Microbial Lipid Production with Oleaginous Yeasts

zur Erlangung des akademischen Grades eines

DOKTORS DER INGENIEURWISSENSCHAFTEN (Dr.-Ing.)

der Fakultät für Chemieingenieurwesen und Verfahrenstechnik des
Karlsruher Instituts für Technologie (KIT)

genehmigte

DISSERTATION

von

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Tag der mündlichen Prüfung: 16.07.2014

Acknowledgement

Preamble

The thesis deals with the microbial lipid production based on renewable raw material.

This thesis is structured into a general introduction about basic knowledge and the research subject, three main chapters (I, II, III) which are partially based on peer-review works and ends with concluding remarks.

The introduction contains excerpts of the section "*Case studies: SCOs as Raw material and Intermediate*" in the book chapter "*Existing Value Chains*" in "*Renewable Raw Materials*" (Wiley VCH).

Chapter I focuses in particular on the fermentative production processes of microbial lipids with the oleaginous yeast *Cryptococcus curvatus* and the recycling of the waste stream CO_2 by coupling the yeast process with the lipid production process of the autotrophic oleaginous microalgae *Phaeodactylum tricornutum.* This chapter contains the main part of the submitted publication *"Combination of algae and yeast fermentation for an integrated process to produce single cell oils"* in *Applied Microbiology and Biotechnology* (2014) which was performed in cooperation with Robert Dillschneider (Institute of Bioprocessing, Karlsruhe Institute of Technology) within the ERA-SME project *BiCycle* funded by BMWI. The author of this dissertation was responsible for the part concerning the yeast process, while Robert Dillschneider worked on the algal part.

Chapter II describes the screening of new oleaginous yeasts via Sudan black B staining technique. Four yeast strains were isolated and characterized in the context of lipid production. This chapter is based on the publication "Characterization of newly isolated oleaginous yeasts - *Cryptococcus podzolicus*, *Trichosporon porosum* and *Pichia segobiensis* in *AMB Express* (2014), and contains additional data concerning the fourth isolated yeast *Candida shehatae.*

Chapter III presents studies for a fast and easy applicable method in 96-well plate format to roughly quantify the lipid content in oleaginous yeast strains in suspension using the fluorescent lysochrome Nile red. This technique was applied to estimate the lipid content of the oleaginous yeast *Cryptococcus curvatus* and for the establishment of a rapid HTP screening assay to identify new oleaginous yeast strains.

Publications and presentations

Original papers:

2014   **Combination of algae and yeast fermentation for an integrated process to produce single cell oils**
Dillschneider, R.; Schulze, I.; Neumann, A.; Posten, C.; Syldatk, C.
Applied Microbiology and Biotechnology (Springer)

2014   **Characterization of newly isolated oleaginous yeasts - *Cryptococcus podzolicus, Trichosporon porosum* and *Pichia segobiensis***
Schulze, I.; Hansen, S.; Großhans S.; Rudszuck T.; Ochsenreither, K.; Syldatk C.; Neumann A.
AMB Express (Springer)

Book chapters:

2011   **Existing Value Chains**
Syldatk, C.; Schaub, G.; Schulze, I.; Ernst, D.; Neumann, A.
in Ulber, R., Sell, D., Hirth, T. (eds.): **Renewable Raw Materials**, Weinheim (Bergstraße): Wiley-VCH

Poster presentations:

2013   **Screening of oleaginous microorganisms for the production of Single Cell Oils as raw material for biofuels or fine chemicals**
VAAM Jahrestagung 2013, Bremen

2012   **Schnelle Quantifizierung des Lipidgehalts in der oleogenen Hefe *Cryptococcus curvatus***
DECHEMA Jahrestagung 2012, Karlsruhe

      Process characterization of microbial oil production by the yeast *Cryptococcus curvatus*
Fette und Öle 2012, Karlsruhe

2011   **Production of microbial lipids from low cost carbon sources with the yeast *Cryptococcus curvatus***
VAAM Jahrestagung 2011, Karlsruhe

      Process characterization of microbial oil production by the yeast *Cryptococcus curvatus*
Fette und Öle 2011, Karlsruhe

2010   **Microbial Production of Single Cell Oils (SCOs) from Low-Cost Carbon Sources and Waste Substrates**
DECHEMA Jahrestagung 2010, Aachen

      Microbial Production of Single Cell Oils (SCOs) from Low-Cost Carbon Sources and Waste Substrates
Microbial Lipids 2010, Wien

Zusammenfassung

Ölhaltige Mikroorganismen sind in der Lage, Kohlenstoffquellen in Speicherlipide umzuwandeln und sie als intrazelluläre Lipidtröpfchen in der Zelle einzulagern. Mikroorganismen werden als oleogen bezeichnet, wenn mehr als 20 % ihrer Biotrockenmasse aus Lipiden besteht. Diese Lipide sind auch als Einzelleröle (SCO) bekannt und werden in der stationären Wachstumsphase unter Stickstofflimitierung mit gleichzeitigem Überschuss einer Kohlenstoffquelle produziert. Abhängig von der Art des Mikroorganismus (Hefe, Mikroalgen, Schimmelpilze und Bakterien) variieren diese mikrobiellen Lipide in der Zusammensetzung ihrer Fettsäureprofile und sind daher für verschiedene industrielle Anwendungen geeignet. Aufgrund der sinkenden Erdöl-Ressourcen, des umstrittenen Einsatzes von Pflanzenölen für die Biodieselproduktion und der Überfischung der Ozeane werden SCOs als Möglichkeit gesehen Erdöl, Pflanzen- und Fischöl teilweise ersetzen zu können. Allerdings ist die mikrobielle Lipidproduktion noch nicht ökonomisch realisierbar, abgesehen von einer kleinen Anzahl an Produktionsanlagen für hochwertige Fettsäuren, wie z.B. Docosahexaensäure (DHA), Eicosapentaensäure (EPA) und Arachidonsäure (ARA). Es sind daher Strategien erforderlich, um die Produktionskosten mikrobieller Öle zu reduzieren und die Produktivität zu erhöhen.

Cryptococcus curvatus ist eine der am besten untersuchten ölhaltigen Hefen und wird daher in dieser Studie als Modellorganismus genutzt. Wie alle heterotrophen Organismen, emittiert auch *C. curvatus* das Treibhausgas CO_2, dessen Ausstoß in industriellen Prozessen verringert werden soll, um der globalen Erderwärmung entgegen zu wirken. Das emittierte Abgas CO_2 des Lipid produzierenden Hefe-Prozesses wurde daher beim Prozess Lipid bildender Mikroalgen als Kohlenstoffquelle genutzt und auf diese Weise recycelt. Es wurde gezeigt, dass eine Kultivierung der ölhaltigen Hefe C. curvatus in einem 1,2 L-Maßstab ausreicht, um eine Kultivierung der ölhaltigen Mikroalge Phaeodactylum tricornutum in einem 21 L-Blasensäulenreaktor mit CO_2 zu versorgen, während in beiden Prozessen gleichzeitig Einzelleröle produziert wurden. Die von *C. curvatus* hergestellten Hauptfettsäuren sind Ölsäure (49,0 %), Palmitinsäure (18,5 %), Stearinsäure (17,7 %) und Linolensäure (8,6 %). Die Mikroalge P. tricornutum produziert hauptsächlich Palmitoleinsäure (44,9 %), Palmitinsäure (25,0 %), Eicosapentaensäure (8,9 %) und Ölsäure (7,6 %).

Vier Hefestämme wurden aus Bodenproben isoliert und mit dem Farbstoff Sudanschwarz B angefärbt, der Neutralfette färbt. Diese vier Stämme wurden als *Cryptococcus podzolicus*, *Trichosporon porosum*, *Pichia segobiensis* und *Candida shehatae* identifiziert und in Bioreaktoren kultiviert, um sie hinsichtlich ihrer Lipidproduktion zu charakterisieren. Mit

Glucose als Kohlenstoffquelle produzierte *C. podzolicus* 31,8 % Lipid pro Biotrockenmasse bei 20 °C, *T. porosum* 34,1 % bei 25 °C und *P. segobiensis* 24,6 % bei 25 °C. Daher können diese drei Isolate als oleogene Hefen klassifiziert werden, wohingegen *C. shehatae* mit 17,8 % Lipidgehalt pro Biotrockenmasse auf Glucose bei 25 °C nicht als oleogen klassifiziert werden kann. Gluconsäure wurde als Nebenprodukt nachgewiesen, wenn *C. podzolicus* und *T. porosum* auf Glucose kultiviert wurden (jeweils 30 g/L und 12 g/L). Wenn Glucose durch Xylose als Kohlenstoffquelle ersetzt wurde, konnte Gluconsäure für beide Stämme nicht nachgewiesen werden. Mit Xylose als Kohlenstoffquelle waren die Lipidgehalte nur geringfügig niedriger als bei der Verwendung von Glucose. Daraus folgt, dass sich Xylose als Kohlenstoffquelle für *C. podzolicus* und *T. porosum* eignet, wenn lediglich Lipide produziert werden sollen. Glucose könnte als Kohlenstoffquelle zum Einsatz kommen, wenn die gleichzeitige Produktion von Gluconsäure als extrazelluläres Produkt und Lipid als intrazelluläres Produkt erwünscht sind. Xylose - als Bestandteil der Hemicellulosen, die Biomasse der zweiten Generation darstellen - ist somit eine lohnende Kohlenstoffquelle für die mikrobielle Lipidproduktion. Die Hauptfettsäure in allen vier Isolaten ist Ölsäure (zwischen 39,6 % und 63,0 %), die z. B. für die Biodiesel-Produktion geeignet ist. Auch Palmitinsäure (zwischen 9,8 % und 21,1 %) und Linolensäure (zwischen 7,5 % und 18,7%) sind in einer angemessenen Menge vertreten, welche für kosmetische Anwendungen wertvoll sind. *P. segobiensis* erzeugt einen erheblichen Anteil an Palmitoleinsäure (16,0 %), die für medizinische Anwendungen geeignet ist.

Zusätzlich zur Lipidfärbung mit dem Farbstoff Sudanschwarz B, eignet sich auch der fluoreszierende Farbstoff Nilrot, um Neutralfette anzufärben, sodass er daher auch für die Färbung der intrazellulären Lipide in oleogenen Mikroorganismen geeignet ist. Die Intensität der Fluoreszenz ist proportional zur Lipidmenge. Daher ist die Intensität der Fluoreszenz ein Kriterium, um den Lipidgehalt pro Biotrockenmasse abschätzen zu können, ohne die zeitintensive Analyse per Gaschromatographie zu verwenden – die Standardmethode zur Quantifizierung und Qualifizierung für Lipide. Zu diesem Zweck wurden Zellsuspensionen oleogener Hefen mit Nilrot in 96-Well-Mikrotiter-Platten angefärbt und sowohl die optische Dichte (OD_{600}) als auch die Fluoreszenz gemessen. Es wurde der Quotient "Fluoreszenz/OD" gebildet und mit dem Lipidgehalt verglichen, der über gaschromatographische Analyse bestimmt wurde. Diese Methode wurde in Mikrotiterplatten erfolgreich angewendet, um den Lipidgehalt der ölhaltigen Hefe *C. curvatus* abzuschätzen. Der Lipidgehalt von *C. curvatus* konnte mittels einer Kalibrierung mit einer Abweichung von 5 % für einen Lipidgehalt im Bereich von 18,3 % und 35,6 % quantifiziert werden. Diese Quantifizierungs-Methode kann nun auf andere oleogene Hefen übertragen werden.

C. curvatus und fünf andere Hefestämme wurden zusätzlich mit der gleichen Methode angefärbt und mit den gleichen Geräteeinstellungen bezüglich Fluoreszenz und optischer Dichte untersucht. Die Quotienten "Fluoreszenz/OD" und "Fluoreszenz/Biotrockenmasse" wurden mit dem Lipidgehalt ins Verhältnis gesetzt und die Ergebnisse wurden zwischen den verschiedenen Hefestämmen verglichen. Es wurde gezeigt, dass sich dieses Verfahren als Methode eignet, um in einem Hochdurchsatz-Verfahren neue oleogene Hefen zu identifizieren.

Abstract

Oleaginous microorganisms are able to convert carbon sources into storage lipids as intracellular lipid droplets yielding in more than 20 % lipid per dry biomass. These lipids are also known as single cell oils (SCOs) and are produced in the stationary growth phase under nitrogen limitation with simultaneous excess of a carbon source. Depending on the species (yeast, microalgae, filamentous fungi or bacteria), these microbial lipids vary in the composition of their fatty acid profiles and are therefore suited for diverse industrial applications. Considering the depletion of crude oil, the controversial use of plant oils for biodiesel production and the overfishing of the oceans, SCOs are considered as suitable oil substitutes for crude, plant and fish oil. However, microbial lipid production is still not economical feasible, apart from a small number of production plants for high value fatty acids, e.g. docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA). Therefore strategies are required to reduce the production costs of SCOs and to increase the productivity.

Cryptococcus curvatus is one of the most examined oleaginous yeasts and was therefore suited as a model organism in this study. Like all heterotrophic organisms, *C. curvatus* emits the greenhouse gas CO_2 which should be decreased in industrial processes to prevent further global warming. Therefore, the emitted gas CO_2 of the yeast process was channeled into a microalgae process in order to recycle the waste stream CO_2. It was shown that the cultivation of the oleaginous yeast *C. curvatus* on a 1.2 L scale was sufficient to supply a culture of the oleaginous microalgae *P. tricornutum* in a 21 L bubble column reactor with CO_2 while SCOs were produced simultaneously in both processes. The main fatty acids produced by *C. curvatus* were oleic acid (49.0 %), palmitic acid (18.5 %), stearic acid (17.7 %) and linoleic acid (8.6 %). The microalgae, *P. tricornutum* produced mainly palmitoleic acid (44.9 %), palmitic acid (25.0 %), eicosapentaenoic acid (8.9 %) and oleic acid (7.6 %).

Four yeast strains were isolated from soil samples and stained by the lipid staining dye Sudan black B. They were identified as *Cryptococcus podzolicus*, *Trichosporon porosum, Pichia segobiensis* and *Candida shehatae* and were cultivated in bioreactors to characterize their lipid producing capacities. When cultured on glucose as sole carbon source *C. podzolicus* yielded in 31.8 % lipid content per dry biomass at 20 °C, while *T. porosum* yielded in 34.1 % at 25 °C and *P. segobiensis* in 24.6 % at 25 °C. Hence, those three yeast isolates can be classified as oleaginous, whereas *C. shehatae* with 17.8 % lipid content on glucose at 25 °C was not classified as oleaginous. Gluconic acid was detected as additional product if *C. podzolicus* and *T. porosum* were cultured on glucose (30 g/L and 12 g/L,

respectively). When glucose was substituted by xylose as carbon source gluconic acid was not detectable for both strains. Using xylose, lipid yields were slightly lower than with glucose. Therefore, it was concluded that when using either *C. podzolicus* or *T. porosum* as the production strain, xylose is the carbon source of choice for exclusive lipid production, but glucose may be used for the simultaneous production of gluconic acid as extracellular product and lipid as intracellular product. Xylose – as a component of the second generation biomass hemicelluloses – is a worthwhile carbon source for microbial lipid production. The main fatty acid in all four isolates was oleic acid (between 39.6 % and 63.0 %) which is applicable for e.g. biodiesel production. A distinctive percentage of palmitic acid (between 9.8 % and 21.1 %) and linolenic acid (between 7.5 % and 18.7 %) was determined, which are valuable for cosmetic applications. *P. segobiensis* produces a considerable percentage of palmitoleic acid (16.0 %) which is suitable for medical applications.

In addition to the lipid staining dye Sudan black B, the fluorescent lysochrome Nile red is suited to stain neutral fats and is therefore also applicable to stain intracellular lipids in oleaginous microorganisms. The intensity of the fluorescence is proportional to the lipid amount. Therefore, the intensity of fluorescence can be used to rapidly estimate the lipid content per dry biomass without use of the time consuming gas chromatographically analysis - the standard lipid quantification and qualification method. For this purpose liquid samples of oleaginous yeasts were stained with Nile red in 96-well microtiter plates and the optical density (OD) and the fluorescence were measured. The ratio fluorescence/OD was formed and compared with lipid quantities gained via gas chromatographically analysis. This technique in 96-well plate format was successfully applied to estimate the lipid content of the oleaginous yeast *C. curvatus*. The lipid content of *C. curvatus* can be quantified via a linear fit with a deviation from 5 % for lipid contents in the range of 18.3 % and 35.6 %. This quantification method can now be transferred to other oleaginous yeasts.

C. curvatus and five other yeast strains were additionally stained with the same method and the same device settings for the measurement of fluorescence and optical density (OD). The ratios fluorescence/OD and fluorescence/dry biomass were set into relation with the lipid content and compared among the various yeast strains. It was shown that this method is suitable to apply for rapid lipid estimation within a high-throughput (HTP) screening assay to identify new candidates of oleaginous yeasts.

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I. Introduction

Crude oil and natural gas are nowadays the main raw materials for the chemical industry and for energy supply. Even 95 % of the worldwide primary building blocks for organic chemicals originate from crude oil and natural gas (Wittcoff et al. 2004). In consideration of the growing world population and the forthcoming exhaustion of crude oil, alternative resources as energy and chemical feedstock have to be explored to meet the needs of the increasing world population (Clark and Deswarte 2008). In addition to the above mentioned reasons, also ecological aspects like the reduction of greenhouse gas (GHG) emissions, which are caused by the combustion of petrol based fuels, should be respected. Therefore bioenergy and biomaterials from renewable resources based on biomass are getting more important since the last decades. It has to be distinguished between two different generations of biomass feedstock, the first and the second generation of biomass feedstock. While first generation biomass originates from edible biomass or rather food or feed crop, second generation biomass includes different non-food feedstock like lignocellulosic material, forest residues or municipal solid wastes (Lee and Lavoie 2013). Biomass in solid form can be directly converted into heat energy by e.g. pelletizing wood. The conversion into liquid fuels as transport fuel, however, is more complex and needs e.g. microbial fermentative conversion technologies such as conversion of sugar and oil into bio-ethanol, biogas or biodiesel. Currently, main biofuel producers of the first generation are Brazil with bioethanol based on sugar cane, USA with bioethanol from corn, Germany with biodiesel from oilseed rape and Malaysia with biodiesel based on palm oil. The production amount of first generation biofuels tripled from 2000 to 2007 and amounted in 2007 even 1.5 % of the global transport fuel (around 37 Mt oil equivalents) (Sims et al. 2008). Even though biofuels represent a renewable feedstock, one main drawback is the fact that first generation biofuels are primarily based on food crops which leads to a competition with feed and food and consequently leads to increasing food prices. Especially in developing countries, increasing food prices, but also the use of scarce water for the cultivation of biofuel's crop lead to famine among the poor population. Further drawbacks are seen in accelerating deforestation, monocultures of biofuel crops and resulting loss of biodiversity (Sims et al. 2008). As the sustainability of first generation biofuels is controversial, second generation biofuels got more important. One advantage is that non-food biomass like cellulosic wastes or forest residuals are less expensive than first generation biomass like vegetable oil, corn or sugar cane (Lee and Lavoie 2013). On the other hand lignocellulosic second generation biomass is more complex than sugar or oil and therefore needs special conversion techniques to be degraded, before being further processed into biofuel. In this context

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biorefineries are refineries which convert biomass – a renewable resource – within multiple parallel processes into several low and high value products, which can be used as material products or for energy supply. Biorefineries need interdisciplinary collaboration as it works in combination of physical, chemical, biotechnological and thermo chemical technologies including pyrolysis, Fischer-Tropsch synthesis and other catalytic reactions to gain all possible chemicals and materials from the rich biomass (Naik et al. 2009). The concept of biorefineries is one possibility to replace fossil feedstock with plant-based feedstock (Clark and Deswarte 2008). Only 3 % of world's biomass, amounting to 170 million tons, is currently used for food and non-food applications, therefore plant-based biorefineries including the production of second generation biofuels are worthwhile for the future. The aim is to maximize the value of biomass and to minimize the waste by recycling certain waste streams within the whole biorefinery (Clark and Deswarte 2008). The biotechnological part of the biorefinery consists of a microbial fermentation using a certain microorganism able to metabolize the carbonic second generation biomass directly or one of its constituents after chemical or thermal treatment into the final product (Lee and Lavoie 2013). One single process in such a whole biorefinery concept may be the microbial production of oil by using so-called *oleaginous microorganisms*. These microorganisms may partially substitute several conventional oil sources like crude oil, fish or vegetable oils for the application in the energy sector, food industry, pharmaceutical or cosmetic industry depending on the fatty acid profile of the microbial oil. The recycling of waste streams within such microbial lipid production processes and the search and determination of new lipid producing microorganisms, which are also able to convert complex second generation biomass, are challenges for the development of microbial oil production processes to compete with conventional methods of oil production.

II. Research subject

Microbial lipids are similar to plant oils. Hence, they are suited to substitute plant oils for industrial applications. However, microbial lipid production is still not economically feasible, therefore strategies are required to reduce the production costs and to increase the productivity. Recycling of waste streams and the identification of new microorganisms with high-value products or/and higher productivities are possibilities.

The first objective of this study was to develop and establish a microbial lipid production process with the well known oleaginous yeast strain *Cryptococcus curvatus* with glucose as carbon source as platform process. The lipid production process was characterized in terms of nitrogen limitation, carbon source consumption and exhaust gas analysis of CO_2. The obtained data were used to couple the yeast process to an algal lipid production process in order to recycle the greenhouse gas CO_2 which is produced by the yeasts to introduce as carbon source for the algae cells. Hence, this study presents an integrated microbial lipid production process with reduced emission of the green house gas CO_2.

The second purpose of this study was to identify new oleaginous yeast strains and to characterize them regarding to their fatty acid profile, lipid content and lipid productivity. A subsequent cultivation in bioreactors gave further information about each single process concerning lipid production and possible by-products with glucose as carbon source. Xylose – as a component of the second generation biomass hemicelluloses – was evaluated as carbon source for the microbial lipid production using the new isolated yeast strains.

To accelerate the lipid quantification, e.g. to monitor the lipid production during a process, the third aim was to establish a rapid lipid quantification method for oleaginous yeast strains in suspension in 96-well plate format using the fluorescent lysochrome Nile red instead of the time and solvent consuming gas chromatographical analysis. This method should also be applicable for a high-throughput-assay to identify new oleaginous yeast strains which produce fatty acids for several industrial applications.

III. Theoretical background

1. Fats and oils

Fats and oils are compounds in plants, animals, fish and microalgae. They belong to the molecular group of lipids and are molecules serving in cells as structure molecules, as energy storage molecules or as molecules for signal transmissions. Lipids can be divided into five subclasses including free fatty acids, triacylglycerols, glycerophospholipids, sphingolipids and steroids (Voet et al. 2002). The main part of plant and animal fats are triacylglycerols (TAGs) which are also called neutral fats (fig. 1) as they do not contain any charged groups (Czabany et al. 2007). They occur by esterification of one glycerol molecule with three free fatty acids (Voet et al. 2002). Glycerol is a trivalent alcohol while fatty acids are carboxylic acids with a long aliphatic tail (chain), which is either saturated or unsaturated. TAGs serve as intracellular energy storage. Because of their lower state of oxidation, they are better applicable for energy storage than storage polysaccharides or proteins (Voet et al. 2002). In case of energy demand, the fatty acids are cleaved from the glycerol backbone and oxidized via β-oxidation to gain energy and generate reducing equivalents.
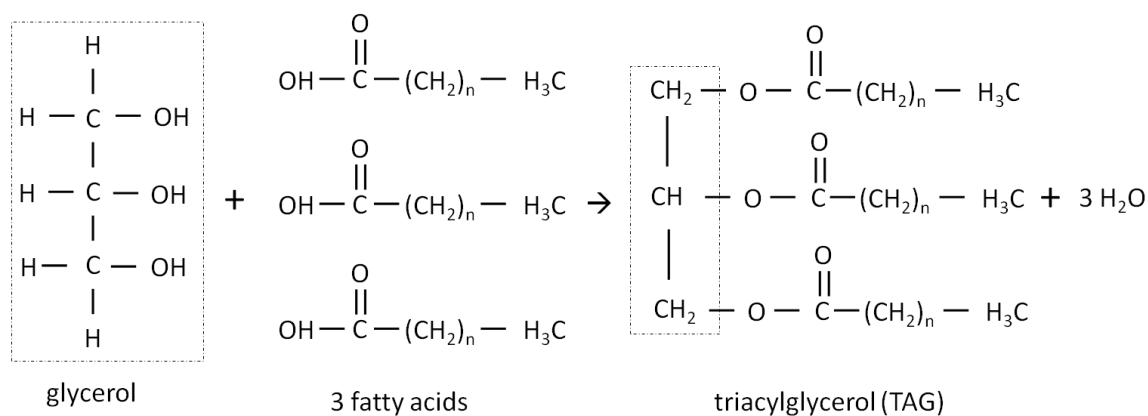
Fig. 1 Schematic illustration of the esterification of a glycerol with three fatty acids to one triacylglycerol (TAG)

Lipids and oils deliver interesting derivatives, also called oleochemicals, for several industrial applications depending on the composition of their fatty acid profiles, on the carbon chain length and the saturation grade of the fatty acids within the TAG. Due to their chemical functionality available in their structure, they are excellent bioresources for the production of detergents, biopolymers and other oleochemicals (Verhé 2010). Those oleochemicals which derive from fat and oil are renewable raw materials and belong to biodegradable substances and are therefore ecologically friendly in contrast to the conventional petrochemicals

(Metzger and Bornscheuer 2006). A summary of oleochemicals and their industrial applications is given in table 1.

Table 1 Applications of oleochemical products (Baerns et al. 2013)

Products	Applications
Fatty acids	Soaps, cleansing agents, laundry detergents, plastics, lubricants, natural rubber, cosmetics, dye, coatings
Fatty acid methyl esters	Cosmetics, cleansing agents, laundry detergents, biodiesel
Fatty alcohols	Cleansing agents, laundry detergents, mineral oil additives, cosmetics, textile and paper industry
Fatty amines	Softener, mineral oil additives, road construction, mining, biocides, fibre industry
Glycerol	Pharmaceuticals, cosmetics, synthetic resin, plastics, toothpaste, tobacco, nutrition, processing of cellulose

One advantage of plant and animal fat is the possibility to gain fatty acids in such a purity to be suitable for chemical conversions and for the synthesis of chemically pure compounds. Plant oils are particularly gained by mechanical extraction under pressure from the plant's seeds or nuts. Most global oil production in 2009/2010 with 84.6 Mt arose from palm oil plant, soybean, rape seed, peanut, sunflower, palm kernel, olives and coconut. In addition 3.8 Mt were produced from sesame, flax, castor oil plant and corn. Animal fat production amounted to 22.1 Mt including butter, lard, schmaltz and fish oil. Primary use of those plant and animal fats is the food industry. However, the production of palm, soybean and rapeseed oil increased in the following decade enormously (fig. 2) due to their use for biodiesel production (Biermann et al. 2011), but with the consequence of increasing prices for agricultural foods. Large agricultural crop lands are used for such energy plants for biodiesel production resulting in increasing prices. The price for soybean and corn e.g. increased up to 25 % from 2001 till 2011 (Hochmann et al. 2012). That leads to nutrition problems especially in developing countries. To avoid price increase for food, which is essential for life, oil based feedstock for biodiesel production should be substituted by non edible and sustainable sources to produce second generation biofuel. *Oleaginous microorganisms*, which are able to produce intracellular storage lipids, represent one promising strategy. High value fatty acids which arise from fish oil originate from microalgae which are in the beginning of the food chain for fish. However, pollution of the environment leads to the accumulation of health hazardous substances like dioxins, chlorinated diphenyls (PCBs) and heavy metals in fish, which makes the use of fish oil as nutrient highly questionable (Ratledge 2004). In addition, if the market for high value fatty acids from fish oil further rises, oceans are endangered of overfishing and the global fish stock will be in severe decline (Venegas-Caleron et al. 2010). Microalgae as lipid producer are therefore worthwhile to examine.
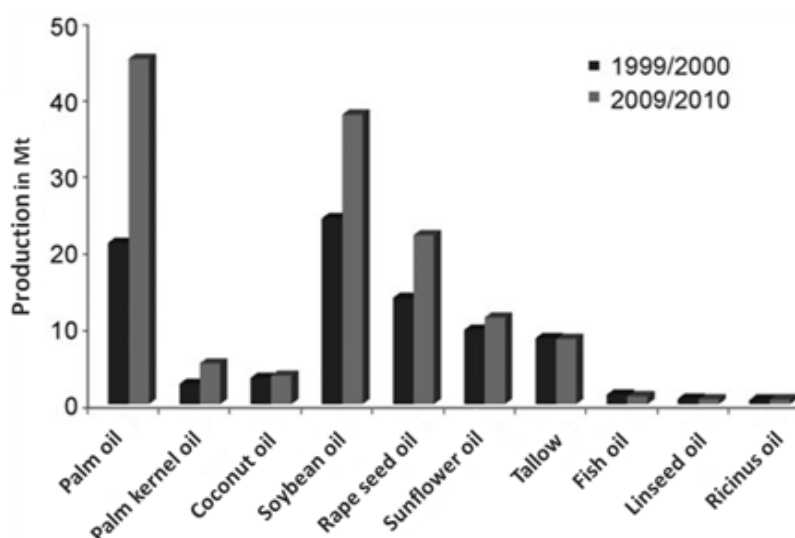
Fig. 2 Production of oils and fats as resource for the oleochemical industry in the years 1999/2000 and 2009/2010 (Biermann et al. 2011)

Castor oil derives from the castor plant *Ricinus communis*, which grows in tropical and sub-tropical regions. It contains 90 % of the mono-saturated fatty acid ricinoleic acid C18:1 (ω9). The castor seeds contain toxic substances which make these seeds poisonous, but the extracted oil contains only trace amounts of these toxins. It has been used as a purgative (Mutlu and Meier 2010), but because of its toxin and allergen characteristics in the seed it is not bearable for large applications in pharmacy, cosmetics or nutrition. Additionally, it is not suited to be cultivated in high amounts because the field workers suffer from these negative health effects (Holic et al. 2012). However, it is well suitable as raw material for the chemical industry e.g. for paints, coatings, inks or lubricants (Ogunniyi 2006) and does not compete with feed or food if gained from *Ricinus communis* plant. Therefore it is especially suited in developing countries as raw material.

