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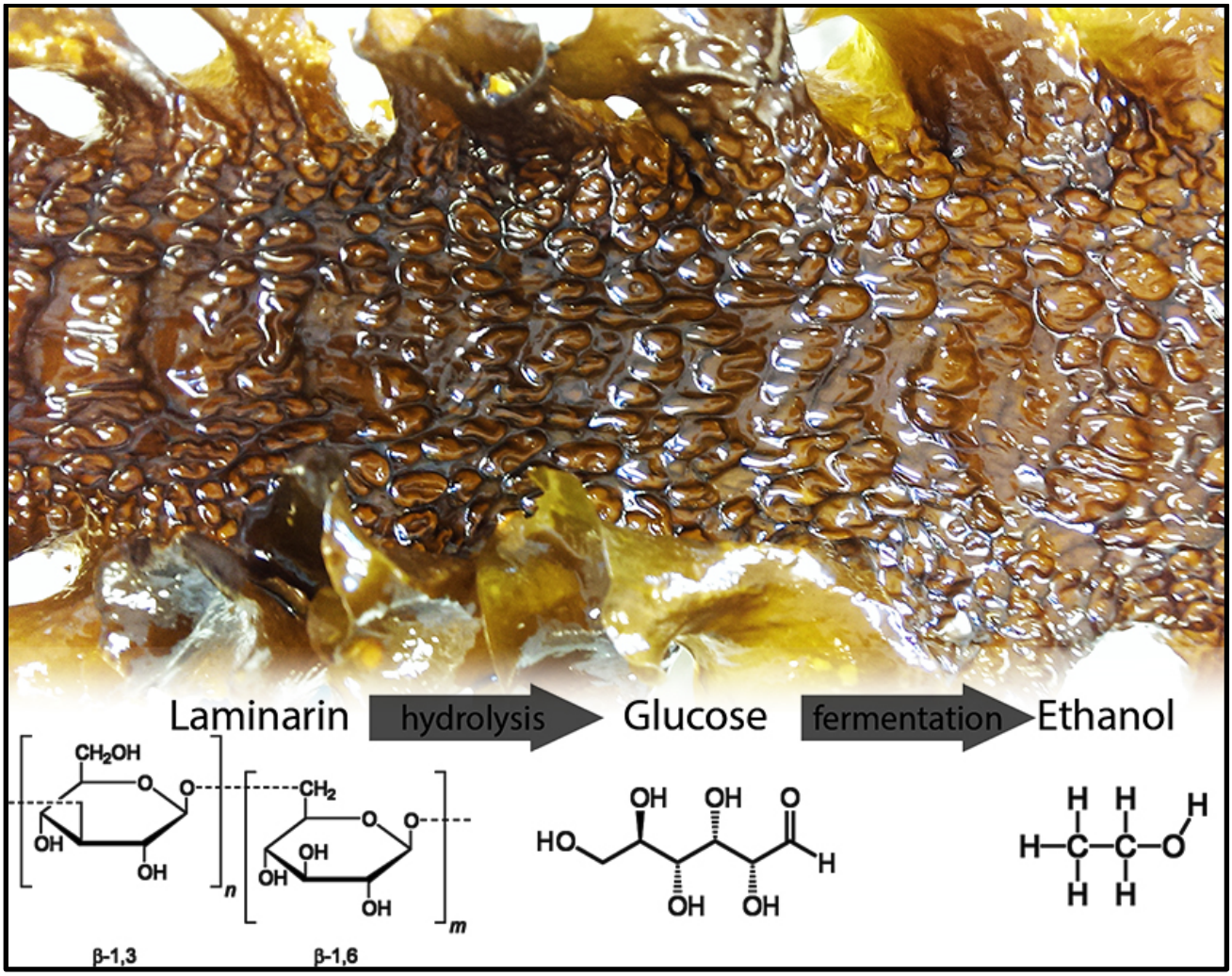

Swedish macroalgae as substrate for bioethanol production

Degree project for Bachelor of Science in Chemical Engineering

CHRISTOFFER DAVIDSON

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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ABSTRACT

This project investigates *Saccharina latissima* seaweeds, harvested in Sweden, as substrate for bioethanol fermentation. *Saccharina latissima* is also called sugar kelp and contains large amounts of the polysaccharide laminarin which was the target of interest in this project. The seaweed was milled before mixed with strong base followed by acid to extract the laminarin into a solution. After extraction, three methods for hydrolysing the laminarin into glucose were evaluated: sulphuric acid and autoclaving, commercial laminarinase enzyme, and enzymes from the naturally occuring bacterium *Pseudoalteromonas sp.* After hydrolysis fermentation was performed in small batches with a commercial strain of brewers' yeast. The results show that both sulphuric acid and enzymes from *Pseudoalteromonas sp*. did release glucose, which was successfully fermented into ethanol.

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1. Introduction

1.1 Purpose

Bioethanol is a hot topic nowadays due to its use as fuel and gasoline blend for vehicles. "Bioethanol" is not any different from just "ethanol", it just tells that it been produced by fermentation of sugar containing biomass rather than synthesis from fossil fuels (Balat et al., 2008). Thus, bioethanol is considered a renewable fuel/chemical and potentially "carbon neutral", thereby more environmentally friendly than fuels derived from fossil resources. Sugars are most often obtained from sugar canes or hydrolysed starch-containing agricultural- or energy crops (Balat et al., 2008). However, if all fossil fuels were to be replaced with bioethanol, enormous areas of land would be required to grow enough crops to sufficiently feed the production needs, thereby competing with food production (Adams et al., 2009). In the light of this, other feedstocks for biofuel production need to be explored. Marine macroalgae (seaweeds) could be an interesting alternative since they do not require arable land.

Seaweeds have a high polysaccharide content, making them a potential substrate for bioethanol fermentation. They grow much faster than agricultural crops and could, if cultivated, theoretically yield almost four times more ethanol per hectare and year compared to sugar canes which is the most efficient substrate today (Adams et al., 2009). They do not require any irrigation or fertilizers. In fact, big scale macroalgae cultivating could help decrease eutrophication in our oceans by using the nutrients in the water (Chojnacka, 2012). However, processes for extracting and hydrolysing the polysaccharide content are still under research.

The Seafarm project is an ongoing collaboration between several universities in Sweden that is investigating the possibilities of cultivating seaweeds for harvesting and what kind of products that could be obtained or synthesised from them. The strategy is to first extract valuable nutrients and components/chemicals then use the leftover biomass for making either biogas or, as here, bioethanol.

The purpose of this degree project was to investigate the possibility to produce bioethanol from an extract of the brown seaweed *Saccharina latissima*, also known as sea belt or sugar kelp. The component of focus has been laminarin, a β-glucan abundant in the seaweed, which could be hydrolysed into glucose and fermented into ethanol.

