Unversität für Bodenkultur Wien

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Optimisation of Media Composition for the Fermentation of Lipids by *Aurantiochytrium limacinum* SR21

Master Thesis

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Abstract

Over the last decades, poly unsaturated fatty acid (PUFA)s have been increasingly recognized for their health benefits and therefore their importance in human diet. Among all ω-3 PUFAs, docosahexaenoic acid (DHA) is the most important one. It is essential for the development and function of brain and eyes in infants and in adults a deficit of DHA is associated with cardiovascular diseases, unipolar depression, attention deficit hyperactivity disorder and so forth. Fatty fish and their derived fish oils used to be the only source of DHA until the late 1990s, when DHA was first produced on an industrial scale using micro algae.

In this study the media composition and cultivation technique of the thraustochytrid *Aurantiochytrium limacinum* SR21 (*A. limacinum*) were optimised for the production of DHA. First attempts to cultivate *A. limacinum* in 9% glucose, 2% yeast extract and peptone from casein (YEP) medium resulted in a low average specific growth rate (μ_{av}) of 0.012 h^{-1} and 0.065 h^{-1} for shaken flask and bioreactor cultures respectively. As the next step, different N-sources (urea, NH_4NO_3 and $(NH_4)_2SO_4$) were tested in different concentrations to find a medium composition that would enable fast cell growth with low lipid production. Using a 2% glucose, 0.05% YEP, 31.2 mM $(NH_4)_2SO_4$ medium with a C:N ratio of 10:1, a μ_{av} of 0.243 h^{-1} and a total fatty acid (TFA) content of 2.24% were achieved with a biomass to substrate yield ($Y_{X/S}$) of 0.46 g g^{-1}. This medium was then used as batch medium for a fed-batch fermentation, which consisted of a biomass and a lipid production phase, which was designed to investigate the lipid production under N-limitation. During the feed phase an average volumetric docosahexaenoic acid productivity (q_{DHA}) of 119 mg L^{-1} h^{-1} was achieved with a final biomass concentration of 19.03 g L^{-1}. Cells at the end of the fermentation had a TFA and DHA content of 45.18% and 20.59% (45.56% of TFA) respectively. The q_{DHA} of 53.1 mg L^{-1} h^{-1} for the whole process is considerable lower compared to published data, due to the rather low biomass concentration.

This study showed that a fed-batch cultivation with a biomass and lipid accumulation phase is clearly preferable to a batch cultivation, NH_3 is well suited as a N-source for *A. limacinum* and a C:N ratio of 10:1 is optimal for the production of biomass with low lipid content. The feed medium should not contain a N-source, as loss of DHA in the cells appeared simultaneous with N uptake.

Zusammenfassung

Im Laufe der letzten Jahrzehnte wurden die gesundheitsfördernden Eigenschaften von mehrfach ungesättigten Fettsäuren (PUFAs) und deren Bedeutung in der menschlichen Ernährung zunehmend erkannt. Unter allen ω-3 PUFAs ist Docosahexaensäure (DHA) die Wichtigste. DHA ist essentiell für die Entwicklung von Gehirn und Augen von Neugeborenen und bei Erwachsenen ist ein DHA-Mangel mit Herz-Kreislauf-Erkrankungen, unipolarer Depression, Aufmerksamkeitsdefizit-/Hyperaktivitätsstörung usw. assoziert. Fette Fische und die von ihnen gewonnenen Fischöle waren die einzigen DHA Quellen bis Ende der 1990er, als DHA erstmals industriell mit Mikroalgen produziert wurde.

In dieser Arbeit wurden die Medienzusammensetzung und Kultivierungstechnik des Thraustochytriden *Aurantiochytrium limacinum* SR21 (*A. limacinum*) hinsichtlich der DHA Produktion optimiert. Erste Versuche, *A. limacinum* in 9% Glukose, 2% Hefeextrakt und Pepton aus Casein (YEP) Medium zu kultivieren, zeigten niedrige mittlere spezifische Wachstumsraten (μ_{av}) von jeweils 0.012 h^{-1} und 0.065 h^{-1} für Schüttelkolben und Bioreaktor Kulturen. Als nächsten Schritt wurden unterschiedliche Stickstoffquellen (Harnstoff, NH_4NO_3, $(NH_4)_2SO_4$) in unterschiedlichen Konzentrationen getestet um eine Medienzusammensetzung zu finden, die schnelles Zellwachstum mit niedriger Lipidproduktion ermöglicht. Mit einem 2% Glukose, 0.05% YEP, 31.2 mM $(NH_4)_2SO_4$t Medium (C:N Verhltnis von 10:1) wurde ein μ_{av} von 0.243 h^{-1}, ein totaler Fettsäuren (TFA) Gehalt der Zellen von 2.24% mit einen Biomasse Ausbeutekoeffizient ($Y_{X/S}$) von 0.46 g g^{-1} erreicht. Dieses Medium wurde anschließend als Batch-Medium in einer Fed-Batch Fermentation verwendet, die aus einer Biomasse- und Lipid-Produktionsphase bestand und deren Ziel die Untersuchung der Lipidproduktion unter N-Limitierung war. Wärend der Feed-Phase wurde eine volumetrische DHA Produktivität (q_{DHA}) von 119 mg L^{-1} h^{-1} mit einer Biomassekonzentration von 19.03 g L^{-1} erreicht. Zellen am Ende der Fermentation wiesen einen TFA- und DHA-Gehalt von jeweils 45.18% und 20.59% (45.56% von TFA) auf. Der gesamte Prozess hatte ein q_{DHA} von 53.1 mg L^{-1} h^{-1}, welches wegen der relativ niedrigen Biomassekonzentration deutlich unter bereits publizierten Produktionsraten liegt.

Diese Studie konnte zeigen, dass ein Fed-Batch Prozess, mit einer Biomasse- und einer Lipid-Produktionsphase, einem Batch-Prozess vorzuziehen ist. Des weiteren ist NH_3 eine gut geeignete N-Quelle und ein C:N Verhältnis von 10:1 scheint optimal fr die Produktion von Biomasse mit niedrigen Lipid-Gehalt zu sein. Das Feed-Medium sollte keine N-Quelle enthalten, da ein Verlust von DHA in den Zellen zeitgleich mit der Aufnahme von N auftrat.

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Contents

1 Introduction

1.1 Docosahexaenoic Acid (DHA)

Docosahexaenoic acid (DHA, $22{:}6^{\Delta 4,7,10,13,16,19}$) is an extreme example of an ω-3 poly unsaturated fatty acid (PUFA), with 22 carbon atoms and 6 cis double bonds, the first double bond beeing located at the third carbon atom, counted from the methyl group (ω end). The chemical structure of DHA is shown in figure 1.1.

Primary produces of DHA are marine microalgae, e.g. *Crypthecodinium cohnii*, *Pavlova lutheri* and certain species of thraustochytrids (Guschina and Harwood, 2006). Deep sea bacteria such as *Moritella marina* or *Vibrio marinus* have aswell been found to produce DHA (Morita et al., 2000, 1999), although they might be of less ecological importance in terms of DHA production.

Beside beeing a storage compound for energy and carbon in micro-organisms (Zweytick et al., 2000), DHA has been demonstrated to change many basic properties of membranes, once incorporated into membrane phospholipids. As summarized by Stillwell and Wassall (2003), DHA significantly alters acyl chain order and "fluidity", phase behavior, elastic compressability, permeability, fusion, flip-flop and protein activity. It is aswell concluded that DHA's interaction with other membrane lipids, especially cholesterol, may play an important role in modulating the local structure and function of cell membranes.

Unlike algae and plants, mammals lack the specific enzymes for the *de novo* synthesis of α-linolenic acid (ALA), which is the precursor of all ω-3 PUFAs. Although humans can endogenously synthesize DHA from ALA, studies have shown that this process is

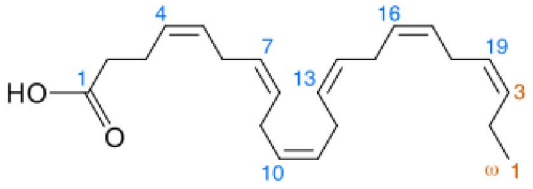


Figure 1.1: Chemical structure of docosahexaenoic acid (DHA). Blue numbers are the carbon atoms counted from the carboxyl group, brown ones are counted from the methyl group (ω end). Figure was taken from Wikipedia (2014).

not efficient. Estimates of the convertion efficiency of ALA to DHA range from 2-5% in healthy adults (Wijendran and Hayes, 2004) to 1% for infants and considerably lower for adults (Brenna et al., 2009). The low conversion of ALA to DHA is of particular concern for vegetarian and other people that don't eat fatty fish or fish oil supplements, due to the association of DHA deficiencies with cardiovascular diseases, unipolar depression, attention deficit hyperactivity disorder etc. (Horrocks and Yeo, 1999) as well as the importance for the growth, functional development and maintenance of the retina (SanGiovanni and Chew, 2005; Litman et al., 2001) and brain (Salem et al., 2001; Birch et al., 2000), of which DHA is the most abundant PUFA in the cell membrane.

1.1.1 Pathways for DHA Synthesis

Aerobic Pathways In almost all eukaryotes, synthesis of DHA starts with stearic acid (18:0) as precursor. Double bonds are inserted successively by $\Delta 9$, $\Delta 12$, $\Delta 15$ and $\Delta 6$ fatty acid desaturases, which require oxygen as cofactor. Mammals lack $\Delta 12$ and $\Delta 15/\omega 3$ fatty acid desaturases and therefore need exogenous α-linolenic acid (ALA, 18:3-9,12,15) as precursor for DHA synthesis. After the $\Delta 6$ desaturation of 18:4-6,9,12,15, an elongation to 20:4-8,11,14,17 and a $\Delta 5$ desaturation takes place, leading to eicosapentaenoic acid (EPA, 20:5-5,8,11,14,17).
In mammals DHA is currently thought to be synthesized by the so called Sprecher's shunt. EPA is elongated twice to 24:5-9,12,15,18,21, $\Delta 6$ desaturated to 24:6-6,9,12,15,-18,21, which is then β-oxidized to DHA (22:6-4,7,10,13,16,19).
In *Thraustochytrium* sp., a member of the Thraustochytriidae, a $\Delta 4$ desaturase has been described (Qiu et al., 2001), which was actually believed to participate in mammalian DHA synthesis, but researchers never managed to demonstrate its activity. Thus *Thraustochytrium* sp. is able to produce DHA in a more elegant way, by simply elongating eicosapentaenoic acid (EPA) (20:5-5,8,11,14,17) once with a fatty acid elongase to 22:5-7,10,13,16,19, which is then converted to DHA (22:6-4,7,10,13,16,19) by the $\Delta 4$ desaturase. Both aerobic pathways are shown in figure 1.2.

Anaerobic Pathway Metz et al. (2001) described a completely oxygen independent polyketide synthase (PKS) based pathway for PUFA synthesis in the marine bacterium *Shewanella* aswell as in the Thraustochytrid *Schizochytrium*. While the exact mechanisms are still not completely elucidated, it is known that the pathway is catalyzed by a multi-subunit PUFA synthase. In *Schizochytrium* the PUFA synthase is composed of three subunits (A, B and C) which are all homolog to those found in the marine bacteria PUFA synthase system (Hauvermale et al., 2006; Metz et al., 2001). Metz et al. (2001) suggested therefore that the PUFA synthase may have undergone lateral gene transfer, which is quite possible because some Thraustochytrids, including *Schizochytrium*, are able to phagocytize bacteria (Raghukumar, 1992). In addition, Metz et al. (2001) demon-

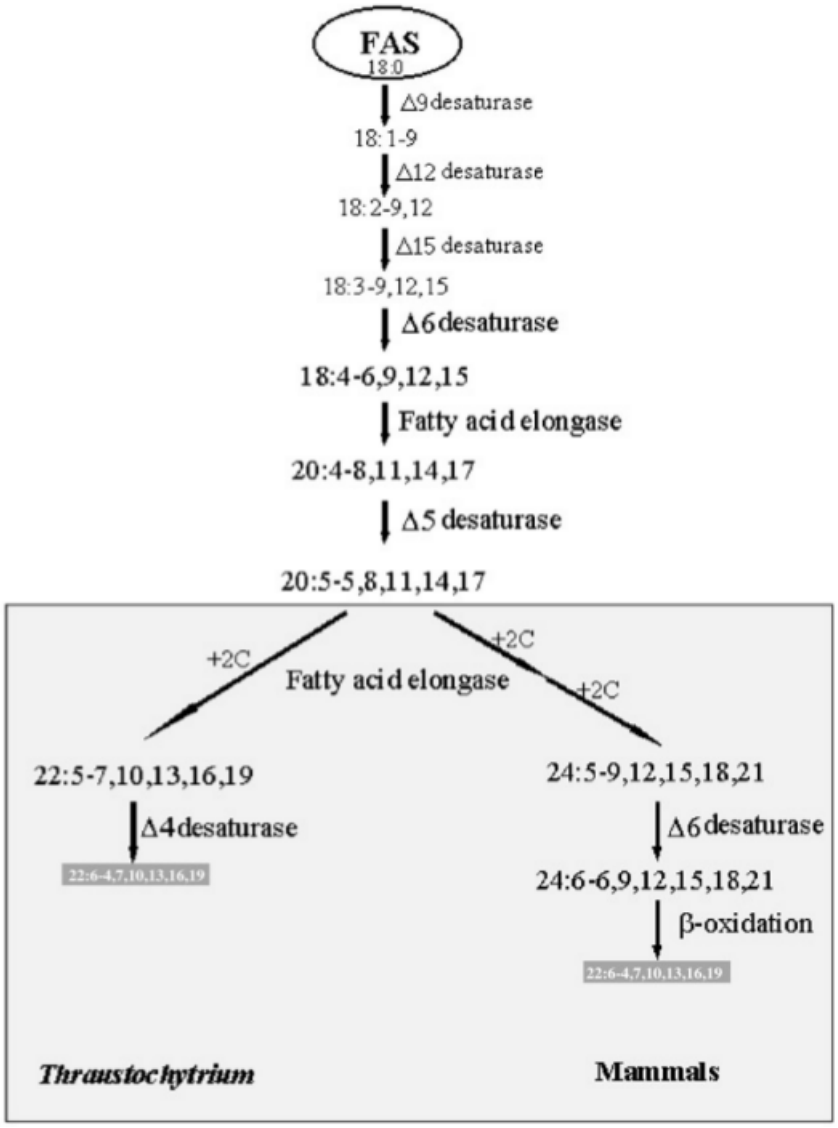


Figure 1.2: Aerobic pathways for docosahexaenoic acid (DHA) biosynthesis. Mammals lack $\Delta 12$ and $\Delta 15/\omega 3$ fatty acid desaturase and therefore require an exogenous supply of α-linolenic acid to synthesize DHA via the $\Delta 6$ desaturates/ β-oxidase pathway. Figure has been taken from Qiu (2003).

strated that 14:0 and 16:0 do not act as precursors for docosapentaenoic acid (DPA) and DHA synthesis in *Schizochytrium*, which was further supported by Hauvermale et al. (2006). It is also worth pointing out that DPA, which occurs in *Schizochytrium* and other Thraustochytrids that use or are thought to use the PKS pathway, is in the ω-6 form and not ω-3. The constant ratio of 5:1 DHA to DPA and labeling experiments with [^{14}C]acetate by Metz et al. (2001) indicate that DPA is produced by the same pathway as DHA and may even be its precursor.

1.1.2 Conventional Sources and Applications of DHA

Conventional Sources of DHA DHA, as other ω-3 PUFAs, is conventionally obtained from fatty fish species such as salmon, herring, mackerel, anchovies, and sardines in form of fish oil (Gunstone, 1996). However, the quality of the fish oil is variable and depends on the used fish species, season and location of the catching site. While crude fish oil is used in aquaculture, it needs extensive refinement until it can be used for human needs, due to contaminations by environmental pollution and problems associated with the typical fishy smell and unpleasant taste.

Fish Oil Production Crude fish oil, which is used for aquaculture and other animal feeds, is produced by cooking the fish material. Afterwards a filter press is used to remove solids from the suspension. The filter cake is used as fish meal. To obtain crude fish oil, the water and other impurities are removed by a centrifugation step. Depending on the later use, further refinement of the crude fish oil is performed. The crude fish oil is winterised to remove waxes or fats naturally contained in the oil to make it limpid. For this procedure the oil is slowly cooled down to obtain a homogeneous cristallisation of waxes and fats. It is then filtered through cloths that retain these waxes or fats. Crude winterised fish oil is then neutralised to remove free fatty acids, either by saponification of the free fatty acids and centrifuged or distillation. The oil is further refined by a bleaching step in which the pigments responsible for the colour are eliminated by the use of bleaching clays (bentonites), which are later removed by filtration. During this stage, it is possible to add active carbons in the oil to capture and eliminate the major part of the contaminants such as dioxins, PCBs (polychlorinated biphenyls) and PAHs (polycyclic aromatic hydrocarbons). These active carbons are later removed by the filtration. Afterwards a deodorisation step is carried out to remove volatile substances responsible for the odours and significantly reduce traces of pesticides (Olvea, 2014).

Applications The major application of fish oil is currently aquaculture. It is not only used to increase the content of PUFAs in the cultured fish, but is also needed for the development and reproduction of decapod crustaceans (Harrison, 1990). A global survey, conducted between December 2006 and October 2007 concerning the use of fish meal

and fish oil within compound aquafeeds, estimated that in 2006 the aquaculture sector consumed 3724 thousand tonnes of fish meal (68.2% total global fish meal production in 2006) and 835 thousand tonnes of fish oil (88.5% total reported fish oil production in 2006), or the equivalent of 16.6 million tonnes of small pelagic forage fish (Tacon and Metian, 2008).
It is due to the high dependancy of aquaculture on fish meal and oil, in conjunction with the globaly declining fish stocks, that the costs for both products are globaly increasing. It is predicted that dietary fish meal and fish oil inclusion levels within compound aquafeeds will decrease in the long term, with fish meal and fish oil usage increasingly being targeted for use as a high value specialty feed ingredient for use within higher value starter, finisher and broodstock feeds, and by doing so extending supply of these much sought after and limited feed ingredient commodities (Tacon and Metian, 2008).
Apart from its use in aquaculture and livestock breeding, DHA and DHA containing oils are used as additives in infant formulas, functional food and dietary supplements. Fish oil has a number of disadvantages, as already mentioned before, that limit its application range (Sijtsma and de Swaaf, 2004). Especially the polluted nature of fish oil raised concerns about the use in infant formulas and thus technologies like single cell oils (SCO) are now a well established alternative sources for DHA and other PUFAs. However, compared to fish oil production, SCO production happens on a rather small scale with about 800 tons in 2003 (Cohen and Ratledge, 2005).

1.2 Single Cell Oils

SCO might be defined as the edible oils obtainable from microorganisms and are similar in type and composition to those oils and fats from plants or animals (Cohen and Ratledge, 2005). Interest in SCO emerged during the early 20^{th} century. Paul Lindner, working at the Institut für Gärungsgewerbe in Berlin, appears to have been the first person to develop a process for fat production using *Trichosporon pullulans*. The real breakthrough for SCO came in the late 1980s and early 1990s with the realization of the importance of PUFAs in the human diet. The presence of arachidonic acid (ARA) and DHA in mother's milk, together with their occurence as major PUFAs of brain lipids and retinal membrane lipids, supported the idea that it would be beneficial to include both PUFAs in the diet of pregnant women and infant formulas. Since fish oil contains DHA and EPA in more or less equal amounts, and EPA having the disadvantageous property to metabolically interfere with the efficacy of DHA uptake and its incorporation into brain and retinal lipids, it was not suitable for the application in infant formulas. Extraction of DHA from fish oil would have required several steps culminating in the use of preparative level high performance liquid chromatography (HPLC), which was simply not economical (Cohen and Ratledge, 2005).

Martek Biosiences Corp., founded in the late 1980s, on the other hand were able to produce DHA in large scale fermentors (200 m^3) using *Crypthecodinium cohnii* as the organism of choice, leading to the product DHASCO® which has since had a major impact on the infant nutrition market. ARA has aswell been produced by Martek, using the fungus *Mortierella alpina* and is sold under the product name ARASCO®. Other companies that are producing SCO are DSM (*M. alpina*, ARA), Wuhan Alking Engineering (*M. alpina*, ARA), Omega-Tech (*Schizochytrium sp.*, DHA) and Nutrinova GmbH (*Ulkenia sp.*, DHA) (Ratledge, 2004).

1.2.1 Generall Information about Thraustochytrids

Thraustochytrids are unicellular marine heterotroph protists, that form together with labyrinthulids the class of Labyrinthulomycetes in the phylum Stramenopiles, which is part of the kingdom Chromalveolata. The family of Thraustochytriaceae is composed of the genera *Althornia*, *Aplanochytrium*, *Elnia*, *Japonochytrium*, *Schizochytrium*,*Thraustochytrium* and *Ulkenia* (Adl et al., 2012). Yokoyama and Honda (2007) proposed an emendation of the genus *Schizochytrium* sensu lato by dividing it into the three genera *Schizochytrium* sensu stricto, *Aurantiochytrium* and *Oblongichytrium* gen. nov. based on their combined morphological characteristics, the PUFA profiles, carotenoid pigments they contain and 18S rRNA. Therefore *Aurantiochytrium limacinum*, which was used in this study, was formerly known as *Schizochytrium limacinum* and both names are still used in the literature.

In contrast to labyrinthulids, thraustochytrids are rarely found on living plants and seem to be inhibited by plant antimicrobials. They are rather found on dead plant material like mangrove leaves submerged in water or on microalgae. Thraustochytrids might play an important role as saprobes in the marine ecosystem, due to their production and secretion of degradative enzymes. They are ubiquitous in marine ecosystems and have been found in oceanic waters, costal waters, sediments, deep sea waters (down to 4,000 m) and even oxygen minimum zones. While thraustochytrid biomass doesn't correlate with algal blooms, the biomass of thraustochytrids is at about the same level as the bacterial during periods of phytoplankton decay. They contribute a significant amount to the mineralisation of photoplankton and zooplankton detritus in the sea (Raghukumar, 2002).

The vegetative life cycle of thraustochytrids consists of single cells with an overall globular shape and a diameter between 4 and 20 m. Most species reproduce by zoospores and the exact way of reproduction is one major distinctive feature between the species. Almost all species produce an ectoplasmatic net (EN) from one or more points of the cell wall. The EN extends the cell surface and improves nutrient uptake from the environment (Raghukumar, 2002).

Thraustochytrids can utilise a broad spectrum of carbon and nitrogen sources, and were thought to always require on a minimum level of sodium ions which cannot be replaced by potassium ions (Raghukumar, 2008). Shabala et al. (2009), however, were able to elucidate the ionic mechanisms of osmotic adjustment in *Thraustochytrium* by using a non-invasive ion-selective microelectrode technique and thus were able to grow *Thraustochytrium* in sodium free media by using mannitol as substitute for sodium ions to adjust the osmotic pressure. Potassium ions cannot replace sodium ions, because potassium only plays a minor role in the process of osmotic adjustment by the cells.

Thraustochytrids became biotechnologically interesting due to their abilities to grow to high biomass concentrations (up to 200 g L^{-1}) and store up to 50% of their biomass as lipids, with a high portion of them being PUFAs. The majority of the lipids occur in the form of triacylglycerol and a smaller part as phospholipids (Lewis et al., 1999).

1.2.2 Aurantiochytrium limacinum SR21

Aurantiochytrium limacinum SR21 (*A. limacinum*) was first isolated in 1994 from the coral reef area of the Yap Islands in the Federated States of Micronesia. In a screening for DHA production of about 400 strains, grown on 20 g L^{-1} glucose, 10 g L^{-1} peptone and 5 g L^{-1} yeast extract agar plates, one strain was identified to produce DHA using high-performance liquid chromatography (HPLC). This isolate, strain SR21, was identified first as *Thraustochytrium* sp., but was thought later to be *Schizochytrium* sp. due to successive binary division of its vegetative cells Nakahara et al. (1996).

After a suitable strain was identified, optimization of culture conditions was done, both in flask and stirred tank fermenter. The high production performance, simple fatty acid profile with a majority in DHA (22:6 ω-3) and DPA (22:5 ω-6) PUFAs and a high specific growth rate made this strain an excellent source of microbial DHA.

As mentioned before, the genus *Schizochytrium* was rearranged by Yokoyama and Honda (2007) and now *Schizochytrium* aswell as *Aurantiochytrium* are both used as genus names for the strain SR21.

The first describtion of the strain, based on morphological features and assimilative profiles of carbon compounds, was done by Honda et al. (1998). The stronges distinctive features to other thraustochytrids are the limaciform amoeboid cells, the size of the released zoospores and the assimilated carbon sources. The best utilized carbon sources are glucose, fructose, mannose, galactose and glycerol.

Strain description by Honda et al. (1998): "Cells spherical, 7-15 μm diameter with ectoplasmic net element; zoosporangium, 12-24 μm diameter, producing 16-64 zoospores; zoospores ovoid, 6-8.5 μm long and 5-7 μm wide with two laterally inserted unequal flagella; in sea water/pine pollen culture and nutrient medium, vegetative cells forming limaciform amoeboid cells, 12-20 μm long and 5-8 m wide with pseudopodia; rounded amoeboid cell forming eight zoospores, 4.5-6 μm long and 3.5-5 μm wide."

1.2.3 Cultivation Parameters Effecting the Production of DHA in Thraustochytrids

Nitrogen Nitrogen limitation has long been recognised to induce the accumulation of fatty acid (FA)s in oleaginous microorganisms (Cohen and Ratledge, 2005). In the abscence of nitrogen, excess carbon/energy is stored in the form of FA which can even be seen under the microscope by the formation of lipid bodies in the cytoplasm (Morita et al., 2006). Once nitrogen is available again, these storage compounds can be methabolized by the cell to fuel cell growth and reproduction. Although there is a lot of information available in the literature on the effect of different nitrogen sources effecting the productivity of DHA in thraustochytrids, some authors do not take different C:N ratios into account when complex nitrogen sources are assessed for their influence on DHA production. Thus e.g. using the same amount of corn steep liquor as yeast extract might simply result in a higher DHA content of the cells due to the lower nitrogen content of corn steep liquor. Nevertheless, the form in which nitrogen is provided to cells by the medium is important for cell growth and reproduction of the cells, as different forms of nitrogen are metabolized differently.

Phosphor Phosphor limitation was shown by Jakobsen et al. (2008), using *Aurantiochytrium* sp. strain T66, to induce lipid accumulation similar to nitrogen limitation. While the ratio of DHA to TFA was higher compared nitrogen limitation, max. biomass concentrations were only about 50% of nitrogen limited cultures. Since phosphate was completely exausted at the first sampling point, 22 h after inoculation, the low biomass concentration was probably a consequence of an earlier onset of limitation, which results in less FA producing biomass.

Oxygen The discrepancy in the literature regarding the the effect of oxygen on the production of DHA is probably due to the fact that some thraustochytrids use oxygen independant PKS like PUFA synthase for the synthesis of DHA, while others use oxygen dependant elongase/desaturase pathway. For thraustochytrids using a PUFA synthase, oxygen limitation below 3% (Bailey et al., 2010) or even below 1% (Jakobsen et al., 2008) can raise the DHA to TFA ratio by depleting monounsaturated FA from the cells, which are produced by oxygen dependent desaturase(s), while thraustochytrids using the aerobic pathway require a high oxygen level for DHA production (Chang et al., 2013).

2 Aim of Work

Over the last years the biotechnological interest in thraustochytrids has been increasing, due to their ability to effectively synthesize polyunsaturated fatty acids, especially docosahexaenoic acid (DHA). Since nitrogen limitation is the most common method to induce lipid production in oleaginous microorganisms, different nitrogen sources and C:N ratios were tested during this study and their effect on the specific growth rate and fatty acid (FA) production were investigated.

During the previous work of Rappel (2013) on *Aurantiochytrium limacinum* SR21, a medium composition for reliable growth of *A. limacinum* was established. In the current study, the medium composition and cultivation technique were further improved in respect of lipid production, in particular docosahexaenoic acid (DHA).

Since the established medium had rather low substrate concentrations to prevent possible inhibition, the first step of this work was to find out how *A. limacinum* reacts to higher substrate concentrations.

In the next step, different chemically defined nitrogen sources like urea, ammonium nitrate and ammonium sulfate were tested in different concentrations with glucose as carbon source. Specific growth rate and total fatty acid (TFA) content at the end of the cultivation period were taken into account for the selection of the medium composition, which would afterwards be used as batch medium in a fed-batch experiment.

The fed-batch experiment was designed to get a basic knowledge about the production of FAs under nitrogen limitation. Therefore a batch medium, that would produce a biomass with a low TFA content, was used. After the batch phase, the culture was fed with a glucose rich, nitrogen poor medium to investigate FA production.

3 Materials and Methods

3.1 Auratiochytrium limacinum SR21

The strain *A. limacinum* was ordered from the American Type Culture Collection (ATCC), described as "*Schizochytrium limacinum* Honda et al. SR21 [IFO 32693]", item description "MYA-1381".

3.2 Media and Stock Solutions

3.2.1 Articifial Seawater

For the cultivation of *A. limacinum*, an artificial sea water (ASW) solution was used, which mimicked the salt composition of undiluted sea water. The final concentration of ASW in the culture medium was 50% (w/w).

Materials

- Salts and stock solutions as listed in table 3.1
- Suitable glassware (500 mL, 1 L or 2 L glass bottles)
- Beakers of appropriate volume
- 1000 μL piston stroke pipette
- 1000 μL plastic tips autoclaved (121 ℃, 1 atm overpressure, 20 min)
- deionised water (DI H_2O)
- Measuring cylinder
- Balance with an accurateness of 0.01 g
- Magnet stirrer and rod
- laminar flow closet with HEPA filter (Laminar)
- Steam autoclave

Method

- All salts as listed in table 3.1 were weighted in beakers and dissolved in a sufficient amount of DI H_2O.
- A vessel of appropriate volume was weighted with a stirring rod inside and after filled with DI H_2O to about 70% of the final mass.
- The vessel was put on the magnet stirrer and all dissolved salt solutions were added in the same order as listed in table 3.1.
- After the vessel was put on the balance, stock solutions (except vitamines and K_2HPO_4 stock solutions) were added using a 1000 μL piston stroke pipette and filled up to the final mass with DI H_2O subtracted by the amount of stock solutions which were added after autoclavation.
- The ASW solution was autoclaved for 20 minutes at 121 ℃ at 1 atm overpressure in a steam autoclave.
- After the solution was cooled down to room temperature, vitamine- and K_2HPO_4 stock solutions were added under the Laminar using a 1000 μL piston stroke pipette with sterile plastic tips. The solution was stored at 4 ℃.