2. Fatty acids

Fatty acids are aliphatic carbon chains with one terminal carboxylic group and are common with chain lengths between 12 and 24 carbon atoms. Fatty acids with 18 and more carbon atoms are named long chain fatty acids (LCFA), fatty acids with less than 18 carbon atoms are named short chain fatty acid (SCFA). The carbon atoms within the carbon chain are covalently linked with single bonds or double bonds. Hence, the fatty acids can be divided into saturated (sFA), mono-unsaturated (MUFA) or poly-unsaturated (PUFA) fatty acids. Saturated fatty acids contain only single bonds, unsaturated fatty acids feature double bonds with *cis*-configuration which leads to a curved form. Hence, the degree of saturation or rather

unsaturation of the fatty acids within a triacylglycerol (TAG) determines the melting point of the lipid. The more double bonds the lower is the melting point. This effect leads to the differentiation into fats and oils. Fats are solid at room temperature while oils are present in liquid form. Most plant oils have a certain amount of mono- and poly-unsaturated fatty acids, which makes it liquid at room temperature while butter, an animal fat with high amounts of saturated fatty acids, tends to be solid. Different fats and oils with their composition of saturated fatty acids (sFA), mono-unsaturated (MUFAs) and poly-unsaturated fatty acids (PUFAs) are listed in table 2. The nomenclature of the unsaturated fatty acids is based on the position number of the double bond within the carbon chain. The position of the double bond, which is next to the methyl end (ω-carbon atom) of the fatty acid, determines the name of the fatty acid, no matter how many double bonds exist (fig. 3). The most important groups are $\omega 3$ and $\omega 6$ fatty acids. There is a number of so called essential fatty acids (eFA) which cannot be synthesized by the human body. As they are important for human health they have to be taken up by nutrition and serve as precursors for other longer chain fatty acids (Voet et al. 2002).

Table 2 Saturation grades of fatty acids included in several plant and animal fats (Hofer, website)

	Fatty acid composition (%)		
	Saturated FAs	MUFAs	PUFAs
Butter	64	33	3
Safflower oil	14	24	62
Peanut oil	19	50	31
Coconut oil	92	6	2
Pumpkin seed oil	18	24	58
Corn oil	17	32	51
Olive oil	19	73	8
Palm kernel fat	83	15	2
Rapeseed oil	8	60	32
Lard	41	49	10
Soy bean oil	14	24	62
Sunflower oil	8	27	65
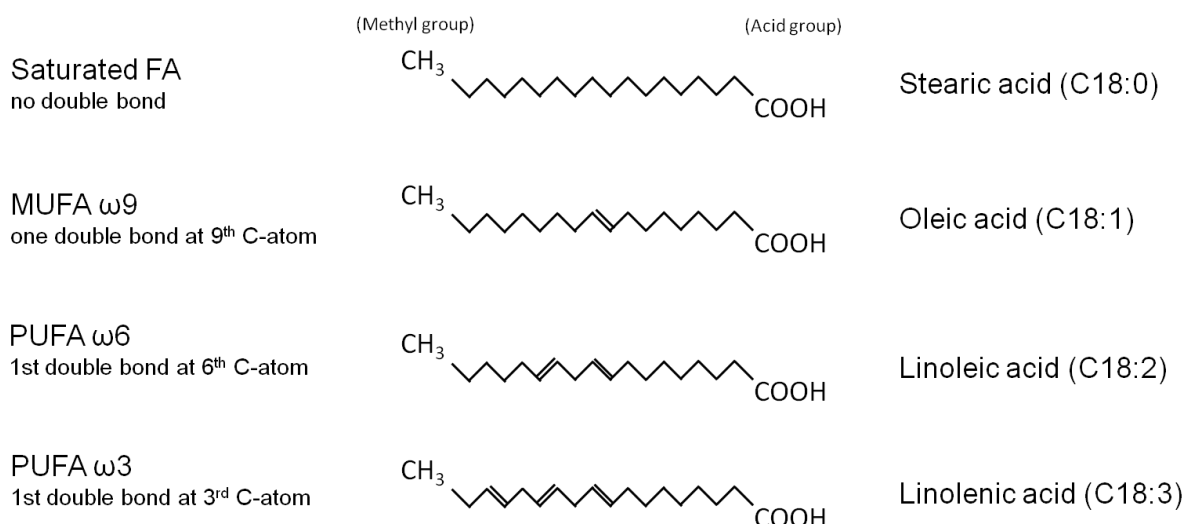
Fig. 3 Examples for ω fatty acids with none to three double bonds with 18 carbon atoms

3. Industrial applications of fatty acids

Fatty acids are mainly produced by hydrolysis of triacylglycerol (TAGs) from plant, fish oil and animal fat and the final industrial applications of each fatty acid depend on their carbon chain length and grade of saturation. A summary of the most important fatty acids and their applications are listed in table 3. Fatty acids can be classified into low- and high-value fatty acids depending on their market value. While nutrition industry favors high-value LC-PUFAs, biodiesel as a transport fuel consisting of fatty acid methyl esters requires fatty acids with C16 and C18 fatty acids in saturated or mono-unsaturated form (Christophe et al. 2012). Highly unsaturated fatty acid methyl esters tend to be oxidized easily during long term storage which could have negative influence to the engine motor. Coconut and palm kernel oil are composed of a high percentage of saturated C12 and C14 fatty acids and are therefore suitable for the production of surfactants (Metzger and Bornscheuer 2006).

Linoleic acid (C18:2) provides the most potential benefit for the skin barrier and is therefore used in the cosmetics industry (Darmstadt et al. 2002). Gamma linolenic acid (GLA, C18:3, ω6) is essential for the brain and the nerve conduction (Coste et al. 1999) and lowers blood pressure (Engler 1992). Palmitoleic acid (C16:1) is known to prevent cerebral- and cardiovascular diseases by enhancing the function of vascular smooth muscle cells. Therefore, this fatty acid seems to find novel and valuable uses in human nutrition and medicine (Matsunaga et al. 1995; Yamori et al. 1986). Even in the therapy against obesity, palmitoleic acid can be applied (Yang et al. 2011). Palmitoleic acid can be extracted from some seed oils, e.g. sea-buckthorn (Fatima et al. 2012) or macadamia (Nestel et al. 1994), but the availability is insufficient for higher medicinal demand in future. Therefore alternative production ways are required (Matsunaga et al. 1995).

Highly poly-unsaturated fatty acids (PUFAs) are used for nutritional and health applications. Fish oil e.g. deriving from the tissue of oily fish, contains predominantly two of those PUFAs, docosahexaenoic acid (DHA, C22:6, ω3) and eicosapentaenoic acid (EPA, C20:5, ω3). These PUFAs are important for human's health, but cannot be synthesized by the human body. That's why they have been longtime used as supplementary compounds in dietary and health applications (Sahena et al. 2009). Studies have shown that these PUFAs may be valuable for the prevention of atherosclerosis, heart attack, hypertension and cancer. Furthermore, they have been suited to medicate patients with rheumatoid arthritis and diabetes and for the protection of human coronary artery (Sahena et al. 2009), (Kremer 2000; Leaf et al. 2008). EPA and DHA are in addition to arachidonic acid (ARA, C20:4, ω6), which is included in low concentrations in meat, egg, poultry and seafood, important for the neurocognitive development and normal brain functions. ARA acts as an important structural component of the lipids in the neural system and "serves as a precursor of several classes of

biologically active molecules" (Streekstra 1997). Therefore, all three fatty acids show benefit even against Alzheimer's disease (Zhang et al. 2011). Arachidonic acid (ARA, C20:4, ω6) and docosahexaenoic acid (DHA, C22:6, ω3), which is found in brain tissue and mother's milk, are used as dietary supplements in infant nutrition (Ratledge 2004). It has been concluded that these two fatty acids are important for the development of neural and retinal functions of newborn babies and hence ensure good memory and good eyesight (Ratledge 2004).

Table 3 Most important fatty acids from conventional sources

Name	Carbon length	Applications	Source	Acronym
Palmitic acid	C16:0	Cosmetics	Fat of animals and plants	
Palmitoleic acid	C16:1 (ω7)	Pharma	Milk fat, train oil, fats of plants and animals	
Oleic acid	C18:1 (ω9)	Biodiesel	All natural oils	
Linolic aicd	C18:2 (ω6)	Cosmetics	Sunflower oil, safflower oil	
α-Linolenic acid	C18:3 (ω3)	Pharma	Linseed oil, palm oil, soybean oil, walnut	ALA
γ-Linolenic acid	C18:3 (ω6)	Pharma	Evening primrose oil, hempseed oil	GLA
Arachidonic acid	C20:4 (ω6)	Dietary supplement	Animal fats, train oil	ARA
Eicosapentaenoic acid	C20:5 (ω3)	Dietary supplement	Fish oils	EPA
Docosahexaenoic acid	C20:6 (ω3)	Dietary supplement	Fish oils	DHA
Docosapentaenoic acid	C22:5 (ω3)	Dietary supplement	Fish oils	DPA

4. Oleaginous microorganisms

Oleaginous microorganisms are a good alternative source for industrial used oil. Lipids are produced by all microorganisms (MOs) usually in the range of 6 to 8 % of the dry biomass, principally as components for the cell membrane. However, oleaginous microorganisms, including yeasts, bacteria, filamentous fungi and microalgae contain more than 20 % lipid per dry biomass as carbon storage. They convert a carbon source available in excess into intracellular triacylglycerol (TAGs) as soon as nitrogen limitation occurs (Ratledge 2002; Ratledge 2004; Ageitos et al. 2011). Therefore the formulated medium should have a high C/N ratio of about 40:1 (Ratledge 2005). These storage lipids are also called single cell oils (SCO) and are stored as lipid droplets within cells. Baker's yeast *Saccharomyces cerevisiae* does not produce intracellular lipid droplets (Vorapreeda et al. 2012), but several other yeast strains are known to belong to the oleaginous microorganisms, e.g. *Cryptococcus* sp*., Yarrowia* sp., *Candida* sp., *Rhodotorula* sp., *Rhodosporidium* sp., *Trichosporon porosum* and *Lipomyces* sp. (Ratledge 1991; Papanikolaou and Aggelis 2011b). A summary about the best examined oleaginous yeasts with lipid percentage and fatty acid profile is given in fig. 4.

Depending on the chosen microorganism, different carbon and energy sources are required and the microbial lipids contain a vast diversity of different fatty acids suitable as alternative base material for industrial applications (Ratledge and Cohen 2005).

Cryptococcus curvatus is one of the best explored oleaginous yeasts able to grow on several carbon sources, e.g. glucose, glycerol or xylose (Meesters et al. 1996; Zhang et al. 2011; Heredia and Ratledge 1988). Hence, it is well suited as a model organism for research in microbial lipid production. It was classified to the genus *Candida* and to the species *curvata*. It is also known as *Apiotrichum curvatum* and *Candida curvata* C, but nowadays *Cryptococcus curvatus* is used as the official name in data bases (Akindumila and Glatz 1998). The lipid composition is similar to that of palm oil with a high amount of oleic acid and is therefore suitable as a sustainable substitute of palm oil (Meesters et al. 1996).

Examples among autotrophic microalgae are *Phaeodactylum* sp., *Chlorella* sp. and *Monodus* sp., which have shown to produce the ω3 fatty acid EPA and *Crypthecodinium cohnii* the ω3 fatty acid DHA (Ward and Singh 2005; Vazhappilly and Chen 1998).

Moreover, heterotrophic marine traustochytrides exist, e.g. *Aurantiochytrium limacinum*, (Nagano et al. 2009; Vazhappilly and Chen 1998) which has also potential to produce DHA.

Advantages of microbial oil production compared to plant oil is the short life cycle of microbes and the possibility of a production process not influenced by external factors such as venue, season or climate (Thiru et al. 2011). The production of single cell oil in large scale is suited to avoid the conflict with plants used for energy and material industry, and to avoid the appearance of mono cultures and exhaustion of the soils. Furthermore, less land is needed for microbial production than for conventional agricultural production (Ratledge and Cohen 2008). Autotrophic microalgae as well as heterotrophic yeasts, bacteria and filamentous fungi implicate still limitations for a commercial SCOs production process. The main reasons are the costly downstream process (Chisti and Moo-Young 1986) of the intracellular SCOs and the high costs of carbon sources for heterotrophic microorganisms. The relation between the costs for carbon sources (1/4 of product oil) and the stoichiometry of carbon to oil conversion is not efficient. Theoretical calculations reveal that 33 g oil can be achieved from 100 g glucose, excluding energy consumption for the maintenance metabolism (Ratledge and Cohen 2008). In principle, conversion from glucose to oil is only a conversion from one agricultural resource into another without economic gain. This problem can only be overcome for heterotrophic organisms if carbon sources are less expensive or if the product is of higher value than usual commodity oils. Possibilities are therefore using carbon sources originating from waste material or constituents of hemicellulosic material like xylose. Many microorganisms, e.g. *Pichia pastoris* (Lee et al. 1986), are able to convert xylanes, a

constituent of hemicelluloses and polymer of xylose molecules. Hemicellulosic material is accessible in large quantities all over the world (Pan et al. 2009) and can therefore be classified as low-cost substrate suitable as carbon source for heterotrophic oleaginous microorganisms.

In contrast, oleaginous microalgae, using CO_2 as carbon source, might become a realistic alternative within the next 10 to 15 years if the price for crude oil continues to increase like it did over the last 12 years. Moreover algal lipids are promising substitutes for fish oils as they contain a lot of poly unsaturated fatty acids (PUFAs) (Ratledge and Cohen 2008). Moreover, oleaginous microalgae might become energy storage systems if wind energy, solar energy or thermal solar energy is excessively available by converting the surplus electricity from light energy into storage lipids.

Strain	Lipid (% w/w)	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Reference
Candida sp. 107[a]	37.1	37	1	14	36	7	T.	Gill et al. [104]
Candida sp. 107	n.r.	28	n.r.	8	41	17	17	Davies [22]
Candida sp.	40.3	23	13	3	54	5	2	Aggelis et al. [24]
Rhodotorula gracilis	41.0	21	T.	13	51	11	3	Choi et al. [111]
Candida curvata[b]	29.1	36	T.	14	40	7	T.	Evans and Ratledge [59]
Candida curvata[b]	28.0	37	T.	10	44	6	T.	Evans and Ratledge [59]
Apiotrichum curvarum[b]	31.0	34	T.	10	43	7	2	Hassan et al. [113]
Cryptococcus curvatus[b]	38.0	24	T.	10	46	9	6	Hassan et al. [114]
Cryptococcus curvatus[b]	25.0	18	T.	16	50	16	T.	Meesters et al. [72]
Cryptococcus curvatus[b]	50.0	31	-	22	42	1	n.r.	Wu et al. [15]
Cruptococcus albidus	46.3	14	T.	9	53	18	2	Hansson and Dostálek [112]
Cruptococcus albidus	n.r	20	n.r	11	59	6	6	Davies [22]
Yarrowia lipolytica	43.2	15	2	11	47	21	3	Papanikolaou and Aggelis [74]
Yarrowia lipolytica	30.7	12	11	9	57	11	T.	André et al. [75]
Yarrowia lipolytica[a]	22.3	13	17	6	55	7	n.r.	Makri et al. [76]
Rhodosporidium toruloides	67.5	20	1	15	47	13	3	Li et al. [9]
Rhodosporidium toruloides	65.2	34	T.	13	48	1	T.	Hu et al. [115]
Rhodosporidium toruloides	62.1	26	2	5	62	3	T.	Wu et al. [14]
Rhodosporidium toruloides	55.6	43	T.	16	35	2	T.	Wu et al. [109]
Lipomyces starkeyi	68.0	56	2	14	26	T.	T.	Angerbauer et al. [10]
Lipomyces starkeyi	61.5	37	4	6	49	1	T.	Zhao et al. [11]
Rhodotorula mucilaginosa	48.6	22	2	9	55	11	T.	Zhao et al. [116]
Trichosporon capitatum	37.6	12	1	2	74	9	n.r.	Wu et al. [16]
Rhodotorula sp.	22.0	22	1	7	56	12	n.r.	Chatzifragkou et al. [83]
Candida oleophila	15.3	13	3	7	66	11	n.r.	Chatzifragkou et al. [83]

T. <0.5% w/w; n.r.: not reported.
[a] Representation of the neutral fraction of microbial lipids produced.
[b] *Cryptococcus curvatus* was formely *Candida curvata* and then *Apiotrichum curvatum*; thus these microorganisms in fact are the same species.

Fig. 4 Overview of various yeast strains grown on substrates like sugars, glycerol or molasses with lipid content per dry biomass and their corresponding fatty acid profiles of the accumulated lipid; References can be found in (Papanikolaou and Aggelis 2011a). Note the use of three different names for *Cryptococcus curvatus = Candida curvata = Apiotrichum curvatum*

5. The biosynthesis of single cell oil

The production of single cell oil in oleaginous microorganisms depends on the composition of the culture medium. Whereas the carbon source has to be available in excess, another cell growth limiting factor like nitrogen (N), phosphor (P), magnesium (Mg), zink (Zn), calcium

(Ca) or vitamins, has to be limited – which is most cases nitrogen. Once nitrogen is limited, the cell growth stops, but available carbon will be assimilated into the cell and stored as SCO. The carbon source is converted into pyruvate in the cytosol, pyruvate is transported into the mitochondrion, decarboxylized to acetyl-CoA (C_2) which reacts with oxalacetat (C_4) and then further to citrate and subsequently usually to iso-citrate within the citrate cycle. In the case of nitrogen limitation the enzyme AMP-deaminase is activated by cleaving adenosin-monophosphat (AMP) into inosin-monophospaht (IMP) and NH_4^+ to provide cell own nitrogen for cell functions. However, AMP is required for the functionality of the enzyme isocitrate-dehydrogenase (ICDH), which converts isocitrate into α-ketoglutarate within the citrate cycle in order to produce $NADH^+ + H^+$ for the production of ATP within the respiratory chain. If no AMP is available, iso-citrate accumulates in the mitochondrion. Because of equilibrium reactions iso-citrate is converted into citrate which accumulates in the mitochondrion as well and is channeled into the cytosol via malat/citrat transporter. At this point citrate is cleaved under the consumption of ATP into oxalacetat (C_4) and one C_2-unit acetyl-CoA which is the chemical precursor for the fatty acid synthesis. This conversion is done by the enzyme ATP-Citrate-Lyase (ACL), which is special in oleaginous microorganisms (Laoteng et al. 2011; Ratledge 2002; Ratledge 2004). The extent of fatty acid production depends on the malic enzyme (ME) concentration which converts malate to pyruvate via NADPH release. This chemical conversion is the sole source of NADPH for the enzyme fatty-acid-synthase (FAS), which is required in the fatty acid biosynthesis. A summary is shown in fig. 5. The fatty acids are constructed by condensations of the C_2-units acetyl-CoA up to the C16 or C18 saturated fatty acids. These saturated fatty acids are extended to longer chain saturated fatty acids (sFA) via the enzyme elongase and can be subsequently converted into unsaturated fatty acids (PUFAs) via the enzyme complex of desaturases. The enzyme complexes of desaturases and elongases are part of the cell membrane in the periplasmatic reticulum (Ratledge 2004; Rossi et al. 2011).

CYTOSOL

MITOCHONDRION

NITROGEN LIMITATION

glucose → glycolysis → pyruvate

ADP + Pi

oxalacetate

NADH

PC + ATP

MDH

NAD$^+$

CO$_2$

malate

ME

NADP$^+$

NADPH

pyruvate → acetyl-CoA → citrate

AMP → AMP-deaminase (+) → IMP + NH$_4^+$

aconitase

isocitrate

TCA

isocitrate dehydrogenase (+)

oxalacetate

α-ketoglutarate

malate

ATP

ADP + Pi

ACL

citrate → acetyl-CoA → LIPID BIOSYNTHESIS

CoA

oxalacetate

malate
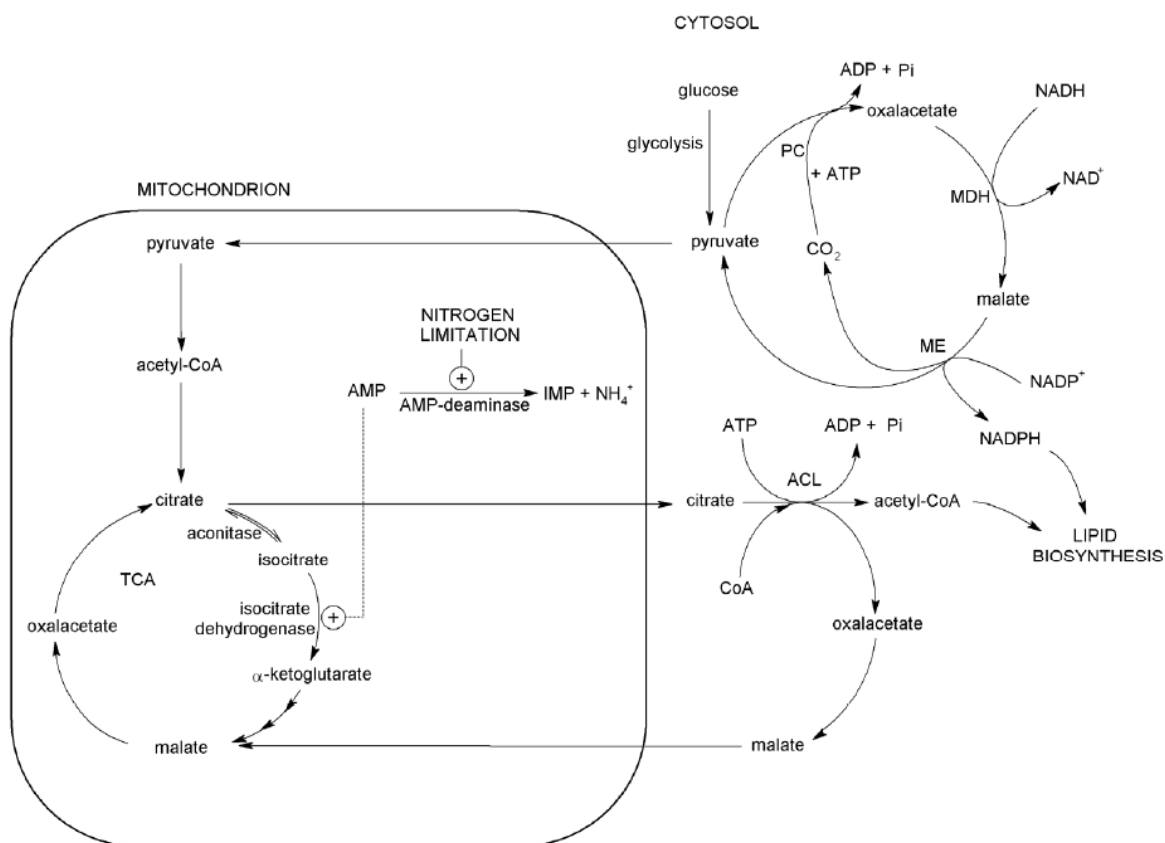
Fig. 5 Overview diagram of the biosynthesis of single cell oils in oleaginous microorganisms (Rossi et al. 2011)

6. Oleaginous microorganisms in industry

The yield and type of lipid in oleaginous microorganisms depend on several factors including the type of microorganism, the culture conditions and the chosen substrates (Li et al. 2008; Griffiths and Harrison 2009). Yeast strains produce mainly fatty acids, which are similar in composition to those in plant oils containing predominantly saturated or mono-unsaturated fatty acids with carbon lengths of C16 and C18 (Papanikolaou and Aggelis 2011a). Other microorganisms, e.g. microalgae or filamentous fungi are also able to produce significant amounts of poly unsaturated fatty acids (PUFAs) like ω3 or ω6 fatty acids, e.g. docosahexaeonic acid (DHA) or eicosapentaenoic acid (EPA) (Ward and Singh 2005), which are usually extracted from fish oil. This knowledge permits the use of different microorganisms for different industrial applications.

Even though much research effort was put into the production of SCOs in the last 30 years, only few processes could be commercialized, including the production of cacao butter

equivalent (CBE), gamma linolenic acid (GLA, C18:3, ω6), docosahexaenoic acid (DHA, C22:6, ω3) and arachidonic acid (ARA, C20:4, ω6). CBE was produced by the yeast *Apiotrichum curvatum* (=*Cryptococcus curvatus*), GLA by the fungi *Mortierella isabellina* and *Mucor circinelloides*. However, most of the commercialization attempts failed because of lower cost alternatives on the market (Kyle 2005). DHA and ARA could be produced since late 1980s successfully by Martek, the new division of DSM's new Nutritional Lipids division. Martek developed economic microbial production processes with the filamentous fungus *Mortierella alpina* to produce fish free arachidonic acid (ARA, C20:4, ω6) and docosahexaenoic acid (DHA, C22:6, ω3) with the use of the microalgae *Crypthecodinium cohnii*. Both fatty acids are used as nutrition supplements which are also applicable for vegetarians under the labels *life's*DHA and *life's*ARA. The non-toxicity of *Mortierella alpina* and *Crypthecodinium cohnii* enables to use these fatty acids as nutrition supplement even in infant formulas (Streekstra 1997). Neste Oil in Porvoo in Finland announced promising patents for the microbial production process from waste and residues with the help of various yeasts and molds for the use as raw material for NExBTL renewable diesel. A pilot plant being tested may be commercialized in 2015. (http://www.nesteoil.com/default.asp?path=1,41,11991,12243,12139,15694; 03.07.2013). Ricinoleic acid (C18:1) was detected in the dark-colored sclerotia, the overwintering form - of the filamentous fungus *Claviceps* spp. also known as ergot (Franzmann et al. 2010). First investigations in early years to produce ricinoleic acid with the fungi *Claviceps purpurea* (Morris et al. 1966) unfortunately failed. Younger studies revealed progress in the microbial production of ricinoleic acid due to genetic engineering manipulation techniques (Holic et al. 2012).

7. Screening methods for oleaginous microorganisms

To identify new oleaginous microorganisms which are applicable for economic industrial applications, suitable screening experiments are required. The most important information is the amount of produced lipid, but also the fatty acid profile is useful for classifying interesting hits for possible industrial applications. In literature several examples are described either the Sudan black B method (Evans et al. 1985; Pan et al. 2009) or Nile red (Greenspan and Fowler 1985). Sudan black B and Nile red are both lysochromes which have been successfully applied to identify new oleaginous microorganisms. Pan et al. (2009) and Evans et al. (1985) focused on the isolation of yeast cells from soil samples whereas Waltermann et al. (2000) screened successfully mutants from the best known oleaginous bacteria *Rhodococcus opacus*. In all above mentioned studies the Sudan black B staining technique was applied on agar plates for a qualitative analysis of lipid production in microorganisms. This described technique enables to stain microorganisms grown on agar plates via a

replicate printing technique on filter paper. Sudan black B dissolves better in oil than in water and stains neutral fats unspecifically. It diffuses from the liquid phase into the oil phase, in the case of oleaginous microorganisms into the lipid containing cells, and leaves a blue staining in those cells. Microorganisms that can be stained blue with Sudan black B are potential lipid producing organisms. To further examine the lipid production capacities of potential strains, cell cultures have to be prepared, lipid containing biomass has to be dried and lipid amount and lipid composition have to be checked via the time-consuming gas chromatographically analysis. Agar plates can be supplemented with either antibiotic or a certain carbon source like xylose to especially screen for yeast and fungi – not for bacteria – and additionally for strains which are able to convert xylose.

Compared to Sudan black B, Nile red ($C_{20}H_{18}N_2O_2$) is a fluorescent lipophilic dye and well described in literature. It is highly fluorescent in organic solvents, but solubility and fluorescence are negligible in water. Its excitation and emission spectra shift to shorter wavelengths with decreasing polarity (Greenspan and Fowler 1985). The fluorescence intensity of lipids composed of unsaturated fatty acids is stronger than that of the saturated fatty acids (Kimura et al. 2004). Due to Kimura et al. (2004) the intensity of fluorescence corresponds to the amount of lipid and should therefore be suited for quantitative analyses. Nile red has been successfully used as a staining method for a rapid estimation of lipid in oleaginous yeast and fungi using a cuvette (Kimura et al. 2004). As a high throughput screening method in suspensions using 96-well microtiter plates Nile red has been used for algal cells (Chen et al. 2009). Mammalian oocytes were checked for lipid with Nile red using a microscope (Genicot et al. 2005). All results of the former mentioned studies conclude that Nile red is a fluorescent lipophilic dye characterized by a shift of emission from red to yellow according to the degree of hydrophobicity of lipids (Diaz et al. 2008). An emission beneath 580 nm was proven to be a good wavelength for neutral lipids whereas emission higher than 590 nm is well suited for polar membrane lipids (Greenspan and Fowler 1985). Hence, polar membrane lipids (phospholipids) are stained red whereas neutral lipids are stained yellow. This knowledge allows the application of Nile red for sensitive measurement of either neutral or polar lipids to roughly quantify the lipid content in *oleaginous microorganisms*.

IV. Main Part

Chapter I:

Combination of algae and yeast fermentation for an integrated process with low CO$_2$ footprint for the production of single cell oils

1 Abstract

Economic and ecological reasons cause the industry to develop new innovative bio-based processes for the production of oil as renewable feedstock. Petroleum resources are expected to be depleted in the near future. Plant oils as sole substituent are highly criticized because of the competitive utilization of the agricultural area for food and energy feedstock production. Microbial lipids of oleaginous microorganisms are therefore a suitable alternative, but their production is still too expensive to compete with plant oils. To decrease production costs of microbial lipids and gain spatial independence from industrial sites of CO$_2$ emission, a combination of heterotrophic and phototrophic cultivation with integrated CO$_2$ recycling was investigated in this study. It was shown that the cultivation of the oleaginous yeast *Cryptococcus curvatus* on a 1.2 L scale was sufficient to supply a culture of the oleaginous microalgae *Phaeodactylum tricornutum* in a 21 L bubble column reactor with CO$_2$ while single cell oils were produced in both processes due to a nutrient limitation.

2 Introduction

Concerns about the increasing emission of greenhouse gases, the resulting global warming and the need to reduce our dependence from crude oil force the industry to develop innovative bio-based industrial processes with reduced CO$_2$ emission (Lee et al. 2002). The substitution of petroleum is a long term objective not only because of its importance as fuel for the transport sector, but it is also main raw material for oleochemicals (Carlsson 2009).

The production of plant based first generation biofuels is increasingly criticized because of the rising competition between agricultural production for the food and for the energy sector. Against this backdrop, single cell oils from microorganisms seem promising substitutes. Heterotrophic oleaginous microorganisms are able to convert a carbon source, which is available in excess, into storage lipids inside the cells as soon as a concurrent nitrogen limitation stops cell growth (Ratledge 2002). Advantages of such a microbial lipid production compared to petroleum production or agricultural production of plant oils are the

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independence from the season and from the location. *Cryptococcus curvatus* is a well examined oleaginous yeast, simple to cultivate in bioreactors with high growth rates, high lipid yields and a cultivation duration of maximum 6 days. It was first isolated 1978 by Moon and Hammond (1978) from cheese plant floors and floor drains during fermentation experiments on lactose. The lipid composition is similar to that of palm oil with high amount of oleic acid suitable for applications as biodiesel. *C. curvatus* is able to convert several various carbon sources into fatty acids (Hassan et al. 1994), e.g. glucose, galactose, cellobiose, sucrose and lactose converts even waste substrates like whey as carbon and nitrogen sources (Ratledge and Cohen 2005).

