Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

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Protein extraction from the brown seaweed *Saccharina latissima* and *Alaria esculenta*

The effect of ultrasonication and enzymatic treatment

Master's thesis in Biotechnology
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PREFACE

This master thesis was written at the Department of Biotechnology and Food Science, Faculty of Natural Sciences, at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway. The lab work was performed at the Food Chemistry lab with Turid Rustad as a supervisor. I would like to thank Turid for solid theoretical background, good discussions, essential problem solving and hours of proofreading. She has been patient and helpful, and a great support through the whole process. I want to thank the laboratory engineer Siri Stavrum for the practical help and advices in the lab, as well as performing all HPLC analyses. Additionally, I want to thank Marte Schei at SINTEF for performing CN analysis.

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ABSTRACT

Worldwide food production is facing a greater challenge than ever before. The population growth increases the demand for food, especially proteins. We must develop new, sustainable food supplies, such as exploiting aquatic plants. Seaweed can have relatively high protein levels and contain other important nutrients, as well as having a low caloric content. Brown algae have a lower protein content compared to red and green species, but they are larger in size, available in high amounts along the coast and can be readily cultivated in Norway. They do not grow very deep and are therefore easy, cheap and fast to harvest. This gives brown algae a high potential for large-scale production for use as food and nutrients.

Despite their appealing characteristics and high potential for use in food and feed, macroalgae are under-exploited resources. This is partly due to lack of knowledge leading to a small market and low demand in the Western world. Challenges are also safety hazards present in macroalgae that might make them unpleasant or dangerous to consume, in addition to the low bioavailability of valuable seaweed nutrients. Both problems can be overcome when extracting nutrients from macroalgae, and there is therefore a need to study and improve specific extraction methods. Extraction assisted by ultrasonic treatment and enzymatic hydrolysis can give effective biomass degradation with reduced time, solvent, and energy consumption. This will be more sustainable, reproducible, and economically favourable than conventional techniques.

The objective of this thesis was to investigate protein extraction from two species of brown macroalgae from the Norwegian coast, *Saccharina latissima* and *Alaria esculenta*. The focus was different methods for pre-treatment of the macroalgal biomass and how this affects the protein extraction. The effects of independent and combined treatment by ultrasonic waves and enzymes was evaluated. Ultrasonic waves were used to collapse the cell wall matrix to release cavitated proteins and thereby increase the protein extraction. The enzymes Alginate lyase, Viscozyme L and Cellic CTec2 were used for degrading the cell wall polysaccharide matrix, and Alcalase was responsible for proteolytic breakdown of peptide bonds in proteins. The protein analysis was mainly done by the Lowry method, but some samples were also measured with CN and total amino acid analyses for comparison.

Results show that enzymes and sonication increase the protein extraction in *S. latissima* and *A. esculenta*. The efficiency of protein extraction depends on water to seaweed ratio, sonication settings, enzyme concentration, polyphenol content in the algae, state of the biomass (wet or

dried), and extent of degradation prior to treatment. Also, the combination of different treatments and treatment order is crucial. There were significant differences between the old and new *S. latissima*, with up to 240 % increase in protein extraction yield in the old batch due to partly degraded biomass. There was a positive correlation between extracted polyphenols and protein for both algae, but with the most potent effect in *A. esculenta*. The enzyme concentrations giving the best protein yield for wet algae was 100 U/g (dw) Alginate lyase and 3 % (ww) Cellic CTec2 for both algal species, and respectively 196 µl/g and 98 µl/g Viscozyme for *S. latissima* and *A. esculenta*.

Combined treatment from enzymes and sonication improved protein yield for both wet and dry algae. Sonication followed by enzymes seemed to be the best treatment order, but this was not conclusive in all cases. Dry algae gave significantly higher yield than wet algae for all compared treatments except for one. For dry algae, sonication combined with enzymes increased protein yield compared to that of enzymes and sonication alone, although not always significantly. The highest protein yield for dry algae was from treatment with US → Alginate lyase for *S. latissim*a with 6.01 ± 0.03 % (dw) and US → Cellic CTec2 for *A. esculenta* with 5.59 ± 0.21 % (dw).

Sequential enzymatic treatment with Cellic and/or Alcalase significantly increased protein yield. Alcalase, alone or in combination with Cellic CTec, gave a significantly higher protein yield compared to that of Cellic CTec alone. The highest protein yield was from treatment by Alcalase for wet *S. latissima* with a protein yield of 11.75 ± 0.63 % (dw) and Cellic CTec + Alcalase for wet *A. esculenta* with yield of 6.05 ± 0.09 % (dw). These were the treatments with the highest extraction yield for all experiments. However, Cellic seemed to reduce protein size more than Alcalase. Amino acid profiles showed that all measured amino acids are present in all extracts, and the most abundant amino acids are alanine, glutamic acid and aspartic acid. Protein yield varies according to the analytical method used. Lowry analysis provided higher yield than CN and total amino acid analysis. The most correct estimation of protein yield is thought to be something between what is recorded from the three methods.

Extraction yield from *S. latissima* was higher than that of *A. esculenta* in all experiments, with few exceptions. Enzymatic treatment was more efficient for *S. latissima*, while ultrasonication affected *A. esculenta* to a greater extent. The overall most promising extraction is seen for biomass with combined treatment, either a combination of sonication and enzymes or several enzymes of different specificity.

SAMANDRAG

Verdsomspennande matproduksjon møter no ei større utfordring enn nokon sinne. Aukande populasjon gjev auka etterspurnad etter mat, spesielt protein. Me må utvikle nye, berekraftige matforsyningar, som for eksempel akvatiske planter. Sjøgras kan ha relativt høgt proteininnhald og inneheld andre viktige næringsstoff, i tillegg til å ha eit lågt kaloriinnhald. Brunalgar har lågare proteininnhald samanlikna med raude og grøne artar, men dei er større, tilgjengelege i store mengder langs kysten, og kan enkelt dyrkast i Noreg. Dei veks ikkje djupt og kan derfor haustast enkelt, billig og raskt. Dette gjev brunalgar eit høgt potensial for storskala produksjon til bruk som mat og næringsstoff.

Trass i sine eigna karakteristikkar og høgt potensial for bruk i mat og fór, er makroalger framleis underutnytta ressursar. Dette er delvis grunna manglande kunnskap som gjev ein dårleg marknad og låg etterspurnad i Vesten. Utfordringar er også uønskte komponentar til stades i makroalger som gjer dei uønskeleg eller farlege å ete, i tillegg til låg biotilgjengelegheit av ønska næringsstoff. Begge desse problema kan ein overvinne ved å ekstrahere næringsstoff frå makroalgane, og det er derfor naudsynt å studere og forbetre spesifikke ekstraksjonsmetodar. Ekstraksjon assistert med ultralydbehandling og enzymhydrolyse kan gi effektiv degradering av biomasse med redusert forbruk av tid, løysemiddel og energi. Dette er meir berekraftig, økonomisk gunstig og reproduserbart samanlikna med konvensjonelle teknikkar.

Målet med denne masteravhandlinga var å undersøke proteinekstraksjon frå to brunalgeartar frå Norskekysten, *Saccharina latissima* og *Alaria esculenta*. Fokus var ulike metodar for forbehandling av makroalgane, og korleis dette påverkar proteinekstraksjon. Effekten av enkel og kombinert behandling ved ultralydbølger og enzym vart drøfta. Ultralydbølger vart brukt til å kollapse celleveggen for å frigjere innfanga protein, og deretter auke proteinekstraksjon. Enzyma Alginat lyase, Viscozyme L og Cellic CTec2 vart brukt til degradering av polysakkarid i celleveggen, og Alkalase skulle stå for proteolytisk nedbryting av peptidbindingar i protein. Proteinanalyse vart hovudsakleg gjort ved Lowry-metoden, men nokre prøvar vart også målt med CN-analyse og total aminosyreanalyse til samanlikning.

Resultat viser at enzym og sonikering aukar proteinekstraksjon i *S. latissima* og *A. esculenta*. Effektiviteten til proteinekstraksjon avhenger av mengdeforholdet mellom vatn og alge, innstillingar for sonikering, enzymkonsentrasjon, polyfenolinnhald i algane, tilstanden til biomassen (våt eller tørr), og graden av degradering før behandling. Også kombinasjonen av ulike behandlingar og behandlingsrekkjefølge er avgjerande. Det var signifikante forskjellar

mellom gamal og ny *S. latissima*, med opp til 240 % auka utbytte frå proteinekstraksjon i det gamle partiet, grunna delvis degradert biomasse. Det var positiv korrelasjon mellom ekstraksjon av polyfenolar og protein i begge algar, men effekten var meir tydeleg i *A. esculenta*. Enzymkonsentrasjonane med best proteinutbytte for våt alge var 100 U/g (tørrvekt) Alginat lyase og 3 % (våtvekt) Cellic CTec2 for begge algeartar, og høvesvis 196 µl/g og 98 µl/g Viscozyme for *S. latissima* og *A. esculenta*.

Kombinert behandling frå enzym og sonikering gav auka utbytte i både våt og tørr alge. Sonikering etterfylgt av enzym såg ut til å vere den beste rekkjefølga for behandling, men dette var ikkje einrøystes for alle tilfelle. Tørr alge gav signifikant høgare utbytte enn våt alge for alle samanlikna behandlingar bortsett frå éi. For tørr alge gav sonikering kombinert med enzym auka proteinutbytte samanlikna med enzym og sonikering aleine, men forbetringa var ikkje alltid signifikant. Høgst proteinutbytte for tørr alge var frå behandling med US → Alginat lyase for *S. latissima* med 6.01 ± 0.03 % (tørrvekt) og US → Cellic CTec2 for *A. esculenta* med 5.59 ± 0.21 % (tørrvekt).

Sekvensiell enzymbehandling med Cellic og/eller Alkalase gav signifikant auke i proteinutbytte. Alkalase, aleine eller kombinert med Cellic CTec, gav signifikant høgre proteinutbytte samanlikna med Cellic CTec aleine. Høgst proteinutbytte var frå behandling av Alkalase for våt *S. latissima* med proteinutbytte på 11.75 ± 0.63 % (tørrvekt) og Cellic CTec + Alkalase for våt *A. esculenta* med utbytte på 6.05 ± 0.09 % (tørrvekt). Dette var behandlingane med høgst proteinutbytte blant alle eksperiment. Likevel såg Cellic CTec ut til å redusere størrelsen på proteina i større grad enn Alkalase. Aminosyreprofilane viste at alle målte aminosyrer er til stades i alle ekstrakt, og det er mest av aminosyrene alanin, glutaminsyre og aspartinsyre. Proteinutbytte varierer i forhold til analysemetoden som vert brukt. Lowry-analysen gav høgare utbytte enn CN- og total aminosyreanalyse. Det beste estimatet for proteinutbytte er antatt å vere ein mellomting mellom det som er målt frå dei tre metodane.

Utbytte ved ekstraksjon frå *S. latissima* var høgre enn for *A. esculenta* i alle eksperiment, med få unntak. Behandling med enzym var meir effektivt for *S. latissima*, mens ultralyd påverka *A. esculenta* i større grad. Totalt sett er ekstraksjon mest lovande frå biomasse som har fått påført kombinert behandling, enten ein kombinasjon av sonikering og enzym eller fleire enzym med ulik spesifisitet.

CONTENT

1 INTRODUCTION

Sustainability is one of the main societal challenges. There is a continuously growing world population of at present about seven billion people, from which about 10 % are undernourished due to inadequate food access or resources (Rutledge *et al.*, 2011; Aizen *et al.*, 2019). Another concern is the increasing obesity due to excessive intake of animal products, inducing various health risks (Springmann *et al.*, 2016). To overcome these challenges, we need to find new food resources that are both sustainable and nutritious, preferably being from plant sources. Agriculture already uses almost half of the arable land in the world and provides most of the world's food. It consumes 90 percent of the water used by humanity, generates 25 % of the global emissions, and leads to use of pesticides that pose a chemical hazard to ecological niches and the health of people and animals (Rutledge *et al.*, 2011; Aizen *et al.*, 2019). By 2050 we need to have doubled the world's food production as the population is estimated to reach 10 billion people (Gibbens, 2018). An accompanying consequence is the shortage of protein sources, which will make it more critical to supply enough protein for both human food and animal feed (Aiking, 2014).

As of now, a general estimate is that 6 kg of plant protein is consumed to produce 1 kg of meat protein (Aiking, 2014). In addition, 100 times more freshwater is needed when producing animal protein compared to the equal amount of plant protein (Pimentel and Pimentel 1996, referred by Pimentel and Pimentel, 2003). It is cheaper to produce proteins from plants than those from animals. Thus, using protein directly from the plants is more beneficial and more sustainable, and should be done to a greater extent (Yun *et al.*, 2005). The biggest problem with plant proteins as food source is that they lack certain essential amino acids (Ufaz and Galili, 2008). It is therefore vital to look at alternatives to proteins from both animal and terrestrial plant sources that are sustainable and economically viable in production as well as having adequate functional properties and fulfilling the nutritional requirements. We must be innovative, and aquaculture might be a solution to the problem (Gibbens, 2018).

Even though water covers approximately 75 % of the earth's surface (Chahine, 1992), only about 6.5 % of the global consumption of protein from food comes from the ocean, mainly from fish and shellfish (Béné *et al*. 2015, referred by Mæhre, Jensen and Eilertsen, 2016). Using more from the potential that lies in the seas and waters of the world can unlock new resources to help sustain our planet. In general, ocean farms are more sustainable than land-based production, as they do not need fresh-water, fertilizers or land area (Tiwari and Declan, 2015).

Ocean farms require minimal human intervention, have fast growth rates, rapid reproduction, and high biomass yield (Torres, Kraan and Dominguez, 2019). For this reason, they do not compete for resources with traditional crops (Bleakley and Hayes, 2017). Also, seaweed can efficiently absorb carbon dioxide from seawater, and therefore contributes to improve the absorption of atmospheric CO_2 (Tang, Zhang and Fang, 2011; Torres, Kraan and Dominguez, 2019). The fast growth rate and photosynthesis gives reduced carbon, and supplies oxygen to the ocean (Kraan, 2013)

Seaweed has appeared to be increasingly used as a food resource, with proteins possibly better suited for human consumption than other vegetable protein sources (Suresh Kumar *et al.*, 2014). Besides, the protein content in seaweed might be relatively high. Some seaweed species have protein levels comparable to that of protein-rich products as e.g. eggs and soybeans (Fleurence, 1999). According to FAO in 2018, the global production of macroalgae was 2.94×10^7 tons (wet weight). The potential for marine macroalgal cultivation is estimated to be in the range of 10^9-10^{11} tons per year (dry weight) (Lehahn, Ingle and Golberg, 2016). Thus, macroalgae are still under-exploited (Bleakley and Hayes, 2017). Fortunately, we have now started to open our eyes for seaweed, understanding that it can be a good source of food and various nutrients for the growing population (Tiwari and Declan, 2015).

The valuable nutritional content, the potential for sustainable growth, and the thought of untouched resources establishes a curiosity for studying macroalgae and how to make it more usable and visible as a food resource. Biomass treatment might be a suitable way to extract seaweed nutrients better, expand the nutritional applications, and make it a resource well suited for use in food and feed.

1.1 Seaweed

As already pointed out, seaweed, also called macroalgae, are more than the wrapping around sushi. Seaweed is a group of marine, multicellular or unicellular, photosynthetic, eukaryotic organisms (Levine, 2016), with around 80 % moisture (Kadam, Álvarez, *et al.*, 2015b). Seaweeds are plants, and they are therefore primary producers of macronutrients by utilizing their photosynthetic and auxiliary pigments for photosynthesis (Jacquin *et al.*, 2014; Mæhre, Jensen and Eilertsen, 2016). They take up elements like carbon, nitrogen and phosphorous from the environment and convert them into macronutrients like proteins, carbohydrates, and lipids. These nutrients are further used for growth or maintenance of the plant, or stored inside the

cells (Mæhre, Jensen and Eilertsen, 2016). They are very diverse due to different shapes, size and composition (Kadam, Álvarez, *et al.*, 2015b).

The varying content of different pigments is what gives the distinct colours (Kadam, Álvarez, *et al.*, 2015b). According to composition and nutrients, seaweeds are classified as red (Rhodophyta), brown (Phaeophyta), and green seaweeds (Chlorophyta) (Belghit *et al.*, 2017). Pigments in seaweed are chlorophyll, carotenoids and xanthophylls. The colour of seaweed mainly comes from the pigments fucoxanthin for brown algae, phycoerythrin and phycocyanin for red algae and chlorophyll for green algae (Aryee, Agyei and Akanbi, 2018). Red and green seaweeds are usually small, measuring between a few centimetres to about a metre in length. Contrary, brown seaweeds are larger and can range from the small 30-60 cm long species, the medium 2-4 m long thick species, to the largest 20 m long ones (FAO, 2003).

As large parts of the world are covered by the ocean with macroalgae in the upper layers, the photosynthesis and primary production of seaweed is crucial for the ecosystem (Mann 1973, referred by Wiencke and Bischof, 2013). In 2016 there were over 10 000 described macroalgal species (Levine, 2016). I Norway, there are 175 brown, 200 red, and 100 green species of marine macroalgae. Several of them are already being cultivated, but there is a great potential for excessive cultivation. This requires new technology for more economical cultivation and better utilization of the whole raw material (Skjermo *et al.*, 2015). This thesis investigates brown seaweed, and they are therefore described more thoroughly. Seaweed has many synonyms, and in this thesis, they will be referred to by seaweed, macroalgae or algae.

1.1.1 Brown seaweeds

Brown seaweed have their distinctive olive-brown colour (Kadam, Tiwari and O'Donnell, 2013) mainly from the presence of xanthophyll and fucoxanthin masking chlorophyll a and c, beta carotenes and other xanthophylls (Bold and Wynne, 1985; referred by El Gamal, 2010). Brown algae are unique only to exist in the multicellular form (Cock *et al.*, 2010). There are 1500-2000 species of brown algae in the world (Hoek, Mann and Jahns, 1995; referred by Bleakley and Hayes, 2017). They usually are large and can range from the smaller species of 30-60 cm to those of 20 m long. The most used species thrive best in cold climates up to 20 °C, but some species are also found in warmer waters (FAO, 2003). Some of them can get large and form underwater forests that are very important in the ecosystem (Foster and Schei, 1985; referred by Bleakley and Hayes, 2017). For centuries, brown seaweeds have been utilized as

animal feed (Fleurence, 1999; Torres, Kraan and Dominguez, 2019), with growing importance also for human consumption (Fitzgerald *et al.*, 2011).

Brown seaweed has a high polyphenol content (up to 15 % dw) (Ragan, Glombitza 1986; Targett and Arnold, 1998; referred by Koivikko *et al.*, 2007) and low protein content (3-15 % dw) (Fleurence, 1999) relative to the red and green species. There are also many bioactive metabolites with different pharmacological activities (El Gamal, 2010). The fraction of various compounds in seaweed shows large seasonal variations due to the differences in temperature and availability of light and nutrients. For protein and amino acid synthesis, a good ambient nitrogen supply is essential, while polysaccharide content is profoundly affected by the carbon availability. An example of wide seasonal variation is seen in *Saccharina latissima*, where the content of mannitol and laminarin can go from total absence in winter to around 26 % (dw) in summer (Wiencke and Bischof, 2013).

Until now, research has mainly been done on red and green macroalgae as protein sources, whereas the brown species are less studied due to the lower protein content. However, using brown algae can still be valuable from an economical and sustainable point of view. They have fast growth (Vilg and Undeland, 2017), large size, are the most familiar and most abundant of the seaweeds. Generally speaking, the red and green species grow deeper and are much smaller, which makes their harvest more complicated, more costly, and more time consuming than that for brown algae (FAO, 1976). Since brown algae are already widely used as sources for i.e. alginates, co-extraction might be an easy way to benefit other nutrients at the same time (FAO, 2003; Vilg and Undeland, 2017). Thus, there are many benefits of using brown algae, and they might be an auspicious food and protein source in the future. Therefore, two species of brown seaweed will be further studied, *Saccharina latissima* and *Alaria esculenta*. These are presented in Figure 1.

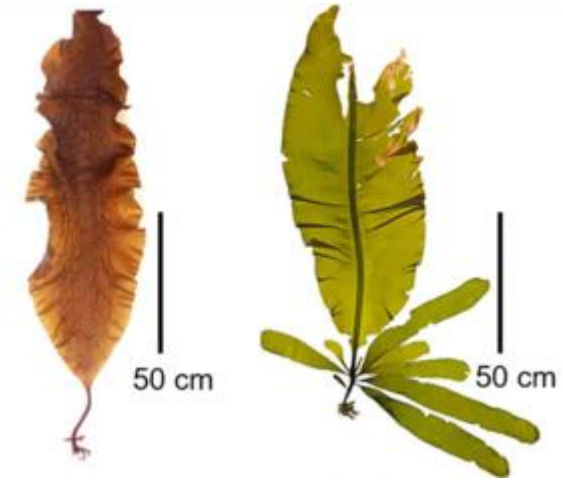

Figure 1: *Saccharina latissima* (left) and *Alaria esculenta* (right). The size is relative to the line of 50 cm (*Innhold av jod i makroalger*, 2020).

1.1.2 *Saccharina latissima*

Saccharina latissima, also called sugar kelp and earlier referred to as *Laminaria saccharina*, is a brown seaweed that can be found in rocky areas in both temperate and polar habitats at the coast of the northern hemisphere (Bolton 2010, Devit and Saunders 2010, referred by Breton *et al.*, 2018). The growth is optimal in cold temperate water with a temperature of 10-15 °C (FAO, 2003). *Saccharina latissima* grows attached to rocks, mussels or other objects from the intertidal zone and down to 26-meter depths (Borom *et al.* 2002, Bartsch *et al.* 2008, Mathieson and Dawes 2017, referred by Breton *et al.*, 2018). It typically has rapid, early seasonal growth, forms sporangia when temperature decrease in the autumn and winter (Luning, 1979; Bartsch *et al.* 2008 and Mathieson and Dawes 2017, referred by Breton *et al.*, 2018), and has a capacity for quick regrowth (Moy *et al.*, 2006). It has a characteristic flexible, short stipe (<60 cm) and elongated frilly fonds that can grow to 2-4 meters in length. In shielded areas with unoccupied space under acceptable environmental conditions, sugar kelp can grow in dense, forest-like assemblages making up a brown sea carpet (Morrissey, Kraan and Guiry, 2001; Moy *et al.*, 2006).

This specie has traditionally been used as fertilizer but is also increasingly sold as the sea vegetable “Sweet Kombu”. The protein content is 6-11 % of dry weight (Morrissey, Kraan and Guiry, 2001). It contains active phenolic compounds with beneficial health effects and has high levels of alginate, mannitol and laminarin that are of commercial interest (Breton *et al.*, 2018). Mannitol gives a sweet taste, and *Saccharina latissima* is therefore a very palatable kelp, that in some areas is eaten fresh or cooked (Morrissey, Kraan and Guiry, 2001). Among the natural

sugar kelp beds in the world, about half of them are situated along the Norwegian coast (Moy *et al.*, 2006), indicating that Norway has optimal habitat for growth, which is very advantageous for increasing seaweed farming (Forbord *et al.*, 2020).

1.1.3 *Alaria esculenta*

Alaria esculenta, also called winged kelp, is a large brown seaweed that grows in the upper part of the sublittoral zone. It exists with a wide distribution in cold waters like in Norway, since it can only grow at temperatures below 16 °C. It can grow up to about 4 meters in length and grow well in rocky coasts in sites exposed to waves (FAO, 2003). The kelp has a long, thin, frond that rises from a short stipe, and a midrib that makes it tolerant to strong waves (Morrissey, Kraan and Guiry, 2001). *Alaria* is said to be the most protein-rich brown algae (Kim, 2011) with protein content up to 9-20 % of dry weight (Morrissey, Kraan and Guiry, 2001). There are also high levels of vitamin B6, vitamin K, iodine and bromine, among other nutritional elements (Morrissey, Kraan and Guiry, 2001).

Winged kelp is commonly known as the sea-vegetable "Atlantic Wakame", with a good tasting, slightly sweetish flavour (Morrissey, Kraan and Guiry, 2001). It has been used as feed for domestic animals in European coastal areas since the fifth century (Kim, 2011) and has gradually become more extensively eaten also by humans (raw or cooked) in some countries (FAO, 2003). It has been successfully cultivated (FAO, 2003) and has great potential for extension to a commercial scale.

1.2 Seaweed in the industry

Seaweeds are essential marine bioresources that are still underutilized, even though they have been used as a vegetable in the Asian diet for a very long time. In contrast, elsewhere in the world it has been used more widely as a source of biochemicals for feed and food, as well as pharmaceuticals, cosmetics (Kadam *et al.*, 2017), textile, paint (FAO, 2003) and also as a fertilizer and for biogas production (Pechsiri *et al.*, 2016). Seaweeds can be harvested wild, but are now increasingly cultivated to provide a massive and stable amount of raw material of good nutritional value (Tiwari and Declan, 2015; Mæhre, Jensen and Eilertsen, 2016; Qin, 2018). Today, seaweed for food purposes comes mainly from cultivation and farming rather than

naturally growing sources (FAO, 2003). Cultivated seaweed is applied to a wide range of industries (Mæhre, Jensen and Eilertsen, 2016; Qin, 2018).

From the global production of seaweed in 2012, around 95 % came from aquaculture with China and Indonesia as the main contributors (FAO, 2014). As a result of the growing industry, seaweed farming took place in over 50 countries in 2016 (FAO, 2016). In Norway, the over 100 000 km long coastline with complex structures, long fjords and islands, is highly suited for aquaculture. The North Atlantic sea is habitat for more than 400 seaweed species (Rueness 1998, referred by Stévant, Rebours and Chapman, 2017), many of them with good commercial value (Stévant, Rebours and Chapman, 2017). There are also numerous fish farms in Norway that discharge organic and inorganic nutrients (Wang *et al.* 2012, referred by Stévant, Rebours and Chapman, 2017), which increases the potential for seaweed growth and production (Stévant, Rebours and Chapman, 2017). In Norway, 130 000-180 000 tonnes of brown seaweed are harvested annually, a number that has been stable for some time (Morrison, 2018). The seaweed cultivation potential in Norway is about 16 000 tonnes (2016), but the actual production output is much lower. In Norway, seaweed is used mainly in food, but also other uses as animal feed and fertilizers are common (Stévant, Rebours and Chapman, 2017).

A significant proportion of seaweed in the world is used for direct human consumption (fresh or dried), or it gets further processed for other food applications (Stévant, Rebours and Chapman, 2017). Seaweed compounds are already used in many different food products with gelling, thickening, emulsifying and stabilizing properties. These properties mainly come from phycocolloids such as alginates, agar and carrageenan (Fleurence, 1999; Stévant, Rebours and Chapman, 2017). The applications in the food industry are of particular interest, using seaweed nutrients to increase the nutritional value of products or to obtain functional ingredients in food with potential health benefits. Seaweeds have a high amount of nutrients together with low caloric content, which makes them appealing for human consumption (Sartal, Alonso and Bermejo Barrera, 2011). In most cases, seaweeds are used due to their content and functional properties of polysaccharides, and rarely due to the nutritional value of their proteins (Fleurence, 1999). Seaweed meal has been produced in Norway from dried and milled brown seaweeds, as an additive to animal feed (FAO, 2003).

Challenges are still present in the seaweed industry. The wide seasonal, geographical and individual variation in the chemical composition of seaweed gives challenges for delivering a constant, stable and chemically optimized biomass (Troy *et al.*, 2015). Also, there are several challenges with seaweed processing as well as lack of appropriate processing technologies that

limits production and holds back production growth. Utilizing nutrients from seaweed requires extraction, where the conventional techniques are usually manual and hard to reproduce. Processing conditions as pH, temperature and pressure may give alterations in sensitive bioactive components in the seaweed, reducing its commercial application. Also, the solvents used in extraction might be unfortunate for the environment (Kadam, Álvarez, *et al.*, 2015b).

With the increasing demand for seaweed, it is crucial to find species that have a beneficial chemical composition with a high content of nutrients and bioactive compounds. The specie must be sufficiently abundant or have the potential for being cultivated to a great extent along the coast (Troy *et al.*, 2015). To give an increased use of seaweed in food, there needs to be developed new, rapid technologies with low energy consumption, high yields, that are economical and eco-friendly. All of this, without compromising quality and taste (Ibañez *et al.*, 2012). Biomass utilization should be maximally optimized by fractionating into a wide range of valuable components during processing, thereby reducing the waste fractions and strengthening the circular economy (Torres, Kraan and Dominguez, 2019).

1.3 Bioactive compounds in seaweed

Seaweeds have been used as animal feed for centuries due to their high level of specific nutrients (Kim, 2011). There is a steadily increasing use of seaweed for human consumption in the world, due to the increasing focus on sustainable growth and healthy "natural food" with a good nutritional composition (Garcia-Vaquero, Lopez-Alonso and Hayes, 2017). Marine algae a have harsh growth environment and a phototrophic life, giving exposure to high oxygen and radical stresses, leading to the evolution of protective systems like pigments and polyphenols (Pulz and Gross, 2004). Seaweed polyphenols are also referred to as phlorotannins. They have essential biological activities in e.g. preventing oxidation, bacterial, fungal, and viral infections, allergies, and cancer (Li et al., 2011).

Other active compounds produced in seaweeds include polysaccharides, fatty acids, vitamins, minerals, sterols, carotenoids, phycobilins, tocopherol, phycocyanins and proteins (Kadam *et al.*, 2017). The content of lipids in seaweed is low, but the fatty acid composition is valuable (Sartal, Alonso and Bermejo Barrera, 2011). Seaweed lipids can be a superior source of ω-3 and ω-6 polyunsaturated fatty acids as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) arachidonic acid (ARA) and stearidonic acid (SDA) (Sartal, Alonso and Bermejo Barrera, 2011; Billakanti *et al.*, 2013). The polysaccharide content can vary over a wide range

in seaweed, with 4-76 % of dry weight (Holdt and Kraan, 2011), and is of high interest for the food industry. As already mentioned, seaweed is increasingly used as a source of protein (Kadam *et al.,* 2017). Seaweed also have high levels of macro elements as Iodine, Potassium, Calcium, Magnesium and Iron compared to many land vegetables, as well as containing most vitamins (Holdt and Kraan, 2011; Torres, Kraan and Dominguez, 2019). Thus, seaweeds are an important source of biologically active compounds for interest in the food and pharmaceutical industries (Chen and Yiang 2001, referred by Sartal, Alonso and Bermejo Barrera, 2011).

Food has been used to give taste and consistency to food, as well as improving health, for a long time. There has been an increasing focus on looking deeper into the bioactive compounds in seaweed, how to make them more bioavailable and how to exploit them in food and feed (Holdt and Kraan, 2011). Some foods only have basic nutritional functions like development, growth, and body maintenance. Other foods or food components provide further health benefits and are called functional foods. Functional foods contribute beneficially to bodily functions, improve general well-being and increase life quality, by reducing the risk of chronic diseases or make the body better fit to manage chronic diseases (Holdt and Kraan, 2011; Troy *et al.,* 2015). Functional foods can also have positive effects on growth and development, and enhance performance (Holdt and Kraan, 2011). As seaweed contains nutrients with several important bioactive functions, seaweed compounds can be used as supplements or ingredients in functional food (Kumar *et al*. 2008, referred by Holdt and Kraan, 2011). Some reported benefits are control of hyperlipidaemia, thrombosis, development of tumours and obesity (Kadam and Prabhasankar, 2010). The composition of nutrients varies depending on the type of seaweed, the season and the area of production (Murata and Nakazoe, 2001; Ne Connan *et al.,* 2004; Marinho-Soriano *et al.,* 2006), and their bioavailability depends on the treatment performed on the plants. Most of the carbohydrates and proteins in seaweed are not digestible. Thus, the nutritional value of seaweed has traditionally been from its contribution of minerals, trace elements and vitamins (Holdt and Kraan, 2011).

1.3.1 Carbohydrates

Brown seaweeds are rich in carbohydrates and contain various polysaccharides. Food reserves are typically complex polysaccharides and higher alcohols (El Gamal, 2010). Laminarin is the primary storage polysaccharide and principal carbohydrate reserve (Mišurcová, 2011). Reserve storage polysaccharides are mannitol and the relatively low molecular β-1,3-1,6-glucans

(laminarins) (Usov, Smirnova and Klochkova, 2001). The major part of the structural polysaccharides in the cell wall consists of cellulose and alginate (El Gamal, 2010), and there are also a significant amounts of fucoidan (Usov, Smirnova and Klochkova, 2001). The cell walls are mainly made up of cellulose embedded in a matrix of acid polysaccharides (Kloareg, Demarty and Mabeau, 1986). Cellulose consists of repeating units of β-(1,4)-linked D-glucose (Mišurcová, 2011). The acid polysaccharides are mainly alginate and fucoidans (Mišurcová, 2011), linked to each other by proteins and strongly associated with proteins (Kloareg, Demarty and Mabeau, 1986; Billakanti *et al.*, 2013). The fucoidans in the cell wall have variable amounts of saccharide units with different degrees of sulfonation, primarily fucose, galactose, mannose and xylose (Berteau, 2003). Alginates are heteropolysaccharides with varying ratio and composition of β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The residues are bound together by glycosidic bonds to homopolymeric or alternating blocks (Kim, Lee and Lee, 2011; Billakanti *et al.*, 2013; Zhu and Yin, 2015).

Alginate is only found in brown seaweeds. It is the dominating polysaccharide, accounting for as much as 70 % of the total polysaccharide in some species (Billakanti *et al.*, 2013), making up about 40 % of the total dry weight of the seaweed (Zhu and Yin, 2015). Thus, the content of alginate is especially high. The other main polysaccharides cellulose, laminarin and mannitol are also present in relatively high amounts in brown algae (Schiener *et al.*, 2015). Fucoidan is also a structural polysaccharide that has shown to contribute significantly to the total carbohydrate content (Usov, Smirnova and Klochkova, 2001). The most important polysaccharides, their monosaccharide units, and content in the relevant algal species are presented in Table 1. Different amount of polysaccharides are reported in literature, and the carbohydrate content depends highly on the algal specie and the harvesting season (Manns *et al.*, 2014). The structural polysaccharides alginate and cellulose are stable throughout seasons, while storage carbohydrates like mannitol and laminarin have more considerable seasonal variations, with lowest levels in winter or early spring (Schiener *et al.*, 2015). The high concentration of alginate and other polysaccharides gives a strong structure that binds the seaweed compounds tightly together (Billakanti *et al.*, 2013). This structure may be degraded with various enzymes as cellulases or alginate lyase (Manns *et al.*, 2016).

Table 1: The main polysaccharides in brown seaweed and their composition of monosaccharide units. The amount of each polysaccharide in the brown seaweed *Saccharina latissima* and *Alaria esculenta*. Sources a) (Bruhn *et al.*, 2017) b) (Manns *et al.*, 2014), c) (Schiener *et al.*, 2015) **and d) (Vishchuk *et al.*, 2012).**

Polysaccharide	Monosaccharide units	*Saccharina latissima* (% dw)	*Alaria esculenta* (% dw)
Alginate	Mannuronic acid and guluronic acid (a)	28.5 ± 3.9 (c)	37.4 ± 4.0 (c)
Cellulose	Glucose (β-1,4) (a)	11.0 ± 1.4 (c)	11.3 ± 1.0 (c)
Laminarin	Glucose (β-1,3 and β-1,6) (a)	8.2 ± 5.3 (c)	11.1 ± 7.2 (c)
Mannitol	Mannose (alcohol form) (b)	18.6 ± 4.7 (c)	12.1 ± 3.5 (c)
Fucoidan	Fucose, galactose, mannose, xylose, glucuronic acid and arabinose (a)	2.3-6.2* (a)	3.8** (d)

*Measured in the frond **Not found for *Alaria esculenta*. Value found for *Alaria* sp., measured in the frond

1.3.2 Proteins

Proteins have an essential role in the human body as enzymes and carriers, as well as being one of the three dietary nutrients that give energy. It crucial to have a diet rich in proteins, but it is also important that they are of good nutritional quality (Mæhre *et al.*, 2018). Seaweed can be a rich protein source, and the content and composition vary depending on geographical location, the season of growth and nutrient availability, and is highly dependent on the species (Fleurence, 1999). The protein fraction is smaller in brown seaweed (up to 24 % dw), compared to green and red species (up to respectively 33 % and 47 % of dw). These can be levels comparable with high-protein vegetables (Holdt and Kraan, 2011; Bleakley and Hayes, 2017). Other sources indicate a maximum of 15 % in brown seaweed (Fleurence, 1999) and up to 50 % in red seaweed (McHugh 2003, referred by Schiener *et al.*, 2015a). The protein content is generally higher during the winter due to lower photosynthetic activity giving a lower rate of carbohydrate production and storage. The wide seasonal variation is a disadvantage when using seaweed as a protein source (Kadam *et al.*, 2017). Another problem is that the high content of insoluble polysaccharides make plant proteins tougher to digest than those from animals (Bleakley and Hayes, 2017).

