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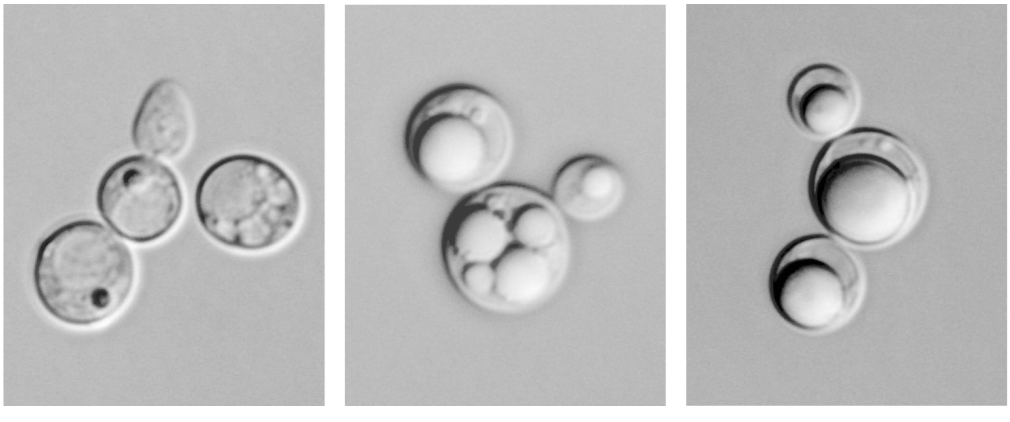
Jule Brandenburg



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Cover: *Lipomyces starkeyi* with increasing intracellular lipid content from left to right (Photo: Jule Brandenburg)

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# Lipid production from lignocellulosic material by oleaginous yeasts

Abstract

Oleaginous yeasts are a potential lipid source for production of fuels, chemicals and food or feed and use of lignocellulosic biomass as feedstock is considered a sustainable approach. Pre-treatment of lignocellulose is required to release the major carbon sources, glucose, xylose and other sugars for lipid production by oleaginous yeasts, but also releases inhibitory compounds. Aim of this thesis was to examine the potential for producing lipids from different lignocellulosic substrates using oleaginous yeasts and to develop analytical methods for monitoring the kinetics of lipid accumulation, as a basis for further investigations of physiological differences in oleaginous yeasts on different substrates.

Investigations of 29 different oleaginous yeast strains revealed considerable differences in xylose utilisation capacity, even among very closely related strains. Some strains were very efficient in accumulating lipids from all carbon sources in lignocellulose hydrolysate, others showed no or only weak growth on xylose and in one case intracellular lipid degradation during consumption of xylose was observed. Further investigation demonstrated that it is possible to combine furfural production from hemicellulose and microbial lipid or ethanol production from the cellulose fraction of wheat straw material. An investigation of lipid production from birch wood hemicellulose containing high amounts of xylose and acetic acid revealed that by establishing a pH-regulated feeding strategy, acetic acid could be utilised as an additional carbon source and no growth inhibition was observed. Target parameters when studying lipid-accumulating yeasts are intracellular lipid content and lipid profile. However, classical extraction-based analytical methods are time- and work-intensive. Therefore, a non-invasive method based on high-throughput Fourier transform infrared (FTIR) spectroscopy was established.

Overall, large diversity among oleaginous yeasts was revealed, especially when converting xylose. Promising strains for lipid production from different substrates were identified, providing a baseline for further studies on the physiology of oleaginous yeasts and on biotechnological production of microbial lipids.

Keywords: microbial lipids, FTIR, lipid extraction, hydrolysate, biorefinery

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# Lipid produktion från lignocellulosisk biomassa med lipidackumulerande jäst

Sammanfattning

Lipidackumulerande jästarter är potentiella källor för lipider till drivmedel, kemikalier och foder- och livsmedelsproduktion och att använda lignocellulosisk biomassa som råvara anses som ett hållbart tillvägagångssätt. Förbehandling av lignocellulosa krävs för att kunna använda det som substrat till lipidackumulerande jästarter men förbehandlingen frigör även inhiberande ämnen.

Målet med denna avhandling var att undersöka potentialen hos lipidackumulerande jästarter att producera lipider från olika lignocellulosiska substrat och att utveckla analytiska metoder för att följa lipidkinetiken som en grund för fortsatta studier av fysiologiska skillnader hos lipidackumulerande jästarter.

En undersökning av tjugonio olika lipidackumulerande jästarter avslöjade anmärkningsvärda skillnader i xylosutnyttjande, även mellan närbesläktade stammar. Några ackumulerade lipider väldigt effektivt från alla sockerarter i lignocellulosiskt hydrolysat, men i ett exempel observerade vi lipidnedbrytning vid xyloskonsumtion. Vidare visades det att det är möjligt att kombinera furfuralproduktion från hemicellulosa och lipid- eller etanolproduktion från den kvarvarande cellulosafraktionen av halm. Ett annat substrat som testades för lipidproduktion var hydrolysat av björk, innehållande stora mängder xylos och ättiksyra. Genom att tillsätta hydrolysatet med hjälp av pH-regleringen, kunde ättikssyra användas som kolkälla av jästen och ingen hämning av tillväxt observerades. Lipidhalt och lipidprofil är parametrar man undersöker hos lipidackumulerande jästarter. Då de klassiska extraktionsbaserade analysmetoderna är både tids -och arbetsintensiva etablerades en icke-invasiv analysmetod baserat på Fourier transform infraröd (FTIR) spektroskopi.

Denna avhandling upptäckte en stor diversitet mellan lipidackumulerande jästarter, speciellt vid xylosanvändning. Lovande stammar för lipidproduktion från olika substrat identifierades. Avhandlingen ger en grund för vidare studier av lipidackumulerande jästarter och utvecklingen av bioteknologisk produktion av mikrobiella lipider.

Keywords: mikrobiella lipider, FT-IR, lipidextraktion, hydrolysat

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To my family

# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Brandenburg, J., Blomqvist, J., Shapaval, V., Kohler, A., Samples, S., Sandgren, M. & Passoth, V. (2021). Different responses of oleaginous yeast to carbon sources present in lignocellulose hydrolysate (manuscript).

II. Brandenburg, J., Poppele, I., Blomqvist, J., Puke, M., Pickova, J., Sandgren, M., Rapoport, A., Vedernikovs, N. & Passoth, V. (2018). Bioethanol and lipid production from enzymatic hydrolysate of wheat straw after furfural extraction. *Applied Microbiology and Biotechnology*, 102 (14), 6269-6277.

III. Brandenburg, J., Blomqvist, J., Pickova, J., Bonturi, N., Sandgren, M. & Passoth, V. (2016). Lipid production from hemicellulose with *Lipomyces starkeyi* in a pH regulated fed batch cultivation. *Yeast* 33 (8), 451-462.

IV. Shapaval, V., Brandenburg, J., Blomqvist, J., Tafintseva, V., Passoth, V., Sandgren, M. & Kohler, A. (2019). Biochemical profiling, prediction of total lipid content and fatty acid profile in oleaginous yeasts by FTIR spectroscopy. *Biotechnology for Biofuels* 10 (1), 1-10.

The contribution of Jule Brandenburg to Papers I-IV was as follows:

I. Took part in planning the project. Performed almost all laboratory work. Main writer of the manuscript.

II. Took part in planning the project. Performed main parts of the laboratory work. Involved in supervision of IP. Took part in writing the manuscript.

III. Took part in planning the project. Performed all laboratory work. Took part in writing the manuscript.

IV. Took part in planning the project. Performed most laboratory work. Involved in computer analysis. Minor part in writing the manuscript.

In addition to Papers I-IV, Jule Brandenburg contributed to the following papers within the timeframe of the project:

Blomqvist, J., Pickova, J., Tilami, S.K., Samples, S., Mikkelsen, N., Brandenburg, J., Sandgren, M. & Passoth, V. (2018). Oleaginous yeast as a component in fish feed. *Scientific Reports* 8 (1), 15945

Tiukova, I., Brandenburg, J., Blomqvist, J., Samples, S., Mikkelsen, N., Skaugen, M., Arntzen, M. Ø., Nielsen, J., Sandgren, M. & Kerkhoven, E.J. (2019). Proteome analysis of xylose metabolism in *Rhodotorula toruloides* during lipid production. *Biotechnology for Biofuels* 12 (1), 1-17.

Chmielarz, M., Sampels, S., Blomqvist, J., Brandenburg, J., Wende, F., Sandgren, M. & Passoth, V. (2019). FT-NIR: A tool for rapid intracellular lipid quantification in oleaginous yeasts. *Biotechnology for Biofuels* 12 (1), 1-9.

# Abbreviations

| | |
|---|---|
| AMP | Adenosine monophosphate |
| ARA | Arachidonic acid |
| ATP-CL | Adenosine triphosphate-citrate lyase |
| CDW | Cell dry weight |
| CoA | Coenzyme A |
| DAG | Diacylglycerol |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| FAME | Fatty acid methyl esters |
| FTIR | Fourier transform infrared |
| FTNIR | Fourier transform near-infrared |
| G-3-P | Glycerol-3-phosphate |
| GC | Gas chromatography |
| GLA | Gamma-linolenic acid |
| HMF | Hydroxymethylfurfural |
| HPLC | High-performance liquid chromatography |
| ICDH | Isocitrate dehydrogenase |
| IMP | Inosine monophosphate |
| LCA | Life cycle assessment |

| | |
|---|---|
| LD | Lipid droplet |
| MAG | Monoacylglycerol |
| ME | Malic enzyme |
| NLM | Nitrogen-limited medium |
| MUFA | Monounsaturated fatty acid |
| NAD | Nicotinamide adenine dinucleotide |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| OD | Optical density |
| PPP | Pentose phosphate pathway |
| PUFA | Polyunsaturated fatty acid |
| RMSE | Root mean square error |
| SAT | Saturated fatty acid |
| TCA | Tricarboxylic acid |
| TAG | Triacylglycerol |
| TLC | Thin layer chromatography |

# 1. Introduction

## 1.1 Context and research project

Modern society is mainly based on fossil resources, for example fuel production, and many branches of the industry rely heavily on petrol polymers. This is problematic due to the finite nature of fossil resources and their alarmingly strong negative influence on climate change. The major challenge for mankind is to change to a bio-based economy, using renewable resources in a sustainable way (Lewandowski, 2018). Biomass in the form of lignocellulose is an excellent precursor for replacing fossil resources. Strictly speaking, fossil resources also derive from biomass origins, but are highly reduced in oxygen and stored for a very long time (Sato, 1990). On utilising fossil fuels, the stored carbon is released to the atmosphere as carbon dioxide, whereas new biomass binds carbon dioxide. Thus using new biomass will lead to a more balanced carbon cycle (Spagnuolo *et al.*, 2019). Lignocellulose is rich in oxygen and also has the potential to generate other chemicals than those generated from substrates of petrochemical origin. However, for fuel production or in the oleochemical industry, there is a need to have a reduced oxygen content in the feedstock (Demirbas, 2011). Oleaginous yeasts are able to convert carbon present in lignocellulose to lipids, as a potential resource for fuels, chemicals and even for food or feed production, due to their general similarity to vegetable oils (Patel *et al.*, 2016; Bharathiraja *et al.*, 2017). Using hydrolysed lignocellulosic material as a feedstock for microbial lipid production is considered a sustainable approach (Huang *et al.*, 2013; Qin *et al.*, 2017; Valdés *et al.*, 2020).

This PhD thesis was part of a research programme examining many aspects of sustainable microbial lipid production from lignocellulosic material. The underlying concept for the programme is shown in Figure 1.

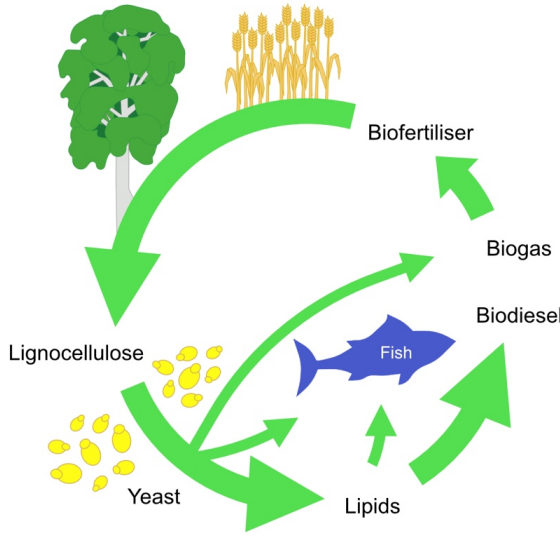



*Figure 1*. Underlying concept of the LipodrivE research programme for sustainable microbial lipid production for fuels and feed in a circular biorefinery approach, including utilisation of the residues for biogas and their recirculation as fertiliser.

Starting a circular production process with the selection of lignocellulosic material obtained as a by-product or waste product from agriculture or forestry was the foundation of the research concept. This approach minimises direct competition with food (or feed) production or undesirable land use change. Lignocellulosic material has to be pre-treated to break up its recalcitrant structure and make the constituent carbohydrates available. These carbohydrates can be converted into microbial lipids with the help of oleaginous yeasts. This thesis investigated specific research questions in this part of the research programme, *e.g.* how different substrates can be converted by different yeasts (Papers I-III). Different applications of microbial lipids were also considered within the research programme, for instance feed production in terms of fish feed (Blomqvist *et al.*, 2018). Left over material obtained after lipid extraction was tested for its biogas potential and residues from biogas plants (biodigestate) can be used as fertiliser to

close the circle to agricultural production chains. System analysis and life cycle assessment (LCA) were used to evaluate the impact of microbial lipid production (Karlsson *et al.*, 2016; Karlsson *et al.*, 2017). Overall, the research programme contributed valuable knowledge needed to create a more sustainable bio-based economy.

## 1.2 Aims

The main aim of this thesis was to expand knowledge about oleaginous microorganisms, especially about their growth and lipid accumulation on lignocellulosic material. Substrate generated from lignocellulosic material is challenging for microorganisms due to presence of (i) diverse sugars, for example xylose, which many microbes can metabolise and others cannot utilise; and (ii) presence of molecules with inhibitory effects on microorganisms, *e.g.* acetic acid, furfural or hydroxymethylfurfural (HMF).

Differences in physiology between several oleaginous yeast species and strains of the same species in terms of lipid accumulation were investigated to identify promising yeast strains for lipid production on hydrolysate generated from wheat straw (Paper I). Splitting the lignocellulosic material in different fractions provides the possibility to combine microbial lipid production with other industrial branches (Papers II & III). Other specific objectives were to investigate whether it is possible to combine furfural production with microbial lipid production (Paper II) and how to use the hemicellulose fraction with high inhibition properties to produce microbial lipids (Paper III).

Target parameters when studying lipid-accumulating yeasts are intracellular lipid content and lipid profile. In this regard, classical analytical methods based on lipid extraction are rather time-consuming and work-intensive, so alternative lipid quantification and characterisation techniques are needed. Therefore, another specific objective of the work in this thesis was to establish a non-invasive method based on high-throughput Fourier transform-infrared (FTIR) spectroscopy for prediction of lipid content and profile of several yeast strains in a robust model (Paper IV).

# 2. Historical background

Lipid accumulation in microorganisms has been in focus of research for a very long time, although research interests and specific research objectives have changed greatly over the years.

Yeasts have long been used for food production in terms of brewing and baking, with utilisation of baker's yeast representing a breakthrough for development in human civilisation (Hayden *et al.*, 2013). Knowledge and usage of microorganisms increased around the start of the 19$^{th}$ century. Lipid accumulation in yeast was first described in 1878, with the observation of intracellular lipid drops (Nägeli & Loew, 1878). However, it was not until the First World War and the nutrition problems of that time that research began to be devoted to this finding. To identify a new source of edible fats, microorganisms with lipid accumulation capacity were studied more closely during that period. These initial investigations of lipid metabolism revealed that some microorganisms were able to accumulate high amounts of lipids, while others could not. As cultivation methods were poorly developed and little was known about lipid metabolism, microbial lipid production for food applications was unsuccessful at that time (Lindner, 1922).

A bit later, already in 1938, low protein content in substrates for yeast cultivation was found to be associated with increased lipid accumulation (Steiner, 1938). Equipped with more knowledge and again with increasing nutrition problems occurring during the following years due to the Second World War, lipid production on an industrial scale was considered again. Molasses and lignocellulosic hydrolysates from wood and straw were tested as a feedstock for lipid-accumulating yeasts. A few years after the Second World War had ended, it had been determined that microbial lipid production was indeed feasible, but uneconomical. This is because microbial lipids could not compete with lipids of animal or vegetable origin, although it was

assumed that they could be interesting due to the presence of unsaturated fatty acids, for example linoleic and linolenic acid (Lundin, 1950; Woodbine, 1959).

Research in the following decades focused on lipid characterisation and similarities to other lipid resources were demonstrated (Deinema & Landheer, 1956). For instance, the structure of triacylglycerols (TAG) was investigated, fatty acids and their positions on the glycerol backbone were characterised and similarities to highly valuable lipids, such as cacao butter, were discovered (Thorpe & Ratledge, 1972). In the early 1980s, research interest shifted towards using microbial oils as cocoa butter equivalents, in order to compensate for higher production costs compared with common vegetable oils (Thorpe & Ratledge, 1972; Moreton, 1988; Ratledge, 2010). Another focus in research at that time was to understand the metabolism of oleaginous yeasts (Holdsworth & Ratledge, 1988; Holdsworth *et al.*, 1988; Ratledge, 1988) and to identify cultivation conditions or possible substrates (Evans & Ratledge, 1983; Ykema *et al.*, 1988; Johnson *et al.*, 1995). Cost efficiency has been found to be the major factor for microbial lipid production, and this can be achieved either by generating high-value products or using extremely cheap substrates (Ratledge & Cohen, 2008).