In addition to heterotrophic oleaginous microorganisms, autotrophic microorganisms like microalgae have shown efficient microbial lipid production. One important advantage is that microalgae do not need any carbohydrates as carbon source, but require just the gas CO_2. They fix CO_2 and hence contribute to CO_2 sequestration. Moreover, they can accumulate lipids to high specific contents up to more than 80 % of their dry weight (Spolaore et al. 2006; Tredici 2010). Most importantly, growth and lipid accumulation are more efficient than that of higher plants, which are cultivated as feedstock for biofuel production (Chisti 2007).

The establishment of economic processes for the production of microbial oil requires energy-efficient and cost-effective strategies. While algae harvesting and cell disruption of the resistant yeast cells are main cost drivers in downstream processing (Cerff et al. 2012; Chisti and Moo-Young 1986), the cultivation itself offers great potential for optimization. CO_2 is generally assumed to be available for free in phototrophic processes as shown by studies assessing the economic viability (Singh et al. 2011). It can potentially be obtained from the flue gas of power plants (Brennan and Owende 2010), but its nitrous gases and sulphur oxides can entail negative effects on growth rates and volumetric productivity of algae (Lee et al. 2002). Moreover, optimal locations of plants for microalgae fuel production are often remote and distant from industrial sites of CO_2 emission (Ratledge and Cohen 2008). In consequence, Feron and Hendriks suggest that a price in the range of 0.15 \$/kg to 0.20 \$/kg should be attached to CO_2 to account for the cost of its capture and transport (Feron and Hendriks 2005). With a demand of approximately 1.8 kg CO_2 for the production of 1 kg algal dry material, this imposes cost for the process in a dimension that cannot be neglected. *Phaeodactylum tricornutum* is suited as oleaginous microalgae since the organism accumulates lipids up to high specific contents. It grows with high growth rates at nutrient replete conditions and showed reasonable photosynthetic efficiency when nutrients were limiting (Dillschneider et al. 2013). Extensive research has been performed with *P. tricornutum* as a model organism, also because it is an interesting producer of

polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA) (Yongmanitchai and Ward 1991).

The purpose of this study was to investigate requirements and implications of the combination of a heterotrophic and a phototrophic single cell oils production process (fig. I.1). Thereby, the heterotrophic process supplies the algae with CO_2 and thus gains a lower CO_2 footprint. On the other hand, the phototrophic lipid production benefits from the cost reduction in terms of the carbon source, but at the same time also gains independence from industrial plants in terms of location.

For this purpose the exhaust gas of the yeast cycle of *C. curvatus* had first to be analyzed to establish a proper process management to combine it with the microalgae cycle of *P. tricornutum*. The course of the carbon dioxide concentration during the process was monitored to plan a time adjusted coupling with the microalgae cultivation adapted to the CO_2 needs for the microalgae cultivation. A further aspect to consider was the concentration of the carbon source glucose for the yeast cultivation which has to be available in excess throughout the whole process, to ensure lipid production, but concurrently to prevent substrate inhibition. However, no online-measurement exists for the measurement of glucose, why glucose is measured and refilled manually. A further important aspect to consider is the influence of glucose deprivation in the yeast process to the emission of CO_2 with regard to a constant CO_2 supply for the microalgae.
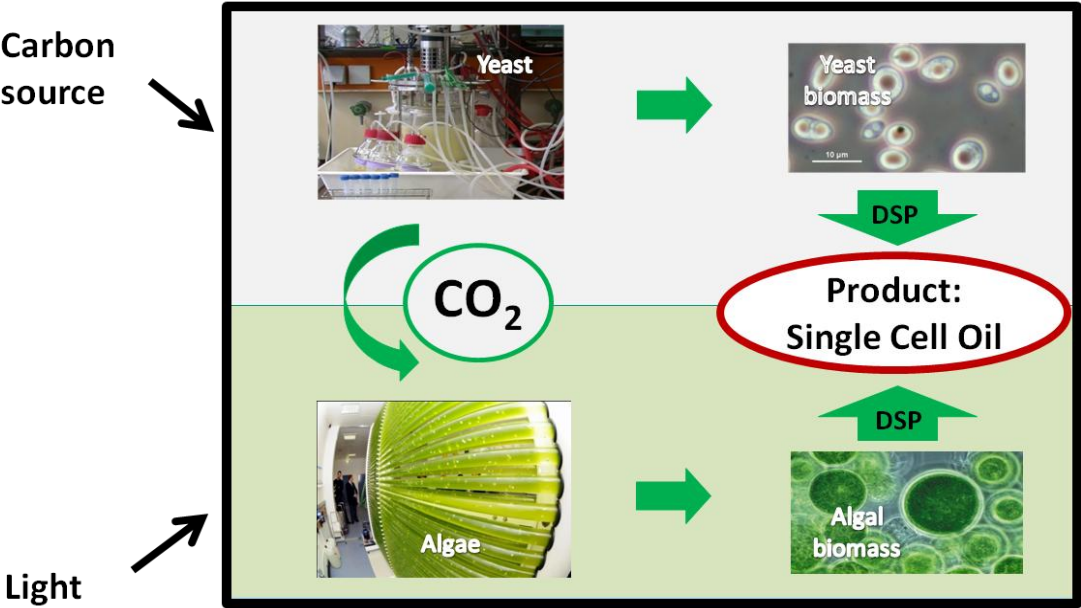

Fig. I.1 Schematic diagram of the coupled process of yeast and microalgae lipid production processes to reuse the emitted CO_2 of the yeast cycle as carbon source for the microalgae

3 Materials and methods

3.1 Microorganisms and culture medium

Cryptococcus curvatus (ATCC 20509) was obtained from the American Type Culture Collection. *Phaeodactylum tricornutum* (SAG 1090-1a) was obtained from the Culture Collection of Algae (*SAG*), University of Göttingen, Germany.

The culture medium for the yeast fermentation was formulated on a phosphate buffer at pH 5 (8.99 g/L KH_2PO_4 and 0.12 g/L $Na_2HPO_4 \cdot 2H_2O$). The medium constituents were 0.1 g/L sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$), 0.1 g/L yeast extract, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 18.9 g/L $(NH_4)_2SO_4$. The initial culture was supplemented with 2 mL trace elements solution (4 g/L $CaCl_2 \cdot 2H_2O$, 0.55 g/L $FeSO_4 \cdot 7H_2O$, 0.475 g/L citric acid, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.076 g/L $MnSO_4 \cdot H_2O$, 100 µL 18 M H_2SO_4) and 1 mL salt solution (20 g/L $MgSO_4 \cdot 7H_2O$, 10 g/L yeast extract) per 100 mL cultivation medium. Trace element and salt solution were supplemented once a day to the culture broth during the whole cultivation time in the fermenter. Initial glucose concentration was 50 g/L and was fed using a stock solution of 500 g/L.

The first preculture was prepared in 20 mL medium in 100 mL conical shake flasks and was inoculated with 100 µL glycerol stock culture (15% w/w, stored at -80 °C). The second preculture was prepared from the first preculture in 200 mL culture medium in 2 L shake flasks with an initial OD_{600} of 1. Both precultures were incubated at 28 °C with 120 rpm for approximately 24 hours.

The culture medium for the phototrophic process was originally published by Mann and Myers (1986). The following modifications were made: NaCl concentration was 27.0 g/L, K_2HPO_4 concentration was 0.15 g/L and $MgSO_4 \cdot 7H_2O$ was reduced to a final concentration of 0.6 g/L. 30 mg/L $Na_2SiO_3 \cdot 5H_2O$ were added. In order to attain a nitrogen limitation early in the process, the $NaNO_3$ concentration was adjusted to 0.4 g/L. Tris buffer was present in the preculture medium, but otherwise omitted in order to prevent growth of heterotrophic organisms (Fábregas et al. 1993).

The algae inoculum for the experiments was cultivated in shaking flask cultures incubated at 21 °C for 3 weeks with LED illumination adjusted to a PFD of 150 µmol/(m²·s). The pH of the preculture medium was adjusted to pH 7.5. During the process the pH was controlled at pH 7.7 as described below.

3.2 Set-up of the integrated bioprocess

The set-up of the integrated bioprocess is depicted in fig. I.2. Yeast fermentation was conducted in a 2.5 L stirred tank reactor (Infors Minifors, left) with 1.2 L culture medium and

an initial OD_{600} of 1. Mixing was achieved by stirring with a rotation speed of 600 rpm. The cell suspension was aerated with compressed air at an aeration rate of 1 vvm and a slight overpressure of maximum $2 \cdot 10^4$ Pa. The pH was set to pH 5.0 and controlled by addition of 4 M H_3PO_4 and 4 M NaOH in the fermenter. Contraspum A 4050 HAC (Zschimmer und Schwarz) was applied as antifoam agent. Initial glucose concentration was 50 g/L. Glucose concentration was measured daily and was replenished to a maximum concentration of 120 g/L if it was depleted in the culture. A minimum of five samples were taken per day (four samples of 3 mL for the determination of OD_{600}, dry biomass (g/L), carbon and nitrogen source and by-products; one sample of 20 mL for lipid analysis (% lipid/dry biomass) via gas chromatography). The partial pressure of oxygen (pO_2) was measured by pO_2-elektrode (Hamilton).

The exhaust gas from the yeast fermenter was filtered (Millex-FG, 0.2 µm, Millipore) and directly fed into the photobioreactor. The latter was an annular bubble column reactor consisting of a 1.3 m long glass cylinder with 0.3 m diameter. The inner cylinder – made from stainless steel – contained the cooling water circulation system. The temperature was controlled at 21 °C. Before inoculation the reactor was autoclaved with a SIP steam generator (DG 7/6, Zirbus) and filled under sterile conditions with the medium, which was separately autoclaved (Vakulab, MMM Group). The pH of the media was adjusted to pH 10.3 with $NaNO_3$ prior to autoclaving, but decreased in the course of the cultivation due to saturation with CO_2. The pH was measured by an Easyferm Plus 425 Sensor (Hamilton) and controlled at pH 7.7. pH-control was implemented by addition of 4 M NaOH and CO_2 by the additional mass flow controller (shown in fig. I.2) which was integrated to supply the algae culture with CO_2 in case the volume fraction in the off-gas of the yeast culture was not sufficiently high. To account for fluctuations in the volume fraction of CO_2 dead zones of the pH-controller were + 0.5 and – 0.3.

The volume fractions of O_2 and CO_2 in the off-gas were analyzed by an off-gas analyzer (Multor 610, Maihak). The annular column reactor was illuminated with 76 halogen spotlights (Decostar 20 W, Osram) which were adjusted circularly around the reactor so that the illumination was as homogenous as possible. The photon flux density amounted to 250 µE/(m²·s).
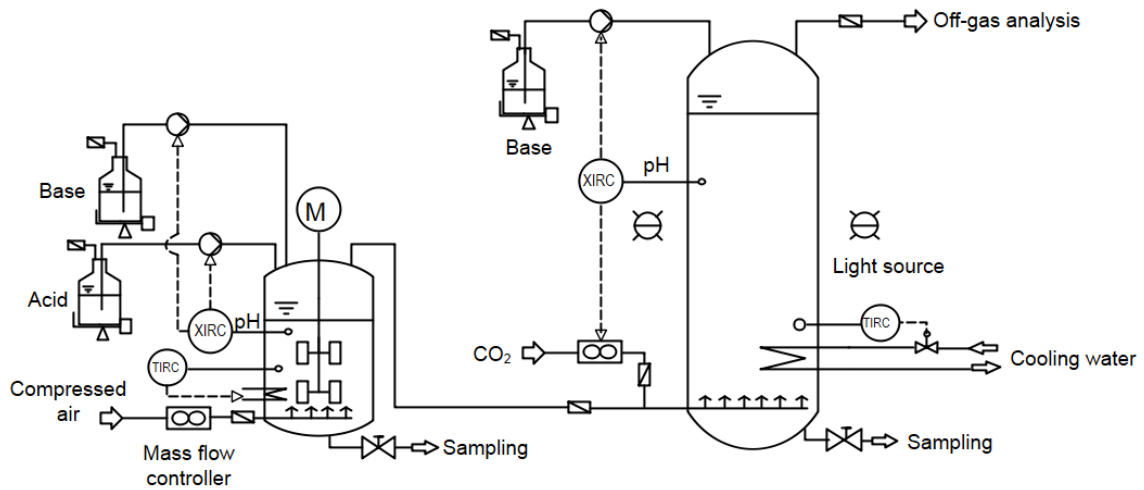
Fig. I.2 Process flow chart of the integrated bioprocess (Dillschneider et al. 2014)

Fig. I.3 Experimental set-up in the laboratory

3.3 Biomass concentration

Yeast dry biomass was analyzed gravimetrically. A 1 mL aliquot of the culture broth was transferred into a pre-dried and pre-weighed 1.5 mL reaction tube and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and used for the determination of

glucose and NH_4^+. The cell pellet was washed with 800 µL saline (0.9 % NaCl), dried at 60 °C for 24 h and weighed.

Algae biomass concentration was determined by spectrophotometry. The absorbance was measured with a T60 U spectrophotometer (PG Instruments) at 750 nm and the calculation of biomass concentration was based on a correlation factor of 0.376 g dry biomass/OD750nm. The correlation factor was obtained from a calibration with 6 duplicate samples of 10 mL. The suspension was centrifuged, the pellet was washed, frozen and freeze-dried (ALPHA 1-2 LDplus, Christ). The weight of the dried biomass samples was determined gravimetrically.

3.4 Glucose concentration

Glucose was enzymatically measured in the supernatant of centrifuged samples for biomass concentration measurement with the D-Glucose kit (r-Biopharm). Glucose was measured in triplicates using microtiter plates.

3.5 Ammonium concentration

Ammonium concentration was measured photometrically with the Spectroquant kit (Merck) in the supernatant of samples for the measurement of biomass concentration. The assay was downsized to 300 µL per sample and measured in microtiter plates in triplicates.

3.6 Nitrate concentration

Nitrate concentration in the supernatant of samples were measured by ion chromatography (882 Compact IC plus, Metrohm) equipped with a conductivity detector (Metrohm). Samples were automatically diluted (1:10) and injected by an autosampler unit (Professional Sample Processor 858). The device was equipped with a Metrosep A Supp 5 column (Metrohm) consisting of polyvinyl-alcohol with quaternary ammonium groups. The elution buffer consisted of 3.2 mM Na_2CO_3, 1.0 mM $NaHCO_3$ and 12.5 % (v/v) acetonitrile in water.

3.7 Lipid quantification

Fatty acid concentrations and total lipid content were measured in a gas chromatograph (Agilent 6890N, Agilent Technologies) equipped with a 30 m DB-Wax column (I: 30 m d: 0.25 mm, Agilent Technologies) and FID detector. For sample preparation duplicates of 20 mg freeze dried biomass samples were transesterified according to the method described by Meesters et al. (1996), but using hexane instead of chloroform. Methyl benzoate (FLUKA) was added as internal standard to a final concentration of 0.5 mg/mL in the lipophilic phase. The temperature program consisted of heating up at a rate of 8 °C/min starting from 40 °C.

Final temperature was held at 250 °C until the end of the measurement. The analytical standards Marine Oil FAME Mix (Restek) and AOCS No. 3 (RM-3, Supelco) were used for calibration and fatty acid identification.

Due to the lower biomass concentration algae lipid quantification was performed with a combination of Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one, Sigma-Aldrich) staining and fluorescence measurement and calibration with data gained from gas chromatography. The protocol was described by Dillschneider et al. (2013).

3.8 Exhaust gas composition

The volume fraction of O_2 and CO_2 in the exhaust gas was analyzed with a Multor 610 System (Maihak).

4 Results

4.1 Preliminary study: Characterization of the cultivation of Cryptococcus curvatus

In order to plan a possible setup for the combination of the yeast and algae cultivation considering the different cultivation durations of both kinds of microorganism, preliminary experiments with the oleaginous yeast *Cryptococcus curvatus* were investigated. The process was characterized with regard to lipid production in response to nitrogen limitation and consumption of the carbon source glucose. Moreover, the time course of the exhaust gases CO_2 and the counterpart O_2 were analyzed with regard to carbon source consumption, depletion and re-feeding.

The cultivation results for the lipid production process of *Cryptococcus curvatus* in a 2.5 L-bioreactor in mineral salt medium are presented in fig. I.4. The initial glucose concentration was 50 g/L and did not exceed 80 g/L during the whole process. The carbon source glucose was added three times after total consumption of glucose and two times before consumption of the glucose to examine the influence of glucose deprivation on the carbon dioxide emission.

The growth rate of biomass was maximal until the nitrogen source was totally consumed. Interpolation of the nitrogen consumption during night shows that the nitrogen limitation was probably reached after 35 hours when no samples were taken. The cessation of base pump to control the pH due to consumption of the base NH_4^+ confirms this time of nitrogen consumption as well (fig. I.4). After 40 hours the growth curve leveled off and the cells

started to accumulate lipids. Fig. I.5 illustrates the course of the emitted gases CO_2 and O_2. Until the maximal growth rate the CO_2 increased to a value of 1.49 % in the exhaust gas and leveled off continuously up to 1 % at the end of the cultivation. As soon as glucose was depleted, the CO_2 value dropped down immediately. By adding glucose CO_2 production did increase and attained the original level. O_2 and the partial pressure of oxygen (pO_2) ran anti-parallel to CO_2. As soon as glucose was consumed, the values for O_2 and pO_2 increased immediately and decreased again as soon as new glucose was added.

Considering possible by-products, organic acids were most probably, but were not produced due to missing pH adaption after NH_4^+ depletion and ethanol was not detected at any time.

One further interesting aspect is the respiration coefficient during the process of the microbial lipid production, an online indicator for the metabolic state. The RQ value was calculated according to equation (1) and is shown in fig. I.6.

$$RQ = \frac{\frac{V(CO_2)}{t}}{\frac{V(O_2)}{t}} \qquad (1)$$

$V(CO_2)$ = produced CO_2; $V(O_2)$ = consumed O_2; t = time

The RQ value increased up to a maximum value of 1 at the time of the maximal growth rate during the growth phase on glucose and runs parallel to the production of CO_2. After approximately 40 hours, when lipid production started, the RQ value started to increase above 1 up to 1.3 and fluctuated between those two values until the total consumption of glucose was reached. At this time point the RQ decreased immediately to values below 1. During the glucose deprivation phase up to the end of the process the RQ value decreased to minimum values of 0.25. Summing up, the course of the RQ value can be used as indication for the lipid production phase in oleaginous yeasts.

Fig. I.4 Cultivation of the oleaginous yeast *Cryptococcus curvatus* in 2.5 L bioreactor in mineral salt medium at pH 5 at 28 °C with manually feeding of glucose; base pump for pH control, NH_4^+: nitrogen source ammonium
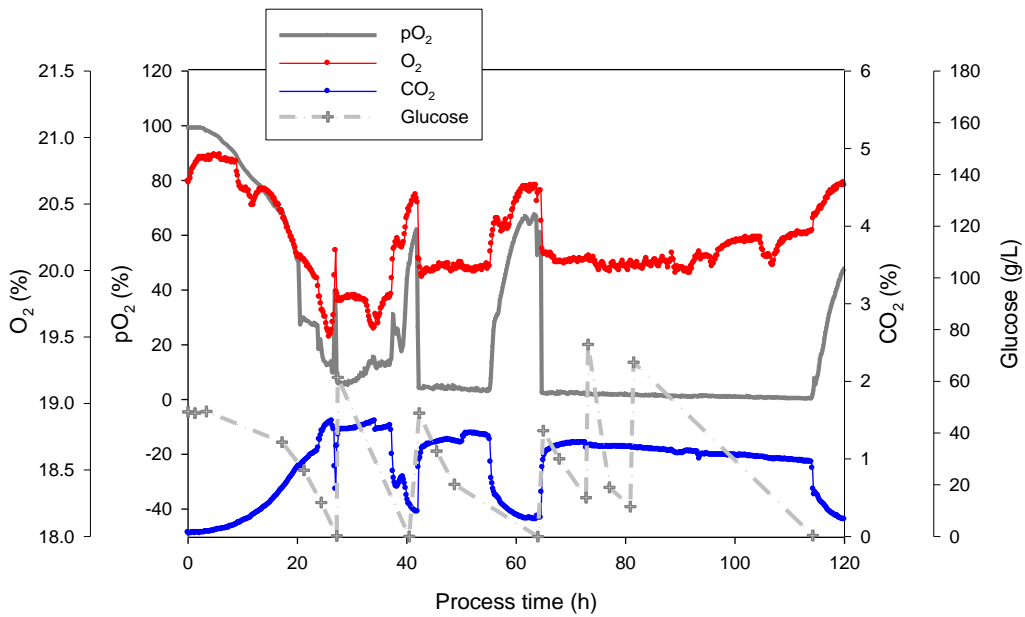
Fig. I.5 Cultivation of the oleaginous yeast *Cryptococcus curvatus* in 2.5 L bioreactor in mineral salt medium at pH 5 at 28 °C with manually feeding of glucose, pO_2: oxygen partial pressure
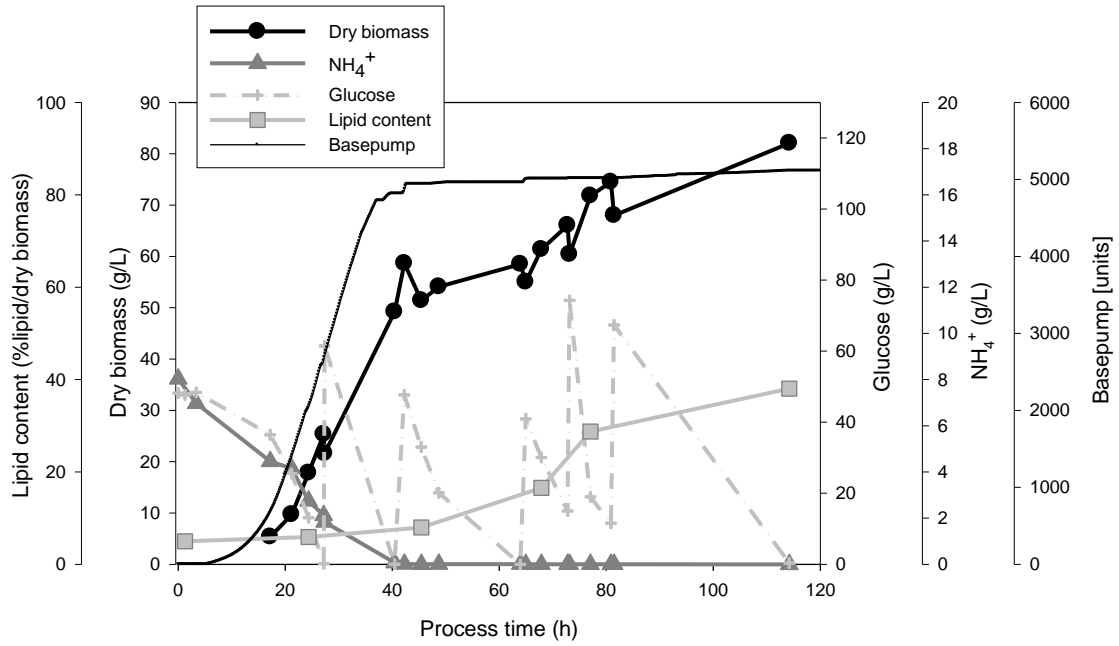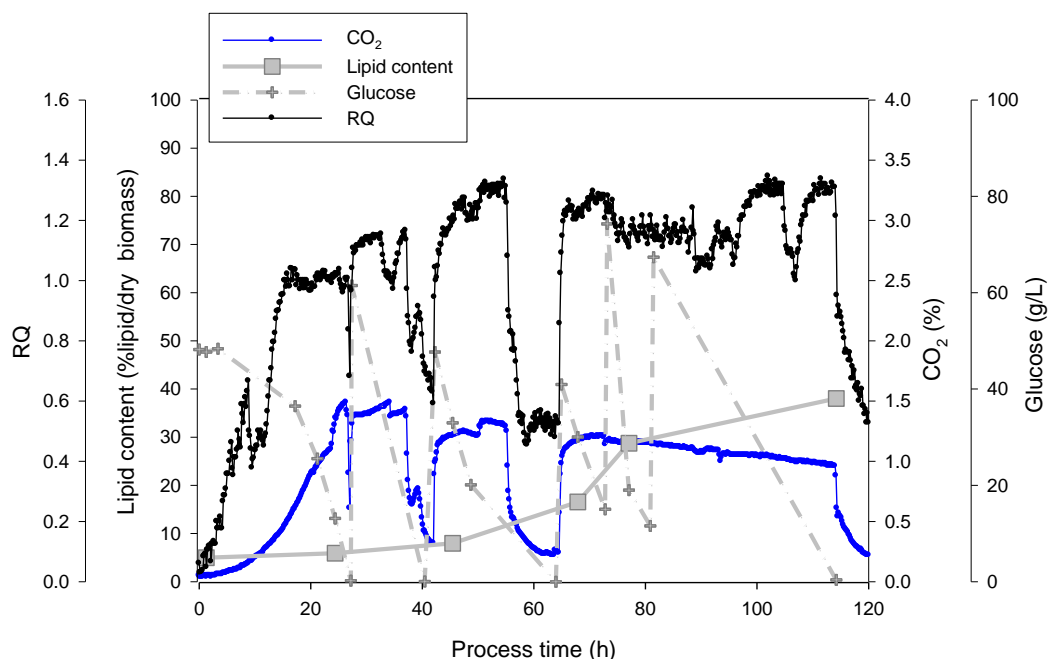
Fig. I.6 Cultivation of the oleaginous yeast *Cryptococcus curvatus* in 2.5 L bioreactor in mineral salt medium at pH 5 at 28 °C with manually feeding of glucose, RQ: respiratory coefficient

4.2 Process integration – Combination of the heterotrophic and phototrophic process

The heterotrophic and phototrophic processes were set up as described in section 2.2. The yeast cultivation was conducted in a repeated fed-batch mode. *C. curvatus* was growing exponentially until nitrogen limitation (36 h), when lipid accumulation started (fig. I.7). Afterwards, the culture was fed every day with a glucose solution to keep the carbon source in excess and sustain lipid synthesis. At day 5 the culture reached a biomass concentration of 76.2 g/L and lipid concentration was 34.5 g/L when the first harvesting of biomass was conducted. 1 L of the culture suspension was harvested and refilled with 1 L fresh culture medium. Glucose was added to a maximum value of 100 g/L. Thereby, a biomass concentration of 15.1 g/L remained in the culture suspension to continue metabolizing the carbon source and hence incessantly supplied the algae culture with CO_2. At this point the yeast cells were also provided with NH_4^+. Renewed availability of the nitrogen source led to the cessation of lipid production and adaption to a new growth phase of the yeast cells. This change in the metabolic state led to a fast drop-down of the CO_2, as the metabolism of the yeast cells had to change from lipid production to growth mode, but CO_2 level increased again as soon as the yeast cells reached growth phase. The maximal volumetric productivity in this second cycle was slightly lower than in the first cycle. During this exponential growth phase the mass fractions of lipids in biomass decreased from initially 45.3 % to a minimum value of 18.2 %. When the culture entered again the nitrogen-limitation, a rapid switch to lipid production occurred. Lipids were then accumulated to a maximum value of 39.3 % before the

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second harvest was performed. Like in the first cycle the intermittent glucose feeding was continued throughout the process until it was stopped at day 15. In the third cycle the lipid content decreased first from 39.4 % to 17.5 % during the growth phase and afterwards reached a maximum lipid content of 34.3 %.
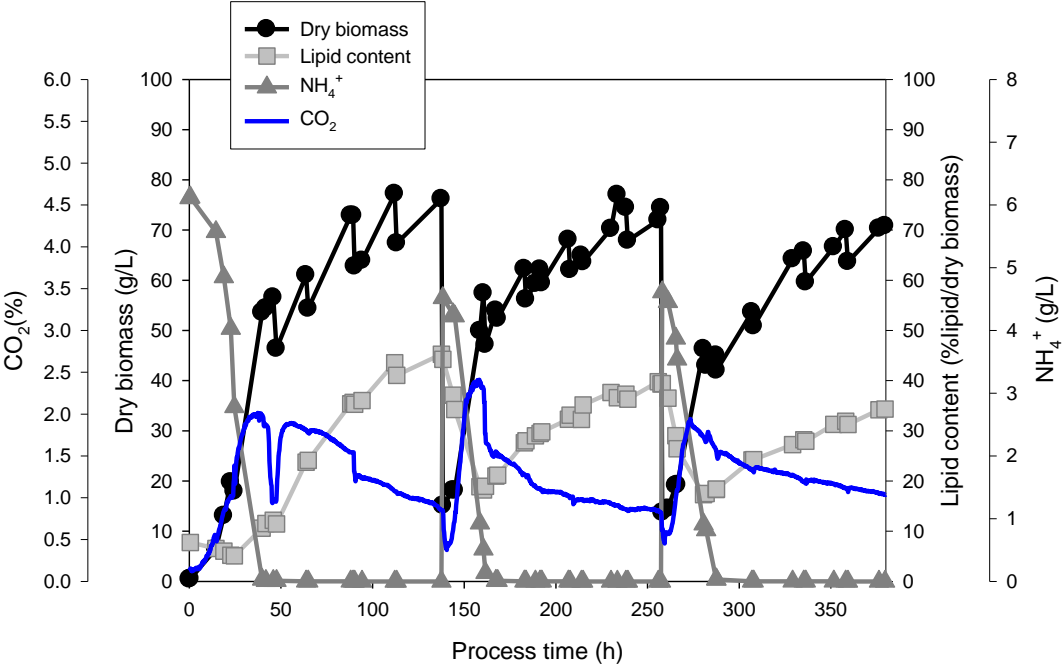
Fig. I.7 Repeated fed-batch process of oleaginous yeast *C. curvatus* in 2.5 L bioreactor in mineral salt medium at pH 5 at 28 °C with regular feeding of glucose (not shown)

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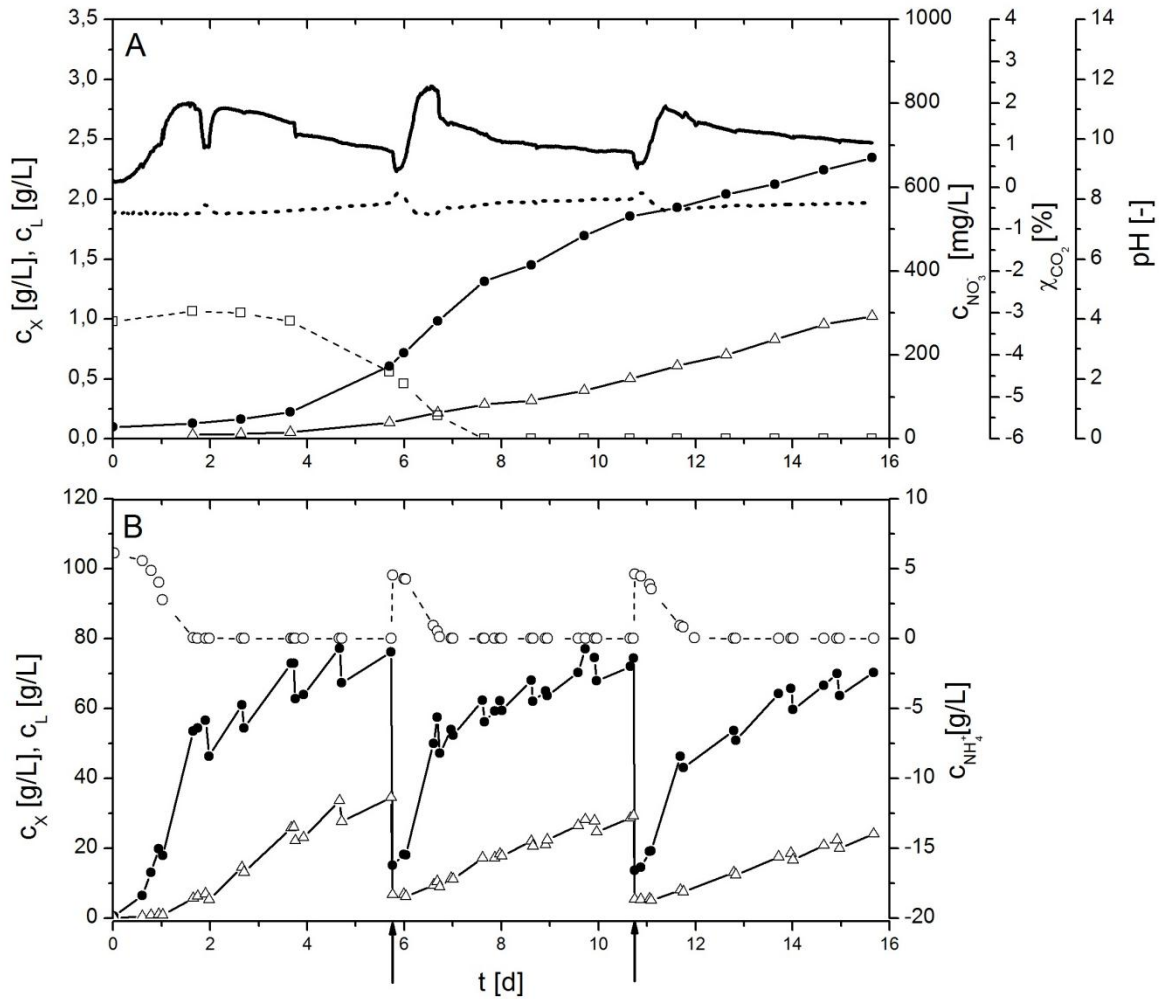
Fig. I.8 Process integration by combination of the heterotrophic and phototrophic process. A: Phototrophic process; B: heterotrophic process. ●: Biomass concentration c_X; Δ: Lipid concentration c_L; □: Nitrate concentration $c_{NO_3^-}$ in the photobioreactor; ○: Ammonium concentration $c_{NH_4^+}$ in the yeast fermenter. Online data: —: Volume fraction of CO_2 in the exhaust gas χ_{CO_2}; ---: pH-value in the photobioreactor. Arrows indicate temporary supply with additional CO_2 from an external source (Dillschneider et al. 2014)

Table I.1 Yield coefficients and volumetric lipid production rates of the heterotrophic repeated fed-batch process

Cycle number	1	2	3
$Y_{x/s}$	0.3	0.34	0.29
$Y_{p/s}$	0.13	0.11	0.09
Q_L (g/(L·h))	0.36	0.21	0.21

Comparing the yield coefficients (biomass and lipid synthesis related to glucose consumption) as shown in table I.1, it can be concluded that the lipid yields decreased slightly from 0.13 to 0.09 from the first to the third cycle. The values for the lipid production

rate Q_L show a similar trend. The first cycle reached a value of 0.36 g/(L·h) and decreased subsequently to 0.21 g/(L·h) in the second and third cycle.