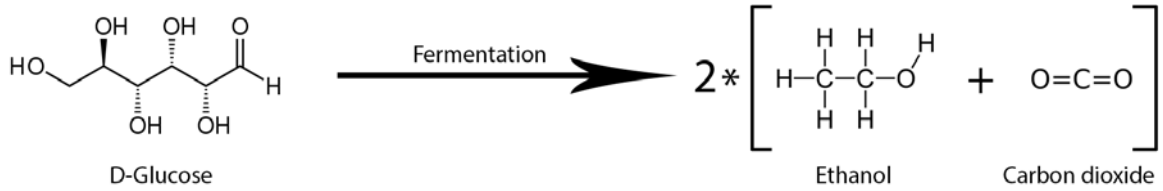
1.2 Boundaries

Due to limited time, only one extraction method was used, according to previous procedures within Seafarm. Although both laminarin and mannitol in brown macroalgae are potential substrates for ethanol production, focus has been on laminarin, as when hydrolysed into its glucose monomers, becomes a good substrate for the widely used ethanol-producing yeast *Saccharomyces cerevisiae*. This was not possible with mannitol which few microorganisms can utilise for ethanol fermentation (Horn et al., 2000b). Three methods of hydrolysis have been be tested, but only one fermentation setup for all hydrolysates.

2. Background

2.1 Bioethanol

Bioethanol is simply ethanol that has been fermented and distilled from a carbohydrate-containing biomass feedstock, as opposed to synthetic ethanol which is produced from hydration of ethene derived from fossil fuels (Eberson & Ohlson, 2014). Ethanol fermentation is the degradation of sugars into ethanol and carbon dioxide by the use of microorganisms, most often yeasts. The process have been known and used for centuries both for bread baking and making alcoholic beverages. The basic formula is as follows:



Saccharomyces cerevisiae (brewer's/baker's yeast) is the most used yeast species and can produce up to just over 20% (v/v) (www.alcotec.co.uk) ethanol under the best conditions before it becomes inhibited by the ethanol concentration. Fermentable sugars for *Saccharomyces cerevisiae* are the monosaccharides glucose, fructose, mannose and galactose as well as the disaccharides sucrose (glucose and fructose) and maltose (two glucose units) (van Maris et al., 2006). However, glucose and fructose are the preferred substrates (Gancedo, 1998).

Sucrose or "table sugar", a disaccharide of glucose and fructose, is refined sugar from sugar beets or sugar canes. Glucose is also found in various glucans which are polysaccharides of glucose monomers. Starch is composed of the α-glucans amylose and amylopectin which are large glucans bonded mainly by α-1,4 bonds with some α-1,6 branches. Starch can be obtained from various crops such as corn, grain or potatoes and hydrolysis into fermentable sugars are fairly straightforward.

2.1.1 Substrates for bioethanol fermentation

Bioethanol made from sucrose or starch containing feedstocks are considered the first generation of bioethanol and the technology to ferment them into ethanol is well known. As an example, whisky manufacturing has been known for hundreds of years and is made by distilling fermented hydrolysed starch from grains (Byrenius & Laurin, 2014). And bioethanol is fundamentally not any different from alcohol for consumption except that it has been distilled to a higher purity.

The most common feedstocks for bioethanol production today are hydrolysed corn starch in the USA (Gray et al., 2006) and sugar canes in Brazil (Balat et al., 2008). However, all sorts of carbohydrate-containing biomass could theoretically be used, as long as the glucans can be hydrolysed into fermentable sugars effectively at a competitive cost.

The second generation of bioethanol refers to lignocellulosic biomass as feedstock which is the most abundant carbohydrate source in the world. Lignocellulosic biomass is a mixture of cellulose, hemicellulose and lignin which composes the structure of the cell walls of almost all sorts of plant material like trees, bushes and grass (Jørgensen et al., 2007).

Cellulose is a very large linear β-1,4-glucan which could be hydrolysed into fermentable sugars by acids or enzymes but its hard structure and insolubility in water makes it resistant to

degradation. Hemicellulose is a highly branched polysaccharide of various size and monosaccharides, such as glucose, xylose, mannose and galactose to name a few. It is hydrogen bonded to cellulose and more easily hydrolysed than cellulose but in contrast does not contain as much readily fermentable sugars. Lignin is a complex structure of aromatic alcohols which act as a glue that holds cellulose and hemicellulose together (Jørgensen et al., 2007; Mosier et al., 2005).

Before hydrolysis can be performed, pretreatment steps must be performed to open up the compact structure of lignocellulosic biomass. Extensive research is put to this and the technology of producing bioethanol from lignocellulosic biomass is soon believed to be commercially possible. Still, remaining issues involves need for high enzyme concentrations, product inhibition, and lignin which both shields cellulose and forms bonds to the enzymes (Jørgensen et al., 2007). SP Processum in Sweden has a pilot plant, the Biorefinery Demo plant, which is producing bioethanol from wood industry residues (www.processum.se).

Algae is often referred to as the third generation of biofuels (Nigam & Singh, 2011). They do not compete with arable land; in fact there are many water environments where they could be cultivated.

2.2 Macroalgae (seaweeds)

Algae are simple organisms which grow by photosynthesis. They can be either uni- or multicellular and live in all kinds of environments where there is moisture. Thus, algae are found almost anywhere all over the world: in oceans, lakes and rivers of course, but also in soil or even inside plants and animals, living in symbiosis.

There are two types of algae: microalgae, such as phytoplankton, are very small and often unicellular while macroalgae are synonymous with seaweeds, such as kelps, with sizes varying from a few centimeters to a staggering 50-60 meters in length (Olsson, 2014).

Macroalgae contain lots of nutrients and are cultivated for use as food in Asia (El Gamal, 2012). Recent claims of beneficial health effects have also been made in popular media suggesting seaweeds-extracts could help reduce obesity or even cure chronic diseases. However, further research is still required to ascertain this (Brown et al., 2014). Macroalgae is divided into three main groups: red (Rhodophyta), green (Chlorophyta) and brown (Phaeophyta).

2.3 Ethanol production from the brown macroalgae*Saccharinalatissima*

Saccharina latissima is a brown macroalgae that grows in shallow waters and could reach a full length of ~4 meters (Adams et al., 2009). Dry content can vary between 8-25% and the largest fraction of it, except for ash, is mannitol and polysaccharides: primarily alginate and laminarin (Black, 1950). Alginate is a structural polysaccharide found in the cell walls and widely extracted and used for its gelling properties in food and cosmetics (Samaraweera et al., 2012), whereas laminarin is a storage glucan that could be hydrolysed to glucose and converted into bioethanol (Adams et al., 2009).

The main challenge in using a seaweed-feedstock for bioethanol production is finding and developing appropriate microorganisms that can metabolise the major part of the polysaccharide and mannitol content into ethanol from the fermentable sugars. Recent articles report achieving up to 80% of theoretical maximum conversion of the sugar content from

brown seaweeds into ethanol, by engineering the alginate and mannitol catabolic pathways (Enquist-Newman et al., 2014; Wargacki et al., 2012).

