan & Lee, 2015). Hence, the purpose of this literature review was to examine the characteristics of macroalgae, focusing on its structural components, as well as the description of the saccharification process, the enzymes used and other pre-treatment steps necessary to obtain the sugars used for fermentation in the production of biofuels.

2.1 Food versus Fuel Debate

The food versus fuel debate started in the last two decades and is based on the assumption that by diverting more crops and crop land into producing biofuels, food insecurity for the world could be promoted and thus, increasing food prices (Ajanovic, 2011; Koizumi, 2015; Zhang *et al*., 2010b). It also debates the ethicality of diverting food for biofuel production (Koizumi, 2015). This debate is still relevant even today as a clear answer or consensus has not yet been reached and there are studies that show benefits and consequences of both sides of the argument (Ajanovic, 2011; Koizumi, 2015; Srinivasan, 2009; Vanthoor-Koopmans *et al*., 2013; Zhang *et al*., 2010b). This is relevant to this project because of the fact that seaweed can be grown at sea, thus, do not compete for land like other traditional biofuel crops do.

On one side of the debate, a 2017 study published in *Earth's Future*, showed that feed production systems waste less energy and affect the environment less than corn-based ethanol production (Richardson & Kumar, 2017). These findings further contributed to the debate that crops should be used for food instead of for biofuels. They state that bioenergy challenges a sustainable food future. This side also claims that although cellulosic biofuels do not use feedstocks that are being diverted from food to fuel production, competition for land use is still likely to occur (Carroll & Somerville, 2009; Madhu, 2008; Searchiner & Heimlich, 2015). This is because the material used for the production of cellulosic biofuels is commonly fast-growing grasses and trees, which grow faster and better on flat, fertile land that could be used for crop farming (Carroll & Somerville, 2009; Searchiner & Heimlich, 2015). Cellulosic biofuels can also rely on harvesting existing forests, or producing trees and grasses on existing forests and savannas, but this can reduce their carbon storage capabilities, and could also impact their ability to support a diverse ecosystem (Madhu, 2008; Searchiner & Heimlich, 2015).

On the other side of the argument, some say that the debate is now a myth. Although grain usage for ethanol production in the United States reached a record high in 2016, grocery prices endured a deflation that had not been experienced since 1967, with global undernourishment occurrence falling to the lowest level since the United Nations started keeping logs more than 25 years ago (RFA, 2017). Furthermore, a study from The Renewable Fuels Association released in 2016 showed that while the increase in ethanol production has indeed made an impact in the supply and demand aspects of corn, and therefore corn prices, ethanol alone is not the only driver of the corn market (RFA, 2016). The corn prices in 2012, for example, were at a record high, but that was partly because of a drought that affected much of the U.S. that year (RFA, 2016). Statistical analysis has shown a weak link between corn usage for ethanol and the prices of food (Gardebroek & Hernandez, 2013; RFA, 2016; Zhang *et al*., 2010b). One of the reasons for this is that for every dollar paid for food by the consumer, only around 10% is paid to the farmers; the majority of the cost comes from transportation of the products from the farm to the retail stores (RFA, 2016).

2.2 Algae

When working with a non-traditional feedstock, such as seaweed, it is of importance to know the biology, structure, and environment where the feedstock grows. This algae are generally thought of as aquatic, oxygen-evolving photosynthetic autotrophs that can be unicellular, colonial, or are constructed of filaments or simple tissue (Guiry, 2012). A more precise definition is that algae are plant-like organisms that contain chlorophyll *a*, partake on oxygenic photosynthesis and are not land plants with specialized tissues (Borowitzka, 2012; Lee, 1989). It encompasses a broad group made up of around 72,000 different recognized species, though some estimate the total number of species to be around one million (Guiry, 2012). They are currently represented in four different kingdoms: Bacteria, Plantae, Chromista, and Protozoa, which leads to great diversity and

variability between species of this group known as algae (Guiry, 2012). A subset of algae also includes seaweed. This project centers on the seaweed species *Ascophyllum nodosum,* a brown seaweed from the Chromista kingdom.

Algae occur most commonly in water, which can be saltwater, freshwater, brackish water, and even in waste water (Borowitzka, 2012; DeWreede, 2001; Lehnberg & Schramm, 1984). However, algae can also be seen growing in almost any other environment, such as the snow, living in bare rocks in lichen associations, hot springs, and have even been observed as unicellular species living in desert soil (DeWreede, 2001; Lee, 1989). They are primary producers in most environments, generating organic matter from water, carbon dioxide, and sunlight, thus forming a basic food source for many food chains (Lee, 1989). Algae can be divided into two cellular types: eukaryotic or prokaryotic (Lee, 1989; Moestrup, 2006). Unlike eukaryotic cells, prokaryotic cells lack organelles that are bound with membranes, like nuclei, Golgi bodies, or plastids (Lee, 1989; Ramus *et al*., 1976).

2.2.1 Eukaryotic Algae

Out of the eukaryotic algae species, there are three main groups: Rhodophyceae (red algae), Chlorophyceae (green algae), and Phaeophyceae (brown algae) (Guschina & Harwood, 2006; Lee, 1989). A defining feature of eukaryotic algae species is the presence of chloroplasts as shown in **Figure 2.1**. Chloroplasts have sac-like structures called thylakoids where the light reactions of photosynthesis take place (Lane & Saunders, 2006; Lee, 1989; Moestrup, 2006). Thylakoids can be free from others or can be arranged together in thylakoid bands. Thylakoids are attached in the stroma where reactions of carbon fixation take place (Lee, 1989). Sometimes, the chloroplasts have pyrenoids, a dense proteinaceous area that is associated with the formation of storage products.

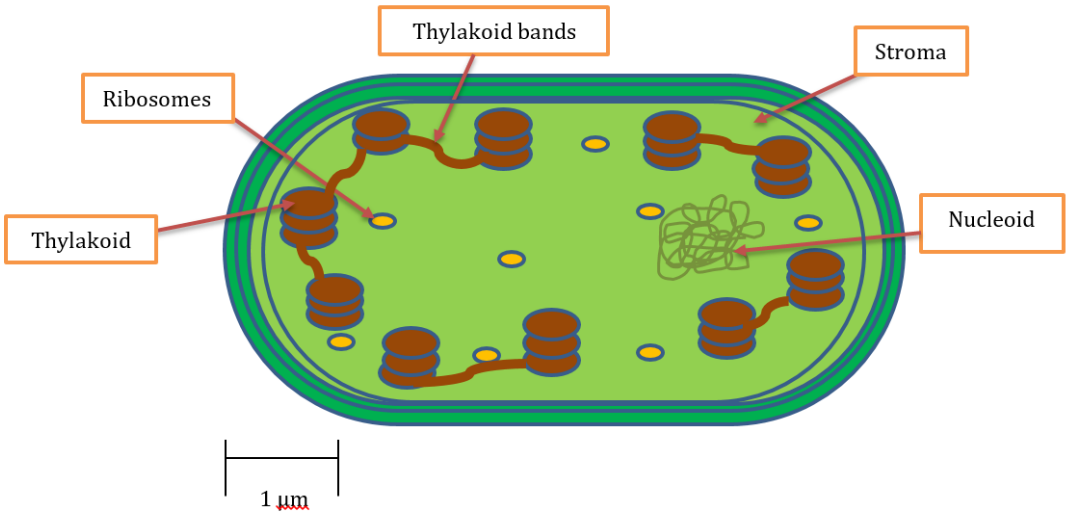


Figure 2.1 Algae Chloroplast

Algae chloroplasts contain four surrounding membranes, the outermost layer connected to the endoplasmic reticulum. They have thylakoids in stacks of three, which are joined by thylakoid bands contained in the stroma. Microfibrils of DNA can also be found in the chloroplasts

There are several different storage products that can occur in algae. For example, one main storage product consists of α-1,4 linked glucans like floridean starch, myxophycean starch, and starch (Lee, 1989). Algae can also have β-1,3 glucans as storage products, such as laminarin, chrysolaminarin (leucosin), and paramylon (Lee, 1989). Fructosans can also occur as a storage product. Other low-molecular weight compounds that can be storage products are sugars (sucrose), glycosides (floridoside and isofloridoside), and polyols (mannitol and free glycerol) (Lee, 1989).

In general, algae cell walls have two main components: the fibrillar component, and the amorphous component (Domozych, 2011; Lee, 1989). The fibrillar component is the "skeleton"

of the cell wall and is what gives the cell its structure and rigidity. The most common fibrillar component in algae is cellulose, a 1,4 linked β-D-glucose polymer also found in terrestrial plants (Domozych, 2011; Lee, 1989). Other fibrillar components that are in fewer species include mannan, a 1,4 linked β-D-mannose polymer, and xylans of different polymers (Lee, 1989). The amorphous component of the cell wall is the matrix in which the fibrillar component is rooted (Lane & Saunders, 2006; Lee, 1989). The amorphous component occurs in the greatest quantity in brown and red algae (Domozych, 2011; Lane & Saunders, 2006; Lee, 1989). Alginic acid and fucoidin are amorphous components that can occur in brown seaweed (Domozych, 2011; Lane & Saunders, 2006). In red algae, both galactans, like agar or carrageenan, or polymers of galactose, can be the amorphous components (Lee, 1989). Although the basic composition of the algae cell walls is known, what they are made up of, literature has yet to shine light on the actual structure and interactions.

2.2.2 Prokaryotic Algae

Blue-green algae are the only group of prokaryotic algae. They are usually referred to as cyanobacteria (Moestrup, 2006). They have an outer plasma membrane enclosing the protoplasm, which contains the photosynthetic thylakoids, 70S ribosomes, and DNA fibrils. The DNA fibrils are not enclosed within a separate membrane, unlike in eukaryotic algae. Cyanobacteria have chlorophyll *a*, phycobiliproteins (proteins that catch light energy), glycogen as a storage product, and their cell walls contain amino sugars and amino acids (Moestrup, 2006).

2.2.3 Red Algae

The majority of all algae species are red algae, or Rhodophyceae, which are one of the most ancient groups of eukaryotic algae (Fredericq & Schmidt, 2016; Lee, 1989). They have phycobiliproteins as accessory pigments that give them their characteristic crimson coloring,

utilize floridean starch as a storage product, only have single free thylakoids in their chloroplasts, and no chloroplast endoplasmic reticulum (Lee, 1989). About 98% of red algae are marine species while only 2% are freshwater species (Fredericq & Schmidt, 2016). Marine red algae occur in all latitudes, but they are more prominent in temperate and tropical climates than in polar and subpolar climates, where green and brown algae tend to dominate (Lee, 1989).

The size of the algae also varies depending on where they are growing. The bigger species of red algae generally grow in cool-temperate areas, while in tropical areas they tend to be small filamentous species. Due to a function of their accessory pigments, they can live in deeper waters than other algae; some red algae can even survive depths of 200 meters. This is because algae can change its pigmentation in order to adapt to different light concentrations (Lee, 1989; Ramus *et al*., 1976; Talarico & Maranzana, 2000). There are also about 200 freshwater red algae species, which grow in sizes smaller than the marine red algae species (Cavalier-Smith & Chao, 1996). The majority of the freshwater species occur in running waters of small and medium sized streams (Lee, 1989).

2.2.4 Green Algae

Green algae, also known as Chlorophyta, consist of mostly freshwater algae species. Chloroplast pigments in green algae are similar to those in higher plants, with chlorophylls *a* and *b* being present (Lee, 1989; Moestrup, 2006). They use starch as their storage product, which, different from other algae, is formed in the chloroplast instead of the cytoplasm (Lee, 1989). Cell walls in green algae usually have cellulose as the main structural component, but xylans and mannans can also replace cellulose in some of the species as the main structural component (Domozych, 2011; Lee, 1989).

The majority of the green algae species live in freshwater environments; only about one-tenth of the green algae species are marine (DeWreede, 2001; Lee, 1989; Moestrup, 2006). The freshwater species are broadly distributed globally, with very few species endemic to certain areas. For the marine species, at warmer climates, all green algae tend to be the same. This is in contrast to cold water species where very different species exist in the Northern and the Southern cold marine climates; the warmer water in the equator is believed to act as a barrier for the green algae living in the colder climates at opposite poles (Lee, 1989).

2.2.5 Brown Algae

Brown algae, also known as Phaeophyceae, are algae that get their brown-yellow color from fucoxanthin, a carotenoid, and/or any phaeophycean tannins that could exist in chloroplasts (Lee, 1989). They have chlorophylls of types *a*, c_1, *and* c_2 (Domozych, 2011; Lee, 1989). They possess fucoxanthin and β-1,3-glucans as their food reserve (Domozych, 2011). They use laminarin as their storage product (the carbohydrate in which they store their energy) (Lane & Saunders, 2006; Lee, 1989). The total polysaccharide concentration in marine species varies from 4–76% dry weight (Kadam *et al*., 2015b). The highest concentrations of polysaccharides are found in *Ascophyllum, Porphyra* and *Palmaria* species (Kadam *et al*., 2015b).

Most species are marine, with only four genera of freshwater species. Some of the species can also be found in brackish water (Lee, 1989). Brown algae are more dominant in colder waters, particularly in the Northern Hemisphere, growing in the intertidal belt and the upper littoral region (Lee, 1989). Brown algae are unique in that they have an uptake of inorganic carbon (separate from photosynthesis), and photosynthetic carbon fixation (Lee, 1989). This can be a limiting factor, as photosynthesis can be halted by the unavailability of inorganic carbon in the environment (Lee, 1989).

2.2.5.1 Brown Algae Cell Wall

Brown algae cell walls normally have two layers, with cellulose making up its predominant structural frame (Domozych, 2011; Lane & Saunders, 2006; Lee, 1989). The cellulose microfibrils that make up the frame of the wall are contained in layers parallel to the cell surface (Domozych, 2011). Though cellulose is the main component of the skeletal structure, it is a small component of the overall cell wall when compared to the matrix polysaccharides (Domozych, 2011). The amorphous region of the wall is made up of alginic acid and fucoidin, whereas the mucilage and cuticle are made up mainly of alginic acid (Domozych, 2011; Lane & Saunders, 2006; Lee, 1989).

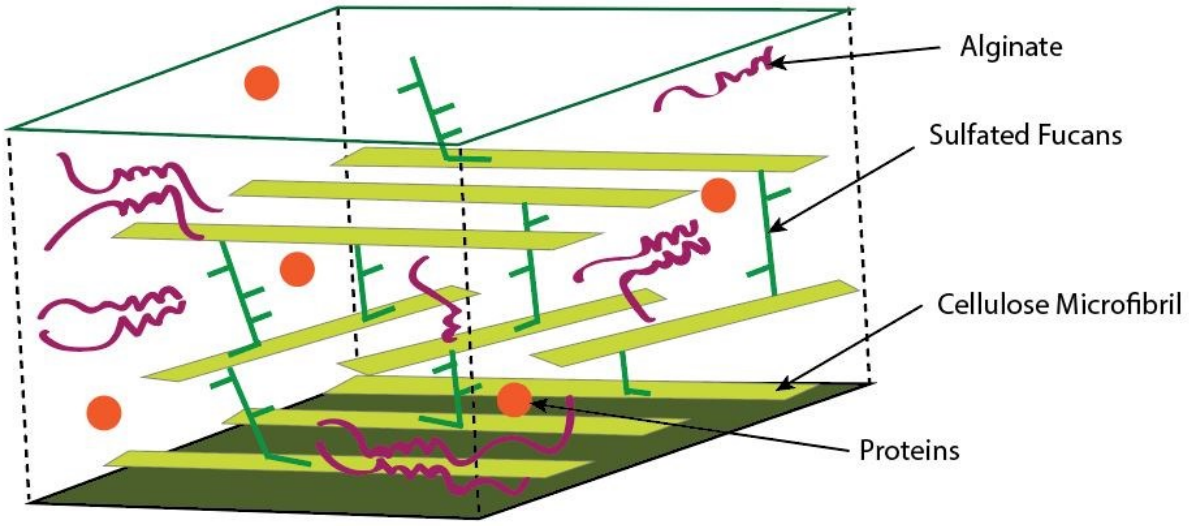


Figure 2.2 Brown Algae Cell Wall Interpretation.

Brown algae cell wall is made up of cellulose microfibrils arranged in layers parallel to the cell surface. Though most of the composition of the cell wall is cellulose, other elements such as proteins, alginate and fucoidin can also be found.

2.2.5.2 Brown Seaweed Chloroplasts

Brown seaweed chloroplasts have three thylakoids per band. These are surrounded by the chloroplast envelope and two membranes of the chloroplast endoplasmic reticulum (Lane & Saunders, 2006; Lee, 1989). Microfibrils of DNA are in the chloroplasts, which contain chlorophylls *a, c_1,* and *c_2,* with fucoxanthin being the major carotenoid (Lee, 1989). Carotenoids are naturally occurring yellow, orange or red pigments that are fat soluble (Stange, 2016). All brown algae species have pyrenoids, sub-cellular micro compartments within their chloroplasts, but their presence can vary depending on the stage of the plant in its lifecycle. Pyrenoids are usually

a structure that looks like a stalk, set off from the main body of the chloroplast. Surrounding the pyrenoid, there are sacs with membranes that contain the long-term storage product of the brown algae, laminarin (Lane & Saunders, 2006; Lee, 1989).

2.2.5.3 Fucales

Fucales is an order of brown seaweed (Lane & Saunders, 2006). The seaweed included in this order grow from an apical cell, which is a cell that divides constantly to form new cells (Lepp, 2012). Their tissue is parenchymatous, a basic tissue of plants which is composed of thin walled cells, and which makes up the non-woody tissue in plants (Morris *et al*., 2016). The organisms of the order of Fucales are widespread throughout the world (Lane & Saunders, 2006; Lee, 1989).

The species of the south temperate waters and the Antarctic are very different from those in the Arctic and northern temperate seas, due to the fact that the high temperature of the equator creates a barrier of sorts which species cannot cross. *Fucus* is a genus that can be commonly found in northern waters. *Sargassum* is a genus of Fucales that can be found in tropical and subtropical waters. *Cystophora* is a genus that can be found in Australian waters as a predominant species of the aquatic flora there, and *Durvillea* is a genus that is common to the sub-Antarctic waters (Lee, 1989). Sequencing of Fucales genes has brought to light that the genus diverged early from the rest of the brown seaweed species (Lee, 1989).

2.2.6 Structural Components of Algae

2.2.6.1 Alginic Acid

Alginic acid is an acid that is made up of β-1,4 linked mannuronic acid units (M units) that have different amounts of guluronic acid units (G units) attached through C-1 and C-4 linkages (Lee, 1989). Alginate is the most common polysaccharide found in brown algae (Ravanal *et al*.,

2016). For commercial use, alginate is commonly transformed into its more common salt, sodium alginate (25). The use of sodium alginate as a micro-encapsulation agent has been studied where sodium alginate is used as a polymer and calcium chloride as the gelling agent in order to encapsulate polyphenols from wine production waste (Aizpurua-Olaizola *et al*., 2016). Alginic acid is a colloid that is often used in the food industry as an emulsifier, thickening agent, stabilizer, and formulation aid. It can also be used in pharmaceuticals, such as Gaviscon, which is an antacid, in order to lower the effects of gastric reflux (Castell *et al*., 1992; McHardy, 1978; NCBI, 2017). Alginic acid helps in the inhibition of gastroesophageal reflux by binding with bicarbonate ions (Castell *et al*., 1992). More recently, alginate has also been studied as a polymer to build bioresorbable, biocompatible scaffolds to generate soft tissue for repair, using tissue cells from the same individual (Hirsch *et al*.). Calcium-alginate hydrogels are also being studied as a medium for high density microalgae production (an alternative to biofilm photobioreactors) and its feasibility for sustained, high density production has been demonstrated (Pierobon *et al*., 2017).

2.2.6.2 Laminarin

Laminarin is a long term storage product of brown seaweed algae (Kadam *et al*., 2015b; Lee, 1989). Laminarin is located in vacuoles present in cells (Kadam *et al*., 2015b). Laminarin has been reported to have biofunctional activities such as antitumoral, anti-apoptotic, anti-inflammatory, anticoagulant and antioxidant activities (Kadam *et al*., 2015b). It is composed of β-(1, 3)-linked glucose, and has sugars in large amounts and small quantities of uronic acids (Ghadiryanfar *et al*., 2016; Moroney *et al*., 2015). There are two types of laminarin chains, M and G, depending upon the reducing end. M-chains end with 1-O-substituted d-mannitol, while G-chains end with glucose (Kadam *et al*., 2015b). The distribution of the two types of laminarin vary

in response to certain environmental factors such as water temperature, salinity, waves, sea current, nutritive salts and depth of immersion (Kadam *et al*., 2015b).

