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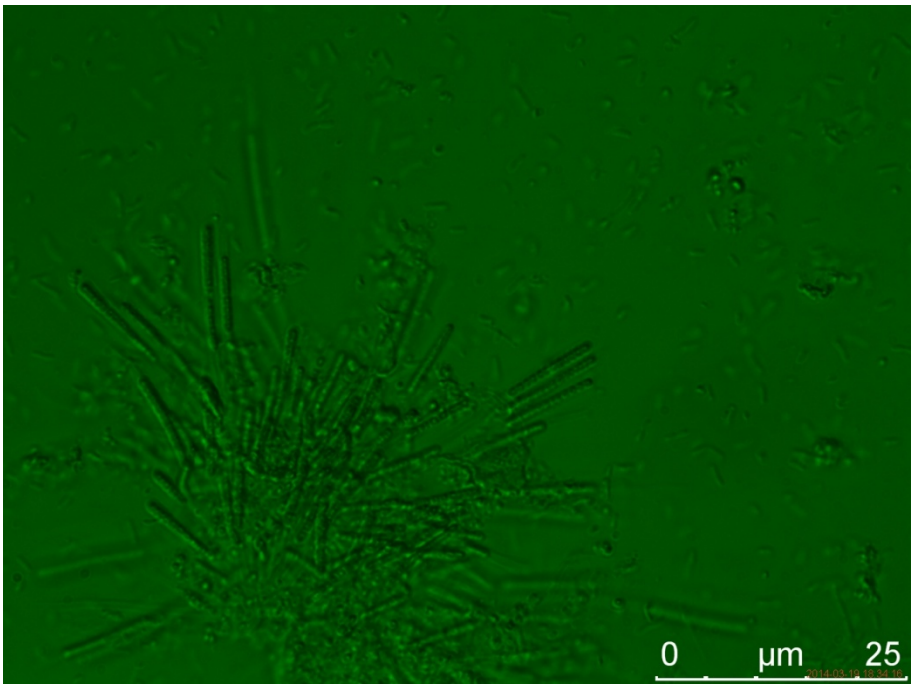

Bioprospecting for novel laminarin-degrading enzymes in marine microorganisms

A step towards the use of macroalgae in bioprocesses

Master of Science Thesis

Joakim Olsson

Department of Biology and Biological Engineering
Division of Industrial biotechnology
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden, 2015

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Department of Biology and Biological Engineering
Chalmers University of Technology
SE-412 96 Göteborg
Sweden
Telephone + 46 (0)31-772 1000

Cover: *Pseudoalteromonas* sp. cultivated on algae extract medium still containing partly intact particles from the macroalgae *Saccharina latissima*.

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Abstract

Currently, efforts are being made to find alternative biomasses for biofuel production, which are not competing with food production. Several kelp species have potential for large-scale cultivation, harvest and biorefinery processes. On the Swedish west coast, *Laminaria digitata* and *Saccharina latissima* are two such species.

These kelps can contain up to 33% of the storage carbohydrate laminarin (dry weight), which can be hydrolysed to glucose and subsequently converted into bioethanol by for instance yeast. Enzymes able to digest the β-1,3 or β-1,6 bonds of the laminarin, to release glucose, are in large unknown and this project has been about finding microorganisms expressing such enzymes, known as laminarases. Samples from partly decomposed *L. digitata* and *S. latissima* specimens were streaked on nutrient agar plates for proliferation and isolation of surface microorganisms. Isolated organisms were subsequently screened for growth on laminarin and promising strains were identified through 16s/18s rRNA sequencing. After growth experiments on liquid medium with different carbon sources, two bacterial strains, *Pseudoalteromonas* ssp., were selected for further characterisation. The two strains were grown in algae extract and the sugar composition was monitored over time. In the extract, mainly mannitol and laminarin were present but glucose was formed during the cultivation, indicating the presence of hydrolytic enzymes, after which the hydrolytic activity on pure laminarin was investigated. The supernatants of one of the strains showed in vitro activity, further indicating the presence of extracellular hydrolytic enzymes. More investigations are needed on whether the enzymes can be used in any type of process. This thesis work has been successful in isolating marine organisms with laminarin-degrading activity.

Keywords: Laminarases, laminarin, enzymes, *Pseudoalteromonas* sp., biorefinery, bioethanol, *Saccharina latissima*, *Laminaria digitata*

Sammanfattning

För en hållbar framtid behöver det finnas biomassor till biobränsleproduktion, som inte konkurrerar med matproduktion. Vissa tångarter visar potential för storskalig odling, skörd och bioraffineringsprocesser. På den svenska västkusten har *Laminaria digitata* och *Saccharina latissima* sådan potential och utvärderas i projektet Seafarm för detta syfte.

Dessa tångarter kan innehålla så mycket som 33% (torrvikt) av kolhydraten laminarin som genom hydrolys kan sönderdelas till glukos och konverteras till bioetanol i en fermenteringsprocess. Få enzymer är kända som kan hydrolysera β-1,3 och β-1,6 bindningarna för att frigöra glukos, så kallade laminaraser. Därför har detta projekt inriktat sig mot att finna organismer som uttrycker denna typ av enzymer. Prover från delvis förruttnad *L. digitata* och *S. latissima* prover ströks ut på näringsrika agarplattor i syfte att isolera potentiellt intressanta nedbrytningsorganismer. De isolerade organismerna screenades för tillväxt på laminarin och lovande mikroorganismer identifierades med 16s/18s rRNA sekvensering. Efter tillväxtexperiment i flytande medium med olika kolkällor valdes två stammar av *Pseudoalteromonas* ssp. för vidare experiment. De två stammarna kultiverades i algextrakt och kolhydratinnehållet undersöktes kontinuerligt. I extraktet fanns till en början mestadels mannitol och laminarin, men mot slutet bildades en del glukos, vilket indikerar närvaro av hydrolyserande enzymer. Efter detta undersöktes supernatantens effekt på rent laminarin och degradering kunde påvisas vilket ytterligare påvisar närvaron av hydrolyserande enzymer. Mer undersökningar krävs för att för att se om enzymerna kan användas i en process. Detta projektarbete har lyckats med att isolera marina mikroorganismer med laminarin degraderande aktivitet.

Acknowledgements

There are so many I would like to thank for their help with the work behind this thesis and for all the support I have been given along the way. First I would like to thank Jenny for all the nice discussions in our office and for always solving my problems. George for patiently answering all my silly practical questions and for staying late with me in the lab. Eva for giving me the opportunity to do my thesis in the industrial biotechnology group. Big thanks also to Cecilia and Valeria Mapelli for lending me primers for PCRs and sequencing and also to David Moreno for all your help with the HPLC (even though we almost destroyed the column with alginate). Everyone else at Indbio also deserves a thank you for making me feel so welcome that I now count many you as good friends. A big thanks also to the Centre for Marine Chemical Ecology for access to the strain collection that made this project possible.

I also want to dedicate a special thanks to my girlfriend Karin, for enduring all of the nights I've spent writing, reading, doing lab work and picking me up at Chalmers with the car after the trams had stopped going. We will soon have a dishwasher so let the next chapter begin!

Contents

1. Introduction

Not once in the last 800 000 years have atmospheric levels of carbon dioxide, methane and nitric oxide been as high as today (Petit et al., 1999, Siegenthaler et al., 2005, Luthi et al., 2008). The Intergovernmental Panel on Climate Change (IPCC) recently reported, once again, that this has caused ocean acidification, raised sea levels, rising temperatures and increased frequency of storms (Stocker et al., 2013). The main contribution to the increased carbon dioxide levels in the atmosphere has been made by the use of fossil oil (Stocker et al., 2013) and a change has to be made to halt the effects. As one possible technological adaptation option the IPCC suggests the transition from fossil fuels to biofuels and biorefinery processes (Edenhofer et al., 2011).

A biofuel that is already available on the market and as well as being used as an additive in gasoline, is bioethanol. Today bioethanol is mainly made from grain and sugar-cane (Mussatto et al., 2010). As these crops could be used to feed the worlds growing population, there is an ongoing "Food vs Fuel" debate, which has given bioethanol a rather bad reputation. To solve this problem new feedstocks for bioethanol production must be explored and cultured brown macroalgae is one interesting alternative. Some of the big advantages with brown macroalgae are that they need no fertilizers, no pesticides or land that can be used to grow food. It also contains no lignin (Holdt and Kraan, 2011), which is a big problem for many other available biomasses such as forestry residues. The algal biomass can contain as much as 74% carbohydrates in dry weight depending on the species and season (Holdt and Kraan, 2011).

The species *Laminaria digitata* and *Saccharina latissima* are of particular interest for this project as they are common on the west coast of Sweden. They are currently the target of a large cooperation project, Seafarm, aiming to farm macroalgae on a larger scale and convert them into useful products in a biorefinery. To convert the carbohydrates into *e.g.* ethanol or biogas they need to be fermented. *Saccharomyces cerevisiae*, which is the most common organism for industrial ethanol production, would be favourable for the fermentation step. However, *L. digitata* and *S. latissima* mainly contain the carbohydrates mannitol, alginate and laminarin, which *S. cerevisiae* cannot digest (Kim et al., 2011, Wargacki et al., 2012, Abdelal and Phaff, 1968).

Alginate and laminarin are polysaccharides, which have to be hydrolysed into monomers for a production organism to consume them. With a long term aim to engineer a *S. cerevisiae* strain that is able to utilize the algae biomass, in a simultaneous saccharification process, this master thesis focuses on finding laminarin-degrading enzymes.

1.2 Aim

The aim of this project was to find and identify marine microorganisms with laminarin-degrading enzymes. In the future these enzymes could be identified and used for simultaneous saccharification and fermentation of macroalgal biomass, rich in laminarin. This would enable the use of algal biomass as a feedstock for bioethanol production or in a biorefinery.

2 Background

To find laminarin-degrading enzymes some basic knowledge is needed on seaweed composition, current state of the research and bioprocesses. The content of *Laminaria digitata* and *Saccharina latissima* is particularly important as well as what context the enzymes will be used in.

2.1 Seaweeds

There are about 9000 different species of seaweed ordered into three different divisions: green (*Chlorophyta*), red (*Rhodophyta*) and brown algae (*Phaeophyta*). In contrast to the microalgae they are multicellular, macroscopic and grow in the benthic zone, *i.e.* near or on the seafloor at 0-200 m depth (Venugopal, 2011). Out of these species over 200 are being utilized for food, alginate extraction, agar extraction, medicinal or agricultural uses (Zemke-White and Ohno, 1999). The amounts produced were as much as 151 million tonnes in 2006 with most of the production in China, the Philippines, Republic of Korea, and Japan (Venugopal, 2011). In Europe however, there has not been much interest in cultivating seaweeds although some wild harvest do occur (Bixler and Porse, 2011).

2.1.1 *Laminaria digitata* and *Saccharina latissima*

In this study two different kinds of macroalgae of similar composition are of interest. They are common on the western coast of Sweden and are the species being evaluated in the Seafarm project.

Saccharina latissima, also called sugar kelp, is a brown algae consisting of a single blade which can be as long as 3.5 m and be 18 cm wide. It grows in the littoral zone at many locations around the world *e.g.* the north Atlantic, the Northern Sea and Japan (Lane et al., 2014).

Laminaria digitata is also a brown algae and its blades grow in finger-like extensions. The alga is common in the littoral zone around the northern Atlantic Ocean and can become 2-3 m (Edwards et al., 2011). Table 1 presents the main carbohydrates of the two algae species. It should be noted that the variations in concentrations depends on the season, as well as location of harvest.

Table 1. Main sugars and protein content in two species of brown algae given in percent of dry weight (Holdt and Kraan, 2011) *(Manns et al., 2014).

Compound	*Saccharina latissima*	*Laminaria digitata*
Alginate	17-33%	30-44%
Laminarin	0-33%	0-18%
Mannitol	2-19%	2.5-20%
Fucoidan	4.1%*	2-5.5%
Protein	3-21%	3-21%

2.1.2 Seafarm

The Seafarm project is an interdisciplinary project which is investigating how macroalgae could be used in a biorefinery. The project includes the evaluation of environmental and ecological effects of the farming process of the macroalgae, as well as how the biorefinery can extract valuable compounds in the algae. The growth of the algae could potentially counteract eutrophication and provide habitats for marine organisms. The algae will not be harvested from the seafloor but will be grown on ropes where it disturbs existing costal ecosystems as little as possible (SEAFARM, 2014).