Fig. I.8 summarizes the coupled process of the yeast *C. curvatus* and the microalgae *P. tricornutum*. The phototrophic process was supplied with CO_2 throughout the entire process by the exhaust gas from the yeast culture. The only exceptions were the time points of harvesting. At these time points the pH value in the culture suspension of the phototrophic process reached the upper limits of the pH-controller, which were defined as described in section 2.2. The automatic controller action prevented the pH-value from increasing and supplied CO_2 from a pressure cylinder through the action of a mass flow controller. At these harvesting points CO_2 had to be supplied from the external source for about 100 min with a flow rate of 2.8 mL/min (average value) during the start of the second cycle of yeast growth. Similarly, CO_2 with an average flow rate of 4.1 mL/min had to be supplied for about 180 min at the beginning of the third cycle.

The phototrophic culture of *P. tricornutum* showed exponential growth during the first 7 days after a short initial lag-phase. At day 7 the nitrate in the culture medium was completely consumed and lipid accumulation started. Biomass was ca. 1 g/L at the onset of the nitrogen-limitation. An early limitation at low concentrations of biomass was aimed to keep the planned time period for the experiment of 16 days. In the following days the biomass growth curve showed a linear evolution and storage lipids accumulated in this phase. Biomass increased to a final value of 2.3 g/L at day 15. The lipid concentration increased from initially 0.2 g/L at the onset of the nutrient limitation to about 1.0 g/L at the end of the process. The volume fraction of CO_2 was measured in the exhaust gas of the photobioreactor. Therewith, the signal comprises the CO_2 emission caused by the yeast respiration and also the effect of CO_2 uptake of the algae. The CO_2 emission was low in the beginning of the cultivation and increased subsequently with exponential growth of the yeast. A maximum value of about 2 % was reached at the onset of the first nitrogen-depletion when metabolic rates slowed down compared to nutrient-replete growth. At the end of the first lipid accumulation phase the volume fraction dropped below 1 %. In the subsequent two cycles maximal values of 2.4 % and 1.9 % were reached. Even though the variation of the volume fraction of CO_2 in the gas stream was considerable, it never fell below a value of 1 % except for the time points of harvesting. Therefore, CO_2 limitation was prevented throughout the entire process and the phototrophic process could be sufficiently supplied with CO_2 by the heterotrophic yeast culture.

Due to the considerable changes in the volume fraction of CO_2 in the gas stream entering the phototrophic reactor moderate changes in the pH-value in the algae culture suspension occurred. The latter varied in a range between pH 7.5 and pH 8.2. Further variation of the pH

was prevented due to the pH-controller settings. During the harvesting process of yeast and the subsequent adaption period the CO_2 flow temporarily decreased slightly so that the pH temporarily reached a value of pH 8.2, where additional CO_2 was added for a short period as described above.

Table I.2 Measured and calculated parameters for the algae cultivation of *P. tricornutum* and one yeast cycle of *C. curvatus*

	Concentration of dry biomass (g/L)	Lipid content (%lipid/dry biomass)	Lipid concentration (g/L)	Culture volume$_{total}$ (L)	Total dry biomass (g)	Total lipid (g)
Algae	2.3	43.5	1	21	48	21
Yeast	74.4	39.3	29.3	3	223.2	87.72

Table I.2 summarizes the produced lipid amounts of the 16 day lasting algae cycle as well one 5-6 days lasting yeast cycle. Although the algal fermenter had a 7-fold volume than the yeast fermenter, one single yeast cultivation resulted in a 4-fold lipid amount. Taking into account that three yeast cultivation cycles were performed, the 12-fold lipid amount could be obtained with yeast.

4.3 Fatty acid profiles of the oleaginous microorganisms

The fatty acid profiles of both production strains were analyzed in order to state possible industrial applications of the microbial oil. The fatty acid profiles are presented in fig I.9. The fatty acid profile of the yeast *C. curvatus* (fig. I.9, A) refers to the last taken sample after 115 hours at the end of the first cycle. The main component of the stored lipid is oleic acid (C18:1) with 48.8 %. Palmitic acid (C16:0) and stearic acid (C18:0) reached values of 18.5 % and 17.7 %, respectively. Linoleic acid (C18:2) with 8.6 % and lignoceric acid (C24:0) with 3 % were less represented. Myristic acid (C14:0), linolenic acid (C18:3), arachidic acid (C20:0) and behenic acid (C22:0) are negligible with less than 1 % of total fatty acids. The fatty acid profile of the microalgae *P. tricornutum* (fig. I.9, B) contains 45 % palmitoleic acid (C16:1) as the main component followed by palmitic acid (C16:0) with 25 %. High amounts of the industrially relevant fatty acids DHA (C20:5, 8.9%) and oleic acid (C18:1, 7.6 %) were obtained. Myristic acid (C14:0), linoleic acid (C18:2) and arachidonic acid (C20:4) are rather negligible with less than 5 %. Fatty acids detected with amounts less than 1 % are combined to "others".
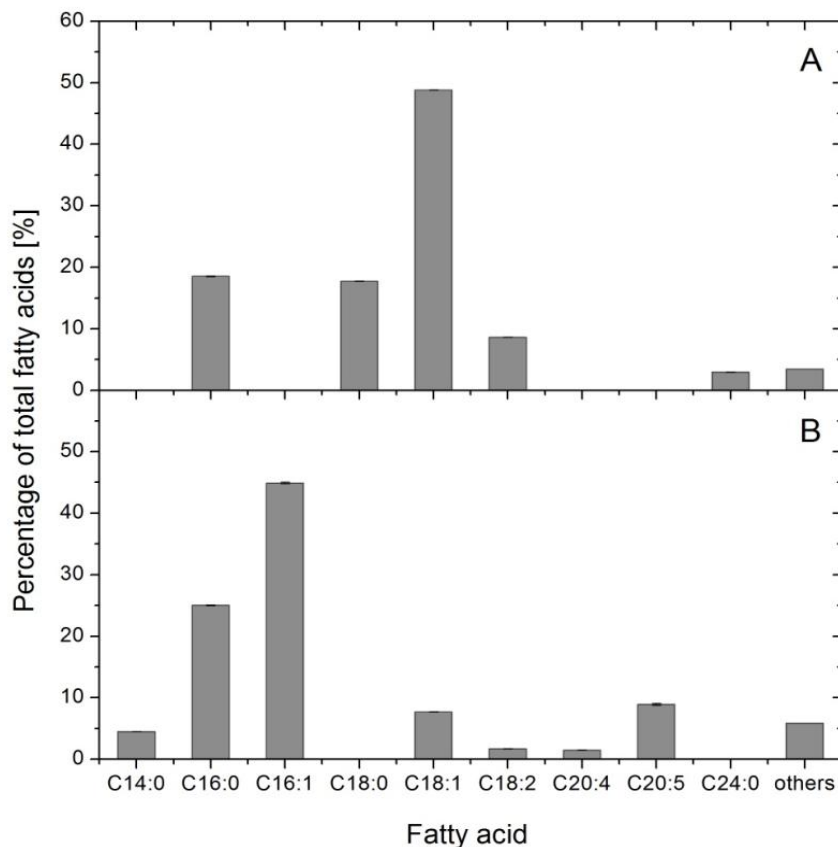
Fig. I.9 Fatty acid profiles of the yeast *Cryptococcus curvatus* (A) and the microalgae *Phaeodactylum tricornutum* (B); fatty acids detected with amounts less than 1 % are combined to "others"; values in detail in appendix; (Dillschneider et al. 2014)

4.4 Variation of the fatty acid profile of C. curvatus during the course of the process

The fatty acid profile of the yeast *C. curvatus* was examined in further detail for the transition phase from the second to the third cycle (fig. I.10). The fatty acid profile started to change as soon as the yeast cells were provided with new medium containing nitrogen in the form of NH_4^+. At this point lipid production stopped and cell growth was initiated. As soon as the refilled nitrogen was totally consumed the fatty acid profile tended to change again to the same profile as before harvesting and refilling with new medium. Oleic acid (C18:1) decreased slightly from 48.5 % to 46.2 %. Palmitic acid (C16:0) decreased from 19.5 % to 15.8 %. A more intense decrease can be measured for stearic acid (18:0) falling from 17.7 % to 12.4 %. By contrast, the percentage of linoleic acid (18:2) and linolenic acid (18:3) increased during the renewed growth phase from 8.6 % to 16.5 % and from 0.6 % to 1.9 %, respectively.
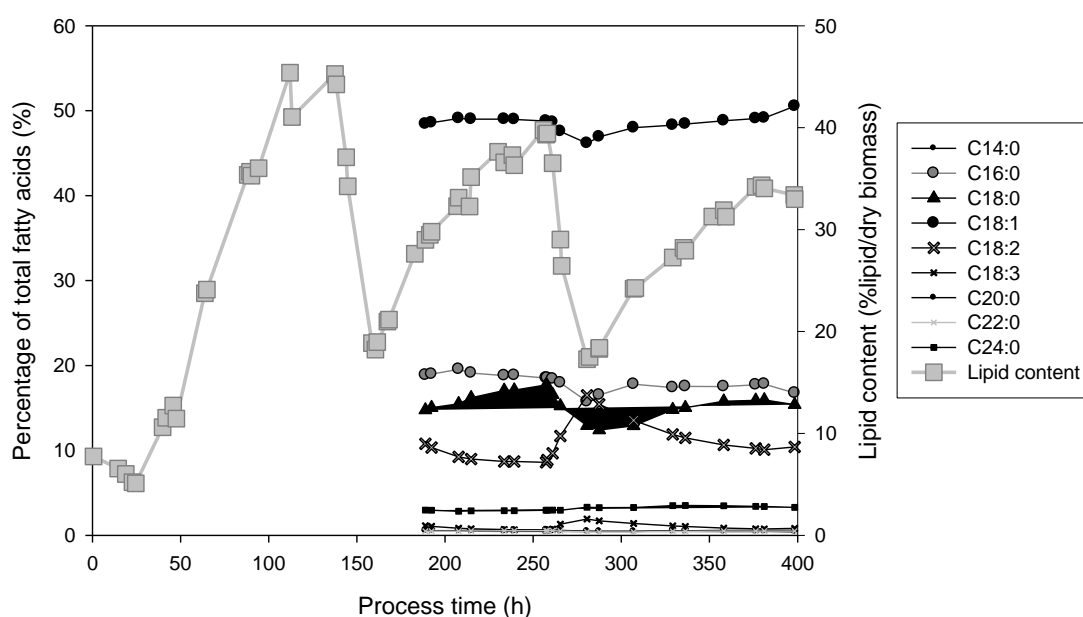
Fig. I.10 Composition of fatty acid profile during the fermentation of the oleaginous yeast *C. curvatus*

5 Discussion

5.1 Preliminary study of Cryptococcus curvatus

C. curvatus has been long proven as a microbial lipid producer and many production processes have been described in literature (Meesters et al 1996; Thiru et al. 2011; Zhang et al. 2011). However, attempts to use those processes economically failed because of the high production costs. Hence, the microbial lipids cannot compete with conventional lipid products arising from plants and fish (Ratledge and Cohen 2008). To reduce the overall costs of a lipid production process, the setup of this study was to recycle the exhaust gas CO_2, one of the resulting waste streams of the yeast cycle. To solve this problem, the lipid production with the yeast *Cryptococcus curvatus* process itself was primarily analyzed with respect to nitrogen consumption and lipid production as well as the course of CO_2 emission during the process and especially the response to carbon deprivation. A constant flow of CO_2 as carbon source for the microalgae is important for a successful cultivation process whereas an excess of the carbon source is necessary for the yeast cultivation to produce microbial lipids.

As shown by Ratledge (2002), the lipid production started as soon as the nitrogen was totally consumed and growth leveled down. The partial pressure of oxygen (pO_2) level decreased to 0 % at high biomass production. Therefore, aeration or stirrer speed may not be optimal for maximal growth or lipid accumulation. However, the pO_2 increased rapidly each time as soon

as the carbon source glucose got totally consumed. This is an indication that the metabolism switches rapidly to a non-glucose-consuming metabolism.

Considering the course of the CO_2, CO_2 behaves anti-parallel to the exhaust gas O_2 and the pO_2. The maximum value for CO_2 was reached at the point of maximal growth and decreased slowly during the lipid production phase. Each time of glucose deprivation, the CO_2 decreased rapidly. With respect to the coupling of CO_2 to a microalgae process such a glucose deprivation is disadvantageous for the algae as they require a constant flow of CO_2. Therefore a continuous CO_2 production is required and thus a continuous and sufficient feeding of the carbon source has to be guaranteed. The best option would be a glucose sensor for an online-measurement. Such a technique would permit to stabilize the level of the carbon source and to hold it constant during the whole process in excess to guarantee lipid production. On the other hand the glucose concentration should not exceed a certain concentration level which may lead to a substrate inhibition and thus to an inhibited lipid production (Zhang et al. 2011). Considering the values of the respiratory coefficient (RQ), RQ and CO_2 exhaustion resemble each other. As it was to be expected (Dilly 2005), RQ is 1 during the maximal growth rate on glucose. Parallel to a rapid decrease of CO_2, a rapid decrease of the RQ value was observed. The calculated RQ value at the point of the maximal growth rate reached 1 which corresponds to the value of the complete oxidation of the carbon source. As soon as the lipid production starts, the RQ value increased to values above 1 up to 1.3. This means that more CO_2 is produced than oxygen is consumed during the lipid production phase. The rapid decrease of CO_2 after deprivation of glucose and the rapid increase after refilling of glucose show that the metabolism of the yeast cells switches rapidly between glucose deprivation state and lipid production phase.

5.2 Fatty acid composition

In general C16 and C18 fatty acids are well suited for the production of biodiesel. Those kinds of fatty acids are main components of plant oils used for biodiesel production (Ma and Hanna 1999) which fit well with the profiles of *C. curvatus* as well as that of *P. tricornutum*. Hence, microbial oil from those two strains can be used for the production of biodiesel. However, biodiesel from microbial oils cannot yet compete with those from plant oils. In addition to the use as biodiesel, the vast amount of different fatty acids in the described process can find applications in other industrial sectors. The main fatty acid in the microalgae, palmitoleic acid (C16:1), is known to prevent not only cerebral diseases, but also cardiovascular diseases by enhancing the function of vascular smooth muscle cells. It can also be applied in the therapy against obesity. Therefore, this fatty acid can find novel and valuable uses in human nutrition and medicine (Yamori et al. 1986; Matsunaga et al. 1995; Yang et al. 2011). Oleic acid, the main fatty acid in the yeast lipid, finds application as

lubricant, for cosmetics and cleansing agents, as food addition and for biodiesel production (Yin et al. 2009; Liebert 1987). Palmitic acid which is found in high concentrations in both cell types, is used for the production of biodiesel (Carmo et al. 2009), soaps, cosmetics, lubricants, release agent and food additions (Thieme Römpp online; Liebert 1987)

The fatty acid composition differs in the course of the cultivation cycles. The increasing percentages of linoleic acid (C18:2) and linolenic acid (C18:3) during the transition phase from lipid production and renewed cell growth can be explained by membrane lipids which are required for cell growth consisting of those unsaturated fatty acids. During the lipid production phase the percentage of those two fatty acids decreased whereas the percentage of C16:0, C18:0 and C18:1 increased again as already shown by Meesters et al. (1996). The important fatty acid profile of the microbial lipid is the moment with a high lipid amount, hence at the end of a cycle, shortly before harvesting. By implementation of a semi-continuous or continuous yeast process for microbial production, the fatty acid profile would be more or less the same corresponding to the composition of the single cell oils in the end of one batch cycle.

5.3 Challenges and chances of the process integration

The coupling of algae and yeast cultivation has been already described by Puangbut and Leesing (2012), but those results are based on shake flask cultivations and the exhaust gas of the yeast cultivation was supplemented by air flow. In contrast, this study focuses on the development of a scalable process strategy which is feasible to supply algae cultures with CO_2. Furthermore, the study investigates on challenges and solutions with regard to an entirely automated process for biodiesel production with both organisms.

The challenge of the process integration in order to achieve CO_2 recycling was the adaptation of the diverging process duration of heterotrophic cultivations and phototrophic processes. Since yeast grows on average with a growth rate of 0.4/h and therewith attains about 10 doublings per day algae usually attain significantly lower growth rates in the range of 1/d for fast growing species. Another significant difference is that phototrophic processes are light dependent and the exponential growth is limited to low biomass concentrations due to shading of the cells. Once light is limited the culture grows more or less linearly.

One possibility to match process durations in order to achieve constant supply of the algae cultures with CO_2 would be the implementation of time delayed independent yeast bioprocesses. In such a set-up several yeast fermenters are started at different time points and in phases of peak metabolic turnover the exhaust gas could be connected with the photobioreactor. A switch would allow using the exhaust gas of the next fermenter, when a limitation occurs in one reactor. The obvious disadvantage of such a process would be the

high investment required for the implementation. The other option was a repeated fed-batch process which was conducted as shown in 4.3. The advantage of such a repeated fed-batch is the possibility to reduce the set-up and maintenance time because the bioreactor does not have to be cleaned and newly autoclaved after each batch process. Instead just fresh medium needs to be added (Zhao et al. 2011). This strategy contributes to cost reduction in terms of cost of operation and besides reduces disbursement for the initial investment of the plant. Furthermore, studies have shown that *C. curvatus* has higher lipid production rates when cultured in fed-batch process (Zhang et al. 2011).

Another challenge was the approach of changing volume fractions of CO_2 in the gas stream. Through the chemical equilibrium of dissolved CO_2, hydrogen-carbonate and carbonate changing partial pressures of CO_2 affect the pH in the solution (Camacho et al. 1999). This problem could be avoided in principle by using a combination of a continuous yeast process and an autotrophic algae process. However, such a strategy was not feasible in the study presented here, since the objective was the production of lipids in both process stages. The approach to deal with the changing CO_2 partial pressures was a pH-control in the photobioreactor that allows minor variations of the pH due to a defined dead band of the controller. Nevertheless, the mere adaptation of the pH-control in the photo bioreactor is not sufficient to achieve constant supply of the algae cultures with their carbon source. Therefore, the preliminary experiment presented in 3.1 was conducted in order to analyze the CO_2 emission in a microbial production process with yeast cells. The study was important to align the yeast process with the need of a relatively constant CO_2 release in the exhaust gas. One basic necessity for continuous respiratory activity and additionally for the lipid production in yeast was that the carbon source has to be available in excess. This necessity entails that the carbon source is regularly added. On the other hand, a maximal concentration of 120 g/L should not be exceeded because of substrate inhibition (Zhang et al. 2011). An exhaustion of glucose leads to an immediate decrease of CO_2 in the exhaust gas and must be avoided in the integrated process. An automatic glucose feed could be installed to avoid such declines of CO_2. Control of the glucose feed could be achieved by immediately feeding of glucose as soon as the CO_2 value decreases. A rapid increase of the pO_2 in the culture suspension could also be used as a controlled variable as it indicates declining respiratory activity. The latter would be advantageous, because other than CO_2 emission, which is measured in the exhaust gas of the phototrophic process, the pO_2 can easily be measured independently of the second process stage. An automatic calculation of the RQ value with the online-measured values O_2 and CO_2 in the exhaust gas could be used as a direct indicator of the metabolic state of the yeast cells. Once an RQ value of 1 is reached, a decrease (RQ<1) could indicate that the carbon source is exhausted, whereas an increase (RQ>1) depicts the lipid production phase.

Complementary to the presented process strategy a semi-continuous fed-batch process with more frequent harvesting might be implemented for the heterotrophic process stage. It was shown that the two harvesting events had the strongest impact on the CO_2 partial pressure. More frequent harvesting of smaller volumes would lead to a more constant CO_2 volume fraction and peaks could be dampened. One further aspect to prove is the vitality of the yeast cells from one to another cycle as it was shown that the volumetric productivity of the yeast cells decreased from the first to the second and third cycle within the repeated-batch process. It could be that a part of the yeast cells of the residual biomass which was used as inoculum for the next cycle were not alive anymore as the aeration conditions were not sufficient. Process optimization regarding oxygen transfer could further improve the lipid yields.

5.4 Potential benefits of the overall process

In general assessments of the economic viability of biodiesel production with microalgae CO_2 is often regarded as a free resource (Singh et al. 2011). It can potentially be obtained from the atmosphere, from the flue gas of power plants, from the by-product streams of other industrial plants or from soluble carbonates (Brennan and Owende 2010). Atmospheric partial pressures are usually too low and allow only growth with reduced growth rates due to underlying kinetic coherences (Doucha and Lívanský 2009). The suitability of flue gas is controversial (Clarens et al. 2010). Experiments were published that have shown a negative impact of nitric oxides and sulphur oxides on growth rates and the volumetric productivity of microalgae (Lee et al. 2002). In other experiments the tolerance of limited contents of nitric oxides and sulphur oxides were demonstrated (Brown 1996). Even though the sequestration of CO_2-emissions from power plants or other industrial plants is desirable, their location is often remote from potential locations of microalgae production facilities. Due to the specific requirements in terms of climate and price of land optimal locations are often distant from urban areas or industrial bases (Ratledge and Cohen 2008). Consequently, the transport of CO_2 to the site of its consumption would be necessary and increases the costs of operation of the biomass production (Feron and Hendriks 2005; García et al. 2003).

In summary, it can be stated that independence from sites of CO_2-emission can be achieved by the scale-up of the integrated bioprocess with the combination of heterotrophic cultivation of yeast and CO_2-recycling in a phototrophic process. Moreover, a significant cost saving potential can be accessed so that process integration can significantly improve the economic viability of the microbial production with oleaginous microorganisms. Furthermore yeast and algae oil are intracellular products which means that the same technique of downstream processing can be used leading to further cost-savings of the overall process.

Chapter II:

Screening for new oleaginous microorganisms

1 Abstract

Four yeast strains were isolated from soil samples and characterized as *Cryptococcus podzolicus*, *Trichosporon porosum, Pichia segobiensis* and *Candida shehatae*. All four isolates were tested positive by Sudan black B staining. Cultivation in bioreactors identified *C. podzolicus*, *T. porosum* and *P. segobiensis* as oleaginous yeast strains. When cultured in bioreactors with glucose as the sole carbon source *C. podzolicus* yielded 31.8 % lipid per dry biomass at 20 °C, while *T. porosum* yielded 34.1 % at 25 °C and *P. segobiensis* 24.6 % at 25 °C. These amounts correspond to lipid concentrations of 17.97 g/L, 17.02 g/L and 12.7 g/L and volumetric productivities of 0.09 g/L*h, 0.1 g/L*h and 0.07 g/L*h, respectively. During the cultivation of *C. podzolicus* and *T. porosum* with glucose gluconic acid was detected as by-product (30 g/L and 12 g/L, respectively).

The production of gluconic acid was not detectable for both strains when glucose was substituted by xylose as the carbon source. Using xylose lipid yields were slightly lower than using glucose. Therefore, it was concluded that when using either *C. podzolicus* or *T. porosum* as the production strain, xylose is the carbon source of choice for exclusive lipid production, but glucose may be used for the simultaneous production of gluconic acid as extracellular product and lipid as intracellular product. *C. shehatae* yielded just 17.8 % lipid content on glucose at 25 °C, but produced more than 5.8 % ethanol as extracellular product.

The fatty acid profile analysis showed that oleic acid was the main component (between 39.6 and 63 %) in all four strains and could be applicable for biodiesel production. Palmitic acid (between 9.8 % and 21.1 %) and linolenic acid (between 7.5 % and 18.7 %) are valuable for cosmetic applications. *Pichia segobiensis* had a considerable percentage of palmitoleic acid (16 %) and may be therefore suitable for medical applications.

2 Introduction

As world population continues to grow, there is an ever-increasing demand on energy and material resources. Therefore, to ensure long-term sustainability suitable, alternative production methods for oil as feedstock for several industrial applications have to be developed. Biodiesel and bioethanol derived from plant oil for example, have been used

since decades, but the disadvantage is the competition with the need to produce feed and food (Ratledge 1993). Therefore, oleaginous microorganisms represent an alternative production system for sustainable lipid production as they share the special feature to produce more than 20 % lipid per dry biomass as carbon storage reserves with similar fatty acid compositions to plant oils.

Lipids are produced by all microorganisms (MOs) usually in the range of 6 to 8 % per dry biomass principally as components for the cell membrane. However, oleaginous microorganisms, belonging to the yeasts, bacteria, filamentous fungi and microalgae, convert a carbon source when it is available in excess into intracellular triacylglycerols (TAG) as soon as a nitrogen limitation occurs (Ratledge 2003). These lipids are also called single cell oils (SCO) and are stored as lipid droplets within cells.

Baker's yeast *Saccharomyces cerevisiae* does not produce any intracellular lipid droplets (Vorapreeda et al. 2012), but several other yeast strains are known to belong to the oleaginous microorganisms, e.g. *Cryptococcus* sp.*,* *Yarrowia* sp., *Candida* sp., *Rhodotorula* sp., *Rhodosporidium* sp., *Trichosporon porosum* and *Lipomyces* sp. (Ratledge 1991). *Cryptococcus curvatus* is one of the most known oleaginous microorganisms able to grow on several carbon sources, e.g. glucose, glycerol or xylose (Meesters et al. 1996; Zhang et al. 2011). Advantages of microbial oil production compared to plant oil is the short life cycle of microbes and the possibility of an in vitro production process not influenced by external factors such as venue, season or climate (Thiru et al. 2011).

The yield and type of lipid depend on several factors including the type of microorganism, the culture conditions and the chosen substrates (Li et al. 2008; Griffiths et al. 2010). Yeast strains produce mainly fatty acids which have a similar composition to those in plant oils containing predominantly saturated or monounsaturated fatty acids with carbon lengths of C16 and C18 (Papanikolaou and Aggelis 2011a). Other microorganisms, e.g. microalgae or molds are also able to produce significant amounts of poly unsaturated fatty acids (PUFAs) like ω-3 or ω-6 fatty acids, e.g. docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) (Ward and Singh 2005) which are usually extracted from fish oil. This knowledge permits the use of different microorganisms for different industrial applications.

Costs for microbial lipid production include the payments of the raw materials (chosen substrates), the payments of the fermentation process (monitoring, control, labour and operating costs) and downstream processing expenses. Fermentation costs are almost unchangeable. Substrate costs can be reduced by using low-cost substrates or waste material as carbon and nitrogen source. The greatest challenge for the downstream

processing of intracellular lipid is overcoming the high energy expenses for the cell disruption.

The establishment of an economic production process entails either attaining higher volumetric output of lipid or producing lipids with high value fatty acids. Therefore, suitable microorganisms have to be identified or new strains have to be isolated from the environment, which are able to grow on low-cost substrates, e.g. hemicellulosic wastes including xylose as monomers.

