Norwegian University of Life Sciences
Faculty of Chemistry, Biotechnology and Food Science

Philosophiae Doctor (PhD)
Thesis 2018:46

Production of microbial protein from brown seaweed and spruce wood and its use as a novel feed ingredient in aquaculture

Produksjon av mikrobielt protein fra tare og granved og dets anvendelse som en ny fôringrediens i akvakultur

Sandeep Sharma

Production of microbial protein from brown seaweed and spruce wood and its use as a novel feed ingredient in aquaculture

Produksjon av mikrobielt protein fra tare og granved og dets anvendelse som en ny fôringrediens i akvakultur

Philosophiae Doctor (PhD) Thesis

Sandeep Sharma

Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Ås 2018

Thesis number 2018:46
ISSN 1894-6402
ISBN 978-82-575-1518-8

Table of Contents

Acknowledgements

The present work was accomplished at the Faculty of Chemistry, Biotechnology and Food Science (KBM) and the Faculty of Biosciences (IHA) of the Norwegian University of Life Sciences (NMBU), Ås, Norway. The PhD research was funded by NMBU, and the Research Council of Norway through grants BIOFEED and Foods of Norway project. Thank you!

Above all, I like to express my immense gratitude to my supervisor, Prof. Svein Jarle Horn for giving me the opportunity to start my PhD on such an exciting research topic. I am grateful to you for your scientific advices, planning, discussions, inputs, and support throughout my PhD. Undertaking this PhD has been a truly life-changing experience in both professional and personal level and I am very thankful to you Svein!

I am deeply thankful to my co-supervisor Prof. Margareth Øverland for introducing me to the aquaculture field. Your scientific advices, management skills, and motivation are truly an inspiration for me. I am also very grateful to my other co-supervisor Dr. Liv Torunn Mydland; you are my primary resource for getting my science questions answered. Your critical scientific inputs and insightful discussions will always be recommendable. Thank you for being very helpful. You have the best professor quality.

I am grateful to Prof. Vincent Eijsink, for all your direct and indirect support, and scientific inputs for the fermentation project. I am particularly thankful to Jon Funderud for your valuable discussion related to the seaweed and providing us seaweed. My special thanks to the all my co-authors for their contribution and for sharing their scientific experiences.

I am thankful to Dr. Kiira Vuoristo and Line Degn Hansen for their contribution in the fermentation work and Dr. Jon Øvrum Hansen for his technical and scientific support in the fish work.

A big “Tusen Takk” to all my colleagues in the PEP, BIORAF, animal nutrition and the fish lab. Thanks to these amazing people for sharing their knowledge and good moments: David, Gerdt, Mirzaman, Wenche, May Helene, Aniko, Magnus, Solfrid, Hanne, Ingrid, Leidy and Felipe.

My gratitude also goes to all my friends in Ås for making my life enjoyable. Thanks to Abhilash, Nivea, Ranju, Malli, Selba, and Mangu for your support, distraction and hospitality.

My deepest gratitude goes to my mummy-papa: your patience and sacrifices will remain my inspiration throughout my life. I am thankful to Sapna didi and Franz for your never-ending support and believing in me. Love to my nephews: Malav and Vinzenz. My sincere thanks to Appa, Amma, and Sridhar bhaiya for your encouragement and unconditional love. I am blessed to have you all.

Finally, and most importantly, I would like to thank my beloved wife, Anu, for your understanding and support. Thanks for believing in me and chasing our dream together. Love you yaar!

Sandeep Sharma
Ås, 2018

Summary

With the world's rapidly expanding population, it is necessary to provide sustainable and nutritious food. Aquaculture is the world's fastest growing food production sector and carries with it some major tradeoffs and constraints. It is facing a major sustainability challenge as it is heavily dependent upon marine-derived feedstocks such as fishmeal. With the inevitable increase in the price of fishmeal, declining supply and rising demand, more emphasis has been given to alternative feed sources. The partial replacement of fishmeal with plant based protein sources in the aquaculture has been steadily increasing, however, many of these ingredients can be used as human food directly. Furthermore, the anti-nutritional factors in many plant ingredients can have negative effects in carnivorous fish such as salmonids. Large efforts have been made to develop the technology to produce alternative protein sources by using unicellular microorganisms such as microalgae, yeasts, fungi or bacteria. In this thesis, we have produced microbial protein from Norwegian bioresources such as brown seaweed and Norway spruce that could partially replace fishmeal in the Atlantic salmon diets. The work included characterization of the feedstocks, enzymatic saccharification of seaweed, fermentation for microbial protein production and fish feed trials. This study is based on five research papers:

A detailed characterization of *Saccharina latissima* biomass is presented in the Paper I. The paper describes the biomass production and chemical composition of *S. latissima* cultivated at different depths and harvested at different time points.

The enzymatic saccharification process of *S. latissima* by using a blend of cellulases and an alginate lyase are described in Paper II. It was shown that the inclusion of alginate lyases improved the saccharification yield of the seaweed, particularly at high solid loading.

The carbohydrate content and the enzymatic saccharification of the brown seaweeds *Macrocystis pyrifera* from Chile and *Saccharina latissima* from Norway was compared in Paper III. For both seaweeds, recombinant alginate lyases and oligoalginate lyases in combination with cellulases gave higher sugar release than using cellulases only. However, for saccharification of pretreated seaweed only cellulases were needed to achieve high sugar release, indicating that the pretreatment partially hydrolysed the alginate. Moreover, it was shown that seaweed hydrolysate could be used as a growth medium for the yeast *Candida utilis*.

The cultivation of microbial yeast (*C. utilis*) from enzymatic hydrolysates of brown seaweed and spruce at different fermentation scales was studied in Paper IV. The yeast product quality in terms of amino acids composition, and mineral content were also studied. A feeding experiment with Atlantic salmon showed that the yeast biomass could partly replace a fishmeal diet, without affecting the growth, but with sub-optimal nutrient digestibility.

The nutrient digestibility of *C. utilis* cultivated from three different carbon and nutrient sources: 1) a blend of woody hydrolysate and molasses, 2) spend sulphite liquor, and 3) a blend of brown seaweed and woody hydrolysate, and the impact of the different yeast biomasses on faecal mineral excretion was evaluated in Paper V. Inclusion of 30 % yeast cultivated on seaweed and spruce hydrolysates resulted in reduced digestibility of protein compared to both the fishmeal-diet and the two other yeast-based diets.

In conclusion, this study demonstrated a proof-of-concept for utilization of brown seaweed and wood biomass for the production of microbial protein ingredients for the aquaculture sector.

Sammendrag

I en verden med en raskt voksende befolkning er det nødvendig å produsere næringsrik mat på en bærekraftig måte. Akvakultur er verdens raskest voksende matproduksjonssektor, noe som medfører store utfordringer. Den står overfor en stor utfordring innenfor bærekraft siden sektoren er sterkt avhengig av marine råvarer som fiskemel. Med den uunngåelige økningen i prisen på fiskemel, fallende fiskebestander og økende etterspørsel, har det vært lagt stor vekt på å utvikle alternative fôrkilder. De siste tiårene har derfor fiskemel blitt erstattet delvis med plantebaserte proteinkilder. Men disse proteinkildene kan også spises direkte av mennesker, og de inneholder dessuten også endel anti-næringsstoffer som kan ha negativ effekt i kjøttetende fisker som salmonider. Det jobbes med å utvikle teknologi for å produsere og bruke encellede mikroorganismer som mikroalger, gjær, sopp eller bakterier som fôringredienser. I denne doktorgraden har vi produsert mikrobielt protein ved å bruke de norske bio-ressursene tare og gran, og brukt dette som en delvis erstatning av fiskemel i laksefôr. Arbeidet omfattet karakterisering av råvarene, enzymatisk sakkarifisering av tare, fermentering for å produsere mikrobielt protein og fôringsforsøk av laks. Denne studien er basert på fem forskningsartikler:

En detaljert karakterisering av *Saccharina latissima* ble utført i Artikkel I. Artikkelen beskriver både biomasseproduksjon og den kjemiske sammensetningen av *S. latissima* som ble dyrket på forskjellige dybder og høstet på forskjellige tidspunkter.

Den enzymatiske sakkarifiseringsprosessen av *S. latissima* ble studert i Artikkel II ved å bruke en blanding av cellulaser og en alginat lyase Studien viste også at inkludering av alginat lyaser var spesielt viktig når konsentrasjonen av tare var høy.

Karbohydratinnholdet og den enzymatiske sakkarifiseringen av tareartene *Macrocystis pyrifera* fra Chile og *Saccharina latissima* fra Norge ble sammenlignet i Artikkel III. Den enzymatiske sakkarifisering ble utført med en blanding av cellulaser og nye rekombinante alginat-lyaser og oligoalginat-lyaser. Hvis taren ble forbehandlet kunne hydrolysen utføres med bare cellulaser, noe som indikerer at forbehandlingen delvis brøt ned alginaten. Studien viste også at tarehydrolysater kan anvendes som vekstmedium for å fremstille gjæren *Candida utilis*.

Dyrking av gjær (*C. utilis*) på enzymatiske hydrolysater fra tare og gran ble demonstrert i Artikkel IV. Kvaliteten på den produserte gjæren i form av aminosyresammensetning og mineralinnhold ble også studert. Et fôringsforsøk med atlantisk laks viste at gjærbiomassen delvis kunne erstatte en fiskemeldiett uten å påvirke veksten, men med sub-optimal fordøyelighet av næringsstoffene.

Fordøyelighet av *C. utilis* dyrket på forskjellige karbon- og næringsstoffkilder; ble studert i Artikkel V. De tre ulike fermenteringmediene var 1) en blanding av hydrolysat av trevirke og melasse, 2) brukt sulfittlut fra cellulose- og papirindustri, og 3) en blanding av enzymatiske hydrolysater fra dyrket sukkertare og gran. Inkludering av 30% gjær dyrket på tare- og gran-hydrolysater i laksedietten, resulterte i redusert fordøyelighet av protein, sammenlignet med både fiskemel-dietten og de to andre diettene med 30% gjær produsert på henholdsvis en blanding av hydrolysat av trevirke og melasse og brukt sulfittlut fra cellulose- og papirindustri.

Oppsummert viser denne studien at tare og trebiomasse kan brukes som råstoff for å produsere mikrobielt protein for akvakultursektoren.

Abbreviations

ABPs	Animal by-products
ADC	Apparent digestibility coefficient
BGs	β-glucosidases
CBHs	Cellobiohydrolases
CBM	Carbohydrate binding module
CDH	Cellobiose dehydrogenase
CM	Cottonseed meal
CUA	*C. utilis* produced in United States of America
CUE	*C. utilis* produced in Estonia
CUN	*C. utilis* produced in Norway
DEHU	4-deoxy-L-erythro-hex-4-enepyranosyluronate
DM	Dry matter
DP	Degree of polymerization
EnGs	Endo-β-(1-4)-glucanases
ExGs	Exo-β-(1-4)-glucanases
FAO	Food and Agriculture Organization
FDA	Food and drug administration
FM	Fishmeal
G	Guluronic acid
GH	Glycoside hydrolase
GRAS	Generally regarded as safe
LPMO	Lytic polysaccharide monoxygenase
LYCC	Lallemand Yeast Culture Collection
M	Mannuronic acid
PBM	Poultry co-products
PL	Polysaccharide lyase
PPC	Potato protein concentrate
PSU	Practical salinity units
RM	Rapeseed meal
SCP	Single cell protein
SEH	*S. latissima* enzymatic hydrolysate
SGA	Solanidine glycoalkaloids
SM	Sunflower meal
SPC	Soy protein concentration
SPH	Spruce enzymatic hydrolysate
YPD	Yeast extract peptone dextrose

List of Papers

Paper I

Sharma, S., Neves, L., Funderud, J., Mydland, L. T., Øverland, M. & Horn, S. J. (2018). Seasonal and depth variations in the chemical composition of cultivated *Saccharina latissima*. *Algal Research*, 32: 107-112.

Paper II

Sharma, S. & Horn, S. J. (2016). Enzymatic saccharification of brown seaweed for production of fermentable sugars. *Bioresource Technology*, 213: 155-161.

Paper III

Sharma, S.*, Ravanal, M. C.*, Gimpel, J., Reveco-Urzua, F. E., Øverland, M., Horn, S. J. & Lienqueo, M. E. (2017). The role of alginate lyases in the enzymatic saccharification of brown macroalgae, *Macrocystis pyrifera* and *Saccharina latissima*. *Algal Research*, 26: 287-293.

Paper IV

Sharma, S., Hansen, D. L., Hansen, Ø. J., Mydland, L. T., Horn, S. J., Øverland, M., Eijsink, G. H. V. & Vuoristo, S. K. (2018). Microbial protein produced from brown seaweed and spruce wood as a feed ingredient in aquaculture. Submitted to *Journal of Agricultural and Food Chemistry*

Paper V

Sharma, S., Hansen, Ø. J., Mydland, L. T., Horn, S. J., Eijsink, G. H. V., & Øverland, M. (2018). Microbial feed ingredients produced from different feedstock in diets for Atlantic salmon (*Salmo salar*): nutrient and mineral digestibility. (Draft Manuscript)

* The authors contributed equally to this work

1. Introduction

1.1. Background

Planet Earth is currently sustaining more than 7 billion people and the population is projected to reach 9.7 billion by 2050, which is an annual increase of 80 million people. According to the Food and Agriculture Organization (FAO) of the United Nations, almost 70 % more food production is required to feed the world population by 2050 (Vasileska & Rechkoska, 2012). The global *per capita* food consumption in terms of kilocalories (kcal) per day was 2358 kcal in 1965, when the world population was 3.3 billion, and is expected to reach 3050 kcal in 2030. In the same manner, the *per capita* fish consumption was 9.9 kg per year in 1965 and reached more than 20 kg per year in 2015 (FAO, 2016a; FAO, 2016b). The world food producing sector has to secure food supply to meet this demand without compromising its' nutritional value or sustainability. Generally, two main food sources exists: plants and animals. The production of agricultural based foods is declining and may not be able to meet the demand of the future human population. Different crops have to meet the demand for food production and for the production of animal feed and biofuels, which may lead to environmental problems.

Aquaculture has become the fastest growing food production industry in the world, currently constituting half of the global food fish production (Francis et al., 2001). The total fish production (captured fish and aquaculture) has experienced a five fold increase from 1960 to 2015, dominated by an increase in aquaculture since the late 1980s. Globally, fish provides 6.7 % of all protein consumed by humans (FAO, 2016b). Fish offers a high quality protein, low saturated fat, and wide range of essential micronutrients, including vitamins (A, D, E and K), minerals (calcium, iodine, zinc, iron, selenium), and omega-3 fatty acids (Miles & Chapman, 2015). Fish as food offers health benefits, such as lowering of blood pressure, reduced risk of heart diseases, aid healthy brain function, lower the risk of depression, ADHD, dementia, diabetes, and may prevent inflammation and arthritis (Riediger et al., 2009). Thus, even small quantities of fish in the diets could be very beneficial, in particular for societies with limited access to food. Fish is providing not only nutritious food, but also income and livelihood for hundred millions of people around the world.

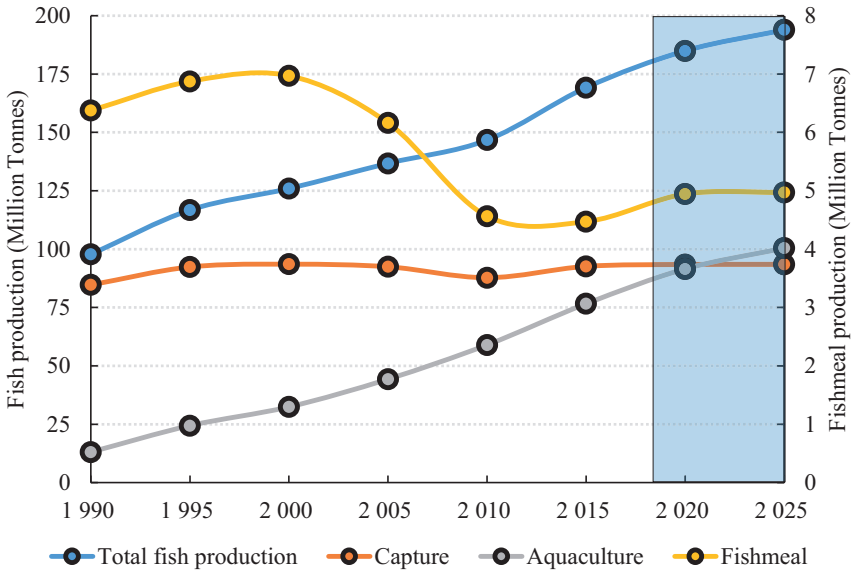


Fig. 1. World capture fisheries, aquaculture and fishmeal production. Adapted figures from food and agriculture organization of the united nations (OECD-FAO, 2018). Note: 2017-2025 are projected values.

As shown in Figure 1, the aquaculture production (both in freshwater and saltwater) is projected to equal the wild fish capture by 2020. This rapid growth in the aquaculture industry increases the demand for fish feed without compromising the environment.

Fish feed costs represent approximately 60 % of the total operating costs of fish farms (Kolstad et al., 2004). Fish feeds were traditionally based on fish meal and fish oil. Fish meal is a high-quality protein source and is mostly derived from wild-caught small marine fish (e.g., anchovies, sardine, horse mackerel, herring, pout, sandeel) that are unsuitable for human consumption. Peru makes nearly one third of the world's fish meal production followed by Chile and China (Deutsch et al., 2007). It is noteworthy that the commodity price of fish meal has increased almost four fold during the last two decades (Figure 2). This trend causes an increase in the prices of aquaculture products. The main reason for this increment is the increase in transportation cost due to increased in fuel price. With the inevitable increase in the price of fish meal, declining supply and rising demand, more emphasis has been given to alternative feed ingredients like plants protein sources, animal co-products, and microbial ingredients.

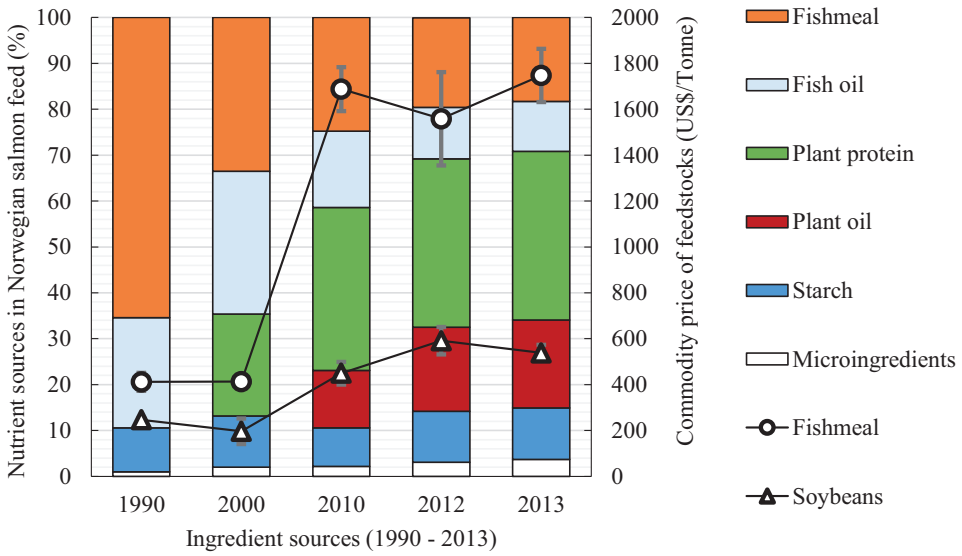


Fig. 2. Ingredient sources in Norwegian salmon feed and commodity price of feedstock (fish meal and soybeans) from 1990 to 2013. Compiled data from (Ytrestøyl et al., 2015), copyright © (2015) Elsevier Ltd and (Fishmeal, 2018; Soybeans, 2018), copyright © (2018) IndexMundi.

1.1.1. Resources used for substitution of fish meal in Atlantic salmon diets

Aquaculture is the major industry in Norway after oil and gas. Norway is known as the largest Atlantic salmon supplier to the global market. In 2015-2016, 1.32 million tonnes of salmonids were harvested in Norway, of which 93 % was Atlantic salmon and 6.6 % rainbow trout (Statistisk Sentralbyrå, 2016). Norway is expected to expand Atlantic salmon production from 1.32 million tonnes to 5 million tonnes by 2050 (Olafsen, 2012). The partial replacement of fish meal with plant protein sources in Norwegian salmon diets have been steadily increased from 2000 (Figure 2). Currently, fish feeds contain less than 29 % of fish meal and fish oil, while the rest are plant ingredients and micro-ingredients. The most common plant protein source used in a fish feed are soy protein concentrate, rapeseed meal, lupin seed, potato protein concentration, wheat gluten and corn gluten. It should be noted that the commodity price of soybeans has increased twice over the period of two decades due to the increase in fuel prices. Several plant based proteins have been introduced as a replacement for fish meal. However, due to the presence of anti-nutritional factors, these replacements may lead to

adverse effects on growth performance and health of fish (Francis et al., 2001; Krogdahl et al., 2010).

Another alternative protein source is animal by-products. Animal by-products (ABPs) include fish by-products, blood meal, poultry by-products and are concentrated sources of protein, vitamins and essential minerals. However, use of ABPs may lead to spread of animal-borne diseases and are prone to contaminations (e.g. dioxins). This could be deleterious for fish health and human consumption (Official Journal of the European Union, 2009). The advantages and disadvantages of plant and animal by-product substitutes used as a partial replacement for fish meal are shown in Table 1.

Microbial ingredients (single cell protein; SCP) refers to edible unicellular microorganisms (microalgae, yeasts, fungi, or bacteria) that are currently under development for replacing fish meal or fish oil. Microalgae such as *Chlorella sp, Spirulina sp* and *Nannochloropsis sp* are widely studied as an alternative resources of fish meal in the salmonid diets (Grammes et al., 2013; Sørensen et al., 2017; Teimouri et al., 2013). The main challenges is to develop cost efficient methods to produce the microbial ingredients, but large efforts have been made to develop this technology and today microalgae are available on the market. Several studies have been performed to the replace of fish meal with bacterial meal such as *Methylococcus capsulatus* in the salmonid diets (Øverland et al., 2010). The main advantages of bacterial cultivation is that it possesses high growth rates, have a high protein content with a favourable amino acid composition. Bacterial meal also contain a wide range of bioactive components with health-beneficial effects in fish (Romarheim et al., 2011; Romarheim et al., 2013a; Romarheim et al., 2013b). The bacterial cultivation required major precautions to avoid contaminations that could be dangerous to the fish health. The health risk of bacterial meal has however been extensively evaluate and it was EU approved in 2009 (Øverland et al., 2010).

Yeast as a protein source in the replacement of fish meal has also gained increasing interest. A variety of yeast species has been studied as dietary protein sources in salmonids (Grammes et al., 2013; Øverland & Skrede, 2017). Yeast has high growth rates and can metabolize a wide range of substrates, and the risk for contamination is low, and toxic compounds are absent, making yeast a suitable protein source in fish diets. (Anupama & Ravindra, 2000).

The common amylolytic yeasts species used in fermentation processes are *Saccharomyces*, *Candida, Hansenula*, *Torulopsis,* and *Pichia* (Anupama & Ravindra, 2000). *Candida utilis* is a protein-rich single-celled yeast that belong to the *Saccharomycetes* class. It has a generally-regarded-as-safe (GRAS) status, it is widely used in animal feed and it can also metabolize a wide range of substrates (FDA, 2018). *C. utilis* has a high content of amino acids and have documented health properties in salmon, and could be an ideal protein source in the fish diets (Øverland & Skrede, 2017). The utilisation of regular carbon sources (glucose) and micronutrients (amino acids and minerals) are not economically feasible for the cultivation of *C. utilis.* The cost of these growth media components constitute more than 50 % of the overall cost for fermentative production (Walker & Stewart, 2016). Therefore, less expensive growth media ingredients for cultivation of *C. utilis* are needed. Several studies have demonstrated biomass conversion of fruits, vegetables, crop residues, and organic wastes as a nutrient sources for the cultivation of *C. utilis* (Bekatorou et al., 2006; Lee & Kyun Kim, 2001; Nigam, 1998; Panda et al., 2018).

Table 1: Advantages and disadvantages of plant and animal by-product substitutes for fish meal.

Sources	Advantages	Disadvantages	Reference
Plant substitute			
Soy protein concentrate (SPC)	Most essential amino acids matches profile of fishmeal	Low in methionine and cysteine content. Crude fat content and minerals is lower	(Dersjant-Li, 2002; Glencross et al., 2007; Rana, 2009)
Rapeseed meal (RM)	Balanced amino acid profile	Poor palatability, glucosinolates, tannins	(Francis et al., 2001; Rana, 2009)
Sunflower meal (SM)	High amino acid than RM and CM	High fibre content, arginate inhibitor	(Francis et al., 2001; Rana, 2009)
Cottonseed meal (CM)	EAA profile matches fishmeal except lysine	Limited amount of lysine. Presence of antivitamins, and phytic acid	(Jiang et al., 2013)
Potato protein concentrate (PPC)	Rich in crude protein (≥85 %). Well balanced amino acid composition	Presence of solanidine glycoalkaloids (SGA) from the skin of potato cause bitter flavoured and toxic substance.	(Hemre et al., 2009)
Lupine seed	High amount of crude protein	Limited amount of lysine and methionine. Poor digestibility. Presence of saponins, and alkaloids	(Francis et al., 2001; Glencross et al., 2007; Rana, 2009)
Wheat	Good binding properties	Low protein content and high starch content	(Rana, 2009)
Animal by-product			
Fish by-products	Best nutritional substitutes	Prone to contamination	(El-Sayed, 1999; Rana, 2009)
Blood meal	Rich in crude protein (≥80 %) good digestibility	Low in methionine. Sensitive to heat and drying conditions	(Kirimi et al., 2017; Rana, 2009)
Poultry co-products (PBM)	Crude protein ≥66%	Low in lysine, methionine and histidine	(Mustafa Erturk & Sevgili, 2003; Rana, 2009)

1.2. Norwegian bioresources

In the emerging bioeconomy, better utilisation of biological resources from agriculture, forestry, aquaculture and different waste streams for the production of conventional and new bioproducts is essential. Norway is rich in bioresources, particular forest, but possesses also large amounts of seaweed along its long coastline.

The country has 83,000 km of coastline (including fjords and island) rich in brown seaweed, a multicellular algae (macroalgae) that is composed of lamina, stipe, and holdfast (Kim & Lee, 2015). In Norway, approximately 0.2 million tonnes of wet wild seaweed are harvested annually, primarily *Laminaria hyperborea* and *Ascophyllum nodosum* (Stévant et al., 2017). Seaweeds are composed of carbohydrates, minerals, amino acids, and lipids, and are clearly a potential source for growth media ingredients for the production of microbial protein.

Norway's largest bioresource is the coniferous forest, covering 37 % of total land area with an annual growth increment of about 25 million m^3 (Scarlat et al., 2011). The total forest cover is 12 million hectares with a standing stock of 910 million m^3. The total annual harvest has been stagnant (6 -10 million m^3) for a century (Trømborg et al., 2008). This coniferous evergreen forest is dominated by two species; Norway spruce and Scots pine (Skrøppa, 2012). These lignocellulosic biomasses are composed of cellulose, hemicellulose and lignin. Borregaard ASA, an advanced Norwegian biorefinery company, manufactures emerging bioproducts based on the different components in the wood (Norwegian spruce). The most important bio-products are cellulose, lignin (lignosulphonates), bioethanol, yeast, yeast extracts and lignin based vanillin (Borregaard, 2018).

1.2.1. Brown seaweed

Seaweed is classified into three groups empirically distinguished on the basis of the thallus (whole algal body) colour: Brown (*Phaeophyta*), Red (*Rhodophyta*) and Green (*Chlorophyta*). Norway has good conditions for the growth of brown seaweed, which grows at the intertidal or upper littoral zones and predominately in relatively cold waters. Brown seaweed is divided into 4 subclasses, 20 orders, 300 genera, and have approximately 2000 known species (Silberfeld et al., 2014). The main brown seaweed species found in Norwegian

waters are *Saccharina latissima*, *Alaria esculenta*, *Ascophyllum nodosum, Laminaria digitata* and *Laminaria hyperborea* (Lindsey Zemke-White & Ohno, 1999; Moy & Christie, 2012; Sivertsen & Bjørge, 2014).

Saccharina latissima belongs to the Laminariaceae order and family. As the name indicates *Saccharina latissima* is one of the brown seaweed with highest sugar content. It is known as sugar kelp and the name is derived from Latin: *saccharum* means sugar and *latissimus* means large. It has a yellowish brown colour with a long narrow blade that can get up to 5 m long and 20 cm broad (Figure 3). *S. latissima* grown in the waters of the colder northern hemisphere waters in the intertidal or littoral zone. The main factors which may influence the growth rate are irradiation, temperature, total inorganic nitrogen (NO_3, NH_4) and salinity. The ideal temperature for the optimal growth of brown seaweed is between 5 – 15 °C, 25 – 35 practical salinity units (PSU), saturated irradiation levels of 2 – 3 $Em^{-2}d^{-1}$, and total inorganic nitrogen from 6.7 to17.8 μgl^{-1} (Dean & Jacobsen, 1984; Wheeler & North, 1981).

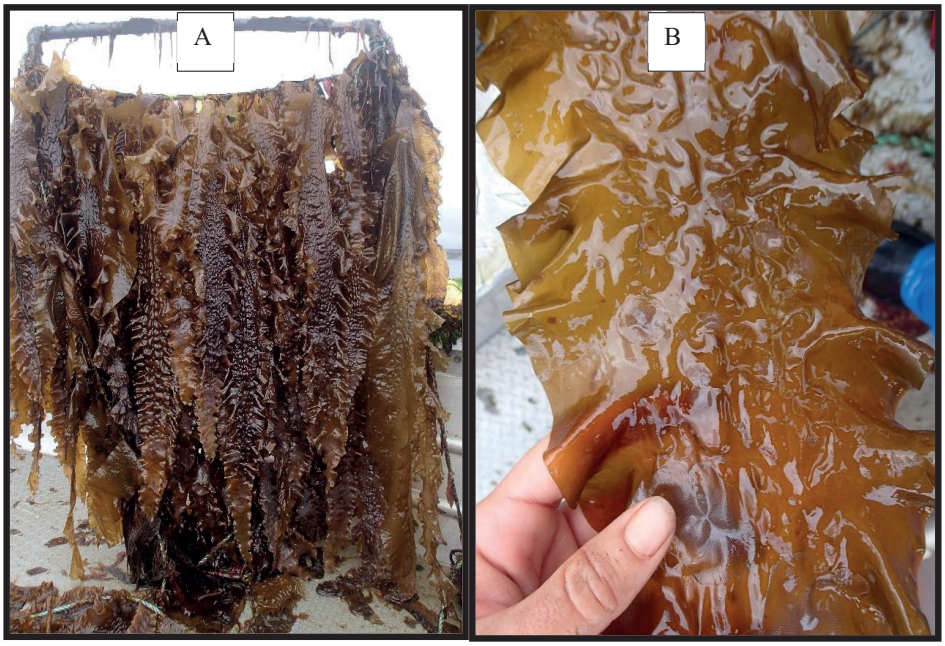


Fig. 3. Images of cultivated *Saccharina latissima.* A) *S. latissima* cultivated at 8 m depth with the attached holdfast on the string frame, B) close view of the blade.

1.2.1.1. Biochemical composition

The moisture content of fresh brown seaweed (*Laminaria* and *Saccharina*) is high, typically up to 84 – 90 % (Schiener et al., 2015). Brown seaweed contains structurally diverse bioactive compounds that are not found in terrestrial plants (Gupta & Abu-Ghannam, 2011; Holdt & Kraan, 2011). It is rich in carbohydrates, protein, minerals, polyphenolic compounds, vitamins, and fat (Figure 4) (Holdt & Kraan, 2011; Kim & Lee, 2015; Manns et al., 2016), and some of these compounds have biological functions including antioxidant, anticancer, antidiabetic, antimicrobial, antitumor, anti-inflammatory, anti-HIV, anticoagulant, antivirus, and prebiotic effects (Deniaud-Bouët et al., 2017; Holdt & Kraan, 2011; Wijesinghe & Jeon, 2012). Brown seaweed contains laminarin which is a storage carbohydrate, while the cell walls are composed of alginate, cellulose and fucoidans as structural carbohydrates.

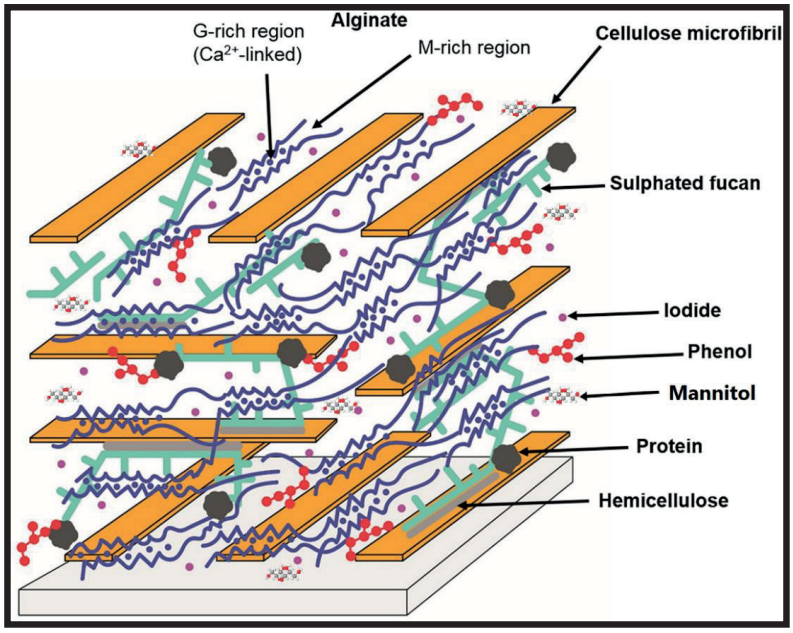


Fig. 4. Cell wall model of brown seaweed (Fucales order); cellulose microfibrils (spares, ribbon shape), hemicellulose, fucans and protein are embedded within the alginate network. Phenols are likely to be associated with alginates and protein. Mannitol are freely associated in the cell wall. Adapted from figure (Charoensiddhi et al., 2017), copyright © (2017) Elsevier Ltd, and (Deniaud-Bouët et al., 2014) copyright © (2014) Oxford University Press. Remark: Fucose-containing sulfate polysaccharides (FCSPs) are relatively higher in the cell wall of Fucales order (Deniaud-Bouët et al., 2017).

1.2.1.1.1. Storage carbohydrates

Laminarin and mannitol are considered as the storage carbohydrates in brown seaweed. Laminarin is a linear polysaccharides of glucan, built up from β-(1-3) and β-(1-6) glucose residues, with β-(1-3):β-(1-6) ratio of 3:1 (Figure 5). It is made up of 25-50 glucose units with different terminal reducing end, which corresponds to a glucose reside in G-type laminarins and mannitol in M-type laminarins (Stiger-Pouvreau et al., 2016). The average molecular weight of laminarin extracted from *L. digitata* is about 5.3 kDa (laminariaceae family). Laminarin content have found to accumulate during summer and autumn season in *S. latissima* species and decline during the dark season (Schiener et al., 2015).

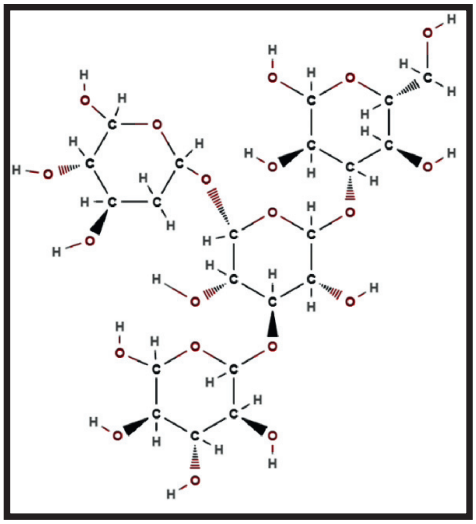


Fig. 5. Laminarin structure: Backbone consists of β-(1-3) linked glucose with β-(1-6) branched glucose substituents.

Laminarin shows anti-coagulant activity after structural modification with sulphation or oxidation (Shanmugam & Mody, 2000). However, the commercial application for the extracted laminarin are limited. It is used as an antiviral agent in agriculture applications (Goëmar, 2013). Laminarins need to be hydrolysed into monomers by enzymatically before fermentation processes and production of chemicals like bioethanol, acetone or butanol.

Mannitol is the sugar alcohol corresponding to mannose (Figure 6). It has numerous applications such as by dehydration process it can be converted into isomannide (Yokoyama

et al., 2017). By microbial fermentation, it can produce bioethanol by *Saccharomyces cerevisiae* and butyric acid and acetic acid via *Lactobacillus acidophilus* bacteria (Enquist-Newman et al., 2014; Liong & Shah, 2005). It has been used in pharmaceuticals, paint, pulp and paper industries. It is also often used as a sweetener in the food industries for people with diabetic and in chewing gums. In laminariales, mannitol is also a storage carbohydrate, with large annual changes in concentration (Adams et al., 2011b; Schiener et al., 2015).

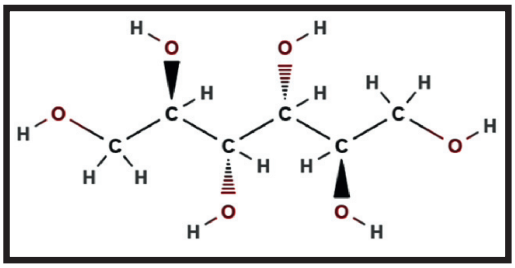


Fig. 6. Chemical structure of Mannitol (sugar alcohol)

1.2.1.1.2. Structural carbohydrates

Alginate is a structural carbohydrates that is distributed widely in the cell walls of brown seaweed. It is made up of linear blocks of covalently β-(1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) (Figure 7). The monomers are present as homopolymeric blocks of G-residues or M-residues, or alternating M and G residues (MG-residues). Normally, the G/M ratio are in the range 1.2 – 2.1 (Percival & McDowell, 1967). The average alginate content in the brown seaweed is 20 – 47 % of dry matter of whole seaweed (Di Filippo-Herrera Dania et al., 2018; Kraan, 2012).

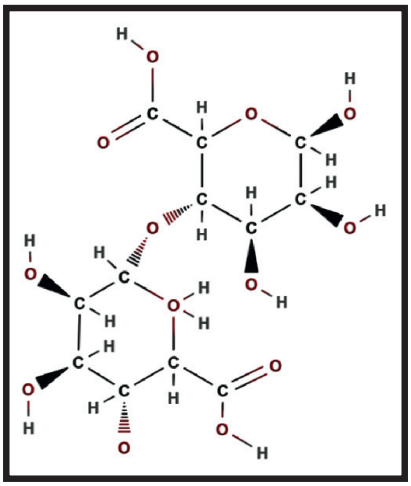


Fig. 7. Chemical structure of alginate (guluronic and mannuronic acids).

Alginate can be extracted from brown seaweed in the form of acid (alginic acid) or salt (calcium alginate). Alginate is used in the food industry as a thickening agent, in ice creams, cosmetics, and in the pharmaceutical industry for the preparation of capsules. The global production of alginate is approximately 26,500 tonnes with the market valued of US $ 318 million annually (Sudha, 2017). By biochemical conversion, alginate can be used for the production of bioethanol and acetic acid (Enquist-Newman et al., 2014; Sawabe et al., 2003). Recent studies have shown that the higher doses of alginates fed to rats, human, and pigs gives significant effects on the carbohydrate digestion (Kimura et al., 1996; Vaugelade et al., 2000).

Fucoidan is a sulfated polysaccharide that has a backbone primary built of α-(1-3)-linked L-fucopyranosyl (Figure 8) or alternating α-(1-4)-linked L-fucopyranosyl. Moreover, it also includes β-(1-6)-linked D-galactose- and/or β-(1-2)-linked D-mannopyranosyl residues, branching and/or glucuronic acid, xylose or glucose substituents. Fucoidan content is relatively high in the cell wall of the *Fucales* order, constituting 1-10 % of the seaweed dry matter (Wang & Chen, 2013).

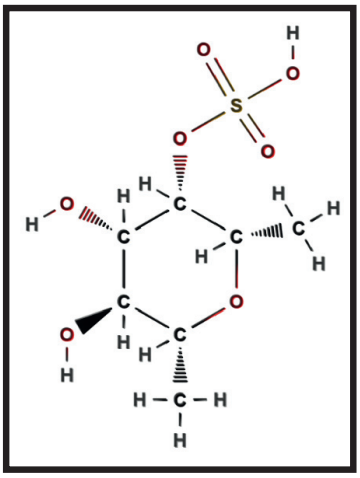


Fig. 8. Chemical structure of Fucoidan (sulphated polysaccharides)

Due to the complex cell wall structure of brown seaweed, it is not easy to extract complete polysaccharides (Fucoidan) using a solvent extraction process. Therefore, enzymatic hydrolysis is needed prior to the solvent extraction process (ethanol, water) (Wijesinghe & Jeon, 2012). Fucoidan is known as a novel functional ingredient in pharmaceutical, cosmeceutical or food industries and have shown to be responsible for biological effects such as anticoagulant, immunomodulation, anti-inflammation, antitumor, angiogenesis, antivirus, gastric mucosal protection, neuroprotection and cardio protection (Deniaud-Bouët et al., 2017; Kumar et al., 2011; Wijesinghe & Jeon, 2012).

1.2.1.2. Minerals, proteins and polyphenolic compounds

Apart from carbohydrates, brown seaweed contains other compounds such as minerals, proteins and polyphenolics. Seaweed is rich in minerals and trace elements, which can account for over 50 % of its dry weight, higher than green and red seaweed (Betty Moss, 1952; Rupérez, 2002). The main cations found in the brown seaweed (*L. digitata*) are sodium, potassium, calcium and magnesium, along with chloride and sulphates as the main anions (Adams et al., 2011b). Seaweeds are considered as one of the most important sources of iodine and calcium, which helps in nutrient metabolic regulation and Ca deficiency risk, especially for pregnant women and adolescent (Hamed et al., 2015). Brown seaweed can be used as

fertiliser and soil improver in agriculture, and animal food additives (Adams et al., 2011b; Schiener et al., 2015).

Another significant part of brown seaweed biomass is its protein fraction, typically accounting for 3 – 15 % of the dry matter (Arasaki & Arasaki, 1983; Kim, 2016). With the presence of all essential amino acids, brown seaweed has several potential uses such as marine vegetables as food proteins and even used as fish feed (Fleurence, 1999).

Brown seaweed also contains phenolic compounds. Phlorotannins are a type of tannins that is commonly found in the brown seaweed, whereas gallic and ellagic acids are found in terrestrial plants. The difference is probably due to the absence of lignin-type materials in seaweed. The polyphenolic compounds in brown seaweed are in the range of 0.2 – 5.3 % of the dry weight (Connan et al., 2006; Holdt & Kraan, 2011; Horn, 2000). Brown seaweed also contains vitamins, especially vitamin B_{12}, and fatty acids that could be used in the food and pharmaceutical industry (Schiener et al., 2015). However, the proportion of vitamins and fatty acids are quite low.

1.2.1.3. Compositional variation

Brown seaweed shows large variation in its biochemical composition. These changes are related to several environmental factors such as water temperature, light, salinity, mineral availability, species, waves and water current (Handå et al., 2013; Marinho et al., 2015; Schiener et al., 2015). The growth rate of brown seaweed varies according to the season, from minimal growth during the dark winter time to rapid growth during spring and summer (Skriptsova et al., 2004; Tønder, 2014). Laminarin and mannitol accumulate in the brown seaweed during the light season (May to September), while consume these storage carbohydrates in the dark season for developing new tissue growth (Adams et al., 2011a; Schiener et al., 2015). This results in highest alginate content late in the winter time (Skriptsova et al., 2004). The ash content in *S. latissima* gradually increase in the winter period to up to 40 % (Schiener et al., 2015). Brown seaweed also has a nitrogen reservoir that can sustain growth in the periods of the summer when the available nitrogen (nitrates) in the sea are low (Dayton et al., 1999; Schiener et al., 2015). These variations in seaweed composition will affect the choice of harvesting time, depending on the intended use of the seaweed

biomass. Two examples are the effect of seasonal variation on the production of bio-oils and bio-ethanol (Adams et al., 2011a; Adams et al., 2011b).

The value chain for the cultivation of brown seaweed in Norway is incomplete and many hurdles need to be solved, technologically, ecologically, financially, marketing, and governmental administration. Several permits for the seaweed cultivation in Norway was granted for six companies in 2014, but has increased to 20 companies by 2016 (Stévant et al., 2017). Seaweed Energy Solutions AS, an early Norwegian seaweed cultivation company, with patented cultivation technology, are cultivating brown seaweed (*S. latissima* and *A. esculenta*) outside Frøya, Norway, aiming for the production of biofuels and feed (Seaweed Energy Solutions AS, 2018). ALGEA AS and FMC Biopolymer has developed the cultivation lines for *A. nodosum* and *L. hyperborean*, respectively, for the production of seaweed meal and alginate (Meland & Rebours, 2012).

1.2.2. Lignocellulosic biomass

Another important bioresource in Norway is lignocellulosic biomass in the form of wood, which is mainly composed of cellulose, hemicellulose and. Cellulose is a linear polysaccharide composed of β-(1-4)-glucose residues. Hemicellulose is heteropolymers like xylan, arabinoxylan, xyloglucan, glucuronoxylan and glucomannan. The dominant hemicellulose in softwoods is glucomannan. Lignin is a complex aromatic polymer that is particularly important in the formation of rigid cell walls. Lignocellulosic dry biomass contains about 50-70 % of sugars in the form of holocellulose (cellulose and hemicellulose) making it an ideal feedstock for sugar-platform biorefinery. The chemical composition of woody lignocellulosic biomass does not vary significantly over the season. However, while this biomass is rich in sugars, it lacks other nutrients that are important in a fermentation medium (N, P, minerals).

1.3. Enzymatic saccharification of biomass

The main aim of the "sugar-platform" type of biorefinery is to efficiently convert sugars to a range of products, typically via fermentation processes. Microorganisms could be used as a biocatalyst to make specific fermentation products like ethanol or acetate, or the microbial biomass itself could be the product (Farzad et al., 2017; Kamm & Kamm, 2004).