2.2.6.3 Fucoidan

Fucoidan is made up "of α-1,2 linked sulfated-fucose units, with a lesser amount of α-1,4 linked sulfated-fucose units" (Lee, 1989). Fucoidan makes up the amorphous part of the cell wall in brown algae. Recently, it has attracted interest because of its use as a natural immunomodulator (Choi *et al*., 2005; Kim & Joo, 2008; Maruyama *et al*., 2003; Vetvicka & Vetvickova, 2017). It has been found that fucoidans have strong immune-stimulating properties, including inhibition of cancer (Choi *et al*., 2005; Maruyama *et al*., 2003; Vetvicka & Vetvickova, 2017). They have also been found to improve acute colitis, by affecting natural killer cell activity, and affect inflammation, as well as vascular physiology and oxidative stress (Choi *et al*., 2005; Maruyama *et al*., 2003; Vetvicka & Vetvickova, 2017). Fucose is a hexose deoxy sugar. It has a chemical formula of $C_6H_{12}O_5$, and is the monomer in fucoidan (NCBI, 2005).

2.2.6.4 Cellulose

Cellulose is a polysaccharide made of chains of β-1, 4 D-glucose monomers. It is prevalent in plants and algae and is the main fiber that makes up the cell wall of most plants comprising around 40% to 50% of cellulosic biomass. Its chemical formula is $(C_6H_{10}O_5)_n$, it is insoluble in water, and soluble with chemical degradation in sulfuric acid (NCBI, 2007; Yang *et al*., 2011).

Cellulose consists of amorphous and crystalline regions (Pérez *et al*., 2002). The crystalline regions of cellulose have strong molecular bonds. Amorphous regions have weaker bonds and absorb moisture easily. Crystallinity has been studied as a factor that could influence enzymatic hydrolysis rate in different cellulose samples (Mélanie *et al*., 2010). Cellulose structures contain

reducing and non-reducing ends. Reducing ends have an OH group, making them capable of reducing other compounds, while non-reducing ends lack this OH group.

2.3 *Ascophyllum nodosum*

The taxonomy of *Ascophyllum nodosum* is shown in **Table 2.1**.

Table 2.1 Taxonomy of *Ascophyllum nodosum*

Category	**Classification**
Domain	Eukariota (Chatton, 1925)
Kingdom	Chromista (Cavalier-Smith, 1986)
Phylum	Ochrophyta (Cavalier-Smith & Chao, 1996)
Class	Phaeophyceae (Yang *et al*., 2012)
Subclass	Fucophycidae (Cavalier-Smith, 1986)
Order	Fucales (Huisman, 2015)
Family	Fucaceae (Silva, 1980)
Genus	*Ascophyllum (Le Jolis, 1863)*
Species	*nodosum (Le Jolis, 1863)*

2.3.1 Description of *Ascophyllum nodosum*

Ascophyllum nodosum is a brown algae that is olive-green in color (Fish & Fish, 2011). They can be up to 2 meters or more in length (Fish & Fish, 2011). The axis is dichotomously

branched, which means that it divides into two. Its main axis and principal branches are flattened lengthwise, with texture resembling that of leather. It has large float bladders, often around 1.5 to 2 centimeters in diameter and 2 to 3 centimeters long, but can be larger at times (Taylor & Rao, 1937). After the algae reaches one to two years of age, it produces roughly one new float bladder per year, which helps estimate the age of undamaged algae. The lifespan of *Ascophyllum nodosum* has been estimated using this method to be around 12 years (Fish & Fish, 2011).

A. nodosum is dioecious, meaning that each plant can be either female or male. Gametes are produced and released in spring by their reproductive bodies, which are golden-yellow colored. After spring, the stalks and the reproductive bodies are shed (Fish & Fish, 2011).

2.3.2 Distribution of *Ascophyllum nodosum*

A. nodosum is widely distributed in north-west Europe (Fish & Fish, 2011). It can be found in Ireland, the Faroe Islands (Børgesen, 1903), Isle of Man, Britain (Hardy *et al*., 2003), the Netherlands (Stegenga *et al*., 1997), and Norway (Round, 1984). In North America, it has been found in Nova Scotia (Lüning, 1990), the Bay of Fundy, Newfoundland, Baffin Island, Labrador, and the Hudson Strait (Taylor & Rao, 1937). This makes it a feedstock that can be easily acquired, especially in the areas mentioned.

2.3.3 Uses of *Ascophyllum nodosum*

2.3.3.1 Animal Feed

A. nodosum is harvested for a plethora of uses. Near the coastal areas, where seaweed is easily available, there is a long history of people and farmers using seaweed as animal feed. Presently, this is even more common as there are now processing plants that can dry up and grind the seaweed (FAO). Therefore, this seaweed can be easily used as a feed additive (FAO). Another positive characteristic besides the abundance of seaweed in coastal areas, is that seaweeds are rich

in minerals, vitamins, and trace elements, making it a valuable feed additive (Colapietra & Alexander, 2005). The value of using seaweed meal as a feed supplement varies depending on the species that you are using and the animal in which the seaweed meal is used. “Incorporation of seaweed meal in the basic hay ration has been shown to improve the fertility of sheep but, in general, with less marked effect than with a supplement of herring meal” (FAO). One of the main problems of using seaweed as a sole feed source is indigestion or poor digestibility problems in the animals. However, studies have shown that seaweed meal could be used as an additive to the animal’s normal feed up to around 10% without encountering any problems, although with no clear advantages over alternative additives (FAO). Regardless, with the projected world food perspectives, the substitution of cereals and other food additives by seaweed meal as a feed additive may be needed in the future.

2.3.3.2 Fertilizer

Wherever seaweed has been available on the coastline, one of the most proven, widespread and ancient uses of it is as a direct fertilizer (FAO). *A. nodosum* is used as an organic fertilizer for a wide selection of crops mainly for its micronutrients (zinc, iron, copper, magnesium, calcium, manganese, sulfur, potassium, phosphorous, and nitrogen content) (Jayaraman *et al*., 2011; Norrie & Hiltz, 1999). Transport problems are the major problems when trying to use seaweed as a fertilizer, as the costs increase proportionally as you go further and further inland, away from the source of the raw material. Liquid seaweed products, extracts, and liquid seaweed-based fertilizers have tried to address this problem since their introduction in the 1950’s. This has made the use of seaweed fertilizers more widespread geographically (FAO).

2.3.3.3 Liquid Extract

Liquid extracts of *A. nodosum* have been used as a fertilizer to grow a wide variety of crops with good results. An *in vitro* study with spinach was performed, showing that the use of a commercial liquid extract from *A. nodosum* affected yield and nutritional value of the product in a positive way, increasing fresh weight by 1.6 times, dry matter content by 1.2 times, and total soluble protein content of the spinach plants by 1.5 times. In addition, the total chlorophyll was increased by 30% fresh weight when compared to the controls. The total antioxidant capacity, phenolics, and flavonoid content was increased too by at least 33%, fresh weight basis (Jayaraman *et al*., 2011). Again, in spinach, another study showed that soil irrigation with the extract enhanced post-harvest quality of the product (Fan *et al*., 2011). Another study done with lilies in pots demonstrated that foliar applications improved the bulb weight, the leaves were bigger and greener, and the stems were higher (De Lucia & Vecchietti, 2012). Liquid extracts have also led to enhanced germination leading to increased shoot growth and root length in potted marigolds (Russo *et al*., 1993), have increased fruit weight and maturity in kiwis (Chouliaras *et al*., 1995), and improved fruit yield on a field experiment in grapes (Colapietra & Alexander, 2005). In strawberries, *A. nodosum* extracts were able to mitigate increased soil salinity (Spinelli *et al*., 2010) and a tolerance in iron deficiency, with increased fruit yields (Spinelli *et al*., 2010). Lettuce plants fed with liquid extracts displayed improved salt stress (Neily *et al*.). In blueberries, there was an increase in berry size and yield (Loyola & Muñoz, 2008). In oranges, it increased bud sprouting, fruit yield, and gibberellin content (Fornes *et al*., 2002). In apples, liquid extracts increased chlorophyll levels, yield, fruit sugar content (Spinelli *et al*., 2009) and improved vegetative growth and flowering (Basak, 2008). Olives had an improved oil quality and increased yields when exposed before full bloom to the extract (Chouliaras *et al*., 2009). Oil quality considers the aroma,

color and taste and it is usually associated with the presence of volatile compounds (Kalua *et al*., 2007). In tomatoes, it improved chlorophyll content in potted plants (Blunden *et al*., 1996).

A. nodosum has been tested on *Arabidopsis thaliana*, where the extract enhanced tolerance against freezing (Rayirath *et al*., 2009). On turfgrass species, it has been used on creeping bentgrass, where it has enhanced drought tolerance, antioxidant activity, and promoted growth (Zhang & Schmidt, 2000). In another study also done using creeping bentgrass, heat stress tolerance was improved (Zhang *et al*., 2010a). Meanwhile, on forestry species, *A. nodosum* extracts have been tested on spruce and pine trees. For spruce trees, spring root development was enhanced (MacDonald *et al*., 2010). On pine trees, the liquid extract enhanced drought resistance and spring root growth (MacDonald *et al*., 2012). *A. nodosum* extracts can also be used on arable crops. For example, it can increase yield in several varieties of potatoes (Blunden & Wildgoose, 1977), and foliar sprays has been studied to control *Verticillium* wilt (Uppal *et al*., 2008). In wheat, barley, and maize, drenching the soil with the extract increased chlorophyll content (Blunden *et al*., 1996). Also, use of a diluted extract has increased the yield of spring barley grown hydroponically (Steveni *et al*., 1992).

The examples mentioned above show the multitude of crops for which *A. nodosum* liquid extract can be applied, with positive results varying from yield increase, abiotic stress tolerance (drought, high salt, heat tolerance), increased micronutrient content (vitamins and minerals act as antioxidants), and pest control.

2.3.4 Ecology of *Ascophyllum nodosum*

A. nodosum grows in the eulittoral zone (line from the low tide to the high tide) of the shore. On the eulittoral zone of a semi-exposed rocky shore, there are three major subzones, the upper zone, which is dominated by barnacles, the mid zone, which is dominated by brown algae

like *A. nodosum* and *Fucus* spp, and the lower zone, which is dominated by red algae (Knox, 2000). Distinct species may dominate a subzone because of the tide characteristics, their water needs, and their resistance to the wave action/force. Either *A. nodosum* or *Fucus* spp. can dominate the mid zone, depending on the amount and size of the waves, and other physical and biological factors. For example, *A. nodosum* thrives in more sheltered sites and shores. As the wave exposure rises, *A. nodosum* is phased out by *Fucus* spp., which is shorter and able to withstand waves.

2.4 Enzyme Hydrolysis

Enzymes are biological catalysts made up of proteins that produce a chemical change by catalysis, and are highly specific to a determined function or reaction (Wright, 1988). Hydrolysis is the breaking down of a compound due to a reaction with water. Enzyme hydrolysis is then a single or a multi-step reaction in which a substrate is broken down into smaller components by the catalytic action of one or more enzymes (Yang *et al*., 2011).

2.4.1 Cellulases

Cellulase is the general term that encompasses all enzymes that are needed to degrade cellulose into monosaccharides, mainly glucose. Cellulases degrade cellulose through hydrolysis. To convert cellulose into monosaccharides, the combined action of three different types of enzymes are needed. These enzymes are endoglucanases or endocellulases (also called endo-β-1, 4-glucanases), exoglucanases or exocellulases (also called exo-β-1, 4-cellobiohydrolases), and β-glucosidases (Bao *et al*., 2011; Liu & Kokare, 2017). Cellulases are commercially readily available and in nature are produced by an array organisms such as fungi and bacteria (Liu & Kokare, 2017). The genera of *Clostridium, Cellulomonas, Thermomonospora, Trichoderma*, and *Aspergillus* are the most extensively studied cellulase producers (Mojsov, 2016).

Cellulase enzymes are used in processes for different markets. They are used in the textile, food, detergent and paper industries (Meenu *et al*., 2014). With the increasing interest in the production of biofuels, cellulases have gained interest due to their ability to contribute to the hydrolysis of lignocellulosic materials (Meenu *et al*., 2014). Lignocellulose is a primary structural component of plant biomass and it is produced by the plant, supported through photosynthesis (Adav & Sze, 2014).

2.5 Saccharification of Cellulose

The saccharification of cellulose is shown in **Figure 2.2**. Endoglucanases break down cellulose chains within the chain, in no specific order (Mélanie *et al*., 2010). Exocellulases cleave off cellobiose from the ends of the cellulose chains (Mélanie *et al*., 2010). Exocellulases can break both amorphous and the crystalline regions of cellulose. This property makes exocellulases extremely important in the saccharification of cellulose as they are the only enzymes that can break down the highly ordered crystalline regions of the cellulose (Bao *et al*., 2011). Exocellulases can be further divided into two classes: the first class cleaves the cellulose from reducing ends, while the second class cleaves the cellulose from the nonreducing ends (Liu & Kokare, 2017; Wilson & Kostylev, 2012). After that, β-glucosidases are free to cleave the β-glucosidic linkages in cellobiose molecules to produce glucose monomers (Jeng *et al*., 2011; Liu & Kokare, 2017).

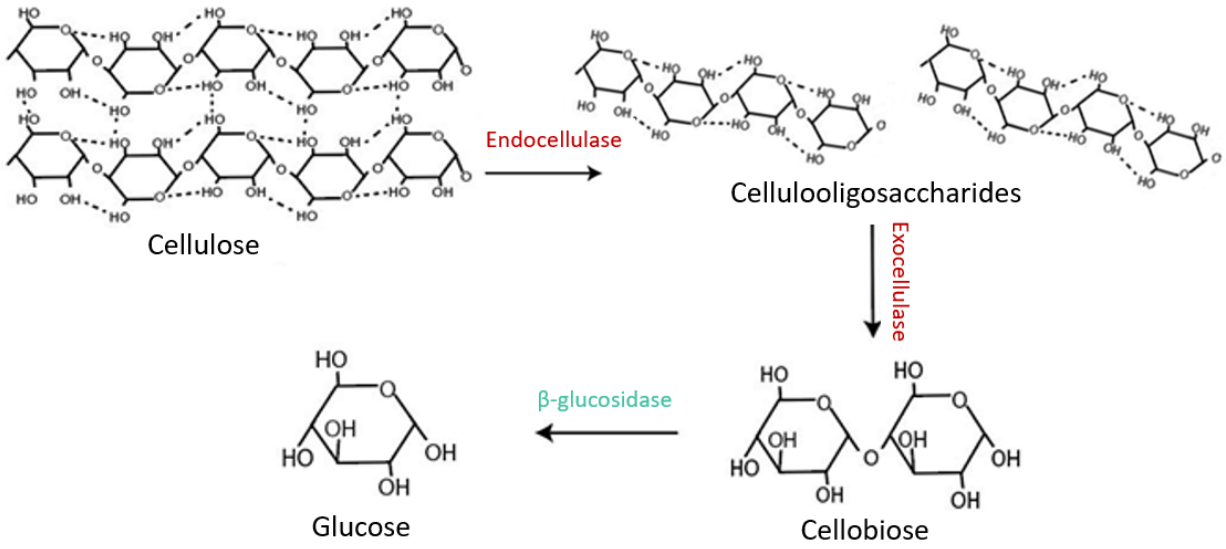


Figure 2.3 Cellulose Saccharification.

Three different enzymes are required to break down cellulose into glucose. Endocellulases break down cellulose chains into cellulooligosaccharides. Exocellulases cleave off cellobiose from the cellulooligosaccharide chains. Lastly, β-glucosidases break down cellobiose into glucose monomers.

The primary purpose of enzymatic hydrolysis of cellulose is to break down cellulose and other carbohydrate polymers into fermentable sugars like glucose and other oligomers that can be converted further into other valuable products through chemical and biological approaches (Yang *et al*., 2011). Fermentable sugars are being used by industry for various reasons, currently one of the main ones being biofuel production.

2.6 Alginate Lyase

Alginate lyases are enzymes that catalyze the breakdown of alginate and alginic acid. These enzymes can be classified as mannuronate lyases or guluronate lyases. Mannuronate lyases have a higher affinity to poly-mannuronate chains, while guluronate lyases have a higher affinity to

poly-gulutonate chains (Wong *et al*., 2000). Both alginate lyases have the same degradation action on alginate. Another important element is that both lyases have some activity in both G and M chains. The alginate lyases degrade alginate by β-elimination, which targets the glycosidic 1, 4 O-linkage between monomers.

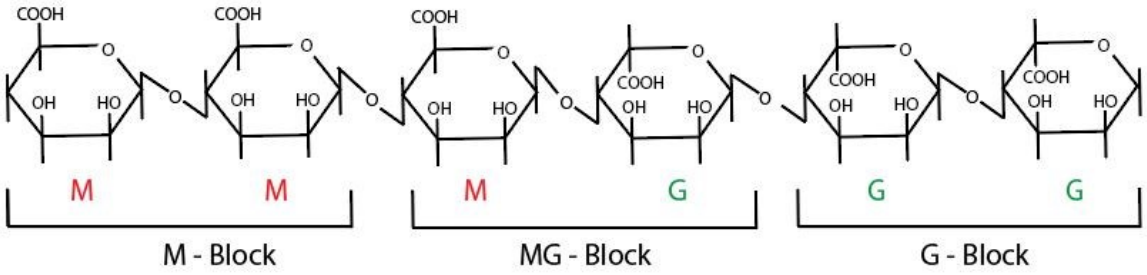


Figure 2.4 Alginate G and M chain structures.

Alginate can be found in 3 main chain structures: 1) M-Blocks, which are chains containing mannuronate 2) MG-Blocks, which are chains containing both mannuronate and guluronate 3) G-Blocks, which are chains containing guluronate. These chains are joined by a 1, 4 glucosidic bond.

2.7 Enzyme Saccharification of Brown Seaweed

Often, a pretreatment is performed on algae prior to enzymatic hydrolysis (Rastogi & Shrivastava, 2017). This pretreatment serves different goals, with the most relevant one being the liberation of polysaccharides (Ravanal *et al*., 2016). There are several common pretreatments used prior to the processing and saccharification of seaweed. These include size reduction pretreatment, chemical pretreatment and hydrothermal pretreatment (Sharma & Horn, 2016). These pretreatments will be discussed in more depth later in this Section.

Enzyme saccharification of brown algae has been carried out using enzymes such as commercial cellulase blends, laminarinases, and alginate lyases. (Sharma & Horn, 2016). In a

study, CellicCTec2 and an alginate lyase from *Flavobacterium multivorum* were used in *Saccharina latissima,* and had a maximum combined concentration of glucose and mannitol of 74 g/L at 25% solid loading. Another studied the use of laminarinases (endo-1,3(4)-β- glucanase) and varied pretreatments (acid pretreatment, temperature pretreatment, and no pretreatment) to study which pretreatment would work better in the algae *Saccharina latissima*, and found out that higher ethanol yields were seen in the non-pretreated samples, yielding 0.45% (v/v). This was almost opposite of what they expected, as heating has been shown help solubilize laminarin, which would in theory aid in hydrolysis, but the opposite happened. As for the acid pre-treatment, it was noted that the low fermentation rate was due to salt inhibition on the yeast (Adams *et al*., 2008). In another study, the authors used *Laminaria digitata* as feedstock for the fermentation of bioethanol and the extraction of proteins. As a pretreatment, only milling was used. For enzymes, they used the commercial enzymes, Celluclast® 1.5L (Novozymes) and alginate lyase (Sigma-Aldrich) to hydrolyze the brown seaweed. They had a maximum recovery of glucose at 84.1%, and managed to increase the protein content in the solids after hydrolysis 2.7 times. They also suggested that *Laminaria digitata* appeared to have some inherent enzymes for glucan hydrolysis. (Hou *et al*., 2015).

A study used the commercial enzymes Celluclast® 1.5L, Viscozyme® L, Novoprime® 959, Novoprime® 969, and AMG® 300L (Novozymes) on *Ulva lactuca*, *Gelidium amansii*, *Laminaria japonica*, and *Sargassum fulvellum*. The aim of the study was to see which algae species could yield more sugar content for later fermentation. The novelty was that the study used *Escherichia coli* K011 for the fermentation, due to the fact that this recombinant *E. coli* has the capacity not only to use glucose as a carbon source, but also mannitol. They got a maximum of 0.4g of ethanol per gram of carbohydrate using *Laminaria japonica.* (Kim *et al*., 2011). Cellic

Ctec2 (Novozymes) has been also tested on the brown seaweed *Laminaria digitata* for enzyme saccharification. In that study, they looked at different milling severities, to see the effect of the milled particle size on enzyme hydrolysis. Using an enzyme loading of 10% (v/w), they released all of the available glucose in 8 h. In this case, they found out that even in the unmilled seaweed, all available glucose was released, but this was probably due to the shape of the algae species, which are flat blades, and when milled, do not increase the surface area significantly. (Manns *et al*., 2016a). Another study used Celluclast® 1.5L (Sigma-Aldrich) and β-glucosidase (Sigma-Aldrich) for the enzymatic saccharification of *Macrocystis pyrifera* to produce sugars for biofuel applications, and used dilute sulfuric acid as a pretreatment. They were successful, as the maximum yield reached was 55.74 ± 0.05 mg of glucose/g algae (Ravanal *et al*., 2016). Another study looked at seaweed solid wastes of *Eucheuma cottonii* after the extraction of carrageenan as feedstock for the production of bioethanol. They used Novozyme 188 and Celluclast® 1.5L (Novozymes) to hydrolyze and further ferment the sugars, and using simultaneous saccharification and fermentation, they got a yield of 90.9% ethanol, compared to 55.9% when using a separated hydrolysis and fermentation approach (Tan & Lee, 2014). For *Saccharina latissima*, another study also used Cellic CTec2 (Novozymes), Cellic HTec2 (Novozymes), and laminarinase (Sigma-Aldrich) in different combinations for its saccharification, the aim being to determine what combination of enzymes worked the best. They found out that laminarinases and Cellic HTec2 yielded the lowest quantities, while Cellic CTec2 and Cellic CTec2 and laminarinases yielded the most amount of sugars (40% and 52% more than the laminarinases alone, respectively) (Scullin *et al*., 2015).