Glucose can be converted by almost every microorganism. Thus, it is suitable for screening experiments. However, many microorganisms, e.g. *Pichia pastoris* (Lee et al. 1986) are able to convert xylanes, a constituent of hemicelluloses and polymer of xylose molecules. Hemicellulosic material is accessible in large quantities all over the world (Pan et al. 2009) and can therefore be classified as low-cost substrate suitable as carbon source for oleaginous microorganisms. Soil samples contain old wood; hence products of decomposition like xylanes might be biotransformed by several soil microbes. Some of those microbes might belong to the group of oleaginous microorganisms showing the ability to convert xylose as carbon source into SCO. New isolates from soil samples may therefore be qualified for the application in biotechnological processes using hemicellulosic feedstock as cost-efficient substrate.

This study aimed to identify new yeast isolates from soil samples for the production of SCO, which can be used as oil substitute for industrial applications in the cosmetic, pharmaceutical, nutritional or energy sectors. Furthermore, xylose as low-cost substrate should be examined and evaluated as carbon source for promising lipid producing isolates.

3 Materials and methods

3.1 Microorganisms

Cryptococcus curvatus (ATCC 20509) as an oleaginous yeast was used as a positive control for SCO production. *Saccharomyces cerevisiae* (DSM 11285) as a non oleaginous yeast was taken as the negative control for yeasts which are not able to produce SCO.

Characterized soil isolates used in this study were deposited at the DSMZ culture collection. CPOH4 *Cryptococcus podzolicus* as DSM 27192, SSOH12 *Pichia segobiensis* as DSM 27193 and TPST6 *Trichosporon porosum* as DSM 27194. CSOH1 *Candida shehatae* has not yet been deposited at any culture collection.

3.2 Soil sample collection

Two samples were taken in summer time (2011) from peat bog soil in Kaltenbronn near Bad Wildbad in the black forest of Germany (sample 1: 48.720°N, 8.471°E, 894.4 m above sea level; sample 2: 48.716°N, 8.456°E, 911.8 m above sea level) and one soil sample was taken in summer time from a grassland in Karlsruhe (Baden-Württemberg, Germany, 48.98989°N, 8.40462°E, 116.7 m above sea level). All samples were taken at a depth of 5 cm. The soil samples were stored at -20 °C.

3.3 Yeast isolation from soil samples

A fraction of the collected soil sample (10 mg) was resuspended in 1 mL sterile demineralized water. An aliquot (100 µL) of the suspension was plated out on YM agar plates (3 g/L yeast extract, 3 g/L malt extract, 5 g/l peptone, 10 g/L glucose, 20 g/L agar, pH 7) containing antibiotics (10 mg/L ampicillin, 20 mg/L tetracycline). Agar plates were incubated at 20 °C until 1 mm diameter colonies became visible. Each colony was picked and looked at under a microscope to determine if it was a yeast. For long-term storage the isolated strains were stored in glycerol stocks (15% w/w) at -80 °C.

3.4 Screening for oleaginous microorganisms with Sudan black B staining

Isolated yeasts were cultivated on YM agar plates for 4 days at 20 °C. Replica plates were prepared by transferring the colonies from the original agar plate to a round filter paper (size of agar plate, GE Healthcare Europe GmbH, Freiburg, Germany, Whatman; Ref No 10311610). The filter paper was dried for 15 min at 60 °C and subsequently stained for 20 min with 0.08 % Sudan black B in 96 %-Ethanol (EtOH) solution under shaking. Afterwards the filter was washed twice for 5 min with 96 % EtOH under shaking. Colonies which were stained blue could be potential oleaginous MOs with intracellular TAGs (Evans et al. 1985).

3.5 Identification of the isolates

Genomic DNA was isolated using the commercial kit "Precellys Bacterial/ Fungal DNA-Kit" (PEQLAB Biotechnologie GmbH, Erlangen, Germany; 12-7511-00). Afterwards Polymerase-chain-reaction (PCR) fragments were produced applying universal primers ITS1 (5'-TCCGTAGGTGAACCTGCG-3') (Eurofins MWG GmbH, Ebersberg, Germany) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Eurofins MWG GmbH, Ebersberg, Germany) (fig. II.1) (Fujita et al. 2001). Polymerase chain reaction (PCR) amplification was performed in a total volume of 50 µL. The composition of each PCR reaction was as followed: 5 µL PCR buffer (Dream Taq Green buffer, Thermo Scientific Fermentas, Schwerte, Germany; ♯B71), 5 µL of dNTP mixture (2 mM each) (Thermo Scientific Fermentas, Schwerte, Germany; #R0241),

1 µL ITS1 primer (10 µM), 1 µL ITS4 primer (10 µM) and 0.5 µL Dream Taq DNA polymerase (Thermo Scientific Fermentas, Schwerte, Germany; ♯EP0701) were filled up with PCR water (Carl ROTH GmbH, Karlsruhe, Germany; T143.4). The PCR amplification started with 95 °C for initial denaturation, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 1 min. The final extension was done at 72 °C for 10 min. PCR products were visualized on 1 % agarose gel (1x TAE-buffer: 40 mM Tris base, 1 mM EDTA, pH 8 adjusted with acetic acid; 0.1 µg/mL ethidium bromide) after carrying out gel electrophoresis of each PCR amplification product and 6 µL Quick Load 1 kb DNA Ladder (New England Biolabs, Frankfurt/Main, Germany; N0468 S) with 1x TAE buffer at 100 V for 1 h. Distilled water was used as negative control. The amplified DNA (including the 5.8 S rDNA) was sequenced by GATC Biotech Corporation (Konstanz, Germany). Alignments were performed via MEGABLAST with NCBI database (http://www.ncbi.nlm.nih.gov/).

Fig. II.1 Illustration of the fungal ribosomal genes containing the primer target areas used in this study (Fujita et al. 2001)

3.6 Cultivation in shake flasks

YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/l peptone, pH 7) was supplemented with glucose to an initial concentration of 50 g/L glucose. 50 mL initial culture volume filled in 500 mL conical shake flasks with an initial optical density (OD_{600}) of 0.5 were incubated at 130 rpm at 25 °C for 120 hours. 35 g/L glucose was daily added to ensure that the carbon source was in excess.

3.7 Cultivation in bioreactors

For the cultivation in the bioreactor a mineral salt medium was used, formulated on a phosphate buffer at pH 5 (8.99 g/L KH_2PO_4 and 0.12 g/L $Na_2HPO_4 * 2H_2O$) which was based on the medium used in Meesters et al. (1996). The medium constituents were 0.1 g/L sodium citrate * $2H_2O$, 0.1 g/L yeast extract, 0.2 g/L $MgSO_4 * 7H_2O$, 4.72 g/L $(NH_4)_2SO_4$ (refers to 1 g/L N). Once a day the culture broth was supplemented with 2 mL trace elements solution (4 g/L $CaCl_2 * 2H_2O$, 0.55 g/L $FeSO_4 * 7H_2O$, 0.475 g/L citric acid, 0.1 g/L $ZnSO_4 * 7H_2O$, 0.076 g/L $MnSO_4 * H_2O$, 100 µL 18 M H_2SO_4) and 2 mL salt solution (20 g/L $MgSO_4 * 7H_2O$,

10 g/L yeast extract) per 100 mL cultivation medium. Precultures were prepared in conical shake flasks with initial OD_{600} of 0.5 and 120 rpm. Fermentation was performed in a 2.5 L fermenter (Infors HT, Bottmingen, Switzerland; Minifors fermenter) with 1 L initial culture medium, initial OD_{600} of 1, at 600 rpm and with 1 vvm aeration rate without control of the pO_2 for at least 188 h. CPOH4 was grown at 20 °C, SSOH12 and TPST6 at 25 °C. The control of pH was done automatically by addition of 4 M H_3PO_4 and 4 M NaOH in each fermenter, Contraspum A 4050 HAC (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) was applied as antifoam agent. Initial glucose or xylose concentration was 50 g/L. Each day the carbon source was replenished to a maximum concentration of 90 g/L after measuring the actual concentration. A minimum of five samples were taken per day (four samples of 3 mL for the determination of OD_{600}, dry biomass (g/L), carbon and nitrogen source and by-products; one sample of 20 mL for lipid analysis (% lipid/dry biomass) via gas chromatography). The exhaust gas O_2 and CO_2 were measured via exhaust gas analyzer (BlueSens).The partial pressure of oxygen (pO_2) was measured by pO_2-elektrode (Hamilton).

3.8 Sample preparation for dry biomass and analysis of supernatant

Dry biomass was analyzed gravimetrically. A 1 mL aliquot of the culture fluid was transferred into a pre-dried and pre-weighed 1.5 mL reaction tube and centrifuged at 13,000 rpm for 5 min. The supernatant was collected and used for the determination of glucose and NH_4^+. The cell pellet was washed with 800 µL saline (0.9 % NaCl), dried at 60 °C for 24 h and weighed.

3.9 Analysis of NH_4^+, glucose, xylose, ethanol and gluconic acid

All components were measured in triplicates with enzymatic test kits. D-Glucose, ethanol and gluconic acid were purchased at R-biopharm (Darmstadt, Germany). NH_4^+ was measured via Spectroquant kit (Merck KGaA, Darmstadt, Germany). D-Xylose assay kit was taken for the concentration of xylose (Megazyme, Bray, Ireland; K-XYLOSE).

3.10 HPLC analysis of organic acids

The pure supernatant was taken to measure five different organic acids (gluconic, malic, citric, succinic and fumaric acid) using a standard high pressure liquid chromatography (HPLC) device (Agilent 1100 Series, Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with a 150 x 4.6 mm HPLC column Synergi™ 4 µm Fusion-RP 80 Å (Phenomenex, Aschaffenburg, Germany; 00F-4424-E0) at 30 °C column temperature. 20 mM KH_2PO_4 pH 2.5 (A) and 100 % Methanol (B) were used as eluents to drive the following temporal gradients. 0-0.5 min 100 % eluent A, 0.5-10 min increase of eluent B from 0 % to 10 %, 10-12 min decrease of eluent B from 10 % back to 0 % and 12-14 min again

100 % eluent A. 10 µL sample was injected, a flow rate of 1 mL/min was adjusted and peaks were detected via UV at 220 nm.

3.11 Lipid analysis

A 20 mL aliquot of the culture broth was centrifuged (4,700 rpm 5 min), the pellet was resuspended in saline (0.9 % NaCl) and again centrifuged (4,700 rpm, 5 min). The supernatant was discarded and the pellet was freeze dried (-30 °C, 0,370 mbar). Preparation for the quantitative and qualitative gas chromatographically analysis was done in a one-step-procedure by direct esterification plus extraction. A portion (20 mg) of freeze dried biomass was weighed into a 15 mL glass falcon with Teflon cap. 1.5 mL hexane and 0.5 mL of 2 mg/mL internal standard (methyl benzoate) dissolved in hexane were added as solvent for the extraction of lipid. In addition, 2 mL 15 % H_2SO_4 in methanol was added for the esterification step. Each sample was heated up to 100 °C for 2 h with continuous shaking. After cooling on ice, 1 mL demineralized water was added. The mixture was centrifuged for 5 min at 2,500 rpm. 1 µL of the upper phase, containing the fatty acid methyl esters extract, was analyzed via chromatography (Agilent Technologies, 6890 N Network GC-System). The instrument was equipped with a DB-Wax column (l: 30m d: 0.25 mm, Agilent Technologies Deutschland GmbH, Böblingen, Germany; 122-7032), a flame ionization detector and working with a pressure of 1.083 bar and initial temperature of 40 °C. The column temperature was increased from 40 °C to 250 °C with a rate of 8 °C/min. The temperature was held at 250 °C for 10 min before cooling down to 40 °C. The total fatty acid content and the identification of fatty acids were performed using the standard RM3 FAME Mix (Sigma Aldrich, Taufkirchen, Germany; 07256-1AMP) and Marine FAME Mix (Acid Methyl Ester Marine Oil FAME Mix) (Restek GmbH, Bad Homburg, Germany; 35066). Fatty acids which represented less than 1 % of total fatty acids were combined to "trace fatty acids".

3.12 Accession numbers

The following EMBL accession numbers have been assigned for the fungal ribosomal genes of the isolates: CPOH4 *Cryptococcus podzolicus* as HG737350, SSOH12 *Pichia segobiensis* as HG737349 and TPST6 *Trichosporon porosum* as HG737348.

4 Results

4.1 Screening and identification of yeast isolates

Four different yeast strains in total could be isolated from three soil samples using agar plates with YM medium supplemented with antibiotic and glucose as carbon source.

Glucose was taken as a standard carbon source as it can be converted by almost every microorganism and is therefore suitable for screening experiments. Three isolates (CPOH4, CSOH1 and SSOH12) arose from peat bog samples and one isolate (TPST6) from grass land soil sample. Peat bog soil is assumed to contain a high level of carbon compared to nitrogen. All four isolates were stainable by lysochrome Sudan black B (Evans et al. 1985), and were therefore regarded as potential lipid producer (fig. II.2).

The sequences of ITS region were compared with the nucleotide data base using the NCBI-blast tool. SSOH12 showed highest genetic agreement with *Pichia segobiensis* closely followed by *Scheffersomyces stipitis* which is a synonym of *Pichia stipitis*. CPOH4 was identified as *Cryptococcus podzolicus*. TPST6 was identified as *Trichosporon porosum* and CSOH1 showed highest agreement to *Candida shehatae*. The results are presented in table II.1.

Fig. II.2 Sudan black B staining of the four isolates CSOH1, SSOH12, CPOH4 and TPST6

Table II.1 Results of the sequencing of ITS region including 5.8 S rRNA of isolates SSOH12, CPOH4 and TPST6

Isolate	Deposited at DSMZ as (EMBL accession number)	Total score	Query coverage [%]	E-value	Max identity	Closest relative in NCBI data bank (Accession number)
CSOH1	-	1044	100	0.0	100	*Candida shehatae* (JQ026374.1)
SSOH12	DSM 27193 (HG737349)	1062	99	0.0	99	*Pichia segobiensis* (DQ409166.1)
		1059	99	0.0	99	*Scheffersomyces stipitis* (JQ026363.1)
CPOH4	DSM 27192 (HG737350)	852	100	0.0	100	*Cryptococcus podzolicus* (HF558652.1)
TPST6	DSM 27194 (HG737348)	878	100	0.0	100	*Trichosporon porosum* (HF558656.1)

DSMZ: German Collection of Microorganisms and Cell Cultures, EMBL: The European Molecular Biology Laboratory, NCBI: National Center for Biotechnology Information

4.2 Shake flask cultivation in YM medium

All four isolates were cultured in 500 mL shake flasks containing 50 mL YM medium supplemented with 50 g/L glucose. The flasks were incubated at 25 °C for 120 hours.

Cryptococcus curvatus - a well examined oleaginous yeast - was taken as positive control and *Saccharomyces cerevisiae* as negative control. Glucose was taken as standard carbon source and was held in excess throughout the fermentation period. The pH value did not decrease below 4 in all cultivations. The results are presented in fig. II.3. As expected, the oleaginous yeast *C. curvatus* yielded more than 40 % lipid per dry biomass whereas *S. cerevisiae*, a typical non-oleaginous microorganism, yielded less than 10 % lipid per dry biomass corresponding to the normal cellular amount of lipids.

The best lipid producer among the four new isolates was CPOH4 with 34.6 % lipid per dry biomass and can therefore be classified as oleaginous under the chosen conditions in YM medium and shake flasks. TPST6 yielded 24.5 % lipid per dry biomass, hence this yeast also belongs to the oleaginous microorganisms. However, CSOH1 yielded 8.5 % and SSOH12 yielded 11.2 % lipid per dry biomass; hence the latter two yeasts cannot be classified as lipid producing strains under the chosen conditions in shake flasks.
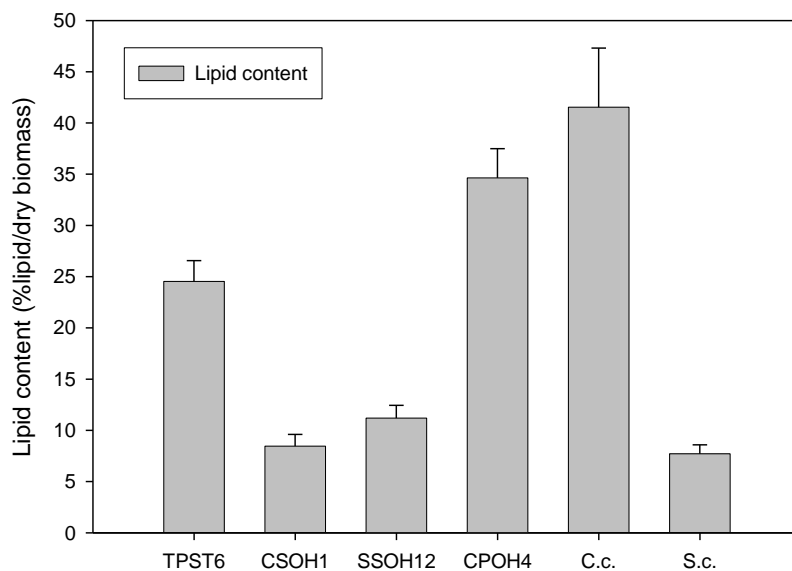
Fig. II.3 Lipid yield in shake flask cultivation at 25 °C in YM medium after 120 h of all four isolates TPST6, CSOH1, SSOH12 and CPOH4 as well as oleaginous yeast *Cryptococcus curvatus* (*C.c.*) and non oleaginous yeast *Saccharomyces cerevisiae* (*S.c.*)

4.3 Cultivation of the isolated yeasts in 2.5 L-bioreactor on glucose or xylose

All four isolates CSOH1, SSOH12, CPOH4 and TPST6 were cultivated in bioreactors at pH 5 and 600 rpm during the whole cultivation. Hence, compared to shake flask cultivation a constant pH and higher aeration rates were ensured. CPOH4 was cultivated at 20 °C as preliminary studies (data not shown) revealed best growth at this temperature. CSOH1, SSOH12 and TPST6 were cultivated at 25 °C as this was the suggested cultivation

temperature for *Trichosporon porosum* (Middelhoven et al. 2001) and described growth conditions for *Scheffersomyces segobiensis* and *Candida shehatae* according to ATCC data bank. The carbon source, glucose or xylose, was held in excess throughout the fermentation, but less than 90 g/L to prevent substrate inhibition. All strains were cultivated on glucose as the carbon source, but CPOH4 and TPST6 were additionally grown on xylose. Table II.2 summarizes the main results of the different fermentations; growth yield coefficient ($y_{x/s}$), product yield coefficient ($y_{p/s}$), volumetric productivity (Q_L), lipid content per dry biomass (% lipid/dry biomass), lipid concentration (g/L) and detected by-products are presented. The data for CPOH4 on glucose and xylose and the data for CSOH1 on glucose in table II.2 represent the mean from two independent fermentations. The data for TPST6 on glucose and xylose as well as the data for SSOH12 on glucose are single fermentations. To illustrate the lipid production processes in more detail the fermentations of all four isolates are exemplarily shown for one single fermentation in fig. II.4 till fig. II.9. The first diagram (a) of each strain presents the lipid production and by-product production compared to nitrogen consumption and dry biomass production. The second diagram (b) focuses on the consumption of the carbon source and the development of the pO_2 compared to dry biomass and by-product development. Due to daily feeding of the carbon source in the case of CSOH1, CPOH4 and SSOH12 the values for the dry biomass are shown before and after feeding. Concerning TPST6 feeding of the carbon source was repeated two times after 29 and 48 h. A third feeding was conducted after 94 h only for the glucose cultivation.

For all isolates the lipid production started as soon as the nitrogen source NH_4^+ was totally consumed (approximately after 50 hours). In addition, the level of pO_2 reached minimum values of 0 % during the maximal growth phase, but increased again as soon as the nitrogen source NH_4^+ was exhausted. At this time point growth rate stagnated and lipid production started.

The three yeast isolates SSOH12, CPOH4 and TPST6 exceeded a lipid content of more than 20 % lipid per dry biomass (table II.2). CSOH1 yielded just less than 18 % lipid content per dry biomass (table II.2). The highest lipid content was reached with yeast isolate TPST6. Using glucose as carbon source this strain produced up to 33.4 % lipid per dry biomass, followed by CPOH4 (31.8 %) and SSOH12 (24.6 %). When cultured on xylose CPOH4 gave 26.8 % lipid per dry biomass, whereas TPST6 yielded even 33.4 %. Under the chosen conditions of the fermentations the isolates SSOH12, SPOH4 and TPST6 may be classified as oleaginous yeasts. However, CSOH1 with 17.8 % lipid content cannot be classified as oleaginous.

The determination of by-products was carried out as there were some indications for ethanol and acid production. In the culture broth of CSOH1 and SSOH12 ethanol was detected

whereas ethanol production by CPOH4 and TPST6 was negligible (table II.2). Instead, CPOH4 and TPST6 showed evidence of acid production when cultured on glucose. This acid was identified as gluconic acid by HPLC analysis and was confirmed with an enzymatic test. Malic, citric, succinic and fumaric acids were not detected. These acids were neither detected for CSOH1 and SSOH12 cultured on glucose nor for CPOH4 and TPST6 cultured on xylose.

When cultured on glucose, CPOH4 yielded the highest lipid concentration of 18.0 g/L and the highest concentration of gluconic acid of 30 g/L, followed by TPST6 with 17.0 g/L lipid concentration and 12 g/L gluconic acid. CSOH1 yielded only 5.6 g/L lipid concentration, but instead reached the ethanol concentrations above 5.8 g/L. SSOH12 resulted only in 12.7 g/L lipid and a minimum of 3.3 g/L ethanol as by-product. Considering the lipid productivity (Q_L), TPST6 grown on glucose may be the best lipid producer (0.10 g/L*h) followed by TPST6 on xylose and CPOH4 on glucose with the same value of 0.09 g/L*h.

Considering the ethanol production in CSOH1 and SSOH12 it is visible that CSOH1 starts to produce ethanol directly from the beginning. At this time the pO_2 is sufficient for aerobic growth which points out that CSOH1 is crabtree positive. In contrast, SSOH12 starts ethanol production under oxygen limitation, hence is a facultative anaerobic strain. As soon as the pO_2 level increased again and sufficient oxygen is available, the ethanol was degraded and decreased to negligible amounts.

Table II.2 Overview about performed fermentations of isolates SSOH12, CPOH4 and TPST6; SSOH12 and TPST6 represent single fermentations, whereas CPOH4 are mean of two independent fermentations

	CSOH1	SSOH12	CPOH4		TPST6	
C-source	Glucose	Glucose	Glucose	Xylose	Glucose	Xylose
Process time [h]	188	188	188		161	
T [°C]	25	25	20		25	
Dry biomass$_{max}$ [g/L]	31.9±0.9	51.7	56.5±5.2	41.5±1.24	49.9	41.5
$Y_{x/s}$ [g/g]	0.174±0.03	0.35	0.50±0.0	0.53±0.02	0.38	0.41
$Y_{p/s}$ [g/g]	0.024±0.003	0.06	0.11±0.01	0.09±0.0	0.11	0.12
Q_L [g/L*h]	0.029±0.002	0.07	0.09±0.04	0.07±0.01	0.1	0.09
Lipid content [%lipid/dry biomass]	17.8±0.07	24.6	31.8±8.0	26.8±1.2	34.1	33.4
Concentration of lipid [g/L]	5.6	12.7	18	11.1	17	13.9
Gluconic acid [g/L]	n.d.	n.d.	30	n.d.	12	n.d.
EtOH [g/L]	> 5.8	>3.3	< 1.0	<1.0	< 1.0	<1.0

Fig. II.4a CPOH4 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 20 °C; glucose was fed daily
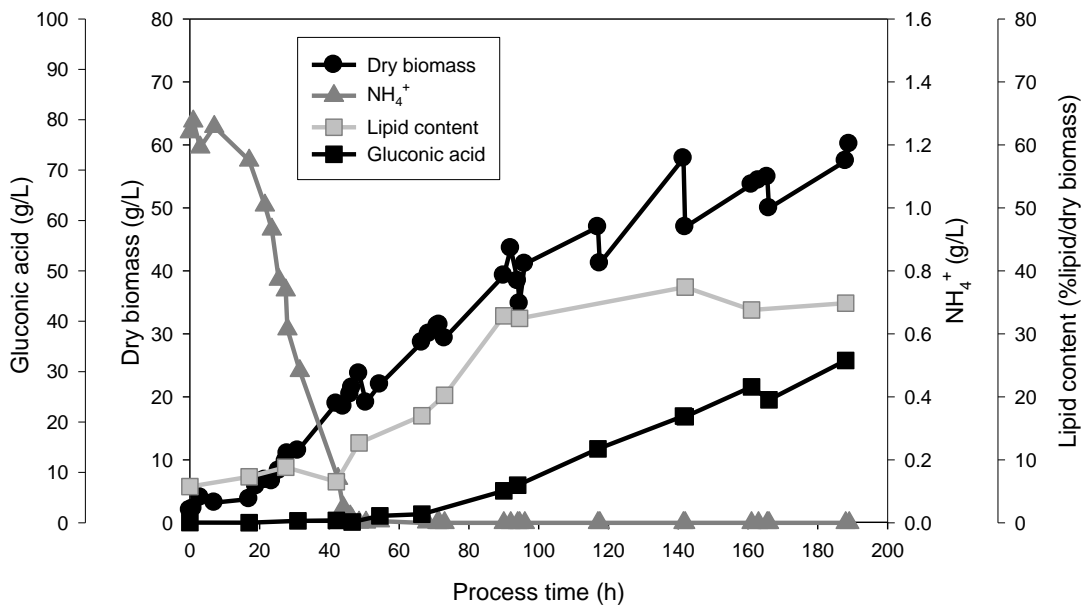