Especially in the past decade, much research has been done on lipid-accumulating yeasts. A review of annual number of publications since 1990 (found on PubMed, 2021) in response to the search query “oleaginous yeast” indicates how this research field is growing (Figure 2).

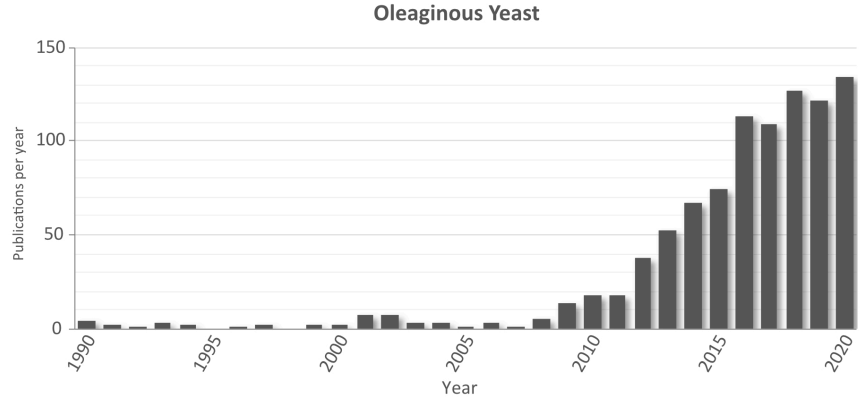



*Figure 2*. Number of publications per year including the key word "oleaginous yeast" found in PubMed on March 2021.

The term oleaginous yeast is a common key word, although of course not all relevant publications can be found in this way. A check of databases for publications about relevant species, *e.g. Lipomyces starkeyi*, *Rhodotorula toruloides* or *Yarrowia lipolytica*, showed the same pattern of increasing numbers of publications and revealed the enormous amount of information generated by research. In particular, *Y. lipolytica* as a model organism has been studied quite extensively (Figure 3).

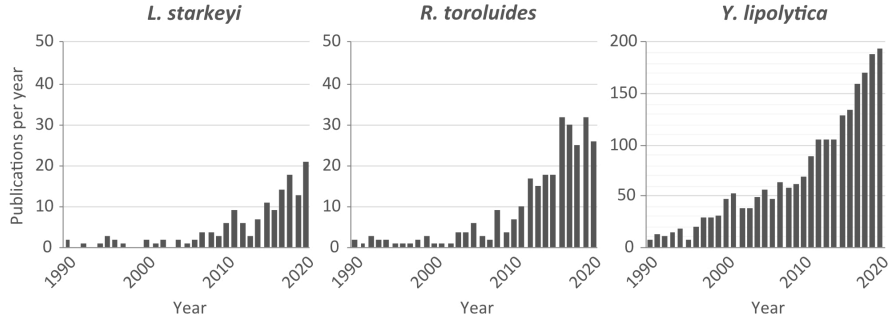



*Figure 3*. Number of publications per year on oleaginous yeast species and including the key word "*L. starkeyi*", "*R. toruloides*" and "*Y. lipolytica*" found in PubMed in March 2021.

Current research is quite diverse and is exploring many different aspects of oleaginous yeasts. For example, some research groups are investigating different yeast species, cultivation conditions or different substrates (Sitepu *et al.*, 2013; Karamerou & Webb, 2019). A variety of -omics studies have been performed, including genomics, transcriptomics, proteomics and metabolomics (Liu *et al.*, 2011; Morin *et al.*, 2011; Coradetti *et al.*, 2018; Tiukova *et al.*, 2019; Kim *et al.*, 2021). Furthermore, work has been done on optimisation of lipid production from several perspectives, such as optimisation of cultivation, strain adaptation or genetic engineering (Shi & Zhao, 2017; Marella *et al.*, 2018). Studies with the focus on modification of lipid composition have also been performed (Görner *et al.*, 2016). Much work has been done on investigating the metabolism of oleaginous yeasts (Pinheiro *et al.*, 2020), for instance in terms of flux calculation (Liu *et al.*, 2016).

In addition, all of this work has been reviewed extensively from different aspects, *e.g*. metabolism (Papanikolaou & Aggelis, 2011; Donot *et al.*, 2014),

individual species (Park *et al.*, 2018; Takaku *et al.*, 2020), substrates (Huang *et al.*, 2013; Qin *et al.*, 2017) or applications (Bharathiraja *et al.*, 2017; Khot *et al.*, 2020). The research field on oleaginous yeasts also extends to system analysis (Biddy *et al.*, 2016; Karlsson *et al.*, 2016; Wang *et al.*, 2018) and life cycle analysis, which are important to get a good perspective on the benefit of microbial lipid production from oleaginous yeasts (Karlsson *et al.*, 2017; Parsons *et al.*, 2018; Bonatsos *et al.*, 2020).

# 3. Oleaginous microorganisms

Microorganisms are able to synthesise lipids for different functions in their cells, for example as membrane components, as storage lipids or for regulatory functions (Sandager *et al.*, 2002; Lingwood & Simons, 2010; Eisenberg & Büttner, 2014). The proportion of lipids in dry cells is usually around 7 – 15% (Kaneko *et al.*, 1976). Oleaginous microorganisms are able to convert carbon into storage lipids and are defined by their ability to accumulate more than 20% of their cell dry weight (CDW) as lipids (Ratledge & Wynn, 2002).

The type and yield of lipids accumulated in microorganisms depends among other factors on the origin, meaning type of organism, the culture conditions and the carbon source provided (Balan, 2019). These lipids are storage lipids and are usually found in lipid droplets inside yeast cells, with only some exceptions. Figure 4 shows *Lipomyces starkeyi* as an example of oleaginous yeast with and without accumulated intracellular lipids.

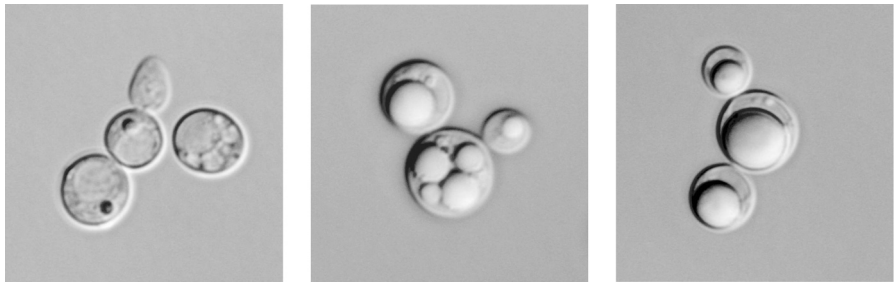


*Figure 4*. Lipid accumulation in the oleaginous yeast species *Lipomyces starkeyi*, with increasing lipid content from left to right.

### 3.1.1 Overview of different oleaginous microorganisms

Oleaginous organisms are widespread in both eukaryotic and prokaryotic organisms and are found among yeasts, filamentous fungi and algae, to a lesser extent in bacteria and even among some marine protists (Balan, 2019).

Many algae are photoautotrophic, using sunlight and carbon dioxide for lipid production, but some are heterotrophic and use organic carbon as a feedstock (Balan, 2019). The suitability of phototrophic algae for biodiesel production is rather poor, due to high cost of production and low productivity rates. However, higher-value compounds produced by these algae are definitely interesting to investigate. A very important example are polyunsaturated fatty acids (PUFAs) produced by oleaginous algae, which are used in the food industry. Pharmaceutical and other industries also have great interest in these products and other compounds with high value, such as carotenoids, astaxanthin, phenols *etc*. (Ranga Rao *et al.*, 2010; Goiris *et al.*, 2012). One example is a Swedish company (Simris) selling n-3 oil produced from algae. Some algae are not strictly phototrophic and are able to grow on different types of wastewater from industries. Cultivation on wastewater and other residues is a desirable way to generate valuable products in a biorefinery approach (Bellou *et al.*, 2014; Bellou *et al.*, 2016).

Lipids generated by filamentous oleaginous fungi contain high amounts of long-chain PUFAs, *e.g.* gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA). For example, *Mortierella alpina* and *Mucor circinelloides* were used commercially to produce ARA and GLA, as high-value products (Yao *et al.*, 2019).

Only a few bacterial species are known to accumulate lipids in high amounts. They have high production rates and require simple cultivation conditions. Some store neutral lipids in the form of TAG in lipid droplets and some others store lipids as polyhydroxyalkanoates in the outer membrane (Alvarez & Steinbüchel, 2002; Meng *et al.*, 2009). Some microbial lipid producers are found among marine protist species (Wang *et al.*, 2019). Oleaginous yeasts are another important group of microbial lipid producers and are described in detail in the following sections.

### 3.1.2 Oleaginous yeasts

*Saccharomyces cerevisiae* is the best known yeast and called conventional yeast, but the diversity of non-conventional yeasts is huge. Among these non-conventional yeasts, oleaginous yeasts can be found. The ability to accumulate lipids in high amounts is known among ascomycetes and basidiomycetes. Many different species have been described, with the most studied yeasts being *Yarrowia lipolytica*, *Lipomyces starkeyi* and *Lipomyces lipofer* belonging to the ascomycetes, and *Rhodotorula toruloides*, *Rhodotorula glutinis*, *Rhodotorula babjevae* and *Cutaneotrichosporon curvatum* (syn. *Cryptococcus curvatus*) belonging to the basidiomycetes (Kurtzman *et al.*, 2011; Sitepu *et al.*, 2014). Many of these yeasts can grow on various carbon sources such as glucose, xylose and other sugars such as cellobiose, and on glycerol, fats or organic acids. Multiple types of substrates have been tested, for instance by-products or wastes from industry such as molasses, whey, pulp and paper mill wastewater, sewage sludge or hydrolysate from different biomass types, such as wheat straw, corn stover, rice straw, sugarcane bagasse, softwoods, hardwoods or grasses (Huang *et al.*, 2013; Qin *et al.*, 2017; Valdés *et al.*, 2020).

## 3.2 Lipid metabolism in oleaginous yeasts

Oleaginous yeasts accumulate lipids as storage lipids, usually in the form of TAGs. It is generally possible to divide oleaginous yeast growth into two phases, a growth phase and a lipid accumulation phase (Morin *et al.*, 2011). In the growth phase, all nutrients are abundant and typical growth in form of generating cell biomass occurs. Lipid accumulation takes place when there is a surplus of a carbon source (glucose *etc*.) combined with limited availability of nutrients, such as nitrogen, phosphorus, sulphur *etc*. (Granger *et al.*, 1993). Under these conditions, carbon flux is directed towards lipid synthesis (Ratledge & Wynn, 2002). The biosynthetic pathway is almost the same as in non-oleaginous yeasts, with some specialities in *e.g.* enzyme activity, as described in the following section using the example of nitrogen limitation.

### 3.2.1 Lipid accumulation

Nitrogen limitation leads to a change in metabolism, as shown in the simplified overview in Figure 5. In order to provide ammonium ions ($NH_4^+$)

for cell maintenance, adenosine monophosphate (AMP) deaminase cleaves AMP into inosine monophosphate (IMP) and $NH_4^+$ to overcome the extracellular nutrient limitation. The associated steep decrease in intracellular AMP influences the activity of parts of the tricarboxylic acid (TCA) cycle, where the isocitrate dehydrogenase (ICDH) in oleaginous yeasts, allosterically activated by AMP, is deactivated. Inside the mitochondria, ICDH is responsible for transforming isocitrate into α-ketoglutarate. On losing this activity, isocitrate and therefore also citrate, which occurs in an equilibrium with isocitrate, are accumulated within the mitochondria. On reaching a critical value, citrate is transported out of the mitochondria into the cytoplasm in an exchange with malate, presumably by citrate/malate shuttle (Evans *et al.*, 1983).

Citrate is subsequently cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase (ATP-CL), a key enzyme present in the cytosol in oleaginous microorganisms. This is at the expense of ATP and the opposite step of citrate synthetase in the TCA cycle. The increasing amount of acetyl-CoA is further shuttled into fatty acid synthesis, which takes place in the cytoplasm. On the other hand, oxaloacetate is converted via malate dehydrogenase to malate, which is used in countering the citrate efflux system (Ratledge & Wynn, 2002; Papanikolaou & Aggelis, 2011; Donot *et al.*, 2014) (Figure 5).

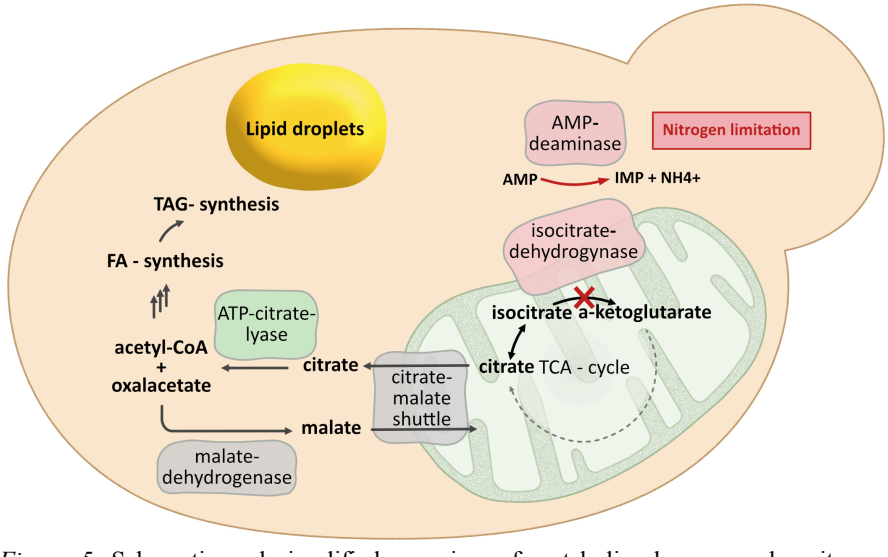



*Figure 5*. Schematic and simplified overview of metabolic changes under nitrogen limitation in oleaginous yeast, leading to intracellular lipid accumulation.

Fatty acid synthesis is carried out via the fatty acid synthase complex. Acetyl-CoA is converted into malonyl-CoA by acetyl-CoA carboxylase. In the next step, the fatty acid synthase complex forms acyl-CoA from acetyl-CoA and malonyl-CoA. For Acyl-CoA formation, NADPH is required. In most cases it is probably generated by 6-phosphogluconate dehydrogenase of the pentose phosphate pathway (PPP) or by maleic enzyme (ME (Ratledge, 2014). In some cases, for example in *L. starkeyi*, ME is NADH-dependent, not NADPH-dependent, and might not be connected to fatty acid synthesis (Tang *et al.*, 2010). The acyl-CoA chains produced are transferred to the endoplasmic reticulum, where esterification with glycerol-3-phosphate (G-3-P) takes place to generate either structural lipids (such as phospholipids or glycolipids) or storage lipids in the form of TAGs (Fakas, 2017).

Synthesis of TAGs takes place via the Kennedy pathway, where one acyl-CoA molecule is first attached to a G-3-P molecule with the help of G-3-P-acyltransferase in the sn-1 position to generate 1-acyl-G-3-P. This molecule is then further acylated in the sn-2 position to form phosphatic acid and further dephosphorylated to release diacylglycerol (DAG), which is acylated by an acyltransferase to generate TAG (Kennedy, 1961; Beopoulos *et al.*, 2009). Lipids are stored in intracellular lipid droplets (LD), also called lipid bodies. Biogenesis of LD starts between the two membrane leaflets of the endoplasmic reticulum. The mechanisms involved are not yet fully understood, but enzymes connected to the TAG synthesis have been found in these organelles. Thus it is proposed that lipid droplets grow by synthesising TAGs on their surface (Athenstaedt *et al.*, 2006; Zanghellini *et al.*, 2010; Garay *et al.*, 2014).

One interesting consideration is the lipid yield generated from carbon sources. It can be calculated from the number of moles of acetyl-CoA generated from carbon sources, from which then the maximum amount of lipids formed can be assessed. Different carbon sources have different theoretical yield, *e.g.* glucose corresponds to 1.1 mole acetyl-CoA and xylose to 1.2 mole, resulting in a maximum theoretical yield for lipid production of 0.32 g lipids / g glucose and 0.34 g lipids / g xylose (Evans & Ratledge, 1984; Ratledge & Cohen, 2008; Papanikolaou & Aggelis, 2011). Taking the origin of NADPH involved in fatty acid synthesis into consideration, these values shift slightly. For example if generated via maleic enzyme, the yield will drop to 0.31 g lipids / g glucose, while if NADPH comes from PPP the theoretical maximum will be 0.27 g lipids / g glucose (Ratledge, 2014).

This is one factor explaining the practical yield obtained in lipid accumulation, which is usually between 20 – 22% because carbon is also used for generating non-lipid biomass (Ratledge & Cohen, 2008). With genetically modified strains of *Y. lipolytica*, values of up to 0.28 respectively 0.29 g lipids / g glucose and 0.28 were achieved (Tai & Stephanopoulos, 2013; Qiao *et al.*, 2017).

### 3.2.2 Lipid degradation/lipid turnover

When the carbon is exhausted or a decrease in carbon uptake takes place, most oleaginous microorganisms start to consume their storage lipids, a process described as lipid degradation or lipid turnover. This was already studied in 1988 in different oleaginous yeast species (Holdsworth & Ratledge, 1988). Lipid degradation was observed in that study when previously accumulated lipids under nitrogen limitation in excess of carbon were produced and then the culture conditions were changed to carbon starvation in the presence of nitrogen. An exception was *Lipomyces starkeyi*, for which no lipid degradation was observed. Further it was observed that *Lipomyces starkeyi* had fewer peroxisomes than other oleaginous microorganisms for which lipid degradation was observed (Holdsworth *et al.*, 1988).