Pretreatment of biomass may be needed prior to enzymatic hydrolysis of polysaccharides to fermentable sugars, in particular for recalcitrant lignocellulosic biomass. It is a technique that opens the structure of the cell wall of biomass to make the polysaccharides more accessible to the enzymes. Pretreatments such as chemical (Adams et al., 2009), hydrothermal (Vivekanand et al., 2012), ultrasonic and alkaline treatment (Park et al., 2009) has been applied to brown seaweed. However, such pretreatment may not be required for the brown seaweed since it lacks lignin in the cell wall and has low cellulose content. Simple size reduction is alone an effective pretreatment prior to biochemical processing (Manns et al., 2016). Compared to acid hydrolysis, enzymatic hydrolysis offers advantages like high yields and minimal by-products formation. However, one major hurdle for application of enzymes is their relative high production cost (Chibata et al., 2013).

Typical pretreatments for lignocellulosic biomass are milling, steam explosion, chemical treatement (acid, alkali, ionic, organosolve) and wet oxidation (Alvira et al., 2010). Recently, the Norwegian biorefinery company Borregaard developed a novel pretretament technology that included a sulphite cooking step utilizing calcium and sodium as a counter ions, which solubilized lignin into water by sulfonation and removes most of the hemicellulose that is washed out and remains solid fraction that majorly consisted of cellulose pulp (Chylenski et al., 2017; Rødsrud et al., 2012).

1.3.1. Cellulases

Cellulases are enzymes produced by fungi, bacteria, and protozoans that cleave β-(1-4)-glycosidic bonds through hydrolysis. Their major catalytic reaction mode is classified into three groups: endo-β-(1-4)-glucanases (EnGs) [EC 3.2.1.4], exo-β-(1-4)-glucanases/cellobiohydrolases (ExGs/CBHs) [EC 3.2.1.91], and cellobiases/ β-glucosidases

(BGs) [EC 3.2.1.21]. EnGs cleave internal bonds in the cellulose chain, ExGs/CBHs hydrolyse cellobiose units from either the reducing or non-reducing ends. BGs hydrolyze short soluble cello-oligosaccharides and cellobiose into glucose. In addition there are some auxiliary enzymes, so called lytic polysaccharide monoxygenase (LPMOs/GH61), which cleaves the glycosidic bond in the presence of molecular oxygen, external electron donors and divalent metal ions (Figure 9) (Vaaje-Kolstad et al., 2010). Companies like Novozymes (Cellic® CTec2 and Cellic® CTec3), and Dupont (previously Genencor International Inc; Accellerase®) have recently made significant progress toward minimizing enzyme cost by streamlining enzyme production and formulation process (Lamers et al., 2016).

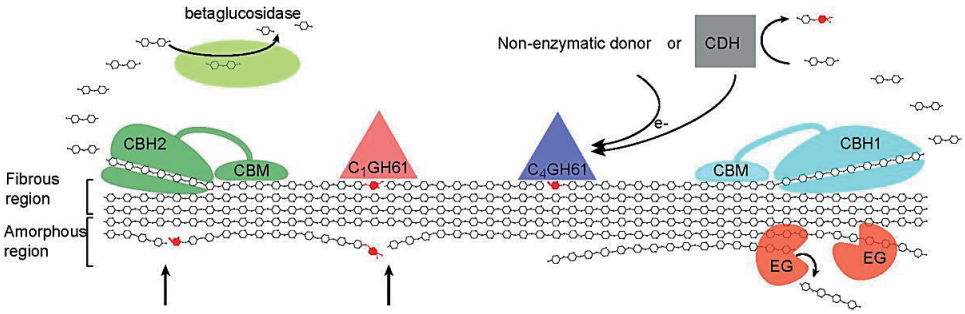


Fig. 9: Model for enzymatic saccharification of cellulose by hydrolytic (cellulases) and oxidative enzymes (here GH61). Figure was taken from (Horn et al., 2012), copyright © (2012) BioMed Central Ltd.

In brown seaweed, laminarin can easily be degraded to glucose by glucanase enzymes such as Laminarinases [EC# 3.2.1.6] and β-glucosidases. Laminarinase is an endo-1,3(4)-β-glucanase that catalyzes the endohydrolysis of 1,3 or 1,4-linkages in β-D-glucans. 1,6- β-glucanases are enzymes that can break down β-1,6-glucan linkages; while β-glucosidases hydrolyse cellobiose to glucose (Adams et al., 2008; Huesemann et al., 2012). Such activities are found in commercial enzyme preparations such as CellicCtec2 (Manns et al., 2014).

1.3.2. Alginate lyase

Alginate lyases are part of the polysaccharide lyase [EC 4.2.2.-] group. These enzymes have been isolated from various sources such as marine algae, marine invertebrates, and a wide range of marine and terrestrial microorganism (Zhu & Yin, 2015). Generally, they can be divided into three main groups: a) G block-specific lyases (polyG lyases) [EC 4.2.2.11], b) M block-specific lyases (polyM lyases) [EC 4.2.2.3], and c) GM/MG block-specific lyases (polyGM/MG lyases) (Kim et al., 2011). In terms of mode of action, alginate lyases can be grouped into endolytic and exolytic enzymes. The alginate lyases assigned to PL-5, PL-7 and PL-18 are endolytic bacterial lyases cleaving alginate in the middle of the chain. The alginate lyases assigned to PL-15 and PL-17 families are exolytic lyases attacking alginate chain ends (Table 2) (Zhu & Yin, 2015).

Table 2: Alginate lyases from different lyase families and their specificities

Microorganism	**Family/Type**	**Substrate specificity**	**Reference**
Pseudoalteromonas sp. SM0524	PL18/Endo	MM, GG, MG	(Li et al., 2015)
Pseudoalteromonas elyakovii	PL18/Endo	MM, GG, MG	(Ma et al., 2008)
Microbulbifer sp. 6532A	PL7/Endo	MM, GG, MG	(Swift et al., 2014)
Sphingomonas sp. A1	PL7/Endo	GG, MG > MM	(Yoon et al., 2000)
Sphingomonas sp. A1	PL7/Endo	MM, GG, MG	(Miyake et al., 2004)
Sphingomonas sp. A1	PL5/Endo	MM, GG	(Zhu & Yin, 2015)
Pseudomonas aeruginosa PAO1	PL7/Endo	MM, GG, MG	(Zhu & Yin, 2015)
Agrobacterium tumefaciens C58	PL15/Exo	MM, GG, MG	(Ochiai et al., 2006)
SCB49 unidentified bacterium	PL7/Exo*	Non- characterized	Unpublished*
Lewinella persica	PL7/Exo*	Non-characterized	Unpublished*
Saccharophagus degradans 2-40	PL17/Exo	MM, GG, MG	(Zhu & Yin, 2015)

Alginate lyases degrade alginate through a β-elimination mechanism, cleaving the glycosidic bond between uronic acids yielding a double bond between the C4 and C5 carbons of the sugar at the new non-reducing end. Endolytic alginate lyases degrade alginate polymers and release unsaturated oligosaccharides. Exolytic alginate lyases (oligoalginate lyases) cleave oligomers to monomers (unsaturated uronate or 4-deoxy-L-erythro-hex-4-

enepyranosyluronate or DEHU) (Figure 10) (Kim et al., 2011; Zhu & Yin, 2015). It has been shown that a combination of a cellulase cocktail and alginate lyases can efficiently degrade seaweed (Manns et al., 2016). It has also been shown that engineered microbes can produce bioethanol from both mannitol, laminarin and alginate (Enquist-Newman et al., 2014; Wargacki et al., 2012).

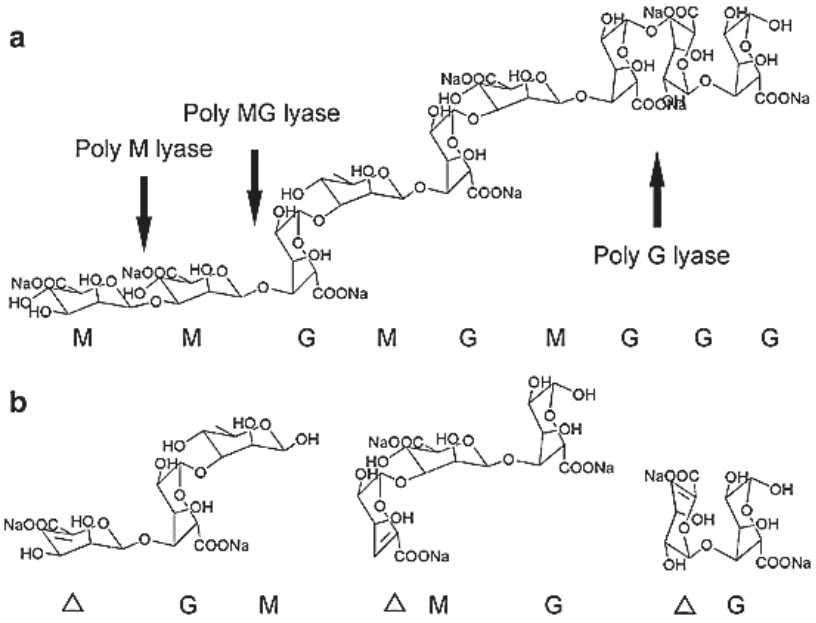


Fig. 10: Alginate degradation by endolytic and exolytic alginate lyases (β-elimination reactions) with different substrate specificities (a) and their degradation products (b). The three kinds of blocks (M, G, GM/MG) in alginate are all cleaved to produce a 4-deoxy-L-erythro-hex-4-enepyranosyluronate moiety (△). Figure was taken from (Zhu et al., 2015), copyright © (2015) Taylor & Francis Group, LLC.

1.4. Microbial protein

Microbial protein or single cell protein (SCP) are terms used when whole cell microbial biomass is used as a protein source. SCP may consist of dried cells of microorganisms such as algae, yeast, fungi or bacteria. It typically has a high protein content (60-82 % of dry matter) but also contains carbohydrates, fats, vitamins, and nucleic acids (DNA and RNA) (Panda et

al., 2018). The building blocks of proteins are made up of amino acids chains. The content of lysine and methionine in SCP are usually higher than that in conventional plants and animal based food, making it a unique feed ingredients. (Anupama & Ravindra, 2000). Cell walls of yeast are composed of 26-32 % of the cell dry matter and contain varying proportion of β-glucan, mannan-oligosaccharide, chitin and nucleic acids (Nguyen et al., 1998).

1.4.1. Nutrients requirement

For the production of SCP, the composition of the fermentation medium is crucial for the microbial growth, metabolism and the quality of the final product. The cost of the fermentation medium may be more than 50 % of the overall cost of a fermentation process (Walker & Stewart, 2016). The main nutritional requirements for the cultivation of yeast are carbon (i.e., sugars), nitrogen (amino acids, small peptides and ammonium salts), oxygen, sulphur, phosphorus, potassium, and magnesium (Kampen, 2014). Carbon is the vital element of the organic cell material that provide the source of energy to the yeasts. Certain other macro elements especially nitrogen, phosphorous and potassium are also required for an anabolic role in the biosynthesis of structural and functional protein and nucleic acid (Kampen, 2014). Oxygen and hydrogen are essential constituents of cellular water and for organic cell materials (Kampen, 2014). Others trace elements include calcium, copper, iron, manganese, and zinc are also needed that acts as cofactor for enzymes and constituent of vitamins.

1.4.2. Production of microbial protein

Microbial protein (SCP) production from all types of biomass proceeds through three steps: a) pretreatment, b) enzymatic saccharification and c) fermentation. After fermentation, SCP are harvested and subjected to downstream processing steps like washing, purification and drying (Anupama & Ravindra, 2000). The common carbon sources (substrates) used industrially for the cultivation of yeast are molasses, cellulosic wastes, sulphite liquor, whey, methanol, brewery waste, fruits and vegetable wastes (Anupama & Ravindra, 2000; Panda et al., 2018). For the SCP production, aerobic fermentation is mostly preferable, where cells metabolize sugars via fermentation in the presence of oxygen and produce biomass instead of ethanol or acetate. Optimum SCP production is dependent upon the culture conditions,

substrate used, media compositions, type of fermentation (batch, fed batch or continuous) and strain improvement (wild or genetic modified strain). In the commercial production of SCP, a limited number of organisms have been used with their product names such as Mycoprotein (*Fusarium*), Toprina (*Candida*), Pekilo (*Paecilomyces variotii*), and Quorn (*Fusarium*) (Lundebye et al., 2006).

1.5. Microbial protein in fish feed

Digestibility of microbial ingredients, thus the availability of nutrients and energy for maintenance and growth, can be affected by several factors including the yeast species, fermentation media, downstream processing and diet formulation (Øverland & Skrede, 2017). Several digestibility studies have been carried out with different yeast strains in diets for various fish species. Results from studies with up to 380 g kg^{-1} of *S. cerevisiae* yeast, replacing fishmeal in diets for pacu (*Piaractus mesopotamicus*), showed no significant difference in protein digestibility, however, the lipid digestibility was significantly higher (Ozório et al., 2010). In most of the studies, the apparent protein and amino acid digestibility of intact and dried *S. cerevisiae* used in diets for salmonids are rather poor (Cheng et al., 2004; Langeland et al., 2016; Rumsey et al., 1991). Another reason for the low digestibility in some fish experiments could be due to differences in enzyme activities (e.g., carbohydrases, lipase, trypsin and chymotrypsin) between fish species (Langeland et al., 2014). Live intact yeast has a thick and rigid cell wall that may limit enzymatic access to cellular contents, which result in lower utilisation of the dietary yeast protein (Murray & Marchant, 1986; Tukmechi & Bandboni, 2014; Yamada & Sgarbieri, 2005). A study with Atlantic salmon showed that partial replacement of high-quality fishmeal (substituting 40% of the crude protein from FM) with spray-dried and inactivated *S. cerevisiae* lowered the crude protein digestibility and specific growth rate, while inactivated *Candida utilis* and *Kluyveromyces marxianus* did not affect the protein digestibility or growth rate (Øverland et al., 2013). In an experiment with Arctic char, 40 % of the fish meal in extruded diets was replaced with intact *S. cerevisiae* without any negative effect on the growth performance (Vidakovic et al., 2016). In another study, increased digestibility of protein and amino acids were observed in the Arctic char fish, fed autolyzed yeast extract with intact cells of *S. cerevisiae* (Langeland et al., 2016). The post processing of yeast (spray drying, autolysis) and the feed preparation (extruder) are an

essential step that should be subjected to preserve nutrient values of ingredients and to promote high digestibility.

Microbial protein ingredients also provides macro and micro minerals to the fish. Mineral digestibility in fish is important for the skeletal formation, regulation of acid-base equilibrium and formation of biological compounds such as hormones and enzymes (Watanabe et al., 1997). Excessive use of inorganic minerals in the diets by adding premix could be avoided by providing organically bound minerals in feed ingredients such as yeast (Sugiura et al., 1998). The most important minerals for fish are iron, copper, manganese, selenium, zinc, chromium, and iodine and their requirements are usually less than 100 mg kg^{-1} dry diet (Watanabe et al., 1997). However, high level of these minerals could reduce growth rate and feed efficiency, and could also be toxic to the fish. There are several other heavy metals such as arsenic, lead, cadmium, mercury and chromium that are well known for their toxicological effects in humans. The mineral requirements of Atlantic salmon has been fairly well investigated and some of the achievements have been highlighted in several publications (Cobelo-García et al., 2017; Nøstbakken et al., 2015; Rombough & Garside, 1982; Svecevicius et al., 2014; Watanabe et al., 1997). In general, organically bound minerals in yeast could be considered as a potential source of minerals with a high bioavailability in fish feeds.

2. Purpose of the work and outline of the thesis

The main objective of this thesis was to demonstrate that brown seaweed and spruce wood can be used to produce microbial protein ingredients for Atlantic salmon diets. To achieve this, the following secondary objectives were set: 1) Perform a detailed characterization of *S. latissima* biomass, 2) Develop efficient enzymatic saccharification of *S. latissima,* 3) Design a growth medium of blends of seaweed and spruce hydrolysates that support growth of yeast, 4) Upscale yeast production to produce sufficient amounts for fish feed trials, and 5) Use yeast as a protein source in fish feed and carry out a feeding experiment with salmon. Thus, this is a proof-of-concept study to investigate if local Norwegian biomass resources can be used to produce fish feed. The thesis is based on five research papers:

In Paper I, the variation in chemical composition of *S. latissima* cultivated at different depths and harvested at different time points is described. This study shows that cultivation depth and harvesting time affect chemical composition, which again will affect potential applications of the feedstock.

In Paper II, different blends of cellulases and an alginate lyase were used to enzymatically saccharify *S. latissima*. Parameters such as enzyme blend ratios, enzyme dose and solid loading were investigated to optimize the extraction of sugars from *S. latissima.*

In Paper III, native and pretreated brown seaweed from Chile and Norway were enzymatically hydrolysed by blends of cellulases and alginate lyases. The focus of this paper was to evaluate and compare five novel recombinant alginate lyases in respect to their effect on sugar release from seaweed. Growth of yeast in seaweed hydrolysates for the production of microbial protein was also investigated.

In Paper IV, a proof-of-concept study for the production of microbial protein (yeast) from brown seaweed and spruce hydrolysates are presented. The production of microbial protein was carried out in fermenters of different size, and the yeast produced in a 27 L fermenter was spray dried. The amino acid composition and mineral content of the final yeast were also studied. A preliminary assessment of the nutrient digestibility of the microbial protein was made in a digestibility trial with Atlantic salmon.

In Paper V, three different yeast biomasses were replaced 30 % of fishmeal-based diet, nutrient and mineral digestibility was determined in Atlantic salmon. The yeast (*C.utilis*) was cultivated using 1) a blend of woody hydrolysate and molasses, 2) spent sulphite liquor, and 3) a blend of brown seaweed and woody hydrolysate.

3. Main results and discussion

3.1. Biomass growth and chemical composition of cultivated *S. latissima* (Paper I)

Mass cultivation of *S. latissima* is emerging in Norway, due to advances in industrial-scale cultivation techniques. To enable efficient processing of *S. latissima* to biofuels, food, feed ingredients, and other value-added products, it is important to obtain detailed information about its growth and chemical composition. It is well-known that the growth rate and the chemical composition of brown seaweed varies according to the season and a range of environmental factors such as salinity, pH, sunlight, mineral availability, and water current (Handå et al., 2013; Marinho et al., 2015; Schiener et al., 2015). However, very few studies have been carried out to investigate the effect of both different cultivation depths and different harvesting times. In Paper I, the biomass growth of cultivated *S. latissima* deployed in February at cultivation depths of 3 and 8 meters (m) were monitored from May to August (Figure S1 in **Paper I**). It was found that *S. latissima* grown at 3 m had a higher biomass production than at 8 m depth at all sampling dates. An important factor that affected the growth was the presence of epibionts such as bryozoans and larvae of barnacles. This results in brittle seaweed prone to defoliation during mechanical events such as storms and strong currents. Other factors affecting growth are the light intensity and availability of nutrients (Cronin & Hay, 1996). These factors not only affect the growth but also the chemical composition of the *S. latissima*, as demonstrated by a detailed chemical analysis of seaweed samples.

The highest content of the fermentable carbohydrates glucose and mannitol was found for the June samples at 3 m depth. The August samples had a low sugar content, probably due to the formation of epibionts which reduce the rate of photosynthesis in the seaweed (Tønder, 2014). The total glucose and mannitol showed large variations over the cultivation period, from 37 to 230 g kg^{-1} DM of *S. latissima*. *S. latissima* is also possess significant amounts of amino acids and minerals. The content of amino acids in cultivated *S. latissima* varied both with cultivation depth and time. It was noticed that the content of amino acids was higher at the deeper cultivation, with a maximum in the 8 m August sample. This might be due to the low

light intensity during the deep cultivation, which is known to result in higher protein content (Cronin & Hay, 1996). Another factor is the presence of epibionts in August which may have contributed to the high protein content. The dominant amino acids present in the cultivated *S. latissima* were aspartic acid, glutamic acid, and alanine, which are in the agreement with literature values (Manns et al., 2014; Marinho et al., 2015). The amino acid profile is generally considered an indication of the nutritional value of protein in seaweed, however, the availability is dependent on protein digestibility (Dawczynski et al., 2007). *S. latissima* has a relatively high ash content, where the most abundant cations are sodium, potassium, calcium and magnesium, associated with the anions, chlorine, bromine, iodine, phosphorous and sulphur.

The total ash content was in the range of 253 – 412 g kg^{-1} DM *S. latissima*. The highest accumulation of total minerals was observed in the 8 m depth August samples, and may be due to several factors, including season, site location, salinity of seawater, sunlight, epibionts, and age of the seaweed (Chan & Matanjun, 2017; Sánchez-Rodríguez et al., 2001). Apart from these factors, the cultivation depth can be considered as a major factor as shown in this study. These high values of ash and mineral content of *S. latissima* are a factor of concern in relation to feed and food industries. For instance, the concentration of iodine in seafood are in the range of 0.03 – 3.5 mg kg^{-1} wet weight, whereas, the content of iodine in *S. latissima* are 1.6 – 4.2 g kg^{-1} DM that is 300-13,000 fold higher than seafood (Duinker et al., 2016). In terms of seaweed used as a food, the main concerning elements that is overtaking the daily nutrition recommendations values are iodine, zinc, selenium, iron, boron, silicon, arsenic, and cadmium (Institute of Medicine Panel on Micronutrients, 2001; Nordic Nutrition Recommendation, 2012). As a result, with a small portion of *S. latissima* intake daily will cover the daily nutritional requirements, whereas, high intake could cause toxicity. However, there are some solution for this problem, for instance, by boiling the seaweed for 5 min, the iodine content can be reduced to approximately one third (Duinker et al., 2016; Lüning & Mortensen, 2015).

Overall, **Paper I** demonstrated the presence of fermentable sugars, nitrogen, phosphorus and macro and trace minerals in *S. latissima.* This makes the seaweed an interesting component of microbial growth media. However, the annual changes in chemical composition is a challenge, in particular the huge variations in fermentable sugars. For a growth medium this may be regulated by blending in sugars from another source such as lignocellulosic biomass, which is the topic of **Paper IV**. Furthermore, the seaweed has to be efficiently saccharified

and liquefied to be part of the microbial growth medium. This is the topic of **Paper II** and **III**.

3.2. Optimization of enzymatic saccharification of *S. latissima* (Paper II)

Brown seaweed is a source of fermentable sugars such as laminarin and mannitol (Adams et al., 2009; Horn et al., 2000). Since, seaweed lack lignin it is mechanically less robust compared to lignocellulosic biomass, which makes it easier to process to fermentable sugars. Enzymatic saccharification of brown seaweed has been investigated using enzymes such as laminarinase, alginate lyases, and commercial glucanases (Adams et al., 2009; Hou et al., 2015; Manns et al., 2014). In these studies, the experiments were performed using dried seaweed at relatively low substrate concentrations and a thorough optimization of enzymatic saccharification was not carried out. In **Paper II**, the effect of drying temperature on enzymatic saccharification was tested prior to the optimization part because drying of biomass may affect the enzymatic accessibility. Samples dried at different temperatures were hydrolysed at 5 % (w/v) of substrate loading with an enzyme blend of alginate lyase and commercial cellulases (CellicCTec2) in a ratio of 1:1 on a protein basis, which was similar to the blend ratio used in literature (Manns et al., 2014). Optimal pH and temperature conditions for the alginate lyase (*Flavobacterium multivolume*) were 6.3 and 37 °C, respectively, whileCellicCTec2 has a relatively broad pH optimum around 5 and an optimum temperature at 50 °C. Therefore, the hydrolysis were carried out in two stage, where we used 37 °C for 3 h and ramped up the temperature to 50 °C for 17 h. The treatment was carried out in 100 mM citric acid-sodium buffer solution (pH of 6.3) in an Eppendorf thermomixer. We observed that 30 °C oven dried *S. latissima* resulted in the highest final concentration of glucose (Fig.1 **Paper II**). It was seen that the glucose yield gradually decreased with increasing drying temperatures, but not as severely as seen for drying of pretreated lignocellulosic materials. Notably, the drying temperature had a very limited effect on the release of mannitol. Seaweed samples used for the optimization of the enzymatic hydrolysis were dried at 30 °C.

The first variable to be investigated was enzyme loading (1 – 10 mg protein g^{-1} DM) of a CellicCTec2 (50 %) and alginate lyase (50 %) blend (Fig. 2 **Paper II**). It was found that 7 mg protein g^{-1} DM was enough to achieve maximum glucose release and this enzyme dose was used in all further experiments. To determine the optimal enzyme ratio for saccharification of

S. latissima a range of experiments were carried out where the amount of alginate lyases was varied from 0 % to 100 %, while keeping the total enzyme dose constant (7 mg g^{-1} DM). A small inclusion of 10 % alginate lyase with 90 % CellicCTec2 gave the highest glucose release from the seaweed (Fig. 3 **Paper II**). A further set of experiments were carried out where the dry matter concentration was increased from 5 to 25 % DM. It was observed that increased the dry matter concentrations reduced the sugar yield (Fig 5 **Paper II**), which is similar to observations done for saccharification of lignocellulosic biomass (Kristensen et al., 2009). Importantly, it was observed that the glucose yield with alginate lyase present always was higher than the reactions with only CellicCTec2, and particularly at high DM concentrations. At 25 % dry matter loading a maximum combined glucose and mannitol concentration of 74 g L^{-1} was achieved. Overall, the results described in **Paper II** show that *S. latissima* can be efficiently enzymatically saccharified by a blend of cellulases and an alginate lyase.

3.3. Enzymatic saccharification of *M. pyrifera* and *S. latissima* (Paper III)

The main objective of this paper was to compare the chemical composition of two different seaweed species, both native and pretreated, and evaluate the saccharification performance of five different recombinant alginate lyases. Moreover, initial evaluation of growth of the yeast *C. utilis* was carried out in the seaweed hydrolysates. The two seaweed species *Macrocystis pyrifera* and *Saccharina latissima* were used in this study. They are phylogenetically closely related and belong to the same Laminariaceae family. *M. pyrifera* was harvested at the coast of Puerto Montt in Chile. These brown seaweeds were pretreated with sulphuric acid and at 120 °C for 1 h. After the incubation, the biomass was washed several times with milli-Q water and two times with McIlvain buffer at pH 7.5, and then centrifuged and dried at 37 °C for 3 days. The native *S. latissima* biomass used in this paper contained 37 % glucose and 25 % mannitol (percentage in carbohydrates), whereas, the composition of native *M. pyrifera* was reciprocal to *S. latissima* with 15 % glucose and 40 % mannitol. It was noticed that the pretreatment had changed the composition of both algae significantly. The pretreatment reduced the ash content in both algae, particularly in *M. pyrifera*, where the ash content became 23 times lower. For enzymatic saccharification, dry algae (both native and pretreated) were incubated in McIlvain buffer with different recombinant alginate lyases (G, H, I, J, K, L), oligoalginate lyases (Atu, Q, and O) and cellulases (CellicCTec2). A description of each

recombinant alginate lyase and oligoalginate lyase are shown in supplementary Table S2 of **Paper III**). In the enzymatic saccharification experiments, a blending ratio of 50:50 % [(25 % alginate lyase + 25 % oligoalginate lyase) and glucanases] was used. The saccharification with recombinant alginate lyases and oligoalginate lyases were carried out at 25 °C for 12 h and then the cellulases were added to the reactions and incubated for 4 h at 50 °C. The highest glucose release from *S. latissima* (native) was 209 g kg^{-1} of DM (94.3 % theoretical yield), and obtained using the recombinant alginate lyase H (*Pseudoalteromonas elyakovvii*), oligoalginate lyases and CellicCTec2. The sugar recovery from alginate lyase was similar. For the pretreated biomass (*S. latissima*) the highest glucose release was observed when using alginate lyase (from Sigma) along with CellicCTec2, yielding 232 g glucose kg^{-1} of DM (94.6 % theoretical yield). In the case of the pretreated samples, all incubations showed similar high sugar yields, even the reaction with CellicCTec2 alone (Fig. 3 **Paper III**). Thus, alginate lyases are helpful for saccharification of native seaweed, while the effect of the enzymes are limited in pretreated seaweed.

For the release of glucose from native *M. pyrifera* inclusion of alginate lyases was positive, but there were no significant differences between the tested lyases. For pretreated *M. pyrifera*, inclusion of alginate lyases did not increase the glucose yield. Finally, a growth test showed that seaweed hydrolysates could support growth of *C. utilis*.

Overall, **Paper III** showed that the use of recombinant alginate lyases and oligoalginate lyases in combination with cellulases increased the glucose release from untreated seaweed. However, for saccharification of pretreated algae only cellulases were needed to achieve high glucose release. Interestingly, this paper also showed that seaweed hydrolysates could be used as growth medium for *C. utilis*.

3.4. Combined seaweed and spruce hydrolysates for production of microbial protein (Paper IV)

The main aim of **Paper IV** was to study the possibility to produce the protein-rich yeast *C. utilis* on a medium consisting of enzymatically hydrolysed sulphite-pulped spruce wood, mainly providing glucose, and enzymatically hydrolysed *S. latissima*, providing both sugars and other nutrients. Moreover, a secondary aim was to evaluate the yeast quality in terms of amino acids and minerals and to carry out a preliminary assessment of nutrient digestibility of the yeast in Atlantic salmon. The fermentations were carried out with preliminary testing in microtiter plates, then shake flask cultures, 1 L and 27 L fermenters. To produce enough fermentation media, relatively large batches of enzymatic hydrolysis of *S. latissima* was performed at 15 % (w/v) dry matter and without using any citric acid-sodium phosphate buffer (only water). The main reason for not using a buffer was to not introduce an extra carbon source in the fermentation. We carried out a preliminary cultivation tests with *C. utilis* using the seaweed hydrolysate (SEH) and a rich yeast extract peptone dextrose (YPD) in microtiter plates, resulting in no significant effect by even the highest inclusion level of SEH of 30 % (v/v). In **Paper III** we observed that *C. utilis* can be cultivated in the SEH medium alone, however, higher cell density can only be achieved by providing an extra carbon source. To provide extra carbon source in the fermentation, spruce enzymatic hydrolysate (SPH) was added along with SEH. SPH was retrieved from the process optimization studies in the BALI pilot plant at Borregaard, Norway. We have observed some variation in the concentration of sugars in blended hydrolysate that could be due to the seasonal variation in the chemical composition of seaweed (**Paper I**) and differences in the SPH batches. The medium consisted of 90 % (v/v) SEH and 10 % (v/v) SPH, giving initial glucose concentration of 32 to 43 g L^{-1} for all fermentation processes. In shake flask culture, it was observed that after a short lag phase, the cells grew rapidly until complete depletion of both glucose and ammonium. Notably, during the fermentation, pH dropped from 5.5 to 3.8, which could be attributed to the production of acetate by the yeast (Christen et al., 1999; Pampulha & Loureiro-Dias, 1989)

Due to the lack of pH control, aeration (pO_2) and stirring in the shake flask, *C. utilis* was then cultivated in 2.5 L fermenters. To increase the concentration of yeast, the fermentation was carried out as a fed-batch. To avoid possible nitrogen limitation, extra nitrogen (ammonium sulphate) was added as per yeast elemental composition ($C_1H_{1.64}N_{0.16}O_{0.52}P_{0.01}S_{0.005}$). It was

observed (Fig. 2 **Paper IV**) that the maximum cell density was 18 g L^{-1} just after feeding (sugar and ammonium sulphate was added between 20 and 23 h) with a protein content of the cell (40 %).

To produce reasonable amount of microbial protein for downstream processing and fish feed trials, the fermentation was scaled up to 27 L. It was observed that the larger scale fermentation gave better results than the 1 L, with a maximum cell mass concentration of 20 g L^{-1} At 20 h, prior to feeding, the protein content reached to 53 %, and the yields were 0.26 g of cells and 0.14 g of protein per g of glucose. These yields were similar to those reported in the literature for the same Candida sp with different type of fermentation media (de Arruda et al., 2011; Gao et al., 2012; Lemmel et al., 1979; Nigam, 2000). The maximum biomass yield for *C. utilis* can be up to 0.36 g g^{-1} glucose in rich media, indicating that further process optimization are required for our type of media (Lee & Kyun Kim, 2001). The process optimization can be done by adaptation of the yeast to the SPH and SEH medium, adjusting the feeding regimes, and culturing conditions. It was observed that the heat inactivation of the yeast reduced both the dry cell mass concentration and the protein content, which could be due to some degree of autolysis (Tanguler & Erten, 2008). The protein content after heat inactivation was reduced from 42 % (w/w) to 36 % (w/w). Prior to spray drying, the yeast cells were centrifuged and washed several times. The resulting spray dried yeast cells that was used for further analyses and in feeding experiments with salmon had a protein content of 33.3 %.

The presence of macro and trace minerals in the native *S. latissima,* SEH, SPH and spray dried *C. utilis* are shown in Table 4 in **Paper IV**. The mineral composition results revealed that most of the nutrients for the fermentation were provided by SEH. It was noticed that the some elements that are scarce in SEH, were present in SPH, meaning that the combination of two hydrolysates to some extent can compensate each other (e.g., calcium and nickel). Amino acid content of the spray dried yeast, fishmeal and native *S. latissima* are presented in Table 5 in **Paper IV**. The relative profile of the amino acids of *C. utlis* was quite similar to the fishmeal, except a lower level of methionine.

Finally, a fishmeal-based reference diet was partially replaced with 30% spray dried *C. utilis* and fed to pre-smolt Atlantic salmon over a period of 48 days. Apparent digestibility coefficients (ADC) for dry matter, ash, protein and starch in the diets are shown in Table 6 in

Paper IV. ADCs for the yeast was sub-optimal which could be due to the rigid cell wall that increases the non-digestible fraction by restricting access to intracellular proteins.

Overall, **Paper IV** demonstrated the possibility of cultivating *C. utilis* on a medium composed of local, simple, and sustainable feedstocks. The amino acid and mineral profile of the microbial biomass is signifying that sufficient amounts of nutrients are present. Moreover, the salmon feeding experiment showed that the yeast protein can partly replace a fishmeal diet without harmful effects, but with sub-optimal ADCs.

3.5. Microbial protein ingredients in diets for Atlantic salmon (Paper V)

The objective of **Paper V** was to investigate the nutrient digestibility of *C. utilis* cultivated from different carbon and nutrient sources and used in diets for pre-smolt Atlantic salmon (*Salmo salar*). *C. utilis* was cultivated using three different feedstocks with different upstream and downstream processing. *C. utilis* from Estonia (CUE) was cultivated by using spruce enzymatic hydrolysate (SPH) and beet molasses sugars with fed batch fermentation. After fermentation, the yeast cells were heat inactivated, washed and dried by drum dryer. *C. utilis* from USA (CUA) was cultivated by using spent sulphite liquor retrieved from pulp and paper mill in a continuous fermentation process. The yeast cells were heat inactivated, washed and dried by spray dryer. The final yeast (CUN) was produced by using *S. latissima* enzymatic hydrolysate (SEH) and spruce enzymatic hydrolysate (SPH) (**Paper IV**) in a fed batch fermentation process. The yeast cells were heat inactivated, washed couple of times and spray dried. One of the main challenge of using these cheaper source of carbon and nutrient in the cultivation of yeast is the risk of incorporation of harmful minerals or polyphenols (e.g., phlorotannis) from media that could reduce the growth performance and nutrient digestibility. Therefore, another aim of our study was to evaluate the impact of the different yeast biomasses on faecal mineral excretion. The control diet was based on high-quality fishmeal (FM). Three experimental diets (one for each type of yeast: CUE30, CUA30, CUN30) were formulated by replacing 30 % of the control diet with yeast meals (*C. utilis*) derived from different feedstocks. Yttrium oxide was added in the diet as an internal marker to evaluate nutrient digestibility. Proximate analysis and minerals content of the control and experimental diets are shown in Table 3 (Table 3 **Paper V**).

Growth performance (final weight, FCR, and SGR) of pre-smolt Atlantic salmon are shown in Table 4 (Table 4 **Paper V**). At the end of experiment (48 days feeding), fish biomass weight increased 1.45 – 1.62 times in all group. It was observed that fish fed the CUN30 diet, had the lowest final weight, however, the CUN biomass had a lower protein level. The apparent digestibility coefficient (ADC) of dry matter, ash, fat, protein and starch of the control and experimental diets are shown in Table 5 (Table 5 **Paper V**). The highest ADC of dry matter was observed in the control diet (81 %), whereas the experimental diets were in the range of 61 – 69 %. The ADC of ash in the CUN30 diet was 1.7 %, which was considerable lower than the three other diets. One factor that can explain the lower digestibility of yeast, is the structure of the yeast cell wall. Yeast cells wall are composed of β-1,3 glucan, which has generally high degree of polymerization (DP) upto 1500. This thick and rigid cell wall may limit enzymes access to the cellular contents (Murray & Marchant, 1986; Tukmechi & Bandboni, 2014; Yamada & Sgarbieri, 2005). It has also been shown that soluble non-starch polysaccharides (NSP) such as alginate and guar gum in plant diets can reduce nutrient digestibilities (Kraugerud et al., 2007). Yeast grown on seaweed hydrolysate and the final CUN30 diet was darker in colour than the other diets. This might be due to presence of phenolic compounds and/or pigments coming from the seaweed, which could inhibit the digestibility of nutrients. Difference in digestibility among the yeast ingredients could also be due to the different upstream and downstream processes used during the production of yeast biomasses. For instance, the CUA ingredient was drum dried, while CUE and CUN was spray dried. Thus, it is important to continue to optimize and improve the downstream processing of yeast. Interesting strategies, could be cell disruption by cell homogenization and fractionation (Baldwin & Robinson, 1994; Bzducha-Wróbel et al., 2013; Rumsey et al., 1991).

The different carbon and nutrient sources that were used for the cultivation of *C. utilis* resulted in some differences in the mineral profile of the diets. The main interest of the elements in this study was phosphorus, potassium, zinc, cadmium, arsenic and iodine.

Elements such as arsenic and cadmium could be toxic for fish and consumer. The maximum permissible level of cadmium and arsenic in diets for fish are 1 and 10 mg kg^{-1} diets, respectively (Commission Regulation (EU), 2013). The content of cadmium in all diets were lower than this, except for the CUN30 diet. An excess dose of cadmium in diets for salmonids could induce hypocalcemia resulting in mortality (Roch & Maly, 1979). However, the absorption of cadmium in all diets were found to be low (percentage excretion > 92.5). The concentration of arsenic in all diets were lower than the permissible level.

Fish is generally considered as a rich source of iodine. Dietary recommendation of iodine for salmonids are reported to be 4 mg kg^{-1} of diet (Watanabe et al., 1997). The diet with yeast grown on the seaweed hydrolysate had an iodine content of 88 mg kg^{-1} of diet. Thus, the high content of iodine in the seaweed could be a limitation for the use of seaweed as a feedstock for the fermentation of yeast. This problem could be reduced by pre-processing of the brown seaweed, such as short washing in boiling water, which can reduce the iodine content with up to 70 % (Duinker et al., 2016).

To conclude, **Paper V** showed that the *C. utilis* cultivated on spend sulphite liquor, woody hydrolysate and molasses could replace 30 % of whole fishmeal diet in the feed for Atlantic salmon, without compromising growth performance, however, there was a small reduction in nutrient digestibility. A detailed growth study of fish fed increasing levels of seaweed-derived yeast diets, where the accumulation of minerals in organs such as kidney, liver and muscles has been performed and will be reported elsewhere.

4. Concluding remarks and perspectives

In this thesis a proof-of-concept for production of microbial protein as a salmon feed ingredient from seaweed and spruce trees was successfully demonstrated. This was possible due to the rich nutrient content of seaweed, which in combination with sugars from spruce trees made a decent growth medium for yeast. Efficient saccharification and liquefaction of brown seaweed was shown by using a combination of cellulases and alginate lyases. Yeast grown on the seaweed-spruce hydrolysates medium was harvested, washed and dried and used as a partial replacement of a fishmeal-based diet in a nutrient digestibility experiments with Atlantic salmon. Based on the results from the present thesis, the following conclusions can be made:

From **Paper I**, it can be concluded that cultivated *S. latissima* differs in biomass production and chemical composition, depending on both the cultivation depth and harvesting time. The optimal time of harvest and cultivation depth has to be decided according to the desired application of feedstock. As a provider of nutrients for fermentation media, *S. latissima* is a rich source and may be harvested for most of the year for this purpose. However, the annual fluctuations in fermentable sugars are large and the media thus need to be balanced with nutrients from a different sources.

The results presented **in Paper II** demonstrate that *S. latissima* can be efficiently hydrolysed into fermentable sugars using a blend of cellulases and an alginate lyase. Particularly at high solid loadings, the inclusion of alginate lyases were shown to improve the saccharification of the seaweed. The highest total concentration of glucose and mannitol of 74 g L^{-1} was achieved at 25 % dry matter loading of *S. latissima*.

The study presented in **Paper III** shows that the chemical composition of brown seaweed from Chile (*M. pyrifera*) and Norway (*S. latissima*) differs; in particular the ratio of mannitol and glucose. For both seaweeds, recombinant alginate lyases and oligoalginate lyases in combination with cellulases gave higher sugar release than using cellulases alone. However, for the saccharification of pretreated seaweed only cellulases were needed to achieve high sugar release, indicating that the pretreatment partially hydrolysed the alginate. It was also demonstrated that seaweed hydrolysates alone could be used as a growth medium for *C. utilis*.

In **Paper IV**, a proof-of-concept study was performed, demonstrating that it is possible to produce *C. utilis* on a medium composed of brown seaweed and wood hydrolysates. A digestibility experiment with salmon showed that the yeast biomass could partly replace a fishmeal-based diet, but with sub-optimal nutrient digestibility. Thus, further optimization of both fermentation and downstream processes are needed to achieve higher yeast protein content and higher digestibility.

In **Paper V**, nutrient and mineral digestibility of *C. utilis* cultivated on different carbon and nutrient source in the diet of Atlantic salmon were investigated. *C. utilis* cultivated on spent sulphite liquor (CUA) or spruce hydrolysate and molasses (CUE) could replace 30 % of a fishmeal-based diet without compromising growth performance, however, there was a small reduction in protein digestibility. The *C. utilis* cultivated on enzymatic hydrolysates of seaweed and wood (CUN) resulted in both reduced nutrient digestibility and growth performance. However, this was not designed as growth experiment, thus a longer growth performance experiment with isonitrogenic and isoenergetic diets is warranted.

This work points out some new directions for future research. Seaweeds are an attractive source of nutrients, which until now has been highly underutilized for development of microbial protein ingredients for the food and feed industry. Seaweed hydrolysates in blend with wood sugars make it possible to produce protein from local Norwegian resources, providing a new opportunity to the aquaculture industry. Several questions are still not answered regarding how this process can be made efficient and commercialized. In the seaweed cultivation sector, the logistics related to harvesting, transport and storage need to be addressed. Moreover, it is a clear need to design a dedicated enzyme cocktail for seaweed liquefaction. The cellulase cocktail used in this study was designed to degrade lignocellulosic biomass, which is more difficult to hydrolyse than seaweed. Thus, a simpler enzyme cocktail, containing a limited number of cellulases and alginate lyases, for seaweed hydrolysis can be envisioned. The blend of seaweed and wood hydrolysates proved to be a good medium supporting yeast growth. However, the fermentation process needs to be optimized to produce higher protein content by adjusting medium composition, the feeding regime, and adaption of the yeast to the medium to avoid long lag phases. The use of inorganic nitrogen could be replaced by nitrogen derived from more sustainable resources such as meat and fish by-products from the food industry. Yeast strains with better growth characteristics and higher protein content could be identified through extensive screening studies. Also, the downstream processing of yeast after fermentation should be addressed to improve yeast digestibility. In

vitro screening of functional properties of different yeasts and yeast treatments should be applied prior to performing a growth performance experiments with fish.

Norway currently produce about 1.33 million tonnes of salmon each year, with the consumption of 1.52 million tonnes of feed. Replacing 10 % of this feed with yeast would require production of 0.15 million tonnes of yeast each year. By applying a yield of 0.26 g of yeast/g of glucose this would mean a need of 0.58 million tonnes of glucose or 0.53 million tonnes of cellulose. With 44 % cellulose in spruce this would translate to 1.21 million tonnes dry spruce, or 2.81 million m^3 wet spruce, about one third the total annual harvest of spruce in Norway. The total annual production of forest in Norway is around 25 million m^3. In our fermentations, we used around 5.8 kg dry seaweed per kg of yeast produced, meaning a need for 0.87 million tonnes dry seaweed or around 4.37 million tonnes wet seaweed. This far exceeds the annual harvest of wild seaweed in Norway which is around 150 000 ton wet weight, mainly used in the alginate industry. Thus, substituting 10 % of the fish feed in Norway with yeast would require consumption of one-third of the annual harvest of spruce, but availability of seaweed would clearly be the limiting factor. For large scale production of yeast, other nutrient resources such as residues from the meat and fish industry need to be considered."

Production of microbial protein could be made more economical in co-production with other products. This is very clear for the Borregaard - BALI process where lignin is first extracted from the wood, and used as a feedstock for a range of products, while the cellulose is hydrolysed and the sugars used for fermentation. A similar approach could be possible for the seaweed industry, which currently mainly utilize the alginate fraction of the seaweed. By developing new processes, alginate production could in principle be combined with production of microbial growth media components.

5. References

Adams, J., Gallagher, J. & Donnison, I. (2009). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *Journal of Applied Phycology*, 21 (5): 569-574.

Adams, J. M., Gallagher, J. A. & Donnison, I. S. (2008). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *Journal of Applied Phycology*, 21 (5): 569.

Adams, J. M. M., Ross, A. B., Anastasakis, K., Hodgson, E. M., Gallagher, J. A., Jones, J. M. & Donnison, I. S. (2011a). Seasonal variation in the chemical composition of the bioenergy feedstock *Laminaria digitata* for thermochemical conversion. *Bioresource Technology*, 102 (1): 226-234.

Adams, J. M. M., Toop, T. A., Donnison, I. S. & Gallagher, J. A. (2011b). Seasonal variation in *Laminaria digitata* and its impact on biochemical conversion routes to biofuels. *Bioresource Technology*, 102 (21): 9976-9984.

Alvira, P., Tomás-Pejó, E., Ballesteros, M. & Negro, M. J. (2010). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology*, 101 (13): 4851-4861.

Anupama & Ravindra, P. (2000). Value-added food: Single cell protein. *Biotechnology Advances*, 18 (6): 459-479.

Arasaki, S. & Arasaki, T. (1983). *Low Calorie, High Nutrition Vegetables from the Sea to Help You Look and Feel Better*: Japan Publications.

Baldwin, C. V. & Robinson, C. W. (1994). Enhanced disruption of *Candida utilis* using enzymatic pretreatment and high-pressure homogenization. *Biotechnology and Bioengineering*, 43 (1): 46-56.

