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Faculty of Chemistry, Biotechnology and Food Science (KBM)
Prof. Svein Jarle Horn

Production of DHA-rich oil by *Aurantiochytrium limacinum* on lignocellulosic sugars and crude glycerol

Marianne Klüver
Chemistry and Biotechnology

Abbreviations

PUFA	Polyunsaturated fatty acids
CDW	Cell dry weight
CSL	Corn Steep Liquor
EPAX	Glycerol/fat rest material from
BALI	Spruce sugars / spruce hydrolysate
NS	Na_2SO_4
SS	Sea salts
C_x	Gram carbon per gram biomass
C_S	Gram carbon per gram substrate
x	Biomass
s	Substrate: glucose/glycerol/spruce sugars (glucose + xylose)
$Y_{x/s}$	Biomass yield on substrate
μ_{max}	Maximum growth rate
HPLC	High Performance Liquid Chromatography

Abstract

The Omega-3 Polyunsaturated fatty acids (PUFAs) Docosahexaenoic (DHA, 22:6n-3) and Eicosapentaenoic (EPA, 20:5n-3) acid are essential nutrients for both fish and humans. These PUFAs cannot be synthesized in sufficient amounts by the beings themselves and must therefore be supplemented through the diet. The main sources of these PUFAs are fish oil and fishmeal, and aquaculture fish is both the biggest user and provider of fish oil for human consumption. However due to a stagnant supply from capture fisheries, a growing population and need for aquaculture volume the content of DHA and EPA have decreased drastically in fish feed. It is therefore required novel sources of these PUFAs. Production of microbial oils by fermentation of the oleaginous marine protists *Thratochcytrids* is a promising solution to this issue.

The *Thratochcytrids* strain *Aurantiochytrium limacinum* (*A. limacinum*) has shown to yield high titres lipids, and especially DHA. In addition, *A. limacinum* is able to grow on a variety of different substrates, in this study it was discovered that a medium consisting of the industrial by-products: spruce sugars (carbon source), and corn steep liquor (nitrogen source) generated decent biomass and lipid titres. There were conducted both batch fermentations (in shake flasks) and fed-batch fermentations (in bioreactors) of *A. limacinum* grown in this medium, where the shake flask cultivations lasted for 144 hours and generated an average biomass concentration of 26.5 g/L, where 51.4% of the biomass was lipids, and 36.1 % of the lipids where DHA. When the spruce sugar medium was used in bioreactors, it was generated a biomass concentration of 33.1 g/L, and a lipid content of 47.8%, with a DHA fraction of 33.1%. This result was achieved after 120 hours. It was concluded that this medium is promising for industrial fermentation of *A.limacinum,* and production of DHA, which in turn can be used in fish feed.

Sammendrag

Omega-3 fettsyrene Dokosaheksaensyre (DHA, 22:6n-3) og Eikosapentaensyre (EPA, 20:5n-3) er essensielle næringsstoffer for både fisk og mennesker. Disse produseres i små mengder i kroppen til mennesker og enkelte fiskearter, men ikke nok til selvforsyning. Disse fettsyrene må derfor tilføres via kosten. Hovedkilden til disse fettsyrene er fiskeolje og fiskekjøtt, der akvakultur er både den største forbrukeren og produsenten. Paradoksalt nok er det et økende problem at innholdet av DHA og EPA i akvakulturfisk har sunket drastisk på grunn av en stillestående tilførsel av fiskeolje fra fangstfiskerier, i tillegg til økende etterspørsel etter akvakultur fisk. Det kreves derfor nye kilder til EPA og DHA. Produksjon av mikrobielle oljer via fermentering av marine protister kalt *Thratochcytrids* er en lovende løsning på dette problemet. *Thratochcytrids* stammen *Aurantiochytrium limacinum (A. limacinum)* har vist seg å være en effektiv produsent av lipider, og da spesielt DHA. Denne protiststammen kan i tillegg vokse i medier med et bredt utvalg substrater.

I denne oppgaven ble det funnet ut at vekstmedie med lignocellulose hydrolysat av gran som karbonkilde, og «corn steep liquor» som nitrogenkilde genererte lovende biomasse og lipid konsentrasjoner. Det ble utført både batch fermenteringer (i ristekolber) og fed-batch fermenteringer (i bioreaktorer) *av A.limacinum*, med dette mediet. Det ga en gjennomsnittlig biomassekonsentrasjon på 26,5 g/L, hvor 51,4 % av biomassen var lipider, og 36,1 % av lipidene var DHA risteflaskeforsøkene (144 timer fermentering), og tilsvarende konsentrasjoner på 33,1 g/L biomasse, 47,8 % lipider, med en DHA-fraksjon på 33,1 %. I bioreaktor (120 timer kultivering). Det ble konkludert med at dette mediet er lovende for industriell fermentering av *A.limacinum*, og produksjon av DHA, som igjen kan brukes i fiskefôr.

Acknowledgements

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1. Introduction

1.1 Omega-3 Polyunsaturated fatty acids

Omega-3 Polyunsaturated fatty acids (omega-3 PUFAs) are essential supplements in a healthy human diet. They contribute to basic cell functions as membrane fluidity, cell metabolism and transport, and are important components of the brain and nervous system (Lopes Da Silva et al., 2019). Furthermore, the PUFAs Docosahexaenoic acid (DHA, 22:6n-3) together with Eicosapentaenoic acid (EPA, 20:5n-3) are crucial for the maintenance of good cardiovascular health (Guo, 2021 , Lopes Da Silva et al., 2019). Humans and some fish species are able to synthesize small amounts DHA of and EPA by themselves, however the quantities are insufficient and these PUFAs must therefore be supplemented by their diets(Tocher et al., 2019). It is therefore recommended by the European Food Safety Authority (EFSA) a daily intake of 250 mg of EPA and DHA for healthy adults and children. Today the main sources of DHA and EPA are fishmeal and fish oil (Tocher et al., 2019). However, the content of EPA and DHA in aquaculture fish, which supplies 50 % of the fish for human consumption, has decreased drastically (Guo, 2021, Sprague et al., 2016)

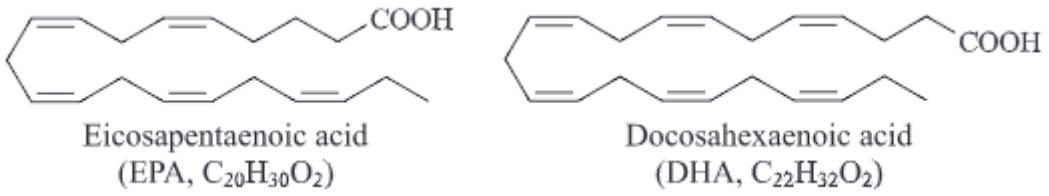

Figure 1.1: Shows the chemical structure of Omega 3 fatty acids Docosahexaenoic acid (DHA, 22:6n-3) and Eicosapentaenoic acid (EPA, 20:5n-3). The figure is obtained from (Yi et al., 2014).

Today about 75% of the fish oil that is obtained worldwide are implemented in aquaculture, where most of it goes into salmon feed and shrimp feed. Nevertheless, the content of fish oil and fishmeal in feed has decreased from approximately 90% in 1990 to only 24% in 2019. The reason for this is that the supply of fish oil and fishmeal from capture fisheries are rather stable, while the demand for aquaculture volume, along with a growing world population is increasing. Consequently, the content of omega-3 fatty acids in salmon from aquaculture has decreased with more than 54% during these years. The gap between the supply and demand of these omega-3 PUFAs requires novel sources of DHA and EPA (Guo, 2021)

1.2 Thraustochytrids

Microbial oils from *Thraustochytrids*, previously known as heterotrophic microalgae, is a promising novel source of omega-3 oils. *Thraustochytrids* are marine, fungus-like protist. Although they were referred to as microalgae, taxonomists hardly use this phrase because they have no plastids and are unable to perform photosynthesis. Due to their fast growth rate and high lipid accumulation potential, these protists have become increasingly popular for production of PUFAs like DHA, EPA and arachidonic acid (ARA), and are supplying food additives-, nutraceutical- and aquaculture

industries (Leyland et al., 2017, Du et al., 2021). Some of the conducted studies on lipid production by cultivation of *Thraustochytrids* strains have reported achieved lipid titres of more than 75% of the biomass. Most *Thraustochytrids* have a fatty acid composition rich in C14:0, C16:0, docoapentaenoic acid (DPA) and DHA, where the C16:0 and DHA content may account for 65% of the total fatty acid content (Du et al., 2021). Cultivations of the strain *Aurantiochytrium limacinum (A. limacinum)* has generated biomass concentrations up to 88.32 g/L and an impressing lipid content of 83.84% of the biomass, when cultivated on glucose followed by glycerol as carbon source (Li et al., 2015 p.57). In addition to producing PUFAs some *Thraustochytrids* strains also make pigments like squalene and catenoids. It has been found in concentrations as high as 30% of CDW in *Aurantiochytrium* (Du et al., 2021)

The reason for *Thraustochytrids* effective DHA production is their ability to produce PUFAs through the polyketide-like synthase pathway (PKS). This differs from many other oleaginous microorganisms that can only produce PUFAs through the other PUFA biosynthesis pathway: the elongase-desaturase pathway (Du et al., 2021). The PKS-pathway is oxygen independent (Napier, 2002), which can be advantageous in production processes where supply of sufficient oxygen for oleaginous microbes sometimes can be a challenge (Sirirak et al., 2021, Bailey et al., 2001). In addition the PKS pathway requires less NADPH than the the elongase-desaturase pathway which also makes it more effective (Du et al., 2021).

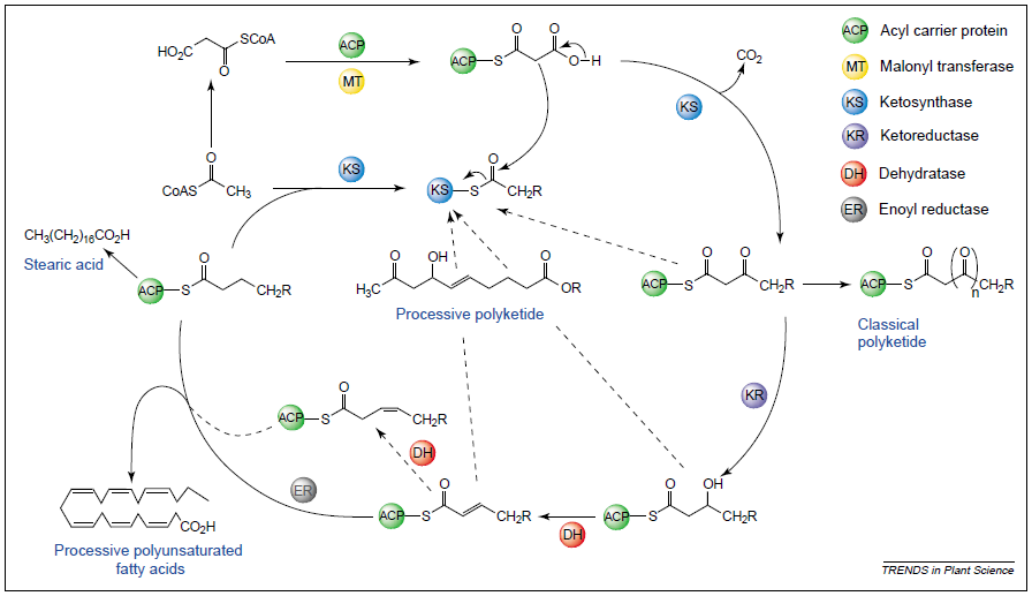

Figure 1.2: The picture shows the PKS-pathway, and is obtained from Napier et al.(2002)

1.3 Cultivation of thraustochytrids

PUFAs from thraustochytrids are produced during aerobic fermentation. In this kind of process, the microbe is typically cultivated in a bioreactor, which is a closed container that makes it possible to precisely control the cultivation environment (Stanbury et al., 2017a p. 402, Yatmaz and Turhan,

2019 p. 136). One of the most crucial functions of the bioreactor is to allow sterile operation of the culture. Thus, contamination by other microorganisms, and associated loss in productivity, is avoided (Vaghari et al., 2019 p. 86). Other important features are mechanisms to provide efficient aeration needed for cell metabolism, and the monitoring and controlling of environmental factors like temperature, pH, and dissolved oxygen level (Stanbury et al., 2017ap. 402)

In order to make Thraustochytrids produce lipids, stress must be inflicted on the cells. This can be done by cultivating the protists in media with excess of carbon source, and a limited amount of an essential nutrient such as phosphorus or nitrogen. When the essential nutrient which is needed for cell growth is depleted, the oleaginous microorganism will start to store the excess carbon source as lipids (Ratledge, 2013 p.535, Patel et al., 2020).

When selecting type the of bioreactor and fermentation procedure several factors must be taken into account, including the biological requirements of the producing organism, the production scale, and costs. The choice of substrate feeding strategy can be an effective tool for increasing productivity and yield (Yatmaz and Turhan, 2019 p. 151), which in turn may improve the production efficiency and therefore reduce the cost of the overall production (Stanbury et al., 1995 p. 331). Batch mode is a fermentation procedure where all nutrients are added to a closed system in finite amounts prior to inoculation (Stanbury et al., 2017b p. 21). The culture stops growing when the carbon source is depleted, and passes through several growth phases: Lag phase, exponential phase, stationary phase, and death phase (Yatmaz and Turhan, 2019). The lag phase which is the start phase where the producing organism adapts to the medium and does not grow much. This phase should be kept as short as possible in an industrial process. This can be a achieved by utilization of a suitable pre-culture (Stanbury et al., 2017b p. 21). Batch fermentation is the oldest and most common technique, due to its simple operating requirements. In addition, this strategy has a low risk of contamination (Yatmaz and Turhan, 2019 p. 151-152).

Continuous feeding of substrate is another fermentation strategy, in which the aim is to prolong the exponential phase of the batch culture (Yatmaz and Turhan, 2019 p. 160). In the exponential phase, the cell population grow in such a way that the increase in biomass is proportional to the biomass concentration, giving an exponential growth where the cells grow at their maximum rate (p. 33, Stanbury et al., 2017b) (Britannica). To achieve this, substrate must be in excess and added in the same rate that the substrate is consumed during the fermentation. If the growth of the producing organism is limited by only substrate, the culture can continue to grow until the vessel is full (Stanbury et al., 2017b p. 21-22, 33, Yatmaz and Turhan, 2019 p. 160). If liquid is removed from the bioreactor (e.g. via an overflow device) at the same rate as new medium is added a continuous fermentation system is established (a chemostat)(Stanbury et al., 2017b p.33).

Fed-batch mode is a mix of the mentioned feeding strategies, where the culture starts as a batch cultivation before new feed is added continuously or sequentially, without removal of fermentation liquid (Stanbury et al., 2017b p. 58). Fed batch mode is favourable when the growth of the producing organism is inhibited by high substrate concentrations, and the product or cell yield is higher at low substrate titres (Yatmaz and Turhan, 2019 p. 163).