2.2 Biorefinary/Bioethanol from algae

A biorefinery is a process which transforms biomass into a products utilizing all the material put in. One classical bio-process is the production of ethanol from starch grain and other starch rich crops. However this process only utilizes the starch-rich part of the plant while the rest becomes residue. In the case of grain, as much as half the crop is straw which is not utilized (Villadsen et al., 2011). The residue however can be treated as a lingocellulosic biomass and be refined into various products making it a whole crop biorefinery (Kamm and Kamm, 2004). A biorefinery could extract protein, lipids and other biomass constituents into separate product fractions but for bioethanol mainly the sugars are of interest. Starch, cellulose and laminarin are all polysaccharides of glucose which have to be degraded before they can be utilized by a production organism such as *S. cerevisiae*. In macroalgae mannitol is also interesting as a substrate. *S. cerevisiae* cannot utilize mannitol but there are trials using metabolically engineered *Escherichia coli* to co-ferment mannitol and alginate (Wargacki et al., 2012, Kim et al., 2011).

2.2.1 Pre-Treatment and hydrolysis

Enzymatic and acidic hydrolysis are two common ways to hydrolyse polysaccharides before fermentation. In the case of starch the biomass itself produces enzymes when it is allowed to germinate breaking down the glucose polymer into glucose. This is the classical approach when making beer or whisky (Chesworth et al., 1998). Large scale industrial productions uses amylases from various fungi and bacteria (Nigam and Singh, 1995). The sugars of lignocellulosic biomass are much harder to get out from the constituents cellulose and hemicellulose. Cellulose is the main component and there are enzymes which are good at degrading this polysaccharide into glucose. However lignin, which is the glue holding the cellulose and hemicellulose together, acts as a recalcitrant by binding enzymes non-specifically. Other treatments such as dilute acid hydrolysis have other problems when it comes to the lignocellulosic biomass as they often form various fermentation inhibitors such a furfurals (Agbor et al., 2011). The laminarin in *Laminaria digitata* and *Saccharina latissima* is a glucose polymer as well, which cannot be utilized unless it is hydrolysed into monomeric sugar. The big advantage over lignocellulosic biomass when it comes to enzymatic hydrolysis is the lack of lignin, but on the other hand few laminarases are known. Various pre-treatments followed by laminarase and yeast addition was tried by (Adams et al., 2009) on *S. latissima* slurries. For them, pre-treatment in room temperature at pH 6 gave the best fermentation results, indicating that only mild pre-treatment is needed for laminarin.

2.2.2 Laminarin

Laminarin is the primary storage glucan of *Laminaria digitata* and *Saccharina latissima*. Laminarin consists of subunits of β-D-glucose linked with β-1,3 or β-1,6, see Figure 1, at a ratio of 3:1(Kraan, 2012). In *Laminaria digitata,* a third of the chains has a β-1,1 linked mannitol at the reducing end and the degree of polymerisation is between 20 and 30 with

a branch every 0-4 residues (average 1.3) (Stone et al., 2009). Unbranched forms of laminarin are present in other seaweeds and this form is insoluble (Stone et al., 2009, Lobban and Harrison, 1994).

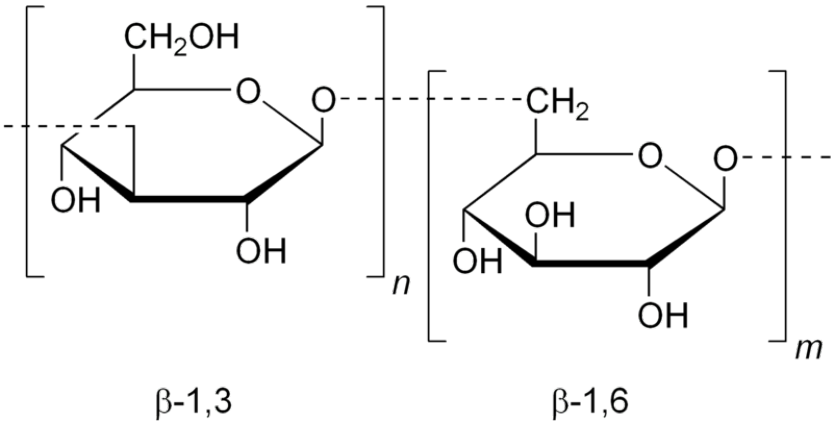

Figure 1. The structure of laminarin with the β-1,3 or β-1,6 at 3:1 ratio.

2.2.3 Laminarases

Laminarases are β-1,3 glucanases and cleave laminarin. One of the searches for laminarases was done using the protoist *Euglena gracilis* which has a storage polysaccharide similar to laminarin (Fellig, 1960). Using crude extracts of the protoist some degradation of laminarin was seen by measuring formed glucose. In fraction experiments it was found out that several enzymes were involved in the degradation. The best result of the study, in a reaction mixture with 0.5% laminarin, was 0.4 mg/ml of formed glucose per hour and they claim than Mn^{2+} can increase the degradation by over 100%.

In another study laminarases excreted by *Trichoderma longibrachiatum* was investigated (Sharma and Nakas, 1987). In this study supernatants were used and released glucose was measured. The supernatant did degrade laminarin and using one of the graphs presented the supernatant formed approximately 1 mg glucose per mg protein and hour in an assay with 125 µg/ml laminarin which was run for ten minutes.

A gene library from *Rhodothermus marinus* was cloned into *E.coli* and screened for lichenase activity (β-1,4-glucan) (Spilliaert et al., 1994). A positive result showed identity to β-1,3-glucanases and the enzyme was tested for laminarin degrading capability. It is claimed in this article that laminarin was degraded, yet no data is shown except a thin-layer-chromatography picture with no laminarin blank. This enzyme was tested for glucose production within the Industrial biotechnology group at Chalmers with very poor results (Davidson, 2015).

In a recent study, the gene for laminarase in a macroalgae-associated marine bacterium *Zobellia galatanivorans* was cloned into *E.coli* for overexpression (Labourel et al., 2014). The purified enzyme was used in laminarin-degrading assays as well as crystallization experiments which resulted in a highly resolved structure of the protein. The native enzyme is predicted to be anchored to the outer membrane and the results indicates that the smallest unit to be degraded is laminaritriose.

Another study found that *Pseudoaltoromonas* sp. contains exo-β-1,3/1,4-glucanase (Nakatani et al., 2010). The investigated enzyme showed broad specificity and is thought

to act together with endo-enzymes in the degradation of laminarin. Through sequence analysis this enzyme was showed to be related to β-1,3-glucanases found in plants.

2.3 Bioprospecting

According to Oxford Dictionaries bioprospecting is “The search for plant and animal species from which medicinal drugs and other commercially valuable compounds can be obtained”. Within this project that means identifying potential organisms based on growth on laminarin and screening them for laminarases, which would be our valuable compound.

2.3.1Bacterial and fungal identification

The 16S rRNA (prokaryotes) and the 18S rRNA (eukaryotes) genes are part of the small subunit of the ribosome. It has been found that these genes are well suited for phylogeny analysis since they are present in almost all prokaryotes and eukaryotes, respectively. These housekeeping genes are 1500 and 2300 bp and have regions that are conserved between all organisms, enabling the use of universal primers. Other regions vary which makes it possible to distinguish different species and their relations to each other by sequencing PCR fragments of the genes (Canfield et al., 2005). There are several different primer options for both 16S and 18S rRNA sequencing (Stackebrandt and Goodfellow, 1991, White et al., 1990).

2.3.2 Marine microorganisms

The phrase marine microorganisms include organisms from all domains of life but for this project only fungi and bacteria are of interest. Coming from an environment with 1-4.4% salinity, pH 7.5-8.4 and large temperature ranges these organisms needs to be handled properly to grow in a laboratory environment (Munn, 2004). Isolation techniques based on plating are not well suited for marine bacteria and studies have shown that only 0.001-1% of the marine bacteria in a sample are captured (Joint et al., 2010). The aquatic environment is mostly low in nutrients and is also affected by currents which make nutrients highly mobile (Rosenberg et al., 2013). However, there are nutrient-rich environments, such as the surface of macroalgae where biofilms can form (Munn, 2004, Martin et al., 2014). Microorganisms living on macroalgae have been studied before and vary more depending on whether the alga is green, brown or red than on the species. Studies have also shown that the bacterial populations are different depending on what part of the algae is sampled and that they produce polysaccharides dependent on the host (Martin et al., 2014).

3 Material and methods

3.1 Collection of bacterial and fungal isolates

Isolates of bacteria and fungi growing on beach-cast, decaying and living samples of *Laminaria digitata* and *Saccharina latissima* were collected between the 24th and 27th of July 2013 at Tjärnö, Sweden. This was done as a mini project within the frame of the Centre for Marine Chemical Ecology, University of Gothenburg. The microorganisms were crudely isolated by streaking parts of algae on plates or by picking interesting colonies with a cotton stick and spreading it on plates. The plates used for the isolation was made out of different minimal media for marine microorganisms and using glucose, mannitol, sucrose or alginate as carbon source. Many were also isolated on an algae extract (AEX) (Appendix 1B. Algae extract), containing large amounts of laminarin. Bacterial colonies formed on the plates were restreaked once before being preserved in marine broth or f/2 minimal medium with 20% glycerol in -80 °C prior to the start of this thesis work. Plates with fungal growth were stored at 4 °C.

3.2 Selection and isolation of bacterial strains

115 of the crude isolates were inoculated from freezer cultures to 200 µl liquid algae extract medium in 96-well plates. The bacteria were incubated on a platform rocker for 72 hours. 1 µl of each microculture was transferred to two 96-well plates containing f/2 agar (Guillard and Ryther, 1962) (Appendix 1C) with 5 g/l laminarin and AEX agar, respectively. The plates were incubated at room temperature for two days and then stored at 4°C until analysed.

The plates were analysed visually and isolates growing well on the laminarin agar were picked for isolation of single species. To account for different inoculation densities the laminarin plates were compared to the AEX plates. Isolates with small colonies on the AEX but large colonies on the laminarin plates were chosen over isolates where the colonies on the plates were similar. In total 16 isolates were chosen for isolation and streaked on f/2 agar plates with 5 g/l laminarin. The plates were incubated at room temperature.

Single colonies were picked after two days and restreaked on AEX agar plates several times to ensure that single species were isolated. Eight of the crude isolates from the 96-well laminarin plates were also streaked on AEX agar plates as they were of particular interest due to observations of fast degradation of the algae they were collected from at Tjärnö. They were also restreaked several times on AEX agar.

3.3 Selection and isolation of fungal strains

The crude samples from Tjärnö had been stored at 4°C for ~6 months and were mixtures of different fungi. Based on visual inspection of colour and shape several morphologically different fungi were isolated be multiple restreaks. This was done using algae extract for the first few restreaks and then Potatoe Dextrose Agar (PDA) (Fluka, United States). The plates were incubated at room temperature for fungal growth, then long term stored at 4°C. The amount of isolates was reduced along the way by ocular inspection of

morphology and colour, as some were obviously the same species. A total of 6 strains which were distinct species were finally isolated.

3.3.1 Stock culture preparation

Of the 16 bacterial strains isolated on laminarin plates, three were excluded due to low growth. They were replaced by the three fastest growing strains from the eight that were not isolated on laminarin first (3.2 Selection and isolation of bacterial strains). Single colonies of these 16 strains were picked from two-day old plates for cultivation in 5 ml AEX media without added N/P source. The inoculated strains were incubated on a rotary shaker at 180 rpm in room temperature overnight. The optical density at 600 nm (OD600) was measured once in the morning and once in the afternoon the day after, to see the growth patterns of the bacteria.100 µl samples were taken and centrifuged at 14800 *x*g for 1 minute, in a microcentrifuge. The supernatant was then discarded while the cells and growth medium debris were resuspended in 1 ml synthetic seawater (Appendix 1A), after which OD600 was measured. The OD measurements showed that the cultures had reached stationary phase and stock cultures were made for all strains except one which had not grown. 700 µl of each culture was mixed with 300 µl of 67% Glycerol in screw cap tubes and frozen in liquid nitrogen. The stock cultures were stored in -80°C for future use and the remaining culture was stored in fridge.

The fungal strains were cultivated in shake flasks containing 50 ml of basal growth medium (BGM) (Appendix 1D). To inoculate the cultures, some of the growth media was poured onto PDA plates with well-grown fungal colonies. The submerged colonies were then grazed with a sterilized spatula so that biomass and spores were released to the BGM medium. The mixtures were poured into shake flasks after which the plates were rinsed once with fresh BGM before the rest of the medium was added. The cultures were incubated in a rotary incubator for three days at 25 °C with 180 rpm shaking.