Bekatorou, A., Psarianos, C. & Koutinas, A. A. (2006). Production of Food Grade Yeasts. *Food Technology and Biotechnology*, 44 (3): 407-415.

Betty Moss, u. (1952). Variations in chemical composition during the development of Himanthalia elongata (L.) S. F. Gray. *Journal of the Marine Biological Association of the United Kingdom*, 31 (1): 29-34.

Borregaard. (2018). *Produkter og Løsninger*. Tilgjengelig fra: https://www.borregaard.com/Products-Solutions [Accessed 20.02.2018].

Bzducha-Wróbel, A., Kieliszek, M. & Błażejak, S. (2013). Chemical composition of the cell wall of probiotic and brewer's yeast in response to cultivation medium with glycerol as a carbon source. *European Food Research and Technology*, 237 (4): 489-499.

Chan, P. T. & Matanjun, P. (2017). Chemical composition and physicochemical properties of tropical red seaweed, *Gracilaria changii*. *Food Chemistry*, 221: 302-310.

Charoensiddhi, S., Conlon, M. A., Franco, C. M. M. & Zhang, W. (2017). The development of seaweed-derived bioactive compounds for use as prebiotics and nutraceuticals using enzyme technologies. *Trends in Food Science & Technology*, 70: 20-33.

Cheng, Z. J., Hardy, R. W. & Huige, N. J. (2004). Apparent digestibility coefficients of nutrients in brewer's and rendered animal by-products for rainbow trout (*Oncorhynchus mykiss* (Walbaum)). *Aquaculture Research*, 35 (1): 1-9.

Chibata, I., Fukui, S. & Wingard, L. B. J. (2013). *Enzyme Engineering*: Springer US.

Christen, P., Domenech, F., Páca, J. & Revah, S. (1999). Evaluation of four *Candida utilis* strains for biomass, acetic acid and ethyl acetate production from ethanol. *Bioresource Technology*, 68 (2): 193-195.

Chylenski, P., Forsberg, Z., Stahlberg, J., Varnai, A., Lersch, M., Bengtsson, O., Saebo, S., Horn, S. J. & Eijsink, V. G. H. (2017). Development of minimal enzyme cocktails for hydrolysis of sulfite-pulped lignocellulosic biomass. *J Biotechnol*, 246: 16-23.

Cobelo-García, A., Morán, P., Almécija, C. & Caballero, P. (2017). Historical record of trace elements (1983–2007) in scales from Atlantic salmon (*Salmo salar*): Study of past metal contamination from a copper mine (Ulla River, NW Iberian Peninsula). *Chemosphere*, 188: 18-24.

Commission Regulation (EU). (2013). amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities. *Official Journal of the European Union*, 328.

Connan, S., Delisle, F., Deslandes, E. & Ar Gall, E. (2006). *Intra-thallus phlorotannin content and antioxidant activity in Phaeophyceae of temperate waters*. Botanica Marina, 49, 1.

Cronin, G. & Hay, M. E. (1996). Effects of Light and Nutrient Availability on the Growth, Secondary Chemistry, and Resistance to Herbivory of Two Brown Seaweeds. *Oikos*, 77 (1): 93-106.

Dawczynski, C., Schubert, R. & Jahreis, G. (2007). Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry*, 103 (3): 891-899.

Dayton, P. K., Tegner, M. J., Edwards, P. B. & Riser, K. L. (1999). Temporal and spatial scales of kelp demography: The role of oceanographic climate. *Ecological Monographs*, 69 (2): 219-250.

de Arruda, P. V., Rodrigues Rde, C., da Silva, D. D. & Felipe, M. (2011). Evaluation of hexose and pentose in pre-cultivation of *Candida guilliermondii* on the key enzymes for xylitol production in sugarcane hemicellulosic hydrolysate. *Biodegradation*, 22 (4): 815-22.

Dean, T. A. & Jacobsen, F. R. (1984). Growth of juvenile *Macrocystis pyrifera* (Laminariales) in relation to environmental factors. *Marine Biology*, 83 (3): 301-311.

Deniaud-Bouët, E., Kervarec, N., Michel, G., Tonon, T., Kloareg, B. & Hervé, C. (2014). Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. *Annals of Botany*, 114 (6): 1203-1216.

Deniaud-Bouët, E., Hardouin, K., Potin, P., Kloareg, B. & Hervé, C. (2017). A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges. *Carbohydrate Polymers*, 175: 395-408.

Dersjant-Li, Y. (2002). The use of soy protein in aquafeeds. Avances en Nutricion Acuicola VI. Memorias del VI Simposium Internacional de Nutricion Acuicola. 3 al 6 de Septiembre del 2002. Cancun, Quintana Roo, Mexico.

Deutsch, L., Gräslund, S., Folke, C., Troell, M., Huitric, M., Kautsky, N. & Lebel, L. (2007). Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal. *Global Environmental Change*, 17 (2): 238-249.

Di Filippo-Herrera Dania, A., Hernández-Carmona, G., Muñoz-Ochoa, M., Arvizu-Higuera Dora, L. & Rodríguez-Montesinos Yoloxochitl, E. (2018). *Monthly variation in the chemical composition and biological activity of Sargassum horridum*. Botanica Marina, 61, 1.

Duinker, A., Roiha, I. S., Amlund, H., Dahl, L., Lock, E.-J., Kögel, T., Måge, A. & Lunestad, B. T. (2016). Potential risks posed by macroalgae for application as feed and food - a Norwegian perspective. *National Institute of Nutrition and Seafood Research (NIFES)*.

El-Sayed, A.-F. M. (1999). Alternative dietary protein sources for farmed tilapia, *Oreochromis spp*. *Aquaculture*, 179 (1): 149-168.

Enquist-Newman, M., Faust, A. M. E., Bravo, D. D., Santos, C. N. S., Raisner, R. M., Hanel, A., Sarvabhowman, P., Le, C., Regitsky, D. D., Cooper, S. R., et al. (2014). Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. *Nature*, 505 (7482): 239-243.

FAO. (2016a). Fisheries and aquaculture software. FishStatJ - software for fishery statistical time series. *Food and Agriculture Organization of the United Nations*.

FAO. (2016b). The state of world fisheries and aquaculture. *Food and Agriculture Organization of the United Nations*.

Farzad, S., Mandegari, M. A., Guo, M., Haigh, K. F., Shah, N. & Görgens, J. F. (2017). Multi-product biorefineries from lignocelluloses: a pathway to revitalisation of the sugar industry? *Biotechnology for Biofuels*, 10 (1): 87.

FDA. (2018). *U.S. Food & Drug Administration, "Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List)"*. Tilgjengelig fra: https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm [Accessed 20.02.2018].

Fishmeal, I. (2018). *Fish meal average annual price - US Dollars per Metric Ton*. Tilgjengelig fra: https://www.indexmundi.com/commodities/?Commodity=fish-meal [Accessed 06.02.2018].

Fleurence, J. (1999). Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science & Technology*, 10 (1): 25-28.

Francis, G., Makkar, H. P. S. & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199 (3): 197-227.

Gao, Y., Li, D. & Liu, Y. (2012). Production of single cell protein from soy molasses using *Candida tropicalis*. *Annals of Microbiology*, 62 (3): 1165-1172.

Glencross, B. D., Booth, M. & Allan, G. L. (2007). A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition*, 13 (1): 17-34.

Goëmar. (2013). *Laminarin used against apple scab*. Tilgjengelig fra: http://www.goemar.com/sites/default/files/fichiers/phytomavacciplantflscab662.pdf [Accessed 21.02.2018].

Grammes, F., Reveco, F. E., Romarheim, O. H., Landsverk, T., Mydland, L. T. & Øverland, M. (2013). *Candida utilis* and *Chlorella vulgaris* Counteract Intestinal Inflammation in Atlantic Salmon (*Salmo salar* L.). *PLOS ONE*, 8 (12): e83213.

Gupta, S. & Abu-Ghannam, N. (2011). Recent developments in the application of seaweeds or seaweed extracts as a means for enhancing the safety and quality attributes of foods. *Innovative Food Science & Emerging Technologies*, 12 (4): 600-609.

Hamed, I., Özogul, F., Özogul, Y. & Regenstein, J. M. (2015). Marine Bioactive Compounds and Their Health Benefits: A Review. *Comprehensive Reviews in Food Science and Food Safety*, 14 (4): 446-465.

Handå, A., Forbord, S., Wang, X., Broch, O. J., Dahle, S. W., Størseth, T. R., Reitan, K. I., Olsen, Y. & Skjermo, J. (2013). Seasonal- and depth-dependent growth of cultivated kelp (*Saccharina latissima*) in close proximity to salmon (*Salmo salar*) aquaculture in Norway. *Aquaculture*, 414–415: 191-201.

Hemre, G., I., Amlund, H., Aursand, M., Bakke, A., M., Olsen, R. E., Ringø, E. & Svihus, B. (2009). Criteria for safe use of plant ingredients in diets for aquacultured fish. *Vitenskapskomiteen for mattrygghet (Norwegian Scientific Commitee for Food Safety)*.

Holdt, S. L. & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, 23 (3): 543-597.
Horn, S. J. (2000). Bioenergy from brown seaweeds.
Horn, S. J., Aasen, I. M. & Østgaard, K. (2000). Production of ethanol from mannitol by *Zymobacter palmae*. *Journal of Industrial Microbiology and Biotechnology*, 24 (1): 51-57.
Horn, S. J., Vaaje-Kolstad, G., Westereng, B. & Eijsink, V. (2012). Novel enzymes for the degradation of cellulose. *Biotechnology for Biofuels*, 5 (1): 1-13.
Hou, X., Hansen, J. H. & Bjerre, A.-B. (2015). Integrated bioethanol and protein production from brown seaweed Laminaria digitata. *Bioresource Technology*, 197: 310-317.
Huesemann, M. H., Kuo, L. J., Urquhart, L., Gill, G. A. & Roesijadi, G. (2012). Acetone-butanol fermentation of marine macroalgae. *Bioresour Technol*, 108: 305-9.
Institute of Medicine Panel on Micronutrients. (2001). I: *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*. Washington (DC): National Academies Press (US) Copyright 2001 by the National Academy of Sciences. All rights reserved.
Jiang, H.-B., Chen, L.-Q., Qin, J.-G., Gao, L.-J., Li, E.-C., Yu, N., Sun, S.-M. & Jiang, X.-Q. (2013). Partial or complete substitution of fish meal with soybean meal and cottonseed meal in Chinese mitten crab Eriocheir sinensis diets. *Aquaculture International*, 21 (3): 617-628.
Kamm, B. & Kamm, M. (2004). Principles of biorefineries. *Applied Microbiology and Biotechnology*, 64 (2): 137-145.
Kampen, W. H. (2014). Chapter 4 - Nutritional Requirements in Fermentation Processes A2 - Vogel, Henry C. I: Todaro, C. M. (red.) *Fermentation and Biochemical Engineering Handbook (Third Edition)*, s. 37-57. Boston: William Andrew Publishing.
Kim, H. S., Lee, C.-G. & Lee, E. Y. (2011). Alginate lyase: Structure, property, and application. *Biotechnology and Bioprocess Engineering*, 16 (5): 843.
Kim, S. K. & Lee, C. G. (2015). *Marine Bioenergy: Trends and Developments*: CRC Press.
Kim, S. K. (2016). *Marine Glycobiology: Principles and Applications*: CRC Press.
Kimura, Y., Watanabe, K. & Okuda, H. (1996). Effects of soluble sodium alginate on cholesterol excretion and glucose tolerance in rats. *J Ethnopharmacol*, 54 (1): 47-54.
Kirimi, J. G., Musalia, L. M. & Munguti, J. M. (2017). Effect Of Replacing Fish Meal With Blood Meal On Chemical Composition Of Supplement For Nile Tilapia (*Oreochromis Niloticus*). *East African Agricultural and Forestry Journal*, 82 (1): 1-9.
Kolstad, K., Grisdale-Helland, B. & Gjerde, B. (2004). Family differences in feed efficiency in Atlantic salmon (*Salmo salar*). *Aquaculture*, 241 (1): 169-177.
Kraan, S. (2012). Algal Polysaccharides, Novel Applications and Outlook. I: Chang, C.-F. (red.) *Carbohydrates - Comprehensive Studies on Glycobiology and Glycotechnology*, s. Ch. 22. Rijeka: InTech.
Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, Å. & Svihus, B. (2007). Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed diets with cellulose or non-starch polysaccharides from soy. *Aquaculture*, 273 (1): 96-107.
Kristensen, J. B., Felby, C. & Jørgensen, H. (2009). Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, 2 (1): 1-10.
Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S. & Bakke, A. M. (2010). Important antinutrients in plant feedstuffs for aquaculture: an update on recent findings regarding responses in salmonids. *Aquaculture Research*, 41 (3): 333-344.

Kumar, M., Kumari, P., Trivedi, N., Shukla, M. K., Gupta, V., Reddy, C. R. K. & Jha, B. (2011). Minerals, PUFAs and antioxidant properties of some tropical seaweeds from Saurashtra coast of India. *Journal of Applied Phycology*, 23 (5): 797-810.

Lamers, P., Searcy, E., Hess, J. R. & Stichnothe, H. (2016). *Developing the Global Bioeconomy: Technical, Market, and Environmental Lessons from Bioenergy*: Elsevier Science.

Langeland, M., Lindberg, J. E. & Lundh, T. (2014). Digestive Enzyme Activity in Eurasian Perch (*Perca Fluviatilis*) and Arctic Charr (*Salvelinus Alpinus*). *Journal of aquaculture research and development*, 5: 208.

Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J. E., Kiessling, A. & Lundh, T. (2016). Digestibility of microbial and mussel meal for Arctic charr (*Salvelinus alpinus*) and Eurasian perch (*Perca fluviatilis*). *Aquaculture Nutrition*, 22 (2): 485-495.

Lee, B.-K. & Kyun Kim, J. (2001). Production of *Candida utilis* biomass on molasses in different culture types. *Aquacultural Engineering*, 25 (2): 111-124.

Lemmel, S. A., Heimsch, R. C. & Edwards, L. L. (1979). Optimizing the Continuous Production of *Candida utilis* and *Saccharomycopsis fibuliger* on Potato Processing Wastewater. *Applied and Environmental Microbiology*, 37 (2): 227-232.

Li, S., Yang, X., Zhang, L., Yu, W. & Han, F. (2015). Cloning, Expression, and Characterization of a Cold-Adapted and Surfactant-Stable Alginate Lyase from Marine Bacterium Agarivorans sp. L11. *J Microbiol Biotechnol*, 25 (5): 681-6.

Lindsey Zemke-White, W. & Ohno, M. (1999). World seaweed utilisation: An end-of-century summary. *Journal of Applied Phycology*, 11 (4): 369-376.

Liong, M. T. & Shah, N. P. (2005). Production of organic acids from fermentation of mannitol, fructooligosaccharide and inulin by a cholesterol removing *Lactobacillus acidophilus* strain. *Journal of Applied Microbiology*, 99 (4): 783-793.

Lundebye, A.-K., Aursand, M., Berntssen, M., Hemre, G. I., Jenssen, B. M., Thomassen, M. S. S., Volden, H., Berdal, K. G., Espe, M., Mjaaland, S., et al. (2006). *Opinion on the safety of BioProtein® by the Scientific Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety. Revised version*. Tilgjengelig fra: http://hdl.handle.net/11250/2469812 [Accessed 28.02.2018].

Lüning, K. & Mortensen, L. (2015). European aquaculture of sugar kelp *(Saccharina latissima)* for food industries: iodine content and epiphytic animals as major problems. *Botanica Marina*, 58, 6.

Ma, L. Y., Chi, Z. M., Li, J. & Wu, L. F. (2008). Overexpression of alginate lyase of *Pseudoalteromonas elyakovii* in *Escherichia coli*, purification, and characterization of the recombinant alginate lyase. *World Journal of Microbiology and Biotechnology*, 24 (1): 89-96.

Manns, D., Deutschle, A. L., Saake, B. & Meyer, A. S. (2014). Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). *RSC Advances*, 4 (49): 25736-25746.

Manns, D., Andersen, S. K., Saake, B. & Meyer, A. S. (2016). Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment. *Journal of Applied Phycology*, 28 (2): 1287-1294.

Marinho, G. S., Holdt, S. L. & Angelidaki, I. (2015). Seasonal variations in the amino acid profile and protein nutritional value of *Saccharina latissima* cultivated in a commercial IMTA system. *Journal of Applied Phycology*, 27 (5): 1991-2000.

Meland, M. & Rebours, C. (2012). *Short description of the Norwegian seaweed industry*.

Miles, R. D. & Chapman, F. A. (2015). The Benefits of Fish Meal in Aquaculture Diets. *University of Florida, IFAS extension* (1).

Miyake, O., Ochiai, A., Hashimoto, W. & Murata, K. (2004). Origin and Diversity of Alginate Lyases of Families PL-5 and -7 in *Sphingomonas sp*. Strain A1. *Journal of Bacteriology*, 186 (9): 2891-2896.

Moy, F. E. & Christie, H. (2012). Large-scale shift from sugar kelp (*Saccharina latissima*) to ephemeral algae along the south and west coast of Norway. *Marine Biology Research*, 8 (4): 309-321.

Murray, A. P. & Marchant, R. (1986). Nitrogen utilization in rainbow trout fingerlings (*Salmo gairdneri* Richardson) fed mixed microbial biomass. *Aquaculture*, 54 (4): 263-275.

Mustafa Erturk, M. & Sevgili, H. (2003). Effects of Replacement of Fish Meal with Poultry By-product Meals on Apparent Digestibility, Body Composition and Protein Efficiency Ratio in a Practical Diets for Rainbow Trout, *Onchorynchus mykiss*. *Asian-Australas J Anim Sci*, 16 (9): 1355-1359.

Nguyen, T. H., Fleet, G. H. & Rogers, P. L. (1998). Composition of the cell walls of several yeast species. *Appl Microbiol Biotechnol*, 50 (2): 206-12.

Nigam, J. N. (1998). Single cell protein from pineapple cannery effluent. *World Journal of Microbiology and Biotechnology*, 14 (5): 693-696.

Nigam, J. N. (2000). Cultivation of *Candida langeronii* in sugar cane bagasse hemicellulosic hydrolyzate for the production of single cell protein. *World Journal of Microbiology and Biotechnology*, 16 (4): 367-372.

Nordic Nutrition Recommendation. (2012). *Nordic Nutrition Recommendations (NNR), Integrating nutrition and physical activity. Copenhagen, Norden Nordic Council of Ministers.*

Nøstbakken, O. J., Hove, H. T., Duinker, A., Lundebye, A.-K., Berntssen, M. H. G., Hannisdal, R., Lunestad, B. T., Maage, A., Madsen, L., Torstensen, B. E., et al. (2015). Contaminant levels in Norwegian farmed Atlantic salmon (*Salmo salar*) in the 13-year period from 1999 to 2011. *Environment International*, 74: 274-280.

Ochiai, A., Hashimoto, W. & Murata, K. (2006). A biosystem for alginate metabolism in *Agrobacterium tumefaciens* strain C58: molecular identification of Atu3025 as an exotype family PL-15 alginate lyase. *Res Microbiol*, 157 (7): 642-9.

OECD-FAO. (2018). *OECD-FAO Agricultural Outlook 2017-2026*. Tilgjengelig fra: http://stats.oecd.org/viewhtml.aspx?datasetcode=HIGH_AGLINK_2017&lang=en# [Accessed 03.03.2018].

Official Journal of the European Union. (2009). *Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation)*. EUR-LEX access to European Union Law. Tilgjengelig fra: http://data.europa.eu/eli/reg/2009/1069/oj [Accessed 20.02.2018].

Olafsen, T. (2012). Verdiskaping basert på produktive hav i 2050. *Rapport fra en arbeidsgruppe oppnevnt av det Kongelige Norske Videnskabers Selskab (DKNVS) og Norges Tekniske Vitenskapsakademi (NTVA)*: 77.

Ozório, R. O. A., Turini, B. G. S., Môro, G. V., Oliveira, L. S. T., Portz, L. & Cyrino, J. E. P. (2010). Growth, nitrogen gain and indispensable amino acid retention of pacu (*Piaractus mesopotamicus*, Holmberg 1887) fed different brewers yeast (*Saccharomyces cerevisiae*) levels. *Aquaculture Nutrition*, 16 (3): 276-283.

Pampulha, M. E. & Loureiro-Dias, M. C. (1989). Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Applied Microbiology and Biotechnology*, 31 (5): 547-550.

Panda, S. K., Ray, R. C., Mishra, S. S. & Kayitesi, E. (2018). Microbial processing of fruit and vegetable wastes into potential biocommodities: a review. *Critical Reviews in Biotechnology*, 38 (1): 1-16.

Park, J.-I., Lee, J., Sim, S. J. & Lee, J.-H. (2009). Production of hydrogen from marine macro-algae biomass using anaerobic sewage sludge microflora. *Biotechnology and Bioprocess Engineering*, 14 (3): 307-315.

Percival, E. & McDowell, R. H. (1967). *Chemistry and enzymology of marine algal polysaccharides*: Academic Press.

Rana, K. J. (2009). *Impact of rising feed ingredient prices on aquafeeds and aquaculture production*. Siriwardena, S. & Hasan, M. R. (red.). Rome: Food and Agriculture Organization of the United Nations.

Riediger, N. D., Othman, R. A., Suh, M. & Moghadasian, M. H. (2009). A systemic review of the roles of n-3 fatty acids in health and disease. *J Am Diet Assoc*, 109 (4): 668-79.

Roch, M. & Maly, E. J. (1979). Relationship of Cadmium-Induced Hypocalcemia with Mortality in Rainbow Trout (*Salmo gairdneri*) and the Influence of Temperature on Toxicity. *Journal of the Fisheries Research Board of Canada*, 36 (11): 1297-1303.

Romarheim, O. H., Overland, M., Mydland, L. T., Skrede, A. & Landsverk, T. (2011). Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. *J Nutr*, 141 (1): 124-30.

Romarheim, O. H., Hetland, D. L., Skrede, A., Overland, M., Mydland, L. T. & Landsverk, T. (2013a). Prevention of soya-induced enteritis in Atlantic salmon (*Salmo salar*) by bacteria grown on natural gas is dose dependent and related to epithelial MHC II reactivity and CD8alpha+ intraepithelial lymphocytes. *Br J Nutr*, 109 (6): 1062-70.

Romarheim, O. H., Landsverk, T., Mydland, L. T., Skrede, A. & Øverland, M. (2013b). Cell wall fractions from *Methylococcus capsulatus* prevent soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*). *Aquaculture*, 402-403: 13-18.

Rombough, P. J. & Garside, E. T. (1982). Cadmium toxicity and accumulation in eggs and alevins of Atlantic salmon *Salmo salar*. *Canadian Journal of Zoology*, 60 (8): 2006-2014.

Rumsey, G. L., Hughes, S. G., Smith, R. R., Kinsella, J. E. & Shetty, K. J. (1991). Digestibility and energy values of intact, disrupted and extracts from brewer's dried yeast fed to rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and Technology*, 33 (3): 185-193.

Rupérez, P. (2002). Mineral content of edible marine seaweeds. *Food Chemistry*, 79 (1): 23-26.

Rødsrud, G., Lersch, M. & Sjöde, A. (2012). History and future of world's most advanced biorefinery in operation. *Biomass and Bioenergy*, 46: 46-59.

Sánchez-Rodríguez, I., Huerta-Diaz, M. A., Choumiline, E., Holguín-Quiñones, O. & Zertuche-González, J. A. (2001). Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue. *Environmental Pollution*, 114 (2): 145-160.

Sawabe, T., Setoguchi, N., Inoue, S., Tanaka, R., Ootsubo, M., Yoshimizu, M. & Ezura, Y. (2003). Acetic acid production of Vibrio halioticoli from alginate: a possible role for establishment of abalone–V. halioticoli association. *Aquaculture*, 219 (1): 671-679.

Scarlat, N., Dallemand, J.-F., Skjelhaugen, O. J., Asplund, D. & Nesheim, L. (2011). An overview of the biomass resource potential of Norway for bioenergy use. *Renewable and Sustainable Energy Reviews*, 15 (7): 3388-3398.

Schiener, P., Black, K., Stanley, M. & Green, D. (2015). The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*,

Saccharina latissima and *Alaria esculenta*. *Journal of Applied Phycology*, 27 (1): 363-373.
Seaweed Energy Solutions AS. (2018). Tilgjengelig fra: http://www.seaweedenergysolutions.com/en [Accessed 25.02.2018].
Shanmugam, M. & Mody, K. H. (2000). Heparinoid-active sulphated polysaccharides from marine algae as potential blood anticoagulant agents. *Current Science*, 79 (12): 1672-1683.
Silberfeld, T., Rousseau, F. & Reviers, B. d. (2014). An Updated Classification of Brown Algae (Ochrophyta, Phaeophyceae). *Cryptogamie, Algologie*, 35 (2): 117-156.
Sivertsen, K. & Bjørge, A. (2014). On the brink of the Arctic: Unusual intertidal sub-Arctic kelp associations in the Porsangerfjord, North Norway. *Marine Biology Research*, 11 (4): 405-413.
Skriptsova, A., Khomenko, V. & Isakov, V. (2004). Seasonal changes in growth rate, morphology and alginate content in *Undaria pinnatifida* at the northern limit in the Sea of Japan (Russia). *Journal of Applied Phycology*, 16 (1): 17.
Skrøppa, T. (2012). State of forest genetic resources in Norway. *Norwegian Forest and Landscape Institute*: 1-56.
Soybeans, I. (2018). *Soybeans average annual price - US Dollars per Metric Ton*. Tilgjengelig fra: https://www.indexmundi.com/commodities/?commodity=soybeans [Accessed 06.02.2018].
Statistisk Sentralbyrå. (2016). *Statistics Norway*. Tilgjengelig fra: http://www.ssb.no/en/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett/aar [Accessed 12.02.2018].
Stévant, P., Rebours, C. & Chapman, A. (2017). Seaweed aquaculture in Norway: recent industrial developments and future perspectives. *Aquaculture International*, 25 (4): 1373-1390.
Stiger-Pouvreau, V., Bourgougnon, N. & Deslandes, E. (2016). Chapter 8 - Carbohydrates From Seaweeds. I: *Seaweed in Health and Disease Prevention*, s. 223-274. San Diego: Academic Press.
Sudha, P. N. (2017). *Industrial Applications of Marine Biopolymers*: CRC Press.
Sugiura, S. H., Dong, F. M., Rathbone, C. K. & Hardy, R. W. (1998). Apparent protein digestibility and mineral availabilities in various feed ingredients for salmonid feeds. *Aquaculture*, 159 (3): 177-202.
Svecevicius, G., Sauliute, G., Idzelis, R. L. & Grigeleviciute, J. (2014). Accumulation of heavy metals in different body tissues of Atlantic salmon, *Salmo salar* L., exposed to a model mixture (Cu, Zn, Ni, Cr, Pb, Cd) and singly to nickel, chromium, and lead. *Bull Environ Contam Toxicol*, 92 (4): 440-5.
Swift, S. M., Hudgens, J. W., Heselpoth, R. D., Bales, P. M. & Nelson, D. C. (2014). Characterization of AlgMsp, an Alginate Lyase from *Microbulbifer sp*. 6532A. *PLOS ONE*, 9 (11): e112939.
Sørensen, M., Gong, Y., Bjarnason, F., Vasanth, G. K., Dahle, D., Huntley, M. & Kiron, V. (2017). Nannochloropsis oceania-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds. *PLOS ONE*, 12 (7): e0179907.
Tanguler, H. & Erten, H. (2008). Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproducts Processing*, 86 (4): 317-321.
Teimouri, M., Amirkolaie, A. K. & Yeganeh, S. (2013). The effects of *Spirulina platensis* meal as a feed supplement on growth performance and pigmentation of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 396-399 (Supplement C): 14-19.
Trømborg, E., Bolkesjø, T. F. & Solberg, B. (2008). Biomass market and trade in Norway: Status and future prospects. *Biomass and Bioenergy*, 32 (8): 660-671.

Tukmechi, A. & Bandboni, M. (2014). Effects of *Saccharomyces cerevisiae* supplementation on immune response, hematological parameters, body composition and disease resistance in rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792). *Journal of Applied Ichthyology*, 30 (1): 55-61.

Tønder, T. S. (2014). A study of the seasonal variation in biochemical composition of *Saccharina latissima*. MSc Thesis. Norwegian University of Science and Technology, Trondheim, Norway.

Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z., Zhai, H., Sørlie, M. & Eijsink, V. G. H. (2010). An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides. *Science*, 330 (6001): 219-222.

Vasileska, A. & Rechkoska, G. (2012). Global and Regional Food Consumption Patterns and Trends. *Procedia - Social and Behavioral Sciences*, 44: 363-369.

Vaugelade, P., Hoebler, C., Bernard, F., Guillon, F., Lahaye, M., Duee, P. H. & Darcy-Vrillon, B. (2000). Non-starch polysaccharides extracted from seaweed can modulate intestinal absorption of glucose and insulin response in the pig. *Reprod Nutr Dev*, 40 (1): 33-47.

Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling, A. & Lundh, T. (2016). Evaluation of growth performance and intestinal barrier function in Arctic Charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*), fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*). *Aquaculture Nutrition*, 22 (6): 1348-1360.

Vivekanand, V., Eijsink, V. G. & Horn, S. J. (2012). Biogas production from the brown seaweed *Saccharina latissima*: thermal pretreatment and codigestion with wheat straw. *Journal of Applied Phycology*, 24 (5): 1295-1301.

Walker, G. & Stewart, G. (2016). *Saccharomyces cerevisiae* in the Production of Fermented Beverages. *Beverages*, 2 (4): 30.

Wang, C.-Y. & Chen, Y.-C. (2013). Extraction and characterization of fucoidan from six brown macroalgae. *Journal of Marine Science and Technology*: 201-219.

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N. S., Kim, P. B., Cooper, S. R., Raisner, R. M., Herman, A., Sivitz, A. B., et al. (2012). An Engineered Microbial Platform for Direct Biofuel Production from Brown Macroalgae. *Science*, 335 (6066): 308-313.

Watanabe, T., Kiron, V. & Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture*, 151 (1): 185-207.

Wheeler, P. A. & North, W. J. (1981). Nitrogen supply, tissue composition and frond growth rates for *Macrocystis pyrifera* off the coast of southern California. *Marine Biology*, 64 (1): 59-69.

Wijesinghe, W. A. J. P. & Jeon, Y.-J. (2012). Biological activities and potential industrial applications of fucose rich sulfated polysaccharides and fucoidans isolated from brown seaweeds: A review. *Carbohydrate Polymers*, 88 (1): 13-20.

Yamada, E. A. & Sgarbieri, V. C. (2005). Yeast (*Saccharomyces cerevisiae*) protein concentrate: preparation, chemical composition, and nutritional and functional properties. *J Agric Food Chem*, 53 (10): 3931-6.

Yokoyama, H., Kobayashi, H., Hasegawa, J.-y. & Fukuoka, A. (2017). Selective Dehydration of Mannitol to Isomannide over Hβ Zeolite. *ACS Catalysis*, 7 (7): 4828-4834.

Yoon, H. J., Hashimoto, W., Miyake, O., Okamoto, M., Mikami, B. & Murata, K. (2000). Overexpression in *Escherichia coli*, purification, and characterization of *Sphingomonas sp.* A1 alginate lyases. *Protein Expr Purif*, 19 (1): 84-90.

Ytrestøyl, T., Aas, T. S. & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture*, 448: 365-374.

Zhu, B.-W., Huang, L.-S.-X., Tan, H.-D., Qin, Y.-Q., Du, Y.-G. & Yin, H. (2015). Characterization of a new endo-type polyM-specific alginate lyase from *Pseudomonas sp. Biotechnology Letters*, 37 (2): 409-415.

Zhu, B. & Yin, H. (2015). Alginate lyase: Review of major sources and classification, properties, structure-function analysis and applications. *Bioengineered*, 6 (3): 125-131.

Øverland, M., Tauson, A.-H., Shearer, K. & Skrede, A. (2010). Evaluation of methane-utilising bacteria products as feed ingredients for monogastric animals. *Archives of Animal Nutrition*, 64 (3): 171-189.

Øverland, M., Karlsson, A., Mydland, L. T., Romarheim, O. H. & Skrede, A. (2013). Evaluation of *Candida utilis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*). *Aquaculture*, 402: 1-7.

Øverland, M. & Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *Journal of the Science of Food and Agriculture*, 97 (3): 733-742.

Paper I

Algal Research 32 (2018) 107–112

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Seasonal and depth variations in the chemical composition of cultivated *Saccharina latissima*

Sandeep Sharma[a], Luiza Neves[b], Jon Funderud[b], Liv Torunn Mydland[c], Margareth Øverland[c], Svein Jarle Horn[a,*]

[a] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway
[b] Seaweed Energy Solutions AS, Trondheim, Norway
[c] Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Ås, Norway

ARTICLE INFO

Keywords:
Seaweed
Cultivation
Sugars
Amino acids
Minerals

ABSTRACT

Sugar kelp (*Saccharina latissima*) is an abundantly available macroalgae species along the Norwegian coast, and there is currently emerging an industry based on seaweed cultivation. In this study, the biomass growth of cultivated *S. latissima* deployed in February was studied at cultivation depths of 3 and 8 meters (m) and monitored over the period of May, June, and August. The highest biomass production was observed in June at the depth of 3 m (38.3 kg wet weight m^{-2}). Furthermore, all seaweed samples underwent a detailed chemical characterization including analysis of carbohydrates (glucose, mannitol, fucose, xylose, uronic acids), amino acids and minerals. The macroalgae deployed in February at 3 m depth and sampled in June had the highest proportion of total sugars (534.5 g kg^{-1} of DM) and the lowest content of ash (252.7 g kg^{-1} of DM). Thus, cultivation at 3 m and harvesting in June are suitable when the feedstock is used for biochemical production of fuels and chemicals. Macroalgae deployed at 8 m depth and harvested in August had the highest proportion of total amino acids (242.4 g kg^{-1} DM) and ash content (411.5 g kg^{-1} DM). This biomass may be suitable as a nitrogen and mineral source in microbial growth media. Overall, the choice of cultivation depth and harvesting time depends on the intended use of the seaweed biomass.

1. Introduction

Macroalgae have gained attention globally as a potential feedstock for production of biopharmaceuticals, food and feed ingredients, biofuels, and bio-fertilizers [1–5]. In 2014, the global production of farmed macroalgae was 27.3 million tons wet weight, of which 99.3% was produced in Asian countries, pre-dominantly China and Indonesia [6]. Macroalgae can exhibit higher growth rates and production yields than terrestrial biomass [1,2]. A relatively small number of macroalgae genera constitute almost 98% of the global production of cultivated seaweed, i.e., *Saccharina/Laminaria* and *Undaria* (brown macroalgae), and *Eucheuma/Kappaphycus*, *Porphyra/Pyropia* and *Gracilaria* (red macroalgae). *Laminaria* spp. are widely distributed in the surface water environments on both sides of the Atlantic Ocean and off the coasts of China and Japan [7].

Saccharina latissima belongs to the Laminariaceae family (Phaeophyceae), and is a perennial species that can grow in sheltered waters attached to the seabed. The main groups of carbohydrates found in *S. latissima* are laminarin, alginate, cellulose, fucoidan, and the sugar alcohol mannitol. Laminarin and mannitol are storage carbohydrates, which accumulate in the seaweed during the light season, while alginate is a structural component with little annual variation [1,4,8,9]. Structurally, alginate is a linear polysaccharide of mannuronic and guluronic acids, which due to its physiological and rheological properties is used as a thickening agent for drinks, ice cream and cosmetic products [10]. Cellulose and laminarin may be hydrolyzed to glucose which, together with mannitol, could be used as a carbon source in fermentation to bioethanol or other valuable products [1,2,11]. Fucoidan is a sulphated polysaccharide composed of L-fucose units and possesses biological activities such as anticoagulant, antioxidant and antibacterial [12].

S. latissima is also known as a source of amino acids, minerals, and phenolic compounds [4,13,14]. *S. latissima* has a nitrogen reservoir that can sustain the growth in the periods of the summer when the available nitrogen (nitrates) in the sea are low [13,15]. The protein fraction of *S. latissima* contains all essential amino acids (EAAs) and non-essential amino acids (NEAAs) and their amount varies over the season [5]. *S. latissima* has a relatively high ash content, where the most important

* Corresponding author.
E-mail address: svein.horn@nmbu.no (S.J. Horn).

https://doi.org/10.1016/j.algal.2018.03.012
Received 27 November 2017; Received in revised form 15 March 2018; Accepted 21 March 2018

cations are sodium, potassium, calcium and magnesium, associated with the anions, chlorine, bromine, iodine, phosphorus and sulfur. The polyphenolic compounds in brown seaweed possess biological activities and are responsible for their inhibitory action towards microorganisms [13]. The growth rate of macroalgae varies according to the season, from hardly any growth during the dark winter time to rapid growth during spring and summer. Generally, macroalgal composition and growth rate vary considerably according to a range of environmental factors such as the salinity of seawater, pH, sunlight, mineral availability, waves, and water current [4,13,14].

In this work, we present a thorough study of the chemical composition of cultivated *S. latissima*. The objective was to investigate the effect of different cultivation depths and harvesting times on seaweed growth and chemical compositions, and how this affected potential applications of the feedstock.

2. Materials and methods

2.1. Seedling preparation, cultivation and measurements

Sorus induction of *S. latissima* was carried out from individuals of wild populations of *S. latissima* near the island of Frøya (63°42′15″N, 8°52′40″E), by Seaweed Energy Solutions AS (Trondheim, Norway). The induced sori of *S. latissima* were used for the release of zoospores. Young sporophytes were seeded onto 'spools' (plastic tubes covered with 60 m of polyester silk string), and incubated for 8 weeks in a 200 liter tank with continuous water flow, under a 16:8 light:dark (L:D) photoperiod. The culture was maintained at 8–9 °C and the light intensity was 30–60 μmol/m^2/s [16]. The young seedlings were transported to Frøya and placed temporarily in the sea at a pier for 2–3 days before being deployed. Frøya has a mild maritime climate with the driest season in May–June and coldest season from January–March. On average the highest tide at Frøya region is 3 m and lowest is 0.5 m. According to a study by Tønder et al. [16], the water salinity and temperature at Frøya over the period of June–August 2013 was 32–34 ppt and 12–14 °C, respectively. On February 16th 2015, the seedlings of *S. latissima* were deployed 3 and 8 m below sea level on 0.5 m^2 plastic frames (see supplementary data Fig. S1), and monitored after 84, 134 and 183 days (May, June and August 2015). Cultivation was carried out in triplicates with a total of 18 frames. In each frame, 20 plants were attached with a distance of 5 cm apart and suspended from a horizontal rope at the surface and down to 3 or 8 m and anchored on the same line. Possible shading of the 3 m frame on the 8 m frame was limited due to the relatively low angle of sun at the cultivation site and scattering of light in the water.

The growth of cultivated *S. latissima* was recorded on every monitoring date. This was done by measuring the total length of 5 random individuals from each replicate. Thallus length was measured from tips of blade to holdfast. Biomass measurement was carried out by weighing the total weight of each frame with cultivated *S. latissima* and subtracting the weight of an unseeded control frame. The weight of each frame was measured with a spring scale and a digital fish scale [16]. For chemical composition analysis of *S. latissima*, 5 random individuals from each replicate frames were arbitrarily sampled.

2.2. S. latissima sample preparation

Samples collected in the field on each monitoring date were immediately frozen once back on the land. The samples constituted the whole plant including blade, stipe and holdfast. The frozen unwashed *S. latissima* samples were later thawed and oven-dried at 50 °C until they reached equilibrium moisture. Samples were homogenized using a MF 10 basic micro-fine electric grinder (IKA, USA), shipped to the Norwegian University of Life Science (Ås, Norway) and stored in a desiccator until chemical analyses were performed. Epibionts were not removed from the samples.

2.3. Carbohydrate analyses

To determine the sugar composition of *S. latissima* samples, monomeric sugars were released by a modified two step acid hydrolysis. Dried ground samples and sugar recovery standards (SRS) were subjected to 72% (w/w) H_2SO_4 at 30 °C for exactly 60 min and then 4% (w/w) H_2SO_4 at 121 °C in an autoclave for 40 min [1]. After hydrolysis, the monosaccharide hydrolysates were filtered through ROBU glassfilter (16–40 μm, ROBU, Germany) and diluted with deionized water as per their standards' concentration range for HPLC and total uronic acid analysis. The released monomeric sugars and SRS were analyzed by a HPLC system equipped with refractive index detector. The separation column was a 300 × 7.8 mm Rezex ROA-Organic Acid H+ fitted with cation-H cartridge guard column. The column temperature was 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1}. Identification and quantification of sugars were carried out against external sugar standard curves (glucose, mannitol, fucose, and xylose), which were of analytical grade procured from Sigma Aldrich (USA). The same hydrolysates were used for quantification of total uronic acids (guluronic and mannuronic acids) by a spectrophotometrical method using absorbance at 550 nm and with carbazole as an indicator. The samples were filtered and diluted prior to the spectrophotometric analysis. The total content of uronic acids was measured as a galacturonic acid (GalA) equivalents. GalA was also used as recovery standard. All necessary chemicals (sulfuric acid, galacturonic acid, sodium borate, and carbazole) were procured from Merck (Germany).

2.4. Proximate, element and mineral analyses

The dry matter content of the samples was determined by a Metrohm Karl Fischer titrator (Florida, USA). Ash content was determined by incineration of the samples in a muffle furnace at 550 °C for 8 h. The content of C, H, and N was determined by elemental analysis (LECO, CHN-1000, USA). For metal analysis (cations) the samples were hydrolyzed with concentrated 65% HNO_3 in a high performance microwave reactor (UltraClave, MLS Milestone, Italy). For halides (anions) analysis the samples were digested with concentrated TMAH (Tetramethylammonium hydroxide). Both cations and anions were analyzed by inductively coupled plasma spectrometry coupled to a mass spectrometric detector (ICP-MS) (Perkin-Elmer, USA).

2.5. Amino acid composition analysis

Amino acid (AA) analysis of the *S. latissima* samples was performed according to Commission dir. No 152/2009/EC on a Biochrom 30 Amino Acid Analyzer (Oxidised Protein Hydrolysate System; Biochrom Ltd., UK) [17]. Tryptophan was analyzed on a Dionex UltiMate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) connected to a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Both amino acids and tryptophan data were analyzed against external standards curves (amino acid standard solutions; Sigma Chemical, St. Louis, Mo., U.S.A.) using the Chromeleon® Chromatography Management Software (Dionex Ltd., Surrey, UK).

2.6. Total phenolic content

Total phenolic content of the *S. latissima* samples were determined using the Folin-Ciocalteu assay [18]. 100 mg of dried *S. latissima* samples were first extracted with 1 ml of 50% methanol (*v*/v) in the dark at ambient temperature for 15 h. Then the reaction mixture containing 100 μl of extracted sample, 500 μl of Folin-Ciocalteu reagent, 1500 μl of 20% Na_2CO_3 and 6000 μl of deionized water was kept for 2 h in the dark. The absorbance at 765 nm was measured using gallic acid as an external standard, and the results are represented as g GAE kg^{-1}(gallic acid equivalents).

2.7. Statistical analysis

A two-way ANOVA was used to analyze the influence of cultivation depth and sampling time on various response parameters: carbohydrates (glucose, mannitol, fucose, xylose, uronic acids), phenolic content, amino acids and minerals. Degree of freedom, F distribution and probability values (*P*-value) of all components except mineral quantification were carried out using MS Excel, data analysis ToolPak (see supplementary Table S1).

3. Results

3.1. Growth of cultivated S. latissima

Seedlings of *S. latissima* were deployed in February and the seaweed was monitored in May, June and August. The biomass growth data showed highest biomass production for *S. latissima* harvested in June at the depth of 3 m (wet weight: 38.3 kg m^{-2}), while the lowest value was found for samples harvested in May at the depth of 8 m (3.0 kg m^{-2}). 38.3 kg m^{-2} biomass was produced in 134 days meaning a productivity of 286 g wet weight m^{-2} day^{-1}, or 57 g dry weight m^{-2} day^{-1} (assuming a typical dry matter content of 20%). The average length of seaweed plants were 153.8 ± 20.1 cm, 126.3 ± 7.9 cm, 124 ± 25.3 cm, and 112.9 ± 15.6 cm for June 3 m, June 8 m, August 3 m and August 8 m, respectively. Due to their small size, the length of the plants was not measured in May. Biomass growth highlighted significant differences between depth at 3 m and 8 m ($P < 0.05$) and between sampling times ($P < 0.05$).

3.2. Content of sugars and phenolics

Laminarin and cellulose content as monomeric glucose in *S. latissima* samples were determined by the two-step acid hydrolysis method [1]. Table 1 shows the content of monomeric sugars for the different cultivated *S. latissima* samples. The highest content of glucose and mannitol was found for the 3 m depth June sample. The concentration of xylose and fucose was similar in May and June, but lower in August. It should be noted that xylose could not be separated from other possible co-eluting sugars in our analysis, and the xylose peak may thus represent a combination of different sugars. Generally, the alginate content (total uronic acids) was similar at the two depths and declined over the cultivation period. Most sugars showed significant differences between sampling times and between depths ($P < 0.05$).The content of total phenolics (Table 1) was rather low and similar over the cultivation period and independent of depth.

3.3. Amino acid content

The amino acid compositions of the different samples are shown in Table 2. The main trend was that the content of both essential amino acids (EAA) and non-essential amino acids (NEAA) increased over the growth season, and that the content was higher at 8 m than 3 m. The levels of EAA and NEAA in our study ranged from 45.9 ± 1.3 to 71.6 ± 5.6 and 78.0 ± 2.6 to 170.8 ± 15.2 g kg^{-1} DM of *S. latissima*, respectively. The concentration of NEAA is approximately two times higher than EAA. A pairwise ANOVA test revealed significant differences in the composition of EAAs ($P = 0.003–0.0001$), NEAA ($P = 0.007–0.0001$) and total amino acids ($P = 0.006–0.0001$) between depths and between sampling times. Aspartic acid, glutamic acid and alanine constituted 35–42% of the total amino acids in all the samples. Moreover, threonine, valin, leucin, and lysin are the major EAAs in cultivated *S. latissima* whereas methionine, histidine and tryptophan are less abundant. There was no significant difference in tryptophan content due to different depth or harvesting time.

Apart from the amino acids, other nitrogenous compounds such as taurine and ammonia were also found in cultivated *S. latissima*. The taurine concentration in May at 3 m depth was 1.1 ± 0.1 and increased to a maximum of 13.6 ± 0.2 g kg^{-1} DM in August at 8 m. The ammonia content was in the range of 3.4 ± 0.1 to 7.6 ± 0.3 g kg^{-1} DM. The concentration of taurine and ammonia highlighted significant differences between depths and between sampling times ($P < 0.05$).