2.3.1 Laminarin

Laminarin, a storage β-1,3-glucan located inside the cells, is a linear polymer of ~20-30 glucose units with variable amounts of β-1,6 branching occurring. The main difference of laminarin as opposed to starch and cellulose is the β-1,3 bonding instead of α- or β-1,4 and the significantly smaller size which could theoretically simplify hydrolysis. The polymer's terminating ends can be either glucose- or mannitol-groups (Chizhov et al., 1998). This variable degree of branching and terminating groups separate laminarins into either soluble or insoluble in water (Adams et al., 2009). Laminarin content in *Saccharina latissima* samples varies with location and season and can be at most around 15-22% dry weight (Black, 1950).

2.3.2 Laminarinase

Laminarinases are enzymes that degrades laminarin, or more explicitly, endo-β-1,3-glucanases which hydrolyse laminarin by cleaving the polysaccharide chain randomly, creating both disaccharides and smaller glucan chains as products (Salyers et al., 1977).

In addition to endo-glucanases, there are exo-glucanases that removes single glucose units from one end of the chain. Glucosidases are one kind of exo-glucanases which specifically cleaves disaccharides. To hydrolyse laminarin effectively, a combination of endo- and exo-β-1,3-glucanases should be considered (Salyers et al., 1977; Alderkamp et al., 2007).

2.3.3 Fermentation of alga-derived glucose

A recent article (Adams et al., 2009), achieves up to 15% (g ethanol/g laminarin) conversion of milled samples of *Saccharina latissima* by *Saccharomyces cerevisiae* in combination with laminarinase. Another (Horn & Aasen, 2000) achieves up to 43% (g ethanol / g laminarin and mannitol) by fermenting with the yeast *Pichia angophorae*.

3. Methods

Wild *Saccharina latissima* samples were harvested at Tjärnö Marine Station, The Sven Lovén Centre for Marine Sciences, Strömstad on the 12th of November 2013, by researchers at the Department of Biological and Environmental Sciences, University of Gothenburg, and then ground, frozen and stored in -20°C. The algae were pretreated to extract laminarin and other water soluble compounds into an extract solution. Laminarin is the component of the macroalgae samples which was to be hydrolysed and fermented. However, the extraction step will also release other soluble components which were present during the remaining processes of hydrolysis and fermentation. Three different hydrolysis methods were investigated; one acidic and two enzymatic. The hydrolysates were fermented anaerobically in small scale with an industrial *Saccharomyces cerevisisae* strain, Ethanol Red®. After fermentation, initial, during and final ethanol and glucose concentrations were analysed with HPLC to measure differences between the methods of hydrolysis.

3.1 Extraction of laminarin from *S. latissima* samples

280.19 g of defrosted and ground(to about 1 mm size) samples of *Saccharina latissima* were put in a beaker. 450 ml of saturated calcium hydroxide solution (pH 12.3) was added and pH dropped to 10. About 1 g of pure calcium hydroxide was added making pH increase to 10.45. 4.7 ml of 5M sodium hydroxide solution was added raising the pH to 12. The solution was incubated at room temperature for 4 hours with stirring. Then the solution was titrated with 73.85 ml 1.85 M sulphuric acid until pH 2 under stirring. After a few minutes, pH was again raised to about pH 6.5 with 6.5 ml 5 M sodium hydroxide before filtering the solution through a sieve cloth and autoclaving the filtrate for 20 minutes in 121°C.

3.2 Hydrolysis of extract

The laminarin of the extract solution was hydrolysed into glucose using three different approaches: Sulphuric acid and heat; a commercial laminarinase; and an enzyme extract from an isolated strain of *Pseudoalteromonas sp.*

3.2.1 Sulphuric acid

1st experiment

Three 250ml blue cap bottles were filled with 40ml each of extract solution. 2.15 ml of 98% (v/v) sulphuric acid was added dropwise under stirring to give final 5% (v/v) sulphuric acid in each bottle. The bottles were autoclaved at 121°C for 1 hour and then cooled before stored in refrigerator until inoculation. Before inoculation the pH was adjusted to about pH 6.5 with 8.6 ml (10 M) and 0.12 ml (5 M) sodium hydroxide solution.

2nd experiment

The second try involved the same procedure except that since we speculated about the high salt concentrations being a possible inhibitor for the yeast, we decided to dilute the hydrolysate prior to fermentation.

80ml of the extract was autoclaved, after adding 4.3ml 98% (v/v) sulphuric acid to the extract to give final 5% (v/v) sulphuric acid. After 1 h autoclavation with subsequent cooling the hydrolysate was mixed with an equal amount of sterile water then titrated to pH 6 by adding 15.5 ml of 10 M and 0.12 ml of 5 M sodium hydroxide solution. This reduced the salt amount added during hydrolysis by half (and also reduced the laminarin in the hydrolysate by half) thus possibly making the environment more friendly for the yeast.

3.2.2 Laminarinase

200 U (5 mg) of "ThermoActive™ Laminarinase" (Prokazyme, Iceland) , in powder form, was ordered specifically for usage in the project. The enzyme is an endo-1,3(4)-β-glucanase obtained from *Rhodothermusmarinus*, a bacterium obtained from a marine alkaline hot spring in Iceland. In this article (Spilliaert, Hreggvidsson, Kristjansson, & Eggertsson, 1994) 83% of the laminarin in the assay is hydrolysed after 16 h in 80°C.

One unit (U) of enzyme is the amount that leads to the release of 1 µmol reducing sugars per minute. According to instructions from Prokazyme the entire amount was mixed with 0.5ml of 0.2M phosphate buffer (pH 7) and 0.5ml of glycerol making 1 ml of 200 U enzyme/ml and 0.1M phosphate that was stored in a vial in-20°C prior to use.

1st experiment

40 ml of seaweed extract was aliquoted in 50 ml falcon tubes, mixed with 1 U of laminarinase, in triplicates, and heated to 80°C for 2 hours with 1100 rpm shaking on a Thermomixer comfort (Eppendorf AG, Germany).

2nd experiment

Since no glucose at all was released during the first hydrolysis experiment with the laminarinase, a test was made to investigate the activity:

Three 1.5 ml eppendorf tubes were prepared with seaweed extract and different amounts of enzyme (1U, 2U and 5U), then incubated in 80°C at 1000 rpm on Thermomixer comfort. Glucose was then measured with glucose test strips (Medi-Test, MACHEREY-NAGEL GmbH & Co., Germany) after 2 h, with negative results. After 24h there was a very weak indication (~0.1% compared to an estimated ~2% maximum) of glucose in all three tubes. They were let to incubate for another 24h, but no more glucose seemed to be released.