The production of PUFAs through fermentation of *Thraustochytrids* can be an expensive process. It is therefore desirable to find solutions that will make the production cost as low as possible. Substrate expenses make up a fair share of the total production cost. Common medium components for

Thraustochytrids cultivation are based on artificial seawater glucose and some kind of nitrogen source, with glucose being the most expensive substrate. For instance, if *Schizochytrium sp.* is cultivated for 120 hours in a 25 L bioreactor the glucose prize makes up 73% of the total substrate cost. Exchanging the glucose with cheaper carbon sources like crude glycerol or lignocellulosic hydrolysate can therefore save a lot of money (Du et al., 2021).

1.4 industrial carbon sources

Lignocellulose is the most abundant of all renewable organic materials. After processing, sugars like glucose, arabinose and xylose can be derived from the biomass and be utilized as carbon sources by microorganisms. *Thraustochytrid*s are able to utilize both glucose and xylose as carbon sources, although xylose can only be exploited when glucose is present (Du et al., 2021). At the biorefinery Borregaard at Sarpsborg in Norway, spruce sugars are produced through the BALI^{TM} (Borregaard Advanced Lignin)-process, which is a concept presently under development (Bredal, 2013). The BALI^{TM} method is a four-step process, aimed to convert 80 % of the biomass into various competitive products. The biomass is first chemically pre-treated, which makes it possible to separate the components lignin, cellulose and hemicellulose and diminish the crystallinity of cellulose. After enzymatic hydrolysation of cellulose and hemicellulose, the spruce sugars are ready to be used as a substrate for fermentation (Rødsrud et al., 2012 p.52-54).

The use of crude glycerol as a carbon source in fermentation mediums for *A.limacinum*, has proven to give decent cell growth and high lipid yields (Liang et al., 2010, Patel et al., 2020) Glycerol is abundant in nature since it is a component in most lipids, due to this many microorganisms can utilize it as a source of carbon (Samul et al., 2014). When oils like vegetable oil or animal fats reacts with an alcohol by means of a catalyst, crude glycerol can be formed. This process also yields bioethanol (J. Van Gerpen et al., 2004), and crude glycerol is thus regarded as a by-product in bioethanol production. Crude glycerol differs from pure glycerol in the way that it contains variable impurities such as alcohol, inorganic salts, fatty acids, methyl esters, water and un unreacted mono-, di-and triacylglycerols (Samul et al., 2014). These impurities can be problematic in microbial fermentation, Liang et al. found that the cell growth of a *A.limacninum* were inhibited by methanol, but grew well on crude glycerol with a glycerol concentration of 25-35 g/L, yielding a lipid content of 73% when grown on the 35 g/L glycerol substrate.

Another strategy that can be used to make the production more economic, is co-production of PUFAs together with high value antioxidants like squalene. The main source of squalene is oil from shark liver or whale, which is not ecologically sustainable (Patel et al., 2020 p.2). Like mentioned earlier Squalene is generated in high titres in *Aurantiochytrium limacinum*, making it a promising source. Downstream processing is also a big part of the production expenses that needs to be accounted for. This production step makes up 20-60% of the overall costs, and different methods should be considered (Du et al., 2021)

The aim of this study was to optimize medium- and fermentation conditions for production of DHA by *A. limacinum* from glucose and glycerol. Furthermore, the feasibly of using crude industrial substrates for DHA production was investigated.

2. Materials and methods

2.1 Materials

In this study various growth medium components were tested for growth of *A. limacinum* in batch and fed-batch fermentation mode. This included spruce sugars produced with the BALI^{TM} method from Borregaard and a glycerol/fat rest material from fish industry, provided by Pelagia, (named EPAX in this study). Furthermore, corn steep liquor (CSL) which is a by-product from corn wet-milling industry, was tested as nitrogen source. This product does also contain a diversity of different vitamins, trace elements and minerals (Merck KGaA, 2021, Roquette). The rest of the medium ingredients are listed in the table below.

Table 2.1: Medium components used for fermentations and their suppliers.

Medium components	Supplier
Spruce sugars (BALI)	Borregaard AS (Sarpsborg, Norway)
Glycerol-fat rest material (EPAX)	Pelagia^{TM}, Epax Norway AS (Ålesund, Norway)
D-(+)-Glucose	Sigma-Aldrich (Missouri, USA)
Glycerol	Sigma-Aldrich (Missouri, USA)
Bacto^{TM}Peptone	Becton, Dickinson and Company (New Jersey, USA)
Yeast extract granulated	Merck Millipore (Massachusetts, USA)
Sea salts	Sigma-Aldrich (Missouri, USA)
Na_2SO_4	Sigma-Aldrich (Missouri, USA)
Corn Steep Liquor (CSL)	Sigma-Aldrich (Missouri, USA)

2.2 Protist strain and preparation of live cultures

The thraustochytrid strain *Aurantiochytrium limacinum (A. limacinum)* ATCC-MYA 1381 was stored in 2 mL cryovials with 20% glycerol (v/v) at -80 degrees Celsius. After thawing, the strain was revived in ATCC By^{+} medium, containing 35 g/L sea salts, 1 g/L yeast extract, 1 g/L peptone and 5 g/L glucose. This was prepared in PS tubes using 4.5 mL By^{+} medium and 0.5 mL thawed cell suspension.

2.3 Preparation of seed culture

Inoculum shake flasks was prepared 1,5 - 2 days before every shake flask or bioreactor experiment. The seed cultures for the shake flasks experiments were prepared in 250 mL baffled shake flasks, using 45 mL of medium and inoculated with 5 mL 3-7 days old *A.limacinum* culture (stored at 21 C). For the bioreactor fermentations, the seed culture was prepared in 500 mL baffled shake flasks, using 95 mL medium and 5 mL of inoculum culture. The seed cultures were prepared in a shaker (Infors HT Multitron Standard / Infors HT Ecotron, Bottmingen, Switzerland) at 25°C and 170 rpm.

Medium for seed culture:

Yeast extract: 5 g/L

Peptone: 5 g/L

Sea salts: 17.5 g/L

Glucose or Glycerol: 30 g/L

2.4 Shake flask experiments

Batch shake flask experiments were performed for medium screening, as well as for growth and lipid accumulation trials. The concentrations of the different medium components are shown in Table 2.4. All media types were tested in either triplicates or duplicates. 250 mL baffled Shake flasks with a working volume of 50 mL were inoculated with 10% seed culture.

Table 2.4 Medium components used in the individual experiments and their concentration in different shake flasks. There were only used on type of carbon source in each shake flask. Yeast and peptone were added as a mixture, making the total concentration of nitrogen source 10 g/L. Sea salts and Na_2SO_4 were added to separate shake flasks and never mixed. Finally, 0.3 mg/L streptomycin and 0.3 mg/L Penicillin G were added to all shake flasks as a general precaution against bacterial contamination. For experiment 2 and 3 there was tested a growth medium without any salts. In the lipid accumulation trial, there was used sea salts and no Na_2SO_4 together with glucose and glycerol. Furthermore, all shake flasks were added KH_2PO_4 solution and vitamins (1mL/L) plus trace elements (5 mL/L). Spruce sugars were tested with both sea salts, Na_2SO_4, and neither.

Substrates	Exp.1 (g/L)	Exp.2 (g/L)	Exp.3 (g/L)	Exp.4** (g/L)
Yeast extract	5			
Peptone	5			
Corn steep liquor (CSL)		10	10	10
Sea salts	35	0/17.5/35	0/17.5/35	17.5
Na_2SO_4		0/17.5/35	0/17.5/35	17.5
KH_2PO_4*				4
Glucose	30			90
Spruce sugars (BALI)	30	30		90
Glyserol	30			90
EPAX	30		30	90

*This solution did also contain 3 g/L $MgSO_4$ and 0,3 g/L $CaCl_2$

** lipid accumulation trial

Before media preparation all components except yeast and peptone were autoclaved separately, at 120°C and 1 bar. Mixing of medium and inoculation were done in a sterile bench, making 45 mL medium, then adding 5 mL seed culture. pH was measured and adjusted to ~pH 7.0 before inoculation, in all shake flask experiments except the first.

The shake flasks were incubated at 25 °C and 170 rpm (Infors HT Multitron Standard / Infors HT Ecotron, Bottmingen, Switzerland) for 3-6 days (growth experiments for 3 days, and lipid accumulation experiment for up to 6 days). Samples for analytics were collected every 24 hours. OD measurements at 680 nm were performed to monitor cell growth and GOPOD or Glycerol assays were utilized to monitor the carbon source level.

2.5 Bioreactor experiment

2.5.1 Bioreactor set-up

The bioreactor fermentations were carried out in 1.5 L glass fermentors (Minifors 2, Infors, Bottmingen, Switzerland) containing two 6 bladed Rushton impellers. The distance between the impellers were adjusted to be 1.25 times the impeller diameter (from one blade tip to the opposite). Prior to inoculation the bioreactors were autoclaved with the media inside for 15 minutes at 121°C at 1 bar. Glucose and BALI were autoclaved separately, and trace elements and vitamins added aseptically after autoclaving. The medium set-up for the bioreactor runs is shown in Table 2.5.2.a and 2.5.2.b below. The fermentations were carried out at 25°C and pH 7.0.

The pH was monitored using a pH probe and adjusted automatically with 2 M H_2SO_4 and 5 M NaOH. A foam sensor was used to monitor the foam level, and 10 times diluted Clerol antifoam (PMC Ouvrie, Carvin, France) were added with a pump whenever the foaming got too extensive. The airflow was set to 0.5 VVM (air volume/reactor volume/minute), and the stirrer speed of the impeller was set to maintain a dissolved oxygen level of 20 %. This was monitored by a pO_2 probe, while the minimum and maximum stirrer speed was set to 300 and 650 rpm respectively and automatically adjusted during experiments. All bioreactor data was recorded using a computer and a customized software (Eve, Infors, Bottmingen, Switzerland).

2.5.2 Bioreactor runs

The bioreactor runs were carried out as fed batch fermentations, running for 120 hours with a start volume of 750 mL. Samples of the fermentation broth were taken every 12 hours. The glucose concentrations in the cultivation broth were monitored during the fermentation with a glucose assay (see chapter 2.7.6.a for detailed instructions). For the first fermentations, pulse feeding was applied, using syringes to add glucose and spruce sugars aseptically, while continuous feed was used for the second run. The continuous feed was performed by a pump, which had been calibrated based on testing of pump capacity (grams of test material pumped per minute, corresponding to percentage pump speed). In the last bioreactor run there were also taken additional samples with an Seg-Mod autosampler from Flownamics (Madison, Wisconsin, USA), coupled with Seg-Flow 4800® Automated On-Line Sampling System (Madison, Wisconsin, USA). A dip probe was utilized, and the samples were stored at 4 °C and collected every 24 hours. This samples were only used for HPLC-analysis, hence there was taken samples every 6 hours for HPLC analysis and every 12 hours for cell dry weight, and additional analytics (see chapter 2.7).

Table 2.5.2.a: The medium components in each fermenter for the first bioreactor run, and their concentration (g/L) in the medium after inoculation. There were also added vitamins (1mL/L), trace elements (5 mL/L) and antibiotics (0.3 mg/L). The composition of the vitamin solution and trace element mix are listed in Appendix A.

Substrates	Bioreactor 1 (g/L)	Bioreactor 2 (g/L)	Bioreactor 3 (g/L)	Bioreactor 4 (g/L)
Glucose	90	90		
BALI			90	90
CSL			20	20
Yeast extract + Peptone	20	20		
KH_2PO_4	4	4	4	4
$MgSO_4$	2	2	2	2
$CaCl_2*2H_20$	0.2	0.2	0.2	0.2
$NaSO_4$			17.5	35
Sea salts	17.5	17.5		

Table 2.5.2.b: The substrates used in each fermenter in the second bioreactor run, plus their final concentrations (g/L) in the medium after inoculation. There were added vitamins, trace elements and antibiotics in the same concentrations as in the first bioreactor run.

Substrates	Bioreactor 1 (g/L)	Bioreactor 2 (g/L)	Bioreactor 3 (g/L)	Bioreactor 4 (g/L)
Glucose	90	90		
Spruce sugars			90	90
CSL			20	20
Yeast extract + Peptone	20	20		
KH_2PO_4	4	4	4	4
$MgSO_4$	2	2	2	2
$CaCl_2*2H_20$	0.2	0.2	0.2	0.2
Sea salts	17.5	17.5		

2.6 Downstream processing

At the end of the bioreactor run approximately 750 mL fermentation broth was collected and centrifuged at 4° C and 8000 g for 30 minutes. The pellet was washed with 0.9 % NaCl solution. The wet biomass was packed in vacuum plastic bags, before air was removed in a Henkelman 300 Vac Packer (Hertogenbosh, The Netherlands), then the plastic bags were stored at -80°C for further analytics.

2.7 Analytics

2.7.1 Microscopy

1 ml of selcted samples from the bioreactors were collected for bright-field (BF) microscopy, and nile red microscopy.

2.7.2 Cell dry weight (CDW) from shake flask samples

Prior to collecting samples, 2 mL eppendorf tubes were weighed. 1 mL broth from the shake flasks were transferred to the pre weighed eppendorf tubes in sterile bench, and then weighed a second time with the broth inside. The samples were centrifuged at 8000 g for 5 minutes. The supernatant was separated from the pellet and transferred to new eppendorf tubes. The pellet was washed with 0,9 g/L saline solution and then centrifuged again. The supernatant was disposed, and pellet was stored in a Freezer at -20° C. Later the samples were freeze dried in a Heto Drywinner model DW 6-85 (Sigma-Aldrich, Missouri, USA) and then the tubes were weighed again. The weights were used to calculate CDW.

2.7.3 CDW from bioreactor samples

The bioreactor samples were transferred to pre weighed 15 mL falcon tubes, before weighing the tubes with broth inside. Subsequently the tubes were centrifuged at 4°C, and 4700 rpm for 10 minutes with VWR, Mega Star 1.6R (Radnor, Pennsylvania, USA). 1 mL of supernatant was collected and transferred to eppendorf tubes. The pellet was washed with 0.9 % saline solution, and then stored at -20°C, before freeze-drying.

2.7.5 Lipid extraction and GC-FID

2.7.5.a Lipid extraction

Lipid extraction was performed according to (Dzurendova et al., 2021) with some modifications as described in this section. A 2-milliliter screw-cap polypropylene tube was filled with approx. 15 mg freeze-dried biomass, approximately 150 mg (710–1180µm diameter) acid-washed glass beads. Then, 0.50-1.00 mg of glyceryl tritridecanoate (C13:0 triacylglycerol- TAG + C:23 trichloroacetate acid) internal standard followed by 500 µL of chloroform was added to the polypropylene tube. The biomass was homogenized in a Percellys Evolution tissue homogenizer at 5500 rpm for 6×20-s cycles. The processed biomass was transferred into a glass reaction tube by washing the polypropylene tube with 2400µL of methanol–chloroform–hydrochloric acid solvent mixture (7.6:1:1v/v) (3×800µL). Finally, 500µL of methanol was added into a glass reaction tube. The reaction mixture was incubated at 90°C for 90 min in a heating block, followed by cooling to room temperature. Then, 1 mL of distilled water was added to the glass reaction tube. Fatty acid methyl esters (FAMEs) were extracted by the addition of 2 mL hexane followed by 10 s vortex mixing. The reaction tube was centrifuged at 3000 rpm for 5 min at 4°C, and the upper (organic) phase was collected in a glass tube. The lower

(aqueous) phase was extracted twice more, this time by the addition of 2 mL hexane–chloroform mixture (4:1v/v). The solvent in the glass tube was evaporated under nitrogen at 30°C, and a small amount of anhydrous sodium sulphate (approx. 5 mg) was added in the glass tube. FAMEs were transferred into a GC vial by washing the glass tube with 1500µL hexane (2×750µL) containing 0.01% butylated hydroxytoluene (BHT, Sigma-Aldrich, St. Louis, MO, USA) followed by 5 s vortex mixing. Lipid contents and fatty acid profiles were analysed using a gas chromatography 7820A System (Agilent Technologies, Santa Clara, CA, USA) as described below.