The protein quality in seaweed can have large variations concerning the amino acid composition and their bioavailability (Boisen and Eggum 1991, referred by Bleakley and Hayes, 2017). Seaweeds have amino acid and protein composition different from those of land proteins (Fleurence, 1999). The nutritional value of proteins is said to be higher in algae than in cereals and vegetables, due to the essential amino acid composition (Holdt and Kraan, 2011). Plant proteins commonly lack one or several essential amino acids and are therefore not considered

as complete protein sources (Young and Pellett 1994, referred by Bleakley and Hayes, 2017). Many seaweeds, especially those from red and brown species, contain all the essential amino acids (Fleurence, 1999; Dawczynski, Schubert and Jahreis, 2007; Holdt and Kraan, 2011), and are meeting FAO requirements as viable protein sources (FAO; WHO, 1991; Fleurence, 1999).

Seaweeds are especially rich in the acidic amino acids aspartic acid and glutamic acid, which can together constitute between 22 and 44 % of the total amino acids (Fleurence, 1999; Holdt and Kraan, 2011). These amino acids largely contribute to the characteristic "umami" taste in seaweed (Macartain *et al.*, 2007). The distinctive taste of nori comes from large amounts of the three amino acids alanine, glutamic acid and glycine (Holdt and Kraan, 2011). Limiting amino acids in algal proteins are typically threonine, lysine, tryptophan, cysteine, methionine, and histidine. Still, the levels of these amino acids are generally higher in seaweeds than in terrestrial plants (Fujiwara-Arasaki *et al.* 1984, referred by Galland-Irmouli *et al.*, 1999). However, the limiting amino acids will differ from plant to plant, and therefore a varied diet will provide sufficient amino acids (Holdt and Kraan, 2011).

Bioactive peptides are amino acid sequences with health benefits beyond their fundamental nutritional value (Hayes 2013, referred by Bleakley and Hayes, 2017). They typically consist of 2-30 amino acids and hold hormone-like properties. The peptides are inactive in their parent protein, but hydrolysis or fermentation can release the bioactive peptides (Bleakley and Hayes, 2017). Seaweed proteins, peptides and amino acids are documented to have positive bioactive effects in the treatment of AIDS, cancer and diabetes and preventing vascular diseases (Holdt and Kraan, 2011), among other health-promoting effects.

Proteins can be extracted and isolated from seaweed and thereby used in food products. Following protein extraction, the conditions employed will lead to different physiochemical characteristics (Mwasaru *et al.*, 1999). The functional properties for proteins depend on factors like amino acid composition, net charge, molecular weight and surface hydrophobicity (Garcia-Vaquero, Lopez-Alonso and Hayes, 2017). In food products, proteins give techno-functional properties and can give emulsification, texture modification and whipping properties, as well as assisting fat and water absorption (Ogunwolu *et al.*, 2009). These are properties that can contribute to the taste and texture of food products, making proteins suitable for use in a wide variety of foods such as sausages, bread, cakes, soups and dressings (Chandi and Sogi, 2007). Seaweed proteins can also be used in vegan products for human consumption (Torres, Kraan and Dominguez, 2019).

1.4 Challenges with seaweed as food material

As pointed out, there are many beneficial compounds in macroalga that make them healthy and favourable for consumption. Still, some seaweed compounds must be considered carefully before eating excessive amounts of seaweed. In fact, seaweed can contain safety hazards with minor, moderate or major impact on the safety of the biomass for food and feed purposes (Banach, 2019). Eating seaweed on a regular base has led to concerns regarding the ingestion of accumulated toxic compounds, heavy metals, contaminants and excessive amounts of iodine (Brandon, Janssen and de Wit-Bos, 2014; Bouga and Combet, 2015). Many factors influence the presence and quantity of hazardous compounds, including seaweed species, physiology, season, environment and any further processing conditions as preparation and cooking. The growth location can also be an essential factor if water quality is negatively influenced by contamination from anthropogenic or industrialized activities (Bouga and Combet, 2015).

One of the possible hazards in seaweed is iodine, which can be useful to consume to prevent iodine insufficiency. Still, in some species and sources of macroalgae, it can also pose a risk for giving too high iodine levels and thyroid disorders (Bouga and Combet, 2015). The content of iodine can vary greatly, with content e.g. from 16 μg/g in *Porphyra tenera* to 8165 μg/g in granules from *Laminaria digitata* (Teas et al. 2004, referred by Bouga and Combet, 2015). Laminariales such as *S. latissima* and *A. esculenta* are reported to have iodine levels one level of magnitude higher than most red and green seaweeds, according to several studies (Saenko *et al.*, 1978; Nitschke and Stengel, 2015, 2016). The daily iodine requirement of 140 μg is easily reached by eating fish, milk or dairy products, and is therefore easily exceeded when eating considerable amounts of seaweed (Bouga and Combet, 2015).

Seaweed has known to accumulate minerals and essential elements from its near environment, which will also result in the accumulation of heavy metals and contaminants. Heavy metals are metals with high density compared to water, that are toxic in low concentrations, and can e.g. bind to proteins and disturb their physiological activity. In macroalgae, heavy metals as Cd, Pb, Hg and As can be found (Bouga and Combet, 2015). There may also be microbiological hazards in seaweed, such as Salmonella, *Escheria coli*, Listeria, Vibrio and different viruses from the cultivation or processing. These are especially harmful in the case of eating uncooked seaweed, like when eaten in sushi. There are also contaminants in the ocean of increasing concern, like e.g. plastic pollution (Bouga and Combet, 2015).

Ingestion of such undesired compounds can lead to adverse health effects if excessively consumed, even though it is unharmful if consumed under certain thresholds (Bouga and Combet, 2015). For this reason, extracting nutrients from seaweed might be an excellent way to avoid overcome the problem of consumption of toxic compounds in seaweed.

1.4 Algal nutrient extraction

The bioavailability of seaweed nutrients is generally low due to the tight polysaccharide matrix (Harnedy and FitzGerald, 2013). Like in other plants, the cells in seaweed are surrounded by a cell wall mainly consisting of complex polysaccharides that give structure and protection to the cell. However, seaweed cell walls and cuticles are even more complex, with sulphated and branched polysaccharides in association with proteins and bound ions (Bleakley and Hayes, 2017). A big fraction of the amino acids and proteins in seaweed are situated intracellularly and can make ionic interactions with the cell wall and intracellular polysaccharides (Jordan and Vilter, 1991). Furthermore, macroalgal proteins might be positioned in cell wall assemblies or they can be crosslinked by disulphide bonds to assembly polysaccharides (Harnedy and FitzGerald, 2013). The proteins can also be strongly bound to polyphenols (Stern *et al.*, 1996), which may limit protein availability (Holdt and Kraan, 2011) and digestibility (Fleurence, 1999; Wong and Cheung, 2001). This is especially limiting for the brown species, with higher levels of polyphenols and lower protein content (Holdt and Kraan, 2011), and can complicate protein extraction (Fleurence *et al.*, 1995; Ragan and Glombitza 1986, referred by Wong and Cheung, 2001).

Humans do not have the enzyme for digesting the cell wall polysaccharides in raw, unprocessed algae, and therefore the bioavailability of the embedded and attached proteins is low (Joubert and Fleurence, 2008; Mæhre, Jensen and Eilertsen, 2016; Bleakley and Hayes, 2017). Therefore, it is beneficial to extract proteins out from the algal matrix to make them more bioavailable and digestible (Mæhre, Jensen and Eilertsen, 2016). After extraction, centrifugation will remove non-soluble matter and give a protein rich supernatant. Proteins can be recovered by ultrafiltration, precipitation or chromatographic techniques (Kadam *et al.*, 2017; Vilg and Undeland, 2017). Powder and extracts of seaweed are more nutrient-dense than the raw material (Vilg and Undeland, 2017), and can be a good way to make seaweed proteins more suited for consumption as ingredients in food and feed (Holdt and Kraan, 2011). An

advantage with extraction is that it is possible to separate several nutrient fractions in parallel, which makes it more sustainable and economically feasible (Vilg and Undeland, 2017).

Various extraction methods have been used to release identified and unidentified bioactive substances from marine algae (Holdt and Kraan, 2011). The efficiency of extracting active compounds from plant material can be profoundly affected by several factors such as time, temperature, pH, extraction solvent and particle size (Bleakley and Hayes, 2017). The selection of an optimal method for maximal yield and high purity varies according to the target compound (Kadam, Álvarez, *et al.*, 2015b). Conventionally, algal proteins are extracted with aqueous acid and alkaline extraction (Kadam *et al.*, 2017). The conventional methods are the most common, as they are both simple and cheap (Rawdkuen and Ketnawa, 2019). However, in some cases, alkaline treatment can negatively affect the nutritional and functional properties of the protein (Fabian and Ju, 2011). Extraction of proteins is time and solvent consuming, gives a limited extraction efficacy and has a high potential for optimization (Rawdkuen and Ketnawa, 2019). New extraction methods are developed to improve the extraction yield while at the same time-saving time and resources (Kadam *et al.*, 2017).

1.5 Biomass pre-treatment

New extraction techniques involve biomass treatment to degrade the cell wall and liberate the intracellular proteins for more efficient extraction (Mæhre, Jensen and Eilertsen, 2016). Different tools are used for pre-treatment, like osmotic shock, mechanical grinding, ultrasonic treatment, microwaves and enzymatic degradation of the cell wall (Barbarino and Lourenço, 2005; Harnedy and FitzGerald, 2013; Rawdkuen and Ketnawa, 2019). These processes can give a better mass transfer rate, more solvent to solute interaction, and thereby more efficient extraction (Vilkhu *et al.*, 2011). Protein extraction commonly involves cell burst following exposure to hypotonic conditions. The plant cell wall holds a defence against osmotic variations and prevents the cell from bursting, making the extraction process inefficient. Enzymatic treatment can be a way of catalysing or accelerating reactions, to overcome the structural and chemical barriers of the cell wall (Mæhre, Jensen and Eilertsen, 2016).

1.5.1 Enzymatic treatment

Enzymes has become an important tool in the food industry. The application of enzymes will enable hydrolysis of the cell wall matrix in a green process with low costs and no toxic waste (Shannon and Abu-Ghannam, 2018). Enzymatic treatment is highly specific, active at low concentrations, requires mild conditions (pH and temperature), and gives few adverse side effects (Menzefricke 1997; Simpson and Haard 1987; referred by Shahidi and Janak Kamil, 2001). In enzymatic extraction, enzymes like proteases, endoproteases, glucanases, amylases or cellulases are used (Kadam, Álvarez, *et al.*, 2015a). The enzymes induce release of proteins by degrading the seaweed matrix they are kept in (Kadam *et al.*, 2017). Enzymes can also be used to break protein-polyphenol interactions or break down large protein complexes into smaller parts to enable extraction (Wang *et al.*, 2010). Bioactive peptides that are inactive in the protein can be released using enzymatic hydrolysis or by the use of digestive enzymes (Bleakley and Hayes, 2017). Enzymatic pre-treatment has shown to effectively increase the extraction of nutrients and the amount of available amino acids for hydrolysis. Therefore, it can increase the extractability and bio accessibility of proteins in seaweed, and thereby enhance their utilization potential (Mæhre, Jensen and Eilertsen, 2016).

The enzyme to use can be carefully selected for the substrate and the nature of the molecules to be extracted (Guerard, 2006), for an optimal breakdown of the structural and chemical barriers of the cell wall (Mæhre, Jensen and Eilertsen, 2016). The enzyme to utilize depends highly on the cell wall composition of each algal specie, and careful selection of enzymes is required for an efficient process (Harnedy and FitzGerald, 2013). Enzymes can be used alone but can be more efficient in combinations, indicating a synergistic effect of varying enzyme combinations for the right algal specie (Denis, Le Jeune, *et al.*, 2009; Denis, Morancais, *et al.*, 2009). Choosing the right conditions for the enzyme is essential to obtain its optimal activity and maximum recovery of active components (Bleakley and Hayes, 2017). Several factors may affect the efficiency of the enzymes, including enzyme to substrate ratio, incubation time, temperature, pH, as well as the state of the substrate (Guan and Yao, 2008). The type and amount of extraction solvent used is also an a critical variable, as well as particle size (Bleakley and Hayes, 2017). Enzymatically assisted extraction can be a sustainable alternative to traditional methods (Kadam *et al.*, 2017), even though it is challenging to transfer to an industrial scale. This is due to high costs, difficulties of finding enzymes with the perfect

substrate specificity, and challenges with maintaining suitable bioreactor conditions (Kadam, Tiwari and O'Donnell, 2013).

Proteases and carbohydrases are two groups of enzymes that have been used in protein extraction from plants, increasing the yield. Proteases work through proteolysis, while carbohydrases work through degradation of the cell wall (Sari *et al.*, 2015). This thesis will further investigate the effect of three carbohydrases, Alginate lyase, Viscozyme L and Cellic CTec2, as well as the protease Alcalase.

Alginate lyase

The cell walls of brown seaweed consist mainly of alginates, which gives a strong barrier against extraction. Alginate lyase can be used to hydrolyse alginate or alginic acid polymers to lower molecular weight oligosaccharides (Billakanti et al., 2013). This carbohydrase can degrade alginate by cleaving the polymer bonds in a β-elimination reaction, producing unsaturated oligosaccharides at the non-reducing end (Kim, Lee and Lee, 2011; Zhu and Yin, 2015). All lyases perform the same action on alginate but may have a preference for a specific G/M block structure (Wong, Preston and Schiller, 2000; Zhu and Yin, 2015). Alginate lyase has shown to have a positive effect on saccharification in macroalgae (Kadam, Álvarez, *et al.*, 2015a; Sharma and Horn, 2016; Ravanal *et al.*, 2017).

Viscozyme® L

The cell walls in brown seaweed contain large amounts of cellulose (El Gamal, 2010), which limits the efficiency of the extraction of algal bioactives (Shannon and Abu-Ghannam, 2018). Viscozyme L is a carbohydrase that hydrolyse plant cell wall polysaccharides effectively and cleave the linkages within the matrix (Guan and Yao, 2008; Majideh Jamshidi, Javad Keramat, 2018). The enzyme is efficient for cell wall degradation due to its composition, which include xylanase, cellulase, hemicellulase, β-glucanase and arabanase activity. This mix of enzymes catalyse bond cleavage between monomers, giving reducing sugars as galactose, glucose and arabinose, and reduces the viscosity of the biomass (Hanmoungjai et al. 2002, referred by Chen *et al.*, 2014; Park et al 2004 and Gupta 2016, referred by Shannon and Abu-Ghannam, 2018). This can be advantageous for liberating intercellular constituents like proteins (Guan and Yao,

2008). Viscozyme L has shown to improve protein extraction from algae (Wijesinghe and Jeon, 2012; Rodrigues *et al.*, 2015; Majideh Jamshidi, Javad Keramat, 2018).

Cellic® CTec2

For complete carbohydrate hydrolysis, synergistic action from different enzymes is necessary (Ribeiro, 2017). Cellic® CTec2 consists of aggressive cellulases, β-glucanases, and hemicellulases, as well as a β-glucosidase and a β-xylosidase. This makes Cellic specialized for liberation of sugars from polysaccharide rich biomass (Manns *et al.*, 2016; Ribeiro, 2017). The Novozymes Cellic CTec2 enzyme mix is suggested to be more specialized to seaweed breakdown than many other enzymes. It degrades polysaccharides into several hexose C6 monosaccharides as e.g. galactose and glucose. Glucose is the primary hydrolysis product to be released, due to the predominance of cellulases in the enzyme mix (Kostas, White and Cook, 2019). Treatment by Cellic CTec2 has shown to release 50 % of available glucose in seaweed within 8 hours and can even release all available glucose in the same time if combined with other enzymes. Cellic is highly mentioned to be used together with Alginate lyase, which improves the accessibility of cellulose by catalysing the degradation of alginate (Manns *et al.*, 2016). Mechanical or thermochemical pre-treatment has shown to be advantageous prior to hydrolysis by Cellic CTec2, to break up the polysaccharide structure for the enzymes better to access its substrates (Ribeiro, 2017).

Alcalase

Proteases are enzymes than can be used for the degradation of proteins in seaweed. They are typically used after polysaccharides have been broken down and proteins are more available. Protease treatment will then give proteolysis, leading to reduced protein size and making proteins more available for extraction. Several studies have recorded combined extraction with carbohydrases and proteases (Sari *et al.*, 2015).

Alcalase is a protease with esterase activity that effectively hydrolyses the peptide bonds between amino acids in a protein. It is further classified as a serine endo-peptidase (Novozymes, 2016). Serine proteinases have specific peptide bond cleavage, different from the other three major classes of proteases (Guerard, 2006). Endoproteinases induce the breakdown of proteins into relatively large peptides by cleaving the peptide bond at specific residues (Kristinsson and

Rasco, 2000). Alcalase addition has shown to increase protein yield with 30-40 % compared to the use of carbohydrases alone. However, the use of Alcalase alone (without carbohydrase) gave almost the same yield (Hanmoungjai, Pyle and Niranjan, 2002). Thus, protease provides more efficient protein extraction (Sari *et al.*, 2015).

1.5.2 Ultrasonic treatment

Another way to increase protein extraction yield is by applying mechanical force to break up the seaweed biomass, e.g. by using ultrasounds (Kadam *et al.*, 2017). Ultrasound is an acoustic wave with a frequency higher than 20 kHz (Huang *et al.*, 2015). It requires a liquid medium to spread, where molecules can be temporarily dislodged from their original position. The ultrasonic waves create alternating cycles of compression and rarefaction (Suslick 1989; referred by Chemat *et al.*, 2017). At high intensity, the rarefaction phase induces formation of dynamic microbubbles that generates a negative pressure. This will pull molecules apart and create cavities in the liquid, called acoustic cavitation. Pressure and temperature inside the bubbles can respectively rise to over 1000 atm and 5000 K and make molecules collide (Suslick 1989, referred by Chemat *et al.*, 2017; Wang *et al.*, 2018). Ultrasound is a mechanical wave, and therefore changes in frequency, wavelength and amplitude can affect the acoustic cavitation and thereby the extraction efficiency (Pingret, Fabiano-Tixier And and Chemat, 2013).

The mechanical effects from ultrasound give accelerated solvent flow and blending of reactants as well as increasing the transfer of heat and mass, leading to a disruption of cell wall material (Kadam *et al.*, 2017; Wang *et al.*, 2018). Collapsing microbubbles generate liquid flowing in high speed, that damage nearby particle surfaces. Cavitation can also give chemical effects, if the bubble collapses and gives dissociation of water and dissolved oxygen, creating highly reactive free radicals (•OH and •OOH). The radicals can further induce different reactions in the cavitation bubble and/or in the bulk solution, which will lead to an increased rate of biomass degradation (Pang, Abdullah and Bhatia, 2011). When the cell wall is degraded by sonication, the solvent can easier penetrate the plant material and is forced into the cell. This will give dissolution of the intracellular components, and release cavitated material as proteins from the matrix (Albu, 2004).

High power ultrasound is based on a transducer as energy source and can be applied by using an ultrasonic bath or an ultrasonic probe. Ultrasonic baths are cheap and easy but have low power and reproducibility compared to the probe (Chemat *et al.*, 2017). High power ultrasonic

probes are generally more effective for extraction purposes, with more intense cavitation and matrix degradation (Kadam *et al.*, 2017). The power is generated through a small surface (only the tip), creates a more direct and powerful delivery of ultrasound, with low energy loss and rapid temperature increase in the medium. Different probe lengths, diameters and tip geometries can be used according to the application and the volume to be sonicated (Chemat *et al.*, 2017).

Ultrasound (US) treatment has shown to increase the liquefaction of complex carbohydrates effectively, and just a short treatment can be enough to fully liquefy tightly packed material (Kunaver, Jasiukaityte and Čuk, 2012). Sensitive compounds from brown seaweeds are more easily extracted after ultrasonic pre-treatment (Kadam, Tiwari, *et al.*, 2015). According to Kadam *et al.* (2017), the degradative effect of ultrasounds has also shown to improve the bioavailability of seaweed proteins, increasing the protein extraction with over 500 %, and reducing the extraction time greatly. Sonication lowers the required amount of acid or alkali (Kadam *et al.*, 2017), and gives the opportunity to use different solvents than in conventional extraction (Mason, Chemat and Vinatoru, 2011). It is also able to preserve the activity of bioactive compounds and give higher yields at a lower cost (Le Guillard *et al.*, 2016).

Ultrasonic treatment can be useful in certain areas of food processing and has increasing attention for pre-treatment in extraction (Phongthai, Lim and Rawdkuen, 2017). It is very beneficial due to its highly reproducible action with short time, energy and solvent consumption (Rawdkuen and Ketnawa, 2019). It can, thus, be an eco-friendly way to enhance the traditional extraction of bioactive compounds (Kadam *et al.*, 2017). Ultrasonic treatment has been performed at an industrial scale for extraction from natural sources, and has a potential to be used similarly for marine algae (Kadam, Tiwari and O'Donnell, 2013).

1.5.3 Combined treatment - enzymes and ultrasound

Enzymatic treatment is a green chemical technique. However, if not optimally customized, it can give low yields, low selectivity and long treatment time (Shannon and Abu-Ghannam, 2018). Enzymatic hydrolysis can be combined with ultrasonic treatment and give an increase of plant biomass liquefaction and solubilization of biomolecules (Le Guillard *et al.*, 2016). Studies have demonstrated that ultrasound can improve enzymatic hydrolysis and enable recovery of valuable components, enhancing the total protein extraction, indicating a synergistic effect between the two treatment techniques (Le Guillard *et al.*, 2016).

The activity of enzymes is highly dependent on enzyme to substrate interaction and the configuration of the active site (Ma *et al.*, 2011). Sonication is believed to increase the mass transfer, and thereby enhance the accessibility of the substrate to the enzyme (Kwiatkowska *et al.*, 2011; Mason, Chemat and Vinatoru, 2011). Sonication can give structural transformations of the active site, giving increased enzyme stability and thereby increased activity (Bashari *et al.*, 2013). Ultrasonic treatment can be done on an enzyme-substrate mix and change the conformation on both the enzyme and substrate and promote their interaction. This can induce enzymatic reactions with higher activity and increased yield (Wang *et al.*, 2018).

Radicals generated during sonication may give protein cross-linking and oxidation of free sulfhydryl groups that might change the enzyme stability (Cavalieri *et al.*, 2008; Mirmoghtadaie, Shojaee Aliabadi and Hosseini, 2016). Short time and low intensity ultrasonication is beneficial to avoid denaturation and inactivation of enzymes while still improving the catalytic activity (Delgado-Povedano and Luque de Castro, 2015). Ultrasound can also be applied before enzymatic hydrolysis, which can be advantageous if there is a protective layer outside the substrate, impeding the reaction. Sonication can break the protective layer, exposing the substrate (Yang and Fang, 2015). It can also degrade biomass to decrease particle size (Gogate and Prajapat, 2015), enhance mass transfer and thereby accelerate an enzymatic reaction (Luo, Fang and Smith, 2014). Even for complex substrates, enzymatic digestion of a material can be done in only a few minutes if the material is pre-treated with ultrasonic waves. Thus, ultrasounds can promote and accelerate enzymatic reactions, with great potential for upscaling to industrial applications (Wang *et al.*, 2018).

1.7 The aim of the study

In this thesis, protein extraction from the macroalgae *Saccharina latissima* and *Alaria esculenta* was studied. These are some of the common seaweed species that grow on the Norwegian coast and are also commonly cultivated. Little research has been done on protein extraction on brown macroalgal species, even though they are valuable sources of food and rich in nutrients. New pre-treatment and extraction techniques need to be developed for providing good quality, well tasting and safe nutrients in a sustainable and affordable way.

The aim of the study was to investigate the effect of different treatment techniques on the protein extraction yield from the two species of macroalgae. Various settings, conditions, and

combinations for enzymatic and ultrasonic treatment in extraction were examined. The effects of independent and combined application of ultrasonic waves and enzymes was tested, as well as sequential enzymatic treatment with enzymes of different specificity. Different protein analysis techniques were compared to evaluate the importance of choosing the best-fitted analysis method.

2. MATERIALS AND METHODS

2.1 Macroalgae samples

Two different batches of *Saccharina latissima* and one batch of *Alaria esculenta* were used. The first batch of *S. latissima* was harvested by Sintef Ocean the 27.06.2019 and was (as far as understood) frozen stored in black bags at Sintef Sealab the 01.07.2019. Thus, it was refrigerated for four days before freezing. The alga was received by NTNU 13.09.2019 and stored at -20 until use. *A. esculenta* and the second batch of *S. latissima* were cultivated and harvested by Seaweed Energy Solutions (SES), and directly frozen in the facility on HitraMat. *S. latissima* was harvested 29.04.2019 and received by NTNU the 18.11.2918, while *A. esculenta* was harvested 06.05.2019 and received by NTNU the 13.09.2019. Both were stored at -20 until use. Different batches of *Saccharina latissima* were used, due to suspicions of started degradation and reduced quality of the first batch.

Before experimental use, macroalgal wet samples were thawed in room temperature for up to 1 hour (until all ice crystals were melted) and cut it with knife to pieces with a diameter of about 0.1 to 1 mm. The finely cut biomass was either used immediately or stored in a cold room for maximum 24 hours until use. Macroalgal dry samples were freeze-dried (Alpha 1-4 LO plus, type GT 5PM-R, Glems-Technik) and further stored at -20 until use. The amount needed experimentally was finely ground by a mortar.

2.2 Enzymes and chemicals

Enzymes used in this thesis are Alginate lyase powder (A1603, Sigma-Aldrich, ≥10.000 units/g solid), Viscozyme® L liquid enzyme mixture (V2010, Sigma-Aldrich), Cellic® CTec2 liquid (Novozymes) and Alcalase liquid (Novozymes). Enzymes will further be referred to by their full names, or Alg.lyase, Viscozyme and Cellic CTec/Cellic. All chemicals used were of analytical (pa) quality. Distilled water was used for all extractions, while de-ionized water (Mili-Q® purification system) was used for diluting samples to be analysed by HPLC.

2.3 Determination of dry matter and ash content

Biomass of known weight was heated in a heat cabinet (Termaks) at 105 °C for about 24 hours, until stable weight was reached. Accurate weight was measured for determination of dry matter

content. Samples were transferred to an ash oven (muffle furnace, Model L5/C6, Nabertherm), and left at 550 °C overnight. Accurate weight was measured for determination of ash content. The dry matter and ash content were measured by another master student for *A. esculenta* and the old *S. latissima*, while the values for the new *S. latissima* was measured by the thesis author.

2.4 Pre treatment

Pre-treatment was performed by enzymatic treatment (E) or ultrasonication using a sonicator (US). Ultrasonic treatment was conducted using a Sonic Dismembrator (Model 505, Fisher Scientific) with a 3.2 mm microtip (size 1/8'') and a maximum applicable amplitude of 40 %. Enzymatic treatment was performed using a heating cabinet (Termaks) for incubation and a water bath for inactivation. The two treatment methods were either used separately or in combination.

2.4.1 Enzymes

Amount of the enzymes used were first estimated with inspiration from literature, and additional enzyme concentrations for comparison were chosen according to experimental results. Altogether, three different enzyme concentrations were used for each of the three carbohydrases (Alginate lyase, Viscozyme and Cellic CTec) and one concentration of the protease (Alcalase). The amount of Alginate lyase by dry weight as well as the enzyme incubation time was adapted from amounts of xylanase and cellulose used by Mæhre, Jensen and Eilertsen (2016). The pH and temperature conditions for Alginate lyase was chosen based on Chen et al. (2018). The amount of Viscozyme by dry weight was adapted from Shannon, Emer and Abu-Ghannam (2018). Optimum conditions for Viscozyme was adapted from Novozymes Viscozyme® L product sheet (Novozymes, 2002). The amount and of Cellic CTec was adapted from wet weight used by Manns *et al.* (2016), and optimum conditions (pH and temperature) from the enzyme bioenergy application sheet by Ribeiro (2017). A citrate phosphate buffer of pH 5.09 was used to lower the pH to optimum conditions for Cellic CTec, based on Manns et al. (2014). The same buffer was also used for lowering pH for Viscozyme as used by Ribeiro Garcia de Figueiredo *et al.* (2018). About 500 µl buffer was used in each sample tube to change pH to around 5. The amount and conditions for Alcalase was selected based on Lyng (2015).

Selected concentrations of enzymes were added directly into the sample tube with algae and water and mixed gently by inverting the tubes. Samples were incubated in heat cabinets (Termaks) at 37 °C for Alginate lyase and Viscozyme, and 50 °C for Cellic CTec. Incubation was done for 18 hours for all experiments, except for the first experiment with combined effect, where incubation was done for 25 hours. Inactivation of enzymes was done in a water bath at >95 °C for 10 minutes. Samples with added Alcalase were additionally incubated for 1 hour at 50 °C before water bath inactivation. Tubes were inverted from time to time during incubation to try to get a good mixing, for more equal accessibility of enzyme to substrate for all the biomass. Table 2 shows the guiding enzyme incubation and inactivation conditions from literature, and the conditions used in the experimental protocol. The reason that the inactivation temperature that was used is not given specifically (denoted >95 °C), is that there was a lot of heat exchange between the water bath and the surrounding air when the lid was removed to place the samples in the water. The temperature dropped but was observed at 95-100 °C during inactivation.

Table 2: Conditions for incubation and inactivation of enzymes, from literature and experimentally used, for the enzymes Alginate lyase, Viscozyme L, Cellic CTec2 and Alcalase.

Enzymes	Literature optimal temperature (°C)	Literature optimal pH	Literature inactivation conditions	Incubation conditions (pH, temperature, time)	Inactivation conditions (temperature, time)	Source(s)
Alginate lyase	30-55 (a)	7.0-10.0 (a)	100°C, 5 min (a)	~7, 37°C, 18/25h	>95°C, 10 min	a: (Chen *et al.*, 2018)
Viscozyme L	25-55 (a)	3.3-5.5 (a)	85°C, 10 min (b)	~5, 50°C, 18h ~7, 37°C, 18/25h	>95 °C, 10 min	a: (Novozymes, 2002) b: (Chen *et al.*, 2014)
Cellic CTec2	45-50 (a)	5.0-5.5 (a)	60-70°C (b)	~5, 50°C, 18h ~7, 50°C, 18h	>95 °C, 10 min	a: (Ribeiro, 2017). b: (Viikari *et al.*, 2007)
Alcalase	30-65 (a)	7-9 (a)	90°C, 15 min (b)	~7, 50°C, 1h	>95 °C, 10 min	a: (Novozymes, 2016). b: (Li, Li and Zhao, 2010)

2.4.2 Ultrasound

The sample tubes were gently inverted to mix the content and placed in ice to avoid unwanted protein denaturation by heat produced from the sonicator. Sonication was conducted by placing the sonication probe directly into the 15 mL sample tubes. Sonication settings were chosen

according to the limitations of the instrument and recommendations from previous users of the tool. In all cases, the sonication interval was performed with pulse 2 seconds and break for 1 second. The sonication probe was wiped with a paper towel with 70 % ethanol between each new sample to avoid contamination between samples.

Most sonication experiments were performed using several sonication runs on each sample. This was done by heightening the sonication probe about 1 cm upwards in the tube between each run, as demonstrated in Figure 2, with arrows marking the probe position for run 1, 2 and 3. For only one sonication run, the probe was lowered almost to the bottom of the tube (position 1) and sonication was conducted. For several sonication runs, the probe was further heightened once (position 2) for two sonication runs and once more (position 3) for three sonication runs. Sonication was conducted for each new position of the probe, giving a total of 1-3 sonication runs per tube. This was done to get maximal expose of the sonication probe to different parts of the biomass in the tube.

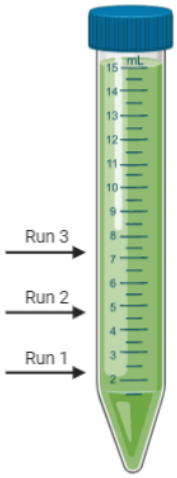

Figure 2: Demonstration of the position of different sonication runs conducted on the sample. The arrows are pointing at the position of the probe tip at the numbered runs. Figure drawn using Biorender online tool (*biorender.com*, 2020).

2.5 Experimental procedure for extraction

2.5.1 Screening for optimal parameters

Extraction was performed with specific ratios of wet algae to distilled water, giving a total weight of 13 g of water and algae. The weight of *S. latissima* and *A. esculenta* were based on equal wet weight, giving slightly different dry weights. Water and algae were mixed in a 15 ml

centrifuge tube and gently inverted to mix the content. Enzymatic and/or ultrasonic treatment was conducted immediately after (or samples were stored at 4 °C for a short period before treatment in a few cases). Control samples with water and algae, and no treatment, were used for comparison. After treatment, samples were centrifuged (Heraeus Multifuge X1R Centrifuge, Thermo Scientific) at 4950 x g for 30 minutes at 4 °C. The supernatant was separated from the pellet by pipetting. Further analysis was conducted immediately, or the supernatant was chilled at 4 °C (for analysis within a short time) or frozen at -18°C/-40°C (for later analysis). The supernatants from extraction were analysed using the Lowry method. Figure 3 shows the flow sheet for the experimental procedure.

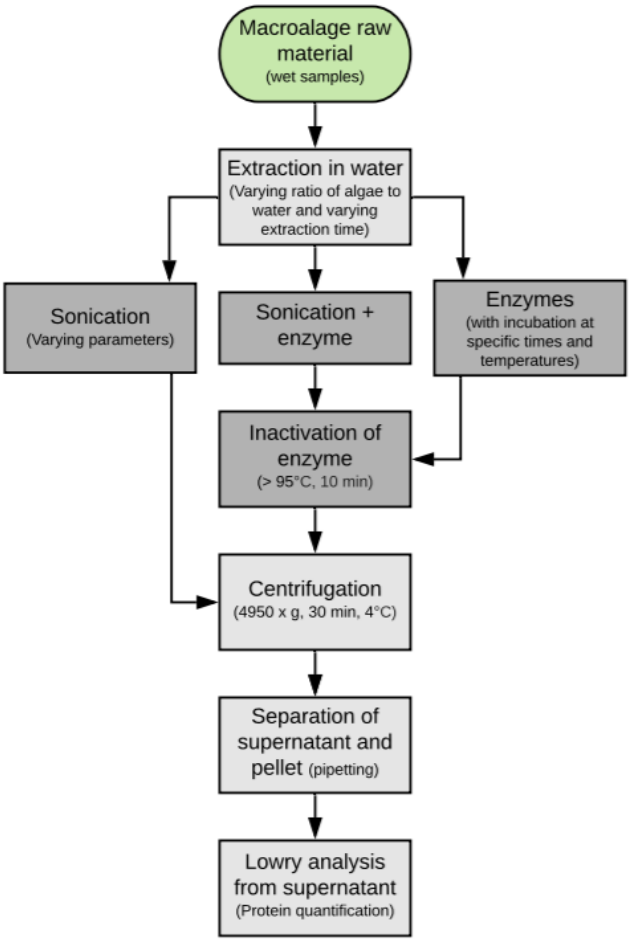

Figure 3: Flow diagram of the extraction procedure performed in the screening phase with pre-treatment with sonication and the enzymes Alginate lyase, Viscozyme L and Cellic CTec2 of the algae *Saccharina latissima* and *Alaria esculenta*. The flow chart is drawn using the online tool lucidchart (*lucidchart.com*, 2020).