The biochemical degradation of lipids is described as β-oxidation. It starts with the activity of TAG lipases, which hydrolyse the ester bonds in TAGs and release fatty acids from the glycerol backbone (Müllner & Daum, 2004). Once fatty acids are released, they are activated by acyl-CoA synthetase to generate acyl-CoA in the cytosol. This acyl-CoA is transported into the peroxisomes. The oxidation cycle is the complete oxidation of a fatty acid molecule, shortening it each time between C2 and C3 and releasing an acetyl-CoA (Hiltunen *et al.*, 2003). Each oxidation cycle is divided into four steps (Fransen *et al.*, 2017). A schematic overview of the process is given in Figure 6. Some investigations have shown mitochondrial β-oxidation for instance in *Yarrowia lipolytica* (Beopoulos et al., 2011). There, the intermediate molecules are the same as in peroxisomal β-oxidation, but the pathway is driven by different enzymes (Kunau *et al.*, 1995).

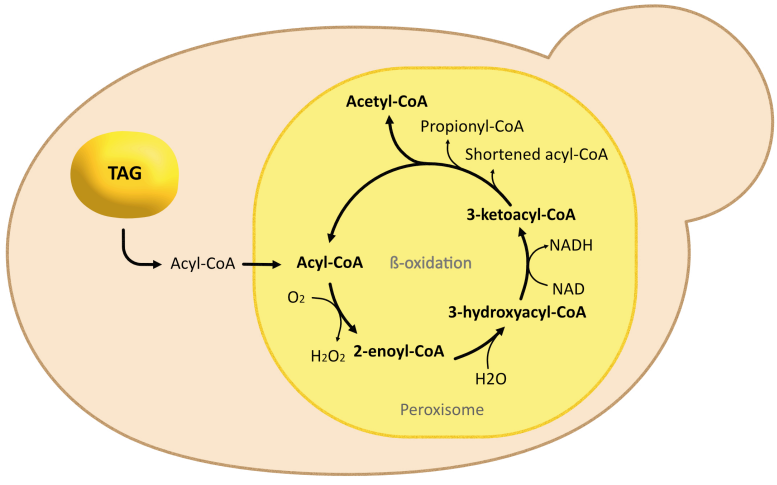



*Figure 6.* Schematic and simplified overview of β-oxidation in peroxisomes in yeasts. The β-oxidation cycle is divided into four steps, which are repeated until acyl chains are oxidised completely. Diagram modified from Fransen *et al.* (2017).

# 4. Yeast lipids and vegetable oils

### 4.1.1 Lipids in yeast

The major form of lipids in oleaginous yeasts, TAGs, are mainly discussed in this thesis. These lipids are also known as neutral lipids and consist of a glycerol backbone with three fatty acid chains (Figure 7). They are often found in lipid droplets as storage lipids. Other important lipids classes in yeasts are phospholipids, sphingolipids, free fatty acids, monoacylglycerols (MAGs), diacylglycerols (DAGs), sterols and wax esters (Rattray *et al.*, 1975).


*Figure 7.* Example of a triacylglycerol (TAG), with the glycerol backbone linked by ester bonds to palmitic acid, oleic acid and linolenic acid.

### 4.1.2 Fatty acid composition in oleaginous yeasts

The fatty acid profiles in oleaginous yeasts can differ between species and culture conditions, but in general they are similar. Present fatty acids are mainly long-chain fatty acids (14 – 20 carbons) and the composition is quite similar to those in vegetable oils. The most prominent fatty acids present in oleaginous yeasts are oleic acid (C18:1), palmitic acid (C16:0), linolenic acid (C18:2), stearic acid (C18:0) and palmitoleic acid (C16:1) (Table 1). Linolenic acid (C18:3), myristic acid (C14:0) and some other fatty acids are also found, but in most cases only in small amounts.

Table 1. *Examples of fatty acid composition in lipids from different oleaginous yeasts grown on nitrogen-limited media containing glucose as a carbon source*[1]

| | Palmitic acid C16:0 | Palmitoleic acid C16:1 | Stearic acid C18:0 | Oleic acid C18:1 | Linoleic acid C18:2 | Linolenic acid C18:3 |
|---|---|---|---|---|---|---|
| ***L. starkeyi*** | 31.7 | 4.1 | 5.8 | 53.2 | 2.8 | 0.3 |
| ***L. lipofer*** | 23.0 | 4.9 | 4.4 | 61.1 | 3.5 | 0.7 |
| ***R. babjevae*** | 22.4 | 2.6 | 1.6 | 54.5 | 11.5 | 4.1 |
| ***R. toruloides*** | 26.6 | 2.1 | 3.1 | 46.2 | 16.4 | 2.2 |
| ***R. graminis*** | 20.3 | 1.6 | 2.5 | 57.4 | 11.4 | 3.5 |
| ***S. terricula*** | 32.9 | 3.2 | 8.9 | 49.6 | 2.8 | 0.0 |

[1]Raw data from Paper IV, summarised in supplementary Table S1 in that paper. Cultivation conditions and extraction method are described in Paper IV.

### 4.1.3 Vegetable oils

Around 205 million tons of vegetable oils are currently produced worldwide per year and the amount is increasing. Five years ago, it was about 177 Mt and 25 years ago only 75 Mt (Figure 8). Thus, vegetable oil production has increased by 15% only within the past five years and has almost tripled within the past 25 years. The main oils produced are palm oil (35.7% of the total amount on the world market), including palm kern oil (39.9%), soybean oil (27.8%), rapeseed oil (13.4%) and sunflower oil (10.2%) (USDA, 2020; Mielke, 2018) (Figure 8).

The main applications of vegetable oils are in food production, but they are also used in industry and increasingly in biofuel production. The main reasons for the drastic increase in oil production are the growing global demand for food, the expansion of oleochemical demand and the rapidly increasing energy market, especially for generation of biodiesel in recent decades (Mielke, 2018). The most dramatic development has occurred in the palm oil sector, where production has doubled every decade in the past 30 years. Soybean oil production has also increased tremendously and it is now the second most abundant vegetable oil.

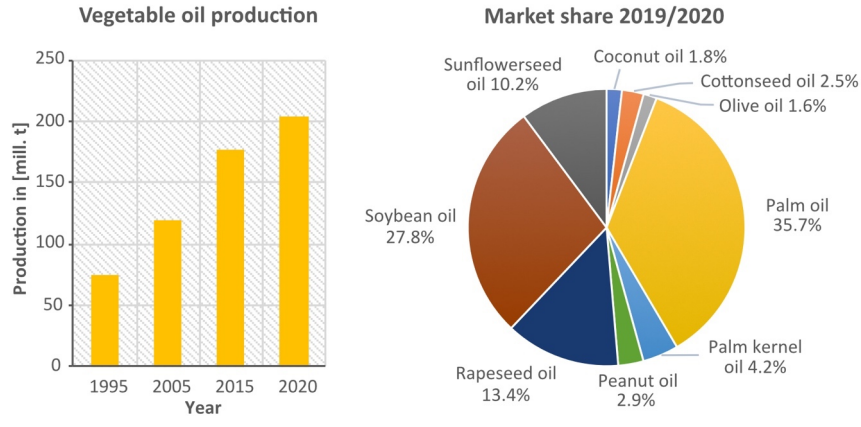



*Figure 8.* Vegetable oil production (Mt per year) and vegetable oil market share (%) in the 2019/2020 season. Data from USDA (2020).

Increasing vegetable oil production is causing different kinds of problems. Agricultural land is needed for planting oil crops. The fastest growing oil commodity, palm oil, is produced mainly in Indonesia and Malaysia. To obtain land for palm tree plantation much deforestation occurs, with 50 – 60% of new plantations established by removing forest, including rainforest. This is reported to have a huge negative impact in terms of greenhouse gas emissions, and consequently a negative climate impact. Furthermore, loss of natural forest causes loss of biodiversity (Koh & Wilcove, 2008). Similar problems are associated with soybean production, which also causes deforestation and land use change (Carvalho *et al.*, 2019). Production of rapeseed oil and sunflower oil is not directly associated with problems such as deforestation or land use change, as it is commonly done on existing agricultural land, but it can still have negative climate impacts (Uusitalo *et al.*, 2014). On the other hand, productivity is lower in rapeseed oil production than in palm oil production. The oil yield generated from palm trees is reported to be 5 – 7 t/ha/year, while the oil yield in rapeseed production is much lower, only 1 – 2 t/ha/year (Zimmer, 2010).

### 4.1.4 Composition of vegetable oils

Plant oils consist mostly of storage lipids in the form of TAGs (95 – 98%). Fatty acid composition is similar in many vegetable oils and the fatty acids most commonly present are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and a-linoleic acid (C18:3) (Table 2) (Dubois *et al.*, 2007).

Table 2. *Overview of fatty acid composition of different plant oils, summarised from Dubios* et al. *(2007)*

| | **Palmitic acid C16:0** | **Stearic acid C18:0** | **Oleic acid C18:1** | **Linoleic acid C18:2** | **Linolenic acid C18:3** |
|---|---|---|---|---|---|
| **Palm oil** | 43.8 | 4.4 | 39.1 | 10.2 | 0.3 |
| **Soybean oil** | 10.8 | 3.9 | 23.9 | 52.1 | 7.8 |
| **Rapeseed oil** | 5.1 | 1.7 | 60.1 | 21.5 | 9.9 |
| **Sunflower oil** | 6.4 | 4.5 | 22.1 | 65.6 | 0.5 |
| **Cacao butter** | 25.1 | 36.4 | 34.1 | 2.8 | 0.3 |

In addition to TAGs, some other components (usually comprising less than 5%) are found in vegetable oils. These components have important biological properties and nutritional value. They can be divided into glycerolipids, such as MAGs, DAGs and phospholipids, and non-glycerolipids, such as sterols, free fatty acids, some vitamins (A, D, E and K) and pigments (Aluyor *et al.*, 2009).

## 4.2 Applications for vegetable oils and microbial lipids

### 4.2.1 General overview

Vegetable oils play an important role in the human diet, as they are used for cooking and as a food ingredient, directly or in industrial food products. As mentioned in Chapter 2 of this thesis, microbial lipids were first considered for food applications over one hundred years ago, but production is so far not economically feasible. The similarity to vegetable oil composition supports their suitability for food applications. Fatty acids in the form of saturated fatty acid (SATs), monounsaturated fatty acids (MUFAs) and PUFAs are important in metabolism for energy supply. They are also involved in cell division and growth, as they are part of cellular membrane systems.

Furthermore, they are components in signalling systems such as hormones or neurotransmitters. The composition of TAGs in the diet has an influence on human health. For instance, high amounts of SATs increase the risk of cardiovascular disease, while increased amounts of PUFAs in the diet can decrease the risk of these diseases, if implemented appropriately in the diet. The PUFAs can be categorised by the position of the double bond, with n-3 or n-6 fatty acids most commonly occurring in the human diet, and the ratio between these has a great impact on human health (a ratio of 4:1 is recommended in food intake to get a beneficial health impact) (Simopoulos, 2002).

Other important fatty acids for human nutrition are some long-chain PUFAs such as arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5) and very long-chain PUFAs (defined by their length with 22 or more carbons) such as docosahexaenoic acid (C 22:6), which are usually derived from marine resources instead of vegetable oils or microbial lipids from yeasts. They are important for brain development and can have a preventive effect on cardiovascular diseases (Yara-Varón *et al.*, 2017).

As predatory fish in marine ecosystems feed on other fish, they naturally have a high intake of long-chain fatty acids in their diet, which is reflected in the fatty acid composition of their muscle. Due to the increased demand for fish for human consumption, aquaculture has increased greatly during recent decades (FAO, 2020). Therefore, there is higher demand for fish feed and, to mimic the natural diet, fish meal or oil is used. With increasing fish production, this is not feasible and/or sustainable, so part of the fish meal and oil are replaced with plant oils and plant-based proteins. Recent studies have also shown the possibility to use insects (Turek *et al.*, 2020) or oleaginous yeast as an alternative to plant-based ingredients in fish diets (Blomqvist *et al.*, 2018). This has the advantage that lipid extraction is not essential, since insects or yeast cells can be included in the fish feed and provide proteins in addition to lipids. Unfortunately, the alternative lipids from plants, insects or yeasts do not contain EPA and DHA, which must be still added as fish oil to maintain fish as a resource for LC-PUFAs in the human diet. Blending in lipids from *e.g.* algae can be an approach to replace even more fish meal and fish oil in fish feed or genetically modified yeasts can be used to produce EPA and DHA, and thereby increase the content of these fatty acids in the feed (Norambuena *et al.*, 2015; Gemperlein *et al.*, 2019).

Besides food and feed applications, vegetable oils and potentially microbial lipids can be used for instance in the oleochemical, pharma and cosmetic industries, in products such as soaps, detergents, lubricants, varnish, paints and other wood treatment products (Koutinas *et al.*, 2014). Another huge application sector is biofuel production, which is discussed in detail in the following section.

### 4.2.2 Oils for biofuel production

Biofuel production has increased tremendously over the past 20 years. Bioethanol is still the most abundant biofuel on the market and its production has increased in recent decades. An even higher increase has occurred on the biodiesel market. Biodiesel is mainly produced from vegetable oils (Figure 9).

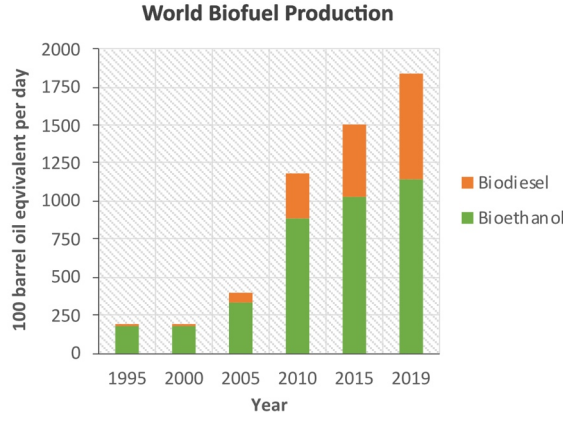



*Figure 9*. Changes in world biofuel production during the period 1995 – 2019. Data from BP (2019).

The biodiesel market was practically non-existent around the turn of this millennium, while in 2019 the overall amount of biodiesel on the market was estimated to be around 45 Mt (Bockey, 2019). Approximately 80% (in 2017) of all biodiesel produced is generated directly from plant oil and an additional 9% from used cooking oils. Thus, a substantial part of the vegetable oil on the market is used for biodiesel production (UFOP, 2017). In 2008, rapeseed oil was the most common feedstock used for biodiesel production in the

European Union, whereas now palm oil and soybean oil are the most used feedstocks (Mielke, 2018).

The increased usage of vegetable oils for biofuel production causes competition between food and feed production and is a driving force for increased vegetable oil production, with all its environmental consequences. Therefore a debate about food versus fuel production started already in the early 2000s and discussion on the feasibility of sustainable biofuel production is ongoing (Thompson, 2012). Within this discussion, biofuels generated from crops are defined as first-generation biofuels, while second-generation biofuels are produced from non-edible resources, for instance lignocellulosic material. In terms of bioethanol production this is an established process, *e.g.* use of hydrolysed sugarcane bagasse for fermentation processes. Biogas production using lignocellulosic material and other waste products, such as manure or organic waste blends, is also becoming more common (Abraham *et al.*, 2020). Microbial lipid production from lignocellulose or waste products can be an alternative resource for biodiesel production, to achieve a more sustainable process. The TAGs produced by oleaginous yeasts need to be transesterified, as it is done for vegetable oils, into fatty acid methyl or ethyl esters, making them similar to the hydrocarbon molecules in diesel (Meher *et al.*, 2006). These fatty acid esters can be blended into diesel without modification of current diesel engines (Patel *et al.*, 2017).

### 4.2.3 Advantages of microbial oils

An advantage of microbial lipid production compared with vegetable oils is that lipids can be produced all year round, irrespective of season or climate. Furthermore, microbial lipids can be produced on land that is unsuitable for agriculture and yields per hectare are higher than in plant oil production. The substrate needed for microbial lipid production can be diverse, *e.g.* by-products and waste products from industry, agriculture or forestry can be used (Bharathiraja *et al.*, 2017).

It is possible to genetically modify the metabolism of yeasts to produce high amounts of specific fatty acids, in order to create high-value products meeting the demands of industry. So-called designer lipids can play an important role for use of lipids in targeted applications, increasing specific yields and decreases the need for refinery processes (Görner *et al.*, 2016).

# 5. Lignocellulose

### 5.1.1 Lignocellulose structure

Lignocellulose is the most abundant biomass on earth but, due to its complex and rigid structure, it is inedible for humans. Lignocellulose is composed of three main components, cellulose, hemicellulose and lignin, in different ratios, depending on its origin (Table 3).