3rd experiment

After correspondence with Prokazyme, total salt concentration in the extract was evaluated. It is hard to know exactly since the seaweeds contain salt from the ocean to start with but summing all the acids and bases added during the extraction it was estimated to 0.3-0.4 M which could act inhibitory on the enzyme. Therefore the 2nd experiment was repeated but with half the amount of extract and the rest distilled water:

Three 1.5 ml eppendorf tubes were prepared with 0.5 ml seaweed extract and 0.5 ml distilled water each. The same amount (1U, 2U and 5U) of laminarinase was dosed again and incubated as before. None of the tubes showed any glucose after 2 hours. After 24h Medi-Test strips indicated somewhere between 0.1-0.25% glucose and the same after 48h.

4th experiment

Due to lack of reasonable glucose production from the alga extract with the laminarinase, one final experiment was done:

Four 1.5 ml eppendorf tubes were prepared with 20 mg Laminarin from *Laminaria digitata* (Sigma-Aldrich Co. LLC) in 1 ml of distilled water. Then laminarinase was dosed 0.5 U, 1 U, 2 U and 5 U in each tube respectively and incubated as before for 24 h.

3.2.3 Enzyme extract from *Pseudoalteromonas* sp.

Joakim Olsson, a master student working on bioprospecting for laminarinases from marine bacteria, provided an enzyme mix consisting of conditioned growth media from a laminarin-

degrading strain of the marine bacterium *Pseudoalteromonas sp*. The enzyme activity of the mixture was not known. The mix was added to an equal volume of seaweed extract in three 100 ml shake flasks, making a total of 40 ml each. These were incubated at 30°C for 2 hours before inoculation of yeast.

3.3 Glucose detection

Prior to inoculating yeast into the hydrolysates two methods for detecting glucose were used, as described below.

3.3.1 ByMedi-Test Glucose

The acid hydrolysates were tested for glucose by adding a few drops of it to Medi-Test Glucose test strips (MACHEREY-NAGEL GmbH & Co., Germany) which indicate glucose by changing color.

3.3.2 By D-Glucose kit

After the difficultieswith the laminarinase hydrolysis, D-Glucose UV-method kit (BOEHRINGER MANNHEIM / R-BIOPHARM) was used to provide a more accurate glucose concentration measurement.

It works by measuring the optical density, as explained in section 3.4.4, of NADPH produced in the sample after adding excessive amounts of ATP, NADP, hexokinase, and glucose-6-phosphate dehydrogenase hereby degrading all the glucose in the sample according to the formulas below:

Hexokinase: D-Glucose + ATP → D-Glucose-6-phosphate (G-6-P) + ADP

G6PDH: G-6-P + $NADP^+$ → D-gluconate-6-phosphate + NADPH + H^+

All the glucose in the sample (if within the maximum limit) is thereby degraded and an equal amount of NADPH is created. It works by first adding ATP and NADP in both sample(s) and a blank sample (water) and measuring absorbance (A_1). Then enzymes are added and after 15 minutes the absorbance is measured again (A_2). Glucose concentration is then calculated with the formula below:

$$c = \frac{V * MW}{\varepsilon * d * v * 1000} * \Delta A \ [g/l]$$

where, V = final volume = 3.020 [ml]
v = sample volume = 0.1 [ml]
MW = molecular weight of glucose = 180.16 [g/mol]
d = light path = 1 [cm]
ε = extinction coefficient of NADPH at 340 nm = 6.3 [l * $mmol^{-1}$ * cm^{-1}]
$\Delta A = (A_2\text{-}A_1)_{sample} - (A_2\text{-}A_1)_{blank}$

$$c = \frac{5.441}{6.3} * \Delta A \ [g/l]$$

3.4 Fermentation of the hydrolysates

3.4.1 Equipment

Fermentation was performed in an incubator at 30°C with 180 rpm shaking in 100 ml shake flasks equipped with rubber stoppers with two drilled holes. The larger hole was for airlock/loops (filled with sterile glycerol). In the small hole a thin metal tube was inserted that reach the bottom of the shake flask, and in the other end (outside the flask) a cutoff needle with a syringe was mounted, see figure 3.4.1 below. This was to be able to take small samples throughout the fermentations without opening the flasks, thereby avoiding introduction of oxygen and/or contaminants to the fermentation.

Figure 3.4.1: 6 fermentors "in action" (Acid- & enzyme hydrolysates fermentations in triplicates) in the incubator.

3.4.2 Sterilisation

All equipment used in the fermentation was autoclaved prior to use. Both preparing the preculture and inoculation of it to the hydrolysates was performed inside LAF (laminar air flow) benches to prevent contaminants disturbing the fermentation.

3.4.3 Yeast and culturing media

Ethanol Red® yeast (Fermentis, France), pregrown on an agar plate in 30 °C until individual colonies were clearly seen by eye, was stored in refrigerator until inoculation. YPD media was prepared by mixing 10 g yeast extract (Difco™, Becton Dickinson and Company, France), 20 g peptone (Bacto™, Becton Dickinson and Company, France) and 20 g glucose (Merck, KGaA, Germany) in one liter of distilled water, then autoclaving. Yeast extract solution (100 g/l) was prepared by mixing 10 g yeast extract in 100 ml distilled water and autoclaving.

3.4.4 Optical density (OD)

Optical density is a measure of how much particles and/or color there are in a media. It is measured in a spectrophotometer, by measuring absorbance at a specific wavelength of samples in cuvettes and comparing it against the absorbance of a background/standard. When starting a fermentation and taking samples frequently for measuring of absorbance, a measure

of yeast growth can be detected as the increasing number of yeast cells make the absorbance of the culture to increase.

3.4.5 Preculture and inoculation

One day prior to inoculating yeast into the hydrolysates, one of the colonies was picked from the agar plate, with the Ethanol Red yeast, and mixed in ~100 ml of YPD solution in a sterile 250 ml shake flask which was incubated in 30°C with shaking overnight. Next morning, OD at 600 nm was measured in the preculture against pure YPD solution as background to calculate what volume to inoculate the hydrolysates to give them an OD_{600nm} of 0.5. Refer to the formulas below. In addition to adding preculture, 4 ml of the yeast extract solution was also added to every fermentation.

$$V_{preculture} * (OD)_{preculture} = (V_{hydrolysate} + V_{preculture}) * (OD)_{final} \longrightarrow$$

$$V_{preculture} = \frac{OD_{final}}{OD_{preculture} - OD_{final}} * V_{hydrolysate} \qquad \text{where,} \quad \left\{ \begin{array}{l} OD_{fermentation} = 0.5 \\ V_{hydrolysate} = 40\ ml \end{array} \right\}$$

3.4.6 Sampling

3.4.6.1 For monitoring OD

When hydrolysate, yeast and yeast extract were added and mixed, the first ~1 ml sample was taken immediately from it, then samples were taken continuously throughout the fermentation period, first every hour, then less frequently covering a time span of 3 days maximum. One part of the sample was filtered through a syringe filter w/ 0.2 µm nylon membrane (VWR International) and used as background. Then the unfiltered part was measured for OD against the background.