Using a sterile Mira-cloth (Calbiochem, 475855, United States) the filamentous fungi and its spores were separated after three days of growth. The spores were put in 30% glycerol and frozen at -80°C as stock cultures. The biomass caught in the Mira-cloth was collected in Eppendorf tubes or aluminium foil packages and frozen together with the stocks.

3.4 Identification of isolates

3.4.1Bacterial DNA isolation

The remainder of the cultures used for stock culture preparation of the bacteria, were used for inoculation of new AEX plate cultures. For the strain that had not grown in liquid AEX an old plate was used as inoculum. After one day of incubation at room temperature, single colonies of each strain were restreaked on fresh AEX. A loopfull of cells were taken from each plate after two days in room temperature and used for DNA purification using the PowerSoil™ DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA). For strains which had not grown enough, the 3 days old AEX plates were used to increase the amount of bacteria used for the DNA isolation. To provide extra harsh conditions for the lysis, the PowerBead Tubes were pre-incubated at 70ºC for 5 min. All but four samples were lysed using FastPrep 120 (Thermo Savant, Cedex, France) at 4 hz for 40s. The remaining four were instead taped vertically to a vortex in an attempt to imitate the method described in the kit, as the fast prep was so harsh the gravel-looking powerbeads in the kit broke. After the lysis step the kit protocol was followed and DNA was successfully isolated from all samples.

3.4.2 16S rRNA amplification

The 16S rRNA genes of the bacterial strains were amplified through PCR using the Phusion polymerase kit of Thermo Scientific (F-530S) with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of primer 27F and 1494R(Appendix 2A) (Stackebrandt and Goodfellow, 1991), 10-16 ng of DNA (0.5 µl) and 1 U of Phusion DNA polymerase in a total reaction volume of 50 µl. The PCR program was run as follows: 98 ℃ for 3 min followed by 35 cycles of 98 ℃ for 30s, 55.2 ℃ for 30s, 75 ℃ for 1min, and when the cykles were completed 72 ℃ for 10 min.

3.4.3 Fungal DNA isolation

Eppendorf tubes containing 100-200 mg of biomass, from the liquid cultivation that was made for the stock culture preparation, was used for DNA extraction. A tungsten carbide bead was added to each tube and they were then put back in -80 °C inside the TissueLyser II (Qiagen) adapter. The cells were then mechanically lysed in the TissueLyser at 25 Hz for 2x1 minute. The remaining extraction was made using the previously used PowerSoil ™ DNA Isolation kit by adding the powerbead solution without powerbeads to the lysed biomass and following the protocol from after the lysis step. Sixty µl of solution C1 was added to the tubes, followed by brief vortexing before centrifugation at 10000 *x*g for 1 minute. The supernatant was transferred to new tubes and the kit protocol was followed from step 7. DNA was successfully isolated from all samples.

3.4.4 18S rRNA amplification

Amplification of the 18S rRNA genes of the fungal strains were done using the DreamTaq DNA polymerase from Thermo Scientific (EP0702) with 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of primer ITS1 and ITS4 (Appendix 2A)(White et al., 1990), 1 µl of template equal to 23-30 ng and 1.25 U DreamTaq DNA polymerase in a total reaction volume of 50 µl. The PCR was run according to the following: 95 ℃ for 5 min followed by 30 cycles of 94 ℃ for 30s, 56 ℃ for 45s, 72 ℃ for 1min and when the cycles were completed 72 ℃ for 7 min (Hanafy and Morsy, 2012).

3.4.5 Sequencing

The PCR products were analysed by running 10-15 µl on a 1.2% agarose gel at 80-100V for 35-75 min and staining with GelRed for 25-35 min. The bands were visualized using GelDoc (Bio-Rad, United States). The remaining reaction volumes were purified by running them on a 1.2% agarose gel for 48-55 minutes and staining with GelRed for 25 minutes. The gel bands were visualized under UV light and cut out. The DNA fragments of the 16S and 18S rRNA genes were purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification kit (GE Healthcare,). The 16s rRNA gene fragments were sequenced using the 27F and 1492R primers while the 18s rRNA gene fragments were sequenced using the ITS1 primer (at Eurofins MWG GmbH, Martinsried, Germany). The partial sequences from the 16s rRNA were unitized into full sequences of about 1400 bp, where possible, using ApE a plasmid editor freely available at http://biologylabs.utah.edu/jorgensen/wayned/ape/. In a few cases unitization was not possible due to low quality sequencing data. All sequences were analysed with BLAST (NCBI) against their library of previously sequenced genomes. In cases where the blast tool gave the same results for several strains these sequences were aligned against each other using ClustalW, a multiple sequence alignment program for DNA or proteins (http://www.ebi.ac.uk/Tools/msa/clustalw2/help/faq.html#5).

3.5 Growth experiments

3.5.1 Fungal growth assay

The growth of the fungi on laminarin was investigated in 24-well plate format. 1 ml of BGA containing just 2% agar or an additional 2% laminarin was added to the wells. Each strain was grown in triplicates in this assay where the agar medium was used as control. The plates were grown in room temperature and was analysed after 10 days.

3.5.2 Carbon source screening in microplates

A Microbiology Reader Bioscreen C (Oy Growth Curves Ab Ltd) was used to investigate the growth of the bacterial strains on different carbon sources in microplate format. Precultures were inoculated from stock cultures and incubated overnight in AEX at 25°C with 180 rpm shaking. The medium used for the experiment was f/2 minimal medium (Appendix 1C) with 5 g/l of either laminarin, mannitol, glucose or 2.5 g/l of laminarin and mannitol. OD600 of the precultures was measured after centrifuging the samples at 14800 *x*g for 1 minute and then resuspending the cells in f/2 medium twice to wash the cells. Each well contained 175 µl of culture medium and was inoculated using 8.75 µl of washed preculture, making the starting OD of the assay ~0.05. All 15 strains were run in triplicates on each carbon source and the experiment was run at room temperature for 72 hours with continuous fast shaking.

3.5.3 300 ml cultivation of two *Pseudoalteromonas* species

From the Bioscreen growth experiment strains MP073 and MP108 were investigated further for presence of laminarin-degrading enzymes. Precultures were made with 30 ml of AEX with added N/P source and incubated overnight at room temperature on an orbital shaker at 200 rpm. The next day OD600 was measured by making large dilutions of the samples with synthetic seawater and not doing a wash step as in previous experiments. The precultures were used to make cultures of 300 ml total volume with a starting OD600 of 0.05. OD600 was measured right after inoculation and once every hour for eleven hours. Each hour samples were taken and centrifuged at 14 800 *x*g for 1 min. The supernatant was stored in freezer for HPLC, High Pressure Anion Exchange Chromatography (HPAEC) and protein analyses. After eleven hours of growth 100 ml was harvested from each culture. The harvest was divided into falcon tubes and centrifuged at 5000 *x*g for 10 minutes. The supernatant was collected and stored in freezer. Roughly 11 and 13 hours later the OD600 was measured again and the remaining volume of MP108 was harvested as before as the OD600 was constant indicating that growth had stopped. MP073 was left to grow another hour to make sure the growth of it had stopped as well before being harvested. Due to this difference between the cultures there is an extra time point of samples for the MP073 culture.

3.6 Hydrolysis and monosaccharide analysis

To thoroughly investigate the growth medium composition, hydrolysed AEX was analysed in both HPAEC and HPLC. 10 ml recovery standards of glucose, mannitol and fucose at 0.67, 0.69 and 0.28 g/l respectively were prepared and hydrolysed as well to account for degradation from the harsh treatment. These concentrations should have been ten times higher though to better match the expected amount of each sugar in the algae extract, but a dilution error was made. As in the standards, 10 ml of AEX was added to a glass tube with a cap before 262 µl of 98% sulphuric acid was added to each tube making the concentration of sulphuric acid in the tubes 4.6%. All the tubes were autoclaved at

121 °C for 3 hours with tightly screwed on caps. When done the tubes were stored in fridge prior to analysis.

Using HPAEC (Dionex ICS-3000, United States), equipped with a pulsed amperometric detector (PAD) and CarboPac PA1 column (4x250mm, Dionex), glucose and fucose were quantified in the hydrolysate samples. Gradient elution was used as is described in Table 2. The flow was 1 ml/min

Table 2. Eluent composition at different time points during investigation of carbohydrates

Time [min]	Sodium Hydroxide [mM]	Sodium Acetate [mM]
-12	160	34
-6	0	0
21	105	0
27	105	150
31	160	34

The mannitol could not be seen with HPAEC. Hence, the hydrolysates were neutralized and analysed with HPLC. This was done by adding 4 M NaOH until the pH was between 3 and 5. Using an Amicon Ultra 0.5 10kDa filter 400 µl AEX was filtered, to remove compounds which could cause harm to the column, and the resulting flow-through was run on the HPLC to see the concentrations of monomeric sugars before hydrolysis. The eluent for the samples was 5 mM H_2SO_4 and they were run on a Rezex ROA-Organic Acid H+ (8%) column (300x7.8 mm) at 80°C using refractive index and UV at 210 nm as detection methods. All samples from the 300 ml cultures were also run on the HPLC to quantify the changes in carbohydrate concentrations during growth.

3.7 Protein analyses of supernatant from the 300 ml cultures.

3.7.1 Lowry assay

To analyse the protein content of the samples from the large cultivation, the DC assay (Bio-Rad, United States) was used in microplate format. A bovine serum albumin standard ranging from 0-1.38 mg/ml was used to make a standard curve for analysis. The kit instructions were followed but the amounts of sample, reagent A and reagent B were 30, 150 and 1200 µl respectively. 200 µl of the reaction volume was added to the microplate wells and absorbance was measured at 750 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Germany). Each reaction was run in duplicates and the absorbance was measured three times in each well to ensure stability.

3.7.2 SDS-PAGE

All samples from the 300 ml cultures were analysed by SDS-PAGE to see if specific proteins were expressed during the growth and if changes could be seen during the cultivation. 75 µl of the samples were mixed with 25 µl of loading dye gifted by Christian Thörn. To denature the proteins, they were heat treated at 95°C for 7 minutes before loading 30 µl on a Mini-Protean TGX precast gels (Biorad, United States). The gels were run at 100 V for 15 min and then 200 V for 27 min in Tris running buffer. Distilled water was used to wash the gels 3x5min, using fresh water for each iteration, before staining with Bio-Safe Comassie stain (161-0786, Bio-Rad, United States). The gels were

incubated for 1 h in the staining solution before being washed and destained in distilled water for 1 h and 40 minutes. The gels were pictured using GelDoc (Bio-Rad, United States) and the used protein ladder for the experiment was Spectra™ Multicolor Broad Range Protein Ladder from Thermo Scientific (SM1841).

3.7.3 Laminarin degradation assay

Two supernatant samples per 300 ml culture were analysed for laminarin degrading capacity. The reaction conditions for the assay were 50 mM sodium phosphate buffer at pH 6, and 6.25g/l laminarin in a total volume of 1.5 ml. As the amount of active proteins was unknown, the amount of protein-containing supernatant added to the assay was varied to 1/4th, 1/3rd and 1/2nd of the total reaction volume. As blanks for the assay, both non-laminarin and non-enzyme controls were used. The supernatant sample was added last to each reaction, which was run for 1 hour before inactivation at 95°C for 10 minutes. The inactivated samples were filtered through 0.2 µm nylon filters into HPLC vials to remove the denatured proteins. The samples were run both in HPLC and HPAEC as described in Section 3.6.

4 Results

4.1 Initial selection

The selection of bacterial strains on laminarin plates and algae extract plates resulted in 16 strains to identify and screen for laminarin growth. These were originally isolated on different substrates and at different surroundings and in Table 3 this information is given for the chosen strains. An important note is that these strains were not the only ones to grow on the laminarin during the selection process but they were selected for having the better growth. All colonies were of similar colour of white or cream.