3.2.2 Stock Solutions

Citric Acid Monohydrate Stock Solution 0.656 g of citric acid monohydrate were solved in 200 mL DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Ferric Ammonium Citrate Stock Solution 0.60 g of ferric ammonium citrate were solved in 200 mL DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Ethylenediaminetetraacetic Acid Stock Solution 0.1005 g of $Na_2EDTA \cdot 2\,H_2O$ were solved in 200 mL DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Sodium Carbonate Stock Solution 4.0 g of Na_2CO_3 were solved in 200 mL DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Dipotassium Phosphate Stock Solution 11.453 g of K_2HPO_4 were solved in 200 mL DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Table 3.1: Preparation of 1 L artificial sea water (ASW)

Compound	$\mathbf{c_{stock}}$ [a] [g L^{-1}]	**Amount**	**Unit**
Components added before heat sterilisation [b]			
NaCl	-	25.00	g
$MgCl_2 \cdot 6\,H_2O$	-	2.00	g
KCl	-	0.50	g
$NaNO_3$	-	0.75	g
$MgSO_4 \cdot 7\,H_2O$	-	3.50	g
$CaCl_2 \cdot 2\,H_2O$	-	0.50	g
Citric acid monohydrate stock	3.28	1.00	mL
Ferric ammonium citrate stock	3.00	1.00	mL
$Na_2EDTA \cdot 2\,H_2O$ stock	0.5025	1.30	mL
Na_2CO_3 stock	20.0	1.00	mL
Trace metal stock	see table 3.2	1.00	mL
Components added after heat sterilisation [b]			
K_2HPO_4	57.265	1.00	mL
Vitamin B_{12}	0.1352	1.00	mL
Biotin	0.0250	1.00	mL
Thiamine-HCl	1.0960	1.00	mL

[a] concentration of the stock solution (c_{stock})

[b] heat sterilisation was done by autoclaving the solution in a steam autoclave (121 ℃, 1 atm overpressure, 20 min)

Table 3.2: Preparation of 1 L trace metal stock solution

Trace Metal	**M** [g mol^{-1}]	**Amount** [g]	**c** [mM]
H_3BO_3	61.83	2.860	46.25
$MnCl_2 \cdot 4\,H_2O$	197.91	1.810	9.15
$ZnSO_4 \cdot 7\,H_2O$	287.56	0.222	0.77
$Na_2MoO_4 \cdot 2\,H_2O$	241.95	0.390	1.61
$CuSO_4 \cdot 5\,H_2O$	249.69	0.079	0.32
$CoCl_2 \cdot 6\,H_2O$	237.93	0.040	0.17

Table 3.3: Vitamin stock solutions

Stock Solution	**M** [g mol^{-1}]	**Amount** [g]	**c** [mM]
Vitamin B_{12}	1355.37	0.027	0.1
Biotin	244.31	0.005	0.1
Thiamine-HCl	337.27	0.22	6.5

Each vitamin was solved in 200 mL 50 mM HEPES buffer (pH 7.8).

Trace Metal Stock Solution 1 L of a trace metal stock solution was prepared by heating up 900 mL of DI H_2O and once boiling point was reached, trace metals as listed in table 3.2 were added under constant stirring. Afterwards the solution was filled up to the final volume, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Phosphate Buffer 1 M 13.61 g of KH_2PO_4 were weighted in a 100 mL glass bottle, filled up to 85 mL with DI H_2O, adjusted to pH 7.0 with saturated NaOH and filled up to the final volume of 100 mL with DI H_2O. The solution was autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

HEPES Buffer 50 mM 5.96 g HEPES were solved in 500 mL DI H_2O, adjusted to pH 7.8 with NaOH, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Vitamin Stock Solutions Amounts of Vitamin B_{12}, Biotin and Thiamin-HCl as listed in table 3.3 were solved each in 200 mL of 50 mM HEPES buffer pH 7.8. To sterilise the stock solutions, each stock was sterile filtrated in the Laminar with a 0.22 μm filter, using 5 mL sterile syringes, into autoclaved (121 ℃, 1 atm overpressure, 20 min) 20 mL flasks with metal caps. All vitamin stocks were stored in the dark at -20 ℃.

3.2.3 Carbon and Nitrogen Sources

For the cultivation 50% (w/w) ASW medium with glucose as carbon source and YEP, as the basic nitrogen source, were used. To investigate the growth behaviour of *A. limacinum* on other N-sources than YEP and also higher C:N ratios, which can't be achieved

Table 3.4: Chemically defined nitrogen sources

N-Source	**M** [g mol^{-1}]	**Amount** [g]	**c** [M]
Urea	60.06	15.02	1.0
Ammonium sulfate	132.14	33.04	1.0
Ammonium nitrate	80.04	20.01	1.0

Listed amounts were solved in 250 mL DI H_2O.

by YEP alone, urea, ammonium nitrate and ammonium sulfate were used in addition to YEP.

Glucose 400 g kg^{-1} Stock Solution Glucose was always used as carbon source for the cultivation of *A. limacinum*. An aqueous stock solution of 400 g kg^{-1} was prepared, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Yeast Extract and Peptone 10 g kg^{-1} Stock Solution 2.5 g of yeast extract and 2.5 g of peptone from casein were weighted in a 500 mL glass bottle and filled up to 500 g with DI H_2O to produce a 10 g kg^{-1} YEP stock solution. The solution was autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Yeast Extract and Peptone 20 g kg^{-1} Stock Solution 5.0 g of yeast extract and 5.0 g of peptone from casein were weighted in a 500 mL glass bottle and filled up to 500 g with DI H_2O to produce a 10 g kg^{-1} YEP stock solution. The solution was autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Yeast Extract and Peptone 100 g kg^{-1} Stock Solution 25 g of yeast extract and 25 g of peptone from casein were weighted in a 500 mL glass bottle and filled up to 500 g with DI H_2O to produce a 10 g kg^{-1} YEP stock solution. The solution was autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃.

Chemically defined Nitrogen Sources For testing different nitrogen souces, 250 mL of 1 M urea (CH_4N_2O) , ammonium sulfate ($(NH_4)_2SO_4$) and ammonium nitrate ($(NH_4)(NO_3)$) stock solutions were prepared, autoclaved (121 ℃, 1 atm overpressure, 20 min) and stored at 4 ℃. Amount weighted in and molecular mass of each substance are listed in table 3.4.

3.3 Shaking Flask Experiments

3.3.1 Media Preparation for Shaking Flask Experiments

Materials

- Laminar
- 70% (w/w) ethanol
- Balance with an accurateness of 0.01 g
- Empty glass bottle of appropriate size with plastic cap, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Erlenmeyer flasks: 300 mL baffled flasks with cotton wool, 500 mL baffled flasks with plastic caps with PTFE membrane for gas exchange, 2000 mL shaking flasks with metal caps, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- ASW, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Glucose stock solution [400 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- YEP stock solution [10 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- YEP stock solution [20 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- YEP stock solution [100 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Urea stock solution [1 M], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Ammonium nitrate stock solution [1 M], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Ammonium sulfate stock solution [1 M], autoclaved (121 ℃, 1 atm overpressure, 20 min)

Method

- Laminar was switched on and allowed to establish a sterile laminar air flow for at least 10 min before anything was put inside.
- Sterile empty glass bottle, ASW, DI H_2O, stock solutions (glucose, YEP, urea, NH_4NO_3, $(NH_4)_2SO_4$), sterile Erlenmeyer flasks and balance were wiped off with 70% (w/w) ethanol and put in the Laminar.

Table 3.5: Shaking flask media

Medium	Glucose	YEP [a]	Urea	NH_4NO_3	$(NH_4)_2SO_4$	Buffer [b]
Inoculum	5 g L^{-1}	1 g L^{-1}	-	-	-	-
Inoculum with Urea	5 g L^{-1}	1 g L^{-1}	1 g L^{-1}	-	-	-
2% Glucose	20 g L^{-1}	4 g L^{-1}	-	-	-	-
9% Glucose	90 g L^{-1}	20 g L^{-1}	-	-	-	-
0.3% Urea	20 g L^{-1}	-	3 g L^{-1}	-	-	-
0.3% Urea with YEP	20 g L^{-1}	0.5 g L^{-1}	3 g L^{-1}	-	-	-
pH Urea 20:1	20 g L^{-1}	0.5 g L^{-1}	15 mM	-	-	-
pH Urea 6:1	20 g L^{-1}	0.5 g L^{-1}	50 mM	-	-	-
pH NH_4NO_3 20:1	20 g L^{-1}	0.5 g L^{-1}	-	15 mM	-	-
pH NH_4NO_3 6:1	20 g L^{-1}	0.5 g L^{-1}	-	50 mM	-	-
pH $(NH_4)_2SO_4$ 20:1	20 g L^{-1}	0.5 g L^{-1}	-	-	15 mM	-
pH $(NH_4)_2SO_4$ 6:1	20 g L^{-1}	0.5 g L^{-1}	-	-	50 mM	-
pH YEP 20:1	20 g L^{-1}	4 g L^{-1}	-	-	-	-
Urea 20:1	20 g L^{-1}	0.5 g L^{-1}	15 mM	-	-	10 mM
Urea 10:1	20 g L^{-1}	0.5 g L^{-1}	31.2 mM	-	-	10 mM
NH_4NO_3 20:1	20 g L^{-1}	0.5 g L^{-1}	-	15 mM	-	10 mM
NH_4NO_3 10:1	20 g L^{-1}	0.5 g L^{-1}	-	31.2 mM	-	10 mM
$(NH_4)_2SO_4$ 20:1	20 g L^{-1}	0.5 g L^{-1}	-	-	15 mM	10 mM
$(NH_4)_2SO_4$ 10:1	20 g L^{-1}	0.5 g L^{-1}	-	-	31.2 mM	10 mM
YEP 20:1	20 g L^{-1}	4 g L^{-1}	-	-	-	10 mM

[a] yeast extract and peptone from casein (YEP)

[b] KH_2PO_4 pH 7.00 (Buffer)

- Amounts (measured with the balance) of each medium component, as listed in table 3.5, were put in the sterile empty glass bottle, mixed by shaking and filled into the Erlenmeyer flasks which were later used for the cultivation of *A. limacinum*.
- The preparation of media with chemicaly defined nitrogen sources was done by filling the amounts of ASW, glucose, YEP and phosphate buffer for all media in an empty glass bottle and splitting it up upon the Erlenmeyer flasks. Each medium was then completed in the Erlenmeyer flasks with the particular amounts of DI H_2O and chemically defined nitrogen source.

3.3.2 Maintenance of the Research Cell Bank

From the already established research cell bank (RCB) by Rappel (2013), a slightly different cryo stock was established. The culture was grown on 2% (w/w) glucose, 0.36% (w/w) urea and 0.05% (w/w) YEP to ensure that cells would also be adapted to urea when testing their growth characteristics on different nitrogen sources.

Materials

- Laminar
- 100 and 500 mL glass bottles with plastic caps, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- 500 mL DURAN® baffled flasks with PTFE membrane screw cap, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- 70% (w/w) ethanol
- ASW
- Glucose stock solution [400 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- YEP stock solution [10 g kg^{-1}], autoclaved (121 ℃, 1 atm overpressure, 20 min)
- DI H_2O, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Cryo stock of *A. limacinum* from the previous RCB
- 200 μL piston stroke pipette
- 200 μL plastic tips, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Balance with an accurateness of 0.01 g
- Orbital shaker Thermo Scientific MaxQ 3000
- 80% (w/w) glycerol, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- 10 mL glas pipettes with cotton wool, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Accu pipettor
- 1.5 mL reaction tubes, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Cryo rack
- -80 ℃ freezer
- Breeding chamber set to 25 ℃

Method

- 500 mL glass bottle, two 100 mL baffled flasks, ASW, glucose stock, YEP stock, DI H_2O, 200 μL piston stroke pipette with tips, balance and an *A. limacinum* cryo stock were wiped off with 70% (w/w) ethanol and put under the Laminar.
- 200 g of medium containing 2% (w/w) glucose, 0.4% (w/w) YEP and 50% (w/w) ASW were prepared in a 500 mL glass bottle, using the balance to measure the amount of each component.
- Two baffled flasks were filled each with 100 g medium and one of the flasks was inoculated with 100 μL *A. limacinum* cryo stock, while the other was kept as a sterile control. Cultures were incubated at 25 ℃ on an orbital shaker at 150 rpm for 30 hours.
- The next day, 500 g of medium with 2% (w/w) glucose, 0.36% (w/w) urea and 0.05% (w/w) YEP were prepared under the Laminar in a 500 mL glass bottle and split up equally upon two 500 mL baffled flasks. One of the flasks was inoculated with 5 mL of the 30 h culture, while the other one served as sterile control.
- After 30 hours, 50 g of the culture were put together with 11.5 g 80% (w/w) glycerol (autoclaved at 121 ℃, 1 atm overpressure for 20 min) in a sterile 100 mL glass bottle to create a 15% glycerol stock. The solution was mixed by shaking and allowed to stay for 30 min to ensure glycerol uptake into the cells.
- Sterile 1.5 mL reaction tubes were put in a cryo rack under the Laminar, labeled and filled with 1 mL glycerol stock each, using a sterile 10 mL glass pipette. The RCB was stored at -80 ℃.
- For cell dry weight (CDW) determination 2 x 10 mL culture were collected and treated as described under 3.5.1.

3.3.3 Sampling for Shaking Flasks Experiments

Materials

- Laminar
- 70% (w/w) ethanol
- 10 mL glas pipettes with cotton wool in a metal cylinder, autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Accu pipettor

- 12 mL centrifugation tubes with appropriate rack
- 50 mL centrifugation tubes with appropriate rack

Method

- Laminar was switched on and allowed to establish a sterile laminar air flow for at least 10 min.
- Shaking flasks, accu pipettor, pipettes and centrifugation tubes were wiped off with ethanol and put inside the Laminar.
- For regular shaking flasks covered with metal caps or cotton wool, 12 mL samples were taken with the sterile pipettes and filled into 12 mL centrifugation tubes. At sampling points for PUFA analysis, additional 40 mL were taken and filled into 50 mL centrifugation tubes. To ensure a homogen biomass distribution, cultures were always shaken before and between every sampling step.
- For baffled flasks with plastic cap and PTFE membrane, samples were taken by pouring the required volume into the centrifugation tubes.
- Sample processing was performed as described under section 3.5.

3.4 Bioreactor Experiments

3.4.1 Bioreactor

A stainless steel bioreactor (Bioengineering AG) with a total volume of 16 L and a maximum working volume of 10 L was used for the experiments. The reactor had a jacket to control temperature by an external supply of cold and hot water (external heating device). An inspection glass provided a view inside the bioreactor to see the broth and check foam development.

In addition, the bioreactor had three fix speed peristaltic pumps (Bioengineering AG, 23430 Peripex W1, 100 rpm), sterile filters (Bioengineering AG) for air in- and outlet, a return cooler to prevent water loss via the offgas, a light source, a manometer and Ingold connections.

A double Rushton 6-blade impeller (80 mm diameter) combined with four baffles, which were arranged in combination with the sparger, were used for homogenisation of the broth. The impeller was powered by an engine (Unitec Motor 1,1 kW), achieving maximum rotational speeds of up to 1500 rpm. Samples were collected through a sampling valve (Bioengineering AG), which could be steam sterilized by an external steaming device (Faber Trevil).

Table 3.6: Inoculum media for bioreactor experiments

Medium	Glucose	YEP [a]	RCB [b]	Volume
Ino Batch	20 g L^{-1}	4 g L^{-1}	1000 μL	2 x 250 mL
Ino Fed-Batch	5 g L^{-1}	1 g L^{-1}	200 μL	2 x 200 mL

Both media had an artificial sea water concentration of 50%.

[a] yeast extract and peptone from casein (YEP)

[b] research cell bank (RCB)

Information about relevant process parameters was obtained from a thermometer (Pt 100), an anti foam level probe (DN19, Bioengineering AG), an oxygen sensor (Mettler Toledo InPro 6820) equipped with Teflon membranes (Mettler Toledo O2 membrane t-96) and a pH electrode (MT 405-DPAS-SC-K85/120 combination pH). Temperature, impeller speed and peristaltic pumps were automatically controlled by a process control unit (ISE GmbH), while oxygen and pH were controlled by Mettler Toledo pH-panel 2100e and Mettler Toledo O2 panel 4100e respectively. All process parameters were also saved on an external PC, with the according software installed (iFIX Startup, Version 3.0, 2002 (Build 5179)).

3.4.2 Inoculum Preparation

Materials Additional to the materials listed under 3.3.1 an autoclaved (121 ℃, 1 atm overpressure, 20 min) 500 mL glass bottle with a biosystem (a plastic cap with two openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter) was used.

Method

- The media were prepared as written under 3.3.1 with media concentrations as listed in table 3.6. 500 mL baffled flasks with plastic caps, with a PTFE membrane for gas exchange, were used for the cultivation of the inoculum cultures.
- After about 30 hours, the inoculum cultures were transfered under the Laminar into the sterile 500 mL bottle with a biosystem.
- To inoculate the bioreactor, the plastic bag of the hypodermic needle was removed, the needle was punched through a silicone septum and the culture broth was pumped into the bioreactor using one of the peristaltic pumps.

3.4.3 Preparation of Batch Media

Materials

- Balance with an accurateness of 0.01 g
- Beakers of a appropriate size
- 4 L plastic beaker
- Magnet stirrer and rod
- 2 L empty glass bottle closed with a biosystem (a plastic cap with two openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter)
- 16 L bioreactor (Bioengineering AG), 10 L working volume
- DI H_2O
- Salts and stock solutions as listed in table 3.1
- Glucose
- Yeast extract
- Peptone from casein
- Ammonium sulfate
- 1000 μL piston stroke pipette
- 1000 μL plastic tips autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Laminar
- 70% (w/w) ethanol
- Steam autoclave

Method

- Salts as listed in table 3.1, yeast extract, peptone and ammonium sulfate (for the 2% glucose medium) were weighted in beakers and presolved in a sufficient amount of DI H_2O.
- 4 L beaker with stirring rod was weighted on the balance and filled with about 2 kg of DI H_2O.

Table 3.7: Composition of batch media

Medium	Glucose	YEP [a]	$(NH_4)_2SO_4$	Buffer [b]	Volume
Batch	100 g L^{-1}	22.2 g L^{-1}	-	-	4.5 L
Fed-Batch	21.05 g L^{-1}	0.526 g L^{-1}	4.34 g L^{-1}	10.53 mM	3.8 L

Both media had an artificial sea water (ASW) concentration of 50%. Glucose and NaCl from the ASW were autoclaved separately to prevent the formation of maillard products (glucose) and corrosion of the bioreactor (NaCl). Concentrations of media components were adjusted to meet the final concentration after adding the inoculum (10% and 5% for Batch and Fed-Batch media respectively).

[a] yeast extract and peptone from casein (YEP)

[b] KH_2PO_4 pH 7.00 (Buffer)

- Presolved salt solutions (except NaCl), ASW stock solutions (added before sterilization), yeast extract, peptone and ammonium sulfate were put in the beaker under constant stirring and afterwards filled up on the balance to a final mass of 3 kg with DI H_2O.
- 450 g (9% glucose medium) or 80 g (2% glucose medium) of glucose were solved together with the NaCl salt solution in DI H_2O in a 2 L glass bottle and filled up to a final mass of 1500 g or 800 g respectively.
- The ASW-N-source-solution was poured into the bioreactor and autoclaved at 121 ℃and 1 atm overpressure for 20 min.
- The glucose-NaCl-solution was closed with the biosystem, the tube with the hypodermic needle was pinched off with a metal clamp and then sterilized in a steam autoclave at 121 ℃ and 1 atm overpressure for 20 min. After the solution was cooled down to room temperature, phosphate buffer (for 2% glucose medium) and ASW stock solutions, which were supposed to be added after sterilization, were put inside under the Laminar.
- To complete the media in the bioreactor, the plastic bag of the hypodermic needle was removed, the needle was punched through a silicone septum and the glucose-NaCl-solution was pumped into the bioreactor using one of the peristaltic pumps.

3.4.4 Preparation of Feed Medium

Materials

- Balance with an accurateness of 0.01 g
- Beakers of a appropriate size
- Magnet stirrer and rod
- 2 L glass bottle with biosystem (a plastic cap with two openings, one with a silicone tube attached, the second opening connected by a short tube to a 0.22 μm sterile filter)
- 6 L glass bottle closed with biosystem (a plastic cap with three openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter, the third opening is connected by a silicone tube to a reusable 0.22 μm sterile filter), autoclaved (121 ℃, 1 atm overpressure, 20 min)
- DI H_2O
- Salts and stock solutions as listed in table 3.1
- Glucose
- Ammonium sulfate
- 1000 μL piston stroke pipette
- 1000 μL plastic tips
- 1000 μL plastic tips autoclaved (121 ℃, 1 atm overpressure, 20 min)
- Laminar
- 70% (w/w) ethanol
- Hand pump

Method

- Salts and stock solutions as listed in table 3.1, glucose and ammonium sulfate were weighted in beakers and presolved in a sufficient amount of DI H_2O.
- A 2 L glass bottle with a stirring rod inside was weighted and put on a magnetic stirrer. Glucose-, ammonium sulfate- and ASW solutions were put inside and filled up with DI H_2O to a final mass of 1 kg.

Table 3.8: Composition of 1 L feed medium

Medium	Glucose	$(NH_4)_2SO_4$	ASW [a]	Volume
Feed	250 g L^{-1}	2.75 g L^{-1}	50%	1 L

Medium was sterilized by filtrating the solution through a reusable 0.22 μm filter.
[a] artificial sea water (ASW)

- Vitamin and K_2HPO_4 stock solutions were pipetted under the Laminar into the bottle using a 1000 μL piston stroke pipette with sterile tips.
- The bottle was closed with the biosystem (two openings) and sterile filtrated into the sterile 6 L glass bottle. This was done by connecting the silicone tube to the reusable filter of the 6 L bottle and creating overpressure with the hand pumb that was attached to the sterile filter of the 2 L bottle.

3.4.5 Preliminary Work and Start of the Fermentation

Materials

- 16 L bioreactor (Bioengineering AG), 10 L working volume
- 3 x fix speed peristaltic pumps (Bioengineering AG, 23430 Peripex W1, 100 rpm)
- Sterile filter (Bioengineering AG) connected by a tube to a hypodermic needle for air inlet
- Sterile filter with return cooler (Bioengineering AG) for air outlet
- Double Rushton 6-blade impeller (80 mm diameter)
- Baffle basket with sparger
- Steam generator (Faber Trevil)
- Sample valve (Bioengineering AG)
- Thermometer (Pt 100)
- Antifoam probe (DN19, Bioengineering AG)
- Clark electrode (Mettler Toledo InPro 6820) with a Teflon membrane (Mettler Toledo O2 membrane t-96)
- pH electrode (MT 405-DPAS-SC-K85/120 combination pH)

- 250 mL glass bottles with biosystem (a plastic cap with two openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter)
- 2 L glass bottle with biosystem (a plastic cap with two openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter)
- 1 L glass bottle with biosystem (a plastic cap with two openings, one connected by a plastic tube to a hypodermic needle (sealed with a plastic bag), the second opening connected by a short tube to a 0.22 μm sterile filter)
- 2 x 500 mL glass bottle with biosystem (a plastic cap with two openings, one with a silicone tube attached, the second opening connected by a short tube to a 0.22 μm sterile filter)
- 2 x silicone tubes with peristaltic pump tube attached and hypodermic needle, both ends sealed by plastic bags
- GLANAPON DG 160 (antifoam)
- NaOH
- KH_2PO_4
- pH calibration solutions with pH 7.01 and 4.01
- Electrolyte for Clark electrode

Method

Preliminary Work

- The rubber septum of the sampling valve was exchanged and the sampling valve was packed in aluminuium foil with all valves openen.
- All hypodermic needles were packed in plastic bags, of which the air was squeezed out, and sealed with autoclavation tape. Also the ends of the two peristaltic pump tubes were put in plastic bags and sealed with autoclavation tape.
- 500 mL of 10% (w/w) NaOH and 10% (w/w) KH_2PO_4 were prepared in 500 mL glass bottles. Biosystems without hypodermic needle were attached to the bottles afterwards.

- 200 mL of 10% (w/w) GLANAPON DG 160 were prepared in a 250 mL glass bottle, which was closed with a biosystem and the tube with the hypodermic needle was closed by a clamp.
- Glucose-NaCl-solution (preparation described under subsection 3.4.3) was filled in a 2 L glass bottle and closed with a biosystem. The tube of the biosystem connected to the hypodermic needle was pinched off with a clamp.
- Sampling valve, peristaltic pump tubes with hypodermic needle, Glucose-NaCl-solution, inoculum bottle with biosystem and antifoam solution were sterilized in a steam autoclave (121 ℃, 1 atm overpressure, 20 min).

Preparation of the Bioreactor

- Steam generator was filled with DI H_2O and switched on.
- Water inside the bioreactor was drained and discarded.
- Before the bioreactor was opened, electric current supply of the motor was switched off. The off gas cooling was removed from the lid and the reactor was opened to check if the baffle basket with sparger and impeller were installed correctly.
- Teflon membrane of the Clark electrode was changed if necessary and electrolyte was refilled.
- pH electrode was calibrated using pH 7.01 and 4.01 buffer solutions.
- Sampling valve was assambled and screwed in the reactor, aswell as the pH electrode and Clark electrode.
- The ASW solution with N-source was poured into the bioreactor.
- Lid was closed, off gas cooling with sterile filter was screwed on, off gas cooling was connected to the cooling water pipes and their valves were opened.
- Autoclavation program was started and at 100 ℃ the valve of the off gas was closed. After autoclaving the bioreactor (121 ℃, 1 atm overpressure, 20 min) the valve was opened again during the cool down after reaching about 100 ℃.
- Medium was completed by pumping the Glucose-NaCl-solution into the bioreactor, using a fix speed peristaltic pump. The tube was then pinched off with a clamp.
- Base and acid (for the batch experiment) were connected with the peristaltic pump tubes, hypodermic needles were punched through the silicon septa and pumps were activated to adjust the pH of the medium to pH 7.

- Antifoam was connected to the bioreactor.
- Sterile filter with hypodermic needle was connected to the air pipe, the hypodermic needle was punched through the silicone septum into the sparger and gas flow was turned on.

Calibration of the Oxygen Sensor

- Once the temperature was constant on 25 ℃, the oxygen sensor was calibrated.
- The air flow was set to 1 vvm and the impeller speed was manualy set to 800 rpm.
- The zero point calibration was carried out with a Mettler Toledo O_2 Simulator 82 by simulating a 0 nA signal and entering the code 1001 on the control panel.
- Afterwards the cable was put back on the Clark electrode and the 100% air saturation calibration was done by entering the the code 1100 on the control panel.

Inoculation and Start of the Fermentation

- The inoculum was transfered under the Laminar into a sterile 500 mL bottle with a biosystem. 40 mL of culture broth were taken to determine the CDW of the inoculum and thus calculate the biomass at the start of the fermentation.
- To inoculate the bioreactor, the plastic bag of the hypodermic needle was removed, the needle was punched through a silicone septum and the culture broth was pumped into the bioreactor using one of the peristaltic pumps.
- With this step the fermentation was started an the first sample was taken.

3.4.6 Experiment Parameters

Parameters for the bioreactor experiments are shown in table 3.9. For the Batch experiment, pH was controlled with 10% (w/w) NaOH and 10% (w/w) KH_2PO_4. For the Fed-Batch experiment, only 10% (w/w) NaOH was used to control the pH. During the Fed-Batch experiment, pH was set from 7.00 to 6.00 because crystals precipitated after the addition of the inoculum, and minimal stirring speed was set later onto 400 rpm to increase the air cooling of the motor.

Table 3.9: Parameters of bioreactor experiments

Experiment	Volume [L]	Temperature [℃]	O_2 air sat. [%]	pH	Stirring Speed
Batch	5	25	30	7.00	300 - 800
Fed-Batch	4 + 1	25	30	7.00	300 - 800

3.4.7 Sampling

Materials

- Bioreactor with sampling valve
- External steaming device
- 50 mL centrifugation tube
- 20 mL plastic syringe

Method

- Before the acctual sample was taken, 5 mL of culture broth were sucked through the sampling valve, using the plastic syringe, and discarded to exchange the dead volume of the sampling valve and prevent sample dilution.
- Afterwards 25 mL of culture broth were collected into a 50 mL centrifugation tube for further analyses. In case of lipid analysis, additional 40 mL of culture broth were collected in another 50 mL centrifugation tube.
- Sampling valve was flushed with steam, with completely opened steam valve, for about 30 seconds and for another 15 minutes with partialy opened steam valve, after which the valve was closed.

3.4.8 Decontamination, Cleaning and Resetting the Bioreactor to the Initial State

After the experiments were finished, the fermentation was stopped and the bioreactor was decontaminated for 20 minutes at 80 ℃. Culture broth was discarded and the bioreactor and all parts were cleaned with water and rinsed with DI H_2O to avoid calcification. To reset the bioreactor to its initial state, all silicone septa were exchanged, all blind plugs were screwed in, the reactor was filled with 4 L of DI H_2O and autoclavation program was started (121 ℃, 1 atm overpressure, 20 min).

3.5 Analytics

3.5.1 Gravimetrical Cell Dry Weight Determination

Materials

- Accu pipettor
- 10 mL volumetric pipette
- 12 mL centrifugation tubes
- DI H_2O
- 20 mL glass flasks closed with a metal cap
- 20 mL glass flasks, dried for 24 hours at 105 ℃, stored inside the desiccator
- Eppendorf Centrifuge 5804
- Rotor: Eppendorf A-4-44
- Desiccator
- Drying chamber set to 105 ℃
- Balance with an accurateness of 0.01 g
- Analytical balance with an accurateness of 0.0001 g

Method

- Culture samples were shaken vigorously and 10 mL (2 x 10 mL for bioreactor experiments) were transfered with a volumetric pipette into a 12 mL centrifugation tube.
- 12 mL tubes were put in centrifugation racks and in each rack one empty 12 mL centrifugation tube was used as tare weight.
- Centrifugation racks were put on the balance and adjusted with DI H_2O to a maximum weight difference of 0.1 g.
- Samples were centrifuged for 10 minutes at 2000 x g.
- Suppernatant of each sample was poured in a 20 mL glass flask, closed with a metal cap, labeled and stored at -20 ℃for further analysis

- Biomass was washed with 10 mL DI H_2O, centrifuged under the same conditions and suppernatant was discarded. This step was done twice.
- The cell pellet was transfered quantitatively into a preweight 20 mL glass flask of the desiccator. For the bioreactor experiments, the two pellets were united into one flask.
- The pellet was dried for 24 hours at 105 ℃, afterwards put in the desiccator to cool down and weighted.
- The biomass concentration in g L^{-1} was calculated from the weight difference.

3.5.2 Optical Density at 600 nm

Materials

- Disposable semi-micro cuvettes
- 15 ml Falcon tubes
- Piston stroke pipettes
- Suitable tips
- Vortex mixer
- Photometer Lange DR 5000
- DI H_2O

Method The sample of culture broth was appropriately diluted in Falcon tubes with DI H_2O to obtain an absorbance at 600 nm between 0.1 0.6. The dilution was vortexed and measured without delay to ensure proper dispersion. For the dilutions, no bigger dilution rate than 1:20 per step was carried out. To receive the OD_{600} the measured absorbance was multiplied by the dilution factor.

3.5.3 Cell Number Determination

Materials

- Thoma counting chamber with cover glass
- 96% ethanol
- Upright microscope, Olympus CH-2
- Lens cleaning tissue

- 20 μL piston stroke pipette with plastic tips
- Vortex mixer
- DI H_2O
- 15 mL Falcon tubes

Method

- Thoma counting chamber was cleaned with 96% ethanol and dried with lens cleaning tissue.
- The bridges of the counting chamber were wetted with a small amount of 96% ethanol, cover glass was put on top and carfully pressed on the bridges, with a slight up and down movement, until Newton's rings occurred.
- Sample, or a dilution made in a 15 mL Falcon tube, was vortexed and loaded with a 20 μL piston stroke pipette.
- Undamaged cells of 8 big squares, each with a volume of 4 nL (0.20 mm x 0.20 mm x 0.10 mm), were counted and the cell concentration was calculated by Formula 3.1.

$$CN = \frac{\sum_{i=1}^{n} CC_i}{n} * \frac{DF}{4 * 10^{-6}} \tag{3.1}$$

CN = cells per mL
CC = cells counted per square
DF = dilution factor of the suspension

3.5.4 Microscopy

Materials

- Upright microscope, Olympus CH-2
- DCM130 Microscope CMOS Camera
- Culture samples
- 20 μL piston stroke pipette with plastic tips
- Microscope slides
- Cover glasses
- Immersion oil
- Lens cleaning tissue

Method To follow the morphological development of the cells during the experiment, pictures were taken of every culture at each sampling point. 10 μL of undiluted cultured broth were pipetted on a microscope slide, using a 20 μL piston stroke pipette, and covered with a cover glass. Pictures of the samples were taken at 10 x 10, 10 x 40 and in one case at 10 x 100 magnification. To take pictures with a 10 x 100 magnification, immersion oil was put on the cover glass. After using the microscope, the lens was cleaned with a lens cleaning tissue.