Fig. II.4b CPOH4 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 20 °C; glucose was fed daily
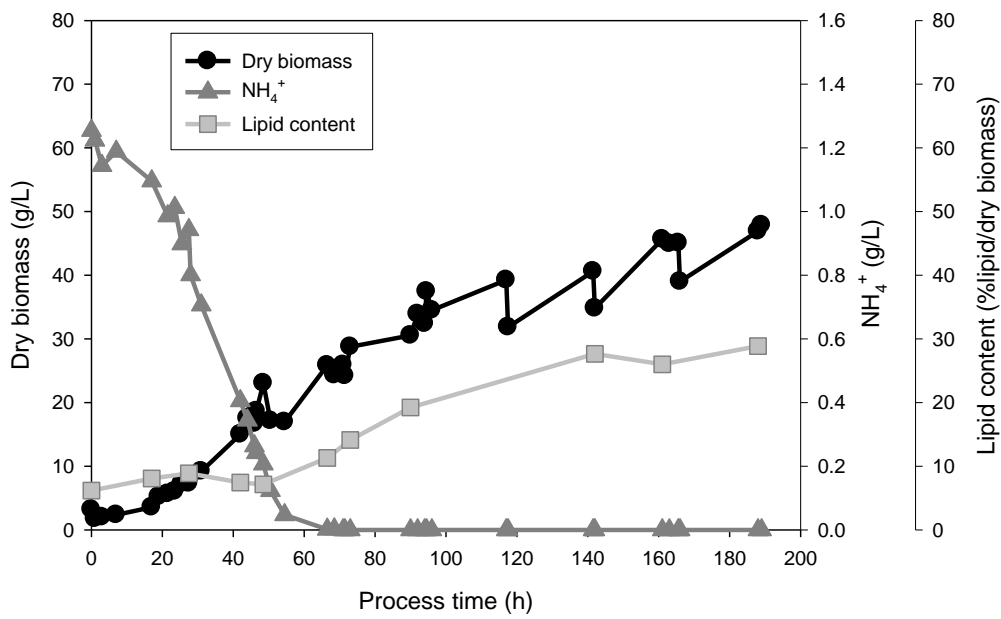
Fig. II.5a CPOH4 cultivated on xylose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 20 °C; xylose was fed daily
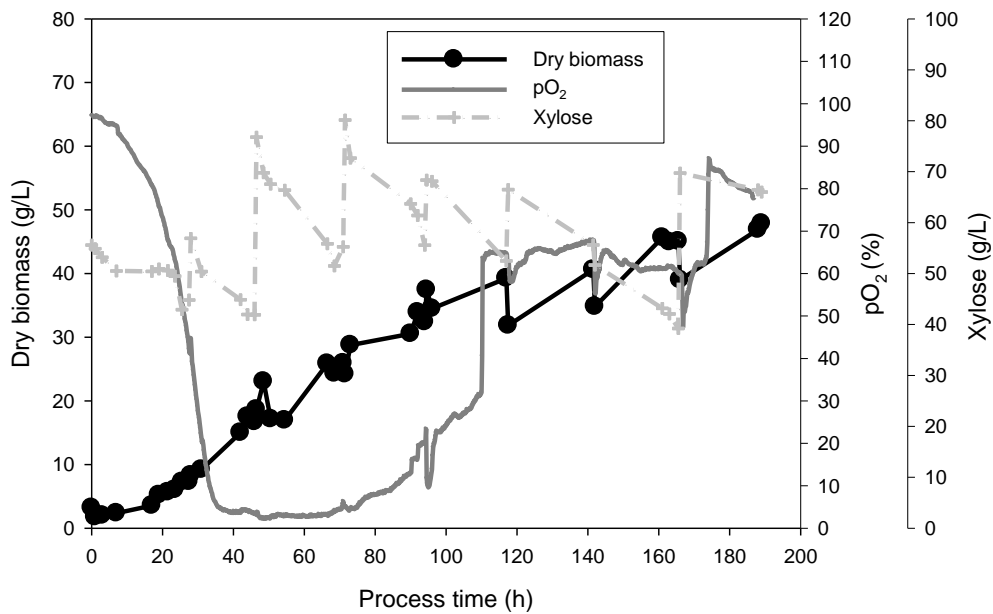
Fig. II.5b CPOH4 cultivated on xylose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 20 °C; xylose was fed daily
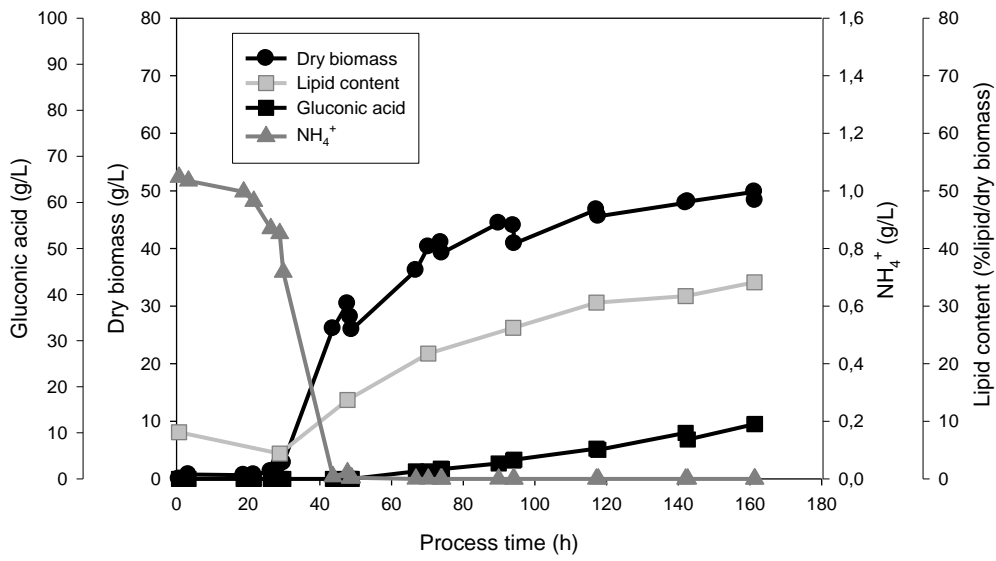
Fig. II.6a TPST6 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 25 °C; glucose was fed daily
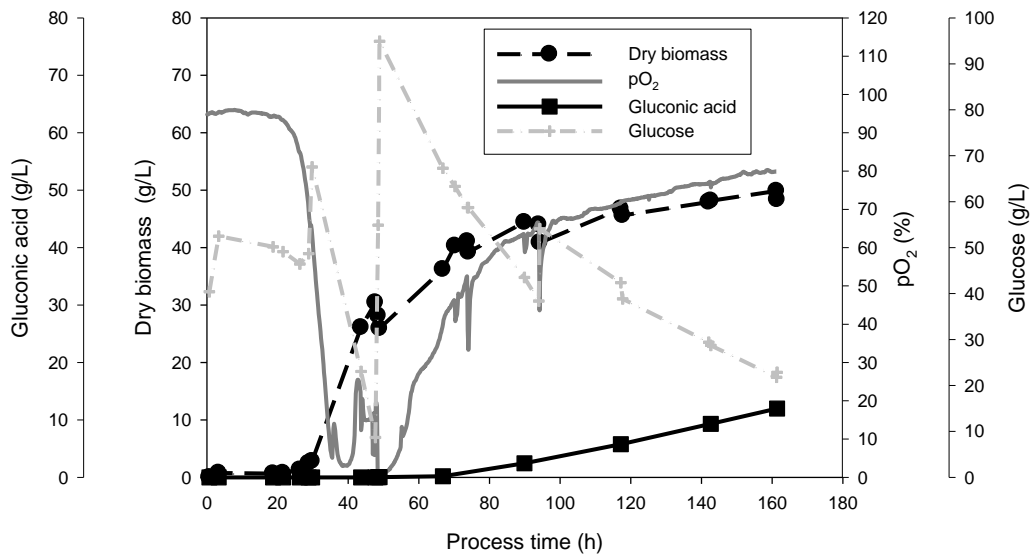
Fig. II.6b TPST6 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5.0 and 25 °C; glucose was fed daily
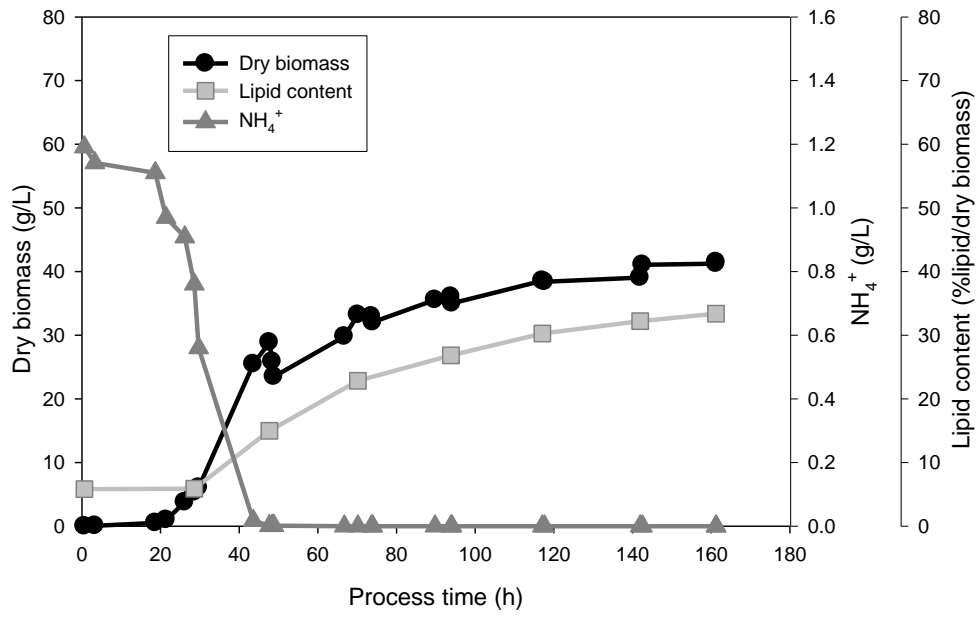
Fig. II.7a TPST6 cultivated on xylose in 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; xylose was fed daily
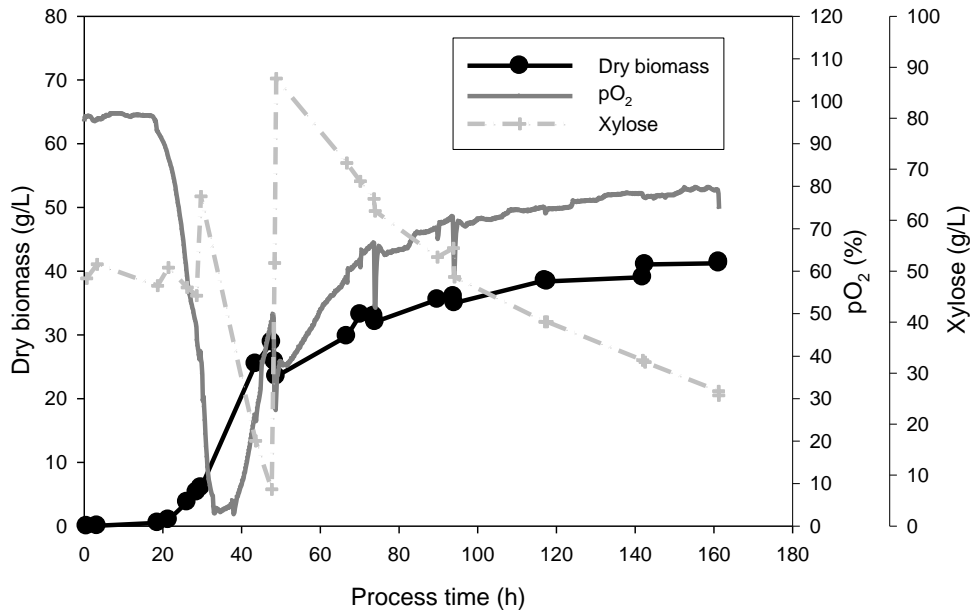
Fig. II.7b TPST6 cultivated on xylose in 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; xylose was fed daily

Fig. II.8a SSOH12 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; glucose was fed daily
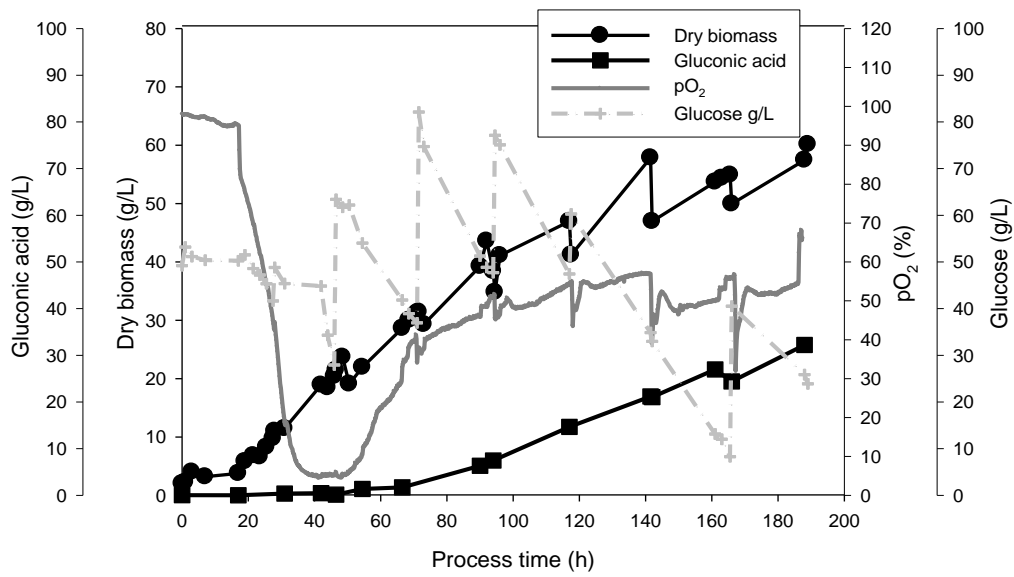

Fig. II.8b SSOH12 cultivated on glucose in 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; glucose was fed daily

Fig. II.9a CSOH1 cultivated on glucose on 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; glucose was fed daily

Fig. II.9b CSOH1 cultivated on glucose on 2.5 L-bioreactor in mineral salt medium at pH 5 and 25 °C; glucose was fed daily
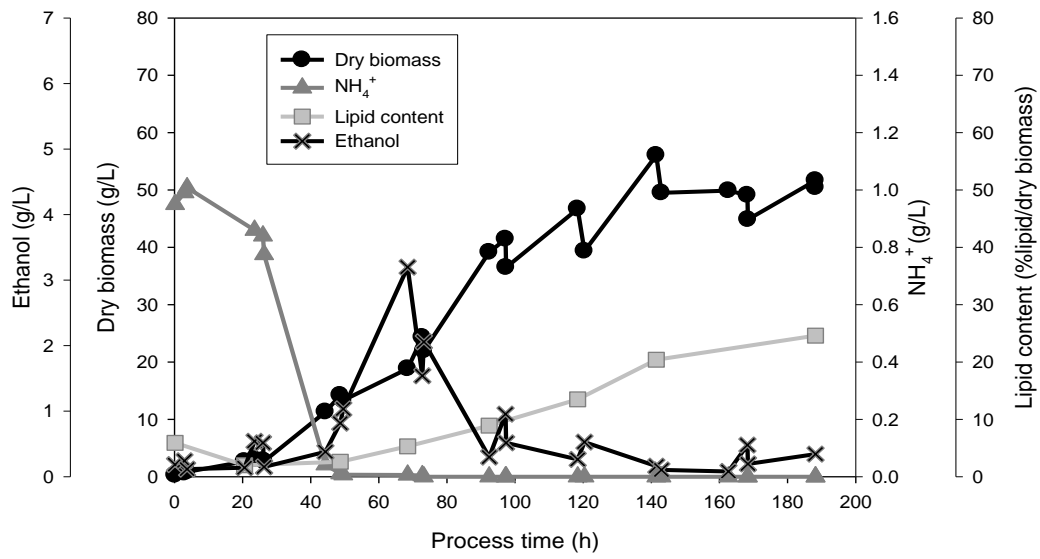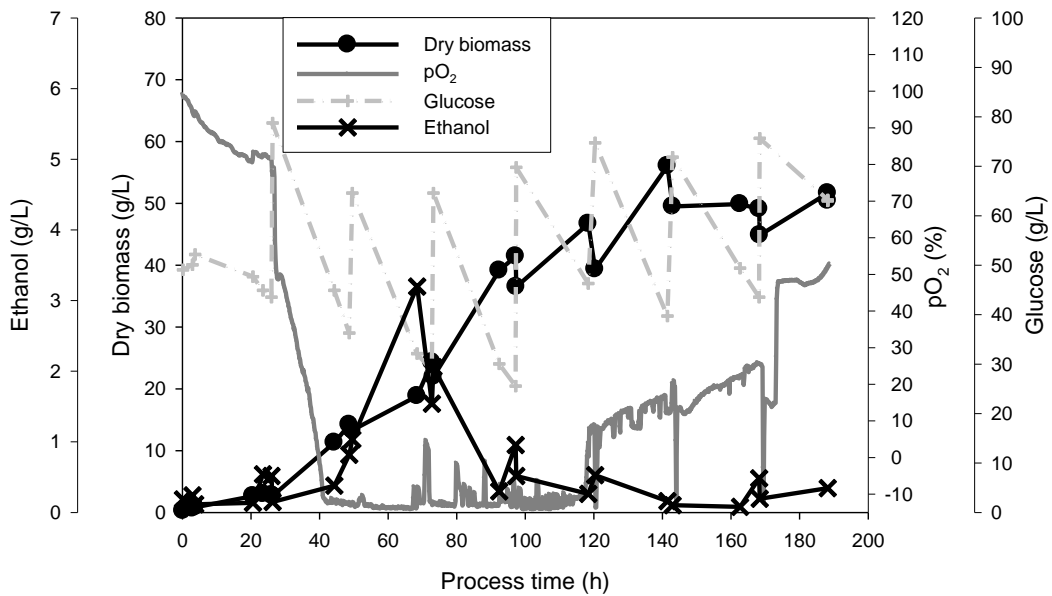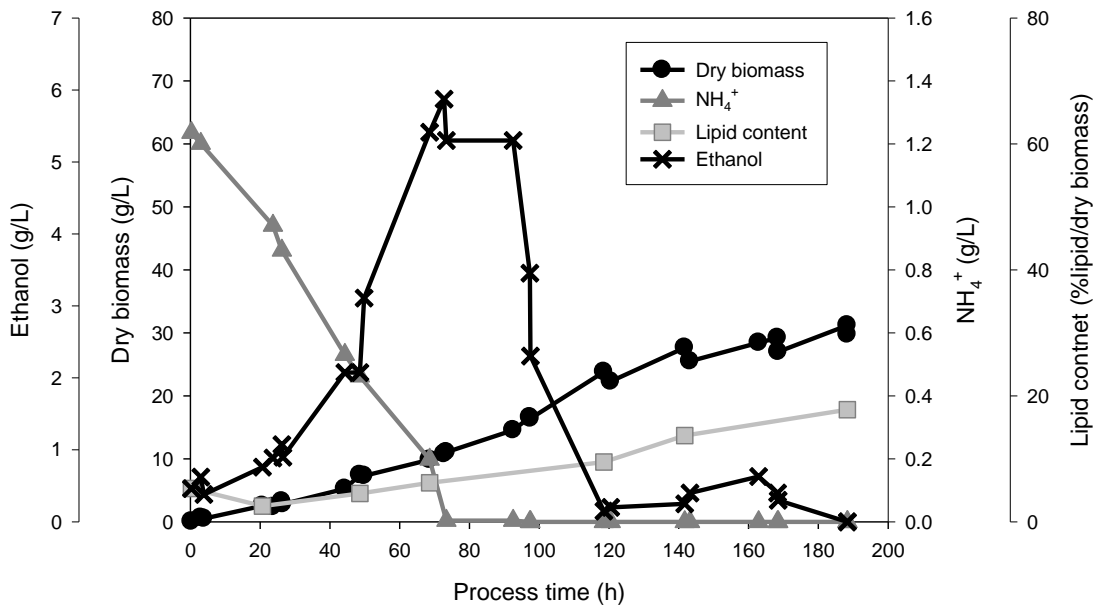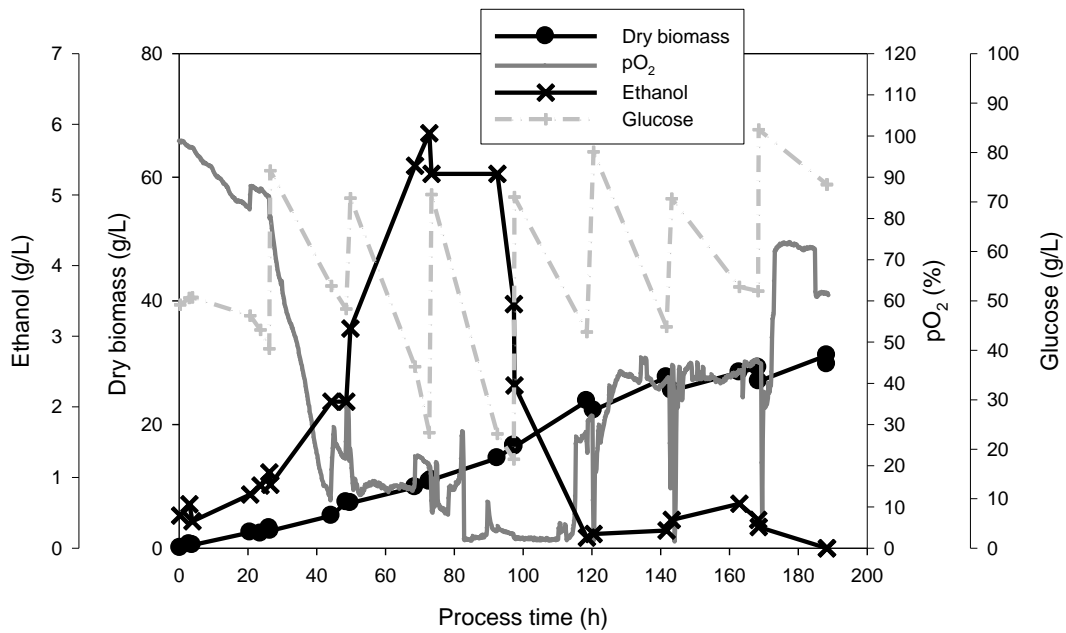
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4.4 Fatty acid profiles

The analysis of the fatty acid profile (table II.3) revealed different profiles for each isolate. The main fatty acid in all isolates was oleic acid (C18:1) with contents ranging between 39.6 % and 63 %. Relatively high yields of palmitic acid (C16:0) ranging from 18.4 % to 21.1 % were obtained for the three best lipid producing isolates SSOH12, CPOH4 and TPST6. In addition to oleic and palmitic acid, palmitoleic acid (C16:1) with 16 % was one of the main products in SSOH12 and at least 10.4 % in CSOH1 fermentation whereas CPOH4 and TPST6 produced only negligible amounts of this fatty acid. In contrast, TPST6 was characterized by high amounts of stearic acid (C18:0) and linoleic acid (C18:2) ranging between 15.5 % and 18.7 %. CPOH4 produced between 4.7 % and 5.3 % of stearic acid (C18:0) and just 8.7 % to 11.1 % of linoleic (C18:2) acid. The amount of linolenic acid (C18:3) was less than 1.4 % and is therefore negligible in all isolates.

No remarkable difference in most of the fatty acid profiles were noticed comparing glucose and xylose as the carbon source. However, for both isolates CPOH4 and TPST6 a slightly higher amount of oleic acid (C18:1) and slightly lower amount of linoleic acid (C18:2) were observed when cultured on glucose.

Table II.3 Fatty acid profile of the four isolates CSOH1, SSOH12, CPOH4 and TPST6 with respect to chosen carbon source employed in growth medium; fatty acids detected with percentages less than 1 % are combined to "others"; grey marked fatty acids indicate saturated fatty acids

Isolate	C source	Type of fatty acid (% of total fatty acids)						
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	others
CSOH1	Glucose	9.8	10.4	2.0	63.0	11.4	1.4	2.0
SSOH12	Glucose	19.1	16.0	2.0	51.8	7.5	0.5	3.1
CPOH4	Glucose	18.4	0.3	5.3	59.4	8.7	0.9	7.0
	Xylose	20.1	0.4	4.7	55.1	11.1	1.1	7.5
TPST6	Glucose	19.5	0.3	17.0	40.4	17.8	1.3	3.7
	Xylose	21.1	0.3	15.5	39.6	18.7	1.0	3.8

5 Discussion

Four yeast strains were isolated from soil samples taken from peat bog and grassland. Peat bog soil as screening source was chosen due to its high amount of carbon arising from decomposed wood and plants accompanied by low nitrogen content. The use of a grassland sample was chosen as a comparison sample without any specific limitations. High carbon content and low nitrogen content as it is found in peat bog samples are important for the production of intracellular lipid in oleaginous strains (Ratledge 2002). The lysochrome Sudan black B stains triacylglycerols (TAG), even TAG which are found in oleaginous

microorganisms. Hence, positive staining with Sudan black B of certain microorganisms may indicate possible intracellular lipid accumulation; therefore, Sudan black B is suitable to screen for oleaginous microorganisms.

In this study four yeast isolates, SSOH12, CPOH4, TPST6 and CSOH1, were tested via Sudan black B staining technique on agar plates, but only three of them, SSOH12, CPOH4 and TPST6, could be finally classified as oleaginous via cultivation in bioreactors and subsequent quantitative lipid analysis with gas chromatography.

The first strain, SSOH12, was identified as *Pichia segobiensis* and produced a considerable amount of 16 % palmitoleic acid (C16:1) of total lipid amount. The second strain CPOH4 was identified as *Cryptococcus podzolicus* and yielded the highest lipid concentration with 18.0 g/L of all isolates. The third strain TPST6 was identified as *Trichosporon porosum* and produced a considerable amount of 17.8 % linoleic acid (C18:2) on glucose and 18.7 % on xylose. All three strains have not been described as oleaginous before. Due to sufficient production of lipid amounts and interesting fatty acid profiles, further studies of all three strains are worthwhile to establish sustainable bioprocesses for the production of adequate amounts of microbial oil for industrial applications.

The fourth strain CSOH1 was identified as *Candida shehatae* which is in particular known for the production of ethanol (Sanchez et al. 2002). This yeast could not be classified as oleaginous yeast under the performed conditions, but an increase of the pO_2 might reduce the ethanol production in favor to increase lipid production.

5.1 Influence of cultivation conditions on lipid production for screening experiments

Different lipid contents per dry biomass were reached depending on cultivation condition, meaning shake flask cultivation with YM medium or a bioreactor cultivation in mineral salt medium with defined pH value and improved supply of oxygen. *C. podzolicus* CPOH4 and *T. porosum* TPST6 were able to produce high amounts of lipid between 24.5 % and 34.1 % lipid per dry biomass in both cultivation methods. In contrast, *P. segobiensis* SSOH12 with just 11.2 % lipid per dry biomass and *C. shehatae* CSOH1 with 8.5 % could not be classified as oleaginous yeast when cultured in shake flasks with the chosen medium. Nevertheless, when cultured in a 2.5 L-bioreactor with defined conditions 24.6 % lipid per dry biomass was realized with *P. segobiensis* SSOH12, which corresponds to twice the amount obtained with shake flask cultivation. The same applies to *C. shehatae* CSOH1. Low aeration rates in shake flasks may be the reason for low lipid level as increasing cell densities lead to less available oxygen. Moreover, a nitrogen limiting mineral salt medium with adequate buffering capacities would be more appropriate for shake flask cultures. Under low aeration rates facultative anaerobic strains start to produce ethanol. The reason, why the oxygen limitation

occurred during the maximal growth in the cultivations with bioreactors, could be explained because of the agitation speed was too low for the present cell density.

In the present study *P. segobiensis* SSOH12 produced ethanol as by-product as soon as the level of pO$_2$ reached 0 % (fig. II.8b). Hence, this strain may be classified as facultative anaerobic. Concerning the isolate *C. shehatae* CSOH1 ethanol was produced directly from the beginning which is an evidence for the crabtree effect. In this case, the increase of the aeration rate and thereby the increase of the pO$_2$ together with a fed-batch cultivation with low initial glucose level might reduce the ethanol production and favor lipid formation. By this way *C. shehatae* CSOH1 may get also oleaginous characteristics. This shows the importance of adequate aeration even for screening experiments. To prevent the production of ethanol and to increase the production of lipid by a higher pO$_2$ value the agitation speed has to be increased. Strains that gave a positive test result with Sudan black B staining on agar plates, but a negative result in shake flasks with YM medium supplemented with glucose, are worthwhile to examine in further detail in a well aerated bioreactor as not only the nitrogen limitation, but also sufficient oxygen supply may be a prerequisite for lipid production.

In addition to the aeration rate, the pH of the medium is also an important parameter to consider during the screening of lipid producing strains as the pH value cannot be controlled in shake flasks. Most strains are acid labile and are not growing well in acidic environment like *Candida shehatae* (Kastner et al. 1996), and therefore will not produce any favored product like lipid. The shake flask cultivation of *Cryptococcus curvatus* and *Cryptococcus podzolicus* CPOH4 in mineral salt medium (data not shown) resulted in a decrease of the pH value from 5.0 to 2.0 within 60 hours. The well known oleaginous yeast *C. curvatus* produced a maximum of 15 % lipid per dry biomass under these conditions whereas *C. podzolicus* CPOH4 ended up with 23 % lipid within 100 h cultivation time (data not shown). The advantage of *C. podzolicus* CPOH4 is its acid resistance, which is beneficial in podsol soil, an acidic environment, from which *C. podzolicus* CPOH4 once had been isolated and from which arose its name (Botes et al. 2005). When cultivating *C. curvatus* in YM medium the pH decreased slightly from 5 to 4 and yielded up to more than 40 % lipid per dry biomass. This shows that the YM medium supplemented with glucose has good buffering capacities and is a useful medium for a first examination of yeast strains.

5.2 Characterization of newly isolated yeast strains

SSOH12 was identified as *Pichia segobiensis*. This strain belongs to the ascomycetes. The second highest agreement of SSOH12 was found with *Scheffersomyces stipitis,* also known as *Pichia stipitis*. *Pichia stipitis* (Nigam 2001; Sanchez et al. 2002) and *Scheffersomyces*

stipitis (Liu et al. 2012) are well described within the context of microbial ethanol production, but have not been mentioned previously with regard to lipid production. Furthermore they are able to assimilate hemicellulosic compounds (Ferreira et al. 2011, Nigam 2001, Sanchez et al. 2002).

Aside from the focus on the production of ethanol under anaerobic conditions, this study has shown that *Pichia segobiensis* SSOH12 is able to produce more than 24 % lipid content under aerobic conditions and sufficient aeration. As the ethanol production of *P. segobiensis* SSOH12 commences only after oxygen limitation occurs, the crabtree effect like in *Saccharomyces cerevisiae* (Al-mhanna 2010) is excluded. This could be confirmed for the closely related yeast, *Scheffersomyces stipitis* (Papini et al. 2012). Under aerobic conditions this yeast can be classified as oleaginous yeast and process optimization with higher pO_2 level might increase the lipid yield.

The special feature of *P. segobiensis* SSOH12, concerning the fatty acid profile, is the different composition to most other oleaginous yeasts containing a fatty acid profile similar to cacao-butter with the main components of C16:0, C18:0, C18:1 and C18:2. In addition, *P. segobiensis* SSOH12 produces 16 % palmitoleic acid (C16:1), an omega-7 mono-unsaturated fatty acid which has been shown to have positive effects against obesity (Yang et al. 2011) and potential for the prevention of brain and cardiovascular diseases (Matsunaga et al. 1995). It is a component of some oil seeds, especially sea-buckthorn (Fatima et al. 2012) or macadamia (Nestel et al. 1994). An alternative source via microbial production is the opportunity to produce palmitoleic acid (C16:1) in sufficient quantities for possible future applications in medicine. *P. segobiensis* SSOH12 has been described as one of the best xylose-converting strains (Liu et al. 2012; Toivola et al. 1984). Hence, the investigation of lipid production with xylose as the carbon source may be a worthwhile exercise.

CSOH1 was identified as *Candida shehatae*. The strain belongs to the ascomycetes. It is well known for the production of ethanol (Sanchez et al. 2002), but has never been mentioned with regard to intracellular lipid production. This study has shown that cultured in shake flasks *Candida shehatae* CSOH1 formed a maximum of 8 % intracellular lipids whereas the cultivation in a bioreactor with pH control and a higher aeration rate than in shake flasks resulted in a lipid content of 17.8 %. Considering that the cultivation was partially oxygen limited it can be assumed that a higher agitator speed leading to a higher level of pO_2 might achieve lipid contents above 20 % which is the threshold for a classification as oleaginous microorganism.

The ethanol production started from the beginning of the cultivation which indicated that *Candida shehatae* CSOH1 is a crabtree positive strain and does not produce ethanol

principally due to anaerobic conditions. The ethanol production, based on the crabtree effect, may be the reason for the low growth rate of CSOH1 compared to SSOH12, which grew twice as fast. One possibility in case of *C. shehatae* CSOH1 would be a controlled fed-batch feeding strategy of glucose to prevent the crabtree effect or to choose xylose as carbon source which is known to be metabolized by *Candida shehatae* (Sanchez et al. 2002), but does not induce the crabtree effect. The ethanol production due to oxygen limited conditions in the case of CSOH1 and SSOH12 could be avoided by increasing the agitator speed and hence to favor the lipid production instead.

The fatty acid profile of *C. shehatae* CSOH1 contains a predominant amount of oleic acid (C18:1) with 63 % whereas the three other important fatty acids C16:0, C16:1 and C18:2 amount only values around 10 %. Oleic acid is the main fatty acid which is present in all plant oils used for biodiesel production (Christophe et al. 2012). Consequently, if the microbial lipid production with isolate *C. shehatae* CSOH1 was optimized to worthwhile amounts, it would be especially qualified as resource for biodiesel.

The two other isolated strains were identified as *Cryptococcus podzolicus* (CPOH4) and *Trichosporon porosum* (TPST6). Both strains belong to the yeast-like anamorphic basidiomycetes and are found in soil (Botes et al. 2005; Colombo et al. 2011). They are also known to assimilate hemicelluloses (Middelhoven et al. 2001; Shubakov 2000).

Trichosporon sp. and *Cryptococcus* sp. in general are known to belong to the oleaginous strains (Gujjari et al. 2011; Zhu et al. 2008; Hu et al. 2011), whereas *Trichosporon porosum* and *Cryptococcus podzolicus* have not been mentioned before in relation to microbial oil production. The results of this study reveal that both strains are able to produce approximately 30 % lipid per dry biomass when grown on glucose or xylose as carbon source. Therefore, *Cryptococcus podzolicus* CPOH4 and *Trichosporon porosum* TPST6 can be characterized for the first time as oleaginous yeasts. *T. porosum* TPST6 produced in this study almost 20 % linoleic acid which makes it unique among other *Trichosporon* species, which generally yield less than 10 % linoleic acid, e.g. *Trichosporon fermentans* less than 8 % linoleic acid (Huang et al. 2012) and *Trichosporon cutaneum* less than 3.4 % linoleic acid (Hu et al. 2011).

The first assumption to explain the observed acid production in the culture broth of *C. podzolicus* CPOH4 and *T. porosum* TPST6 was that excessive citric acid may be secreted into the medium which serves as a precursor for acetyl-CoA and further for the production of triacylglycerols in oleaginous strains (Ratledge 2002). However, no citric acid could be determined in the culture broth. Instead, gluconic acid was measured as additional by-

product with high concentrations up to 30 g/L for *C. podzolicus* CPOH4 and 12 g/L for *T. porosum* TPST6. Both strains are simultaneous producers of lipid and gluconic acid.