The carbon content was in the range of 28.6–32.9% (Table 2). These values are similar to data in other *S. latissima* studies (21–37%) [13,19,20]. The amount of carbon in *S. latissima* was gradually decreased during the cultivation period and depth of cultivation. Generally, the carbon content was lower in cultivated *S. latissima* at 8 m compared to 3 m. A similar observation was observed for the hydrogen content. The carbon and hydrogen content followed the same general trend as glucose, xylose, mannitol and uronic acids (Table 1). The nitrogen content in cultivated *S. latissima* was highest in August at 8 m.

3.4. Mineral content

The ash content of cultivated *S. latissima* varied from 253 to 412 g kg^{-1} DM as shown in Table 3. Two-way ANOVA and pairwise comparison of ash content showed significant differences between depths and between sampling times ($P < 0.05$). Among the trace minerals present in *S. latissima*, strontium was the most abundant in the cultivated as well as in wild type *S. latissima* followed by silicon > boron > iron > arsenic > zinc. The amount of sulfur, calcium and trace minerals was in general as gradually increased during the cultivation period.

4. Discussion

Seaweed cultivation is a new emerging industry in Norway, and it is a growing interest to utilize seaweed components such as carbohydrates, amino acids, and minerals for biofuels, food and feed ingredients, therapeutic products and other value-added products. This study shows that *S. latissima* grown at 3 m had a higher biomass

Table 1
Monosaccharides, total phenolics and ash content of cultivated *S. latissima* at different time and depth.

Chemical constituent[a]	May 3 m	May 8 m	June 3 m	June 8 m	August 3 m	August 8 m
Glucose	140.8 ± 2.0	120.1 ± 2.2	173.4 ± 5.5	112.4 ± 3.0	66.8 ± 1.3	37.7 ± 0.5
Mannitol	140.7 ± 2.7	115.4 ± 2.5	158.4 ± 5.2	96.5 ± 1.2	68.9 ± 0.2	20.5 ± 0.5
Uronic acids	170.7 ± 3.1	170.6 ± 4.4	148.5 ± 2.3	127.9 ± 3.3	64.7 ± 5.4	63.7 ± 4.3
Xylose	41.6 ± 1.5	42.4 ± 1.4	31.9 ± 2.4	41.2 ± 1.5	18.9 ± 0.3	18.4 ± 0.4
Fucose	28.5 ± 0.2	25.5 ± 0.8	22.3 ± 2.7	27.9 ± 1.6	13.9 ± 0.2	13.1 ± 0.3
Total sugars	522.3 ± 9.5	474 ± 11.3	534.5 ± 18.1	405.9 ± 10.6	233.2 ± 7.4	153.4 ± 6.0
Total phenolic content[b]	0.9 ± 0.04	1.1 ± 0.04	1.0 ± 0.1	0.7 ± 0.01	1.3 ± 0.01	1.4 ± 0.01
Ash	272.8 ± 5.6	275.9 ± 2.8	252.7 ± 10.0	327.4 ± 4.9	366.6 ± 4.4	411.5 ± 9.6

[a] The values are means of triplicates with standard deviation and given as g kg^{-1} DM.

[b] Concentration given as gallic acid equivalents in g kg^{-1} DM.

Table 2
Amino acid profiles and elemental composition of cultivated *S. latissima* harvested at different time and depth.

Amino acids[a]	May 3 m	May 8 m	June 3 m	June 8 m	Aug 3 m	Aug 8 m	*Wild S. latissima*[b]	Literature values
Essential amino acids (EAAs)								
Leucine	10.7 ± 0.5	11.0 ± < 0.1	8.3 ± 0.6	9.5 ± 0.9	11.7 ± 0.1	13.8 ± 1.0	4.7 ± 0.2	7.4–7.8 [4,19]
Valine	7.3 ± < 0.1	7.4 ± 0.5	6.2 ± < 0.1	6.7 ± 0.4	9.4 ± 0.1	11.1 ± 0.8	4.0 ± 0.3	1.9–5.6 [4,19]
Lysine	7.3 ± < 0.1	7.9 ± 0.1	7.7 ± 0.1	7.4 ± 0.3	11.0 ± 0.1	13.6 ± 1.0	4.3 ± 0.2	4.0–5.4 [4,19]
Threonine	6.6 ± 0.1	7.0 ± 0.3	5.5 ± 0.6	6.4 ± 0.4	9.1 ± 0.1	11.9 ± 1.3	3.8 ± 0.2	2.0–5.2 [4,19]
Isoleucine	6.0 ± 0.6	6.3 ± < 0.1	4.9 ± 0.1	5.6 ± 0.2	7.2 ± 0.1	8.6 ± 0.6	2.8 ± 0.2	0.9–4.1 [4,19]
Methionine	3.0 ± < 0.1	2.8 ± 0.1	1.9 ± 0.2	2.9 ± 0.3	4.0 ± 0.1	5.1 ± 0.3	1.6 ± 0.1	2.2–2.3 [4,19]
Tryptophan	2.7	2.8	2.1	2.4	2.9	3.3	1.1	3.5 [19]
Histidine	2.1 ± 0.1	2.3 ± < 0.1	2.1 ± 0.1	2.1 ± < 0.1	3.2 ± 0.2	4.0 ± 0.5	1.1 ± < 0.1	0.8–1.8 [4,19]
Total EAAs	45.9 ± 1.3	47.5 ± 1.0	38.8 ± 1.6	43.1 ± 2.5	58.6 ± 0.8	71.6 ± 5.6	23.4 ± 1.2	19.9–35.2
Non-essential amino acids (NEAAs)								
Glutamic acid	26.0 ± 0.5	26.6 ± 0.4	16.8 ± 0.1	16.8 ± 1.0	29.7 ± 0.2	36.5 ± 2.7	15.6 ± 0.5	12.3–15.2 [4,19]
Aspartic acid	14.0 ± 0.8	14.8 ± 0.1	14.4 ± 1.4	15.8 ± 0.4	23.8 ± 0.7	30.2 ± 3.6	9.8 ± 0.4	12.1–12.8 [4,19]
Alanine	19.1 ± 0.3	19.2 ± 0.9	9.2 ± < 0.1	10.0 ± 0.2	14.8 ± 0.1	19.0 ± 1.9	7.6 ± 0.1	11.0–11.9 [4,19]
Glysine	6.5 ± 0.1	6.8 ± 0.4	8.9 ± < 0.1	8.4 ± 0.2	17.3 ± 0.1	23.1 ± 2.3	3.5 ± 0.1	5.1–8.4 [4,19]
Arginine	7.3 ± 0.5	7.7 ± 0.5	6.5 ± 0.3	6.9 ± 0.1	10.9 ± 0.1	14.0 ± 1.7	4.1 ± 0.3	4.5–4.8 [4,19]
Phenylalanine	7.3 ± 0.2	7.6 ± 0.1	6.3 ± 0.1	7.1 ± 0.1	9.2 ± 0.2	11.6 ± 0.6	4.0 ± < 0.1	–
Proline	7.1 ± 0.1	7.8 ± 0.1	6.6 ± 0.2	6.9 ± < 0.1	10.2 ± 0.3	12.9 ± 0.6	4.1 ± < 0.1	3.9–4.6 [4,19]
Serine	5.8 ± 0.6	6.5 ± 0.4	5.4 ± 0.3	6.0 ± 0.3	9.3 ± 0.4	12.4 ± 1.2	3.6 ± 0.3	4.7–5.1 [4,19]
Cyst(e)in	1.3 ± < 0.1	1.2 ± < 0.1	1.8 ± < 0.1	2.0 ± < 0.1	3.8 ± 0.1	4.7 ± 0.3	2.2 ± 0.1	0.3–1.9 [4,19]
Tyrosine	0.8 ± 0.1	1.0 ± < 0.1	0.7 ± < 0.1	0.6 ± < 0.1	0.7 ± < 0.1	1.0 ± 0.1	0.7 ± < 0.1	2.2–3.5 [4,19]
Hydroxyproline	< 0.01	< 0.01	1.4 ± 0.1	1.1 ± 0.1	4.7 ± < 0.1	5.3 ± 0.3	< 0.1	–
Total NEAAs	95.2 ± 3.3	99.3 ± 2.9	78.0 ± 2.6	81.6 ± 2.4	134.4 ± 2.3	170.8 ± 15.2	55.1 ± 1.7	61.6–63.6
Total (g kg^{-1} of DM)	141.1 ± 4.6	146.9 ± 3.9	116.8 ± 4.1	124.7 ± 4.9	193.0 ± 3.1	242.4 ± 20.8	78.5 ± 2.9	81.5–98.8
Taurine[c]	1.1 ± 0.1	1.4 ± 0.1	2.9 ± 0.5	3.8 ± 1.3	10.5 ± 0.6	13.6 ± 0.2	1.6 ± < 0.1	7.4 [23]
Ammonia[c]	4.8 ± 0.1	5.2 ± < 0.1	3.6 ± < 0.1	3.4 ± 0.1	6.1 ± < 0.1	7.6 ± 0.3	3.0 ± 0.2	–
Nitrogen-to-Protein ratio[d]	4.4	4.4	4.6	4.6	4.3	4.7	5.2	3.83 [19]
Carbon[e]	32.9	32.7	32.7	30.7	30.4	28.6	33.8	26.5 [13]
Hydrogen[e]	4.56	4.53	4.62	4.20	4.01	3.73	–	–
Nitrogen[e]	3.17	3.33	2.55	2.73	4.47	5.17	1.5	1.5–2.6 [1,19]

[a] The amino acids values are in terms of g kg^{-1} of DM. All amino acids analyses were carried out in duplicate with standard deviation except Tryptophan analysis.
[b] Wild *Saccharina latissima* harvested in July 2014 and dried at 30 °C, provided by SES (Norway).
[c] Taurine and ammonia were analyzed together with amino acids by HPLC.
[d] Nitrogen to protein ratio is total amino acids divided by Nitrogen.
[e] The element values are expressed in % of DM.

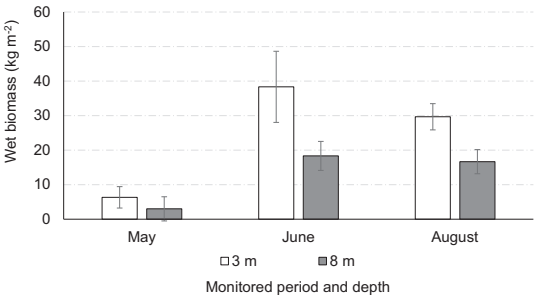


Fig. 1. Biomass growth of cultivated *S. latissima* deployed in February. Data are shown as means of three replicates ± standard deviation.

production than seaweed cultivated at 8 m depth at all sampling dates (Fig.1). This is in agreement with Handå et al. [14], which in a study of cultivation of *S. latissima* at 2, 5, and 8 m found that significantly longer seaweed plants were found at 2 m and 5 m depth.

A factor that can negatively affect seaweed growth is the settlement of epibionts such as bryozoans, filamentous microorganisms and larvae of barnacles. These epibionts have been shown to form brittle macroalgae resulting in defoliation during mechanical events such as storms and strong currents, and thereby loss of biomass [21]. Epibionts were observed on the samples harvested in June and August at both depths (see Fig. S2), indicating that erosion and/or lamina breakage caused the decrease in biomass production in August. A recent study of cultivated *S. latissima* deployed at the same location at Frøya showed that the typical bryozoans species were *Membranipora membranacea and Electra pilosa* [22]. This study also showed that seaweed grown at 15 m generally had less biofouling than seaweed grown at shallower water.

Other factors that affect seaweed growth are light and nutrient accessibility [23]. In our study, there is no reason to expect any difference in nutrient concentrations at 3 and 8 m. However, less light will be available at 8 m compared to 3 m.

Generally, the sugar content found in the algal tissue in the present study is close to those noted earlier for *S. latissima* [1,2] and L. *digitata* [3,19]. The observed annual changes in sugar content (Table 1) are in agreement with Handå et al. [14] which found 350, 470, and 150 g carbohydrates kg^{-1} DM in May, June, and August, respectively [14]. The low glucose and mannitol content in our August samples were probably due to the formation of epibionts, which reduce the rate of photosynthesis in the macroalgae [16] and also reduce the relative content of sugars.

Both cultivation depth and time significantly affected amino acid content in *S. latissima.* The highest level of amino acids was found in the 8 m August sample (Table 2) with 242.4 g kg^{-1} DM, which is approximately three times higher than the content in wild *S. latissima* (harvested in July 2014) [4,24]. The observed higher level of amino acids at 8 m than at 3 m can be related to reduced light. Cronin et al. [23] also stated that reduced light during deep cultivation of *D. ciliolate* and *S. filipendula* (Phaeophyceae) led to an increase in the protein content. The higher density of cultivated seaweed may also result in reduction of light, but also the considerable amounts of epibionts in August may have contributed to the high protein content. Seaweed with a high protein content may potentially be used as a feed ingredient or to

Table 3
Major mineral and trace elements determined by ICP-MS of cultivated *S. latissima* harvested at different time and depth.

	May 3 m	May 8 m	June 3 m	June 8 m	Aug 3 m	Aug 8 m	*Wild S. latissima*[a]	Literature values
Macro minerals[b]								
Potassium	80.5	82.1	52.4	81.1	32.1	33.9	58.9	25.5–109.0 [19,32]
Sodium	48.1	44.9	50.2	46.3	43.9	52.5	33.3	12.3–60.0 [19,32]
Calcium	7.9	8.6	27.9	24.2	89.8	109.3	17.8	1.3 [19]
Magnesium	7.2	7.1	7.5	7.1	6.3	7.0	6.0	7.9 [19]
Phosphorus	2.8	2.8	2.6	2.4	3.7	4.4	1.9	4.0–4.4 [19,32]
Sulfur	8.8	8.4	9.9	10.1	11.8	14.2	9.7	11.0–12.1 [19,32]
Trace minerals[c]								
Strontium	648.8	722.1	958.8	957.9	2032.1	2404.4	688.9	661 [13]
Silicon	548.1	536.1	947.6	1157.9	406.4	623.0	366.6	51.1 [19]
Boron	105.1	109.4	144.9	147.4	107.0	91.8	122.2	142.4 [19]
Iron	53.7	55.8	108.1	115.8	66.3	96.2	111.1	133.9 [19]
Arsenic	55.9	54.6	44.5	52.6	30.3	23.3	92.5	63.5–88 [13,34]
Zinc	44.7	54.7	40.9	73.7	46.3	58.3	33.7	44.4–81.0 [19,35]
Aluminum	17.9	18.6	107.0	74.7	43.9	74.3	58.8	106.5 [19]
Barium	8.4	9.4	24.5	34.7	11.8	14.2	10.4	39.3 [19]
Manganese	5.6	5.6	4.8	5.4	3.6	4.0	4.2	10.4 [19]
Copper	1.3	1.2	2.3	2.2	3.1	4.0	4.0	2.3 [19]
Vanadium	0.9	1.0	1.9	2.3	3.1	4.0	2.4	–
Cadmium	0.8	0.9	0.8	0.9	1.7	2.0	1.0	0.37 [34]
Nickel	0.2	0.5	0.6	0.6	1.0	1.3	1.4	0.6 [13]
Selenium	0.1	0.1	1.4	1.2	2.9	4.2	0.1	0.05 [35]
Molybdenum	0.2	0.2	0.3	0.3	0.5	0.6	0.3	0.4 [13]
Lead	0.1	0.1	4.5	1.6	0.2	0.2	1.2	0.46–1.5 [19,34]
Chromium	0.1	0.3	1.1	0.5	0.4	0.4	1.1	0.4–5.9 [19]
Cobalt	0.1	0.1	0.1	0.1	0.1	0.2	0.2	–
Silver	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	–
Mercury	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.02 [34]
Thallium	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	–
Halides[b]								
Chlorine	89.5	87.5	85.8	103.2	77.0	81.4	67.7	122.0 [33]
Bromine	0.6	0.7	1.2	1.4	1.4	1.5	0.6	–
Iodine	3.6	3.6	3.9	4.2	2.0	1.6	3.6	2.7–3.5 [13,35]
Total minerals[d]	157.1	155.9	153.1	174.1	190.6	224.8	155.4	64.8–192.9
Total halides	93.7	91.8	91.0	108.7	80.4	84.5	74.4	–
Total elements[e]	250.5	247.3	243.8	282.5	270.8	309.1	203.5	186.8–314.9
Ash	273	276	253	327	367	412	248	346 [19]

All analyses were carried out in single.

[a] Wild *Saccharina latissima* harvested in July 2014 and dried at 30 °C, provided by SES (Norway).

[b] The concentration of all macro minerals and halides are in g kg^{-1} of DM.

[c] The concentration trace minerals are in mg kg^{-1} of DM.

[d] Sum of 57 minerals analyzed by ICP-MS.

[e] Sum of 57 minerals and 3 halides analyzed by ICP-MS.

produce microbial growth media.

The dominant amino acids in cultivated *S. latissima* were aspartic acid, glutamic acid, and alanine, which are in agreement with literature data [4,24]. The unique taste and flavor called "umami" is attributed to the high levels of aspartic and glutamic acids [25]. Generally, the amino acids profiles changed over the season and with depth, which agrees with observations by other authors for several species; *S. latissima* [4], *L. digitata* [24], *U. lactuca* [26], and *J. rubens* [26]. Generally, amino acids profiles are an indication of the nutritional value of proteins in macroalgae. However, the availability of amino acids is dependent on protein digestibility [5].

Brown seaweed biomass has a high content of non-protein nitrogen, and by using the common conversion factor for crude protein (nitrogen content × 6.25), the protein content will be overestimated. Lourenço et al. [27] reported the average conversion factor for brown seaweed was 5.38. In this study, the relative nitrogen-to-protein factor for cultivated *S. latissima* was estimated to be in the range 4.3 to 4.7 (Table 2), lower than for the wild seaweed control of 5.2.

The high mineral content of *S. latissima* (Table 3) is a factor of concern in relation to feed and food applications. The concentrations of iodine in seafood are reported [28] in the range of 0.03 to 3.5 mg kg^{-1} wet weight. However, it is known that *S. latissima* has the potential to concentrate halides from seawater. So, the high content of iodine in the algae (1.6–4.2 g kg^{-1} DM) that is 300–13,000 times higher than in seafood is not surprising. The concentration of cadmium in the cultivated *S. latissima* is in the range of 0.8 to 2.0 mg kg^{-1} DM, which is below the limit of found in EU regulations (3.0 mg kg^{-1} DM) [28,29]. According to the Nordic Nutrition Recommendations, the recommended daily intake levels for adolescents are: iodine (150 μg day^{-1}), zinc (9000 μg day^{-1}), copper (9000 μg day^{-1}), selenium (60 μg day^{-1}), and iron (9000 μg day^{-1}) [30]. According to the Institute of Medicine (US) Panel on Micronutrients, the adult upper intake levels are: boron (20 mg day^{-1}), manganese (11 mg day^{-1}), molybdenum (2 mg day^{-1}), nickel (1 mg day^{-1}), vanadium (1.8 mg day^{-1}) and silicon (40 mg day^{-1}) [31]. The adequate intake of arsenic for young women and men is 0.002 to 0.003 mg day^{-1} and chromium for young women and men is 0.025 to 0.035 mg day^{-1}, respectively [31]. Thus, for many minerals and elements, a small portion of seaweed will cover the daily requirements, whereas a high intake over time could cause toxicity. *S. latissima* is invaluable source of these micro elements. However, the high iodine content in the alga can be a problem, but it should be noted that the high iodine content may be easily reduced (up to 70%) by boiling water treatment [28].

Accumulation of minerals in the *S. latissima* depends on several

factors, including season, site location, the salinity of seawater, current, sunlight, nitrogen and carbon availability, metabolic processes and age of the seaweed [32,33]. In addition to these factors, cultivation depth can be considered as a major factor, as shown in the current study. Taking into account that the concentrations of most of the amino acids and essential minerals for humans were increased from May to August and with depth, we suggest harvesting *S. latissima* at 8 m in late summer if the purpose is human consumption. *S. latissima* is a superior source of minerals in comparison with terrestrial biomass. As a result, it could also be used as a bio-fertilizers, ingredient in microbial growth media, mineral supplement in animal feeds, therapeutic products and other value added products [1,29,34,35].

5. Conclusion

Cultivated *S. latissima* shows differences in its biomass production and chemical composition depending on the depth of cultivation and time of harvest. The optimal harvest time (and depth) must be decided according to the desired application of the feedstock. The macroalgae deployed in February at 3 m depth and harvested in June contained the highest amount of sugars, which could be ideal for the production of fuels and chemicals via fermentation processes. The seaweed deployed at 8 m depth and harvested in August contained the highest proportion of amino acids, minerals, and phenolic compounds. This biomass could be considered for the production of nutritional supplements, feeds, microbial growth media or fertilizers.

Authors' contributions

SS, JF and SJH conceived and planned the experiments. LN and JF carried out cultivation and sample preparation. SS performed the analysis of all samples and interpreted the results. SS wrote the manuscript in consultation with LN, JF, LTM, MØ, and SJH. SJH supervised the project. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgement

This study was funded by a PhD fellowship from the Norwegian University of Life Sciences to S.S. and by the Research Council of Norway (Grant 239003; BIOFEED and Grant 237841; Foods of Norway). The authors thank two anonymous reviewers for their valuable comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.03.012.

References

[1] S. Sharma, S.J. Horn, Enzymatic saccharification of brown seaweed for production of fermentable sugars, Bioresour. Technol. 213 (2016) 155–161.
[2] C. Scullin, V. Stavila, A. Skarstad, J.D. Keasling, B.A. Simmons, S. Singh, Optimization of renewable pinene production from the conversion of macroalgae *Saccharina latissima*, Bioresour. Technol. 184 (2015) 415–420.
[3] J. Adams, A. Schmidt, J. Gallagher, The impact of sample preparation of the macroalgae Laminaria digitata on the production of the biofuels bioethanol and biomethane, J. Appl. Phycol. 27 (2014) 985–991.
[4] G.S. Marinho, S.L. Holdt, I. Angelidaki, Seasonal variations in the amino acid profile and protein nutritional value of *Saccharina latissima* cultivated in a commercial IMTA system, J. Appl. Phycol. 27 (2015) 1991–2000.
[5] C. Dawczynski, R. Schubert, G. Jahreis, Amino acids, fatty acids, and dietary fibre in edible seaweed products, Food Chem. 103 (2007) 891–899.
[6] FAO, The State of World Fisheries and Aquaculture, Food and Agriculture Organization of the United Nations, 2016.
[7] B. Santelices, The discovery of kelp forests in deep-water habitats of tropical regions, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 19163–19164.
[8] S.J. Horn, Bioenergy from Brown Seaweeds, (2000).
[9] S.J. Horn, I.M. Aasen, K. Østgaard, Ethanol production from seaweed extract, J. Ind. Microbiol. Biotechnol. 25 (2000) 249–254.
[10] S.K. Kim, Marine Glycobiology: Principles and Applications, CRC Press, 2016.
[11] M.C. Ravanal, S. Sharma, J. Gimpel, F.E. Reveco-Urzua, M. Øverland, S.J. Horn, M.E. Lienqueo, The role of alginate lyases in the enzymatic saccharification of brown macroalgae, Macrocystis pyrifera and *Saccharina latissima*, Algal Res. 26 (2017) 287–293.
[12] S.H.S. Khalafu, W.M. Wan Aida, S.J. Lim, M.Y. Maskat, Effects of deodorisation methods on volatile compounds, chemical properties and antioxidant activities of fucoidan isolated from brown seaweed (Sargassum sp.), Algal Res. 25 (2017) 507–515.
[13] P. Schiener, K. Black, M. Stanley, D. Green, The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, *Saccharina latissima* and Alaria esculenta, J. Appl. Phycol. 27 (2015) 363–373.
[14] A. Handå, S. Forbord, X. Wang, O.J. Broch, S.W. Dahle, T.R. Størseth, K.I. Reitan, Y. Olsen, J. Skjermo, Seasonal- and depth-dependent growth of cultivated kelp (*Saccharina latissima*) in close proximity to salmon (Salmo salar) aquaculture in Norway, Aquaculture 414–415 (2013) 191–201.
[15] P.K. Dayton, M.J. Tegner, P.B. Edwards, K.L. Riser, Temporal and spatial scales of kelp demography: the role of oceanographic climate, Ecol. Monogr. 69 (1999) 219–250.
[16] T.S. Tønder, A study of the seasonal variation in biochemical composition of *Saccharina latissima*, MSc Thesis, Norwegian University of Science and Technology, Trondheim, Norway, 2014.
[17] Commission Directive, 152/2009/EC of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, Off. J. Eur. Union 006 (2009) 3–132.
[18] O.A. Fawole, S.O. Amoo, A.R. Ndhlala, M.E. Light, J.F. Finnie, J. Van Staden, Anti-inflammatory, anticholinesterase, antioxidant and phytochemical properties of medicinal plants used for pain-related ailments in South Africa, J. Ethnopharmacol. 127 (2010) 235–241.
[19] T. Boderskov, P.S. Schmedes, A. Bruhn, M.B. Rasmussen, M.M. Nielsen, M.F. Pedersen, The effect of light and nutrient availability on growth, nitrogen, and pigment contents of *Saccharina latissima* (Phaeophyceae) grown in outdoor tanks, under natural variation of sunlight and temperature, during autumn and early winter in Denmark, J. Appl. Phycol. 28 (2016) 1153–1165.
[20] M.M. Nielsen, D. Manns, M. D'Este, D. Krause-Jensen, M.B. Rasmussen, M.M. Larsen, M. Alvarado-Morales, I. Angelidaki, A. Bruhn, Variation in biochemical composition of *Saccharina latissima* and Laminaria digitata along an estuarine salinity gradient in inner Danish waters, Algal Res. 13 (2016) 235–245.
[21] G. Sogn Andersen, H. Steen, H. Christie, S. Fredriksen, F.E. Moy, Seasonal patterns of sporophyte growth, fertility, fouling, and mortality of *Saccharina latissima* in Skagerrak, Norway: implications for forest recovery, Journal of Marine Biology 2011 (2011) 8.
[22] H. Førde, S. Forbord, A. Handå, J. Fossberg, J. Arff, G. Johnsen, K.I. Reitan, Development of bryozoan fouling on cultivated kelp (*Saccharina latissima*) in Norway, J. Appl. Phycol. 28 (2016) 1225–1234.
[23] G. Cronin, M.E. Hay, Effects of light and nutrient availability on the growth, secondary chemistry, and resistance to herbivory of two brown seaweeds, Oikos 77 (1996) 93–106.
[24] D. Manns, A.L. Deutschle, B. Saake, A.S. Meyer, Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae), RSC Adv. 4 (2014) 25736–25746.
[25] O.G. Mouritsen, L. Williams, R. Bjerregaard, L. Duelund, Seaweeds for umami flavour in the New Nordic Cuisine, Flavour 1 (2012) 4.
[26] H.M. Khairy, S.M. El-Shafay, Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt, Oceanologia 55 (2013) 435–452.
[27] S.O. Lourenço, E. Barbarino, J.C. De-Paula, L.O.d.S. Pereira, U.M.L. Marquez, Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds, Phycol. Res. 50 (2002) 233–241.
[28] A. Duinker, I.S. Roiha, H. Amlund, L. Dahl, E.-J. Lock, T. Kögel, A. Måge, B.T. Lunestad, Potential Risks Posed by Macroalgae for Application as Feed and Food - A Norwegian Perspective, National Institute of Nutrition and Seafood Research (NIFES), 2016.
[29] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation, J. Appl. Phycol. 23 (2011) 543–597.
[30] Nordic Nutrition Recommendations (NNR), Integrating Nutrition and Physical Activity, Norden Nordic Council of Ministers, Copenhagen, 2012.
[31] Institute of Medicine (US) Panel on Micronutrients, Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc, National Academies Press (US), Washington (DC), 2001.
[32] I. Sánchez-Rodríguez, M.A. Huerta-Diaz, E. Choumiline, O. Holguín-Quiñones, J.A. Zertuche-González, Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue, Environ. Pollut. 114 (2001) 145–160.
[33] P.T. Chan, P. Matanjun, Chemical composition and physicochemical properties of tropical red seaweed, Gracilaria changii, Food Chem. 221 (2017) 302–310.
[34] S. Kumar, D. Sahoo, A comprehensive analysis of alginate content and biochemical composition of leftover pulp from brown seaweed Sargassum wightii, Algal Res. 23 (2017) 233–239.
[35] P. Rupérez, Mineral content of edible marine seaweeds, Food Chem. 79 (2002) 23–26.

Seasonal and depth variations in the chemical composition of cultivated *Saccharina latissima*

Sandeep Sharma[1], Luiza Neves[2], Jon Funderud[2], Liv Torunn Mydland[3], Margareth Øverland[3] and Svein Jarle Horn[1,*]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

[2]Seaweed Energy Solutions AS, Trondheim, Norway

[3]Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, Ås, Norway

*Corresponding author. Tel.: +47 67232488. E-mail address: svein.horn@nmbu.no

Supplementary material

Figure S1

Figure S2

Table S1

Figure S1. Illustrated figure of frame setup deployed for *S. latissima* cultivation

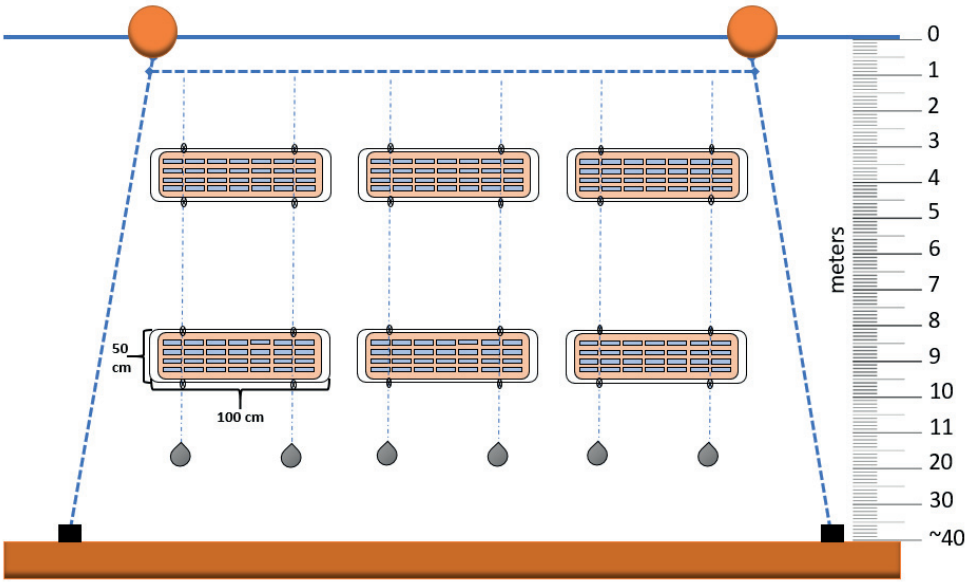


Figure S2. *Saccharina latissima* deployed in February and monitored in June 2015. a, frame with seaweed from 3m depth; b, formation of epibionts (circled) and other filamentous microorganisms on *S. latissima* (3m depth); c, frame with seaweed from 8m depth; d, formation of epibionts and other microorganisms (circled) on lamina part of *S. latissima* (8m depth).

Table S1. Statistical evaluation of cultivated *S. latissima* at different depth, seasons and interactions by using ANOVA two-way with replication.

Source of Variation	*df*	*F*	*P-value*
Growth			
Depth	1	23.2	< 0.01
Seasons	2	32.7	< 0.01
Depth x Seasons	2	3.7	0.056
Glucose			
Depth	1	241.6	< 0.01
Seasons	2	569.4	< 0.01
Depth x Seasons	2	26.8	< 0.01
Mannitol			
Depth	1	424.9	< 0.01
Seasons	2	637.4	< 0.01
Depth x Seasons	2	23.8	< 0.01
Uronic acids			
Depth	1	< 0.01	0.966
Seasons	2	14.0	< 0.01
Depth x Seasons	2	0.1	< 0.01
Fucose			
Depth	1	0.3	0.617
Seasons	2	59.1	< 0.01
Depth x Seasons	2	5.5	< 0.01
Essential amino acids			
Depth	1	21.4	< 0.01
Seasons	2	113.7	< 0.01
Depth x Seasons	2	6.2	0.035
Non-essential amino acids			
Depth	1	16.4	< 0.01
Seasons	2	146.1	< 0.01
Depth x Seasons	2	8.9	0.016
Ash			
Depth	1	105.3	< 0.01
Seasons	2	324.5	< 0.01
Depth x Seasons	2	27.1	< 0.01

df: degree of freedom, F: F-distribution

Paper II

Bioresource Technology 213 (2016) 155–161

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enzymatic saccharification of brown seaweed for production of fermentable sugars

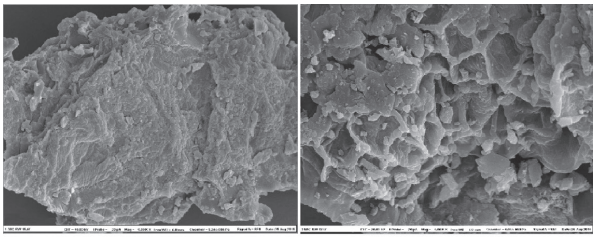
Sandeep Sharma, Svein Jarle Horn *

Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P.O. Box 5003, N-1432 Ås, Norway

HIGHLIGHTS

- *Saccharina latissima* can be efficiently hydrolysed to fermentable sugars.
- High drying temperatures negatively affect enzymatic saccharification yield.
- The efficiency of a cellulase cocktail was improved by inclusion of alginate lyase.
- At 25% solid loading a sugar concentration of 74 g/L was achieved.

GRAPHICAL ABSTRACT



Seaweed before saccharification | Seaweed residue after saccharification

ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 19 February 2016
Accepted 20 February 2016
Available online 27 February 2016

Keywords:
Mannitol
Laminarin
Biorefining
Macroalgae
Kelp

ABSTRACT

This study shows that high drying temperatures negatively affect the enzymatic saccharification yield of the brown seaweed *Saccharina latissima*. The optimal drying temperature of the seaweed in terms of enzymatic sugar release was found to be 30 °C. The enzymatic saccharification process was optimized by investigating factors such as kinetics of sugar release, enzyme dose, solid loading and different blend ratios of cellulases and an alginate lyase. It was found that the seaweed biomass could be efficiently hydrolysed to fermentable sugars using a commercial cellulase cocktail. The inclusion of a mono-component alginate lyase was shown to improve the performance of the enzyme blend, in particular at high solid loadings. At 25% dry matter loading a combined glucose and mannitol concentration of 74 g/L was achieved.

1. Introduction

In the emerging bio-economy, fossil resources will be substituted by biomass for the production of renewable fuels and chemicals. In particular, biomass rich in carbohydrates has been in focus as a basis for a "sugar-platform" type of biorefinery, where one of the main challenges is saccharification of the biomass to fermentable sugars (Sharma et al., 2015). Seaweed biomass has a high content of carbohydrates and offer major advantages, such as no requirement for agricultural land, no need of fertilizer supplements, no need of fresh water, and high growth yields (Kraan, 2013).

The dominant group of seaweed along the Norwegian coast are brown seaweed such as *Saccharina latissimi*, containing high levels of the carbohydrates laminarin, mannitol and alginate (Horn, 2009; Peinado et al., 2014). Laminarin is a glucose polymer consisting of a (1,3),-β-D-glucan backbone with β (1,6) branches, while mannitol is a sugar alcohol. Alginate is a linear copolymer composed of (1,4)-β-D-mannuronic acid (M) and (1,4)-α-L-guluronic acid (G) (Leal et al., 2008). Laminarin and mannitol are storage carbohydrates that accumulates in the seaweed during the light season, while alginate is a structural component with little annual variation. Regarding brown seaweed as a source of fermentable sugars, laminarin and

* Corresponding author. Tel.: +47 67232488.
E-mail address: svein.horn@nmbu.no (S.J. Horn).

http://dx.doi.org/10.1016/j.biortech.2016.02.090
0960-8524/

mannitol has been shown to be good fermentation substrates for several microorganisms (Horn et al., 2000a,b), while alginate fermentation is challenging and seems to require genetically engineered microorganisms (Wargacki et al., 2012; Enquist-Newman et al., 2014).

Compared to lignocellulosic biomass, seaweed does not contain lignin and is mechanically less robust. This make seaweed an easier biomass to process to fermentable sugars. The most common pretreatments used prior to biochemical processing are size reduction treatment (Manns et al., 2015), chemical treatment (Adams et al., 2009), hydrothermal (Vivekanand et al., 2012), ultrasonic and alkaline treatment (Park et al., 2009). Enzymatic saccharification of brown seaweed has been carried out using enzymes such as laminarinases, alginate lyases, and commercial cellulase blends (Adams et al., 2009; Hou et al., 2015; Kim et al., 2011; Manns et al., 2015; Ravanal et al., 2016; Scullin et al., 2015; Tan and Lee, 2014). In these previous studies, saccharification is typically performed using dried seaweed at relatively low substrate concentrations.

The purpose of this study was threefold: (a) investigate the effect of drying temperature on enzymatic sugar release from seaweed, (b) optimize dose and blends of cellulases and alginate lyase to achieve high saccharification yields, and (c) study enzymatic saccharification at high dry matter loading to achieve high concentrations of fermentable sugars.

2. Methods

2.1. Seaweed biomass

Natural growing *Saccharina latissima* were harvested in the Trondheimsfjord (63°N, 10°E) in July 2014, by Seaweed Energy Solutions (Trondheim, Norway). The fresh and unwashed seaweed biomass was shipped frozen to the Norwegian University of Life Science in Ås and stored −20 °C. Part of the seaweed was grinded and divided into three samples. One sample was dried under ambient temperature (21 °C), the second was freeze dried at −20 °C for 72 h, and the third sample was split and dried in a conventional oven at different temperatures (30–105 °C). The dried samples were further milled in a Retsch Ultra Centrifugal Mill ZM 200 (Haan, Germany) and screened through a 1 mm sieve. The milled seaweed samples were stored in a desiccator at room temperature until use.

2.2. Chemicals and enzyme preparations

All HPLC standards and chemicals (i.e. glucose, mannitol, fucose, xylose, sulphuric acid, galacturonic acid, laminarin, β-glucan, sodium borate, carbazole, sodium azide and ethanol) were analytical grade procured from Sigma Aldrich (USA), Merck (Germany) or Megazyme (USA).

The enzyme preparations used in this study were CellicCTec2 (Novozyme A/S, Denmark) and an alginate lyase from *Flavobacterium multivorum* (Sigma Aldrich, Norway).

2.3. Chemical composition of seaweed biomass

The carbohydrate composition of *S. latissima* was analyzed applying the two steps sulphuric acid hydrolysis method developed by NREL (Sluiter et al., 2004). Dried grounded samples and sugar recovery standards (glucose, xylose, mannitol, fucose, and galacturonic acid) was exposed to 72% (w/w) H_2SO_4 at 30 °C for exactly 1 h and then further hydrolyzed in 4% (w/w) H_2SO_4 at 121 °C in an autoclave for 40 min. After complete hydrolysis, the hydrolysates were filtered through Duran sintered filter (4 μm, Sigma Aldrich) and diluted with deionised water. The hydrolysates were stored at −20 °C.

2.4. Enzymatic saccharification

For most of the experiments, the enzymatic treatment of *S. latissima* was carried out at 5% (w/v) substrate concentration in 100 mM citric acid-sodium phosphate buffer (pH of 6.3) at 1400 rpm using an Eppendorf thermomixer. For the high substrate loading experiments, a maximum dry matter (DM) content of 25% (w/v) was applied. The incubation temperature of enzymatic treatment was 50 °C unless otherwise noted. For hydrolysis experiments, 6.3 mg protein of CellicCTec2 (Novozymes A/S, Denmark) and 0.7 mg protein of alginate lyase (Sigma Aldrich, Germany) per g of DM of seaweed were used, unless otherwise noted. The protein concentration of CellicCTec2 was 82 mg of protein per mL of enzyme preparation. The protein content was determined using the Bradford method (Bradford, 1976). During the enzymatic treatment samples were taken and enzymes deactivated by incubating at 100 °C for 15 min. Prior to sugar analyses the samples were filtered through a 0.2 μm centrifuge filter (Merck Millipore, Germany) and diluted appropriately in deionized water.

All experiments were performed in triplicate and the standard deviations are illustrated as error bars in the figures.

2.5. Analysis

2.5.1. Moisture and ash content

Moisture content of untreated *S. latissima* samples was determined by a Metrohmn Karl Fischer titrator (Florida, USA) (Agger et al., 2014). Ash content was determined by a NREL method (Sluiter et al., 2008), weighing samples before and after heating in a furnace at temperature of 550 °C for 8 h.

2.5.2. Carbohydrate analysis

Samples containing soluble monomeric sugars were filtered through a 0.22 μm micro-filter before subjecting them to high performance liquid chromatography (HPLC). The concentrations of monomeric sugars were analyzed by a HPLC system equipped with refractive index detector. The separation was made using a 300 × 7.8 mm Rezex ROA-Organic Acid H+ analytical column fitted with cation-H cartridge guard column at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL per min. Calibration standards were made for glucose, xylose, fucose and mannitol at 0.10, 0.25, 0.50, 1.00, and 2.50 g/L. For identification and quantification of the sugars, the Dionex software Chromeleon 7.2 was used.

Quantification of total uronic acids (mannuronic and guluronic acids) present in hydrolysate of *S. latissima* was carried out by a spectrophotometric method using absorbance at 550 nm and with Carbazole as indicator (Bitter and Muir, 1962). Prior to the spectrophotometric analysis samples were filtered and diluted 10 times. 50 μl of the samples were added 25 mM sodium borate in concentrated sulphuric acid. The concentrated blend was incubated for 10 min at 100 °C in a water bath and then cooled down to room temperature on ice. Then 0.125% (w/v) carbazole made up in ethanol was added into the mixture and incubated for 10 min at 100 °C. After incubation absorbance of the cooled sample mixtures and standards (GalA) were measured at 550 nm. Total uronic acids were determined by applying a calibration curve of galacturonic acid (GalA) with recovery factor correction.

Total nitrogen and carbon content was determined by Dumas combustion (AOAC 990.03).

3. Results and discussion

3.1. Compositional analysis

Compositional analysis showed that the *S. latissima* biomass used in this study consisted of 23.0% glucose (laminarin and cellulose), 20.5% mannitol, 20.0% uronic acids (alginate), 5.2% fucose (fucoidan) and 24.8% ash on a dry weight basis (Sluiter et al., 2008, 2004). The original DM content of the fresh seaweed was 25.3%. Similar chemical composition of ash and total carbohydrates has previously been reported for *Laminaria digitata* and *S. latissima* (Peinado et al., 2014; Schiener et al., 2015). Elemental analysis showed a carbon content of 33.8% and a nitrogen content of 1.5%.

3.2. Effect of drying on saccharification yield

To produce a homogenous substrate that could be used for small-scale enzymatic studies, the seaweed material was dried and milled to pass a sieve of 1 mm. As drying might affect the enzymatic accessibility of the substrate, it was decided to investigate the effect of drying temperature on enzymatic saccharification. The theory is that the amount of hydrogen bonds between biopolymers may increase in the drying process, and these bonds are not broken during rewetting, making the dried biomass less accessible for enzymatic degradation. A range of different drying temperatures were applied and compared to freeze dried and wet *S. latissima*.

To test the effect of drying on enzymatic saccharification, we applied an enzyme blend of alginate lyase and CellicCTec2 in a ratio of 1:1 on a protein basis, which is similar to the blend ratio used in a recent study (Manns et al., 2015). Fig. 1 shows the concentration of glucose and mannitol released from the *S. latissima* samples. The highest glucose and mannitol yields were 225 and 141 g/kg of DM of *S. latissima*, respectively, achieved for the 30 °C oven dried biomass. This corresponds to 97.8% and 68.9% of theoretical yields for glucose and mannitol, respectively. For the wet sample, the concentration of glucose and mannitol was 22.3% and 12.4% lower than the 30 °C sample, respectively. This lower saccharification yield may be attributed to a larger particle size of the wet substrate. Similar low yields were achieved for the freeze dried, 100 °C and 105 °C dried *S. latissima* samples. Overall, the enzymatic sugar yield decreased with increasing drying temperature, but not as severely as seen for drying of lignocellulosic materials and its associated hornification reactions. It should be noted that glucose release was more affected by drying temperature than mannitol release. This is probably because glucose originates from biopolymers (cellulose and laminarin) that may form hydrogen bonds during drying and become less accessible for enzymes. Even though drying of seaweed is very common in the literature, very little data exist on the effect of drying on subsequent processing. According to previous work (Adams et al., 2014), freeze-dried *L. digitata* offered higher fermentation yield than oven-dried seaweed at 70 °C.

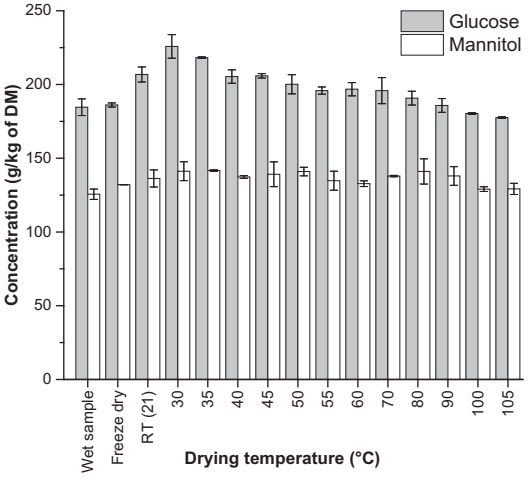


Fig. 1. Effect of drying conditions on enzymatic saccharification. Bars show concentration of glucose and mannitol obtained after enzymatic saccharification with CellicCTec2 and alginate lyase. Enzyme dosage was 7 mg of protein per g of DM, pH 6.3, temperature at 37 °C for 3 h, then 50 °C for 17 h. Each data point represents the mean value of independent triplicates ± SD.