2.7.5.b GC-FID

The GC-FID analysis was caried out as stated in (Langseter et al., 2021). Determination of total lipid content (expressed as the wt% of total fatty acid methyl esters (FAMEs) of sample dry weight) and fatty acid composition (expressed as wt% of individual FAME of total FAMEs) were performed by using gas chromatography 7820A System (Agilent Technologies, USA), equipped with an Agilent J&W 121–2323 DB-23 column, 20m × 180 µm × 0.20 µm and a flame ionization detector (FID). Helium was used as a carrier gas. The total runtime for one sample was 36 min with the following oven temperature increase: initial temperature 70 °C for 2 min, after 8 min to 150 °C with no hold time, 230 °C in 16 min with 5 min hold time, and 245 °C in 1 min with 4 min hold time. The injector temperature was 250 °C and 1 µl of a sample was injected (30:1 split ratio, with split flow 30 ml/min). For the identification and quantification of fatty acids, the Supelco 37 Component FAME Mix (C4–C24 FAME mixture, Sigma-Aldrich, USA) was used as an external standard, in addition to C13:0 TAG and C:23 internal standards. Measurements were controlled by the Agilent Open LAB software (Agilent Technologies, USA).

2.7.6 GOPOD and Glycerol assay

To monitor the carbon source concentrations Glucose Oxidase PerOxiDase (GOPOD) (Megazyme, Bray Ireland) and GK Glycerol (Megazyme, Bray Ireland) enzymatic colorimetric assays were applied to dilutions of supernatants collected after sample centrifugation. GOPOD assay was used for the samples that contained glucose or spruce sugars and the glycerol assay was used for the samples containing glycerol or glycerol-fat rest material.

Initially there was made a calibration curve for the GOPOD assay using known concentrations of glucose ranging from 0 - 1.5 g/L glucose. There was also made a calibration curve for the glycerol assay using known concentrations of glycerol ranging from 0 – 0.2 g/L.

2.7.6.a GOPOD assay

The supernatant from the samples were diluted 100 times (or 10 times when the glucose level was expected to be low) with distilled water to a concentration of 1.5 g/L at the most. A 96 well titre plate was utilized, and each sample was analysed in duplicates. There was added 190 µl of GOPOD reagent to each well, before pipetting 10 µl sample dilution and mixing with pipette. A plate cover

was applied, and the plate was incubated at 50°C for 20 minutes at 700 rpm in an Eppendorf thermomixer C (Eppendorf, Hamburg, Germany) The plate cover was removed and bubbles if any, were eliminated. Absorbance was measured at 510 nm in a Synergy H4 plate reader (BioTek, Winooski, Vermont, USA). The average absorbance of two replicates were used for calculation of the glucose concentration in each sample, using the calibration curve.

2.7.6.b Glycerol assay

The samples were diluted 500 times with distilled water, to a concentration of 0.2 g/L at most. Then there was added 150 µl distilled water to the wells of a 96-well titre plate, before mixing with 10 µl of sample dilution. Each sample were analysed in duplicates. Subsequently 20 µl from Bottle 1 (Buffer pH 7.4) was added to and mixed with the content in each well. Afterwards 20 µl from Bottle 2 (NAD+/ATP/Glucose) was added, and then 2 µl from Bottle 3 (ADP-GK/G6P-DH). The plate was covered and incubated at room temperature for 4 minutes with a shaking speed of 500 rpm in an Eppendorf thermomixer C (Eppendorf, Hamburg, Germany). There were checked for bubbles afterwards, which had to be removed before measuring the absorbance. Absobance 1 was measured at 340 nm using a Synergy H4 plate reader (BioTek, Winooski, Vermont, USA). Following this 2 µl of Bottle 4 (Glycerokinase) was mixed in all wells, and the plate was covered and incubated at 37° C for 10 minutes at 500 rpm in the Eppendorf Thermomixer C. Plate cover was removed and absorbance 2 was measured at 340 nm with the Synergy H4 plate reader (BioTek, Winooski, Vermont, USA). The total absorbance of each sample was calculated subtracting A1 from A2 and calculating the average value of the two replicates. Concentrations were found using the absorbance values and the respective calibration curve.

2.7.7 Willis Ammonia assay

Table 2.7: Chemicals used for preparation of Willis Ammonia assay, and their suppliers.

Chemicals	Supplier
Sodium salicylate	Sigma-Aldrich (Missouri, USA)
Trisodium phosphate (TSP, Na_3PO_4, $12H_2O$)	Sigma-Aldrich (Missouri, USA)
Sodium Nitroprusside (Sodium nitroferricyanide)	Sigma-Aldrich (Missouri, USA)
Chlorix (5-5.25% sodium hypochlorite)	Supermarket

Willis's method was performed to determine the ammonium concentration in the samples. This analysis gave an indication of when the nitrogen source in the culture was depleted, by testing supernatant from different timepoints. At first calibration curves were made for ammonium, yeast, peptone, mixture of yeast and peptone, and CSL. The known concentrations that were utilized ranged from 0 – 100 mg/L for ammonium, and 0 – 5 g/L for the rest.

A reagent consisting of 32 g/L sodium salicylate, 40 g/L TSP, and 0.5 g/L sodium nitroprusside was mixed in advance. For the analysis a 96 well titre plate was utilized. The supernatant was diluted, so the maximum concentration of ammonium would not exceed 20 mg/L. 10 µL of diluted supernatant was added to each well, then 200 µL of reagent, added and mixed using a multichannel pipette. Afterwards 50 µL of hypochlorite was mixed with the samples. The time between addition of reagent and hypochlorite should be minimized. The plate was Incubated at room temperature for 12 minutes with an Eppendorf thermomixer C (Eppendorf, Hamburg, Germany) and then the absorbance was measured at 685 nm using a Synergy H4 plate reader (BioTek, Winooski, Vermont, USA).

Remarks: due to the risk of HCN gas formation sodium nitroprusside should never be acidified. Cyanide is a toxic compound, and the waste should be disposed properly.

2.7.8 HPLC

The supernatant collected after centrifugation of the samples were analysed for glucose, glycerol and xylose using High Performance Liquid Chromatography (HPLC). There were made standard solutions for these substances with concentrations ranging from 0.5 – 10 g/L. These solutions were used for calibration. The samples were diluted 10 times with distilled water, using 20 µL sample and 180 µL distilled water, on a filtered 96-well microtiter plate (0,45 µL). Afterwards they were vacuum filtered and permeates were analysed with a Rezex ROA-organic acid H+, 300 x 7.8 mm2 analytical column (Phenomenex, Torrance, CA, USA) connected to a cation-H cartridge guard column. The column temperature was set to 65 °C and 5 mM H_2SO_4 was used as mobile phase with a flow rate of 0.600 mL/min. It should be noted that xylose will co-elute with mannose in this HPLC setup, but in this study these sugars are quantified as xylose only.

2.8 Equations

The following equations were applied for calculations of the maximum growth rate (C.1), and biomass on carbon source yield (C.2):

$$\mu = \frac{\ln(\frac{C_x}{C_0})}{t - t_0} \qquad \text{C.1}$$

$$Y_{x/s} = \frac{\Delta C_x}{\Delta C_s} \qquad \text{C.2}$$

The substrate concentrations were found by HPLC analysis of samples. The different substrate used for yield calculations were glucose, glycerol (for EPAX and glycerol medium) or glucose and xylose for the spruce sugar medium.

3. Results

3.1 Batch fermentations in shake flasks

3.1.1 Carbon source screening

The media contained 30 g/L of carbon source, 5 g/L of yeast extract, 5 g/L of peptone and 35 g/L of sea salts (SS). The screening was performed in triplicates.

Table 3.1.1: CDW (g/L) after 24, 48 and 72 hours of batch fermentation, for shake flasks with a medium consisting of yeast extract, peptone, sea salts and different carbon sources (glucose, glycerol, spruce sugars, or EPAX. The CDW was corrected by subtraction of start biomass concentration (CDW at zero hours). In addition, the cell yields (g_x/g_s) are shown. Yields were calculated based on increase in CDW and depletion of glucose/glycerol. When calculating the yield for the spruce sugar-medium both glucose and xylose consumption were included, while for EPAX the glycerol consumption was used.

	Carbon source	Time (h)	CDW (g/L)	$Y_{x/s}$ (g_x/g_s)
1	Glucose	24	7.4 ± 0.1	0.31
		48	12.1 ± 0.4	
		72	11.7 ± 0.4	
2	Glycerol	24	6.5 ± 0.7	0.33
		48	11.4 ± 0.3	
		72	11.3 ± 0.2	
3	Spruce sugars	24	6.5 ± 0.3	0.40
		48	14.4 ± 1.1	
		72	11.2 ± 1.0	
4	EPAX	24	5.9 ± 0.5	0.43
		48	12.2 ± 0.5	
		72	13.0 ± 0.8	

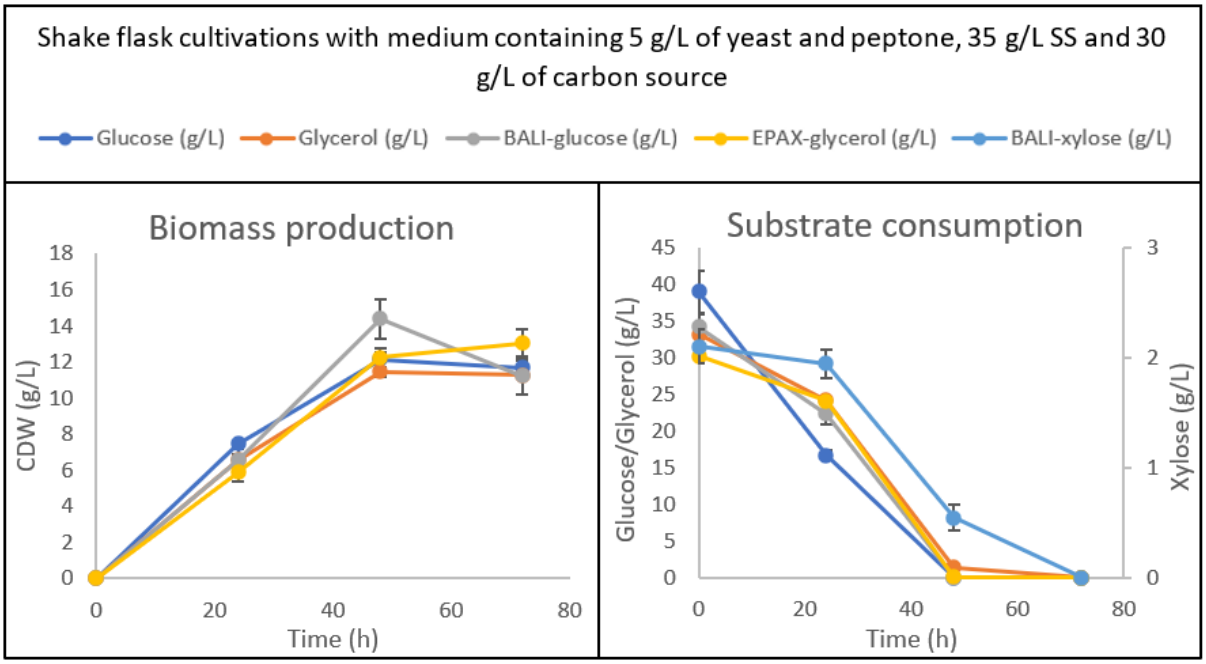

Figure 3.1.1: Biomass production in CDW (g/L) for media containing different carbon sources (Left panel) and the corresponding consumption of glucose, glycerol and xylose (g/L; Right panel).

In general, the cell growth was quite similar for all medium types (**Figure 3.1.1**). *A. limacinum* seemed to grow fastest in the glucose medium the first 24 hours, corresponding also to a fast glucose consumption. Between 24 hours and 48 hours, the growth was highest in the spruce sugar medium, resulting in the highest final CDW value of this experiment with a concentration of 14.4 g/L of biomass after 48 hours. The CDW graphs indicates that *A. limacinum* stopped growing somewhere between 48 and 72 hours in all medium types except EPAX. After 48 h of cultivation the substrate was depleted in all shake flasks, except for some remaining xylose in the spruce sugar medium.

The biomass yields in Table 3.1.1 showed that *A. limacinum* achieved the highest cell biomass per gram carbon source consumed in the EPAX medium, while the yield was lowest in the glucose medium.

3.1.2 Effect of salt in combined spruce and CSL media

In the second medium screening the effect of using different salts and salt concentrations on growth performance in combined spruce sugar- and CSL media was investigated. The media consisted of 30 g/L spruce sugar (based on glucose concentration) and 10 g/L of CSL, and each combination were performed in triplicates (see Table 3.1.2).

Table 3.1.2: The different types of salts, CDW (g/L) and yields (g_x/g_s) of *A. limacinum* in batch fermentations in combined BALI and CSL media. The CDW values were corrected by subtraction of the CDW concentrations of the time zero samples. The yields were calculated using CDW increase and glucose plus xylose consumption from 0 to 48 hours, except for the medium with 35 g/L of Na_2SO_4 (NS where 72 h values were used.