However, due to the high amount of particles in the hydrolysate, another approach was used when monitoring the hydrolysate from the enzyme extract from *Pseudoalteromonas sp.*: Before pitching the preculture, a small sample of the hydrolysate (including yeast extract addition) was stored and used as background when measuring the unfiltered samples taken throughout fermentation.

3.4.6.2 For HPLC analysis

As with the OD samples, one sample was taken immediately after pitching. Then samples were taken, first every 2 hours then in declining frequency, continuously throughout the fermentation until the last sample after 3-5 days (depending on "batch").

About 1.5 ml, taken with the syringe arrangement, was immediately filtered through a 0.2µm nylon membrane syringe filter into 1.5 ml eppendorf tube that was frozen instantly in liquid nitrogen preventing ethanol from evaporating. Then tubes were placed in freezer at -20°C until analysis. When the fermentations were all done and all samples gathered, all the tubes were analysed by HPLC to detect and monitor glucose, ethanol and mannitol concentrations.

3.4.7 HPLC analysis

HPLC analysis was performed with a Rezex ROA-Organic Acid H+ (8%) 300x7.8mm column with 80°C temperature. Two detectors were used: refractive index and UV (210 nm). The eluent was 5 mM sulphuric acid and eluent speed 0.8 ml/min.

4. Results

The results are divided into four different fermentations, all with different hydrolysates. Both OD and concentrations of glucose, mannitol and ethanol are shown in individual plots for all experiments.

Keep in mind when viewing the HPLC plots that according to the general formula at section 2.1, there are 2 molecules of ethanol and 2 of carbon dioxide per molecule of glucose which means the theoretical maximum concentration (w/v) of ethanol can be at most 51.19% of the initial glucose concentration (w/v).

4.1 Sulphuric acid hydrolysate

The frozen samples of *Saccharina latissima* were pretreated into an extract solution, then autoclaved after adding concentrated sulphuric acid to a final 5% (v/v) as explained in section 3.1 and 3.2.1. After raising the pH back to ~pH 6Medi-Test strips indicated about 0.5-2% glucose in the hydrolysate, and the hydrolysate was inoculated with yeast and yeast extract, in three replicates. The fermentors were put in incubation at 30°C with shaking for almost 4 days, although only the first 45 h are shown in figure 4.1 below.

The OD measurements were taken every hour at first, and HPLC samples every second hour the first day of fermentation. However, the OD measurements did not show very much due to high amount of particles from the beginning. Therefore it was believed that there was not any activity of the yeast and samples were only taken during the first day and then after two days.

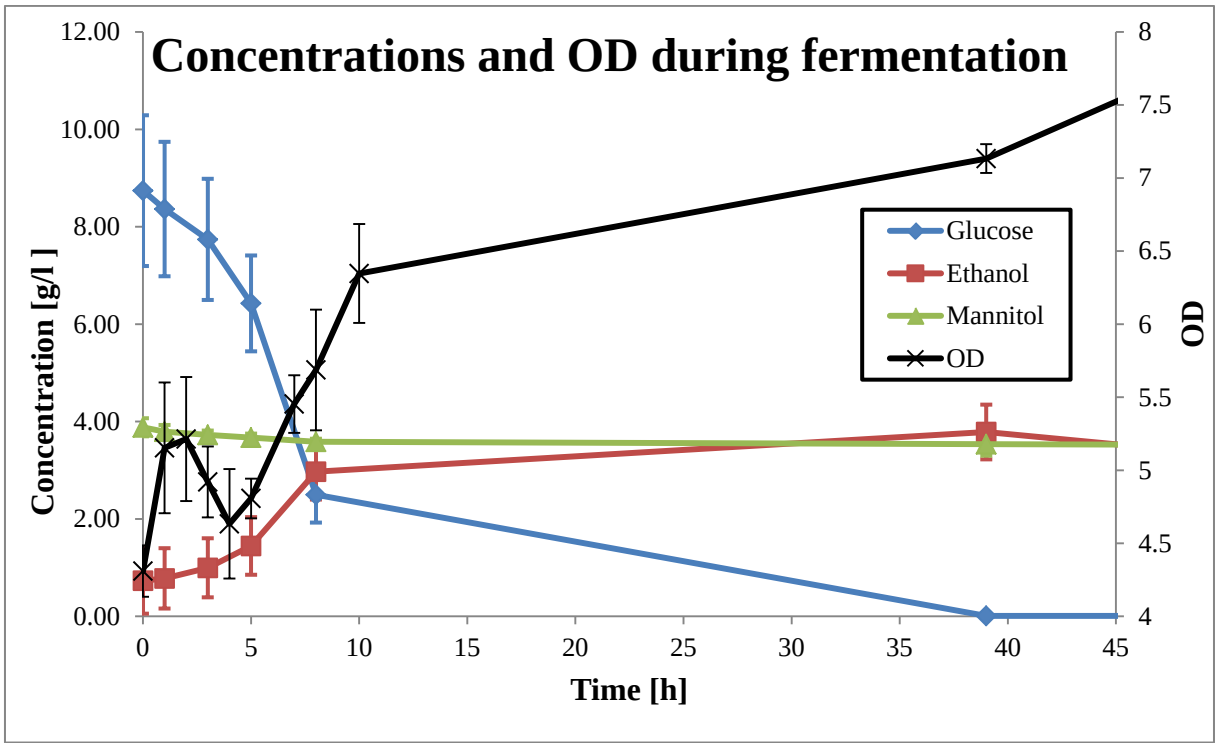


Figure 4.1: Concentrations from HPLC analysis on the left axis, which are averages from the three replicates with error bars indicating the standard deviation. OD is on the right axis and also averaged from the three replicates. Unluckily, there was not a sample taken at around time=10 where the OD plot peaked.

However, the HPLC results show that there was indeed fermentation activity going on and if samples had been continued to been taken at the same interval there had probably been a maximum in ethanol somewhere after 8-10 hours. When concentrations and OD are compared they correspond well: even though the OD has an odd peak at ~2.5 h, the maximum is at 10 h; at the same time glucose and ethanol concentrations reaches their minimum and maximum, respectively.

The maximum yield possible of ethanol from glucose (as according to the formula at section 2.1) is 0.51 g ethanol/g glucose and the rest is carbon dioxide. Dividing the maximum ethanol concentration by the initial glucose concentration gives the ethanol yield of the cultivation as 0.43 g/g which divided and multiplied by 100 gives 85% of maximum yield.

4.2 Sulphuric acid hydrolysate diluted with 50% (v/v) distilled water

This time the same procedure was done, except that half the volume was used. Then, after autoclaving with sulphuric acid, the hydrolysate was diluted with an equal volume of water.

The reason for this was that it was believed that the first fermentation had not worked. All HPLC measurements were performed at the same time: last week of the project, so before that one could only guess if glucose had been fermented into ethanol and the OD measurements gave reason for uncertainty.