3.5.5 pH Measurement

The pH of the Erlenmeyer flask samples were measured with a benchtop pH electrode (Thermo Scientific ORION 4 Star). Bioreactor experiments were monitored online with a pH electrode (MT 405-DPAS-SC-K85/120 combination pH).

3.5.6 Non Purgeable Organic Carbon (NPOC) and Total Nitrogen (TN)

Materials

- Potassium hydrogen phthalate ($C_8H_5KO_4$), dried in the desiccator
- Potassium nitrate (KNO_3), dried in the desiccator
- 1000 mL, 100 mL, 50 mL volumetric flasks
- 5 mL measuring pipette
- 20 mL, 10 mL, 5 mL volumetric pipettes
- 1000 μL piston stroke pipette with plastic tips
- Accu pipettor
- 20 mL TOC-flasks with open plastic caps (Shimadzu)
- Parafilm
- Shimadzu TOC-$L_{CPH/CPN}$ analyzer
- Shimadzu ASI-L autosampler
- Analytical balance with an accurateness of 0.0001 g
- 0.22 μm sterile filters
- 5 mL plastic syringe

Generation of the non purgeable organic carbon (NPOC) Calibration Curve

- 2.125 g of dried potassium hydrogen phthalate were weighted in, transfered into a 1000 mL volumetric flask which was filled to the mark with DI H_2O and shaken. This stock solution served as the 1000 ppm standard solution.
- 100 ppm standard solution: 10 mL of the stock solution were transfered with a 10 mL volumetric pipette into a 100 mL volumetric flask and filled up to the mark with DI H_2O.
- 300 ppm standard solution: 30 mL of the stock solution were transfered with a 10 mL volumetric pipette into a 100 mL volumetric flask and filled up to the mark with DI H_2O.
- 600 ppm standard solution: 60 mL of the stock solution were transfered with a 20 mL volumetric pipette into a 100 mL volumetric flask and filled up to the mark with DI H_2O.
- For the preparation of 10, 30 and 60 ppm standard solutions, 5 mL of the 100, 300 and 600 ppm standard solutions were transfered into 50 mL volumetric flasks and filled up to their mark with DI H_2O.
- All standard solutions were shaken vigorously before 20 mL of each were transfered into 20 mL TOC-flasks, which were sealed with Parafilm and afterwards plastic caps were srewed on top of the Parafilm. The standard solutions were stored at 4 ℃.
- The flasks were placed in the autosampler, measured and two calibration curves were generated. The first ranged from 10 to 100 ppm and the second from 100 to 1000 ppm.

Generation of the total nitrogen (TN) Calibration Curve

- 7.219 g of dried potassium nitrate were weighted in, transfered into a 1000 mL volumetric flask which was filled to the mark with DI H_2O and shaken. This stock solution served as the 1000 ppm standard solution.
- 100 ppm standard solution: 10 mL of the stock solution were transfered with a 10 mL volumetric pipette into a 100 mL volumetric flask, filled up to the mark with DI H_2O and mixed by shaking.
- 60 ppm standard solution: 3 mL of the stock solution were transfered with a 1000 μL piston stroke pipette into a 50 mL volumetric flask, filled up to the mark with DI H_2O and mixed by shaking.

$$Y_{x/s} = \frac{x - x_0}{s_0 - s} \tag{3.2}$$

$Y_{x/s}$ = biomass to substrate yield
x = final CDW concentration
x_0 = start CDW concentration
s_0 = substrate concentration at the start of the fermentation

- 30 ppm standard solution: 1.5 mL of the stock solution were transfered with a 1000 μL piston stroke pipette into a 50 mL volumetric flask, filled up to the mark with DI H_2O and mixed by shaking.
- For the preparation of 1, 3, 6 and 10 ppm standard solutions, 5 mL of the 10, 30, 60 and 100 ppm standard solutions were transfered into 50 mL volumetric flasks, filled up to their mark with DI H_2O and mixed by shaking.
- All standard solutions were shaken vigorously before 20 mL of each were transfered into 20 mL TOC-flasks, which were sealed with Parafilm and afterwards plastic caps were srewed on top of the Parafilm. The standard solutions were stored at 4 ℃.
- The flasks were placed in the autosampler, measured and two calibration curves were generated. The first ranged from 1 to 10 ppm and the second from 10 to 100 ppm.

Preparation of the Samples The supernatant of each culture broth sample, taken after the centrifugation step as described under subsection 3.5.1, was filtered with 0.22 μm sterile filters (to remove any particles left after the centrifugation step) and then diluted with DI H_2O to be in the range of the calibration curves. To estimate the proper dilution, the biomass to substrate yield was first calculated by the formula 3.2. Then the residual carbon of each sample was calculated with the formula 3.3 and a proper dilution was chosen to get a final concentration of about 500 ppm NPOC in the measured sample.

The samples were transfered into 20 mL TOC-flasks, which were sealed with Parafilm and afterwards plastic caps were srewed on top of the Parafilm. Samples were put in the autosampler and measured.

Paramaters of the NPOC and TN Measurements Parameters as shown in table 3.10 were used for the NPOC and TN measurements.

$$s_i = s_0 - \frac{x_i - x_0}{Y_{x/s}} \tag{3.3}$$

$Y_{x/s}$ = biomass to substrate yield
x_i = CDW concentration of the sample
x_0 = CDW concentration at the start of the fermentation
s_i = substrate concentration of the sample
s_0 = substrate concentration at the start of the fermentation

Table 3.10: Parameters for the non purgeable organic carbon (NPOC) and total nitrogen (TN) measurements

Unit	mg L^{-1}
Number of injections	2/3
Number of washing steps	3
Maximum standard deviation	0.1
Maximum CV	2%
Acid added	1.5%
Sparge gas flow	80 mL min^{-1}
Sparging time	600 sec
Automatic dilution	no
Injection volume	20 μL
Multiple injections	no
Min. integration time	0 sec
Max. integration time	290 sec

Table 3.11: Parameters of the residual glucose determination

Autosampler temperature	10 ℃
Column temperature	25 ℃
RI-Detector temperature	25 ℃
Gradient	isocratic flow
Flow	0.450 mL min^{-1} ($\tilde{5}$0 bar)
Injection volume	20 μL
Retention time (glucose)	11.5 min
Calibration range	100 mg L^{-1} - 2000 mg L^{-1}

3.5.7 Residual Glucose Determination

Materials

- 5 mL plastic syringe
- disposable 0.22 μm filter
- 2 mL GC vials and membrane caps
- High pressure pump (Agilent, G1310A IsoPump)
- Column oven (TEC, Jetstream 2 Serie)
- Precolumn (Micro-Guard cartridge (BIO RAD, Cat. #125-0129))
- Column (Aminex HPX 87H, 300 x 7.8 mm (BIO RAD, Cat. #125-0140))
- RI-Detector (Agilent, G1362A RID; 25 ℃)
- Autosampler (Agilent, G1329A ALS; 10 ℃)
- Carrier solvent (0.005 M H_2S_{04})

Method The measurement of the residual glucose was performed by the Institute of Applied Microbiology using a standardized HPLC method. The supernatant of each sample was diluted with DI H_2O to an expected concentration of 1 g L^{-1} glucose and filtered with 0.22 μm filter to remove any particles. Dilutions were transfered into 2 mL GC-vials and sealed air tight with metal caps with teflon membrane. Parameters for the residual glucose determination are shown in table 3.11.

3.5.8 Lyophilisation

Materials

- 50 mL Falcon tubes with washed cell pellets, stored at -20 ℃
- Freeze-drier
- polycarbonate containers

Method

- Samples were put on ice in a polystyrene foam box and brought to the freeze-dryer.
- Polycarbonate containers were filled with the sample tubes. The tube lids were slightly opened to enable pressure equalisation within the tubes.
- Lids of the containers were attached to the freeze-drier.
- Containers were attached to the container lids and the valves to the vacuum pump were opened.
- Pressure was observed until it reached less than 2 mbar.
- Samples were freeze-dried for at least overnight and stored afterwards at 4 ℃.

3.5.9 Extraction and Transesterification

Materials

- Waterbath
- Toluol
- Heptadecanoic acid
- Acetyl chloride
- Methanol
- Potassium carbonate
- Sodium sulfate
- DI H_2O
- 25 mL glass tubes with plastic caps
- 10 mL glass tubes with plastic caps

- Pasteur pipettes (glass)
- Lyophilised biomass samples
- Analytical balance with an accurateness of 0.0001 g
- Fume hood
- 250 mL glass bottle
- 100 mL volumetric flask
- 500 mL volumetric flask
- Measuring cylinder
- Box filled with ice
- 2 mL GC vials and membrane caps

Method

Preparation of 10% Methanolic HCl Under the fume hood 100 mL of methanol were poured in a 250 mL bottle. The bottle was placed in an ice box and 10 mL of acetyl chloride were added slowly. The solution was stored at 4 ℃.

Preparation of the Internal Standard 496.7 mg of heptadecanoic acid were weighted in, transfered in a 100 mL volumetric flask which was then filled to the mark with toluol. Solution was mixed and stored at 4 ℃.

Preparation of 6% Potassium Carbonate Solution 30 g of potassium carbonate were weighted in, transfered in a 500 mL volumetric flask which was then filled to the mark with DI H_2O. Solution was mixed and stored at 4 ℃.

Extraction and Transesterification

- Waterbath was set to 70 ℃ and switched on.
- 10% methanolic HCl, internal standard and 6% potassium carbonate solution were taken out of the 4 ℃ room and allowed to warm up to room temperature.
- 25 mL glass tubes (external screw thread intact) were labeled and between 20 and 50 mg of lyophilised sample (depending on the total amount of sample available) were weighted in and the exact weight was marked down. Of each sample quadruplets were prepared for the analysis, unless there was not enought biomass available.

- 2 mL of internal standard were added under the fume hood.
- 3 mL of methanolic HCl were added under the fume hood.
- Headspace of the tubes were flushed with nitrogen and tubes closed with plastic caps.
- Glass tubes were shaken and put in the waterbath.
- After ca. 1 minute the tubes were shaken again and placed back in the waterbath for 2 hours.
- Glass tubes were taken out of the waterbath and allowed to cool down to room temperature before 5 mL of 6% potassium carbonate solution were added.
- Glass tubes were shaken and after the two phases separated each from another, the upper phase was pipetted with Pasteur pipettes in 10 mL glass tubes.
- Samples were centrifuged at 1100 rpm for 10 min.
- A small amount of sodium sulfate (water free) was put in 2 mL GC vials.
- Samples were transfered with Pasteur pipettes in the prepared GC vials which were closed air tightly with a membrane cap.

3.5.10 Gas Chromatography-Mass Spectrometry (GC-MS)

Materials

- Agilent Technologies 7890A GC-System
- Column J&W 19091N-202Innowax 260 C: 25 m x 200 m x 0.4 m
- Agilent Technologies 7693 Autosampler
- Agilent Technologies 5975C Inert MSD with Triple-Axis Detector
- GC vials
- Volumetric flasks, 5 ml, 6 ml
- Toluol
- F.A.M.E. Mix C4-C24; Supelco Cat. No. 18919-1AMP
- F.A.M.E. Mix GLC-90; Supelco Cat. No. 1896-1AMP
- n-Hexane

Table 3.12: Composition of the FAME Mix GLC-90

Name	Fraction
Methyl heneicosanoate	20% (w/w)
Methyl heptadecanoate	20% (w/w)
Methyl nonadecanoate	20% (w/w)
Methyl pentadecanoate	20% (w/w)
Methyl tridecanoate	20% (w/w)

Software

- Agilent Technologies MSD Chem Station E.02.01.1177
- Agilent Technologies Enhanced Chem Station E.02.01.1177

Method

Preparation of the Fatty Acid Methyl Ester Mix Calibration Standard To ensure a fatty acid methyl ester (FAME) mix standard with comparable concentrations of the desired FAMEs the following procedure was carried out:

- 100 mg of F.A.M.E. Mix C4-C24 were dissolved in 5 mL n-hexane in a volumetric flask. This gave a concentration of the F.A.M.E. Mix C4-C24 of 20 mg mL^{-1}.
- 100 mg of F.A.M.E. Mix GLC-90 were dissolved in 5 mL n-hexane (20 mg mL^{-1}) and afterwards 1 mL of that solution was taken and filled up to 6 mL with n-hexane. This gave a concentration of F.A.M.E. Mix GLC-90 of 3.33 mg mL^{-1}.
- A single calibration standard was prepared by mixing the two F.A.M.E. mixes in the ratio 1:1 giving final concentrations of 10 mg mL^{-1} and 1.67 mg mL^{-1} respectively.

Preparation of the Docosahexaenoic Acid Methy Ester Standard Since DHA was of main interest for the PUFA analysis, a standard solution containing only DHA methyl ester (ME) was prepared. 4 mg of DHA ME were solved in 2 mL n-hexane to get a 2000 ppm standard.

Measurement Calibration standards and samples were measured with the parameters shown in table 3.14.

Table 3.13: Composition of the FAME Mix C4-C24

Name	**Fraction**
cis-13,16-Docosadienoic acid methyl ester	2% (w/w)
cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester	2% (w/w)
cis-11,14-Eicosadienoic acid methyl ester	2% (w/w)
cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester	2% (w/w)
cis-8,11,14-Eicosatrienoic acid methyl ester	2% (w/w)
cis-11,14,17-Eicosatrienoic acid methyl ester	2% (w/w)
Methyl arachidate	4% (w/w)
Methyl arachidonate	2% (w/w)
Methyl behenate	4% (w/w)
Methyl butyrate	4% (w/w)
Methyl decanoate	4% (w/w)
Methyl cis-13-docosenoate	2% (w/w)
Methyl dodecanoate	4% (w/w)
Methyl cis-11-eicosenoate	2% (w/w)
Methyl elaidate	2% (w/w)
Methyl heneicosanoate	2% (w/w)
Methyl heptadecanoate	2% (w/w)
Methyl cis-10-heptadecenoate	2% (w/w)
Methyl hexanoate	4% (w/w)
Methyl linoleate	2% (w/w)
Methyl linolelaidate	2% (w/w)
Methyl linolenate	2% (w/w)
Methyl γ-linolenate	2% (w/w)
Methyl myristate	4% (w/w)
Methyl myristoleate	2% (w/w)
Methyl octanoate	4% (w/w)
Methyl oleate	4% (w/w)
Methyl palmitate	6% (w/w)
Methyl palmitoleate	2% (w/w)
Methyl pentadecanoate	2% (w/w)
Methyl cis-10-pentadecenoate	2% (w/w)
Methyl stearate	4% (w/w)
Methyl tetracosanoate	4% (w/w)
Methyl cis-15-tetracosenoate	2% (w/w)
Methyl tricosanoate	2% (w/w)
Methyl tridecanoate	2% (w/w)
Methyl undecanoate	2% (w/w)

Table 3.14: Parameter for the measurement of fatty acid methyl esters

Oven Program	140 ℃ for 5 min, then 4 ℃ per min to 240 ℃ for 20 min
Run time	50 min
Injection volume	1 μL
Washing solvent	n-Hexane for standards and toluol for samples
Measuring mode	Total Ion Chromatogram (TIC)
Split mode / Split ratio / Split flow	Split / 100:1 and 10:1 / 30 mL min^{-1}
Total flow	33.3 mL min^{-1}

Table 3.15: Retention times of fatty acid methyl esters

FAME	**Structural Formula** [a]	**Lipid Numbers** [a]	**RT** [b] [min]
TDA ME [c]	$CH_3(CH_2)_{12}COOH$	C14:0	17.20 - 17.32
HDA ME [d]	$CH_3(CH_2)_{14}COOH$	C16:0	22.10 - 22.30
HpDA ME [e]	$CH_3(CH_2)_{15}COOH$	C17:0	24.40 - 24.65
ODA ME [f]	$CH_3(CH_2)_{16}COOH$	C18:0	26.70 - 26.90
DPA ME [g]	$CH_3(CH_2)_4(CH{=}CHCH_2)_5CH_2COOH$	C22:5(n-6)	43.10 - 43.40
DHA ME [h]	$CH_3CH_2(CH{=}CHCH_2)_6CH_2COOH$	C22:6(n-3)	46.70 - 47.10

[a] structural formulas and lipid numbers refering to the fatty acids of the methyl esters.

[b] retention time (RT)

[c] tetradecanioc acid methyl ester (TDA ME)

[d] hexadecanoic acid methyl ester (HDA ME)

[e] heptadecanoic acid methyl ester (HpDA ME)

[f] octadecanoic acid methyl ester (ODA ME)

[g] docosapentaenoic acid methyl ester (DPA ME)

[h] docosahexaenoic acid methyl ester (DHA ME)

$$c = \frac{p \cdot m}{V} \cdot DF \tag{3.4}$$

c = concentration of FAME Mix C4-C24 component [mg L^{-1}]
p = purity of FAME Mix C4-C24 component
m = FAME mass in the calibration standard
V = volume of n-hexane in which the standard was solved (0.005 L)
DF = dilution factor of 0.5 from mixing the standard solutions

$$c = \frac{p \cdot m}{V \cdot DF_1} \cdot DF_2 \tag{3.5}$$

c = concentration of FAME Mix GLC-90 component [mg L^{-1}]
p = purity of FAME Mix GLC-90 component
m = FAME mass in the calibration standard
V = volume of n-hexane in which the standard was solved (0.005 L)
DF_1 = dilution factor of 6 from the 1:6 dilution of the standard
DF_2 = dilution factor of 0.5 from mixing the standard solutions

3.5.11 Calculation of Fatty Acid Methyl Ester Parameters

DHA ME Standard Concentration The concentration value was multiplied by the purity of the DHA ME to get the actual concentration of the standard solution.

FAME Mix C4-C24 Components Concentrations in the Calibration Standard Concentration of FAME Mix C4-C24 components were calculated as shown in formula 3.4.

FAME Mix GLC-90 Components Concentrations in the Calibration Standard Concentration of FAME Mix GLC-90 components were calculated as shown in formula 3.5.

Calculation of Fatty Acids present in both FAME Mixes For FAs present in both FAME mixes, the results of formula 3.4 and 3.5 were added to calculate the concentration in the calibration standard.

Purities and Mass Fractions of Standard FAMEs Purities and mass fractions of FAMEs in the standard solution, which were used later for the calculation of FA contents of biomass samples, are shown in table 3.16.

Linear Calibration and Calculation of Analyte Concentrations in the Samples Based on the formula 3.6 the proportionality constant k was calculated for each FAME as shown in formula 3.7. The proportionality constant was then used to determine the

Table 3.16: Purities and mass fractions of standard FAMEs

FAME	Purity	Mass Fraction [%]	Standard
TDA ME [a]	0.998	4.04	FAME Mix C4-C24
HDA ME [b]	0.992	5.93	FAME Mix C4-C24
HpDA ME [c]	0.994	1.98 / 20	FAME Mix C4-C24 / FAME Mix GLC-90
ODA ME [d]	0.997	3.95	FAME Mix C4-C24
DHA ME [e]	0.98	100	DHA ME

[a] tetradecanioc acid methy ester (TDA ME)

[b] hexadecanoic acid methy ester (HDA ME)

[c] heptadecanoic acid methy ester (HpDA ME)

[d] octadecanoic acid methy ester (ODA ME)

[e] docosahexaenoic acid methy ester (DHA ME)

$$\frac{Y_{fame}}{Y_i} = k \cdot \frac{c_{fame}}{c_i} \tag{3.6}$$

$$k = \frac{\frac{Y_{fame}}{Y_i}}{\frac{c_{fame}}{c_i}} \tag{3.7}$$

$$c_{fa} = \frac{c_i \cdot \frac{Y_{fame}}{Y_i}}{k} \cdot \frac{M_{fa}}{M_{fame}} \tag{3.8}$$

k = proportionality constant
Y = gas chromatography-mass spectrometry (GC-MS) signal
c = concentration [mg L^{-1}]
fame = fatty acid methy ester
fa = fatty acid
i = internal standard
M = molar mass

concentration of the corresponding FAME. To get the actual FA concentration, the FAME concentration was multiplied by the FA to FAME ratio (formula 3.8).

Calculation of the Fatty Acid Content in Lyophilised Biomass Samples To calculate the mass fraction of each FA for each sample, the concentration was multiplied by the extraction volume (2 mL toluol), divided by the lyophilised biomass weighted in and multiplied by 100 (formula 3.9).

3.5.12 Calculation of Culture Derived Parameters

Specific Growth Rate Formula 3.10 was used for the calculation of specific growth rate (μ).

$$\omega = \frac{c_{fa} \cdot V}{m} \cdot 100 \tag{3.9}$$

ω = mass fraction of the fatty acid [%]
c_{fa} = fatty acid concentration [mg L^{-1}]
V = volume of toloul in which the fatty acids were extracted (2 mL)
m = mass of the sample weighted in [mg]

$$\mu = \frac{\ln(X_{n+1}) - \ln(X_n)}{t_{n+1} - t_n} \tag{3.10}$$

μ = specific growth rate [h^{-1}]
X = cell dry weight [g or g L^{-1}]
t = time [h]

Uptake Rates Volumetric uptake rates for glucose, carbon and nitrogen were calculated as shown in formula 3.11.

Specific Uptake Rates Specific uptake rates for glucose, carbon and nitrogen were calculated as shown in figure 3.12.

Product Formation Rates The docosahexaenoic acid formation rate (P_{DHA}) and total fatty acid formation rate (P_{TFA}) between two sampling points were calculated with the formula 3.13. To determine the average formation rate over a longer time period formula 3.14 was used.

Volumetric Product Formation Rates (Space Time Yield) The volumetric docosahexaenoic acid productivity (q_{DHA}) and volumetric total fatty acid productivity (q_{TFA}) were calculated as shown in formula 3.15 and the average values were calculated by formula

$$UR = \frac{\Delta c}{\Delta t} \tag{3.11}$$

UR = volumetric uptake rate [g L^{-1} h^{-1}]
c = concentration of the substrate [g L^{-1}]
t = time [h]

$$sUR = \frac{c_n - c_{n-1}}{(t_n - t_{n-1}) \cdot (CDW_n - CDW_{n-1})} \tag{3.12}$$

sUR = specific uptake rate [g L^{-1} h^{-1}]
c = concentration of the substrate [g L^{-1}]
t = time [h]
CDW = cell dry weight [g L^{-1}]

$$P_n = \frac{CDW_n \cdot \omega_n - CDW_{n-1} \cdot \omega_{n-1}}{(t_n - t_{n-1}) \cdot 100} \tag{3.13}$$

P = product formation rate [g h^{-1}]
CDW = cell dry weight [g]
ω = mass fraction of the fatty acid [%]
t = time [h]

$$P_{av} = \frac{\sum P_n \cdot \Delta t_n}{\sum \Delta t_n} \tag{3.14}$$

P_{av} = average product formation rate [g h^{-1}]
t = time [h]

$$q_n = \frac{CDW_n \cdot \omega_n - CDW_{n-1} \cdot \omega_{n-1}}{(t_n - t_{n-1}) \cdot 100 \cdot V_n} \tag{3.15}$$

q = volumetric product formation rate [g L^{-1} h^{-1}]
CDW = cell dry weight [g]
ω = mass fraction of the fatty acid [%]
t = time [h]
V = volume [L]

$$q_{av} = \frac{\sum q_n \cdot \Delta t_n}{\sum \Delta t_n} \tag{3.16}$$

q_{av} = average volumetric product formation rate [g L^{-1} h^{-1}]
t = time [h]

4 Experiments and Results

4.1 High Glucose Medium Cultivation in Shaken Flasks

4.1.1 Experiment

A. limacinum was grown on 9% (w/w) glucose, 2% (w/w) YEP medium to gather a basic knowledge about the development of biomass and the corresponding fatty acid profile in a high glucose medium. Two 2 L shaken flasks were filled with 450 mL medium and inoculated with 50 mL of a 30 h old 0.5% (w/w) glucose and 0.1% (w/w) YEP culture. Samples were taken in irregular intervals over a period of 20 days for the determination of CDW, optical density at 600 nm wavelength (OD_{600}), cell number (CN), residual glucose, NPOC and TN. In addition, at each sampling point, 40 mL of culture were taken for fatty acid analysis.

4.1.2 Results

Development of Biomass Specific Parameters As shown in figure 4.1 both cultures had a very similar development of CDW, OD_{600} and CN. The CDW increased gradually until 338 h, when it reached a maximum of 28.96 and 29.12 g L^{-1} for culture A and B respectively. At the last sampling point both cultures had less CDW and were also contaminated with a filamentous fungi.

The OD_{600} followed the CDW development over the first 72 h, after which it increased with a higher rate until it reached a maximum of 58.7 and 59.0 at 287 h in culture A and B respectively, which was followed by a decline of the OD_{600}, while the CDW was still increasing.

The CN had a quite different trend compared to the CDW and OD_{600} measurements. At the first sampling point after 22 h, culture A was at $6.80 \pm 0.36 \cdot 10^7$ cells mL^{-1} and B at $5.83 \pm 0.31 \cdot 10^7$ cells mL^{-1}, from which both decreased until 143 h to minima of 2.97 ± 0.87 and $3.05 \pm 0.51 \cdot 10^7$ cells mL^{-1}. After that the CN increased again in both cultures to it's maxima of 1.38 ± 0.34 and $1.22 \pm 0.14 \cdot 10^8$ cells mL^{-1} at 287 h, followed by a decline of the CN. At the last sampling point the CN could not be determined, because *A. limacinum* cells formed agglomerates with the contaminating filamentous fungi.

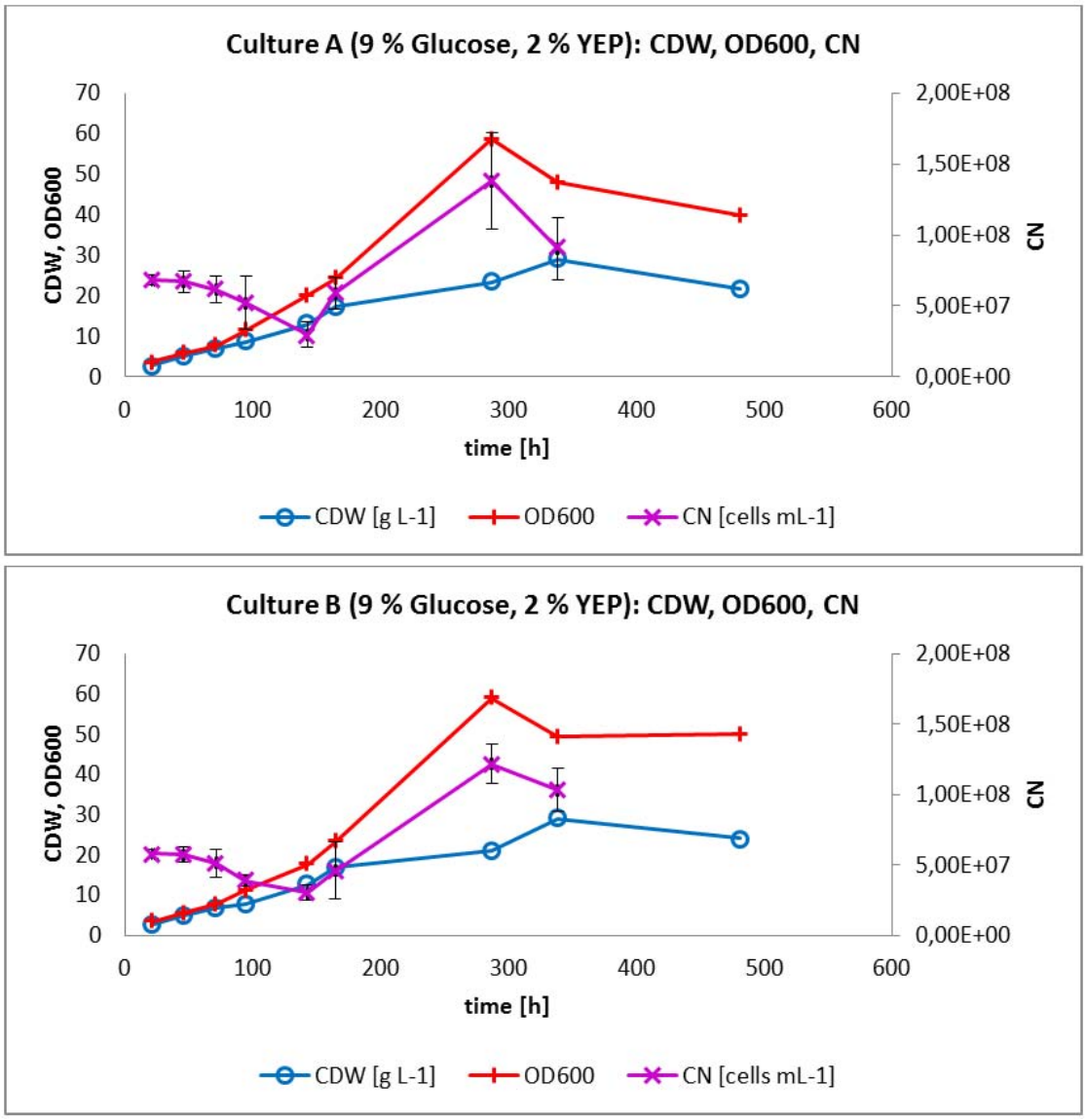


Figure 4.1: Development of biomass specific parameters of shaken flask cultures (9% (w/w) glucose, 2% (w/w) YEP). The cell dry weight (CDW) increased over a period of 338 h to a maximum of 28.96 and 29.12 g L^{-1} for culture A and B respectively. The optical density at 600 nm wavelength (OD_{600}) followed the CDW development over the first 72 h, after which it increased with a higher rate till it reached its maxima at 287 h of 58.7 and 59.0 for culture A and B respectively. The cell number (CN) of culture A and B of 6.80 ± 0.36 and $5.83 \pm 0.31 \cdot 10^7$ cells mL^{-1} at 22 h, decreased till 2.97 ± 0.87 and $3.05 \pm 0.51 \cdot 10^7$ cells mL^{-1} at 143 h, from which it increased again to it's maximum values of 1.38 ± 0.34 and $1.22 \pm 0.14 \cdot 10^8$ cells mL^{-1} at 287 h. At the last sampling point, both cultures were contaminated with a filamentous fungi, which prevented the CN determination.

Development of Substrate Specific Parameters Figure 4.2 shows the courses of the residual glucose, NPOC and TN of culture A and B. The glucose concentration decreased, after the first three sampling points, in both cultures linear.

The TN curves show slight differences between the two cultures. For culture A, the TN decreased from 1.96 g L^{-1} at 22 h to 0.90 g L^{-1} at 165 h in a rather linear way. The lowest concentration of TN was measured at 287 h with 0.73 g L^{-1}, from which it increased again to 1.44 g L^{-1} at 481 h. Culture B shows a clearer two stage nitrogen consumption process. First the TN concentration deceased linear from 2.02 g L^{-1} at 22 h to 1.09 g L^{-1} at 143 h. After this phase the TN decreased linear with a lower rate until it reached its minimum of 0.73 g L^{-1} at 338 h. At the last sampling point at 481 h the TN increased again to 0.85 g L^{-1}. Since all samples were diluted 1:5 with DI H_2O, the measered values were not in the range of the calibration curve (10-100 mg L^{-1}) and thus are prone to error. The consequences for the NPOC analysis were even more severe, because most samples had NPOC concentrations exceeding the upper detection limit of the Shimadzu TOC-$L_{CPH/CPN}$ analyzer. For this reason the NPOC measurements do not show the actual concentrations of the samples.