	Salt	Time (h)	CDW (g/L)	$Y_{x/s}$ (g_x/g_s)
1	Sea salts 35 g/L	24	5.0 ± 0.2	0.37
		48	12.8 ± 0.3	
		72	12.0 ± 0.5	
2	Sea salts 17.5 g/L	24	6.2 ± 0.4	0.38
		48	13.3 ± 0.4	
		72	12.6 ± 0.2	
3	No sea salts or Na_2SO_4	24	6.6 ± 0.5	0.42
		48	14.5 ± 0.1	
		72	13.7 ± 0.1	
4	Na_2SO_4 35 g/L	24	4.6 ± 0.2	0.38
		48	12.8 ± 0.3	
		72	13.4 ± 0.3	
5	Na_2SO_4 17.5 g/L	24	6.7 ± 0.7	0.41
		48	14.2 ± 0.3	
		72	13.6 ± 0.4	

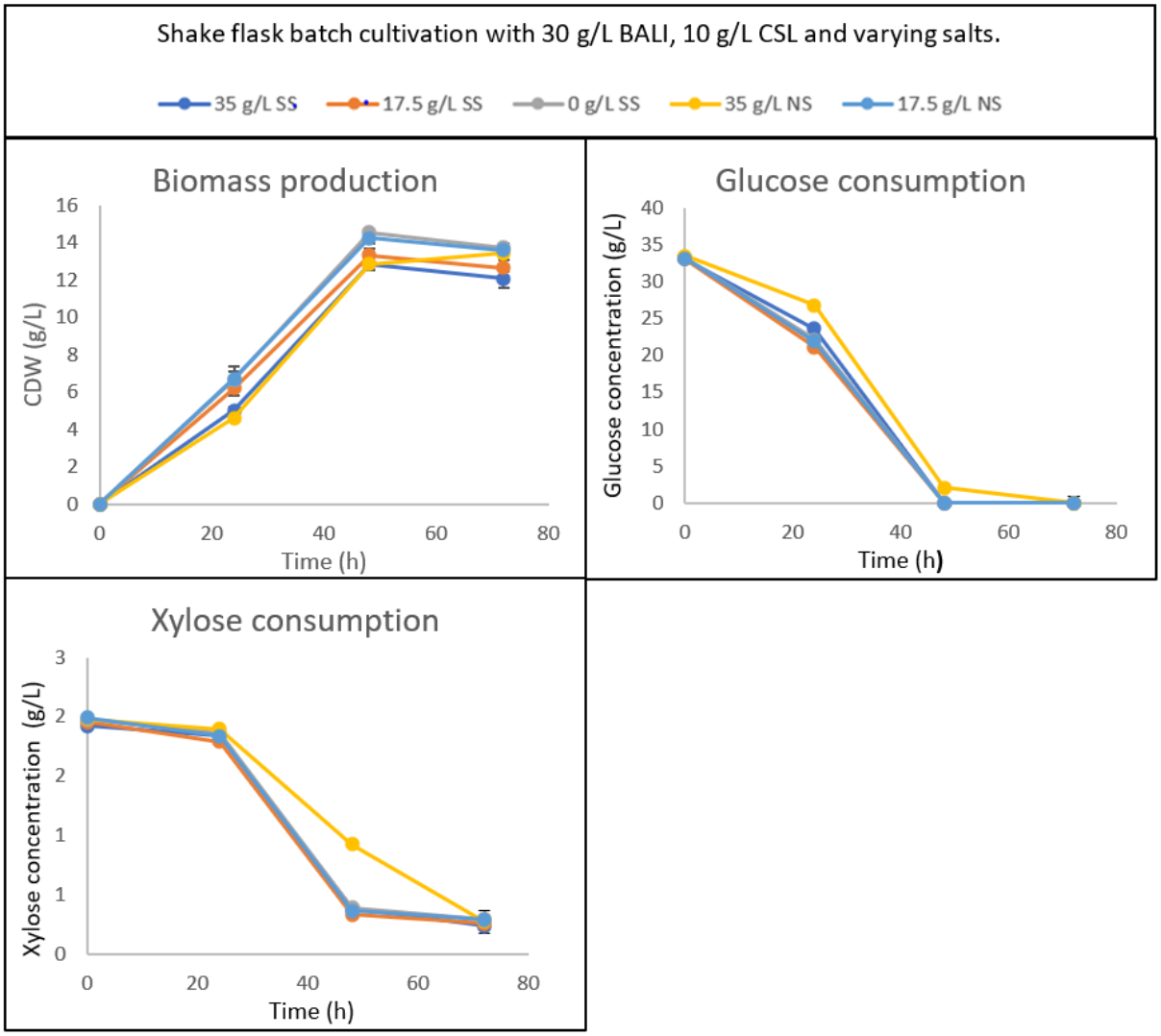

Figure 3.1.2: CDW (g/L), glucose concentration (g/L) and xylose concentration (g/L) for all media, at 0, 24, 48 and 72 hours of cultivation.

The CDW data in Figure 3.1.2 show that the spruce sugar medium without any salts and the spruce sugar medium with 17.5 g/L of Na_2SO_4 (NS) had the best growth performance, reaching biomass concentrations of 14.5 g/L and 14.2 g/L, respectively (Table 3.1.2). Compared to the spruce sugar medium in the first screening experiment (Table 3.1.1), the CDW of the medium with CSL and no salts had a slightly higher value at the same time. The yield for this medium was also higher than the yield produced in the first shake flask experiment, and the highest for this experiment, with a value of 0.42 as compared to 0.40 g_x/g_s.

The medium with 35 g/L of Na_2SO_4 where the only medium in which *A. limacinum* continued growing after 48 hours of fermentation. This was the medium resulting in slowest growth together with the 35 g/L SS medium. Additionally, the 35 g/L of Na_2SO_4 medium had the lowest consumption rates of both glucose and xylose by far. It appears that the higher the salt concentration was, the lower the biomass production. Judging by the biomass concentration it also seems like the spruce sugar media generated more biomass with Na_2SO_4 in general, than with sea salts.

3.1.3 Cultivations with EPAX and CSL medium

The third shake flask experiment was performed in the same manner as the second experiment, with the same substrates and concentrations, except that EPAX was used as carbon source.

Table 3.1.3: The medium type, indicated by the salt concentration, CDW (g/L), and yields on glycerol (g_x/g_s), at 24, 48 and 72 hours of fermentation. The yields are calculated using CDW increase and glycerol consumption from 0 to 72 hours.

	Salts	**Time (h)**	**CDW (g/L)**	**$Y_{x/s}$ (g_x/g_s)**
1	Sea salts 35 (g/L)	24	5.1 ± 0.2	0.41
		48	12.2 ± 0.2	
		72	12.7 ± 0.1	
2	Sea salts 17.5 (g/L)	24	3.3 ± 0.1	0.43
		48	11.1 ± 0.8	
		72	13.2 ± 0.1	
3	No sea salts	24	0.0 ± 0.2	0.36
		48	0.9 ± 0.1	
		72	5.3 ± 0.3	
4	Na_2SO_4 35 g/L	24	2.6 ± 0.2	0.41
		48	8.2 ± 0.4	
		72	12.5 ± 0.3	
5	Na_2SO_4 17.5 g/L	24	2.7 ± 0.3	0.44
		48	9.5 ± 0.7	
		72	13.3 ± 0.1	

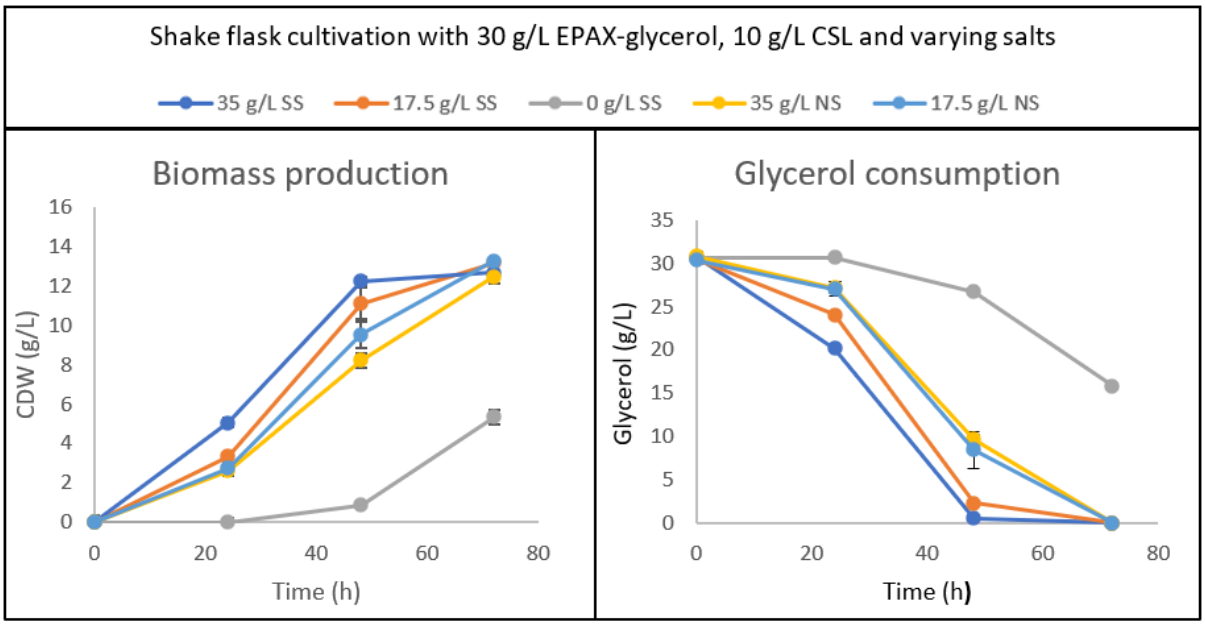

Figure 3.1.3: The CDW (g/L) and glycerol concentrations (g/L) for all media, at 0, 24, 48 and 72 hours of cultivation.

In contrast to the shake flask experiment with spruce sugars, the EPAX medium barely generates cell growth in the medium type with no sea salts. The biomass concentration was only 0.87 g/L after 48 hours, and 5.35 g/L after 72 (Table 3.1.3). It is also clear that *A. limacinum* grows better in the mediums containing sea salts than the ones with Na_2SO_4. Furthermore, the strain consumed glycerol faster in the sea salt media (Figure 3.1.3). However, at 72 hours all cultivations resulted in quite similar CDW values: 12.7, 13.2,12.5 and 13.3 g/L for medium 1, 2, 4, and 5 respectively. At the end of the fermentation the medium with the NS concentration at 17,5 g/L produced the highest biomass concentrations and generated the highest yield of 0.44 (g_x/g_s), followed by the media 2 generating a yield value of 0.43 (g_x/g_s) (Table 3.1.3).

3.1.4 Lipid accumulation trial

The fourth shake flask experiment was run for a longer period (120 hours for medium 1 and 2, and 144 hours for the rest), while using a higher start concentration of the carbon source (90 g/L), with the aim to accumulate lipids in the biomass. 10 g/L CSL was used as nitrogen source in all media. Further medium specifications are listed in table 2.4. The cultures were run as batch fermentations like the rest of the shake flask experiments and performed in duplicates.

Table 3.1.4: Medium components, CDW (g/L), lipid content (%), DHA content (%), yield (g_x/g_s) and the maximum growth rate (h^{-1}). The CDW values are average end results after 120 hours of cultivation (media 1 and 2), and 144 hours of cultivation for media 3-7. The DHA values are percentage DHA of the lipid content. Yields are calculated based on CDW increase and carbon source depletion.

	Medium	CDW (g/L)	Lipids (%)	DHA (%)	$Y_{x/s}$ (g_x/g_s)	μ_{max} (h^{-1})
1	Glucose-SS	35.2 ± 0.3	61.4 ± 1.2	32.9 ± 0.2	0.35	0.06
2	Glycerol-SS	31.2 ± 0.8	61.7 ± 1.3	34.5 ± 0.5	0.36	0.02
3	BALI-SS	12.7 ± 0.2	28.3 ± 3.5	36.9 ± 0.3	0.35	0.03
4	BALI-no-SS/NS	26.5 ± 0.1	51.4 ± 0.5	36.1 ± 0.5	0.31	0.05
5	BALI-NS	15.1 ± 0.8	39.4 ± 0.5	37.6 ± 0.3	0.31	0.07
6	EPAX-SS	15.0 ± 1.1	56.9 ± 1.1	28.6 ± 0.6	0.31	0.03
7	EPAX-NS	15.6 ± 0.1	56.9 ± 1.0	27.4 ± 0.1	0.41	0.01

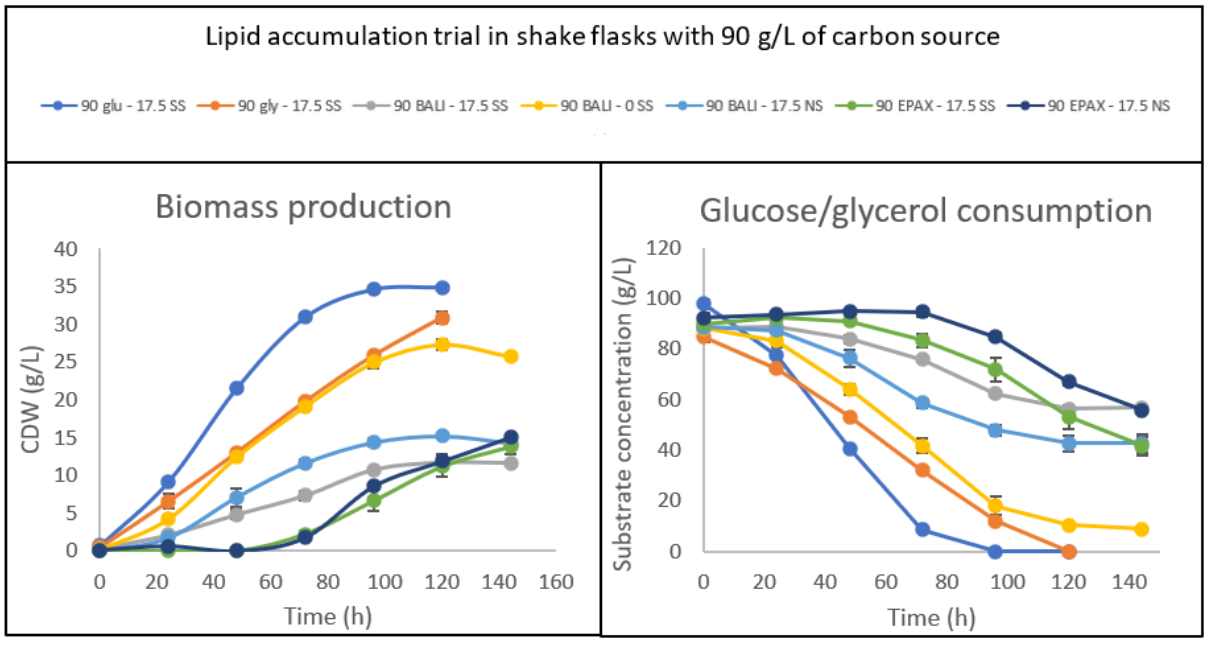

Figure 3.1.4: CDW (g/L), and glycerol/glucose concentration (g/L) every 24 hours in 6 days (5 days for medium 1 and 2), for all medium types.

Figure 3.1.4 shows that the glucose medium resulted in the fastest growth rate. Table 3.1.4 shows that this medium also achieved the highest maximum growth rate at 0.06 h^{-1}. By the end of the cultivation, it had produced 35.2 g/L of biomass, which was the highest concentration reached. Furthermore, *A.limacinum* consumed the glucose faster than all of the other substrates. The glycerol and spruce sugar media without SS or NS had the highest growth rates following glucose, yielding biomass concentrations of 31.2 and 26.5 g/L, respectively. However, their maximum growth rates differed noticeably with values of at 0.05 h^{-1} for the Spruce sugar medium, and only 0.02 h^{-1} for the glycerol medium.