3.3. Optimization of enzymatic saccharification

3.3.1. Effect of enzyme dose

For the following experiments on enzymatic saccharification, the 30 °C dried *S. latissima* sample (Section 3.2) was used as a substrate. Initially, pure β-glucan (5% w/v) was incubated with CellicCTec2 (5 mg of protein per g of substrate, pH 6.3, 50 °C for 10 h) to check for the presence of endo-(1,3(4)-β-glucanase activity in the enzyme preparation. The substrate was completely hydrolysed by CellicCTec2, confirming the presence of endo-(1,3(4)-β-glucanase activity, which is needed to degrade *S. latissima* laminarin. Optimal pH and temperature of the alginate lyase (from *Flavobacterium multivolume*) applied in this study is 6.3 and 37 °C (Ochi et al., 1995). Since CellicCTec2 has an optimum around 50 °C, blends of alginate lyase and CellicCTec2 needed to be run at 37 °C or in two steps where the temperature is increased to 50 °C after alginate lyase has done its job first at 37 °C. Initial time course experiments (data not shown) with alginate lyase and CellicCTec2 blends showed that 3 h incubation time at 37 °C was adequate to take advantage of alginate lyase activity. This protocol was applied in the following experiments.

Enzymatic saccharification of *S. latissima* at enzyme loadings ranging from 1 to 10 mg of protein per g of DM was investigated

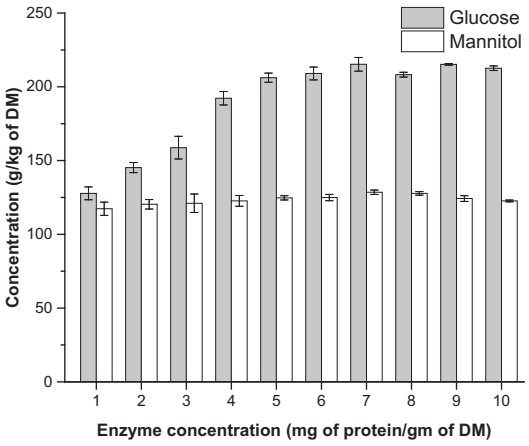


Fig. 2. Concentration (g/kg of DM of *S. latissima*) of glucose and mannitol as function of enzyme loading (50% alginate lyase and 50% CellicCTec2 on a protein basis). Conditions: pH 6.3, temperature at 37 °C for 3 h, then 50 °C for 17 h. Each data point represents the mean value of independent triplicates ± SD.

(see Fig 2). For the lowest enzyme dose (1 mg of protein per g of DM) the glucose release after 20 h of incubation was 127 g per kg of DM. Increasing doses of enzymes lead to increased release of glucose with a maximum of 225 g per kg of DM when using 7 mg of protein per g of DM. Higher enzyme doses did not increase the final glucose yield. Mannitol release also increased with enzyme dose up to 7 mg of protein per g of DM, but to a much lesser extent than glucose (increased from 117.3 to 128.5 g/kg of DM). Thus, 7 mg of protein per g of DM was found to be the optimum enzyme dose.

To investigate possible problems with microbial contamination during the enzymatic hydrolysis reactions, a set of experiments were carried in the presence of 2% (w/v) sodium azide. For 20 h incubations there were no significant differences between reactions with and without azide inclusion.

3.3.2. Optimization of alginate lyase – Cellulase ratio

In the above described experiments we used a 50–50% ratio of alginate lyase and CellicCTec2, which is similar to what have been used previously (Manns et al., 2015). To determine optimal enzyme ratio for saccharification of *S. latissima* a range of experiments were carried out where the amount of alginate lyase was varied from 0% to 100%, while keeping the total enzyme dose constant at 7 mg/g of DM. Fig. 3 shows the sugar release as function of CellicCTec2/alginate lyase ratio. A clear trend was that total saccharification yield increased with the amount of CellicCTec2 in the blend up to 90% CellicCTec2 and 10% alginate lyase. Thus, CellicCTec2 could alone efficiently degrade the seaweed, but a 10% incorporation of alginate lyase seems to be beneficial. Interestingly, alginate lyase alone could assist the release of some glucose and mannitol. This might be due to release of some free glucose and mannitol embedded in the alginate matrix, or due to endogenous glucanase activity. Such autolytic enzyme activity has been observed previously for degradation of *L. digitata* (Hou et al., 2015).

3.3.3. Saccharification kinetics

The saccharification process was further investigated by looking at the kinetics of the degradation by CellicCTec2 alone or CellicCTec2 with 10% alginate lyase. Fig. 4A shows that after 4 h of hydrolysis the glucose yield by CellicCTec2 was 25.6% higher than the enzyme blend containing alginate lyase. This was probably due to the higher temperature (50 °C) in the CellicCTec2 reaction during the first 3 h, and thus faster laminarin hydrolysis. The reaction with 10% alginate lyase was run at 37 °C for 3 h to make sure that the alginate lyase was active. Interestingly, the reaction with 10% alginate lyase showed highest glucose release between 5 and 8 h of incubation, where the biggest difference was after 7 h (14.3% higher than CellicCTec2 alone). The final glucose yield after 20 h of incubation was almost similar for the two reactions. For mannitol release (Fig. 4B), the kinetics was somewhat different, where the initial release was similar for the first 4 h. From 5 to 20 h the reaction with 10% alginate lyase yielded higher release of mannitol. After 7 h of incubation, the total sugar release was 13.8% higher in the reaction with alginate lyase present. Thus, the inclusion of alginate lyase makes the sugar release faster, and also results in a higher total sugar release, mainly due to higher concentrations of mannitol.

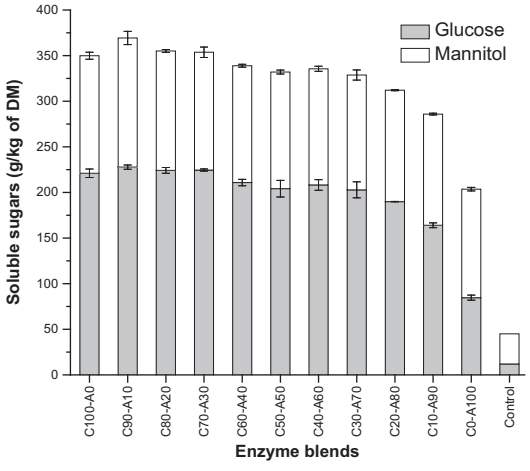


Fig. 3. Enzymatic sugar release (g/kg of DM of *S. latissima*) of glucose and mannitol as function of enzyme blend ratios (alginate lyase and CellicCTec2). Total enzyme dose was 7 mg of protein per g of DM. Conditions: pH 6.3, temperature at 37 °C for 3 h, then 50 °C for 17. Each data point represents the mean value of independent triplicates ± SD.

3.4. Saccharification at high solids loading

All the above experiments were carried out at 5% DM concentration. Obviously, for practical applications, it is important to achieve high concentrations of fermentable sugars after a saccharification process. This means running the saccharification process at high DM concentrations. Thus, a set of experiments were run where the DM concentration was increased from 5% to 25% (see Fig. 5). It is clearly seen that increasing DM concentrations lead to a decrease in sugar yield. This is similar to observations done for saccharification of lignocellulosic materials (Kristensen et al., 2009). Another trend is that yields in the reactions with alginate lyase present (Fig. 5A) is always higher than the reactions with only CellicCTec2 (Fig. 5B). The biggest difference was found for the 25% DM reaction at the 24 h time point where the alginate lyse containing reactions gave 26% higher sugar yield. For longer incubation times the yields for all reactions got more similar. The highest concentration of sugars was achieved in the 25% DM reaction with alginate lyase that reached an end concentration of 74 g/L of total sugars (see Fig. 6). Hydrolysis experiments with *L. digitata* (with an extremely high glucan content of 69%) at the same solid loading reported a final glucose concentration of 119 g/L (Alvarado-Morales et al., 2015). It should also be noted that for practical applications running saccharification at 25% DM might require drying of the seaweed. Natural *S. latissima* typically contain from 15% to 25% DM, meaning still lower sugar concentrations based on wet feedstock. Sugar concentrations could be increased if alginate could be hydrolysed to uronic acids. This approach has been explored recently applying genetically modified organisms able to hydrolyse alginate and ferment uronic acids. Using a genetically engineered *Escherichia coli* Wargacki et al. (2012) was able to produce 0.28 g ethanol/g DM of seaweed. In another study (Enquist-Newman et al., 2014) it was demonstrated that a genetically engineered *Saccharomyces cerevisiae* could utilize mannitol and alginate monomers for ethanol production. This shows that developing enzyme cocktails for total saccharification of alginate could increase the yields of fermentation products from seaweed.

It should be mentioned that CellicCTec2 belongs to a new generation of cellulase cocktails that contains auxiliary enzymes called lytic polysaccharide monooxygenases (LPMO), which cleave cellulose by oxidative mechanism using molecular oxygen and an electron donor (Müller et al., 2015; Vaaje-Kolstad et al., 2010). Thus, the presence of oxygen and an electron donor is important to take full advantage of these enzyme preparations. However, the role of LPMOs in saccharification of macroalgae has not been studied. To

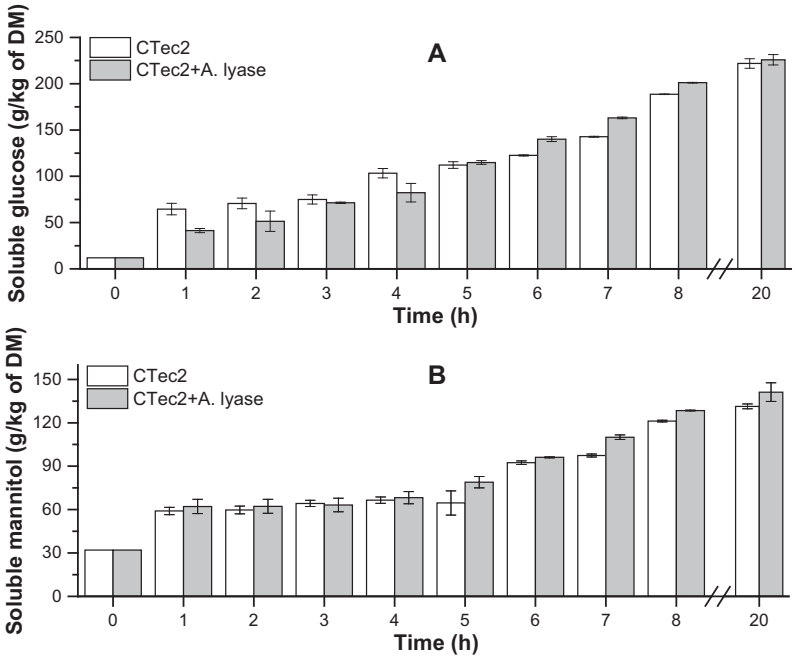


Fig. 4. Kinetics of glucose (A) and mannitol (B) release from seaweed by CellicCTec2 + alginate lyase and CellicCTec2. Concentration of protein was 7 mg of protein per g of DM and inclusion of alginate lyase was 10%. Conditions: pH 6.3, for reaction with alginate lyase temperature was at 37 °C for 3 h, then 50 °C for 17; for CellicTec2 only reaction temperature was at 50 °C for 20 h. Each data point represents the mean value of independent triplicates ± SD.

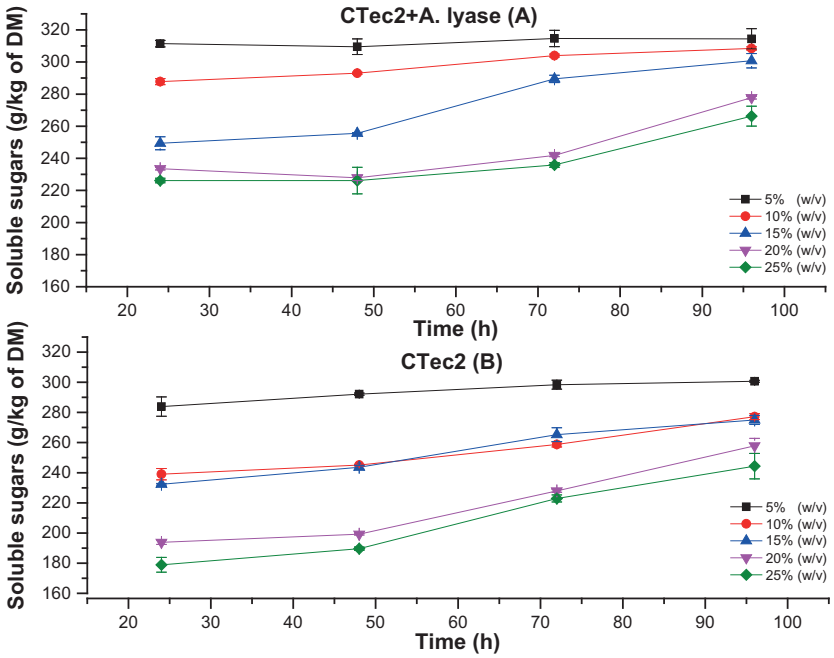


Fig. 5. Saccharification of seaweed at different biomass loadings (w/v) by CellicCTec2 + alginate lyase (A) and CellicCTec2 (B). Concentration of protein was 7 mg of protein per g of DM and inclusion of alginate lyase was 10%. Conditions: pH 6.3, for reaction with alginate lyase temperature was at 37 °C for 3 h, then 50 °C for 17; for CellicTec2 reaction temperature at 50 °C for 20).

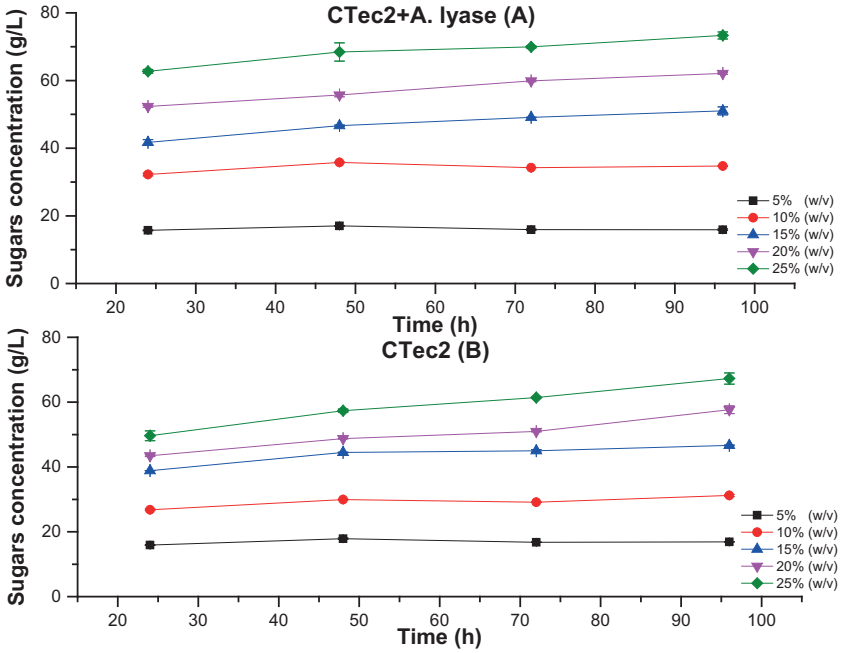


Fig. 6. Total sugar concentration as a function of different solid loading (w/v) for reactions with CellicCTec2 + alginate lyase (A) and CellicCTec2 (B). Same conditions as in Fig. 5.

investigate this a small set of saccharification experiments were carried out under aerobic and anaerobic conditions with and without an electron donor (Müller et al., 2015). After 20 h of incubation, no significant differences were seen between the reactions (data not shown), indicating that LPMO activity is not beneficial for degradation of seaweed, probably due to its low content of cellulose. This points to the more general issue that CellicCTec2 is not designed to degrade seaweed, but agricultural lignocellulosic feedstocks like corn stover. Thus, the potential for designing new more efficient enzyme cocktails for seaweed saccharification is large. Our results indicate that it would be important to include an alginate lyase that could work at 50 °C. The achieved sugar concentration of 74 g/L is too low to economically produce bioethanol (Galbe et al., 2007), but such a hydrolysate could be applied as a fermentation medium to produce higher value chemicals or single cell protein. Additionally, and in contrast to lignocellulosic hydrolysates, seaweed hydrolysates also contain phosphorus, nitrogen and minerals that are important ingredients in fermentation media.

4. Conclusions

This study shows that high drying temperatures negatively affect the enzymatic saccharification yield of *S. latissima*. The seaweed biomass could be efficiently hydrolysed into fermentable sugars using a commercial cellulase cocktail. The inclusion of a mono-component alginate lyase was shown to improve the performance of the enzyme blend, in particular at high solid loadings. At 25% DM loading a combined glucose and mannitol concentration of 74 g/L was achieved.

Acknowledgements

This work was funded by a PhD fellowship from the Norwegian University of Life Sciences to S.S. and by the Research Council of Norway (Grant 239003; BIOFEED – Novel salmon feed by integrated bioprocessing of non-food biomass).

References

Adams, J., Gallagher, J., Donnison, I., 2009. Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. J. Appl. Phycol. 21 (5), 569–574.

Adams, J., Schmidt, A., Gallagher, J., 2014. The impact of sample preparation of the macroalgae *Laminaria digitata* on the production of the biofuels bioethanol and biomethane. J. Appl. Phycol. 27 (2), 985–991.

Agger, J., Nilsen, P., Eijsink, V.H., Horn, S., 2014. On the determination of water content in biomass processing. Bioenergy Res. 7 (1), 442–449.

Alvarado-Morales, M., Gunnarsson, I.B., Fotidis, I.A., Vasilakou, E., Lyberatos, G., Angelidaki, I., 2015. *Laminaria digitata* as a potential carbon source for succinic acid and bioenergy production in a biorefinery perspective. Algal Res. 9, 126–132.

Bitter, T., Muir, H.M., 1962. A modified uronic acid carbazole reaction. Anal. Biochem. 4, 330–334.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1), 248–254.

Enquist-Newman, M., Faust, A.M.E., Bravo, D.D., Santos, C.N.S., Raisner, R.M., Hanel, A., Sarvabhowman, P., Le, C., Regitsky, D.D., Cooper, S.R., Peereboom, L., Clark, A., Martinez, Y., Goldsmith, J., Cho, M.Y., Donohoue, P.D., Luo, L., Lamberson, B., Tamrakar, P., Kim, E.J., Villari, J.L., Gill, A., Tripathi, S.A., Karamchedu, P., Paredes, C.J., Rajgarhia, V., Kotlar, H.K., Bailey, R.B., Miller, D.J., Ohler, N.L., Swimmer, C., Yoshikuni, Y., 2014. Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. Nature 505 (7482), 239–243.

Galbe, M., Sassner, P., Wingren, A., Zacchi, G., 2007. Process engineering economics of bioethanol production. In: Olsson, L. (Ed.), Biofuels, vol. 108. Springer, Berlin Heidelberg, pp. 303–327.

Horn, S.J., 2009. Seaweed Biofuels: Production of Biogas and Bioethanol from Brown Macroalgae. VDM Publishing.

Horn, S.J., Aasen, I.M., Østgaard, K., 2000a. Production of ethanol from mannitol by Zymobacter palmae. J. Ind. Microbiol. Biotechnol. 24 (1), 51–57.
Horn, S.J., Aasen, I.M., Østgaard, K., 2000b. Ethanol production from seaweed extract. J. Ind. Microbiol. Biotechnol. 25 (5), 249–254.
Hou, X., Hansen, J.H., Bjerre, A.-B., 2015. Integrated bioethanol and protein production from brown seaweed *Laminaria digitata*. Bioresour. Technol. 197, 310–317.
Kim, N.-J., Li, H., Jung, K., Chang, H.N., Lee, P.C., 2011. Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. Bioresour. Technol. 102 (16), 7466–7469.
Kraan, S., 2013. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. Mitig. Adapt. Strat. Glob. Change 18 (1), 27–46.
Kristensen, J.B., Felby, C., Jørgensen, H., 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol. Biofuels 2 (1), 1–10.
Leal, D., Matsuhiro, B., Rossi, M., Caruso, F., 2008. FT-IR spectra of alginic acid block fractions in three species of brown seaweeds. Carbohydr. Res. 343 (2), 308–316.
Manns, D., Andersen, S., Saake, B., Meyer, A., 2015. Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment. J. Appl. Phycol., 1–8
Müller, G., Várnai, A., Johansen, K.S., Eijsink, V.G.H., Horn, S.J., 2015. Harnessing the potential of LPMO-containing cellulase cocktails poses new demands on processing conditions. Biotechnol. Biofuels 8, 187.
Ochi, Y., Takeuchi, T., Murata, K., Kawabata, Y., Kusakabe, I., 1995. A simple method for preparation of poly-mannuronate using poly-guluronate lyase. Biosci. Biotechnol. Biochem. 59 (8), 1560–1561.
Park, J.-I., Lee, J., Sim, S.J., Lee, J.-H., 2009. Production of hydrogen from marine macro-algae biomass using anaerobic sewage sludge microflora. Biotechnol. Bioprocess Eng. 14 (3), 307–315.
Peinado, I., Girón, J., Koutsidis, G., Ames, J.M., 2014. Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds. Food Res. Int. 66, 36–44.
Ravanal, M.C., Pezoa-Conte, R., von Schoultz, S., Hemming, J., Salazar, O., Anugwom, I., Jogunola, O., Mäki-Arvela, P., Willför, S., Mikkola, J.-P., Lienqueo, M.E., 2016. Comparison of different types of pretreatment and enzymatic saccharification of *Macrocystis pyrifera* for the production of biofuel. Algal Res. 13, 141–147.
Schiener, P., Black, K., Stanley, M., Green, D., 2015. The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta*. J. Appl. Phycol. 27 (1), 363–373.
Scullin, C., Stavila, V., Skarstad, A., Keasling, J.D., Simmons, B.A., Singh, S., 2015. Optimization of renewable pinene production from the conversion of macroalgae *Saccharina latissima*. Bioresour. Technol. 184, 415–420.
Sharma, S., Kumar, R., Gaur, R., Agrawal, R., Gupta, R.P., Tuli, D.K., Das, B., 2015. Pilot scale study on steam explosion and mass balance for higher sugar recovery from rice straw. Bioresour. Technol. 175, 350–357.
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2004. Determination of Structural Carbohydrates and Lignin in Biomass [Electronic Resource]. National Renewable Energy Laboratory, Laboratory Analytical Procedure (LAP).
Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Determination of Ash in Biomass [Electronic Resource]. National Renewable Energy Laboratory, Laboratory Analytical Procedure (LAP).
Tan, I.S., Lee, K.T., 2014. Enzymatic hydrolysis and fermentation of seaweed solid wastes for bioethanol production: an optimization study. Energy 78, 53–62.
Vaaje-Kolstad, G., Westereng, B., Horn, S.J., Liu, Z., Zhai, H., Sørlie, M., Eijsink, V.G.H., 2010. An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. Science 330 (6001), 219–222.
Vivekanand, V., Eijsink, V.G., Horn, S.J., 2012. Biogas production from the brown seaweed *Saccharina latissima*: thermal pretreatment and codigestion with wheat straw. J. Appl. Phycol. 24 (5), 1295–1301.
Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D., Yoshikuni, Y., 2012. An engineered microbial platform for direct biofuel production from brown macroalgae. Science 335 (6066), 308–313.

Paper III

Algal Research 26 (2017) 287–293

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

The role of alginate lyases in the enzymatic saccharification of brown macroalgae, *Macrocystis pyrifera* and *Saccharina latissima*

María Cristina Ravanal[a,b,1], Sandeep Sharma[c,1], Javier Gimpel[a], Felipe E. Reveco-Urzua[d], Margareth Øverland[d], Svein Jarle Horn[c], María Elena Lienqueo[a,*]

[a] Institute for Cell Dynamics and Biotechnology (ICDB), Centre for Biochemical Engineering and Biotechnology (CeBIB), Department of Chemical Engineering and Biotechnology, Universidad de Chile, Beauchef 850, Santiago, Chile
[b] Center for Bioinformatics and Integrative Biology, Facultad de Ciencias Biológicas, Universidad Andrés Bello, Avenida República 239, Santiago, Chile
[c] Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Aas, Norway
[d] Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, 1433 Aas, Norway

ARTICLE INFO

Keywords:
Brown algae
Macrocystis pyrifera
Saccharina latissima
Pretreatment
Alginate lyase
Microbial ingredients

ABSTRACT

In this work, we have compared the carbohydrate content and the enzymatic saccharification of the brown algae *Macrocystis pyrifera* from Chile and *Saccharina latissima* from Norway. *M. pyrifera* contained 40% mannitol, 31% uronic acids and 15% glucose, while *S. latissima* contained 37% glucose, 30% uronic acids and 25% mannitol. Thus, the ratio between mannitol and glucose was much higher for *M. pyrifera*. Acid pre-treated and untreated algae were enzymatically saccharified in two steps; first at pH 7.5, 25 °C for 12 h with a blend of recombinant alginate and oligoalginate lyases, then the pH was changed to 5.2, a commercial cellulase cocktail was added and saccharification continued at 50 °C for 4 h. These experiments showed that the use of recombinant alginate lyases and oligoalginate lyases in combination with cellulases increased the release of glucose from untreated seaweed. However, for saccharification of pretreated algae, only cellulases were needed to achieve high glucose yields. Finally, it was shown that brown algae hydrolysates could be used as a growth medium to produce microbial ingredients, such as *Candida utilis* yeast.

1. Introduction

Marine macroalgae such as brown algae are currently receiving a lot of attention as a source of renewable biomass for the production of a wide range of products, including food, nutraceutical, pharmaceutical, fine chemical, biofuel, fertilizer, as well as feed [1]. Macroalgae are among the fastest growing organisms in the world, and produce large amounts of biomass without the use of fresh water, agricultural land, fertilizer, or pesticides [2]. The cold-temperate growth conditions in Norway and Chile offer large potential for cultivation of macroalgae for the production of sustainable biomass. The brown algae *Macrocystis pyrifera* and *Saccharina latissima* are phylogenetically closely related and belong to the family Laminariaceae [3]. *M. pyrifera* is the dominant brown macroalgae found at the coast of south of Chile, and the largest and fastest-growing seaweed species on earth [4]. *S. latissima* is one of the main seaweeds found along the Norwegian coast and preparations for large scale cultivation are currently underway [5,6]. The main carbohydrates found in brown algae are alginate, cellulose, laminarin, fucoidan and mannitol. Alginate is a *co*-polymer of the uronic acids α-L-guluronate (G) and β-D-mannuronate (M), being arranged as homopolymeric G blocks, homopolymeric M blocks, alternating GM blocks or random heteropolymeric G/M stretches [7]. Both laminarin and cellulose are polysaccharides composed of glucose; laminarin consists of a β(1 → 3)-glucan backbone with β(1 → 6)-branches, while cellulose is a linear chain of β-1,4-linked D-glucose units that shows an x-ray diffraction pattern similar to that of native cellulose from land plants [8]. These polysaccharides from algae can be converted to fermentable sugars by enzymatic saccharification [9,10] that can be further used as a carbon and energy source in the fermentation of yeast. The main enzymes used for saccharification of cellulose are endoglucanases (EC 3.2.1.4), which randomly cleave internal glucosidic linkages of this polysaccharide, cellobiohydrolases (EC 3.2.1.91); β-glucosidase, which hydrolyze cellobiose to glucose (EC 3.2.1.21); and lytic polysaccharide monooxygenases (LPMOs), which cleave cellulose by an oxidative mechanism [11]. Mannuronate lyases (EC 4.2.2.3) and guluronate lyases (EC 4.2.2.11) cleave alginate within the chain producing unsaturated uronic acid oligomers with a double bond between C4 and C5 at the non-reducing end. Oligoalginate lyases (EC 4.2.2.-) cleave these

* Corresponding author.
E-mail address: mlienque@ing.uchile.cl (M.E. Lienqueo).
[1] These authors contribute equally to this work.

http://dx.doi.org/10.1016/j.algal.2017.08.012
Received 23 December 2016; Received in revised form 3 August 2017; Accepted 3 August 2017
Available online 09 August 2017

oligomers to produce monosaccharides (unsaturated uronate) [12]. The enzymatic saccharification of the macroalgae was carried out using a combination of a commercial cellulase cocktail and recombinant alginate lyases and oligoalginate lyases [13].

The aims of this study were: (a) comparison of the chemical composition of the brown algae *M. pyrifera* from Chile and *S. latissima* from Norway, (b) evaluation of five different recombinant alginate lyases for their effect on enzymatic liberation of glucose from the macroalgae, and (c) evaluation of the growth of the yeast *Candida utilis* in algae hydrolysate for the production of microbial ingredients.

2. Materials and methods

2.1. Feedstock and compositional analysis

Two brown algae species from the family Laminariaceae were evaluated in this study performed at the Norwegian University of Life Sciences: *M. pyrifera* from Chile and *S. latissima* from Norway. *M. pyrifera* samples were identified based on blade and holdfast morphology (Buschmann A., personal communication). *S. latissima* was identified based on overall morphology, and verified by genetic analysis (Seaweed Energy Innovations, personal communication). Both algae are closely related phylogenetically [1] and they were collected in the summer months of each country. *M. pyrifera* were collected by scuba diving 30 km southwest of Puerto Montt in Chile. The seaweed was harvested in January 2015 (average temperature: 16.5 °C), by Professor Buschmann, University of Los Lagos, Chile. *S. latissima* was harvested in the Trondheims fjord in Norway In July 2014 (average temperature 15.0 °C), by Seaweed Energy Solutions. The fresh and unwashed seaweed was shipped, frozen and stored at − 20 °C until use. Both seaweed were dried at room temperature, grounded by an electric grinder and passed through a 1-mm sieve. Elemental analysis of the untreated, sulfuric acid pre-treated, and enzymatic residue of *S. latissima* and *M. pyrifera* samples were performed by a Vario EL cube elemental analyzer (Hanau, Germany). All samples were dried at 105 °C and homogenized before analysis.

The carbohydrate composition of *M. pyrifera* and *S. latissima* were analyzed using a two-step sulphuric acid hydrolysis method [14] modified from the one developed by NREL [15]. Dried grounded samples were exposed to 72% (*w*/w) H_2SO_4 at 30 °C for exactly 1 h and then further hydrolyzed in 4% (*w*/w) sulphuric acid at 121 °C in an autoclave for 40 min. After complete hydrolysis, the hydrolysates were filtered with syringe filter (0.22 μm). The supernatant was analyzed for sugar content by High Performance Liquid Chromatography (HPLC) and High Performance Anion Exchange Chromatography (HPAEC).

2.2. Pre-treatments and enzymatic saccharification

For the pretreatment, 30 g of dry *M. pyrifera* and *S. latissima* were pretreated in a 1:3 ratio (wt) with 2% (*v*/v) sulfuric acid diluted in milli-Q water. Algae and solvent were placed into tubes followed by incubation in a thermostated oil bath at 120 °C for 1 h. After incubation, the tubes were removed from the oil bath and the algae were first washed six times using milli-Q water, then two times with McIlvaine Buffers [16] at pH 7.5 (for neutralization), later centrifuged and finally dried at 37 °C for 3 days.

For enzymatic saccharification, 80 mg dry algae (both native and pretreated with diluted sulphuric acid) were incubated at 25 °C for 12 h in a 0.45 M McIlvaine buffer pH 7.5 with 0.2 mg protein of recombinant alginate lyases (alginate lyase H from *Pseudoalteromonas elyakovii* [17], alginate lyase G from *Pseudoalteromonas sp.* [18], alginate lyase I from *Microbulbifer sp.* 6532A [19], alginate lyase K from *Sphingomonas sp.* [20] or alginate lyase L from *Sphingomonas sp.* [21]), and 0.2 mg protein of a blend of recombinant oligoalginatelyases; oligoalginatolyase Atu from *Agrobacterium tumefaciens* strain C58 [12], oligoalginatelyase Q from *eubacterium* SCB49 and oligoalginatelyase O from *Lewinellapersica*. Then, the pH was adjusted to 5.2 using 6 M of HCl and 0.4 mg protein from cellulases (CellicCTec2 from Novozymes) was added to the samples which were incubated at 50 °C for 4 h. Thus, a 50:50% ratio of alginases (25% alginate lyase + 25% oligoalginate lyase) and cellulases on a protein-basis was applied. The biomass loading in the reaction was 5% (*w*/*v*). Four control incubations were performed; a blank without enzymes, a control with a commercial alginate lyase (from Sigma) and two controls with only cellulases (Celluclast (from Novozymes) + β-glucosidase (from Megazyme) or CellicCTec2 (from Novozymes). In each control, 0.4 mg cellulases were used. After enzymatic saccharification, the samples were incubated at 100 °C for 10 min, centrifuged and filtered through a 0.2-μm centrifuge filter. All experiments were performed in duplicate.

2.3. Analysis of soluble sugars

The release of monomeric sugars was quantified by a HPLC. The separation was made using a 300 × 7.8-mm Rezex ROA-Organic Acid H^+ analytical column fitted with a cation-H cartridge guard column at 65 °C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min. Calibration standards were made for glucose, xylose, mannitol and fucose in the concentration range 0.1 to 2.5 g/l. Dionex software Chromeleon 7.2 had been used for identification and quantification of the sugars.

Quantification of uronic acid (mannuronic acid and guluronic acid) were performed by HPAEC. The mobile phase was 0.1 M NaOH and 1 M NaAc at a flow rate of 0.25 ml/min. The standards of guluronic acid and mannuronic acid (Carbosynth, Berkshire, UK) were made in the concentration range 0.1 to 2.5 g/l.

2.4. Ash content

Ash content of *M. pyrifera* and *S. latissima*, both native and pretreated, were determined by a NREL method [22], weighing samples before and after heating in a furnace at temperature of 600 °C for 4 h. This analysis was performed in duplicates.

2.5. Recombinant alginate lyases

Previously, an *E. coli* expression protocol has been developed for the expression of alginate lyases from polysaccharide lyases families; PL7 and PL18 according to Carbohydrate-Active enzymes database [23]. These alginate lyases are highly active and soluble enzyme preparations [13]. These same parameters were utilized for expressing the enzymes in this work. Endo-alginate lyases were expressed using vector pETG41 containing an N-terminal His-tag/Maltose-binding protein (MBP) partner [24]. Exo-alginate lyases were cloned into vector pNative, which has no fusion protein partner (modified pet22b + with a stop codon before the His-tag, Merck, MA, USA). After chemical transformation of BL21 (DE3) Rosetta2, the complete mix was transferred to grow overnight in 7 ml Luria-Bertani (LB) liquid medium plus 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The OD600 of the cultures were then recorded, and the expression cultures were inoculated to a 0.05 final OD600. Expression cultures consisted of 20 ml of ZYM-5052 autoinduction medium [25] in 60 ml Erlenmeyer flasks, grown at 25 °C, and 200 RPM orbital shaking for 14 h. 20 ml of the autoinduction cultures were centrifuge and resuspended in 6 ml Tris-buffered saline (TBS) pH 7.5. Samples were sonicated three times for 45 s alternating with incubations on ice, with a 1/8″ tip at 20 W power using a XL-2000 sonicator (Misonix, Farmingdale, NY, USA). Samples were then centrifuged at 9289 g for 10 min at 4 °C. The clear supernatant was then recovered and used for the enzymatic assays. A description of each recombinant alginate lyase is shown in Supplementary Table S2.

2.6. Fermentation for microbial ingredient production

To obtain hydrolysates as suitable media for fermentation experiments *M. pyrifera* (pretreated) and *S. latissima* (native) were enzymatically saccharified, using the best conditions determing in 2.2. The saccharification was carried out at 15% Dry Mass (DM) loading in a 0.45 M McIlvaine buffer pH 7.5 with alginate lyase G for pretreated *M. pyrifera* and with alginate lyase H for native *S. latissima* at 25 °C for 12 h. Then the pH was changed to 5.2 using 6 M of HCl and the samples were incubated with 0.4 mg protein of CellicCTec2 at 50 °C for 4 h.

The obtained hydrolysates were centrifuged and the resulting liquid fractions were used for the yeast fermentation experiments. To increase the nitrogen content of the hydrolysates, ammonium sulphate was added as per yeast elemental composition ($C_1H_{1.64}N_{0.16}O_{0.52}P_{0.01}S_{0.005}$). A preculture of *Candida utilis* was grown in yeast extract-peptone-dextrose (YPD) broth at 30 °C for 24 h. Fermented hydrolysates were produced by combining 2 ml of the pre-culture with 20 ml of hydrolysate media followed by incubation at 30 °C for 42 h. After that, the fermented hydrolysates were centrifuged and the resulting pellets were washed once using milli-Q water. The pellets were then dried in a freeze drier (0,042 mbar to − 80 °C) for two days prior to nitrogen quantification by Kjeldahl nitrogen analyzer (Kjeltec™ 8400, Sweden).

2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) of native, pretreated and saccharified *S. latissima* and *M. pyrifera* were analyzed by a Zeiss Evo (50EP, UK) instrument at excitation voltage of 10 kV. The dried samples were prepared by sputter coating with a thin layer of gold-palladium. The micrographs were taken at 5000 × magnification.

3. Results and discussion

3.1. Chemical characterization of macroalgal biomass

Carbohydrates, protein and ash were quantified for the algal biomasses (Fig. 1). The values are given as dehydrated monomers (by applying a conversion factor for dehydration on polymerization: glucose, fucose, guluronic, mannuronic = 0.9; xylose = 0.88; and mannitol = 1). Supplementary Table S1 shows the concentration of carbohydrates (g/kg of DM) and percentage of carbohydrates from *S. latissima* and *M. pyrifera* biomass without pretreatment and pretreatment with 2% sulphuric acid. The composition of *S. latissima* biomass used in this study consisted of 37% glucose and 25% mannitol. For *M. pyrifera* biomass consisted of 15% glucose and 40% mannitol. Fig. 1 shows that the pretreatment changed the composition of both algae biomasses. The pretreated *S. latissima* contained 247 g glucose/kg of DM and 94.3 g alginate/kg of DM. Pretreated *M. pyrifera* contained 217.8 g glucose/kg of DM, 167.9 g alginate/kg of DM. The main carbohydrate lost during pretreatment was mannitol. This sugar alcohol is free in algae or it is part of laminarin [18]. The pre-treatment reduced the ash content in both algae biomass, and in particular for *M. pyrifera*, where the ash content became 23 times lower. Interestingly, the pretreatment led to a more than doubling of the crude protein content in *S. latissima*.

Pretreatment changed the α-L-guluronic acid (G) and β-D-mannuronic acid (M) ratio. The alginates are made up of different blocks of guluronic and mannuronic acids, the blocks are referred to as MM blocks, GG blocks and MG blocks [7]. The change of G:M ratio could be because the MM blocks are more exposed to pretreatment and, therefore, the amount of M decreases after the pretreatment

M. pyrifera and *S. latissima* are closely related phylogenetically, belonging to the family Laminariaceae [1]; however, their chemical composition differs and both respond differently to the applied pretreatment.

3.2. Enzymatic saccharification and released of glucose

Native and pretreated *S. latissima* and *M. pyrifera* were saccharified with recombinant alginate lyases plus oligoalginate lyases at 25 °C for 12 h; then a cellulase cocktail was added to the reactions and further incubated for 4 h at 50 °C. Five different recombinant alginate lyases were used to compare their effect in the release of glucose. Figs. 2 and 3 show the enzymatically released glucose from native algae and pretreatment algae, respectively. The highest glucose release (209 g/kg DM; 94.3% of theoretical yield) for *S. latissima* (native) was obtained

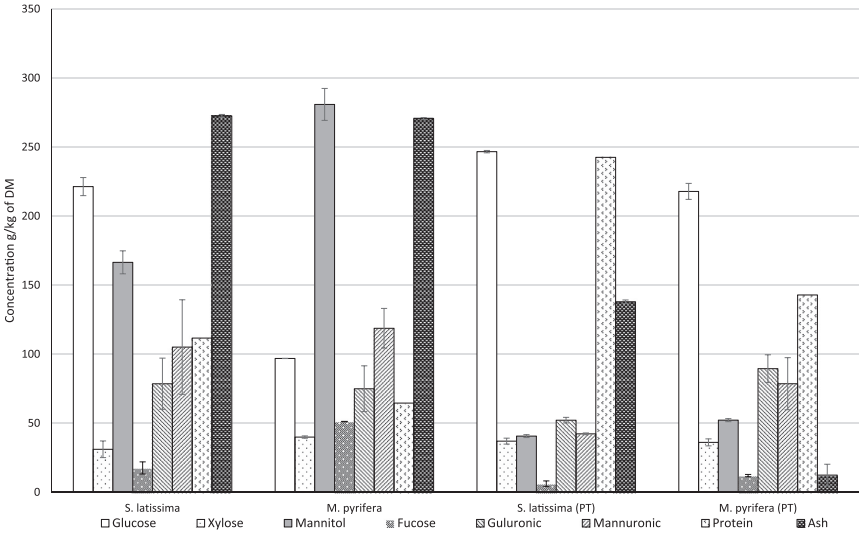


Fig. 1. Carbohydrate, protein and ash contents (g/Kg of dry mass) in *S. latissima* and *M. pyrifera* native and pretreated form. Monosaccharide content was analyzed after of acid hydrolysis by high performance High-performance Liquid Chromatography (HPLC) and High-performance Anion Exchange Chromatography (HPAEC). Each data point represents the mean value of independent duplicates ± SD.

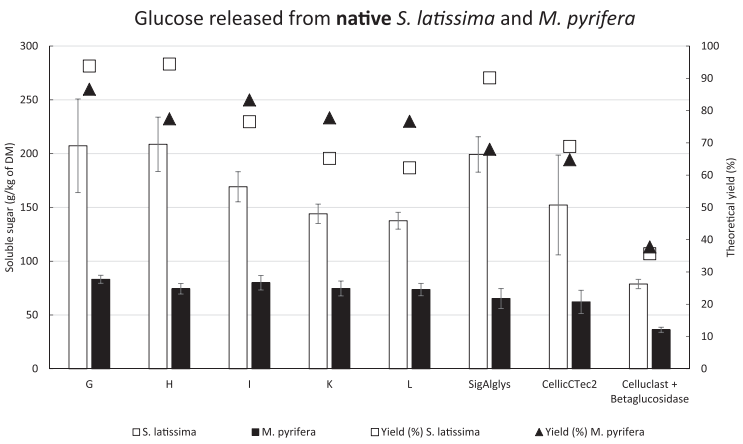


Fig. 2. Enzymatic glucose released from native *S. latissima* and *M. pyrifera*. Bars show concentration of glucose obtained after enzymatic saccharification with alginases (G: Aly-SJ02, H: AlyPEEC, I: AlgmsP, K: A1-II and L: A1-II′) and cellulases as described in methods, and glucose was quantified by High-performance Liquid Chromatography (HPLC). Each data point represents the mean value of independent duplicates ± SD. Symbols □ and ▲ represent percentage of theoretical yield of glucose released after enzymatic hydrolysis of *S. latissima* and *M. pyrifera*, respectively.

using the recombinant alginate lyase H (from *Pseudoalteromonas elyakovii*) plus oligoalginate lyases and CellicCTec2. However, the reactions with alginate lyase G and the Sigma alginate lyase showed glucose release at similar high levels. When the *S. latissima* was saccharified with only CellicCTec2, it released 68.8% of the glucose. The reactions with the alginate lyases I, K and L showed similar levels of glucose release as CellicCTec2 alone. Thus, only two of the recombinant lyases increased the glucose release. For native *M. pyrifera*, the results were very different. As expected, the glucose concentrations obtained after enzymatic processing of *M. pyrifera* were much lower than for *S. latissima* (Fig. 2) as *M. pyrifera* has lower initial content of glucose. It has also been shown that all alginate lyases had a positive effect on glucose release, where the highest yield was achieved with alginate lyase G.

Fig. 3 shows that the saccharification results were very different for pretreated algae. In this case, *M. pyrifera* also showed high yields of glucose release. This reflects the high glucose content in the pretreated *M. pyrifera* (more than twice the concentration found in the untreated *M. pyrifera*; Fig. 1). The highest glucose yield (207.2 g/kg of algae, 95.1%) was obtained with the recombinant alginate lyase G (from *Pseudoalteromonas sp.*) plus oligoalginate lyases and CellicCTec2. However, all incubations showed high yields, even the reaction with CellicCtec2 alone. This was also the case when *S. latissima* was degraded, showing that the cellulase cocktail alone can efficiently saccharify pretreated algae. Figs. 2 and 3 show that alginate lyases are only helpful for saccharification of native macroalgae, while the effect of this enzyme is limited in pretreated algae. Interestingly, enzymes G and H seemed to be better suited for saccharification of non-pretreated *S. latissima*. This could be due to their particular preference for the different glycosidic bonds within alginate, although all *endo*-lyases in this work where selected for their flexibility to cleave GG, MM, and GM glycosidic bonds (Table S2). On the other hand, these two endo-lyases are the only ones from the Polysaccharide Lyase family 18 (Table S2), which could be related to a structural or catalytic feature that confers them an advantage for degrading alginate molecules that are not readily exposed (non-pretreated biomass), although this would have to be tested through structure/function studies.

Additionally, mannitol released from native as well as pretreated *S. latissima* and *M. pyrifera* with the theoretical yields are shown in Figs. S2 and S3.

Usually, saccharification of brown macroalgae is carried out by a two-step process of biomass treatment. In a first step, biomass is processed with acid and subsequently treated with enzymes. Acid for the first step is generally sulfuric acid [26–31], but cases of pretreatment with hydrochloric acid have also been reported [32]. In general, conditions for acid hydrolysis with sulfuric acid vary between 0.1 and 2 wt % at 120–121 °C for 30 to 60 min. Considering that the acid is not

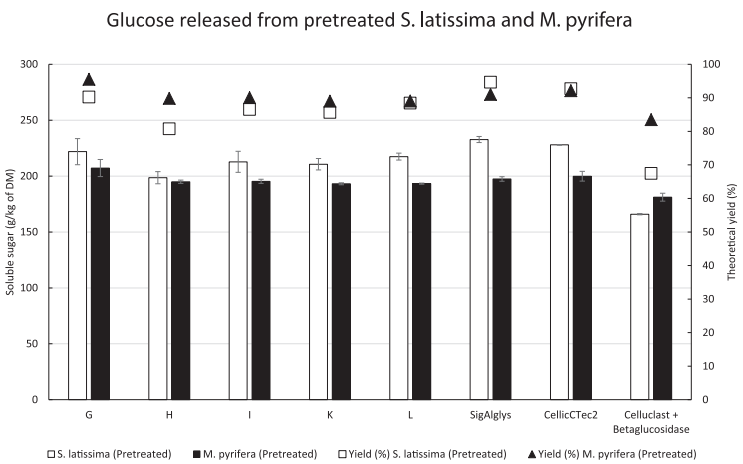


Fig. 3. Enzymatic glucose released from pretreated *S. latissima* and *M. pyrifera*. Bars show concentration of glucose obtained after enzymatic saccharification with alginases (G: Aly-SJ02, H: AlyPEEC, I: AlgmsP, K: A1-II and L: A1-II′) and cellulases as described in methods, and glucose was quantified by High-performance Liquid Chromatography (HPLC). Each data point represents the mean value of independent duplicates ± SD. Symbols □ and ▲ and represent percentage of theoretical yield of glucose released after enzymatic hydrolysis of *S. latissima* and *M. pyrifera*, respectively.