Table 3. Strains that were selected due to their growth on laminarin agar and data on their original isolation

Strain	Isolated from	Originally selected with
MP005	Beach cast *S. latissima*	Laminarin extract
MP020	Beach cast *S. latissima*	Mannitol
MP027	Beach cast *S. latissima*	Glucose
MP028	Beach cast *S. latissima*	Glucose
MP035	Beach cast *S. latissima*	Glucose
MP038	Offshore *L. digitata*	Laminarin extract
MP068	Offshore *L. digitata*	Laminarin extract
MP073	Offshore *L. digitata*	Glucose
MP083	Beach cast *S. latissima*	Marine Broth
MP084	Beach cast *S. latissima*	Marine Broth
MP091	Beach placed *L. digitata*	Laminarin extract
MP099	Beach placed *L. digitata*	Laminarin extract
MP102	Beach placed *L. digitata*	Laminarin extract
MP107	Beach cast *S. latissima*	Alginate
MP108	Offshore *S. latissima*	Alginate
MP111	Offshore *S. latissima*	Alginate

4.2 Identification of bacterial and fungal strains

The sequences obtained from sequencing of the 16S and 16S rRNA genes were analysed and the results are summarised in Table 4. For the bacterial strains the number of 99-100% identity hits were very large and the specie identity of the strains could not be determined. However, the genera could be determined with some certainty for the *Pseudoalteromonas* and *Paracoccus* below. The full sequences can be found in Appendix 2B. All the genera and species found are represented in the World Register of Marine Species (WoRMS Editorial Board, 2015) except the *Cobetia* hits which have been isolated from marine environments by Romanenko et al., (2013). The PCR reaction for strain MP083 was unsuccessful.

Table 4. High identify hits from BLAST searches of the bacterial strains

Strain	High identity Genera	High identity species
MP005	*Halomonas, Cobetia, Bacillus, Actinomycetales(order)*	*Bacillus subtilis, Cobetia marina*
MP020	*Halomonas, Cobetia, Bacillus, Actinomycetales(order)*	*Bacillus subtilis, Cobetia litoralis, Cobetia marina*
MP027	*Pseudoalteromonas*	-
MP028	*Paracoccus*	*Paracoccus homiensis*
MP035	*Halomonas, Cobetia, Bacillus, Actinomycetales(order)*	*Bacillus subtilis, Cobetia marina*
MP038	*Pseudoalteromonas*	-
MP068	*Cobetia, Halomonas, Actinomycetales(order)*	*Cobetia amphilecti, Cobetia marina*
MP073	*Pseudoalteromonas*	*Pseudoalteromonas arctica partial*
MP083	-	-
MP084	*Pseudoalteromonas*	*Pseudoalteromonas*
MP091	*Cobetia, Halomonas, Actinomycetales(order)*	*Cobetia litorali, Cobetia amphilecti, Cobetia marina*
MP099	*Cobetia, Halomonas, Actinomycetales(order)*	*Cobetia litorali, Cobetia amphilecti, Cobetia marina*
MP102	*Cobetia, Halomonas, Actinomycetales(order)*	*Cobetia litorali, Cobetia amphilecti, Cobetia marina*
MP107	*Pseudoalteromonas*	-
MP108	*Pseudoalteromonas*	-
MP111	*Halomonas, Cobetia, Bacillus, Actinomycetales(order)*	*Bacillus subtilis, Cobetia litoralis, Cobetia marina*

As with the bacteria the sequencing data from the fungal isolates gave many hits of high identity and the possible species and genera are many. However, MF8 is most likely *Penicillum brevicompactum* as all high identity hits from the BLAST search are different strains of this fungus. Though not regarded a marine species it has previously been isolated from marine sources (Rovirosa et al., 2006). A summary of the high identity hits for all the fungi are in Table 5 and the full sequences from the PCR reactions are in Appendix 2B. Most of genera found are listed in (WoRMS Editorial Board, 2015) and have marine species listed. *Davidiella* was not listed and the high identity species *Davidiella macrospora* is synonomous to *Cladosporium iridis* (Schubert et al., 2007) so it is very likely that the MF1isolate is a *Cladosporium* species. *Paradendryphiella* is another genus missing in the WoRMS (World Registry of Marine Species) but according to (Woudenberg et al., 2013) certain *Dendryphiella* strains are now sorted as *Paradendryphiella* making the two listed high identity species for MF3 synonomous.

Table 5. High identify hits from BLAST searches of the fungal strains

Strain	High identity Genera	High identity species
MF1	*Cladosporium, Dothideomycetes, Davidiella*	*Cladosporium macrocarpum, Cladosporium cladosporides, Cladosporium herbarum Davidiella macrospora*
MF3	*Dendryphiella, Paradendryphiella*	*Dendryphiella salina, Paradendryphiella salina*
MF5	*Cladosporium, Davidiella, Dothideomycetes(Class)*	*Davidiella macrospora, Cladosporium cladosporides, Cladosporides macrocarpum, Cladosporides herbarum*
MF6	*Cladosporium*	*Cladosporium cladosporides*
MF7	*Acremonium, Emericellopsis*	*Acremonium potronii, Acremonium zonatum, Emericellopsis pallida*
MF8	*Penicillum*	*Penicillium brevicompactum*

4.3 Bacterial growth in microcultures on different substrates

Growth was determined with all 16 strains using f/2 medium with one of four different carbon source compositions: laminarin, mannitol, glucose or a mix between laminarin and mannitol. Growth was followed over 72 hours at room temperature. Due to missing replicates and unclear results the strains MP005, MP068, MP073, MP107, MP108 and MP111 were rerun under the same conditions.

In Figure 2 growth curves of strain MP073 and MP108 from the second set of cultures are shown. These strains were chosen for further studies based on the results of the first run (not shown) as they grew to a higher density and at a faster rate on laminarin than other strains. Despite identical 16S genes and both being identified as *Pseudoalteromonas* sp. the two showed clearly different growth characteristics. This indicates that they are different species or at least different strains of the same species. The two-phased growth curves on laminarin (blue) are interesting and could be a clue as to how the laminarin is degraded. Note also the purple line which shows poor growth on glucose for MP073. During the first growth assay six strains grew on laminarin, with similar growth patterns as MP073 and MP108. Strains that did not grow on laminarin had similar growth patterns to MP091shown in Figure 2.

During the repetition of the growth experiment the growth curves for some of the strains that previously did not grown on laminarin looked as MP005 in Figure 2. An additional three strains that grew on laminarin were thus found. These three however, only showed a single exponential phase but similar final OD600 in half the time, indicating a different way of growing on laminarin. Graphs for the strains not presented here can be found in

Appendix 3A. In Table 6 the laminarin-degrading strains from both runs are listed. It is also shown whether the growth curve had a single or two exponential growth phases.

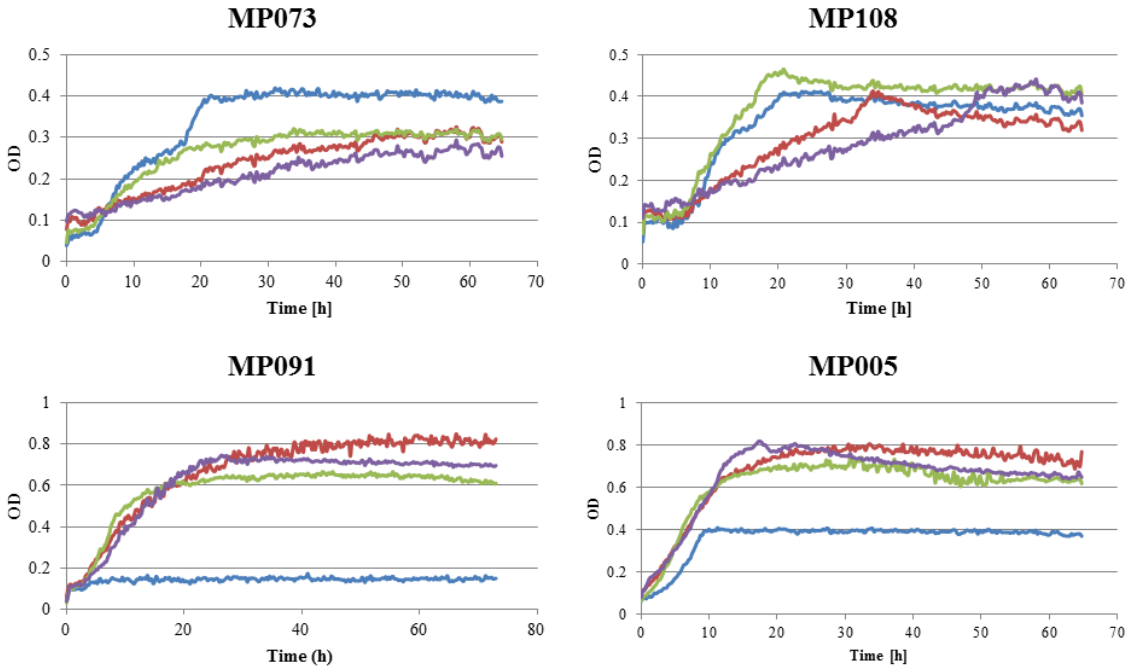

Figure 2. The bacterial strains MP073, MP108, MP091 and MP005 were cultivated in f/2 medium (Appendix 1C. f/2) with one of four different carbon source compositions; 5 g/l of either laminarin (blue), mannitol (red), glucose (purple) or 2.5 g/l of laminarin and mannitol (green).

Table 6. List of all strains found to grow on laminarin and the number of exponential phases they showed during growth.

Strain	Number of exponential phases
MP005	1
MP027	2
MP068	1
MP073	2
MP083	2
MP084	2
MP107	2
MP108	2
MP111	1

4.5 The growth of fungal isolates on laminarin

The growth of the fungal strains after 10 days growth on laminarin and agar as carbon source or just agar can be seen in Figure 3 and Figure 4. As can be seen in the figures, there were differences in the growth between the different fungi and also between the different media compositions. The first row had growth in the first well and an infinitesimal growth in the second well but no growth in the last laminarin well nor the agar wells. This indicated that MF1 could not grow on agar and if at all poorly on laminarin. The MF3 strain formed bigger colonies on the agar controls but it is hard to say whether this is due to stress induced morphology changes or better growth on agar without incubating the colonies for a longer period of time. MF5 showed clear signs of stress in the agar wells as it grew thin and wispily over a large area indicating that it was trying to find a better growth environment. In the laminarin wells however, the colonies were dense and extensively sporulating which showed that this fungi could grow on laminarin. In row 4 MF6 grew equally poor in all wells. MF7 in the first row on the second plate, showed signs of being stressed in all wells but the colonies on laminarin are bigger, indicating some ability to grow on it. In the last row with inoculated fungi the MF8 strain had grown a lot in both the laminarin and the agar controls. However, the difference in size of the colonies between the two is large and MF8, which is *Penicillum brevicompactum,* showed capability of growing on laminarin. This can be said although there were contamination by a fungi with yellow spores, as the green colonies of *Penicillum brevicompactum,* are clearly visible and can be compared.

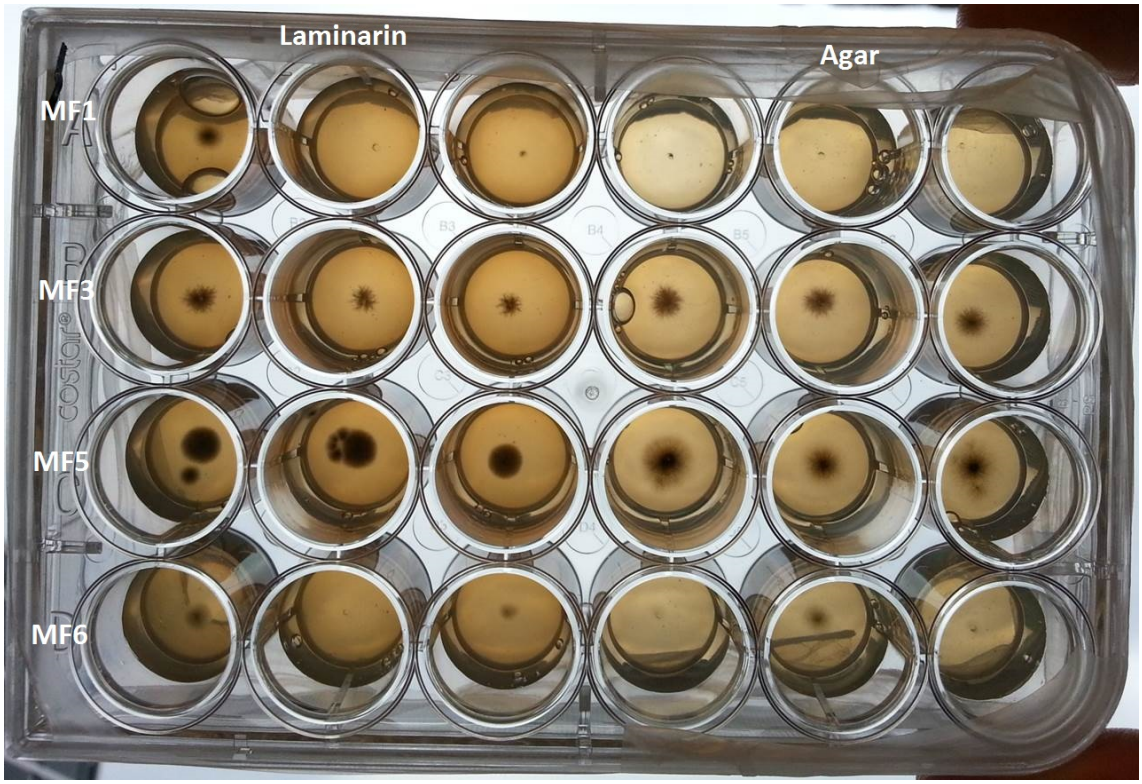

Figure 3. On each row a fungal strain was grown starting with MF1 at the top then MF3, MF5 and on the bottom row MF6. They were grown on agar made from BGA (Appendix 1D) where the first three wells on each row contained 2%laminarin in addition to the 2% agar while the three wells to the right only had 2% agar.