Since brown seaweed have important amounts of alginate, alginate lyase is an enzyme that was also of interest. One study tried to see the effect of the addition of alginate lyase during the

saccharification of *Macrocystis pyrifera* and *Saccharina latissima*. They used both acid pretreated samples, as well as samples with no pretreatment, and used alginate and oligoalginate lyases and several commercial cellulase enzyme blends. The results were that on the sample with no pretreatment, the addition of alginate lyase to the cellulases did yield a higher amount of glucose than using the cellulases alone. On the contrary, for the acid pretreated samples, cellulases alone were needed. (Ravanal *et al*., 2017). Another study aimed to determine if the addition of alginate lyase increased sugar yields in enzyme hydrolysis, and to find the ratio of cellulase: alginate lyase that should be added for maximum release of sugars. It was determined that cellulases alone were needed to release sugars, but also found that a ratio of 9:1 cellulases to alginate lyase increased the yield of sugars. (Sharma & Horn, 2016).

2.7.1 Seaweed Pretreatments

2.7.1.1 Size Reduction Pretreatment

Size reduction pretreatment, or mechanical pretreatment, involves the use of mechanical force to degrade the cell wall (Razif *et al*., 2014). This pretreatment can include cutting the sample into smaller pieces, milling the sample into fine bits, ultrasonication, or crushing the sample in a mortar and pestle or a press (Rastogi & Shrivastava, 2017; Razif *et al*., 2014). Ultrasonication is the use of ultrasonic waves in order to disrupt the integrity of the cell walls. This pretreatment is especially important for macroalgae, as it increases the surface area for future enzyme hydrolysis (Daroch *et al*., 2013). This type of treatment can be less expensive compared to others that require specialized equipment and materials, especially when using methods like mortar and pestle. It is expected for milling to be a key pretreatment in the goal to extract value added components, as it increases the surface area that the sample is in contact with the treatments.

2.7.1.2 Chemical Pretreatment

Chemical pretreatment can include alkaline or acid pretreatment. This type of pretreatment is highly dependent on operating conditions, including temperature, concentration of solution, and time. Alkaline pretreatment consists of the solubilization of hemicelluloses through the destruction of ester links (Ling *et al*., 2017). Alkaline pretreatment disrupts the crystalline structure of cellulose, making it more digestible by enzymes. Alkaline pretreatment is done using NaOH solutions with varying concentrations (Lee *et al*., 2018). Harun *et al.* found release of fermentable sugars when using 0.75% (w/v) of NaOH at 120 °C for 30 min (Harun *et al*., 2011).

Acid pretreatment is another method used with algae. One of the most common is extremely low acid pretreatment (Razif *et al*., 2014). During this process the cell wall is destroyed due to the breaking of inter and intra molecular hydrogen bonds (Razif *et al*., 2014). Nguyen *et al.* found a 58% (w/v) release of glucose by using 3% sulfuric acid at 110 °C for 30 min (Nguyen *et al*., 2009). Acid pretreatments are used widely on algae, and it could possibly be a good pretreatment for use in *Ascophyllum nodosum*.

2.7.1.3 Hydrothermal Pretreatment

Hydrothermal pretreatment consists of heating the sample in water to open up the substrate. Some of the techniques used are hydrothermal treatment and steam explosion. Hydrothermal pretreatment fractionates the algal biomass into lipid and sugar phases (Razif *et al*., 2014). The thermal energy and pressure used in steam explosion results in the degradation of cell walls. Choi *et al.* found that optimal pretreatment conditions in order to maximize glucose conversion yields when using steam explosion were 180 °C (temperature) and 10 bar for 8 min (Choi *et al*., 2013). Hydrothermal treatments might not be ideal to use in algae, as they are adapted to live in water, but steam explosion, while needing special equipment, has been shown to enhance the enzymatic

hydrolysis of lignocellulosic material (Pielhop *et al*., 2016), and has also been used on algae, leading to believe it could be a good pretreatment on *Ascophyllum nodosum.*

2.8 Algae to Biofuels

Algae is a promising solution as a feedstock for biofuel production. It has several advantages against other first and second generation of biomass from food crop and lignocellulosic sources. It can be grown under water and nutrient conditions that are typically considered to be suboptimal (Aitken *et al*., 2014). Algae contains a high amount of carbohydrates, making it suitable for bioethanol production (John *et al*., 2011). It has virtually no lignin or hemicellulose, so it presents less resistance to conversion into monosaccharides in comparison to biomass used for second generation biofuels (Daroch *et al*., 2013; Wei *et al*., 2013). Though research is being done, the technology is still in its early stages, and a lot still needs to be done (Tan & Lee, 2015). Potential obstacles exist, as the complexity of the arrangement of the algae cell walls is still do not fully understood. A life cycle assessment (LCA) on algae grown in an open pond pitched some doubts on the viability of using macroalgae to produce biofuels, ranking it lower than other sources in net energy consumption (Clarens *et al*., 2010), but other LCAs that were based on marine environments have cast a more positive light on macroalgae (Aitken *et al*., 2014; Fernand *et al*., 2017). One LCA, for example, considered the energy return and the impacts on the environment of the cultivation and processing of macroalgae to biogas and bioethanol, focusing specifically on the species *Gracilaria chilensis* and *Macrocystis pyrifera*, and on two different cultivation methods: bottom planting and long-line cultivation. They found that the cultivation of *Gracilaria chilensis* using bottom planting and the further processing of the algae for the extraction for bioethanol and biogas was the one with the most sustainability, with an Energy Return On Investment (EROI) of 2.95, meaning that they were able to get out 2.95 MJ for every 1 MJ used

for the process (Aitken *et al*., 2014). Some of the doubts mentioned by the hesitant LCA study included the need for CO_2 and fertilizer, which increases the environmental footprint (Clarens *et al*., 2010). It is mentioned that waste water could be utilized, but its acquisition and relocation to manufacturing locations might be difficult. Ethanol yields from macroalgae have already been reported in many species and with the fermentation of several microorganisms. In *Laminaria hyperborea*¸ a yield of 0.43g/g of substrate has been reported (Horn *et al*., 2000). In *Gracilaria salicornia*, a bioethanol yield of 79.1g/kg of dry biomass was reported (Wang *et al*., 2011). In *Laminaria digitata*, a 167 mL yield per kg of biomass was reported (Adams *et al*., 2011), just to name a few successful cases.

Coupled with fermentable sugars for biofuel production, algal processing platforms can produce other byproduct streams that either have value and/or could be readily converted to value-added chemicals and materials. In the case of algae, one intriguing possibility is the co-production of sugars and biostimulants from algal biomass. This co-production strategy could lead to significant improvements to process economics.

2.9 Lignocellulosic biomass vs algae biomass

One of the main differences in the saccharification of lignocellulosic material and algae is the difficulty to break down cellulose into glucose monomers by enzymes due to the cell wall's components. In the case of lignocellulosic materials, the cell wall contains, besides cellulose, lignin chains which act as a barrier that prevents enzymes from easily accessing the cellulose (Sartori *et al*., 2015; Welker *et al*., 2015). The presence of lignin makes the use of pre-treatments necessary in order to remove the lignin, which adds steps and cost to the process (Robak & Balcerek, 2018; Sartori *et al*., 2015). Saccharification of algae is easier than that of lignocellulosic material because of the lack of lignin in the cell wall (Welker *et al*., 2015). Sometimes pre-treatments might be

needed in the saccharification of algae, but this is mainly used to disrupt the cell wall and give enzymes a more accessible surface area to act on (Martin *et al*., 2016). The use of algae for the production of biofuels, especially macroalgae, though promising, is still a developing field. As such, it is still not clear which specific pre-treatments and enzymatic cocktails are the most efficient in order to address the structural differences of algae and lignocellulosic materials, which is a potential downside to third generation biofuels.

3. Materials and Methods

3.1 Seaweed Characterization

Ascophyllum nodosum (brown algae) was acquired from collaborators at Acadian Seaplants Ltd. in Nova Scotia. Prior to any experimentation, the acquired seaweed was subjected to characterization experiments, which included proximate analysis of the sample (moisture analysis, ash content, crude fat, and crude protein), elemental analysis, and a two-step acid hydrolysis for analysis of monosaccharide content. This was done in order to further understand the seaweed sample received, and try to build a mass balance.

3.1.1 Moisture and Ash Content

Moisture and ash content of the algae samples were assessed using a modified method from the National Renewable Energy Laboratory (NREL) (Van Wychen & Laurens, 2013). First, moisture analysis was done to provide the water content of the samples, which needs to be accounted for in downstream compositional analyses. It should be noted that the seaweed samples were already pre-dried and thus the values obtained in these experiments do not equate to water content in the native seaweed. Porcelain crucibles were preconditioned at 550 °C in a muffle furnace overnight to remove any contaminants and were then stored in a desiccator to prevent any water absorption. 100 mg of biomass was then weighted into the pre-weighted and preconditioned crucibles and placed in a convection oven at 60 °C for 18 h. After that, the crucibles were weighted again. The difference between the weight of the crucible before and after heating was used to determine the moisture content.

Ash analysis is a simple procedure that gives an idea of the overall elemental content of the sample, which was defined further through subsequent elemental analysis (Section 3.1.2). After weighing for moisture analysis, the crucibles were placed in a muffle furnace at 550 °C for 8 h.

The weight difference of the crucible before and after treatment in the muffle furnace was determined to be the ash content. All analyses were performed in triplicate.

3.1.2 Elemental Analysis

Elemental analysis was done to determine the specific levels of various elements in the seaweed sample, and also help identify early possible future inhibition of the enzymes due to high salt concentrations. This analysis was done in the Natural Resources Analytical Laboratory (NRAL) in the Department of Renewable Resources, at the University of Alberta. The samples were first dried overnight at 60 °C in a convection oven. The next day, the samples were packaged in individual glass vials and sent to NRAL for analysis. There, a microwave-assisted nitric acid digestion was performed on the dry biomass using 5 mL of trace-metal grade HNO_3. After this sample preparation, the Thermo iCAP6300 Duo inductively coupled plasma optical emission spectrometer (ICP-OES; Thermo Fisher Corp, Cambridge, United Kingdom) was used to quantify the following metals: calcium, iron, magnesium, manganese, sodium, potassium, copper, zinc, and nickel. Elemental analysis was performed in triplicate.

3.1.3 Crude Fat Analysis

Crude fat analysis is a proximate analysis, as it does not divide or identify the specific fats. This procedure was done using petroleum ether (certified ACS; Fisher Scientific, Fairlawn, New Jersey, United States) as a solvent and in accordance to the AOAC method 945.16 (AOAC, 1990), using a Goldfisch Fat Extraction Apparatus (Laboratory Construction Company, Model 35001; Kansas City, Missouri, United States). Biomass (2 g) was weighed into Whatman cellulose extraction thimbles (Cat No: 2810228; Sigma-Aldrich, St. Louis, Missouri, United States). After weighing, the thimbles were covered using a small amount of glass wool. This was done to minimize any loss of sample due to transportation of the thimbles and during the actual extraction

process. Afterwards, the weight of the extraction beakers was recorded; this weight was compared to the weight of the extraction beakers after the extraction in order to get the crude fat weight. The cellulose thimbles were then fastened to the Goldfisch Extraction Apparatus using the sample holders, and 40 mL of petroleum ether were added to each extraction beaker inside a fume hood. The beakers were then attached to the Goldfisch apparatus and the heaters were turned on, which facilitated evaporation of the petroleum ether. During the course of the experiment, the gaseous solvent was condensed to prevent changes in sample volume due to solvent evaporation and loss. The extraction was allowed to proceed for 6 h. Afterwards, the heaters were shut down and the beakers were allowed to cool down. The cellulose thimbles were then replaced with glass collector tubes, in order to recover the petroleum ether solvent from the extraction beakers. The heat was turned on again, but this time, the petroleum ether was recovered in the glass collector tubes. When all petroleum ether was recovered in the collector tubes (none of it remained in the extraction beakers), the heaters were turned off and the extraction beakers were allowed to cool. Then, they were transferred to a desiccator. Lipid content was determined gravimetrically by weighting the extraction beakers after the experiment and subtracting the weight of the extraction beakers before the extraction. This analysis was performed in triplicate.

3.1.4 Crude Protein Analysis

Crude protein analysis was done by measuring the amount of nitrogen in the sample, and then calculating the amount of protein using a conversion factor. Similar to the crude fat analysis, this method does not separate or identify specific amino acid amounts or specific protein amounts. A sample of seaweed (~0.5 g) was dried in a convection oven at 60 °C overnight. Afterwards, 0.1 g of the dried seaweed was then weighed in a tin foil cup. The cups were sealed and then pressed using a pellet press (Parr Instrument Company; Moline, Illinois, United States). The pelleted tin

cups containing the seaweed sample were then loaded into the LECO automatic sampler (Humphrey; Kalamazoo, Michigan, United States). The nitrogen content was then determined using a LECO TruSpec CN Carbon/Nitrogen Determinator (LECO; St. Joseph, Michigan, United States). Analyses were performed in triplicate. The crude protein content was then calculated using a conversion factor of 4.92, which is the accepted average conversion factor for seaweed (Maehre *et al*., 2014).

3.1.5 Monosaccharide Analysis

Part of the characterization steps to build a mas balance, a two-step acid hydrolysis was used to release monosaccharides from the seaweed, after which High Performance Liquid Chromatography (HPLC) using a refractive index detector was used to quantify glucose, xylose, fucose, and mannitol in the seaweed samples. This was done following a procedure from the National Renewable Energy Laboratory (NREL) (Sluiter *et al*., 2008).

Three hundred mg of the algae sample was weighted into ACE pressure tubes (120 mL capacity, Sigma-Aldrich; St. Louis, Missouri, United States), and 3 mL of 72% sulfuric acid (Sigma-Aldrich; St. Louis, Missouri, United States) was added. The tubes were then placed in a water bath at 30 °C for 1 h to facilitate the first hydrolysis step. The sulfuric acid was then diluted to 4% (w/w) by adding 84 mL of water.

A set of Sugar Recovery Standards (SRS) were also prepared in pressure tubes by adding 10 mL of water, 348 µL of 72% sulfuric acid, and the monosaccharides to be quantified (all from Sigma-Aldrich; St. Louis, Missouri, United States). In this case, D-(+)-glucose (≥99.5%), D-(+)-xylose (≥99.0%), L-(-)-fucose (≥99%), and D-mannitol (≥98%) were used as standards, and were added to the SRS at a final concentration of 1 mg/mL. The SRS were prepared in order to correct

for losses due to the destruction of monosaccharides during the dilute acid hydrolysis (the second hydrolysis step).

Pressure tubes containing the SRS or the various samples were then securely closed and placed in an autoclave at 121 °C for 40 min. After autoclaving, the samples were left to cool down at room temperature. The solids were then filtered using filtering crucibles (Pyrex 50 mL ASTM 40-60; Sigma-Aldrich, St. Louis, Missouri, United States) and a vacuum line. From the liquid component, a 5 mL aliquot was taken and then neutralized using calcium carbonate (≥99%, Sigma-Aldrich, St. Louis, Missouri, United States) to a pH between 5 and 6. To check the pH of the aliquot, pH paper was used (colorpHast range 0-6, EMD Chemicals Inc.; Gibbstown, New Jersey, United States). The tubes were centrifuged for 5 min at 2900 x *g* (accuSpin 400; Fisher Scientific, Osterode, Germany). The liquid fraction was transferred to another 50 mL conical tube. Three mL of water was then added to the original tube (the tube containing the pellet) to facilitate washing of the pellet and collection of all monosaccharides. The pellet was resuspended in the added water, and then the mixture was centrifuged; the liquid supernatant was pooled with the first liquid supernatant. This wash was repeated two more times.

The monosaccharides in the pooled liquid fractions were then analyzed using HPLC equipped with a Biorad Aminex HPX-87P column (Biorad; Hercules, California, United States) and a refractive index detector (Agilent Technologies 1200 Series; Waldbronn, Germany). The samples were passed through a 0.22 µm filter (Nylon Syringe Filter; Mandel Scientific, Guelph, Ontario, Canada) into an HPLC vial. D-(+)-glucose, D-(+)-xylose, L-(-)-fucose, and D-mannitol were used as monosaccharide standards for the HPLC. An injection volume of 20 µL was used. The mobile phase used was HPLC grade water that had been filtered (0.2 µm) and degassed. The

flow rate of the mobile phase was 0.6 mL/min. The column temperature used was 80 °C, while the detector temperature was 35 °C, and the run time for each sample was 35 min.

3.2 Enzyme Activity Assays

Enzyme activity assays help as evidence that the enzymes that will be used for hydrolysis are in fact working as advertised by the companies, and also help determine our enzyme loadings so we add enzyme depending on its activity, so a more fair comparison can be made when comparing several different enzymes.

3.2.1. Sodium Citrate Buffer Preparation

To facilitate buffer preparation, 100 mL stock solutions of 0.1 M citric acid (ACS reagent ≥99.5%; Sigma-Aldrich, St. Louis, Missouri, United States) and 0.1 M sodium citrate dihydrate (≥99%; Sigma-Aldrich, St. Louis, Missouri, United States) were prepared. Then, depending on the final pH desired, 0.05 M sodium citrate buffers were prepared by adding and mixing the two stock solutions in the proportions indicated below, and adjusting the final volume to 100 mL with deionized water in a volumetric flask.

For pH 4.8: 20 mL 0.1 M citric acid + 30 mL 0.1M sodium citrate dihydrate

For pH 5.8: 7 mL 0.1 M citric acid + 43 mL 0.1 M sodium citrate dihydrate

3.2.2. Dinitrosalicylic Acid (DNS) Reagent Preparation

Phenol (≥99%, Sigma-Aldrich; St. Louis, Missouri, United States) was first melted in a 50 °C water bath. 708 mL of deionized water, 5.3 g of 3,5-dinitrosalicylic acid (98%; Acros Organics, New Jersey, United States), and 9.9 g of sodium hydroxide (Certified ACS; Fisher Scientific, Fair Lawn, New Jersey, United States) were then added. After the contents were dissolved, 153 g of sodium potassium tartarate (Certified ACS; Fisher Scientific, Fair Lawn, New Jersey, United

States) were added to 3.8 g of the previously melted phenol and 4.15 g of sodium metasulfite (Certified ACS; Fisher Scientific, Fair Lawn, New Jersey, United States), and all components were dissolved.

3.2.3 Filter Paper Units Assay for Celluclast® 1.5L and Cellic CTec 2

The filter paper assay employed was modified from a protocol from the International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987), which uses Whatman No. 1 filter paper and dinitrosalicylic acid (DNS) reagent to measure the amount of reducing monosaccharide yield, allowing for calculation of activity in terms of filter paper units per milliliter of undiluted enzyme.

First, Whatman No. 1 filter paper (Fisher Scientific; Ontario, Canada) was cut into 1 x 6 cm strips. One mL of previously prepared 0.05 M sodium citrate buffer at pH 4.8 was added to a test tube, along with 0.5 mL of the enzyme to be tested. Celluclast® 1.5L and Cellic CTec2 were tested separately (both from Sigma-Aldrich; St. Louis, Missouri, United States). For the purposes of the experiment, at least two dilutions of the enzyme must be used; one must release less than 2 mg of glucose, and the other must release more than 2 mg of glucose. For Celluclast® 1.5L, dilutions ranging from 1/100 to 1/450 were used. For Cellic CTec2, dilutions ranging from 1/400 to 1/850 were used.

The test tubes containing buffer and enzyme were then preconditioned at 50 °C for 10 min in a water bath. One of the previously cut filter paper strips was then added to each test tube and incubated at 50 °C for 1 h in a water bath. After the hour, the tubes were taken out of the water bath, and 3 mL of previously prepared DNS reagent was added to the mix. The tubes were placed in boiling water for 5 min, then transferred to a cool water bath. When samples reached room temperature, 20 mL of water was added to each tube, and the pulp in the tubes was then left to settle for at least 20 min. The color formed was measured using a spectrophotometer (Biochrom

Ltd, Ultrospec4300 pro UV/Vis spectrometer; Cambridge, England) at 540 nm. Glucose standards (glucose and buffer, no filter paper or enzymes) and enzyme blanks (enzyme and buffer only, no filter paper) were also prepared and treated in the same way and at the same time as the filter paper samples. All analyses were done in triplicate.