Development of Fatty Acids The development of the FA profile of both cultures is shown in figure 4.3. The fatty acid (FA) data of culture A has very hight standard deviations and therefore won't be taken into further considerations. For culture B the data looks quite reliable, however for some sampling points the standard deviation is still quite high. DHA was the most abundant FA in all analysed samples, with the highest mass fraction of docosahexaenoic acid to cell dry weight (ω_{DHA}) of $18.4 \pm 2.9\%$ (w/w) CDW after 338 h. Apart from DHA, hexadecanoic acid (HDA) and DPA were produced in mentionable quantities, with maximum values of $6.9 \pm 1.0\%$ (w/w) and $5.0 \pm 0.8\%$ (w/w) respectively. Over the whole cultivation period tetradecanoic acid (TDA) was not detected at all and octadecanoic acid (ODA) was only present in very small amounts (max. $0.6 \pm 0.1\%$ (w/w)) during the late phase of the experiment.

4.2 High Glucose Medium Cultivation in a Bioreactor

4.2.1 Experiment

To see if the cultures in the shaking flask experiment were limited by the availability of oxygen, *A. limacinum* was cultivated again on 9% (w/w) glucose, 2% (w/w) YEP medium in a bioreactor. Total culture volume was planed to be 5 L, 4.5 L medium and 0.5 L inoculum. Inoculum cultures were grown in two 500 mL baffled shaking flasks, each filled with 250 mL medium (2% (w/w) glucose, 0.4% (w/w) YEP) and inoculated with 1 mL RCB. The final inoculum was only 250 mL, because one of the flasks broke by

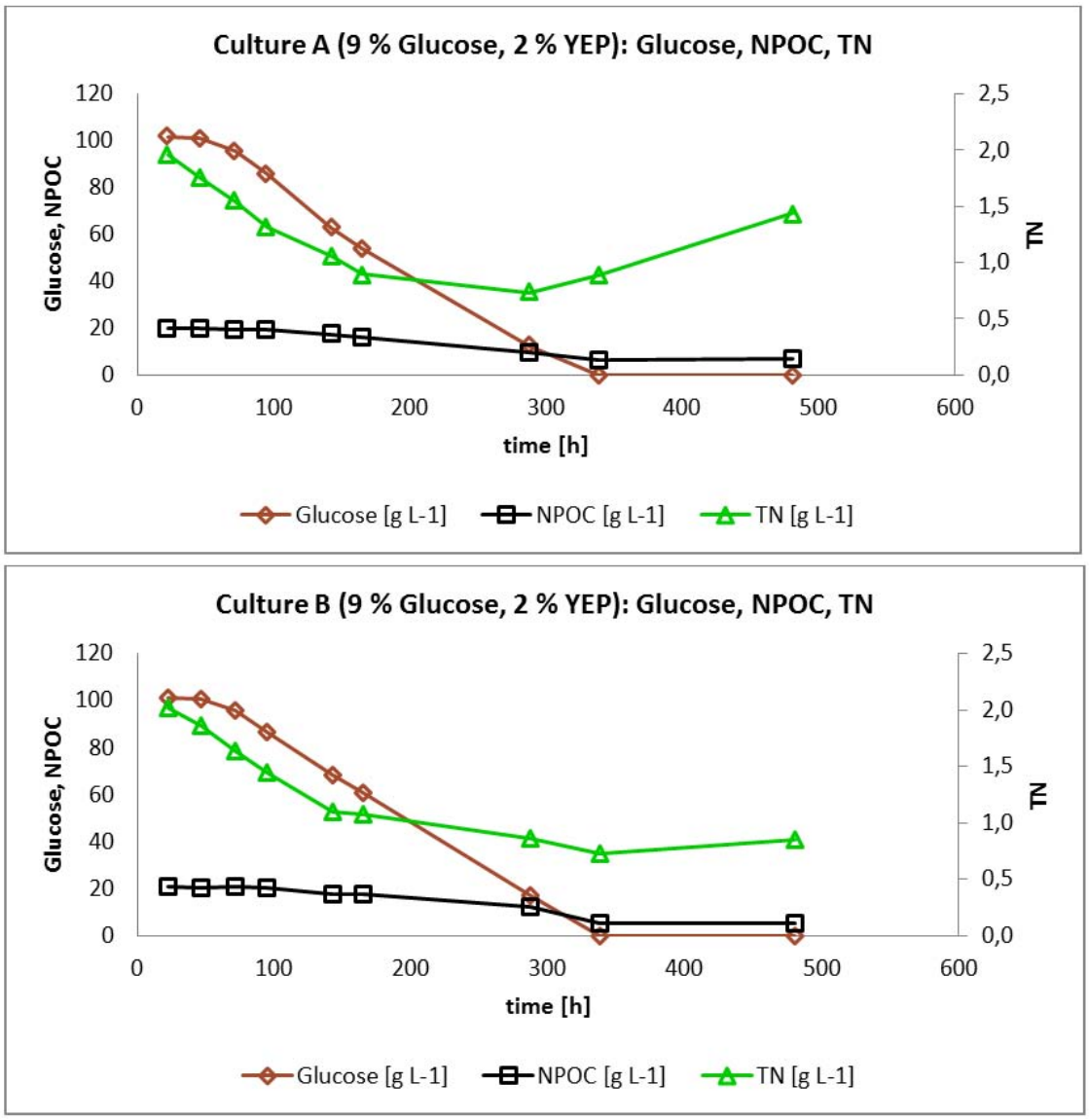


Figure 4.2: Substrate specific parameters of shaken flask cultures (9% (w/w) glucose, 2% (w/w) YEP). Glucose was consumed linear after the third sampling point in both cultures. The total nitrogen (TN) of culture A decreased to a minimum of 0.90 g L^{-1} at 165 h, after which it rose again to 1.44 g L^{-1} at 481 h. In culture B the TN appears to be consumed in a two step process (1.09 g L^{-1} at 143 h and 0.73 g L^{-1} at 338 h). The non purgeable organic carbon (NPOC) measurements do not reflect the actual concentrations, because sample concentrations exceeded the detection limit.

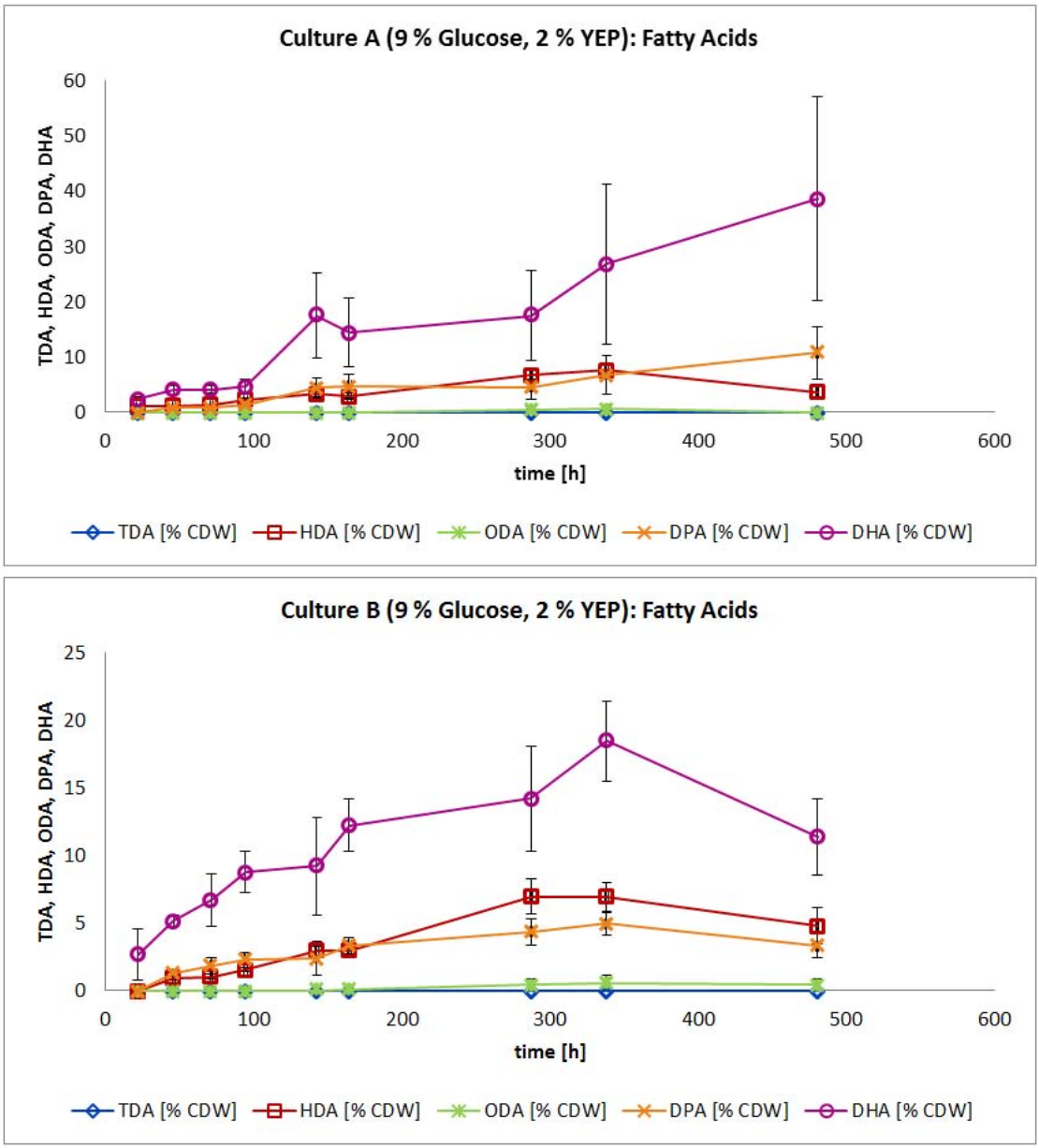


Figure 4.3: Production of fatty acids by shaken flask cultures (9% (w/w) glucose, 2% (w/w) YEP). The fatty acid (FA) measurements of culture A have very high standard deviations and thus are not taken into consideration. The development of the FA profile of culture B shows that docosahexaenoic acid (DHA) was the most abundant FA in all analysed samples, making up to $18.4 \pm 2.9\%$ (w/w) of cell dry weight (CDW) after 338 h. Hexadecanoic acid HDA and docosapentaenoic acid (DPA) were also produced in relevant quantities, reaching maximum values of $6.9 \pm 1.0\%$ (w/w) and $5.0 \pm 0.8\%$ (w/w) respectively.

accident. This led to slightly higher glucose and YEP concentrations in the beginning, because the concentrations of the 4.5 L medium in the reactor were calculated to be 9% (w/w) glucose and 2% (w/w) YEP after adding 500 mL of inoculum.

Samples were taken in irregular intervals and CDW, NPOC and TN were measured. FA analysis was not performed, because from 63 h on, the culture was contaminated with rods. Samples were taken until 71 h to gather information about the carbon and nitrogen consumption behavior of the culture.

As online parameters dissolved oxygen (DO), pH, temperature and stirring speed were automatically controlled and recorded from the bioreactor every 10 minutes. Gassing rate was set to 3 L min^{-1} (0.63 vvm with a total culture volume of 4.75 L) 20 minutes after inoculation, when the DO level reached 30% air saturation. The DO level was controlled by the stirring speed (300-800 rpm) to a minimum level of 30% air saturation, pH was controlled with 10% (w/w) NaOH and 10% (w/w) KH_2PO_4, temperature was set to 25 ℃ and controlled by the heating jacket of the reactor.

4.2.2 Results

As can be seen in figure 4.4, the CDW developed, quite similar to the shaken flask experiment, in a linear way for the first 43 h. After that the culture samples were contaminated with small rods, which led to a faster biomass accumulation.

The NPOC measurements show a increasing rate of consumption, especially from 63 h onwards, due to the contamination.

The TN however decreased again in a rather linear way, as it was also the case for the shaken flask experiment, even after the culture was contaminated.

The recorded online parameters show that temperature and pH were successfully controlled at their preset values of 25 ℃ and 7. The dissolved oxygen (DO) control didn‘t work very well, because the controller got in a swinging state about 10 h after inoculation, which caused hight fluctuations of the DO.

4.3 Cultivation with Urea

4.3.1 Experiment

As a preliminary experiment for testing different nitrogen sources beside YEP, *A. limacinum* was grown in two 500 mL baffled flasks, each filled with 200 mL medium (2% (w/w) glucose, 0.3% (w/w) urea). One medium contained, in addition to glucose and urea, 0.05% (w/w) YEP to see if biomass accumulation would be effected. Samples were taken in irregular intervals and CDW and OD_{600} were measured. Samples at 37 and 43 h were taken by different operators and are probably effected by pipetting error, as well as OD_{600} was not measured from the 37 h samples.

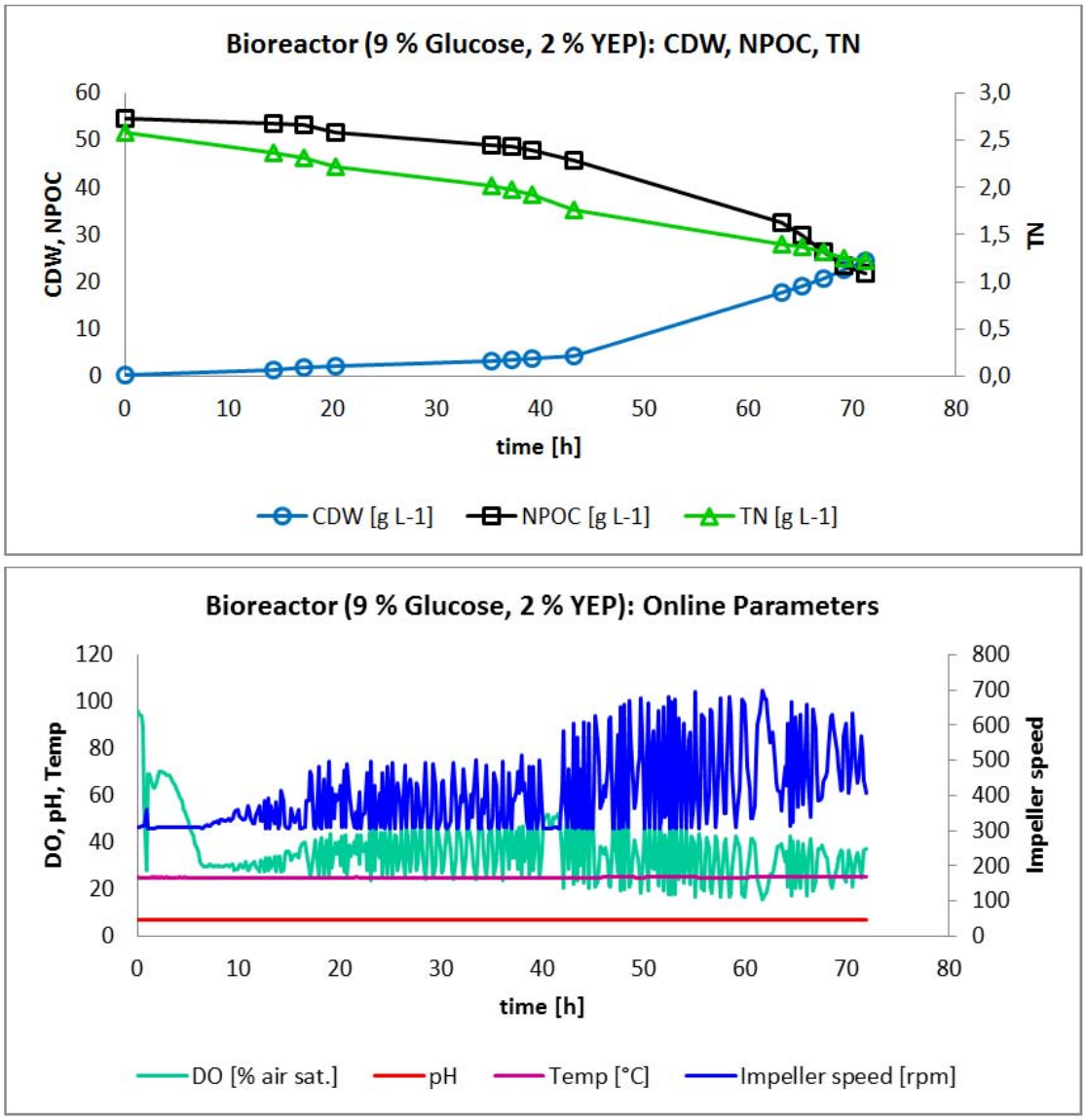


Figure 4.4: Bioreactor batch experiment (9% (w/w) glucose, 2% (w/w) YEP). The cell dry weight (CDW) accumulates in a linear way over the first 43 h, after which it increased faster due to a contamination of rods. The non purgeable organic carbon (NPOC) appears to decrease over the cultivation period with an increasing rate, while total nitrogen (TN) decreases linear. The data of the online parameters show that temperature and pH were stable over the whole process, while dissolved oxygen (DO) was fluctuating from 10 h until the end of the process, because the controler got into a swinging state, which is shown by the fluctuation of the impeller speed.

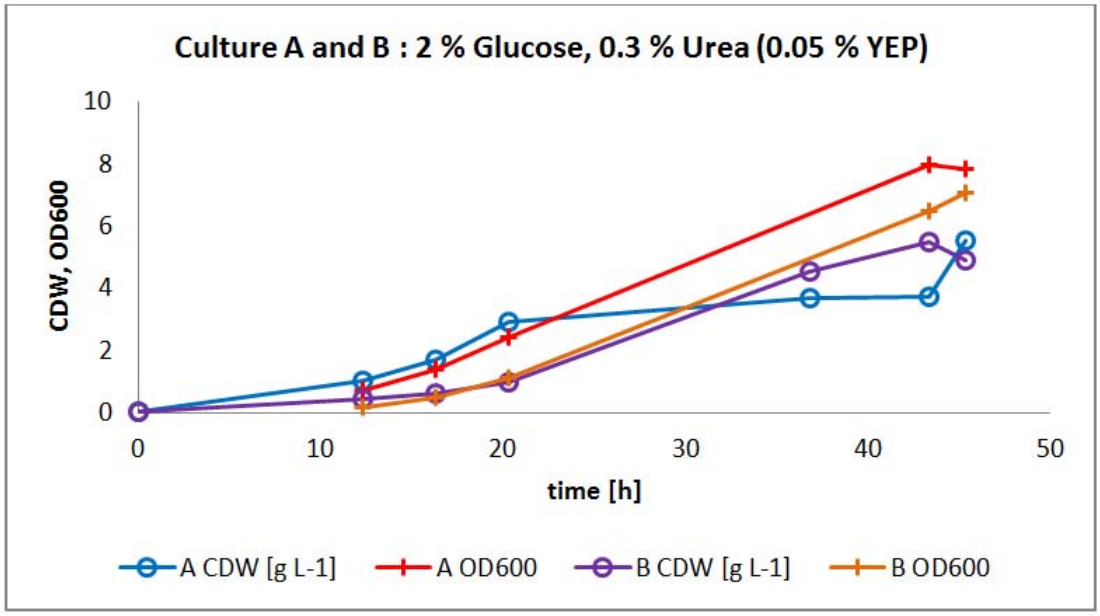


Figure 4.5: Cultivation with urea as primary nitrogen source with and without yeast extract and peptone from casein (YEP). The cell dry weight (CDW) of culture A (with 0.05% (w/w) YEP) accumulates faster than culture B. Final CDW shows only a minor difference between the two cultures (5.5 and 4.9 g L^{-1} for culture A and B respectively). The optical density at 600 nm wavelength (OD_{600}) measurements confirm the trend of the CDW development. CDW measurements at 37 and 43 h may be distorted, because they were done by different operators.

4.3.2 Results

The CDW developments of culture A (with 0.05% (w/w) YEP) and B in figure 4.5 show clearly, that even a small amount of YEP in the medium increased the specific growth rate (μ). However the final CDW values of both cultures are quite similar with 5.5 and 4.9 g L^{-1} for culture A and B respectively. CDW values at 37 and 43 h should rather be ignored, because they were taken by different operators and may be effected by pipetting error.

OD_{600} measurements confirm the trend of the CDW determination, but show an increasing ratio to CDW with increasing biomass.

4.4 Cultivaton with Chemically Defined Nitrogen Sources

4.4.1 Experiment

To find out a medium composition, which would result in a fast accumulation of biomass with minimal generation of FAs, urea, ammonium nitrate and ammonium sulfate were

tested, each in two different concentrations, in addition to YEP. The media contained 2% (w/w) glucose, 0.05% (w/w) YEP and 15 or 50 mM of one of the N-sources mentioned above. One culture with 2% (w/w) glucose and 0.4% (w/w) YEP, with a C:N ratio of 20:1 (without considering the carbon from YEP), was used as reference for the 15 mM cultures. The 50 mM cultures were prepared to have a C:N ratio of 6:1 as shown by Rosa et al. (2010) to be the optimal C:N ratio for biomass production.

To determine the nitrogen and carbon content of the YEP stock solution, 1:50 and 1:100 dilutions were prepared with DI H_2O and the NPOC and TN were measured. The NPOC and TN of 1:300 and 1:600 dilutions of the other N-stock solutions were also measured to check if they lost any nitrogen during the autoclavation process.

The experiment was performed two times, because the first time the media were not buffered and growth was inhibited in the cultures containing ammonium sulfate or ammonium nitrate by low pH. The second time, the media were buffered with 10 mM phosphate buffer pH 7.0 and addjusted during the experiment with a saturated NaOH solution. The C:N ratio was increased from 6:1 to 10:1 to decrease the acidification during the cultivation even further.

From the samples of the first experiment only CDW and pH were measured. From the samples of the second experiment CDW, NPOC, TN, pH and residual glucose were determined. To follow the developmental stages of the cells, microscopic pictures were taken of each sample with 10 x 10 and 10 x 40 magnification. At the last sampling point, 40 mL of each culture were taken for FA analysis.

4.4.2 Results

Experiment without Phosphate Buffer

The results of NPOC and TN measurements of the stock solutions are shown in table 4.1. From the 10 g L^{-1} YEP stock solution, 4.64 g L^{-1} were carbon and 1.18 g L^{-1} were nitrogen. Figure 4.6 shows the variation of CDW and pH of all seven cultures over a cultivation period of 93 h. The Urea 20:1 culture started with pH 8.46 and grew without any delay to a maximum CDW of 6.0 g L^{-1} at 46 h. Urea 6:1 culture had an initial pH of 8.99 and did not grow for the first 22 h. When the pH had decreased to 7.97 the culture grew quite fast to a CDW of 5.1 g L^{-1} at 46 h. All cultures with an anorganic nitrogen sources (NH_4NO_3 and $(NH_4)_2SO_4$) show very similar pattern. The pH was about 6.6 at the start and had decreased to 2.2 at 18 h, after which biomass production nearly stopped. To test if cells were still alive, 5 mL of the $(NH_4)_2SO_4$ 6:1 culture were used to inoculate 100 mL of a 0.5% (w/w) glucose, 0.1% (w/w) YEP medium in a 300 mL baffled flask after 46 h. The cells resumed growth and reverted to their normal cell shape as shown by the microscopic pictures of figure 4.7.

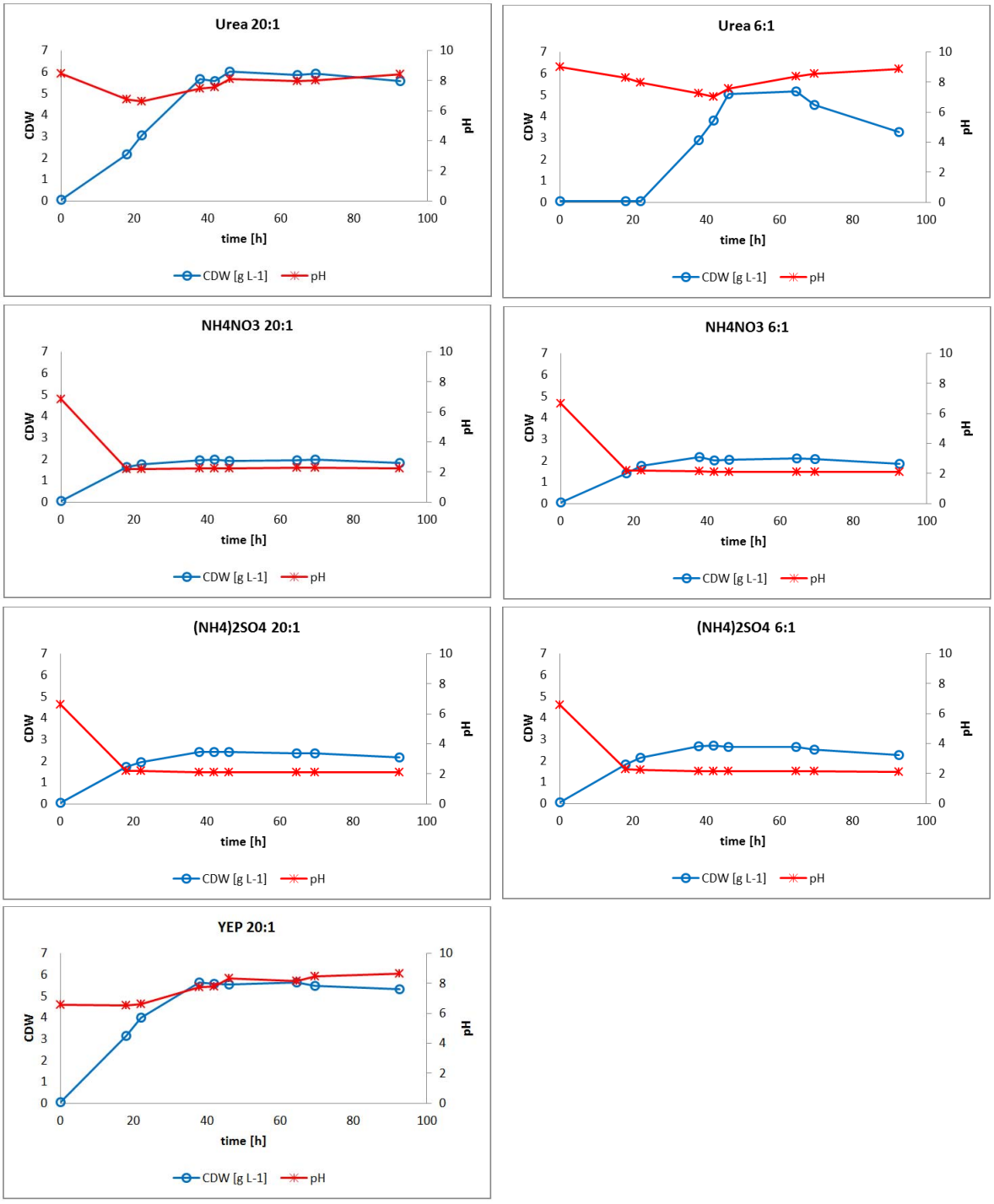


Figure 4.6: Cultivation with chemically defined N-sources without phosphate buffer. All media contained 2% (w/w) glucose, 0.05% (w/w) yeast extract and peptone from casein (YEP) and 15 or 50 mM of the N-source shown in the title of each diagram with the corresponding C:N ratio. The YEP culture was used as a reference for the 15 mM cultures.

Table 4.1: NPOC and TN measurements of nitrogen stock solutions

Stock Solution	**Concentration**	**Molar Mass** [g mol^{-1}]	**NPOC** [a] [g L^{-1}]	**TN** [b] [g L^{-1}]
YEP	10 g L^{-1}	-	4.64	1.18
Urea	1 M	60.06	13.71	29.58
NH_4NO_3	1 M	80.052	0	30.6
$(NH_4)_2SO_4$	1 M	132.14	0	33.59

[a] non purgeable organic carbon (NPOC)

[b] total nitrogen (TN)

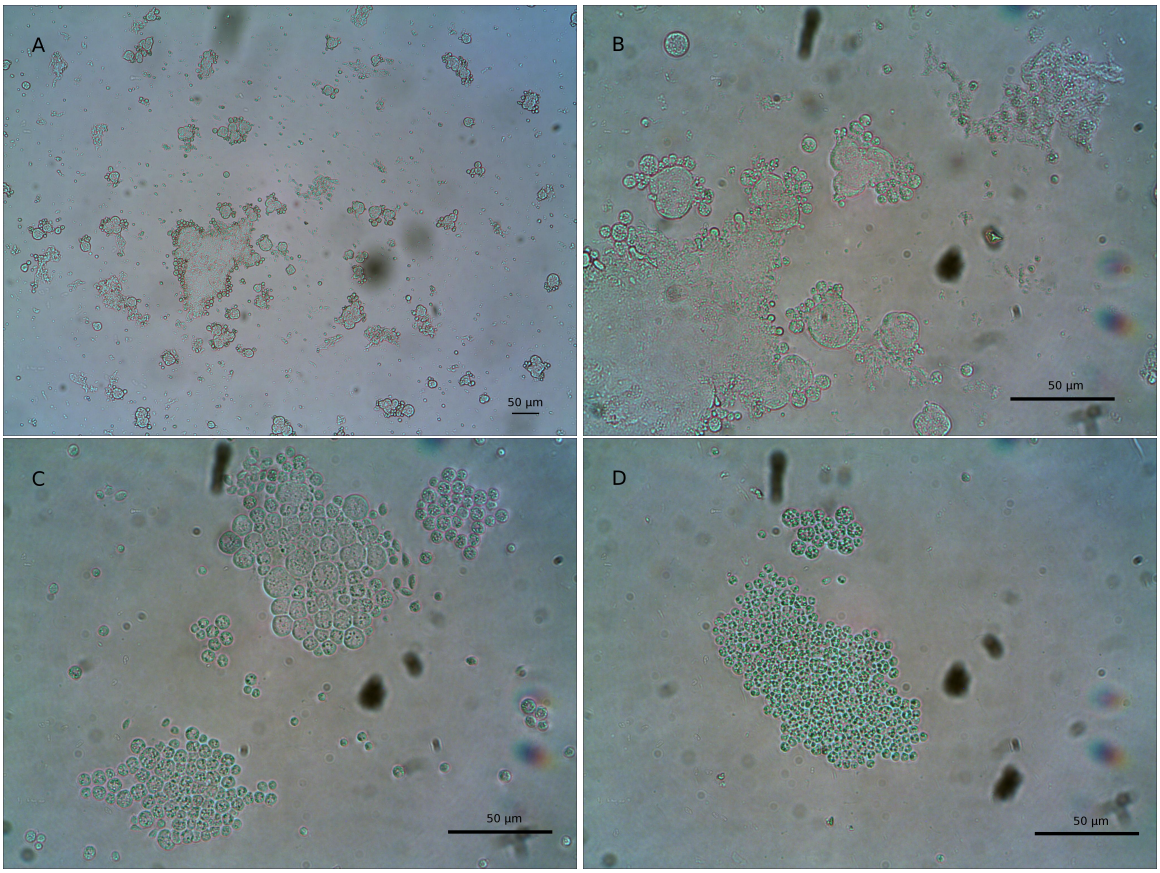


Figure 4.7: 50 mM Ammonium sulfate culture. A and B show the cells after 46 h of cultivation at a pH of 2.16 with a 10 x 10 and 10 x 40 magnification. 5 mL of that culture were used as an inoculum for 100 mL of 0.5% (w/w) glucose and 0.1% (w/w) yeast extract and peptone from casein (YEP) medium. C and D show the regenerated culture after 19 and 24 h respectively in 10 x 40 magnification.