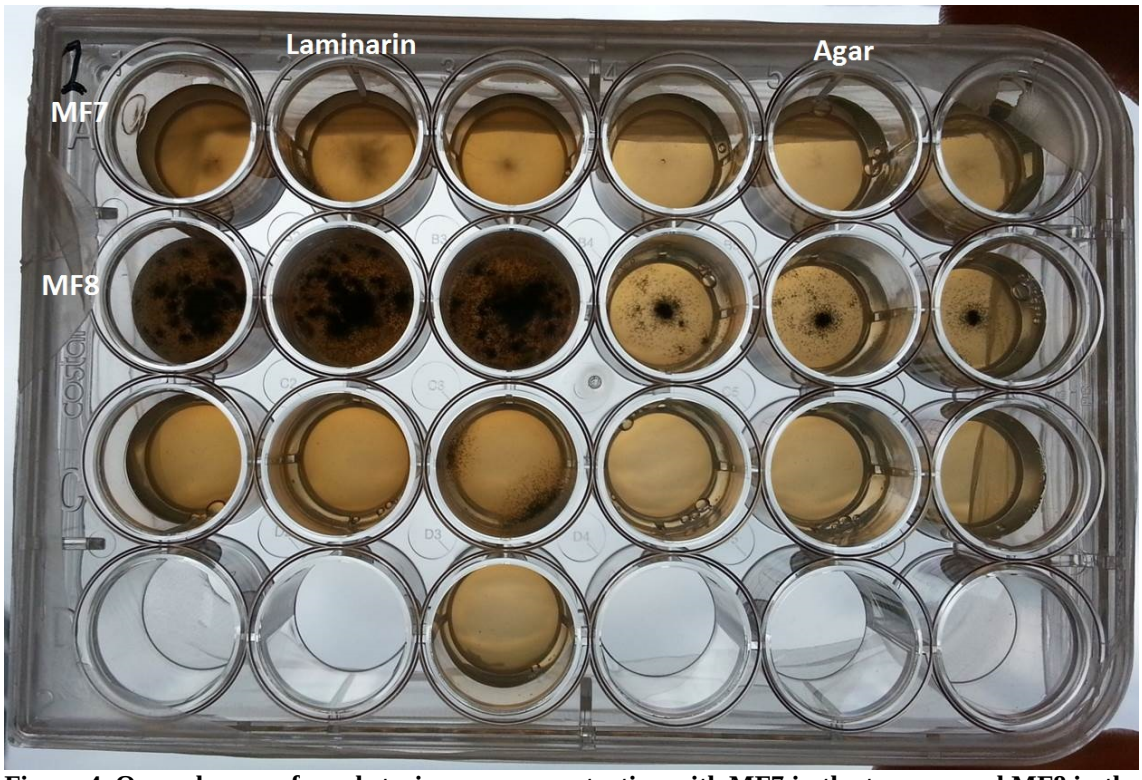

Figure 4. On each row a fungal strain was grown starting with MF7 in the top row and MF8 in the second while the rest of the wells were kept in reserve. The strains were grown on agar made from BGA (Appendix 1D) where the first three wells on each row contained 2%laminarin in addition to the 2% agar while the three wells to the right only had 2% agar.

4.6 *Pseudoalteromonas* growth on algae extract

The strains MP073 and MP108 were grown in 300 ml algae extract medium to investigate more growth characteristics and to analyse the supernatant for extracellular enzymes.

The hydrolysis and analysis by HPLC and HPAEC determined the starting composition of the algae extract medium. In Figure 5 these results are shown but it should be kept in mind no measurement of total sugars was done and hence the chart only shows what was detected and identified during analysis.

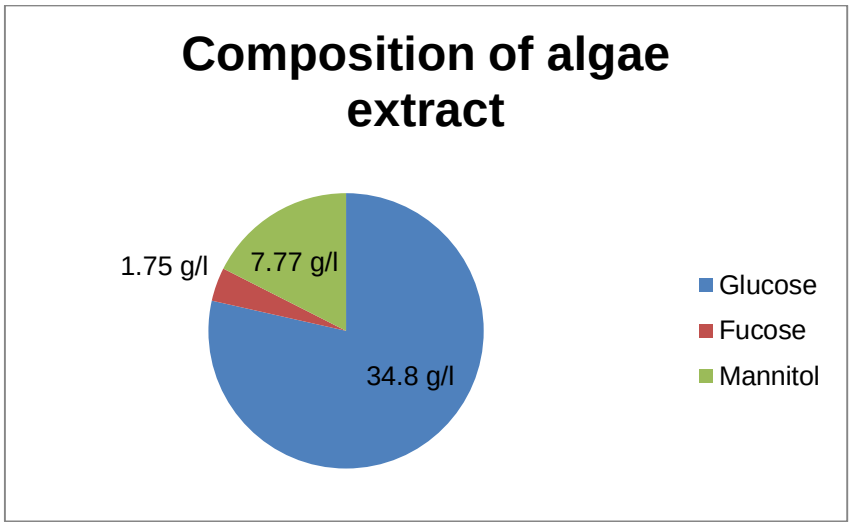

Figure 5. The chart shows the sugar composition of algae extract medium (Appendix 1B). A sample of the medium was hydrolysed with 4.6% H_2SO_4 for three hours at 121 °C and 2.1 bar in an autoclave. The sample was analysed using HPLC and HPAEC.

As previously described in Section 4.3 the strains MP073 and MP108, both *Pseudoalteromonas* spp., were chosen for investigation of presence of extracellular laminarin-degrading activity. The two strains were cultivated in 300 ml of AEX for 25 and 23 hours respectively with continuous sampling of supernatant for analysis of sugar content. At two time points, over 100 ml of supernatant was collected for the cell-free enzyme assay Section 4.8. In Figure 6 the growth curves of the two strains are shown and note the large difference in final OD600.

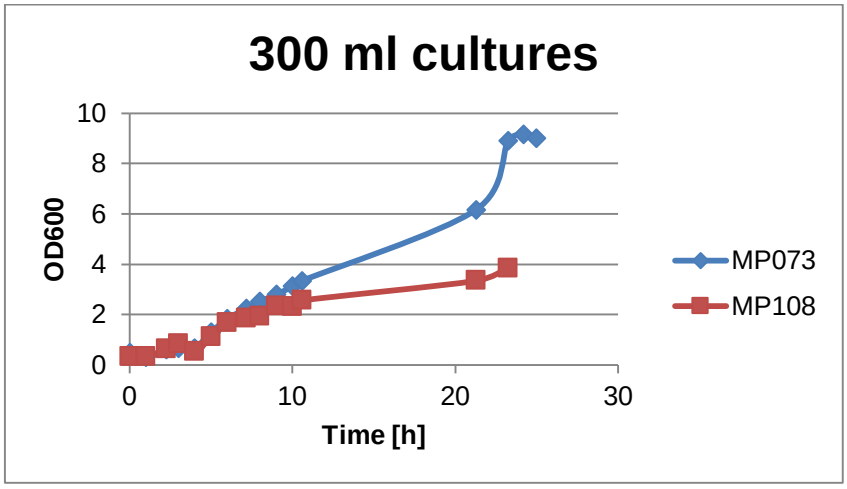

Figure 6. The strains MP073 and MP108 were grown in 300 ml cultures of algae extract (Appendix 1B) and samples were continuously taken to monitor medium composition changes and OD600 as well as save supernatant for protein analyses.

The samples taken during the growth were analysed with HPLC to see the changes in mannitol and glucose content during growth. This data, Figure 7, clearly shows that both strains consume mannitol during growth. Interestingly enough the glucose concentration increased at the end of the experiment. The glucose concentration in the supernatants is low but not zero except in the last samples taken.

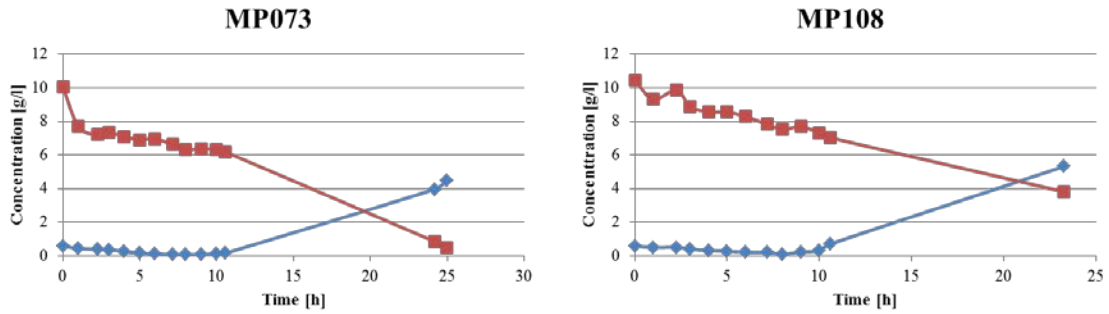

Figure 7. Bacterial strain MP073 and MP108 was grown in a 300 ml culture in algae extract medium (Appendix 1B). Samples were continuously taken and glucose (blue) and mannitol (red) were quantified using HPLC and plotted over time.

4.7 Protein analysis

To monitor the production of extracellular proteins during growth, the protein content of the samples from the 300 ml cultures of MP073 and MP108 were analysed, Figure 8. For both strains, the initial protein concentration of the algae extract medium was around 2 mg/ml and slowly decreased during 10 hours of growth, before starting to increase again by the end of the experiment, which confirmed that the two investigated strains excrete proteins to the surrounding medium.

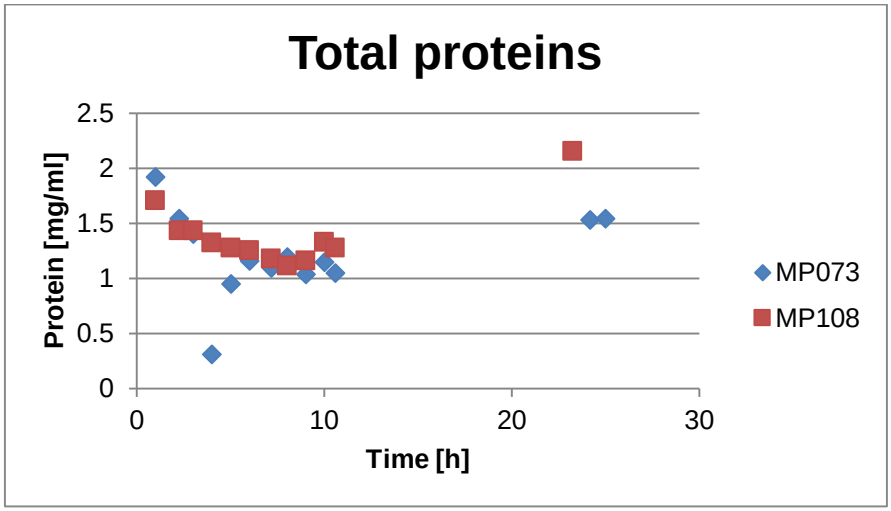

Figure 8. Bacterial strain MP073 and MP108 were grown in 300 ml culture on algae extract medium (Appendix 1B). Samples were continuously taken and the total protein content was analysed in a Lowry assay.