A glucose standard curve was generated using the glucose standards, which was then used to convert the absorbance values from the sample tubes into the glucose amount produced during the enzyme reaction. The activity in Filter Paper Units (FPU) was then calculated using the following equation:

$$FPU = \frac{0.37}{enzyme\ concentration\ to\ release\ 2.0\ mg\ glucose} units\ mL^{-1}$$

3.2.4 Alginate Lyase Activity Assay

Alginate lyase activity can be measured by direct spectrophotometric measurement of uronic acid products that are generated by the enzyme-mediated depolymerization of alginate. The activity of alginate lyase was measured by monitoring the increase in absorbance using a spectrophotometer (Biochrom Ltd, Ultrospec4300 pro UV/Vis spectrometer; Cambridge, England) set at a wavelength of 230 nm. The activity was measured at room temperature and in 1 mL quartz spectrophotometer cuvettes. 700 µL of 0.05 M sodium citrate buffer at pH 5.8, 180 µL of an alginic acid (≤100%, Sigma-Aldrich; St. Louis, Missouri, United States) solution dissolved in water at a concentration of 4 mg/mL, and 10 µL of alginate lyase (*Sphingomonas sp*., from Megazymes; Chicago, Illinois, United States) were added to each cuvette. Absorbance was then measured every minute for the first 15 min, and then every 5 min for another 25 min, for 40 min in total. All analyses were performed at least in triplicate. A curve of time and absorbance was

then plotted. When there were no further increases in absorbance observed, the mg of alginic acid broken down per min per μL of alginate lyase was calculated.

3.3 Enzyme Hydrolysis

The method proposed as an alternative to alkaline extraction is enzyme hydrolysis. Several enzymes were used throughout this project. They are identified in **Table 3.1** below:

Table 3.1 Enzymes used in this study for the hydrolysis of algal biomass

Enzyme	**Supplier**
Celluclast® 1.5L	Sigma-Aldrich (St. Louis, Missouri, United States)
Alginate lyase	Megazymes (Chicago, Illinois, United States)
Viscozyme® L	Sigma-Aldrich (St. Louis, Missouri, United States)
Cellic CTec2	Sigma-Aldrich (St. Louis, Missouri, United States)

3.3.1 Celluclast® 1.5L and Alginate Lyase

The first approach at hydrolyzing *A. nodosum* algae involved the use of the commercially proven cellulase enzyme blend, Celluclast® 1.5L, and a commercially available alginate lyase in order to break down and extract monosaccharides from the biomass. As stated previously, alginate is a major component in the algae cell wall, so alginate lyase was implemented in order to break down and facilitate the monosaccharide extraction process. Since alginate lyase is an enzyme that works at lower temperatures than Celluclast® 1.5L, hydrolysis was first attempted at 37 °C, after which the temperature was increased to 50 °C, allowing both enzyme systems to work at their optimal temperatures. Both enzymes were added at the beginning of the incubation. Initially, a solid loading of 10% was used for enzyme hydrolysis. This proved to be a problem as the resulting liquid hydrolysate immediately clogged the 0.2 μm filters, even after centrifuging and diluting. Thus, a 1% solid loading (0.1 g seaweed in 10 mL buffer) was employed for the following experiments.

The method for enzymatic hydrolysis was modified from an NREL method (Resch *et al*., 2015). Of the sample, 0.1 g was placed in a 50 mL glass test tube. Moisture content determination was done on the same day to correct for % solids. Substrate blanks (substrate and buffer, no enzyme) and enzyme blanks (enzyme and buffer, no substrate) were also prepared. 10 mL of 0.05 M sodium citrate buffer (pH 4.8) were added to each test tube, which were then preconditioned in a water bath set at 37 ± 2 °C for 20 min so that the tubes reach the desired temperature. Afterwards, the test tubes were removed from the water bath, and without letting them cool, added the necessary enzymes to each tube. For these experiments, 43 FPU/g of sample was used for Celluclast® 1.5L, and 0.82 units/g of sample for alginate lyase. For samples where both enzymes were used, a ratio of 4:1 (v/v; Celluclast® 1.5L:alginate lyase) was used (Sharma & Horn, 2016). The reason for using different amounts when both enzymes were used was that if there was a synergistic effect, there would be an increase in the amounts of monosaccharides released, even if the overall enzyme loading for each enzyme was lower. The tubes were placed in the water bath for incubation at 37 °C. After 3 h, the temperature of the water bath was raised to 50 °C ± 2 °C. Then, after another 20 h, The tubes were removed from the water bath and the reaction terminated by submerging the test tubes in boiling water for 15 min. The test tubes were then left to cool to room temperature.

Afterwards, the contents were transferred to 50 mL conical plastic tubes. The glass test tubes were washed with another 2 mL of water to ensure complete transfer of the sample. The tubes were then centrifuged for 7 min at 2900 x *g* (accuSpin 400, Fisher Scientific; Osterode, Germany) to create a pellet. The liquid fraction was transferred to a new 50 mL conical tube. The pellet was resuspended in 2 mL water using a vortex (Fisher Scientific; Fair Lawn, New Jersey, United States) and then subjected to centrifugation. This wash process was repeated a total of 3

times, with all liquid fractions being pooled with the liquid fraction isolated from the initial centrifugation of the enzyme hydrolysate. After washing, the sample was prepared for HPLC analysis by filtering 1 mL of the pooled liquid fraction using disposable syringes and syringe filters (1 mL, fitted with 0.2 μm syringe filters) and placing samples into an autosampler vial. HPLC analysis was then performed as described in *Section 3.1.5*.

3.3.2 Freeze/Thaw Pretreatment

As substantial amounts of monosaccharides were not extracted by the enzymes alone, the next step was to assess whether a simple freeze/thaw treatment would increase the amount of monosaccharides released from the seaweed samples. As a sample is frozen, ice crystals are formed and rupture the cells (Smichi *et al*., 2016). 0.1 g of seaweed was processed as described in *Section 3.3.1*. After the addition of 0.05 M sodium citrate, half of the samples were then stored at 4 °C, while the other half was subjected to three consecutive freeze/thaw cycles. For this, freezing was performed by placing samples in a -20 °C freezer and then allowing them to thaw at room temperature, and repeating this process for a total of three times. Afterwards, tubes from both treatment conditions were then preconditioned at 37 °C for 30 min, after which the samples were subjected to enzyme treatment and downstream processing as described in *Section 3.3.1*.

3.3.3 Addition of Viscozyme® L

Viscozyme® L is an enzyme complex which contains carbohydrases including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase, and also has activity against pectin-like materials from plant cell walls. Knowing this, the next step was to assess if this enzyme cocktail could enhance hydrolysis of the seaweed, as it is an enzyme complex used in the breaking down of plant cell walls for the extraction of components, especially in the alcohol, brewing, and starch industries (Novozymes, 2002). For this approach, the methodology described in *Section 3.3.1* was

again used. The only difference was the addition of 50 μL of Viscozyme® L to half of the samples, while the other half did not have Viscozyme® L added. For Viscozyme® L, 60 Fungal Beta-Glucanase Units per gram of sample used (activity taken from the manufacturer) was used. For clarity, it should be noted that all samples in these studies were simultaneously treated with both Celluclast® 1.5L and alginate lyase.

3.3.4 Alginate Lyase and Cellic CTec 2

Cellic Ctec2 is another enzyme blend consisting of cellulases and is known to be much more aggressive than the Celluclast® 1.5L enzyme blend. To assess activity of Cellic Ctec2 on algal biomass, the exact same experiments described in *Section 3.3.1* were performed, but substituted Cellic Ctec2 (47 FPU/g of sample) for Celluclast® 1.5L.

3.3.5 Addition of carboxymethyl cellulose (CMC) to the enzyme systems

To explore the possible inhibition of Celluclast® 1.5L and Cellic Ctec2 due to compounds formed and/or released during the breakdown of seaweed samples, the samples were spiked with carboxymethyl cellulose (sodium salt, DS=1.2, 100% purity, Acros Organics; New Jersey, United States). Carboxymethyl cellulose (CMC) is cellulose in which carboxymethyl groups are attached to the hydroxyl groups of the glucose monomers. The methodology described in *Section 3.3.1* was used, but for half of the samples, 0.1 g of CMC was added to the 50 mL glass test tube.

3.3.6 Celluclast® 1.5L, Alginate Lyase, and Cellic Ctec 2, pH 5.8

In an attempt to improve breakdown of seaweed, enzymatic hydrolysis was performed using three different enzyme cocktails: Celluclast® 1.5L, alginate lyase, and Cellic Ctec 2. These experiments (enzyme hydrolysis and monosaccharide analysis) were performed as described in *Section 3.3.1*, with slight modifications. All of the volumes and the amount of sample used was doubled. This was done in order to ensure that samples of the hydrolysate could be used in mung

bean assays at Acadian Seaplants Ltd., as well as for Scanning Electron Microscopy (SEM), which are described in *Sections 3.4 and 3.5*, respectively. Furthermore, in these experiments, 0.1 g of carboxymethyl cellulose (sodium salt, 100% purity, Acros Organics; New Jersey, United States), and 0.1 g alginic acid (≤100%, Sigma-Aldrich; St. Louis, Missouri, United States) were added to half of the reactions as model substrates to monitor hydrolysis rates. Enzyme activities were standardized for the cellulase enzymes (both enzymes at 15 FPU/g sample), and the enzymes were added at different times during the reaction: alginate lyase (0.13 units) was added first at 37 °C, then when the temperature was ramped up to 50 °C, Celluclast® 1.5L and Cellic Ctec2 (15 FPU/g of seaweed for both) were added. Prior to hydrolysis, the seaweed was suspended in 20 mL of 0.05 M sodium citrate buffer (pH 5.89). The change in pH was due to the fact that alginate lyase works at a higher pH than Celluclast® 1.5L and Cellic Ctec2. Also, following centrifugation, pellets were washed with 4 mL of water rather than 2 mL.

For analysis of alginate, HPLC was performed using a Biorad Aminex HPX-87H column (Biorad; Hercules, California, United States) equipped with a refractive index detector. The injection volume was still 20 µL, but the mobile phase was 5 mM sulfuric acid and the flow rate of the mobile phase was 0.5 mL/min. The column temperature used was 60 °C, while the detector temperature was 35 °C. The run time for each sample was 25 min.

3.4. Scanning Electron Microscopy

No substantial amounts of monosaccharides were being released from our treatments. To determine whether enzyme-induced physical damage to the algal biomass could be observed, scanning electron microscopy (SEM) was performed. This was done at the Scanning Electron Microscope Lab, in the Department of Earth & Atmospheric Sciences, at the University of Alberta using a Zeiss Sigma 300 VP-FESEM (Zeiss; Oberkochen, Germany). The sample preparation and

the selection of the images was done by myself, but the actual operation of the SEM equipment was done with the support of the SEM lab technician. The solid component recovered after hydrolysis of *A. nodosum* (*Section 3.3.6*; solid pellet after centrifugation) was dried at 40 °C overnight in a convection oven and mounted in SEM stubs. The technician from the Scanning Electron Microscope Lab carbon coated the samples using a Leica EM SCD005 evaporative carbon coater (Leica; Concord, Ontario, Canada). After coating of the samples, SEM was performed. Pictures were taken of various samples at various magnifications. Seven treatments from the preparations made in *Section 3.3.6* were pictured using SEM: 1) Celluclast® 1.5L; 2) Cellic CTec2; 3) Alginate Lyase; 4) Celluclast® 1.5L and Alginate Lyase; 5) Cellic CTec2 and Alginate Lyase; 6) Seaweed only (mock treatment, no enzymes); and 7) Native seaweed (the untreated, raw milled seaweed from Acadian Seaplants Ltd.). The other samples were not processed due to the fact that they were enzyme blanks, and thus contained no seaweed, or were the carboxymethyl cellulose controls.

3.5. Mung Bean Rooting Assay

To test the second hypothesis of this research project, that enzyme hydrolysis will rupture the cells enough to release plant biostimulants into the hydrolysate, a rooting assay was performed. Liquid fractions collected from hydrolyzed samples in *Section 3.3.6* were shipped to collaborators at Acadian Seaplants Ltd. in Nova Scotia who performed the mung bean rooting assay at their facilities. This assay was done in order to assess plant biostimulant activity in the enzyme hydrolysates. In this case, scientists at Acadian Seaplants Ltd. measured rooting activity in mung beans.

The first step of the assay was the seedling initiation. Five liters of Pro-Mix BX potting soil (Pro-Mix Gardening; Mississauga, Ontario, Canada) was mixed with 5 L of medium grade

vermiculite and 1.6 L of distilled water. Two 1020 standard seed trays then were filled with the potting mix. The mung bean seeds were then planted across each tray. The seeds were then covered with 1.5 L of the Pro-Mix/vermiculite/water mix, and each tray was then watered with 600 mL of distilled water. The trays were covered with clear plastic covers and moved to a controlled environment room set at 27 °C and with a 24 h photoperiod. The plastic covers were then taken off after two days. The trays were watered with 600-800 mL of distilled water after the removal of the plastic covers; this was repeated every other day, until the seedling transfer.

The second step was the seedling transfer. After six days of the seedling initiation, the plants were cut at soil level, and the cotyledons were removed by twisting off with fingers. Using a ruler, the hypocotyls were cut 4 cm below the cotyledons using a scalpel. The epicotyls should be between 2.5 and 3.5 cm. Each cut seedling was placed in a 20 mL glass scintillation vial containing 12 mL of each treatment solution. Acadian Seaplants Ltd. also added a deionized water treatment, a nutrient treatment (K, N, and P), and a biostimulant (SSEP) treatment as controls. Ten vials were prepared for each treatment. 24 hours later, each bottle was topped up with 6 mL of distilled water, a process that was repeated every 2 days for 7 days. The vials were placed in the controlled environment room at 27 °C with a 24 h light photoperiod and rotated daily to reduce some chamber effects that could occur.

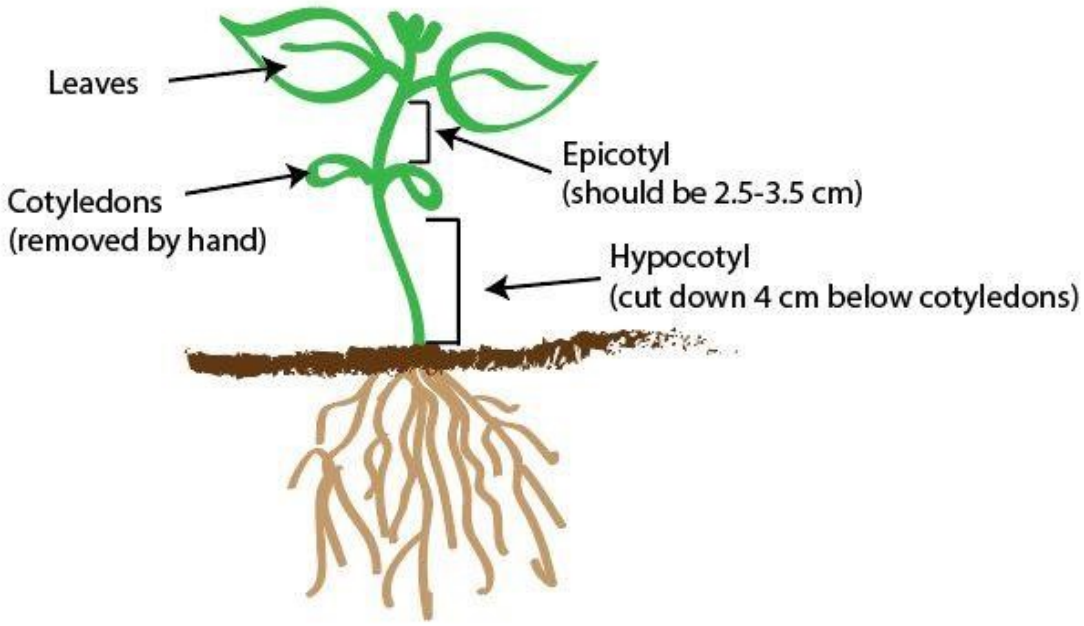


Figure 3.1. Diagram of seedlings and the cutting procedure for seedling transfer.

After the plants are cut at ground level, the cotyledons are removed, and the hypocotyl is cut 4 cm below cotyledons, while the epicotyl should be between 2.5 and 3.5 cm.

The last step was data collection. After 7 days, the roots were cut 2.5 cm up from the bottom of the stem and all 10 roots from each treatment were scanned together using a WinRHIZO root scanner. The average root length, surface area, and tip number were measured from each treatment. All assays were done in triplicate, but the measurements using the WinRHIZO root scanner where done 10 times to each of the three replicas, giving 30 information points for each treatment. Acadian Seaplants does this in order to take into account the error within the root scanner machine and reduce its uncertainty.

3.6 Carboxymethyl Cellulose (CMC) Assay

Since the CMC was not hydrolyzed in a substantial way when used as a positive control in the hydrolysis, there was doubt that it would be a good positive control in the first place, so an activity assay was done to evaluate CMC as a substrate for the enzymes used. To assess activities of Celluclast® 1.5L and Cellic CTec2 on CMC, a method from the International Union of Pure and Applied Chemistry (IUPAC) was adapted (Ghose, 1987). Various concentrations of enzyme were added to 0.05 M citrate buffer at pH 4.8. Three different enzyme dilutions were made for both Celluclast® 1.5L and Cellic CTec2: 1/100, 1/250, and 1/400. The tubes were preconditioned at 50 °C for 15 min, and then 0.5 mL of 2% CMC diluted in 0.05 M citrate buffer (pH 4.8) was added to each tube. The tubes were then incubated at 50 °C for 30 min. Following this incubation, 3 mL of DNS reagent was added to each tube. The tubes were then boiled for 5 min in boiling water, and after, the tubes were transferred to a cool water bath. After cooling to room temperature, 20 mL of water was added to each tube, and the tubes were left to settle for at least 20 min. 200 µL samples were then taken from each tube and placed in a 96-well plate. The color formed was then measured in a spectrophotometer (Ultrospec4300 pro UV/Vis spectrometer; Cambridge, England) at a wavelength of 540 nm. D-(+)-glucose standards, substrate standards, and enzyme blanks were also prepared and treated in the same way. All analyses were done in triplicate.

3.7 Enzyme Hydrolysis of Wood Pulp

As another method for assessment of enzyme activity, hydrolysis of hardwood pulp of poplar/aspen (Alberta Pacific Forest Industries Inc.; Edmonton, Alberta, Canada) was also examined. 1 g of wood pulp was weighted into each test tube. Ten mL of 0.05 M sodium citrate buffer (pH 4.8) was then added. The tubes were preconditioned at 50 °C in a water bath for 20 min. Based on data from the filter paper activity tests previously performed (*section 3.2.1*), 15

FPU/g of substrate was then added for each enzyme treatment (Celluclast® 1.5L or Cellic CTec2). The tubes were incubated at 50 °C for 6 h, then submerged in boiling water for 15 min to terminate the reaction. Subsequent downstream processing, collection of pooled liquid hydrolysates, and HPLC analysis was performed as previously described (*Section 3.3.1*).

3.8 Effect of Autoclaving on Enzyme Hydrolysis

In viewing the results of the previous experiments, there could possibly be an inhibitory effect on the enzymes produced by the algae itself. Since in *Section 4.3.5* the positive control (carboxymethyl cellulose) was not ideal, the experiment was recreated, using wood pulp now as the positive control, which was concluded by seeing the results in *Section 4.7* that it was a much better substrate for the enzymes. Not only that, but already suspecting an inhibition effect from polyphenols, the potential to reduce this inhibition effect by autoclaving the seaweed samples as also assessed.

From literature, it is known that *A. nodosum* contains a lot of polyphenols (Kadam *et al*., 2015a). A study revealed that *A. nodosum* could have up to 9% dry weight in tannins, a type of polyphenol, and from 3-10% volume of physodes, special vacuoles where phenols reside in the algae cell (Baardseth, 1970). Polyphenols have been studied in the past for their role in the inhibition of cellulases (Qin *et al*., 2016; Sineiro *et al*., 1997; Ximenes *et al*., 2010). While the effect of *A. nodosum* specific polyphenol extracts have not been studied on cellulases, they have been studied that it inhibits lipase activity *in vitro* (Austin *et al*., 2018). This leads us to believe that the polyphenols in *A. nodosum* could potentially inhibit other types of enzymes, like cellulases. Taking that into account, the objective was now to reduce the polyphenols in the algae and test if that reduced the suspected inhibition effect in the enzymes. The methodology was the same as in *Section 4.3.5*, but replacing carboxymethyl cellulose with wood pulp as a positive control, and also

adding a new factor, autoclaving. For the temperature treatment, the seaweed sample was autoclaved inside ACE pressure tubes (120 mL capacity, Sigma-Aldrich; St. Louis, Missouri, United States), at a temperature of 121 °C for 30 min. The same enzymatic treatments to the autoclaved seaweed was then applied.

4. Results

4.1 Seaweed Characterization

Seaweed are a very diverse group of plants. In order to get a better understanding of the algal feedstock used in this research project, a detailed characterization was performed. This included moisture and ash content, elemental analysis, crude fat analysis, crude protein analysis, and monosaccharide analysis. The details of these experiments are described below.