Gluconic acid and its derivates find wide application in the food and pharmaceutical industries. Therefore, it could be worthwhile to improve gluconic acid production with the newly isolated yeasts *Cryptococcus podzolicus* CPOH4 or *Trichosporon porosum* TPST6. The ascomycete *Aspergillus niger* (Ramachandran et al. 2006) and yeast like *Aureobasidium pullulans* (Anastassiadis and Rehm 2006) are other examples of gluconic acid producers with high production rates of 120-140 g/L and up to 370 g/L, respectively.

The production of gluconic acid by the fungus *Aspergillus niger* is favored at high glucose concentrations between 110 and 250 g/L, at low concentrations of nitrogen and phosphorus, at pH values between 4.5 and 6.5 and high aeration rates (Ramachandran et al. 2006). In this study the requirements for low nitrogen conditions and acid conditions (pH 5) are met. The glucose concentration of 90 g/L is rather minor, but sufficient for the production of gluconic acid and lipid production.

C. podzolicus CPOH4 and *T. porosum* TPST6 give the opportunity to favor either the production of intracellular oil or the production of gluconic acid or rather the simultaneous production. An advantage of the simultaneous production of lipid and gluconic acid would be the easy separation of both products as the oil is produced intracellular, whereas the gluconic acid is secreted into the culture broth. Higher aeration rates to prevent oxygen limitation are required in any case to increase the product levels, although ethanol production is negligible. This low ethanol production, which started just at the stage of oxygen limitation, verifies that both strains are crabtree negative. To avoid the production of gluconic acid as by-product in a lipid production process, xylose may be the carbon source of choice.

However, another interesting approach could be the combined feeding of glucose and xylose as both carbon sources are components of hydrolyzed wood and straw waste. If glucose and xylose were consumed simultaneously hydrolyzed straw and wood wastes could be used as low-cost carbon source. *Trichosporon cutaneum* (Hu et al. 2011) and *Candida curvata* D (Heredia and Ratledge 1988) could be described as such strains. The use of other low-cost carbon sources and further process optimization to increase the lipid yield are further possible approaches.

Both isolates *C. podzolicus* CPOH4 and *T. porosum* TPST6 are suitable for a lipid production bioprocess, but *T. porosum* TPST6 shows a more interesting fatty acid profile with 18.7 % linoleic acid (C18:2). Moreover, xylose as carbon source favors the lipid production whereas glucose as carbon source leads to a simultaneous production of gluconic acid and intracellular lipid. If further optimized *C. shehatae* CSOH1 may produce a sufficient amount

of intracellular lipid with high amount of oleic acid (C18:1) on glucose or xylose. Therefore, it could be worthwhile to further examine CSOH1 as lipid producer for subsequent processing to biodiesel. *P. segobiensis* SSOH12 is worthwhile to be further investigated because of its considerable amount (16 %) of palmitoleic acid (C16:1), which may be suitable for medical applications.

IV. Chapter III

Establishment of an easy lipid quantification method and a rapid screening assay for oleaginous yeasts using the fluorescent dye Nile red in microtiter plates

1 Abstract

The fluorescent lysochrome Nile red stains neutral fats and is therefore also applicable to stain intracellular lipids in oleaginous microorganisms. The staining intensity can be used to rapidly estimate the lipid content per dry biomass without use of the time consuming analysis by gas chromatography.

The dye Nile red was taken for the quantification of intracellular lipid of the oleaginous yeast *Cryptococcus curvatus* grown in liquid culture. For this purpose, the optical density and the fluorescence of a defined volume of the yeast culture were measured in a 96-well microtiter plate; the ratio fluorescence/OD was formed and compared with the lipid content per dry biomass gained via gas chromatographically analysis – the standard lipid quantification method. The lipid content could be quantified via a linear fit with a deviation from 5 % for lipid content in the range of 18.3 % to 35.6 %.

To test the transferability of the method, five other yeast strains were stained by the same method and the same device settings for the measurement of fluorescence and optical density (OD). The ratio fluorescence/OD was set into relation to the lipid content and the results were compared among the various yeast strains. It was concluded that the ratio fluorescence/OD for intracellular lipids is proportional to the lipid content within one species, but not among various oleaginous yeast strains. The optical density as well as the measured values for the fluorescence depend on the shape and the size of the yeast cells and is influenced by cell agglomeration. However, this method is suitable for rapid lipid estimation within a high-throughput (HTP) screening assay to identify new oleaginous microorganisms.

2 Introduction

The quantification and qualification of fatty acids in form of fatty acid methyl esters via gas chromatography (GC) with flame ionization detection (FID) is a well-established method and gives precise results. It is the common method in biochemical, biomedical, microbiological, agricultural and ecological research (Dodds et al. 2005). Several oleaginous microorganisms have been characterized via GC relating to their intracellular lipid content

(single cell oil = SCO) and their corresponding fatty acid profile (Alvarez and Steinbüchel 2002; Sorger and Daum 2003; Sakuradani and Shimizu 2009; Hu et al. 2008). However, some disadvantages relating to the GC procedure for fatty acid analysis exist. The sample preparation takes a lot of time as the biomass cannot be analyzed in suspension, but has to be dried in advance over-night. Furthermore, two hours are required for the final sample preparation to extract the intracellular lipids from the biomass and to transesterify them to fatty acid methyl esters, which is the required chemical structure to detect them via GC. In addition, the samples are measured consecutively taking 30 to 40 minutes each. Furthermore, a plenty of solvents (in total 2 mL per sample) and a minimum of 20 mg of freeze-dried biomass is required for one single measurement.

For the determination of the fatty acid profile in oleaginous microorganisms GC is the only method, but the quantification of lipid is more often the value required. Therefore, alternative methods for the quantification of lipid content are required which need less sample volume, less solvent and less time: a quantitative assay which is applicable for suspensions with low required volume and with less or without solvent use. Furthermore, it should be possible to measure several samples simultaneously, preferably in a 96-well microtiter plate with minimal use of other additional consumables. Such a method could be applicable to establish a high-throughput-assay to check a high number of different strains from e.g. strain collections as candidates for lipid production. In addition, the influence of different cultivation conditions (carbon source, nitrogen source, concentration of substrates, temperature, pH) on the lipid content could be rapidly determined.

In literature, two staining dyes for neutral lipids are described. They are suitable for screening approaches aiming the identification of new oleaginous microorganisms. Sudan black B - a lipophilic lysochrome – was successfully applied to screen microorganisms cultivated on solid media (Evans et al. 1985), but this is a qualitative approach. Nile red - a fluorescent lipophilic dye ($C_{20}H_{18}N_2O_2$) - is also able to stain neutral fats and intracellular lipid droplets (Greenspan et al. 1985). Due to Kimura et al. (2004) the intensity of fluorescence corresponds to the amount of lipid and should therefore be suitable for quantitative analyses (Kimura et al. 2004). Nile red has been successfully used for the staining of intracellular lipid droplets in various oleaginous microorganisms in suspensions. In most studies the cell suspension was taken and the fluorescence of a single sample was measured using the fluorescence microscope or fluorescence photometer including a cuvette as reaction vessel (Kimura et al. 2004). Chen et al. (2009) developed a high throughput screening method using Nile red in a 96-well microtiter plate for algal cells. Sitepu et al. (2012) disproved the results of Kimura et al. (2004). Due to Sitepu et al. (2012) the emission maxima of the fluorescent dye Nile red vary between different yeast species. Hence, the procedure with one specific

wavelength for excitation and one specific wavelength for emission could not be used for the exact lipid quantification of various oleaginous yeast strains. Reasons might be that the penetration of Nile red into the cells depends on the thickness of the cell wall and the cytoplasmic membrane. Chen et al. (2009) and Sitepu et al. (2012) added dimethyl sulfoxide (DMSO) as a solvent to improve cell permeability of the dye. Because of the toxic characteristics of DMSO, in this study acetone was used according to Kimura et al. (2004).

This study includes two subjects. In both parts the optical density (OD_{600}) of a culture broth and the fluorescence after staining with Nile red were measured to form the ratio fluorescence/OD as a measure of the lipid content per dry biomass.

The first subject was to determine a correlation factor for the rough quantification of the lipid content in the oleaginous yeast *Cryptococcus curvatus* in suspension using Nile red in a microtiter plate. The application in a microtiter plate enabled parallel measurements of several samples or multiple determinations. The correlation factor is applicable for future experiments with *C. curvatus* in which the approximate estimation of intracellular lipids will be of interest, e.g. for process optimizations. The established technique for *C. curvatus* in this study acetone was used according to Kimura et al. (2004) can be easily transferred to other strains, which will be of importance for experiments with lipid quantification.

For the second subject the same device settings for the fluorescence measurement were adopted. The established quantification method with Nile red was applied to other yeast strains and compared with each other to evaluate this Nile red staining technique for a high-throughput-assay to screen for new oleaginous microorganisms.

3 Materials and methods

3.1 Applied strains

The oleaginous yeast *C. curvatus* (ATCC 20509) was used as positive control for SCO production. The non-oleaginous yeast *Saccharomyces cerevisiae* (DSM 11285) was taken as negative control for yeasts which do not produce any SCO. The isolates which are described in chapter II were used as additional lipid producer strains to test the method: CPOH4 *Cryptococcus podzolicus* (DSM 27192), SSOH12 *Pichia segobiensis* (DSM 27193) and TPST6 *Trichosporon porosum* (DSM 27194) and CSOH1 *Candida shehatae* (not yet deposited at any strain collection).

3.2 Cultivation in shake flasks

Part I (lipid quantification in *C. curvatus*)

C. curvatus was cultured in YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, pH 7) in 2 L conical shake flasks in a total volume of 400 mL supplemented with 50 g/L glucose for four days at 28 °C at 120 rpm with an initial OD_{600} of 1. The culture broth was daily supplemented with 35 g/L glucose as carbon source to ensure lipid accumulation within the yeast cells. Three shake flasks were prepared and 10 samples were taken in total from all shake flasks within the four day cultivation to obtain cells with different lipid contents and from different growth phases.

Part II (screening assay for oleaginous yeast)

50 mL YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/l peptone, pH 7) with 50 g/L glucose in 500 mL conical shake flasks were inoculated directly with a colony from an agar plate and incubated at 130 rpm at 25 °C for 120 hours. 35 g/L glucose was daily added to ensure that the carbon source was in excess. The OD_{600} was measured once a day, whereas the lipid content via GC was only determined on day 4 and day 5.

YM medium did not show any fluorescence in preliminary studies with Nile red (results not shown); therefore a washing step of the cells was not required.

3.3 Microscopic observation

Microscopic observation of the lipid accumulating yeast cells was performed using Eclipse E200 (Nikon) with a total enlargement of 400 x.

3.4 Lipid analysis via gas chromatography

A 20 mL aliquot of the culture broth was centrifuged (4,700 rpm, 5 min) and the cell pellet was resuspended in saline (0.9 % NaCl) and again centrifuged (4,700 rpm, 5 min). The supernatant was discarded and the pellet was freeze dried (-30 °C, 0,370 mbar). Preparation for the quantitative and qualitative gas chromatographically analysis was performed in a one-step-procedure by direct esterification plus extraction. A portion (20 mg) of freeze dried biomass was weighed into a 15 mL glass falcon with Teflon cap. 1.5 mL hexane and 0.5 mL of 2 mg/mL internal standard (methyl benzoate) dissolved in hexane were added as solvent for the extraction of lipid. In addition, 2 mL 15 % H_2SO_4 in methanol were added for the esterification step. Each sample was incubated at 100 °C for 2 h with continuous shaking. After cooling on ice 1 mL distilled water was added. The mixture was centrifuged for 5 min at 2,500 rpm. 1 µL of the upper phase, containing the fatty acid methyl esters extract, was

analyzed via GC (Agilent Technologies, 6890 N Network GC-System). The instrument was equipped with a DB-Wax column (l: 30 m d: 0.25 mm, Agilent Technologies) and a flame ionization detector. It worked at a pressure of 1.083 bar and initial temperature of 40 °C. The column temperature was increased from 40 °C to 250 °C with a rate of 8 °C/min. The temperature was held at 250 °C for 10 min before cooling down to 40 °C. The total fatty acid content and the identification of fatty acids were performed using the standard RM3 FAME Mix (Sigma Aldrich) and Marine FAME Mix (Restek).

3.5 Nile red assay

The optical density (OD_{600}) and the fluorescence of the Nile red stained lipid cell culture were measured using the Fluorescence Photometer Infinite®M200PRO (Tecan). All measurements for optical density (OD_{600}) and fluorescence were performed in black 96-well-microtiter plates (3603, Costar). The OD_{600} of each undiluted sample was adjusted with saline (0.9 % NaCl) to a maximum value of 0.7 measured in a microtiter plate with a volume of 200 µL to stay within the linear range of OD_{600} measurements (data not shown). 200 µL of each diluted culture were filled into the wells of a microtiter plate in six copies for a six-fold determination. Saline was used as negative control. After 15 s of shaking, OD_{600} was measured. Afterwards 10 µL freshly prepared Nile red solution (0.1 g/L in acetone, stored on ice) were added into each sample. The microtiter plate was covered with aluminum foil to prevent evaporation of acetone and was shaken for 15 min at room temperature. Thereafter, fluorescence was measured at 490 nm extinction and 580 nm emission. The settings of the device were 10 flashes for the OD_{600} and 25 flashes for fluorescence (z-position 19,441, gain 94). Average values were calculated by subtracting the negative control and standard deviations were determined. To normalize the results, the obtained values for fluorescence were divided by the values for the OD_{600}.

4 Results

4.1 Lipid estimation in the oleaginous yeast Cryptococcus curvatus

C. curvatus was cultured in YM medium and the lipid content per dry biomass was measured via chromatography (GC), which is a very accurate, but time consuming method. The value for the lipid content measured via GC and the ratio fluorescence/OD were set into relation for each sample to create a correlation factor which should enable a rapid determination of the lipid content via the Nile red staining method. Fig. III.1 shows the results which originate from ten single samples from a four day cultivation period. A linear fit of the Nile red values was possible for lipid contents between the minimum value 18.3 % and the maximum 35.6 %.

Fig. III.1 presents the resulting calibration. The linear equation of the fit was determined as:

$$y = 8087(\frac{units}{\%})x - 136{,}318 \; units \quad R^2 = 0.976$$

This equation was used to calculate the lipid content at day 4 (second part, Fig. III.3 and Fig. III.4) with the measured value of 118,540.2 units.

$$x = \frac{y + 136{,}318 \; \text{units}}{8{,}087(\frac{\text{units}}{\%})} = \frac{118{,}540.2 \; \text{units} + 136{,}318 \; \text{units}}{8{,}087(\frac{\text{units}}{\%})} = 31.5\%$$

The determination of lipid content via gas chromatography gives a value of 33.1 %. Compared to the above calculated value of 31.5 % this corresponds to a deviation from 4.7 %.
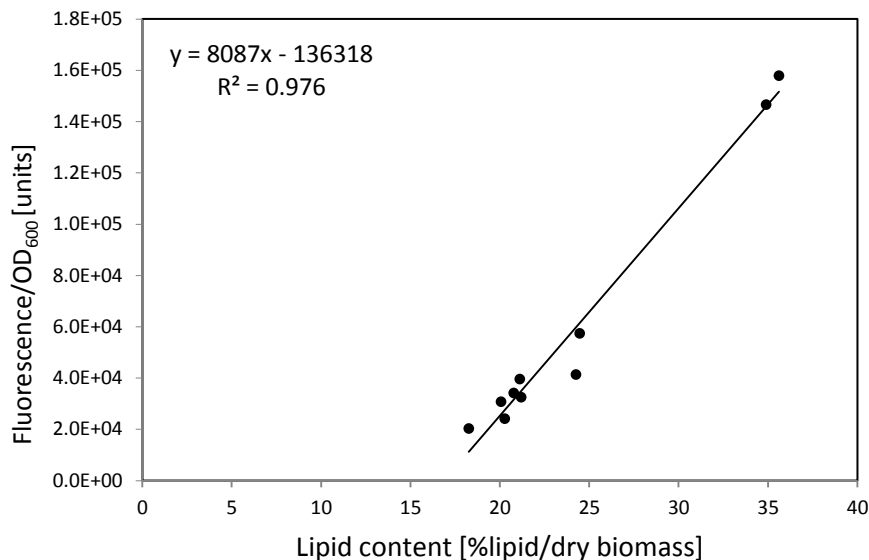
Fig. III.1 Regression line to determine the lipid content in the yeast *Cryptococcus curvatus* via measuring the fluorescence/OD_{600}; data result from a four day cultivation (96 h); Fluorescence/OD_{600} mean of six measurements; Lipid content mean of three measurements

4.2 Rapid screening assay in microtiter plates

Cryptococcus curvatus (*C.c.*) - an oleaginous yeast - was taken as positive control, *Saccharomyces cerevisiae* (*S.c.*) - a non oleaginous yeast - was taken as negative control and all four newly isolated yeasts from Chapter II (CSOH1, SSOH12, CPOH4 and TPST6) were used to test the determination of lipid content using the fluorescent dye Nile red.

All yeast strains were cultured in shake flasks using YM medium and were incubated at the same temperature of 25 °C, as this is the average optimum temperature for yeast cells. The OD_{600} of all six strains developed differently (Fig. III.2).

Fig. III.2 Growth curve of a five day shake flask cultivation in YM medium of six different yeast strains (TPST6, CSOH1, SSOH12, CPOH4, *Cryptococcus curvatus* (*C.c.*), *Saccharomyces cerevisiae* (*S.c.*)); OD_{600} mean of two measurements
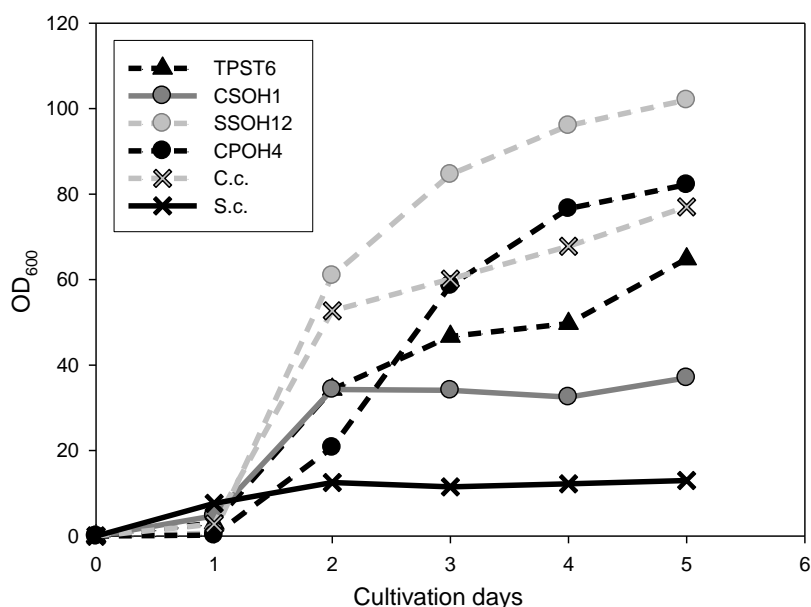
While *S. cerevisiae* and CSOH1 stopped growing after two days, the OD_{600} of the four other strains was still increasing up to the fifth cultivation day to values between 60 and 100. The highest value for the optical density with final values of 102 was obtained by strain SSOH12. TPST6 reached 65, *C. curvatus* 77 and CPOH4 82. CSOH1 stopped growing at an OD_{600} of 37 and *S. cerevisiae* stopped growing even at a value of 13. The lipid content per dry biomass was determined for all strains of samples taken at day 4 and 5. The ratio fluorescence/OD was determined for all strains at day 2, 3, 4 and 5.

Considering the lipid content per dry biomass (Fig. III.3), which was measured via GC, an increase from day 4 to day 5 for all six strains was recognized. After five cultivation days TPST6, CPOH4 and *C. curvatus* reached more than 20 % lipid content, which classifies these three strains as oleaginous microorganisms. TPST6 reached 24.5 %, CPOH4 34.6 % and the oleaginous yeast *C. curvatus* reached 41.5 % lipid per dry biomass. CSOH1 and SSOH12 reached low values between 7.9 % and 11.2 % under the given conditions in shake flasks, similar to the non-oleaginous yeast *S. cerevisiae* which reached only 7.7 % lipid content. That means that the two strains CSOH1 and SSOH12 cannot be classified as oleaginous under the chosen conditions in shake flasks.
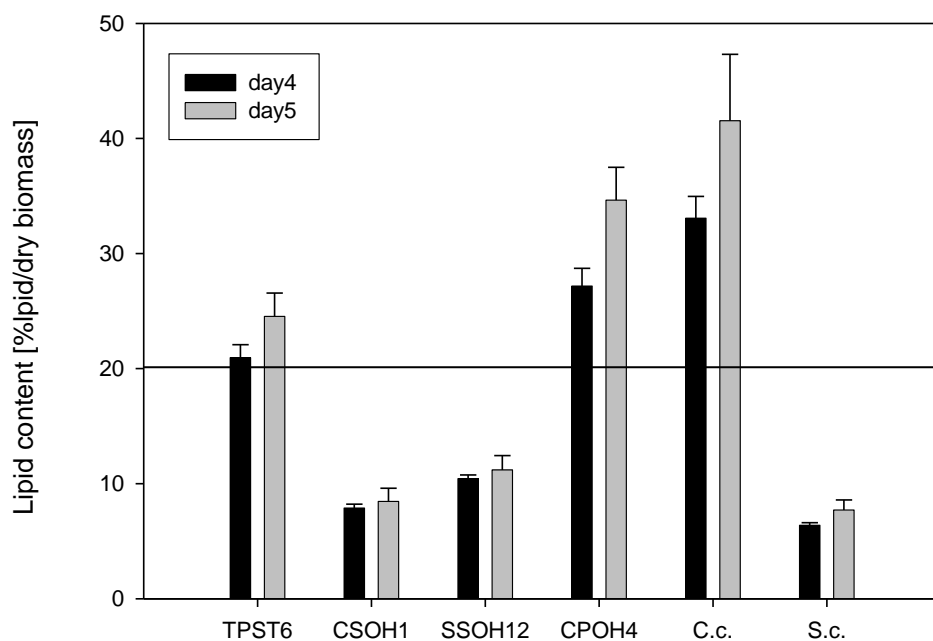
Fig. III.3 Lipid content [%lipid/dry biomass] at 4th and 5th day of a five day shake flask cultivation in YM medium of six different yeast strains measured via GC (TPST6, CSOH1, SSOH12, CPOH4, *Cryptococcus curvatus* (*C.c.*), *Saccharomyces cerevisiae* (*S.c.*)); the line indicates the threshold of 20 % lipid content to be classified as oleaginous; lipid content mean of three measurements; values in detail are found in appendix

Fig. III.4 Fluorescence/OD_{600} determined for daily samples of a five day shake flask cultivation in YM medium of six different yeast strains (TPST6, CSOH1, SSOH12, CPOH4, *Cryptococcus curvatus* (*C.c.*), *Saccharomyces cerevisiae* (*S.c.*)); Fluorescence/OD_{600} mean of six measurements; values in detail are found in appendix

Considering the ratio fluorescence/OD (Fig. III.4), *C. curvatus* reached after four days of cultivation the highest value with 118,540 units, CPOH4 reached with a value of 50,970 units nearly the half of *C. curvatus*. TPST6 resulted just in 9,259 units which is roughly a twelfth of *C. curvatus*. Nevertheless, TPST6 reached 16,638 units after five days cultivation which is nearly a doubling within one day. The negative control *S. cerevisiae* remained the same value with an average of around 6,439 units. Even smaller values were obtained by CSOH1 and SSOH12 with maximum values after five days cultivation of 3,611 units and 1,546 units, respectively. Strikingly, the value for *C. curvatus* at the second day reached already 56,096 units, more than a five-fold higher value than CPOH4.

Table III.1 Linear fit between dry biomass and optical density (OD_{600}); data are based on fermentations of Chapter II

	Regression line for OD_{600}/dry biomass [L/g]	R^2
C. curvatus	$y = 4.3195x$	$R^2 = 0.9524$
CSOH1	$y = 5.7514x$	$R^2 = 0.9823$
SSOH12	$y = 5.5454x$	$R^2 = 0.9739$
CPOH4	$y = 3.3056x$	$R^2 = 0.9336$
TPST6	$y = 4.5266x$	$R^2 = 0.9828$

Table III.1 presents the linear fit between dry biomass and optical density (OD_{600}) for *C. curvatus* and for each isolate of Chapter II (CSOH1, SSOH12, CPOH4 and TPST6) to calculate the dry biomass directly from the OD_{600}. The data of *C. curvatus* arises from the fermentation described in chapter I and the data of the four isolates (CSOH1, SSOH12, CPOH4 and TPST6) from the fermentations described in chapter II. All five correlation factors are different which means that each yeast strain has another OD_{600} for one defined biomass.

The correlation factors from table III.1 enable the calculation of the dry biomasses for each day corresponding to the OD_{600} of each yeast strain. Hence, the factor [fluorescence/dry biomass] can be calculated (Fig. III.5). *C. curvatus* and CPOH4 achieved by far the highest values with 430,753 units/(g/L) and 357,313 units/(g/L) at day 4, respectively. Slightly lower values, about ¾ of day 4 were reached at day 3. *C. curvatus* showed already 5-fold higher values than CPOH4 on the second cultivation day. The values of TPST6 are very low compared to *C. curvatus* and CPOH4. They increased continuously within the five days from 7,271 units/(g/L) to 52,620 units/(g/L). The values for CSOH1 and SSOH12 are very low; they did not even reach the minimum values of TPST6 at day 2.

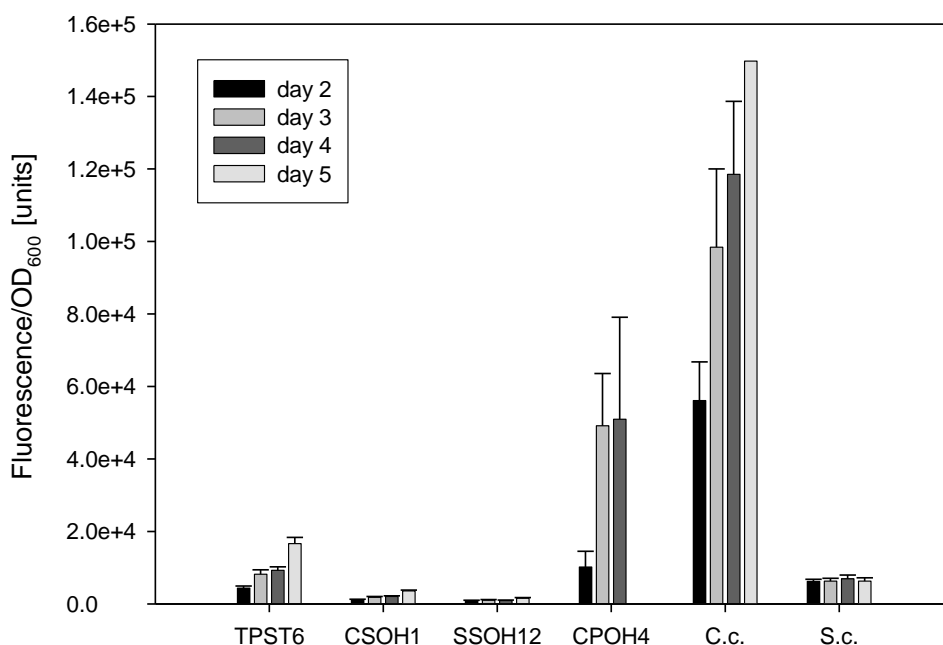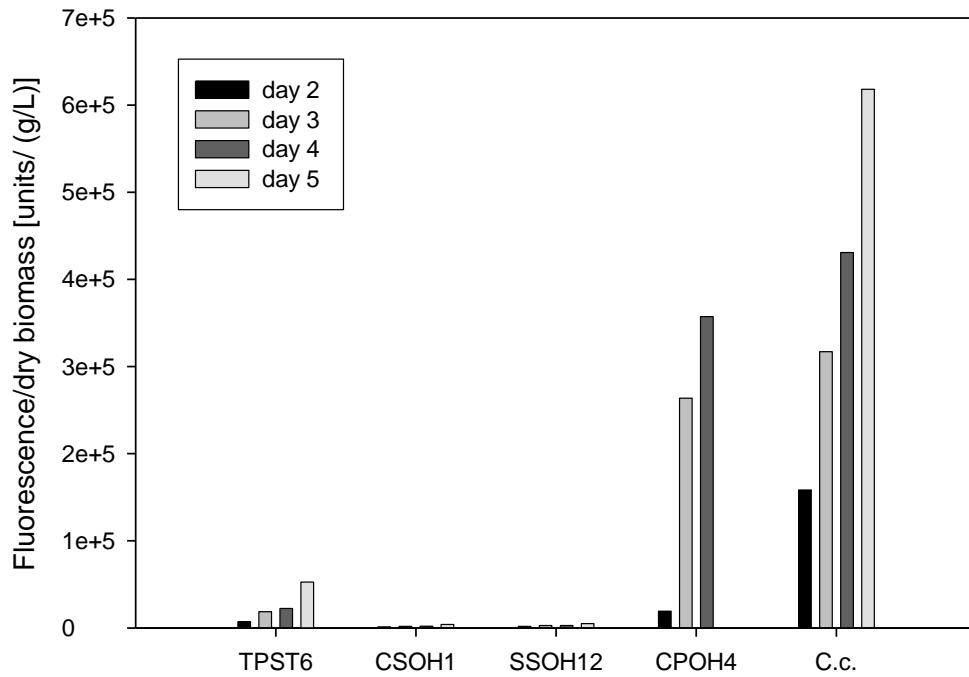**Fig. III.5** [Fluorescence/dry biomass] for daily samples of a five day shake flask cultivation in YM medium of six different yeast strains (TPST6, CSOH1, SSOH12, CPOH4, *Cryptococcus curvatus* (*C.c.*)) – dry biomass was calculated via correlation factors from table III.1 using the average values of OD_{600}; values in detail are found in appendix