At first, a combined treatment of enzymes and sonication was conducted in duplicate on *Saccharina latissima* and *Alaria esculenta* with algae to water ratio 1:2. The two enzymes

Alginate lyase and Viscozyme were used in a concentration of 0.2 % of added algae (ww). Samples were prepared and incubated for 25 hours. The impact of enzymes on the total protein yield was measured. This was done by performing the experimental procedure as for the other samples but with samples of only water and enzymes and measuring the protein yield in the Lowry method.

Further, screening of water to algae ratio, extraction time, sonication settings and algae to enzyme ratio was done to optimize individual parameters before using them together. Experiments were done with wet *Saccharina latissima* and *Alaria esculenta*. Enzyme experiments were done in duplicate, while the rest were done without parallels. Ratio of algae to water was tested by performing extraction with the ratios 1:2, 1:4, 1:6, 1:10, 1:15, 1:20 and 1:25. Extraction time was tested by extracting algae in water for 0, 1, 2, 3, 4, 5, 6 and 19 hours, with algae to water ratio 1:10.

Sonication settings were tested with both ratio 1:10 and 1:20. Amplitude and time settings were changed to see the optimal conditions within the range of the limitations for the equipment (maximum 40 % amplitude). Amplitudes of 30-40 % were tested against sonication times of 20-35 seconds, as shown in Table 3. The settings chosen for further use is highlighted. Also, constant amplitude and time (for the chosen settings) were tested against multiple (1-3) sonication runs per sample.

Table 3: Different amplitude and time settings tested for pre-treatment of *Saccharina latissima* and *Alaria esculenta*. The highlighted setting is the one to be further used in the thesis work.

Amplitude (%)	Time (seconds)
40	**20**
37	25
34	30
30	35

Enzymatically assisted extraction was done with three different concentrations of the three enzymes Alginate lyase, Viscozyme and Cellic CTec as shown in Table 4. The enzyme concentrations chosen for further use are highlighted. Treatments with Cellic CTec were done adding 413 µl citrate phosphate buffer of pH 5.09 for a more optimized incubation pH. All enzymes were incubated for 18 hours. The amounts of enzymes added was at this moment based on the amount of alga added in wet weight to be the same for *S. latissima* and *A. esculenta*. This gives different concentrations added for the two algae of Alginate lyase and Viscozyme, and the same concentration for both algae added of Cellic CTec.

Table 4: Different enzyme concentrations for pre-treatment of *Saccharina latissima* and *Alaria esculenta*. The amount of added enzyme is given in enzyme unit (U) for Alginate lyase with known activity, and volume (µl) and weight (g) respectively for Viscozyme L and Cellic CTec2 with unknown activity. The highlighted concentrations are the ones to be further used in the thesis work.

Enzyme	Amount in *S. latissima* (U, g or µl)	Amount in *A. esculenta* (U, g or µl)	Basis for calculation	Source
Alginate lyase (A1603), ≥10,000 units/g solid	4.03 U / 0.0004 g	3.09 U / 0.0003 g	50 U/g dry algae	Mæhre, Jensen and Eilertsen, 2016
	8.06 U / 0.0008 g	**6.18 U / 0.0006 g**	100 U/g dry algae	
	12.09 U / 0.0012 g	9.27 U / 0.0009 g	150 U/g dry algae	
Viscozyme L (V2010), unknown activity	**7.88 µl / 0.0095 g**	6.01 µl / 0.0072 g	98 µl/g dry algae	Shannon, Emer; Abu-Ghannam, 2018
	11.8 µl / 0.0142 g	9.02 µl / 0.0108 g	147 µl/g dry algae	
	15.8 µl / 0.0190 g	**12.0 µl / 0.0144 g**	196 µl/g dry algae	
Cellic CTec2, unknown activity	0.01 g	0.01 g	1.5 % of wet algae weight	Ribeiro, 2017
	0.02 g	**0.02 g**	3 % of wet algae weight	
	0.03g	0.03 g	4.5 % of wet algae weight	

Extraction was performed with a control sample and a buffer control sample (control sample with added only buffer) to see the impact of the buffer on the Lowry measurements. The effect of adjusting pH with buffer was tested for treatment with Cellic CTec and Viscozyme. Buffer was added before enzymatic incubation, and either adjusted back or not before Lowry analysis. This was compared with an unadjusted control sample (pH 7). All samples were incubated for 18 hours.

Two different batches of *Saccharina latissima* were compared. Experiments were performed for US with 1-3 sonication runs and Viscozyme with concentration 1-3 for the new *Saccharina latissima*, to be compared with results conducted earlier for the old *Saccharina latissima*. Samples treated with Viscozyme were incubated for 18 hours.

2.5.2 Further extractions

The optimal settings from the screening survey were evaluated and further used. In the following extractions, algae to water ratio was 1:20 (ww), and sonication was performed with three runs of 40 % amplitude for 20 seconds, still with pulse 2 seconds and 1 second break. Enzyme concentrations were chosen based on their optimal amounts found for the different combinations of algal species and enzymes (Table 4). All incubation with enzymes was conducted for 18 hours. As the use of citrate phosphate buffer did not increase the protein yield, all enzymes were incubated without adjusting pH. Different experiments were conducted to see the combined effect of the parameters from the screening experiments. Combination of

sonication and enzymes with varying treatment order was conducted on both wet and dry algae. Dry alga was also singly treated with sonication and enzymes. The adapted protocol is shown in Figure 4.

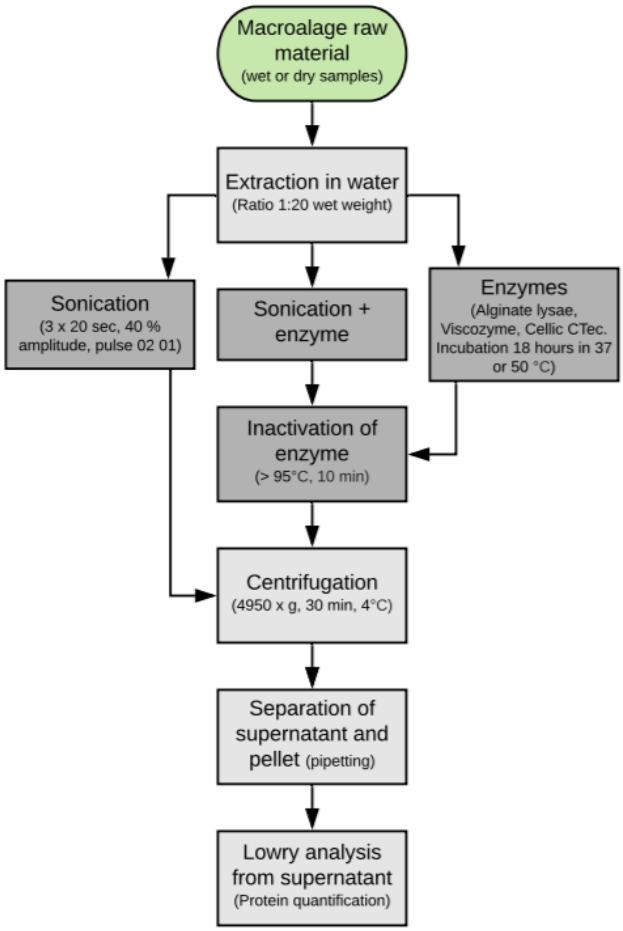

Figure 4: Flow diagram of the extraction procedure with pre-treatment from sonication and the enzymes Alginate lyase, Viscozyme L and Cellic CTec2 of the algae *Saccharina latissima* and *Alaria esculenta*. The flow chart is drawn using the online tool lucidchart (*lucidchart.com*, 2020).

For the last experiment, a sequential enzymatic treatment with Cellic CTec and Alcalase was performed. Samples were incubated with Cellic for 18 hours and/or thereby incubated with Alcalase for additional 1 hour. For a better comparison between the two species of algae, water to algae ratio was calculated based on dry weight. The new ratio was calculated to be about 1:177 (dw), corresponding to 1:20 (ww) for *S. latissima* and about 1:18 (ww) for *A. esculenta*. Thus, the amounts of wet algae added was slightly adjusted for *A. esculenta*, and it was slightly different between the two species. The amount of enzyme added would then directly correspond

to the dry weight of both algae. The amounts of enzyme added was 28.57 % Cellic (per dry weight algae) and/or 500 µl Alcalase per g of dry algae.

In addition to Lowry analysis of the supernatant, the samples were prepared for further quantitative and qualitative analyses. Due to this, a bigger volume of supernatant was needed. In additional to the regular small scale batch, a larger batch was also performed in 200 mL bottles (~148.2 mL water). Thus, the exact same experiment was done in both a big and a small batch. For the trials done in big batches, separation of pellet and supernatant after extraction was done using special folded filters with good flow for viscous liquids (185 mm, Schleicher and Schuell MicroScience), due to difficulties with upscaling the pipetting technique. Freeze-drying was done with a vacuum freeze-dryer (Alpha 1-4 LO plus, type GT 5PM-R, Glems-Technik). Freeze-dried material was frozen at -18 °C for later analysis.

The supernatants from the small batch was used as regularly for Lowry analysis, but additionally also for analysis of acid soluble peptides and free amino acids. The freeze-dried supernatant from the big batch was used for total amino acid and CN analyses. Freeze-dried pellet from extraction and algal raw material were also prepared for CN analysis. The adapted protocol (Figure 5) was applied in the further work.

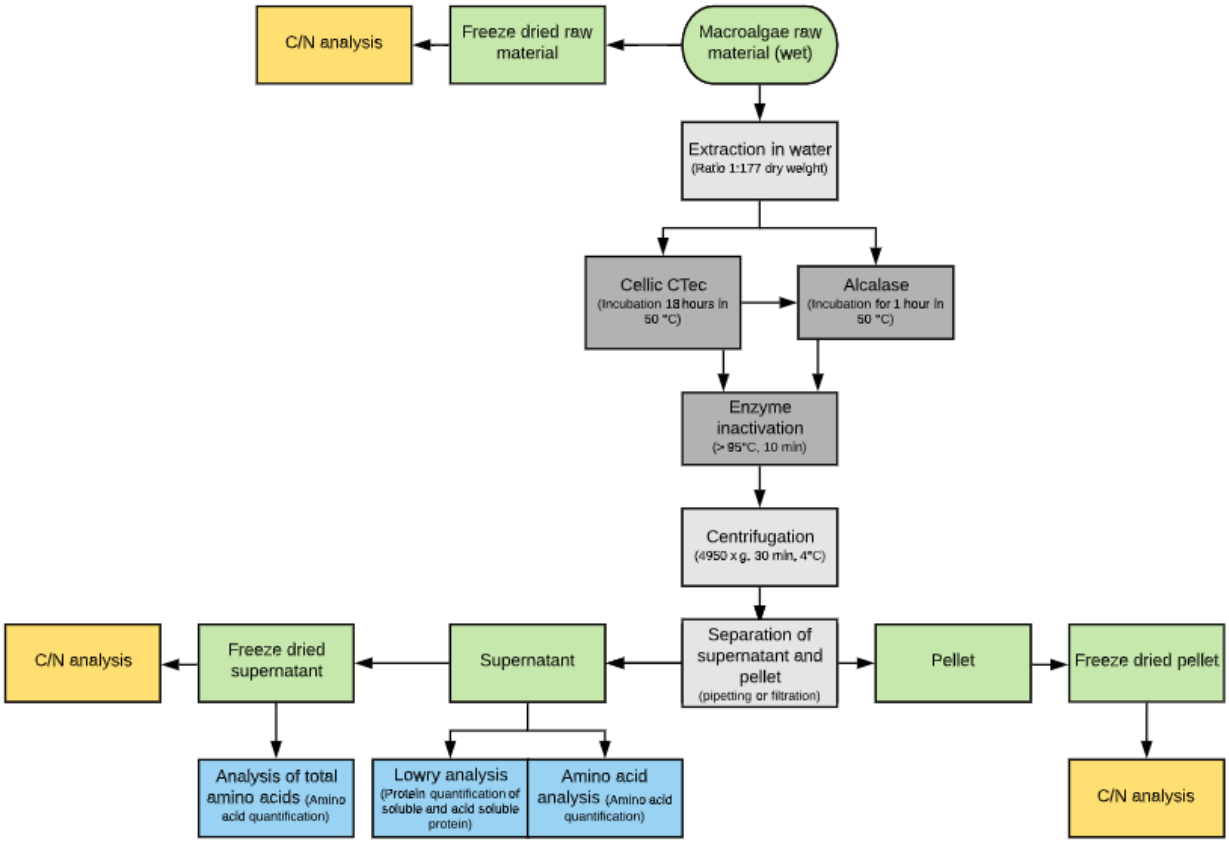

Figure 5: Flow diagram of the extraction procedure for sequential enzymatic treatment with pre-treatment by enzymes Cellic CTec2 and Alcalase of the algae *Saccharina latissima* and *Alaria esculenta*. The flow chart is drawn using the online tool lucidchart (*lucidchart.com*, 2020).

2.6 Protein and amino acid analysis

2.6.1 Quantification of proteins

Protein content was determined with the Lowry analysis method (Lowry *et al.*, 1951), due to its sensitivity and ability to detect protein at low concentrations. Three parallels were analysed for each sample. Samples of diluted supernatant were treated with an alkaline copper reagent and a diluted Folin reagent, resulting in a blue colour from reduction of phosphomolybdic-tungstic mixed acid in the Folin phenol reagent. Standard curves were made of BSA (Bovine serum albumin) for comparison, due to similarities with algae in the amino acid composition. The method depends on the pH of the sample, and maximum colour occurs at about pH 10 with complete mixing of solutions immediately after addition. This is since copper ions from the alkaline reagent needs high pH to react with peptide bonds (Lowry *et al.*, 1951). Therefore, samples with added buffer were adjusted to neutral or weakly basic for the analysis. Absorbance was measured at 750 nm using a UV-VIS spectrophotometer (Genesys 10 S, Thermo

Scientific). The same cuvettes were re-used for the three parallels for the same sample. Measured absorbance was used to calculate the protein yield, where the volume of the supernatant was defined as the volume of added water (plus buffer in a few cases). The extra volume of added enzyme and extracted protein was considered insignificantly small and hard to exactly measure, and therefore not added to the total volume.

2.6.2 Quantification of acid soluble peptides

Analysis of acid soluble peptides was done as described by Hoyle and Merritt (1994). Supernatant from extraction was treated with 20 % trichloroacetic acid (TCA), filtered (70 mm filter paper, S&S Roundfilter, Schleicher & Schüll) for removing precipitated protein, and analysed by the Lowry method as described in section 2.4.1.

2.6.3 Composition of total amino acids

Analysis of total amino acids was performed according to Blackburn (1978), with two parallels. Freeze-dried supernatant from extraction was treated with 6M HCl and hydrolysed at 105 °C for 22 hours. Samples were neutralized with NaOH, filtered (Whatman glass microfiber filter GF/C) using a vacuum filtering pump and suitably diluted with de-ionized water. Samples were filtered through 0.22 μm filters, with the same syringe used for the two parallels, while the filter tip was changed between parallels. Samples were analysed by reverse phase HPLC using a Nova-Pak C18 column on an UltiMate® 3000 HPLC (Thermo Scientific) with a Dionex RF 2000 fluorescence detector. This analysis was performed by Siri Stavrum at NTNU. The total weight of freeze-dried supernatant was not measured before used, and an approximate weight was measured afterwards by summing up the amount left and what had been used (not accounting weight of spilt matter etc). This approximate weight was used to calculate the concentration of total amino acids (% dw), which might give small variations to the results.

2.6.4 Composition of free amino acids

Analysis of free amino acids was done as described by Osnes and Mohr (1985). Supernatant from extraction was treated with 10 % sulphosalisylic acid, and after 30 minutes protein precipitate was removed by centrifugation. The samples were suitably diluted with de-ionized water and filtered through 0.22 μm filters. The same syringe was used for the two parallels,

while the filter tip was changed between parallels. Samples were analysed by HPLC as described in section 2.6.3.

2.6.5 CN analysis

Samples were prepared for CN analysis by accurately measuring 300-800 µg sample into a small tin capsule and packing it tightly. Analysis was performed in a CN-analyser (ECS 4010 CHNSO analyser, Costech, Italy) by Marte Schei at SINTEF Fisheries and Aquaculture. Two parallels for each treatment was analysed in three parallels in the CN-analyser, giving a total of 6 parallels of each sample.

2.7 Statistical analysis

In the experiments examining water ratio, incubation time and sonication settings, there was only one parallel since it was used as a screening for conditions to use for further work. The rest of the experiments were conducted with two parallels (to reduce the enzyme consumption). Statistical analysis was conducted with two parallels using Excel functions. Average values with corresponding standard deviations and confidence intervals were calculated. To determine if the differences between results were significant or not, Student t-test was performed, with $p < 0,05$ as a limit for significance. For further details about statistical analysis, see Appendix A.

3 RESULTS AND DISCUSSION

The results are presented in this section as a percentage of the dry weight of the algae used for extraction (% dw). The most discussed results with corresponding standard deviations are also given in Appendix C. It is seen that the protein yield from the control samples vary between experiments, and in some cases the variation is relatively large. This variation partly comes from a heterogeneous chemical structure of the seaweed biomass, which is expected for such a small sample size. It can also be a result of treatment conditions having varying effects on different control samples. To make it easy to compare corresponding control samples, some of the figures have columns divided in two parts. In all figures, orange colour represents *S. latissima* and green is *A. esculenta*. The darkly orange/green is wet algae, the lighter colour is dry algae, while the patterned columns are for different treatments or conditions for the same type of algae.

For the screening experiments with enzymatic treatment, results are displayed in several ways. This was done because it was first assumed (from results in section 3.2.1) that the enzymes contributed little/nothing to the protein yield. Later analyses showed that their contribution was bigger than expected, which changed the results. Different interpretations are presented to explain the basis of the choices made, as well as the true results.

3.1 Dry matter and ash

Dry matter and ash content of *A. esculenta* and old/new *S. latissima* were measured. These are compared with values from literature (Table 5). Literature values are presented from three different papers, where Schiener *et al.* (2015a) has an average of samples harvested regularly throughout the whole year, between August 2010 and September 2011. The other two authors, Reissiger (2016) and Stévant *et al.* (2018), have averages of samples harvested at specific months of the year. From the two latter, the best comparison is chosen from the available results to best match the harvesting season of the algae used in this thesis. Therefore, the experimental *A. esculenta* harvested 29th of April is compared with literature with harvested algae from May, since it is thought that data can be quite similar from the very end of April compared to May. The two experimental *S. latissima* are compared with algae harvested in the same month, respectively being June (old batch) and May (new batch). Results from Schiener *et al.* (2015a) and Stévant *et al.* (2018) are given as average with standard deviations, while results from Reissiger (2016) are given as intervals with the range of all values.

Table 5: Dry matter and ash content of wet *S. latissima* and *A. esculenta* compared between experimentally measured values and literature. The experimentally measured algae were *S. latissima* (old and new batch) harvested respectively the 27th of June and 6th of May 2019 and *A. esculenta*, harvested the 29th of April 2019. Values are reported as an average of three values (new *S. latissima*) and an average of two values (*A. esculenta* and old *S. latissima*) with the confidence interval. Literature values are (a) average of samples harvested in august 2010 to September 2011 (Schiener *et al.*, 2015)**. Values (b) are samples harvested in specific months of the year in 2015, (b.1) being *A. esculenta* harvested in May, and *S. latissima* harvested in June (b.2) and May (b.3) (Reissiger, 2016). Values (c) are samples harvested in 2015, being *A. esculenta* harvested in May (c.1), and *S. latissima* in June (c.2) and May (c.3) (Stévant *et al.*, 2018)**

Alga specie	Literature dry matter (% of ww)	Literature ash content (% dw)	Experimental dry matter content (% of ww)	Experimental ash content (% dw)
Alaria esculenta (Harvest April)	14.5 ± 2.5 (a) 8.5 - 17.6 (b.1) 17.2 ± 0.8 (c.1)	25.3 ± 5.8 (a) 24.5 - 30.9 (b.1) 24.2 ± 1.4 (c.1)	9.5 ± 1.7	37.8 ± 1.1
Old *S. latissima* (Harvest June)	15.1 ± 2.9 (a) 8.8 - 10.3 (b.2) 13.8 ± 0.8 (c.2)	31.7 ± 7.6 (a) 37.8 - 44.4 (b.2) 30.5 ± 1.1 (c.2)	12.4 ± 0.8	46.4 ± 0.3
New *S. latissima* (Harvest May)	15.1 ± 2.9 (a) 7.0 - 11.5 (b.3) 16.1 ± 1.2 (c.3)	31.7 ± 7.6 (a) 36.7 - 47.0 (b.3) 26.2 ±2.6 (c.3)	10.7 ± 0.4	41.4 ± 0.1

Results from Schiener *et al.* (2015a) and Stévant *et al.* (2018) are very similar for both dry matter and ash, while results from Reissiger (2016) are in general different from the other two, but closer to the results from this thesis. It must be noted that values from Reissiger (2016) are given as intervals, and cover a wider range of values, thereby more easily covering the experimental values, but also having a higher standard deviation. Comparing dry matter values, *A. esculenta* and the new *S. latissima* is closer to Reissiger (2016), while the old *S. latissima* is more like results from Stévant *et al.* (2018). When it comes to ash content, all experimental values are more similar to results from Reissiger (2016). Thus, overall, dry matter is lower and ash content is higher compared to the average of the other literature values. The results for *S. latissima* are generally more similar to the literature values than *A. esculenta*.

The variations between the three batches of algae is assumed to be explained by the different harvesting season, location, and compositional differences. It is recorded in literature that ash content in brown seaweed can be higher than 50 % (dw) (Moss 1952, referred by Schiener *et al.*, 2015a). Both ash content and dry matter will vary over a wide range due to the species, seasonal differences (Schiener *et al.*, 2015) and the depth they have been growing at (Reissiger, 2016). No information on depth have been compared and there might be differences between the algae used in this thesis. So even though both *S. latissima* and *A. esculenta* are brown algae and have quite similar data, it is still reasonable that there are variations between the two species. The difference between the two batches of *S. latissima* can emphasize variations in the

properties of the old and the new batch of algae and indicate that storage conditions affects the nutrient composition in the algae. It is recorded in literature that storage of *S. latissima* in seawater for just a short time can lead to changed nutrient composition, including increased ash content (Stévant *et al.*, 2017). The increased ash content is seen in the old batch of algae compared to the new one and might substantiate the suspicions of changed nutrient composition due to inappropriate preservation and excessive biomass breakdown.

3.2 Protein extraction from screening experiments

3.2.1 Combined effect of enzymes and US

The effect of sonication alone was compared with sequential enzymatic and sonication treatment, using the enzyme Alginate lyase. In the sequential treatment, enzymatic incubation was performed before conducting sonication (E→US). The impact of the enzyme itself on the protein yield was found from samples of only water and enzyme. These were measured by the Lowry analysis and showed that 12.07 % of the added Alginate lyase was detected in the supernatant. This amount was subtracted from the measured protein for an accurate measure of the extracted protein. Figure 6 shows the results.

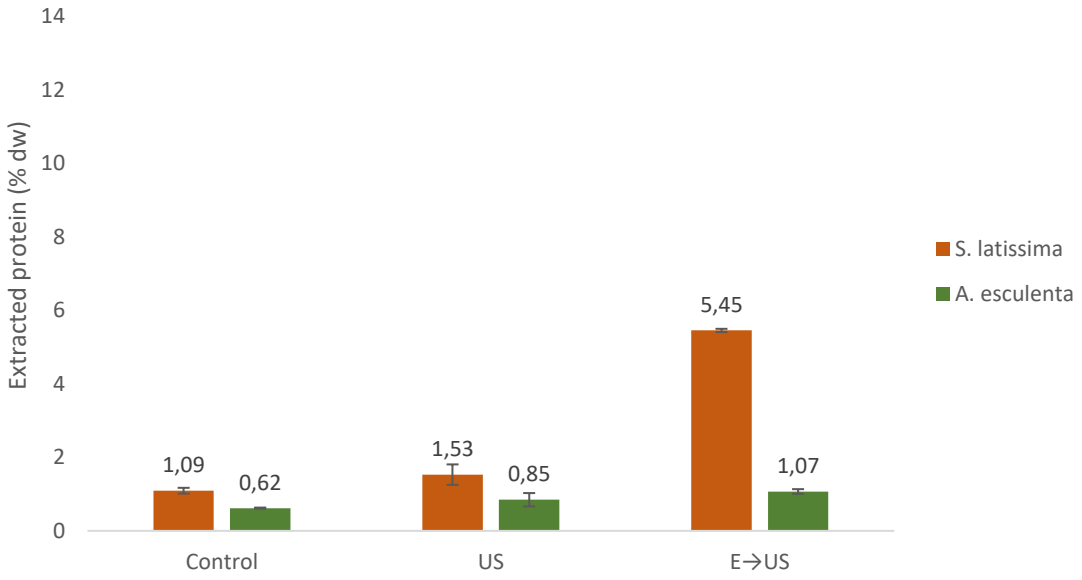

Figure 6: Extracted protein from *S. latissima* and *A. esculenta* in water with different treatments. US is sonication and E → US is enzymatic treatment (Alginate lyase) followed by sonication. Values are average of two parallels, with calculated standard deviation given as error bars.

The same experiment was performed using the enzyme Viscozyme, and sequential enzymatic treatment was conducted in both treatment orders. Sonication followed by enzymatic treatment is further denoted by US→E, while E→US denotes enzymatic treatment before sonication. No control samples were used in this experiment. The impact of the enzyme on the protein yield was measured as explained for Alginate lyase and showed that 4.02 % of the added Viscozyme was detected in the supernatant. This amount was subtracted from the measured total protein. Figure 7 shows the results.

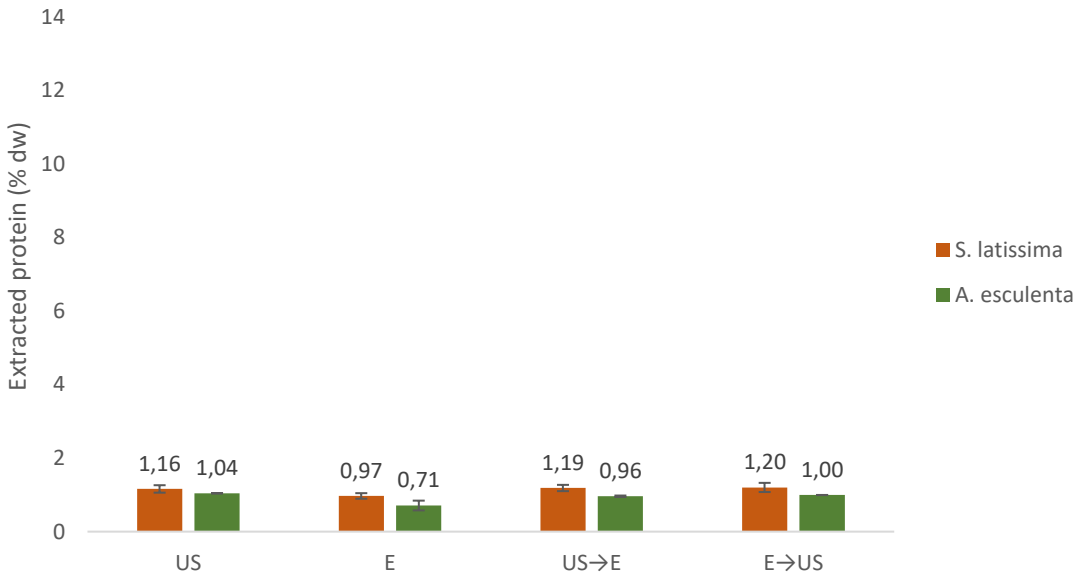

Figure 7: Extracted protein from *S. latissima* and *A. esculenta* in water with different treatments. On the x-axis, US and E respectively denotes sonication and enzymatic treatment by Viscozyme L. E ↔ US indicates the conducted order in the sequential treatment with enzymes and sonication. Values are average of two parallels, with calculated standard deviation demonstrated by error bars.

Figure 6 shows consistently higher protein yield in extraction from *Saccharina latissima* than *Alaria esculenta*. This difference is significant for the control sample and E→US. For both species, the yield increases from the control sample to the sample treated with ultrasonication, indicating that sonication improves protein extraction. For *A. esculenta*, there is a steady linear increased yield by further treatment with both enzymes and sonication, suggesting that combining these two treatment methods further improves extraction. Contrary, for *S. latissima*, the yield increases markedly for the combined enzymatic and sonication treatment, indicating that this alga was more prone to degradation by Alginate lyase, thereby releasing more protein. The increase is significant between control and E→US for both algae and additionally between US and E→US for *S. latissima*.

As the results displayed in Figure 7 do not contain control samples, it is harder to draw conclusions in comparing treatments, and which effect they have relative to untreated samples. On the other hand, the yield from sonication when comparing the same algal species in Figure 7 and Figure 6 are relatively similar, but protein yield is slightly lower for *S. latissima* and higher for *A. esculenta* in Figure 7. Therefore, it is likely to think that control samples would therefore have similar but slightly lower/higher values. Considering this, extraction yield seems to be increased from treatment with Viscozyme for both algae and further improves by the other treatments. For both algae, the protein yield is very similar for sonication alone and the two different combined enzymatic and sonication treatments (E→US and US→E). Significance is shown with higher yield in *A. esculenta* for sonication compared to all treatments and for E→US compared to enzymatic treatment alone for *S. latissima*. This suggests that *A. esculenta* is less prone to degradation by Viscozyme and more affected by sonication. The lower effect of enzymes in *A. esculenta* is seen despite of the fact that more enzyme is added per dry weight of algae (since it has a lower dry weight) than for *S. latissima*. The protein yield is higher for *Saccharina latissima* than *Alaria esculenta*, but only significant for US→E treatment.

Even though it is hard to fully interpret these results, they might suggest possible different effects from the distinct treatment methods. Combined sonication with treatment from Alginate lyase seems to improve protein yield compared to sonication alone. Alginate lyase appears to have a stronger degrading effect, especially on *Saccharina latissima*. The stronger effect might be explained by possibly higher activity of the enzyme (this value is unknown for Viscozyme), thereby having a higher degradative impact from the same amount of enzyme. Results from both experiments highly suggest that both sonication and enzymatic treatment improves protein extraction. Still, the magnitude of improvement seems to depend on the type of enzyme and alga specie.

3.2.2 Effect of algae to water ratio

The effect of the ratio of algae and water is shown in Figure 8. Due to miscommunications, some parallels from certain samples were thrown away before the absorbance was measured in the Lowry analysis. For this reason, samples from *S. latissima* with ratios 1:2, 1:4, 1:6, 1:10 and 1:15 had only one parallel in the analysis. For the rest of the samples from *S. latissima* (ratios 1:20 and 1:25) and all samples for *A. esculenta*, three parallels were measured from Lowry analysis as usual. Despite this, in agreement with my supervisor, it was decided that there was

no need for repeating the experiment, since it was meant as a screening experiment and results showed quite clear trends anyway.

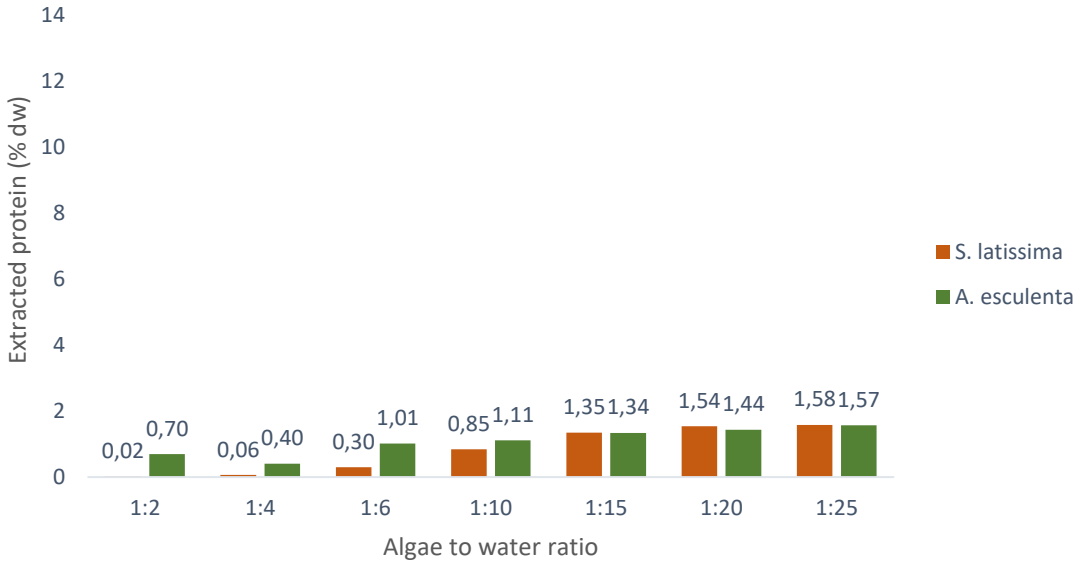

Figure 8: Extracted protein from *S. latissima* and *A. esculenta* with different ratios of algae to water (marked on the x-axis). Values originate from one parallel for each of the two algae.

For the results in Figure 8 there are no parallels, and therefore no statistical measurements. Since two different algae are examined, trends can still indicate the relation between ratio and resulting protein extraction. For the selected ratios, a higher ratio of water gives a higher value of extracted protein. This is likely due to more water in contact with the algae giving a higher concentration gradient, inducing increased protein extraction. According to Stefansson and Hultin (1994) the ratio affects the extraction yield because more water allows for better solubility. Optimally, high amounts of extracted protein should be combined with sustainable and up scalable methods. Thus, excessively increasing the water concentration would possibly give a higher protein yield but also gives limitations for industrial upscaling due to increased time and space requirements and will therefore be unsuitable. Therefore, both ratios 1:10 and 1:20 were chosen for further work for comparison.

3.2.3 Extraction time

The effect of different durations of extraction in water was tested, with algae to water ratio 1:10 for 0, 1, 2, 3, 4, 5, 6 and 19 hours, as shown in Figure 9. When measuring the absorbance of the samples, for some reason, one of the parallels for *S. latissima* extracted for 19 hours was emptied before being measured, and therefore this result only has two Lowry analysis parallels. Results were still considered valid without being repeated since the resulting values agreed well with the expected outcome.

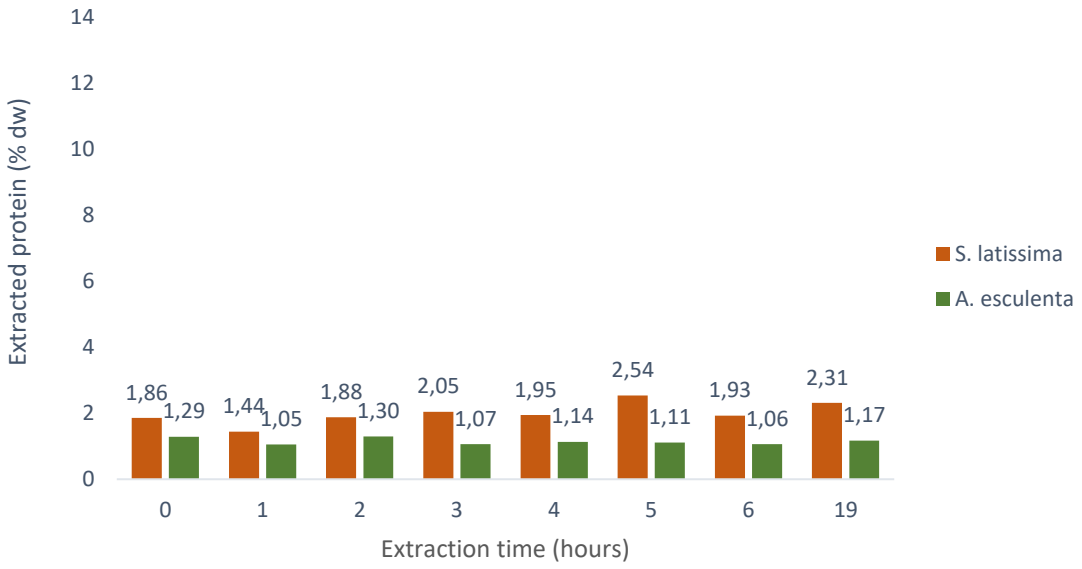

Figure 9: Extracted protein from *S. latissima* and *A. esculenta* with different durations of extraction in water. Extraction time in hours is marked on the x-axis. Values originate from one parallel for each of the two algae.