4.1.1 Moisture and Ash Content

Moisture and ash content was done to determine the amount of water and the amount of ash in the seaweed samples, respectively. Moisture analysis is very important, as it is needed for many other experiments in order to convert results to a dry basis. The weights of the crucibles before and after the convection oven, and before and after the muffle furnace were recorded, and the data represented the moisture content and ash content in % weight, respectively (**Table 4.1**). The moisture content was calculated at 4.1 ± 0.2% weight, fresh basis, while the ash content was much higher, at 21.3 ± 1.2% weight, dry basis. As mentioned in *Section 3.1.1*, it should be noted that the seaweed samples were already pre-dried when they were received at the University of Alberta, and thus the values obtained in these experiments do not equate to water content in the native seaweed, only in the specific sample received.

Table 4.1 Moisture and Ash Content

Analysis	Percent weight (%)
Moisture Content	4.1 ± 0.2
Ash Content	21.3 ± 1.2

**Data expressed as mean ± standard deviation*

4.1.2 Elemental Analysis

Given the high ash content that was found, which is attributable to inorganic material, elemental analysis was performed in order to further characterize the more than 20% dry weight that the samples had in ash content. This analysis was done in the Natural Resources Analytical Laboratory (NRAL), in the Department of Renewable Resources at the University of Alberta using inductively coupled plasma optical emission spectrometry (ICP-OES). The summarized data for the elemental analysis were divided into two figures, **Figure 4.1** and **Figure 4.2**. This was done in order to separate the high quantity elements (**Figure 4.1**), from the trace elements (**Figure 4.2**) that were found in the samples. Sodium was the most abundant element at 3.17 ± 0.08% dry weight, followed by potassium and sulfur, at 2.47 ± 0.03% dry weight and 2.49 ± 0 .05% dry weight, respectively. Looking at the trace elements (**Figure 4.2**), it can be seen that the highest was iron at 149 ± 13 parts per million (ppm), while the lowest element recorded in the ICP-OES was copper, at 12 ± 3 ppm. It should be noted that the data reported here does not account for any anions present. This is because of the operating costs of renting the ICP-OES equipment services from NRAL, so not all elements could be determined. This could be a possible explanation to why the elemental content does not add up to the 20% ash content described above. This results could

help in the future Sections to identify if there could possibly be an inhibition on the enzymes because of high salt content.

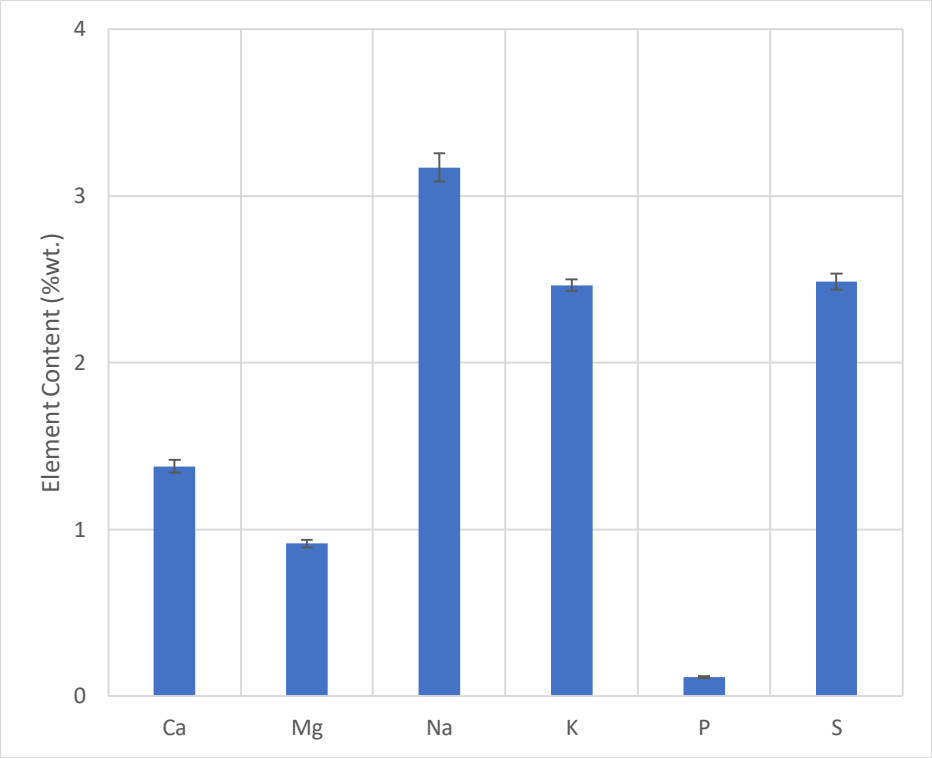


Figure 4.1. Elemental Analysis.

Elemental content (% weight, dry basis) of Ascophyllum nodosum was determined using inductively coupled plasma optical emission spectrometry (ICP-OES). Data is shown as means ± standard deviations of triplicate experiments.

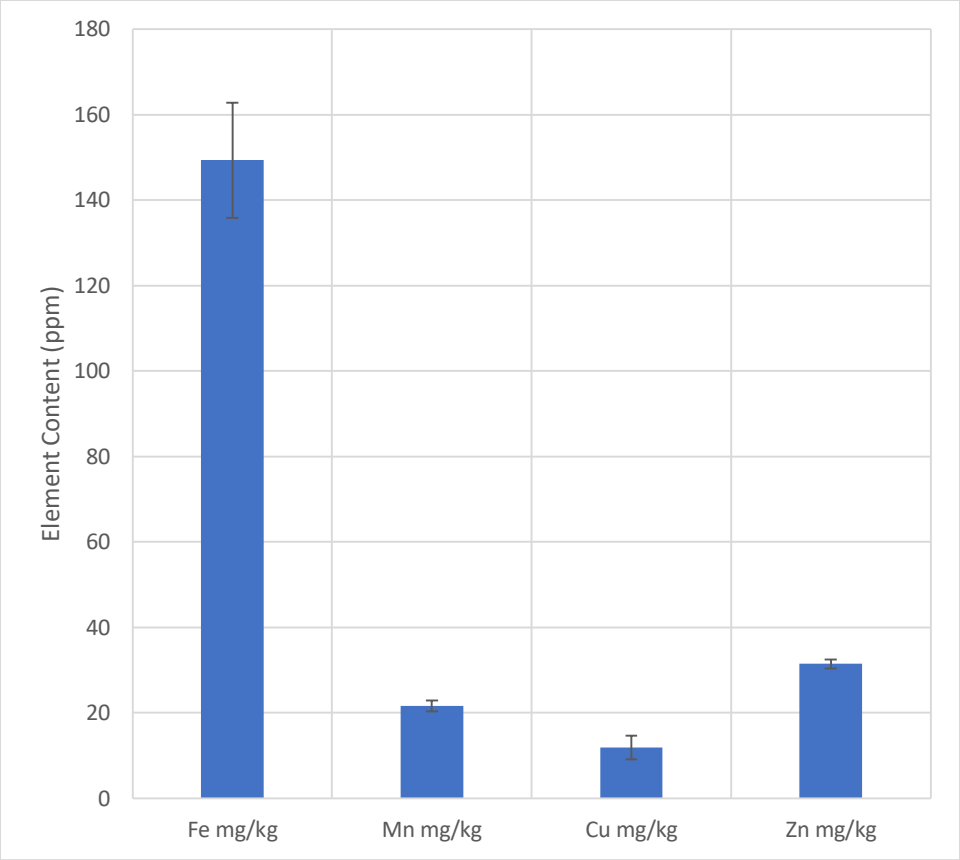


Figure 4.2. Trace Element Analysis.

The trace elemental content of Ascophyllum nodosum is presented as parts per million. Values were determined using inductively coupled plasma optical emission spectrometry (ICP-OES). Data is shown as means ± standard deviations of triplicate experiments.

4.1.3 Crude Fat Analysis

Lipids are one of the major constituents of cells. To determine the crude fat contained in the seaweed samples, a Goldfisch Extraction Apparatus was employed to isolate the crude fat. The beakers containing the crude fat were weighted and compared to the weight of the beaker before the experiment. The crude fat content of *Ascophyllum nodosum* was determined to be 3.1 ± 0.4 % dry weight. As this was a proximate analysis, it should be noted that this analysis does not divide

or identify the specific fats, in this case, a solvent extraction was used in order to quantify the fraction of the sample that was soluble in the petroleum ether solvent used.

4.1.4 Crude Protein Analysis

Proteins are macromolecules that are formed through the bonding of various amino acids. Similar to lipids, they are one of the main components in cells (Cooper, 2000). In order to determine the amount of protein in the samples, crude protein analysis was performed using a LECO TruSpec CN Carbon/Nitrogen Determinator. This experiment provided the nitrogen content of the samples. The crude protein content was then calculated using a conversion factor of 4.92, which is the average conversion factor for seaweed (Maehre *et al*., 2014). The result for the average crude protein content of *Ascophyllum nodosum* was 7.4 ± 0.2 % dry weight.

4.1.5 Monosaccharide Analysis

Carbohydrates are also one of the major macromolecules in cells. Polysaccharides are made from the joining of simple sugars, also known as monosaccharides, such as glucose. The breakdown of glucose gives energy to the cells, and provides materials for the synthesis of other cell components, while polysaccharides are used for storage and as structural components in the cells (Cooper, 2000). To assess the monosaccharide content of the seaweed, a two-step acid hydrolysis was used to release the monosaccharides, then used HPLC with a Biorad Aminex HPX-87P column and a refractive index detector, as a way to quantify glucose, xylose, fucose, and mannitol in the seaweed samples (**Table 4.2)**. All of the monosaccharides analyzed were found in the sample. Fucose was the most prominent monosaccharide in the algae, found at 22.1 ± 4.5 mg, followed by mannitol (14.9 ± 1.0 mg) and glucose (12.5 ± 1.0 mg), with xylose being the least abundant monosaccharide (8.2 ± 0.7 mg). The original amount of substrate used was 300 mg of seaweed as it was received. Other important sugars that could be found in *A. nodosum* would

definitely be the uronic acids that constitute alginate, D-mannuronic and L-guluronic acids. Alginate constitutes up to 30% dry weight in this seaweed species. Uronic acids were not characterized due to a lack of an accurate method for their quantification with the equipment available in our lab at the time of the making of this project (Baardseth, 1970).

Table 4.2 Monosaccharide Analysis Results

Monosaccharide	Milligrams of monosaccharide (mg)
Glucose	12.5 ± 1.0
Xylose	8.2 ± 0.7
Fucose	22.1 ± 4.5
Mannitol	14.9 ± 1.0

Data expressed as mean ± standard deviation.

4.2 Enzyme Activity Assays

In order to effectively quantify the activity of the enzymes, which is very important for determination of how much enzyme to add in the subsequent enzyme hydrolysis experiments, several activity assays were conducted as described below.

4.2.1 Filter Paper Units for Celluclast® 1.5L and Cellic CTec 2

The filter paper assay uses Whatman No.1 filter paper and dinitrosalicylic acid (DNS) reagent to measure the amount of reducing sugar produced, which is then used to determine enzymatic activity in terms of filter paper units per milliliter of undiluted enzyme. The resulting filter paper units for Celluclast® 1.5L and Cellic Ctec2 using 0.05 M sodium citrate buffer (pH 4.8) were: 108 FPU for Celluclast® 1.5L, and 277 FPU for Cellic Ctec2. The substrate for both assays was Whatman No. 1 filter paper strips, which is highly refined cellulose. It can be seen that Cellic Ctec2 has more than double the activity of Celluclast® 1.5L when using filter paper as substrate.

4.2.2 Alginate Lyase Activity Assay

The activity of alginate lyase was measured by monitoring the increase in absorbance in a quartz cuvette at 230 nm in a spectrophotometer by measuring the uronic acid products that are generated by the depolymerization of alginate made by the enzyme. When the absorbance did not increase any further, the amount of alginic acid broken down per minute per microliter of alginate lyase added was calculated (Tondervik *et al*., 2010). The absorbance at 230nm vs time graph can be seen below in **Figure 4.3**. The activity of the alginate lyase was calculated at 2.88 mg alginic acid digested per minute, per mL of alginate lyase used. To each cuvette, 180 µL of alginic acid (4mg of alginic acid/mL) solution was added to 10 µL of enzyme and 700 µL of sodium citrate buffer (pH = 5.8). The calculations were then done by calculating the amount of alginic acid present (4mg/mL x 0.180mL = 0.72 mg of alginic acid), then using 25 min as the peak on the chart (where the absorbance reaches an equilibrium and does not increase further) and taking that as the moment where all alginic acid is consumed, then calculate the mg of alginic acid digested per minute (0.72mg alginic acid / 25 min = 0.0288 mg alginic acid/min), then the activity per microliter of enzyme used (0.0288 mg alginic acid/min / 10 µL enzyme used = 0.00288 mg alginic acid digested per minute, per microliter of alginate lyase used), then converting microliters of alginate lyase to milliliters (2.88 mg alginic acid digested per minute, per mL of alginate lyase used).

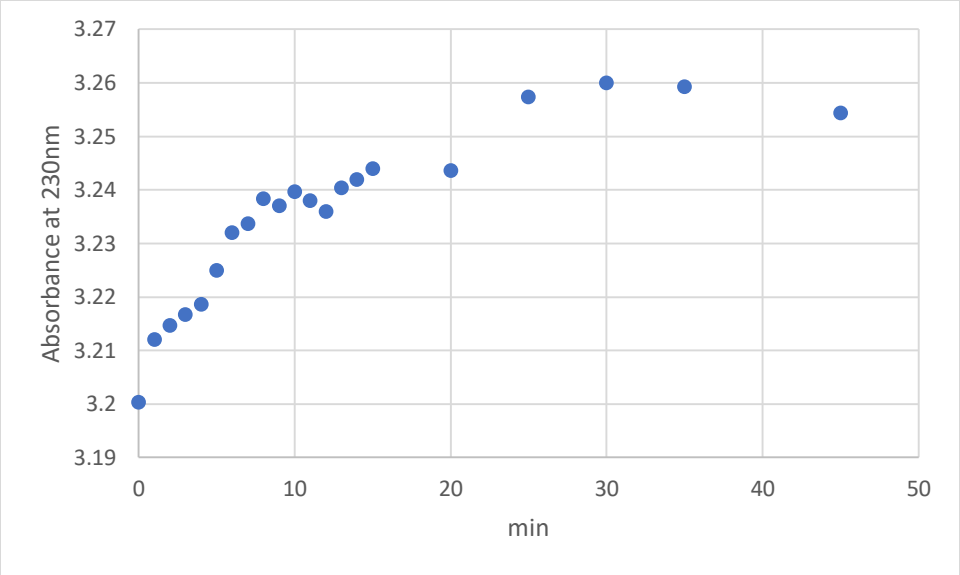


Figure 4.3 Alginate Lyase Activity.

The increase in absorbance (230 nm) due to alginate being broken down was recorded over time to establish the activity of alginate lyase.

4.3 Enzyme Hydrolysis

4.3.1 Celluclast® 1.5L and Alginate Lyase

This was the first approach at hydrolyzing the *A. nodosum* brown seaweed. An important component was to determine the amount of monosaccharides that would be released by hydrolysis of the seaweed using these enzymes. For this, the impact of enzyme treatment on the algal biomass was assessed through analysis of released monosaccharides via HPLC (**Table 4.3)**. There was no xylose or fucose released by enzyme hydrolysis in either of the two systems. For mannitol, there was no statistical difference between the monosaccharide of the seaweed only control and the various enzyme treatments, except for Celluclast® 1.5L + Alginate Lyase. Conversely, for glucose, it can be seen that the Celluclast® 1.5L treatment and the Celluclast® 1.5L + Alginate Lyase treatment were statistically different than the controls. The Celluclast® 1.5L treatment measured 1.96 ± 0.09 mg glucose, while the Celluclast® 1.5L + Alginate Lyase measured 1.77 ±

0.09 mg of glucose. The Alginate Lyase treatment alone did not seem to have any effect on releasing any of the monosaccharides and was not statistically different from the controls, which was already expected, as alginate lyase does not break down cellulose, so it was not expected that any of the monosaccharides measured would increase compared to the seaweed only control.

Table 4.3 Celluclast® 1.5L and Alginate Lyase Monosaccharide Analysis Results

Sample	***Glucose (mg)***	***Mannitol (mg)***
Celluclast® 1.5L + Seaweed	1.96 ± 0.09^{a}	6.84 ± 0.04^{ab}
Alginate Lyase + Seaweed	0.92 ± 0.02^{c}	6.83 ± 0.07^{ab}
Celluclast® 1.5L + Alginate Lyase + Seaweed	1.77 ± 0.09^{b}	6.89 ± 0.02^{a}
Seaweed only (No enzymes)	0.91 ± 0.06^{c}	6.58 ± 0.19^{b}
Celluclast® 1.5L (No Seaweed; Enzyme Blank)	0.34 ± 0.02^{d}	ND
Alginate Lyase (No Seaweed; Enzyme Blank)	ND	ND
Celluclast® 1.5L + Alginate Lyase (No Seaweed; Enzyme Blank)	0.29 ± 0.05^{d}	ND

***** *ND: Not detected. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. The values that share the same letter are not significantly different from each other.*

4.3.2 Freeze/Thaw Pretreatment

The purpose of this experiment was to see if the freezing and thawing of the algal substrate would make it more amenable to enzyme treatment through further disruption of cell walls, yielding more monosaccharides during enzyme hydrolysis. The data for the monosaccharide analysis using the frozen/thaw pretreatment is summarized in **Table 4.4** below. Looking at glucose, the frozen/thaw pretreatment group was statistically similar to the group receiving no

pretreatment. The same can be seen when looking at mannitol. Thus, based on the monosaccharides examined, the freeze/thaw treatment did not impact monosaccharide yields.

Table 4.4. Celluclast® 1.5L and Alginate Lyase; Freeze/Thaw Pretreatment Results

Pretreatment	Sample	Glucose	Mannitol
None (4 °C fridge)	Seaweed + Celluclast® 1.5L + Alginate Lyase	1.73 ± 0.10^{a}	6.84 ± 0.52^{a}
	Seaweed only (No enzymes)	0.92 ± 0.03^{b}	6.49 ± 0.19^{ab}
	Celluclast® 1.5L + Alginate Lyase (No Seaweed; Enzyme Blank)	0.27 ± 0.01^{c}	0.08 ± 0.01^{c}
Frozen/Thaw	Seaweed + Celluclast® 1.5L + Alginate Lyase	1.84 ± 0.11^{a}	6.93 ± 0.02^{a}
	Seaweed only (No enzymes)	0.89 ± 0.06^{b}	5.89 ± 0.01^{b}

***** *ND: Not detected. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. Xylose and fucose were also determined in this analysis, but not detected, so they were excluded for clarity. The values that share the same letter are not significantly different from each other.*

4.3.3. Addition of Viscozyme® L

Since the initial approach used released low levels of monosaccharides, the addition of another enzyme cocktail commonly used in the cellulosic ethanol world, Viscozyme® L, could possibly help increase the release of monosaccharides. As stated in *Section 3.3.3* Viscozyme® L is an enzyme complex used in the breaking down of plant cell walls for the extraction of components, especially in the alcohol, brewing, and starch industries, and contains several carbohydrases (Novozymes, 2002). The data for the monosaccharide analysis is summarized in

Table 4.5. The addition of Viscozyme® L to the enzyme hydrolysis released 2.06 ± 0.13 mg of glucose, compared to the 2.05 ± 0.06 mg of glucose when only Celluclast 1.5L + Alginate Lyase were used to hydrolyze seaweed, which are not statistically different. This confirms that the addition of Viscozyme® L did not have any effect on the amount of monosaccharides measured.

Table 4.5. Celluclast® 1.5L, Alginate Lyase, and Viscozyme® L Monosaccharide Analysis Results

Sample	Glucose	Mannitol
Seaweed + Celluclast® 1.5L + Alginate Lyase	2.05 ± 0.06^{a}	7.00 ± 0.14^{a}
Seaweed + Celluclast® 1.5L + Alginate Lyase + Viscozyme® L	2.06 ± 0.13^{a}	6.48 ± 0.18^{b}
Seaweed only (No enzyme)	0.95 ± 0.05^{b}	6.37 ± 0.15^{b}
Celluclast® 1.5L + Alginate Lyase only (No Seaweed; Enzyme Blank)	0.36 ± 0.04^{c}	0.13 ± 0.01^{c}
Celluclast® 1.5L + Alginate Lyase + Viscozyme® L (No Seaweed; Enzyme Blank)	0.36 ± 0.06^{c}	0.09 ± 0.01^{c}

***** *Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. Xylose and fucose were also determined in this analysis, but not detected, so they were excluded for clarity. The values that share the same letter are not significantly different from each other.*

4.3.4 Alginate Lyase and Cellic Ctec 2

Cellic Ctec 2 is a highly processive cellulase cocktail that has demonstrated superior ability to hydrolyze cellulosic materials, including highly recalcitrant crystalline regions of cellulose (Rodrigues *et al*., 2015). To examine whether this enzyme cocktail could help improve hydrolysis of algal biomass, enzymatic hydrolysis was performed using Cellic Ctec2, with and without addition of alginate lyase. The data for the monosaccharide analysis is summarized in **Table 4.6**. Similar to previous enzyme hydrolysis experiments, no xylose or fucose were released in any of the treatments. Although 6.30 ± 0.20 mg of glucose and 7.19 ± 0.24 mg of mannitol were observed after treatment with Cellic Ctec2, comparison to the enzyme blanks revealed no significant

difference with regards to glucose levels. Also, the addition of alginate lyase had no statistical effect on the amount of monosaccharides released.