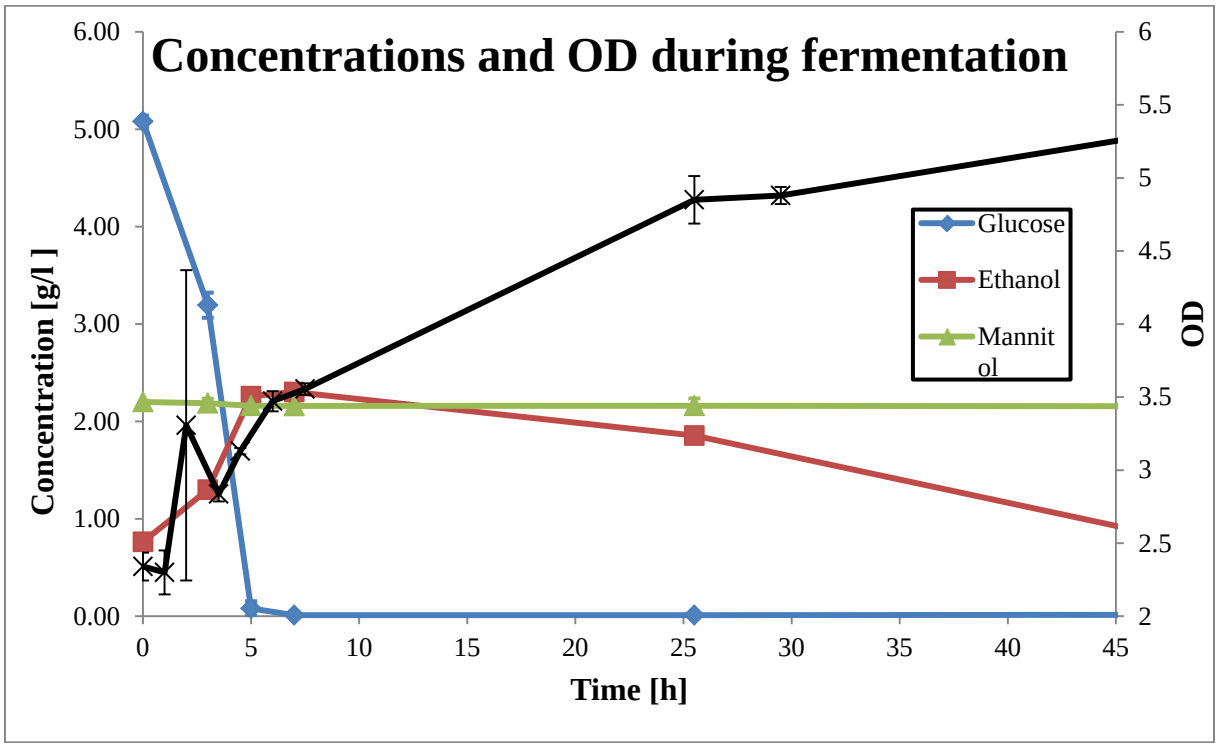


Figure 4.2: Concentrations from HPLC analysis on the left axis, which are averages from the three replicates with error bars indicating the standard deviation. OD is on the right axis and also averaged from the three replicates. Luckily, the ethanol peak and glucose depletion is captured at 7 h where OD is at its maximum.

Fortunately in these fermentations, the sampling intervals better captured the depletion of glucose (at 5-7 h) and the peak of ethanol better. Why the OD still increases after here is probably due to the yeast growing on the ethanol, which can be seen decreasing after the peak. This could happen if the fermentors are not 100% air tight, allowing for respiration of the ethanol.

The ethanol yield of this cultivation was calculated to be 0.45 g/g, which divided and multiplied by 100 gives 88% of maximum yield.

4.3 Laminarinase

1 U of laminarinase was added to the *Saccharina latissima* extract solution and was incubated for2 h as described in section 3.2.2. Then glucose content was measured with Medi-Test strips to be 0%, but since there was not enough time for a new hydrolysation experiment it was inoculated and fermented anyway, just in case the laminarinase would begin/continue to hydrolyse the laminarin during fermentation. The OD measurements show a slow but steady increase during the entire fermentation period. Consequently, HPLC samples were also taken continuously. However, it was later discovered the enzyme did not work, hence only the first and last sample were actually analysed by HPLC to save time.

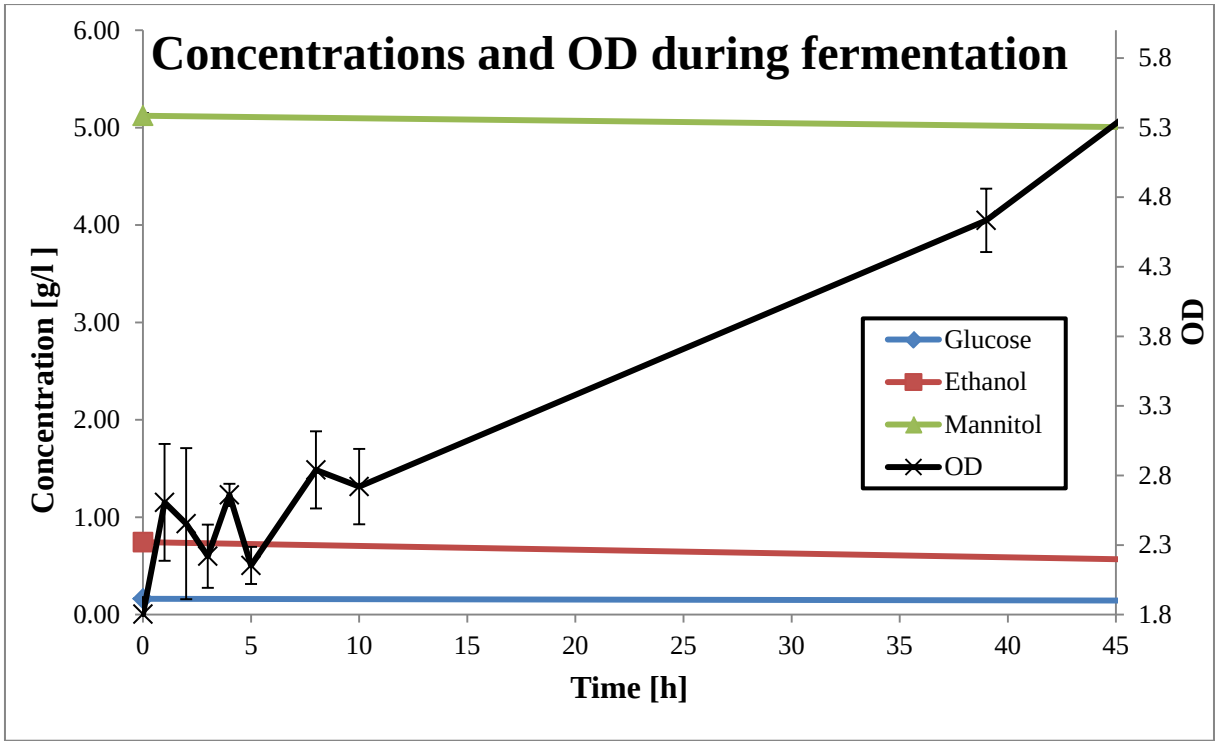


Figure 4.3: Concentrations from HPLC analysis on the left axis, which are averages from the three replicates. Only the first and last sample were analysed. OD was measured during the entire fermentation and is shown on the right axis