TPST6
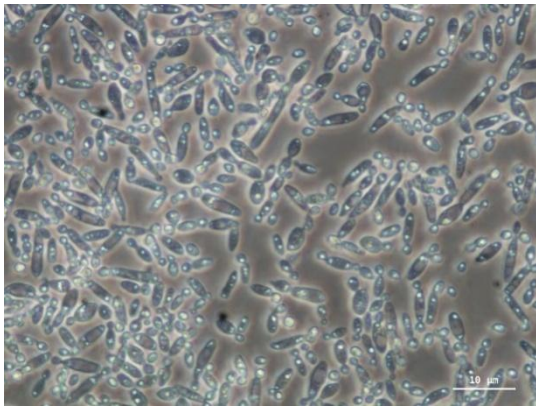
CSOH1
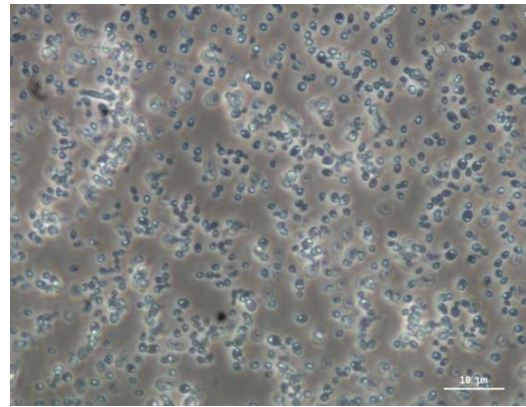
SSOH12
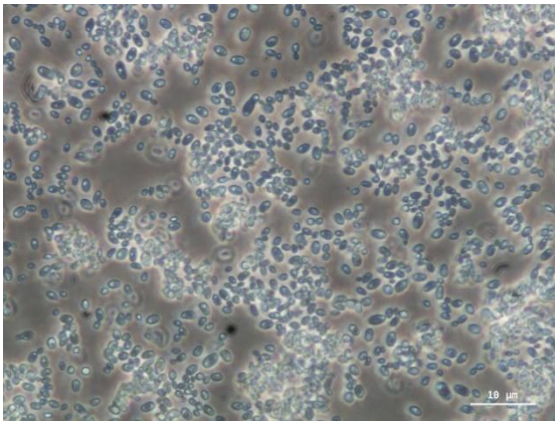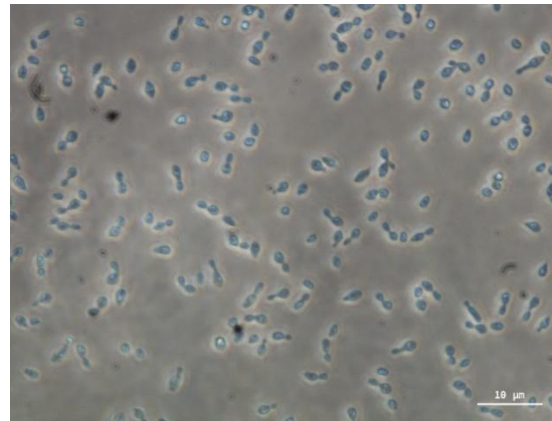
CPOH4

Cryptococcus curvatus
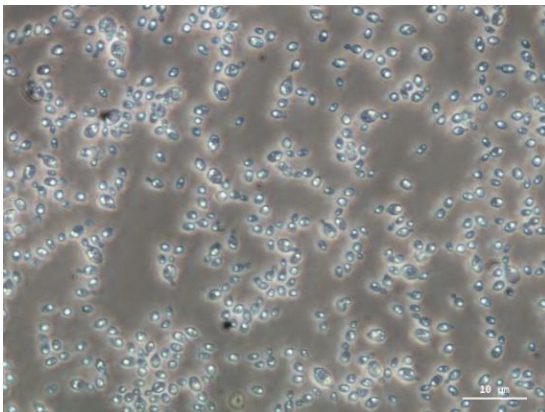
Saccharomyces cerevisiae
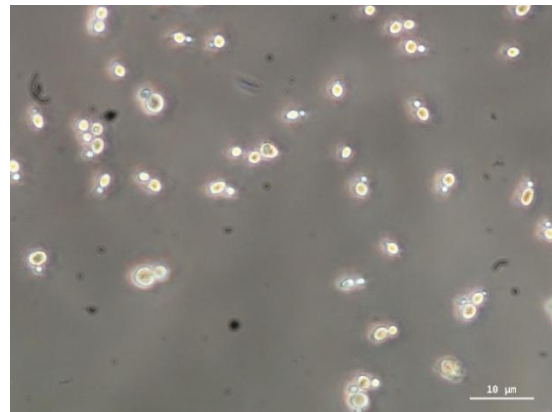
Fig. III.6 Microscopic pictures of a 48 h shake flask cultivation in YM medium with 400 x enlargement

TPST6
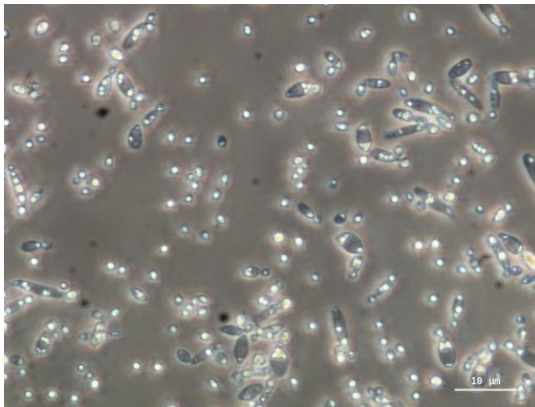
CSOH1
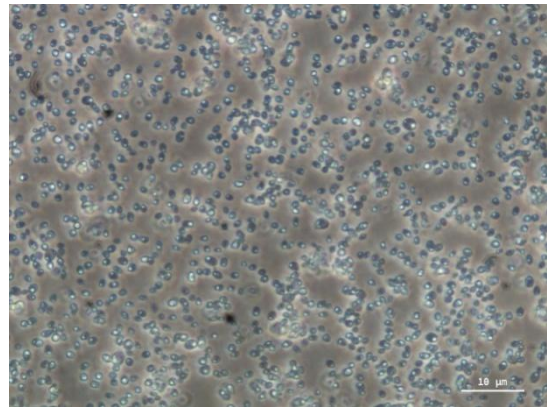
SSOH12
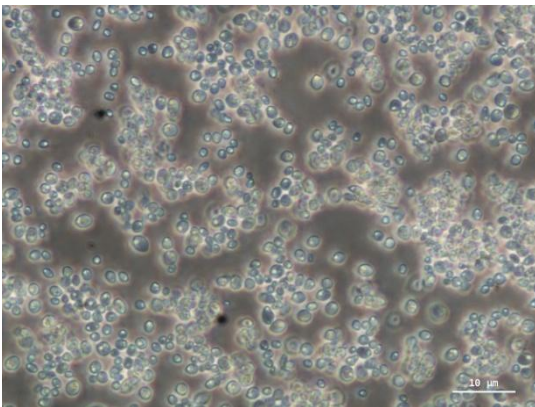
CPOH4
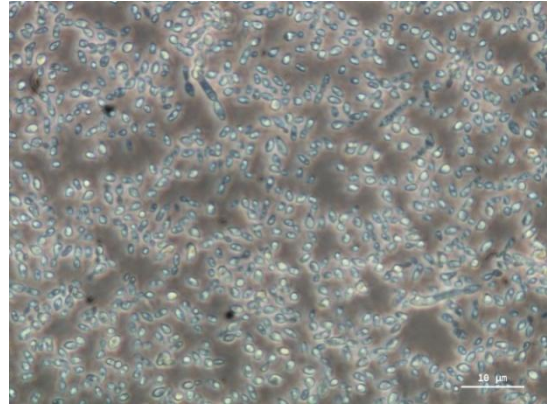
Cryptococcus curvatus
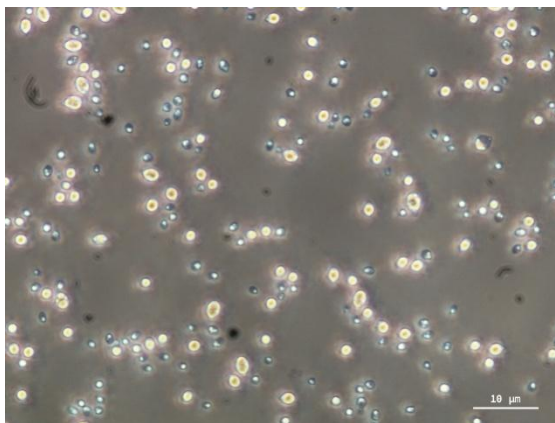
Saccharomyces cerevisiae
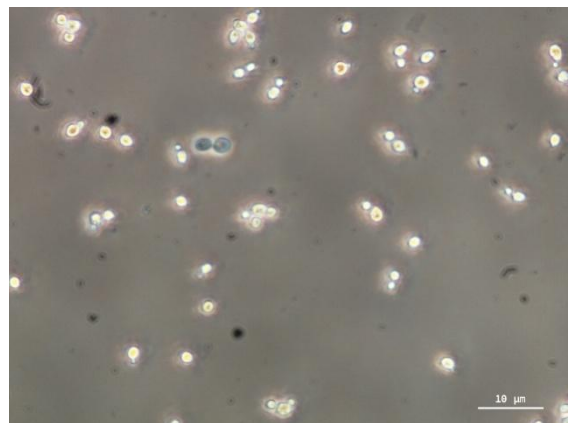
Fig. III.7 Microscopic pictures of a 120 h shake flask cultivation in YM medium with 400 x enlargement

Fig. III.6 depicts the microscopic images of all six examined yeast strains after 48 h cultivation in shake flasks, fig. III.7 after 120 h cultivation. The lipid droplets within the cells indicate the lipid content. Enormous differences among the different yeast species were detected and differences in shape and size were observed. The cells of CSOH1 are significant smaller than the other strains. TPST6 e.g. shows round cells as well as bigger oval cells. Most cells of SSOH12 are quite big and round shaped while CPOH4 forms smaller and oval cells. The cells of SSOH12 tend to agglomerate.

In conclusion, the ratio fluorescence/OD is specific for each single strain. To use this factor as lipid quantification method, GC analysis for the lipid quantification is unavoidable to create calibration for each single strain using the here described method with Nile red in microtiter plates.

Taking into consideration that different optical densities represent different biomasses, the factor fluorescence/dry biomass was divided by the lipid content to make comparisons between the fluorescence and the lipid content normalized to the biomass. The results are listed in table III.2. It is noticeable that the two yeast strains CSOH1 and SSOH12 with low lipid contents below 11 % reached low values below 300 units/(g/L) at the fourth day, whereas those with lipid amounts above 20 % reach more than three to 45 times. TPST6 amounts around 1,070 units/(g/L), *C. curvatus* 13,025 units/(g/L) and CPOH4 even 16,878 units/(g/L). The value of TPST6 is another dimension compared to *C. curvatus* and CPOH4. The same tendency can be recognized for the fifth cultivation day.

Table III.2 Calculation of the ratio "Fluorescence/dry biomass]/[lipid content/dry biomass]"

Yeast strain	Lipid content [%/dry biomass]		[Fluorescence/dry biomass]/[lipid content/dry biomass] [units/(g/L)]	
	day4	day5	day4	day5
TPST6	21.0	24.5	1,072	2,144
CSOH1	7.9	8.5	257	477
SSOH12	10.4	11.2	282	450
CPOH4	21.2	34.6	16,878	n.d.
C.c.	33.1	41.5	13,025	14,880

5 Discussion

5.1 Quantification of lipid content using Nile red

The quantification of lipid in oleaginous yeasts using the fluorescent lysochrome Nile red was successfully applied for the oleaginous yeast *C. curvatus*. A linear correlation between fluorescence/OD_{600} and the lipid content was identified between 18.3 % and 35.6 % lipid content (fig. III.1) and can be used to calculate the lipid content. The approximate deviation was shown to be 5 % when using the fluorescence photometer Infinite®M200PRO (Tecan) with the setting parameters of 10 flashes for the OD_{600} and 25 flashes for the fluorescence measurement (z-position 19,441; gain 94). Further studies are necessary to determine the exact threshold upwards and downwards, which delimit the linear range of the calibration. Using this calibration, the lipid contents can only be calculated from the values for the ratio fluorescence/OD between 20,293 and 157,878 including the range of the determined fit. The quantification method via Nile red is suitable for HTP assays in which an approximate value of lipid content is of interest, e.g. for a medium or pH optimization. If the exact value of lipid content is important, gas chromatographical analysis is required which gives simultaneous information about the fatty acid profile.

5.2 Screening assay for oleaginous yeast using Nile red

The ratio fluorescence/OD was evaluated as a measurement technique for the lipid content in oleaginous yeast cells in order to be applied for a high throughput (HTP) screening assay for oleaginous yeasts in suspension. Such an assay should be easily and rapidly feasible to check a vast number of yeast strains contemporaneous for lipid production.

The fluorescence of Nile red stained yeast cells and the OD_{600} are easily measurable in a 96-well microtiter plate. The ratio fluorescence/OD (fig. III.4) would therefore be a perfect measure to quantify the lipid content in oleaginous yeast. However, the measured values of the various yeast strains TPST6, CSOH1, SSOH12, CPOH4, *C. curvatus* and *S. cerevisiae* (fig. III.4) did not show any proportionality to the lipid content (fig. III.3) which was measured via GC analysis. These differences may be achieved either by the measured OD_{600} or by the fluorescence intensity. The optical density is influenced by the size and shape of the yeast cells and also if the cells agglomerate. Those differences of the cells can be considered on the microscopic images (fig. III.6 and fig. III.7). The reasons for varying fluorescence intensities at same lipid contents are different compositions of the cell membrane, the thickness of the cell wall and also the size of the cells. This phenomenon has been proven already on algal cells by Chen et al. (2009). This explains that the ratior fluorescence/dry biomass (table III.2) is also not proportional to the lipid content among various yeasts with different sizes and shapes. This confirms the results described by Sitepu et al. (2012).

In particular TPST6 with lipid amounts of 24.5 % at day 5 gives very low values for the ratio fluorescence/OD of 16,639 units, whereas CPOH4 at day 4 with a similar value of 27.2 % lipid content reached even 50,971, which represents the 3-fold amount. However, the values for TPST6 from the third day are all higher compared to the constant values of the non-oleaginous yeast *S. cerevisiae*. Hence, it can be concluded that values for fluorescence/OD which are higher than those measured for *S. cerevisiae* are promising lipid producers and are worthwhile to be further examined via GC. Therefore *S. cerevisiae* should be always measured as a reference strain when screening for new oleaginous microorganisms using the technology described in this study. Another influencing factor is the degree of saturation of the fatty acids produced by the different strains. Kimura et al. (2004) showed that the fluorescence intensity of lipids is higher the more unsaturated fatty acids are present. That may explain the lower fluorescence values of TPST6 whose lipids are composed of 59.8 % unsaturated fatty acids (Chapter II table II.3) compared to CPOH4 with 69.3 % unsaturated fatty acids (Chapter II, table II.3). Concerning *C. curvatus* with 57.9 % unsaturated fatty acids (Chapter I, fig. I.9), the grade of saturation cannot be the reason for the higher ratio fluorescence/OD. In this case, Nile red might better penetrate into the cells and leads therefore to higher values.

As a conclusion, the Nile red staining of unknown yeast strains can be used for a high-throughput (HTP) screening approach for oleaginous yeast strains in 96-well microtiter plates by measuring the fluorescence and the optical density when compared to a known non-oleaginous yeast, e.g. *S. cerevisiae*. The here described approach is only suited to check if the unknown strains might be lipid producers. As next step, to use Nile red staining for a rough quantification of the lipid content in each isolate, new correlations have to be created for each new strain.

V. Concluding Remarks

The aim of this work was to develop strategies for the economic and ecological production of microbial lipids, which are also named single cell oils (SCO) to partially substitute plant oil, crude oil or fish oil as renewable raw material. Prerequisites for an economic and ecological process are high lipid yields, high volumetric productivities, low-cost substrates and high-value products. Another aspect can be to recycle all the waste streams, e.g. the CO_2 or the residual biomass leftover after single cell oil extraction.

In this work, the recycling of CO_2 was realized. Therefore, the typical oleaginous yeast *Cryptococcus curvatus* was used as a model organism to establish a platform process, based on a fed-batch process with glucose as carbon source, transferable to other oleaginous yeast strains. The process was characterized due to nitrogen limitation, carbon source consumption and the analysis of the exhaust gas CO_2. The data were used to establish a set-up for an integrated bioprocess with lower ecological impact by reducing the overall CO_2 emission. Therefore, the emitted greenhouse gas CO_2 was channeled into the lipid production process of the microalgae *Phaeodactylum tricornutum* in order to supply the microalgae with the required carbon source CO_2. One challenge in this coupled process was to keep the yeast's emission of CO_2 constant to guarantee a constant supply for the microalgae in order to keep the pH value constant. Depletion of the carbon source or harvesting a certain quantity of the yeast cells within the repeated fed-batch process, led to a sudden decrease of the CO_2 emission. An automatic glucose feed or a more frequent harvesting of less biomass may prevent those sudden declines of CO_2. Consequently, a semi-continuous production process may be worthwhile to be investigated. If no glucose sensor is available, an automated glucose feed may be controlled via the respiration coefficient (RQ) or the pO_2 value in the yeast process.

A screening strategy, using the lysochrome Sudan black on solid media, was applied to identify new oleaginous yeast strains applicable for an economic lipid production. Four promising yeast strains were cultured in bioreactors according to the platform process mentioned above and their lipid contents and fatty acid profiles were analyzed. Three yeast strains, - *Cryptococcus podzolicus*, *Trichosporon porosum* and *Pichia segobiensis* - were classified as oleaginous, yielding 31.8 %, 34.1 % and 24.6 %, respectively. In addition to glucose, *C. podzolicus* and *T. porosum* were also cultivable on xylose with similar lipid productivities as with glucose. Xylose and glucose are both components of hydrolyzed straw and wood wastes, hence oleaginous microorganisms converting both substrates are

worthwhile for lipid production processes with hydrolyzed hemicellulosic waste material. That's one further possibility to reduce process costs.

To control the lipid content during a fermentation process like those described above, a fast analysis of the SCO-content of the biomass is necessary. Gas chromatographical analysis is one accurate method to determine the quantity and quality of the SCO, but is far too slow for process control. At least 20 mg over-night dried biomass, extraction and transesterification of the SCO are required to finally analyze one sample via GC, taking in total 10 hours each. Therefore, a fast assay (30 min duration) with the fluorescent lysochrome Nile red on 96-well plate format for a rough SCO quantification was developed. With this assay the lipid content of *C. curvatus* with lipid contents between 18 and 36 % per dry biomass could be analyzed with a deviation from 5 %. This method is based on the measurement of the optical density, subsequent staining of the cell suspension with Nile red and the measurement of the fluorescence. Subsequently, a correlation of the ratio fluorescence/OD and the lipid content measured via GC was created. It was shown that the ratio fluorescence/OD is proportional to the lipid content within the same yeast strain, but differs among various strains due to different cell sizes, different shapes and cell agglomerations. Therefore, specific correlations can be determined for each oleaginous yeast strain whenever a rough quantification method for e.g. process optimizations is required. The developed quantification method was transferred to a high-throughput (HTP) screening assay to easily identify promising oleaginous yeast strains. Comparing the obtained values for fluorescence/OD of various unknown strains with those of the non oleaginous yeast *Saccharomyces cerevisiae*, it was possible to screen for promising oleaginous yeast strains which are worthwhile to be further examined for microbial lipid production processes. This HTP-screening-assay supplies a basis for a vast screening approach to identify promising oleaginous microorganisms with high lipid yields, high volumetric productivities, high-value fatty acids or even microorganisms able to grow on hydrolyzed hemicellulosic wastes. If those oleaginous microorganisms are supplied with world's vast amounts of non edible hydrolyzed hemicellulosic waste biomass as carbon source in bioprocesses with reduced CO_2 emission, an economic and ecological large-scale production of microbial lipids as renewable raw material for oleochemicals will be possible.

VI. References

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VII. List of abbreviations

ACL	ATP-citrate lyase
AMP	Adenosine-monophosphat
ARA	arachidonic acid
ATCC	American Type Culture Collection
ATP	adenosintriphosphat
C. curvatus	*Cryptococcus curvatus*
C. podzolicus	*Cryptococcus podzolicus*
C. shehatae	*Candida shehatae*
C.c	*Cryptococcus curvatus*
C/N	carbon to nitrogen ratio
CBE	cacao butter equivalent
CO_2	carbon dioxide
CoA	coenzyme A
CPOH4	yeast isolate identified as *Cryptococcus podzolicus*
CSOH1	yeast isolate identified as *Candida shehatae*
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell cultures
e.g.	exempli gratia; for example
eFA	essential fatty acid
EMBL	The European Molecular Biology Laboratory
EPA	eicosapentaenoic acid
FA	fatty acid
FAME	fatty acid methyl esters
FAS	fatty acid synthase
FID	flame ionization detector
GC	gas chromatography
GHG	green house gas
GLA	gamma linolenic acid
HPLC	high pressure liquid chromatography
HTP	high throughput
ICDH	isocitrate-dehydrogenase

IMP	Inosin-monophosphat
ITS	internal transcribed spacer
LCFA	long chain fatty acid
LC-PUFA	long-chain poly-unsaturated fatty acid
ME	malic enzyme
MO	microorganism
MUFA	mono-unsaturated fatty acid
$NADH^+ + H^+$	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
O_2	oxygen
OD	optical density
P. segobiensis	*Pichia segobiensis*
P. tricornutum	*Phaeodactylum tricornutum*
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
pO_2	partial pressure of oxygen
PUFA	poly-unsaturated fatty acid
Q_L	volumetric lipid productivity
RNA	ribonucleic acid
rpm	revolutions per minute
RQ	respiratoy coefficient
rRNA	ribosomal ribonucleic acid
SAG	Culture Collection of Algae at Göttingen University
SCFA	short chain fatty acid
SCO	single cell oil
sFA	saturated fatty acid
SIP	sterilization in place
SSOH12	yeast isolate identified as *Pichia segobiensis*
T	temperature
t	duration time of cultivation/ process
T. porosum	*Trichosporon porosum*
TAG	triacylglycerol
TPST6	yeast isolate identified as *Trichosporon porosum*
YM	yeast extract
$y_{p/s}$	product yield coefficient
$y_{x/s}$	growth yield coefficient

VIII. Appendix

Chapter I, II and III

Composition of FAME Mix standards

Fatty acid methyl ester	Carbon length and degree of saturation	Marine FAME mix (Restek) (%)	FAME MIX RM3 (Supelco) (%)
Methyl myristate	C14:0	6	1
Methyl myristoleate	C14:1	1	-
Methyl palmitate	C16:0	16	4
Methyl palmitoleate	C16:1	5	-
Methyl stearate	C18:0	8	3
Methyl oleate	C18:1(1)	13	45
Methyl vaccenate	C18:1(2)	4	-
Methyl linoleate	C18:2	2	15
Methyl linolenate	C18:3	2	3
Methyl arachidate	C20:0	1	3
Methyl-11-eicoenoate	C20:1	9	-
Methyl 11-14 eicosadienoate	C20:2	1	-
Methyl aradichonate	C20:4	3	-
Methyl 11-14-17 eicosapentaenoate	C20:3	1	-
Methyl eicosapentaenoate	C20:5	10	-
Methyl behenate	C22:0	1	3
Methyl erucate	C22:1	3	20
Methyl lignocerate	C24:0	1	3
Methyl docosahexaenoate	C22:6	12	-
Methyl nervonate	C24:1	1	-

Chapter I

Fatty acid profile of *C. curvatus*

(Standard deviation <0.05), grey marked fatty acids indicate saturated fatty acids

Fatty acid	Percentage of fatty acid of total fatty acids (%)
14:0	0.5
16:0	18.5
18:0	17.7
18:1	48.7
18:2	8.6
18:3	0.6
20:0	0.7
22:0	0.5
24:0	3.0
others	1.2

Fatty acid profile of *P. tricornutum*

(Standard deviation <0.05), grey marked fatty acids indicate saturated fatty acids

Fatty acid	Percentage of fatty acid of total fatty acids [%]
14:0	4.5
16:0	25.0
16:1	44.9
18:1	7.6
18:2	1.7
20:4	1.5
20:5(n-3)	8.9
others	5.8

Chapter II

Lipid content shake flask

	lipid content [%lipid/dry biomass]	Standard deviation
TPST6	24.5	2.0
CSOH1	8.5	1.1
SSOH12	11.2	1.2
CPOH4	34.6	2.9
C.c.	41.5	5.8
S.c.	7.7	0.9

Sequences of the intervening 5.8S rDNA of the new isolates using primer ITS1 and ITS4

All sequences were accessed on 03.04.2013 at NCBI; all nucleotide blast exclude uncultured/model organisms

TPST6 *Trichosporon porosum* DSM 27194

TATATCCATTTACACCTGTGAACCGTTTGATTGACACTCTGTGTTGATTTTACAAACAATATGTAAA
GAAAGTCAAGTTATTATAACAAAAAATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTGTCATGAAATCTCAACCATT
AGGGTTTCTTAATGGCTTGGATTTGGGTGTTGCCAGTCTCTGGCTCGCCTTAAAGGAGTTAGCGA
GTTTAACAATGTCGTCTGGCGTAATAAGTTTCGCTGGTAAGACTTGTGAAGTTTGCTTCTAATCGT
CTTCGGACAATTACTTTGACTCTGGCCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATC
AATAAGCGGAGGAA

SSOH12 *Pichia segobiensis* (DSM 27193)

GCGCGCTTACTGCGCGGCGAAAAAACCTTACACACAGTGTTTTCTTTATTAGAAACTATTGCTTTG
GTTTGGCTCAGAAATGAGTTGGGCCAGAGGTTTACCAAACTTCAATTTTATTGAATTGTTATTTTAT
TAATTTGTCAATTTGTTGATTAAATTCAAAAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTC
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCG
AATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTC
TCTCAAACCCTCGGGTTTGGTATTGAGTGATACTCTTAGTCGAACTAGGCGTTTGCTTGAAAAGTA
TTGGCACGAGTGGTACTAAATAGTACTGACAGAATATTTCAATGTATTAGGTTTATCCAACTCGTT
GAGACTTCTGGCGGTGAATTTTTGGTATATTGGCTTTGCCTTACAAAACAACAAACAAGTTTGACC
TCAAATCAGGTAGGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

CPOH4 *Cryptococcus podzolicus* (DSM 27192)

TATTCCAAACCTCTGTGAACCGTGCCCTTCGGGGCTATTTTACAAACATGGTGTAATGAACGTCAT
ATATCATAACAAAACAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
CCCTCTGGTATTCCGGAGGGCATGCCTGTTTGAGTGTCATGTAGACTCAATCCCTCGGGTTTCCG
AGGAGATTGGACTTGGGTGTTGCCGCTCTGCCGGCTCGCCTTAAAAGACTTAGCGGGATAGCAC
CGTAGTCGGCGTAATAAGTTTCGTCGGTGAAGGTTGTGATGACTGCTTACAATCGCCCTCGGGCA
ATTTTTGACTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAG
GAA

CSOH1 *Candida shehatae*

CCTTACACACAGTGTTTTCTTTATTAGAAACTATTGCTTTGGTCTGGCTTAGAAATAAGTTGGGCC
AGAGGTTTAACTAAACTTCAATTTTATTATTGAATTGTTATTTTATTTAATTTGTCAATTTGTTGATTA
AATTCAAAAAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATATGAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCACATTGC
GCCCTCTGGTATTCCAGAGGGCATGCCTGTTTGAGCGTCATTTCTCTCTCAAGCCCTCGGGTTTG
GTATTGAGTGATACTCTTAGTCAGACTAGGCGTTTGCTTGAAAAGTATCGGCATGAGTAGTACTAG
ATAGTGCTTTCAGGATATTTCAATGTATTAGGTTTATCCAACTCGTTGAGAATTCTTGGTAGTGAAT
TTTTAGTATCATGGCTCTGCCTTACAAAACAACAAACAAGTTTGACCTCAAATCAGGTAGGATTAC
CCGCTGAACTTAAGCATATCAATAAGCGGAGGAA

Chapter III

Part II: Screening assay

Lipid content

		TPST6	CSOH1	SSOH12	CPOH4	*C.c.*	*S.c.*
day 4	%lipid/dry biomass	20.95	7.88	10.44	27.17	33.07	6.39
	Standard deviation	1.12	0.34	0.32	1.55	1.90	0.22
day 5	%lipid/dry biomass	24.54	8.46	11.20	34.63	41.54	7.72
	Standard deviation	2.03	1.14	1.23	2.86	5.77	0.87

Fluorescence/OD$_{600}$

		TPST6	CSOH1	SSOH12	CPOH4	*C.c.*	*S.c.*
day 2	units	4,344	1,180	914	10,179	56,096	6,211
	Standard deviation	594	104	84	4,343	10,651	578
day 3	units	8,213	1,807	1,038	49,164	98,416	6,314
	Standard deviation	1,205	269	164	14,387	21,607	760
day 4	units	9,259	2,061	942	50,971	118,540	6,935
	Standard deviation	981	193	118	28,144	20,111	1,021
day 5	units	16,639	3,611	1547	n.d.	149,778	6,300
	Standard deviation	1736	201	195	n.d.	0.0	914

Fluorescence/dry biomass

		TPST6	CSOH1	SSOH12	CPOH4	*C.c.*
day2	units/(g/L)	7,271	1,224	1,810	19,284	158,444
day3	units/(g/L)	18,719	1,863	2,854	263,658	317,012
day4	units/(g/L)	22,459	2,025	2,942	357,313	430,753
day5	units/(g/L)	52,620	4,039	5,040	n.d.	618,119

IX. Curriculum Vitae

Ines Schulze

Email: Schulze.Ines@googlemail.com

Personal details

Date of birth   20th August 1982
Place of birth   Neuwied
Nationality   German

Professional experience

08/2009-01/2013	***Karlsruhe Institute of Technology (KIT), Institut of Process Engineering in Life Sciences Section II: Technical Biology, Karlsruhe*** - Scientific assistant at the chair of Prof. Dr. Christoph Syldatk
02/2008 - 05/2009	***Fraunhofer Institute for Systems and Innovation Research (ISI) Competence Center Emerging Technologies, Karlsruhe*** - Scientific employee at the department of Prof. Dr. Thomas Reiß

Academic training (international)

Since 08/2009	***Karlsruhe Institute for Technology (KIT), Institute of Process Engineering in Life Sciences Section II: Technical Biology, Karlsruhe*** - Dissertation at the chair of Prof. Dr. Christoph Syldatk *„Microbial lipid production with oleaginous yeasts"*
10/2013-12/2013	***University of Modena and Reggio Emilia, Faculty of Bioscience and Biotechnology, Modena, Italy*** - Research stay at the chair of Prof. Dr. Maddalena Rossi *„Oleaginous yeasts"*
01/2007 - 09/2007	***Fraunhofer Institute for Molecular biology and applied ecology (IME), Aachen*** - Diploma thesis at the chair of Prof. Dr. Stefan Schillberg *„Construction, expression and characterization of a recombinant fusion protein for the targeted inhibition of colon cancer and metatasis"*
07/2005 - 08/2005	***Institute for Molecular Bioscience, Brisbane, Australia*** - Research internship at the department of Prof. Dr. Robert Capon *„Anticancer agents from Australian marine biodiversity"*
10/2004 - 09/2007	***Ecole Supérieure de Biotechnologie (ESBS), Strasbourg, France*** - Tri-national study course **Biotechnology** (Master/ Diploma) of the European universities Strasbourg, Karlsruhe, Freiburg und Basel
10/2002 - 10/2004	***TH Karlsruhe (since 2009 KIT), Karlsruhe*** - Intermediate diploma of the study course **Bioengineering**