The rest of the media resulted in much lower growth performance, yielding biomass concentrations of around 12-16 g/L, where spruce sugar with sea salts had the lowest value of 12.7 g/L. These cultivations failed to consume a lot of the available substrate in these cultivations. The EPAX media had the slowest growth rates at the start of the cultivations but seemed to grow better after 60 hours of cultivation, judging by figure 3.1.4. The growth on the other spruce sugar media stagnated the last two days of cultivation while it continued in the EPAX media. Reviewing the growth results it seems like the *A.limacinum* had a slower but more stable growth in glycerol media than media containing glucose.

Table 3.1.4 show that the lipid content achieved at the end of the cultivation was especially high for pure glycerol and glucose media, with lipid percentages of 61.7 and 61.4 of the biomass, respectively. Biomass produced on EPAX media had a lipid content of 56.9%. The spruce sugar medium without SS/NS produced cells with a slightly smaller lipid fraction of 51.4%. Considering that the spruce sugar medium generated significantly more biomass than the EPAX media, the total amount of lipids produced is higher for spruce sugar media than for EPAX. Looking at the DHA percentage, which is the fractions of the lipid amount, the spruce sugar media generates the highest concentrations of DHA, although glucose and glycerol produces higher amounts due to higher biomass content, and therefore higher total amount of lipids including DHA.

The yields show that EPAX with NS produced the highest amount of biomass per gram substrate consumed (0.41 g_x/g_s), followed by glycerol with a yield of 0.36 g_x/g_s and glucose with a yield of 0.35 g_x/g_s. However, Figure 3.1.4 shows that the EPAX with NS did not consume all the substrate.

In summary, glucose, glycerol and spruce sugars without SS/NS gave the overall most promising results regarding lipid and DHA production. It was decided to continue with these media in further experiments in bioreactors. The first bioreactor experiments used glycerol as substrate but failed due to toxicity of the applied antifoam (Glanapon DB 870). Thus, it was decided to focus on glucose and spruce sugars as substrates in the following bioreactor experiments with a new antifoam type (Clerol).

3.2 Bioreactor experiments

3.2.1 Fed-batch fermentation with glucose and spruce sugar media

The first bioreactor run was performed in Fed-batch mode using pulsed feeding. GOPOD assay was used to monitor the glucose concentration in the bioreactors, and concentrated substrate was added aseptically with a syringe. All media were initially composed of 90 g/L carbon source, and 10 g/L nitrogen source, in addition to different salts (Table 2.4), trace elements and vitamin solution (Appendix A). Bioreactors 1 and 2 were replicates, containing media with glucose and yeast extract/peptone-blend (medium 1 in table 3.2.1). Bioreactor 3 and 4 contained spruce sugar medium with CSL and 17.5 Na_2SO_4 or 35 g/L Na_2SO_4. The choice of media was based on the results from the lipid accumulation trials in shake flasks, and the best choice would have been spruce sugar medium without SS/NS. However, due to a mix up in labelling of samples in an excel file NS containing media were used.

Table 3.2.1: Fed-batch cultivation of *A. limacinum* in medium containing glucose and yeast-peptone blend as nitrogen source, medium 1, and medium containing spruce sugars, CSL as nitrogen source, and 17.5 or 35 g/L NS, in medium 3 and 4, respectively. CDW (g/L), lipid (%), DHA (%), yield (g_x/g_s), and maximum growth rate (h^{-1}) after 96 and 120 hours of cultivation for glucose and spruce sugar fermenters respectively. Yields were calculated with data from fermentation start to stop (0-96 hours).

	Medium	Time (h)	CDW (g/L)	lipid (%)	DHA (%)	$Y_{x/s}$ (g_x/g_s)	μ_{max} (h^{-1})
1	Glucose-YP	96	57.0 ± 2.2	74.0 ± 1.8	32.8 ± 0.0	0.24 ± 0.01	0.13 ± 0.01
2	BALI-17.5 g/L NS	120	33.1	47.8	31.1	0.30	0.09
3	BALI-35 g/L NS	120	30.4	43.8	31.6	0.34	0.08

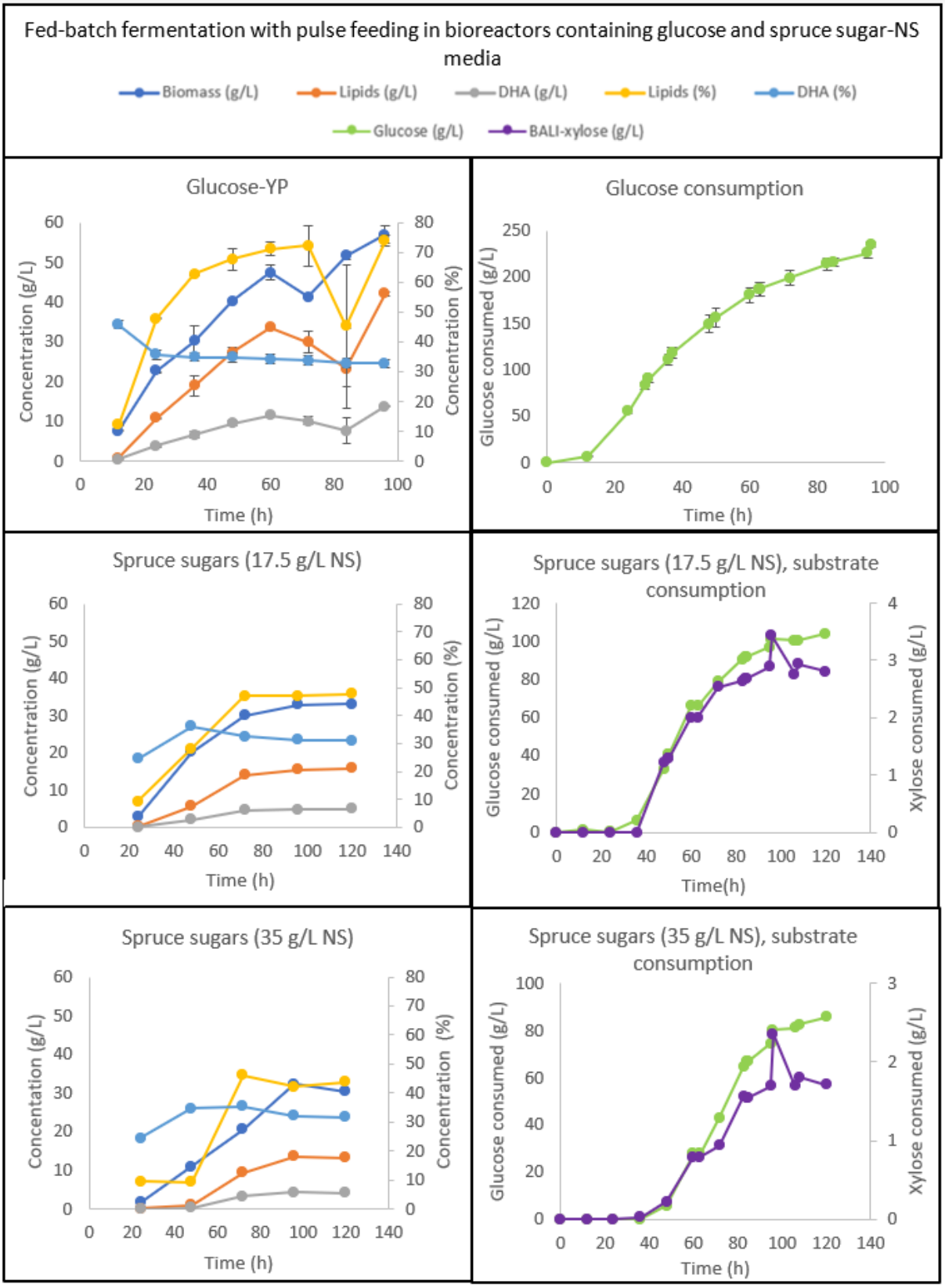

Figure 3.2.1: Biomass concentration (g/L), lipid content (g/L), DHA content (g/L), lipid fraction of the biomass (%), and DHA percentage of the lipid amount (%), for bioreactor samples taken every 12 hours (Left panels). Furthermore, accumulated glucose consumption (g/L) for all media, in addition to the xylose consumption in the spruce sugar media, are displayed (Right panels).

Table 3.2.1 shows that the bioreactors with glucose as substrate generated significantly higher end concentrations of biomass compared to the spruce sugar bioreactors. Looking at the CDW graphs shown in figure 3.2.1, it seems like the growth speed was generally higher in the glucose vessels than in spruce vessels. This corresponds with the maximum growth rates which had an average value of 0.13 for the glucose media, and 0.08-0.09 for the spruce sugar media. In conclusion, *A. limacinum* grew faster in the glucose medium than the spruce sugar media, similar to the observation in shake flask cultivations. The yields, however, are noticeably higher for the spruce sugar media.

Comparing the two spruce sugar media, the medium containing 17.5 g/L NS had a higher biomass concentration at the end of the cultivation than the medium containing 35 g/L of NS, with 33.1 and 30.4 g/L, respectively. The maximum growth rate for the first mentioned medium were also a little bit higher with 0.09 h^{-1}), compared to 0.08. This indicates that the growth was better in the spruce sugar medium with 17.5 g/L NS, which corresponds with the result of the spruce sugar shake flask experiment.

The lipid content was also higher in the fermenters containing glucose than the ones with spruce sugars. Table 3.2.1 shows that the glucose fermentations generated biomass with an average lipid content of 74%. In comparison, the spruce sugar bioreactor with the highest biomass titre had a lipid content of 47.8. The different cultivations achieved similar percentages of DHA, in general approximately a third of the lipid content were DHA. The total amount of DHA however is not the same considering that both the CDW and the lipid percentage is higher for the glucose medium. This can also be seen in Figure 3.2.1 (DHA content as grey graph). Table 3.2.1 and Figure 3.2.1 show that the spruce sugar medium with 17.5 g/L NS produced a higher amount of lipids and DHA than the one with 35 g/L NS. There were also consumed more glucose and xylose in the 17.5 g/L NS medium.

3.2.1.1 Nile red microscopy of bioreactor samples

Harvested cells were visualized using red nile microscopy, which was performed at Nofima in Ås. The nile red staining makes lipids glow yellow when examining with a fluorescence microscope.

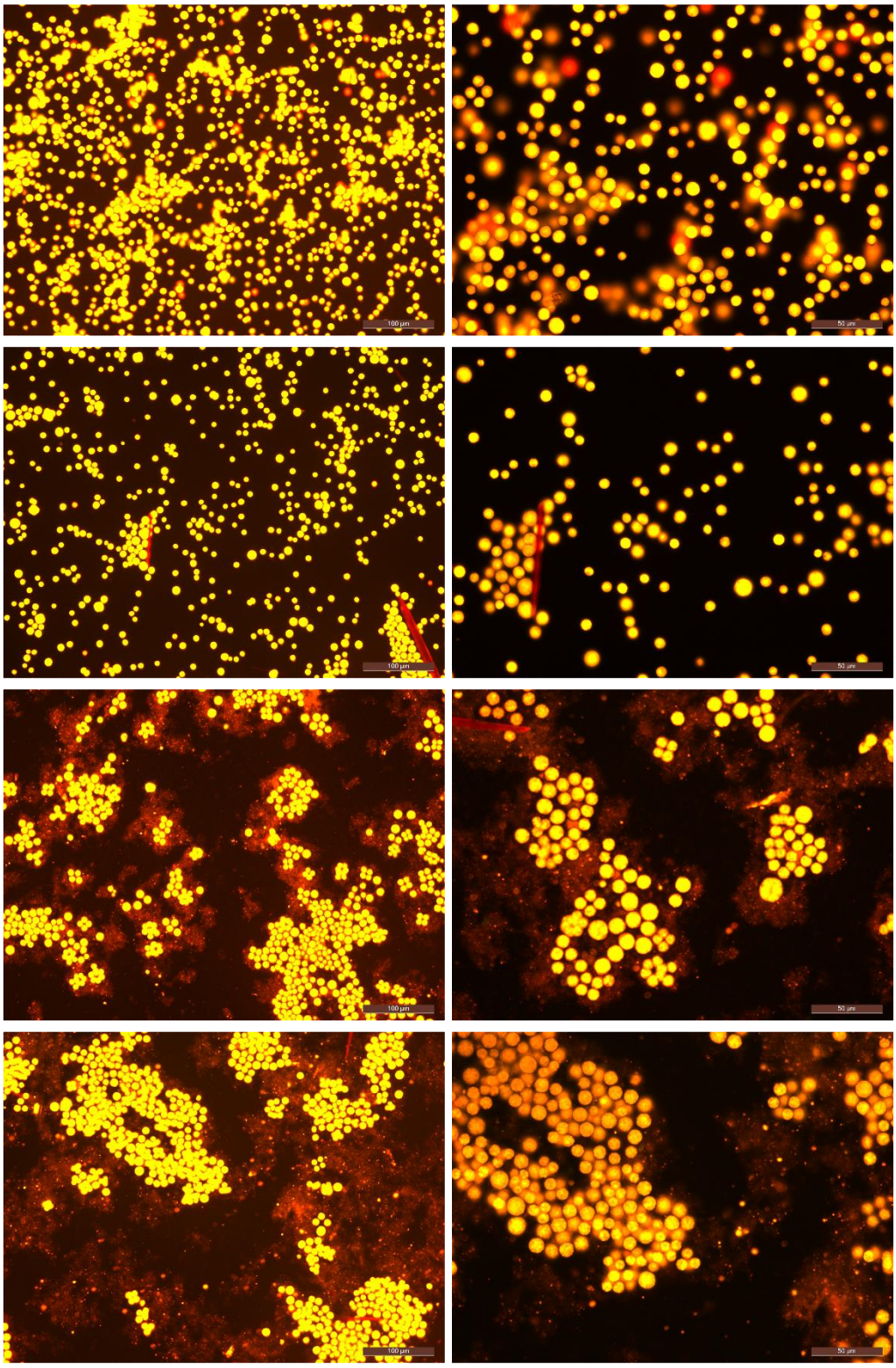

Figure 3.2.1.1: Pictures of *A. limacinum* cells (in yellow) taken during nile red microscopy of 96 hours old glucose media, and 120 hours old BALI media from bioreactors. From the top to the bottom photos of

fermentation broth from bioreactors 1, 2, 3, and 4 respectively are displayed. Left panel photos are taken with 200 times magnification, while right panels are showing 400 times magnfication.

The bright yellow colour of the cells in Figure 3.2.1.1 show that they have accumulated lipids. The top 4 pictures indicates that there was a higher cell density in glucose vessel 1, than 2, which corelates with the CDW concentrations. In addition, it seems like *A. limacinum* tend to form clusters when cultivated in the Spruce sugar medium, which is not the case for the glucose media.

3.2.2 Fed-batch cultivation with glucose medium and batch cultivation with BALI-no SS/NS, using continuous feeding.

The aim of this experiment was to repeat the previous bioreactor run using spruce sugar medium with no sea salts or Na_2SO_4. The same glucose medium was therefore applied to bioreactor 1 and 2 and is referred to as medium 1 in Table 3.2.2 below. Bioreactor 3 and 4 contained spruce sugar medium without SS/NS and were replicates a well, referred to as medium 2.