While fermenting, the glucose production in the control experiments (4th experiment) with the Prokazyme laminarinase was analysed by D-Glucose kit, as described in section 3.3.2, and is shown in table 4.3.1 below. The exact laminarin mass is shown plus 1 ml of water, as well as glucose concentration measured after 24 h in 80°C. The extent of hydrolysis is simply

glucose measured divided by laminarin weighted, since if hydrolysis were to be complete, the entire weight of laminarin should have been hydrolysed into glucose.

Table 4.3.1

	sample 1	sample 2	sample 3	sample 4
Laminarin [mg]	20,5	19,7	20,3	20,1
milliQwater [ml]	1	1	1	1
Glucosemeasured [mg/ml]	3,32	3,94	3,63	2,87
Extent of hydrolysis	**16%**	**20%**	**18%**	**14%**

4.4 *Pseudoalteromonas sp.*

As described in section 3.2.3: these fermentations were made with 50% (v/v) seaweed extract and 50% (v/v) uncharacterised extract of laminarin-degrading enzymes from *Pseudoalteromonas sp.* The enzymatic hydrolysis was performed for 2 h in the same environment as the fermentation, and then yeast was inoculated.

One day into fermentation, the pressure inside the fermentors was seen pushing the glycerol in the airlocks. This was not observed in any of the other fermentations, and a small sample was taken for differential interference contrast (DIC) microscopy. In the microscope, see figure 4.4.1 below, yeast cells were clearly seen budding/reproducing, as well as bacteria.

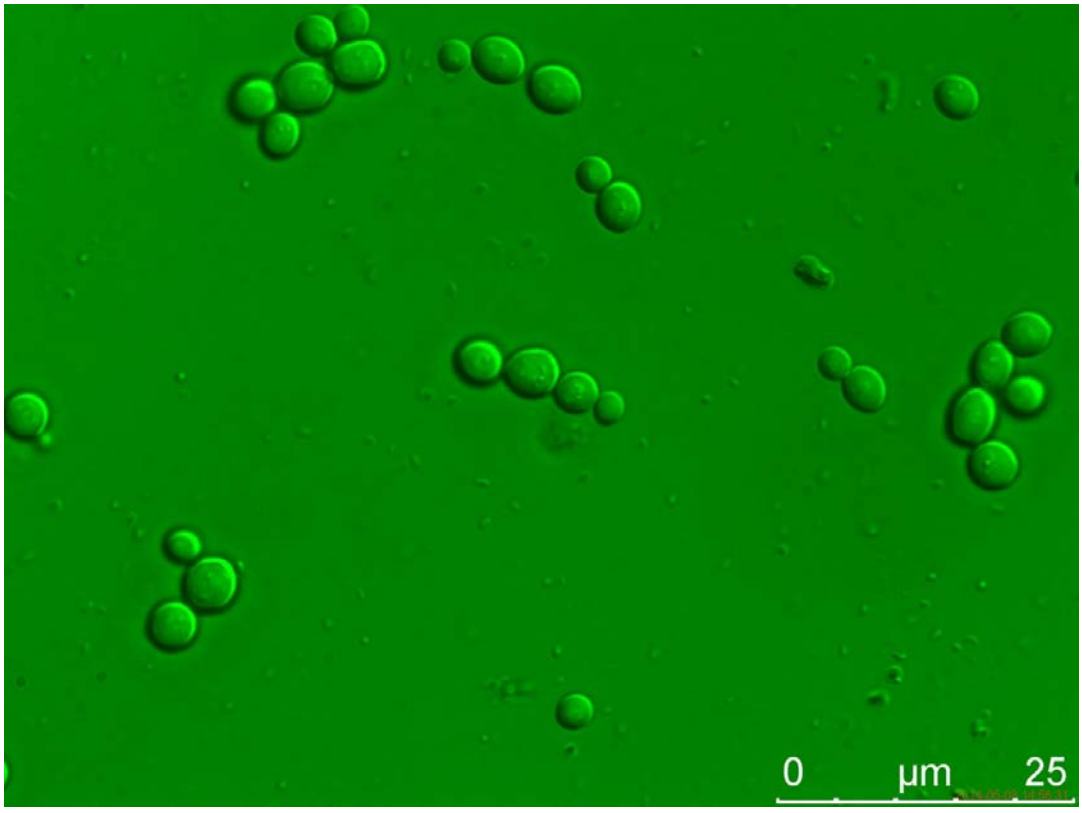


Fig 4.4.1: 1000x Differential interference contrast (DIC) microscope image from one of the fermentations with Pseudoalteromonas sp. hydrolysate. The yeast can be seen "budding"/reproducing. The smaller particles are bacteria from the Pseudoalteromonas sp. extract.

Note that the plot in figure 4.4.2 below has a much longer time scale than in the earlier plots. In the earlier plots the HPLC measurements after a given time are not shown since nothing interesting happened. However, here the last HPLC measurement shows the best achieved ethanol concentration of all the experiments made. In fact, if fermentation had gone on it looks like even more ethanol could be produced.

In contrast to the other experiments, where glucose is depleted at the same time ethanol peaks, here the glucose concentration is decreased fast but then stays at a low concentration while ethanol concentration continues to increase. The reason for this is probably due to the enzymes continuing to hydrolyse laminarin into glucose at the same time the yeast is metabolising it. This is a so-called simultaneous saccharification and fermentation (SSF) process (Poth et al., 2011).