For the results in Figure 9 there are no parallels, and therefore no statistical measurements. Since two different algae are examined, trends can still show a relation between ratio and resulting protein extraction. For *A. esculenta*, the amount of extracted protein stays rather constant for all durations, indicating a small or no effect of extraction time. For *S. latissima* the relation is less clear, but still indicating little effect of extraction time. For this reason, it is further assumed that extraction time has no or little effect on the protein extraction yield, and that results from experiments with different extraction time can be correctly compared without considering extraction time as an extra factor.

3.2.4 Sonication settings

Sonication settings were evaluated to see the effect of different sonication protocols on the amount of extracted protein. Figure 10 shows the effect of variation in amplitude and time settings, and Figure 11 shows the effect of different number of sonication runs, both performed with algae to water ratio 1:10 and 1:20. In the results disclosed in Figure 11, some measured absorbances were very far from the other parallels and far from expected outcome. These values were not used in the results. This counts for two Lowry parallels for *S. latissima* control sample 1:20 and one parallel for *A. esculenta* sample USx2 1:20. Therefore these results only have respectively one and two Lowry analysis parallels. Results were still considered valid without being repeated since the resulting values agreed well with the expected outcome. All measured absorbances for results in Figure 11, including the ones removed, are shown in Appendix B.1.

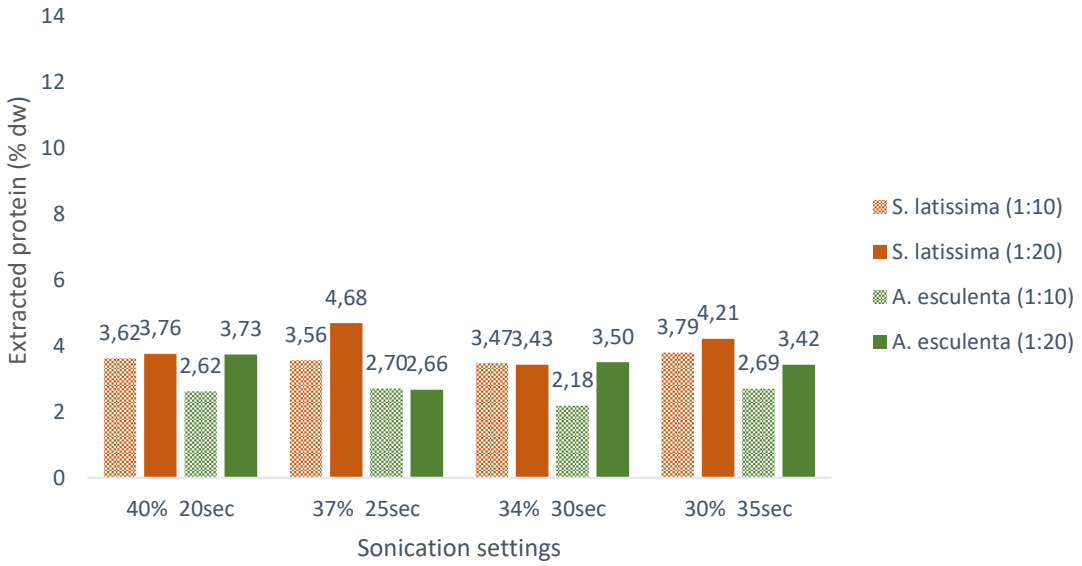

Figure 10: Extracted protein from *S. latissima* and *A. esculenta* with varying amplitude and time settings two different algae to water ratios. The x-axis is marked with amplitude (%) and corresponding time of sonication (seconds). Values originate from one parallel for each of the two algae.

Figure 10 shows no apparent effect of the changed amplitude and time on the amount of extracted protein. There seems to be a linear, horizontal relationship, indicating little or no effect of the changing parameters. The sonicator and tip used for this thesis could not allow an amplitude exceeding 40 %, no higher amplitude settings could be evaluated. Thus, time-to-amplitude settings could only be changed by reducing amplitude and thereby increasing the

sonication time. Increasing the sonication duration would get very time-consuming in such an experiment but might be a suggestion for a larger scale extraction. Since 40 % amplitude for 20 seconds gave an overall good extraction, in addition to being the most time efficient of the combinations, this was the one chosen to be used in further experiments.

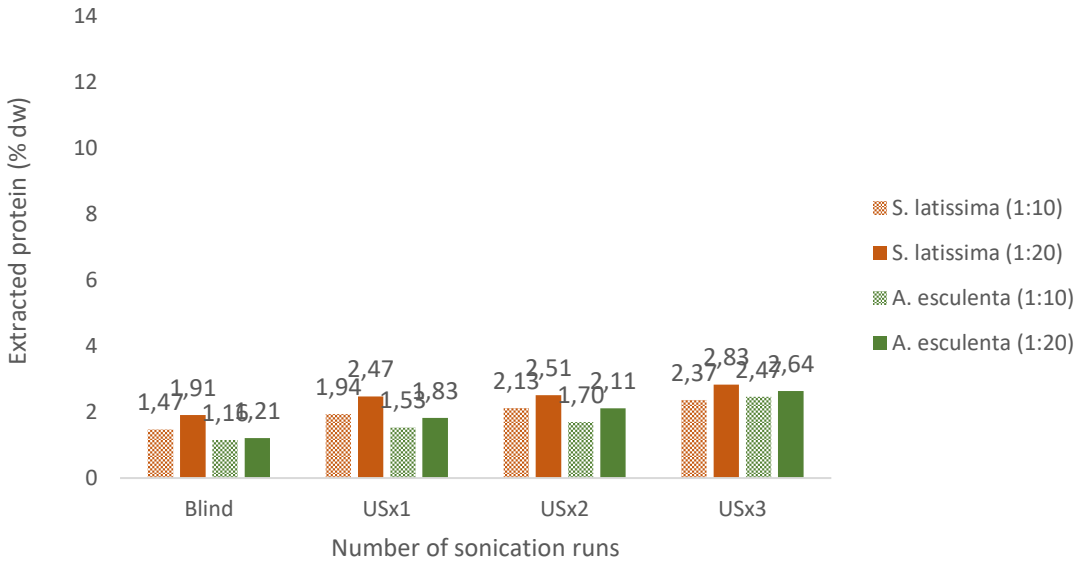

Figure 11: Extracted protein from *S. latissima* and *A. esculenta* with varying number of sonication runs for two different algae to water ratios. US x 1-3 is marked on the x-axis, corresponding to sonication conducted 1, 2 and 3 times on the same sample. Sonication was performed with amplitude 40 % for 20 seconds. Values originate from one parallel for each of the two algae.

Figure 11 shows an approximately linearly increasing relationship between the number of sonication runs and the amount of extracted protein. For both concentrations for both algae, the control sample has the lowest yield, and the amount of extracted protein further increases with an increasing number of sonication runs. USx3 is assumed to be the best of the three treatments.

The results suggest that conducting sonication on biomass from *S. latissima* and *A. esculenta* helps to break up the tightly packed matrix, thereby allowing higher protein solubility. There are several possible reasons for this increase in protein extraction. First, several sonication runs gives a higher sonication time, and thereby a possibility for accelerated solvent flow, blending of reactants as well as increasing heat and mass transfer. This might lead to a stronger disruption

of the cell wall material. Second, conducting numerous sonication runs also includes changing the position of the probe in the sample tube, thereby exposing more of the biomass to the ultrasonic waves. This allows better disruption of the biomass and more solved protein. These assumptions were strengthened by the visual appearance changes of the supernatants after sonication. The more sonicated sample, the stronger green/brown and more cloudy appearance of the supernatant.

It is likely to think that further increasing number of sonication runs would have given a further linear increase. The curve would at some point level out possibly due to excessive heat generation by the sonicator and excessive protein denaturation. Or there might be only a certain amount of protein that can possibly be extracted using this method, which will also give a flattened curve over time. Since sonication in a small and manually performed experiment is time consuming, increasing number of sonication runs was not further tested. Anyhow, there seems to be increased protein extraction for increased sonication. This might be very up scalable to industry if the process of changing position of the sonication probe could be automatized, or a constant movement of the biomass was performed.

3.2.5 Enzyme concentrations

Without considering enzyme contribution

The effect of different enzyme concentrations of the enzymes Alginate lyase, Viscozyme and Cellic CTec was tested. Initially, the contribution of the added enzyme was not accounted when calculating the total protein yield. The extracted protein was calculated as a percentage of algae dry mass, and the control sample was subtracted from each sample for a better comparison. This was done because there was a separate control sample for most enzyme concentrations, with relatively high variations in the protein yield. As enzymes are costly, which will also be multiplied in a big scale, it was also desirable to see the maximum yield of protein as a function of the amount of enzyme added. Therefore, the amount of protein was divided on the weight of enzyme added. As the amount of Viscozyme added was measured in volume, the weight was calculated from the density of the liquid (approximately 1.2 g/ml), from the Novozymes product sheet (Novozymes, 2002). Results are shown in Figure 12 for *S. latissima* and in Figure 13 for *A. esculenta*.

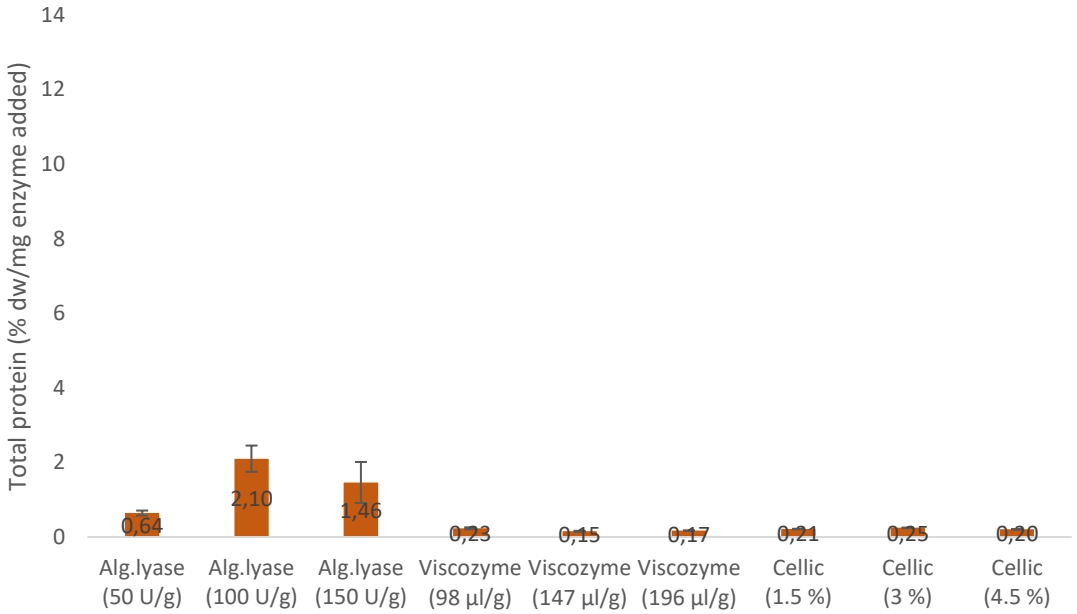

Figure 12: Extracted protein from *S. latissima* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The value of the control sample was subtracted from each sample, and the remaining total protein was divided on the amount of enzyme added. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

From Figure 12, there is one concentration of each enzyme that stands out with a higher protein yield than the other two. For alginate lyase the difference is relatively large, while for Viscozyme and Cellic the extracted protein is similar between concentrations. Concentration 2 of Alginate lyase is significantly higher than concentration 1. For respectively Viscozyme and Cellic, concentration 1 and concentration 2 are significantly higher than the two other concentrations. Overall, the highest amount for total extracted protein for *S. latissima* is for concentration 2 of Alginate lyase and Cellic CTec, and concentration 1 of Viscozyme. These concentrations were the ones further used in the thesis work.

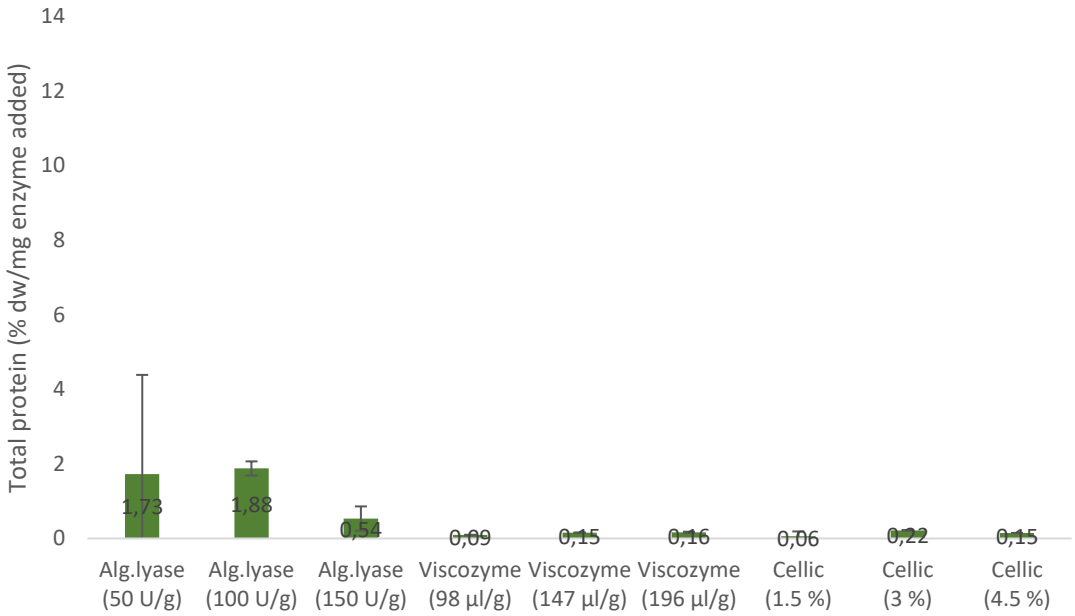

Figure 13: Extracted protein from *A. esculenta* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The value of the control sample was subtracted from each sample, and the remaining total protein was divided on the amount of enzyme added. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

From Figure 13, there is one concentration of each enzyme that stands out with a higher protein yield than the other two. The difference is more prominent for Alginate lyase than for Viscozyme and Cellic. For Alginate lyase and Cellic, concentration 2 is significantly higher than concentration 3. For Viscozyme, both concentration 2 and 3 are significantly higher than concentration 1. Overall, the highest total extracted protein for *A. esculenta* is shown for concentration 2 of Alginate lyase and Cellic CTec, and concentration 3 of Viscozyme. These concentrations were the ones further used in the thesis work.

Taking enzyme contribution into account

At the end of the experimental work, a new experiment was performed to re-evaluate the contribution of enzymes to the measured protein. The results were different than the first time, and enzymes showed to have a more significant contribution to the measured protein yield. The new enzyme contribution will be discussed more thoroughly later. The new enzyme contribution was subtracted from the results, giving changed protein yield compared to earlier. The protein yield from extraction with different enzyme concentrations was re-evaluated.

Results are shown in Figure 14 for *S. latissima* and Figure 15 for *A. esculenta*. The total protein yield was divided on the enzyme weight, but the control sample was not subtracted this time.

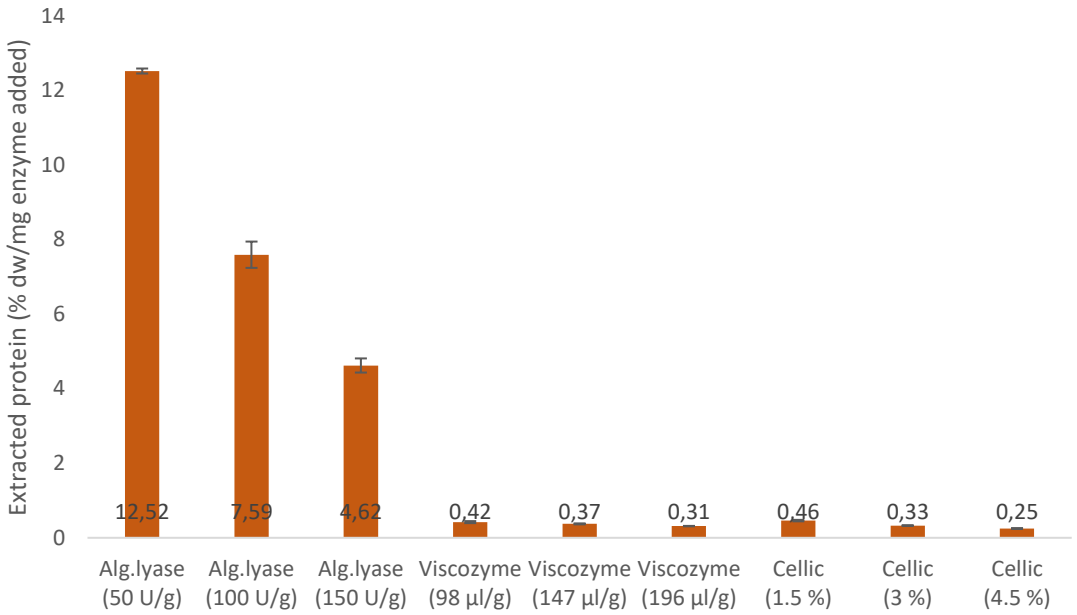

Figure 14: Extracted protein from *S. latissima* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2, taking into account the contribution of the enzyme to the total protein. The total protein was divided on the amount of enzyme added. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

From Figure 14, there is one concentration of each enzyme that stands out with a higher protein yield than the other two. For alginate lyase the difference is still quite large, while for Viscozyme and Cellic the extracted protein is similar between concentrations. For each enzyme, the difference is significant between all three concentrations except for between Viscozyme concentration 1 and 2. Thus, the highest total extracted protein yield for *S. latissima* is now shown for concentration 1 of all enzymes (not significantly for Viscozyme). Unfortunately, this was discovered too late to take into consideration in further lab work.

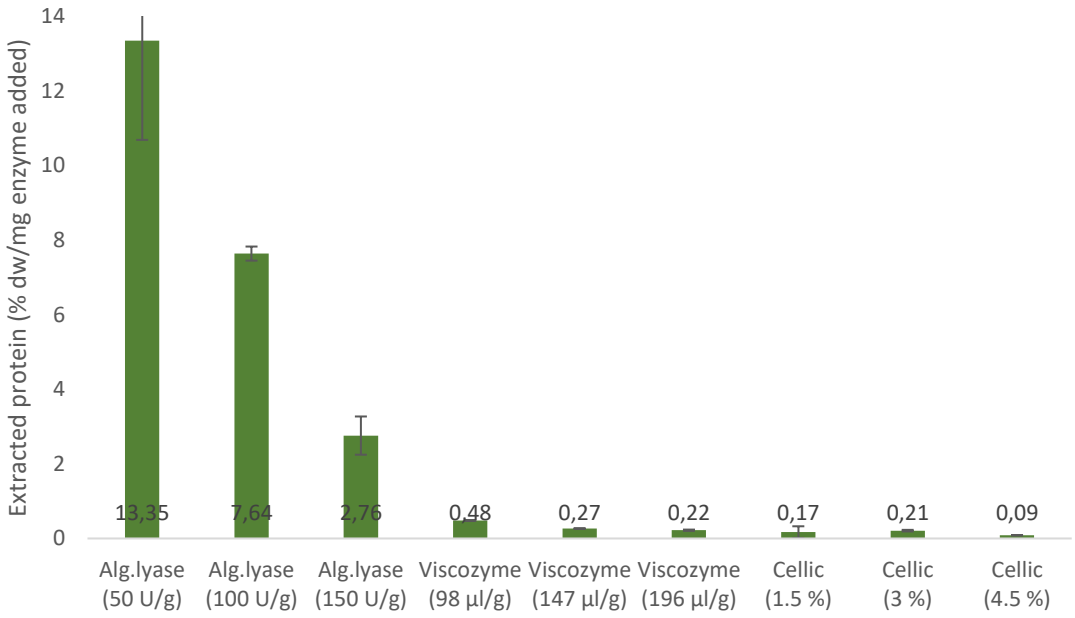

Figure 15: Extracted protein from *A. esculenta* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2, taking into account the contribution of the enzyme to the total protein. The total protein was divided on the amount of enzyme added. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

From Figure 15, there is one concentration of each enzyme that gives higher protein yield than the other two. For Alginate lyase the difference is bigger than for Viscozyme and Cellic. The differences are significant between all three concentrations for Alginate lyase and Viscozyme, while for Cellic there is only a significant difference between concentration 2 and 3. Thus, the highest protein yield from *A. esculenta* is significantly shown for concentration 1 of Alginate lyase and Viscozyme, and concentration 2 of Cellic CTec. Unfortunately, this was discovered too late to take into consideration in further lab work.

Extracted protein yield was also calculated without dividing by the amount of enzyme used, to see the actual protein yield from treatments. Results are shown in Figure 16 for *S. latissima* and Figure 17 for *A. esculenta*. In both figures, the grey part represents the yield of the control value, while the coloured part shows the total protein yield with the subtracted control value. Thus, the full column represents the total extracted protein. In Figure 17, the control sample had a higher yield than the enzymatically treated samples for three treatments. These are Viscozyme 98 µl/g, Cellic 1.5 % and Cellic 4.5 %. In these cases, the green part of the column is the total extracted protein and the light grey part represents the additional yield in the control sample for comparison. The light grey parts are not labelled with the yield since they do not contribute to the protein yield in the sample.

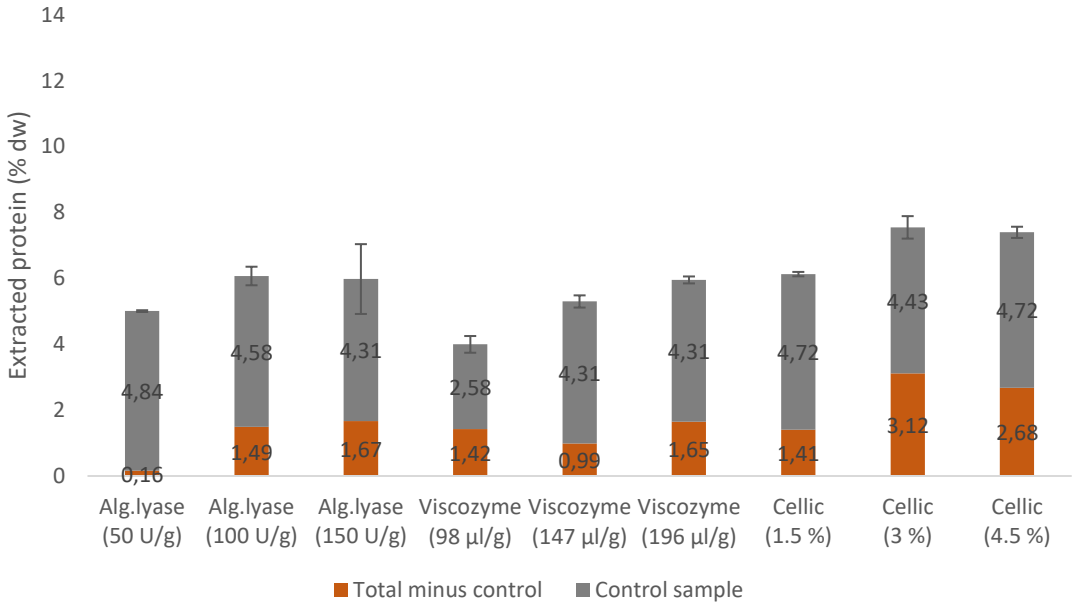

Figure 16: Extracted protein from *S. latissima* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 16 shows that the enzyme concentrations giving the highest protein yield vary slightly from what is seen in Figure 14. For Alginate lyase, concentration 2 gives the highest protein yield, but it is only significantly higher than concentration 1. For Viscozyme, concentration 3 is significantly higher than the others. For Cellic CTec, concentration 2 gives higher protein yield, but only significantly higher than concentration 1. All concentrations of Cellic CTec give significantly higher protein yield than the two other enzymes, except for concentration 3 of Alginate lyase. Also, the difference between the lowest yield in Cellic and the highest yield in Viscozyme is not significant. The highest yield for Alginate lyase is higher than all yields from Viscozyme, but only significantly higher than concentration 1 and 2. The lowest yield from Alginate lyase is significantly higher than Viscozyme concentration 1 and significantly lower than Viscozyme concentration 3. Overall, the protein yield seems to be best from treatment with Cellic CTec, followed by Alginate lyase and Viscozyme.

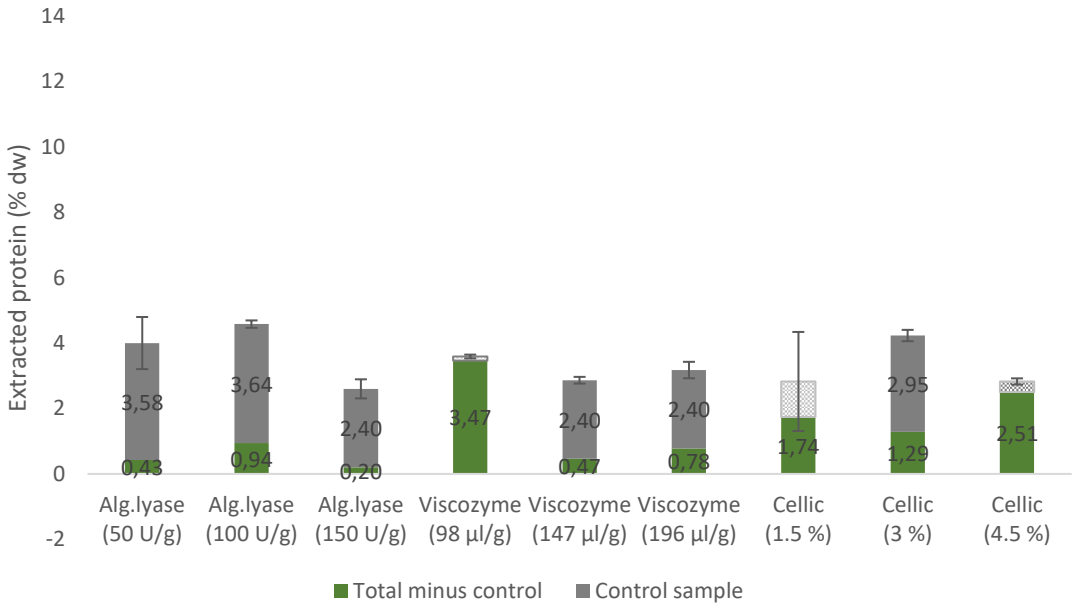

Figure 17: Extracted protein from *A. esculenta* with different enzyme concentrations of the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. Exceptions are for Viscozyme 98, Cellic 1.5 and Cellic 4.5, where the green part is the total extracted protein from enzymatic treatment and the light grey part is the additional yield in the control sample. The enzymes and their concentrations are marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 17 shows that the enzyme concentrations giving the highest protein yield vary slightly from what is seen in Figure 15. For Alginate lyase, concentration 2 gives the highest protein yield, but it is only significantly higher than concentration 3. For Viscozyme, concentration 1 is significantly higher than concentration 2. For Cellic CTec concentration 2 gives the highest protein yield, but only significantly higher than concentration 3. Overall, Alginate lyase concentration 2 has the highest protein yield, significantly higher than all Viscozyme as well as Cellic concentration 3. The second highest yield is for Cellic concentration 2, which is significantly higher than all Viscozyme as well as Alginate lyase concentration 3. The best yield for Viscozyme is significantly higher than Alginate lyase 3 as well as Cellic concentration 2 and 3.

Viscozyme concentration 1 and Cellic 1 and 3 have lower protein yield than their control samples. This would mean that enzymatic treatment in these cases aggravates protein extraction. Cellic concentration 1 has a very high standard deviation and is not significantly different than any other values. It is believed that experimental variations might be the reason for these unexpected results and that enzymatic treatment would improve protein yield in all

cases if experiments were repeated with a higher number of parallels or conducted in a bigger scale. It is demonstrated that differences between control samples may affect the results. The changed enzyme contribution shows how large differences can get between experiments conducted at different times, and how much this can also affect the results. From the two representations with the right subtracted enzyme contribution, it is seen that the lowest concentration of enzymes dominates as the most cost efficient (when divided on the amount of enzyme), while the middle concentration dominates as the one giving the total highest yield.

3.2.6 The effect of buffer

The trials using treatment from Cellic CTec in section 3.2.5 were performed using a citrate phosphate buffer to optimize the pH. To check the effect of the buffer on results from the Lowry analysis, a control sample was compared to a control sample with added buffer (Figure 18).

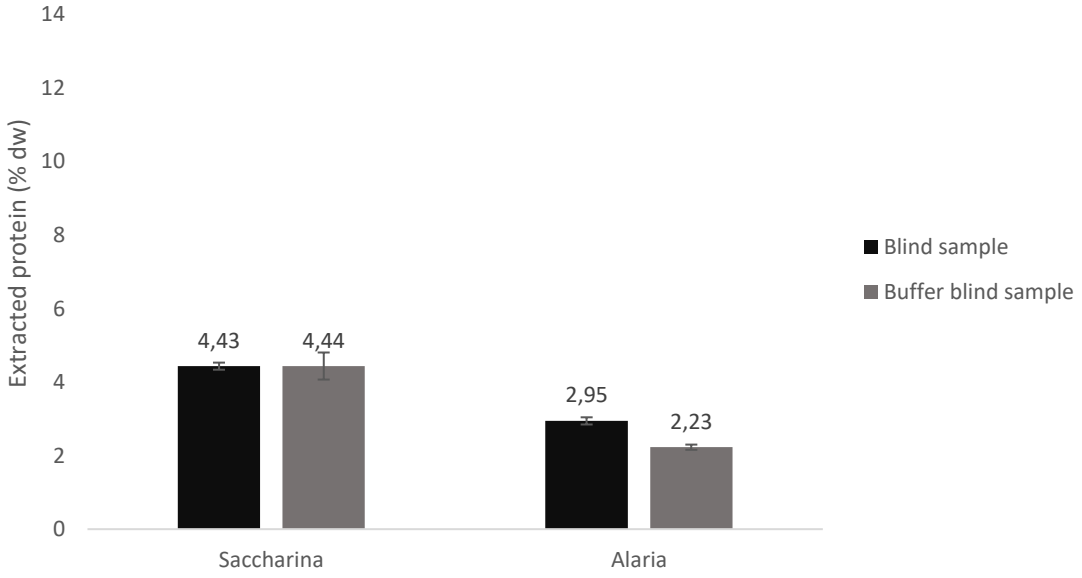

Figure 18: Extracted protein from *S. latissima* and *A. esculenta*. Control samples are compared to control samples with added citrate phosphate buffer. The x-axis marks the algae species *S. latissima* and *A. esculenta*. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 18 shows that the yield from the two blind samples is very similar. There was no significant difference for *S. latissima*, while for *A. esculenta* the control sample without buffer was significantly higher than the one containing buffer. Results from *A. esculenta* might

indicate that measured protein is reduced with buffer. Since the difference is rather small and disagrees with what is observed for *S. latissima*, the effect of buffer is therefore not further considered to be affecting analyses.

There was also a question if the buffer had the desired effect on the enzyme activity. Also, in the experiments where buffer was used with Cellic CTec, the pH was not adjusted back to neutral before performing the Lowry analysis, even though neutral to basic pH is optimal. Therefore, the effect of different pH adjustments on protein yield and Lowry measurements was tested. It was recorded that lowering pH using Viscozyme gave increased polysaccharide breakdown in algal biomass (Shannon and Abu-Ghannam, 2018). Therefore, the effect of lowering pH with buffer was performed for both Cellic CTec and Viscozyme. Only *S. latissima* was used for this experiment, assuming the effect to be the same in *A. esculenta*. Results are shown in Figure 19.

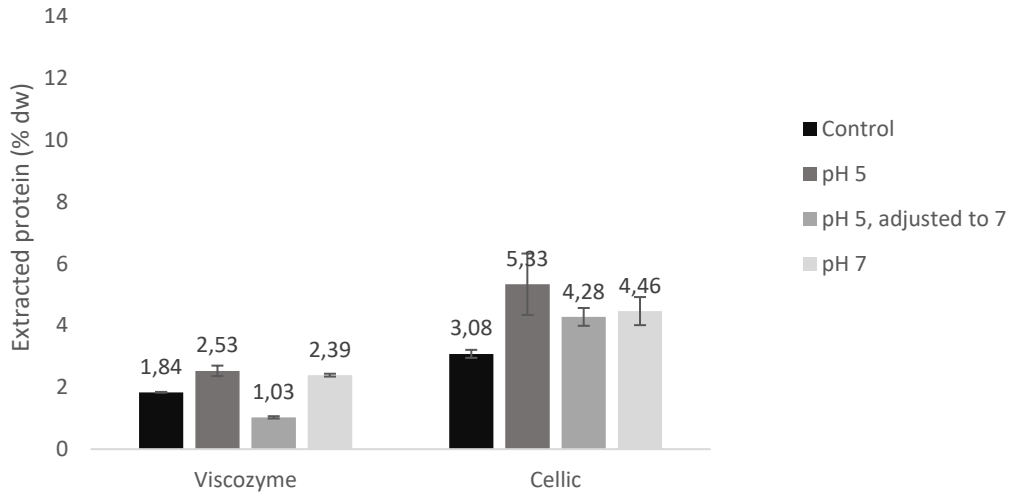

Figure 19: Extracted protein from *S. latissima*. Different pH adjustments were performed in protein extraction with pre-treatment from Viscozyme L and Cellic CTec2. The x-axis marks the two enzymes. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Results (Figure 19) show that the sample with pH 5 adjusted to 7 before Lowry analysis has the lowest protein yield of all treatments, and it is even lower than the control value in the experiment with Viscozyme. The difference is significant for all results for Viscozyme, but only significantly different from the control sample for Cellic. The samples with pH 5 and pH 7 give the highest protein yield with very similar values, and there is no significant difference

for any of the two enzymes. For Viscozyme, these two are significantly higher than both the other samples. It is considered to be unnecessary to lower the pH in enzyme incubation, as it does not seem to increase the protein yield. For this reason, buffer was not used in further work.

3.2.7 Comparing old and new *S. latissima*

Until this point, almost all results from experiments with *S. latissima* gave an unexpectedly higher protein yield than those from *A. esculenta*, even though the latter algae specie has a higher content of protein. It is also reported by other authors that *A. esculenta* has a higher protein extractability than *S. latissima* for different treatment methods (Lyng, 2015; Reissiger, 2016). It was expected that the extracted protein yield would be higher than the one for *S. latissima*, or at least more similar. It was discovered that the used *S. latissima* had not been appropriately cooled after harvest, and it was assumed that this had affected the state of the raw material, and thereby the extraction yield. A new batch of *S. latissima* was received from another supplier. Protein extraction was compared between the two batches of *S. latissima* under identical treatment conditions. This was done by ultrasonic and enzymatic treatment with respectively varying number of sonication runs (US x 1-3) and enzyme concentrations (1-3). Results are shown for sonication in Figure 20 and for enzymatic treatment in Figure 21.