Experiment with Phosphate Buffer

Final CDWs with corresponding mass fraction of total fatty acid to cell dry weight (ω_{TFA}) are shown in figure 4.8. Figures 4.9 to 4.12 show the course of CDW, residual glucose, NPOC, TN, pH and specific growth rate (μ) of Urea 20:1, Urea 10:1, NH_4NO_3 20:1, NH_4NO_3 10:1, $(NH_4)_2SO_4$ 20:1, $(NH_4)_2SO_4$ 10:1 and YEP 20:1 cultures over a cultivation period of 44 h. The FA profiles and the corresponding values with standard deviation are shown in figure 4.13 and table 4.2.

pH For both urea cultures and the YEP culture the pH was quite stable during the whole experiment, decreasing only slightly during the first 16 h after which it increased again. At the last sampling point, crystals precipitated in culture Urea 10:1 (31.2 mM urea), due to a pH of 8.18, which led to an error in the determination of the CDW and also the calculation of the mass percentage of FAs. The ammonium nitrate and ammonium sulfate cultures showed strong acidification after 12 h and the pH had to be adjusted manually with a saturated NaOH solution. In each culture, acidification stopped together with nitrogen consumption.

TN In both urea cultures, nitrogen was consumed with a quite constant rate. From the ammonium nitrate, only about 50% of the nitrogen was consumed quite fast, while the rest remained in solution until the end of the experiment. Both ammonium sulfate cultures consumed nitrogen quite fast and in the $(NH_4)_2SO_4$ 10:1 culture, nitrogen consumption ended when all glucose was gone. After that point the nitrogen increased again in the solution. On the other hand, in the Urea 20:1 culture, urea was still consumed even after all glucose had been metabolized. The YEP culture showed a nitrogen uptake behaviour, quite similar to the previous experiments. The first fraction of nitrogen was consumed rather fast, after which the nitrogen concentration decreased very slowly until it reached a constant value of about 0.22 g L^{-1}.

Residual Glucose and NPOC Instead of 20 g L^{-1} glucose, the residual glucose determination showed only 14 g L^{-1} in all cultures as a starting concentration, which is further supported by the NPOC measurements with starting values of about 6 g L^{-1}. In the cultures YEP 20:1, NH_4NO_3 10:1 ,$(NH_4)_2SO_4$ 10:1 and 20:1, glucose was completely consumed at 24 h. Glucose was gone after 28 h in the NH_4NO_3 20:1 and Urea 10:1 cultures, and after 31 h in the Urea 20:1 culture. The NPOC curves show the same trend as the glucose curves, but in all cultures a minimum level of about 0.6 g L^{-1} remained, except the YEP culture remained at 1.1 g L^{-1}.

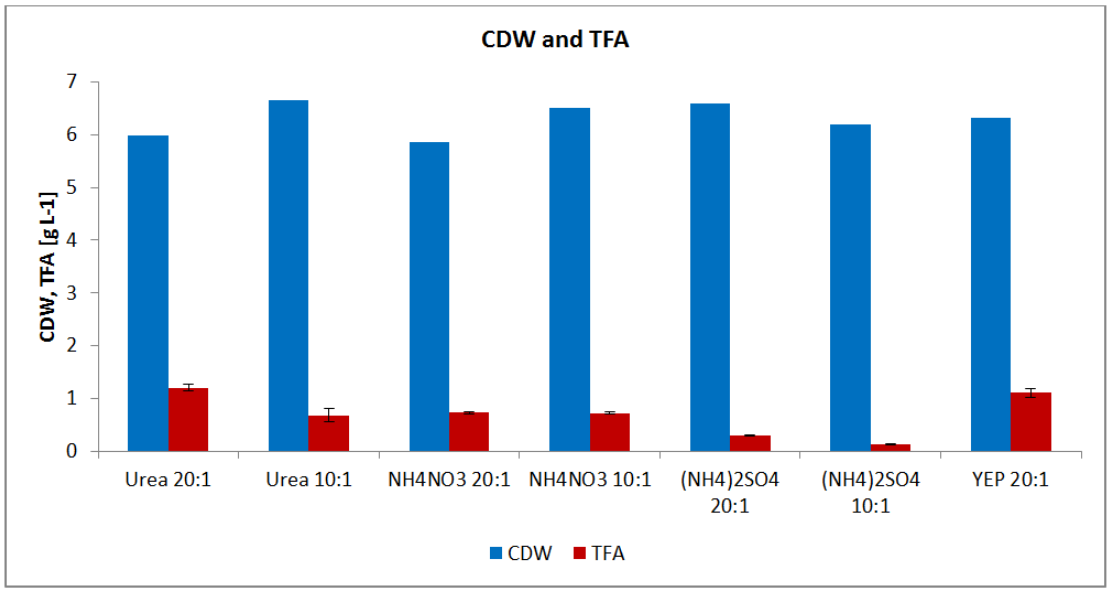


Figure 4.8: Final cell dry weight (CDW) and total fatty acid (TFA) of all tested media compositions. The CDW and TFA of the Urea 10:1 culture were biased by precipitated crystals.

Cell Dry Weight and Total Fatty Acid Content The Urea 20:1 and 10:1 cultures achieved maximum CDWs of 5.98 g L^{-1} and 6.65 g L^{-1} with ω_{TFA} of 20.13 ± 1.09 and $10.31 \pm 1.94\%$ CDW respectively. The CDW determination of the final sample of the Urea 10:1 culture was biased by precipitated crystals. The highest CDW without crystals was 5.85 g L^{-1}. The ω_{TFA} of $20.13 \pm 1.09\%$ CDW of the Urea 20:1 culture was the highest content measured in this experiment.
NH_4NO_3 20:1 and NH_4NO_3 10:1 cultures had maximal CDWs of 5.86 and 6.69 g L^{-1} with ω_{TFA} of 12.48 ± 0.44 and $11.17 \pm 0.39\%$ CDW respectively. While the maximum biomass of the NH_4NO_3 20:1 culture was measured at the end of the experiment (44 h), the biomass of the NH_4NO_3 10:1 culture decreased slightly from 6.69 g L^{-1} at 24 h to 6.51 g L^{-1} at 44 h.
From all tested N-sources, $(NH_4)_2SO_4$ produced the highest maximum CDW with the lowest lipid yield. The effect of different C:N ratios was only observeable in ω_{TFA}, which was 4.52 ± 0.22 and $2.24 \pm 0.11\%$ CDW for the $(NH_4)_2SO_4$ 20:1 and 10:1 cultures respectively, while the difference of the maximum CDW was negligible with maximum CDWs of 6.71 and 6.73 g L^{-1} for $(NH_4)_2SO_4$ 20:1 and 10:1 cultures respectively. However the CDW decreased slightly in both cultures until the end of the experiment to 6.60 and 6.19 g L^{-1} for $(NH_4)_2SO_4$ 20:1 and 10:1 cultures respectively.
The YEP 20:1 culture had a maximum biomass of 6.43 g L^{-1}, which decreased to 6.32 g L^{-1} until the end of the experiment, and had a ω_{TFA} of $17.50 \pm 1.34\%$ CDW.

Table 4.2: Fatty acid compositions of cultures grown on different nitrogen sources

Culture	ω_{TDA} [a] [% CDW]	ω_{HDA} [b] [% CDW]	ω_{ODA} [c] [% CDW]	ω_{DPA} [d] [% CDW]	ω_{DHA} [e] [% CDW]	ω_{TFA} [f] [% CDW]
Urea 20:1	1.15 ± 0.15	8.19 ± 0.58	0.22 ± 0.02	1.66 ± 0.23	8.92 ± 1.01	20.13 ± 1.09
Urea 10:1	0.34 ± 0.05	3.78 ± 0.67	0.17 ± 0.03	0.78 ± 0.20	5.23 ± 1.18	10.31 ± 1.94
NH_4NO_3 20:1	0.64 ± 0.04	11.50 ± 0.44	0.33 ± 0.03	0	0	12.48 ± 0.44
NH_4NO_3 10:1	0.20 ± 0.02	4.46 ± 0.11	0.20 ± 0.02	0.98 ± 0.09	5.33 ± 0.32	11.17 ± 0.39
$(NH_4)_2SO_4$ 20:1	0.19 ± 0.02	4.13 ± 0.20	0.19 ± 0.02	0	0	4.52 ± 0.22
$(NH_4)_2SO_4$ 10:1	0.02 ± 0.03	2.15 ± 0.14	0.07 ± 0.06	0	0	2.24 ± 0.11
YEP 20:1	0.54 ± 0.11	8.99 ± 0.68	0.27 ± 0.02	1.17 ± 0.11	6.54 ± 0.80	17.50 ± 1.34

[a] mass fraction of tetradecanoic acid to cell dry weight (ω_{TDA})

[b] mass fraction of hexadecanoic acid to cell dry weight (ω_{HDA})

[c] mass fraction of octadecanoic acid to cell dry weight (ω_{ODA})

[d] mass fraction of docosapentaenoic acid to cell dry weight (ω_{DPA})

[e] mass fraction of docosahexaenoic acid to cell dry weight (ω_{DHA})

[f] mass fraction of total fatty acid to cell dry weight (ω_{TFA})

Fatty Acid Analysis Figure 4.13 shows the FA composition of every culture after 44 h. Corresponding values with standard deviation can be seen in table 4.2. Highest ω_{TFA} was achieved with the Urea 20:1 culture with 20.13 ± 1.09% CDW and the lowest with the $(NH_4)_2SO_4$ 10:1 culture with 2.24 ± 0.11% CDW. Interestingly the FA composition of the NH_4NO_3 20:1 and 10:1 cultures look very different from each other, while urea and $(NH_4)_2SO_4$ cultures only differ from their counterpart by different ω_{TFA}. Cultures, except NH_4NO_3 cultures, with a C:N ratio of 20 had about twice the ω_{TFA} than cultures with a C:N ratio of 10. NH_4NO_3 cultures on the other hand had nearly the same ω_{TFA} of 12.48 ± 0.44 and 11.17 ± 0.39% CDW for NH_4NO_3 20:1 and NH_4NO_3 10:1 respectively. While HDA was by far the most abundant FA in the NH_4NO_3 20:1 culture (11.50 ± 0.44% CDW), DHA was the most abundant FA in the NH_4NO_3 10:1 culture with 5.33 ± 0.32% CDW and HDA was present with 4.46 ± 0.11% CDW.

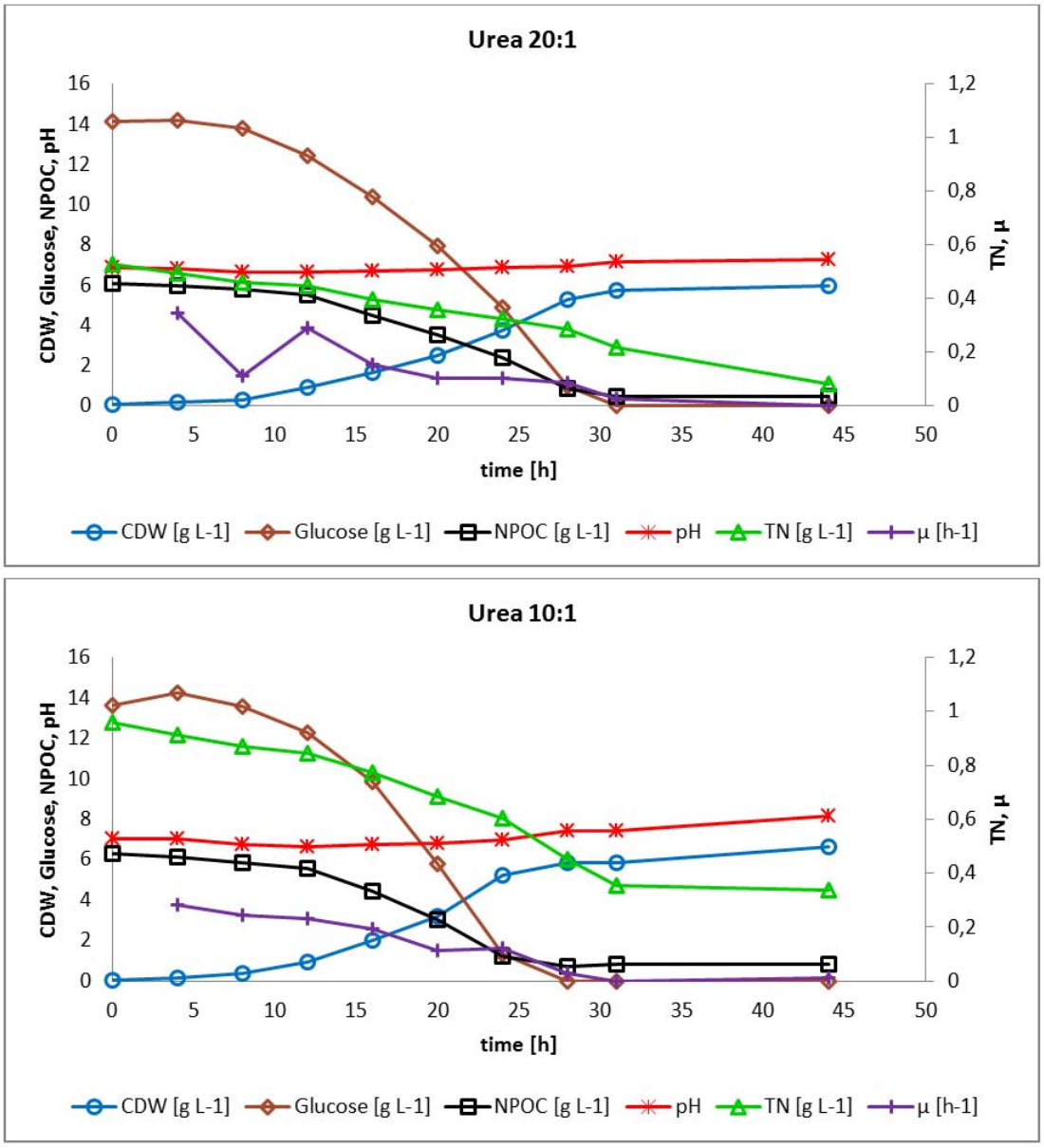


Figure 4.9: Development of cell dry weight (CDW), residual glucose, non purgeable organic carbon (NPOC), total nitrogen (TN), pH and specific growth rate (μ) of cultures with urea as primary N-source with a C:N ratio of 20:1 and 10:1. The last CDW determination of the Urea 10:1 culture was biased by precipitated crystals.

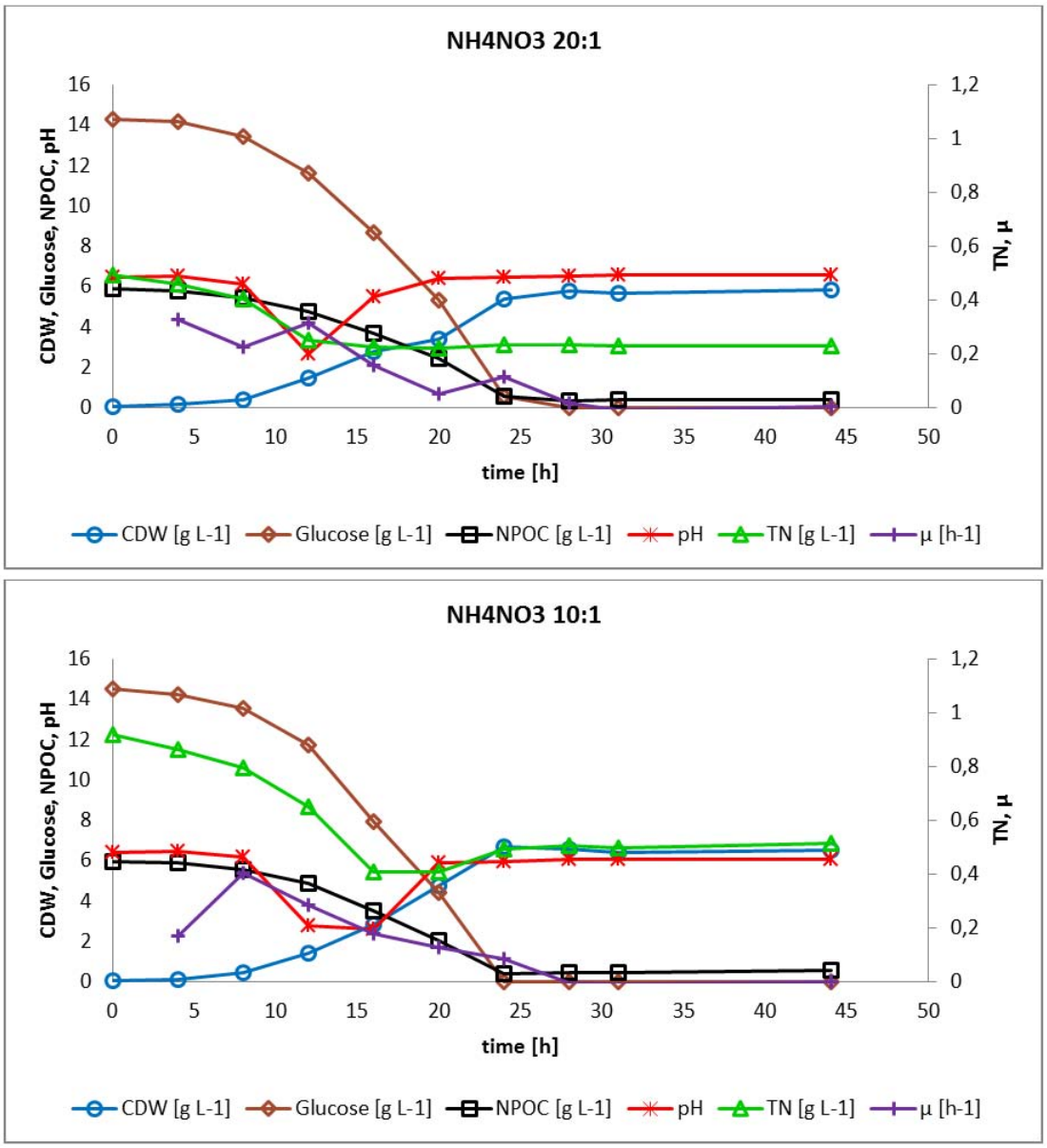


Figure 4.10: Development of cell dry weight (CDW), residual glucose, non purgeable organic carbon (NPOC), total nitrogen (TN), pH and specific growth rate (μ) of cultures with ammonium nitrate as primary N-source with a C:N ratio of 20:1 and 10:1.

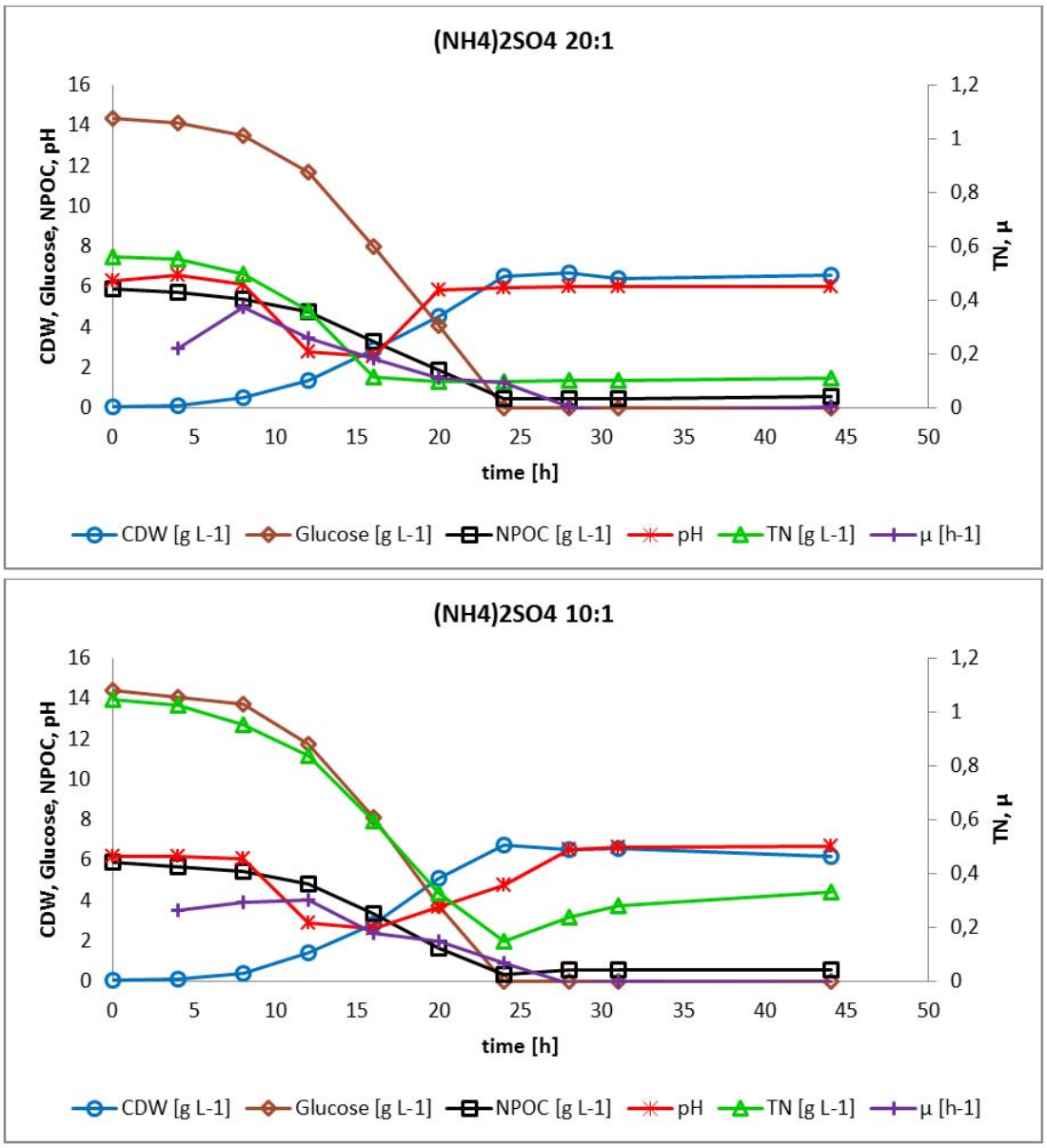


Figure 4.11: Development of cell dry weight (CDW), residual glucose, non purgeable organic carbon (NPOC), total nitrogen (TN), pH and specific growth rate (μ) of cultures with ammonium sulfate as primary N-source with a C:N ratio of 20:1 and 10:1.

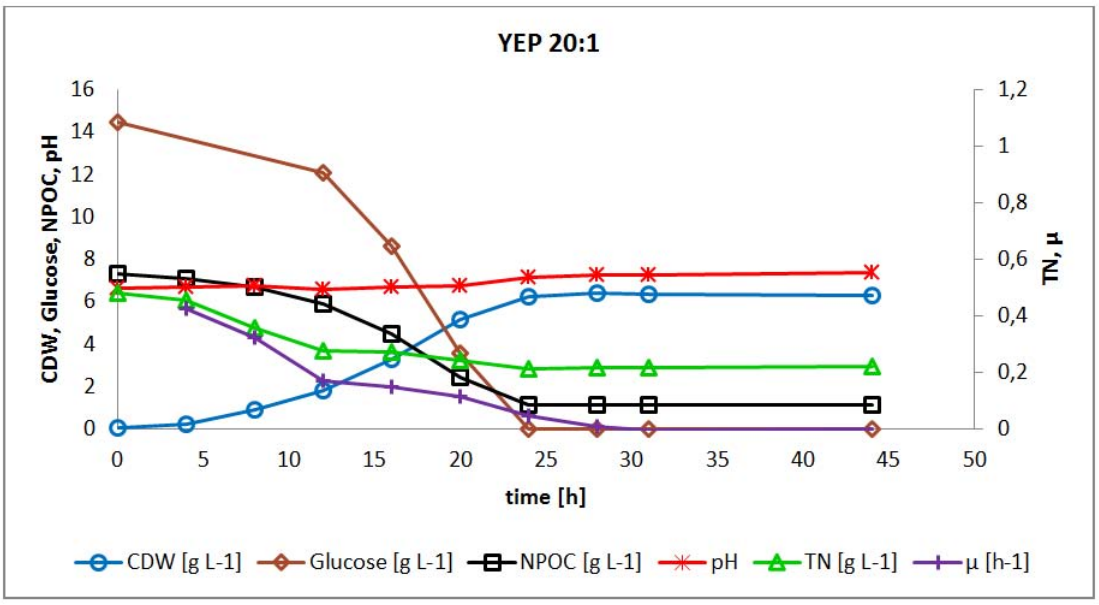


Figure 4.12: Development of cell dry weight (CDW), residual glucose, non purgeable organic carbon (NPOC), total nitrogen (TN), pH and specific growth rate (μ) of the culture with yeast extract and peptone from casein (YEP) as primary N-source with a C:N ratio of 20:1.

4.5 Investigation of Fatty Acid Production in a Fed-Batch

4.5.1 Experiment

The aim of this experiment was to investigate the generation of FAs in a fed-batch, starting with a culture with a low ω_{TFA} from the batch. Based on the results of the previous experiment, 4 L of $(NH_4)_2SO_4$ 10:1 medium were chosen as batch medium to generate a biomass with a low ω_{TFA}. The medium was inoculated with 200 mL of a 30 h old culture, grown on 2% (w/w) glucose, 0.4% (w/w) YEP medium. During the feed phase the culture was fed exponentially ($4000\ mL \cdot e^{0.044\ h^{-1}}$) with 1 L of 25% (w/w) glucose, 20.8 mM $(NH_4)_2SO_4$ medium (200 C:N) over five hours to increase the glucose concentration by 1% (w/w) per hour.

During the batch phase about 25 mL of culture broth were taken every four hours to measure CDW, residual glucose, NPOC and TN. Microscopic pictures were taken from every sample in 10 x 10 and 10 x 40 magnification. DO and pH were measured online and controlled at 30% air saturation and 7. The set value of the pH had to be changed to 6 after inoculation, because crystals precipitated. Since only a 10% (w/w) NaOH solution was used for controlling the pH, the pH was decreased by the culture due to the acidification of the medium, which took more than 12 h until all crystals were dissolved. For this period an aqueous solution of pH 2.5 (pH adjusted with HCl) was used to wash

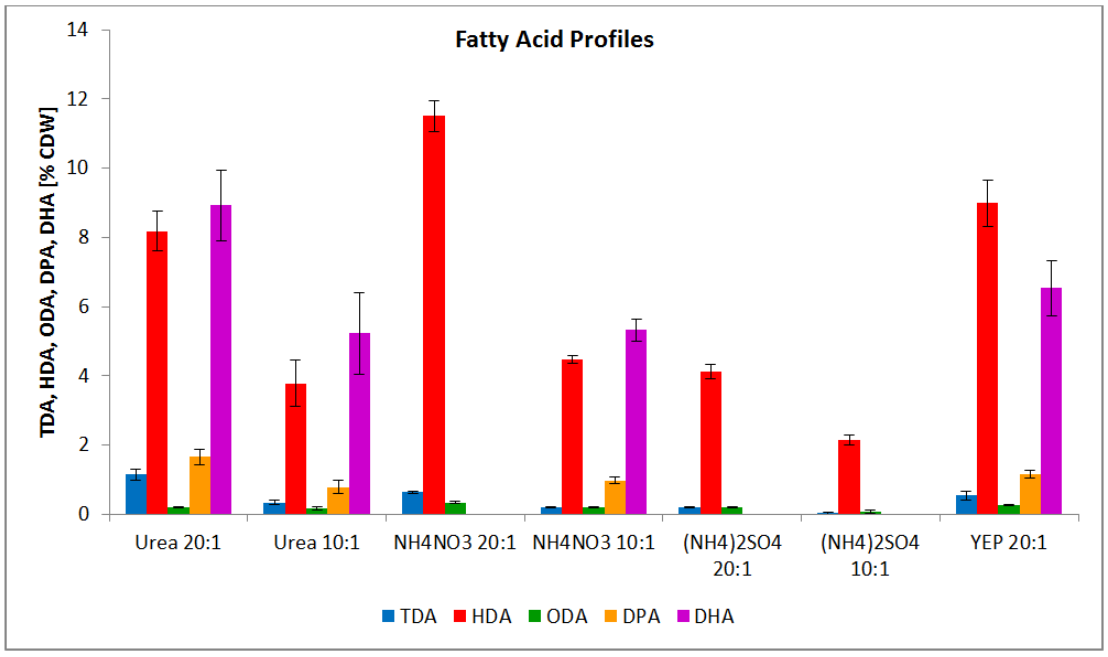


Figure 4.13: The fatty acid (FA) composition of each culture after 44 h with tetradecanoic acid (TDA), hexadecanoic acid (HDA), octadecanoic acid (ODA), docosapentaenoic acid (DPA), docosahexaenoic acid (DHA). The lyophilised biomass of the Urea 10:1 culture contained crystals and thus FA mass percentages are biased.

the cell pellet, in order to remove all phosphate crystals. This was also checked by microscopy of the washed cells. The end of the batch phase was indicated by an increase of DO and pH. Before the feed phase was started, a last sample of the batch culture was taken for analysis, from which also the FAs were determined. During the feed phase, samples were taken every hour and in addition to all parameters measured during the batch phase, 40 mL of culture broth were taken for FA analysis.

After the feed phase the air supply was turned off for one hour to see if oxygen limitation would have an effect on FA production. The air supply was turned on again and the last sample was taken on the next day.

4.5.2 Results

The development of CDW, residual glucose, NPOC, TN and specific growth rate (μ) of the batch phase and the whole fed-batch are shown in figure 4.14.

Batch Phase After a lag phase of 4 h the cells grew exponentially for the rest batch phase, which can be seen by a rather constant μ. The decrease of μ from 20 to 24 h was probably caused by the formation of zoospores, which is shown by the microscopic pictures in figure 4.17. The course of glucose, NPOC and TN concentrations are almost identical, showing that the C:N ratio was quite constant for the first 32 h of the batch phase. At the end of the batch phase all glucose had been consumed, NPOC and TN remained at 0.6 g L^{-1} and 0.2 g L^{-1} respectively and 7.2 g L^{-1} CDW were produced. The biomass had a ω_{TFA} of $14.28 \pm 0.53\%$ (w/w), consisting of $0.35 \pm 0.05\%$ (w/w) TDA, $10.29 \pm 0.29\%$ (w/w) HDA, $0.35 \pm 0.05\%$ (w/w) ODA, $0.58 \pm 0.06\%$ (w/w) DPA and $2.71 \pm 0.28\%$ (w/w) DHA. The end of the batch phase can be seen in figure 4.15 by the increase of the DO level.

Feed Phase The feed phase started 41.75 h after the inoculation of the medium. From the start of the feed phase until the end of the experiment the CDW accumulated rather linear, with a maximum of 94.8 g or 19.0 g L^{-1} (4.98 L culture volume) at the end of the experiment. The glucose concentration increased with about 1% (w/w) per hour, indicating minor glucose uptake by the cells during the feed phase. TN increased during the first hour from 0.20 g L^{-1} to 0.22 g L^{-1}. Over the next 4 h, the TN concentration decreased slightly to 0.21 g L^{-1}, showing nitrogen uptake from the cells during the feed phase. However the total mass of nitrogen in the reactor was increasing.