Table 3. *Composition of lignocellulosic material of different origins. Modified from Cai et al.,* (2017)

| | Cellulose | Hemicellulose | Lignin |
|---|---|---|---|
| **Wheat straw** | 35.0 – 39.0 | 23.0 – 30.0 | 12.0 – 16.0 |
| **Hardwood - birch** | 40.3 – 46.8 | 23.6 – 28.2 | 25.1 – 25.2 |
| **- poplar** | 50.8 – 53.3 | 26.2 – 28.7 | 15.5 – 16.3 |
| **Softwood - pine** | 45.0 – 50.0 | 25.0 – 35.0 | 25.0 – 35.0 |
| **Corncob** | 33.7 – 41.2 | 31.9 – 36.0 | 6.1 – 15.9 |
| **Sugarcane bagasse** | 25.0 – 45.0 | 28.0 – 32.0 | 15.0 – 25.0 |

Cellulose is a homo-polysaccharide composed of D-glucose linked by β-1,4-glycosidic bonds, packed tightly in cellulose fibres. It has a crystalline structure and is composed of several hundred to tens of thousands of glucose monomers. Native cellulose has a high degree of polymerisation, is insoluble in water and is difficult to hydrolyse. Moreover, in most conditions cellulose is encased in hemicellulose and lignin.

Hemicellulose is a hetero-polysaccharide with differing composition and structure in different plants (Gírio *et al.*, 2010). These polysaccharides have a lower degree of polymerisation, are often branched and their side-chains can be acetylated. Hemicelluloses are classified by their main sugar polymer

backbone, *e.g.* xylan, which is composed of β-1,4-linked xylose and may also contain some sugars, such as L-arabinose, D-galactose, D-glucose and D-mannose, in the sugar backbone. Wheat straw, belonging to the grass family, is mainly composed of glucuronoarabinoxylan, whereas birch, belonging to hardwoods, is mostly composed of 4-O-methyl-glucuronoxylans, with both containing mainly xylose. Softwood instead contains more C-6 sugars, in this case mannose in the form of galactoglucomannan. The hemicellulose structure is more easily hydrolysable than the cellulose fraction.

Lignin is a complex aromatic polymer composed of three different types of phenyl propane units, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, connected by alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds. The lignin structure can form covalent bonds to the hemicellulose structure and cover the cellulose structure, protecting it from microbial or chemical degradation (Jørgensen *et al.*, 2007; Jönsson & Martín, 2016; Cai *et al.*, 2017).

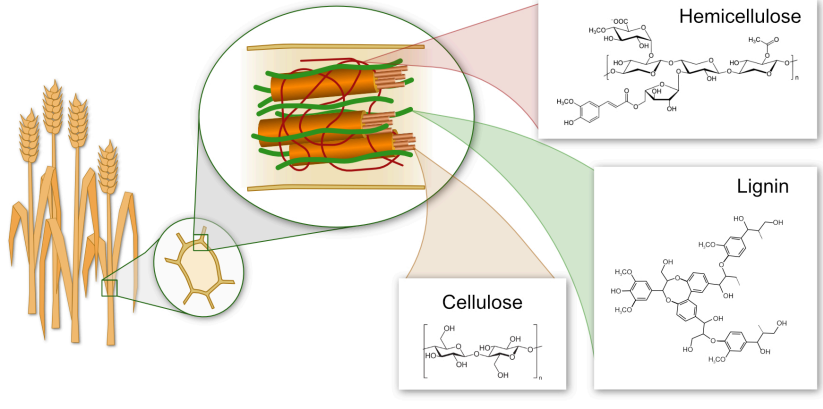



*Figure 10*. Structure of lignocellulosic biomass and its three main components, hemicellulose, cellulose and lignin.

### 5.1.2 Pre-treatment of lignocellulose

The recalcitrant structure of lignocellulose protects it from degradation. A pre-treatment is required to break up the structure and improve the accessibility to enzymes for saccharification. Pre-treatment can be physical, chemical or a combination of these. Mechanical pre-treatment such as milling, grinding or cutting reduces the particle size and increases the surface of the complex structure by reducing the crystallinity and the degree of polymerisation (Volynets & Dahman, 2011).

Mechanical pre-treatment is commonly followed by a thermochemical pre-treatment such as acid-catalysed steam explosion, hot water extraction, or lime or alkaline pre-treatment. All these different methods have their own advantages and drawbacks (*e.g.* formation of inhibitors) (Zheng & Rehmann, 2014).

To finally release the sugar monomers, the pre-treated material is exposed to enzymatic degradation. Commonly used enzyme cocktails contain cellulases, including endoglucanases, to reduce the degree of polymerisation by cleaving β-1,4 glyosidic bonds within the cellulose chains, exoglucanases to degrade cellulose from the ends and release cellobiose, and β-glucosidases to cleave cellobiose into glucose monomers (Jørgensen *et al.*, 2007). Enzyme cocktails also contain hemicellulases, including endoxylanases to hydrolyse internal bonds within the xylan chain, xylosidases to release xylose molecules from xyloologosaccharides (respective enzymes for mannan) and other enzymes to remove side-groups like galactosidases or glucuronidases (Jørgensen *et al.*, 2007; Biely *et al.*, 2016).

## 5.2 Lignocellulose conversion to microbial lipids and other chemicals

During this thesis work, several different substrates were used to produce microbial lipids, representing several different ways to connect microbial lipid production to other industrial branches. In the work, hydrolysate generated from wheat straw was used as the main feedstock (Papers I, II & IV), where either the hemicellulose and cellulose fraction was used for lipid production or only the cellulose fraction. One study was performed on hydrolysate generated from birch wood and only the hemicellulose fraction was used (Paper III). The solid fractions, containing most of the left-over

lignin, were not used in these studies. The lignin fraction has possible applications, but these were beyond the scope of the thesis.

Wheat straw as a waste or by-product from agriculture was pre-treated with steam explosion to use the whole hydrolysate containing the cellulose and hemicellulose fraction (Paper I). Wheat straw was also pre-treated by acid-based thermochemical steam extraction, but in this study the hemicellulose fraction was removed to generate furfural as a high-value chemical. The cellulose fraction was hydrolysed to complete the biorefinery concept with the prospect of microbial lipid/fuel generation from the residues from the furfural production process (Paper II). In the study presented in Paper III, the hemicellulose fraction from birch hydrolysate was used for microbial lipid production. It derived from hot water extraction and was similar to wastewater from the pulp and paper industry, connecting microbial lipid production to that industry (Paper III).

### 5.2.1 Yeasts for lipid production on lignocellulose hydrolysate

Many previous studies have evaluated microbial lipid production from different substrates. Metabolic pathways for carbon source assimilation, lipid accumulation and degradation have been investigated. Much is known about regulation of these pathways, but some steps are still not entirely understood.

Combined understanding of sugar assimilation and lipid accumulation, especially on hydrolysate generated from lignocellulosic material, is a key element for future sustainable microbial lipid production. In the present work, the utilisation capacity of glucose and xylose in combination with lipid production by 29 yeast strains belonging to five different species was evaluated. Variability in xylose utilisation capacity was observed even among very closely related strains, including strains not previously described as differing in xylose utilisation capacity in the literature (Kurtzman *et al.*, 2011). For instance, the ascomycete *Lipomyces starkeyi* has been used for xylose conversion and studied intensively (Anschau *et al.*, 2014; Sitepu *et al.*, 2014; Probst & Vadlani, 2017; Xavier *et al.*, 2017), but anyhow a number of *L. starkeyi* strains were found not to be capable of utilising xylose when presented as a sole carbon source (Paper I). Understanding the genetic basis of these differences would enable theory-based selection of promising lipid producers from among a multitude of strains. Similar findings were obtained for the basidiomycetes species, *R. babjevae* and *R. glutinis* (Paper I; Sitepu *et al.*, 2014).

A variety of strains were also tested on wheat straw hydrolysate containing the cellulose and hemicellulose fractions and showed quite diverse responses to the harsh growth conditions. Some were found to produce high amounts of lipids (Table 4), whereas others did not, although they were all able to do so on artificial culture medium. The effect on growth of *R. toruloides* of several inhibitory compounds, present in hydrolysate from lignocellulosic material, in different concentrations and synergistic effects, has been studied previously (Hu *et al.*, 2009; Zhao *et al.*, 2012). For instance, Sitepu *et al*. (2014) investigated 45 strains and their ability to utilise different carbon sources, including glucose and xylose, and their tolerance to inhibitors commonly present in hydrolysate from lignocellulose, including furfural, HMF or acetic acid. Several promising strains were suggested as possible lipid producers on different substrates but the importance of testing several strains of one species was emphasised, due to variable responses of tested factors and variable descriptions in the literature (Sitepu *et al*., 2014). However, yeast growth on hydrolysate was not tested in that study. A similar approach involving cultivation on hydrolysate was performed by Chen *et al*. (2009), who tested 10 different strains for their ability for sugar utilisation and inhibitor tolerance, followed by cultivation of one strain on diluted corn stover hydrolysate (Chen *et al.*, 2009). In another study, several strains were tested on hydrolysate from corn stover and switch grass and two promising strains were identified and used for further investigations (Slininger *et al.*, 2016). In a two-stage fermentation process, high lipid concentrations of up to 29 g/L lipids from *L. starkeyi* and 26 g/L lipids from *R. toruloides* were reached, with sugar conversion yields of up to 0.15 g lipids/g sugar (Slininger *et al.*, 2016). A more recent study was able to increase the yield to 0.24 g lipids/g sugar for *C. curvatum* and *R. toruloides* and 0.16 g lipids/g sugar for *Trichosporon guehoae*, and achieved high intracellular lipid content of 63%, 61% and 48%, respectively (i Nogué *et al.*, 2018). These values are in line with results obtained in Paper I generated with *R. babjevae* from wheat straw hydrolysate (Table 4).

Table 4. *Growth and lipid production by five oleaginous yeast strains on undiluted wheat straw hydrolysate. Cultivations were performed in triplicate. Lipid yield was calculated as g lipids per g carbon source consumed (*lipid content determined gravimetrically by lipid extraction, **lipid content determined by Fourier transform near-infrared (FTIR) spectroscopy). Standard deviation for all lipid yield values was ±0.01*

| Strain | Time [h] | CDW [g/L] | Final lipid content [% of CDW] | Final lipid concentration [g/L] | Lipid yield [g/g] |
|---|---|---|---|---|---|
| ***R babjevae*** DVBPG 8058 | 96 | 28.02 (±1.03) | 64.81 (±2.97)* | 18.12 (±0.63) | 0.24 |
| ***R. glutinis*** CBS 2367 | 168 | 17.76 (±1.14) | 12.11 (±1.10)* | 2.04 (±0.17) | 0.03 |
| ***R. toruloides*** CBS 14 | 91 | 29.82 (±0.25) | 39.31 (±2.65)** | 11.72 (±0.64) | 0.15 |
| ***L. starkeyi*** CBS 1807 | 120 | 17.76 (±0.37) | 33.21 (±2.13)** | 5.9 (±0.54) | 0.07 |
| ***L. starkeyi*** CBS 7544 | 138 | 30.90 (±0.50) | 38.48(±1.67)* | 11.9 (±0.56) | 0.16 |

Another interesting aspect of this study was observed lipid degradation of intracellular lipids in *R. glutinis* CBS 2367 (Paper I). The lipids were generated while glucose was assimilated and subsequently degraded while still carbon was present in the substrate in the form of xylose. Lipid degradation occurred at the same time as xylose was being assimilated. Lipid degradation has been observed previously when nitrogen limitation was lifted and under exhaustion of the carbon source (Holdsworth & Ratledge, 1988) or under starvation and at low substrate uptake rates. Xylose as a carbon source can be a challenging substrate. For example, in a engineered *S. cerevisiae* strain used for xylose fermentation, a starvation response was observed although the yeast contained all genes required for xylose assimilation (Bergdahl *et al.*, 2012). In *R. toruloides*, induction of proteins involved in β-oxidation has been observed (Tiukova *et al.*, 2019), which may also indicate a starvation response.

### 5.2.2 Lignocellulose conversion to furfural and potential biofuels

Wheat straw from agriculture was used for furfural production and combined with production of two possible fuel products in a biorefinery approach, presented in Paper III (Figure 11).

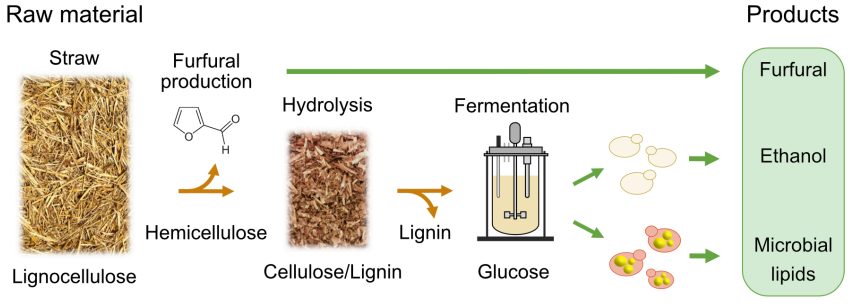



*Figure 11.* Conversion of wheat straw to furfural and biofuels.

Furfural is one of the oldest renewable chemicals and is only derived from lignocellulose biomass (Peters, 1936). It is generated by dehydration of sugars, mainly xylose, present in this biomass (Zeitsch, 2000). Furfural is a natural precursor for furan-based chemicals and is considered as a promising renewable platform chemical (Cai *et al.*, 2014). It can be converted into a variety of solvents, polymers, fuels and other useful chemicals by a range of catalytic reductions (Chen *et al.*, 2018). Production of furfural dates back to the early 20$^{th}$ century and has been performed at industrial scale since 1922. As furfural is generated from the hemicellulose fraction of lignocellulosic material, the residues are usually burned to generate heat for the production process, or may even be used for electricity supply. A disadvantage of production stream was the low yield, which was around 50% of the theoretical yield. Some more recent attempts during the past decade have achieved production yields of up to 70-80% (Cai *et al.*, 2014). A second disadvantage in the production process was that the cellulose fraction may be destroyed. A method developed by (Vedernikovs *et al.*, 2010), which leaves the cellulose mainly intact, was used in Paper III for possible microbial lipid production from wheat straw after furfural production.

The solid fraction obtained was enzymatically hydrolysed to release the glucose present. Conversion of the cellulose fraction of lignocellulosic material into ethanol is an established process for second-generation biofuel production (Lynd *et al.*, 2017). Problematic for ethanol production using

*S. cerevisiae* or *D. bruxellensis* is the low nitrogen content of the hydrolysate after furfural production. Lignocellulosic material generally tends to have low nitrogen content and needs adding or blending for industrial applications in ethanol production or biogas production (Abraham *et al.*, 2020). After adding a nitrogen source, complete conversation of glucose to ethanol was observed (Paper III).

In order to use the hydrolysate as it is, without adding any nitrogen, biolipid production with oleaginous yeast can be considered. The low nitrogen content can even be regarded as an advantage, because it triggers lipid production inside the yeast cells.

Two different strains for lipid production were tested. It was observed that this substrate did not cause any problems for cultivation, with *L. starkeyi* and *R. babjevae* being able to utilise all the carbon present. The main differences found were in cultivation time and conversion rate. With *R. toruloides*, yield of 0.17 g lipids per g glucose was reached, with a total amount of 7.14 g/L lipids, whereas *L. starkeyi* produced 0.09 g lipids per g glucose, resulting in 3.48 g/L lipids (Paper III). Time is an important factor in industrial applications to create cost- and energy-efficient production chains (Karlsson *et al.*, 2016).

### 5.2.3 Hemicellulose conversion to microbial lipids

In another experiment reported in Paper III, birch wood hydrolysate was used as substrate. Birch wood chips were pre-treated with hot water extraction to release the hemicellulose fraction (Helmerius *et al.*, 2010). It then resembled a waste fraction from the pulp and paper industry. Even when the final pH was increased to pH 3 by addition of calcium hydroxide (CaOH), it was still quite inhibitory to yeasts (Paper III). The final composition was $45.06 \pm 0.44$ g/L xylose, $0.46 \pm 0.60$ g/L glucose, $13.07 \pm 0.16$ g/L acetic acid and $4.7 \pm 0.04$ g/L furfural. Different attempts to deal with highly toxic substrates are reviewed in the literature (Chandel *et al.*, 2013). Detoxification may be an option, but can be challenging to implement in a scaled-up process. Furthermore, it is quite expensive and laborious (Moreno *et al.*, 2017). Increasing pH was observed to be an option to increase at least the tolerance to acetic acid (Paper III). At lower pH, the proportion of undissociated acetic acid increases and this undissociated form can more or less freely diffuse through the cell membrane and has strong inhibitory potential (Sugiyama *et al.*, 2015). This was in line with observations made

for oleaginous yeasts tested for acetic acid tolerance by Sitepu *et al*. (2014), who found presence of 2.5 g/L acetic acid to be inhibitory to all strains tested (45 in total), when the pH was set to 3. This is rather low, but usually not a problem for oleaginous yeasts (Sitepu *et al.*, 2014). On increasing the pH to 6, it was found that 4 g/L acetic acid in substrate was not inhibitory for *L. starkeyi* (Paper II) and that 5 g/L acetic acid was not inhibitory for *R. glutinis*, *R. babjevae*, *R. toruloides* and *L. starkeyi* (Paper I), all grown at pH 6. To overcome strong inhibitory effects of a substrate, dilution is an option, but this unfortunately also decreases the carbon source concentration within the substrate, which may result in carbon limitation in batch cultures. Strain adaptation to a substrate can be useful. For instance, the hemicellulose fraction of sugarcane bagasse has been used to produce lipids, using *R. toruloides* (Bonturi *et al.*, 2017). However, the concentrations of components like sugars and inhibitors were much lower than in experiments presented in this thesis. Furthermore, the substrate was blended with an additional carbon source. Fed batch cultures have been shown to be an efficient way to obtain high-density cultures and high lipid yields (Li *et al.*, 2007; Wiebe *et al.*, 2012; Probst & Vadlani, 2017). It is commonly performed with concentrated pure sugar solutions to optimise the outcome of cultivation. Implementing a strategy to feed cultivations with pure hydrolysate as presented in Paper II made it possible to produce lipids from the hemicellulose fraction. Other attempts to use hemicellulose fraction from wheat straw include a study by Yu *et al*. (2011) in which several strains were tested, revealing one *R. toruloides*, one *L. starkeyi* and one *C. curvatum* as promising strains, also able to utilise undetoxified hydrolysate. However, the concentrations of inhibitors and carbon were about three-fold lower than in Paper III. Recently, a large study investigated the use of wheat straw hydrolysate as substrate, both the cellulose and hemicellulose fraction. *L. starkeyi* and *R. toruloides* were tested in differently concentrated hydrolysates, both detoxified and not detoxified (Liu *et al.*, 2020). Using instead the hemicellulose fraction from sugarcane bagasse for microbial lipid production with *L. starkeyi* led to a yield of 0.14 g lipids/g sugars, while a fed batch approach increased the yield to 0.18 g lipids/g sugars (Xavier *et al.*, 2017). However, the hemicellulose fraction had approximately three fold lower concentrations of sugars and inhibitors than hydrolysate used in this thesis. This can be one reason why it was possible to grow yeasts on undiluted substrate (Xavier *et al.*, 2017).