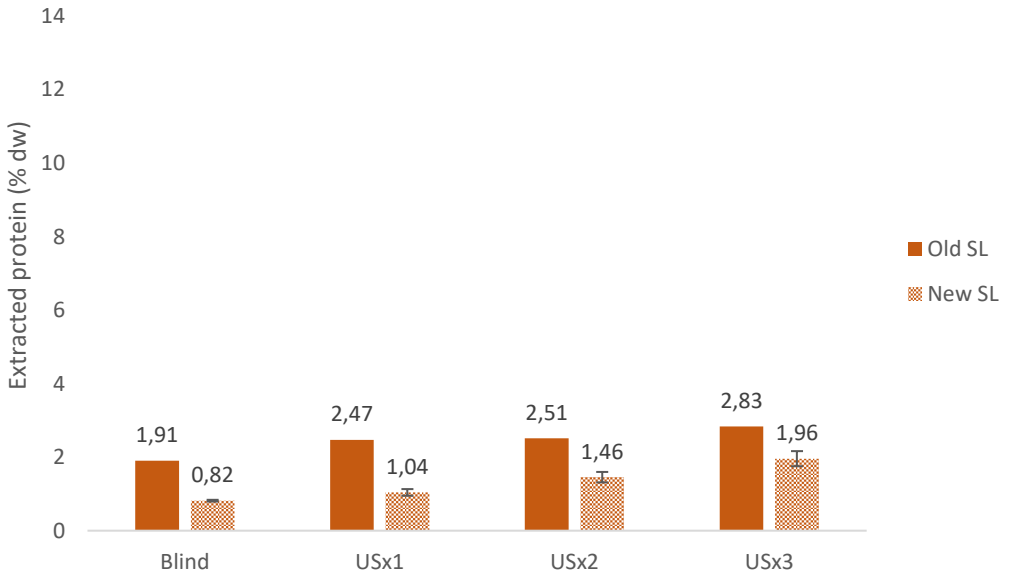

Figure 20: Extracted protein from two different *S. latissima* with different amount of ultrasonic treatment. The strongly coloured columns are the old *S. latissima*, while the patterned columns represent the new *S. latissima*. Values of *old S. latissima* originate from one parallel for each sample. Values of new *S. latissima* originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

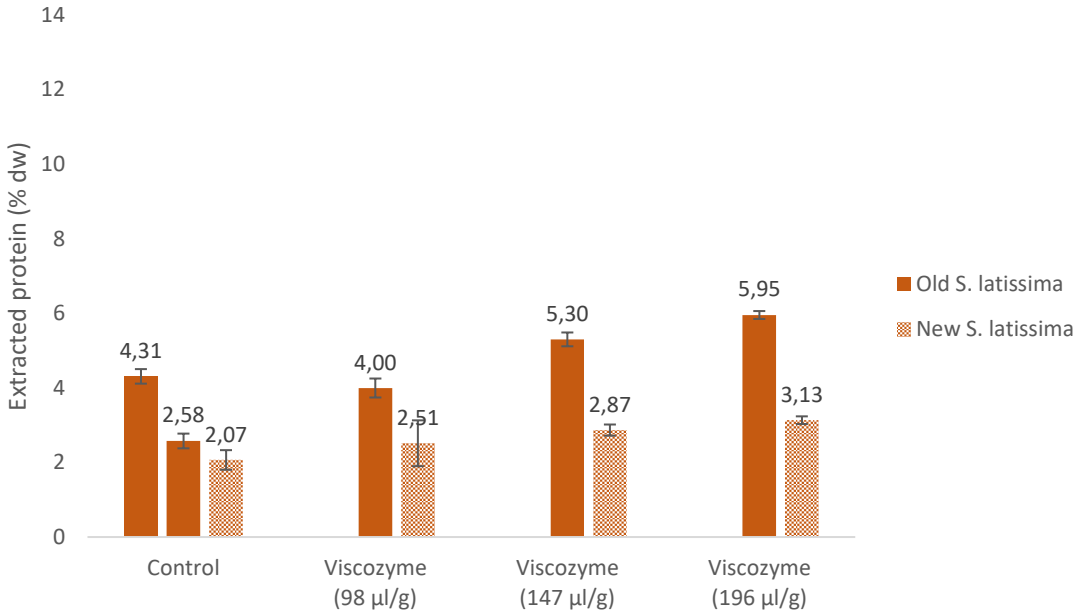

Figure 21: Extracted protein from two different *S. latissima* treated with different concentrations of the enzyme Viscozyme. The strongly coloured columns are the old *S. latissima*, while the patterned columns represent the new *S. latissima*. There are two control samples for the old *S. latissima*. The first (taller) one is for Viscozyme L concentrations 2 and 3, while the second (lower) is for Viscozyme L concentration 1. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Looking at Figure 20 and Figure 21, there is strong evidence that protein yield is highly improved in the old batch compared to the new one. It is reasonable to assume that in the old algae that had not been properly cooled, the breakdown of plant biomass had already started, inducing an improved protein extraction.

From Figure 20 it is highly suspected that there is a difference between the two batches of *S. latissima*, even though no statistical analysis was conducted. The extraction yield increased highly in the new *S. latissima* compared to the old one, with 0.87 increase (1.4 times) for USx3, 1.05 increase (1.7 times) for USx2, 1.09 increase (2.3 times) for the control sample and 1.43 increase (2.4 times) for USx1. The difference is large, especially for control and USx1, which are the least treated samples. The smallest increase is for USx3, which is also the most treated sample. It seems like the final extracted protein yield in the least treated samples are more affected by the difference between the two batches of algae, while the more treated samples are less affected. This is reasonable, since the already slightly broken down biomass will be less affected by the further breakdown by sonication, and the difference will thereby decrease for

more ultrasonic treatment. For the less treated samples, the contribution of already started biomass breakdown in the old *S. latissima* gives a bigger difference between the two batches.

Seen from Figure 21, the extraction yield also had highly increased protein extraction from the old *S. latissima* compared to the new one. The difference is significant for all enzyme concentrations except for the two control samples with the lowest protein yield (new control and control number 2 for old batch). In fact, all extractions from the old algae give significantly higher yield than all extractions from the new algae, except for the control samples mentioned. There was 0.51 increase (1.2 times) for control sample 2, 1.49 increase (1.6 times) for enzyme concentration 1, 2.24 increase (2.2 times) for control sample 1, 2.43 increase (1.8 times) for enzyme concentration 2 and 2.82 increase (1.9 times) for enzyme concentration 3. Thus, except for control sample 1, there is a higher increase between the old and new batch of algae for more enzymatically treated samples. This might indicate that the final extracted protein yield in the least enzymatically treated samples is less affected by the difference between the two batches of algae, while the more treated samples are more affected. In addition, the yield from the new *S. latissima* increases only slightly for increasing enzyme concentration, while the old batch has quite a steep linear raise for increasing enzyme concentrations. It seems like the new algae is more "resistant" to the enzymatic breakdown, while the old alga is more susceptible to the action of enzymes. This might demonstrate that the biomass that is more broken down gives better exposure of the substrate to the enzyme, and thereby giving a highly increased protein yield.

The trend in Figure 21 (enzymatic treatment) is quite the opposite than the one from Figure 20 (ultrasonic treatment). For the ultrasonic treatment, a more treated sample gives a smaller difference between the protein yield of the two batches. Contrary for the enzymatic treatment, where more potent treatments give larger differences between the protein yield of the two batches. This gives a reason to think that enzymatic treatment with Viscozyme and ultrasonication induces quite different reactions in the plant biomass. The sonication seems to break up plant biomass more unspecific and unaffected by the state of the algae. For longer sonication time, further sonication gives less effect since most of the biomass is already broken down. Viscozyme is an enzyme that catalyses bond cleavage between glucose monomers in cellulose, and therefore specifically uses cellulose as its substrate. It is more specific and might therefore be more affected by the state of the algae, and the exposure of the right substrate. More degraded biomass (the old batch) might expose more of the biomass and give access to the cellulose for the enzyme. This might give a higher effect of the enzymatic treatment.

It might seem that storing the algae in elevated temperature before treatment and extraction might in fact improve the protein yield. It also affects the impact of further treatment. However, inappropriate preservation might reduce the quality of the plant material and the extracted nutrients, which is not beneficial. Seaweeds start decomposition quickly after harvest (Enriquez, Duarte and Sand-Jensen, 1993), and it is important with proper preservation for good quality and safety of the biomass. With the purpose of using seaweed in food and feed products, rapid biomass degradation may affect the nutritional value as well as the total biomass quality (Stévant *et al.*, 2017). For this reason, despite of the lower protein yield, the new and properly preserved *S. latissima* was used in the further work.

3.2.8 The correlation between protein and polyphenol extraction

Two of the experiments with enzymatic treatment from Alginate lyase were done in cooperation with another student that was working with the extraction of polyphenols. The experimental procedure for pre-treatment and extraction was performed together, and supernatant from the extraction was analysed separately. Results from polyphenol analysis were retrieved by the other student, analysed by the Folin-Ciocalteu assay (Singleton, Orthofer and Lamuela-Raventós, 1999; Nenadis, Lazaridou and Tsimidou, 2007). Protein extraction yield from these experiments is already presented as some of the samples from the "combined effect of enzymes and US" shown in section 3.2.1 and "enzyme concentrations" in section 3.2.5. From the former section, samples (from Figure 6) are E→US with pre-treatment from alginate lyase and the corresponding control sample. From the latter section, samples (from Figure 16 and Figure 17) are those treated with alginate lyase with 50 U/g and 100 U/g and corresponding control samples.

Extracted protein yield is plotted as a function of extracted polyphenol yield, shown in Figure 22 for *Saccharina latissima* and Figure 23 for *Alaria esculenta*. It must be highlighted that comparison is made to understand the relationship between extraction yield of polyphenols and protein, and it is not a comparison between different enzyme concentrations or treatment conditions. Therefore, despite being results from different treatments, they are compared together only based on their protein and polyphenol extraction yield.

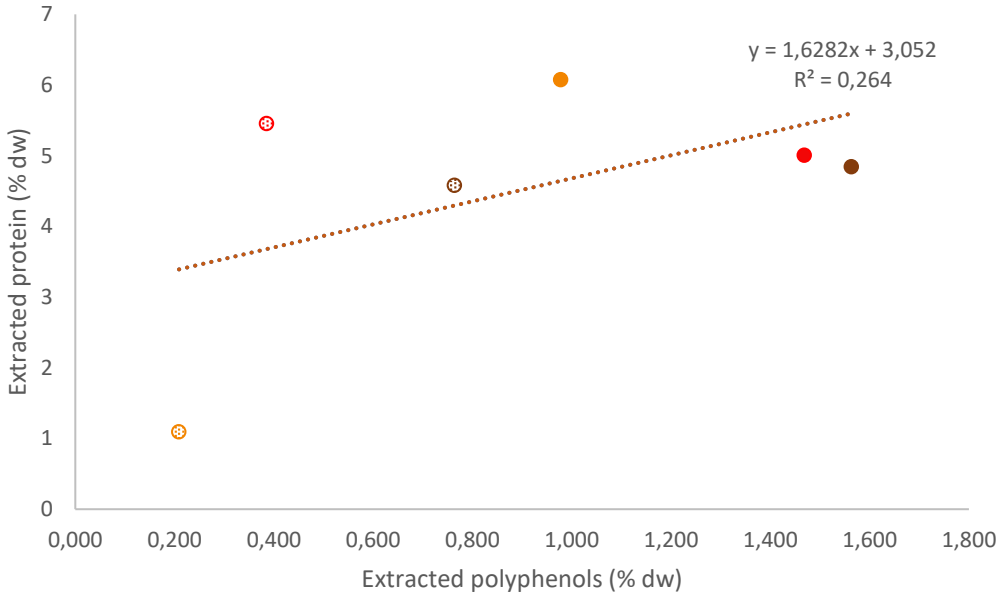

Figure 22: Extracted nutrients from *Saccharina latissima* with different treatment conditions. Extracted protein yield is plotted as a function of extracted polyphenol yield. Fully coloured dots represent treated samples and patterned dots represent control samples (corresponding colour for the corresponding sample). Yellow is for "combined effect of enzymes and US". Red and brown are respectively for treatment with Alginate lyase, 50 U/g and 100 U/g.

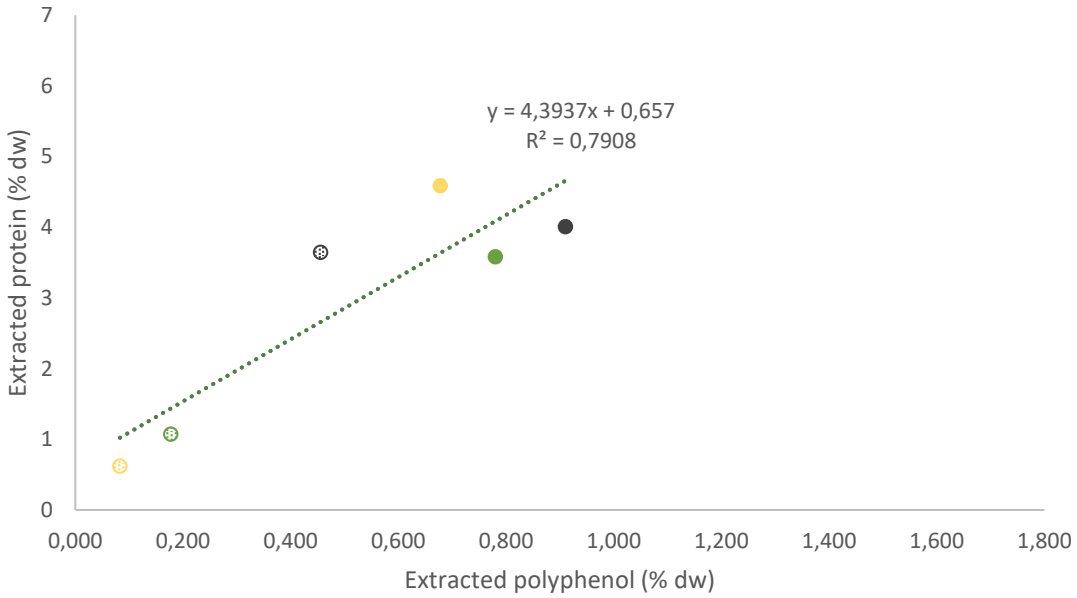

Figure 23: Extracted nutrients from *Alaria esculenta* with different treatment conditions. Extracted protein yield is plotted as a function of extracted polyphenol yield. Fully coloured dots represent treated samples and patterned dots represent control samples (corresponding colour for the corresponding sample). Yellow is for "combined effect of enzymes and US". Green and grey are respectively for treatment with Alginate lyase, 50 U/g and 100 U/g.

First, the theoretical polyphenol content in the two species must be disclosed. *Saccharina latissima* and *Alaria esculenta* are recorded in literature to have a polyphenol content of respectively 0.41±0.15 % (dw) and 0.87±0.52 % (dw) for samples harvested regularly throughout the year (only March, May and July for *Alaria esculenta*). Results for *Saccharina latissima* are higher in the relevant harvesting season (calculated to around 0,57 % in June) and for *Alaria esculenta* it is similar to average (about 0,90 % in May) (Schiener *et al.*, 2015). Another source has recorded polyphenol contents of 0.5-1.5 % (dw) and 1.4-6.1 % (dw) for respectively *S. latissima* and *A. esculenta* in April to October (Roleda *et al.*, 2019). It is said that high-phenolic species have phenolic content higher than 2% (algal dw) (Van Alstyne and Paul, 1990). Thus, *A. esculenta* has a higher polyphenolic content than *S. latissima* and might be described as a high-phenolic specie.

Figure 22 and Figure 23 show the relation between protein yield and polyphenol yield in the two species of brown algae. Even though there is no clear-cut linear relationship, both figures show a positive correlation between the amount of extracted polyphenol and extracted protein. For *Saccharina latissima* in Figure 22, all differences in protein yield are significant except between control Alg.lyase 50 (light red) and control Alg.lyase 100 (light brown)/Alg.lyase 50 (red), as well as control Alg.lyase 100 (light brown) and Alg.lyase 50 (red). The polyphenol yield is significantly different for all except control Alg.lyase 50 (light red) and Alg.lyase 50 (red). For *Alaria esculenta* in Figure 23, all differences in protein yield are significant except the same samples as mentioned for *S. latissima*, and also between sample Alg.lyase 50 (green) and Alg.lyase 100 (grey). The polyphenol yield is significantly different for all except control Alg.lyase 50 (light green) and Alg.lyase 50 (green)/Alg.lyase 100 (grey) as well as between Alg.lyase 50 (green) and Alg.lyase 100 (grey). The insignificant differences are few, and they are seen among the values that give the highest deviations in the regression line, which can make it even more likely that there is a correlation.

The correlation can also be seen by looking at the regression lines with corresponding regression coefficients. From Figure 22 (*S. latissima*), the regression line has the equation $y = 1{,}6282x + 3{,}052$ and R-squared is 0,264. From Figure 23 (*A. esculenta*), these are $y = 4{,}3937x + 0{,}657$ and 0,7908. The equations disclose positive correlations between the two variables for both algae, and the increase is steeper for *A. esculenta*, meaning that there is a higher increase in protein extraction resulting from increased polyphenol extraction in this specie. The R-squared shows that the data for *A. esculenta* are closer to the fitted regression line, where 79 % of the variation is explained by the model, compared to 26 % for the fitted

line for *S. latissima*. Thus, the correlation between extracted protein and polyphenols is better explained by the linear model for *A. esculenta*, and the protein extraction yield is more strongly affected by increased polyphenol yield compared to *S. latissima*. Still, there is evidence that might suggest correlation between variables for both species, where increasing levels of extracted polyphenols give increasing levels of extracted protein.

The positive correlation between extracted protein and polyphenols is logical, since the samples have undergone treatment that breaks up the biomass, which enables more efficient nutrient extraction. Enzymatically assisted extraction is said to induce breakage of the complex bonding between phenolics and proteins and is suitable for extraction of phenolic compounds (Kadam, Álvarez, *et al.*, 2015a) and proteins from seaweeds (Sari *et al.*, 2015; Mæhre, Jensen and Eilertsen, 2016). Also, sonication using an ultrasonic probe system is recorded to give increased extraction efficiency of polyphenols (Fang *et al.*, 2014) and proteins (Kadam *et al.*, 2017). More treatment gives more released nutrients, and therefore it is likely to think that the extractability of both proteins and polyphenols increases in a similar manner.

However, it is reported in literature that seaweed with high content of polyphenols might result in lower yield in protein extraction, due to a high negative correlation between phenolic content and protein yield (Wong and Cheung, 2001). This is because proteins and polyphenols in seaweed might be strongly bound (Stern *et al.*, 1996). According to this statement, it would be likely to think that there would be a negative correlation between polyphenol and protein in this experiment. On the other hand, there is a difference between the content of nutrients present and the portion that is extractable in water. It is recorded that phenolic compounds are generally more soluble in organic solvents than in water (Wang, Jónsdóttir and Ólafsdóttir, 2009), while proteins are more easily extracted with water as only solvent (Chirinos *et al.* 2007, referred by Wang, Jónsdóttir and Ólafsdóttir, 2009). This difference in water solubility might explain the positive correlation between polyphenols and protein. The proteins might have been extracted to a greater extent, while a smaller portion of the total polyphenol content has been extracted.

These results might also explain the higher protein extractability of protein from *S. latissima* compared to *A. esculenta*, even though the latter contains more protein. As mentioned, *A. esculenta* has a higher polyphenol content, and might be classified as a high-phenolic specie. The higher polyphenol content might reduce the efficiency in protein extraction and give a lower protein yield. Then, the reduced extractability of proteins might affect the polyphenol extractability and lower the polyphenol yield. Because proteins and polyphenols bind tightly together, their presence will induce binding to the other one and lower the other's extractability

even more. This will give worsened extractability of both nutrients, and thereby lower extraction in *A. esculenta*. Biomass treatment will affect both nutrients at the same time and increases the extractability of proteins and polyphenols together. According to findings in this section, this effect seems to be even stronger in *A. esculenta* than in *S. latissima*.

3.3 Further protein extractions

3.3.1 Treatment order for sonication and enzymes

Results in section 3.2.1 showed that combining sonication and enzymes might improve extraction yield. Also, there was evidence from section 3.2.7 that enzymatic treatment might be more efficient for biomass that has already been broken down to some extent. This was further tested by combining enzymes and US, with sequential treatment by enzymes and US in different orders. This was performed for both wet and dry algae with the three enzymes Alginate lyase, Viscozyme and Cellic CTec. Enzymatic treatment before sonication is denoted E→US, while US→E is the opposite direction.

After conducting all treatment, there were visible differences between the samples, see Figure 24. The figure shows control samples compared with samples treated with E→US and US→E with the enzyme Alginate lyase, for wet and dry algae. The control samples have a bright supernatant, while the treated samples are more coloured green/brownish. The samples for E→US are more strongly coloured, and the biomass looks more homogenously blended than in samples for US→E. This is partly because the samples with US conducted lastly are probably inverted and mixed more recently before the picture is taken. It could also suggest that the samples treated with E→US are more degraded. The samples with dry biomass have more coloured supernatants, also for the control samples. This might suggest that extraction is more efficient with dry biomass.

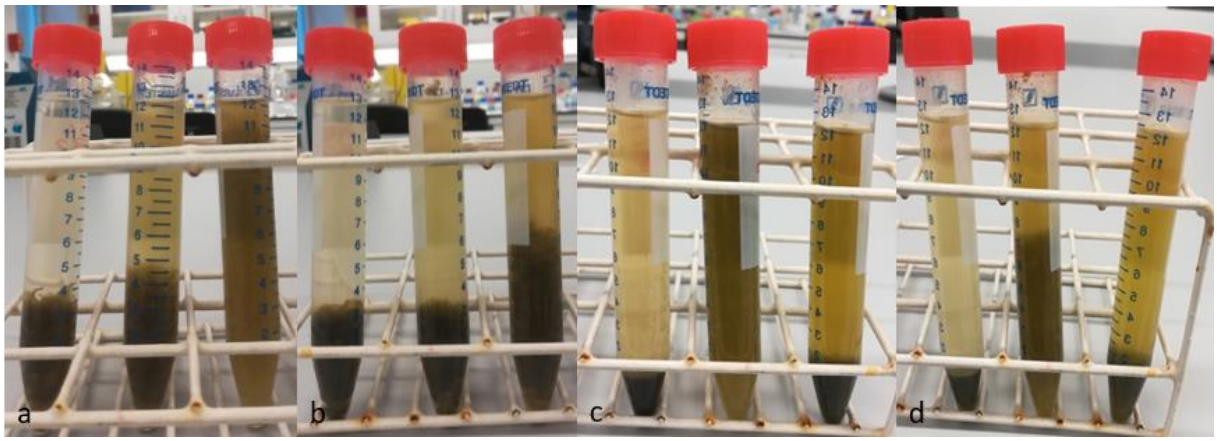

Figure 24: Shows differences in the appearance of samples from different treatments. Part a) shows wet *S. latissima*, b) shows wet *A. esculenta*, c) shows dry *S. latissima* and d) shows dry *A. esculenta*, all with corresponding treatments. For a) and b), the first sample is a control sample with only water and algae, the second is treatment with US → Alginate lyase and the third is Alginate lyase → US. For c) and d) the first sample is control sample, the second is Alginate lyase → US and the third is US → Alginate lyase.

Protein yield from extraction is presented in Figure 25 and Figure 27, respectively for extracted protein from wet and dry *S. latissima*. Figure 26 and Figure 28 respectively present wet and dry *A. esculenta*. In all figures, the grey part represents the yield of the control sample, while the coloured part shows the protein yield with subtracted control value. Thus, the full column represents the total extracted protein. Due to limited stock of *S. latissima* at the point of this experiment, only *A. esculenta* (Figure 26 and Figure 28) had extra control samples for Cellic CTec at 50 °C. It is nevertheless assumed that control sample values are quite similar at 37 °C and 50 °C. Thus, for *S. latissima* (Figure 25 and Figure 27), control sample values at 37 °C are also used for Cellic CTec.

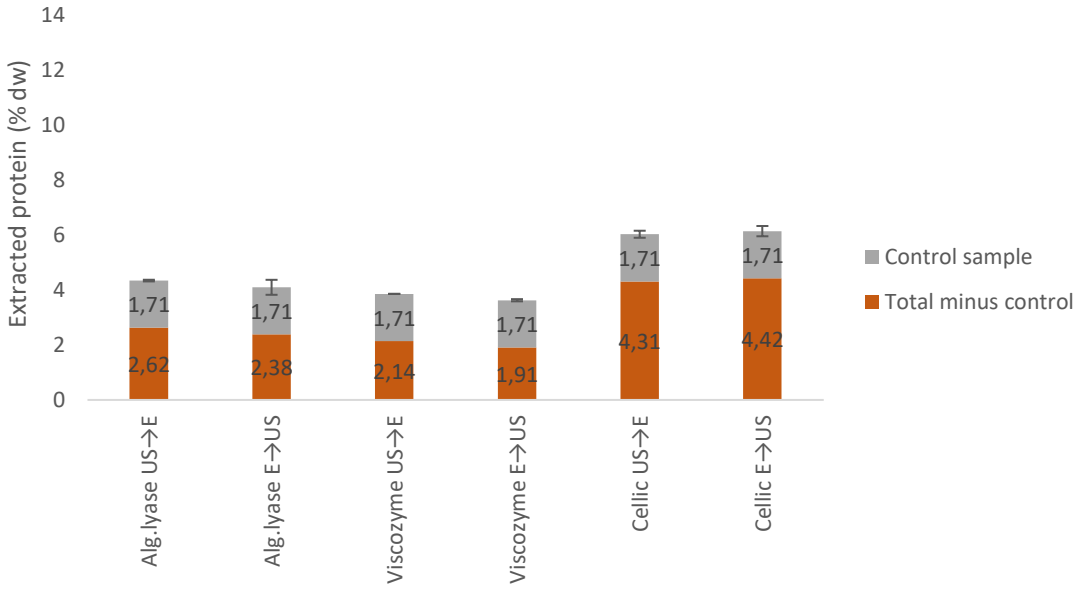

Figure 25: Extracted protein from wet *S. latissima* with sequential enzymatic and sonication treatment, with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The direction of sequential treatment and the enzyme used is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 25 shows an unexpectedly small difference in the protein yield by changing the order of treatment by enzymes and US for wet *S. latissima*. For Alginate lyase and Viscozyme US→E gives a slightly higher yield, while for Cellic there is a somewhat higher yield for E→US. The difference is minimal for all enzymes and only significant for Viscozyme.

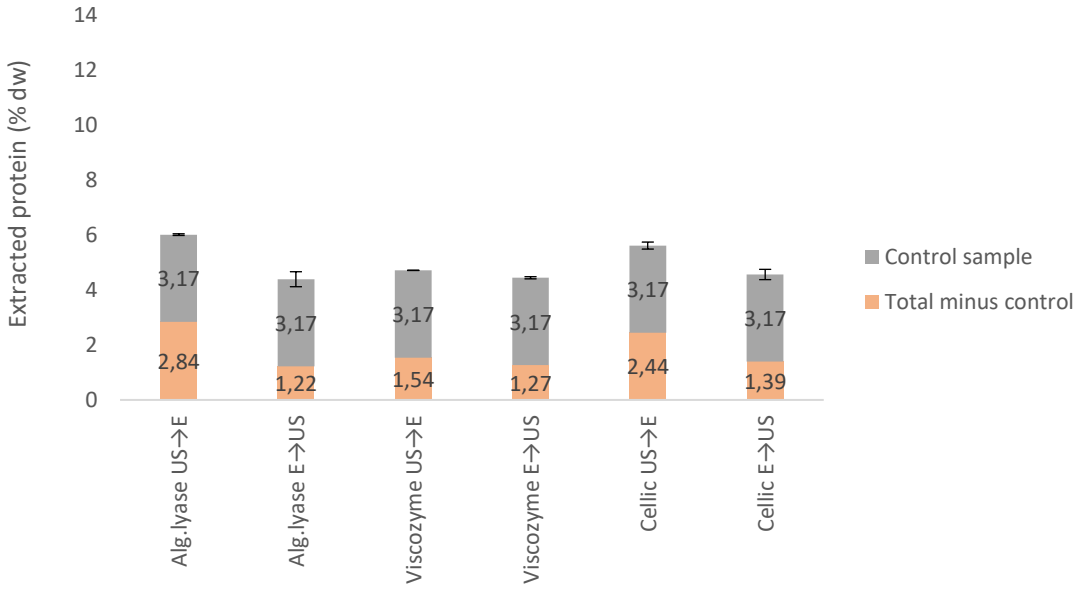

Figure 26: Extracted protein from dry *S. latissima* with sequential enzymatic and sonication treatment, with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The direction of sequential treatment and the enzyme used is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 26 shows a more apparent effect on the protein yield by changing the order of conducted treatment by enzymes and US for dry *S. latissima*. For all enzymes, the yield is higher for US→E, and this is significant for treatment by Alginate lyase and Cellic CTec.

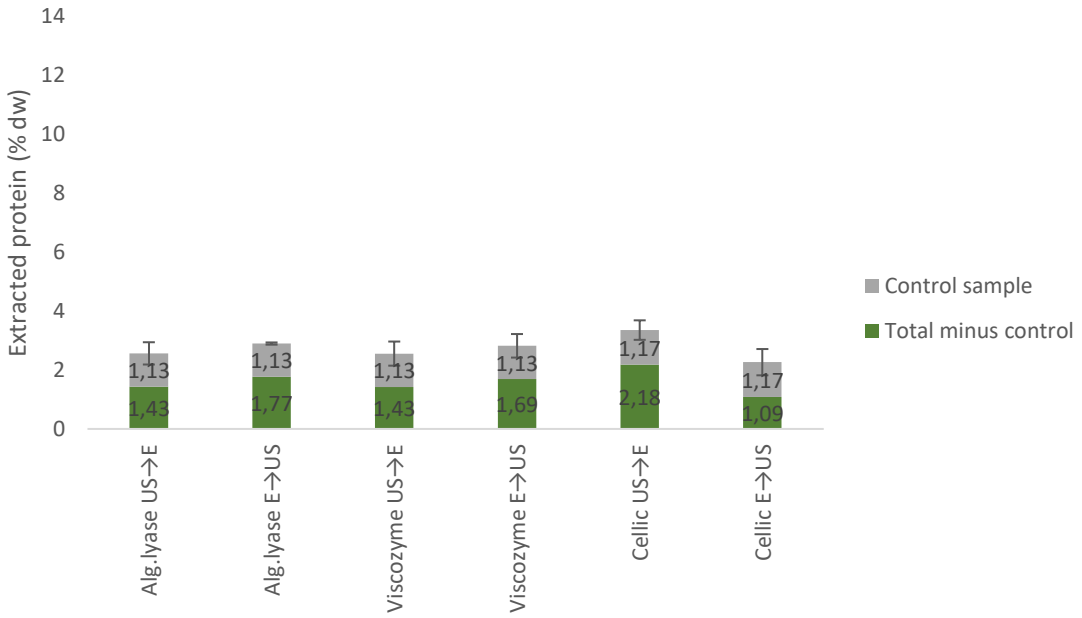

Figure 27: Extracted protein from wet *A. esculenta* with sequential enzymatic and sonication treatment, with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The direction of sequential treatment and the enzyme used is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 27 also shows variations in the protein yield by changing the order of conducted treatment by enzymes and US for wet *A. esculenta*, but no clear pattern. There are high standard deviations in these results, and no significant differences between different treatment orders.

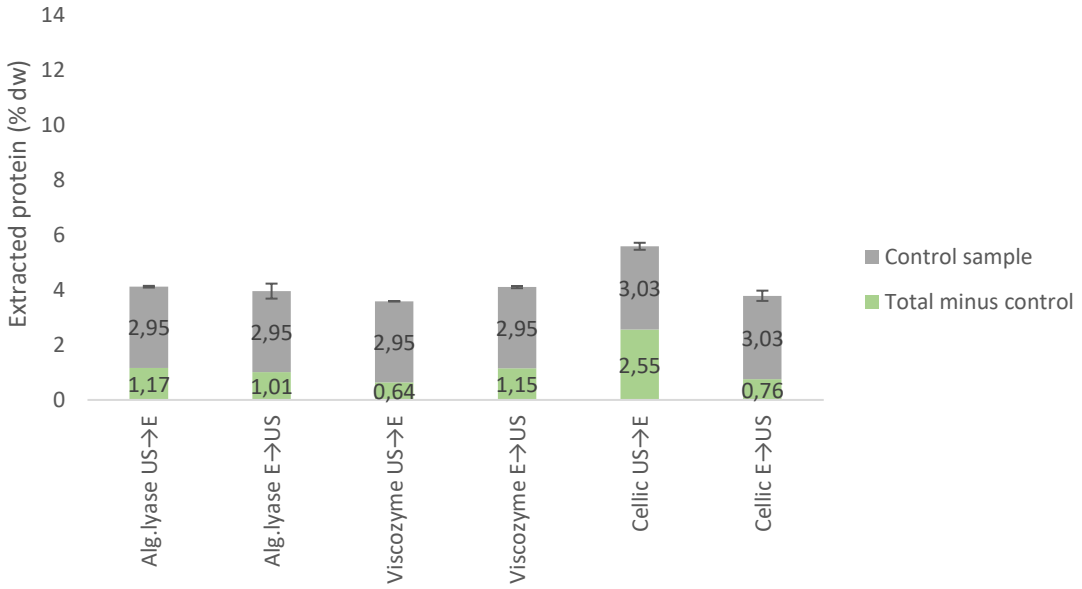

Figure 28: Extracted protein from dry *A. esculenta* with sequential enzymatic and sonication treatment, with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The direction of sequential treatment and the enzyme used is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars

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Figure 28 shows a similar trends for dry *A. esculenta* as seen in Figure 27 for wet *A. esculenta* on the effect of the order of conducted treatment by enzymes and US for protein yield. For Viscozyme, E→US gives higher yield, while for Alginate lyase and Cellic there is a higher yield for US→E. This difference is only significant for treatment by Cellic CTec, where the difference is quite evident.

Looking at the overall results from the diagrams in Figure 25, Figure 26, Figure 27 and Figure 28, there are different trends for *S. latissima* and *A. esculenta* on the effect of the order of treatments on protein yield. From Figure 25 and Figure 27 with wet and dry *S. latissima* half of the results show significantly that an improved protein yield when the biomass is sonicated before enzymatic treatment. This also agrees with assumptions from section 3.2.7 (comparison of old and new *S. latissima*), where already degraded biomass was more susceptible to further breakdown by enzymes. For *A. esculenta* (Figure 26 and Figure 28) with Cellic CTec, the protein yield was higher when conducting sonication before enzymatic treatment, though only significantly for dry algae. No other significant differences are shown for *A. esculenta*. This can indicate that the optimal order of sequential treatment for *A. esculenta* is small or might

vary according to the enzyme that is used. For both dry and wet algae for all treatments, *S. latissima* shows higher protein extraction yield than *A. esculenta.*

Combined sonication and enzymatic treatment by Alginate lyase, Viscozyme and Cellic CTec improves protein yield. All treatments give significantly higher protein yield compared to the corresponding control sample, except for Viscozyme US→E for dry *A. esculenta.* There are also significant variations in the yield from treatment by different enzymes. For wet *S. latissima* (Figure 25), Cellic CTec significantly gives the highest protein yield, followed by Alginate lyase and Viscozyme, even though only Alginate lyase US→E is significantly higher than Viscozyme. For wet *A. esculenta* (Figure 27), there are no significant differences between different enzymes. For dry *S. latissima* (Figure 26), both Alg.lyase and Cellic US→E gives significantly higher yield than treatment by Viscozyme and US. Dry *A. esculenta* (Figure 28) has a significantly higher protein yield in Cellic US→E than both concentrations for Viscozyme and E→US for Alginate lyase, and seems to give the overall highest yield.

3.3.2 Treatment by sonication and enzymes on dry and wet algae

Results from the experiment shown in section 3.3.1 (treatment order of sonication and enzymes) were further processed to analyse the effect of the state of the algae (wet or dry) on the protein yield. The results from different order of sequential treatment (E→US and US→E) were put together, making a total average for combined enzymatic and ultrasonic treatment, independent of the treatment order (E and US). For simplicity, only the control at 37 °C is used since values for 37 °C and 50 °C are very similar. This was done with results from wet and dry algae for both *S. latissima* (Figure 29) and *A. esculenta* (Figure 30). This gives more variation among results and thereby higher standard deviations but can still provide reasonable comparisons between the yield of wet and dry algae.