Online Parameters 1.5 h after the start, the minimal stirring speed was set from 400 to 300 rpm to decrease the DO level and the minimal pH was set from pH 7.00 to 6.00 to solve the precipitated crystals. At 9.3 h and 13.75 h the ractor was aerated shortly with 3 $L\,min^{-1}$. From 19 h the minimal stirring speed was reset to 400 rpm to avoid overheating of the motor and airation rate was set to 3 $L\,min^{-1}$. The end of the batch is indicated by an increase of the dissolved oxygen (DO) and pH at about 41 h. After

Table 4.3: Development of the fatty acid composition after the batch phase

Time [h]	ω_{TDA} [a] [% CDW]	ω_{HDA} [b] [% CDW]	ω_{ODA} [c] [% CDW]	ω_{DPA} [d] [% CDW]	ω_{DHA} [e] [% CDW]	ω_{TFA} [f] [% CDW]
41.75 (0)	0.35 ± 0.05	10.29 ± 0.29	0.35 ± 0.01	0.58 ± 0.06	2.71 ± 0.28	14.28 ± 0.53
42.75 (1)	0.35 ± 0.04	10.67 ± 0.58	0.39 ± 0.02	1.64 ± 0.19	8.16 ± 0.90	21.22 ± 0.89
43.75 (2)	0.43 ± 0.06	11.86 ± 0.66	0.40 ± 0.02	1.80 ± 0.14	9.04 ± 0.46	23.53 ± 1.08
44.75 (3)	0.39 ± 0.04	11.46 ± 0.55	0.40 ± 0.02	1.92 ± 0.11	9.73 ± 0.54	23.89 ± 0.78
45.75 (4)	0.36 ± 0.05	10.34 ± 0.75	0.38 ± 0.02	0.14 ± 0.06	0.49 ± 0.09	11.71 ± 0.75
46.75 (5)	0.42 ± 0.03	11.83 ± 0.45	0.42 ± 0.02	1.75 ± 0.22	8.41 ± 1.19	22.83 ± 1.49
47.75 (6)	0.41 ± 0.03	11.57 ± 0.30	0.41 ± 0.02	2.08 ± 0.11	10.57 ± 0.73	25.06 ± 0.62
73.75 (32)	0.81 ± 0.08	18.94 ± 0.53	0.68 ± 0.03	4.16 ± 0.32	20.59 ± 1.71	45.18 ± 1.85

[a] mass fraction of tetradecanoic acid to cell dry weight (ω_{TDA})

[b] mass fraction of hexadecanoic acid to cell dry weight (ω_{HDA})

[c] mass fraction of octadecanoic acid to cell dry weight (ω_{ODA})

[d] mass fraction of docosapentaenoic acid to cell dry weight (ω_{DPA})

[e] mass fraction of docosahexaenoic acid to cell dry weight (ω_{DHA})

[f] mass fraction of total fatty acid to cell dry weight (ω_{TFA})

the end of the feed phase, the air supply was completely closed from 46.75 to 47.75 h and reset to 3 $\mathrm{L\,min^{-1}}$ (0.6 vvm) afterwards. The progression of DO, impeller speed, pH and temperature are shown in figure 4.15.

Accumulation of Fatty Acids The progression of the FA composition and the FA production are shown in figure 4.16 and the corresponding data for the profile is shown in table 4.3. The ω_{DHA} increased during the first hour from $2.71 \pm 0.28\%$ (w/w) CDW to $8.16 \pm 0.90\%$ (w/w) CDW. Over the next two hours ω_{DHA} increased to $9.73 \pm 0.54\%$ (w/w) CDW from which it decreased during the next hour to $0.49 \pm 0.09\%$ (w/w) CDW and then increased again to $8.41 \pm 1.19\%$ (w/w) CDW. During the oxygen limitation phase ω_{DHA} increased further to $10.57 \pm 0.73\%$ (w/w) CDW, while neither glucose nor nitrogen were consumed in reasonable amounts. The last sample, taken on the next day, had the highest ω_{DHA} of $20.59 \pm 1.71\%$ (w/w) CDW. The development of DPA was very similar to that of DHA, with a DHA:DPA ratio of about 5:1. ω_{TDA} and ω_{ODA} remained constant, both at about 0.4% (w/w) CDW, for the first 6 h after the start of the feed phase and increased in the last sample to $0.81 \pm 0.08\%$ (w/w) CDW and $0.68 \pm 0.03\%$ (w/w) CDW respectively. ω_{HDA} was about 11% (w/w) CDW for the first 6 h and increased in the last sample to a maximum of $18.94 \pm 0.53\%$ (w/w) CDW. The total amount of HDA remained between 4.3-4.4 g during the oxygen limitation phase.

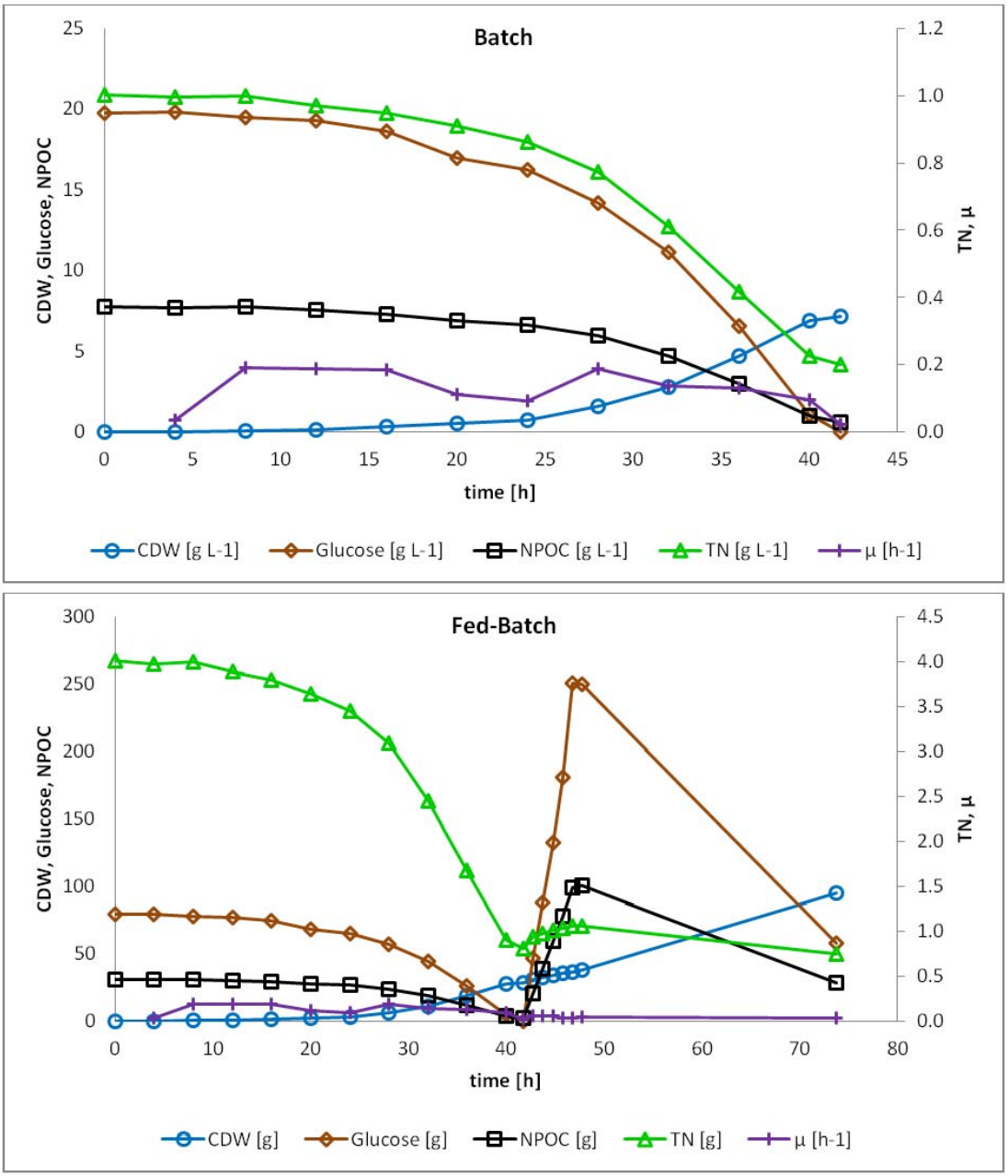


Figure 4.14: Progression of cell dry weight (CDW), glucose, non purgeable organic carbon (NPOC), total nitrogen (TN) and specific growth rate (μ) during the batch phase and the entire fed-batch.

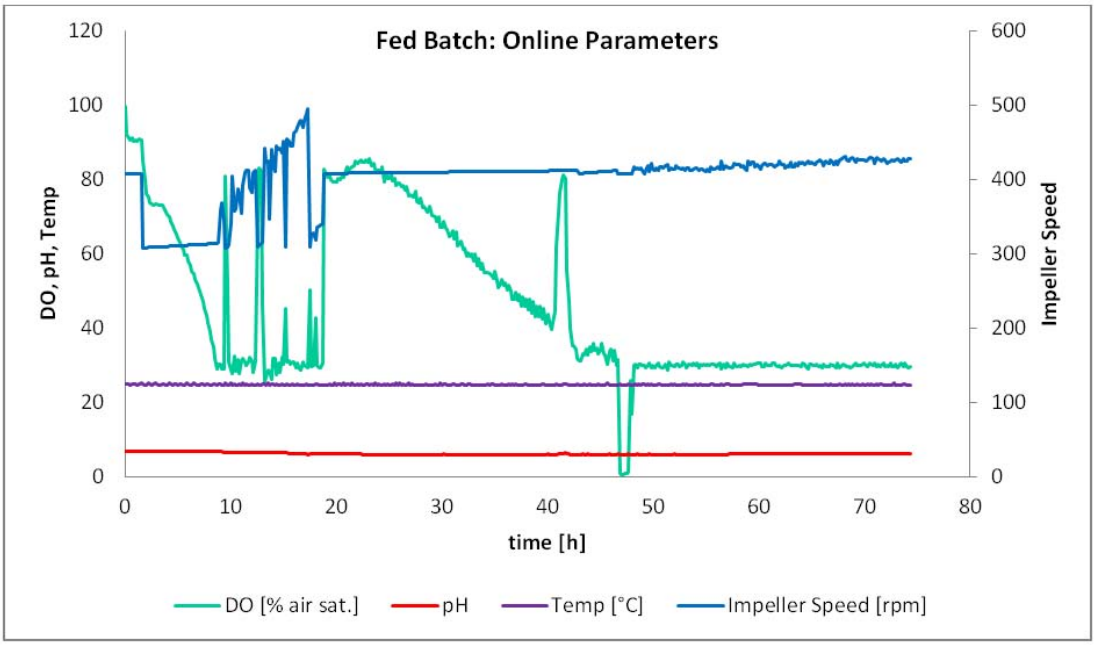
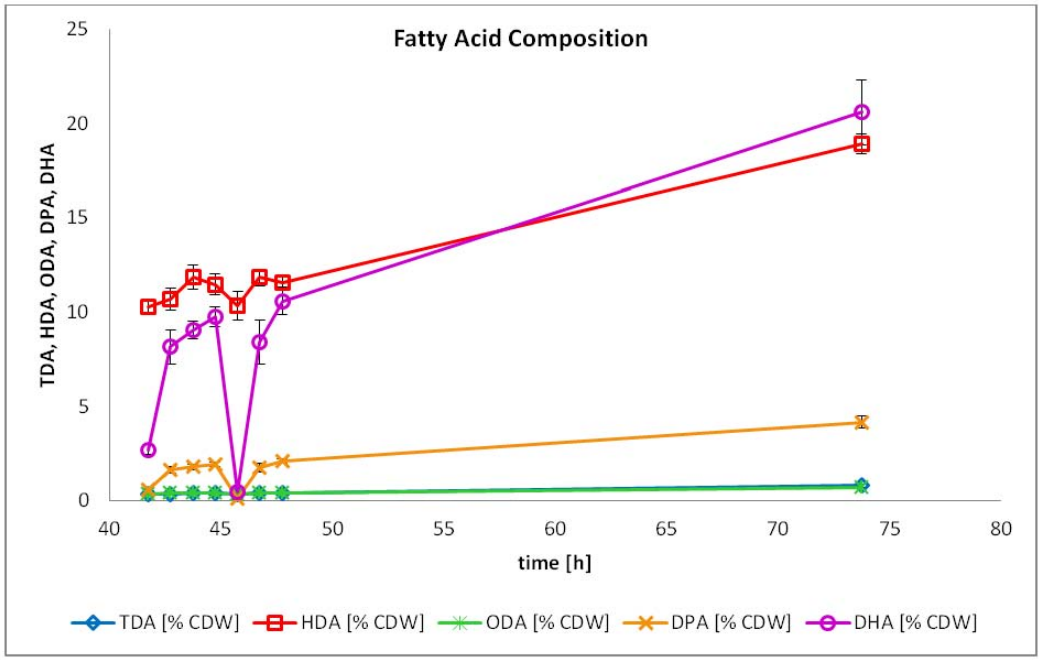


Figure 4.15: Online parameters of the $(NH_4)_2SO_4$ 10:1 Fed-Batch. The end of the batch is indicated by an increase of the dissolved oxygen (DO) and pH at about 41 h. After the end of the feed phase, the air supply was completely closed from 46.75 to 47.75 h and reset to 3 $L\,min^{-1}$ (0.6 vvm) afterwards.



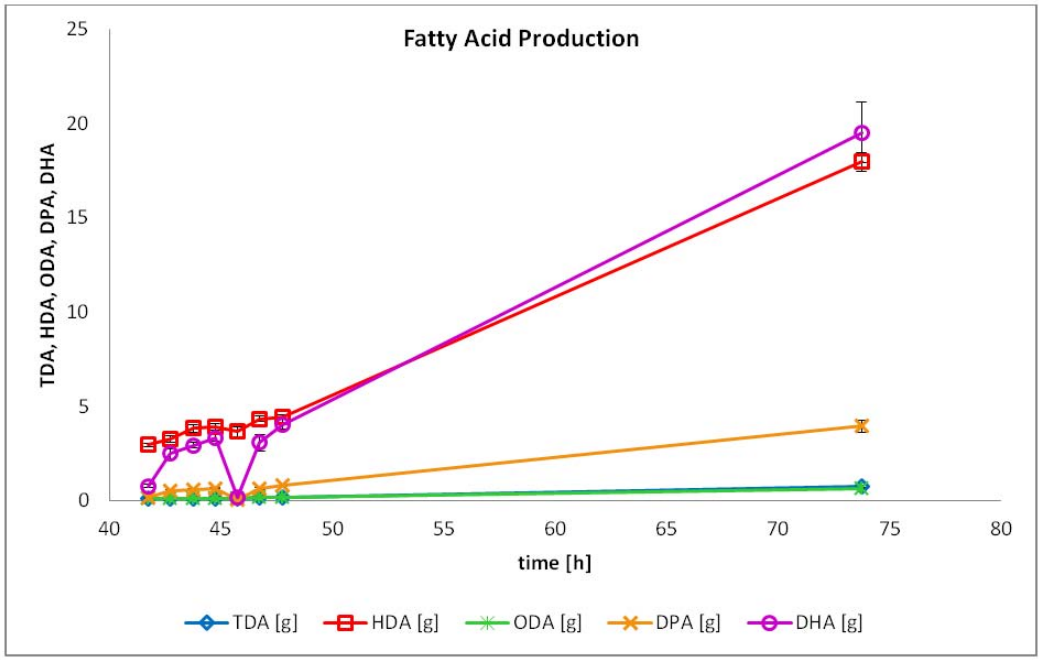


Figure 4.16: Development of the fatty acid composition and the production of fatty acids after the batch phase. At 45.75 h docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) were only marginal abundant, while tetradecanoic acid (TDA), hexadecanoic acid (HDA) and octadecanoic acid (ODA) remained nearly uneffected.

Docosahexaenoic Acid and Total Fatty Acid Production Rates The development of pruductivities and volumetric pruductivities for DHA and TFA are shown in table 4.4. The docosahexaenoic acid formation rate (P_{DHA}) and volumetric docosahexaenoic acid productivity (q_{DHA}) start off with 1.71 g h^{-1} and 0.409 g L^{-1} h^{-1} respectively. For the next two hours the P_{DHA} and q_{DHA} remained rather constant at around 0.4 g h^{-1} and 0.1 g L^{-1} h^{-1}, after which they changed to -3.15 g h^{-1} and -0.661 g L^{-1} h^{-1}. Over the next hour P_{DHA} and q_{DHA} were at their maximum of 2.91 g h^{-1} and 0.585 g L^{-1} h^{-1} respectively. During the oxygen limitation phase P_{DHA} and q_{DHA} were at 0.96 g h^{-1} and 0.193 g L^{-1} h^{-1} and over the next 26 hours they were 0.59 g h^{-1} and 0.119 g L^{-1} h^{-1}, which were also the average values for P_{DHA} and q_{DHA}.

The progression of total fatty acid formation rate (P_{TFA}) and volumetric total fatty acid productivity (q_{TFA}) was very similar to those of DHA, starting with 2.37 g h^{-1} and 0.5689 g L^{-1} h^{-1}, followed by 1.16 g h^{-1}, 0.264 g L^{-1} h^{-1} and 0.55 g h^{-1}, 0.120 g L^{-1} h^{-1} over the next two hours. Then they changed to -4.02 g h^{-1} and -0.843 g L^{-1} h^{-1}, after which they increased again to 4.23 g h^{-1} and 0.849 g L^{-1} h^{-1}. While the oxygen supply was turned off, P_{TFA} and q_{TFA} were at 1.22 g h^{-1} and 0.244 g L^{-1} h^{-1}. For the next 26 hours P_{TFA} and q_{TFA} were slightly higher than during the oxygen limitation phase with 1.28 g h^{-1} and 0.257 g L^{-1} h^{-1}. The average values were 1.21 g h^{-1} and 0.246 g L^{-1} h^{-1} for P_{TFA} and q_{TFA} respectively.

Microscopic Observations Microscopic pictures where taken from every sample in 10 x 10 and 10 x 40 magnification. Cells from the inoculum culture were quite small, with vegetative cells having about the same size as the zoospores with 4.8 ± 0.5 and 3.9 ± 0.6 μm respectively. Over the next 12 h the cell size increased to 11.2 ± 0.9 μm, the cytoplasm of the cells looked quite homogen and no zoospore pruduction was observed. In the next sample, 16 h after inoculation, the cells had segmented cytoplasm with multiple nuclei, indicating zoospore formation. In the next two samples many zoospores were visible, both settled and actively moving. Moving zoospores were attracted to liquid gas interfaces, such as air bubbles trapped under the cover glas, showing attraction to oxygen rich zones. It is however difficult to distinguish between settled zoospores and small vegetative cells that are produced by successive bipartition, since they are very similar in size. Pictures of the formation of zoospores and samples with zoospores are shown in figure 4.17.

Over the following 4 h the cell size of mature and young cells decreased to 7.3 ± 0.8 and 3.5 ± 0.2 μm respectively. After that the cell size of mature cells increased again and remained between 9 to 11 μm for the rest of the experiment. The size of young cells was rather constant over the whole process and ranged between 3 to 4 μm.
From the late batch phase to end of the experiment, an accumulation of lipid bodies in the cytoplasm of large/mature cells was observed, which is shown in figure 4.18.

Table 4.4: Production rates of docosahexaenoic acid and total fatty acid

Time [h]	**Volume** [L]	**CDW** [a] [g]	$\boldsymbol{\omega_{DHA}}$ [b] [% CDW]	$\boldsymbol{\omega_{TFA}}$ [c] [% CDW]	$\mathbf{P_{DHA}}$ [d] [g h^{-1}]	$\mathbf{P_{TFA}}$ [e] [g h^{-1}]	$\mathbf{q_{DHA}}$ [f] [g L^{-1} h^{-1}]	$\mathbf{q_{TFA}}$ [g] [g L^{-1} h^{-1}]
41.75 (0)	4.000	28.68	2.71 ± 0.28	14.28 ± 0.53	-	-	-	-
42.75 (1)	4.180	30.47	8.16 ± 0.90	21.22 ± 0.89	1.71	2.37	0.409	0.568
43.75 (2)	4.368	32.39	9.04 ± 0.46	23.53 ± 1.08	0.44	1.16	0.101	0.264
44.75 (3)	4.564	34.19	9.73 ± 0.54	23.89 ± 0.78	0.40	0.55	0.087	0.120
45.75 (4)	4.770	35.44	0.49 ± 0.09	11.71 ± 0.75	-3.15	-4.02	-0.661	-0.843
46.75 (5)	4.984	36.71	8.41 ± 1.19	22.83 ± 1.49	2.91	4.23	0.585	0.849
47.75 (6)	4.984	38.30	10.57 ± 0.73	25.06 ± 0.62	0.96	1.22	0.193	0.244
73.75 (32)	4.984	94.82	20.59 ± 1.71	45.18 ± 1.85	0.59	1.28	0.119	0.257
				average	0.59	1.21	0.119	0.246

[a] cell dry weight (CDW)

[b] mass fraction of docosahexaenoic acid to cell dry weight (ω_{DHA})

[c] mass fraction of total fatty acid to cell dry weight (ω_{TFA})

[d] docosahexaenoic acid formation rate (P_{DHA})

[e] total fatty acid formation rate (P_{TFA})

[f] volumetric docosahexaenoic acid productivity (q_{DHA})

[g] volumetric total fatty acid productivity (q_{TFA})

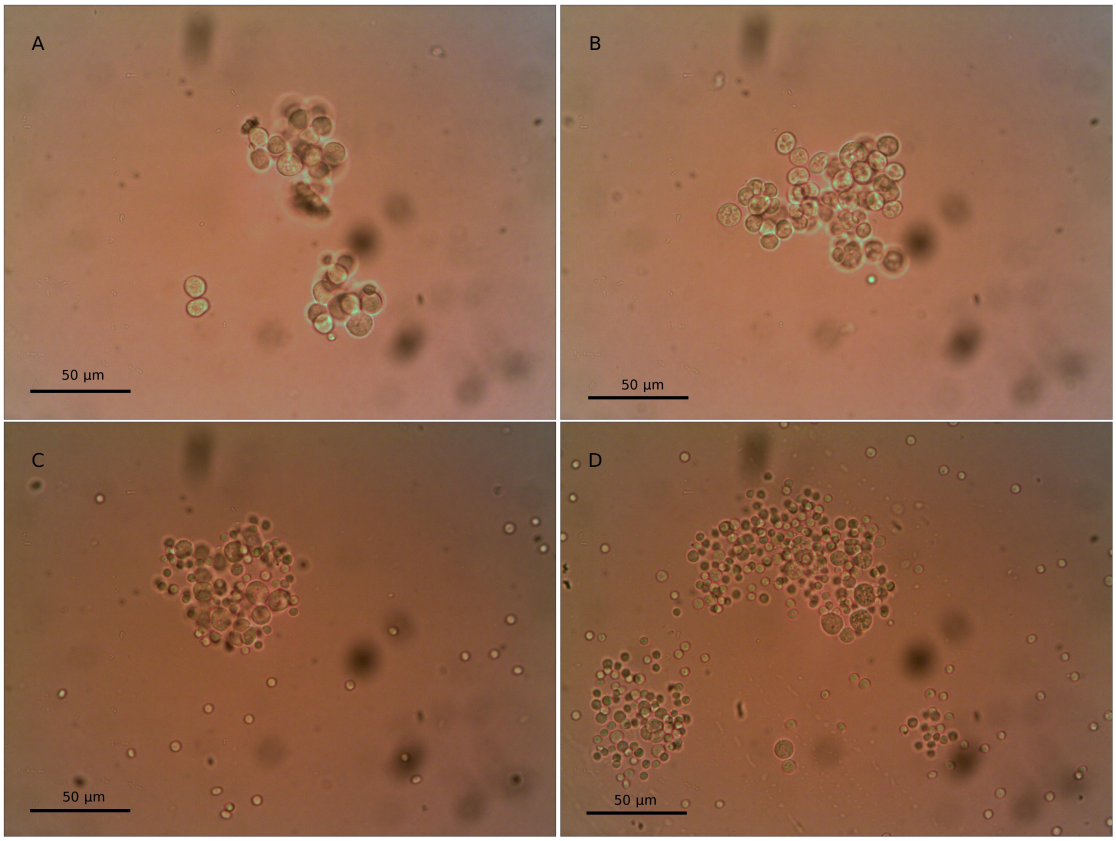


Figure 4.17: Zoospore development of *A. limacinum*. Microscopic pictures of the culture in 10 x 40 magnification after 12 h (A), 16 h (B), 20 h (C) and 24 h (D).

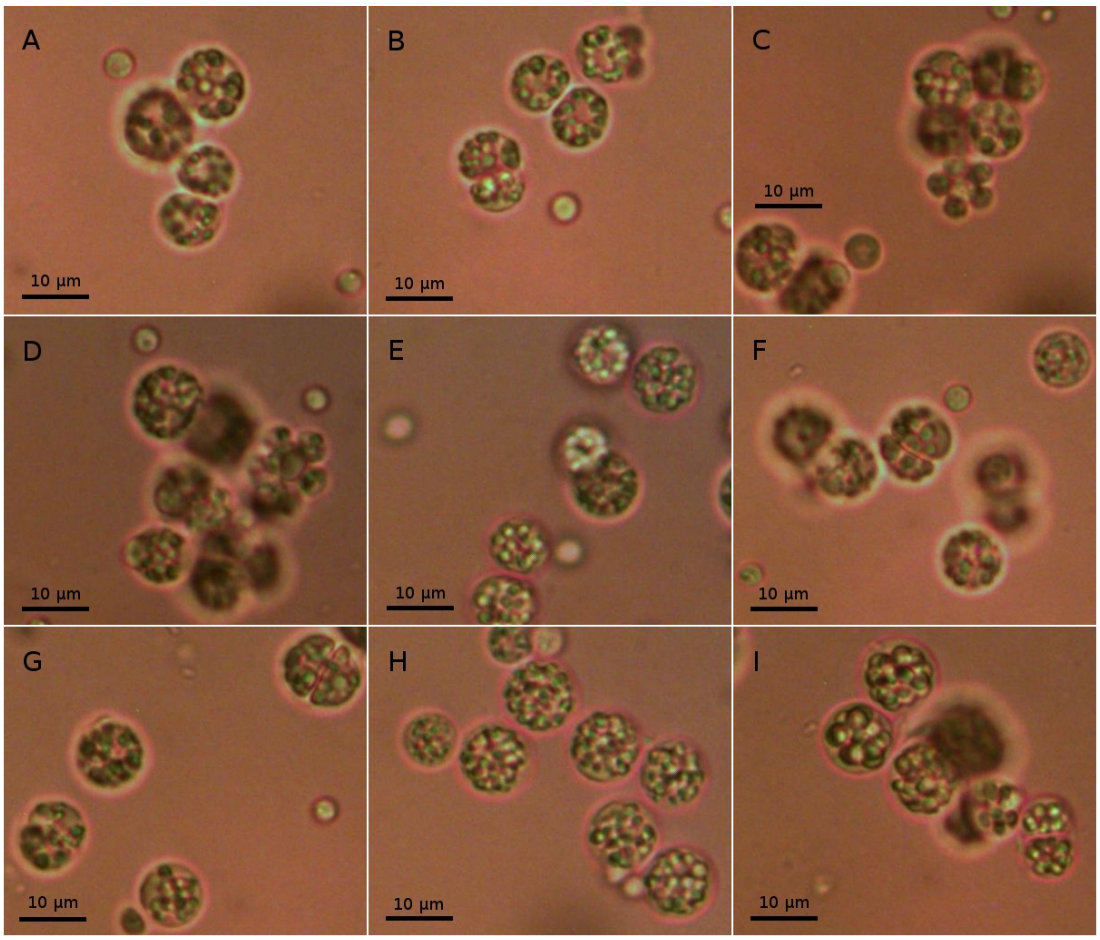


Figure 4.18: Accumulation of spherical vacuoles in the cell's cytoplasm after 40 h (A), 41.75 h (B), 42.75 h (C),43.75 h (D), 44.75 h (E), 45.75 h (F), 46.75 h (G), 47.75 h (H) and 73.75 h (I).

Limaciform Cells in Liquid Culture Limaciform cells were observed five times during the experiment at 32, 40, 43.75, 44.75 and 47.75 h which are shown in figure 4.19. The limaciform cells of the 40, 44.75 and 43.75 h samples were only discovered afterwards on the microscopic pictures and thus they are only available in 10 x 10 magnification. The cells of the 40, 43.75 and 44.75 h samples were 26.5 x 11.5 μm, 37.9 x 12.0 μm and 18.4 x 6.5 μm respectively, and the cell of the 43.75 h sample appeared to engulf a vegetative cell. Unfortunately due to the low magnification of the pictures, no further insights can be gained.

The cytoplasm of the limaciform cells at 32 and 47.75 h appears very different compared to vegetative cells and may be best discribed as amorph. Small pseudopods are also visible and in both cases two limaciform cells are in conjunction with each other. The two cells from the 32 h sample are connected by a septum, which appears very bright in the picture. Behind the first septum, another barrier is visible, which is probably the cell membrane. The bigger cell had a diameter of 17.6 μm, the smaller cell was 10.9 μm long and 4.6 μm wide.

In the picture of the 47.75 h sample, both cells were much larger than usual vegetative cells. The spherical shaped cell had a diameter of 29.7 μm and the elongated cell was measuring 24.7 μm in length and 13.2 μm in width. Both cells are attached to each other and there appears to be a small opening in the membrane between the cells, which connects the cytoplasm of both cells. There is also a spherical organelle in each of the cells, which could be a vacuole or a nucleus.

4.6 Comparison of Culture Parameters

4.6.1 Growth Rates

The maximal specific growth rate (μ_{max}) and average specific growth rate (μ_{av}) of all cultures, except the preliminar urea experiment cultures, were determined by plotting the natural logarithm of CDW vs. time. The slope of the linear regression is μ_{av} and μ_{max} is the maximal slope between two points. A comparison of the growth rates is shown in table 4.5 and the corresponding half logarithmic plots are shown in figures 4.20 to 4.26.

The highest μ_{max} of 0.426 h^{-1} was achieved with the YEP 20:1 culture during the first 4 h of cultivation. The highest μ_{av} of 0.260 h^{-1} was achieved with the NH_4NO_3 20:1 culture over a period of 16 h. In all shaken flask cultures, the growthrate declines in the late exponential phase. In the YEP 20:1 culture, the μ_{av} is composed of two different growth rates. For the first 8 h, the culture was growing with a first average specific growth rate (μ_{av1}) of 0.375 h^{-1} and for the next 12 h with a second average specific growth rate (μ_{av2}) of 0.145 h^{-1}. Similar behaviour was observed with the 9% Glucose Batch culture grown in the bioreactor. During the $(NH_4)_2SO_4$ 10:1 Fed-Batch experiment, *A. limacinum* grew with a μ_{max} of 0.191 h^{-1} and μ_{av} of 0.145 h^{-1}

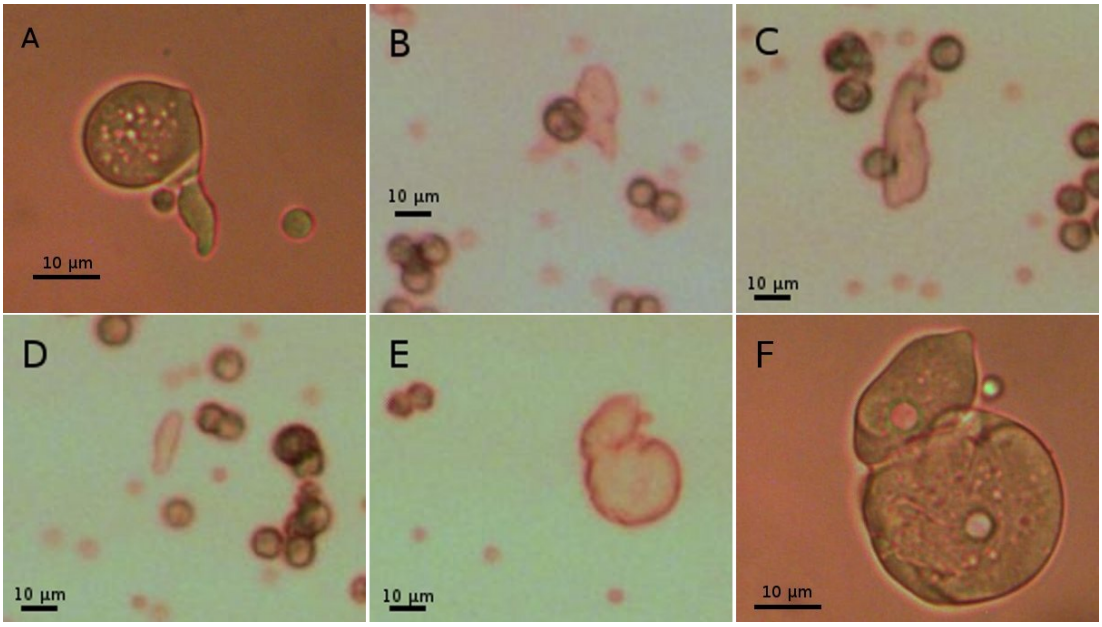


Figure 4.19: Limaciform cells in liquid culture samples after 32 h (A), 40 h (B), 43.75 h (C), 44.75 h (D) and 47.75 h (E and F). Picture E and F show the same cells in 10 x 10 and 10 x 40 magnification respectively.

during the batch phase, excluding the first 4 h of lag phase. Zoospore production is again visible by a slight decrease of the slope from 16 h to 24 h.