There was also tested a new feeding strategy, continuous feed, in which a pre-calibrated pump added feed with a speed calculated to maintain a stable substrate level. The glucose concentrations in the vessels were monitored using GOPOD assay as in the previous bioreactor experiment. However, the tubes connected to the pumps that controlled the spruce sugar feed, got clogged due to clumps in the CSL and spruce sugar substrates, combined with too small tubes. Hence the spruce sugar fermentations ended up being more like a batch fermentation than a fed-batch cultivation. Because of the failed feeding, the lipid accumulation in the spruce sugar bioreactors were considered to be low, and it was decided to not analyse the lipid content in the respective samples.

Table 3.2.2: Shows the results after 84 hours of fermentation for medium 1, and the results after 96 hours of fermentation for medium 2. CDW (g/L), lipid content (%), DHA (%), yields (g_x/g_s), and μ_{max} (h^{-1}) are displayed. The CDW values are corrected in the same way as the previous bioreactor experiment, and yields are calculated with data from cultivation start to cultivation stop (0-96 hours).

	Medium	**Time(h)**	**CDW (g/L)**	**Lipid (%)**	**DHA (%)**	**$Y_{x/s}$ (g_x/g_s)**	**μ_{max} (h^{-1})**
1	Glu-YP	84	52.7 ± 1.4	75.1 ± 0.8	33.8 ± 0.1	0.24 ± 0.02	0.12 ± 0.00
2	BALI-no SS/NS	96	34.7 ± 5.3				0.08 ± 0.00

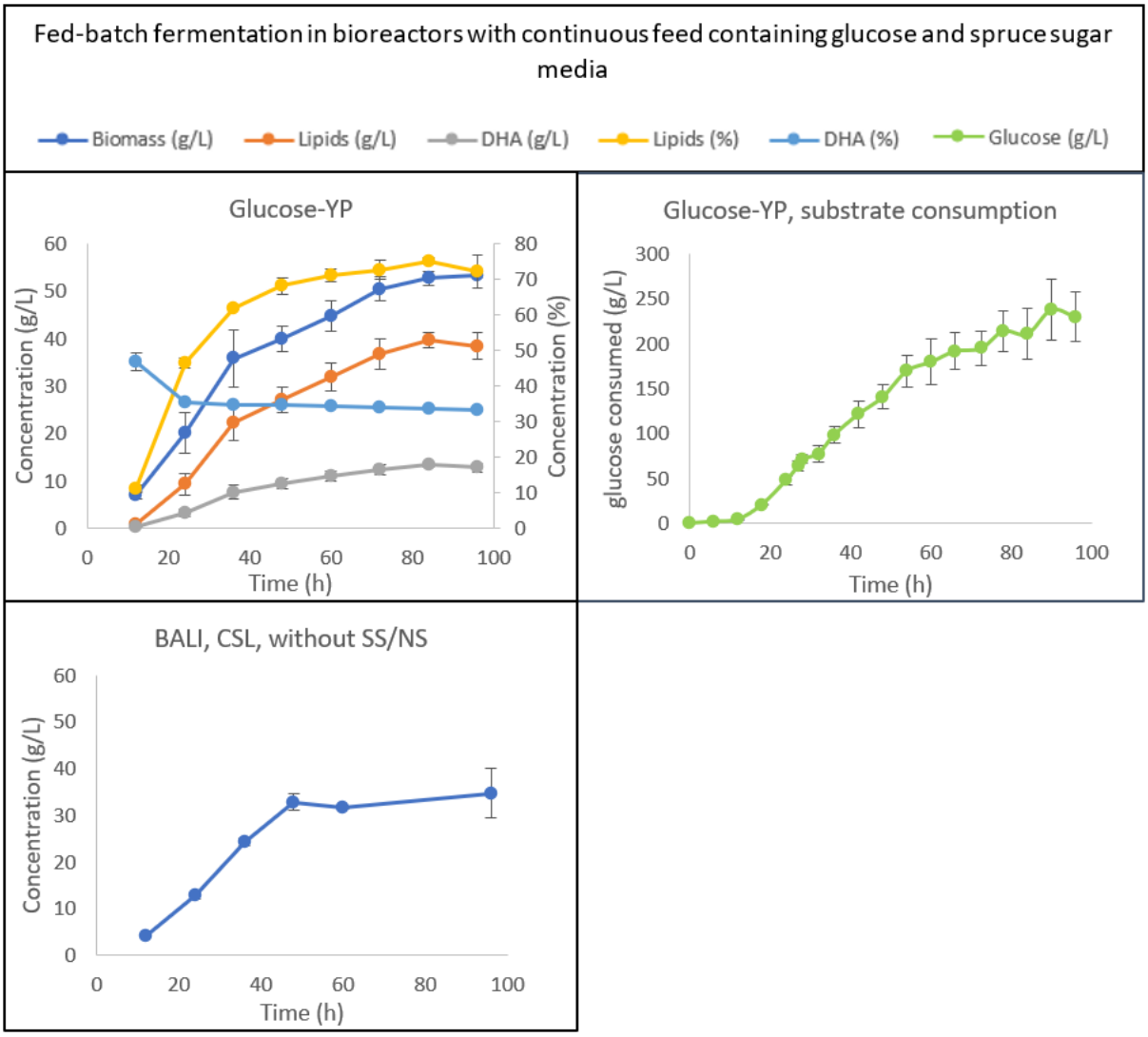

Figure 3.2.2: shows the CDW/biomass (g/L), lipid content (g/L), DHA content (g/L), lipid fraction of the biomass (%), and DHA percentage of the lipid amount (%), for bioreactor samples taken every 12 hours.

Overall, the results from this experiment does not differ much from the first bioreactor experiment. The biomass titres from the glucose media are a little bit lower for this experiment with values of 52.7 compared to 57.0 g/L respectively. The maximum growth rates for these cultivations are similar, having a value of 0.12 for this experiment and 0.13 h^{-1} for the last. The growth speed can therefore be considered almost equal but giving slightly higher biomass titres in the previous experiment. There should also be noted that the biomass concentrations was at its highest at the end of the fermentation (at 96 hours) for the previous experiment, while *A.limacinum* stopped growing at 84 hours for this experiment. The lipid production of the glucose vessels of this run is comparable to those of the previous run, although the final concentration is lower for this run due to a lower biomass concentration.

The spruce sugar fermentations in this experiment gained a higher average biomass concentration than both spruce sugar mediums in the previous run with 34.7, compared to 33.1 and 30.4 for BALI-sugar mediums with 17.5 ang 35 g/L of NS respectively. Nevertheless, the standard deviation of this

run is considerably higher at 5.3 g/L. The maximum growth rates were more or less the same in the bioreactors containing spruce sugars without SS/NS as in the bioreactor containing 35 g/L of NS. The bioreactor containing spruce sugars with 17.5 g/L of NS in the previous experiment had a maximum growth rate of approximately 0.01 (h^{-1}) more. In conclusion the spruce sugar medium with 35 g/L NS produced the lowest biomass concentration of all spruce sugar media tested in the bigger scale fermentations and achieved the lowest lipid titres of the two mediums with NS. Regarding the results from shake flask experiments, *A. limacinum* was expected to grow better on the spruce sugar medium used in this bioreactor run, than the last. Nevertheless, the failed spruce sugar feeding during this experiment, and the missing CDW measurements between 60 – 96 hours of this run, must be taken into consideration.

4. Discussion

A. limacinum was selected for this study because of its well documented ability to produce high titres of lipids and in particular DHA. In addition, initial experiments in the same laboratory had suggested that the strain grew well when cultivated in medium where BALI sugars was used as carbon source. There is a growing need for new sources of omega 3 fatty acids in feed, in particular for fish feed, and *A. limacinum* is a promising candidate for fermentative production of DHA.

In the initial media screening in shake flasks, all the media generated similar biomass concentrations and had similar growth performance (Figure 3.1.1). This shows that the carbon sources derived as by-products from industrial production can perform similar as pure carbon sources in combination with a yeast/peptone blend. The biomass concentrations where even a little higher for the by-products, which corresponds with their higher yield on substrate values. This could be due to content of small amounts of other not identified carbon sources in these substrates.

The utilization of different salts and salt concentrations was tested in combination with spruce sugars and CSL, as medium for *A. limacinum* in the second shake flask experiments (Figure 3.1.2). The yeast extract/peptone blend was here exchanged with CSL as nitrogen source, due to CSL being a cheaper substrate as a by-product from corn milling industry (ROQUETTE, 2018). The content of vitamins, different trace elements, and minerals in this product (ROQUETTE, 2018, Merck, 2021) should also be beneficial for the growth of *A. limacinum*.

Both sea salts (SS) and Na_2SO_4 (NS) were tested in the media and at different concentrations. It was observed in the first shake flask experiment that the strain grew well in spruce sugar media that contained SS. This combination was thus optimal to use when screening the effects of CSL alone on the cell growth. In addition, this medium was used as reference when testing the utilization of the new salt NS. This was tried to potentially substitute SS which is corrosive to bioreactors made of steel, a problem in industrial fermentations (Haque et al., 2020)

When the spruce sugar substrate was combined with CSL and different salts in the second shake flask experiment, the results indicated that a high salt concentration inhibited the growth of *A. limacinum* in spruce sugar media. The biomass titres were noticeably higher for the media with a salt concentration of 0 and 17.5 g/L than those for the media with a salt concentration of 35 g/L. There

was also a difference in biomass titres between the spruce sugar medium with 35 g/L sea salt, and corresponding medium from the carbon source screening, where the yeast and peptone were used instead of CSL. The reason for the higher CDW production in the yeast extract and peptone medium, might be that CSL contains some salts as well (ROQUETTE, 2018), which then adds to the effect of the sea salt. This in turn led to the question if there are any salts or minerals in the spruce sugar substrate too. Indeed, previous work analysing BALI hydrolysates have shown that it contains e.g. 15 g/kg DM Na, 17 g/kg DM S and 3.6 g/kg DM Ca. (Sharma et al., 2018)

In the lipid accumulation trial, CSL was used as nitrogen source for all media. The same effects of spruce sugar media and different salt concentrations were apparent in that experiment as well (Figure 3.1.4). The spruce sugar medium without SS/NS clearly resulted in higher biomass titres than the ones with NS or SS. In this experiment the glucose media that contained salt and CSL, grew better than the spruce medium without SS/NS and with CSL, indicating that salts/impurities might have an additive negative effect on the growth. It should be noted that there were added additional minerals in the lipid experiment as well.

In the third shake flask experiments where EPAX glycerol were combined with CSL and the same salt concentrations as in the second shake flask experiment, the effects were opposite (Figure 3.3.3). *A. limacinum* did not grow well at all in the medium with zero SS or NS and grew fastest in the EPAX medium with 35 g/L of sea salt. Seen in context with the spruce sugar shake flask experiment, this suggests that EPAX lacks some salts or minerals that are present in the spruce sugar substrate and needed for the growth of *A. limacinum*. However, the medium containing no SS or NS, generated a faster growth and consumes more glycerol after 48 hours.

In the lipid accumulation experiment (Figure 3.1.4) the medium was added vitamin solution, trace elements, KH_2PO_4, $CaCl_2$, and $MgSO_4$, in addition to the other salts. Furthermore, CSL was used as nitrogen source, like in the EPAX shake flask experiment (third experiment). None of the additional salts, trace elements or vitamins seemed to help the growth of *A. limacinum* in EPAX and CSL media. Nevertheless, these media generated a decent growth after 48 hours and could have continued growing, judged by the trends of the CDW curves in Figure 3.1.4. There could be various reasons for the lacking growth in the beginning of these cultivations. Even though the EPAX medium with 35 g/L sea salt generated the best cell growth in the third shake flask experiment, the additional salts and trace elements in this experiment may have caused the concentration of one or some elements to get too high and created an inhibitory effect. It should be noted that the experiments described in Figure 3.1.4 used 3 times more concentrated EPAX than the previous experiment (Figure 3.1.3). Possible impurities in crude glycerol substrates can cause problems in microbial fermentations (Samul et al., 2014). Table B.3 (Appendix B) shows that the glycerol concentration in EPAX is quite high (approximately 91%) which indicates that there are only small amounts of impurities. This was also emphasized by the supplier.

The second most abundant component of EPAX is fish oil, making up approximately 5% of the crude glycerol substrate. Venkataramanan et al.(2012) found that for long chained free fatty acids (C:18), the most unsaturated fatty acids have strong inhibitory effects on the glycerol consumption by bacteria. Less unsaturated fatty acids have a milder effect. The free fatty acids are integrated in the cell membranes of bacteria, and thereby interfere with metabolite production (Venkataramanan et al., 2012, Samul et al., 2014). Fish oil is known to contain several highly unsaturated fatty acids, as it is a source of PUFAs like DHA and EPA (Tocher et al., 2019). It is therefore possible that the glycerol

uptake could have been inhibited by fatty acids from the EPAX substrate, and thus hindered cell growth until most of the fatty acids in the fish oil were absorbed. The concentration of the fish oils would have been 3 times higher in in the Figure 3.1.4 experiment as compared to Figure 3.1.3.

Liang et al.(2010) reported that they achieved the highest cell growth and best lipid accumulation results when applying crude glycerol concentrations of 25 – 35 g/L in batch fermentations of *A. limacinum.* The raw material was derived from yellow grease or animal fats. It was also claimed that higher substrates concentrations had an inhibitory effect and contained methanol titres that were harmful for the strain. In this study there were used crude glycerol concentrations of 30 g/L for the first shake flask fermentations and 90 g/L for the lipid accumulation trial. As can be seen in Appendix B, the crude glycerol used for this study does not contain methanol, only small amounts of ethanol. However, the fish oil content is significantly higher, and when the substrate concentration was increased in the lipid accumulation trial, so was the concentration of fish oil. In conclusion the most logical reason for the growth inhibition of *A.limacinum* in these substrates is the presence of free fatty acids in the EPAX substrates that reach inhibitory values in the 90 g/L fermentations. It also seemed like the strain grew best in the EPAX mediums when sea salts were added to the medium.

Because of the poor growth generated by EPAX-glycerol media in the lipid accumulation trial, and because it grew best in the presence of sea salt, while spruce sugar media grew best without SS/NS, this substrate was not prioritized for the bioreactor experiments. It was considered more interesting to continue with fermentations grown on spruce sugars in bioreactor experiments, due to its ability to generate high biomass and lipid titres, without the need of additional sea salts nor Na_2SO_4 to the medium when CSL was used as nitrogen source. This is a sustainable medium combination with opportunities for industrial scale fermentations. The highest biomass concentration generated for this combination was 26.5 g/L, which was produced after 144 hours with a start concentration of 90 g/L BALI-glucose (Table 3.1.4). Patel et al.(2020) achieved similar results: 26.9 g/L biomass for *A.limacinum* cultivated on spruce sugars in shake flask. This was however with peptone as nitrogen source and with the addition of sea water.

Glucose with yeast/peptone blend and SS was used as reference medium for bioreactor fermentations. This combination had given the highest biomass and lipid concentrations in the shake flask experiments and was therefore ideal for comparing results. It is also suitable for comparison with spruce sugar fermentations because spruce sugars consist of mainly glucose (Appendix B). Furthermore, based on the shake flask experiments this medium combination seemed reliable for achieving a decent cell growth, and more robust than the other media. Since the bioreactor vessels used in this study was made of glass, the medium would not cause harm to vessels in this case.