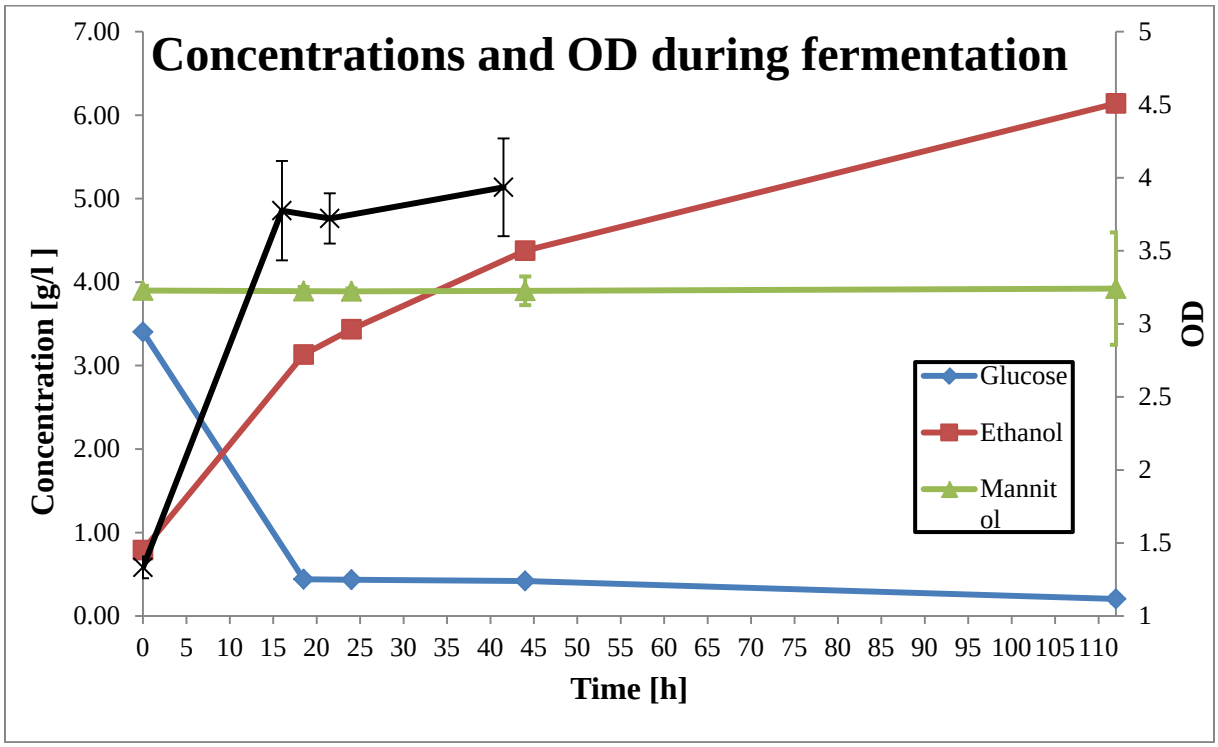


Fig 4.4.2: Concentrations from HPLC analysis on the left axis, which are averages from the three replicates with error bars indicating the standard deviation. OD is on the right axis and also averaged from the three replicates. Note that the time scale is longer in this figure since the ethanol still increases after 45 h. The OD measurements were performed with the same background the whole time, according to section 3.4.6.1.

5. Discussion

The aim of this study was to ferment sugars obtained from the seaweed *Saccharina latissima* into ethanol. Three hydrolysis methods were tested of which two worked: sulphuric acid and an enzyme extract from *Pseudoalteromonas sp.*

Two hydrolysates with sulphuric acid were fermented with the only difference that the second one was diluted with 50% (v/v) water. This was just in case of high salt concentrations being an inhibitor to the yeast. When they are compared, similar results were obtained; the concentrations in the diluted experiment of glucose, ethanol and mannitol are half of the concentrations in the undiluted experiment, as expected. In both experiments glucose concentrations are depleted at the same time ethanol concentrations peak. In the second (diluted) fermentation, samples were luckily taken at the crucial point were glucose is fully depleted and ethanol concentration peaks. If a sample had been taken after approximately 10 h in the first experiment ethanol concentration would probably have peaked somewhere over 4 g/l. New approaches, for trying to get more glucose, could be other concentrations of sulphuric acid, other acids, and other temperatures and autoclaving times.

Four experiments were made with the laminarinase from Prokazymeand in the last one it became evident that a maximum of 20% of the laminarin was degraded into glucose under the best conditions: pure laminarin from Sigma in distilled water plus laminarinase solved in phosphate buffer pH 7 was incubated in 80°C for 24 h. Thus, high salt concentrations as mentioned earlier were not the problem during the experiments. One theory could be that the enzyme only had endo-activity, which means it cleaves the polysaccharide randomly in the chain. The products from the endo-glucanase (laminarinase) may have been β-1,3-disaccharides that can be compared to cellobiose (β-1,4-disaccharide) which the yeast cannot cleave (Ha et al., 2011). A β-glucosidase or exo-glucanase in addition to the laminarinase may had helped driving the hydrolysis from laminarin to glucose completely.

The best results were obtained from the *Pseudoalteromonas sp.* hydrolysate, with a final ethanol concentration of around 6 g/l, but with indications that even higher ethanol concentrations could be obtained with a longer fermentation time. Plot 4.4.2 shows that glucose is decreasing to about 0.5 g/l within the first 15 h, then there is a low but only slowly decreasing glucose concentration throughout the whole fermentation. This, in combination with the fact that ethanol concentration continues to increase throughout the fermentation period, suggests that the laminarin is being hydrolysed into glucose continuously as the yeast is using it. This is typical for a so called simultaneous saccharification and fermentation process (SSF) (Ask et al., 2012). This process is advantageous since it minimises the risk of product inhibition on the enzymes. In the microscope image, substantial amounts of bacteria can be seen which is because the enzyme extract still contained the *Pseudoalteromonas sp.* bacteria. The presence of bacteria may have been favorable since the enzymes could have been bound to the cell walls as described in this article (Salyers et al., 1977). *Pseudoalteromonas sp.* are aerobic bacteria, which according to Joakim Olsson who cultured them also metabolises mannitol (Olsson, 2014). The concentration of mannitol was however constant throughout the fermentation (figure 4.4.2); thus the anaerobic environment during fermentation probably inhibited the bacteria. In fact, mannitol concentrations remained the same throughout all of the fermentations, as expected since the yeast (*Saccharomyces cerevisiae)* does not ferment mannitol (Adams et al., 2009).

The results show that *Saccharina latissima* should be considered as a potential substrate for bioethanol production. However, the content of hydrolysable polysaccharides in the macrolalgae is only a fraction of the content in for example wheat and corn (Adams et al., 2009) so for the process to be profitable the rest of the macroalgae has to be utilized as well. As mentioned in section 2.3 alginate and mannitol could be utilised as well, raising the amount of fermentable components a lot.

6. Conclusion

The results show that the extraction method used for the seaweed *Saccharina latissima* yielded polysaccharides which after hydrolysis could be fermented into ethanol. The hydrolysate from the *Pseudoalteromonas sp.* enzymes yielded more than three times the amount of ethanol compared to the sulphuric acid hydrolysate, indicating that a mixture of enzymes hydrolysing the extract simultaneous as the yeast metabolises it works better. The laminarinase from Prokazyme may only have endo-activity and should probably be tested in combination with exo-glucanases such as β-glucosidase to effectively hydrolyse the entire polysaccharide into glucose monomers.

The concentration of fermentable sugars in seaweeds is lower than in sugar canes or other land-based crops. However, alginate is already a valuable compound that is extracted from seaweeds, and if other compounds were to be extracted as well, laminarin could be considered fermenting into bioethanol as a by-process.

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