Table 4.6 Alginate Lyase and Cellic Ctec2 Monosaccharide Analysis Results

Sample	Glucose	Mannitol
Seaweed + Cellic Ctec2	6.30 ± 0.20^{a}	7.19 ± 0.24^{a}
Seaweed + Cellic Ctec2 + Alginate Lyase	5.93 ± 0.14^{a}	7.54 ± 0.34^{a}
Seaweed only (No enzyme)	1.07 ± 0.00^{b}	7.16 ± 0.18^{a}
Cellic Ctec2 only (No seaweed; Enzyme Blank)	5.43 ± 0.15^{a}	ND
Cellic Ctec2 + Alginate Lyase only (No seaweed; Enzyme Blank)	5.14 ± 1.40^{a}	ND

***** *ND: Not detectable. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. Xylose and fucose were also determined in this analysis, but not detected, so they were excluded for clarity. The values that share the same letter are not significantly different from each other.*

4.3.5 Addition of carboxymethyl cellulose (CMC) to the enzyme systems

None of the enzyme studies described above generated substantial monosaccharide yields. To confirm whether there was any enzyme inhibition arising from the seaweed substrate, the enzyme systems was benchmarked with carboxymethyl cellulose (CMC), which is a substrate typically used to measure endoglucanase activity. The data for the monosaccharide analysis is summarized in **Table 4.7**. Interestingly, mannitol quantities detected were lower in the treatments where CMC was added, with the greatest measurement of mannitol observed when treating seaweed with Cellic Ctec2 + Alginate Lyase (9.85 ± 0.19 mg) or Celluclast® 1.5L + Alginate Lyase (9.39 ± 0.12 mg), but it can be observed that practically all of the mannitol is coming from the enzyme blank and the seaweed control, meaning that mannitol release was not affected by the

enzymes. Addition of CMC to the Celluclast® 1.5L + Alginate Lyase or the Cellic Ctec2 + Alginate Lyase systems resulted in increased glucose production, demonstrating that the cellulase cocktails were active and able to hydrolyze CMC to its constituent glucose. However, it should be noted that complete hydrolysis of CMC was not observed, as 100mg of CMC was added, and less than 5% of the CMC was hydrolyzed from the controls. This could mean that the enzymes are not working correctly in that substrate. As a point of comparison, a study found that indeed Celluclast 1.5L and Cellic CTec2 did have hydrolyzing activity on CMC. As the positive controls were not hydrolyzed in any substantial way, it cannot be concluded whether there was any inhibition from the seaweed itself, as the low CMC hydrolysis in the treatments with both seaweed and CMC could be that the CMC is not a good substrate for our enzymes. Comparing with the literature, a study got activity on CMC from Celluclast 1.5L and Cellic CTec2 of 26.76 ± 0.13 and 34.81 ± 2.10 μmol glucose min^{-1} mg^{-1} protein (Harrison et al., 2013). One limitation regarding this study, however, is that they never reported the degree of substitution of the CMC that was used. The enzymatic hydrolysis of CMC is dependent on the degree of substitution of the CMC that is used (Lee et al., 2007).

Table 4.7. Enzyme hydrolysis systems spiked with CMC

Sample	Glucose	Mannitol
Seaweed + Celluclast® 1.5L + Alginate Lyase	2.80 ± 0.19^{e}	9.39 ± 0.12^{ab}
Seaweed + Celluclast® 1.5L + Alginate Lyase + CMC	6.32 ± 0.31^{c}	8.58 ± 0.12^{cd}
Seaweed + Cellic Ctec2 + Alginate Lyase	9.89 ± 0.37^{b}	9.85 ± 0.19^{a}
Seaweed + Cellic Ctec2 + Alginate Lyase + CMC	12.03 ± 0.42^{a}	8.94 ± 0.36^{bc}
Celluclast® 1.5L + Alginate Lyase + CMC (No seaweed)	3.85 ± 0.09^{d}	1.28 ± 0.06^{f}
Cellic Ctec2 + Alginate Lyase + CMC (No seaweed)	10.48 ± 0.35^{b}	0.46 ± 0.04^{g}
Seaweed only (No enzyme)	1.09 ± 0.02^{f}	8.47 ± 0.13^{d}
CMC only (No enzyme)	ND	ND
Seaweed + CMC only (No enzyme)	1.11 ± 0.01^{f}	6.01 ± 0.12^{e}
Celluclast® 1.5L + Alginate Lyase (Enzyme blank, No seaweed)	0.55 ± 0.02^{f}	1.10 ± 0.06^{f}
Cellic Ctec2 + Alginate Lyase (Enzyme blank, No seaweed)	6.94 ± 0.10^{c}	ND

***** *ND: Not detectable. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. Xylose and fucose were also determined in this analysis, but not detected, so they were excluded for clarity. The values that share the same letter are not significantly different from each other.*

4.3.6 Celluclast® 1.5L, Alginate Lyase, and Cellic Ctec 2, pH 5.8

In order to assess whether the enzyme treatment of seaweed resulted in structural damage to the biomass and release of biostimulant into the liquid phase, scanning electron microscopy and mung bean assays were performed, respectively. Again, Carboxymethyl cellulose (CMC) was used as a positive control. For these experiments, higher volume enzyme hydrolysis was performed to ensure that enough liquid and solid product would be obtained. To ensure that scale-up did not have a major effect of the enzyme hydrolysis, monosaccharide analysis was performed so that monosaccharide yields could be compared with past experiments. The data for the monosaccharide analysis is summarized in **Table 4.8**.

Table 4.8. Monosaccharide analysis of samples for Mung Bean Assay and Scanning Electron Microscopy

Sample	Glucose	Mannitol
Seaweed + Celluclast® 1.5L	2.24 ± 0.70^{cd}	16.2 ± 1.63^{a}
Seaweed + Cellic Ctec2	6.36 ± 0.21^{ab}	17.3 ± 1.37^{a}
Seaweed + Alginate Lyase	1.61 ± 0.39^{cd}	15.8 ± 0.35^{a}
Seaweed + Celluclast® 1.5L + Alginate Lyase	2.55 ± 0.17^{cd}	15.9 ± 1.18^{a}
Seaweed + Cellic Ctec2 + Alginate Lyase	3.85 ± 0.93^{bc}	11.5 ± 0.81^{b}
CMC + Celluclast® 1.5L (no seaweed)	2.70 ± 0.15^{cd}	ND
CMC + Cellic Ctec2 (no seaweed)	7.6 ± 0.25^{a}	11.01 ± 1.67^{b}
CMC + Alginate Lyase (no seaweed)	1.60 ± 2.58^{cd}	ND
CMC + Celluclast® 1.5L + Alginate Lyase (no seaweed)	1.15 ± 0.31^{cd}	ND
CMC + Cellic Ctec2 + Alginate Lyase (no seaweed)	6.31 ± 3.04^{ab}	ND
Seaweed only (no enzyme)	1.80 ± 0.41^{cd}	16.3 ± 0.36^{a}
CMC + Sodium Alginate (no enzyme, no seaweed)	ND	ND
Celluclast® 1.5L (Enzyme Blank, no seaweed)	0.31 ± 0.12^{d}	ND
Cellic Ctec2 (Enzyme Blank, no seaweed)	3.85 ± 0.15^{bc}	ND
Alginate Lyase (Enzyme Blank, no seaweed)	ND	ND
Celluclast® 1.5L + Alginate Lyase (Enzyme Blank, no seaweed)	0.28 ± 0.08^{d}	ND
Cellic Ctec2 + Alginate Lyase (Enzyme Blank, no seaweed)	4.31 ± 0.98^{abc}	ND

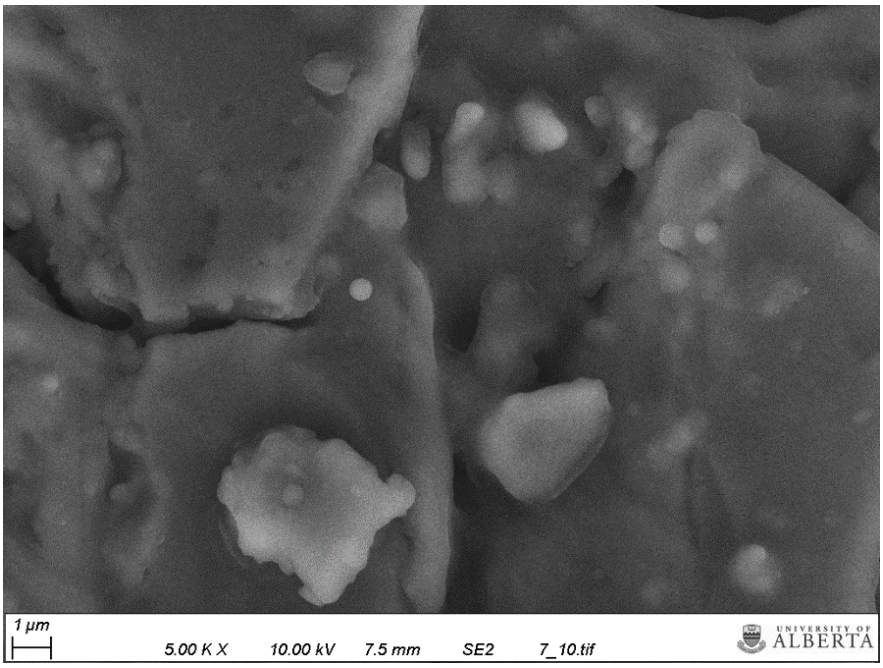
***** *ND: Not detectable. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. Xylose and fucose were also determined in this analysis, but not detected, so they were excluded for clarity. The values that share the same letter are not significantly different from each other.*

Looking at mannitol, most of the treatments had no statistical difference in the amount of mannitol measured, with the exception of the Cellic Ctec2 + Alginate Lyase and the CMC + Alginic Acid + Cellic Ctec2 (no seaweed) systems, which had the least amount of mannitol at 11.5 ± 0.81 mg and 11.0 ± 1.67 mg respectively. For glucose, the largest amount was found using CMC + Alginic Acid + Cellic Ctec2 (no seaweed) at 7.55 ± 0.25 mg. As observed in smaller scale experiments, the positive control (CMC) was not completely degraded in any of the treatment systems, even after 20 h of hydrolysis.

4.4 Scanning Electron Microscopy

In order to assess physical damage incurred by the enzyme treatments to the surface of the seaweed, Scanning Electron Microscopy (SEM) was utilized. The solid residues obtained through enzyme treatment as described in *Section 4.3.6* were subjected to Scanning Electron Microscopy to determine if any damage caused by the enzyme treatments could be seen in the imaging equipment. Smooth surfaces that after hydrolysis get rougher would suggest that the enzymes are indeed acting on the substrate, with little to no changes on the physical structure would mean that the enzymes did not have a big impact. The images taken are shown in **Figure 4.4**, taken at 5,000X magnification. In general, it is very difficult to make any concrete conclusions just from looking at pictures from Scanning Electron Microscopy. It would seem that in panels A) and B) from **Figure 4.4**, which are the native seaweed (milled raw seaweed, without treatment) and the Seaweed only treatment (mock treated seaweed, no enzymes added), the surfaces are a bit smoother and less porous. The enzyme treatments, panels C, D, E, F, and G from **Figure 4.4** seem to result in degradation of the seaweed cell walls, when compared to the images obtained from mock-treated or native seaweed, as the enzyme treated images seem to be rougher and with higher porosity, which could possibly be explained by the enzyme degrading the cell walls.

A)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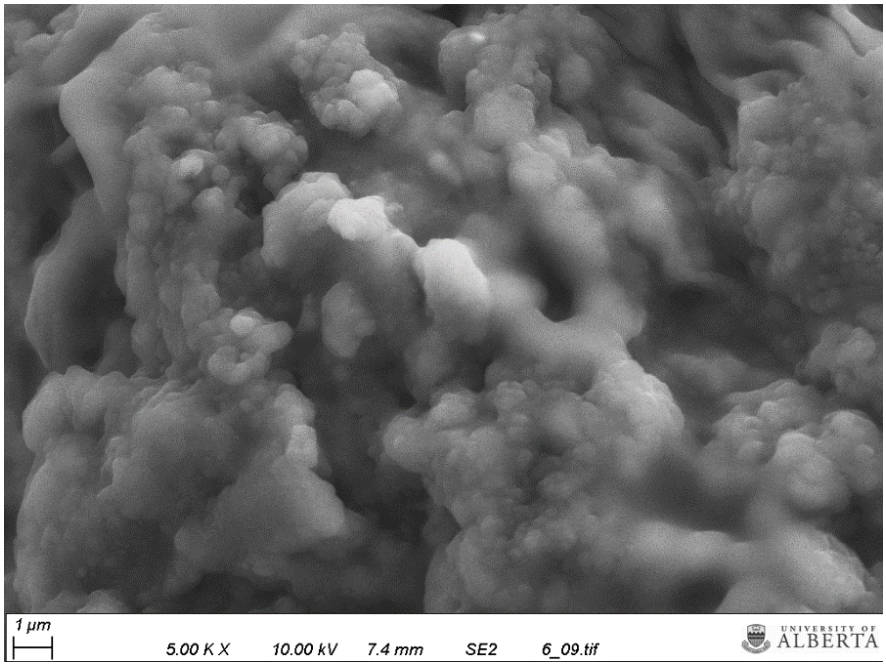
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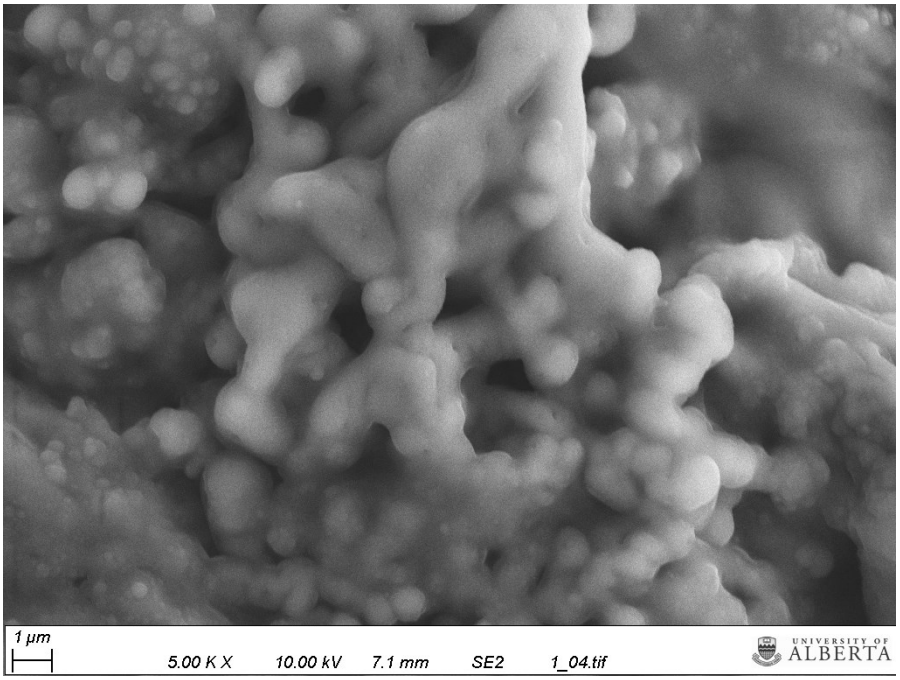
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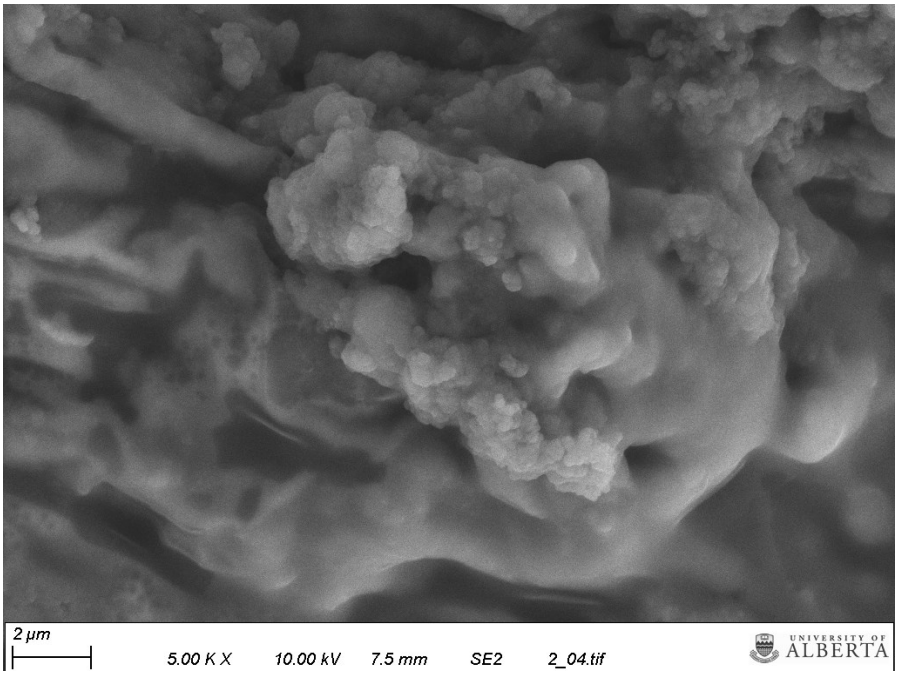
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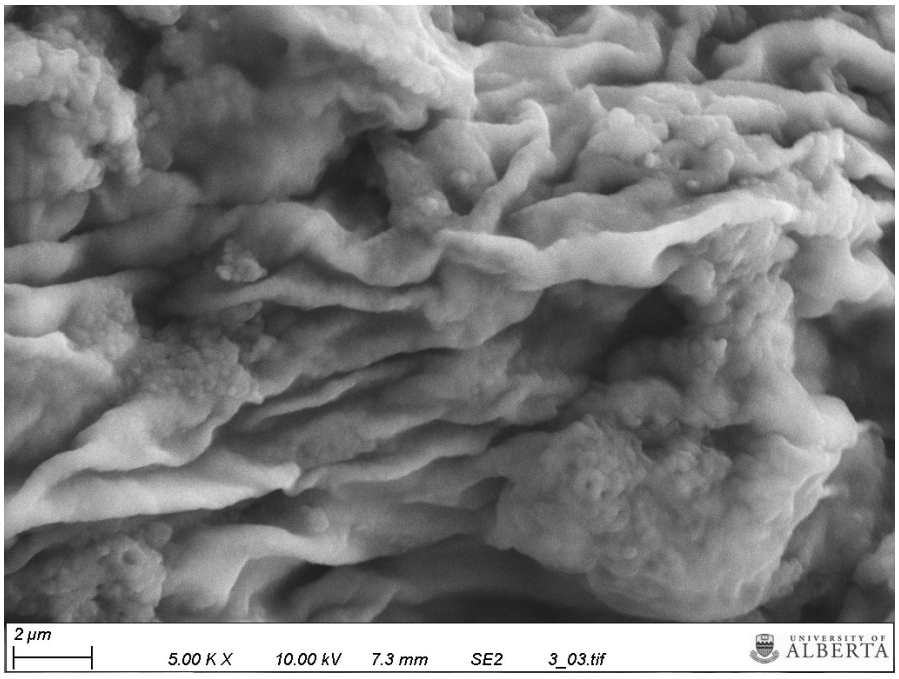
E)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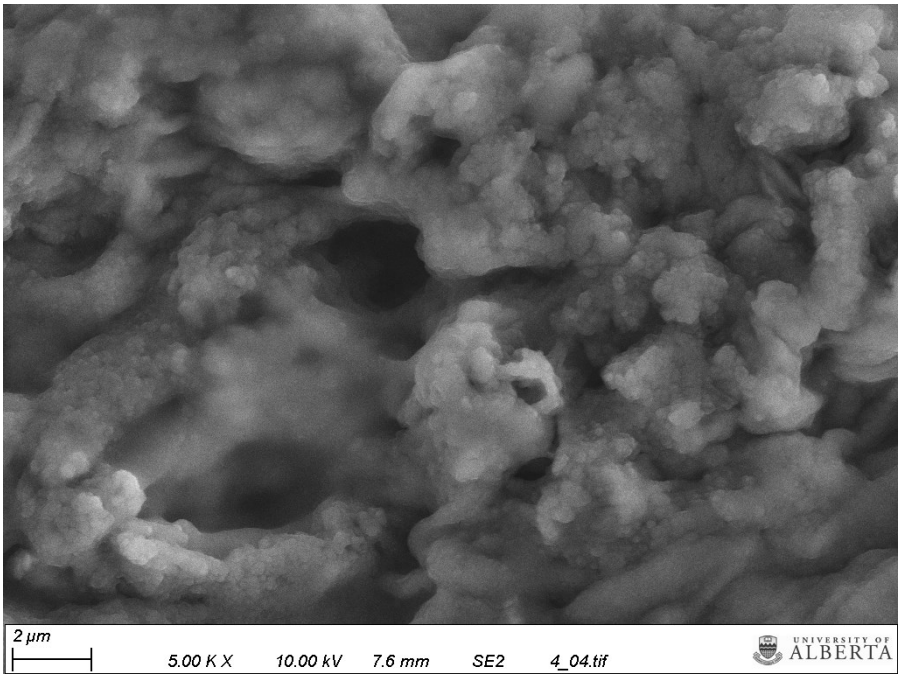
F)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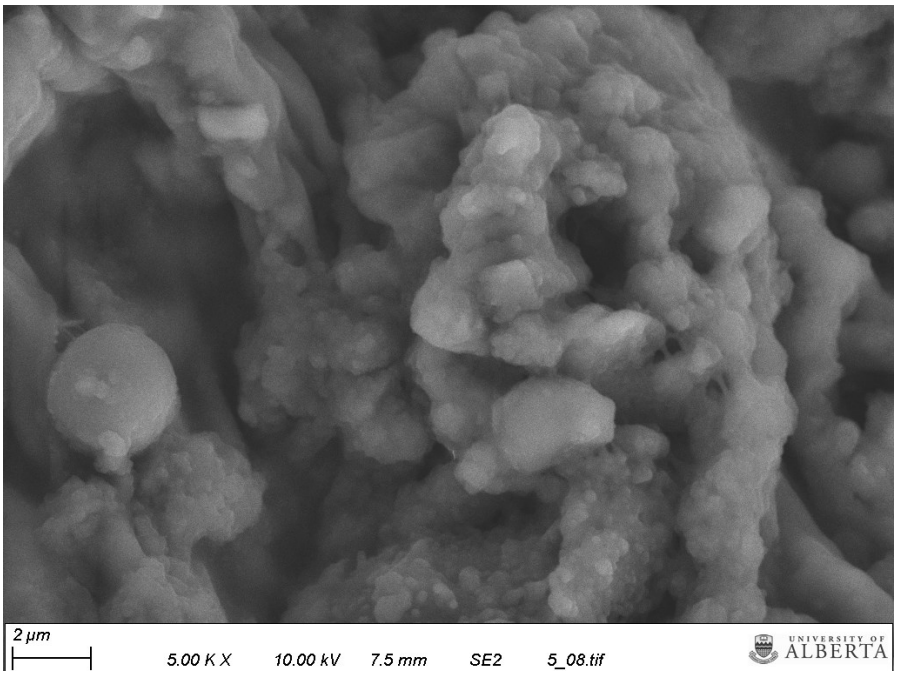
G) 

Figure 4.4 SEM Images

A) Native Seaweed (no treatment, raw seaweed milled); B) Seaweed only (mock treatment, no enzymes added); C) Celluclast® 1.5L treatment; D) Cellic Ctec2 treatment; E) Alginate Lyase treatment; F) Celluclast® 1.5L + Alginate Lyase treatment; G) Cellic Ctec2 + Alginate Lyase treatment; all SEM images were taken at 5000X magnification.