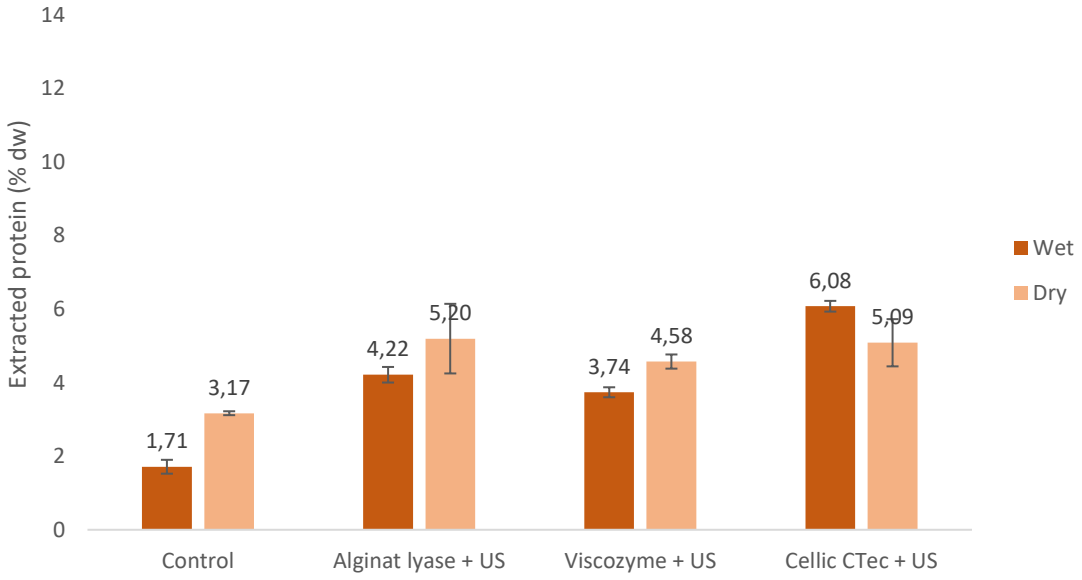

Figure 29: Extracted protein from wet and dry *S. latissima* after sequential enzymatic and sonication treatment with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The x-axis shows the conducted treatments. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

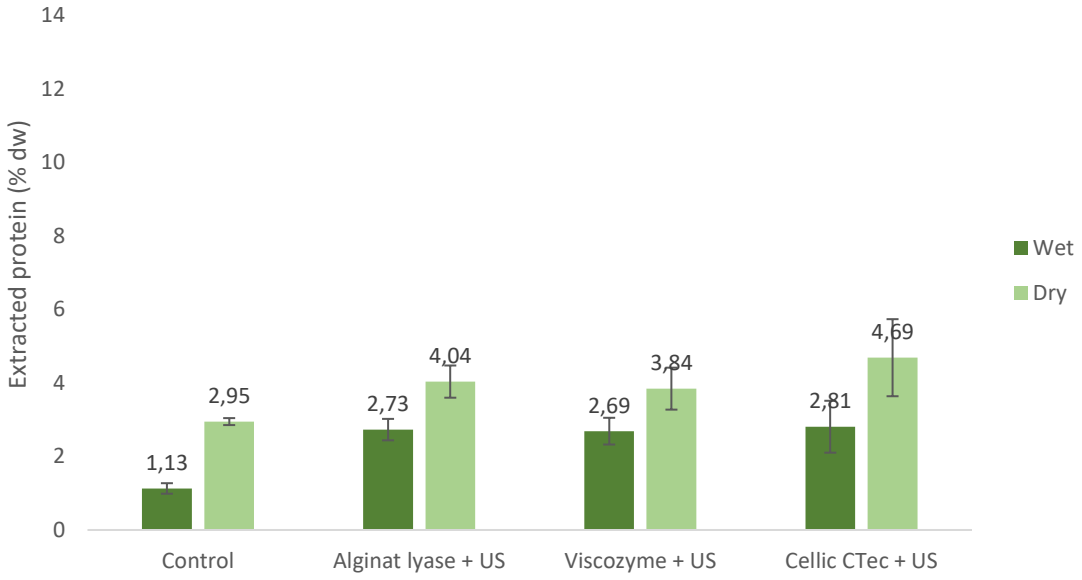

Figure 30: Extracted protein from wet and dry *A. esculenta* after sequential enzymatic and sonication treatment with the enzymes Alginate lyase, Viscozyme L and Cellic CTec2. The contribution of enzymes was subtracted from the measured protein yield. The x-axis shows the conducted treatments. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

From Figure 29 and Figure 30, it can be seen that for both *S. latissima* and *A. esculenta*, the protein yield is higher when extracting from dry algae. All differences between wet and dry

algae for the same treatment are significant. These results are as expected, as dry algae will have more exposure of the tightly packed polysaccharide matrix directly to the solvent, the ultrasonic waves, and the enzymes. This makes the biomass more prone to be affected by the treatment and thereby more protein will be extracted. Wet biomass has a higher content of water bound in the matrix, and water will probably shield the polysaccharide matrix to a greater extent. Also, the freeze-dried algae are generally more easily ground into smaller pieces by a mortar than the wet algae that are cut with a knife. Thus, the dry algae used in the experiment might be overall more finely ground, which will give larger surface area of the biomass to the conducted treatment. There is one exception, where wet alga gives a significantly higher yield than dry algae, which is seen for *S. latissima* treated with Cellic CTec. It might seem like wet *S. latissima* is more susceptible to degradation by Cellic CTec.

There are some significant differences between the yield from treatment by different enzymes. For wet *S. latissima* all treatments are significantly different, and Cellic CTec gives the highest extraction yield, followed by Alginate lyase and thereby Viscozyme. Dry *S. latissima* and wet/dry *A. esculenta* give no significant differences, but still show similar trend, except that dry *S. latissima* indicate that Alginate lyase is more efficient than Cellic CTec.

3.3.3 Treatments for dry algae

More attention was paid to the effect of different treatments on dry algae. The effect of the three enzymes Alginate lyase, Viscozyme and Cellic CTec, as well as ultrasonic treatment, was tested. Results are shown in Figure 31 and Figure 33 for respectively *S. latissima* and *A. esculenta*. In both figures, the grey part represents the yield of the control value, while the coloured part shows the total protein with subtracted control value. Thus, the full column represents the total extracted protein. These results are further compared with results from the combined sequential treatment of sonication and enzymes (E and US) on dry algae, from section 3.3.2. It is worth noticing in Figure 32 and Figure 34 that protein yield for only sonication treatment (US) is the same value in all cases, and does not involve any enzymatic treatment. It is added for a better comparison of the different treatments. For the same figures, for simplicity, only the control sample at 37 °C is used since values for 37 and 50 °C are very similar.

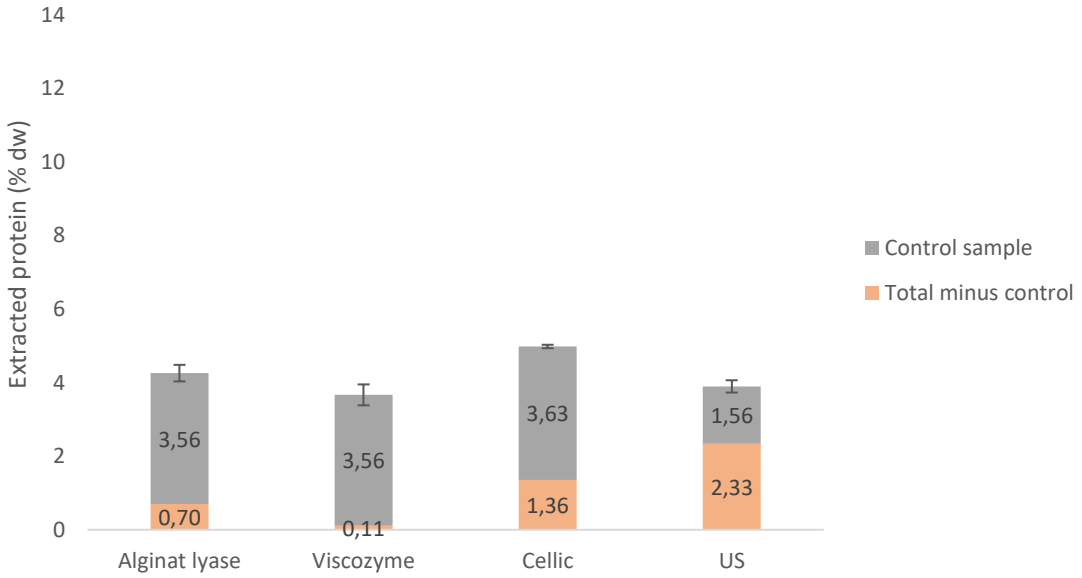

Figure 31: Extracted protein from dry *S. latissima* treated by the enzymes Alginate lyase, Viscozyme L and Cellic CTec2 as well as sonication. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The treatment (and if enzymatic treatment) the enzyme name is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 31 first shows that the control value for the ultrasonic treatment is significantly different from the control value for the enzymatic treatments. Looking at the total extracted protein, Cellic gives significantly higher yield than all other treatments. No other treatments show significant differences. Disregarding the value of the control samples (looking at only the coloured part), sonication shows by far the highest yield. The strong difference between control samples might be explained by the high efficiency of the increased incubation temperature and time on the control sample for enzymatic treatment. A similar difference between enzyme and US control samples is in fact also seen for wet algae, and the influence from time and temperature seems to be present for both wet and dry algae.

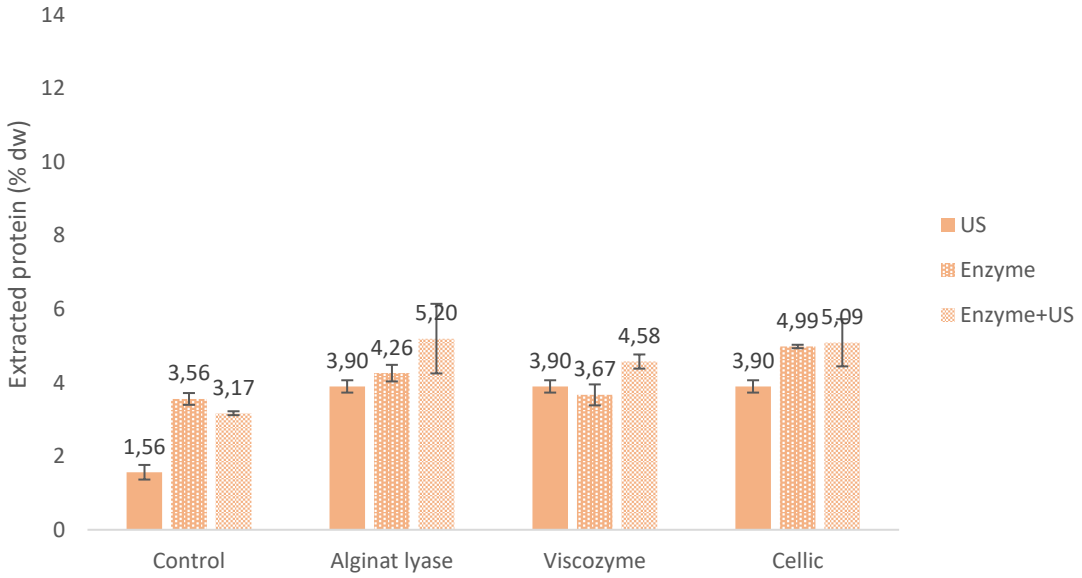

Figure 32: Extracted protein from dry *S. latissima* treated by enzymes, sonication and combined sequential enzyme and sonication treatment. The contribution of enzymes was subtracted from the measured protein yield. The enzyme name is marked on the x-axis. The columns representing only sonication (US) are the same for all enzymes and do not involve any enzyme, even though they are marked with enzyme names. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 32 also demonstrates that the control values vary. All control samples are significantly different, and the protein yield in the US control sample is significantly lower than in all other samples. Also, all enzymatic treatments give higher yield than their control sample, except for treatment by Viscozyme. Different treatments for the same enzyme show no significant differences for Alginate lyase. For Viscozyme, enzyme + US gives significantly higher yield than the two other treatments. For Cellic, US gives significantly lower yield than the two other treatments. From treatment by enzymes alone, Cellic gives significantly higher yield than the two other enzymes. For treatment by enzyme + US there are no significant differences between enzymes.

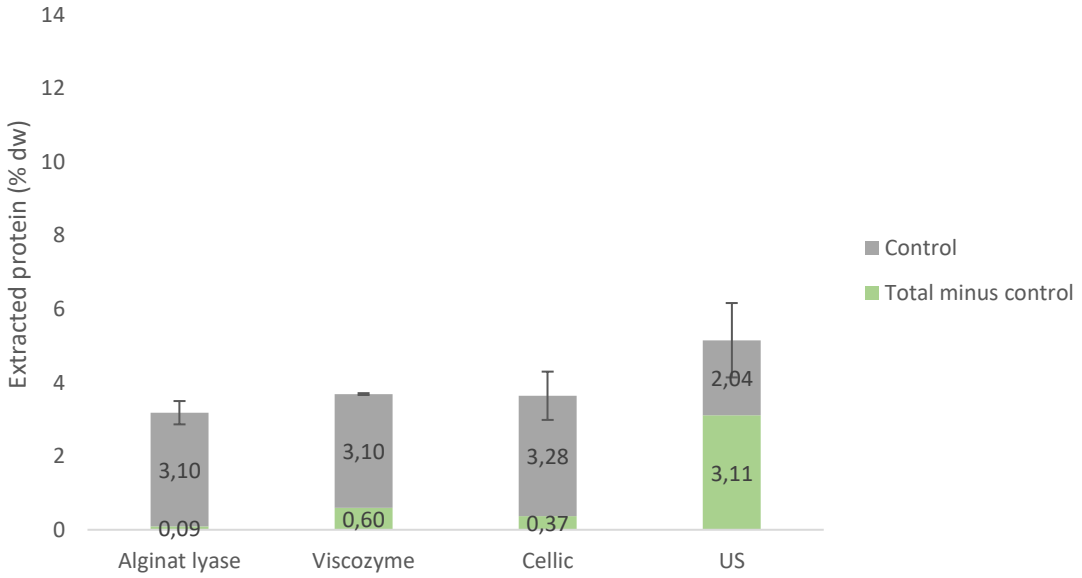

Figure 33: Extracted protein from dry *A. esculenta* treated by the enzymes Alginate lyase, Viscozyme L and Cellic CTec2 as well as sonication. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The treatment (and if enzymatic treatment) the enzyme name is marked on the x-axis. Values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 33 firstly shows that the control value for the ultrasonic treatment is significantly different from the control value for the enzymatic treatments. This is probably due to the same causes as discussed for *S. latissima*. There are no significant differences between any of the treatments due to high standard deviations. Anyhow, the order of efficiency in extraction seems to be US > Viscozyme > Cellic > Alginate lyase.

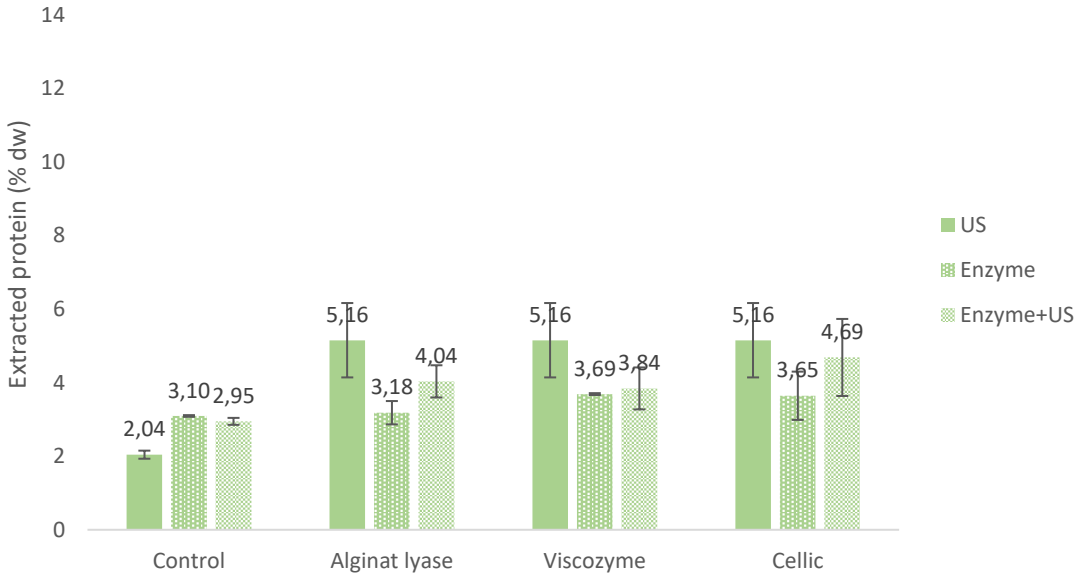

Figure 34: Extracted protein from dry *A. esculenta* treated by enzymes, sonication and combined sequential enzyme and sonication treatment. The contribution of enzymes was subtracted from the measured protein yield. The enzyme name is marked on the x-axis. The columns representing only sonication (US) are the same for all enzymes and do not involve any enzyme, even though they are marked with enzyme names. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 34 also demonstrates that the control values vary. The US control sample has significantly lower yield than all other results. For Viscozyme, the enzymatic treatment is significantly higher than the control sample. For Alginate lyase and Cellic, treatment by enzyme + US are significantly higher than the control samples. Different treatments for the same enzyme show no significant differences for Cellic. For Alginate lyase, enzyme + US gives significantly higher yield than enzymatic treatment. For Viscozyme, US gives significantly higher yield than enzyme + US. There are no significant results between the protein yield from different enzymes for neither enzyme nor enzyme + US. Comparing results between the two algal species, treatment with US gives higher protein yield for *A. esculenta,* while treatments involving enzymes (E + US and only enzyme) give higher yield for *S. latissima*.

It was demonstrated in section 3.3.2 that dry alga gives higher protein yield than wet alga and is more effective in protein extraction of *S. latissima* and *A. esculenta*. Since macroalgae are harvested only at certain periods of the year and drying is a common preservation technique, it is crucial with good extraction techniques for dry algae. It must still be taken into consideration that the process of drying seaweed is quite expensive and energy-intensive, which conflicts with concepts of sustainability and economic optimization. Drying will also affect chemical

composition (Chan, Cheung and Ang, 1997; Gupta, Cox and Abu-Ghannam, 2011), which can affect the nutritional value of the product (Stévant *et al.*, 2017). Economically and environmentally speaking, it is advantageous to also find good extraction methods for wet seaweed. If this is done in an easy and effective way, it can be more sustainable and economical, and therefore more beneficial. Further extractions were done using wet algae.

3.3.4 Sequential enzymatic treatment

Sequential enzymatic treatment with several enzymes has been reported to give a synergistic effect and increased protein extraction yield in algae (Denis, Le Jeune, *et al.*, 2009; Denis, Morancais, *et al.*, 2009). According to Ribeiro (2017), synergistic action is even necessary for complete carbohydrate hydrolysis from enzymes. Sequential treatment can be done with enzymes of similar specificity, for example two polysaccharidases (Manns *et al.*, 2016). Or it can be done with enzymes of different nature, for example combining a polysaccharidase with a protease (Hanmoungjai, Pyle and Niranjan, 2002; Kadam, Álvarez, *et al.*, 2015a; Sari *et al.*, 2015).

It was desired to look at the effect of sequential treatment with two enzymes of different specificity, thereby choosing a carbohydrase and a protease. Cellic CTec was used since it seemed to be the enzyme with the highest effect from earlier experiments, and Alcalase was chosen due to its efficiency in extraction of seaweed proteins recorded in literature. Extraction was performed like in earlier experiments, and protein analysis was done with Lowry, acid soluble peptide, free amino acid, total amino acid, and CN analyses. Two different control samples were used, since treatment with only Alcalase was done in a separate experiment. Control sample 1 belongs to Cellic and Cellic + Alcalase, while control sample 2 belongs to Alcalase. Control sample 2 was not analysed by the total amino acid and CN analyses.

In the results presented in this section, one of the absorbances measured was very far from the other parallels and far from what was expected, and this value was not used in the results. This counts for one of the three Lowry parallels for one of two treatment parallels for *S. latissima* with Cellic and Alcalase. All measured absorbances from these results, including the one removed, will be shown in Appendix B.2. Results from the retained values agreed well with the expected outcome and corresponded well between the two species of algae.

Total extracted protein from Lowry analysis

Results from Lowry analysis are shown respectively in Figure 35 and Figure 36 for *S. latissima* and *A. esculenta*. The grey part represents the yield of the control value while the coloured part shows the total protein with subtracted control value. Thus, the full column represents the total extracted protein.

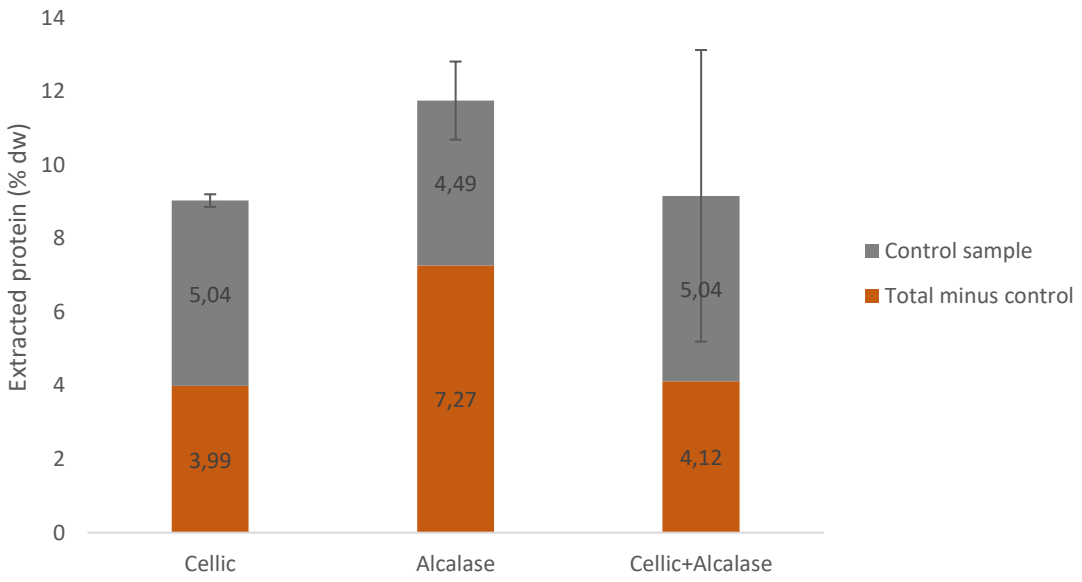

Figure 35: Extracted protein from wet *S. latissima* with single enzymatic and combined sequential enzymatic treatment. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The enzyme names are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 35 shows that the protein yield is relatively similar for all control samples. Total extracted protein is significantly higher than the control sample with treatment from Cellic and Alcalase, but not for Cellic + Alcalase due to high standard deviation. Treatment by Alcalase gives the highest extracted protein yield, but only significantly higher than treatment by Cellic. Cellic + Alcalase and Cellic have very similar protein yields.

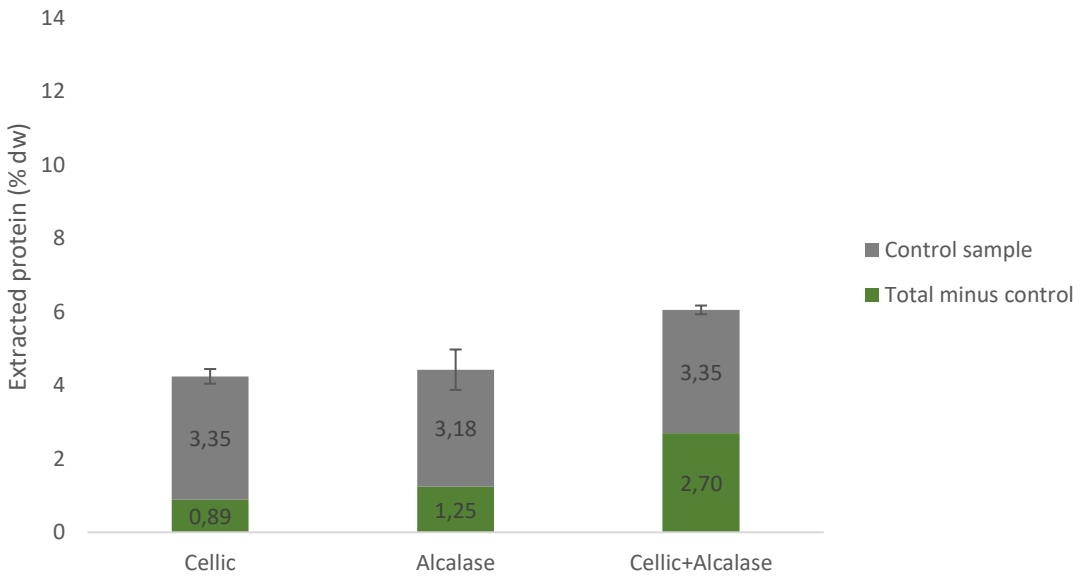

Figure 36: Extracted protein from wet *A. esculenta* with single enzymatic and combined sequential enzymatic treatment. The contribution of enzymes was subtracted from the measured protein yield. The grey part of the column represents the control value, while the coloured part shows the total protein yield with subtracted control value. The full column represents the total extracted protein. The enzyme names are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 36 shows that the protein yield is relatively similar for all control samples. From all the enzymatic treatments, the total extracted protein is significantly higher than the control sample. Treatment from Cellic + Alcalase gives the significantly highest extracted protein yield. Alcalase and Cellic have very similar protein yields.

All treatments for *S. latissima* give higher protein yield than all treatments for *A. esculenta*, though this is not significant for treatment by Cellic + Alcalase, with high standard deviation in *S. latissima*. For both *Saccharina latissima* and *Alaria esculenta*, enzymatic treatment by Cellic, Alcalase and Cellic + Alcalase increases the extracted protein yield. In addition, treatment by Alcalase (alone or in combination with Cellic) seems to increase protein yield compared to the use of Cellic alone. This increased protein extraction yield by Alcalase and sequential Alcalase and carbohydrase treatment is in line with what was discovered by Hanmoungjai, Pyle and Niranjan (2002) and Sari *et al.* (2015).

For *S. latissima*, the high standard deviation from treatment by Cellic + Alcalase makes it hard to draw conclusions on which treatment gives the highest yield. It seems like Alcalase alone

gives the highest yield in *S. latissima*. Similar results are also recorded by Hanmoungjai, Pyle and Niranjan (2002), where the use of sequential carbohydrase and Alcalase treatment gave lower protein yield compared to treatment by Alcalase alone. Hanmoungjai, Pyle and Niranjan (2002) explained this by a detrimental effect by the presence of other enzymes, where two enzymes may completely adsorb on the substrates and inhibit the action of the other enzyme, thereby reducing the protein yield. The same effects were also asserted to be confirmed by additional literature (Hanmoungjai, Pyle and Niranjan, 2002). For *A. esculenta*, Cellic + Alcalase gave the significantly highest protein yield. Similar result is also recorded by Sari *et al.* (2015), where sequential carbohydrase and Alcalase treatment gave higher protein extraction yield than Alcalase alone, yet the difference was not very big. Again, there seems to be a different effect of different treatment conditions between the two algal species.

Acid soluble peptides

TCA is an acid that induces protein precipitation due to the three chlorine atoms in the molecule. Precipitation happens to different extent according to the nature of the present peptides (Sivaraman *et al.*, 1997). The analysis depends on the composition of amino acids and the content of bound/free amino acids, which will give slightly changed colour that affect the absorbance (Peterson, 1979). Acid soluble peptides are smaller than ~1,000 Da (Lyng, 2015). TCA was added to the supernatant from extraction to precipitate the large proteins so that only the acid soluble peptides are left in the solution and their content can be determined. Results for acid soluble peptides are compared with total extracted protein, shown respectively in Figure 37 and Figure 38 for *S. latissima* and *A. esculenta*. It is assumed that there are few or no acid soluble peptides in the added enzymes, and they thereby contribute little or nothing to the measured acid soluble peptides. Therefore, no enzyme contribution is subtracted.

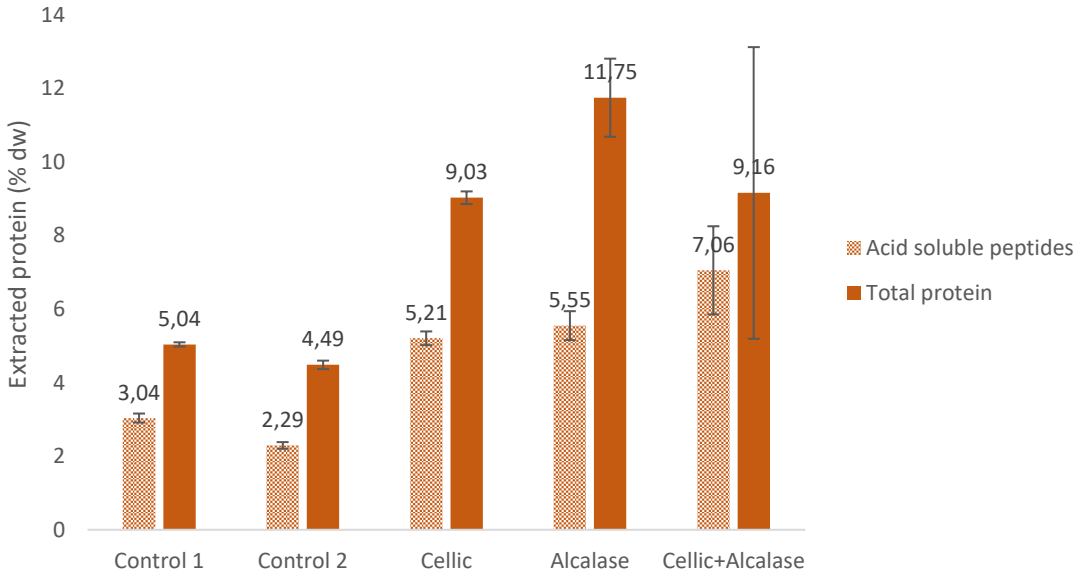

Figure 37: Extracted total protein and acid soluble peptides from wet *S. latissima* with single enzymatic and combined sequential enzymatic treatment. The contribution of enzymes was subtracted only from the total protein. The enzyme names are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 37 shows the relation between total extracted protein and acid soluble peptides, where acid soluble peptides constitute a share of 57.7 % in Cellic, 47.2 % in Alcalase, 77.0 % in Cellic + Alcalase and 60.3 % and 51.1 % respectively in control sample 1 and 2. The difference between total extracted protein and acid soluble peptides is significant in Cellic and Alcalase as well as the two control samples. Thus, the highest percentage of acid soluble peptides is shown in Cellic + Alcalase, followed by control 1, Cellic, control 2 and Alcalase.

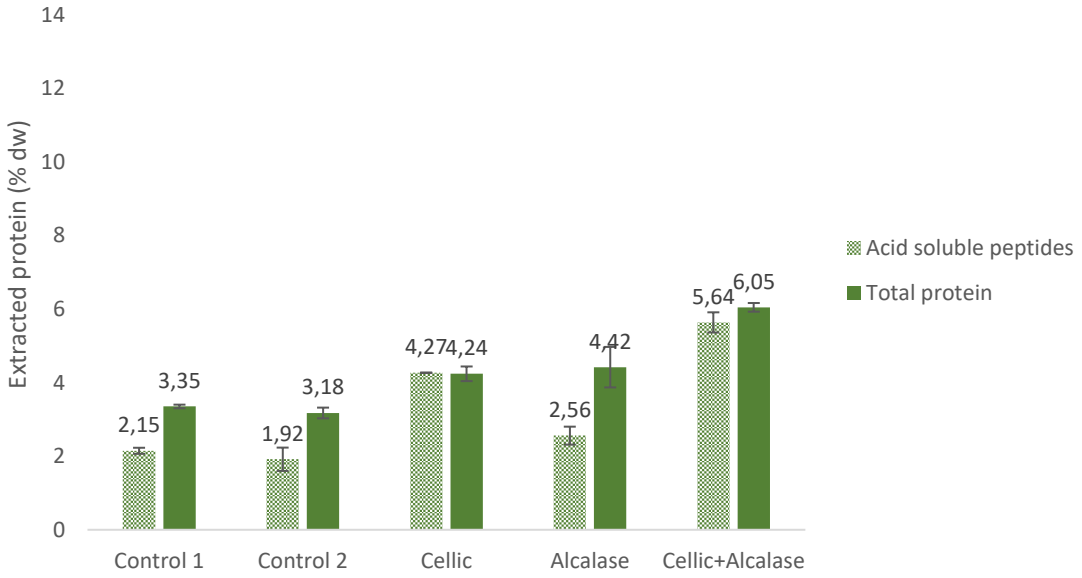

Figure 38: Extracted total protein and acid soluble peptides from wet *S. latissima* with single enzymatic and combined sequential enzymatic treatment. The contribution of enzymes was subtracted only from the total protein. The enzyme names are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 38 shows the relation between total extracted protein and acid soluble peptides, where acid soluble peptides constitutes a share of 100.7 % in Cellic, 57.9 % in Alcalase, 93.2 % in Cellic + Alcalase and 64.0 % and 83.6 % respectively in control sample 1 and 2. The difference between total and acid soluble peptides is significant in Alcalase as well as the two control samples. Thus, the highest content of acid soluble peptides is seen in Cellic, followed by Cellic + Alcalase, control 2, control 1 and Alcalase.

The recorded proportion of acid soluble peptides in the control samples are relatively high for both algae (between 51.1 and 83.6 %) compared to results from Lyng (2015), which is 11-17 % and 4-12 % for respectively *S. latissima* and *A. esculenta*. Anyhow, the proportion of acid soluble peptides increases greatly for enzymatically treated samples compared to the control, which agrees well with what is also seen for Lyng (2015). This is reasonable since enzymes are added to degrade the algal biomass and will thereby release and degrade proteins more.

According to these results, all extracted protein in the sample treated with Cellic and almost all for Cellic + Alcalase was acid soluble in *A. esculenta*. High proportions of acid soluble peptides are also seen from *S. latissima* for the same treatments. For these two treatments, the fraction of acid soluble peptides is higher than in the control samples. There is a reason to believe that for these treatments, there is more reduced protein size due to enzymatic treatment, incubation

over time and elevated temperature. This reduction in protein size is even more evident in *A. esculenta*, where it seems like close to all the extracted protein is acid soluble. Alcalase has an acid soluble peptide fraction very close to but even lower than the control values, which might indicate that this enzyme breaks up the proteins to a lesser extent. Thus, it seems like Cellic breaks down proteins to smaller peptides to a greater extent than Alcalase, and that proteins in *A. esculenta* is more broken down than those in *S. latissima*.

This was not as expected since Cellic is a carbohydrase that hydrolyses carbohydrates while Alcalase is a protease that is supposed to degrade proteins and reduce protein size. Since Alcalase is a endoproteinase, it cleaves the peptide bond at specific residues and induces breakdown of proteins into relatively large peptides (Kristinsson and Rasco, 2000). These peptides might be large enough to get precipitated by the acid, and in that case, they are not measured in the acid soluble peptide analysis. This might explain why the proportion of acid soluble peptides from Alcalase treatment is similar with the control sample. Another explanation is that Cellic does not necessarily need pre-treatment (Kostas, White and Cook, 2019) while Alcalase works much better after e.g. sonication (Ma *et al.*, 2011). Thus, Cellic might have better accessed its substrate, and carbohydrate breakdown may also have induced high degree of protein breakdown due to tight bonding. Alcalase might have poorly accessed its substrate and thereby broken down proteins to small peptides to a lesser extent, even though it releases equal/more proteins.

An increased content of acid soluble peptides from enzymatic treatment might give good nutritional effects, e.g. as bioactive peptides. These are small peptides of typically 2-30 amino acids. They are inactive in their parent protein, but can be released by different reactions, for example by enzymatic hydrolysis (Bleakley and Hayes, 2017). This can increase the nutritional quality of the extracted protein and give health promoting effects. Thus, even though Alcalase releases a bigger quantity of proteins, Cellic may seems to induce the release of more bioactive peptides.