# 6. Lipid determination

Lipid quantification and determination of lipid composition are commonly based on lipid extraction. Lipophilic solvents are used in existing methods for lipid extraction (Folch *et al.*, 1957; Bligh & Dyer, 1959), now usually combined with some pre-treatment of cell biomass prior to extraction. Thereafter, lipid composition can be further analysed by *e.g.* gas chromatography (GC) or thin layer chromatography (TLC). Lipid quantification is crucial in many types of studies investigating oleaginous yeast physiology in terms of lipid accumulation kinetics, conversation rates, yield determination or lipid composition. Due to the robustness of yeast cells, lipid extraction is challenging and requires a harsh pre-treatment to break the cell structure. Extraction and analysis methods are labour-intensive, time-consuming and a large amount of cell material is needed. Furthermore, large amounts of solvents, which in most cases are highly toxic, are required for these determination methods. This creates a need for alternative lipid analysis tools that avoid these disadvantages.

### 6.1.1 Lipid extraction

Most studies investigating lipids in oleaginous yeasts use lipid extraction methods based on a modified Folch extraction method (Folch *et al.*, 1957). The method was originally developed to extract lipids from animal brain tissues and uses a chloroform/methanol mixture. Cell material is homogenised and the lipid fraction is separated from the rest by phase separation and collected in the chloroform layer. Total fat content determination is done by gravimetric measurement. For lipid extraction from oleaginous yeasts, some optimisation and pre-treatment of the material is needed to enable extraction of most intracellular lipids, since intracellularly stored lipids are protected by the complex and tight cell wall structure and

membrane system in yeast cells (Jacob, 1992). Various mechanical, chemical and enzymatic pre-treatments of cell biomass are currently used to disrupt the cells and render most of the intracellular lipids accessible. Methods include acid-catalysed hot water treatment, autoclaving, bead beating, homogenisation, microwave radiation, ultrasonication or thermolysis (Patel *et al.*, 2019). Much work has been done on optimisation of lipid extraction (Bonturi *et al.*, 2015; Patel *et al.*, 2019). In our lab we established a working protocol including certain modifications from the original Folch method. The final protocol is described in Paper II. In brief, these modifications involve freeze-drying the cells prior to extraction, dissolving them in 1 M HCl and incubating them for 1 hour at 75°C. This was found to improve lipid extraction compared with other cell disruption methods such as sonication or French press or using wet cell material. According to results obtained in some pre-experiments, higher acid concentration did not increase lipid extraction efficiency, but using a temperature of 75°C for incubation was found to increase lipid extraction efficiency compared with lower temperatures.

### 6.1.2 Lipid analysis

*Fatty acid profile*

Fatty acid composition can be analysed in different ways and is often performed using GC or HPLC analysis. For GC analysis, the extracted lipids, specifically the fatty acids, need to be transesterified, for example by methylation, prior to separation (Appelqvist, 1968; Paper III). The prepared sample is injected into the GC, separated while being transported with a carrier gas through a column and subsequently recognised by a detector (Raja, 2019). Alternative analytical methods, for example HPLC, LC/MS or NMR analysis, share the requirement for extraction of lipids prior to analysis. These techniques were not used during this thesis work and are thus not addressed further, but they have their own advantages (and drawbacks). The most common fatty acids in oleaginous yeasts are palmitic acid, palmitoleic acid, stearic acid, oleic acid and linolenic acid. All are possible to separate by GC and identifiable and quantifiable by comparing with standards. A detailed analysis of lipid composition is in many cases of great interest, but is labour- and time-intensive. In some cases, the distinction between saturated, monosaturated and polyunsaturated fatty acids already gives the

most interesting information. This analysis is currently performed in the same way as complete fatty acid profile determination. Using a different tool for evaluating the proportion of saturation would speed up the process and provide the most important information on lipid composition already, for example when using spectroscopic analysis of the sample (Paper III).

*Lipid classes*

Lipid class analysis can be performed by TLC, a simple and efficient method. The extracted lipid fraction is applied to a thin plate, usually coated with silica gel, and placed with one edge in a solvent solution. By capillary forces, the solvent and the applied sample are pulled upwards, during which the polarity of the compounds causes interaction with the stationary phase and leads to separation of different molecules (Olsen & Henderson, 1989).

*Challenges*

As mentioned above, lipid extraction is time-consuming and laborious. Freeze-drying the cells prior to extraction also delays determination by at least one day. Faster determination is desirable, especially in screening experiments, in kinetic studies where certain growth stages are analysed or in cases when cultivations are monitored practically online.

The main analytical methods for faster lipid quantification being more intensively studied at present are (a) methods using lipophilic fluorescent dyes, (b) methods based on flow cytometry and (c) methods based on FTIR/FTNIR spectroscopy.

*Lipid staining*

Nile red is a lipophilic lysochrome dye that stains different types of lipids and even neutral lipids inside cells. Nile red staining has promise, but it has limits when it comes to quantification of intracellular lipids in oleaginous yeasts because the accuracy between different tested yeast strains is rather low. This method is quite sensitive to differences in cell culture preparation, strain variations, incubation time (Kimura *et al.*, 2004; Sitepu *et al.*, 2012), and to our experience not easily comparable between different experiments (unpublished results in this thesis). On the other hand, Nile red staining gives impressive results when using fluorescent microscopy for visualisation of intracellular lipid bodies and could be used in screening studies (Shi *et al.*, 2016). However, it has been reported that the emission maximum of different yeast species varies between yeast species and that Nile red staining cannot

be used for exact lipid quantification (Sitepu *et al.*, 2012). Several explanations are possible, for example differences in cell wall structure might lead to differences in stain penetration into the cells (Chen *et al.*, 2009).

Sudan black has been tested for staining intracellular lipids in yeast, but staining could not be established for several different lipid-accumulating yeast strains (Thakur *et al.*, 1989). Recently, LipidTOX™ Green has been described as an alternative dye to stain lipid bodies inside yeast cells, with image processing to determine lipid accumulation, which seems to be a powerful and fast tool for screening but still does not give quantitative lipid content values (Patel *et al.*, 2019).

All these staining techniques have the great advantage of being fast and only require small sample volume and minimal preparation. Staining methods can give an estimate of lipid content.

## 6.2 FTIR/FTNIR

Spectroscopy is a non-destructive method that allows fast and non-invasive investigation of biomolecules, for instance proteins or lipids. Near infra-red (NIR) or mid infra-red (MIR) light is close to the visible light spectra, with wave numbers between 12 500 and 4000 $cm^{-1}$, respective 4000 and 400 $cm^{-1}$. MIR spectroscopy, commonly referred to as IR spectroscopy, can be used to study fundamental vibrations of molecules (El - Azazy, 2018). With NIR spectroscopy, overtones and combination bands can be detected. The advantage of MIR is that the absorption spectrum provides clearer peaks that refer specifically to functional groups. With NIR, the spectra are broader and more undefined, but may hold more information. In comparison with IR/NIR spectroscopy, FTIR/FTNIR spectroscopy uses an additional device, an interferometer, which generates a signal that allows very fast (N)IR spectrum acquisition. However, the interferograms generated need additional “calculations” to generate a full (N)IR spectrum (Raja, 2019).

Any quantitative measurements must always be calibrated against a set of reference values, determined by classical lipid extraction and analysis. Water molecule vibrations cause huge responses in FTIR/FTNIR spectroscopy, strongly interfering with lipid prediction models, so it is important to dry the cells prior to measurement. Advantages of this type of spectroscopy are that almost no sample preparation is required, apart from simple washing and

drying, and that results are given almost in real time. This reduces costs, labour, analysis time and solvent usage (Paper IV). In addition, the sample volume needed can be greatly decreased, making it possible to work in small-scale experiments for screening or lipid kinetics studies (Ami *et al.*, 2014; Forfang *et al.*, 2017; Kosa *et al.*, 2017).

Already in 2013, FTIR has been used to quantify total lipids and unsaturated fatty acids (UFA) in yeasts, *Rhodotorula* species and protists (*Thraustochytrium*). Furthermore, the technique is used for lipid quantification, calibrated against classical lipid analysis, and for species identification using the fingerprint region of the spectra (Vongsvivut *et al.*, 2013). Previously, lipid monitoring using FTIR spectroscopy was implemented by correlating some lipid-correlated peaks against protein-related peaks (Gupta *et al.*, 2012). This work was further developed for three different yeast species (*S. cerevisiae*, *C. curvatum* and *R. toruloides*) by Ami *et al*. (2014). Monitoring of lipid accumulation with FTIR was found to be fast and beneficial, without much sample preparation, but exact lipid quantification was not possible (Ami *et al.*, 2014). For a long time, these were the only attempts at analysing intracellular lipids in oleaginous yeasts by FTIR. In contrast, FTIR lipid determination has been more intensively studied and further developed for analysing intracellular lipids in filamentous fungi (Zimmermann & Kohler, 2013; Kosa *et al.*, 2017).

In Paper IV in this thesis, a wide range of oleaginous yeasts (in total 13 strains belonging to seven different species) were used to establish a prediction model for intracellular lipid content and lipid profile. During this work, different spectral regions and several spectral processing methods were investigated in attempts to build a robust and accurate prediction model for total lipid amount and lipid profile. Figure 12 shows the processed spectra obtained, indicating peaks caused by vibrations of functional groups and the biomolecules to which they mainly refer.

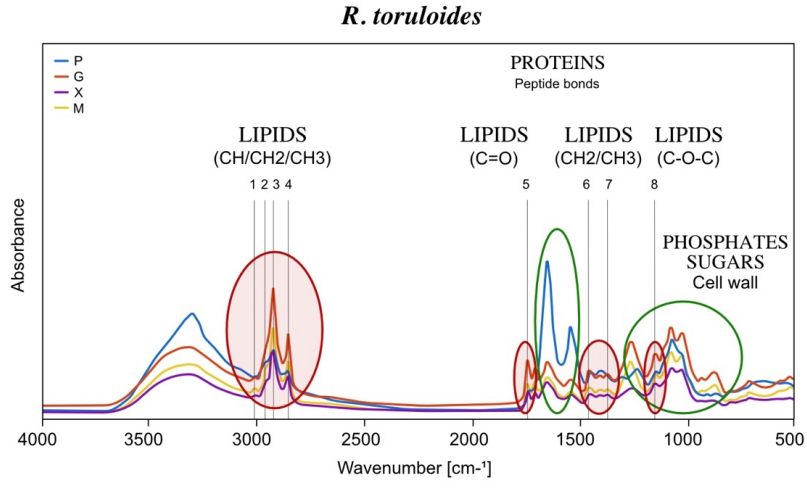



*Figure 12*. Fourier transform infrared (FTIR) spectra of *Rhodotorula toruloides* grown on: YPD medium (P); nitrogen-limited medium (NLM) containing glucose (G); NLM containing xylose (X); and NLM containing glucose and xylose (M). Lipid-relevant peaks corresponding to 1) =C-H stretching, 2) C-H asymmetric stretching of $CH_3$, 3) asymmetric stretching $CH_2$ of acyl chains, 4) symmetric stretching of $CH_2$ of acyl chains, 5) C=O stretching, 6) $CH_2$ deforming, 7) $CH_3$ bending and 8) C-O-C stretching are indicated. Modified from Paper IV.

Spectral region evaluation showed that the spectral range for 3100 - 2800 $cm^{-1}$ was most reliable for prediction of total lipids, SAT, MUFA and PUFA, including peaks corresponding to =C-H stretching, C-H asymmetric stretching of $CH_3$, asymmetric stretching >$CH_2$ of acyl chains and symmetric stretching of $CH_2$ of acyl chains. For total lipid prediction, additionally the spectral region 1800-700 $cm^{-1}$ was also used, including the peak for C=O stretching, regions that refer to proteins, $CH_2$ deforming, $CH_3$ bending, C-O-C stretching, carbohydrates and polyphosphates (Figure 12, for detailed description see Paper IV).

Table 5: *Overview of established lipid prediction models (RMSE = root mean square error, CV = cross validation)*

| Prediction model | Spectral region | RMSE CV | RMSE Test | $R^2_{CV}$ | $R^2_{Test}$ | No. of factors |
|---|---|---|---|---|---|---|
| **Total fat** | 3100 – 2800<br>1800 – 700 | 4.12 | 19 | 0.92 | 0.67 | 10 |
| **SATs** | 3100 – 2800 | 4.50 | 6.70 | 0.78 | 0.73 | 11 |
| **MUFAs** | 3100 – 2800 | 4.99 | 4.34 | 0.79 | 0.79 | 11 |
| **PUFAs** | 3100 – 2800 | 4.58 | 2.43 | 0.73 | 0.75 | 7 |

Four prediction models were established, to quantify total lipid content, SATs, MUFAs and PUFAs (Table 5). Advantages of this method are the small sample volume (<50 µL suspended cell material) and the short time needed before analysis (only one hour for drying the samples). Furthermore, the technique is applied to multi-plates (405) and suitable for high-throughput measurements.

FTNIR/ NIR spectroscopy has been used recently for determining intracellular lipid production in yeasts, using five respective six different yeast strains (Chmielarz *et al.*, 2019; Laurens *et al.*, 2019). Both methods require freeze-drying of the yeast cells prior to analysis. However, compared with conventional lipid extraction, small sample volumes are needed and time and labour requirements are greatly reduced. In addition, no solvents are needed and 96-well high-throughput analysis is possible (Laurens *et al.*, 2019). Accuracy of lipid prediction and profile analysis can be improved by adding more references to the prediction model. These two studies have demonstrated as well that prediction accuracy can be improved while building strain-specific models for lipid production (Chmielarz *et al.*, 2019; Laurens *et al.*, 2019). A robust prediction model including several strains can be beneficial when analysing unknown samples.

*Further work*

Recently, an improved method based on FTNIR spectroscopy was established in our laboratory. It has the advantage of replacing the freeze-drying step currently needed in FTNIR analysis (Chmielarz *et al.*, 2019) with oven-drying of the samples for only 5 - 10 minutes and reducing the amount of sample material down to approximately 100 µl wet cell material. In that way, sample preparation is minimised to washing the cells, transferring 50 µL cell suspension onto a small glass plate, drying and cooling the sample to room temperature before measurement. With this protocol, intracellular lipid content can be predicted within 30 minutes after sampling (Figure 13).

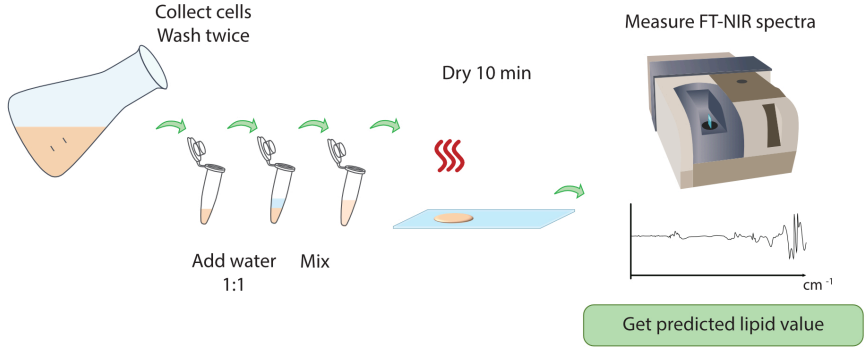



*Figure 13.* Sample preparation for Fourier transform-near infrared (FTNIR) lipid prediction.

These results are preliminary and are based on 161 independent spectra of three strains belonging to different yeast species, *L. starkeyi*, *R. toruloides* and *R. babjevae*. The prediction models were established on pre-processed spectra, using vector normalisation and first derivate, and evaluated with cross-validation, due to lack of samples for test set validation so far. Preliminary results show good correlation for test sets and extension of the prediction models is ongoing. Similar to the two previously established FTNIR/ NIR-based protocols, the improved FTNIR-based method gives higher accuracy in strain-specific lipid prediction models, compared to strain-unspecific prediction models. Still, we have good usage of a robust prediction model which is strain-unspecific for unknown samples, or for lipid prediction of other species/strains not included in the model. Figure 14 shows

two examples of established models, a lipid strain-unspecific prediction model and a strain-specific prediction model for *Lipomyces starkeyi.*

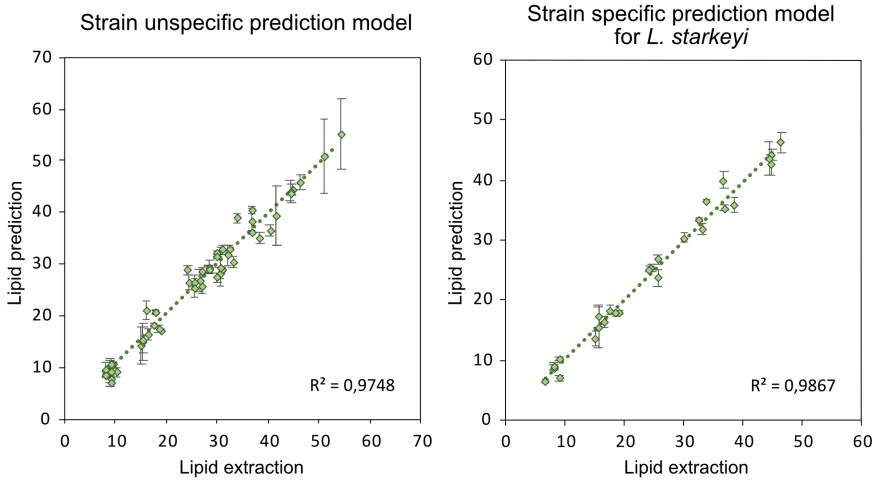



*Figure 14.* Calibration of two Fourier transform near infrared (FTNIR) lipid prediction models. (Left) A strain-unspecific lipid prediction model and (right) a lipid prediction model for *Lipomyces starkeyi*.

# 7. Conclusions and further perspectives

Microbial lipids produced from lignocellulosic materials have potential as alternatives to non-sustainable oil products from fossil resources or vegetable oils. This thesis provided new knowledge on how to utilise hydrolysates, generated from various lignocellulosic materials, as feedstock for microbial lipid production.

Examination of different yeasts cultivated on hydrolysates revealed wide variation in *e.g.* growth and xylose assimilation capacity, even among closely related yeast strains. Following lipid kinetics of these yeast strains showed different responses to similar growth conditions. Some of the yeast strains tested accumulated high amounts of intracellular lipids with high carbon to lipid conversion yields. On the other hand, in one case lipid degradation was observed even in the presence of a carbon source in the form of xylose. Identifying the physiological basis for these differences, for instance by studying transcriptome, proteome and metabolome in conjunction with monitoring of lipid formation kinetics, could generate a knowledge base for genetic modification of yeast strains optimised for lipid accumulation. The acquired knowledge reported in this thesis can also help in selection of suitable yeast strains for microbial lipid production on a more theoretical basis. The work in this thesis has already contributed to the development of a method for monitoring lipid formation kinetics using FTIR spectroscopy. This method allows determination of important aspects of lipid composition easily, without time-consuming sample preparation and extraction, e.g. the degree of unsaturation. The lipid content and profile prediction tool will be further developed in future work. Extending the reference database will increase the accuracy of the prediction models, and thus improve the accuracy of lipid determination.

Embedding microbial lipid production systems in existing biorefinery production chains can improve the sustainability of these processes. The biorefinery concept was tested within this thesis, for instance by combining furfural production, using wheat straw as feedstock, with either ethanol or microbial lipid production. Yeast cultivation on highly toxic hemicellulose material as substrate for the cultivation was also tested, with a pH-regulated feeding strategy. These approaches make it possible to use waste hemicellulose from other biorefinery production chains. Both approaches can also be further refined, *e.g*. by using oleaginous yeast strains with higher productivity and yield to obtain higher final lipid concentrations and by establishing sophisticated lipid extraction methods.

Even in its current form, biofuel production from lignocellulose using a *Lipomyces starkeyi* strain with comparatively low lipid yield was found to have a similar energy balance to second-generation bioethanol production. This indicates that there is great potential for lipid production by oleaginous yeasts and this thesis provides tools and a platform for further exploring these yeasts.

# References


Abraham A., Mathew A. K., Park H., Choi O., Sindhu R., Parameswaran B., Pandey A., Park J. H. & Sang B.-I. (2020) Pretreatment strategies for enhanced biogas production from lignocellulosic biomass. *Bioresource technology* 301: 122725.

Aluyor E., Ozigagu C., Oboh O. & Aluyor P. (2009) Chromatographic analysis of vegetable oils: A review. *Scientific Research and Essay* 4: 191-197.

Alvarez H. & Steinbüchel A. (2002) Triacylglycerols in prokaryotic microorganisms. *Applied microbiology and biotechnology* 60: 367-376.

Ami D., Posteri R., Mereghetti P., Porro D., Doglia S. M. & Branduardi P. (2014) Fourier transform infrared spectroscopy as a method to study lipid accumulation in oleaginous yeasts. *Biotechnology for biofuels* 7: 1-14.

Anschau A., Xavier M. C., Hernalsteens S. & Franco T. T. (2014) Effect of feeding strategies on lipid production by *Lipomyces starkeyi*. *Bioresource technology* 157: 214-222.

Appelqvist L.-Å. (1968) Rapid methods of lipid extraction and fatty acid methyl ester preparation for seed and leaf tissue with special remarks on preventing the accumulation of lipid contaminants. *Arkiv för kemi* 28: 551-570.

Athenstaedt K., Jolivet P., Boulard C., Zivy M., Negroni L., Nicaud J. M. & Chardot T. (2006) Lipid particle composition of the yeast *Yarrowia lipolytica* depends on the carbon source. *Proteomics* 6: 1450-1459.

Balan V. (2019) *Microbial Lipid Production: Methods and Protocols*. Springer.

Bellou S., Baeshen M. N., Elazzazy A. M., Aggeli D., Sayegh F. & Aggelis G. (2014) Microalgal lipids biochemistry and biotechnological perspectives. *Biotechnology Advances* 32: 1476-1493.

Bellou S., Triantaphyllidou I.-E., Aggeli D., Elazzazy A. M., Baeshen M. N. & Aggelis G. (2016) Microbial oils as food additives: recent approaches for improving microbial oil production and its polyunsaturated fatty acid content. *Current opinion in Biotechnology* 37: 24-35.

Beopoulos A., Nicaud J.-M. & Gaillardin C. (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. *Applied microbiology and biotechnology* 90: 1193-1206.

Beopoulos A., Cescut J., Haddouche R., Uribelarrea J.-L., Molina-Jouve C. & Nicaud J.-M. (2009) *Yarrowia lipolytica* as a model for bio-oil production. *Progress in lipid research* 48: 375-387.

Bergdahl B., Heer D., Sauer U., Hahn-Hägerdal B. & van Niel E. W. (2012) Dynamic metabolomics differentiates between carbon and energy starvation

in recombinant *Saccharomyces cerevisiae* fermenting xylose. *Biotechnology for biofuels* 5: 1-19.

Bharathiraja B., Sridharan S., Sowmya V., Yuvaraj D. & Praveenkumar R. (2017) Microbial oil–a plausible alternate resource for food and fuel application. *Bioresource technology* 233: 423-432.

Biddy M. J., Davis R., Humbird D., Tao L., Dowe N., Guarnieri M. T., Linger J. G., Karp E. M., Salvachúa D. & Vardon D. R. (2016) The techno-economic basis for coproduct manufacturing to enable hydrocarbon fuel production from lignocellulosic biomass. *ACS Sustainable Chemistry & Engineering* 4: 3196-3211.

Biely P., Singh S. & Puchart V. (2016) Towards enzymatic breakdown of complex plant xylan structures: State of the art. *Biotechnology Advances* 34: 1260-1274.

Bligh E. G. & Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian journal of biochemistry and physiology* 37: 911-917.

Blomqvist J., Pickova J., Tilami S. K., Sampels S., Mikkelsen N., Brandenburg J., Sandgren M. & Passoth V. (2018) Oleaginous yeast as a component in fish feed. *Scientific Reports* 8: 15945.

Bockey D. (2019) The significance and perspective of biodiesel production–A European and global view. *OCL* 26: 40.

Bonatsos N., Marazioti C., Moutousidi E., Anagnostou A., Koutinas A. & Kookos I. K. (2020) Techno-economic analysis and life cycle assessment of heterotrophic yeast-derived single cell oil production process. *Fuel* 264: 116839.

Bonturi N., Crucello A., Viana A. J. C. & Miranda E. A. (2017) Microbial oil production in sugarcane bagasse hemicellulosic hydrolysate without nutrient supplementation by a *Rhodosporidium toruloides* adapted strain. *Process Biochemistry* 57: 16-25.

Bonturi N., Matsakas L., Nilsson R., Christakopoulos P., Miranda E. A., Berglund K. A. & Rova U. (2015) Single cell oil producing yeasts *Lipomyces starkeyi* and *Rhodosporidium toruloides*: selection of extraction strategies and biodiesel property prediction. *Energies* 8: 5040-5052.

BP (2019) BP Statistical Review of World Energy 2019, No. 68. *British Petroleum https://www.bp.com/en/global/corporate/energy-economics/statistical-review-of-world-energy.html/*

Cai C. M., Zhang T., Kumar R. & Wyman C. E. (2014) Integrated furfural production as a renewable fuel and chemical platform from lignocellulosic biomass. *Journal of Chemical Technology & Biotechnology* 89: 2-10.

Cai J., He Y., Yu X., Banks S. W., Yang Y., Zhang X., Yu Y., Liu R. & Bridgwater A. V. (2017) Review of physicochemical properties and analytical characterization of lignocellulosic biomass. *Renewable and Sustainable Energy Reviews* 76: 309-322.

Carvalho W. D., Mustin K., Hilário R. R., Vasconcelos I. M., Eilers V. & Fearnside P. M. (2019) Deforestation control in the Brazilian Amazon: A conservation struggle being lost as agreements and regulations are subverted and bypassed. *Perspectives in Ecology and Conservation* 17: 122-130.

Chandel A. K., Da Silva S. S. & Singh O. V. (2013) Detoxification of lignocellulose hydrolysates: biochemical and metabolic engineering toward white biotechnology. *BioEnergy Research* 6: 388-401.

Chen S., Wojcieszak R., Dumeignil F., Marceau E. & Royer S. (2018) How Catalysts and Experimental Conditions Determine the Selective Hydroconversion of Furfural and 5-Hydroxymethylfurfural. *Chemical Reviews* 118: 11023-11117.

Chen W., Zhang C., Song L., Sommerfeld M. & Hu Q. (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of microbiological methods* 77: 41-47.

Chen X., Li Z., Zhang X., Hu F., Ryu D. D. & Bao J. (2009) Screening of oleaginous yeast strains tolerant to lignocellulose degradation compounds. *Applied biochemistry and biotechnology* 159: 591-604.

Chmielarz M., Sampels S., Blomqvist J., Brandenburg J., Wende F., Sandgren M. & Passoth V. (2019) FT-NIR: a tool for rapid intracellular lipid quantification in oleaginous yeasts. *Biotechnology for biofuels* 12: 1-9.

Coradetti S. T., Pinel D., Geiselman G. M., Ito M., Mondo S. J., Reilly M. C., Cheng Y.-F., Bauer S., Grigoriev I. V., Gladden J. M., Simmons B. A., Brem R. B., Arkin A. P. & Skerker J. M. (2018) Functional genomics of lipid metabolism in the oleaginous yeast *Rhodosporidium toruloides*. *eLife* 7: e32110.

Deinema M. H. & Landheer C. (1956) Composition of fats, produced by *Lipomyces starkeyi*, under various conditions. *Archiv für Mikrobiologie* 25: 193-200.

Demirbas A. (2011) Competitive liquid biofuels from biomass. *Applied Energy* 88: 17-28.

Donot F., Fontana A., Baccou J., Strub C. & Schorr-Galindo S. (2014) Single cell oils (SCOs) from oleaginous yeasts and moulds: production and genetics. *Biomass and Bioenergy* 68: 135-150.

Dubois V., Breton S., Linder M., Fanni J. & Parmentier M. (2007) Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *European Journal of Lipid Science and Technology* 109: 710-732.

Eisenberg T. & Büttner S. (2014) Lipids and cell death in yeast. *FEMS yeast research* 14: 179-197.

El - Azazy M. (2018) Introductory Chapter: Infrared Spectroscopy - A Synopsis of the Fundamentals and Applications. IntechOpen. London

Evans C. T. & Ratledge C. (1983) A comparison of the oleaginous yeast, Candida curvata, grown on different carbon sources in continuous and batch culture. *Lipids* 18: 623-629.

Evans C. T. & Ratledge C. (1984) Induction of xylulose-5-phosphate phosphoketolase in a variety of yeasts grown on D-xylose: the key to efficient xylose metabolism. *Archives of microbiology* 139: 48-52.

Evans C. T., Scragg A. H. & Ratledge C. (1983) Regulation of citrate efflux from mitochondria of oleaginous and non-oleaginous yeasts by long-chain fatty acyl-CoA esters. *European journal of biochemistry* 132: 617-622.

Fakas S. (2017) Lipid biosynthesis in yeasts: A comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast *Saccharomyces cerevisiae* and the model oleaginous yeast *Yarrowia lipolytica*. *Engineering in Life Sciences* 17: 292-302.

FAO (2020) The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome.

Folch J., Lees M. & Stanley G. (1957) A simple method for total lipid extraction and purification. *J Biol Chem* 226: 497-509.

Forfang K., Zimmermann B., Kosa G., Kohler A. & Shapaval V. (2017) FTIR spectroscopy for evaluation and monitoring of lipid extraction efficiency for oleaginous fungi. *PLoS One* 12: e0170611.

Fransen M., Lismont C. & Walton P. (2017) The peroxisome-mitochondria connection: how and why? *International journal of molecular sciences* 18: 1126.

Garay L. A., Boundy-Mills K. L. & German J. B. (2014) Accumulation of high-value lipids in single-cell microorganisms: a mechanistic approach and future perspectives. *Journal of agricultural and food chemistry* 62: 2709-2727.

Gemperlein K., Dietrich D., Kohlstedt M., Zipf G., Bernauer H. S., Wittmann C., Wenzel S. C. & Müller R. (2019) Polyunsaturated fatty acid production by Yarrowia lipolytica employing designed myxobacterial PUFA synthases. *Nature communications* 10: 1-12.

Gírio F. M., Fonseca C., Carvalheiro F., Duarte L. C., Marques S. & Bogel-Łukasik R. (2010) Hemicelluloses for fuel ethanol: a review. *Bioresource technology* 101: 4775-4800.

Goiris K., Muylaert K., Fraeye I., Foubert I., De Brabanter J. & De Cooman L. (2012) Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *Journal of applied phycology* 24: 1477-1486.

Görner C., Redai V., Bracharz F., Schrepfer P., Garbe D. & Brück T. (2016) Genetic engineering and production of modified fatty acids by the non-conventional oleaginous yeast Trichosporon oleaginosus ATCC 20509. *Green Chemistry* 18: 2037-2046.

Granger L.-M., Perlot P., Goma G. & Pareilleux A. (1993) Effect of various nutrient limitations on fatty acid production by *Rhodotorula glutinis*. *Applied microbiology and biotechnology* 38: 784-789.

Gupta A., Vongsvivut J., Barrow C. J. & Puri M. (2012) Molecular identification of marine yeast and its spectroscopic analysis establishes unsaturated fatty acid accumulation. *Journal of bioscience and bioengineering* 114: 411-417.

Hayden B., Canuel N. & Shanse J. (2013) What was brewing in the Natufian? An archaeological assessment of brewing technology in the Epipaleolithic. *Journal of Archaeological Method and Theory* 20: 102-150.

Helmerius J., von Walter J. V., Rova U., Berglund K. A. & Hodge D. B. (2010) Impact of hemicellulose pre-extraction for bioconversion on birch Kraft pulp properties. *Bioresour Technol* 101: 5996-6005.

Hiltunen J. K., Mursula A. M., Rottensteiner H., Wierenga R. K., Kastaniotis A. J. & Gurvitz A. (2003) The biochemistry of peroxisomal β-oxidation in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* 27: 35-64.

Holdsworth J. E. & Ratledge C. (1988) Lipid turnover in oleaginous yeasts. *Microbiology* 134: 339-346.

Holdsworth J. E., Veenhuis M. & Ratledge C. (1988) Enzyme activities in oleaginous yeasts accumulating and utilizing exogenous or endogenous lipids. *Microbiology* 134: 2907-2915.

Hu C., Zhao X., Zhao J., Wu S. & Zhao Z. K. (2009) Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. *Bioresource technology* 100: 4843-4847.

Huang C., Chen X.-f., Xiong L., Ma L.-l. & Chen Y. (2013) Single cell oil production from low-cost substrates: the possibility and potential of its industrialization. *Biotechnology Advances* 31: 129-139.

i Nogué V. S., Black B. A., Kruger J. S., Singer C. A., Ramirez K. J., Reed M. L., Cleveland N. S., Singer E. R., Yi X. & Yeap R. Y. (2018) Integrated diesel production from lignocellulosic sugars via oleaginous yeast. *Green Chemistry* 20: 4349-4365.

Jacob Z. (1992) Yeast Lipids: Extraction, Quality Analysis, and Acceptability. *Critical Reviews in Biotechnology* 12: 463-491.

Johnson V. W., Singh M., Saini V. S., Adhikari D. K., Sista V. & Yadav N. K. (1995) Utilization of molasses for the production of fat by an oleaginous yeast, *Rhodotorula glutinis* IIP-30. *Journal of Industrial Microbiology and Biotechnology* 14: 1-4.

Jönsson L. J. & Martín C. (2016) Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. *Bioresource technology* 199: 103-112.

Jørgensen H., Kristensen J. B. & Felby C. (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining* 1: 119-134.

Kaneko H., Hosohara M., Tanaka M. & Itoh T. (1976) Lipid composition of 30 species of yeast. *Lipids* 11: 837-844.

Karamerou E. E. & Webb C. (2019) Cultivation modes for microbial oil production using oleaginous yeasts–a review. *Biochemical Engineering Journal* 151: 107322.

Karlsson H., Ahlgren S., Sandgren M., Passoth V., Wallberg O. & Hansson P.-A. (2016) A systems analysis of biodiesel production from wheat straw using oleaginous yeast: process design, mass and energy balances. *Biotechnology for biofuels* 9: 1-13.

Karlsson H., Ahlgren S., Sandgren M., Passoth V., Wallberg O. & Hansson P.-A. (2017) Greenhouse gas performance of biochemical biodiesel production from straw: soil organic carbon changes and time-dependent climate impact. *Biotechnology for biofuels* 10: 1-15.

Kennedy E. P. (1961) Biosynthesis of complex lipids. Vol. 20. 934-940.

Khot M., Raut G., Ghosh D., Alarcón-Vivero M., Contreras D. & Ravikumar A. (2020) Lipid recovery from oleaginous yeasts: perspectives and challenges for industrial applications. *Fuel* 259: 116292.

Kim J., Coradetti S. T., Kim Y.-M., Gao Y., Yaegashi J., Zucker J. D., Munoz N., Zink E. M., Burnum-Johnson K. E., Baker S. E., Simmons B. A., Skerker J. M., Gladden J. M. & Magnuson J. K. (2021) Multi-Omics Driven Metabolic Network Reconstruction and Analysis of Lignocellulosic Carbon Utilization in *Rhodosporidium toruloides*. *Frontiers in bioengineering and biotechnology* 8: 612832-612832.

Kimura K., Yamaoka M. & Kamisaka Y. (2004) Rapid estimation of lipids in oleaginous fungi and yeasts using Nile red fluorescence. *Journal of microbiological methods* 56: 331-338.

Koh L. P. & Wilcove D. S. (2008) Is oil palm agriculture really destroying tropical biodiversity? *Conservation letters* 1: 60-64.

Kosa G., Kohler A., Tafintseva V., Zimmermann B., Forfang K., Afseth N. K., Tzimorotas D., Vuoristo K. S., Horn S. J. & Mounier J. (2017) Microtiter plate cultivation of oleaginous fungi and monitoring of lipogenesis by high-throughput FTIR spectroscopy. *Microbial cell factories* 16: 1-12.

Koutinas A. A., Vlysidis A., Pleissner D., Kopsahelis N., Lopez Garcia I., Kookos I. K., Papanikolaou S., Kwan T. H. & Lin C. S. K. (2014) Valorization of industrial waste and by-product streams via fermentation for the production of chemicals and biopolymers. *Chemical Society Reviews* 43: 2587-2627.

Kunau W.-H., Dommes V. & Schulz H. (1995) β-Oxidation of fatty acids in mitochondria, peroxisomes, and bacteria: a century of continued progress. *Progress in lipid research* 34: 267-342.

Kurtzman C., Fell J. W. & Boekhout T. (2011) *The yeasts: a taxonomic study*. Elsevier.

Laurens L. M., Knoshaug E. P., Rohrer H., Van Wychen S., Dowe N. & Zhang M. (2019) Solvent-free spectroscopic method for high-throughput, quantitative screening of fatty acids in yeast biomass. *Analytical Methods* 11: 58-69.

Lewandowski I. (2018) *Bioeconomy: Shaping the transition to a sustainable, biobased economy*. Springer Nature.

Li Y., Zhao Z. K. & Bai F. (2007) High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme and microbial technology* 41: 312-317.

Lindner P. (1922) Das Problem der biologischen Fettbildung und Fettgewinnung. *Angewandte Chemie* 35: 110-114.

Lingwood D. & Simons K. (2010) Lipid Rafts As a Membrane-Organizing Principle. *Science* 327: 46.

Liu H., Zhao X., Wang F., Jiang X., Zhang S., Ye M., Zhao Z. K. & Zou H. (2011) The proteome analysis of oleaginous yeast *Lipomyces starkeyi*. *FEMS yeast research* 11: 42-51.

Liu N., Qiao K. & Stephanopoulos G. (2016) 13C Metabolic Flux Analysis of acetate conversion to lipids by *Yarrowia lipolytica*. *Metabolic Engineering* 38: 86-97.

Liu Z., Feist A. M., Dragone G. & Mussatto S. I. (2020) Lipid and carotenoid production from wheat straw hydrolysates by different oleaginous yeasts. *Journal of Cleaner Production* 249: 119308.

Lundin H. (1950) Fat synthesis by micro-organisms and its possible applications in industry. *Journal of the Institute of Brewing* 56: 17-28.

Lynd L. R., Liang X., Biddy M. J., Allee A., Cai H., Foust T., Himmel M. E., Laser M. S., Wang M. & Wyman C. E. (2017) Cellulosic ethanol: status and innovation. *Current opinion in Biotechnology* 45: 202-211.

Marella E. R., Holkenbrink C., Siewers V. & Borodina I. (2018) Engineering microbial fatty acid metabolism for biofuels and biochemicals. *Current opinion in Biotechnology* 50: 39-46.

Meher L. C., Sagar D. V. & Naik S. (2006) Technical aspects of biodiesel production by transesterification—a review. *Renewable and Sustainable Energy Reviews* 10: 248-268.

Meng X., Yang J., Xu X., Zhang L., Nie Q. & Xian M. (2009) Biodiesel production from oleaginous microorganisms. *Renewable energy* 34: 1-5.

Mielke T. (2018) World Markets for Vegetable Oils: Status and Prospects. *Encyclopedia of Sustainability Science and Technology,*(Meyers RA, ed.) 1-38. Springer New York, New York, NY.

Moreno A. D., Alvira P., Ibarra D. & Tomás-Pejó E. (2017) Production of Ethanol from Lignocellulosic Biomass. *Production of Platform Chemicals from Sustainable Resources,*(Fang Z, Smith JRL & Qi X, eds.), p.^pp. 375-410. Springer Singapore, Singapore.

Moreton R. (1988) *Single cell oil*. Longman Scientific and Technical.

Morin N., Cescut J., Beopoulos A., Lelandais G., Le Berre V., Uribelarrea J.-L., Molina-Jouve C. & Nicaud J.-M. (2011) Transcriptomic analyses during the

transition from biomass production to lipid accumulation in the oleaginous yeast *Yarrowia lipolytica*. *PLoS One* 6: e27966.

Müllner H. & Daum G. (2004) Dynamics of neutral lipid storage in yeast. *Acta Biochimica Polonica* 51: 323-347.

Nägeli C. & Loew O. (1878) Über die chemische Zusammensetzung der Hefe. *Justus Liebigs Annalen der Chemie* 193: 322-348.

Norambuena F., Hermon K., Skrzypczyk V., Emery J. A., Sharon Y., Beard A. & Turchini G. M. (2015) Algae in fish feed: performances and fatty acid metabolism in juvenile Atlantic salmon. *PLoS One* 10: e0124042.

Olsen R. & Henderson R. (1989) The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. *Journal of Experimental Marine Biology and Ecology* 129: 189-197.

Papanikolaou S. & Aggelis G. (2011) Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. *European Journal of Lipid Science and Technology* 113: 1031-1051.

Park Y.-K., Nicaud J.-M. & Ledesma-Amaro R. (2018) The engineering potential of *Rhodosporidium toruloides* as a workhorse for biotechnological applications. *Trends in biotechnology* 36: 304-317.

Parsons S., Chuck C. J. & McManus M. C. (2018) Microbial lipids: Progress in life cycle assessment (LCA) and future outlook of heterotrophic algae and yeast-derived oils. *Journal of Cleaner Production* 172: 661-672.

Patel A., Pruthi V. & Pruthi P. A. (2019) Innovative screening approach for the identification of triacylglycerol accumulating oleaginous strains. *Renewable energy* 135: 936-944.

Patel A., Arora N., Pruthi V. & Pruthi P. A. (2019) A novel rapid ultrasonication-microwave treatment for total lipid extraction from wet oleaginous yeast biomass for sustainable biodiesel production. *Ultrasonics sonochemistry* 51: 504-516.

Patel A., Arora N., Sartaj K., Pruthi V. & Pruthi P. A. (2016) Sustainable biodiesel production from oleaginous yeasts utilizing hydrolysates of various non-edible lignocellulosic biomasses. *Renewable and Sustainable Energy Reviews* 62: 836-855.

Patel A., Arora N., Mehtani J., Pruthi V. & Pruthi P. A. (2017) Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production. *Renewable and Sustainable Energy Reviews* 77: 604-616.

Peters J. F. N. (1936) The Furans: Fifteen Years of Progress. *Industrial & Engineering Chemistry* 28: 755-759.

Pinheiro M. J., Bonturi N., Belouah I., Miranda E. A. & Lahtvee P.-J. (2020) Xylose Metabolism and the Effect of Oxidative Stress on Lipid and Carotenoid Production in *Rhodotorula toruloides*: Insights for Future Biorefinery. *Frontiers in bioengineering and biotechnology* 8: 1008.

Probst K. V. & Vadlani P. V. (2017) Single cell oil production by *Lipomyces starkeyi*: Biphasic fed-batch fermentation strategy providing glucose for growth and xylose for oil production. *Biochemical Engineering Journal* 121: 49-58.

Qiao K., Wasylenko T. M., Zhou K., Xu P. & Stephanopoulos G. (2017) Lipid production in Yarrowia lipolytica is maximized by engineering cytosolic redox metabolism. *Nature Biotechnology* 35: 173-177.

Qin L., Liu L., Zeng A.-P. & Wei D. (2017) From low-cost substrates to single cell oils synthesized by oleaginous yeasts. *Bioresource technology* 245: 1507-1519.

Raja P. M. V., Barron, Andrew R. (2019) *Physical Methods in Chemistry and Nano Science*. http://cnx.org/content/col10699/1.25/

Ranga Rao A., Raghunath Reddy R., Baskaran V., Sarada R. & Ravishankar G. (2010) Characterization of microalgal carotenoids by mass spectrometry and their bioavailability and antioxidant properties elucidated in rat model. *Journal of agricultural and food chemistry* 58: 8553-8559.

Ratledge C. (1988) Yeasts for lipid production. Portland Press Ltd.

Ratledge C. (2010) Single cell oils for the 21st century. *Single cell oils* (second Edition). 3-26. Elsevier.

Ratledge C. (2014) The role of malic enzyme as the provider of NADPH in oleaginous microorganisms: a reappraisal and unsolved problems. *Biotechnology letters* 36: 1557-1568.

Ratledge C. & Wynn J. P. (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Advances in applied microbiology* 51: 1-52.

Ratledge C. & Cohen Z. (2008) Microbial and algal oils: do they have a future for biodiesel or as commodity oils? *Lipid Technology* 20: 155-160.

Rattray J., Schibeci A. & Kidby D. K. (1975) Lipids of yeasts. *Bacteriological Reviews* 39: 197.

Sandager L., Gustavsson M. H., Ståhl U., Dahlqvist A., Wiberg E., Banas A., Lenman M., Ronne H. & Stymne S. (2002) Storage lipid synthesis is non-essential in yeast. *Journal of Biological Chemistry* 277: 6478-6482.

Sato M. (1990) Thermochemistry of the formation of fossil fuels. *Fluid-Mineral Interactions: A Tribute to HP Eugster* 2: 271-283.

Shi S. & Zhao H. (2017) Metabolic Engineering of Oleaginous Yeasts for Production of Fuels and Chemicals. *Frontiers in microbiology* 8.

Shi S., Ji H., Siewers V. & Nielsen J. (2016) Improved production of fatty acids by *Saccharomyces cerevisiae* through screening a cDNA library from the oleaginous yeast Yarrowia lipolytica. *FEMS yeast research* 16.

Simopoulos A. P. (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedicine & pharmacotherapy* 56: 365-379.

Sitepu I., Selby T., Lin T., Zhu S. & Boundy-Mills K. (2014) Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. *Journal of Industrial Microbiology and Biotechnology* 41: 1061-1070.

Sitepu I., Ignatia L., Franz A., Wong D., Faulina S., Tsui M., Kanti A. & Boundy-Mills K. (2012) An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of microbiological methods* 91: 321-328.

Sitepu I. R., Garay L. A., Sestric R., Levin D., Block D. E., German J. B. & Boundy-Mills K. L. (2014) Oleaginous yeasts for biodiesel: Current and future trends in biology and production. *Biotechnology Advances* 32: 1336-1360.

Sitepu I. R., Sestric R., Ignatia L., Levin D., German J. B., Gillies L. A., Almada L. A. & Boundy-Mills K. L. (2013) Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. *Bioresource technology* 144: 360-369.

Slininger P. J., Dien B. S., Kurtzman C. P., Moser B. R., Bakota E. L., Thompson S. R., O'Bryan P. J., Cotta M. A., Balan V. & Jin M. (2016) Comparative lipid production by oleaginous yeasts in hydrolyzates of lignocellulosic biomass and process strategy for high titers. *Biotechnology and bioengineering* 113: 1676-1690.

Spagnuolo M., Yaguchi A. & Blenner M. (2019) Oleaginous yeast for biofuel and oleochemical production. *Current opinion in Biotechnology* 57: 73-81.

Steiner M. (1938) *Ernährung und Fettbildung bei Endomyces vernalis*.

Sugiyama M., Sasano Y. & Harashima S. (2015) Mechanism of Yeast Adaptation to Weak Organic Acid Stress. *Stress Biology of Yeasts and Fungi: Applications for Industrial Brewing and Fermentation,* (Takagi H & Kitagaki H, eds.), 107-121. Springer Japan, Tokyo.

Tai M. & Stephanopoulos G. (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast Yarrowia lipolytica for biofuel production. *Metabolic Engineering* 15: 1-9.

Takaku H., Matsuzawa T., Yaoi K. & Yamazaki H. (2020) Lipid metabolism of the oleaginous yeast *Lipomyces starkeyi*. *Applied microbiology and biotechnology* 104: 6141-6148.

Tang W., Zhang S., Tan H. & Zhao Z. K. (2010) Molecular cloning and characterization of a malic enzyme gene from the oleaginous yeast *Lipomyces starkeyi*. *Molecular biotechnology* 45: 121-128.

Thakur M., Prapulla S. & Karanth N. (1989) Estimation of intracellular lipids by the measurement of absorbance of yeast cells stained with Sudan Black B. *Enzyme and microbial technology* 11: 252-254.

Thompson P. B. (2012) The agricultural ethics of biofuels: the food vs. fuel debate. *Agriculture* 2: 339-358.

Thorpe R. & Ratledge C. (1972) Fatty acid distribution in triglycerides of yeasts grown on glucose or n-alkanes. *Microbiology* 72: 151-163.

Tiukova I. A., Brandenburg J., Blomqvist J., Sampels S., Mikkelsen N., Skaugen M., Arntzen M. Ø., Nielsen J., Sandgren M. & Kerkhoven E. J. (2019) Proteome analysis of xylose metabolism in *Rhodotorula toruloides* during lipid production. *Biotechnology for biofuels* 12: 1-17.

Turek J., Sampels S., KHALILI TILAMI S., Červený D., Kolářová J., Randák T., Mráz J., Másílko J., Steinbach C. & Burkina V. (2020) Insects in the feed of rainbow trout, *Oncorhynchus Mykiss* (Actinopterygii, Salmonidae): Effect on growth, fatty acid composition and sensory attributes. *Acta Ichthyologica et Piscatoria* 50.

UFOP (2017) Supply report 2016/2017. https://www.ufop.de/files/7814/9977/4144/UFOP_supply_report_20162017.pdf/ Union zur Förderung von Oel- und Proteinpflanzen e.V., Berlin.

USDA (2020) Oil Crops Data: Yearbook Tables. Vol. 2020 https://www.ers.usda.gov/data-products/oil-crops-yearbook/ United States Department of Agriculture, Economic research Service.

Uusitalo V., Väisänen S., Havukainen J., Havukainen M., Soukka R. & Luoranen M. (2014) Carbon footprint of renewable diesel from palm oil, jatropha oil and rapeseed oil. *Renewable energy* 69: 103-113.

Valdés G., Mendonça R. T. & Aggelis G. (2020) Lignocellulosic biomass as a substrate for oleaginous microorganisms: A review. *Applied Sciences* 10: 7698.

Vedernikovs N., Kampars V., Puķe M. & Krūma I. (2010) Changes in the birch wood lignocellulose composition in the pretreatment process. *Materials Science and Applied Chemistry* 22:68–73.

Volynets B. & Dahman Y. (2011) Assessment of pretreatments and enzymatic hydrolysis of wheat straw as a sugar source for bioprocess industry. *International Journal of Energy & Environment* 2.

Vongsvivut J., Heraud P., Gupta A., Puri M., McNaughton D. & Barrow C. J. (2013) FTIR microspectroscopy for rapid screening and monitoring of polyunsaturated fatty acid production in commercially valuable marine yeasts and protists. *Analyst* 138: 6016-6031.

Wang D.-S., Yu X.-J., Zhu X.-Y., Wang Z., Li H.-J. & Wang Z.-P. (2019) Transcriptome Mechanism of Utilizing Corn Steep Liquor as the Sole Nitrogen Resource for Lipid and DHA Biosynthesis in Marine Oleaginous Protist *Aurantiochytrium* sp. *Biomolecules* 9: 695.

Wang Y., Zhang S., Zhu Z., Shen H., Lin X., Jin X., Jiao X. & Zhao Z. K. (2018) Systems analysis of phosphate-limitation-induced lipid accumulation by the oleaginous yeast *Rhodosporidium toruloides*. *Biotechnology for biofuels* 11: 1-15.

Wiebe M. G., Koivuranta K., Penttilä M. & Ruohonen L. (2012) Lipid production in batch and fed-batch cultures of *Rhodosporidium toruloides* from 5 and 6 carbon carbohydrates. *BMC biotechnology* 12: 1-10.

Woodbine M. (1959) Microbial fat: Microorganisms as potential fat producers. *Progress in industrial microbiology* 1: 181-245.

Xavier M. C. A., Coradini A. L. V., Deckmann A. C. & Franco T. T. (2017) Lipid production from hemicellulose hydrolysate and acetic acid by *Lipomyces starkeyi* and the ability of yeast to metabolize inhibitors. *Biochemical Engineering Journal* 118: 11-19.

Yao Q., Chen H., Wang S., Tang X., Gu Z., Zhang H., Chen W. & Chen Y. Q. (2019) An efficient strategy for screening polyunsaturated fatty acid-producing oleaginous filamentous fungi from soil. *Journal of microbiological methods* 158: 80-85.

Yara-Varón E., Li Y., Balcells M., Canela-Garayoa R., Fabiano-Tixier A.-S. & Chemat F. (2017) Vegetable oils as alternative solvents for green oleo-extraction, purification and formulation of food and natural products. *Molecules* 22: 1474.

Ykema A., Verbree E. C., Kater M. M. & Smit H. (1988) Optimization of lipid production in the oleaginous yeast *Apiotrichum curvatum* in wheypermeate. *Applied microbiology and biotechnology* 29: 211-218.

Zanghellini J., Wodlei F. & Von Grünberg H. (2010) Phospholipid demixing and the birth of a lipid droplet. *Journal of theoretical biology* 264: 952-961.

Zeitsch K. J. (2000) *The chemistry and technology of furfural and its many by-products*. Elsevier.

Zhao X., Peng F., Du W., Liu C. & Liu D. (2012) Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast *Rhodosporidium toruloides* and preparation of biodiesel by enzymatic transesterification of the lipid. *Bioprocess and biosystems engineering* 35: 993-1004.

Zheng J. & Rehmann L. (2014) Extrusion pretreatment of lignocellulosic biomass: a review. *International journal of molecular sciences* 15: 18967-18984.

Zimmer Y. (2010) Competitiveness of rapeseed, soybeans and palm oil. *Journal of Oilseed Brassica* 1: 84-90.

Zimmermann B. & Kohler A. (2013) Optimizing Savitzky-Golay parameters for improving spectral resolution and quantification in infrared spectroscopy. *Applied spectroscopy* 67: 892-902.

# Popular science summary

Many parts of the modern economy are currently linear processes based on non-renewable fossil resources, which has negative consequences for the environment and the global climate. It is desirable to change these linear processes into circular processes and generate a sustainable bio-based economy. For this, alternatives to fossil-based fuels and chemicals are needed. For instance, the biofuel market is already developing rapidly. Biofuels such as ethanol and biodiesel currently produced mainly from food crops, *e.g*. corn or sugarcane are used for ethanol production and oil plants, such as rapeseed or palm tree, for biodiesel production. However, these so-called 'first-generation biofuels' may compete with food production, as they are generated from edible plants. Furthermore, increased demand for oils to generate biofuels can lead to deforestation of rainforest areas for palm plantations. Using biomass that currently is regarded mainly as waste products, such as wheat straw or forest residues, has great potential as an alternative renewable resource for production of biofuels, chemicals and other materials.

Oils, or generally speaking lipids, can be produced by specific microorganisms, called oleaginous microorganisms. In this thesis, oleaginous yeasts, defined by their ability to accumulate more than 20% of their dry matter as lipids, were used for microbial lipid production. The conceptual system for using straw as a substrate for microbial lipid production in a circular and sustainable approach is illustrated in Figure 1. The microbial lipids produced can be used as fuels, in lipid-based industries or even for food or feed production. Residues from this process can be used for biogas production and the biodigestate can be recirculated as fertiliser.

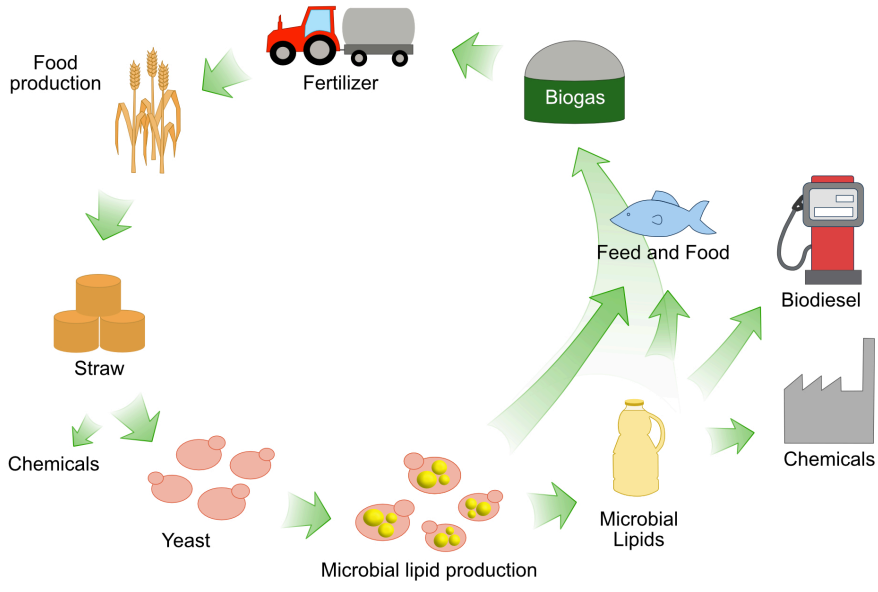



*Figure1*. Sustainable microbial lipid production for fuels, chemicals or food and feed production in a circular biorefinery approach where wheat straw is used as a substrate for microbial lipid production with yeast. Recycling the residues for biogas and the biodigestate as fertiliser closes the circle.

This thesis examined lipid production by yeasts growing on different kinds of substrates generated from biomass, in particular from wheat straw or birch wood. Biomass, or lignocellulosic material, is a complex and robust plant structure that cannot be used directly for human food or for yeast lipid production. To break up the robust structure, harsh pre-treatment is required so that sugar molecules, mainly glucose and xylose, are released into the liquid hydrolysate, where they can be utilised by the yeast. Unfortunately, pre-treatment of biomass also releases inhibitory compounds, which can cause difficulties in yeast cultivation.

In this thesis, 29 different yeast strains were tested for their ability to grow and accumulate lipids from sugars present in hydrolysate produced from wheat straw. Some yeast strains were found to be very efficient in accumulating lipids from all sugars present in the hydrolysate, while other yeast strains could not use xylose at all and in one yeast strain intracellular lipid degradation while consuming xylose was observed.

Combined production of a highly valuable chemical (furfural) from wheat straw and cultivation of yeast was examined, where xylose from lignocellulosic material was used for furfural production and the residues from this process were hydrolysed to make the glucose available as substrate for yeast cultivation. Production of microbial lipids and bioethanol by yeast from this hydrolysate resulted in efficient conversion of sugars into two possible fuel products. For bioethanol production additional nitrogen was required, as a nutrient to achieve efficient ethanol production. In contrast, microbial lipid production was possible from the hydrolysate without any a need for additives. Two different oleaginous yeast species, *Lipomyces starkeyi* and *Rhodotorula babjevae*, were tested for microbial lipid production, showing that *R. babjevae* converted the substrate faster than *L. starkeyi*. High lipid production rate is an important factor for a cost- and energy-efficient process.

Production of microbial lipids from a highly inhibiting hydrolysate generated from birch wood, which contained several inhibitory compounds (for instance acetic acid) was also tested. By adding the hydrolysate as feed slowly to a pre-grown yeast cultivation, it was found that inhibitory compounds could be kept at low levels and that the acetic acid was utilised as an additional carbon source for the yeast. This investigation demonstrated that this type of hydrolysate, which resembles waste from the pulp and paper industry, can be successfully used for microbial lipid production.

Another aspect of the work was to develop tools for intracellular lipid determination. Lipids produced by yeasts are stored intracellularly and need to be extracted before the lipid content and composition can be analysed, but lipid extraction is laborious and time-consuming. Fourier transform-infrared (FTIR) spectroscopy can be used to detect molecular structures, such as those occurring in lipids, by measuring absorption spectra when a sample is exposed to IR light. By combining the spectra with reference values obtained from lipid extraction of the same samples, a prediction model based on FTIR was established in this thesis.

Overall, this thesis provides more knowledge about oleaginous yeasts and microbial lipid production on different lignocellulosic materials. Furthermore, analytic tools were established as a basis for future research work.

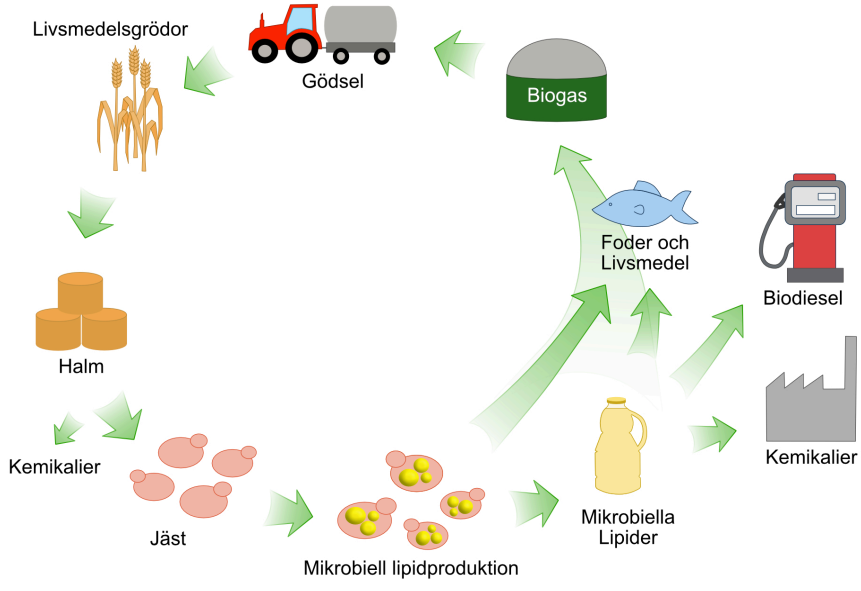
# Populärvetenskaplig sammanfattning

Många delar av vår ekonomi består av linjära processer som bygger på icke-förnybara fossila resurser, och dessa har ofta negativa konsekvenser för vår miljö och det globala klimatet. Det är därför önskvärt att omvandla dessa linjära processer till cirkulära för att därmed uppnå en mer hållbar och biobaserad ekonomi. Att hitta hållbara alternativ till fossilbaserade bränslen och kemikalier är någon som ofta framställs som mycket önskvärt. Ett exempel på nya cirkulära processer är biobränslemarknaden som utvecklas snabbt. Biodrivmedel såsom etanol och biodiesel produceras för närvarande huvudsakligen från livsmedelsgrödor. Majs och sockerrör används exempelvis för etanolproduktion och oljeväxter, såsom raps eller palmträd, för produktion av biodiesel. Dessa så kallade ”första generationens biobränslen” riskerar att konkurrera med viktig livsmedelsproduktionen om råvara eftersom dessa biobränslen ofta produceras från grödor som även kan användas till livsmedel. Dessutom kan ökad efterfrågan på bio-olja som råvara till biodiesel leda till en ökad avverkning av regnskog för att kunna plantera oljeväxter. Däremot kan användande av biomassa som för närvarande främst anses vara avfallsprodukter, exempelvis vetehalm eller skogsrester, har en stor potential som en alternativ, förnybar resurs för produktion av biodrivmedel, kemikalier och andra biobaserade material.

Oljor, ofta lipider, kan produceras av specifika mikroorganismer, så kallade lipidackumulerande mikroorganismer. I denna avhandling, används lipidackumulerande jästarter, definierad av deras förmåga att ackumulera lipider till mer än 20 % av dess biomassa, för mikrobiell lipidproduktion.

En schematisk översikt över hur halm används som substrat för mikrobiell lipidproduktion i en cirkulär och hållbart tillvägagångssätt illustreras i Figur 1. De mikrobiellt producerade lipiderna kan användas till bränslen, i lipidbaserad industri, eller till och med livsmedels- eller

foderproduktion. Rester från denna process kan även användas till biogasproduktion och recirkuleras som gödningsmedel.




*Figur 1.* Hållbar mikrobiell lipidproduktion för bränslen, kemikalier eller livsmedels- och foderproduktion i ett cirkulärt bioraffinaderi. Därför används vetehalm som substrat för mikrobiell lipidproduktion med jäst som produktionsorganism. Ytterligare användningen av resterna för biogas och återcirkulation av dem som gödselmedel stänger cirkeln för ny livsmedelsproduktion.

Ett av huvudmålen för forskningsarbetet som presenteras i denna avhandling var att undersöka lipidproduktionen hos jäst odlade på olika odlingssubstrat producerad från biomassa, i detta fall från vetehalm eller björkträ. Biomassan består till stor del av lignocellulosa, som är en komplex och robust struktur i växter och träd, och kan inte användas som den är till livsmedel för människor eller för produktion av jästlipider. För att bryta upp den robusta strukturen används flera relativt kraftiga förbehandlingar för att frigöra sockermolekyler, främst glukos och xylos, till en vätska som vi kallar hydrolysat. Detta hydrolysat kan sedan används som substrat när vi odlar jäst. Förutom socker så frisläpps även inhiberande ämnen under förbehandlingen av växtbiomassan som kan orsaka stora svårigheter vid odling av jäst och andra mikroorganismer.

Inom ramen för detta avhandlingsarbete har tjugonio olika jäststammar testades med avseende på deras förmåga att växa på och ackumulera lipider från sockerarter som finns i hydrolysat producerat från vetehalm. Vissa jäststammar ackumulerade lipider väldigt effektivt från alla sockerarter som finns i de hydrolysatet som vi har testat, medan andra jäststammar inte kunde använda xylos alls. I en jäststam observerade vi till och med lipidnedbrytning vid konsumtion av xylos.

I den andra studien visar vi på möjligheten att kombinera produktionen av den ekonomiskt värdefulla kemikalien furfural från vetehalm och odling av jäst. Xylosen från det lignocellulosiska materialet har använts till produktion av furfural, och det material som har blivit kvar efter processen har sedan förbehandladlats för att därmed göra glukos tillgängligt som substrat vid odling av jäst. Under denna studie testades mikrobiell lipidproduktion och etanolproduktion av jäst, vilket resulterade i en effektiv omvandling av hydrolysatet till två möjliga bränsleprodukter. Att producera bioetanol krävde ytterligare tillskott av kväve som näringsämne för att nå en effektiv etanolproduktion. Däremot behövdes inget tillskott av kväve till den mikrobiella lipidproduktionen. Två olika jästarter, *Lipomyces starkeyi* och *Rhodotorula babjevae* testades för mikrobiell lipidproduktion och *R. babjevae* omvandlade substratet snabbare än *L. starkeyi*. Hög lipidproduktionshastighet är en viktig faktor för att uppnå en kostnads- och energieffektiv process.

I en tredje studie, testades ett annat mycket hämmande hydrolysat som substrat för produktion av mikrobiella lipider. Detta hydrolysat kom från björkved och innehöll många inhibierande ämnen, till exempel ättiksyra. Genom att långsamt tillsätta hydrolysatet till jästodlingen kunde de hämmande föreningarna hållas på en låg nivå och ättiksyran kan därmed användes som en ytterligare kolkälla för jästen. Vi visade att denna typ av hydrolysat, som mycket liknar avfall från massa- och pappersindustrin, kan användas för mikrobiell lipidproduktion.

Ett annat syfte med det forskningsarbete som presenteras i denna avhandling var att utveckla olika verktyg för intracellulär lipidbestämning. Lipider som produceras av jäst lagras intracellulärt och behöver extraheras för att både mängden och kompositionen ska kunna analyseras. Dessa analyser genomförs vanligen genom kemisk lipidextraktion, vilket är både en tids- och arbetskrävande process. Fourier transform Infraröd (FTIR) spektroskopi är en teknik som använder infrarött (IR) ljus för att detektera

molekylära strukturer, tex de som finns i lipider. Genom att mäta absorptionsspektra när ett prov exponeras för IR-ljus, kan mycket information genereras om cellulära komponenter. Genom att kombinera de IR-spektra som man får med referensvärden erhållna från lipidextraktion av samma prover kan en prediktionsmodell för den analyserade biomolekylen erhållas.

Generellt bidrar det presenterade doktorandarbetet ny kunskap om lipidackumulerande jästarter och produktion av mikrobiella lipider med olika lignocellulosarikamaterial som råmaterial. Dessutom har nya analysmetoder tagit fram som kan användas som verktyg inom framtida forskningsarbete.

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**Tack så mycket…**

Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2021:30

This thesis addresses microbial lipid production from lignocellulosic material using oleaginous yeast. Results show microbial lipid production from cellulose and hemicellulose using different yeast strains and cultivation strategies. It investigates the diversity among closely related strains and includes the possible coproduction of other high value chemicals. Furthermore, an intracellular lipid determination tool was developed. The thesis contributes with knowledge towards generating new sustainable lipid recourses to transform our fossil-based economy into a bio-based economy.

**Jule Brandenburg** received her postgraduate education at the Department of Molecular Sciences, SLU Uppsala, Sweden. She holds a Master of Science in Microbiology from the Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany.

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SLU generates knowledge for the sustainable use of biological natural resources. Research, education, extension, as well as environmental monitoring and assessment are used to achieve this goal.

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