4.5 Mung Bean Rooting Assay

To determine if any plant biostimulants could be found in the enzyme hydrolysates (from Section 3.3.6), liquid fractions were sent to the collaborators at Acadian Seaplants Ltd. (Cornwallis, NS) who measured the effect of hydrolysate application on rooting activity in mung beans. The results of these experiments are summarized in **Figures 4.5** and **4.6**. In **Figure 4.5**, the mung bean root length that was measured for each of the treatment applications. In these figures, SSEP (Soluble Seaweed Extract Powder) served as the positive control and is a commercially available biostimulant produced by Acadian Seaplants Ltd. The root length observed using SSEP

was significantly different from those obtained using the treatment systems. Interestingly, the liquid fraction obtained from mock-treatment of the seaweed also demonstrated comparable biostimulant activity to most of the enzyme treatments with the exception of Cellic Ctec2 + Alginate Lyase, which was worse. The mock treatment of the seaweed consisted on only adding buffer to the seaweed sample (no enzymes) and subjecting it to the same water treatment and temperature as the enzyme treatments. In **Figure 4.6**, the results regarding the number of root tips for each treatment are presented. In this figure, the number of root tips resulting from treatment with SSEP was also significantly different from those obtained using any of the hydrolysates. Again, the liquid fraction isolated from mock-treated seaweed samples also demonstrated similar results as the enzymatic hydrolysates, except for Cellic Ctec2 + Alginate Lyase, which, similar to the root length, was statistically worse than the mock-treated seaweed sample. As a note, while all assays were done in triplicate, the measurements using the WinRHIZO root scanner where done 10 times to each of the three replicas, giving 30 information points for each treatment. Acadian Seaplants does this in order to take into account the error within the root scanner machine and reduce its uncertainty.

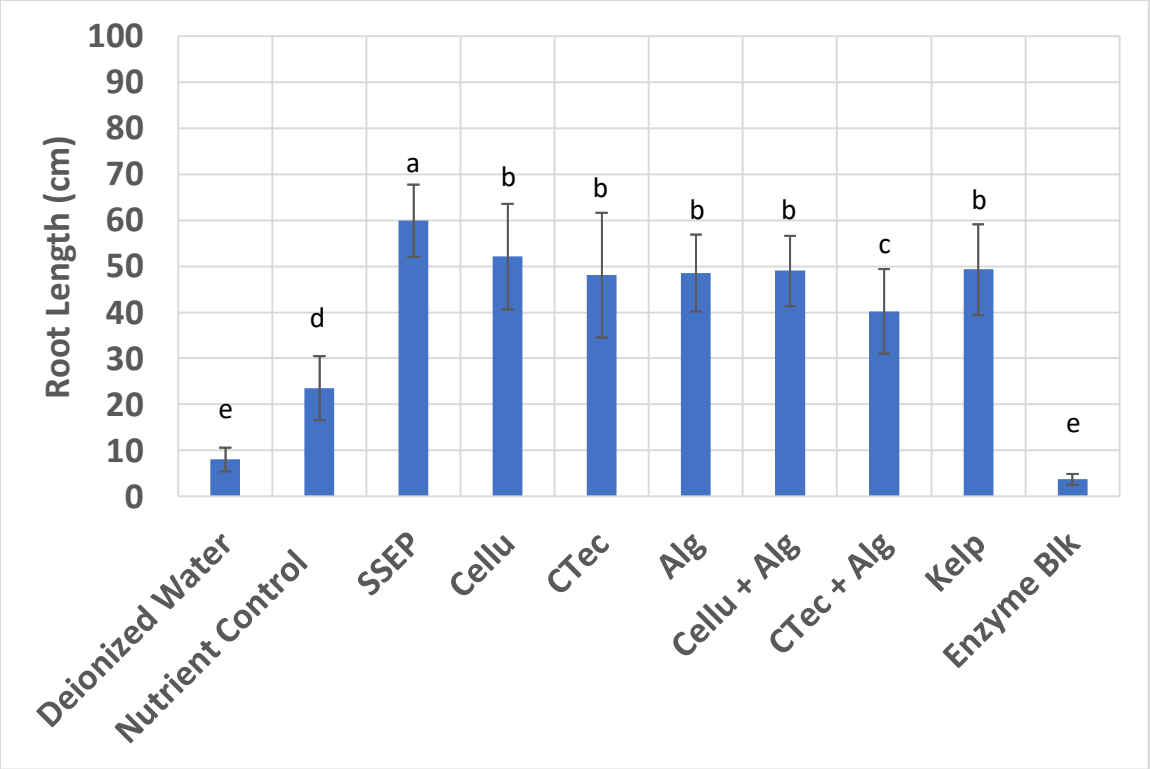


Figure 4.5 Mung Bean Assay, Root Length.

Liquid hydrolysates from the various enzyme treatments were applied to mung bean samples. The root length (cm) for all specimens were measured after incubation at 27 °C for 7 days in a controlled environment room. The various enzyme treatments are as follows: Celluclast® 1.5L (Cellu); Cellic Ctec2 (Ctec); alginate lyase (Alg). Several controls were also used. Deionized water, a nutrient replacement control (K, N, and P), SSEP (Soluble Seaweed Extract Powder; commercial biostimulant from Acadian Seaplants Ltd.), Kelp (seaweed only control; no enzymes), and an enzyme blk (Celluclast® 1.5L + Cellic Ctec2 + Alginate Lyase; enzyme blank, no seaweed). Statistics shown were done using Tukey's HDS test ($\alpha = 0.05$). The values that share the same letter are not significantly different from each other. The comparisons were made between treatments. Treatments that share the same letter mean they are not significantly different.

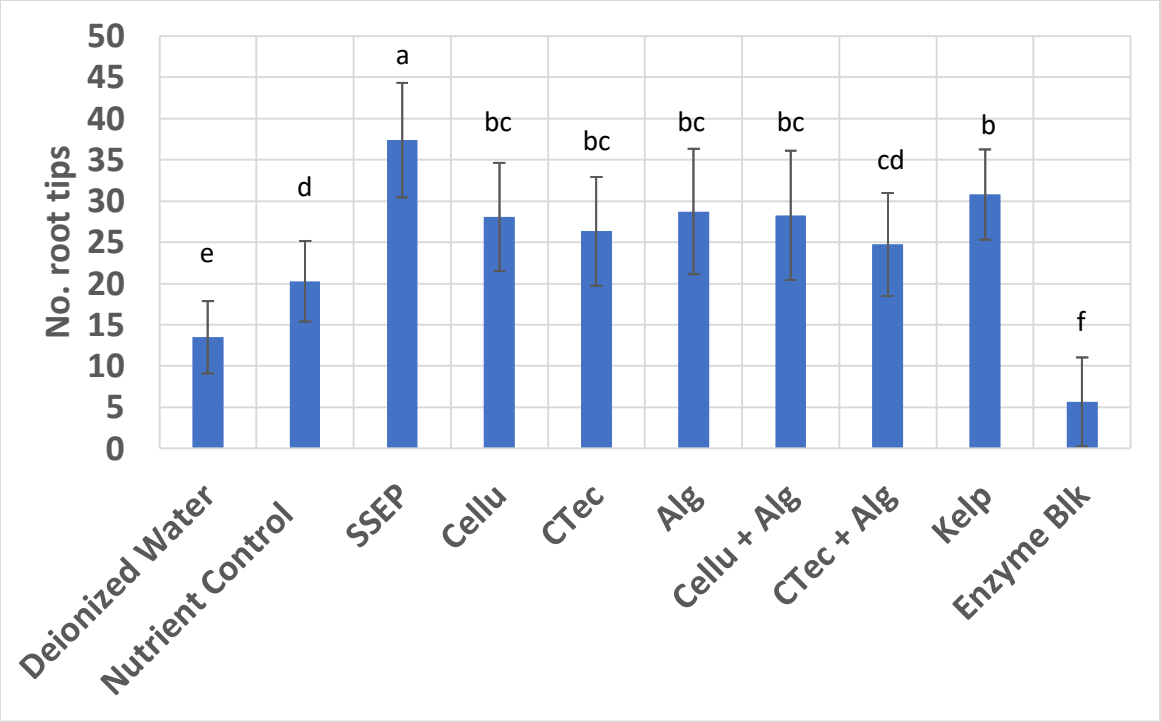


Figure 4.6 Mung Bean Assay, Number of Root Tips.

The liquid hydrolysates from the enzyme treatments were applied to the mung bean samples. The number of root tips was then measured after incubation at 27 °C for 7 days in a controlled environment room. The enzyme treatments used were: Celluclast® 1.5L (Cellu); Cellic Ctec2 (Ctec); alginate lyase (Alg). Several controls were also used: deionized water, a nutrient replacement control (K, N, and P), SSEP (Soluble Seaweed Extract Powder; commercial biostimulant from Acadian Seaplants Ltd.), Kelp (seaweed only control; no enzymes), and enzyme blk (Celluclast® 1.5L + Cellic Ctec2 + Alginate Lyase; enzyme blank, no seaweed). Statistics shown were done using Tukey's HDS test (α = 0.05). The values that share the same letter are not significantly different from each other. The comparisons were made between treatments. Treatments that share the same letter mean they are not significantly different

4.6 Carboxymethyl Cellulose (CMC) Assay

In the enzyme hydrolysis experiments described above, carboxymethyl cellulose (CMC) was used as a positive control for the Celluclast® 1.5L and Cellic Ctec2 enzyme cocktails. However, these studies showed that, under the conditions examined, the two enzyme hydrolyzed CMC very poorly, reaching maximum hydrolysis levels of less than 10%. In order to confirm that carboxymethyl cellulose was a suitable substrate for the enzymes, and thus, a good positive control, the activity of the enzymes when using CMC as a substrate was calculated. This assay is similar to the Filter Paper Units activity assay, and is based on a procedure in the literature (Ghose, 1987). The results from the CMC assay showed that Celluclast® 1.5L had an activity of 6.6 CMC units/mL of enzyme, while Cellic Ctec2 had an activity of 4.4 CMC units/mL of enzyme. Thus, both enzyme cocktails displayed low levels of activity on the carboxymethyl cellulose, demonstrating that carboxymethyl cellulose was not a suitable substrate for a positive control in previous experiments looking at activity of these enzymes in the presence of seaweed. From literature, it is known that the higher the degree of substitution of the CMC, the harder it is to hydrolyze, and degrees of substitution higher than 1 are especially difficult (Lee et al., 2007). The CMC used for these experiments has a degree of substitution of 1.2, making it hard for enzymes to break down, and could explain the low activity in that substrate.

4.7 Enzyme Hydrolysis of Wood Pulp

After analyzing the results from the previous experiment on carboxymethyl cellulose, the activity of the enzymes using another substrate was needed to be confirmed. For these experiments, the enzymatic hydrolysis of 1 gram of hardwood pulp (poplar/aspen) was examined. The glucose and xylose released through these enzyme treatments are summarized in **Table 4.9**. Cellic Ctec2 was the more active enzyme, measuring 238 ± 14.0 mg of glucose and 59.8 ± 4.3 mg of xylose. In

comparison, Celluclast® 1.5L only measured 61.0 ± 15.6 mg of glucose and 21.0 ± 4.9 mg of xylose. Based on previous analysis by another student in Dr. Bressler's lab using the same pulp, the composition of the pulp was known to be 79.1 ± 1.0% cellulose, 21.2 ± 0.6% hemicellulose (xylan), and 4.0 ± 0.1% lignin (Beyene *et al*., 2017). The amount of glucose from the enzyme blanks was then subtracted into its corresponding treatment, and the % cellulose conversion and % xylan conversion were calculated, using an anhydrous correction factor of 0.9 for cellulose and 0.88 for xylan. The cellulose conversion % of the Cellic Ctec2 treatment was 24.9 ± 1.8 %, and the xylan conversion % was 24.8 ± 2.3 %. For Celluclast® 1.5L, cellulose conversion was 6.1 ± 1.7 %, while xylan conversion was 8.7 ± 2.0 %. These experiments confirmed that the enzymes used in this study were indeed active, even though it can be seen that the Celluclast® 1.5L performed worse than Cellic Ctec2. The hydrolysis was only 6 h long, and complete hydrolysis was not expected, this was just a test to see if enzymes were active in the wood pulp. Comparing to literature, a study using an enzyme loading of 35 mg g^{-1} glucan of Celluclast 1.5L and 20 mg g^{-1} glucan of Novozym 188 on wood pulp got a cellulose-to-glucose conversion rate of ~84% after 96 h of incubation (Álvarez *et al*., 2016). A study using Cellic Ctec2 on wood pulp resulted in 76.3% conversion of cellulose to glucose in 72 h. (Aierkentai *et al*., 2017).

Table 4.9 Hydrolysis of Wood Pulp

Monosaccharides measured in HPLC (mg)		
Sample	**Glucose**	**Xylose**
Wood Pulp + Celluclast	61.0 ± 15.6[b]	21.0 ± 4.9[b]
Wood Pulp + Cellic Ctec2	238 ± 14.0[a]	59.8 ± 4.3[a]
Wood Pulp only	ND	ND
Celluclast® 1.5L Only	0.7 ± 0.4[c]	ND
Cellic Ctec2 Only	19.4 ± 2.4[c]	ND

**ND: Not detectable. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The values that share the same letter are not significantly different from each other. The comparisons were made between treatments for each monosaccharide.*

Table 4.10 Percent Conversion of Cellulose and Xylan in Wood Pulp

Percent Conversion		
Sample	**Cellulose conversion (%)**	**Xylan conversion (%)**
Wood Pulp + Celluclast	6.1 ± 1.7 [b]	8.7 ± 2.0 [b]
Wood Pulp + Cellic Ctec2	24.9 ± 1.8 [a]	24.8 ± 2.3 [a]

**Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The values that share the same letter are not significantly different from each other. The comparisons were made between treatments for each polymer*

4.8 Effect of Autoclaving on Enzyme Hydrolysis

To test the previous considerations about the seaweed having some inhibitory effect on the enzymes, the potential to reduce this inhibition effect by autoclaving the seaweed samples was

studied. 100 mg of seaweed sample was used for the treatments, and for the spiked samples and the positive controls, 100 mg of wood pulp was used.

The results for the monosaccharide analysis are summarized in **Table 4.11**. Focusing first on glucose, the highest measurements were from the positive controls (wood pulp + enzymes), which is a good sign that the enzymes were working. This also suggests that there is indeed an inhibitory effect, as the spiked seaweed samples had 100mg of seaweed and 100mg of wood pulp, and the glucose measured was even lower than in the wood pulp positive controls, which only had 100 mg of wood pulp. Next in measurement, is the Autoclaved Seaweed + Wood Pulp + Cellic Ctec2 + Alginate Lyase sample, with 8.54 ± 1.56 mg. This is promising, as it is statistically different from its non-autoclaved counterpart. In fact, a total of five of the autoclaved treatments were statistically different from their non-autoclaved counterparts: Autoclaved Seaweed + Celluclast® 1.5L, Autoclaved Seaweed + Celluclast® 1.5L + Alginate Lyase, Autoclaved Seaweed + Wood Pulp + Celluclast® 1.5L, Autoclaved Seaweed + Wood Pulp + Celluclast® 1.5L + Alginate Lyase, and Autoclaved Seaweed + Wood Pulp + Cellic Ctec2 + Alginate Lyase. On the substrate blanks (only seaweed, no enzymes), there is no statistical difference between the seaweed and its autoclaved counterpart. This shows that the autoclaving had a positive effect on half of the autoclaved seaweed treatments, when compared to its non-autoclaved treatments. For xylose, none was released on the non-autoclaved seaweed treatments, only on the wood pulp positive control, which was expected, and also on the autoclaved seaweed treatments spiked with wood pulp. This helps the previous thinking that there is indeed some inhibition of the enzymes by the seaweed, and that the autoclave is indeed reducing this inhibition, as the enzymes were able to release xylose only after the autoclave treatment. The mannitol, as in previous experiments, has shown that it is

mostly unaffected by enzyme treatments, and can be released in high amounts even in the seaweed only controls.