Figure 9 shows an SDS-PAGE gel for samples taken during the 300 ml culture growth of MP108 on AEX medium. Only the samples from the last two time points (10.6 h and 23.3 h) of the growth have a faint band visible after staining, at about 140 kDA, in lanes marked 1 and 2. After image processing some additional bands can be outlined and other proteins that might be present in the supernatants are of too low concentration to be visible on the gel. This was probably the case with the gel for MP073 which showed no bands. It should be noted that the gel did not run properly, hence the skewed lanes and the curved bottom line of the gel.

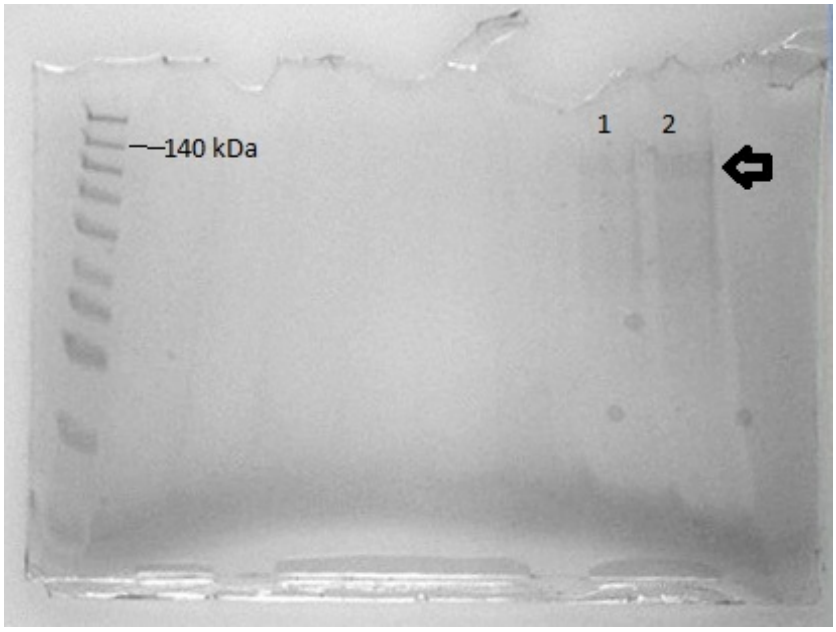

Figure 9. Shows a gel of an SDS-page where supernatant samples from varying time points of a cultivation on algae extract of the *Pseudoalteromonas* sp. strain MP108. Lane one and two represent samples from 10.6 and 25.0 hours into the cultivations, all other time points does not produce visible bands. To the left the ladder, Spectra™ Multicolor Broad Range Protein Ladder from Thermo Scientific (SM1841), was run with the relevant 140kDa band is marked.

4.8 Enzyme assay

Samples taken at 10.6 hours for both strains and samples from 23.3 and 25.0 hours for MP073 and MP0108 respectively were analysed for laminarin degrading activity using 6.25g/l laminarin in 50 mM sodium phosphate buffer at pH 6. Three different concentrations of bacterial culture supernatant was used, 50%, 37.5% or 25% of the total reaction volume. Samples for MP108 showed a significant increase in glucose (α=0.05) in the performed assay and the trend of the remaining were in line with the significant results, as can be seen in Figure 10. MP073 however, did not yield any statistically significant results. The deviation is large in several of the runs with MP073. For MP108 the trend of increasing glucose concentration is very clear and a few samples are significant while others are very close. This shows that these cell free supernatants contain laminarin-degrading enzymes.

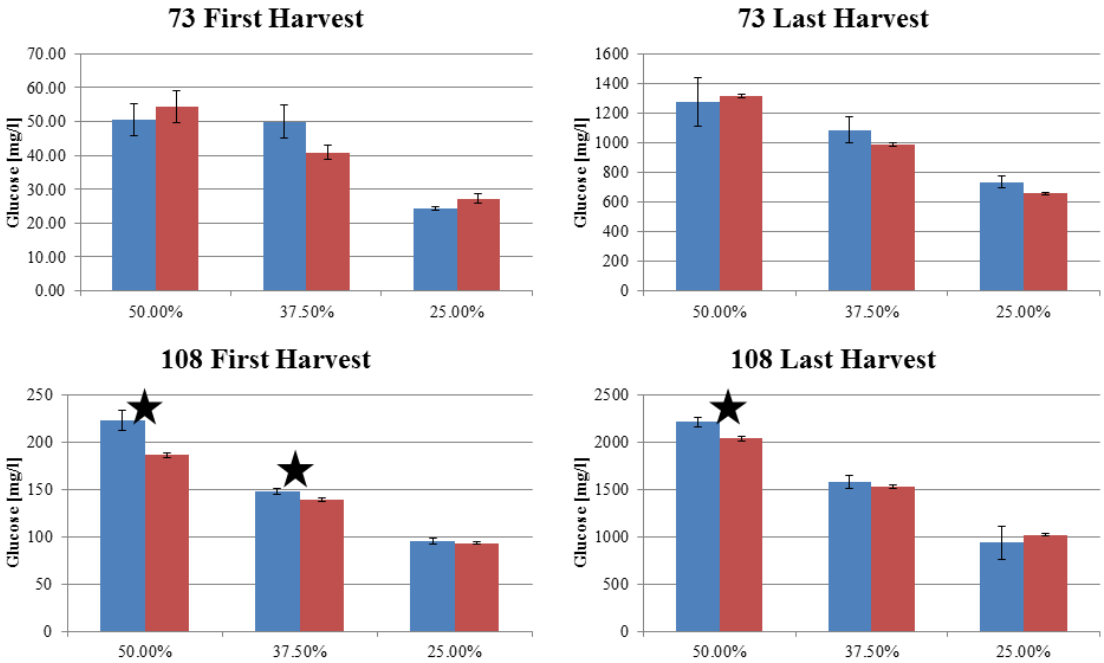

Figure 10. Bacterial strains MP073 and MP108 were grown in a 300 ml culture on algae extract medium (Appendix 1B). The samples taken after 11, 23 or 25 hours (called first and last harvest) for MP073 and MP108 respectively, were used for the enzyme assay presented here. The reaction conditions for the assay were 50 mM Sodium Phosphate, 6.25g/l laminarin at pH 6 in a total volume of 1.5 ml. Three different concentrations of supernatant, 50%, 37.5%, and 25%, were incubated in triplicates at room temperature for one hour before being inactivated by boiling for 10 minutes. The laminarin-degradation was then analysed by measuring the glucose with HPAEC. As blank a reaction mixture with no added laminarin and 50% supernatant in triplicate was used. The blanks for the lower supernatant content were calculated from this value. Statistical significance (α=0.05) calculated using student's paired t-test are shown with *.

4.8.1 Activity

The activity per mg of total extracellular protein and hour was calculated for the activity assays that showed statistically significant differences in glucose concentrations as compared to the controls. In Figure 11 it can be seen that the last harvest of the 300 ml was the one that had the highest activity. It also has the highest total protein although the amount of the enzyme/enzymes responsible for the degradation in either assay is unknown. It should be remembered when looking at this data that the blank samples for the 25% supernatant assays were calculated from blank samples run with 50% of supernatant. Since the supernatant is used algae extract medium the results might be a bit biased as there might be laminarin left and therefore the proteins in the 50% assays have more laminarin to degrade.

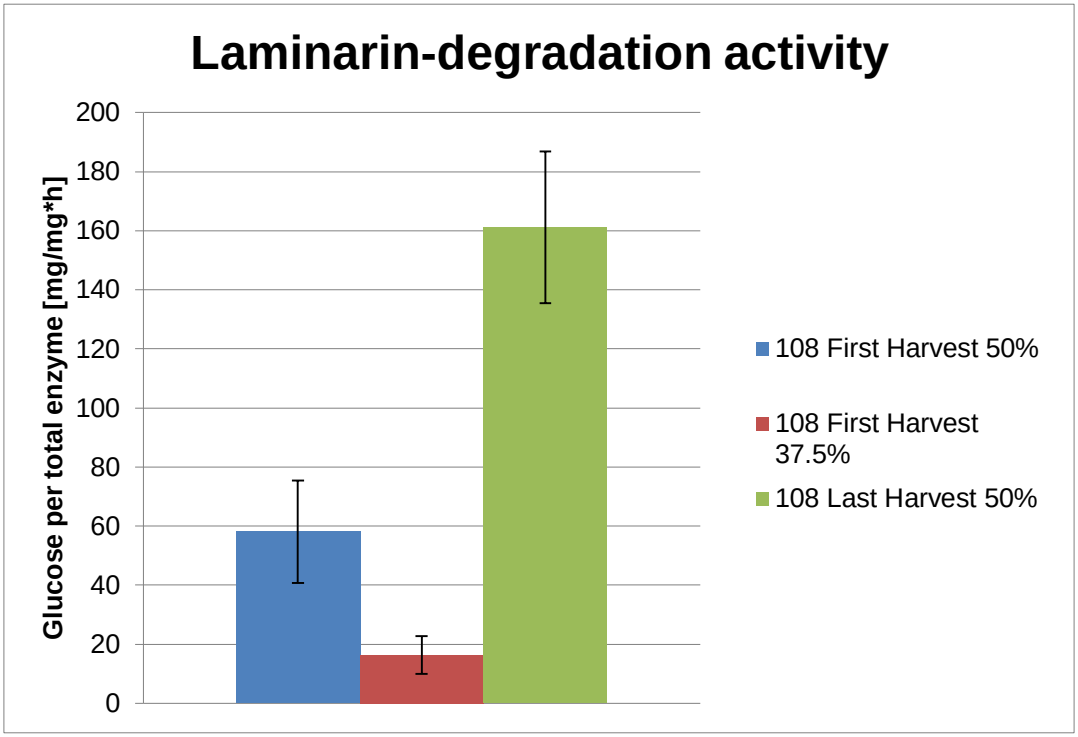

Figure 11. Samples with a statistically significant glucose increase from Figure 10 were used when calculating the glucose formed in mg per hour and total protein. The reaction conditions for the assay were 50 mM sodium phosphate buffer at pH 6, 6.25 g/l laminarin in a total volume of 1.5 ml. Three different concentrations of supernatant, 50%, 37.5%, and 25%, were incubated in triplicates at room temperature for one hour before being inactivated by boiling for 10 minutes. The laminarin-degradation was analysed by measuring the glucose with HPAEC.

5 Discussion

5.1 Isolating laminarin-degrading organisms

To isolate individual species from the crude microbial isolates collected at Tjärnö, the bacteria were streaked in two steps on laminarin agar for single colonies. Despite the selection process several strains did not grow on laminarin during the first growth experiment when screening for growth on different carbohydrates, Figure 2. When repeted however, a few of them did, which puts up the question of whether or not the first growth experiment is trustworthy. As the strains picked for further characterization has the same growth characteristics (3.5.2 Bioscreen and Appendix 3B – Bioscreen run), though with a few replicates missing, the first assay should be trustworthy. The day after the first run, though, there was a mechanical error with the Microbiology Reader Bioscreen C (Oy Growth Curves Ab Ltd) used which could have affected the run. It could be so that all the tested isolates would have grown on laminarin if they had been run a second time. As the preceding isolation and selection was done on laminarin this would be the most logical conclusion.

The fungal strains were never selected for on laminarin plates during this project. Instead the fungi were isolated based on differences in morphology. As the isolation was done on a complex algae extract many potential laminarin-degrading fungi might have been missed due to other fungi growing faster on more easily available sugars (mannitol and fucose). As presented in Section 4.2 the isolated organisms were either of marine origin or, even though terrestrial, previously isolated from marine environments.

5.2 Growth analysis of isolated organisms

From the Bioscreen growth experiments on the four different carbon source compositions of laminarin, mannitol, glucose and a mix of mannitol and laminarin the strains MP073 and MP108 were chosen for further investigation. As can be seen in Figure 2 they both have two exponential phases. This characteristic is usually associated with cultures containing two substrates where the microorganism prefers one over the other but switches metabolism when the favourite is out (Galdieri et al., 2010). In these cultures however the only energy source provided is laminarin which indicates that it is the laminarin in combination with the organisms' degradation pathway that makes it behave like two different substrates. It could be that there is β-1,3-glucanase and β-1,6-glucanase acting at different time points during the cultivation. This would result in one growth phase where the β-1,3 bound glucose is used for growth and one where the remaining β-1,6 bound glucose is used.