Free amino acids

Sulfosalicylic acid is an effective precipitation agent for proteins in aqueous solution, by binding to proteins and making a complex (Bates, Waldren and Teare, 1973). By precipitating proteins, only free amino acids are soluble, and free amino acid content can thereby be measured. The amino acids cysteine, proline and tryptophan are not measured. Free amino acid

content is shown for both species in Figure 39 and amino acid composition is shown in Figure 40 and Figure 41 respectively *S. latissima* and *A. esculenta*. For the amino acid composition (Figure 40 and Figure 41), standard deviations are high, and no statistical differences are therefore discussed. It is assumed that there are little or no free amino acids in the added enzymes, and they thereby contribute little or nothing to the measured acid soluble peptides. Therefore, no enzyme contribution is subtracted from the measured values.

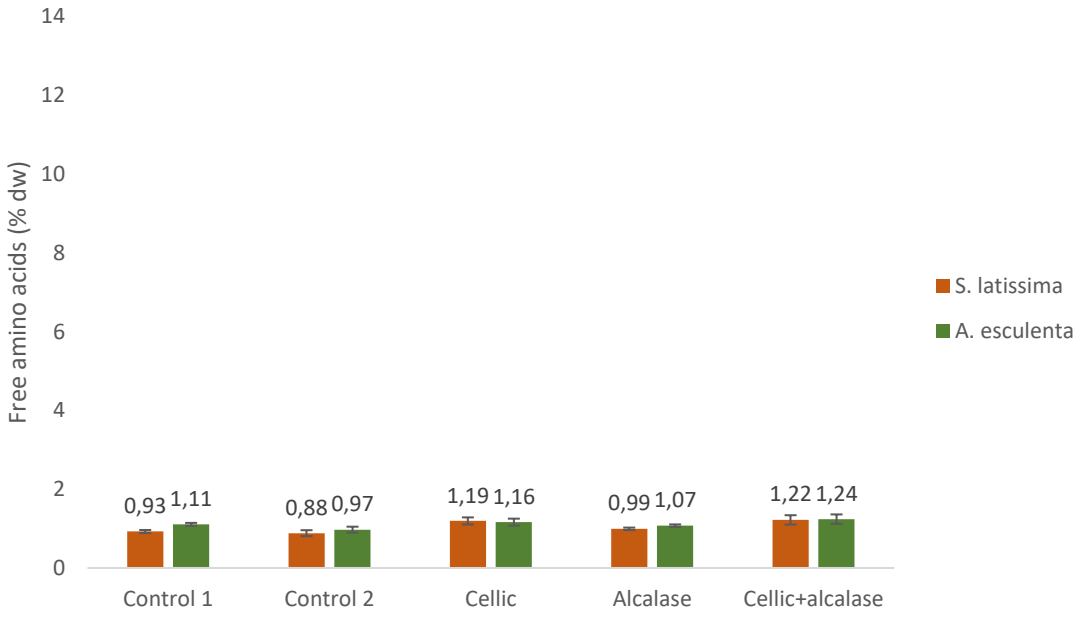

Figure 39: Free amino acid content in wet *Saccharina latissima* and *Alaria esculenta* after single enzymatic and combined sequential enzymatic treatment. The enzyme names are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 39 shows that the free amino acid content is very similar in *A. esculenta* and *S. latissima*, but significant differences between the two species are seen in the sample treated with Alcalase as well as the two control samples, where *A. esculenta* has the highest yield. There are also small differences between distinct treatments for the same alga. In *S. latissima*, control sample 2 has significantly lower yield than all enzymatically treated samples and control sample 1 is significantly lower than treatment by Cellic and Cellic + Alcalase. For *A. esculenta*, control sample 2 has significantly lower yield than all other samples, and Cellic + Alcalase is significantly higher than control sample 1 and Alcalase. Thus, all enzymatic treatments significantly increase amino acid content compared to control sample 2 and almost all treatments have higher yield than control sample 1, even though this is not significantly for most treatments.

The yield from enzymatically treated samples seem to follow the order Cellic + Alcalase > Cellic > Alcalase for both algae. The differences are not significant, except that treatment by Cellic + Alcalase significantly increases yield compared to Alcalase for *A. esculenta*. Thus, there seems to be a higher degree of breakdown of proteins to free amino acids in Cellic + Alcalase and Cellic compared to that of Alcalase alone. This is very similar to the results shown for acid soluble peptides, where Cellic + Alcalase and Cellic also seemed to give more finely degraded proteins. Thus, these results support each other, giving a consensus that Cellic degrades proteins to smaller units to a greater extent than Alcalase.

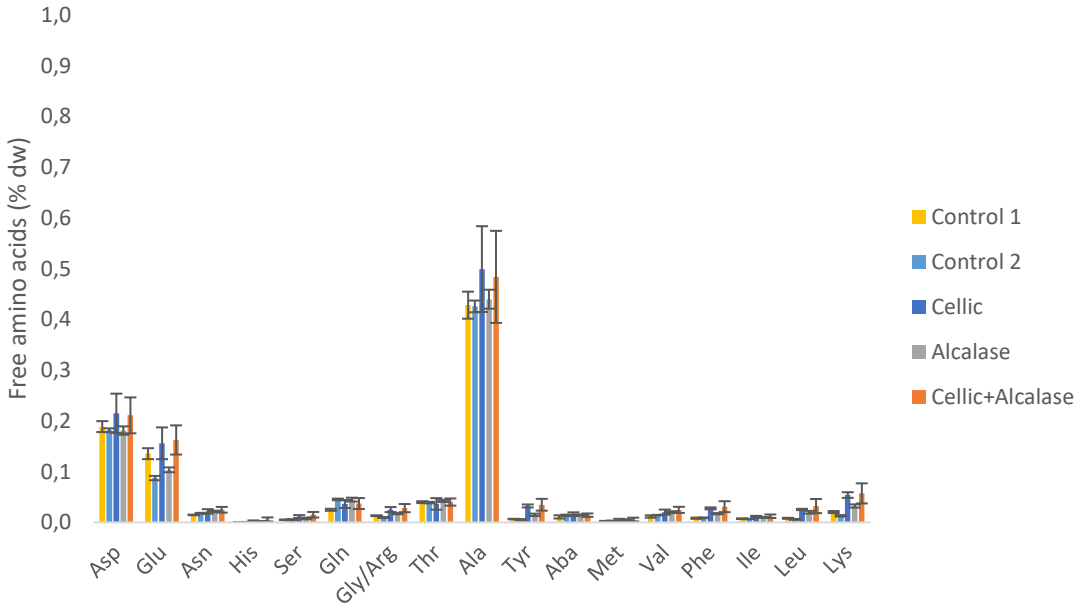

Figure 40: Free amino acid profile in wet *Saccharina latissima* after single enzymatic and combined sequential enzymatic treatment. The amino acids are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

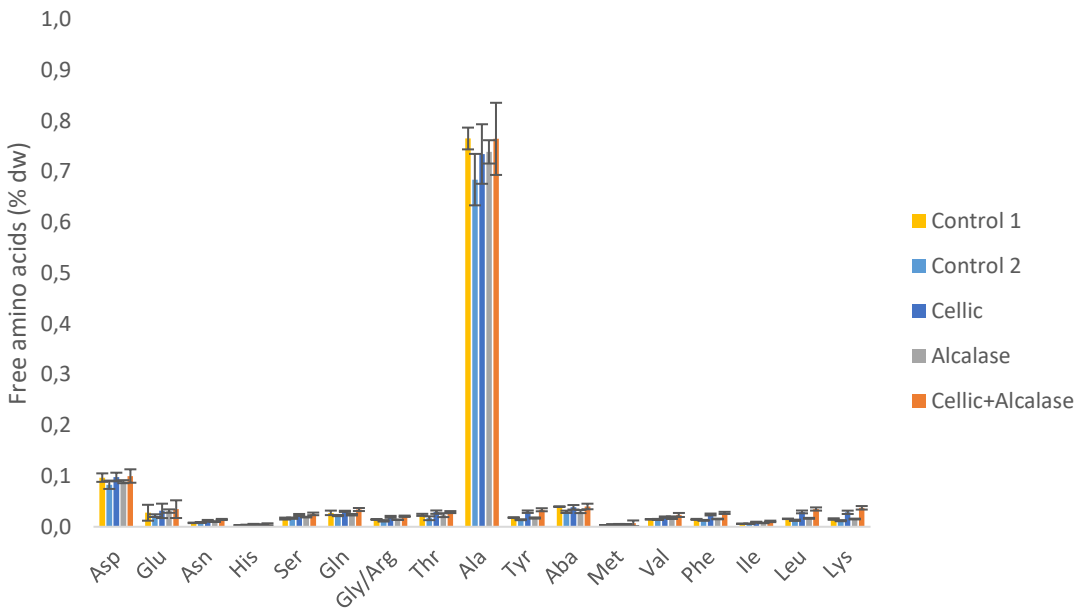

Figure 41: Free amino acid profile in wet *Alaria esculenta* after single enzymatic and combined sequential enzymatic treatment. The amino acids are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

In Figure 40 and Figure 41 there is one clearly dominating free amino acid, which is alanine for all treatments for both algae. Alanine is more abundant in *A. esculenta* than in *S. latissima*. Glutamic acid and aspartic acid are also present in high amounts in *S. latissima* extracts, while only the latter is momentously present for *A. esculenta*. For both algae, all other amino acids are present in quite similar concentrations, and only in trace amounts. There are differences in the yield between different treatments, indicating that the enzymatic treatments degrade proteins to a different extent. For *A. esculenta*, the content of alanine is in fact highest in control sample 1, which is not as expected, but standard deviations are high, and the difference is not significant. Even though there are varying amounts of amino acids in the two algae, the pattern of degradation of proteins and the relative differences between amino acids is very similar.

The free amino acid profile with high amounts of alanine, some aspartic acid and glutamic acid and trace amounts of other amino acids is consistent with results from previous results reported by Lyng (2015) and Kopczyk (2020) for *S. latissima* and *A. esculenta*, and (Mæhre *et al.*, 2014) for *A. esculenta*. The free amino acid profile in this study is very similar to the profile from Lyng (2015) for the same algal species. The most abundant free amino acids are non-essential, even though all the essential amino acids (His, Ile, Met, Phe, Thr, Val, Lys and Trp) are present, except for tryptophan that is not measured. Essential or not, the free amino acids are important taste substances in food (Kato, Rhue and Nishimura, 1989). The abundant amino acids aspartic

acid and glutamic acid will contribute to the umami taste that is characteristic for seaweed (Macartain *et al.*, 2007). A characteristic flavour of seaweed also typically comes from alanine and glycine (Holdt and Kraan, 2011). This makes them well suited for use in food and flavourings.

Total amino acids

The total amino acid composition was measured through acid hydrolysis by HCl, and further quantification with HPLC. The acidic HCl releases protons that break down the peptide bonds in a protein. Complete hydrolysis liberates all amino acids in the samples. Under conventional acidic hydrolysis conditions, glutamine and asparagine are completely hydrolysed to glutamic acid and aspartic acid. Threonine and serine are partially hydrolysed. Tyrosine is partially destroyed, while tryptophan is completely destroyed. Cysteine cannot be directly determined. For the remaining amino acids, precise quantification can be performed (Fountoulakis and Lahm, 1998). The amino acids cysteine, proline and tryptophan are not measured. The total amino acid content is shown for both species in Figure 42 and amino acid composition is shown in Figure 43 and Figure 44 respectively for *S. latissima* and *A. esculenta*. For the amino acid composition (Figure 43 and Figure 44), standard deviations are high, and no statistical differences are therefore discussed. It is uncertain which amino acids are present in the added enzymes, and therefore enzyme contribution is not taken into account in the results with measured amounts of each amino acid (Figure 43 and Figure 44). Anyhow, enzyme contribution is considered in the quantification of total amino acids (Figure 42), and therefore this value is lower than the sum of all amino acids.

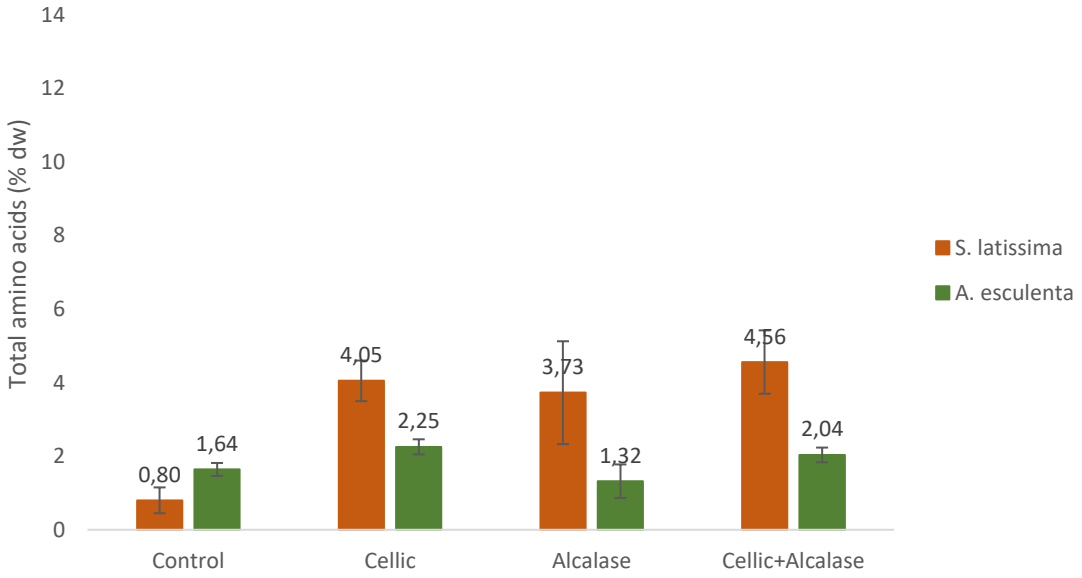

Figure 42: Total amino acid content in wet *Saccharina latissima* and *Alaria esculenta* after single enzymatic and combined sequential enzymatic treatment. The contribution of enzymes was subtracted from the measured protein yield. The enzyme names are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

Figure 42 shows that the protein content in *A. esculenta* and *S. latissima* varies between different treatments. All extraction yields from different treatment are significantly different between the two species. *A. esculenta* has significantly higher protein yield in the control sample, while *S. latissima* has significantly higher yield in the enzymatically treated samples. For *S. latissima*, all enzymatic treatments significantly increase protein yield compared to the control sample. The yield for *S. latissima* seems to follow the order Cellic + Alcalase > Cellic > Alcalase > control, though not significantly. For *A. esculenta*, treatment by Cellic and Cellic + Alcalase significantly increases protein yield compared to the control sample and treatment by Alcalase alone. The yield for *A. esculenta* seems to follow the order Cellic > Cellic + Alcalase > control > Alcalase, though not significantly. Thus, the most efficient enzymatic treatment differs between the two species.

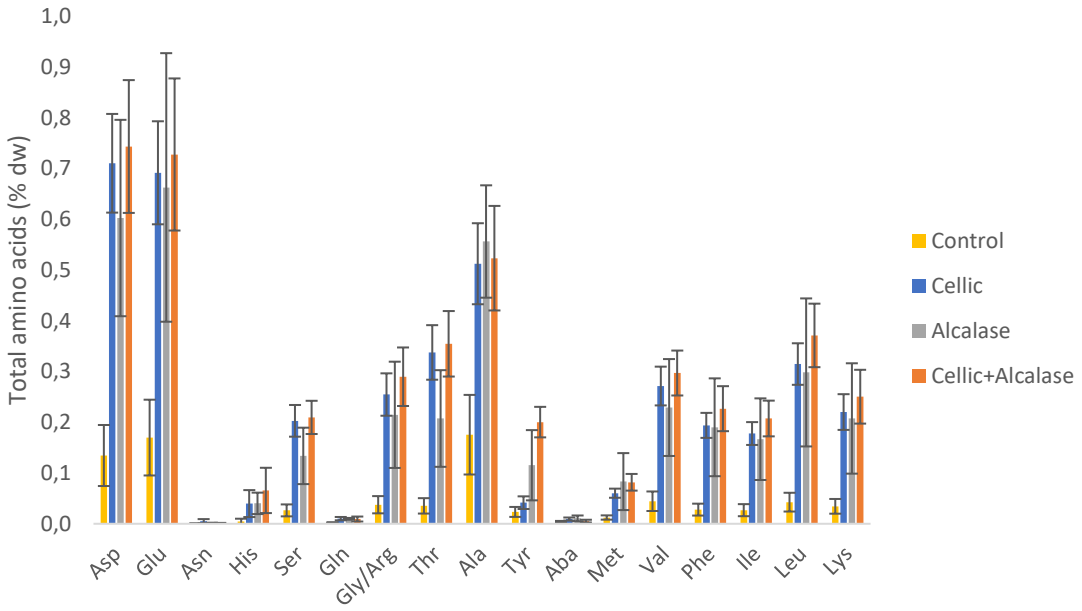

Figure 43: Total amino acid profile in wet *Saccharina latissima* after single enzymatic and combined sequential enzymatic treatment. The amino acids are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

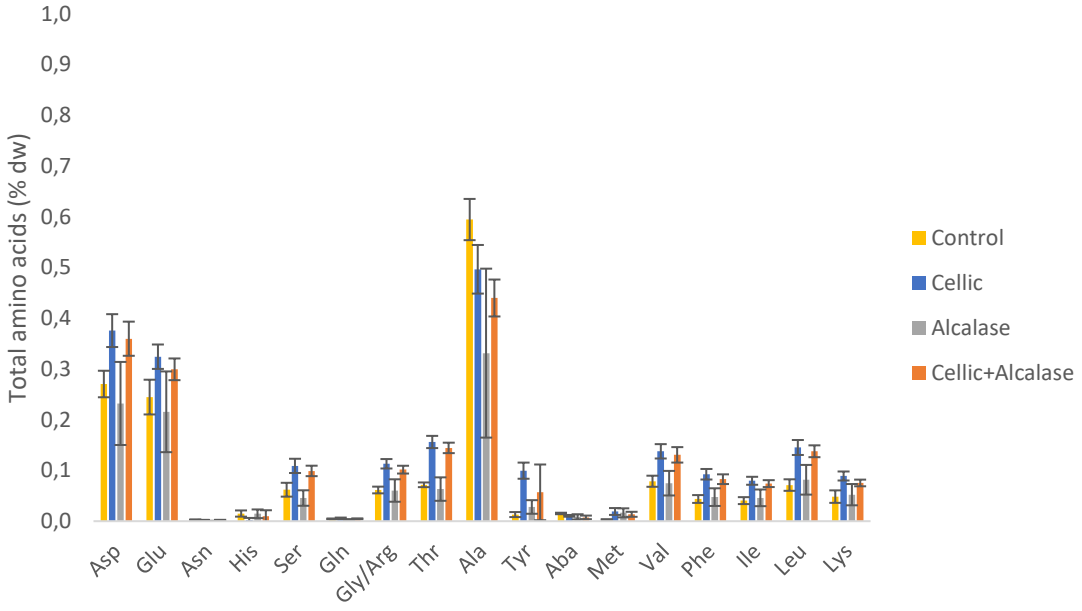

Figure 44: Total amino acid profile in wet *Alaria esculenta* after single enzymatic and combined sequential enzymatic treatment. The amino acids are marked on the x-axis. All values originate from four parallels for each sample with corresponding standard deviations demonstrated by error bars.

As seen in Figure 43 and Figure 44 there is a higher variation in total amino acids than what is seen for free amino acids, even though the most abundant amino acids (alanine, aspartic acid

and glutamic acid) are the same. Another apparent observation when comparing these two results is that some of the amino acids have higher yield of free amino acids than in total amino acids. This was not as expected and might result from not taking enzyme contribution into account, the weight of the freeze-dried supernatant that was not measured accurately, or the amino acids that were destroyed by acid hydrolysis. These factors might give lower results for the total amino acid analysis.

The three amino acids alanine, glutamic acid and aspartic acid are in general the dominating ones for all treatments of both algal species. Serine, glycine/arginine, threonine, tyrosine, valine, phenylalanine, isoleucine, leucine and lysine are also relatively abundant. There are low concentrations of the amino acids asparagine, glutamine and α-aminobutyric acid, and relatively low levels of also histidine and methionine. In *A. esculenta* (Figure 44), alanine is the most abundant amino acid for almost all treatments, while for *S. latissima* (Figure 43), aspartic acid and glutamic acid are the most abundant. The content of alanine is very similar in the two algae. The control sample in *S. latissima* has a low yield compared to the enzymatically treated samples. All enzyme combinations give relatively similar yield, even though the highest is in general seen for Cellic + Alcalase and Cellic. In *A. esculenta*, the control sample has a more similar yield as the enzymatically treated samples, and the control sample even has the highest content of alanine. The control sample and Alcalase have slightly lower yield of most amino acids compared to Cellic + Alcalase and Cellic. Again, *S. latissima* seems to have a higher effect from enzymatic treatment. Even though there are different amounts of amino acids in the two algae, the relative differences between amino acids is very similar.

These results harmonize well with earlier findings that seaweeds are rich in aspartic acid, glutamic acid, alanine and glycine among others, as mentioned in the theory section. Results from previous studies also show similar total amino acid profile with high amounts of alanine, aspartic acid and glutamic acid and lower amounts of the other amino acids in both *Saccharina latissima* and *Alaria esculenta* (Reissiger, 2016), *S. latissima* (Manns *et al.*, 2014; Marinho, Holdt and Angelidaki, 2015) and *A. esculenta* (Mæhre *et al.*, 2014). According to these authors, leucine is among the dominating amino acids, which is also correct for the total amino acid profile in this study. Even though the relative abundancies are similar, the content of many amino acids from this thesis is lower than that recorded by Reissiger (2016) and Mæhre *et al.* (2014). The dominating amino acids are non-essential, but all the essential amino acids (His, Ile, Met, Phe, Thr, Val, Lys and Trp) are present, except from tryptophan that is not measured. Of the essential amino acids, leucine, phenylalanine and lysine dominate, and high amounts of

leucine, threonine and valine are also recorded. Reissiger (2016) shows similar results for essential amino acids, and additionally records high amounts of threonine in *A. esculenta*.

CN analysis

In seaweed biomass, the total nitrogen content will reflect the total protein content accurately. Thus, measuring the amount of nitrogen in a sample can be used to determine the total protein content, assuming that the proportion of non-protein nitrogen in a plant is very small and will not significantly affect the results. The proportion of nitrogen in most proteins is known to be approximately 16 % (by weight), and therefore a conversion factor can be used to calculate the amount of protein in a sample. The amino acid composition (and therefore the nitrogen content) varies in different materials, but a conversion factor of 6.25 is widely used (Coultate, 2002). According to Mariotti, Tomé and Mirand (2008), the general conversion factor is too high and gives an overestimation of protein content for most materials. This might be due to the presence of non-protein N-containing compounds or that the nitrogen concentration in protein is different than 16 % (Lourenço *et al.*, 1998). An average conversion for seaweed has been calculated from 19 seaweed species to be 4.92 (Lourenco *et al.*, 2002) and an adjusted conversion factor for *Saccharina latissima*, *Alaria Esculenta* and *Palmaria palmata* was calculated to 4.8 (Reissiger, 2016). Separate conversion factors were calculated by Schiener *et al.* (2015a) to be 5.3 for *S. latissima* and 6.0 for *A. esculenta*. Table 6 shows the amount of specific elements and nutrients in *S. latissima* and *A. esculenta*, and the conversion factor (Jones' factor) chosen to be used for calculations in this thesis.

Table 6: Different elements and nutrients as well as conversion factor for *S. latissima* and *A. esculenta* (Schiener *et al.*, 2015).

Nutrients and conversion factor	*Saccharina latissima*	*Alaria esculenta*
Carbon (%)	26.6±3.2	30.3±1.5
Nitrogen (%)	1.5±0.5	1.9±0.3
Protein (%)	7.1±1.7	11.0±1.4
Jones' factor	5.3	6.0
Polyphenols (%)	0.41±0.15	0.87±0.52

Thus, conversion factors of 5.3 and 6.0 was respectively used for *S. latissima* and *A. esculenta* to calculate the protein content from the nitrogen content in the samples. Results are shown respectively in Figure 45 and Figure 46 for *S. latissima* and *A. esculenta*, and the total measured

protein summed from the pellet and supernatant is shown in Table 7. The contribution of enzymes was only measured for the supernatant and not the pellet, due to limited lab access. Since the content of enzyme in the enzyme powder/liquid is not known, the contribution in the pellet cannot be calculated. Therefore, in these results it was chosen not to consider the enzyme contribution in neither the pellet nor the supernatant to avoid misleading results. Results considering the enzyme contribution in the supernatant will be presented later.

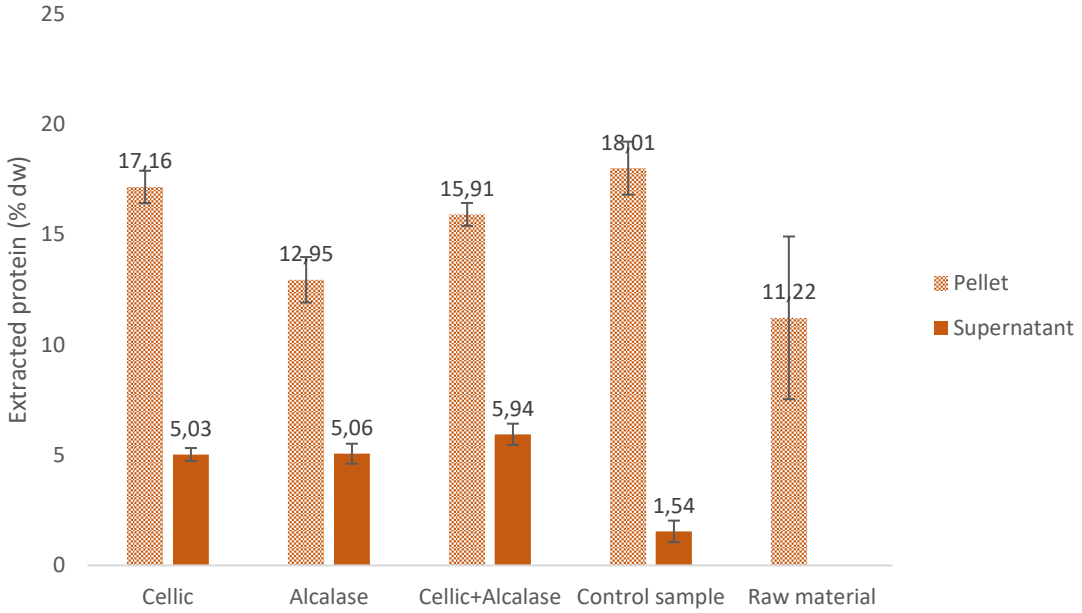

Figure 45: Protein content measured by CN analysis from freeze-dried pellet and supernatant from extraction of wet *S. latissima* with single enzymatic and combined sequential enzymatic treatment, including the control sample with only water and algae. Freeze-dried raw material was also analysed. The enzyme names, control sample and raw material are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

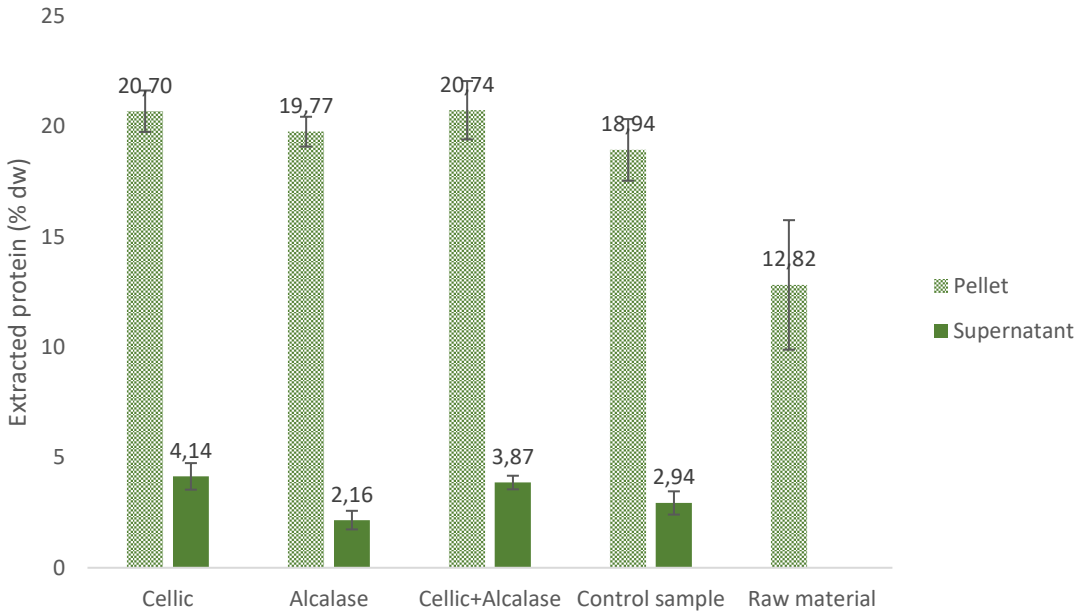

Figure 46: Protein content measured by CN analysis from freeze-dried pellet and supernatant from extraction of wet *A. esculenta* with single enzymatic and combined sequential enzymatic treatment, including the control sample with only water and algae. Freeze-dried raw material was also analysed. The enzyme names, control sample and raw material are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Table 7: Total protein measured by CN analysis from freeze-dried pellet and supernatant from extraction of wet *S. latissima* and *A. esculenta* with single enzymatic and combined sequential enzymatic treatment, including the control sample with only water and algae. The total protein is the sum of the measured protein in the pellet and supernatant. Freeze-dried raw material was also analysed.

	***Saccharina latissima* (% dw)**	***Alaria esculenta* (% dw)**
Cellic	22.19	24.84
Alcalase	18.01	21.93
Cellic + Alcalase	21.86	24.61
Control	19.55	21.88
Raw material	11.22	12.82

Figure 45 and Figure 46 shows varying fractions of protein in the supernatant and pellet for different treatments. For all treatments, there is a significantly higher protein yield in the pellet than in the supernatant. The protein content measured in the raw material agree well with literature values for protein content in the algae (Table 6), though a bit higher than expected for *S. latissima*.

The sum of protein in pellet and supernatant for the treated samples (Table 7) was expected to give a similar protein yield as for the raw material. These are significantly different for all treatments, and in general the total protein is about the double of the raw material. The enzyme

contribution (which is not considered) is thought to be the biggest reason to this. The inaccurate weighing of the freeze-dried supernatant might also affect results. In addition, the conversion factor used to calculate the protein content is adjusted for the protein content in the two algal species, but not suited for the additional protein content in the enzymes. Results from Lyng (2015) showed that after enzymatic treatment, about half of the protein remained in the pellet, but in general a bigger fraction was extracted to the supernatant. From the control sample (with water as solvent), about 90 % of the protein remained in the pellet. Using these fractions to calculate the actual protein content in the pellet from the yield in the supernatant for this experiment would give a more reasonable total protein content (pellet + supernatant). This was not done, since it is hard to know if it is also a good measure for this experiment.

There is still a high uncertainty, and no results are discussed in detail. However, it is interesting to look at the relation between the fraction of measured protein in the pellet and the supernatant, since this can say something about the extractability of proteins. In *S. latissima*, the protein content in the supernatants is around 30-40 % of the protein content in the pellet, while the same value for *A. esculenta* is around 10-20 %. Thus, the extractability seems to be better in *S. latissima* than *A. esculenta*. The highest percentage are seen for treatment by Alcalase and Cellic + Alcalase for *S. latissima*, and for Cellic and Cellic + Alcalase for *A. esculenta*. These are considered the most efficient treatments for the respective algal specie in this case.

Comparing protein analysis methods

Plant biomass is a heterogeneous material, and factors as composition, structure and nutrient interactions will affect the protein analysis. Distinct analysis methods use various analytical principles (Mæhre *et al.*, 2018), and may give different results for the same sample. Therefore, it is interesting to compare results from different methods to see their correlation as well as their strengths and weaknesses. Three different methods were used to measure the total extracted protein in this thesis: Lowry analysis, CN analysis and total amino acid analysis. Results from these analysis methods will be compared, respectively in Figure 47 and Figure 48 for *S. latissima* and *A. esculenta*. The contribution from enzymes is considered in all results.

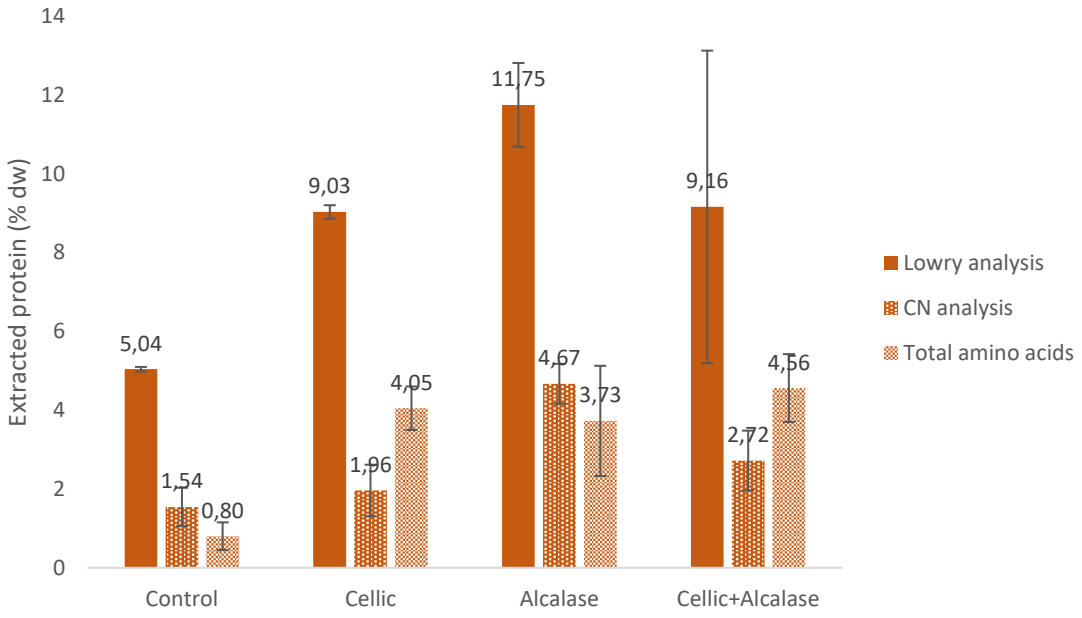

Figure 47: Comparing measured total protein yield from Lowry analysis, CN analysis and total amino acid analysis of extracted protein from wet *S. latissima*. Single enzymatic and combined sequential treatment has been performed. The contribution of enzymes was subtracted from the measured protein yield. The enzymes used are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

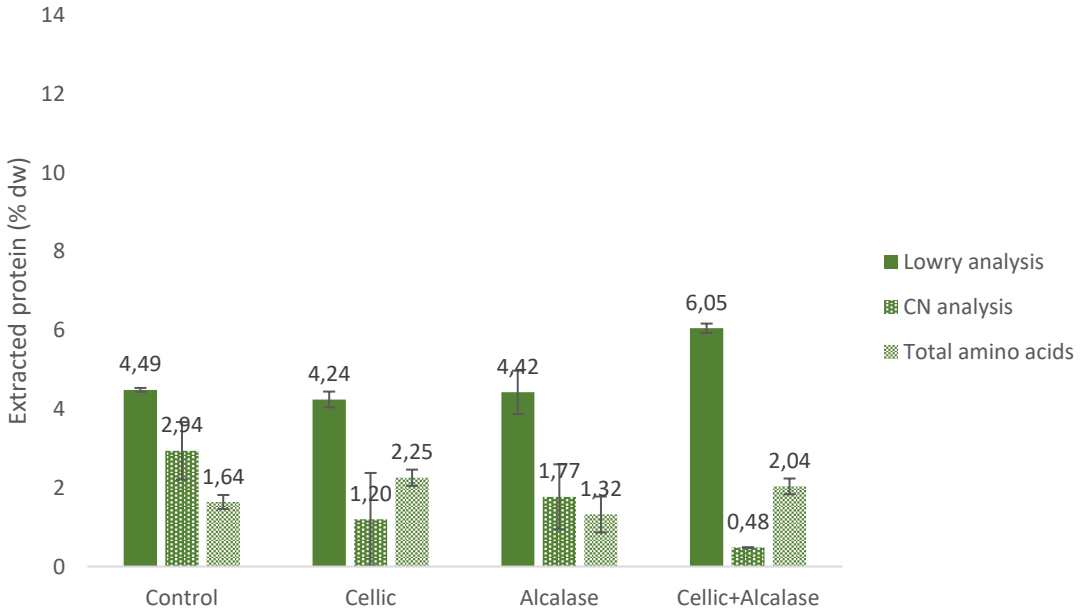

Figure 48: Comparing measured total protein yield from Lowry analysis, CN analysis and total amino acid analysis of extracted protein from wet *A. esculenta*. Single enzymatic and combined sequential treatment has been performed. The contribution of enzymes was subtracted from the measured protein yield. The enzymes used are marked on the x-axis. All values originate from two parallels for each sample with corresponding standard deviations demonstrated by error bars.