Even though two different feeding strategies were used for the bioreactor runs, the results for the glucose fermentations and the spruce sugar fermentations were quite similar from one experiment to the other. The glucose fermentations in the pulsed feed fermentation generated an average biomass concentration of 57.0 g/L, while the glucose fermenters in the continuous feeding experiment achieved an average biomass concentration of 52.7 g/L (Table 3.2.1 and Table 3.2.2). Furthermore, the lipid content was 74. 0 and 75.1%, and DHA content 32.8 and 33.8% respectively. The glucose media used in these experiments where exactly the same, which shows that the change in feeding strategy did not make such a big impact. Reduced standard deviations for the glucose fermentations in the continuous feeding however, showed that the reproducibility was better when using a continuous feed. Since the produced biomass titres were approximately 4 g/L higher for the

pulsed fed fermentations, the lipid amount and DHA amount was also slightly higher using this feeding technique.

The spruce sugar fermentations in the last bioreactor experiment were intended to be a continuously fed fermentation like the glucose fermentations of that run. Unfortunately, the feeding of the cultivations failed, due to clogging of the feed-tubes. This was discovered at a later stage in the fermentation process, and the spruce sugar fermentations were assumed to be more like batch fermentations. Nevertheless, the growth results of these fermentations did not differ much from the growth results of the spruce sugar media in the pulsed fed bioreactor run. The biomass concentration for the batch fermentations were even a little higher than the fed-batch cultivations, generating titres of 34.7 g/L compared to 33.1 and 30.4 g/L. It could be considered more logical that the media provided with additional feed during the cultivation would produce higher biomass titres. Nevertheless, the nitrogen source in these media was in shortage compared to the rest of the substrates, and nitrogen are needed to build proteins which in turn are needed for generation of new cells. Another significant difference between these cultivations was that different salt concentrations were used in these fermentations. The spruce sugar media used in the first bioreactor experiment was with 17.5 and 35 g/L NS. These media generated less cell growth in the shake flask experiments than the medium containing no SS nor NS and were thus used in bioreactor fermentations by mistake. The inhibitory effect of salts combined with the spruce sugar- CSL media could therefore be the reason why the fed batch culture did not generate more cell growth than the batch culture.

Comparing the bioreactor results from the glucose and the spruce sugar media, the glucose media generates almost twice as high biomass titres as the spruce sugar media. There could be several reasons for this. Observing the results from the fourth shake flask experiment, the difference in biomass production was not that big between glucose and the spruce sugar medium without SS/NS. This indicate that the biomass concentration might have been higher for the spruce sugar media in the last bioreactor run if the fermentations had the same growth conditions, and the feeding of spruce sugar cultivation did not fail. However, it should be taken into account that there was used CSL as a nitrogen source for the glucose media in that experiment and not yeast extract/peptone blend as in the bioreactor fermentation, this could have limited the growth of the glucose media in the shake flask experiment. Another reason for the big difference in biomass accumulation between the glucose media and spruce sugar media in bioreactor cultivations, may be that the spruce sugar solution and CSL made a more viscous medium than the glucose and yeast extract/peptone medium. This could have caused a slower movement of the liquid flow in the spruce sugar cultivation, and thus caused less oxygen to reach all cells (Subramaniam, 2019). It is stated in Sirirak et al.(2021) that the main reason for limitations of cell growth of *A.limacinum* in bioreactors are insufficient oxygen availability. Reviewing the online data in Appendix D it is not much difference in stirrer speed or pO_2 level between the glucose reactors and the spruce sugar reactors. Since the stirrer speed is supposed to adjust itself to maintain a dissolved oxygen level of 20 % in the fermentation broth, the stirrer speed would have increased if the oxygen level was lower, which in turn mean that the cells had consumed more oxygen. Even though stirrer speed and pO_2 graphs are not very different between the different fermentations, it is hard to conclude anything due to changes in the graphs all the time. Hence, the difference in density between the media are probably not that big, but this may be part of the explanation.

The yields achieved by the glucose bioreactor fermentations were 0.24 for both experiments, while the yields for the spruce sugar fermentations were 0.30 and 0.34 for the medium containing 17.5 and 35 g/L NS respectively. One possible reason for higher yields in the spruce sugar fermentations was that it contained low levels of sugars that might have been used as substate. This was not accounted for in the calculation of yields. Furthermore, the glucose fermentations generated significantly more lipids. Karamerou et al.(2017) showed that the yields generated by fed-batch cultivations of the oleaginous yeast *Rhodotorula glutinis* differed greatly in the growth proliferation phase and the oil accumulation stage. There were produced much more biomass per gram carbon source consumed in the proliferation stage than in the lipid accumulation stage. This does also seem logical considering that lipids are energy dense (Britannica(2003)). Hence, the higher lipid content of the glucose fermentations could be a reason why they gave lower biomass yields than the spruce sugar fermentations.

Patel et al. (2020) reported an achieved biomass yield of 0.48 $g/g_{substrate}$ when spruce sugars were utilized as carbon source for a 72 hour batch fermentation of *A.limacinum* in bioreactors. In comparison the highest achieved biomass yield on spruce sugars for this study was 0.42 $g/g_{substrate}$, achieved in the second shake flask experiment (72 hours, batch fermentation) where spruce sugar medium with CSL and no SS/NS was used.

The lipid production of the glucose fermentations was quite high, with an average lipid content of 75.1% of the biomass for the second bioreactor experiment, and 74.0% in the first. These results were obtained at 84 and 96 hours of cultivation respectively. In comparison Li et al.(2015) achieved a lipid content of 83.8% in a bioreactor fermentation where glucose was used in the beginning of the cultivation, and thereafter glycerol. This was achieved in a 96 hours fed-batch fermentation. Comparing the DHA fraction of the lipids, it was 33.3% in this study while Li et al.(2015) achieved 43.7%.

The lipid content of the spruce sugar cultivation, in the end of the first bioreactor run, was 47.8% for the medium containing 17.5 g/L NS, where 31.1% of the produced lipids were DHA (Figure 3.2.1). Patel et al.(2020) cultivated a strain of Aurantiochytrium for 72 hours where spruce sugars was used as carbon source in batch fermentations. This resulted in a lipid content of 52.3% when cultivated in bioreactor and 47.9% when cultivated in shake flask. These results where similar to those achieved by the spruce sugar fermentations of this study. The lipid percentage generated by the spruce sugar without SS/NS-medium in the lipid shake flask experiment (batch culture, 144 hours) of this study, resulted in a lipid content of 51.4%, which was higher than the lipid percentage generated in the shake flask experiments of Patel et al(2020). If the bioreactor fermentations of this medium had been successful it may have produced an even higher lipid fraction than those of Patel et al.(2020). However, the DHA content produced in the Patel study were considerably higher, 66.7% and 45.5% of the total lipids, when cultivated in bioreactor and shake flask experiments, respectively.

One strategy that could have been utilized to improve the DHA yields in this study is the strategy of oxygen limitation. Even though sufficient oxygen supply is required for a high growth rate, limiting the oxygen availability in a later stage of the fermentation process can initiate lipid production in thraustochytrids, and increase the DHA fraction of the lipid that is produced (Chi et al., 2009). The reason for this is that DHA can be produced through the PKS-pathway that is oxygen independent (Napier, 2002), while fatty acids with shorter carbon chains like C14:0, and C16:0 are produced through the fatty acid synthase (FAS) pathway, and unsaturated C18 or C20 fatty acids by the

additional elongase desaturase pathway, which are oxygen dependent (Heggeset et al., 2019). However, this strategy has varying success, and several studies have contradicting statements regarding whether the oxygen availability should be as high as possible or limited to produce the highest DHA titres (Patel et al., 2020, Heggeset et al., 2019, Chi et al., 2009). The adjustments of oxygen supply could therefore be a parameter to investigate.

5. Conclusion

This study has shown that *A. limacinum* can grow well on glucose and glycerol as substrates and generate biomass with high content of lipids. Crude industrial substrates were also shown to be able to support growth of *A. limacinum*. Some salt content is needed in the media, which seems to be provided by the spruce hydrolysates but not the EPAX glycerol substrate. Corn steep liquor, a by-product of corn milling, worked well as a nitrogen source. Regarding processing conditions, batch processes worked fine, but might be challenging at high concentrations due to impurities in the industrial substrates that inhibit growth. This was especially clear for EPAX that clearly was inhibitory at 90 g/L glycerol. Fed-bath processes can solve this issue and was successfully demonstrated in this study. Continuous pumping of new substrate into the bioreactor is probably the best strategy since it can be adjusted to give a substrate concentration at relative constant level. Problems related to clogging of tubes by pumping spruce hydrolysate can probably be solved by using tubes with a larger diameter.

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Appendices: Table of Contents

Appendix A: Composition of trace element mix and vitamin solution

Table A.1: The components of the trace element mix and their tires.

Components	Concentration (g/L)
Na_2EDTA	6
$FeCl_3 \times 6H_2O$	0.29
$CoCl_2 \times 6H_2O$	0.026
$MnCl_2 \times 4H_2O$	0.86
$ZnCl_2$	0.06
$NiSO_4 \times 6H_2O$	0.052
$CuSO_4 \times 5H_2O$	0.002
$Na_2MoO_4 \times 2H_2O$	0.005
H_3BO_3	6.95

Table A.2: The composition of the vitamin solution

Components	Concentration (mg/L)
Thiamine chloride	113.24
Biotin	0.5
Cyanocobalamin (Vitamin B12)	0.5

Appendix B: Composition of spruce sugar solution and EPAX-glycerol

Table B.1: The main components of the spruce sugar solution provided by Borregaard, displayed as concentration in g/L:

Component	g/L
Dry matter	673. 5
Glucose	502.4
Xylose	24.9
Galactose	2.0
Mannose	24.2
Fructose	1.3
Total monomeric sugar	555.6
Density (kg/L)	1.2449

Table B.2: The main components of the spruce sugar solution provided by Borregaard, displayed as concentration in %:

Component	%
Dry matter	54.1
Glucose	74.6
Xylose	3.7
Galactose	0.3
Arabinose	0.1
Mannose	3.6
Fructose	0.2
Total monomeric sugar	82.5

Table B.3: The composition of EPAX-glycerol obtained from data safety sheet:

Component	%
Glycerol	~ 91
Fishoil	~ 5
Sodium ethanolate	~ 4
Water	< 0.1
Ethanol	~ 0.03

Appendix C: Equations and calculations

μ_{max}

As an example of how μ_{max} was calculated, the plot of CDW data from one of the glucose fermentations of the first bioreactor experiment, and its ln CDW from the exponential growth rate is shown in figure C.1. The μ_{max} values were obtained from the slope of the ln curve. The average μ_{max} of both glucose fermentations, are presented in Table 3.2.1 The CDW data used for the calculation of these plots are shown in Appendix E.

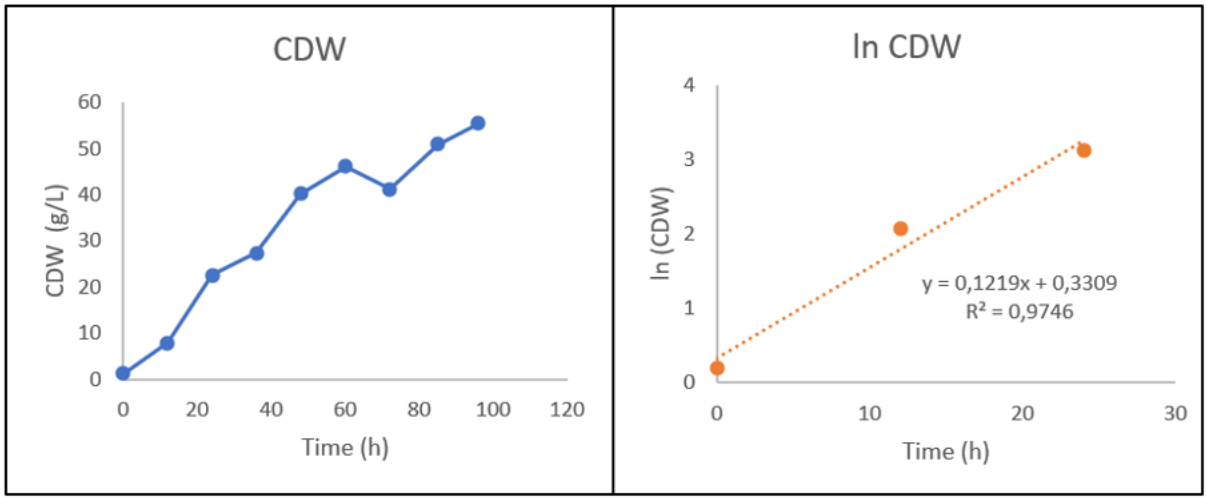

Figure C.1: Shows CDW (g/L) data (after medium correction) taken from glucose fermentation 1 in the first bioreactor experiment (Left panel), and the corresponding ln (CDW) from the exponential growth stage.

$Y_{x/s}$

$$Y_{x/s} = \frac{\Delta C_x}{\Delta C_s} \qquad \text{C.2}$$

The substrate concentrations were found by HPLC analysis of samples. The different substrate used for yield calculations were glucose, glycerol (for EPAX and glycerol medium) or glucose and xylose for the spruce sugar medium.

Appendix D: Stirrer speed and dissolved oxygen level in bioreactor fermentations

The stirrer speed and dissolved oxygen level (pO_2) for all bioreactors in both bioreactor experiments are shown in Figure D.1-4 below.