4.6.2 Uptake Rates

Volumetric and specific uptake rates of glucose, carbon and nitrogen were calculated from all cultures (except from the preliminar urea experiment) and the maximal values are shown in table 4.6. Data from very early samples (first 8 h of cultivation) were not taken into account for the calculation of specific uptake rates, because measurement errors would have a very strong effect due to the low CDW and small changes in substrate concentrations.

The maximal glucose uptake rates ranged between 0.39 g L^{-1} h^{-1} for the 9% Glucose experiment and 1.37 g L^{-1} h^{-1} during the batch phase of the $(NH_4)_2SO_4$ 10:1 Fed-Batch experiment. From the shaken flask cultures, YEP 20:1 had the highest volumetric glucose uptake rate (UR_{Gluc}) of 1.26 g L^{-1} h^{-1}. The results from UR_{Gluc} were further supported by the volumetric carbon uptake rate (UR_C), which ranged between 0.38 g L^{-1} h^{-1} for the Urea 20:1 culture and 0.54 g L^{-1} h^{-1} for the 9% Glucose Batch experiment.

The maximum volumetric nitrogen uptake rate (UR_N) ranged between 0.0087 g L^{-1} h^{-1} for the 9% Glucose and 0.067 g L^{-1} h^{-1} for the $(NH_4)_2SO_4$ 10:1 shaken flask cultures.

The highest specific uptake rates were achieved in the $(NH_4)_2SO_4$ 10:1 Fed-Batch

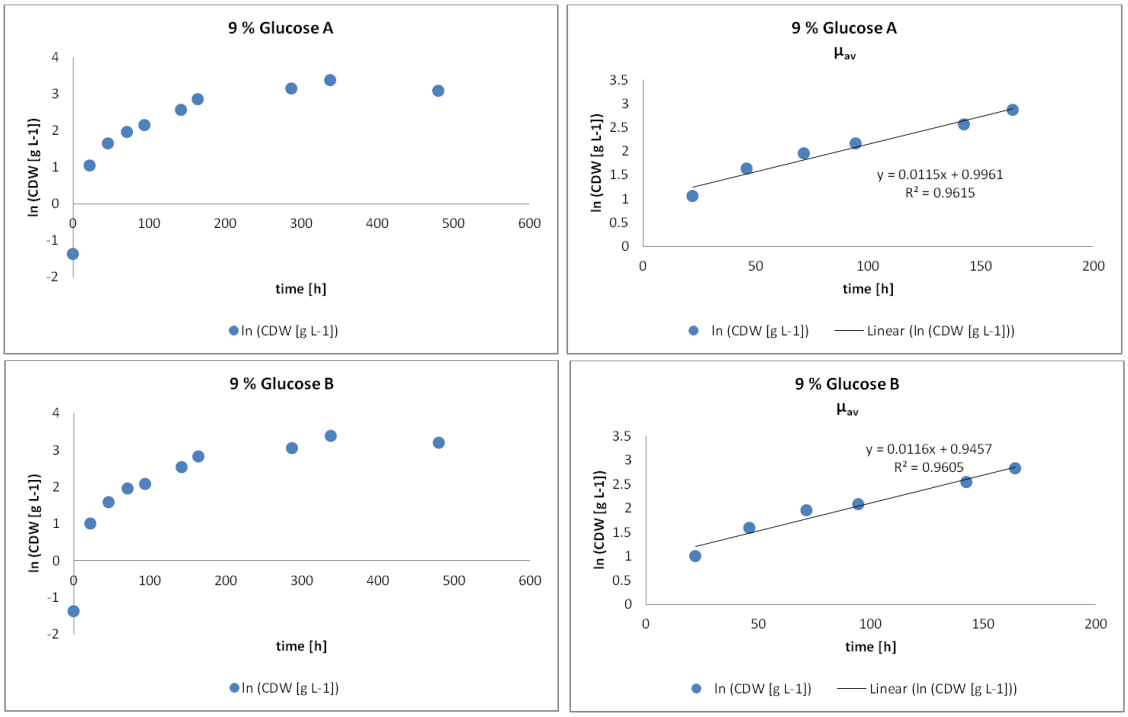


Figure 4.20: Determination of the average specific growth rate (μ_{av}) from 9% Glucose shaken flask cultures.

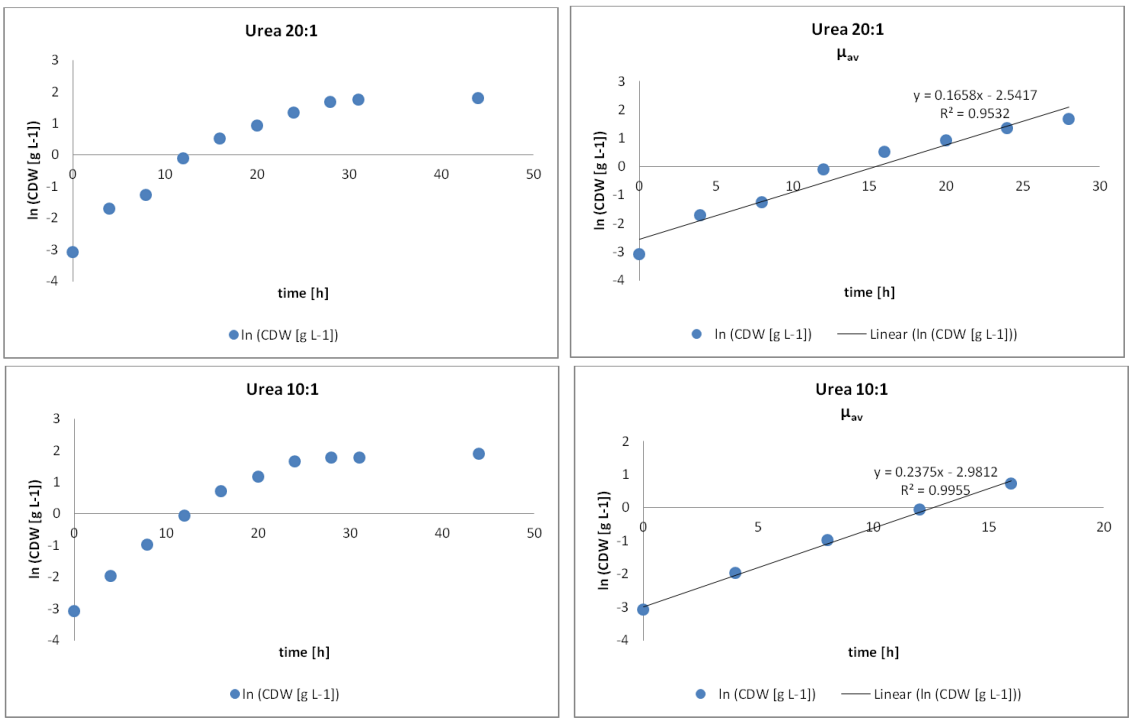


Figure 4.21: Determination of the average specific growth rate (μ_{av}) from Urea 20:1 and 10:1 shaken flask cultures.

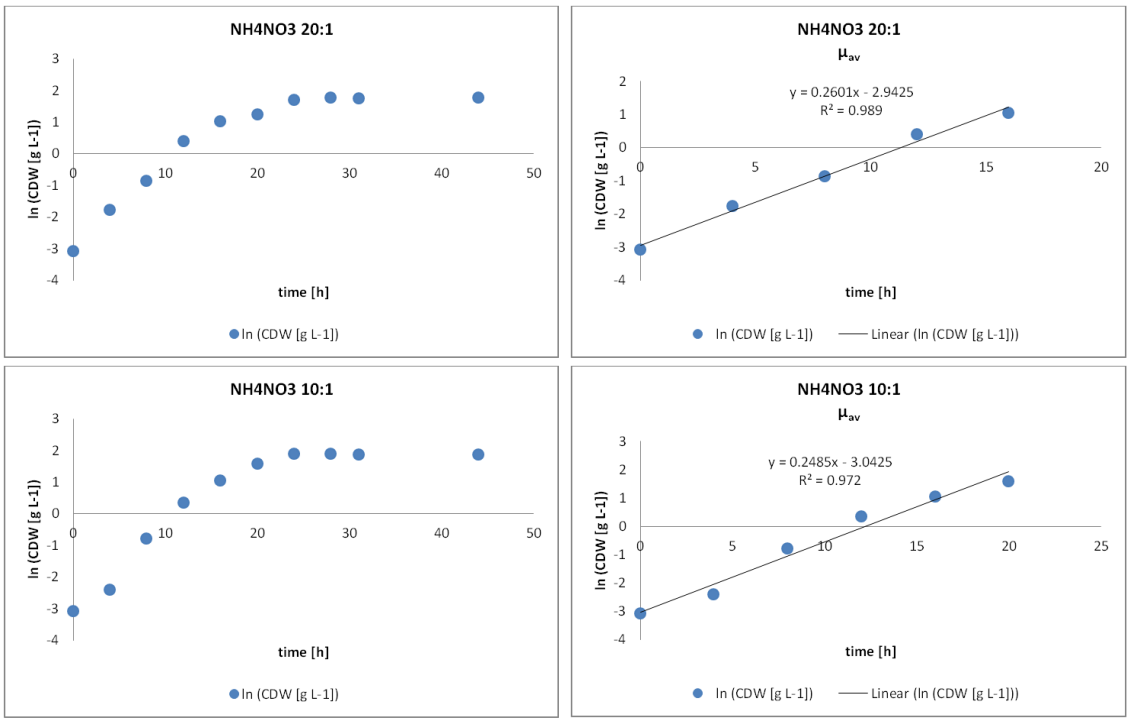


Figure 4.22: Determination of the average specific growth rate (μ_{av}) from NH_4NO_3 20:1 and 10:1 shaken flask cultures.

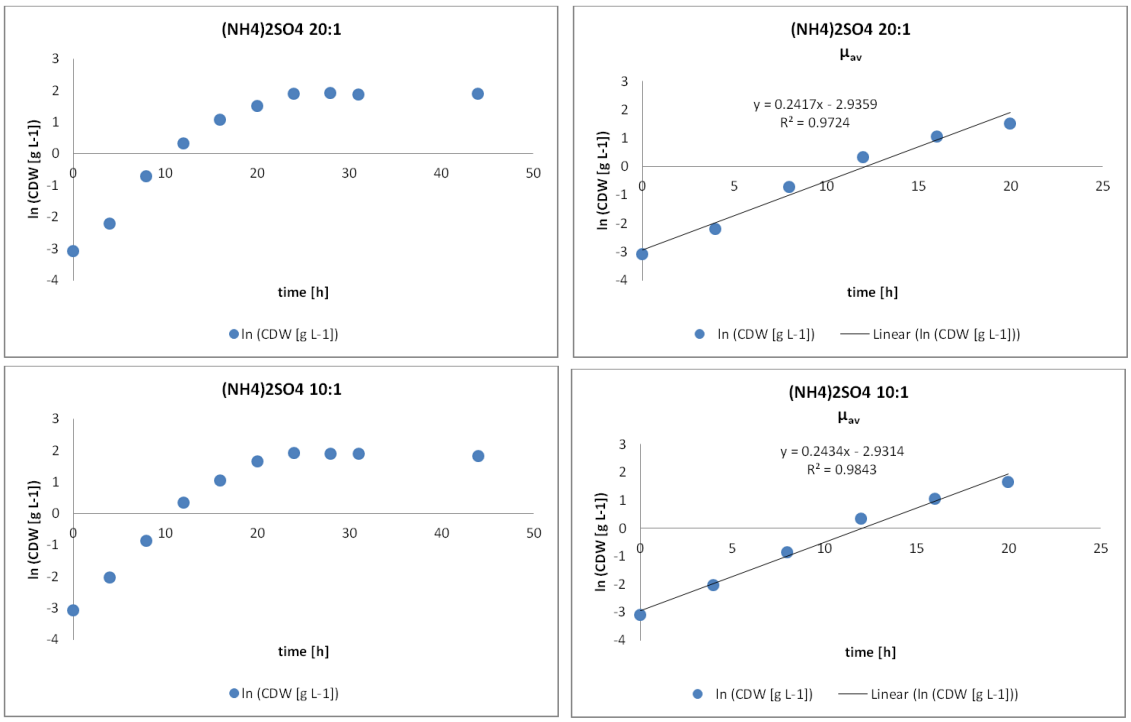


Figure 4.23: Determination of the average specific growth rate (μ_{av}) from $(NH_4)_2SO_4$ 20:1 and 10:1 shaken flask cultures.

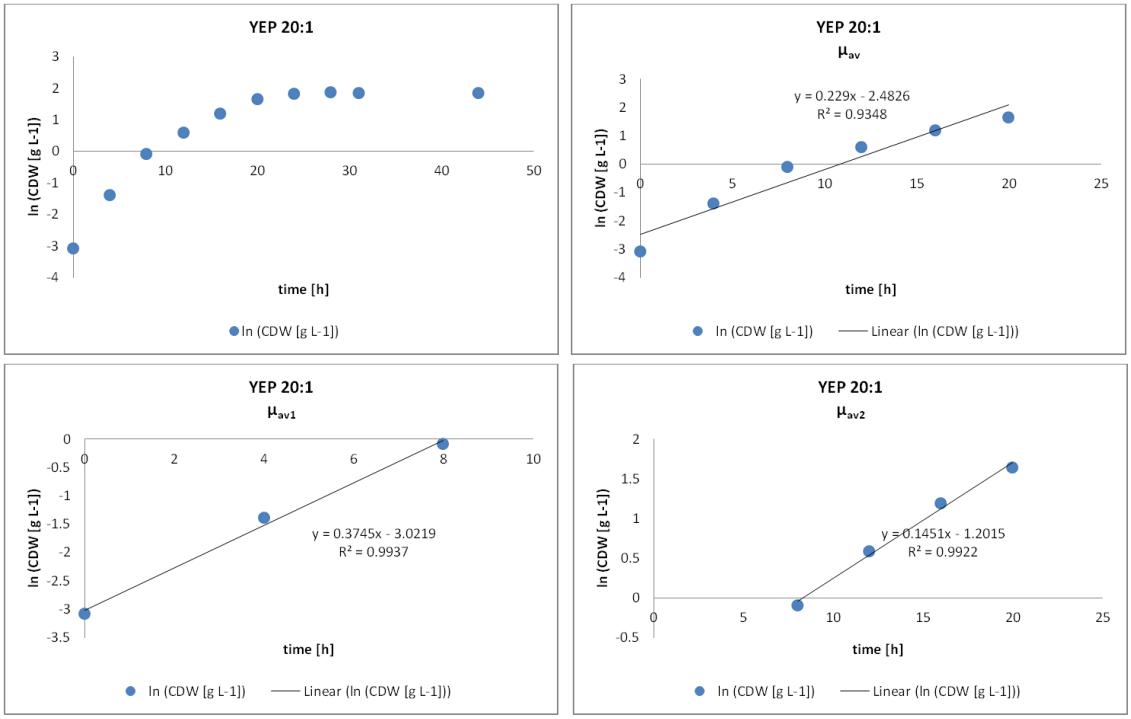


Figure 4.24: Determination of the average specific growth rate (μ_{av}), first average specific growth rate (μ_{av1}) and second average specific growth rate (μ_{av2}) from YEP 20:1 shaken flask culture.

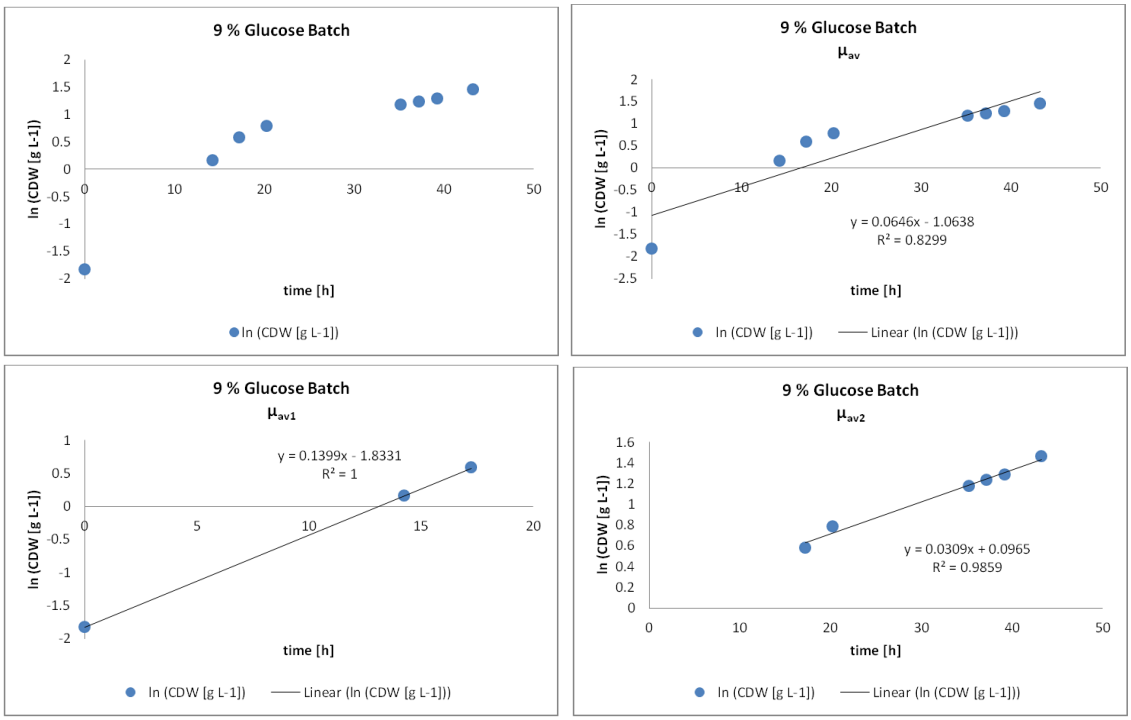


Figure 4.25: Determination of the average specific growth rate (μ_{av}), first average specific growth rate (μ_{av1}) and second average specific growth rate (μ_{av2}) from 9% Glucose Batch culture.

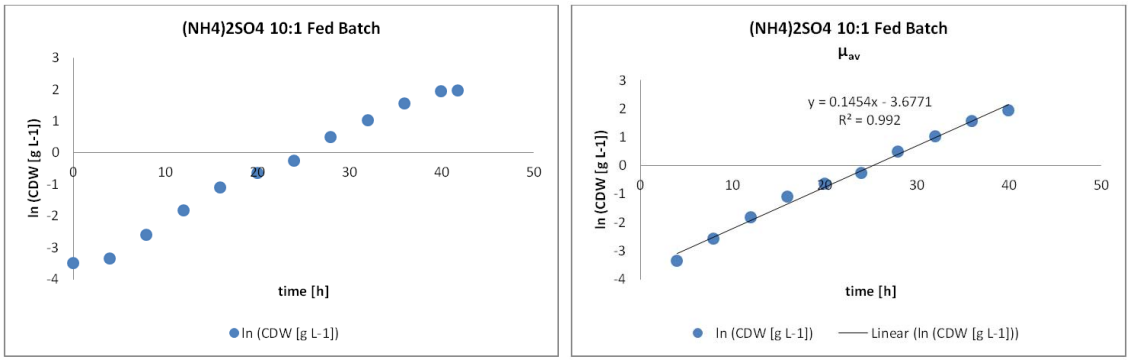


Figure 4.26: Determination of the average specific growth rate (μ_{av}) from $(NH_4)_2SO_4$ 10:1 Fed-Batch culture.

Table 4.5: Comparison of growth rates

Culture	μ_{max} [h^{-1}] [a]	μ_{av} [h^{-1}] [b]	μ_{av1} [h^{-1}] [c]	μ_{av2} [h^{-1}] [d]
Shaken Flask Cultures				
9% Glucose A	0.110	0.012	-	-
9% Glucose B	0.108	0.012	-	-
Urea 20:1	0.344	0.166	-	-
Urea 10:1	0.281	0.238	-	-
NH_4NO_3 20:1	0.330	0.260	-	-
NH_4NO_3 10:1	0.402	0.249	-	-
$(NH_4)_2SO_4$ 20:1	0.373	0.242	-	-
$(NH_4)_2SO_4$ 10:1	0.301	0.243	-	-
YEP 20:1	0.426	0.229	0.375	0.145
Bioreactor Cultures				
9% Glucose Batch	0.142	0.065	0.140	0.031
$(NH_4)_2SO_4$ 10:1 Fed-Batch	0.191	0.145	-	-

[a] maximal specific growth rate (μ_{max})

[b] average specific growth rate (μ_{av})

[c] first average specific growth rate (μ_{av1})

[d] second average specific growth rate (μ_{av2})

experiment, with 0.77, 0.34 and 0.044 h^{-1} for the specific glucose uptake rate (sUR_{Gluc}), specific carbon uptake rate (sUR_C) and specific nitrogen uptake rate (sUR_N) respectively. Except for the 9% Glucose shaken flask cultures, which both had a sUR_{Gluc} of 0.049 h^{-1}, the sUR_{Gluc} was between 0.27 h^{-1} and 0.37 h^{-1} for the YEP 20:1 and Urea 20:1 culture respectively. This was again supported by the specific carbon uptake rates. From the nitrogen source experiment, the Urea 20:1 and 10:1 cultures had the lowest specific nitrogen uptake rate (sUR_N) with 0.0078 and 0.0089 h^{-1} respectively. Highest rates were achieved with the NH_4NO_3 20:1 and 10:1 cultures, both with 0.026 h^{-1}.

4.6.3 Comparision of Yields

Most cultures had a biomass to substrate yield ($Y_{X/S}$) around 0.4 as shown in table 4.7. The highest $Y_{X/S}$ was achieved with the NH_4NO_3 10:1, $(NH_4)_2SO_4$ 20:1 and 10:1 cultures with 0.46 and the lowest $Y_{X/S}$ of 0.26 with 9% Glucose cultures. The $Y_{X/S}$ of 0.49 of the Urea 10:1 culture is not taken into consideration, because it was biased by the precipitated crystals. The $Y_{X/S}$ derived from the last biomass determination (5.85 g L^{-1}) is 0.43 and is thus very much in line with the other cultures.

The highest docosahexaenoic acid to substrate yield ($Y_{DHA/S}$) and total fatty acid to substrate yield ($Y_{TFA/S}$) were achieved during the feed phase of the $(NH_4)_2SO_4$ 10:1 Fed-Batch experiment, with 0.061 g g^{-1} and 0.106 g g^{-1} respectively. The $Y_{X/S}$ of the batch and feed phase were almost the same, with 0.36 g g^{-1} and 0.34 g g^{-1} respectively.

Table 4.6: Comparison of maximum uptake rates

Culture	$\mathbf{UR_{Gluc}}$ [a] [g L^{-1} h^{-1}]	$\mathbf{UR_C}$ [b] [g L^{-1} h^{-1}]	$\mathbf{UR_N}$ [c] [g L^{-1} h^{-1}]	$\mathbf{sUR_{Gluc}}$ [d] [h^{-1}]	$\mathbf{sUR_C}$ [e] [h^{-1}]	$\mathbf{sUR_N}$ [f] [h^{-1}]
Shaken Flask Cultures						
9% Glucose A	0.48	-	0.0098	0.049	-	0.0016
9% Glucose B	0.39	-	0.0087	0.049	-	0.0014
Urea 20:1	0.98	0.38	0.022	0.37	0.16	0.0078
Urea 10:1	1.12	0.45	0.039	0.34	0.14	0.0089
NH_4NO_3 20:1	1.20	0.46	0.039	0.31	0.11	0.026
NH_4NO_3 10:1	1.11	0.41	0.061	0.33	0.12	0.026
$(NH_4)_2SO_4$ 20:1	1.03	0.37	0.061	0.33	0.13	0.025
$(NH_4)_2SO_4$ 10:1	1.10	0.42	0.067	0.35	0.13	0.021
YEP 20:1	1.26	0.52	0.024	0.27	0.11	0.011
Bioreactor Cultures						
9% Glucose Batch	-	0.54	0.042	-	0.24	0.013
$(NH_4)_2SO_4$ 10:1 Fed-Batch	1.37	0.50	0.049	0.77	0.34	0.044

[a] volumetric glucose uptake rate (UR_{Gluc})

[b] volumetric carbon uptake rate (UR_C)

[c] volumetric nitrogen uptake rate (UR_N)

[d] specific glucose uptake rate (sUR_{Gluc})

[e] specific carbon uptake rate (sUR_C)

[f] specific nitrogen uptake rate (sUR_N)

Table 4.7: Comparison of biomass and product yields

Culture	$\mathbf{CDW_{start}}$ [a] [g L^{-1}]	$\mathbf{CDW_{end}}$ [a] [g L^{-1}]	$\mathbf{S_{start}}$ [b] [g L^{-1}]	$\mathbf{S_{end}}$ [b] [g L^{-1}]	**DHA** [c] [% CDW]	**TFA** [d] [% CDW]	$\frac{DHA}{TFA}$ [e] [%]	$\mathbf{Y_{X/S}}$ [f] g g^{-1}	$\mathbf{Y_{DHA/S}}$ [g] g g^{-1}	$\mathbf{Y_{TFA/S}}$ [h] g g^{-1}
9% Glucose A	2.86	28.96	101.85	0	-	-	-	0.26	-	-
9% Glucose B	2.73	29.12	101.10	0	18.44	30.91	59.65	0.26	0.053	0.089
Urea 20:1	0.045	5.98	14.15	0	8.92	20.13	44.32	0.42	0.038	0.085
Urea 10:1	0.045	6.65 [i]	13.61	0	5.23	10.31	50.76	0.49	0.026	0.050
NH_4NO_3 20:1	0.045	5.86	14.29	0	0.00	12.48	0.00	0.41	0.000	0.051
NH_4NO_3 10:1	0.045	6.69	14.52	0	5.33	11.17	47.67	0.46	0.025	0.051
$(NH_4)_2SO_4$ 20:1	0.045	6.71	14.34	0	0.00	4.52	0.00	0.46	0.000	0.021
$(NH_4)_2SO_4$ 10:1	0.045	6.73	14.44	0	0.00	2.24	0.00	0.46	0.000	0.010
YEP 20:1	0.045	6.43	14.47	0	6.54	17.50	37.34	0.44	0.029	0.078
$(NH_4)_2SO_4$ 10:1 Batch Phase	0.030	7.17	19.78	0	2.71	14.28	19.00	0.36	0.010	0.052
	[g]	[g]	[g]	[g]						
$(NH_4)_2SO_4$ 10:1 Feed Phase	28.68	94.82	250	57.72	20.59	45.18	45.56	0.34	0.061	0.106

[a] cell dry weight (CDW)

[b] glucose as substrate (S)

[c] docosahexaenoic acid (DHA)

[d] total fatty acid (TFA)

[e] mass fraction of docosahexaenoic acid (DHA) to total fatty acid (TFA)

[f] biomass to substrate yield ($Y_{X/S}$)

[g] docosahexaenoic acid to substrate yield ($Y_{DHA/S}$)

[h] total fatty acid to substrate yield ($Y_{TFA/S}$)

[i] CDW with crystals

5 Discussion

5.1 Glucose and Yeast Extract and Peptone Medium

The shaken flask experiment with 9% (w/w) glucose and 2% (w/w) YEP was performed to gather basic information about the growth behaviour and FA accumulation of *A. limacinum* in high concentrated medium. For the first 22 h, the growthrates were about 0.11 h^{-1} in both cultures, which fits well with already reported data of 0.11 h^{-1} by Rosa et al. (2010) and 0.14 h^{-1} by Jakobsen et al. (2008). Afterwards the growthrate decreased and growth was continued with an average specific growth rate (μ_{av}) of 0.012 h^{-1}, which is the worst growth rate observed in all performed experiments. To exclude a possible oxygen limitation as the reason for the low μ_{av}, another experiment was done with the same medium composition but in a bioreactor, in which the DO was kept at a minimum level of at least 30% air saturation. The growthrates were successfully increased to a μ_{max} of 0.142 h^{-1} and μ_{av} of 0.065 h^{-1} by the cultivation in the bioreactor. The μ_{av} was actually composed of two different growth rates, named μ_{av1} and μ_{av2}. For the first 17 h the cells were growing with μ_{av1} of 0.140 h^{-1} and after with μ_{av2} of 0.031 h^{-1}. This growth behaviour was also observed in the YEP 20:1 culture (2% (w/w) glucose and 0.4% (w/w) YEP), but the actual growth rates were much higher. The μ_{max} was at 0.426 h^{-1}, which was also the highest growthrate observed in all performed experiments, and μ_{av} was at 0.229 h^{-1}. *A. limacinum* was growing with μ_{av1} of 0.375 h^{-1} for the first 8 h and with μ_{av2} of 0.145 h^{-1} for another 12 h.

The reason for this two phase growing behaviour is probably the availability of nitrogen in different forms, both in yeast extract and peptone. The yeast extract, used in the experiments, contained 10.5% TN (no information available about amino N), and peptone from casein had 12-13% TN and 3.0-5.0% amino N. With a TN of about 11% (11.8% measured from the YEP stock solution), the growth rate of the culture will always suffer from nitrogen limitation after a first period of fast growth, which is due to the easily available nitrogen in form of amino acids. The nitrogen limitation was also most likely the reason why cultures grown on YEP medium had a rather high lipid content, because it has been shown to be the driving factor of fatty acid accumulation in several studies (Yokochi et al., 1998; Jakobsen et al., 2008; Rosa et al., 2010; Bailey et al., 2010).

The low growth rates with 9% (w/w) glucose medium can be explained by substrate inhibition. Growth inhibition by high glucose concentration was mentionend by Yokochi et al. (1998) for 12% glucose, but only the final CDW was determined after 5 days and

thus no information about the effect on the growth rate by the glucose concentration can be obtained from that data. It is however mentioned by Bailey et al. (2010) that high substrate concentrations is believed to have a detrimental effect on cell growth, which is further supported by the data of this study.

9% (w/w) glucose and 2% (w/w) YEP medium is not suited for the production of biomass and FAs, although the obtained CDW concentrations of 29 g L^{-1} and TFA content of 31% (w/w) may appear good, but simply take too much time to generate.