Results from Figure 47 and Figure 48 show that there are wide variations in the protein content when measured by different methods. For *S. latissima* in Figure 47 there are significant differences between methods in all cases except between total amino acids (total aa) and CN for the control sample and treatment by Alcalase. For *A. esculenta* in Figure 48, all methods give significantly different protein yield, except between total aa and CN for treatment by Alcalase. Results from the Lowry analysis have the significantly highest yield in all cases. Measured protein from CN and total aa are much lower compared to Lowry, and relatively similar. It varies which of the two analyses give the highest yield. CN analysis gives the higher yield for Alcalase treatment and control sample (only significantly for control with *A. esculenta*) and total aa gives significantly higher yield for treatment by Cellic + Alcalase and Cellic.

Measured protein from different methods have much larger variations in *S. latissima* than in *A. esculenta*, but the pattern is the same in the two algae. All results from the same analysis and treatment are significantly different between the two species, except with Lowry analysis for Cellic + Alcalase due to high standard deviation. This emphasizes the fact that methods are species sensitive and vary according to compositional differences. The higher gap between methods in *S. latissima* might suggest that protein content has been overestimated in results from the Lowry method. This can help to explain the fact that the protein yield has generally been lower in *A. esculenta* than in *S. latissima*, even though it has a higher protein content. Since Lowry analysis is used as the only method for most results in this thesis, protein yield for *A. esculenta* might have been excessively underestimated relative to *S. latissima*. From CN and total aa analysis, *S. latissima* has higher protein yield than *A. esculenta* in all samples, except in the control samples (about doubled yield in *A. esculenta*). Thus, according to these two analysis methods, the extraction of proteins in the untreated sample is higher in *A. esculenta*, but as soon as enzymatic treatment in conducted, protein extraction in *S. latissima* gets higher. This agrees with previous results, where *S. latissima* has shown to be more prone to enzymatic degradation.

The results might be partly affected by the uncertainty in the enzyme contribution, which might affect results differently among methods. Enzyme contribution is measured only with the Lowry method, and the same enzyme contribution is subtracted in all analysis methods even though the contribution might differ between methods. In addition, there are some challenges and disadvantages with all three methods that must be addressed.

The Lowry analysis method is said to be the most sensitive and commonly employed assay for protein determination (Bensadoun and Weinstein, 1976). It is a very simple, sensitive and

specific method for extracts with a mixture of different proteins, solutions with small amounts of protein, proteins mixed with coloured substances or large numbers of similar protein samples (Lowry *et al.*, 1951). The Lowry method uses the Folin phenol reagent that has the active constituent phosphomolybdic-tungstic mixed acid. This reduction is affected by proteins, with increased sensitivity by copper that chelates in the peptide structure and facilitates electron transfer to the mixed acid chromagen close to the functional groups of the amino acids. The main chromogenic amino acids are tyrosine and tryptophan, but also histidine, cystine and cysteine. Peptide linkages are also chromogenic (Peterson, 1979). It measures both small and large proteins and peptides. Also free amino acids are measured, but with less pronounced colour yield (Lowry *et al.*, 1951; Peterson, 1979). A drawback of the Lowry method is that it is not completely accurate. The resulting colour measured at 750 nm is not strictly proportional to concentration and might depend on the sequence of amino acids, chain length, aromaticity, and exposure of functional groups in different proteins. Complete hydrolysis of a protein reduces colour yield. Several substances in biological material cause interference and give colour errors. These substances give less constant results (Lowry *et al.*, 1951; Bensadoun and Weinstein, 1976; Peterson, 1979), by interfering with various chemicals used in the procedure (Bensadoun and Weinstein, 1976). There are many possible interfering compounds in the Lowry method. Relevant in this experiment are phenols, glycine and the monosaccharides mannose, glucose and xylose (Bensadoun and Weinstein, 1976), that are present in the different macroalgal polysaccharides. In addition, if solutions are not completely mixed after addition or if the samples have low pH, this may give lower colour yield. Errors can also come from the fact that a reference protein is used for making the standard curve.

The CN analysis is a good alternative to measure protein content in a sample, as the protein content is calculated accurately from the measured nitrogen content. The biggest problem with this method is that there are many different conversion factors mentioned in literature, and the exact relation between nitrogen and protein is highly dependent on specie, season, and individual differences between plants. This method can be extremely accurate if the exact relation between nitrogen and protein is known and the best conversion factor is used. In the opposite case, results can be misleading. For the results in this thesis, many different conversion factors were considered, and the one thought to give the best results was chosen. An additional problem is that the conversion factors used are fitted to the algae used, and do not involve the enzymes that are also present. An adjusted conversion factor taking enzyme addition into account would be higher than the ones used and thereby result in a higher protein content.

Measuring the total amino acid content by acid hydrolysis is also described as a good measure of protein content. It is a direct protein determination method with no interfering substances affecting the results (Mæhre *et al.*, 2018). A drawback of this method is that it uses acid hydrolysis to release amino acids, which is a critical part of the analysis that might lead to errors in the analysis. The hydrolysis completely destroys tryptophan, which can therefore not be measured (Coultate, 2002), and cysteine cannot be directly determined (Fountoulakis and Lahm, 1998). Thus, the content of some amino acids is eliminated or reduced (Mæhre *et al.*, 2018). The sequence of amino acids is also crucial for the degree of hydrolysis, as some bonds are stronger than others due to tertiary structure and steric hindrance. Conditions employed might not always be strong enough to cleave all bonds (Fountoulakis and Lahm, 1998), which can also give errors. In addition, some of the amino acids from the enzyme might be partly or completely degraded by the acid hydrolysis and are therefore not measured. Thus, the enzyme contribution in the total aa method is likely smaller than what is estimated from the Lowry analysis, and a more correct enzyme contribution would probably give a higher protein yield.

Other authors have compared different methods for protein analysis. From experiments with Lyng (2015), it was concluded that acid hydrolysis for determination of total amino acids is a good and reliable method. The Lowry method is also described as reliable, despite of different results from the two methods (Lyng, 2015). Reissiger (2016) compared protein determination by CN and total amino acid analysis and recorded that protein yield was lower when obtained with the CN method than total aa, but this was probably due to a low conversion factor used. Reissiger (2016) also reported that the total aa method gave higher results than the ones recorded in literature. According to a study by Mæhre *et al.* (2018), protein content is often overestimated when measured by nitrogen content and spectrophotometric protein determination (Lowry) compared to total amino acid analysis, and therefore total amino acid analysis is recommended for food protein determination.

Thus, there is a consensus that there are many methods for measuring protein yield, and they all give varying results. In this thesis, the Lowry analysis seems to have overestimated protein content, especially for *S. latissima*. This method depends highly on the composition of the algae and interfering substances, and therefore affects the two species differently. The two other methods seem to underestimate the protein content mostly due to an overestimated enzyme contribution, but also due to destroyed amino acids and uncertain conversion factor. This is partly in agreement with findings from Mæhre *et al.* (2018), but unlike what is recorded by Lyng (2015) and Reissiger (2016). However, the protein yield from total amino acid analysis

in this thesis is low when comparing to results from the same method by all these authors. It is hard to decide which method is the fittest for this experiment. In fact, it seems that none of the methods give the perfect measure, whereas the protein content most likely lies somewhere between the results from the three methods.

3.3.5 Effect of enzymes

It is known that the added enzymes will contribute to the total protein yield measured by the different analysis techniques. The contribution of enzymes in the supernatant was measured using the Lowry analysis. First, this was done in the same way as in section 3.2.1, by adding water and enzyme to a sample tube and measuring the protein content with the Lowry analysis. This time, measured enzyme contribution was much higher than what was seen in section 3.2.1, and subtracting this contribution gave improbable low yield for all enzymatically treated samples. It was thought that this method gave an overestimation of the contribution of enzymes in the total yield. This was assumed because the samples that are enzymatically treated in the experiments do not only contain water and enzymes, but also contain alga in the sample tube, which might trap more of the enzyme and reduce the amount of solved enzyme that stays in the supernatant and affects protein analysis.

Therefore, another method was developed. The relevant amount of alga, water and enzymes was added to a tube as usual, but instead of incubating the samples, they were inactivated immediately after adding enzyme. In this way, the conditions were identical to the usual, but the enzymes were not able to start degrading the biomass, and thereby did not induce extraction of protein from the biomass. The measured protein content was compared with a control sample without enzymes, and enzyme contribution was thereby seen as the difference between these samples. The same experiment was done with three parallels for both wet and dry algae since they were assumed to show some differences in results. The contribution of each enzyme is shown as the percentage of the weight of the added enzyme (Table 8).

Table 8: The contribution of added enzymes on the total protein yield measured by the Lowry analysis. Results are average of three parallels.

Enzyme	Contribution of enzymes to the measured protein (% weight)	
	Wet algae	Dry algae
Alginate lyase	19.05	15.49
Viscozyme L	6.74	6.87
Cellic CTec2	10.19	9.80
Alcalase	9.46	Not relevant

Table 8 shows that the measured contribution varies quite a lot among the enzymes. Still, there is a big difference in the added amount of enzyme due to different activity of the different enzymes. Alginate lyase is added in the magnitude of 10^{-4} g, Alcalase is added in the magnitude 10^{-3} g, while Viscozyme and Cellic CTec are added in the magnitude of 10^{-2} g. This makes the contribution to the total protein by Viscozyme and Cellic CTec much larger than that for Alginate lyase and Alcalase. Following, results from treatments using these enzymes will also be more affected by variations in the enzyme contribution. This contribution of enzymes is used for all experiments including enzymes, except in some cases (it is mentioned where relevant).

4 SUMMING UP DISCUSSION

A remarkedly darker green and more viscous appearance of the supernatant was observed from both sonication and enzymatic treatment. This is likely due to a high content of extracted carbohydrates and increased viscosity. Significantly increased protein yield was measured for almost all conducted treatments, because of more dissolved carbohydrates inducing the release of more proteins from the algal biomass.

In general, there was higher protein yield by extraction from *Saccharina latissima* that that of *Alaria esculenta*, even though the latter has a higher protein content. Additionally, *Alaria esculenta* has a higher content of phlorotannins than *S. latissima*. A high phlorotannin content is said to limit protein extractability, due to their strong bonding to proteins that seem to keep them more trapped in the polysaccharide matrix. Besides, if phlorotannins are bound to extracted proteins, they can shield the proteins from reagents in the Lowry analysis, resulting in a lower measured protein content. The higher abundance of carbohydrates in *A. esculenta*, especially Alginate lyase, might make the proteins more tightly bound in the polysaccharide matrix, leading to decreased extractability. Moreover, biomass treatment by ultrasound and

enzymes might lead to the breakdown of polysaccharides into smaller, optically active units that might disturb the Lowry analysis differently in the two species of algae.

It was assumed that biomass treatment with a higher enzyme concentration would give higher protein yield. This is partly correct, as protein extraction increased for the higher carbohydrase concentrations. However, in five out of six cases the middle enzyme concentration gave better protein yield than the highest concentration. Thus, increased enzyme concentration increases extraction, but there may be a point where adding more enzyme will contribute little or nothing to the yield, and it will therefore not be profitable. It is important to keep enzyme consumption as low as possible, since there is no point in adding massive amounts of enzymes (proteins) if the protein yield does not increase significantly. Looking at the protein yield as a function of the amount of enzyme used, the lowest enzyme concentration was the most economical efficient in most cases. There were small differences for Cellic CTec and Viscozyme, but the differences are rather large for Alginate lyase. This might be due to an assumed higher activity of this enzyme, where small changes in concentration might give higher impact on the protein yield. There is also a high industrial cost of enzymes in a big scale. Thus, finding the optimal concentration of enzymes is a delicate balance between high yield and low expenses, both considering the direct high cost of enzymes but also the time consumption for treatment. Optimized protocol and setup are crucial for big scale extraction.

The protein yield is also improved by extraction from dry algae compared to wet algae. Both dry and wet algae seem to have systematically much higher protein yield from enzyme control samples than US control samples, indicating a high effect by the incubation conditions temperature and time. The difference in yield of the control samples and the enzymatically treated samples that had been incubated together was rather small, indicating that the effect of incubation conditions might be equally or even more important than the actual enzymatic degradation for these samples. On the other hand, Vilg and Undeland (2017) recorded that extraction temperature has little effect on the extraction yield of dry algae, which makes it more likely to think that extraction time has a more substantial influence on dry algae than assumed. Extraction time was tested in the screening period of this thesis and assumed to have little effect on protein yield. Thus, it is uncertain what causes this effect.

The difference between the new and old batch of *S. latissima* was unexpectedly large, confirming the suspicions that the biomass had been stored at elevated temperatures, spoiling the raw material and thereby affecting the protein extraction yield greatly. The yield was significantly higher in the spoilt raw material, indicating that the already more degraded

biomass was more susceptible to further breakdown by sonication and enzymatic treatment from Viscozyme. There was suggested different breakdown mechanisms by US and enzymes. Sonication seemed to give a breakdown pattern more like the natural biomass degradation, and therefore the effect decreased for increasing sonication treatment. Viscozyme action seemed to be enhanced when biomass was already broken down to some extent before enzymatic treatment, and therefore the effect increased with more treatment.

All the carbohydrases have good potential to break down polysaccharide structures in both *Saccharina latissima* and *Alaria esculenta* but induce different extent of biomass breakdown in the two species. *S. latissima* generally has higher protein yield from treatments in the order Cellic CTec > Alginate lyase > Viscozyme, while the order for *A. esculenta* is Alginate lyase > Cellic CTec > Viscozyme, even though this order varies more in the latter specie from different experiments. The same order counts for both treatment by enzyme and enzyme + sonication. The effect of different enzymes used in this thesis can reflect certain differences in the carbohydrate extractability between the two species of seaweed. Alginate lyase hydrolyses alginate, and the specific Alginate lyase used might be more suited for breakdown of certain G/M block structures. Viscozyme and Cellic CTec are enzyme mixes with enzymes that mainly degrade cellulose, fucoidan and laminarin. The content of cellulose is very similar between the two species, but laminarin and fucoidan are present at different levels, which might give varying susceptibility for different enzymes on the two species.

Alginate lyase might be the most efficient enzyme for *A. esculenta* since it contains a higher amount of alginate, and possibly the more suited G/M block structure. Cellic CTec is good for both species, since it is an enzyme blend with many different enzymes and degrades a bigger variation of the polysaccharides. Viscozyme has similar degradation pattern as Cellic CTec, but generally gives a lower yield. This might be simply due to a lower enzyme activity of Viscozyme or a less optimal blend of enzymes. Also, it is recorded that Cellic CTec2 releases sugars so effectively that it does not necessarily need pre-treatment (Kostas, White and Cook, 2019), which seems different for Viscozyme according to the results from comparison of old and new *S. latissima*. Thus, using a higher concentration of Viscozyme or combining it with other treatments, e.g. US, might have improved its efficiency. According to Shannon and Abu-Ghannam (2018), reducing pH down to 4,5 for Viscozyme could also enhance extraction.

The treatment order of carbohydrases and sonication showed no clear pattern in *A. esculenta*. For *S. latissima* all results showed (half of them significantly) better protein extraction when sonication was performed before enzymatic treatment. Anyhow, treatment by enzymes and

sonication significantly improves protein extraction in both algae compared to the control samples. The effect of treatments by carbohydrases and sonication seemed to vary greatly between the two species of algae. *A. esculenta* is in most cases more strongly affected by sonication than single enzymatic treatment. For dry algae, *A. esculenta* has especially increased yield from sonication compared to enzymes, and the yield from sonicated *A. esculenta* is even higher than that of sonicated *S. latissima*. High yield for *A. esculenta* is also seen for combined treatment by E + US. *S. latissima* is generally more affected by enzymatic treatment than sonication, but the combination of the two gives a higher yield. The large effect of sonication on *A. esculenta* might be explained by the higher total polysaccharide and polyphenol content. This might hinder enzyme action to a bigger extent and enable better solubility by unspecific breakdown of biomass by sonication.

From the sequential enzymatic treatment with Cellic CTec and Alcalase, all combinations give significantly improved protein extraction. Alcalase alone gives the significantly highest protein extraction from *S. latissima*, while for *A. esculenta*, the significantly highest yield is seen with Cellic + Alcalase. These treatments give the highest yield from all experiments conducted in this thesis. Overall Alcalase, alone or in combination with Cellic, unanimously shows improved protein yield compared to Cellic alone, though only significantly shown by half of the cases. This agrees with literature, where Alcalase in many cases has appeared to be more effective than carbohydrases for protein accessibility (Hanmoungjai, Pyle and Niranjan, 2002; Rodrigues *et al.*, 2015), but its efficiency can also be improved in combination with a carbohydrase (Sari *et al.*, 2015). The high efficiency of Alcalase might be explained by results from Wang *et al.* (2010), where activity from protease gave up to tripled polyphenol extraction. Since there is a positive correlation between polyphenol and protein extraction, the high polyphenol extractability of Alcalase will likely also have a positive impact on protein extraction. This might also be the reason that Alcalase treatment is also relatively efficient on *A. esculenta*.

Alcalase efficiency did not necessarily improve for biomass that has been pre-treated by a carbohydrase. The explanation might be that *S. latissima* has shown to be generally more open for degradation while *A. esculenta* biomass has been harder to degrade. Therefore, for *S. latissima* no carbohydrase pre-treatment is needed, and might also be an advantage since enzymes will not have to compete for the substrate. On the other hand, for *A. esculenta*, prior carbohydrase treatment is necessary for Alcalase to access the proteins. Acid soluble peptide and free amino acid analyses show that treatments involving Cellic (Cellic + Alcalase and Cellic alone) gives a higher proportion of small peptides and free amino acids compared to Alcalase

and control samples. Cellic seems to reduce protein size to a bigger extent, especially in *A. esculenta*. Thus, Alcalase treatment improves extraction yield the most, but does not seem to reduce protein size much compared to the untreated sample. Cellic gives a lower extraction yield but reduces protein size more, which might be beneficial for releasing more bioactive peptides with good health promoting effects. Thus, combining Cellic and Alcalase gives efficiency in both quantitative and qualitative yield. This might make them a good combination for use in extraction for food purposes. It is said that Alcalase activity could be highly improved if combined with sonication to change the structure of the active site (Ma *et al.*, 2011). This might be a way to enable Alcalase to access the proteins and degrade them to a greater extent.

The amino acid composition in foodstuffs is an important part of the measure of food quality, digestibility and choosing the suitable processing techniques (Coultate, 2002). Both free and total amino acid profiles show that the most abundant amino acids are alanine, glutamic acid and aspartic acid. The main portion of amino acids are non-essential, but all measured essential amino acids are represented in smaller concentrations. The enzymatically treated samples have higher amino acid content than the control sample for *S. latissima*, while the opposite is shown for *A. esculenta*. Thus, the extracted amino acid composition in *A. esculenta* is less affected by the enzymatic treatments. This might also reflect the more packed and polyphenol rich biomass of *A. esculenta* that seems to resist treatments more than *S. latissima*. This is different from what is seen by other literature (Lyng, 2015; Reissiger, 2016), but might be partly result of using only water for extraction, since not all proteins are water soluble (Mæhre *et al.*, 2018). A better extractability might have been discovered for *A. esculenta* using other solvents.

In the comparison of different analysis methods, it was discovered that results varied over a wide range between the three methods used. Total amino acid and CN analyses gave low yield compared to literature on extraction from the same algae and compared to results from the Lowry analysis in this thesis. Different explanations for these differences were discussed thoroughly. A factor affecting the results from all analysis methods is the subtracted enzyme contribution, which was very demanding to measure precisely. This was attempted to do in different ways several times but results still varied from time to time. This is a source of error that was challenging to overcome with the methods used in this thesis and might affect results differently according to the measured yield and the amount of enzyme added. Protein contribution was still thought to be overestimated for many of the experiments and have a big impact on the results. Also, the scale that was used for weighing the enzymes was not accurate for small concentrations, which was especially a point of error when using Alginate lyase, since

only a small amount was used. It was hard to know the exact weight of enzyme added, and it was thereby hard to subtract the right enzyme contribution amount.

Moreover, the experiments were conducted in a small scale, and there were probably significant differences in the chemical composition of the algal biomass used for different experiments. Since only small amounts of the sample is analysed, there might also be heterogenous protein content affecting the yield from different samples. In the experiments involving sonication, contamination might have occurred between samples from the sonication probe. Some variations might also come from fouled biomass, where proteins or other nutrients originate from epibionts (Forbord *et al.*, 2020).

5 FURTHER WORK

Increasing extraction yield from pre-treatment by enzymes and sonication is shown in this thesis and widely described in literature. This has a high potential for being used in bigger scale in the industry when cheap and efficient techniques are developed. With a growing market for macroalgae and high cultivation potential, it is therefore necessary to establish protocols for scalable, economical and sustainable protein extraction, for optimized breakdown of plant material giving increased extraction.

The enzymes and enzyme mixtures used in this thesis are efficient, but do not completely degrade macroalgal biomass, and thereby do not release maximal amounts of proteins. The consumption of enzymes is high and is not economically feasible in bigger scale. For an optimized efficiency, many enzymes need to be combined and new enzyme blends should be designed especially for this use. Enzymatic treatment should also be combined to a higher extent with ultrasonic treatment, especially for Alcalase and Viscozyme. With a more optimized equipment, different sonication parameters like increased amplitude and time settings could be tested as well as a constant blending of biomass during sonication. Bigger scale sonication could possibly give very high extraction yield in a time and energy efficient way.

6 CONCLUSION

Screening of parameters

The efficiency of protein extraction depends on many factors. Algae to water ratio was an important extraction parameter, where higher ratio of water gave increased protein extraction. The state of the biomass also had a big impact. More degraded biomass gives a higher protein yield and extraction from freeze-dried algae was more efficient than that from wet algae. There is a positive correlation between the extractability of proteins and polyphenols, even though a higher polyphenol content seems reduce protein extraction. Treatment by enzymes and sonication increases protein extraction, and their effect can be highly optimized when used under the right conditions and combinations. Longer sonication duration and higher blend of biomass improves extraction. Enzyme concentration affects the extraction yield, but the highest concentration does not necessarily give the highest protein yield. Lowering incubation pH closer to the optimum conditions for Cellic and Alcalase did not increase protein yield. Treatment by the enzymes Alginate lyase and Viscozyme was more effective on *S. latissima*, while sonication gave a better effect on *A. esculenta*.

There were significant differences between the old and new *S. latissima*, with up to 240 % increase in protein extraction yield in the old one due storage under unfavourable temperature conditions, resulting in partly degraded biomass. This demonstrated that partly degraded raw material gives more available protein and increased protein extraction.

The enzyme concentrations giving the best protein yield for wet algae was 100 U/g (dw) Alginate lyase and 3 % (ww) Cellic CTec2 for both algae and respectively 196 µl/g and 98 µl/g Viscozyme for *S. latissima* and *A. esculenta*. From these concentrations, the best protein yield was measured in respectively *S. latissima* and *A. esculenta* to be 6.07 ± 0.23 % (dw) and 4.58 ± 0.11 % (dw) with Alginate lyase, 5.95 ± 0.09 % (dw) and 3.47 ± 0.07 % (dw) with Viscozyme L and 7.55 ± 0.025 % (dw) and 4.23 ± 0.17 % (dw) with Cellic CTec2. Note that numbers are from screening of parameters with the old batch of *S. latissima* and are not directly comparable with results from further extractions. The highest protein yield was seen for the middle enzyme concentration, and the most efficient enzyme concentration relative to the amount of enzyme added was for the lowest concentration.

Further extractions

Combination of enzymes and sonication gave improved protein extraction for both wet and dry algae. Treatment by sonication followed by enzymes seemed to be the best treatment order, but this was not conclusive in all cases. The best yield for sequential enzymatic and sonication treatment was Cellic CTec2 → US with 6.14 ± 0.15 % (dw) for wet *S. latissima*, US→ Cellic CTec2 with 3.35 ± 0.37 % (dw) for wet *A. esculenta*, US→ Alginate lyase with 6.01 ± 0.03 % (dw) for dry *S. latissim*a and US→Cellic CTec2 with 5.59 ± 0.21 % (dw) for dry *A. esculenta*. For all compared treatments, dry algae gave significantly higher yield than wet algae in all except for one case, where Cellic CTec2 + US gave significantly higher yield in wet algae. Both dry and wet algae were affected by increased extraction time and elevated temperature. This was one explanation for the increased extraction during enzymatic treatment, in addition to the degradative effect of the enzyme itself. For dry *S. latissima*, combined US and enzymes was more efficient than enzymes and sonication alone. For dry *A. esculenta*, US alone was the most efficient treatment, except when combined with Cellic. Dry *A. esculenta* treated with sonication gave a yield of 5.16 ± 0.89 % (dw).

Sequential enzymatic extraction with Cellic and/or Alcalase gave significantly increased protein extraction for all treatment combinations. The highest protein yield from Lowry analysis was seen for treatment by Alcalase for *S. latissima* with yield of 11.75 ± 0.63 % (dw) and Cellic CTec + Alcalase for *A. esculenta* with yield of 6.05 ± 0.09 % (dw). These were the treatments giving the overall highest extraction yield for both algae for all experiments conducted in this thesis. Thus, Alcalase, alone or in combination with Cellic CTec, gives a significantly higher protein yield compared to that of Cellic CTec alone. According to acid soluble peptide and free amino acid analyses, Cellic seem to induce more breakdown of proteins to smaller peptides and free amino acids than Alcalase. This might be due to the endoproteinase action of Alcalase, giving breakdown of proteins into relatively large peptides. It might also suggest that Alcalase activity is not optimized, and its potential in protein extraction is high if used under optimal conditions. The acid soluble peptides can be sources of bioactive peptides.

Extraction of free and total amino acids improves by enzymatic treatment. Amino acid profiles showed that all measured amino acids are present in all samples, and the most abundant amino acids are alanine, glutamic acid and aspartic acid. These give umami taste and good flavour characteristics of the extracted protein. All essential amino acids are present and will contribute to the nutritional quality of the extracted protein. These are characteristics that make extracted proteins highly suited for use in food.

Protein yield varies largely according to the analysis method chosen. Lowry analysis provide higher protein yield than that measured by CN and total amino acid analysis. Lowry analysis is thought to overestimate results, due to interfering substances increasing the measured absorbance. CN results are likely underestimated due to erroneous conversion factor used, since the enzymes present in the algae will change the nitrogen-to-protein ratio. Total amino acid analysis was underestimated, probably because of some amino acids being destroyed by the acid hydrolysis and the subtracted enzyme contribution that is overestimated for this method. All methods have their sources of error, and the most correct estimation of protein yield is thought to be something between what is recorded from all three methods.

All experiments

Almost all treatments significantly increased protein extraction. *S. latissima* showed higher extraction yields than *A. esculenta* in all experiments with a few exceptions, even though protein content is higher in *A. esculenta*. Different treatments also affected the two algal species overall differently. Enzymatic treatment was most efficient for *S. latissima* while *A. esculenta* was more affected by degradation from ultrasonication. The positive correlation between extracted polyphenols and protein was present for both algae but had the most potent effect in *A. esculenta.* The difference in the protein extractability of the two species was concluded to partly result from the higher carbohydrate and polyphenol content.

The most efficient extraction is seen for biomass with several pre-treatments, either combination of sonication and enzymes or several enzymes. Enzymes work better on previously degraded biomass, which makes the substrate more exposed. The highest extraction yield was seen in samples treated with Alcalase alone or combined with Cellic CTec.

7 LITTERATURE LIST

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APPENDICES

Appendix A – Statistical analysis

A.1 Standard deviation

Standard deviation was calculated using the built in Excel function STDEV, that uses the following formula:

$$s = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}}$$

Here, x is the sample mean average and n is the sample size.

A.2 Confidence interval

Confidence interval was calculated using the built in Excel function KONFIDENS, that uses the following formula:

$$\bar{x} \pm 1.96\left(\frac{\sigma}{\sqrt{n}}\right)$$

Here, 1.96 is given since alfa is set to 0.05, giving a 95 % confidence interval with the value ± 1.96.

A.3 Significance analysis

Statistic significance was calculated using the built in Excel function T.TEST. Datasets were compared using an unpaired two-sample t-test. Significant results are chosen to be $\alpha \leq 0.05$

Appendix B – Measured absorbances with removed values

B.1 Sonication settings

Sonication settings were tested in section 3.2.4, and certain values were removed before further calculations due to illogical results. Thus, these values were not further included in the results. Table 9 discloses all measured values for absorbance, and the values that were removed are marked with red font.

Table 9: Measured absorbance at 750 nm in Lowry analysis of extracted protein from varying sonication settings for treatment in *S. latissima* and *A. esculenta*. Samples with ratio 1:20 are undiluted before Lowry, while 1:10 are diluted 1:2. Values that were removed from the results are marked with red font.

Algae species	Treatment	Algae to water ratio	Measured absorbance at 750 nm (three parallels)		
S. latissima	Control	1:10	0.246	0.249	0.240
		1:20	**1.094**	0.294	**0.491**
	US x 1	1:10	0.319	0.313	0.316
		1:20	0.356	0.395	0.355
	US x 2	1:10	0.353	0.333	0.335
		1:20	0.376	0.374	0.382
	US x 3	1:10	0.371	0.370	0.378
		1:20	0.420	0.417	0.427
A. esculenta	Control	1:10	0.161	0.165	0.166
		1:20	0.158	0.156	0.160
	US x 1	1:10	0.207	0.197	0.202
		1:20	0.217	0.217	0.233
	US x 2	1:10	0.221	0.220	0.218
		1:20	0.252	0.255	**0.403**
	US x 3	1:10	0.303	0.297	0.300
		1:20	0.315	0.303	0.308

B.2 Sequential enzymatic treatment

Sequential enzymatic treatment was tested in section 3.3.4, and one value was removed before further calculations due to illogical results. Thus, this value was not further included in the results. Table 10 discloses all measured values for absorbance, and the value that was removed is marked with red font.

Table 10: Measured absorbance at 750 nm in Lowry analysis of extracted protein from sequential enzymatic treatment in *S. latissima* and *A. esculenta*. Control samples are diluted 1:2 before Lowry and enzymatically treated samples are diluted 1:6. The value that was removed from the results is marked with red font.

Algae specie	Treatment	Treatment parallel	Measured absorbance at 750 nm (three parallels)		
S. latissima	Cellic CTec2	1	0.260	0.258	0.258
		2	0.254	0.258	0.260
	Alcalase	1	0.290	0.289	0.290
		2	0.263	0.260	0.260
	Cellic CTec2 +Alcalase	1	0.326	0.329	0.325
		2	0.281	0.282	**0.608**
	Control 1	1	0.312	0.315	0.310
		2	0.321	0.317	0.319
	Control 2	1	0.308	0.308	0.308
		2	0.293	0.293	0.306
A. esculenta	Cellic CTec2	1	0.167	0.166	0.166
		2	0.163	0.169	0.168
	Alcalase	1	0.116	0.116	0.115
		2	0.132	0.132	0.133
	Cellic CTec2 +Alcalase	1	0.210	0.211	0.212
		2	0.210	0.212	0.208
	Control 1	1	0.217	0.217	0.223
		2	0.221	0.225	0.223
	Control 2	1	0.219	0.216	0.215
		2	0.231	0.226	0.229

Appendix C – Protein yield for the most important results

The most important results are shown for an easy comparison of protein yield from different treatments. Results are from section 3.2.5 (enzyme concentrations) in Table 11, 3.3.1/ 3.3.2/3.3.3 (US and E for dry/wet algae) in Table 12 and 3.3.4 (sequential enzymatic treatment) in Table 13.

Table 11: Protein yield and standard deviations from enzymatically treated *S. latissima* and *A. esculenta*. The extraction is done using the old batch of partly degraded *S. latissima*. Enzymes are Alginate lyase, Viscozyme L and Cellic CTec2 with three concentrations each.

Enzyme	Amount of enzyme	Results *S. latissima*		Results *A. esculenta*	
		Protein yield (% dw)	Stdev	Protein yield (% dw)	Stdev
Alginate lyase	50 U/g	5.00	0.03	4.00	0.80
	100 U/g	6.07	0.28	4.58	0.11
	150 U/g	5.98	1.06	2.60	0.29
Viscozyme L	98 µl/g	4.00	0.25	3.47	0.06
	147 µl/g	5.30	0.19	2.87	0.10
	196 µl/g	5.95	0.11	3.18	0.25
Cellic CTec2	1.5 %	6.13	0.07	1.74	1.52
	3 %	7.55	0.03	4.23	0.17
	4.5 %	7.40	0.17	2.51	0.10

Table 12: Protein yield and standard deviations from *S. latissima* and *A. esculenta* treated with enzymes and sonication alone and combined in different treatment orders. Enzymes are Alginate lyase, Viscozyme L and Cellic CTec2. Results for wet algae with only enzymatic treatment is not reported, since this was done in the screening period, with only the old batch of *S. latissima*.

Condition (dry/wet)	Enzyme	Treatment order	Results *S. latissima*		Results *A. esculenta*	
			Protein yield (% dw)	Stdev.	Protein yield (% dw)	Stdev.
Wet	Alginate lyase	US → E	4.34	0.03	2.56	0.38
		E → US	4.10	0.27	2.90	0.04
	Viscozyme L	US → E	3.86	0.00	2.56	0.41
		E → US	3.62	0.04	2.82	0.40
	Cellic CTec2	US → E	6.02	0.13	3.35	0.33
		E → US	6.14	0.19	2.27	0.45
	Np.	US	1.96	0.21	3.13	0.10
Dry	Alg.lyase	US → E	6.01	0.04	4.12	0.71
		E → US	4.39	0.24	3.96	0.20
		E	4.26	0.23	3.18	0.32
	Viscozyme L	US → E	4.71	0.17	3.59	0.83
		E → US	4.44	0.10	4.10	0.14
		E	3.67	0.29	3.69	0.03
	Cellic CTec2	US → E	5.61	0.32	5.59	0.24
		E → US	4.56	0.17	3.79	0.01
		E	4.99	0.05	3.65	0.66
	N.p.	US	3.80	0.17	5.16	1.01

Table 13: Protein yield and standard deviations (in parenthesis) from enzymatically treated *S. latissima* and *A. esculenta*. Enzymes are Cellic CTec2 and Alcalase, used alone and combined in sequential enzymatic treatment.

Enzyme	Results Lowry Protein yield, % dw (Stdev.)		Results CN Protein yield, % dw (Stdev.)		Total amino acids Protein yield, % dw (Stdev.)	
	S. latissima	***A. esculenta***	***S. latissima***	***A. esculenta***	***S. latissima***	***A. esculenta***
Cellic CTec2	9.03 (0.17)	4.24 (0.20)	7.58 (0.34)	8.01 (0.59)	4.05 (0.55)	2.26 (0.21)
Alcalase	11.75 (1.06)	4.42 (0.55)	9.55 (0.52)	7.90 (0.42)	3.73 (1.40)	1.32 (0.46)
Cellic CTec2 + Alcalase	9.16 (3.97)	6.05 (0.12)	8.81 (0.55)	7.69 (0.31)	4.56 (0.86)	2.04 (0.2)

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Protein extraction from the brown seaweed *Saccharina latissima* and *Alaria esculenta*

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