Another theory is that there is an exo-β-1,3-glucanase with glucosidase activity and then a β-1,6-glucosidase accounting for the second growth phase. Both theories would result in the observed growth pattern. Any of the theories could explain why the first growth phase is approximately three times larger as digesting the more common bond first should release more glucose. It is, however, also possible that the last growth phase is due to consumption of some unidentified product formed by the bacteria themselves. In which case the enzyme/enzymes' mode of action could be numerous.

The 300 ml AEX cultures of MP073 and MP108 were made to continuously monitor growth and protein concentrations. As can be seen in Figure 6, the OD600 values were

very different for these two *Psudoalteromonas* spp. with MP073 having more than double the final OD600 of MP108. This result provides an interesting difference between the two strains and even though it was hard to measure OD600 in this medium and get consistent values in the measurements, this does not account for this large difference in final OD.

Instinctively one would think that the more cells there are, the more protein there would be and also that the two chosen strains would be similar in protein excretion. Especially since these strains are likely closely related or even of the same species. But this is not the case and as can be seen in Figure 8 where MP108 yielded as much as 0.6 mg/ml higher total protein concentration in the final sample, despite a lower OD.

In Figure 7 the mannitol and glucose concentrations in the cultures can be followed over time. The mannitol is steadily declining while the glucose is at a constantly low level but never reaches zero, indicating continuous production of glucose from hydrolytic enzymes and co-utilization mannitol. At the end of the growth the glucose concentration increased to 4.5-5.3 g/l which is a significant concentration compared to being almost depleted in the middle of the experiment. The most reasonable conclusion here is that some other medium component required for growth is depleted which has stopped the growth of the cells, as can be seen in Figure 6, while the extracellular hydrolytic enzymes are still degrading the laminarin in the solution, forming glucose. One problem with this theory is that the data does not confirm that the cells have stopped utilizing mannitol which puts this theory to question. The formation of glucose confirms that both these organisms contain complete pathways for degradation of laminarin into glucose even though it remains unknown what enzymes are involved, their mode of action as well as their regulation.

The consumption of mannitol before glucose could indicate a preference for mannitol over laminarin. For this to be investigated properly the amount of laminarin in the culture must also be investigated.

The fungal growth experiment which can be viewed in Figure 3 and Figure 4 was just a screen to see if the different strains could grow on laminarin as a carbon source. It turned out that M5 and MF8 grew better on laminarin than the control agar which shows that these two strains are very likely to have interesting enzymes with laminarin-degrading activity. Another interesting prospect from this assay is the growth on agar as sole carbon source by MF3, 5, 6, 7 and 8 as agarolytic enzymes are sought after for food and medical applications (Giordano et al., 2006).

5.3 Protein assays

5.3.1 Total protein determination

The total protein levels in the samples from the 300 ml cultures are presented in Figure 8. In the beginning the protein content most likely originates from the algae and it seems like the bacterial strains utilize these as the protein levels decrease during the first hours of cultivation. After about 9 hours the trend changes and the amount of protein in the supernatants start to increase. For the strain MP108 the final concentration is as high as 2.15 mg/ml in the supernatant, compared to 1.7 mg/ml one hour into the cultivation. The trend changes for the strains, as well as the high final concentrations, proves that both strains release extracellular enzymes during the cultivation.

The SDS-page of strain MP108 in Figure 9 showed bands in the two latest sample points while the gel for MP073 was empty. It is interesting that bands are only visible in the two last samples and not in any previous ones as the total concentration is almost the same. In the early samples most proteins originate from the algae and very little from the bacteria. As the algae proteins has gone through both autoclavation and the extraction process to make the AEX they could be too degraded to show individual bands on the SDS-gel. The proteins released by the bacteria are probably not reaching high enough concentrations to be visible on the gels until the cell density is high in the cultures. Hence, bands are only visible in the last samples.

It should be noted though that by concentrating the samples or by using silver staining, which is a more sensitive detection method, bands in all lanes could become visible on either gel. It is unlikely that the bands seen are the only enzymes in the supernatants and some residuals should be seen from the degraded seaweed proteins. If these methods had been tried a lot more insight could have been gained.

5.3.2 Laminarin-degradation assay

The degradation assay produced good results for MP108, Figure 10, where three out of six assays showed statistically significant increase in glucose and except for one outlier the non-significant assays still followed the same trend as the significant. The increase in glucose verifies that, in the supernatant of MP108, there are extracellular enzymes that break down laminarin into glucose. One of the reasons why degradation was not seen in all samples is that the assay was only run for one hour. This is probably why the significant assays are of either the highest supernatant concentration or the intermediate concentration as the enzyme levels might be too low in the other assays to degrade enough glucose in just one hour.

None of the MP073 assays produced a significant increase in glucose and when comparing the samples in Figure 10 it is not possible to see any trends indicating degradation of laminarin. This could be due to the lower total enzyme concentration in these supernatants compared to MP108, see Figure 8, or to the incubation time of the assay being too short. A scenario which explains why no significant glucose concentrations are seen as well as the glucose formed in the large cultures, Figure 7, is that the enzymes degrading laminarin into glucose are membrane bound. If there had not been glucose formed in the supernatants during the last part of the growth experiments, a possible scenario would have been the formation of bioses as a degradation product which could be taken up by the cells. That would have made degradation-activity impossible to see with the experimental setup used here. This should not be the case though, as glucose is formed in the supernatant at the end of the 300 ml growth experiment.

Another reason for the lack of significant results might be product inhibition. In Figure 7 it can be seen that the last samples has high glucose content. Hence, if the hydrolytic enzymes degrading the laminarin are sensitive to glucose inhibition, the lack of degradation seen in MP073, could be explained. It could also affect the amount of glucose formed in the MP108 assays. The fact that the higher dilutions did not have a significant increase in glucose speaks against this in the case of MP108, but as previously discussed it might also be due to the lower protein concentration.

The enzymes were added as an extract from the bacterial cultures, potentially containing residual laminarin. Due to the different dilutions of the extracts in the assay the laminarin

concentrations could be different. There might also be differences between the different strains depending on how much laminarin was left in the supernatant after the cultivation. Judging by the OD600 measurements in Figure 6, MP073 might have the lower laminarin concentration if it has utilized the laminarin through the whole cultivation but it is impossible to say.

An important note to make is that neither the buffer nor the water used for dilutions in the assay contained any salt or other potential co-factors present in seawater. This is something that could potentially affect these marine enzymes and is something to consider for the future. If these enzymes function better in an environment with high salt concentrations this has to be considered in future uses of the enzymes.

5.3.3 Comparison to other laminarases

To compare the potential laminarases found in this project to other laminarases described in the literature, is very hard as the assays are quite different. However, nothing is suggesting that the crude enzyme mix studied here is performing poorer than either of the enzymes in the studies described in 2.2.3 Laminarases, there is just no way of knowing without knowing how much of the active enzyme there is in the work performed here on *Pseudoalteromonas* sp.

The laminarin-degrading enzyme studied by Labourel et al., (2014) degrading laminarin into trioses, seems to suggest that there are several enzymes involved in the degradation into glucose in their organisms, just as this project suggests for. *Pseudoalteromonas* sp. This conclusion is also confirmed by Nakatani et al., (2010) who found that endo- and exo-glucanases are responsible for the laminarin degradation in *Pseudoalteromonas* sp. Before continuing too far with the isolates of this project they should be compared to the *Pseudoalteromonas* sp. of (Nakatani et al., 2010) to keep this research novel.

5.4 Outlook

The crude assay made in this thesis using supernatant from growth on a complex media might have been perfect for screening as potential cofactors for the degrading enzymes are unknown. However, for identification purposes and for proteomic analyses it might be better to use a defined minimal medium with laminarin as sole carbon source to not have a background of kelp proteins, which are of no interest to us. Remembering the two-sloped growth curves of Figure 2 it could be very interesting to analyse samples from different time points of growth to see if β-1,3-glucanase and a β-1,6-glucanase are present in the supernatant at different time points during the growth as well as studying which (if any) metabolites are present. The substrate specificity should also be investigated for the enzymes trying them on other polysaccharides of glucose such as cellulose.

When the laminarin degradation into glucose has been confirmed, isolation and identification of the active enzymes is a key part of continuing working with the *Pseudoalteromonas* sp. studied here. Identification of the genes encoding the enzymes is needed for them to be produced in *e.g. E. coli,* so that they can be used at industrial scale in the biorefinery. This might also make it possible to express the degradation pathway in *S. cerevisiae,* which could be a solution for industrial use.

For the fungi included in this thesis more work needs to be done to see which of them has laminarin degrading capabilities. Congo red staining of the laminarin to screen them for laminarase activity on plates would be a first step. Then, culturing on liquid media with

laminarin as the sole carbon source could provide supernatants that could be tested for extracellular enzymes, making the same tests as for the bacteria.

5.4.1 Future use of the enzymes and microorganisms

Out of all the crude bacterial isolates from Tjärnö only a few was selected for continuation within this project based on their ability to grow on laminarin. Not only did the selected ones come from both *S. latissima* and *L. digitata*, they were also separated on various medium compositions and carbon sources. Comparing Table 3 and Table 4 also shows that strains of the same genera were isolated on different carbon sources. This shows the versatility of these isolates, which makes them interesting for the search of enzymes degrading and metabolizing not only the laminarin but also the other saccharides that are part of the algal biomass. Mannitol is one such example and a pathway enabling growth on mannitol without presence of oxygen would be very interesting as this is missing in *S.cerevisiae*. Organisms utilizing alginate, fucoidan or cellulose could also be of interest.

In a parallel thesis, one of the supernatants from this project was used in an SSF process on a similar type of algae extract to make ethanol (Davidson, 2015). That work showed that *S. cerevisiae* was able to grow on laminarin when supplied with supernatants from *Pseudoalteromonas sp.* grown on laminarin containing algae extract. These supernatants also contained some cells but the bacteria did not grow anaerobically and did not interfere with the fermentation.

6 Conclusions

Organisms with laminarin-degrading properties were found and crude supernatants from two of the cultures were used for enzymatic assays. One of the strains was proven to have laminarase activity since significant concentrations of glucose was produced in the laminarin-degradation assay. Therefore the enzyme mixtures, whether cell-bound or in free solution, are very interesting to investigate further for hydrolysis of the laminarin into glucose, enabling fermentation processes using the laminarin in *Laminaria digitata* and *Saccharina latissima*. More work remains to be done to design a biorefinery process where these enzymes can be of use and a lot of work also needs to be put into enabling utilization of all the components of the macroalgae. The work presented within this thesis is but one piece of the puzzle towards utilization of laminarin containing seaweeds within a biorefinery concept. It is however an important piece and one step has been taken towards fermentations utilizing laminarin.

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Appendix 1 – Media

1A. Synthetic seawater

To 1 l of distilled water 30 ml of Instant Ocean aquarium salt (Aquarium systems, Sarrebourg Cedex, France).

1B. Algae extract

1 L	synthetic seawater
500 g	*Saccharina latissima* or *Laminaria digitata,* cut in fine pieces

Heat for 2 hours at 70° with stirring.
Sieve off the biomass and keep the liquid. Let cool.

(Add 18 g agar per litre for plates)

Autoclave.
Add 1 ml sterile N/P stock per litre extract.
Let cool or poor plates.