5.2 Assessment of Tested Nitrogen Sources

In addition to YEP, urea, NH_4NO_3 and $(NH_4)_2SO_4$ were investigated for their effect on the maximum CDW, specific growth rate and lipid synthesis in order to find a medium composition that would promote fast production of biomass with a low lipid content. The low lipid content was required to gather information, in the following experiment, about the generation of lipids in a fed-batch, in which the biomass was produced during the batch-phase and lipids were generated during the feed-phase. In addition, a low lipid content indicate that cell growth is not limited by the availability of nitrogen, which is required for fast biomass production.

A glucose concentration of 20 g L^{-1} was used to prevent possible substrate inhibition. Measurement of the residual glucose showed that all cultures had a starting concentration of about 14 g L^{-1}. Although the correct amount of glucose stock solution was added to the media, it is possible that a gradient of glucose formed in the stock solution during the storage period, which was not completely abolished by shaking. The actual C:N ratios were therefore approximately 12:1 and 6:1 instead of 20:1 and 10:1.

5.2.1 pH-Range

The first test of different nitrogen sources failed due to unadjusted pH. However, the pH measurements of the samples showed that *A. limacinum* can survive within a range of pH 2.1-9.0. Growth was only observerd below pH 8.0 and a pH around 2 is tolerated for a short period of time, as can be seen from the second experiment, but prevents cell growth if it's not adjusted. A similar pH range was reported by Perveen et al. (2006) with a similar organism, that was also isolated from a mangrove area. In that case, strain 12B could not grow at pH 2 and 9 and had a increasing production of biomass and TFA from pH 3 up to pH 8, where it achieved maximal biomass, TFA and DHA yield.

5.2.2 Urea

The Urea 20:1 and 10:1 cultures produced 5.98 and 6.65 g L^{-1} CDW with a TFA content of $20.13 \pm 1.09\%$ and $10.31 \pm 1.94\%$ respectively. The 6.65 g L^{-1} CDW of the Urea 10:1 culture was biased by precipitated crystals, and thus the highest measured CDW was 5.85 g L^{-1}. The final CDW was mostly influenced by the availability of glucose, while the TFA content was in a clear relationship to the C:N ratio. Interestingly the consumption rate of urea did not increase with increasing biomass, which may have led to an intercellular nitrogen limitation that resulted in a higher TFA content at the end of the experiment. Nitrogen consumption stopped in the Urea 10:1 culture after all glucose was consumed, instead it continued in the Urea 20:1 culture even after all glucose was gone, indicating a deficit of nitrogen in the Urea 20:1 medium. A compareable result was published by Yokochi et al. (1998), which showed a CDW, TFA and DHA production of approximately 24.7 g L^{-1}, 2.7 g L^{-1} and 0.4 g L^{-1} respectively, with a medium composition of 6% glucose, 0.1% yeast extract, 0.1% corn steep liquor and 15.1 mM urea. Although the ratio of about 66 C:N (neglecting yeast extract and corn steep liquor) was much higher than both media of this study, the TFA content was just about 11%, which is similar to that of the Urea 10:1 culture. One reason for this difference may be the time after which the samples were collected for the analysis of FAs, which was 5 days in the study of Yokochi et al. (1998) and 44 h in this present study. However since the biomass was only measured once after 5 days, it is not possible to tell if the biomass and TFA content decreased due to nutrient starvation.

In the terms of fast biomass production with a low FA content, urea supported neither fast production of biomass (μ_{av} of 0.166 and 0.238 h^{-1} for Urea 20:1 and 10:1 respectively) nor a low lipid content and thus it was not chosen as nitrogen source for the fed-batch experiment.

5.2.3 Ammoniumnitrate

The NH_4NO_3 20:1 and 10:1 cultures produced 5.86 and 6.69 g L^{-1} CDW with a TFA content of $12.48 \pm 0.44\%$ and $11.17 \pm 0.39\%$ respectively. In contrast to the Urea 20:1 and 10:1 cultures, the maximum CDW was effected by the C:N ratio. In addition both NH_4NO_3 cultures had quite similar ω_{TFA}, but with a different FA composition. While HDA was the most abundant FA in the NH_4NO_3 20:1 culture (92.1% of TFA) and DHA was not detectable, DHA was the most abundant FA in the NH_4NO_3 10:1 culture with 47.7% of TFA and HDA was making up for 39.9% of TFA. This results are actually quite suprising, because in all other cultures the FA composition was very similar between the two cultures grown on the same nitrogen source, and the different concentration of the nitrogen source was reflected in the TFA content of the biomass. While the FA composition of the NH_4NO_3 20:1 culture was similar to those of the $(NH_4)_2SO_4$ cultures (DHA not detectable and HDA making up for over 90% of the TFA content), the FA

composition of the NH_4NO_3 10:1 culture was similar to the Urea 20:1, 10:1 and YEP 20:1 cultures. Yokochi et al. (1998) reported a final CDW, after 5 days of cultivation in a 6% glucose, 0.1% yeast extract, 0.1% corn steep liquor and 15.1 mM NH_4NO_3 medium, of about 21.7 g L^{-1} with 4.0 g L^{-1} TFA (18.4% CDW) and 1.24 g L^{-1} DHA (31.0% of TFA). This data appears to be compareable to the NH_4NO_3 10:1 culture in terms of TFA and DHA content, considering the different C:N ratio.

It is also worth mentioning, that both cultures only consumed about half of the nitrogen available. Since the TN measurements of the $(NH_4)_2SO_4$ cultures show that nearly all nitrogen in form of ammonium was consumed by the cells, the residual nitrogen in the NH_4NO_3 cultures is most likely in form of nitrate. Yokochi et al. (1998) reported that *A. limacinum* can utilise nitrate, although with a low biomass yield, the results of this study do not support it. It may very well be the case, that nitrate is slower metabolized by *A. limacinum* and thus Yokochi et al. (1998) were able to see growth due to the much longer cultivation period of 5 days.

Although the NH_4NO_3 20:1 achieved the highest average specific growth rate of 0.260 h^{-1}, NH_4NO_3 did not appear to be a suitable nitrogen source, because of its high residual nitrogen. It may howerver indicate that *A. limacinum* prefers lower ammonium concentrations.

5.2.4 Ammoniumsulfate

A. limacinum produced a maximum CDW of 6.71 and 6.73 g L^{-1} and a TFA content of $4.52 \pm 0.22\%$ and $2.24 \pm 0.11\%$ of CDW in the $(NH_4)_2SO_4$ 20:1 and 10:1 media respectively. Similar to the Urea 20:1 and 10:1 cultures, $(NH_4)_2SO_4$ 20:1 and 10:1 cultures had very similar maximum CDWs and lipid compositions but different ω_{TFA}. HDA accounted for 91.4% and 95.9% of TFA of the $(NH_4)_2SO_4$ 20:1 and 10:1 biomass samples respectively.

Both cultures grew with very similar μ_{av} of 0.242 and 0.243 h^{-1} for $(NH_4)_2SO_4$ 20:1 and 10:1 respectively. $(NH_4)_2SO_4$ 20:1 had a μ_{max} of 0.373 h^{-1}, while the μ_{max} of the $(NH_4)_2SO_4$ 10:1 culture only reached 0.301 h^{-1}. The high μ_{max} of the $(NH_4)_2SO_4$ 20:1 may have been caused by a CDW measurement error, because it's just between the 8 and 12 h measurement points. Instead the the μ_{max} of the $(NH_4)_2SO_4$ 10:1 was maintained over the first 16 h.

Yokochi et al. (1998) reported a biomass and TFA production of about 24 g L^{-1} and 1.67 g L^{-1} respectively, with a DHA content of 0.67 g L^{-1} (40% of TFA). In contrast to that, DHA was not detectable in both cultures of this experiment. However in the fed-batch experiment, DHA was detected at the end of the batch phase.

The $(NH_4)_2SO_4$ 10:1 medium composition was chosen as the batch medium for the fed-batch experiment, because it produced the highest maximum CDW (6.73 g L^{-1}) with the lowest lipid content ($2.24 \pm 0.11\%$ of CDW), aswell as a very good μ_{av} (0.243 h^{-1}).

5.2.5 Yeast Extract and Peptone

The YEP 20:1 culture, which served as reference, produced a maximum CDW of 6.43 g L^{-1} and a TFA content of $17.50 \pm 1.34\%$ of CDW. The lipids were composed of 51.4% HDA and 37.3% DHA, quite similar to the lipid composition of the NH_4NO_3 10:1 culture. The μ_{max} of 0.426 h^{-1} was the highest observed during this study, but the specific growth rate decreased gradually over the cultivation period, which led to a μ_{av} of 0.229 h^{-1}. As mentioned already before, μ_{av} was composed of μ_{av1} of 0.375 h^{-1} and μ_{av2} of 0.145 h^{-1}. The stong decrease of the specific growth rate was most likely caused by the fast depletion of easy utilisable nitrogen in form of amino acids.

YEP was not chosen as primary nitrogen source of the fed-batch medium, because it did not enable the cells to grow with a constant specific growth rate and the produced biomass had a high TFA content.

5.3 Production of DHA in a Fed-Batch

5.3.1 Batch-Phase

Based on the results of the nitrogen source experiment, *A. limacinum* was cultivated in a bioreactor in 4 L of $(NH_4)_2SO_4$ 10:1 medium at pH 7.00, 25℃ and at a minimum DO level of 30% air saturation. Upon inoculation of the medium, crystals precipitated due to the pH, most likely phosphate which was used as a buffer. Since only NaOH was used to control the pH, the set point for the pH control was adjusted to 6.00 and acidification of the medium was done by the cells over the next 24 h. The crystals dissolved after 16 h at pH 6.4. To prevent errors of the CDW determination, the the first three biomass samples where washed with acidified DI H_2O pH 2.00 (adjusted with HCl), to dissolve the crystals. CDW determination may have been slightly negatively biased by this treatment, because some of the cell membranes appeared ruptured under the microscope. On the other hand, except for the first measurement after 4 h, the specific growth rate was quite constant (about 0.19 h^{-1}) over this particular time frame and also until the first CDW determination with regular DI H_2O as washing solution. The low growthrate at the beginning of the fermentation may have been caused by destruction of cells due to the crystals in solution, which where highly abundant for the first 4 h.

The decline of the growth rate, after 16 h from 0.185 h^{-1} to 0.112 h^{-1} at 20 h and 0.094 h^{-1} at 24 h, was coincident with the formation of zoospores and an increase of the specific glucose uptake rate (sUR_{Gluc}). After the release of zoospores, the specific growth rate increased again to 0.186 h^{-1}, followed by a successive decrease until the end of the batch.

The μ_{av} of 0.145 h^{-1} and $Y_{X/S}$ of 0.36 g g^{-1} observed in this experiment, were considerably lower than the μ_{av} of 0.243 h^{-1} and $Y_{X/S}$ of 0.46 g g^{-1} from the $(NH_4)_2SO_4$

10:1 shaken flask culture. The reason for this was most likely the destruction of cells by the high shear force of the Rushton impeller blades as already shown by Yaguchi et al. (1997). Similar observations were reported by W. Hong and Seo (2013) with a closely related strain *Aurantiochytrium* sp. KRS101. When cultivated in a 5000 L (3000 L working volume) scale stirred-type bioreactor, *Aurantiochytrium* sp. KRS101 achieved a specific growth rate of 0.057 h^{-1}, compared to 0.090 h^{-1} when grown in a 6000 L (3000 L working volume) scale airlift-type bioreactor.

The C:N ratio of the batch medium appears suitable for the production of non lipid biomass, since the C:N ratio was at a constant level of about 7.8 over the first 32 h of cultivation and only decreasing during the last 10 h of the batch phase. This shows that C and N were taken up by the cells in the same ratio as they were supplied by medium and thus neither C nor N was limiting growth until the end of the batch.

CDW (7.17 g L^{-1}) and ω_{TFA} (14.28% CDW) of the bioreactor culture were higher than those of the $(NH_4)_2SO_4$ 10:1 shaken flask culture (6.73 g L^{-1} CDW and a ω_{TFA} of 2.24% CDW), which can be explained by the higher C:N ratio. Jakobsen et al. (2008) report a very similar lipid content of exponentially growing cells with 13% of CDW. However the concentrations of C and N may still not be optimal, because NH_4NO_3 20:1 and 10:1 cultures of the prior experiment achieved the highest average specific growth rate of 0.260 h^{-1} and 0.249 h^{-1} respectively, indicating that *A. limacinum* may prefer ammonia concentrations around 7.5 mM or even lower. Another explanation for the higher TFA content of the bioreactor culture could be the pH, which was controlled at pH 6.00, while the shaken flask culture grew interimly at pH 2.6.

5.3.2 Feed-Phase

During the feed-phase, the generated biomass was fed exponentially with 1 L of a 250 g L^{-1} glucose, 2.75 g L^{-1} $(NH_4)_2SO_4$ medium (200 C:N) over 5 h, increasing the glucose concentration by 1% per hour. To test if DHA production is enhanced by oxygen limitation, the air supply was completely turned off for 1 h after the 5 h feed-phase. The air supply was turned on afterwards and the fermentation continued with a dissolved oxygen level of 30% air saturation for another 26 h.

The CDW increased in a rather linear fashion due to the nitrogen limitation, quite similar to results shown by Jakobsen et al. (2008). Despite the rather low biomass concentration (7.17 g L^{-1} at the beginning and 19.03 g L^{-1} at the end), the volumetric docosahexaenoic acid productivity (q_{DHA}), 119.4 mg L^{-1} h^{-1} for just the feed-phase and 53.1 mg L^{-1} h^{-1} for the whole process, appears quite good compared to published data. Already reported q_{DHA} values with corresponding biomass concentrations are e.g. 122.62 mg L^{-1} h^{-1}, 61.76 g L^{-1} (Huang et al., 2012); 119 mg L^{-1} h^{-1}, 71 g L^{-1} (Ren et al., 2010); 116.67 mg L^{-1} h^{-1}, 21.45 g L^{-1} (Perveen et al., 2006); 138.54 mg L^{-1} h^{-1}, 48.1 g L^{-1} (Yaguchi et al., 1997).

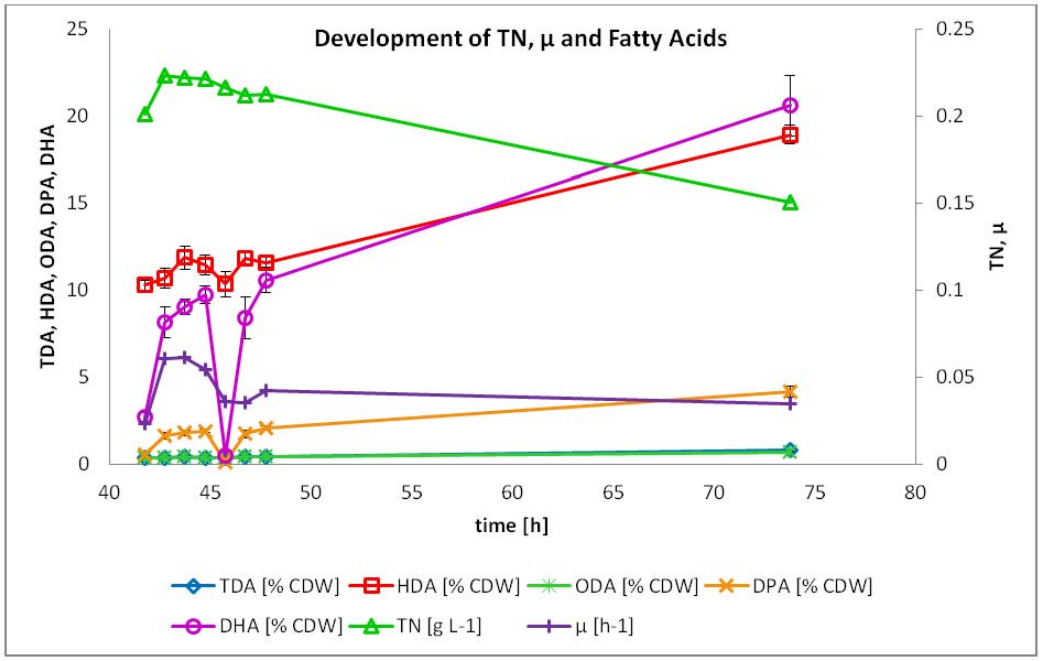


Figure 5.1: Development of the fatty acid composition, total nitrogen (TN) and specific growth rate (μ).

At the beginning of the feed-phase and also after the loss of almost all DHA, DHA increased very rapidly to 8.16 ± 0.90 and $8.41 \pm 1.19\%$ CDW within one hour. This shows that *A. limacinum* can produce DHA up to about 8% of its CDW very fast, after which saturation is reached and DHA accumulation continues with a much lower rate. The drop of the DHA and its precursor DPA was coincident with a decrease of the specific growth rate (μ) and a weak increase in the nitrogen consumption as shown in figure 5.1. Interestingly, TDA, HDA and ODA were almost not effected. This data indicates that the sudden loss of PUFAs may have been caused by methabolic activities of the cells, induced by the availability of nitrogen. It may also be a hint to the function of DHA in *A. limacinum* as a quickly metabolizeable storage compound. Based on this results, it is probably a better strategy to use a nitrogen free feed-medium to avoid depletion of already generated DHA by the cells.

During the oxygen limitation period, the q_{DHA} of 193.4 mg L^{-1} h^{-1} was about 62% higher than the average q_{DHA} of 119.4 mg L^{-1} h^{-1}. This results are in accordance with those published by Jakobsen et al. (2008), which showed that nitrogen starvation in combination with oxygen limitation give the highest lipid content, aswell as oxygen limitation increasing the DHA to TFA ratio. As already inferred by Jakobsen et al. (2008), this is most likely due to the fact, that HDA and DHA are synthesized by two

different pathways (Hauvermale et al., 2006; Metz et al., 2001) in the genus *Aurantiochytrium*, which was formerly known as *Schizochytrium*. *A. limacinum* uses an oxygen independant polyunsaturated fatty acid synthase for the production of DPA and DHA and thus excess carbon may be directed towards the production of DPA and DHA under oxygen limitation. However, this implies that the fatty acid synthesis pathway for TDA and HDA is oxygen dependent or at least affected. Since there is only few information available about the biosynthesis of FAs in *Aurantiochytrium*, further research will be needed to clarify this topic.

5.4 Microscopic Observations

During the experiments, chemotaxis of zoospores towards liquid/air interfaces was observed. The same observation was already reported by Chi et al. (2009), which gave rise to the hypothesis, that a high dissolved oxygen (DO) level may promote zoospore production and thus generate higher cell numbers during the biomass production phase, which would be favorable for the sequential lipid production phase. In the $(NH_4)_2SO_4$ 10:1 Fed-Batch experiment, the release of zoospores was also coincidental with the rise of the DO level, due to an increase of the minimum impeller speed from 300 to 400 rpm.

At the end of the batch-phase, formation of sperical vacuoles were observed mostly in large/old cells, while small/young cells remained with a quite homogen cytoplasm. The vacuoles grew in nummber and size during the feed-phase and are thus most likely lipid bodies as shown by Morita et al. (2006), using Nile red staining. Based on this observations, in contrast to Chi et al. (2009), it appears that small/young cells are contributing only in a minor way to the lipid production and the major part is accomplished by large/old cells. It may therefore be advantageous to avoid zoospore formation by a reduced DO level during the biomass production phase.

Another very interesting observation were the limaciform cells in the $(NH_4)_2SO_4$ 10:1 Fed-Batch culture. This cell type was mentioned by Honda et al. (1998) and was characterized as elongated amorphous cells, that were observed at the margins of colonies on nutrient agar plates. So far there is neither information available about the function of this cell type nor about the environmental circumstances under which they form. In this study, the first limaciform cells were observed 32 h after inoculation. At that time, nutrients were still available, which means that nutrient limitation may not be a factor that induces the formation of limaciform cells. One very interesting picture was taken from the culture after the oxygen limitation period. Two limaciform cells are tightly connected to each other and in both cells a rather transparent spherical vacuole is visible. At the area were both cells connect to each other, an opening in both cell's membranes appears to connect both cell's cytoplasm. This may be the first evidence of sexual reproduction in the family of Thraustochytriaceae, which is so far described as asexual (Adl et al., 2012).

6 Conclusion

The present study shows that for the production DHA with *Aurantiochytrium limacinum* SR21 (*A. limacinum*) a two stage process, which consists of a biomass accumulation and a fatty acid (FA) production phase, is clearly preferable to the use of media with high substrate concentrations. Shaken flask cultures with high concentrated medium (9% glucose, 2% yeast extract and peptone from casein (YEP)) had a low specific growth rate (μ) aswell as a low volumetric total fatty acid productivity (q_{TFA}). While the μ_{av} could be increased from 0.012 h^{-1} to 0.065 h^{-1} by the use of a bioreactor, in which the dissolved oxygen (DO) level was kept at a minimum of 30% air saturation, it was still much lower compared to experiments using similar media compositions with lower concentrations. Highest maximal specific growth rate (μ_{max}) of 0.426 h^{-1} was achieved using a 2% glucose, 0.4% YEP medium, however the μ_{av} was just about 0.229 h^{-1}. Since the reason of the substrate inhibition wasn't further investigated, it is not clear if the low μ was caused by the high glucose or YEP concentration. According to Yokochi et al. (1998), a glucose concentration of 9% should not inhibit growth of the culture, while Bailey et al. (2010) mention that high substrate concentration can have a detrimental effect on cell growth. High concentrations of YEP could very well have an inhibitory effect, due to side products that can form during the autoclavation process. Another drawback of YEP was also that high residual concentrations of carbon and nitrogen remained in the medium after cell growth ceased, which means that substrate utilization of YEP by *A. limacinum* is rather poor. However, a small amount of YEP (0.05%) had a beneficial effect on cell growth and was thus kept in the media compositions of later experiments.

N-Sources and C:N Ratios Urea, NH_4NO_3 and $(NH_4)_2SO_4$ were tested as possible nitrogen sources for a batch medium, that whould support fast cell growth and low FA producion. Thus, substrate concentrations needed to be in a range where no inhibitory effects would occur, aswell as a proper C:N ratio had to ensure that no substrate limitation takes place until the end of the batch. Both requirements were met by the $(NH_4)_2SO_4$ 10:1 medium composition (2% glucose, 31.2 mM $(NH_4)_2SO_4$). In shaken flask, the culture achieved a μ_{av} of 0.243 h^{-1} and had a TFA content of 2.24% of CDW. Residual glucose and ammonium exhausted simultaneously, thus no substrate limitation took place during the whole cultivation period. However, it's worth mentioning that

the highest μ_{av} of 0.260 h^{-1} was observed with NH_4NO_3, using a C:N ratio of 20:1. Therefore the ammonium concentration of $(NH_4)_2SO_4$ 10:1 medium might cause slight inhibition of cell growth. In both NH_4NO_3 cultures, the residual nitrogen concentration was about half of the start concentration. This suggests that nitrate was not consumed by the cells, even though cells in both cultures experienced nitrogen limitation. Thus NH_4NO_3 did not appear to be a suitable nitrogen source for *A. limacinum*. Urea, on the other hand, was chosen as a nitrogen source, because biomass of Urea 20:1 and Urea 10:1 cultures had a TFA yield of about 20% CDW and 10% CDW respectively. For unknown reason, urea was consumed in a rather linear fashion, which explains the high TFA content. The biomass to substrate yield ($Y_{X/S}$) of tested media was between 0.41 (NH_4NO_3 20:1) and 0.46 (NH_4NO_3 10:1, $(NH_4)_2SO_4$ 10:1, $(NH_4)_2SO_4$ 20:1). A summary of all performed experiments is shown in table 6.1.

Production of DHA in a Fed-Batch Fed-batch cultivation of *A. limacinum* was chosen to investigate the production of FAs under nitrogen limitation. Cells grew, after a short lag phase, with an μ_{av} of 0.145 h^{-1} in 4 L of $(NH_4)_2SO_4$ 10:1 medium and had a biomass to substrate yield ($Y_{X/S}$) of 0.36. The decreased μ_{av} and $Y_{X/S}$, compared to the shaken flask culture, indicate that cells might have been destroyed during the fermentation by the high shear force created by the Rushton impeller blades . Since oxygen supply doesn't appear to be a problem with *A. limacinum*, a different impeller type such as pitched-blade or marine-blade impellers would be advantageous to prevent the destruction of cells by reducing the shear force.

Analysis of the biomass, taken during the feed phase, revealed that *A. limacinum* can accumulate lipids up to TFA content of about 20% very fast, with q_{TFA} and q_{DHA} of 568 mg L^{-1} h^{-1} and 409 mg L^{-1} h^{-1} during the first hour. The average q_{DHA} of 119.4 mg L^{-1} h^{-1} (feed phase) and 53.1 mg L^{-1} h^{-1} (whole process) are quite remarkable, considering the rather low biomass concentration of 19.03 g L^{-1} at the end of fermentation. Oxygen limitation, which was originally not planned for the fermentation but rather performed out of curiosity, appeared to be aswell beneficial for DHA production, with a q_{DHA} of 193 mg L^{-1} h^{-1}. It should however be further investigated, since one sampling point is far too few to draw any conclusions from it. Nitrogen, however, should not be included in the feed medium, because it was probably the reason for the sudden loss of DHA and DPA. It is probably worth spending additional research into the optimisation of the culture conditions of *A. limacinum* for the production of DHA, using on a double fed-batch strategy. Possibly inhibitory effects of high ammonia concentrations could be avoided by the use of a feed-medium with a C:N ratio of about 7.8 (measured C:N ratio of the $(NH_4)_2SO_4$ 10:1 medium) for the fast generation of biomass with low TFA content. A second nitrogen free feed-medium could then be used, in combination with oxygen limitation, for the production of DHA.

Table 6.1: Summary of Experiments

Culture	$\boldsymbol{\mu_{max}}$ [a] [h^{-1}]	$\boldsymbol{\mu_{av}}$ [b] [h^{-1}]	$\mathbf{CDW_{end}}$ [c] [g L^{-1}]	$\mathbf{Y_{X/S}}$ [d] g g^{-1}	$\boldsymbol{\omega_{DHA}}$ [e] [% CDW]	$\boldsymbol{\omega_{TFA}}$ [f] [% CDW]	$\frac{\boldsymbol{DHA}}{\boldsymbol{TFA}}$ [g] [%]
Shaken Flask Cultures							
9% Glucose A	0.110	0.012	28.96	0.26	-	-	-
9% Glucose B	0.108	0.012	29.12	0.26	18.44	30.91	59.65
Urea 20:1	0.344	0.166	5.98	0.42	8.92	20.13	44.32
Urea 10:1	0.281	0.238	6.65 [h]	0.49	5.23	10.31	50.76
NH_4NO_3	0.330	0.260	5.86	0.41	0.00	12.48	0.00
NH_4NO_3 10:1	0.402	0.249	6.69	0.46	5.33	11.17	47.67
$(NH_4)_2SO_4$ 20:1	0.373	0.242	6.71	0.46	0.00	4.52	0.00
$(NH_4)_2SO_4$ 10:1	0.301	0.243	6.73	0.46	0.00	2.24	0.00
YEP 20:1	0.426	0.229	6.43	0.44	6.54	17.50	37.34
Bioreactor Cultures							
9% Glucose Batch	0.142	0.065	-	-	-	-	-
$(NH_4)_2SO_4$ 10:1 Fed-Batch	0.191	0.145	19.03	0.35	20.59	45.18	45.56

[a] maximal specific growth rate (μ_{max})

[b] average specific growth rate (μ_{av})

[c] cell dry weight (CDW)

[d] biomass to substrate yield ($Y_{X/S}$)

[e] mass fraction of docosahexaenoic acid to cell dry weight (ω_{DHA})

[f] mass fraction of total fatty acid to cell dry weight (ω_{TFA})

[g] mass fraction of docosahexaenoic acid (DHA) to total fatty acid (TFA)

[h] CDW with crystals

Microscopic Observations Due to the complex life cycle of *A. limacinum*, microscopic pictures have been quite helpful to further understand some observed growth behaviour. The decline of specific growth rate (μ) together with an increase of the specific glucose uptake rate (sUR_{Gluc}) was coincidental with the formation of zoospores during the batch phase of the fed-batch experiment. Based on the microscopic observations, settled zoospores did not seem to accumulate lipids during nitrogen limitation, as no lipid bodies could be seen within the cells. If this is truly the case, it may be worth developing cultivation techniques that would prevent zoospore formation like e.g. using a low DO level.

The observation of limaciform cells in liquid culture was also a quite unexpected finding. Described by Honda et al. (1998) as elongated amorphous cells, that were observed at the margins of colonies on nutrient agar plates, there is literally nothing known about their function or the circumstances under which they form. They serve, however, as the stronges distinctive features to other thraustochytrids and are eponymous for the species *A. limacinum*. In this study they were only found during the fed-batch experiment. While the environmental conditions, that are responsible for their formation, are still unclear, their function may include sexual reproduction. During the fermentation two times two limaciform cells, tightly connected to each other, were observed. In one case, each of the cells had a spherical vacuole inside the cytoplasm, which appeared optically transparent in contrast to the quite optically dense amorphous cytoplasm. In addition a channel, connecting both cells cytoplasm is visible. While further genetical studies would be required to clearly proof sexual reproduction, those pictures are a good indication.

7 Appendix

Abbreviations

A. limacinum	*Aurantiochytrium limacinum* SR21
ALA	α-linolenic acid
ARA	arachidonic acid
ASW	artificial sea water
ATCC	American Type Culture Collection
CDW	cell dry weight
CN	cell number
DI H_2O	deionised water
DHA	docosahexaenoic acid
DO	dissolved oxygen
DPA	docosapentaenoic acid
EN	ectoplasmatic net
EPA	eicosapentaenoic acid
FA	fatty acid
FAME	fatty acid methyl ester
GC-MS	gas chromatography-mass spectrometry
HDA	hexadecanoic acid
Laminar	laminar flow closet with HEPA filter
ME	methyl ester
μ	specific growth rate
μ_{av}	average specific growth rate
μ_{av1}	first average specific growth rate
μ_{av2}	second average specific growth rate
μ_{max}	maximal specific growth rate
NPOC	non purgeable organic carbon
ODA	octadecanoic acid
OD_{600}	optical density at 600 nm wavelength
ω_{DHA}	mass fraction of docosahexaenoic acid to cell dry weight
ω_{DPA}	mass fraction of docosapentaenoic acid to cell dry weight
ω_{HDA}	mass fraction of hexadecanoic acid to cell dry weight
ω_{ODA}	mass fraction of octadecanoic acid to cell dry weight
ω_{TDA}	mass fraction of tetradecanoic acid to cell dry weight
ω_{TFA}	mass fraction of total fatty acid to cell dry weight

PKS	polyketide synthase
PUFA	poly unsaturated fatty acid
$\mathbf{P_{DHA}}$	docosahexaenoic acid formation rate
$\mathbf{P_{TFA}}$	total fatty acid formation rate
$\mathbf{q_{DHA}}$	volumetric docosahexaenoic acid productivity
$\mathbf{q_{TFA}}$	volumetric total fatty acid productivity
RCB	research cell bank
SCO	single cell oils
$\mathbf{sUR_C}$	specific carbon uptake rate
$\mathbf{sUR_{Gluc}}$	specific glucose uptake rate
$\mathbf{sUR_N}$	specific nitrogen uptake rate
TDA	tetradecanoic acid
TFA	total fatty acid
TN	total nitrogen
$\mathbf{UR_C}$	volumetric carbon uptake rate
$\mathbf{UR_{Gluc}}$	volumetric glucose uptake rate
$\mathbf{UR_N}$	volumetric nitrogen uptake rate
YEP	yeast extract and peptone from casein
$\mathbf{Y_{DHA/S}}$	docosahexaenoic acid to substrate yield
$\mathbf{Y_{TFA/S}}$	total fatty acid to substrate yield
$\mathbf{Y_{X/S}}$	biomass to substrate yield

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