Table 1
Elemental and ash (in % weight dry mass basis) analysis of brown macroalgae in native form, sulfuric pretreated, residue and supernatant after enzymatic saccharification.

Brown algae	Carbon	Hydrogen	Nitrogen	Sulfur	Oxygen[a]	Ash
S. latissima	29.8	4.9	1.6	1.1	35.3	27.2
M. pyrifera pretreated	38.8	5.4	2.3	0.7	51.6	1.3
S. latissima (*Residue after saccharification*)	18.3	2.8	1.7	0.6	33.8	42.8
M. pyrifera pretreated (*Residue after saccharification*)	35.8	4.5	4.0	0.5	37.8	17.3
S. latissima (*Supernatant after saccharification*)	48.5	NA	1.0	0.1	NA	4.8
M. pyrifera pretreated (*Supernatant after saccharification*)	45.1	NA	6.4	0.06	NA	5.6

NA is not applicable.
[a] Calculated oxygen value by subtracting all other elements by 100%.

selective, it is difficult to identify the optimal parameters for acidic saccharification and to control the experimental conditions. As a result, there is a non-negligible risk of either partial release of some monosaccharides, or production of toxins derived from the toughness of the treatment. Even so, acid hydrolysis is still chosen as a good option to be used in combination with enzymes and to reduce their use.

After pretreatment, hydrolysis of the seaweed biomass is necessary to release the sugars locked up in the structural polysaccharides. Enzymatic treatment of brown algae is best reported for Laminaria sp. For instance, cell walls of *Laminaria hyperborea* and *Laminaria digitata* were successfully saccharified using laminarinase after acid pre-treatment [33], releasing 92.5% glucose/g algae. On the other hand, to address heterogeneity of algal carbohydrates, multienzymatic preparations containing predominantly cellulase and cellobiase were successfully applied. That is the case for *Laminaria japonica*, which was saccharified with a mixture of fungal cellulase and cellobiase [30]. In the case of *Saccharina japonica*, acid pretreatment and Thermamyl 120 l, a commercial cocktail containing a thermostable amylase, released 70% of total carbohydrates in the biomass [27], but when pretreated with acid and then with cellulase and glucosidase, 84% of carbohydrates were extracted [31].

With the appearance of the biorefinery concept, in the last few years, much interest has been placed on recovery of non glucose-producing carbohydrates. That is how the use of alginate lyases combined with oligoalginate lyases became popular in order to take advantage of monomers of alginate. The alginate lyases have been applied either individually [26,34] or combined with cellulolytic enzymes [14,35–36]. The new generation of cellulolytic enzymes represented by Cellic CTec2 and Cellic HTec2 from Novozymes, have also contributed to increase the recovery of carbohydrate components present in seaweed algae polysaccharides, such as, laminarin and cellulose [14,37]). The use of alginate lyase improves the cellulase catalyzed degradation of laminarin and cellulose in the material by selective removal of alginate, as was suggested by results showing that cellulase alone released only half of the available glucose [14].

Table 1 shows elemental and ash (in % weight dry basis) analysis of brown macroalgae in native form, sulfuric pretreated, residue and supernatant after enzymatic saccharification. The increase in carbon, hydrogen, nitrogen and partial sulfur content have been observed in the pretreated macroalgal compared with the native one, resulting from the release of the ash in the hydrolysate. It must be noted that the carbon and hydrogen content of *S. latissima*, in the residue after enzymatic saccharification was less than the native *S. latissima* due to the release of sugars during saccharification. However, the trend of elements present in *M. pyrifera* in the residue after saccharification was similar to the *S. latissima*. Additionally, Supplementary Fig. S1 shows the mass balance and process scheme for *S. latissima* and *M. pyrifera* (pretreated by sulfuric acid) during enzymatic saccharification. This Fig. S1 shows the amount of dry matter and carbon in the substrates (*S. latissima* and *M. pyrifera* pretreated), the enzyme hydrolysates (solubilized) and the solid residues.

3.3. Scanning electron microscopy analysis

Scanning electron microscopy is an analytical technique that has great versatility and importance when studying biomass structure. The results of this analysis are shown in Fig. 4. The images of particles of *S. latissima* native (SL), *S. latissima* pretreated (SLP), enzymatic residue of *S. latissima* (SLS), *M. pyrifera* native (MP), *M. pyrifera* pretreated (MPP), and enzymatic residue of *M. pyrifera* pretreated (MPPS) were obtained by Scanning electron microscopy (SEM) at 5000 ×. The analysis of the images shows a contrast between before and after enzymatic saccharification, also a contrast between untreated and pretreated and between algae species, where the saccharified biomass has a rough surface with a lot of holes demonstrating enzymatic removal of parts of the biomass, possibly glucose was removed. As expected, the results show greater

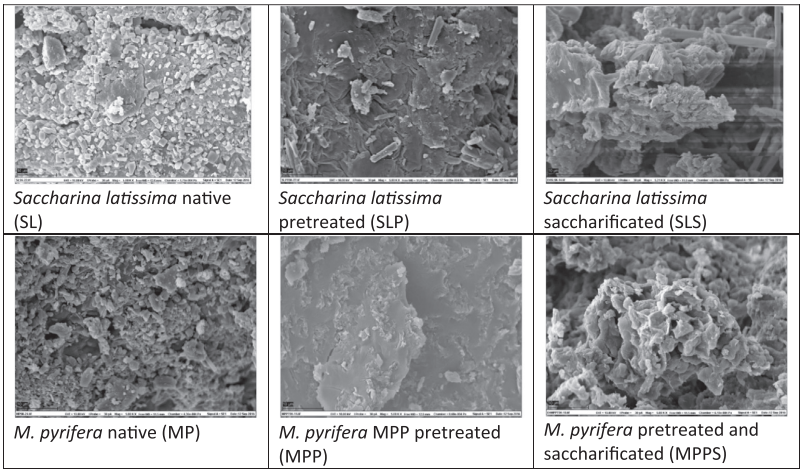


Fig. 4. Images by Scanning electron microscopy (SEM) at 5000 × of: (a) particles of *S. latissima* native (SL), (b) *S. latissima* pretreated (SLP), (c) enzymatic residue of *S. latissima* (saccharified, SLS), *M. pyrifera* native (MP), *M. pyrifera* pretreated (MPP), and enzymatic residue of *M. pyrifera* pretreated (saccharified, MPPS).

Table 2
Candida utilis production from *S. latissima* native and *M. pyrifera* pretreated with 2% sulphuric acid and both brown algae enzymatic hydrolysate.

Brown algae	Initial concentration of glucose (g/l) C_0	Concentration of yeast (g/l)	Yeast nitrogen (% of DM)	Nitrogen to protein factor	Yeast protein (% of DM*)
S. latissima native	15.4 ± 3.1	6.3 ± 0.2	5.72	5.38	30.77
M. pyrifera pretreated with 2% sulphuric acid	16.3 ± 1.6	8.2 ± 0.1	3.34	5.38	17.96

* DM: Dry Mass.

degradation of fibers when the algae were saccharified with different enzymes.

3.4. Biomass loading and S. latissima and M. pyrifera fermentation

Enzymatic hydrolysates were prepared by taking high solid content (15%) of pretreated *M. pyrifera* and native *S. latissima* in order to investigate the potential use of these macroalgae as sources of sugars, nitrogen and other nutrients in the production of yeast. The yeast *Candida utilis*, which has been shown to be a suitable protein source for farmed fish [38], was successfully grown aerobically for 48 h in these hydrolysates. The initial concentration of glucose, final concentration of yeast biomass and its protein content are shown in the Table 2. Algal biomass composed of non protein nitrogen (NPN) such as pigments, nucleic acids, nucleotides, inorganic nitrates (NO_3), ammonium (NH_4) and free amino acids could be produce an overestimation of their protein content value. The nitrogen-to-protein conversion factor of 5.38 for brown algae has been taken for this study [39]. Table 2 shows that 6.3 and 8.2 g of dried yeast per liter were produced in the *S. latissima* (native) and *M. pyrifera* (pretreated) hydrolysates, respectively. The concentration of yeast and protein content in the yeast is highly dependent upon the available carbon and nitrogen in the algae hydrolysates. While the pretreated *M. pyrifera* had a higher protein content than the native *S. latissima*, the yeast produced in the *S. latissima* hydrolysates achieved a much higher nitrogen content. This could be due to the pre-treatment and washing of *M. pyrifera* that may have removed easily available nitrogen.

4. Conclusions

This work shows that the chemical composition of brown algae from Chile (*M. pyrifera*) and Norway (*S. latissima*) differs; in particular, in the ratio between mannitol and glucose, which is higher in *M. pyrifera* than in *S. latissima*. The two brown algae species responded differently to acid pretreatment and washing, in when, for instance, most of the ash was removed from *M. pyrifera* while the ash content S. *latissima* was only slightly reduced. The use of recombinant alginate lyases and oligoalginate lyases in combination with cellulases increased the glucose release from untreated macroalgae. However, for saccharification of pretreated algae only cellulases were needed to achieve high glucose release. Finally, it was shown that algae hydrolysates could be used as growth medium for *Candida utilis* culture for the production of microbial ingredients for use in the diets for farmed fish.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2017.08.012.

Acknowledgement

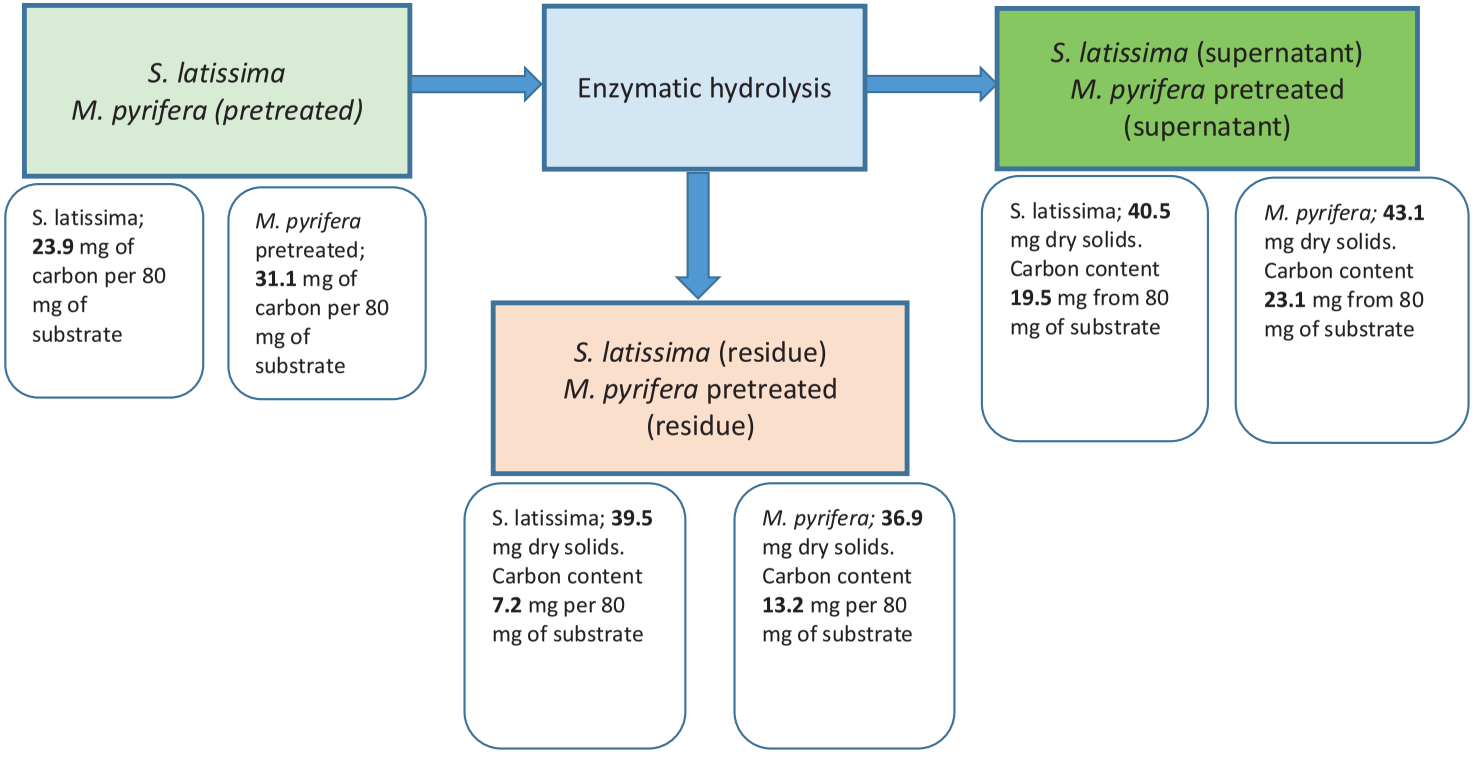
This research was supported by a grant from CONICYT-REDES150014, Algal Biorefinery Network: Foods of Norway-ICDB of Chile and Centre for Biotechnology and Bioengineering (CeBiB) FB-0001, and Foods of Norway, a Centre for Research-based Innovation (the Research Council of Norway; grant no. 237841/030) and BIOFEED-Novel salmon feed by integrated bioprocessing of non-food biomass (the Research Council of Norway; grant no. 239003). We are thankful to Professor Alejandro Buschmann from the University of Los Lagos and Seaweed Energy Solutions AS from Throndheiem, Norway for providing the macroalgae M. pyrifera and S.latissima used in this study.

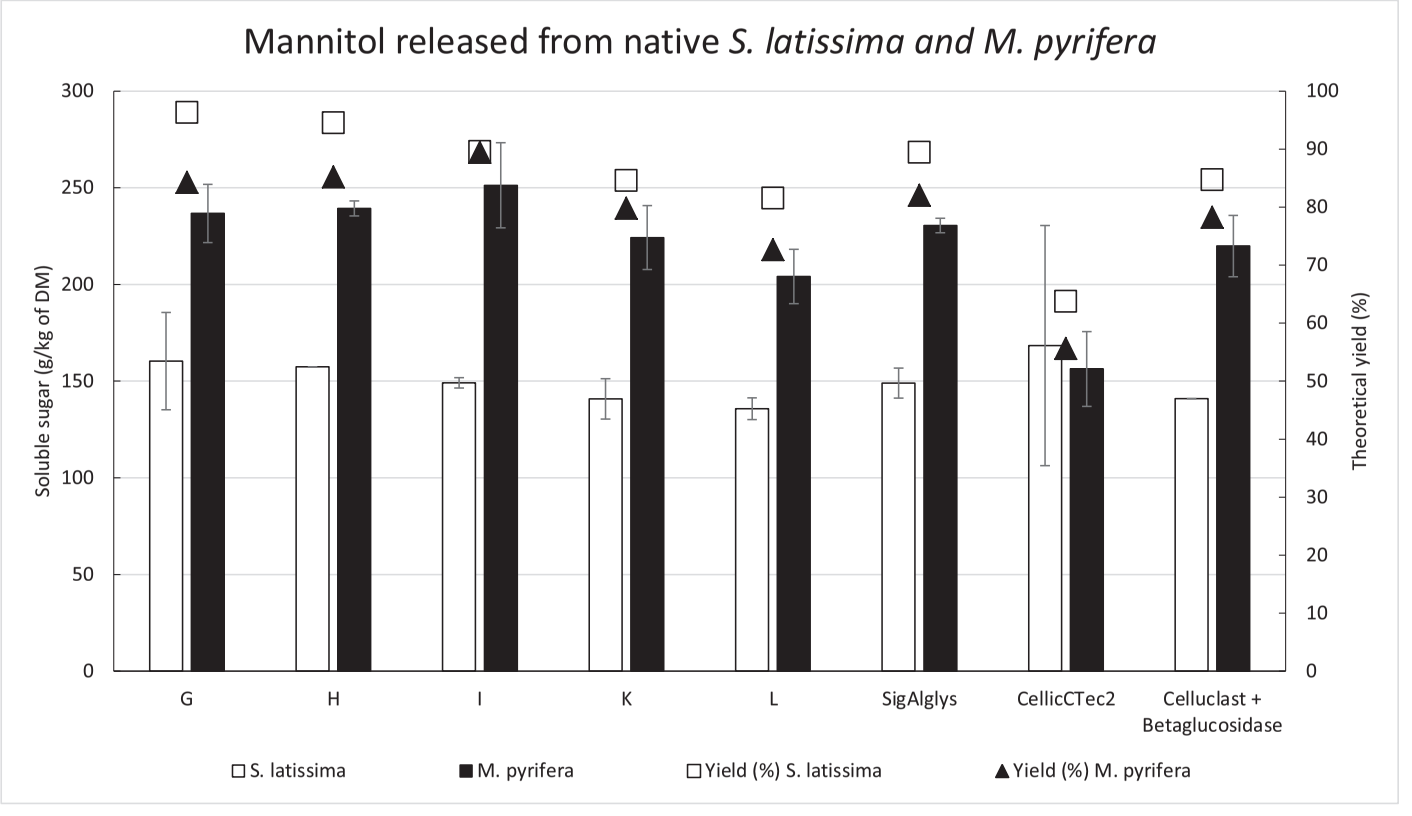
References

[1] F. Masarin, F. Cedeno, E. Solorzano, L. Oliveira, V. Cress, R. Monti, Chemical Analysis and Biorefinery of red Algae Kappaphycusalvarezii for Efficient Production of Glucose From Residue of Carrageenan Extraction Process, (2016), http://dx.doi.org/10.1186/s13068-016-0535-9.
[2] M. Song, H.D. Pham, J. Seon, H.C. Wood, Renew. Sust. Energ. Rev. 50 (2015) 782–792.
[3] H. Kawai, Perspectives in Phycology, vol. 1, (2014), pp. 27–40 Issue 1. Article Published online June 2014 Recent advances in the phylogeny and taxonomy of Laminariales, with special reference to the newly discovered basal member Aureophycus.
[4] C. Camus, P. Ballerino, R. Delgado, A. Olivera-Nappa, C. Leyton, A. Buschmann, Scaling up Bioethanol Production From the Farmed Brown Macroalga *Macrocystis pyrifera* in Chile Biofpr, (2016), http://dx.doi.org/10.1002/bbb.1708.
[5] S.J. Horn, Seaweed Biofuels: Production of Biogas and Bioethanol from Brown Macroalgae, VDM Publishing, 2009.
[6] M.M. Nielsen, D. Krause-Jensen, B. Olesen, R. Thinggaard, P.B. Christensen, A. Bruhn, Growth dynamics of *Saccharina latissima* (*Laminariales*, *Phaeophyceae*) in Aarhus Bay, Denmark, and along the species' distribution range, Mar. Biol. 161 (2014) 2011–2022.
[7] P. Gacesa, Alginates, Carbohydr. Polym. 8 (1988) 161–182.
[8] G. Michel, T. Tonon, D. Scornet, J. Mark Cock, B. Kloareg, The cell wall polysaccharide metabolism of the brown alga *Ectocarpus siliculosus*. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes, New Phytol. 188 (2010) 82–97.
[9] S. Sharma, S.J. Horn, Enzymatic saccharification of brown seaweed for production of fermentable sugars, Bioresour. Technol. 213 (2016) 155–161.
[10] M. Enquist-Newman, A.M.E. Faust, D.D. Bravo, C.N.S. Santos, R.M. Raisner, A. Hanel, P. Sarvabhowman, C. Le, D.D. Regitsky, S.R. Cooper, Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform, Nature 505 (2014) 239–243.
[11] S.J. Horn, G. Vaaje-Kolstad, B. Westereng, V.G.H. Eijsin, Novel enzymes for the degradation of cellulose, Biotechnol. Biofuels 5 (2012) 1–12.
[12] A. Ochiai, W. Hashimoto, K. Murata, A biosystem for alginate metabolism in *Agrobacterium tumefaciens* strain C58: molecular identification of Atu 3025 as an exotype family PL-15 alginate lyase, Res. Microbiol. 157 (2006) 642–649.
[13] J. Gimpel, O. Salazar, M.E. Lienqueo. Optimization of recombinant soluble expression of alginate lyases: an essential tool for brown algae biotechnology, Bioresour. Technol. (submitted).
[14] D. Manns, S.K. Andersen, B. Saake, A.S. Meyer, Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment, J. Appl. Phycol. 28 (2016) 1287–1294.
[15] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of Structural Carbohydrates and Lignin in Biomass [Electronic Resource], National Renewable Energy Laboratory, Laboratory Analytical Procedure (LAP), 2004.
[16] T.C. McIlvaine, A buffer solution for colorimetric comparison, J. Biol. Chem. 49 (1921) 183–186.
[17] L. Ma, Z. Chi, J. Li, L. Wu, Overexpression of alginate lyase of *Pseudoalteromonas elyakovii* in *Escherichia coli*, purification, and characterization of the recombinant alginate lyase, World J. Microbiol. Biotechnol. 24 (2008) 89–96.
[18] J.W. Li, S. Dong, J. Song, C.B. Li, X.L. Chen, B.B. Xie, Y.Z. Zhang, Purification and characterization of a bifunctional alginate lyase from *Pseudoalteromonas sp.* SM0524, Mar. Drugs 9 (2011) 109–123.
[19] S.M. Swift, J.W. Hudgens, R.D. Heselpoth, P.M. Bales, D.C. Nelson, Characterization of AlgMsp, an alginate lyase from *Microbulbifer sp.* 6532A, PLoS One 9 (2014) e112939.
[20] H.J. Yoon, W. Hashimoto, O. Miyake, M. Okamoto, B. Mikami, K. Murata, Overexpression in *Escherichia coli*, purification, and characterization of *Sphingomonas sp.* A1 alginate lyases, Protein Expression and Purification, 19 2000, pp. 84–90.
[21] O. Miyake, A. Ochiai, W. Hashimoto, K. Murata, Origin and diversity of alginate lyases of families PL-5 and-7 in *Sphingomonas sp.* strain A1, J. Bacterial 186 (2004)

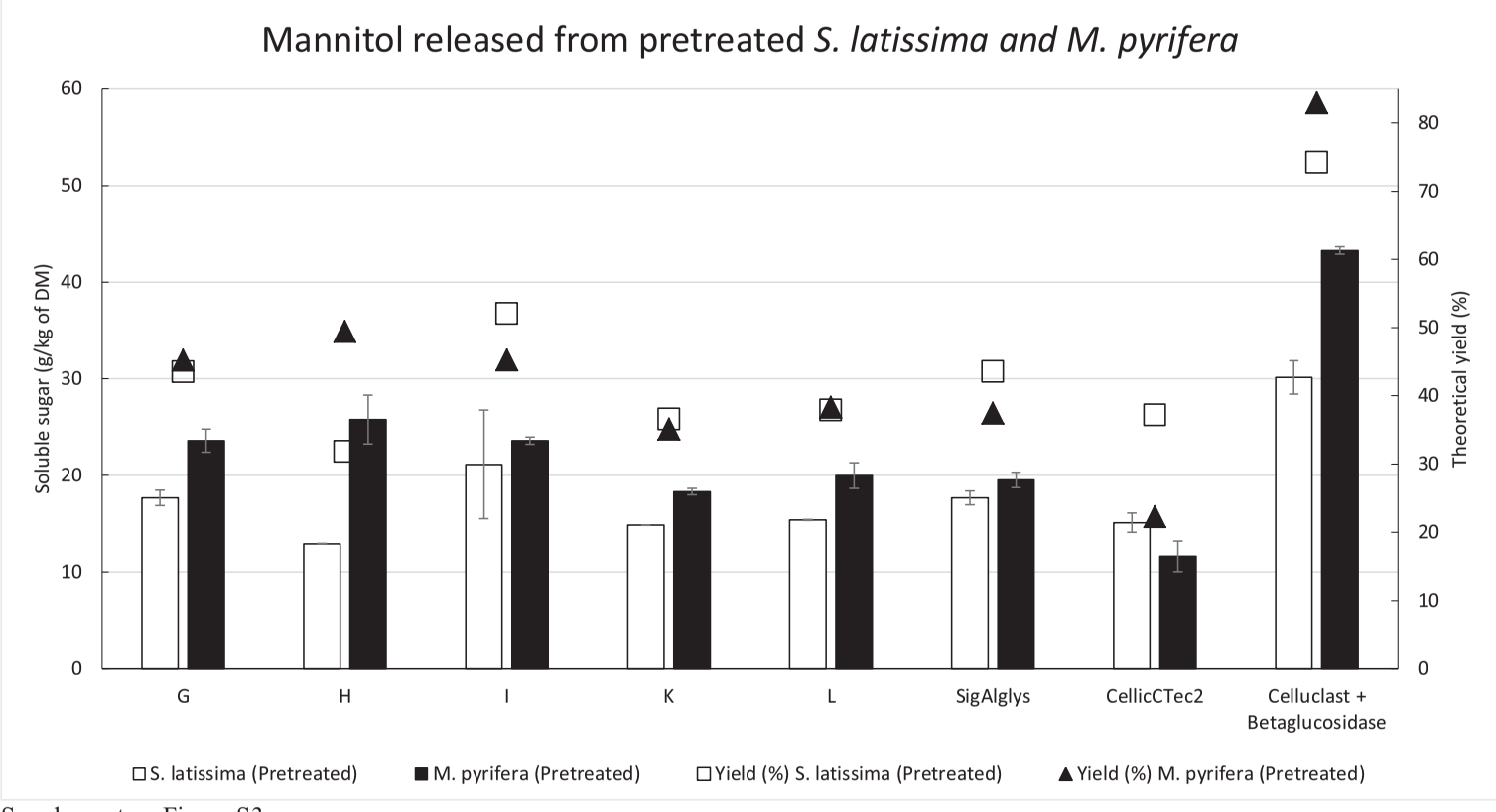
2891–2896.
[22] A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, Determination of ash in Biomass [Electronic Resource], (2008) National Renewable Energy Laboratory, Laboratory Analytical Procedure (LAP).
[23] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy), Nucleic Acids Res. 42 (2013) D490–D495.
[24] R. Vincentelli, A. Cimino, A. Geerlof, A. Kubo, Y. Satou, C. Cambillau, High-throughput protein expression screening and purification in Escherichia coli, Methods 55 (2011) 65–72.
[25] F.W. Studier, Protein production by auto-induction in high-density shaking cultures, Protein Expr. Purif. 41 (2005) 207–234.
[26] S. Jang, Y. Shirai, M. Uchida, M. Wakisaka, Production of L(+)-lactic acid from mixed acid and alkali hydrolysate of brown seaweed, Food Sci. Technol. Res. 17 (2011) 155–160.
[27] S. Jang, Y. Cho, G.T. Jeong, S.K. Kim, Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*, Bioprocess Biosyst. Eng. 35 (2012) 11–18.
[28] P. Yazdani, K. Karimi, M.J. Taherzadeh, Improvement of enzymatic hydrolysis of a marine macro-alga by dilute acid hydrolysis pretreatment, Bioenergy Technology (World Renewable Energy Congress), 2011, pp. 186–191.
[29] M. Yanagisawa, K. Nakamura, O. Ariga, K. Nakasaki, Production of high concentrations of bioethanol from seaweeds that contain easily hydrolyzable polysaccharides, Process Biochem. 46 (2011) 2111–2116.
[30] L. Ge, P. Wang, H. Mou, Study on saccharification techniques of seaweed wastes for the transformation of ethanol, Renew. Energy 36 (2011) 84–89.
[31] J. Lee, Y. Kim, B. Um, K.K. Oh, Pretreatment of Laminaria japonica for bioethanol production with extremely low acid concentration, Renew. Energy 54 (2013) 196–200.
[32] J. Adams, T.A. Toop, I. Donnison, J.A. Gallagher, Seasonal variation in *Laminaria digitata* and its impact on biochemical conversion routes to biofuels, Bioresour. Technol. 102 (2011) 9976–9984.
[33] J. Adams, J. Gallagher, I. Donnison, Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments, J. Appl. Phycol. 21 (2009) 569–574.
[34] X. Hou, J.H. Hansen, A.B. Bjerre, Integrated bioethanol and protein production from brown seaweed *Laminaria digitate*, Bioresour. Technol. 197 (2015) 310–317.
[35] M. Alvarado-Morales, I.B. Gunnarsson, I.A. Fotidis, E. Vasilakou, G. Lyberatos, I. Angelidaki, *Laminaria digitata* as a potential carbon source for succinic acid and bioenergy production in a biorefinery perspective, Algal Res. 9 (2015) 126–132.
[36] M.C. Ravanal, R. Pezoa-Conte, S. von Schoultz, J. Hemming, O. Salazar, I. Anugwomc, O. Jogunola, P. Mäki-Arvela, S. Willför, J.P. Mikkola, M.E. Lienqueo, Comparison of different types of pretreatment and enzymatic saccharification of *Macrocystis pyrifera* for the production of biofuel, Algal Res. 13 (2016) 141–147.
[37] C. Scullin, V. Stavila, A. Skarstad, J.D. Keasling, B.A. Simmons, S. Singh, Optimization of renewable pinene production from the conversion of macroalgae *Saccharina latissima*, Bioresour. Technol. 184 (2015) 415–420.
[38] M. Øverland, A. Skrede, Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture, J. Sci. Food Agric. (2016), http://dx.doi.org/10.1002/jsfa.8007.
[39] H.P.S. Makkar, G. Tran, V. Heuzé, S. Giger-Reverdin, M. Lessire, F. Lebas, P. Ankers, Seaweeds for livestock diets: a review, Anim. Feed Sci. Technol. 212 (2016) 1–17.

Supplementary Figure 1





Supplementary Figure S2



Supplementary Figure S3.

Supplementary Table S1: Concentration of carbohydrate (g/kg of DM*) and percentage of carbohydrate from *S. latissima* and *M. pyrifera* biomass without pretreatment and pretreatment with 2% sulphuric acid.

Carbohydrate	***S. latissima***	***M. pyrifera***	**Pretreated *S. latissima***	**Pretreated *M. pyrifera***
Glucose g/kg of DM*	221.3	96.8	246.6	217.8
%	37%	15%	59%	45%
Alginate g/kg of DM*	183.6	193.6	94.3	167.9
%	30%	31%	22%	35%
Mannitol g/kg of DM*	149.8	252.7	36.6	46.9
%	25%	40%	8.7%	9.8%
Xylose g/kg of DM*	31.1	39.9	36.9	36.1
%	5.1%.	6.3%	8.8%	7.5%
Fucose g/kg of DM*	17.5	51.1	6.1	11.9
%	2.9%	8.1%	1.5%	2.5%

*DM: Dry Mass

Supplementary Table S2. Description of recombinant alginate lyases used in this study.

Code Name	Enzyme name	Microorganism	Family/Type	Expressed amino acids	NCBI Proteins	Glycosidic bond cleavage	Reference
G	Aly-SJ02	*Pseudoalteromonas* sp. SM0524	PL18/Endo	175-400 (226)	618885355	MM, GG, MG	[40]
H	AlyPEEC	*Pseudoalteromonas elyakovii* IAM	PL18/Endo	166-398 (233)	4322372	MM, GG, MG	[41]
I	AlgmsP	*Microbulbifer* sp. 6532A	PL7/Endo	80-358 (279)	319993261	MM, GG, MG	[42]
K	A1-II	*Sphingomonas* sp. A1	PL7/Endo	414-641 (228)	BAB03312.1	GG, MG > MM	[43]
L	A1-II'	*Sphingomonas* sp. A1	PL7/Endo	81-308 (228)	BAD16656.1	MM, GG, MG	[44]
Atu	Atu3025	*Agrobacterium tumefaciens* C58	PL15/Exo	1-776	NP_357573.1	MM, GG, MG	[45]
Q*	ScbB*	SCB49 unidentified bacterium	PL7/Exo*	17-339 (323)	EDM45391.1	Non-characterized	Unpublished*
O*	Lpe*	*Lewinella persica*	PL7/Exo*	20-349 (330)	WP_026231793.1	Non-characterized	Unpublished*

*:

These enzymes have been predicted to be endo-alginate lyases from the PL7 family by sequence homology based on the structure of AlyA5 [46]. The production of uronic acid monomers from these enzymes has been confirmed by thin-layer liquid chromatography.

References

[40] S. Li, X. Yang, L. Zhang, W. Yu, F. Han, Cloning, Expression, and Characterization of a Cold-Adapted and Surfactant-Stable Alginate Lyase from Marine Bacterium Agarivorans sp. L11. Journal of microbiology and biotechnology 25 (2015) 681-86.

[41] L. Ma, Z. Chi, J. Li, L. Wu, Overexpression of alginate lyase of Pseudoalteromonas elyakovii in Escherichia coli, purification, and characterization of the recombinant alginate lyase. World Journal of Microbiology and Biotechnology 24 (2008) 89-96.

[42] S.M. Swift, J.W. Hudgens, R.D. Heselpoth, P.M. Bales, D.C.Nelson, Characterization of AlgMsp, an alginate lyase from Microbulbifer sp. 6532A. PloS one 9 (2014) e112939.

[43] H.-J.Yoon, W. Hashimoto, O. Miyake, M. Okamoto, B. Mikami,, K. Murata, Overexpression in *Escherichia coli,* purification, and characterization of Sphingomonas sp. A1 alginate lyases. Protein expression and purification 19 (2000) 84-90.

[44] O. Miyake, A. Ochiai, W. Hashimoto, K. Murata, Origin and diversity of alginate lyases of families PL-5 and-7 in Sphingomonas sp. strain A1. Journal of bacteriology 186 (2004) 2891-96.

[45] A. Ochiai, W. Hashimoto, K Murata, A biosystem for alginate metabolism in Agrobacterium tumefaciens strain C58: molecular identification of Atu3025 as an exotype family PL-15 alginate lyase. Research in microbiology 157 (2006) 642-49.

[46] F. Thomas, C.E:L- Lena, J. Murielle,J. Alexandra, B. Tristan, S. Corine, M. Gurvan, C. Mirjam, Comparative characterization of two marine alginate lyases from Zobellia galactanivorans reveals distinct modes of action and exquisite adaptation to their natural substrate, Journal of Biological Chemistry. 288 (2013) 23021-23037.

Paper IV

Microbial protein produced from brown seaweed and spruce wood as a feed ingredient

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	jf-2018-01835b
Manuscript Type:	Article
Date Submitted by the Author:	10-Apr-2018
Complete List of Authors:	Sharma, Sandeep; Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science Hansen, Line; Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science Hansen, Jon; Norges miljo- og biovitenskapelige universitet Fakultet for veterinarmedisin og biovitenskap, Animal and Aquacultural Sciences Mydland, Liv ; Norwegian University of Life Sciences, Animal and Aquacultural Sciences Horn, Svein J.; Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science Øverland, Margareth; Norwegian University of Life Sciences, Animal and Aquacultural Sciences Eijsink, Vincent; Norwegian University of Life Sciences, Chemistry, Biotechnology and Food Science Vuoristo, Kiira; Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science

Microbial protein produced from brown seaweed and spruce wood as a feed ingredient

Sandeep Sharma[1], Line D. Hansen[1], Jon Ø Hansen[2], Liv Torunn Mydland[2], Svein J. Horn[1], Margareth Øverland[2], Vincent G. H. Eijsink[1], Kiira S. Vuoristo[1*]

[1]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Aas, Norway

[2]Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, 1433 Aas, Norway

[*] Corresponding author: Kiira S. Vuoristo kiira.vuoristo@nmbu.no

Abstract

The conversion of non-edible biomass to protein for use in feed is an attractive strategy towards improved sustainability in aquaculture. We have studied the possibility to produce the protein-rich yeast *Candida utilis* on a medium consisting of enzymatically hydrolysed sulphite-pulped spruce wood, mainly providing glucose, and enzymatically hydrolysed brown seaweed, supplemented with ammonium sulphate. The results show that this blend constitutes a complete medium that allows reaching good growth rates and cell yields. Results from a salmon feeding trial showed that the yeast can replace parts of a traditional fishmeal diet without harmful effects, although the apparent protein digestibility coefficient for the yeast was sub-optimal. While further optimization of both the fermentation process and down-stream processing is needed, the present proof-of-concept study shows a path to the production of microbial protein based on a simple, local and sustainable fermentation medium.

Keywords: microbial protein; seaweed, spruce; enzymatic hydrolysate; yeast, fermentation; feed; aquaculture

1. Introduction

The demand for high-quality protein sources in the aquaculture industry is rapidly increasing, and the need for sustainable protein sources has urged researchers to explore alternatives for fishmeal, which is a commonly used but limited resource [1]. Substituting fishmeal with plant-based protein has achieved considerable success, but raises issues because plant production requires water, arable land and pesticides, and because of a potential conflict between food and feed production [1-2]. Furthermore, plant-based feeds raise issues related to digestibility and health, especially for carnivorous fish such as Atlantic salmon (*Salmo salar*) [3].

Microbial proteins produced by fungi, algae or bacteria are of interest as possible replacement for fishmeal and plant based feeds, as demonstrated in several studies [4-5]. Of the varying possibilities for producing microbial protein, sources, production of yeast is receiving increasing attention [6-7]. Yeasts grow fast, cultures have low risk of contamination, and the produced biomass is easy to recover [8]. Furthermore, studies with Atlantic salmon have shown that certain yeast types have high nutritional value and beneficial effects on gut health [9]. Hence, yeasts seem an attractive alternative protein source for fish feed [3, 10].

Candida utilis is a protein-rich yeast with a generally-regarded-as-safe (GRAS) status that can metabolise a wide range of substrates. *C. utilis* could serve as a high-quality protein source in fish diets because it contains high concentrations of essential amino acids and has documented health-promoting effects [11-13]. For *C. utilis* to become an economically viable and sustainable source of protein, it is important to develop cheap and sustainable fermentation media. Instead of using glucose from conventional

sources ("first generation sugar"), one could use "second generation" sugar, derived from non-edible biomass such as lignocellulose. Likewise, rich media components, such as amino acids, vitamins and minerals, often supplied in the form of (expensive) peptones and yeast extract, should come from cheap sources, such as protein-rich non-edible by-products from food production. Studies have shown successful production of *C. utilis* using fruits and vegetable wastes, agricultural crop residues and other organic wastes [14-16]. In the present study, as a step towards more sustainable yeast production, we have analysed the potential of using lignocellulosic and seaweed biomass in the cultivation of *C. utilis*.

Large areas of the Nordic countries are covered by coniferous evergreen forests, which, in Norway, are dominated by Norway spruce and Scots pine [17]. This biomass is primarily composed of cellulose, hemicellulose, and lignin. Holocellulose, i.e. the cellulose and hemicellulose-rich fraction emerging after lignin removal, is a co-polymeric structure enriched in cellulose, and several hemicelluloses, such as xylans, arabinoxylans, xyloglucans, glucuronoxylans, and/or glucomannans [18-19]. The types and fractions of hemicelluloses depend on the raw material and on the method used to de-lignify this material. For example, the BALI process [20] used in the present study to pre-treat Norwegian spruce, removes most of the lignin, but also most of the hemicellulose. Enzymatic hydrolysis of polysaccharide-rich pre-treated lignocellulosic biomass yields sugar solutions that may be used for growing *C. utilis*. Notably, these sugars solutions are poor in nitrogen, [21].

Seaweed provides another possible resource in countries such as Norway, which has one of the longest coastal lines in Europe. Seaweeds growing along the coast are

dominated by brown macroalgae such as *Saccharina latissima* [22-23]. The main structural components of *S. latissima* are alginate and cellulose, while the main energy storage compounds are laminarin and mannitol [24]. Laminarin is a branched polymer of glucose, comprising a β-(1-3)-glucan backbone chain with β-(1-6) branches. Mannitol is a sugar alcohol that can be fermented by some microbes such as *Zymobacter palmae* and *Escherichia coli* [25-26]. Next to fermentable carbohydrates, in particular, the glucose in cellulose and laminarin, *S. latissima* contains nitrogen, phosphorus, amino acids, vitamins, and minerals needed for microbial growth [27]. *S. latissima* does not contain lignin and, therefore, no pre-treatment is required prior to the enzymatic conversion of its polysaccharides to obtain fermentable sugars [28-29].

Considering the above, it is of interest to explore whether combinations of lignocellulosic biomass (Norway spruce) and seaweed biomass (*S. latissima*) could be used to produce microbial protein. Therefore, as a first step towards the conversion of seaweed and spruce to protein, we have studied growth of *C. utilis* on a combination of enzymatically generated spruce and seaweed hydrolysates. Next to monitoring the production and protein content of the produced microbial biomass, we also assessed its amino acid composition and mineral content. Finally, a preliminary assessment of the digestibility of the produced yeast cells was made in a digestibility trial with salmon.

2. Materials and methods

2.1 Materials

Culture media, yeast nitrogen base without or with amino acids (L-histidine, DL-methionine, DL-tryptophan), sugars, amino acid standards, and sulphuric acid were procured from Sigma Aldrich (Missouri, USA). Ammonium sulphate was purchased from

Merck (Darmstadt, Germany). Kjeltabs for Kjeldahl analysis were purchased from Thompson & Capper Ltd (Cheshire, UK).

2.2 Enzymatic hydrolysis of seaweed and spruce

Wild and cultivated *S. latissima* were harvested near the island of Frøya (63°42'15"N, 8°52'40"E), in July 2014 and June 2015, by Seaweed Energy Solutions AS (Trondheim, Norway) and frozen immediately until further use. Enzymatic hydrolysis of thawed, wild *S. latissima* substrate was carried out in 100 mL Erlenmeyer flasks, at 15 (w/v) dry matter, for subsequent use in 96-well plate cultures or shake flask cultures. For use in larger fermentations, enzymatic hydrolysis of cultivated, thawed *S. latissima,* at 15 % (w/v) dry matter was performed in 5 L glass bottles. Enzymatic hydrolysis of *S. latissima* was performed in distilled water with 10 mg g^{-1} of Cellic®CTec2 (on a protein basis; Novozymes A/S, Copenhagen, Denmark) and 0.13 mg g^{-1} of alginate lyases (EC 4.2.2.3) (on a dry powder basis; Sigma Aldrich, Missouri, USA). Incubation was carried out in a rotary shaker (Infors HT, Bottmingen, Switzerland) set at 140 rpm, initially at 37 °C for 4 hours, followed by 16 h at 50 °C. The starting and final pH of the reaction mixture were 6.0 and 6.3, respectively. The enzymatic hydrolysates were centrifuged at 4700 rpm, 4 °C for 10 min using a centrifuge (Multicentrifuge X1R, Thermo Scientific, Waltham, USA) and were stored at 5 °C until further use.

Enzymatic hydrolysates of BALI pre-treated spruce [20, 30] were kindly provided by Borregaard AS (Sarpsborg, Norway). The carbohydrate compositions of the *S. latissima* and spruce hydrolysates are shown in Table 3.

2.3 Yeast cultivation *Candida utilis* (LYCC 7549; Lallemand Yeast Culture Collection) was provided by Lallemand Inc (Salutaguse, Estonia) and stored in a medium

containing 15 % (v/v) glycerol at -80 °C. The yeast was propagated on yeast extract-peptone-dextrose (YPD, Sigma Aldrich, Missouri, USA) agar plates containing 20 g L^{-1} glucose, 10 g L^{-1} peptone, 10 g L^{-1} yeast extract and 20 g L^{-1} agar at 30 °C. Cultures were started by using a single colony to inoculate sterile test tubes containing YPD medium, followed by incubation for 24 h, at 30 °C and 180 rpm. To prepare the inoculum for 1 L fermentations, 2 mL of an overnight pre-culture was used to inoculate 48 mL of YPD in a 200 mL flask (same culturing conditions). To prepare an inoculum for 27 L fermentations, 50 mL of a shake flask culture was used to inoculate 950 mL of YPD in a 2 L bottle. In all cases, cultures were incubated at 30 °C, 180 rpm, for 24 h. All media and enzymatic hydrolysates were sterilised by autoclaving at 121 °C for 20 min.

2.4 Microbial growth on spruce and seaweed hydrolysates

2.4.1 Preliminary growth assessments in microtiter plates

C. utilis was grown on YPD with different inclusion levels of SHE (up to 30 % (v/v) in sealed 96 well microtiter plates (200 µL) and growth was measured using a Multiskan FC microplate reader with incubator (Thermo Scientific, Vantaa, Finland). The plate was incubated at 30 °C and shaken vigorously (with 5 sec intervals). The optical density of samples was observed at 595 nm every hour for 24 h using SKANIT software version 2.5.1 for data handling.

2.4.2 Shake flask cultures

Shake flask batch fermentations were conducted using a medium composed of 90 % (v/v) seaweed hydrolysate (SEH) and 10 % (v/v) spruce hydrolysate (SPH), yielding an initial glucose concentration of 43 g L^{-1}, with addition of 2.04 g L^{-1} of YNB (corresponding to 1.5 g L^{-1} ammonium sulphate). The fermentations were done using Erlenmeyer baffled flasks (200 mL) containing 45 mL of the medium inoculated with 5

mL of an overnight test tube culture grown on YPD medium. The cultures were incubated at 30 °C, 180 rpm.

2.4.3 1 L and 27 L fed-batch fermentations

Fermentations in 1 L scale were carried out using 2.5 L Minifors bench-top glass fermenters (Infors HT, Bottmingen, Switzerland) equipped with two 6-bladed Rushton impellers. Fermentations in 27 L scale were carried out in a 42 L Techfors S stainless steel bioreactor (Infors), equipped with three 6-blade Rushton impellers. Fermentations were carried out at 30 °C, and the pH was kept at 5.5 by addition of 2 M HCL or 2 M NaOH, using a pH controller (Mettler Toledo, Greifensee, Switzerland). Dissolved oxygen was set at 20 % saturation and regulated by automatic adjustment of the stirrer speed (100 - 700 rpm) and, when needed, the aeration. Dissolved oxygen (pO_2) was monitored using a pO_2 controller (Hamilton, Bonaduz, Switzerland). Off gas (CO_2) analysis was carried out using a Fermac 368 gas analyser (Electrolab Biotech, Gloucestershire, UK) for 2.5 L fermenters and using an Infors HT Gas Analyser (Infors) for the 42 L fermenter. Formation of foam was controlled by an antifoam-sensor and automatic addition of a 10 % w/v solution of the antifoam Glanapon DB-870 (Busetti, Vienna, Austria). Fermentation data were recorded using IRIS process control software (Infors).

The initial fermentation medium consisted of a 90 % - 10 % (v/v) mixture of SEH and SPE, the inoculum and 4.41 g L^{-1} ammonium sulphate. Fermenters were fed after 20 hours by pumping in a mixture of additional sugar (SPH) and ammonium sulphate. For 1 L fermentations the starting volume was 1 L, including 100 mL inoculum. The feed consisted of 100 mL SPH containing 4.41 g ammonium sulphate. For 27 L

fermentations the starting volume was 24 L, including 1 L inoculum and the feed consisted of 3 L SPH containing 105.8 g ammonium sulphate. The feed was applied gradually by a peristaltic pump, with the pump rate of 35 mL h^{-1} for the 1 L fermenter and 750 mL h^{-1} for 27 L fermenter. During the fermentation at 1L scale, samples were taken out using the Super Safe Sampler (Infors). In 27 L fermentation, samples were taken out aseptically by flushing the sampling line with steam before and after sampling. The samples were stored in 50 mL falcon tubes on ice prior to analysis.

2.5 Downstream processing

Yeast cells obtained at the end of 27 L fermentations were inactivated by heating the fermenter to 70 °C for 30 min. The cells were collected by centrifugation for 10 min at 4 °C, using a Beckman Coulter Avanti J-26S XP centrifuge equipped with a JLA 8.1000 rotor (Indianapolis, Indiana, USA) at 8000 rpm. The pellet was re-suspended and washed three times with distilled water, and stored as a cell paste at 5 °C prior to spray drying. The spray drying was carried out using a Mobile Minor TM spray dryer (GEA, Soeborg, Denmark). The inlet air temperature was set to 180 °C and the outlet air temperature was set to 80 °C. The evaporation rate of the dryer was approximately 2 L h^{-1}.

2.6 Analytical methods

2.6.1 Dry cell mass, cell growth and pH

Dry cell mass was determined by centrifuging culture samples at 4700 rpm, 4 °C for 10 min using a centrifuge (Multicentrifuge X1R, Thermo Scientific, Waltham, USA). Supernatants were collected for analysis of sugars and ammonium (see below). The cell pellets were re-suspended in distilled water and washed three times prior to drying.

Dry matter was determined by weighing samples before and after drying at 105 °C. Cell growth was monitored by recording the optical density at 595 nm using a UV/Visible spectrophotometer (Hitachi U1900 spectrophotometer, Tokyo, Japan). The pH of the broths of shake flask cultures was measured using a pH meter (827 pH lab, Metrohm AG, Herisau, Switzerland).

2.6.2 Sugars

Sugars present in the enzymatic hydrolysate of *S. latissima* and fermentation broth were analysed by high performance liquid chromatography (HPLC) with refractive index detection. The samples were filtered through a 0.22 µm filter and supernatants were separated on a Rezex ROA-organic acid H+, 300 x 7.8 mm (Phenomenex, Torrance, CA, USA) analytical column fitted with a cation-H cartridge guard column. Analysis was performed at a column temperature of 65 °C, with 5mM H_2SO_4 as the eluent applied at a flow rate of 0.6 mL min^{-1}. For quantification, the area of peaks corresponding to glucose, xylose, and mannitol were compared to calibration standard curves generated with known concentrations of sugars (in the range of 0.1 – 10 g L^{-1}). The sugar analysis of the spruce enzymatic hydrolysate was carried out according to a previously described procedure [20].

2.6.3 Ammonium

The concentration of ammonium in supernatants was determined according to [31]. 50 µl samples, including calibration standards with known ammonium sulphate concentrations, were mixed with 1 ml of Willis reagent in a 24 well microtiter plate, followed by brief mixing at 400 rpm at room temperature using a thermomixer (Eppendorf, Hamburg, Germany). After mixing, 0.25 ml of a 5 % hypochlorite solution

was added to the samples, followed immediately by fast mixing. After incubation for 12 min at room temperature, the absorbance at 685nm was measured using a microtiter plate reader (Synergy H1, BioTek, Vermont, USA).

2.6.4 Proteins

The protein concentration in the Cellic®CTec2 preparation was determined by using the Bio-Rad Protein Assay (Bio-Rad, USA), which is based on the Bradford method, using Bovine Serum Albumin (BSA) as a standard. Nitrogen contents of solid materials were analysed according to the Kjeldahl method, using a Kjeltec TM 8400 (FOSS, Tecator, Hoganas, Sweden) after acid digestion in an auto-digestor (FOSS, Tecator, Hoganas, Sweden).

2.6.5 Minerals

The mineral contents of oven dried native *S. latissima*, enzymatic hydrolysates (SPH and SEH) and spray dried yeast were analysed by inductively coupled plasma spectrometry, with mass spectrometric detection (ICP-MS) (Perkin-Elmer, Massachusetts, USA). For chlorine, bromine, and iodine analysis, the samples were hydrolysed by concentrated tetramethylammonium hydroxide and for other analyses, samples were digested by 65 % HNO_3 in a high performance microwave reactor (UltraClave, MLS Milestone, Sorisole, Italy).

2.6.6 Amino acids and lipids

Amino acid analysis (except tryptophan) of spray dried yeast and oven dried native *S. latissima* was performed according to European Commission (EC) regulation No: 152/2009 (L54/23-32) on a Biochrom 30 amino acid analyser (Biochrom Ltd, Cambridge, UK). Tryptophan was analysed according to EC No: 152/2009 (L54/32-37)

on a Dionex Ultimate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) connected to a Shimadzu RF-535 fluorescence detector (Shimadzu RF-535, Shimadzu Corp, Kyoto, Japan). Both amino acid and tryptophan data were analysed against external standard curves using the Chromeleon® Software (Dionex Ltd, Surrey, UK)

The crude lipid content of native *S. latissima* and spray dried yeast was analysed gravimetrically after extraction at 125 °C and 1500 psi with petroleum ether and acetone (70:30, v:v) using an accelerated solvent extractor (Dionex ASE 200, Dionex Corp, Sunnyvale, California, USA).

2.7 Digestibility trial

2.7.1 Feed formulation and preparation

Data on the ingredients and diet used in a feeding trial with pre-smolt Atlantic salmon is provided in Table 1. The standard diet was composed as follows: Fish meal (Table 1), 481.8 g kg-1; wheat gluten, 130.0 g kg-1; gelatinized potato starch, 120.0 g kg-1; fish oil, 150.0 g kg-1; vitamin and mineral premix, 70.0 g kg-1; MCP (Bolifor®Monocalcium phosphate-F from KPP Oy, Animal Nutrition, Helsingborg, Sweden), 0.2 g kg-1; choline chloride, 2.0 g kg-1; yttrium oxide (Y2O3, from Metal Rare Earth Limited, Shenzhen, Guangdong, China), 1.5 × 10-3 g kg-1. The vitamin and mineral premix was Farmix, from Trouw Nutrition, LA Putten, The Netherlands, and was added to give the following values per kg feed: retinol, 2500.0 IU; cholecalciferol, 32400.0 IU; α-tocopherol SD, 0.2 IU; menadione, 40.000 mg; thiamine, 15.0 mg; riboflavin, 25.0 mg; d-Ca-pantothenate, 40.002 mg; niacin, 150.003 mg; biotin, 3000.0 mg; cyanocobalamin, 20.0 mg; folic acid, 5.0 mg; pyridoxine, 15.0 mg; ascorbate polyphosphate, 0.098 g; Cu: CuSulfate.5H2O, 11.998 mg; Zn: ZnSulfate, 89.992 mg; Mn: Mn(II)Sulfate, 34.993 mg; I: K-Iodine, 1.999

mg; Se: Na-Selenite, 0.200 mg; Cd below 0.0003 mg; Pd below 0.028 mg; Ca, 0.915 g; K 1.380 g; Na 0.001 g; Cl 1.252 g. In the experimental diet, 30% of the standard diet was replaced by yeast collected from 27 L fermentations as described in section 2.4.3, and spray dried according to section 2.5. Yttrium was added in the diet as an internal marker to examine nutrient digestibility [32]. Diets were prepared using a blender (Grain, Rome, Italy) and pasta machine (Italgi, Carasco, Italy). The feeds were kept frozen at -20 °C until use. The proximate analysis of the diets is shown in Table 2.

2.7.2 Fish feeding trials and faecal collection

The fish trials were performed at the Norwegian University of Life Sciences. The experimental procedures were in accordance to the institutional and national guidelines for the care and use of animals (the Norwegian Animal Welfare Act and the Norwegian Regulation and Animal Experimentation). Pre-smolt Atlantic salmon were distributed in six 200 L circular, fiberglass tanks operated with a continuous recirculating water system. The fish were randomly assigned to three replicate tanks per diet (40 fish per tank). The fish were fed the experimental diets for 2 h every day for 48 days through automatic feeders. The water quality parameters measured were temperature (13.9 °C ± 0.5), pH (7.3 ± 0.4), alkalinity (0.9 mmol L^{-1}), ammoniacal nitrogen (NH4-N, 0.08 mg L^{-1}) and nitrates (0.04 mg L^{-1}), which were all within acceptable levels for salmon. The initial weight of the pre-smolt salmon was approximately 65 grams.

For assessment of digestibility, faecal samples were collected by stripping the fish on day 30, 36, 43, and 48. The faecal samples collected from fish from the same tank were pooled, frozen and freeze-dried. Prior to the stripping, the fish were anesthetised with tricaine methane sulfonate (Syndel, Washington, USA).

272 2.7.3 Growth performance and digestibility

273 The growth performance of the fish was evaluated by weight gain. The nutrient apparent
274 digestibility coefficients were calculated by using equation 1 [33],

275 $$ADC\ (\%) = 100 * \frac{(a-b)}{a} \quad (1)$$

276 Where *a* represents (nutrient in feed / yttrium in feed) and *b* represents (nutrient in
277 faeces/ yttrium in faeces). The apparent digestibility coefficient of the test ingredient was
278 calculated using equation 2.

279 $$ADC_{ingredients}(\%) = ADC_{testfeed} + \left(ADC_{testfeed} - ADC_{control\ feed}\right) x\ \frac{0.7\ x\ Nutrient_{ref}}{0.3\ x\ Nutrient_{ingredient}} \quad (2)$$

280 2.7.4 Analyses

281 All diets, yeasts and faeces samples were finely ground by mortar and pestle and
282 homogenised prior to analysis. The proximate analysis and gross energy values of diets
283 and samples were performed following established methods for the official control of
284 feed regulation EC No 152/2009: dry matter (L54/12-14), ash (L54/50-51) crude protein
285 (L54/15-19, Kjeldahl method, N x 6.25). Crude fat was analysed using the Accelerated
286 Solvent Extractor (ASE 200) from Dionex, starch according to the method described by
287 [34], and gross energy by bomb calorimetry (Parr 1281 Bomb Calorimeter, Parr
288 Instruments, Illinois, USA) .

289 **3. Results and discussion**

290 3.1 Characterization of seaweed and spruce hydrolysates

291 Table 3 shows data on the composition of the seaweed hydrolysates (SEH) and the
292 spruce hydrolysates (SPH). The data show that the hydrolysates differ in sugar
293 concentration and that their composition varies between batches. The variation in the

concentration of sugars in the seaweed is caused by seasonal variation in the chemical composition [24]. The spruce hydrolysates are derived from process optimization studies in the BALI pilot plant at Borregaard, and concentration differences result from minor variation in process parameters.

3.2 Preliminary testing of seaweed hydrolysate for growing *C. utilis*

Initially various experiments were carried out in a microtiter plate format showing that growth of *C. utilis* on the rich YPD medium was not affected by addition of SEH, not even at the highest tested inclusion levels of 30 % (v/v) (results not shown).

3.3 Shake flask cultures

As a first step towards fermenting *C. utilis* on a combination of SEH and SPH, experiments were conducted in shake flasks and the results of a representative experiment are shown in Fig. 1. The medium contained 90 % (v/v) SEH and 10 % (v/v) SPH, giving an initial glucose concentration of 43 g L^{-1}. After a short lag phase, the cells grew rapidly leading to depletion of both glucose and ammonium, whereas mannitol was hardly consumed (Fig. 1). The pH value during fermentation varied from 3.8 to 5.5. The drop in pH value to 3.8 can be attributed to the formation of acetate during the fermentation process [35-36]. The nutrient consumption profiles and growth curves shown in Fig. 1 indicate that *C. utilis* grows well on a mixture of SEH and SPH.

3.4 Fed-batch fermentations

3.4.1 One liter fed-batch fermentations

Encouraged by the shake flask experiments, which suffer from a lack of pH control and sub-optimal aeration (pO_2) and stirring, *C. utilis* was then cultivated in 2.5 L fermenters in fed-batch cultures. Instead of YNB, ammonium was added in the form of ammonium sulphate. The fermenters were fed between 20 and 23 hours with additional SPH

(equalling addition of 30 g L^{-1} glucose) and ammonium sulphate (4.41 g L^{-1}) and maximum levels of dry cell mass were generally obtained a few hours after feeding. In the experiment depicted in Fig. 2 the maximum cell density was 18 g L^{-1}, and this level was reached three hours after the start of the feeding and consumption of in total approximately 51.0 g L^{-1} glucose. At this point, the protein content of the cells (40 %) had started to decrease. Overall, the approximate yields at this point were 0.35 g of cell mass and 0.14 g protein per gram of consumed glucose. The highest yields of yeast biomass per consumed glucose and the highest yeast protein content (44 %) were both observed just before the feeding, at 20 hours, when the concentration of microbial biomass was 14 g L^{-1}. At this point, the yields were 0.37 g of cell mass and 0.16 g of protein per g of consumed glucose.

3.4.2 27 liter fed-batch fermentations and downstream processing of yeast

Figure 3 shows fermentation parameters typically observed when upscaling the 1 L fermentations described above to 27-liter scale. On the one hand, the larger scale fermentations seemingly gave better results than the 1 L fermentations, with cell mass levels reaching 20 g L^{-1} and protein contents reaching 53 %. On the other hand, however, we observed a larger trade-off between cell mass and protein content. Maximum cell mass (20 g L^{-1}) was reached at 27 hours when the protein content was 43%, corresponding to yields of 0.26 g of microbial biomass and 0.11 g of protein per g of glucose. At 20 hours, just before feeding, when the protein content reached 53 %, the yields were 0.26 g of cells and 0.14 g of protein per g of glucose.

The results in Fig. 3 show that it was possible to produce reasonable amounts of a protein-rich yeast on a medium comprised of seaweed and spruce derived components.

341 Notably, the present yields are similar to those reported in the literature (de Arruda et
342 al., 2011; Gao et al., 2012; Lemmel et al., 1979; Nigam, 2000) for the same Candida sp.
343 using other types of fermentation media. Still, it is known that *C. utilis* can achieve
344 biomass yields up to 0.36 g/g glucose [14] in rich media, indicating that further process
345 optimization, such as adaptation of the yeast to the SPH+SEH medium and adjusting
346 the feed regimes and culturing conditions, are needed and possible.

347 Heat inactivation of the cells at 27 h led to a reduction in dry cell mass and protein
348 content (Fig. 3), likely as a consequence of some degree of autolysis [37]. The protein
349 content of yeast dropped from 42 % (w/w) to 36 % (w/w). This protein content, as well
350 as the protein contents generally observed in the experiments depicted in Figures 2 and
351 3 fall within the range of previously reported crude protein contents of *C. utilis*, which
352 vary between 26 to 53 % [15, 38-39]. Nevertheless, investigation of other, more gentle
353 inactivation strategies seems useful, although most alternative methods, such as
354 irradiation, addition of chemicals such as chlorine and hydrogen peroxide [40-43] seem too
355 harsh and unfavourable for our purpose. Cells from multiple 27 L fermentations were
356 collected and subjected to spray drying, with short residence time. The resulting dried
357 cells had a protein content of 33.3 % and were used for further characterization and a
358 fish feed trial.

359 3.4.3 Mineral and amino acid content of spray-dried *C. utilis*

360 Cultivation of *C. utilis* and other yeasts requires a wide variety of nutrients, next to
361 carbon and nitrogen (e.g. [44]). In our fed batch fermentations, these nutrients were
362 mainly provided by the seaweed hydrolysate. Table 4 provides an overview of selected
363 minerals that are important both for yeast growth and in fish nutrition, in several of the

process fractions (seaweed, SEH, SPH, SEH+SPH with inoculum, and the spray-dried yeast). Interestingly, some elements that are scarce in SEH do also occur in SPH, meaning that the two hydrolysates to some extend can compensate each other (e.g. Ca and Ni). Further optimization of the yeast production process may be achieved by adjusting nutrient concentrations. For example, [45] showed that addition of $CaCl_2$ increased both biomass production and protein content for *Candida* sp. Lee et al showed similar results for phosphorus [14]. The amino acid compositions, as well as the nitrogen and crude lipid contents of spray dried *C. utilis*, native *S. latissima*, and fish meal are presented in Table 5. The data show that the amino acid composition of *C. utilis* produced on SEH and SPH is similar to the amino acid composition of *C. utilis* produced on other substrates [39, 46]. As noted before [47-48], this composition is quite similar to that of fishmeal, although there are some potentially important deviations, such as for methionine.

3.5 Fish growth trial

The spray-dried yeast was used in a preliminary fish feed trial to assess potential harmful effects on the fish and digestibility. A full-scale comparative fish growth trial; including several batches of *C. utilis* is in progress and will be described elsewhere. Details of the digestibility trial are provided in the Materials and methods section, including Tables 1 and 2. In short, 30 % of a standard control diet was replaced by spray-dried *C. utilis* and pre-smolt Atlantic salmon were fed the diets during a period of 48 days. Both control and yeast-fed fish showed similar performance with respect to weight gain during these 48 days and no irregularities, such as non-healthy or dying fish, were observed.

Digestibility data (Table 6) confirm that the fish in both groups grew well, as shown by reasonable apparent protein digestibility values. Not unexpectedly, but importantly, the data also show that part of the added yeast is not digested, including the yeast protein. This is likely due to the presence of cell walls that increase the non-digestible fraction of the dry matter, while at the same time restricting access to intracellular protein. These are well known issues when working with yeast-based microbial protein sources that need to be addressed by developing improved down-stream processing strategies, which may include fractionation and cell lysis [49-50].

The present proof-of-concept study shows that it is possible to produce *C. utilis* on a medium composed only of "local" renewable resources that are widely available in countries such as Norway. Notably, the fermentations were done without using food crops. It was encouraging to see that the use of seaweed did not pose significant problems and that the seaweed hydrolysate seemed to contain sufficient amounts of the most required micronutrients. Despite a lack of systematic optimisation, yeast production levels were promising and the fish fed diets with the yeast seemed to thrive.

As discussed above, further improvements are needed. These improvements concern the fermentations themselves (medium composition, feeding regime, timing, micronutrients), as well as down-stream processing of the yeast. Another issue concerns the use of nitrogen, which in the 1 and 27 liter fermentations described above was provided in the form of ammonium sulphate and was not limiting. Minimizing nitrogen consumption without compromising protein content would be another useful optimisation parameter. As another step towards a more sustainable process, one could consider replacing the inorganic nitrogen with nitrogen derived from by-products from

410 e.g. the food industry. Hydrolysates from protein-rich by-products could also supply
411 additional micronutrients to the process.

412 **Acknowledgements**

413 This research was supported by a PhD fellowship from NMBU, the Norwegian
414 University of Life Sciences, to S.S. and by the Research Council of Norway through
415 grants 229003, BIOFEED – Novel salmon feed by integrated bioprocessing of non-food
416 biomass, and 237841, Foods of Norway, a centre for research-based innovation.
417 Infrastructure was funded in part by the Norwegian Research Council through grants
418 226247 (NorBioLab) and 208674 (Pilot plant facilities for food processing). The authors
419 would like to thank Dr. Volkmar Passoth (SLU, Sweden) and Dr. Oskar Bengtsson
420 (Borregaard, Norway) for helpful discussions.

References

1. Øverland, M.; Skrede, A., Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *Journal of the Science of Food and Agriculture* **2017,** *97* (3), 733-742.
2. Naylor, R. L.; Hardy, R. W.; Bureau, D. P.; Chiu, A.; Elliott, M.; Farrell, A. P.; Forster, I.; Gatlin, D. M.; Goldburg, R. J.; Hua, K.; Nichols, P. D., Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (36), 15103-15110.
3. Øverland, M.; Karlsson, A.; Mydland, L. T.; Romarheim, O. H.; Skrede, A., Evaluation of Candida utilis, Kluyveromyces marxianus and Saccharomyces cerevisiae yeasts as protein sources in diets for Atlantic salmon (Salmo salar). *Aquaculture* **2013,** *402*, 1-7.
4. Anupama; Ravindra, P., Value-added food:: Single cell protein. *Biotechnology Advances* **2000,** *18* (6), 459-479.
5. Alriksson, B.; Hörnberg, A.; Gudnason, A. E.; Knobloch, S.; Arnason, J.; Johannsson, R., Fish feed from wood. *Cellulose Chemistry and Technology* **2014,** *48* (9-10), 843-848.
6. Aggelopoulos, T.; Katsieris, K.; Bekatorou, A.; Pandey, A.; Banat, I. M.; Koutinas, A. A., Solid state fermentation of food waste mixtures for single cell protein, aroma volatiles and fat production. *Food Chemistry* **2014,** *145*, 710-716.
7. Groenewald, M.; Boekhout, T.; Neuveglise, C.; Gaillardin, C.; van Dijck, P. W.; Wyss, M., Yarrowia lipolytica: safety assessment of an oleaginous yeast with a great industrial potential. *Critical reviews in microbiology* **2014,** *40* (3), 187-206.
8. Ritala, A.; Häkkinen, S. T.; Toivari, M.; Wiebe, M. G., Single Cell Protein—State-of-the-Art, Industrial Landscape and Patents 2001–2016. *Frontiers in Microbiology* **2017,** *8* (2009).
9. Refstie, S.; Baeverfjord, G.; Seim, R. R.; Elvebø, O., Effects of dietary yeast cell wall β-glucans and MOS on performance, gut health, and salmon lice resistance in Atlantic salmon (Salmo salar) fed sunflower and soybean meal. *Aquaculture* **2010,** *305* (1), 109-116.
10. Shurson, G. C., Yeast and yeast derivatives in feed additives and ingredients: Sources, characteristics, animal responses, and quantification methods. *Animal Feed Science and Technology* **2018,** *235*, 60-76.
11. Bekatorou, A.; Psarianos, C.; Koutinas, A. A., Production of Food Grade Yeasts. *Food Technology and Biotechnology* **2006,** *44* (3), 407-415.
12. Buerth, C.; Tielker, D.; Ernst, J. F., Candida utilis and Cyberlindnera (Pichia) jadinii: yeast relatives with expanding applications. *Applied Microbiology and Biotechnology* **2016,** *100* (16), 6981-6990.
13. Grammes, F.; Reveco, F. E.; Romarheim, O. H.; Landsverk, T.; Mydland, L. T.; Overland, M., Candida utilis and Chlorella vulgaris counteract intestinal inflammation in Atlantic salmon (Salmo salar L.). *PLoS One* **2013,** *8* (12), e83213.
14. Lee, B.-K.; Kyun Kim, J., Production of Candida utilis biomass on molasses in different culture types. *Aquacultural Engineering* **2001,** *25* (2), 111-124.

15. Nigam, J. N., Single cell protein from pineapple cannery effluent. *World Journal of Microbiology and Biotechnology* **1998,** *14* (5), 693-696.
16. Panda, S. K.; Ray, R. C.; Mishra, S. S.; Kayitesi, E., Microbial processing of fruit and vegetable wastes into potential biocommodities: a review. *Critical Reviews in Biotechnology* **2018,** *38* (1), 1-16.
17. Skrøppa, T., State of forest genetic resources in Norway. *Norwegian Forest and Landscape Institute* **2012**, 1-56.
18. Lundqvist, J.; Teleman, A.; Junel, L.; Zacchi, G.; Dahlman, O.; Tjerneld, F.; Stålbrand, H., Isolation and characterization of galactoglucomannan from spruce (Picea abies). *Carbohydrate Polymers* **2002,** *48* (1), 29-39.
19. Timell, T. E., Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology* **1967,** *1* (1), 45-70.
20. Rødsrud, G.; Lersch, M.; Sjöde, A., History and future of world's most advanced biorefinery in operation. *Biomass and Bioenergy* **2012,** *46*, 46-59.
21. Krajnc, N. *Wood Fuels Handbook*; 978-92-5-108728-2; Food and Agriculture Organization of the United Nations (FAO): 2015; p 31.
22. Maehre, H. K.; Malde, M. K.; Eilertsen, K. E.; Elvevoll, E. O., Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. *J Sci Food Agric* **2014,** *94* (15), 3281-90.
23. Handå, A.; Forbord, S.; Wang, X.; Broch, O. J.; Dahle, S. W.; Størseth, T. R.; Reitan, K. I.; Olsen, Y.; Skjermo, J., Seasonal- and depth-dependent growth of cultivated kelp (Saccharina latissima) in close proximity to salmon (Salmo salar) aquaculture in Norway. *Aquaculture* **2013,** *414–415*, 191-201.
24. Schiener, P.; Black, K.; Stanley, M.; Green, D., The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta. *J Appl Phycol* **2015,** *27* (1), 363-373.
25. Horn, S. J.; Aasen, I. M.; Østgaard, K., Production of ethanol from mannitol by Zymobacter palmae. *Journal of Industrial Microbiology and Biotechnology* **2000,** *24* (1), 51-57.
26. Kim, N.-J.; Li, H.; Jung, K.; Chang, H. N.; Lee, P. C., Ethanol production from marine algal hydrolysates using Escherichia coli KO11. *Bioresource Technology* **2011,** *102* (16), 7466-7469.
27. Adams, J.; Gallagher, J.; Donnison, I., Fermentation study on Saccharina latissima for bioethanol production considering variable pre-treatments. *J Appl Phycol* **2009,** *21* (5), 569-574.
28. Manns, D.; Andersen, S. K.; Saake, B.; Meyer, A. S., Brown seaweed processing: enzymatic saccharification of Laminaria digitata requires no pre-treatment. *J Appl Phycol* **2016,** *28* (2), 1287-1294.
29. Sharma, S.; Horn, S. J., Enzymatic saccharification of brown seaweed for production of fermentable sugars. *Bioresource Technology* **2016**.
30. Chylenski, P.; Petrovic, D. M.; Muller, G.; Dahlstrom, M.; Bengtsson, O.; Lersch, M.; Siika-Aho, M.; Horn, S. J.; Eijsink, V. G. H., Enzymatic degradation of sulfite-pulped softwoods and the role of LPMOs. *Biotechnology for biofuels* **2017,** *10*, 177.
31. Willis, R. B.; Montgomery, M. E.; Allen, P. R., Improved Method for Manual, Colorimetric Determination of Total Kjeldahl Nitrogen Using Salicylate. *Journal of Agricultural and Food Chemistry* **1996,** *44* (7), 1804-1807.

32. Austreng, E.; Storebakken, T.; Thomassen, M. S.; Refstie, S.; Thomassen, Y., Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture* **2000,** *188* (1), 65-78.
33. Cho, C. Y.; Slinger, S. J., Apparent digestibility measurements in feed stu1 s for rainbow trout. In: Halver, J.E., Tiews, K. (Eds.), Berlin, Germany: Heinemann. *Finfish Nutrition and Fish feed Technology* **1979,** *2*, 239-247.
34. McCleary, B. V.; Solah, V.; Gibson, T. S., Quantitative Measurement of Total Starch in Cereal Flours and Products. *Journal of Cereal Science* **1994,** *20* (1), 51-58.
35. Pampulha, M. E.; Loureiro-Dias, M. C., Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Applied Microbiology and Biotechnology* **1989,** *31* (5), 547-550.
36. Christen, P.; Domenech, F.; Páca, J.; Revah, S., Evaluation of four Candida utilis strains for biomass, acetic acid and ethyl acetate production from ethanol. *Bioresource Technology* **1999,** *68* (2), 193-195.
37. Tanguler, H.; Erten, H., Utilisation of spent brewer's yeast for yeast extract production by autolysis: The effect of temperature. *Food and Bioproducts Processing* **2008,** *86* (4), 317-321.
38. Zheng, S.; Yang, M.; Yang, Z., Biomass production of yeast isolate from salad oil manufacturing wastewater. *Bioresource Technology* **2005,** *96* (10), 1183-1187.
39. Ibrahim Rajoka, M.; Tariq Kiani, M. A.; Khan, S.; Awan, M. S.; Hashmi, A.-S., Production of single cell protein from rice polishings using Candida utilis. *World Journal of Microbiology and Biotechnology* **2004,** *20* (3), 297-301.
40. Earnshaw, R. G.; Appleyard, J.; Hurst, R. M., Understanding physical inactivation processes: combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology* **1995,** *28* (2), 197-219.
41. Garcia Carrillo, M.; Ferrario, M.; Guerrero, S., Effectiveness of UV-C light assisted by mild heat on Saccharomyces cerevisiae KE 162 inactivation in carrot-orange juice blend studied by flow cytometry and transmission electron microscopy. *Food Microbiol* **2018,** *73*, 1-10.
42. Guillou, S.; El Murr, N., Inactivation of Saccharomyces cerevisiae in solution by low-amperage electric treatment. *Journal of Applied Microbiology* **2002,** *92* (5), 860-865.
43. Roohinejad, S.; Koubaa, M.; Sant'Ana, A. S.; Greiner, R., Chapter 4 - Mechanisms of Microbial Inactivation by Emerging Technologies. In *Innovative Technologies for Food Preservation*, Academic Press: 2018; pp 111-132.
44. Kampen, W. H., Chapter 4 - Nutritional Requirements in Fermentation Processes A2 - Vogel, Henry C. In *Fermentation and Biochemical Engineering Handbook (Third Edition)*, Todaro, C. M., Ed. William Andrew Publishing: Boston, 2014; pp 37-57.
45. Gao, Y.; Li, D.; Liu, Y., Production of single cell protein from soy molasses using Candida tropicalis. *Annals of Microbiology* **2012,** *62* (3), 1165-1172.
46. Yunus, F.-u.-N.; Nadeem, M.; Rashid, F., Single-cell protein production through microbial conversion of lignocellulosic residue (wheat bran) for animal feed. *Journal of the Institute of Brewing* **2015,** *121* (4), 553-557.
47. Hansen, J. Ø.; Penn, M.; Øverland, M.; Shearer, K. D.; Krogdahl, Å.; Mydland, L. T.; Storebakken, T., High inclusion of partially deshelled and whole krill meals in diets for Atlantic salmon (Salmo salar). *Aquaculture* **2010,** *310* (1), 164-172.

48. Skrede, A.; Berge, G. M.; Storebakken, T.; Herstad, O.; Aarstad, K. G.; Sundstøl, F., Digestibility of bacterial protein grown on natural gas in mink, pigs, chicken and Atlantic salmon. *Animal Feed Science and Technology* **1998,** *76* (1), 103-116.
49. Baldwin, C. V.; Robinson, C. W., Enhanced disruption of Candida utilis using enzymatic pretreatment and high-pressure homogenization. *Biotechnology and Bioengineering* **1994,** *43* (1), 46-56.
50. Bzducha-Wróbel, A.; Kieliszek, M.; Błażejak, S., Chemical composition of the cell wall of probiotic and brewer's yeast in response to cultivation medium with glycerol as a carbon source. *European Food Research and Technology* **2013,** *237* (4), 489-499.

Table 1. Composition of the fishmeal and the yeast, in g kg^{-1}.

	Fish meal	***C. utilis***
Dry matter	926	964
Ash	154	98
Crude protein[a]	684	333
Starch	0	36
Crude fat	75	21
Gross energy[b]	19	19

[a] Protein content (N x 6.25)

[b] Energy content is in MJ kg^{-1}

Table 2. Proximate analysis of the feeds

Ingredient	**Control (g kg^{-1})**	**Diet with *C. utilis* (g kg^{-1})**
Dry matter	979 ± 1	985 ± 1
Ash	83 ± 1	79 ± 1
Crude protein[a]	582 ± 3	499 ± 8
Starch	13 ± 1	10 ± 1
Crude fat	166 ± 5	155 ± 8

[a] Protein content (N x 6.25)

Table 3. Characteristics of seaweed and spruce hydrolysates

Content	**Seaweed hydrolysate (SEH)**	**Spruce hydrolysate (SPH)**
Dry matter (%)	Appr. 10	Appr. 50
Glucose (g L^{-1})	7 – 12	249 – 317
Xylose (g L^{-1})	0 – 1	18 – 20
Mannitol (g L^{-1})	4 – 9	–
Mannose (g L^{-1})	–	26 – 27
Fructose (g L^{-1})	–	1 – 28
Other sugars (g L^{-1})[a]	–	3 – 6
Other acids (g L^{-1})[b]	–	4 – 5

[a] sum of arabinose, galactose and fucose

[b] sum of lactic and acetic acid

Table 4 Macro and trace minerals determined by ICP-MS in native *S. latissima*, enzymatic hydrolysates of spruce and *S. latissima*, the culture medium at the start of a 27 L fermentation (t_0) and spray-dried yeast.

		Enzymatic hydrolysate		Fermentation	
	Native *S. latissima*	Spruce	*S. latissima*	Initial Medium[a] (t_0)	Spray dried yeast
Macro minerals (g kg^{-1})					
B	0.1	0.0	0.0	0.0	0.0
Na	49.0	15.0	12.5	16.5	7.2
Mg	7.0	0.1	1.5	1.6	1.1
Al	0.1	0.0	0.2	0.0	0.0
Si	0.8	0.0	0.1	0.1	0.0
P	4.4	0.3	0.4	0.4	3.7
S	9.9	17.0	2.1	8.4	8.6
K	96.0	0.3	22.5	24.9	7.8
Ca	48.0	3.6	1.7	2.5	2.4
Fe	0.1	0.0	0.0	0.0	0.2
Ni	1.0	1.6	0.1	0.3	2.6
Zn	0.1	0.0	0.0	0.0	0.1
Sr	1.3	0.0	0.1	0.1	0.0
Cl	130.0	0.0	36.0	45.0	11.0
I	3.9	0.0	0.8	0.9	0.4
Trace minerals (mg kg^{-1})					
V	2.2	0.0	0.1	0.1	0.3
Cr	1.9	0.1	0.0	0.1	3.3
Mn	4.7	5.2	0.5	2.7	7.7
Co	0.1	0.0	0.0	0.3	0.
Cu	2.4	<0.1	0.2	1.1	13.0
As	44.0	0.0	9.8	11.1	3.8
Se	2.1	0.0	0.2	0.2	0.6
Mo	0.4	0.0	0.1	0.2	1.1
Ag	0.0	0.0	0.0	0.0	0.0
Cd	1.1	0.0	0.1	0.1	0.3
Hg	0.0	0.0	0.	0.0	0.0
Tl	0.0	0.0	0.0	0.0	0.

Pb	0.4	0.0	0.0	0.0	0.0
Br	1600.0	0.0	285.0	300.0	120.0
Total elements (g kg^{-1})	353.4	38.0	78.1	101.	45.4

[a] after inoculation

Table 5. Contents of amino acids, nitrogen and crude fat in *Candida utilis* grown on seaweed and spruce hydrolysate, *S. latissima* and a typical fishmeal.

Amino acids[a]	*Candida utilis*[b]	*S. latissima*[c]	Fishmeal[d]
Essential amino acids (EAAs)			
Methionine	4.5 ± 0.1	1.6 ± 0.1	16.1
Threonine	20.6 ± 0.1	3.8 ± 0.2	25.4
Valine	20.9 ± 0.1	4.0 ± 0.3	26.4
Isoleucine	17.4 ± 0.1	2.8 ± 0.2	23.7
Leucine	25.8 ± 0.2	4.7 ± 0.2	42.0
Histidine	6.3 ± 0.1	1.1 ± <0.1	11.8
Lysine	22.8 ± 0.2	4.3 ± 0.2	45.5
Tryptophan	5.3 ± 0.1	1.1	6.9[e]
Total EAAs	123.5 ± 0.7	23.4 ± 1.2	197.8
Non-essential amino acids (NEAAs)			
Aspartic acid	34.1 ± 0.2	9.8 ± 0.4	54.7
Serine	18.7 ± 0.1	3.6 ± 0.3	25.3
Glutamic acid	43.3 ± 0.3	15.6 ± 0.5	83.9
Proline	14.4 ± 0.5	4.1 ± <0.1	23.1
Glycine	15.9 ± 0.2	3.5 ± 0.1	30.8
Tyrosine	10.6 ± 0.1	0.7 ± <0.1	15.2
Arginine	17.3 ± 0.1	4.1 ± 0.3	35.3
Cysteine	2.8 ± 0.1	2.2 ± 0.1	5.7
Alanine	21.4 ± 0.2	7.6 ± 0.1	32.6
Phenylalanine	15.0 ± 0.1	4.0 ± <0.1	22.0
Total NEAAs	193.4 ± 1.6	55.1 ± 1.7	328.6
Total (g kg^{-1} of DM)	316.9 ± 2.3	78.5 ± 2.9	526.4
Nitrogen (g kg^{-1})	52.0 ± 0.1	15	113.3
Crude lipid (g kg^{-1})	21.8 ± 0.2	8.11	88.0

[a] All values are in g kg^{-1} of DM with standard deviation. All analyses were carried out in duplicate except tryptophan and the nitrogen content of seaweed.
[b] Spray dried *Candida utilis* yeast biomass from a 27 L fermentation on SEH +SPH.
[c] *Saccharina latissima* seaweed dried at 30 °C
[d] Contents of amino acids (except tryptophan), nitrogen and crude lipid in fishmeal are taken from [47].
[e] The content of tryptophan in fishmeal is taken from [48].

Table 6. Apparent digestibility coefficients of dry matter, ash, protein and starch, and apparent digestibility of *C. utilis* protein.

Diets	**Control diet**	**Diet with *C. utilis***
Dry matter	81.0 ± 0.3	60.7 ± 1.1
Ash	22.1 ± 1.9	1.7 ± 3.4
Protein	89.7 ± 0.7	80.0 ± 1.2
Starch	68.1 ± 1.6	42.2 ± 4.1
C. utilis protein[a]	-	40.5 ± 5.9

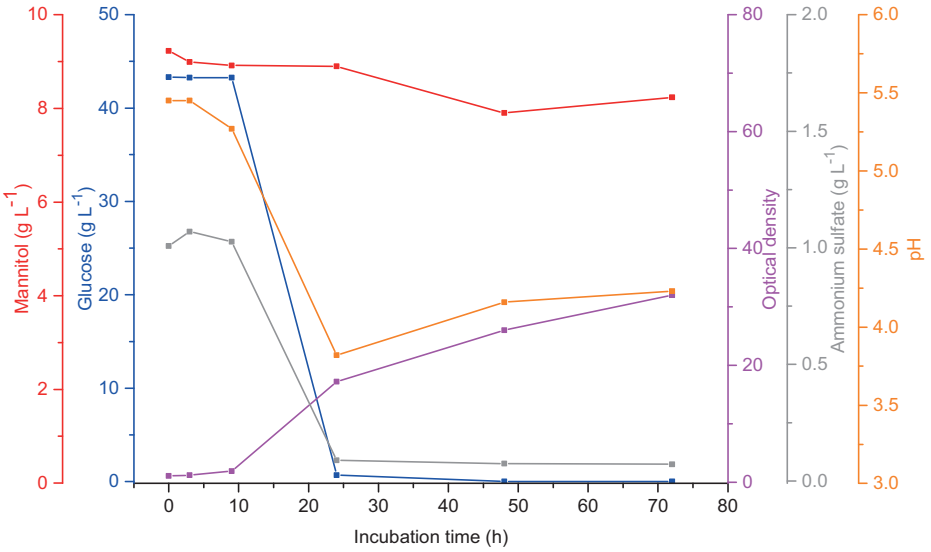
[a] Calculated according to equation 2

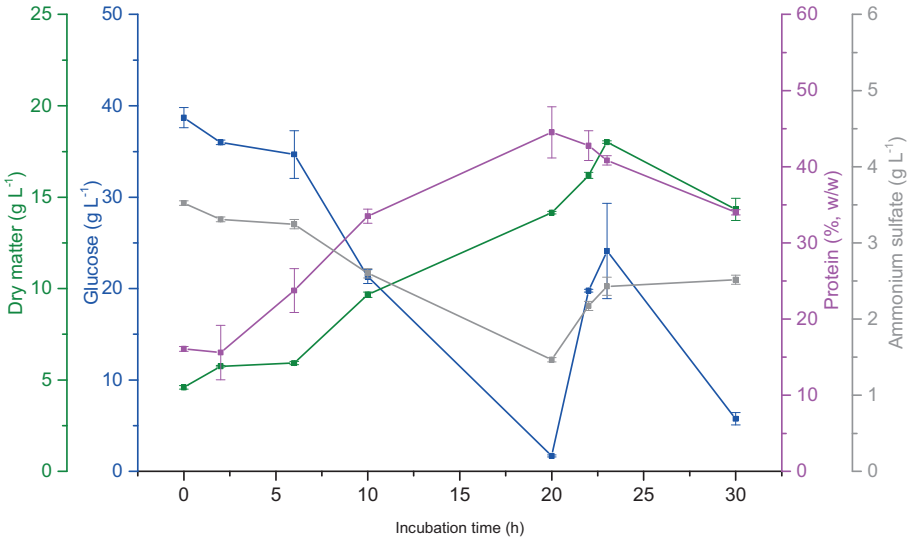
Figure legends

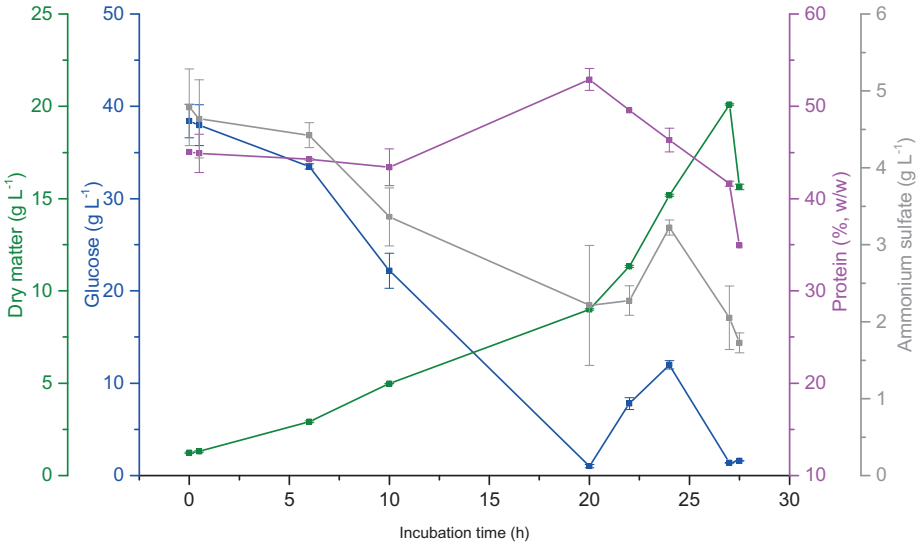
Fig 1. Growth of *C. utilis* on a medium comprised of SEH and SPH in a shake flask. The medium contained 90 % (v/v) SEH and 10 % (v/v) SPH and was supplemented with 2.04 g L^{-1} of YNB salts (corresponding to 1.5 g L^{-1} ammonium sulphate). Growth was monitored by recording the optical density at 595 nm.

Fig 2: One-liter fed-batch fermentation of *C. utilis* on a medium containing SEH, SPH and ammonium sulphate. The starting glucose concentration was 38 g L^{-1} and the reactor was fed between 20 and 23 h at the pump rate of 35 mL h^{-1}. The figure shows the mean of duplicate fermentations ± standard deviation.

Fig 3: Twenty seven liter fed-batch fermentation of *C. utilis* on a medium containing SEH, SPH and ammonium sulphate. The starting glucose concentration was 38 g L^{-1}. The reactor was fed between 20 and 24 h using a pumping rate of 750 mL h^{-1}. The last point of the curves, drawn at 27.5 h, does not represent a sample from the fermentation but represents the situation after heat inactivation of the cells, which was started at 27 h. The figure shows the mean of duplicate measurements for a typical fermentation +/- standard deviation.


Mannitol (g L-1)
Glucose (g L-1)
Optical density
Ammonium sulfate (g L-1)
pH
Incubation time (h)
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
0
10
20
30
40
50
0
20
40
60
80
0.0
0.5
1.0
1.5
2.0
3.0
3.5
4.0
4.5
5.0
5.5
6.0


Dry matter (g L-1)
Glucose (g L-1)
Protein (%, w/w)
Ammonium sulfate (g L-1)
Incubation time (h)
0
5
10
15
20
25
30


Dry matter (g L-1)
Glucose (g L-1)
Protein (%, w/w)
Ammonium sulfate (g L-1)
Incubation time (h)
0
5
10
15
20
25
30
0
10
20
30
40
50
0
5
10
15
20
25
0
10
20
30
40
50
60
0
1
2
3
4
5
6

Paper V

Microbial feed ingredients produced from different feedstock in diets for Atlantic salmon (*Salmo salar*): nutrient and mineral digestibility

Sandeep Sharma[1], Jon Øvrum Hansen[2], Liv Torunn Mydland[2], Svein J. Horn[1], Vincent G. H. Eijsink[1], Margareth Øverland[2*]

[1]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1433 Aas, Norway

[2]Department of Animal and Aquacultural Sciences, Faculty of Biosciences, Norwegian University of Life Sciences, 1433 Aas, Norway

*Corresponding author: margareth.overland@nmbu.no

Abstract

Candida utilis was cultivated using three different feedstocks; 1) a blend of woody hydrolysate and molasses (CUE), 2) spent sulphite liquor (CUA), and 3) a blend of brown seaweed and woody hydrolysate (CUN). A high-quality fish meal-based reference diet (FM; control) and three experimental diets that consisted of a 70:30 mixture of the reference diet to yeast ingredient (CUE30, CUA30 or CUN30) were prepared. The diets were fed for 48 days to triplicate groups of pre-smolt Atlantic salmon (*Salmo salar;* initial body weight = 65 g) kept in freshwater. Faecal samples were collected by stripping and yttrium was used as indigestible marker to determine nutrient and mineral digestibility.

There was no significant differences in the final weight, feed conversion ratio or specific growth rate of fish fed the CUN30 diet and the control. The CUN30 diet resulted in reduced digestibility of dry matter, ash, and protein compared to the three other diets, and digestibility of both fat and starch was lower than for the FM diet. The percentage excretion of all minerals was the same for fish fed the CUE30 and CUA30 diets, except for chromium and iodine. However, there was differences in excretion of all minerals between fish fed the FM and CUN30 diets, except for cadmium, chlorine, selenium and iodine. In conclusion, feeding 30% of CUN resulted in reduced growth performance and digestibility of nutrients, while CUE and CUA were shown to be promising protein sources in diets for salmonids.

Keywords: Atlantic salmon, *Candida utilis*, yeast, Nutrient, Mineral, Digestibility

1. Introduction

The partial replacement of fishmeal with plant-based ingredients in Norwegian Atlantic salmon (*Salmo salar*) diets have been steadily increasing since 2000 (Ytrestøyl et al., 2015). However, decreased growth performance and lower protein utilization have been observed in carnivorous fish, such as the salmon, when plant proteins have been used to replace fishmeal in the feeds (Aslaksen et al., 2007; Refstie et al., 2006). This could be due to imbalanced amino acid composition and the presence of antinutritional factors, which can inhibit digestive enzymes, essential metabolic pathways, and result in negative health effects (Francis et al., 2001; Krogdahl et al., 2010).

Lately, there have been great interest in using microbial ingredients such as microalgae, yeasts and bacteria, in both terrestrial farm animal and fish diets (Aas et al., 2006; Sørensen et al., 2017; Øverland & Skrede, 2017). Yeast represent a potential ingredient in fish feeds due to its high protein content with a favorable amino acid composition that has shown to support high growth rates (Anupama & Ravindra, 2000). In addition, these microbial ingredients are a sustainable alternative as they do not compete with human food production. However, the cost of growth media components constitute more than 50 % of the overall cost for fermentative production of microbial biomass (Walker & Stewart, 2016). Hence, less expensive feedstock for the cultivation of yeast needs to be explored.

Candida utilis is an amyloytic, single-celled, protein-rich yeast, belonging to the *Saccharomycetes* class. It has a status of generally-regarded-as-safe (GRAS), can metabolize a wide range of substrates and has been widely used as a fodder yeast (Bekatorou et al., 2006; FDA, 2018). Molasses is a cheap by-product from the sugar industry, and has been used worldwide for the production of both bioethanol and for the cultivation of *C. utilis* (Gönen & Aksu, 2008; Lee & Kyun Kim, 2001). In other studies, the cultivation of *C. utilis* was performed using wood hydrolysates and sulphite spent liquor (Brenne et al., 1974; Mikulášová et al., 1990). In a recent study, the cultivation of *C. utilis* was carried out using enzymatic hydrolysate of *Saccharina latissima* as a source of nutrients and spruce hydrolysate as the main source of sugars for the fermentation (Sharma et al., 2018a). One of the main challenges of using these cheaper sources of

carbon and nutrients to cultivate *C. utilis* is the risk of incorporation of unwanted components from the media that could adversely affect the growth performance and the safety and nutritional value of the fish product. In this context, heavy metals, such as cadmium and arsenic, as well as iodine, derived from the seaweed feedstock could be assimilated by *C. utilis,* and be potentially harmful for fish and consumers. The inclusion level of yeast in the diets could affect the level of unwanted minerals in the fish flesh. Studies have shown that substitution levels from 30 to 50 % of *C. utilis*, replacing fishmeal in fish diets, did not significantly affect growth performance (Martin et al., 1993; Olvera-Novoa et al., 2002; Øverland et al., 2013). Prior to commercialization of yeast grown on novel substrates as a protein source, it is necessary to perform a thorough evaluation of the nutritional value in targeted fish species and ensure the safety of such ingredients with respect to content of potential harmful components in fish flesh.

The aims of the present study was to investigate the nutrient digestibility of *C. utilis* cultivated on different carbon and nutrient sources, and to evaluate the impact of these differently produced yeast biomasses on faecal mineral excretion.

2. Materials and methods

2.1. Cultivation of yeast

Three different media were used to cultivate the same yeast strain (*Candida utilis*; Torula; LYCC 7549; Lallemand Yeast Culture Collection):

1. *C. utilis* produced in Estonia (CUE): The LYCC 7549 yeast strain was cultivated by using woody hydrolysate (supplied by Borregaard, Norway) and molasses. In brief, wood chips from Norwegian spruce trees was used in a biorefinery (BALI) process at the Borregaard pilot plant (Rødsrud et al., 2012) (Sarpsborg, Norway). The BALI-sugars was mixed (1:1, v/v) with sugars from beet molasses and used as the principal carbon source for cultivating *C. utilis*. Fed-batch fermentation was carried out at Lallemand (Salutaguse, Estonia). After fermentation, the yeast cells were heat-inactivated, centrifuged, and washed before drum drying.
2. *C. utilis* produced in USA (CUA): The LYCC 7549 yeast strain was cultivated by using spent sulfite liquor retrieved from the pulp and paper industry in a continuous fermentation process (Lallemand, Wisconsin, USA). Harvested cells were heat-inactivated, centrifuged, washed couple of times, and then spray dried.
3. *C. utilis* produced in Norway (CUN): The LYCC 7549 yeast strain was cultivated on a blend of brown seaweed and woody enzymatic hydrolysates as a carbon and nutrient source. After fermentation, the yeasts cell were heat-inactivated, centrifuged, suspended in water, and washed 3 times prior to spray drying (Sharma et al., 2018a).

The proximate compositions and amino acid profiles of all three yeast biomasses and fishmeal (FM) are presented in Table 1.

2.2. Diet preparation

The apparent digestibility coefficients (ADC) for each yeast ingredient was determined using the inclusion method (Bureau & Hua, 2006). The reference (control) diet was based on fishmeal (FM) as the main protein source, and the diet formulation is presented in Table 2. Yttrium oxide (0.1 g kg^{-1}) was added in the diet as an internal marker to examine digestibility (Austreng et al., 2000). Three experimental diets (one for each yeast preparation; CUE30, CUA30 and CUN30) were produced by replacing 30 % of the control diet with yeast. The diets were prepared at the feed laboratory of the Norwegian University of Life Sciences, Ås, Norway.

All dry ingredients (excluding gelatin) were mixed in a blender (GRAIN, Italy). The fish oil was added to the blender and mixed thoroughly. Dissolved gelatin (60 °C) was added into the mixture resulting in a homogenous dough. For pelleting, the dough was passed through a pasta extruder (ITALGI, Italy) equipped with a 3 mm die. The diets were kept frozen at -20 °C until further use. The proximate analysis and minerals present in the experimental diets are shown in Table 3.

139 Table 1. Proximate composition and amino acids present in the fish meal and yeasts used
140 in the present experiment (CUE = *C. utilis* from wood hydrolysate and molasses, CUA =
141 *C. utilis* from spent sulphite liquor from pulp and paper mill, CUN = *C. utilis* from brown
142 seaweed and woody hydrolysates)

Ingredient (g kg^{-1})	**Fish meal**	**CUE**	**CUA**	**CUN**
Proximate analysis				
Dry matter	926	970	921	964
Ash	154	78	67	98
Crude protein[a]	684	470	391	333
Crude fat	75	16	21	21
Gross Energy (MJ kg^{-1})	19	20	18	19
Amino acids (g 16 g N^{-1})				
Indispensable amino acids				
Arginine	6.6	5.2	4.4	5.0
Histidine	2.3	1.8	1.7	1.8
Isoleucine	4.7	4.6	3.8	5.0
Leucine	8.0	6.7	5.8	7.5
Lysine	8.9	6.5	6.0	6.6
Methionine	3.1	1.1	1.1	1.3
Phenylalanine	3.8	3.9	3.6	4.3
Threonine	4.8	5.4	4.5	6.0
Tryptophan	1.0	1.3	1.1	1.5
Valine	5.2	5.5	4.5	6.1
Dispensable amino acids				
Cysteine	1.3	0.8	0.8	0.8
Aspartic acid	10.9	8.9	7.8	9.9
Serine	5.0	5.1	4.2	5.4
Glutamic acid	14.8	14.3	14.4	12.5
Proline	4.5	3.5	2.7	4.2
Glycine	6.9	4.1	3.3	4.6
Alanine	6.5	6.0	4.8	6.2
Tyrosine	2.7	2.9	2.9	3.1
Total amino acids	101.1	87.8	77.4	91.9

143 [a] Protein content (N x 6.25)
144

145

146 Table 2. Formulation of the fishmeal reference (control) diet

Ingredient (g kg^{-1})	FM
Fish meal[a]	481.15
Wheat gluten[b]	130.0
Gelatinised potato starch[c]	120.0
Gelatin[d]	110.0
Fish oil[e]	150.0
Vitamin & mineral premix[f]	7.0
MCP[g]	0.2
Choline chloride[h]	1.5
Yttrium oxide[i]	0.15

147 [a] Norse LT 16-001, Norsildmel, Egersund Sildoljefabrikk AS, Egersund, Norway.
148 [b].Vital Wheat Gluten, Aminlina, Panevezys, Lithuania.
149 [c] Lygel F 60, Lyckeby Culinar, Fjälkinge, Sweden.
150 [d] Rousselot® 250 PS, Rousselot SAS, Courbevoie, France.
151 [e] NorSalmOil, Norsildmeld, Egersund, Norway.
152 [f] Farmix, Trouw Nutrition, LA Putten, The Netherlands. Per kg feed. Retinol 2500.0 IU, Cholecalciferol
153 32400.0 IU, α-tocopherol SD 0.2 IU, Menadione 40.000 mg, Thiamin 15.0 mg, Riboflavin 25.0 mg, d-Ca-
154 Pantothenate 40.002 mg, Niacin 150.003 mg, Biotin 3000.0 mg, Cyanocobalamin 20.0 mg, Folic acid 5.0
155 mg, Pyridoxine 15.0 mg, Ascorbate polyphosphate 0.098 g, Cu: CuSulfate $5H_2O$ 11.998 mg, Zn:
156 ZnSulfate 89.992 mg, Mn: Mn(II)Sulfate 34.993 mg, I: K-Iodine 1.999 mg, Se: Na-Selenite 0.200 mg, Cd
157 Max. 0.0003 mg, Pd Max. 0.028 mg, Ca 0.915 g, K 1.380 g, Na 0.001 g, Cl 1.252 g.
158 [g] Bolifor®Monocalcium phosphate-F.KPP Oy, Animal Nutrition, Helsingborg, Sweden.
159 [h] Choline chloride, 70 % Vegetable, Indukern S.A. Spain.
160 [i] Yttrium oxide (Y_2O_3), Metal Rare Earth Limited, Shenzhen, Guangdong, China.
161

162

163 **2.3. Feeding and sampling**

164

165 The diets were fed to triplicate tanks of pre-smolt Atlantic salmon with an initial body
166 weight of 65.0 ± 0.5 g. A total of 480 fish were randomly distributed into 12 fiberglass
167 tanks with 40 fish per tank, each with 300 L capacity. The fish were kept under continuous
168 light in recirculated fresh water with a water supply of 6 - 7 L min^{-1}. The four experimental
169 diets were fed to the fish over a period of 2 h (11:15 to 13:15) daily by automatic belt
170 feeders, and uneaten feed was collected after every meal according (Helland et al., 1996).
171 The fish were initially fed the control diet at a rate of approximately 1 % of the biomass
172 for 21 days and then with experimental diets for 48 days. The water quality parameters

throughout the experiment were all within acceptable levels for salmon (temperature 13.9 °C ± 0.5; pH 7.3 ± 0.4; alkalinity 0.9 mmol L^{-1}; ammonia-nitrogen NH_4-N, 0.08 mg L^{-1}; and nitrates 0.04 mg L^{-1}). Dissolved oxygen levels were measured throughout the study and were kept above 8.5 mg L^{-1}. Faecal samples from each fish were collected by stripping according to the procedures (Austreng, 1978). Prior to stripping, the fish were anesthetized with tricaine methanesulfonate (MS222, USA). Stripping were carried out at day 30, 36, 43 and 48. The faecal samples were pooled per tank, immediately frozen, and kept at -20 °C before freeze-drying. At the end of the experiment, all fish were weighed.

2.4. Chemical analyses

All diets, yeasts and faeces samples were finely ground by mortar and pestle prior to analysis. Dry matter was determined by weighing samples before and after drying at 105 °C for 8 h, and ash was measured by incinerating the samples at 550 °C for 16 h. Nitrogen contents were analysed by the Kjeldahl method according to the European Commission (Commission, 2009) and crude protein was calculated by multiplying the nitrogen value with 6.25. Crude fat content of samples was analysed gravimetrically with petroleum ether and acetone (70:30, v/v) using an accelerated solvent extractor (Dionex ASE 200, Dionex Corp, USA). Starch was analysed enzymatically (McCleary et al., 1994). The gross energy was measured by using bomb calorimetry (Parr 1281, Parr Instruments, USA) (ISO, 1998). Amino acids (except tryptophan) in the ingredients were analyzed according to Commission regulation (EC) No 152/2009 on a Biochrom 30 Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed according to Commission regulation (EC) No 152/2009 on a Dionex UltiMate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Mineral content in diets and faecal samples were analysed by inductively coupled plasma spectrometry combined with mass spectrometric detector (ICP-MS) (Perkin-Elmer, USA)

203 Table 3. Proximate analysis and mineral content of the experimental diets
204

Diets (g kg^{-1})	Control[1]	CUE30[1]	CUA30[1]	CUN30[1]
Dry matter	979	980	964	985
Protein	582	536	512	499
Ash	83	74	71	79
Fat	166	151	149	155
Starch	13	10	11	10
Macro minerals (g kg^{-1})				
Na	6.0	4.4	3.9	5.4
Mg	1.3	1.2	1.2	1.1
Al	0.07	0.05	0.06	0.04
P	12.0	11.7	11.0	9.8
S	5.8	4.7	4.5	8.2
K	6.8	13.0	11.8	7.7
Ca	20.0	12.7	12.5	17.3
Fe	0.12	0.11	0.13	0.10
Zn	0.21	0.19	0.17	0.15
Cl	10.0	6.9	7.0	9.1
Br	0.04	0.02	0.02	0.05
Trace mineral (mg kg^{-1})				
Cr	2.1	1.4	6.1	1.9
Mn	26.0	21.3	20.5	30.3
Co	1.2	0.9	0.8	0.8
Ni	1.2	0.9	3.9	1.2
Cu	12.0	11.0	8.5	11.0
As	6.3	3.9	3.9	5.2
Cd	0.09	0.08	0.08	0.31
Pb	0.27	0.10	0.10	0.16
Se	1.2	0.7	0.8	1.0
I	6.1	6.4	3.0	87.8

205 [1] Control = fishmeal-based reference diet, CUE30, CUA30 and CUN30 = 70% reference diet mixed with
206 30% of the yeast ingredients; CUE = *C. utilis* from woody hydrolysate and molasses; CUA = *C. utilis* from
207 spent sulphite liquor, and CUN = *C. utilis* from brown seaweed and woody hydrolysate.
208 All analyses were carried out in triplicates.

2.5. Calculations and statistical analyses

The growth performance of fish were evaluated by the indices final weight, g; weight gain (g) = (final weight of fish, g – initial weight g). Feed utilization was evaluated by feed conversion ratio (FCR); FCR = feed consumed x weight gain^{-1}. Fish growth was evaluated as specific growth rate (SGR) according to the following equation:

SGR = 100 x [(ln final mean body weight – ln initial mean body weight) x day^{-1}].

The nutrient apparent digestibility coefficients (ADCs) were calculated by using equation 1 (Cho & Slinger, 1979).

$$ADC\ (\%) = 100 * \frac{(a-b)}{a} \quad (1)$$

Where a represents (nutrient in feed / yttrium in feed) and b represents (nutrient in faeces/ yttrium in faeces). The apparent digestibility coefficients of test ingredients was calculated using equation 2 (Bureau & Hua, 2006).

$$ADC_{ingredients}(\%) = ADC_{testfeed} + \left(ADC_{testfeed} - ADC_{control\ feed}\right) x\ \frac{0.7\ x\ Nutrient_{ref}}{0.3\ x\ Nutrient_{ingredient}} \quad (2)$$

The faecal excretion of minerals was calculated by using equation 3.

$$Faecal\ excretion\ of\ minerals\ (\%) = \left(-100 * \frac{(a-b)}{a}\right) + 100 \quad (3)$$

All statistical analyses were performed using SAS (one-way analysis of variance to differentiate between the diets). Significant differences among ADC and mineral excretion values of diets were determined by the Tukey's multiple range test and are indicated in the tables with superscript [a,b,c]. Results are presented as average and pooled standard errors of means (s.e.m) with the significant ($p<0.05$) differences.

3. Results

3.1. Composition of diets

Table 1 shows the proximate composition and amino acid profile of the fishmeal and the yeasts used in this experiment. The dry matter and the gross energy of all ingredients were similar. The protein content in the yeast ingredients were in the range of 33.3 – 47.0 %. The yeast ingredients had similar amino acid composition. However, the methionine content in the yeast ingredients was lower than the fishmeal. The fishmeal also has a higher content of protein, fat and ash. Table 3 shows the proximate composition and mineral content of the control and experimental diets. The protein and lipid content in the control diet was somewhat higher than in the three yeast diets. The CUN30 diet was higher in some of the minerals, particularly sulfur, cadmium and Iodine, whereas the control diet contained more lead.

3.2. Growth performance of salmon

Growth performance of pre-smolt Atlantic salmon fed the experimental diets for 48 days are shown in Table 4. Fish biomass increased between 1.45 – 1.62 times in the different feeding groups.

Table 4. Growth performance of pre-smolt Atlantic salmon (*Salmo salar*) fed the experimental diets for 48 days

Diets	**Control**	**CUE30**	**CUA30**	**CUN30**	**s.e.m.**[1]	***P*-value**
Initial weight (g)	65.2	64.9	64.8	65.2	0.58	0.73
Final weight (g)	97.1^{ab}	105.1^{a}	102.0^{ab}	94.8^{b}	3.33	0.01
FCR[2]	0.75^{ab}	0.68^{b}	0.70^{b}	0.84^{a}	0.41	0.005
SGR[3]	0.82^{bc}	1.00^{a}	0.94^{ab}	0.78^{c}	0.06	0.006

[1] Pooled standard error of mean. Different letters ($^{a, b, c}$) denote significant ($p<0.05$) difference among treatments. n = 3 replicates per treatment.
[2] Feed conversion ratio (FCR) = feed consumed x weight gain^{-1}.
[3] Specific growth rate (SGR) (%) = 100 x [(ln final mean body weight – ln initial mean body weight) x day^{-1}]

At the end of the experiment, final body weight for fish fed the CUN30 was not significantly different from the control diet. The FCR and SGR followed the same pattern. The CUE30 and CUA30 fed fish had a lower FCR than fish fed the CUN30 diet.

3.3. Digestibility

3.3.1. Nutrient digestibility

The ADCs of dry matter (DM), ash, fat, protein and starch of the control and experimental diets are given in Table 5. The ADC of DM was highest in the control diet (81 %), whereas the ADC of DM of the experimental diets was ranging from 60.7 – 68.9 %. There was no significant difference in ADC of DM in fish fed the CUE30 and CUA30 diets. The ADC of ash was similar for all the diets, except CUN30, which was considerably lower (1.7%). The highest ADC value of protein was observed in the control diet (89.7 %), followed by CUA30, CUE30 and CUN30, which were in the range of 80.0 – 86.4 %. The CUN30 diet gave a lower digestibility of fat and starch compared with the control diet. However, on ingredient level, the ADC of protein in fish fed the CUN was significant lower than the other two yeast (CUE and CUA).

3.3.2. Faecal excretion of minerals

The percentage faecal excretion of minerals from fish fed the control and the three experimental diets is presented in Table 5. There was no significant differences in excretion of minerals between fish fed the CUE30 and CUA30 diets, except chromium which had higher excretion in CUE30 fed fish and iodine which had higher excretion in fish fed the CUA30 diet. However, there was difference in digestibility of most minerals between fish fed the control diet and the CUN30 diets, except for cadmium, chlorine, and iodine. In, general a high excretion was observed for cadmium, which was in the range of 92.5 – 99.7 %.

Table 5. Apparent digestibility coefficients of dry matter, ash, fat, protein and starch, apparent digestibility of test ingredients and faecal mineral excretion (%) of the experimental diets

Diets	Control	CUE30	CUA30	CUN30	s.e.m.[1]	*P*-value
Dry matter	81.0^{a}	68.9^{b}	66.0^{b}	60.7^{c}	1.79	<0.001
Ash	22.1^{a}	22.5^{a}	21.0^{a}	1.7^{b}	4.45	<0.001
Fat	97.8^{a}	96.4^{ab}	96.3^{ab}	96.1^{b}	0.60	0.028
Protein	89.7^{a}	85.2^{b}	86.2^{b}	80.0^{c}	1.03	<0.001
Starch	68.1^{a}	51.5^{ab}	47.6^{ab}	42.2^{b}	9.42	0.045
Apparent digestibility of test ingredients						
Protein	-	72.3^{a}	74.0^{a}	40.5^{b}	5.11	<0.001
Faecal minerals excretion (%)						
Mg	33.7^{b}	33.7^{b}	30.5^{b}	43.1^{a}	1.79	<0.001
P	54.9^{a}	48.2^{c}	47.2^{c}	51.3^{b}	1.01	<0.001
S	32.9^{c}	43.4^{b}	42.8^{b}	59.8^{a}	1.84	<0.001
K	4.8^{b}	4.0^{b}	4.7^{b}	7.1^{a}	0.41	<0.001
Cr	51.4^{b}	48.5^{b}	10.2^{c}	85.4^{a}	8.39	<0.001
Zn	65.0^{a}	61.4^{a}	60.9^{a}	55.5^{b}	1.62	<0.001
As	12.8^{c}	21.3^{b}	23.8^{b}	34.7^{a}	1.11	<0.001
Cd	97.4	98.8	99.7	92.5	3.11	0.083
Cl	23.1^{c}	57.4^{ab}	72.9^{a}	42.0^{bc}	8.03	<0.001
Se	44.0^{b}	57.3^{a}	60.2^{a}	58.2^{a}	2.98	<0.001
Br	17.3^{c}	39.3^{ab}	48.7^{a}	34.8^{b}	4.28	<0.001
I	23.4^{b}	18.9^{b}	48.3^{a}	23.5^{b}	2.43	<0.001

[1] Pooled standard error of mean. Different letters in a row denote significant ($p<0.05$) difference among treatments. n = 3 tank replicates per dietary treatment.

4. Discussion

4.1. Nutrient digestibility

The present study evaluated the growth performance and ADCs of main nutrients in Atlantic salmon fed diets containing 30 % of *C. utilis* fermented on three different carbon substrates. Utilization of conventional carbon substrate such as glucose are suitable for lab scale studies, however it is not economically viable to support the production at commercial scale. Thus, there is a need to explore possibilities to utilize novel low-cost carbon substrates in fermentation media. Inclusion of yeast as a protein source is not yet commonly used in salmonid diets, although several trials have been performed evaluating growth performance, digestibility and health to validate its inclusion in salmonid diets (Grammes et al., 2013; Huyben et al., 2017; Vidakovic et al., 2016; Øverland et al., 2013). Several approaches have been carried out to use alternative carbon and nutrient sources for cultivation of yeast on a pilot scale level (Brenne et al., 1974; Gönen & Aksu, 2008; Lee & Kyun Kim, 2001; Mikulášová et al., 1990). Microbial protein ingredients derived from these feedstocks would not only provide high-quality protein, but also other macro and micronutrients such as minerals and vitamins (Anupama & Ravindra, 2000; Ritala et al., 2017).

Notably, it has been reported that yeast (*Saccharomyces cerevisiae*) can replace 50 % of fishmeal in diets for seabass and pacu, with no adverse effects on growth performance (Oliva-Teles & Gonçalves, 2001; Ozório et al., 2010). In our case, the present diets were designed to evaluate nutrient digestibility, thus the diets were not formulated to contain equal amounts of protein and essential amino acids. Thus, the present experiment cannot directly compare the growth potential of these diets in Atlantic salmon.

The digestibility value for protein in the control diet are in line with those reported by others for FM-based diets (Gong et al., 2018; Øverland et al., 2013). The results demonstrated that the salmon fed CUE30 and CUA30 had lower ADC of protein compared to the FM diet, and the CUN30 diet obtained a protein digestibility digestibility that was lower than all the other diets.

One important factor that could explain with the lower protein digestibility of the yeast-based diets (CUN, CUA, and CUE), is the structure of the yeast cell wall. Yeast has thick and rigid cell walls that may limit enzymatic access to the cellular contents (Murray & Marchant, 1986; Tukmechi & Bandboni, 2014; Yamada & Sgarbieri, 2005). For instance, higher digestibility of protein and amino acids has been reported in Arctic char fed autolyzed yeast extract compared to intact cells of *S. cerevisiae* (Langeland et al., 2016). Furthermore, the diets in the present experiment were not extruded, thus feed technology methods such as extrusion may increase the digestibility of protein (Carter & Hauler, 2000).

It is known that the yeast cell wall thickness can vary according to the growth stage, and yeast cells grown in minimal medium have been shown to have a significant reduction in cell wall thickness when phosphate was added to the medium (Blaize et al., 2009). The CUN was produced in several batches in a small fermenter without optimization of nutrients such as N and P, whereas the CUA and CUE fermentations were carried out in large industrial fermenters. Thus, the differences in protein digestibility among the three yeast products could possibly also be partly due to differences in growth stage at harvest, and thereby the cell wall thickness. In this study, apart from the different carbon and nutrient sources used for the cultivation of yeast, the downstream processing of the yeast candidates was different. For instance, the CUA was drum dried, while the CUE and CUN yeast were spray dried. Drying method of the yeast could be a major factor affecting nutrient digestibility. In the drum drying, yeast are in direct contact with the highly heated vessel, which may result in functional damage of the protein. During spray drying, the fermentation broth is atomized to small droplets that are further sprayed into a stream of hot air, which is known as a gentle drying process (Labuza et al., 1972). These are well known issues that are associated with many microbial protein sources that has to be addressed by developing improved down-stream processing strategies, which may include disruption of the cell wall by cell homogenization and fractionation (Baldwin & Robinson, 1994; Bzducha-Wróbel et al., 2013; Rumsey et al., 1991).

The reason for the low ADC of the CUN yeast in the present study could be multifactorial as discussed above. However, it was observed that the color of the yeast grown on

353 seaweed hydrolysate and the final CUN30 diet was noticeably darker and greener than
354 the other diets. This could be due to the presence of polyphenolic compounds, and/or
355 pigments retrieved from the seaweed, which could inhibit digestibility of nutrients.
356 Seaweed also contain non-starch polysaccharides (NSP), and studies have shown that
357 soluble NSPs, such as alginate and guar gum in diets of fish can cause low nutrient
358 digestibility (Kraugerud et al., 2007).

359

360 **4.2. Mineral digestibility**

361

362 Fish ingest minerals from both the diet and the surrounding water. Excessive use of
363 inorganic minerals in the diets by adding premix minerals could be avoided by providing
364 organically bounded minerals in feed ingredients (Sugiura et al., 1998). In this study, the
365 different carbon and nutrient sources that were used for the cultivation of *C. utilis,* resulted
366 in differences in the mineral profiles of the diets. However, in general, the FM control have
367 higher levels of all minerals with some exceptions for the CUA and CUN diet. This is
368 mainly due to the lower inclusion level of FM, which have high ash level and the 30%
369 reduction in mineral mix due to the experimental design. Of particular interest are the
370 elements phosphorus, potassium, zinc, cadmium, arsenic and iodine. Faecal excretion of
371 phosphorus ranged from 47.2 to 51.3 % in the experimental diets, which was lower than
372 the control diet (54.9 %). The relatively low availability of phosphorus in all diets is in line
373 with previously experiments with salmonid (Hansen & Storebakken, 2007; Riche & Brown,
374 1996; Storebakken et al., 1998). Sodium and potassium are essential minerals that helps
375 fish to activate adenosine triphosphatase of their gills, which acts as a major role in
376 adaptation of teleosts to sea water (Epstein et al., 1967). Dietary potassium requirement
377 of fish is reported to be 7 to 12 g kg^{-1} diet (Davis & Gatlin, 1996; Shearer, 1988), and
378 content in the diets ranged from 6.8 to 13 g kg^{-1} . ADC of potassium were high in all diets
379 (>93 %). This observation was in agreement with another study, where they observed
380 that by feeding potassium rich diets to carp, the digestibility increased up to 88 % (Sugiura
381 et al., 1998). Zinc is an important element in fish nutrition that is involved in various
382 metabolic pathways as specific cofactor for several enzymes. The content of zinc in all

383 diets were above the recommended level for fish (Watanabe et al., 1997), but the
384 availability of zinc was relatively low for all diets, however, it is in line with previous
385 salmonid experiments (Denstadli et al., 2006; Hansen & Storebakken, 2007).

386 Apart from all essential minerals, both FM and the microbial protein ingredients also
387 contain toxic elements such as arsenic and cadmium. The maximum permissible level of
388 cadmium and arsenic in the diet for fish are 1 and 10 mg kg^{-1} diet, respectively
389 (Commission Regulation (EU), 2013). Except the CUN30 diet, all diets had cadmium
390 levels below 1 mg kg^{-1} diet. Interestingly, the excretion of cadmium from all diets was
391 above 93 %, thus, a very small amount of the dietary cadmium was absorbed. It has been
392 observed that excess dosage of cadmium in the diets of salmonids could induce
393 hypocalcemia resulting in mortality (Roch & Maly, 1979). The concentration of arsenic in
394 all diets were lower than the permissible level, but the percentage excreted (12.8 – 34.7
395 %) were lower than for cadmium. However, it has been shown that almost 90 % of arsenic
396 accumulated in fish muscle, are in the non-toxic arsenobetaine form (Bosch et al., 2016).

397 Marine fish is considered as a rich source of iodine. Iodine is highly related to thyroid
398 hormones that regulates metabolic processes essential for normal growth and
399 development as well as regulating metabolism in the fish. Dietary iodine requirement of
400 salmonid is reported to be 4 mg kg^{-1} diets (Watanabe et al., 1997). The amount of iodine
401 in CUN30 diet was almost 20 times higher than the recommended levels. The source for
402 this iodine is the yeast grown in the seaweed hydrolysates, as seaweed is known to have
403 a high iodine content (Sharma et al., 2018b). According to study, usage of iodine
404 supplemented diets (20 mg iodine kg^{-1} diet) could reduce stress responses and promote
405 growth in salmonids (Gensic et al., 2004). The high content of minerals derived from the
406 brown seaweed in the present study, could be a limitation for the use of seaweed as a
407 feedstock for the fermentation of yeast as a feed resource for fish. This problem could be
408 eliminated by performing a pre-processing of the brown seaweed to reduce the content
409 of minerals. It has been shown that the high content of iodine in brown seaweed could
410 easily be reduced by up to 70% by a short washing in boiling water (Duinker et al., 2016).
411 High content of minerals are also dependent upon the inclusion level in the diets. Hence,
412 a detailed growth study of fish fed increasing levels of seaweed-derived yeast diets,

where the accumulation of minerals in organs such as kidney, liver and muscles has been performed and will be reported elsewhere.

5. Conclusion

In conclusion, *C. utilis* cultivated on spent sulphite liquor (CUA) or woody hydrolysate and molasses (CUE) could replace 30 % of a fishmeal-based diet for Atlantic salmon without affecting growth performance and digestibility of DM, ash, fat and starch. However, the digestibility of protein was lower in both, compared to the fishmeal-based control diet. Furthermore, feeding *C. utilis* cultivated on enzymatic hydrolysates of seaweed and wood (CUN) resulted in reduced nutrient and mineral digestibility. Further optimization studies are needed in the use of seaweed hydrolysate as a carbon and nutrient source for the cultivation of the yeast. On ingredient level, the ADC of protein in fish fed the CUN was significantly lower than the other two yeast biomasses (CUE and CUA).

Acknowledgment

The authors would like to acknowledge the staff of the fish laboratory at the Norwegian University of Life Sciences for excellent support during the course of the feeding trial. This research was supported by a PhD fellowship from the Norwegian University of Life Sciences (NMBU), to S.S. and by the Research Council of Norway through grants 229003, BIOFEED – Novel salmon feed by integrated bioprocessing of non-food biomass, and 237841, Foods of Norway, a centre for research-based innovation.

References

Aas, T. S., Grisdale-Helland, B., Terjesen, B. F. & Helland, S. J. (2006). Improved growth and nutrient utilisation in Atlantic salmon (*Salmo salar*) fed diets containing a bacterial protein meal. *Aquaculture*, 259 (1): 365-376.

Anupama & Ravindra, P. (2000). Value-added food: Single cell protein. *Biotechnology Advances*, 18 (6): 459-479.

Aslaksen, M. A., Kraugerud, O. F., Penn, M., Svihus, B., Denstadli, V., Jørgensen, H. Y., Hillestad, M., Krogdahl, Å. & Storebakken, T. (2007). Screening of nutrient digestibilities and intestinal pathologies in Atlantic salmon, *Salmo salar*, fed diets with legumes, oilseeds, or cereals. *Aquaculture*, 272 (1): 541-555.

Austreng, E. (1978). Digestibility determination in fish using chromic oxide marking and analysis of contents from different segments of the gastrointestinal tract. *Aquaculture*, 13 (3): 265-272.

Austreng, E., Storebakken, T., Thomassen, M. S., Refstie, S. & Thomassen, Y. (2000). Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture*, 188 (1): 65-78.

Baldwin, C. V. & Robinson, C. W. (1994). Enhanced disruption of *Candida utilis* using enzymatic pretreatment and high-pressure homogenization. *Biotechnology and Bioengineering*, 43 (1): 46-56.

Bekatorou, A., Psarianos, C. & Koutinas, A. A. (2006). Production of Food Grade Yeasts. *Food Technology and Biotechnology*, 44 (3): 407-415.

Blaize, J., L'Amoreaux W, J., Downey, M. & McCoy, E. C. (2009). Dibutylphthalate and Tween 80 alter ultrastructure in *Candida albicans*: implications for peroxisome proliferation. *Can J Microbiol*, 55 (4): 437-49.

Bosch, A. C., O'Neill, B., Sigge, G. O., Kerwath, S. E. & Hoffman, L. C. (2016). Heavy metals in marine fish meat and consumer health: a review. *Journal of the Science of Food and Agriculture*, 96 (1): 32-48.

Brenne, T., Naess, B. & Farstad, L. (1974). The Nutritive Value, for Growing Pigs, of Single Cell Protein (*Candida Utilis*)Produced from Sulphite Spent Liquor. *Acta Agriculturae Scandinavica*, 24 (1): 3-6.

Bureau, D. P. & Hua, K. (2006). Letter to the Editor of Aquaculture. *Aquaculture*, 252 (2): 103-105.

Bzducha-Wróbel, A., Kieliszek, M. & Błażejak, S. (2013). Chemical composition of the cell wall of probiotic and brewer's yeast in response to cultivation medium with glycerol as a carbon source. *European Food Research and Technology*, 237 (4): 489-499.

Carter, C. G. & Hauler, R. C. (2000). Fish meal replacement by plant meals in extruded feeds for Atlantic salmon, *Salmo salar* L. *Aquaculture*, 185 (3): 299-311.

Cho, C. Y. & Slinger, S. J. (1979). Apparent digestibility measurements in feed stuffs for rainbow trout. In: Halver, J.E., Tiews, K. (Eds.), Berlin, Germany: Heinemann. *Finfish Nutrition and Fish feed Technology* 2: 239-247.

Commission, E. (2009). Commission Directive 152/2009/EC of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. *Official Journal of the European Union*, 54: 1-130.

Commission Regulation (EU). (2013). amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities. *Official Journal of the European Union*, 328.

Davis, D. A. & Gatlin, D. M. (1996). Dietary mineral requirements of fish and marine crustaceans. *Reviews in Fisheries Science*, 4 (1): 75-99.

Denstadli, V., Skrede, A., Krogdahl, Å., Sahlstrøm, S. & Storebakken, T. (2006). Feed intake, growth, feed conversion, digestibility, enzyme activities and intestinal structure in Atlantic salmon (*Salmo salar* L.) fed graded levels of phytic acid. *Aquaculture*, 256 (1): 365-376.

Duinker, A., Roiha, I. S., Amlund, H., Dahl, L., Lock, E.-J., Kögel, T., Måge, A. & Lunestad, B. T. (2016). Potential risks posed by macroalgae for application as feed and food - a Norwegian perspective. *National Institute of Nutrition and Seafood Research (NIFES)*.

Epstein, F. H., Katz, A. I. & Pickford, G. E. (1967). Sodium- and Potassium-Activated Adenosine Triphosphatase of Gills: Role in Adaptation of Teleosts to Salt Water. *Science*, 156 (3779): 1245-1247.

FDA. (2018). *U.S. Food & Drug Administration, "Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List)"*. Tilgjengelig fra: https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/MicroorganismsMicrobialDerivedIngredients/default.htm [Accessed 20.02.2018].

Francis, G., Makkar, H. P. S. & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199 (3): 197-227.

Gensic, M., Wissing, P. J., Keefe, T. R. & Mustafa, A. (2004). Effects of iodized feed on stress modulation in steelhead trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture Research*, 35 (12): 1117-1121.

Gong, Y., Guterres, H. A. D. S., Huntley, M., Sørensen, M. & Kiron, V. (2018). Digestibility of the defatted microalgae *Nannochloropsis sp*. and *Desmodesmus sp*. when fed to Atlantic salmon, *Salmo salar*. *Aquaculture Nutrition*, 24 (1): 56-64.

Grammes, F., Reveco, F. E., Romarheim, O. H., Landsverk, T., Mydland, L. T. & Øverland, M. (2013). *Candida utilis* and *Chlorella vulgaris* Counteract Intestinal Inflammation in Atlantic Salmon (Salmo salar L.). *PLOS ONE*, 8 (12): e83213.

Gönen, F. & Aksu, Z. (2008). Use of response surface methodology (RSM) in the evaluation of growth and copper(II) bioaccumulation properties of *Candida utilis* in molasses medium. *Journal of Hazardous Materials*, 154 (1): 731-738.

Hansen, J. Ø. & Storebakken, T. (2007). Effects of dietary cellulose level on pellet quality and nutrient digestibilities in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, 272 (1): 458-465.

Helland, S. J., Grisdale-Helland, B. & Nerland, S. (1996). A simple method for the measurement of daily feed intake of groups of fish in tanks. *Aquaculture*, 139 (1): 157-163.

Huyben, D., Nyman, A., Vidaković, A., Passoth, V., Moccia, R., Kiessling, A., Dicksved, J. & Lundh, T. (2017). Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. *Aquaculture*, 473: 528-537.

526 ISO. (1998). International Organization for Standardization (ISO), ISO 9831:1998. Animal
527 Feeding Stuffs, Animal Products, and Faeces or urine. Determination of Gross
528 Calorific Value. Bomb Calorimeter Method. ISO, Geneva, Switzerland.
529 Kraugerud, O. F., Penn, M., Storebakken, T., Refstie, S., Krogdahl, Å. & Svihus, B.
530 (2007). Nutrient digestibilities and gut function in Atlantic salmon (*Salmo salar*) fed
531 diets with cellulose or non-starch polysaccharides from soy. *Aquaculture*, 273 (1):
532 96-107.
533 Krogdahl, Å., Penn, M., Thorsen, J., Refstie, S. & Bakke, A. M. (2010). Important
534 antinutrients in plant feedstuffs for aquaculture: an update on recent findings
535 regarding responses in salmonids. *Aquaculture Research*, 41 (3): 333-344.
536 Labuza, T. P., Jones, K. A., Sinskey, A. J., Gomez, R., Wilson, S. & Miller, B. (1972).
537 Effect of drying conditions on cell viability and functional properties of single-cell
538 protein. *Journal of Food Science*, 37 (1): 103-107.
539 Langeland, M., Vidakovic, A., Vielma, J., Lindberg, J. E., Kiessling, A. & Lundh, T. (2016).
540 Digestibility of microbial and mussel meal for Arctic charr (*Salvelinus alpinus*) and
541 Eurasian perch (*Perca fluviatilis*). *Aquaculture Nutrition*, 22 (2): 485-495.
542 Lee, B.-K. & Kyun Kim, J. (2001). Production of *Candida utilis* biomass on molasses in
543 different culture types. *Aquacultural Engineering*, 25 (2): 111-124.
544 Martin, A. M., Goddard, S. & Bemibster, P. (1993). Production of *Candida utilis* biomass
545 as aquaculture feed. *Journal of the Science of Food and Agriculture*, 61 (3): 363-
546 370.
547 McCleary, B. V., Solah, V. & Gibson, T. S. (1994). Quantitative Measurement of Total
548 Starch in Cereal Flours and Products. *Journal of Cereal Science*, 20 (1): 51-58.
549 Mikulášová, M., Vodný, Š. & Pekarovičová, A. (1990). Influence of phenolics on biomass
550 production by *Candida utilis* and *Candida albicans*. *Biomass*, 23 (2): 149-154.
551 Murray, A. P. & Marchant, R. (1986). Nitrogen utilization in rainbow trout fingerlings
552 (*Salmo gairdneri* Richardson) fed mixed microbial biomass. *Aquaculture*, 54 (4):
553 263-275.
554 Oliva-Teles, A. & Gonçalves, P. (2001). Partial replacement of fishmeal by brewers yeast
555 (S*accaromyces cerevisae*) in diets for sea bass (*Dicentrarchus labrax*) juveniles.
556 *Aquaculture*, 202 (3): 269-278.
557 Olvera-Novoa, M. A., Martinez-Palacios, C. A. & Olivera-Castillo, L. (2002). Utilization of
558 torula yeast (*Candida utilis*) as a protein source in diets for tilapia (*Oreochromis*
559 *mossambicus Peters*) fry. *Aquaculture Nutrition*, 8 (4): 257-264.
560 Ozório, R. O. A., Turini, B. G. S., Môro, G. V., Oliveira, L. S. T., Portz, L. & Cyrino, J. E.
561 P. (2010). Growth, nitrogen gain and indispensable amino acid retention of pacu
562 (*Piaractus mesopotamicus*, Holmberg 1887) fed different brewers yeast
563 (*Saccharomyces cerevisiae*) levels. *Aquaculture Nutrition*, 16 (3): 276-283.
564 Refstie, S., Førde-Skjærvik, O., Rosenlund, G. & Rørvik, K.-A. (2006). Feed intake,
565 growth, and utilisation of macronutrients and amino acids by 1- and 2-year old
566 Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal.
567 *Aquaculture*, 255 (1): 279-291.
568 Riche, M. & Brown, P. B. (1996). Availability of phosphorus from feedstuffs fed to rainbow
569 trout, *Oncorhynchus mykiss*. *Aquaculture*, 142 (3): 269-282.

570 Ritala, A., Häkkinen, S. T., Toivari, M. & Wiebe, M. G. (2017). Single Cell Protein—State-
571 of-the-Art, Industrial Landscape and Patents 2001–2016. *Frontiers in*
572 *Microbiology*, 8 (2009).
573 Roch, M. & Maly, E. J. (1979). Relationship of Cadmium-Induced Hypocalcemia with
574 Mortality in Rainbow Trout (*Salmo gairdneri*) and the Influence of Temperature on
575 Toxicity. *Journal of the Fisheries Research Board of Canada*, 36 (11): 1297-1303.
576 Rumsey, G. L., Hughes, S. G., Smith, R. R., Kinsella, J. E. & Shetty, K. J. (1991).
577 Digestibility and energy values of intact, disrupted and extracts from brewer's dried
578 yeast fed to rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and*
579 *Technology*, 33 (3): 185-193.
580 Rødsrud, G., Lersch, M. & Sjöde, A. (2012). History and future of world's most advanced
581 biorefinery in operation. *Biomass and Bioenergy*, 46: 46-59.
582 Sharma, S., Hansen, L., Øvrum, J., Mydland, L., Horn, S., Øverland, M., Eijsink, V. &
583 Vuoristo, K. (2018a). Microbial protein produced from brown seaweed and spruce
584 wood as a feed ingredient. *Unpublished research*.
585 Sharma, S., Neves, L., Funderud, J., Mydland, L., Øverland, M. & Horn, S. (2018b).
586 Seasonal and depth variations in the chemical composition of cultivated
587 *Saccharina latissima*. *Algal Research*, 32: 107-112.
588 Shearer, K. D. (1988). Dietary potassium requirement of juvenile chinook salmon.
589 *Aquaculture*, 73 (1): 119-129.
590 Storebakken, T., Shearer, K. D. & Roem, A. J. (1998). Availability of protein, phosphorus
591 and other elements in fish meal, soy-protein concentrate and phytase-treated soy-
592 protein-concentrate-based diets to Atlantic salmon, *Salmo salar. Aquaculture*, 161
593 (1): 365-379.
594 Sugiura, S. H., Dong, F. M., Rathbone, C. K. & Hardy, R. W. (1998). Apparent protein
595 digestibility and mineral availabilities in various feed ingredients for salmonid
596 feeds. *Aquaculture*, 159 (3): 177-202.
597 Sørensen, M., Gong, Y., Bjarnason, F., Vasanth, G. K., Dahle, D., Huntley, M. & Kiron,
598 V. (2017). *Nannochloropsis* oceania-derived defatted meal as an alternative to
599 fishmeal in Atlantic salmon feeds. *PLOS ONE*, 12 (7): e0179907.
600 Tukmechi, A. & Bandboni, M. (2014). Effects of *Saccharomyces cerevisiae*
601 supplementation on immune response, hematological parameters, body
602 composition and disease resistance in rainbow trout, *Oncorhynchus mykiss*
603 (Walbaum, 1792). *Journal of Applied Ichthyology*, 30 (1): 55-61.
604 Vidakovic, A., Langeland, M., Sundh, H., Sundell, K., Olstorpe, M., Vielma, J., Kiessling,
605 A. & Lundh, T. (2016). Evaluation of growth performance and intestinal barrier
606 function in Arctic Charr (*Salvelinus alpinus*) fed yeast (*Saccharomyces cerevisiae*),
607 fungi (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*). *Aquaculture Nutrition*,
608 22 (6): 1348-1360.
609 Walker, G. & Stewart, G. (2016). *Saccharomyces cerevisiae* in the Production of
610 Fermented Beverages. *Beverages*, 2 (4): 30.
611 Watanabe, T., Kiron, V. & Satoh, S. (1997). Trace minerals in fish nutrition. *Aquaculture*,
612 151 (1): 185-207.
613 Yamada, E. A. & Sgarbieri, V. C. (2005). Yeast (*Saccharomyces cerevisiae*) protein
614 concentrate: preparation, chemical composition, and nutritional and functional
615 properties. *J Agric Food Chem*, 53 (10): 3931-6.

Ytrestøyl, T., Aas, T. S. & Åsgård, T. (2015). Utilisation of feed resources in production of Atlantic salmon (*Salmo salar*) in Norway. *Aquaculture*, 448: 365-374.

Øverland, M., Karlsson, A., Mydland, L. T., Romarheim, O. H. & Skrede, A. (2013). Evaluation of *Candida utilis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* yeasts as protein sources in diets for Atlantic salmon (*Salmo salar*). *Aquaculture*, 402: 1-7.

Øverland, M. & Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *Journal of the Science of Food and Agriculture*, 97 (3): 733-742.

ISBN: 978-82-575-1518-8
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

98827 / ANDVORDGRAFISK.NO