Table 4.11 Monosaccharide Analysis Results, Effect of Autoclave in Enzyme Hydrolysis

Sample	Glucose (mg)	Xylose (mg)	Mannitol (mg)
Seaweed + Celluclast® 1.5L	1.08 ± 0.05^{ij}	ND	5.65 ± 0.09^{g}
Seaweed + Celluclast® 1.5L + Alginate Lyase	1.31 ± 0.08^{ij}	ND	5.75 ± 0.05^{defg}
Seaweed + Cellic Ctec2	3.51 ± 0.10^{fgh}	ND	6.13 ± 0.10^{abcd}
Seaweed + Cellic Ctec2 + Alginate Lyase	3.79 ± 0.16^{efgh}	ND	6.18 ± 0.04^{abc}
Seaweed + Alginate Lyase	0.70 ± 0.07^{ij}	ND	5.81 ± 0.15^{cdefg}
Seaweed + Wood Pulp + Celluclast® 1.5L	1.23 ± 0.04^{ij}	ND	5.76 ± 0.05^{defg}
Seaweed + Wood Pulp + Celluclast® 1.5L + Alginate Lyase	1.41 ± 0.17^{ij}	ND	5.74 ± 0.03^{defg}
Seaweed + Wood Pulp + Cellic Ctec2	3.47 ± 0.05^{fgh}	ND	6.31 ± 0.36^{ab}
Seaweed + Wood Pulp + Cellic Ctec2 + Alginate Lyase	3.78 ± 0.08^{efgh}	ND	6.12 ± 0.08^{abcd}
Seaweed + Wood Pulp + Alginate Lyase	0.71 ± 0.03^{ij}	ND	5.69 ± 0.19^{fg}
Autoclaved Seaweed + Celluclast® 1.5L	3.26 ± 0.09^{gh}	ND	5.70 ± 0.13^{fg}
Autoclaved Seaweed + Celluclast® 1.5L + Alginate Lyase	3.39 ± 0.21^{fgh}	ND	5.58 ± 0.14^{g}
Autoclaved Seaweed + Cellic Ctec2	4.16 ± 0.16^{efg}	ND	6.06 ± 0.04^{abcdef}
Autoclaved Seaweed + Cellic Ctec2 + Alginate Lyase	5.35 ± 0.14^{de}	ND	6.29 ± 0.30^{ab}

Sample	Glucose (mg)	Xylose (mg)	Mannitol (mg)
Autoclaved Seaweed + Alginate Lyase	1.00 ± 0.03^{ij}	ND	6.10 ± 0.15^{abcde}
Autoclaved Seaweed + Wood Pulp + Celluclast® 1.5L	4.94 ± 0.71^{efg}	0.51 ± 0.13^{d}	5.80 ± 0.11^{cdefg}
Autoclaved Seaweed + Wood Pulp + Celluclast® 1.5L + Alginate Lyase	6.99 ± 1.26^{cd}	1.33 ± 0.48^{c}	5.93 ± 0.02^{bcdefg}
Autoclaved Seaweed + Wood Pulp + Cellic Ctec2	5.07 ± 0.14^{ef}	0.37 ± 0.07^{de}	6.37 ± 0.14^{a}
Autoclaved Seaweed + Wood Pulp + Cellic Ctec2 + Alginate Lyase	8.54 ± 1.56^{c}	1.32 ± 0.50^{c}	6.45 ± 0.10^{a}
Autoclaved Seaweed + Wood Pulp + Alginate Lyase	0.83 ± 0.02^{ij}	ND	5.71 ± 0.14^{efg}
Wood Pulp + Celluclast® 1.5L	21.64 ± 0.53^{b}	6.95 ± 0.13^{b}	ND
Wood Pulp + Cellic Ctec2	40.96 ± 1.99^{a}	10.81 ± 0.24^{a}	ND
Seaweed only (no enzyme)	0.75 ± 0.02^{ij}	ND	5.70 ± 0.13^{fg}
Autoclaved Seaweed only (no enzyme)	0.75 ± 0.05^{ij}	ND	6.17 ± 0.12^{abc}
Wood Pulp only (no enzyme)	ND	ND	ND
Celluclast® 1.5L (Enzyme Blank, no seaweed)	0.18 ± 0.03^{j}	ND	ND
Celluclast® 1.5L + Alginate Lyase (Enzyme Blank, no seaweed)	0.14 ± 0.00^{j}	ND	ND

Sample	Glucose (mg)	Xylose (mg)	Mannitol (mg)
Cellic Ctec2 (Enzyme Blank, no seaweed)	2.33 ± 0.06^{hi}	ND	ND
Cellic Ctec2 + Alginate Lyase (Enzyme Blank, no seaweed)	2.27 ± 0.03^{hi}	ND	ND
Alginate Lyase (Enzyme Blank, no seaweed)	ND	ND	ND

***** *ND: Not detectable. Data expressed as mean ± standard deviation. Statistics shown were done using Tukey's HDS test (α = 0.05). The comparisons were made between treatments for each monosaccharide. The values that share the same letter are not significantly different from each other. Fucose was also determined in this analysis, but not detected, so it was excluded for clarity.*

5. Discussion and Conclusions

5.1 Discussion

Algae as biomass for the production of ethanol has been studied for years (Hannon *et al*., 2010). Countries with large amount of coastline, such as Canada, have an abundance of this resource. The use of microalgae has its advantages, such as availability, but it comes with its disadvantages as well, the most important being the current economic feasibility. The use of enzymatic hydrolysis has been successful in the past at hydrolyzing several species of algae. The research in this thesis was focused on studying the use of *Ascophyllum nodosum*, a brown algae, as feedstock for the extraction of monosaccharides using a more environmentally friendly methodology, as well as the co-production of a value-added plant biostimulant. This would help address economic feasibility as mentioned previously. In this case, environmentally friendly refers to the fact that it doesn't use toxic chemicals or high quantities of acid and/or base that could yield harmful wastes. In this research, enzyme hydrolysis was used as the algal processing method of choice.

Four different enzyme systems were chosen and used in this study: Celluclast® 1.5L, Cellic Ctec2, Viscozyme® L, and Alginate Lyase, and different pretreatments were used (milling, freeze/thaw, autoclaving). It should be noted that Celluclast® 1.5L and Cellic Ctec2 were chosen due to their widespread use in literature and in the saccharification of lignocellulosic biomass. That being said, the enzymes used were not made specifically to hydrolyze seaweed, which has a different structural composition than terrestrial plants.

The first endeavor of this study focused on the characterization of the seaweed. Proximate analysis (moisture, ash, crude fat and crude protein) were performed, as well as an elemental analysis using ICP-OES (Ca, Mg, Na, K, P, S, Fe, Mn, Cu, and Zn) and a monosaccharide analysis

using HPLC. The elemental analysis was mainly done, to study the seaweed sample given, but also to help identify possible inhibition of the enzymes due to high salt concentrations. In this project, it was concluded that it was not the case, as the solid loading was too little for the enzymes to be affected. There are few studies that have gone into the characterization of *A. nodosum* specifically. One of them is the one of Lorenzo *et al.,* 2017 (Lorenzo *et al*., 2017). They performed proximate analysis and elemental analysis on their sample. The results in this study for crude fat and crude protein were 3.07 ± 0.39 % dry wt. and 7.35 ± 0.21 % dry wt., which are fairly similar to the results found in the study by Lorenzo *et al*., 2017, at 3.62 ± 0.17 dry %wt. and 8.70 ± 0.07 dry %wt., respectively. Moisture and ash content results were 4.1 ± 0.2 %wt. and 21.3 ± 1.2 dry %wt. from this study, which are fairly different from the ones on the Lorenzo *et al*., 2017 research, which were 11.08 ± 0.53 %wt. for moisture content and 30.89 ± 0.06 dry %wt. for ash content. This difference in moisture content will depend a lot on the starting material used and its initial water content. The difference in ash content could be due to seasonal variations. Looking at the elemental analysis performed by Lorenzo *et al*., 2017, it is fairly similar to the one presented in this research project. Sodium was the highest elemental present, followed by potassium, calcium, then magnesium, with a little bit of iron and manganese. The difference was that little amounts of phosphorous, zinc, and copper were found in this research, while none was found in the other study (Lorenzo *et al*., 2017). Another similar study found ash content to be from 18 to 27% of dry matter (Baardseth, 1970). Another study found Strontium as a trace element in 2600 ppm, which is an element that was not accounted for in the ICP-OES in this project (Black & Mitchell, 1952). Alginate has been found to be from 22-30 % weight dry matter. (Baardseth, 1970). Another study finds protein content at 5-10 %, and ether extracts (crude fat) at 2-4 % (Jensen *et al*., 1968). One study did saccharification of *A. nodosum*, and found 29 mg of glucose per gram of seaweed, or 2.9

% dry wt. (Yuan & Macquarrie, 2015). The results for the monosaccharide analysis performed in *Section 4.1.5* show a little bit more, at 4.2 ± 0.3 % dry wt. of glucose (12.5 ± 0.96 mg of glucose/300 mg of seaweed sample used *x* 100). Another study got a glucose content of 4.48 % dry weight, similar to the results in this research (Dierick *et al*., 2009). In terms of mass balance, we have accounted for around 55.2% of the seaweed. As stated earlier, tannins can contribute up to 9% weight and alginates up to 30% (Baardseth, 1970) adding up to around 94.2% of accounted mass. While not the focus of this research project, the high quantities of alginate may make it attractive to diversify into other high value added products by its extraction.

Next was the enzyme hydrolysis. Based on the various experiments that were performed, while there was a statistical difference between some of the monosaccharide measurements of the enzyme treatments and the controls (enzyme blanks and mock treatments), the enzyme treatments used did not release all of the available monosaccharides when compared to the total monosaccharide content measured using a two-step acid hydrolysis. Based on the results of the monosaccharide characterization analysis in *Section 4.1.5*, 4.18 ± 0.32 % dry wt. of glucose (12.5 ± 0.96 mg of glucose/300 mg of seaweed sample used *x* 100) was present in the seaweed. Conversely, in *Section 4.3.5,* it can be seen that 9.89 ± 0.37 mg of glucose was observed in the enzyme hydrolysate when Cellic CTec2 + Alginate Lyase were used. If the glucose is taken into account (i.e. subtracted) from the enzyme blank (6.94 ± 0.10 mg of glucose), the amount of glucose released from Cellic CTec2 + Alginate lyase treatment of seaweed was 2.95 ± 0.38 mg of glucose, or 2.95 ± 0.38 % dry weight of glucose (2.95 ± 0.38 mg of glucose/100mg of seaweed sample used *x* 100). Thus, compared to the amount of glucose present in the seaweed, this enzyme treatment released 70 ± 9% of the glucose present in the acid hydrolysis. Of the various enzyme treatments, this was the best result obtained. Comparing to the literature, Cellic Ctec2 has been also tested on

the brown seaweed, *Laminaria digitata,* for enzyme saccharification. In that study, an enzyme loading of 10% (v/w) released all of the available glucose in the seaweed sample in 8 h (Manns *et al*., 2016a). Similarly, another study used Celluclast® 1.5L and alginate lyase to hydrolyze again *Laminaria digitata*, with a maximum recovery of glucose at 84.1% (Hou *et al*., 2015). One core difference from these results, when compared to the results in this study, is that their enzyme loading was very high compared to what was used in this thesis, the first study using 10% (v/w) in enzyme loading. Another difference, and one of the reasons the enzymes may have had reduced activity, is that *Acophyllum nodosum* is a very good source of polyphenols (Kadam *et al*., 2015a). Polyphenols have been studied for their role in inhibition of cellulases (Qin *et al*., 2016; Sineiro *et al*., 1997; Ximenes *et al*., 2010). While the effect of *Ascophyllum nodosum* polyphenol extracts have not been studied on cellulases, it has been studied that it inhibits lipase activity *in vitro* (Austin *et al*., 2018), which means that it could potentially inhibit other types of enzymes, like cellulases, which in *Section 4.8*, autoclaving the seaweed was used as a way to reduce this inhibition. This is discussed further at the end of this Section.

Xylose and fucose levels were also measured, both of which could not be detected in the enzyme treatment experiments. This could be because of several reasons. Xylose is the main component in hemicellulose, and xylose was indeed found in the characterization step, pointing to there being at least a small amount of hemicellulose. Consequently, since there was little glucose released in the seaweed by the enzyme treatments, it could also mean that the hemicellulose was not broken down, yielding levels of xylose lower than the detectable limit of the RID detector used in the HPLC. Also, in the experiment using Celluclast® 1.5L and Cellic CTec2 on wood pulp, it was found that Celluclast® 1.5L had more trouble on the wood pulp (which contains hemicellulose) than Cellic CTec2, even when the same activity (FPU/g) was added for each

enzyme, which leads to the thought that Celluclast® 1.5L may not be very suitable to degrade hemicellulose in the first place. Moving to fucose, when other literature has tried the saccharification of seaweed, they do not use any specific enzymes for fucose (Sharma & Horn, 2016), as the goal of most of those studies is trying to use seaweed as feedstock for bioethanol production, and the fermentation of fucose produces 1,2-propanediol (Badía *et al*., 1985), not ethanol. In a study, Cellic CTec2 and alginate lyase were used, and while fucose was also not found in the liquid hydrolysate after enzyme hydrolysis, further acid hydrolysis treatment using sulfuric acid after enzyme hydrolysis did yield fucose (Manns *et al*., 2016b). This suggests that while cellulase enzyme blends like Cellic CTec2 do not break the fucoidan down to its fucose monomers, the fucoidan could be present in the liquid hydrolysate after enzyme hydrolysis. This would explain the missing fucose in the enzymatic experiments in this thesis, but also opens other future possibilities for this project, which will be discussed further in the next Section, *5.2.2*. Mannitol was the last monosaccharide that was measured. Mannitol was largely unaffected by any enzyme treatment, and was actually present even in mock treated seaweed in significant quantities, which could mean that mannitol could be extracted without the aid of enzymes, only milling to open up the structure and a mild water hydrolysis is needed.

The freeze/thaw pretreatment was chosen because it is simple and did not involve the addition of chemicals for the pretreatment. Ice crystals are created while slowly freezing the sample, which breaks the cell walls, leaving the substrate more accessible to enzymatic attack. Based on the results in this study, the freeze/thaw pretreatment did not have any effect on the amount of monosaccharides released by the enzymes. There is a study using freeze thaw pretreatment in ethanol production using *Juncus maritimus*, a land plant that grows near the ocean. In their case, it proved to be a good method of pretreatment, even better than dilute acid

pretreatment (Smichi *et al*., 2016). This could be an isolated case of success, as most cases of the use of freeze thaw pretreatment are in non-plant cell disruption, such as animal cells or bacterial cells (Chaiyarit & Thongboonkerd, 2009; Johnson & Hecht, 1994; Shehadul Islam *et al*., 2017; Tansey, 2006). Furthermore, as a disadvantage to using this pretreatment method, it should be taken into consideration that freeze thaw, while at lab scale can work, can be a major problem when scaling up due to the enormous amounts of energy consumption and time that it could add to the complete process.

Due to the low quantities of monosaccharides being released by our enzymes, another method of evaluating the effect of the enzyme treatments would be to see the actual physical damage that was caused by the enzymes. For this, scanning electron microscopy (SEM) was used. When comparing the native untreated seaweed image to the treated seaweed images, it can be seen that the surface gets rougher and looks more porous. While that comparison can be subjective, it could suggest that the enzymes are working on the seaweed. When comparing the results in this research to the literature, other SEM images of seaweed pretreated with acid followed by an enzyme hydrolysis, the after images on the study look considerably more porous and rough than those of the enzyme treatments in this project (Azizi *et al*., 2017). This may suggest that the enzyme treatments are not having the same impact on the seaweed's structure. The difference could also be because of the acid pretreatment, as it has been known to increase enzyme hydrolysis efficiency and increase monomeric sugar release (Azizi *et al*., 2017). This could then lead to better enzyme hydrolysis and thus, a more porous and rough look in the SEM images after the hydrolysis.

To address the second part of this study, the co-production of value-added plant biostimulants, enzyme treated samples were sent to Acadian Seaplants Ltd. In their facilities, they conduct several different assays to test for plant bioactivity, one of them being the mung bean

rooting assay described previously in *Section 3.5*. The enzyme treated samples, while statistically different (and inferior) from the commercial extract used as a control, performed better than all of the other controls, as described in *Section 4.5*, meaning that there are one or more bioactive components that produce a rooting effect on plants. The Kelp mock treatment control also performed as well as the enzyme treatments. This means that milling and incubation at a mild temperature was sufficient to release cellular components that have a biostimulant effect on the roots. Also relevant is that the least effective enzyme treatment was Cellic CTec2 + Alginate lyase. This could have been because of alginate lyase is breaking down the seaweed's alginate, which is believed to have a negative effect on the bioactivity of the extracts, as seaweed alginates are being studied for their biostimulant effects on plants (du Jardin, 2015).

Based on the interpretation of the enzyme hydrolysis studies, one of the concerns was that the activities of the enzymes used were not as high as anticipated. To prove that the enzymes used were indeed active, it was decided to spike seaweed samples with carboxymethyl cellulose as a positive control. In these experiments, the enzymactic activities were low. This suggested that either there were problems with the enzymes (loss of activity or possible inhibition), or that the carboxymethyl cellulose was not an ideal substrate for the enzymes. Since significant hydrolysis of the CMC positive controls was not observed, the first thought was either low enzyme activity or wrong substrate. The filter paper activity tests showed that the enzymes indeed had activity, so the next step was to see if the substrate was the problem. A carboxymethyl cellulose activity test was performed to measure the compatibility of the substrate with the enzymes (Ghose, 1987). The results were of very low activity of the enzymes in the CMC, so it was decided to change the positive control.

A material that was on hand in the lab from other studies was wood pulp, which had previously been worked with successfully using cellulases. First was to try hydrolyzing wood pulp with the enzymes. The results from these experiments, *Section 4.7*, were positive and demonstrated that the enzymes were active on that substrate, although it can also be seen that Celluclast® 1.5L is not as good at breaking down the wood pulp compared to Cellic CTec2, even though the same activity (15 FPU/g of wood pulp) was used for both. This difference in monosaccharide measurements is likely due to the fact that the FPU assay is done with filter paper (highly refined cellulose), while, wood pulp has a more complex structure (hemicellulose), which Celluclast® 1.5L may not be as well suited to degrade.

After having a better substrate to use as a positive control, it was decided to retest one of the questions that the CMC experiment could not prove because the control substrate was not ideal. *Is the seaweed inhibiting the cellulases?* As stated previously, *A. nodosum* is known to have significant quantities of polyphenols, and these have been shown to inhibit certain enzymes. It is also known from literature that a way to reduce polyphenol content is through temperature treatment, as seen on grape seed extracts and grape pomace (Chamorro *et al*., 2012), thyme (Vergara-Salinas *et al*., 2012) and on other brown seaweed species (Rajauria *et al*., 2010). The effect of autoclaving seaweed as a pretreatment before enzyme hydrolysis was studied, now using wood pulp as the positive control.

5.2 Future Recommendations

5.2.1 Separation of the plant biostimulants and the sugars

It is already known that there are, in fact, plant biostimulant components released even when the seaweed is only milled and incubated in a buffer at relatively low temperatures with no enzymes. This means that separating the bioactive components could be a relatively simple

process. After milling and incubation, the supernatant, which contains the biostimulants, could be separated from the solids. Afterwards, a second hydrolysis step could be incorporated to extract the sugars from the seaweed, without needing to worry about destroying the biostimulant as they have already been recovered.

5.2.2 Focus on Fucose

Looking back at the initial characterization, fucose is the most abundant sugar of those identified (i.e. glucose, xylose, mannitol, and fucose). It may be possible to extract this sugar as a fucoidan polysaccharide, or as fucose, its monomer. Fucoidans are being studied for their potential uses in various therapies as they function as anti-coagulants and anti-inflammatories. It could also have potential anti-proliferatory activity on cancerous cells (Ale & Meyer, 2013). Fucose as a monomer could also be fermented using *Salmonella typhimurium* or *Klebsiella pneumoniae* to produce 1,2-propanediol (Badía *et al*., 1985), which is used as a non-toxic antifreeze in various foods, for the production of resins, added to food as humectant or added as a solvent for other flavoring agents (Szajewski, 2009). As stated previously in *Section 5.1*, there was a recent study that discovered that while fucose was not present in the enzyme hydrolysate when using Cellic CTec2 and alginate lyase on the brown seaweed, *Laminaria digitata*, acid hydrolysis on the liquid hydrolysates did in fact release fucose, meaning that the fucoidan was extracted into the liquid hydrolysate on the enzymatic hydrolysis step (Manns *et al*., 2016b). Enzymes that modify fucoidan are classified as fucoidanases (E.C. 3.2.1.44), which are extracted from various sources, including the bacteria *Fucophilus fucoidanolyticus* (Ale & Meyer, 2013). These enzymes could be further used to modify the properties of the fucoidan, or break it down to its monomer to produce fucose.

5.2.3 Chemical pretreatments

In the scope of this study, a more environmentally friendly method to extract sugars from seaweed was tried using no harsh chemicals. That being said, addition of mild chemical pretreatments, such as alkaline and acid pretreatments, could help increase the amount of sugars extracted by enzymes by opening up the structure of the seaweed and making it more accessible for enzymatic hydrolysis. Such experiments may help in the development of strategies to improve enzyme treatment of seaweed.

5.3 Conclusion

In summary, this work showed that enzymes that perform really well in terrestrial plants and cellulosic material from those plants, and that have been studied and used extensively throughout literature, namely, Celluclast® 1.5L and Cellic CTec2, do not work as well with the brown algae *Ascophyllum nodosum.* The several enzyme hydrolysis treatments that were performed did not release significant amounts of monosaccharides, even though Scanning Electron Microscopy showed that there were some changes in the superficial structure of the algae before and after the enzyme treatments. Also, mild pretreatments such as freeze/thaw did not have any impact on the release of monosaccharides, so stronger pretreatments should be used such as acid pretreatment for better results. It was also discovered through testing that the low activity of the enzymes may be caused by inhibition of the cellulases by a component in the seaweed itself, possibly by the polyphenols that can be found in *Ascophyllum nodosum*. By autoclaving the seaweed as a pretreatment, comparatively more monosaccharide release could be seen in some of the enzyme treatments, suggesting that it was an effective method to reduce the inhibitors in the seaweed and increase the enzymatic hydrolysis efficiency, which later studies can help improve upon with this previous knowledge. It was also discovered that enzymes, or any other chemical

digestion, were not needed in order to release plant biostimulants, only milling and a mild incubation. This biostimulants that were extracted did increase the rooting activity of the mung bean plants they were tested with. This has solid implications in the industries of algae fertilizers, such as Acadian Seaplants Ltd. Although further study and optimization is needed, the implication that plant biostimulants can be extracted from *Ascophyllum nodosum* without using a harsh chemical extraction, such as alkaline extraction, makes downstream valorization easier, as the solid seaweed by-product is not filled with salts and of very high pH, but it also has the potential to cut down production costs and energy consumption, as most chemical extractions are done at very high temperatures. The data presented in this thesis highlight the industrial potential for the co-production of biostimulants and monosaccharides from abundant and renewable seaweed feedstocks.

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