N/P stock

3.75 g	$NaNO_3$
0.28 g	$NaH_2PO_4 \cdot 2H_2O$
50 ml	water

1C. f/2 medium

As made by (Guillard and Ryther, 1962). In 1 l of synthetic seawater (Appendix 1A):

75 mg/l	$NaNO_3$
5 mg/l	$NaH_2PO_4 \cdot H_2O$
3.15 mg/l	$FeCl_3 \cdot 6H_2O$
4.36 mg/l	$Na_2EDTA \cdot 2H_2O$
0.18 mg/l	$MnCl_2 \cdot 4H_2O$
22 µg/l	$ZnSO4 \cdot 7_2O$
10 µg/l	$CoCl2 \cdot 6H_2O$
9.8 µg/l	$CuSO4 \cdot 5H_2O$
6.3 µg/l	$Na2MoO4 \cdot 2H_2O$
0.2 mg/l	Thiamine·HCl
1 µg/l	Biotine
1 µg/l	Cyanocobalamin

1D. Basal growth medium

As made by (Anasontzis et al., 2011). For each liter of media made the following was added to distilled water:

7 g $NaH_2PO_4 \cdot 2H_2O$
9.5 g $Na_2HPO_4 \cdot 2H_2O$
1 g KH_2PO_4
10 g $(NH_4)_2HPO_4$
0.3 g $MgSO_4 \cdot 7H_2O$
0.3 g $CaCl_2 \cdot 2H_2O$
10 g glucose

Appendix 2 – Sequences

2A. Primers

27f	AGA GTT TGA TCM TGG CTC AG	(M=A:C)
1494r	CTA CGG CTA CCT TGT TAC GA	
ITS 1	TCC GTA GGT GAA CCT GCG G	
ITS 4	TCC TCC GCT TAT TGA TAT GC	

2B. Full sequences of isolates

MF1

TAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGC
GGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTA
AACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC
ATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAC
GCGGTCCGCCGCGTGCCTCAAATCGTCCGGCTGGGTCTTCTGTCCCCTAAGC
GTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGGCTACGCCGTAAAACA
ACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCATATCAATAAGCGGAGGAA

MF3

TCTCGGTGGGGGCTCCAGCTTGTCTGAATTATTCACCCATGTCTTTTGCGCA
CTTCTTGTTTCCTGGGCGGGTTCGCCCGCCACCAGGACCCAACCATAAACCT
TTTTTTGTAATTGCAATCAGCGTCAGTAAACAATGTAATTATTACAACTTTC
AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGA
TACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA
TTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACC
CTCAAGCTTTGCTTGGTGTTGGGCGTCTTTGTCTCTCACGAGACTCGCCTTAA
AATGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAATCTTGCACTT
CTGATCAGCCATGGTTGAGCATCCATCAAGACCACATTTTTCTCACTTTTGA
CCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGGCGG
AGGAAAAAATTT

MF5

TAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACCCTGCCTTCGGGC
GGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGCAGTCTGAGTA
AACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGCAT
CGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGC
ATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAA
CGCGGTCCGCCGCGTGCCTCAAATCGTCCGGCTGGGTCTTCTGTCCCCTAAG
CGTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGGCTACGCCGTAAAAC
AACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT
AAGCATATCAATAAGCGGAGGAA

MF6

CCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGACC
CTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTT
GCAGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCT
TGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGT
ATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCACCACTCAAGCCTCGCTT
GGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAATCGACCGGCTGGGTCT
TCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGTTCGGGAGGCT
ACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGAT
ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

MF7

CCCTGTGACATACCTATGTTGCTTCGGCGGGCCGTCCCGCGGCGCGCCCACG
TGGCGTGACCCGGAACCAGGCGCCCGCCGGGGACCCAAACTCTTGCCTTTTT
AGTGTCTCCTCTGAGTGGCATAAGCAAAAATAAACAAAACTTTCAGCAACG
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CCTCGCAGAAGGGACGAGCGGGCTGGCCACGCCGTAAAACCCCCAACTTCT
CCAGGTTGACCTCAGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCA
ATAA

MF8

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AGCGGAGGAA

MP005

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MP020

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MP027

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MP028

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CGGCAACGAGCGCAACCCACGTCCCTAGTTGCCAGCATTCAGTTGGGCACT

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GAGCATGAAGTTGGAATCGCTAGTAATCGCGGAACAGCATGCCGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTCT
ACCCGACGACGCTGCGCTAAC

MP035

Part one, second part of sequencing not long enough to connect to this one.
GTCGAGCGGAACGATCCTAGCTTGCTAGGAGGCGTCGAGCGGCGGACGGGT
GAGTAATGCATGGGAATCTGCCCGATAGTGGGGGACAACCTGGGGAAACTC
AGGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCT
TGCGCTATCGGATGAGCCCATGTCGGATTAGCTTGTTGGTGAGGTAATGGCT
CACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGG
ACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTT
CGGGTTGTAAAGCACTTTCAGCGAGGAAGAACGCCTTGGGACTAATACTCC
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GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTA
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CGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG

Part two
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AGTCCCGTAACGAGCGCAACCCCTATCCTTATTTGCCAGCGAGTAATGTCGG
GAACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACG
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CTTCGGGAGGGCG

MP038
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MP068

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MP073

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MP084

Part1:

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Part 2:

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AGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCAT
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MP091

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MP099

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MP102

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MP107

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MP108

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MP111

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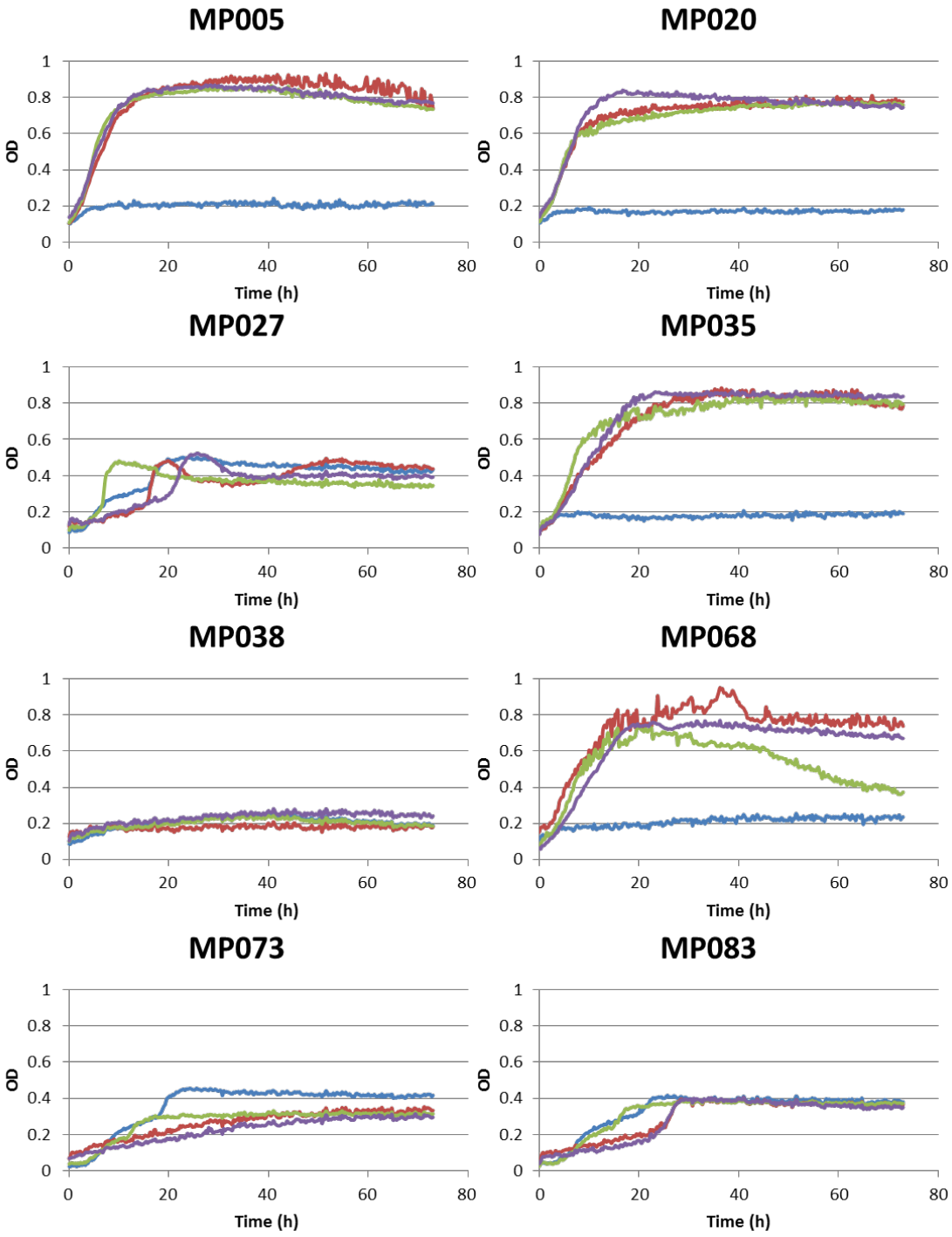
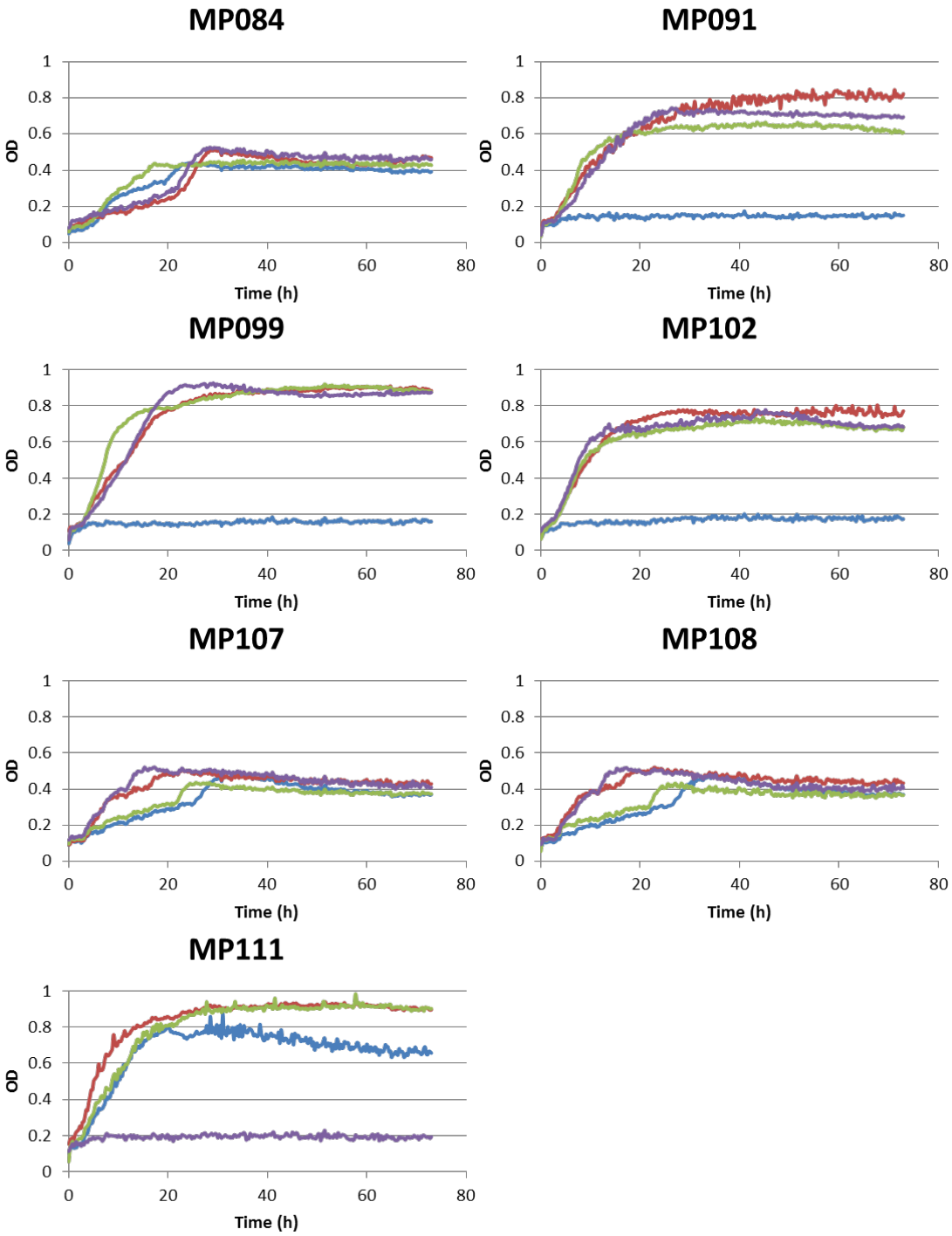
GG

Appendix 3 – Bioscreen

3A – Bioscreen run 1

The bacterial strains were cultivated in f/2 medium (Appendix 1C. f/2) with one of four different carbon source compositions; 5 g/l of either laminarin (blue), mannitol (red), glucose (purple) or 2.5 g/l of laminarin and mannitol (green). Every graph below is an average of three replicates. Except MP005 glucose, MP068 mannitol, MP073 mannitol, MP107 laminarin and mannitol, MP108 glucose and MP111 glucose which are all with two replicates due to errors.




MP084
MP091
MP099
MP102
MP107
MP108
MP111
OD
Time (h)
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80

3B – Bioscreen run 2

The bacterial strains were cultivated in f/2 medium (Appendix 1C. f/2) with one of four different carbon source compositions; 5 g/l of either laminarin (blue), mannitol (red), glucose (purple) or 2.5 g/l of laminarin and mannitol (green). These runs are repetitions due to mistakes in the ones shown in Apendix 3A – Bioscreen run 1.