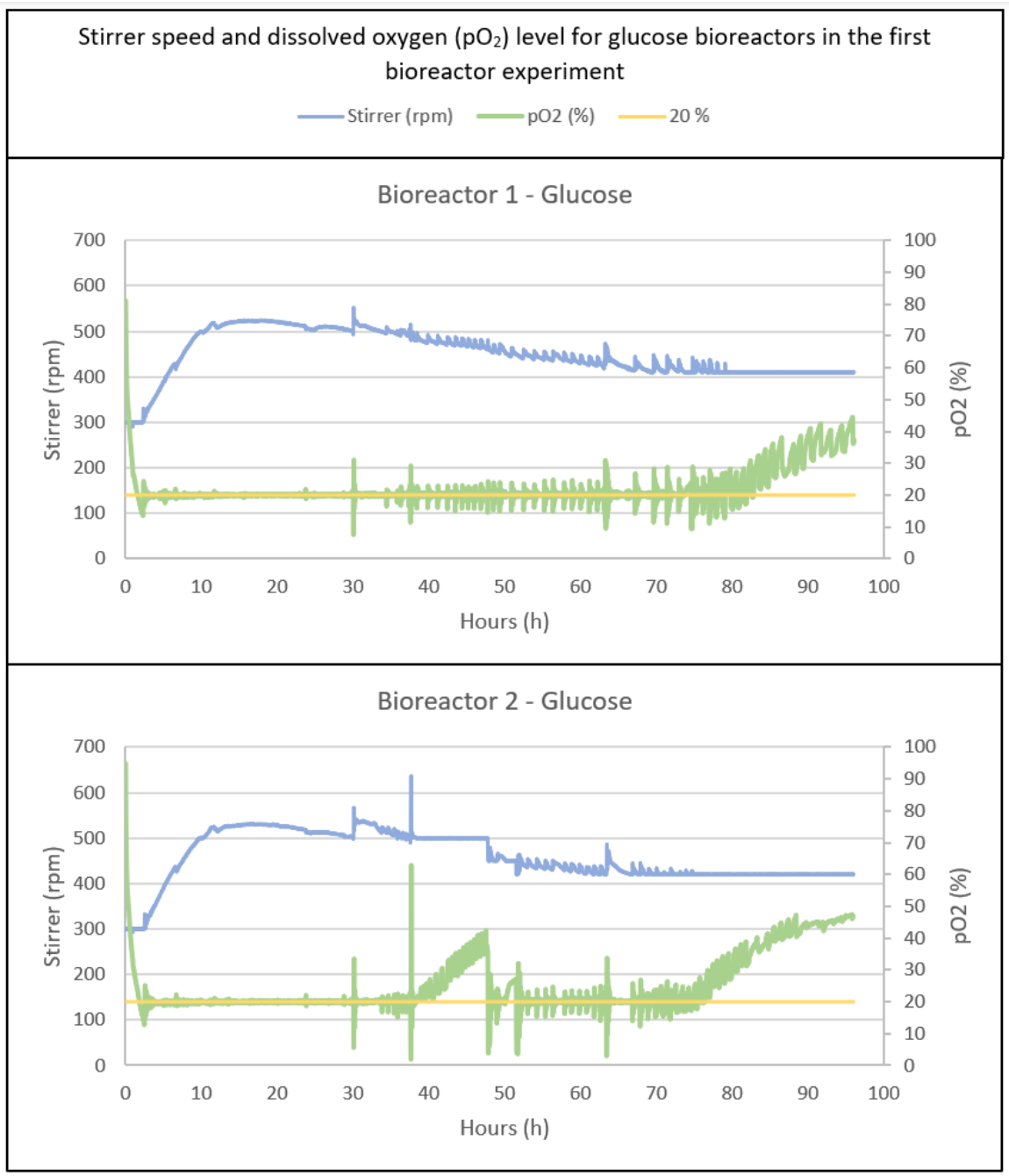

Figure D.1: Shows the stirrer speed (rpm), and pO_2 (%) level for bioreactors containing glucose medium, from the first bioreactor experiment.

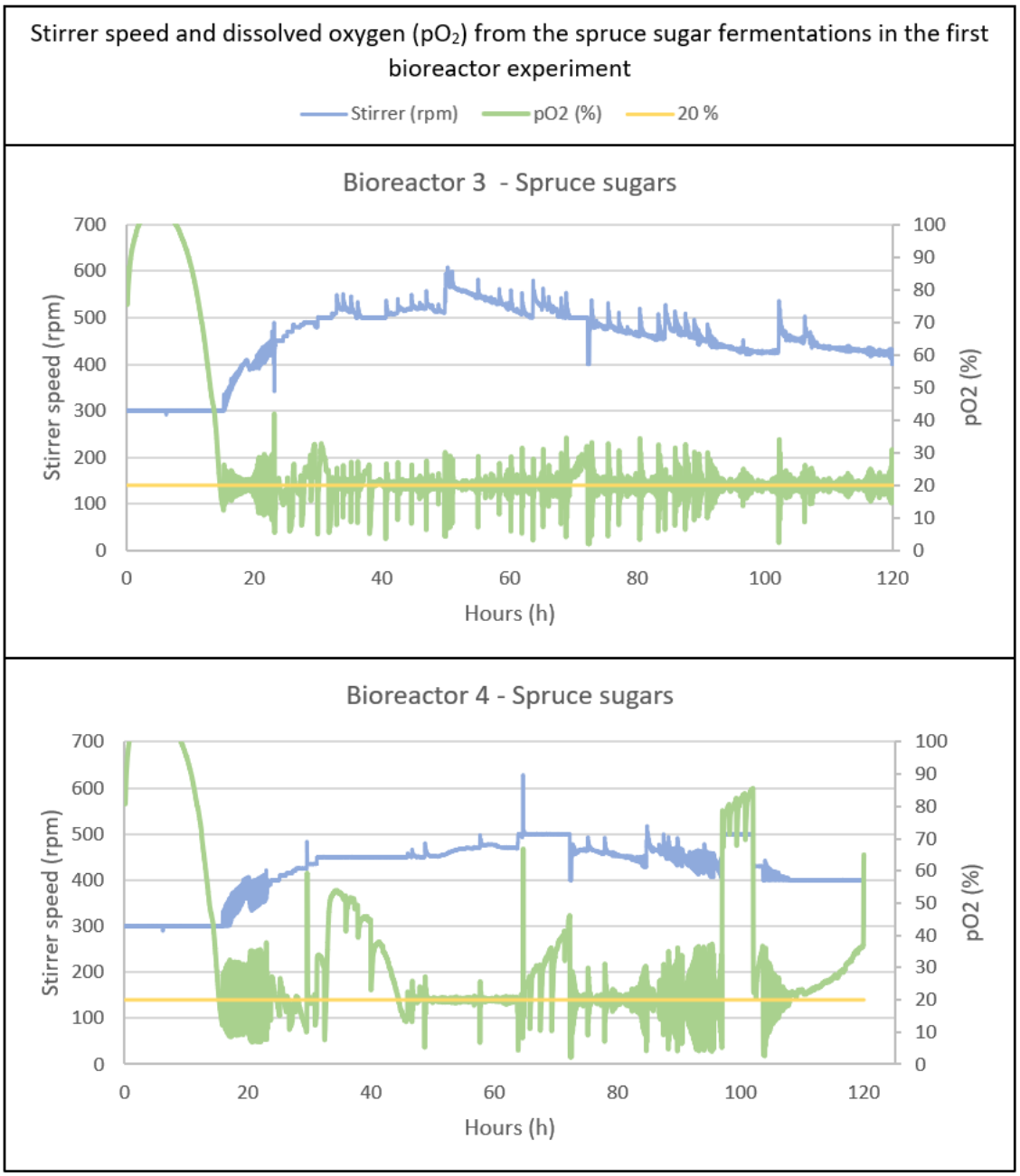

Figure D.2: Shows the stirrer speed (rpm), and pO_2 (%) level for cultivations containing spruce sugar medium, from the first bioreactor experiment.

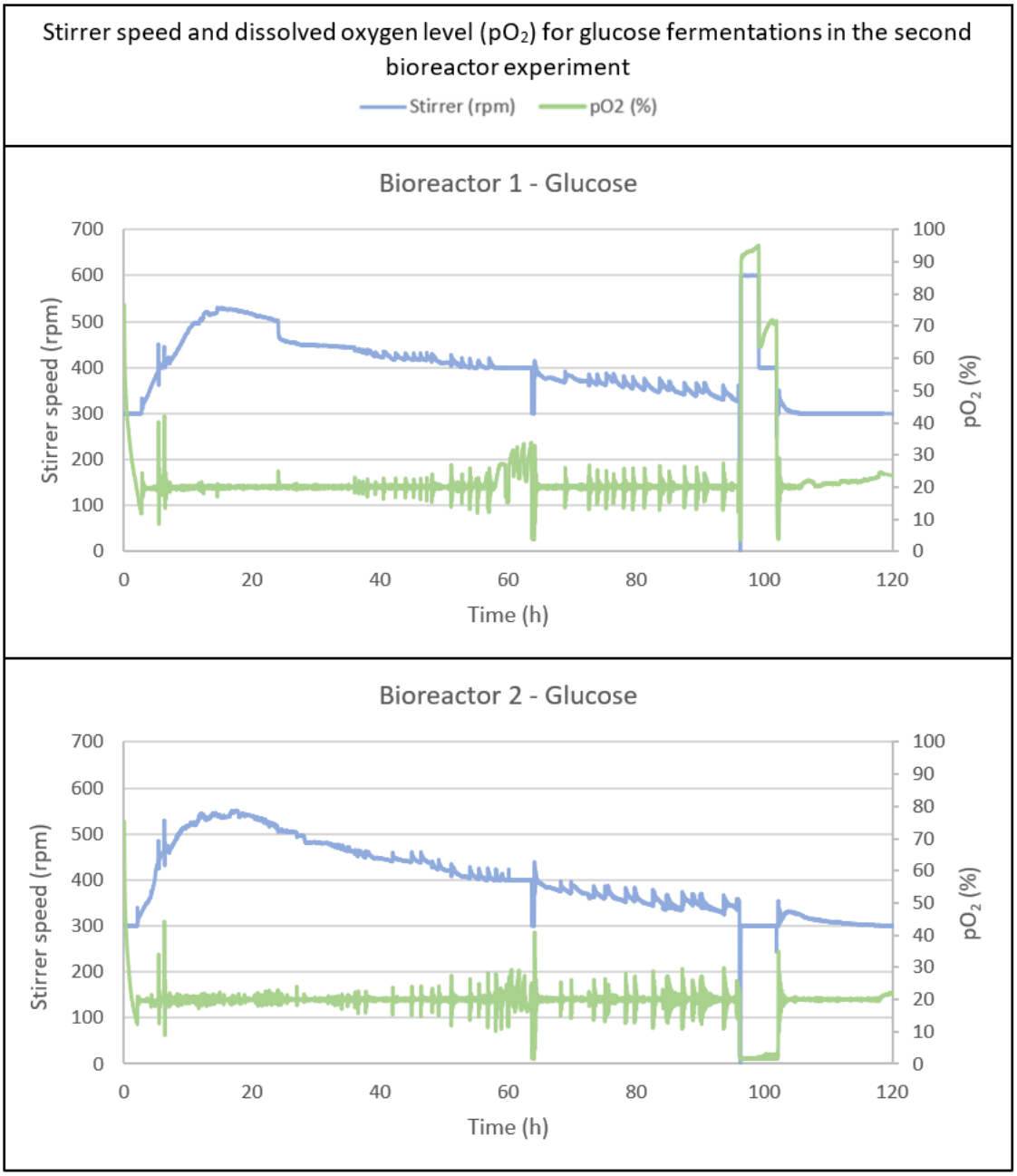

Figure D.3: Shows the stirrer speed (rpm), and pO_2 (%) level for fermentations containing glucose medium, from the second bioreactor experiment.

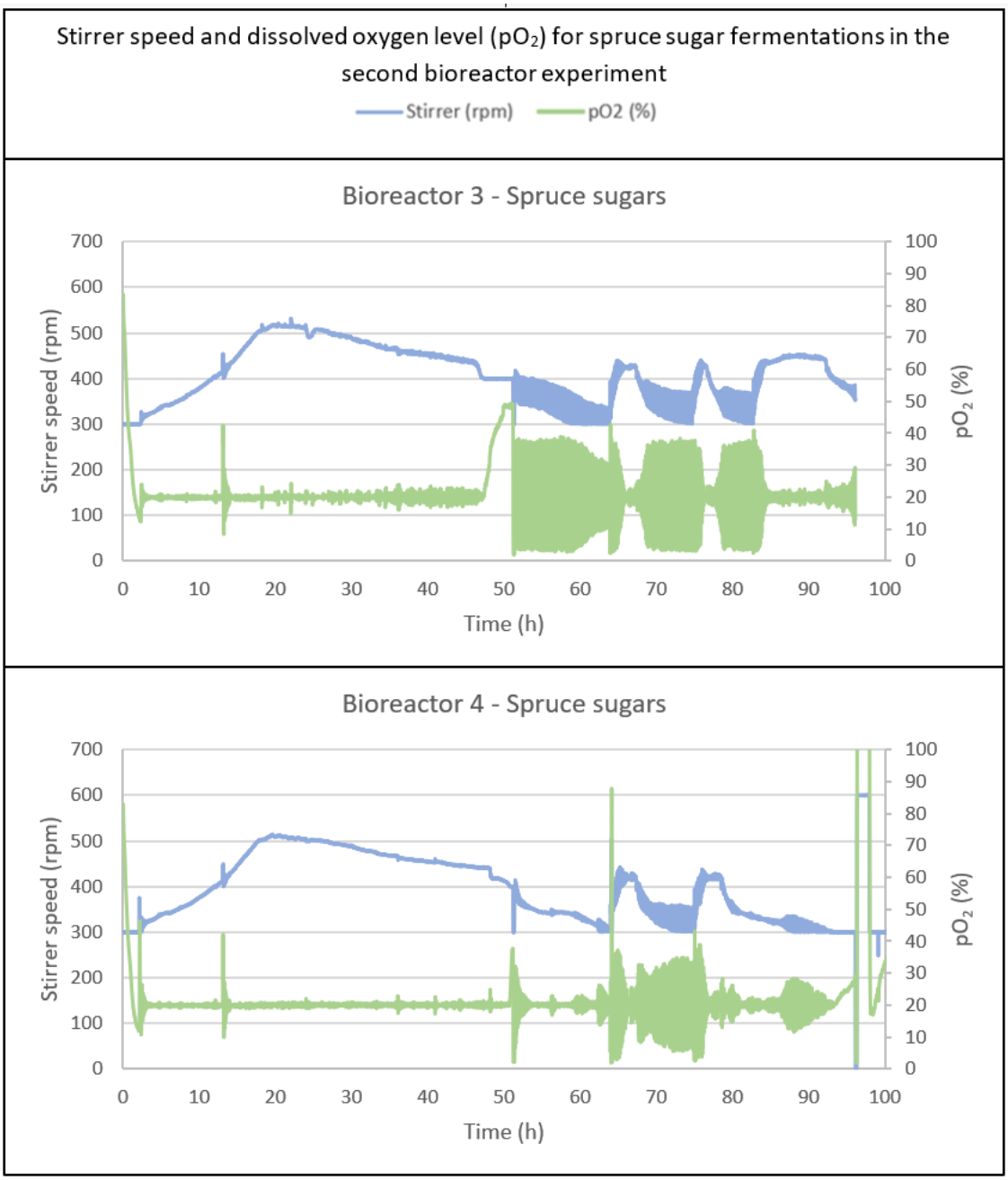

Figure D.4: Shows the stirrer speed (rpm), and pO_2 (%) level for fermentations containing spruce sugar medium, from the second bioreactor experiment.

Appendix E: CDW used as example for μ_{max} calculations

In table E.1 the CDW and the natural logarithm of these values, from glucose vessel 1 in the first bioreactor run are shown. The first three ln (CDW) values were plotted as a function of time, to obtain the graph in the right panel of Figure C.1.

Table E.1: Shows the CDW (g/L) and ln (CDW) for samples taken every 12 hours in the glucose fermentation in Bioreactor 1, of the first bioreactor run.

Time (h)	Corrected CDW (g/L)	ln (CDW)
0	1,2	0,19
12	7,9	2,07
24	22,6	3,12
36	27,4	3,31
48	40,2	3,69
60	46,1	3,83
72	41,2	3,72
85	50,9	3,93
96	55,4	4,02

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway