Conversion of lignocellulose and crude glycerol to lipids by oleaginous yeasts

Physiology and diversity

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Cover: *Rhodotorula toruloides* from left to right: cultivated on plate, micrograph from culture, extracted yeast oil.
(photo: Mikołaj Chmielarz)

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Conversion of lignocellulose and crude glycerol to lipids by oleaginous yeasts - physiology and diversity

Abstract

Oleaginous yeasts are organisms capable of accumulating lipids. Some of them can grow on various substrates considered to be too toxic for many other microorganisms. Crude glycerol (CG) and hemicellulose hydrolysate (HH) are two examples of such substrates.

Lipid quantification by extraction is a time-consuming process which requires usage of organic solvents and strong acids when applied on yeasts. To address this, we developed a new method using Fourier-transform near infra-red (FT-NIR) spectroscopy to quantify lipids within yeast cells. The resulting model for *Rhodotorula toruloides* had a R^2 of 98% and a 5% error in prediction when compared with the traditional lipid extraction method. The method was used to follow lipid formation kinetics in subsequent experiments.

Out of 27 tested oleaginous yeast strains, less than half could grow on crude glycerol. Two strains, *R. toruloides* CBS14 and *Rhodotorula glutinis* CBS3044 grew well on this substrate, and were even activated when it was supplemented with hemicellulose hydrolysate (producing up to 12.5 g/L lipids). RNA sequencing in *R. toruloides* CBS14 revealed increased transcription of genes related to energy metabolism, mitochondrial enzymes and genes involved in protein synthesis. There were only little differences in genes related to glycerol metabolic pathways. Probably, cells grown in CGHH have a more efficient energy metabolism and thus, more ATP to build up biomass and lipids, and to take up substrate from the medium.

The new method for lipid quantification and the identified regulated genes can be the basis for further manipulations of yeast metabolism to reach sustainable microbial oil production from residual substrates.

Keywords: oleaginous yeast, crude glycerol, lignocelluloses, FT-NIR, lipids

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Omvandling av lignocellulosa och råglycerol till lipider med lipidackumulerande jäst - fysiologi och diversitet

Sammanfattning

Lipidackumulerande jäst kan ackumulera lipider till mer än 20% av sin torrvikt. Vissa sådana jäststammar kan växa på substrat som är giftiga för många andra mikroorganismer, till exempel råglycerol (CG) och hemicellulosahydrolysat (HH).

Lipidkvantifiering genom extraktion är en tidskrävande process och som när den ska appliceras på jäst kräver användning av organiska lösningsmedel och starka syror. I detta arbete har vi utvecklat en ny metod där vi använder Fouriertransform nära infraröd (FT-NIR) spektroskopi för att kvantifiera lipider direkt i jästceller utan föregående extraktion. Den framtagna FT-NIR modellen för *Rhodotorula toruloides* hade R^2 på 98% och 5% fel i prediktion jämfört med lipidextraktion och användes för att följa lipidproduktionen i följande odlingar.

Från totalt 27 undersökta lipidackumulerande jäststammar kunde mindre än hälften växa på råglycerol. Två stammar, *R. toruloides* CBS14 och *Rhodotorula glutinis* CBS3044 växte bra på detta substrat och blev även aktiverade när hemicellulosahydrolysat tillsattes (produktion av lipider upp till 12.5 g/L). RNA-sekvensering av *R. toruloides* CBS14 avslöjade en ökad transkription av gener relaterade till energimetabolism, mitokondriella enzymer och gener involverade i proteinsyntes, och vi ser bara små skillnader i uttryck av gener relaterade till glycerolmetaboliska vägar. Förmodligen har celler som odlas i CGHH en mer effektiv energimetabolism och därmed mer ATP för att bygga upp biomassa och lipider samt ta upp substrat från mediet.

Den nya metoden för lipidkvantifiering och de gener som identifierats kan bli bas för ytterligare manipulering av jästmetabolism för att nå en hållbar mikrobiell oljeproduktion från restprodukter.

Keywords: Lipidackumulerande jäst, råglycerol, lignocellulosa, FT-NIR, lipider

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To my family, bez was ta praca by nigdy nie powstała

Things are only impossible until they're not!
Jean-Luc Picard (Star Trek; The Next Generation)

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. Mikołaj Chmielarz, Sabine Sampels, Johanna Blomqvist, Jule Brandenburg, Frida Wende, Mats Sandgren, Volkmar Passoth (2019). FT-NIR: a tool for rapid intracellular lipid quantification in oleaginous yeasts. Biotechnology for Biofuels, 12 (169)

II. Mikołaj Chmielarz, Johanna Blomqvist, Sabine Sampels, Mats Sandgren and Volkmar Passoth (2021). Microbial lipid production from crude glycerol and hemicellulosic hydrolysate with oleaginous yeasts. Biotechnology for Biofuels, 14 (65)

III. Giselle C. Martín-Hernandez, Mikołaj Chmielarz, Martin Hölzer, Christian Brandt, Adrian Viehweger, Bettina Müller, Volkmar Passoth. Differential gene transcription of *Rhodotorula toruloides* CBS14 during growth in crude glycerol and a mixture of crude glycerol and hemicellulose hydrolysate (manuscript)

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The contribution of Mikołaj Chmielarz to the papers included in this thesis was as follows:

I. Took part in planning the project. Performed majority of laboratory work. Main writer of the manuscript. Involved in supervision of IP.

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

III. Took part in planning the project. Performed laboratory part of the work. Major contribution to writing manuscript.

In addition to Papers I-III, Mikołaj Chmielarz contributed to the following papers within the timeframe of the project:

Jitka Laurent; Nore Struyf; An Bautil; Albina Bakeeva; Mikołaj Chmielarz; Marika Lyly; Beatriz Herrera-Malaver; Volkmar Passoth, Kevin J. Verstrepen, Christophe M. Courtin (2021). The potential of *Kluyveromyces marxianus* to produce low FODMAP breads: A pilot scale study. Food and Bioprocess Technology, (in press)

Giselle C. Martín-Hernández, Bettina Müller, Mikołaj Chmielarz, Christian Brandt, Adrian Viehweger; Martin Hölzer, Volkmar Passoth (2021). Chromosome-level genome assembly and transcriptome-based annotation of the oleaginous yeast *Rhodotorula toruloides* CBS 14. Genomics, (submitted)

Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CDW	Cell dry weight
CG	Crude glycerol
CoA	Coenzyme A
DHA	Docosahexaenoic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAME	Fatty acid methyl esters
FT	Fourier transform
FT-NIR	Fourier transform near infra-red
G-3-P	Glycerol-3-phosphate
HH	Hemicellulose hydrolysate
IR	Infra-red
NAD	Nicotinamide adenine dinucleotide
NIR	Near infra-red
OD	Optical density
PUFA	Poly unsaturated fatty acids

TAG	Triacylglycerol
VO	Vegetable oils

1. Introduction

1.1 Societal challenges

Global sustainability is one of the most important goals of humanity in coming years. In context of this study - sustainability can be defined as never harvesting more than environment is able to regenerate – this concept was coined in Germany as *Nachhaltigkeit* in 1713 (Kuhlman & Farrington 2010). When looking at current world state – we are facing sustainability concerns when it comes to overexploitation of natural resources, excessive waste production and greenhouse gas emissions, the last one being one of the biggest factors in global warming for which humanity is responsible with 95% confidence for it (Nuccitelli 2020). One of the factors is our dependence on fossil fuels, which are a finite resource that have to be replaced– their excavation, mining and combustion emits greenhouse gasses. Our goal as humanity should be to avoid or at least limit the use of fossil fuels by introducing green and carbon neutral alternatives before we reach the point of no return in temperature after which global warming might be irreversible, go into positive feedback loop and endanger our existence (Masson-Delmotte 2018). There are ways to address this in form of renewable energy sources like solar, wind and geothermal or low carbon emitting nuclear power. Unfortunately, none of these solutions are without drawbacks. Sun does not always shine, wind does not always blow, not everywhere geothermal power can be utilised, and nuclear power has very high initial cost, problematic waste and regulatory obstacles (Whittington 2002; Zou *et al.* 2019).

There are also other viable alternatives that could be utilised along existing solutions. Biofuels are a potential way to re-circulate existing carbon

in atmosphere without adding more and are an important part of sustainable biobased economy. Biofuels are mainly produced from plant- based raw materials, food wastes or microorganisms, and subsequently further refined to end products. Currently the most used biofuels are bioethanol and 1st generation biodiesel made from vegetable oils (VO). Currently, most used VO are palm oil, soybean oil, rapeseed oil, sunflower oil and peanut oil (Schmidt 2015). Europe imports over 40% of its VO for biofuels. One of the drawbacks of 1st generation of biofuels is that they could directly compete with food production and already have been connected with large deforestation of rainforests (Konečná 2017).

One of the ways to reduce the utilisation of food- grade substances in biofuel production is converting waste products already present in the industry to biofuels, to enable a more sustainable, circular economy.

These challenges lead to the aim of this thesis project. To investigate the potential to use two waste substrates - hemicellulose and crude glycerol, as substrates for oleaginous yeasts to produce lipids. This PhD thesis project was a part of LipoDrivE and Edible Wood research programs designed to introduce recyclable and sustainable conversion of lignocelluloses to high value lipids for biofuel production and fish feed (Figure 1).

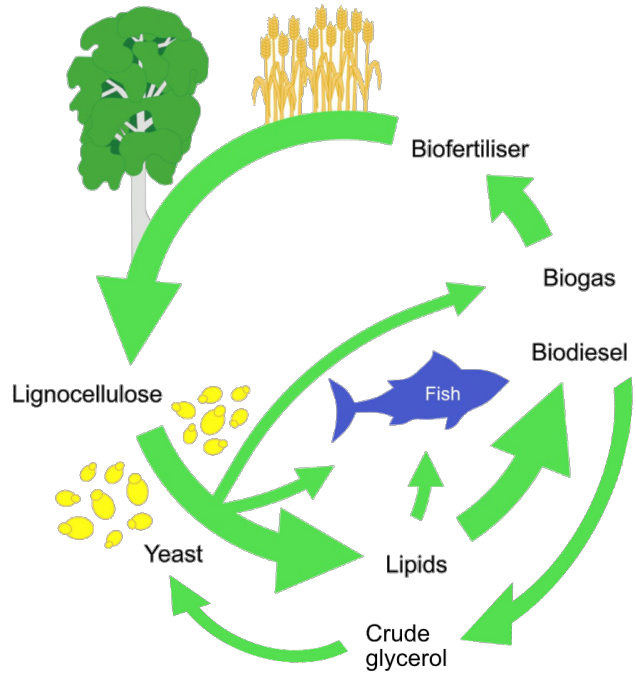

Figure 1. Concept behind the LipoDrivE research programme for sustainable microbial lipid production for fuels and feed in a circular biorefinery approach. This version has an updated path including crude glycerol as a new potential pathway in utilisation of residues from biofuel in addition to biofertilizer for recirculation.

1.2 Residual raw materials as feedstock

1.2.1 Crude glycerol

Biodiesel was considered a great alternative to fossil fuels and is currently used worldwide as blend with fossil diesel in the transportation sector (Chozhavendhan *et al.* 2020). Crude glycerol is a waste by-product of biodiesel production, which is released in the transesterification of triglycerides to generate fatty acid methyl esters (FAME). For example, to create biodiesel through transesterification for every mole of biodiesel one mole of crude glycerol is produced (Demirbas 2005).

Increased demand for biodiesel resulted in large quantities of crude glycerol being available. Unfortunately, in its raw form it does not have any use – it needs to be extensively purified to find use in mostly pharma industry. Currently to dispose it, it is either flushed into water or stored in tanks for later use or treatment. It is not easy to burn glycerol, since it has a very high viscosity (1030 cP), high ignition temperature of 370°C and thus requires special furnace which in the end results in a not very efficient process (Bohon *et al.* 2011).

1.2.2 Lignocellulose

Another residual raw material is lignocellulose. It is the most abundant biomass on earth – it is the main component of plant cell wall which is resilient and sturdy. It mainly consists of three biopolymers: cellulose, hemicellulose and lignin (Isikgor & Becer 2015).

Cellulose is a homo-polysaccharide consisting of D-glucose monomers linked by β-1,4-glycosidic bonds. Cellulose fibres are tightly packed, forming a crystalline structure. It can contain thousands of monomers of glucose. In its native form it is hard to hydrolyse and is insoluble in water. It is most often encased in hemicellulose and lignin (Isikgor & Becer 2015).
Hemicellulose is a heteropolysaccharide with a different composition in different plant groups. Hardwood hemicellulose consists mainly of xylose, while softwood mainly contains glucomannan. Hemicellulose derived from monocotyledons such as straw, contains arabinose and a xylose base with glucuronic acid side chains (Isikgor & Becer 2015; Biely *et al.* 2016). In the pulp and paper industry, hemicellulose is a by-product and is used to generate steam. However, for this application it is quite inefficient due to its low calorie value (13.5 MJ / kg) (Li *et al.* 2017).

One of the biggest industries in Sweden is wood industry. Wood is used for buildings, furniture and paper production from which most is exported - however, from that industry there are waste biomasses not utilised to their full potential - in form of lignin and hemicellulose. Additional potential sources of this residual biomass are straw and energy willow. Lignocellulosic biomasses are not easily usable, as lignocellulose needs several treatment steps to release fermentable sugar monomers (Figure 2). Hemicellulose used in this project was obtained by steam explosion with acid treatment (Brandenburg *et al.* 2016).

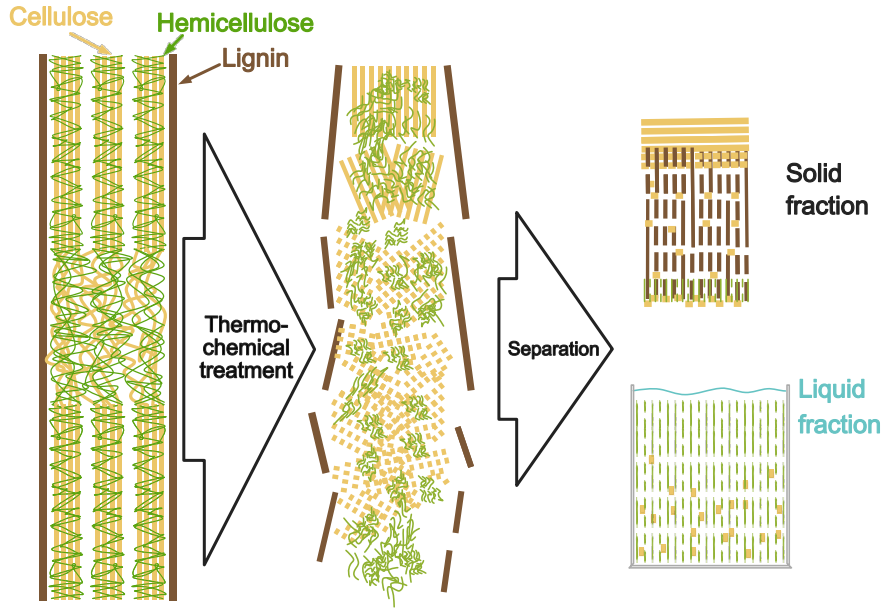

Figure 2. Simplified lignocellulose structure and example hydrolysis steps. After thermochemical pre-treatment (for ex. steam explosion with acid treatment) residues are separated into liquid and solid fractions. The solid fraction contains lignin, cellulose fibres, and non-digestible polysaccharides, the liquid fraction contains hydrolysed hemicellulose and soluble cellulose. Both can then be further treated enzymatically and will have different ratios of glucose, xylose and acetic acid etc. depending on residues present.

In the production of lignocellulose-based bioethanol, it may be advantageous to separate hemicellulose from the cellulose fraction, as the conversion of pentose, the main monomer of hemicellulose sugar, to ethanol is still a problem (Xin *et al.* 2016; Gajdos *et al.* 2020). When lignocellulose is thermochemically treated by acid hydrolysis, hemicellulose is dissolved and hydrolysed to release monosaccharides (Girio *et al.* 2010). Since the major parts of cellulose and lignin are still in the solid phase, it is easy to separate the hemicellulose hydrolysate from the other components of lignocellulose hydrolysate. In addition to released sugars, hemicellulose hydrolysate contains many inhibitors such as furaldehydes, weak organic acids and phenolic compounds (Jonsson & Martin 2016). In order to enable bioconversion of hemicellulose hydrolysates it is necessary to either remove those inhibitors, find more resilient strains or dilute hemicellulose

hydrolysate (Brandenburg *et al.* 2016; Brandenburg *et al.* 2018; Diaz-Fernandez *et al.* 2019).

Thus, crude glycerol and hemicellulose represent underutilised waste streams with some inhibitory potential against fermentation organisms. Mixing both substrates might be a way of utilising their potential as substrate for microbial growth, as they contain different inhibitory compounds, which might then be diluted and thus have a less negative effect. This will then make it possible to repurpose them into high value compounds like proteins, carotenoids and oil.

1.3 Oleaginous microorganisms

The term "oleaginous" means that organisms are capable of accumulating over 20% of their dry mass (DM) as lipids. The lipids usually serve as storage compounds or for regulatory functions. The type and amount of produced lipids is dependent on the organism. Oleaginous organisms are present in both eukaryotic and prokaryotic domains. Among them there are several notable for application by humans like microalgae, bacteria, filamentous fungi and yeasts.

Microalgae, which are usually photosynthetic microorganisms, were already historically used for food supplementation since the 1960s (Pulz & Gross 2004). Unfortunately, while promising, they have low sustainability due to low productivity and high cost of operation if used for biofuel production. However, microalgae like *Chlorella* are capable of producing high value compounds like β-carotene, astaxanthin and polyunsaturated fatty acids (PUFAs) in form of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Wijffels *et al.* 2013). There are several known oleaginous bacteria strains, most well-known are *Rhodococcus opacus, Arthrobacter* sp. and *Acinetobacter calcoaceticus*. There were reports of components of fatty acids taking up to 87% of cell dry weight (CDW) with short cultivation time thanks to bacteria's high growth rate (Kumar *et al.* 2020). Filamentous fungi are another excellent producer of microbial oils. They are capable of producing long chain PUFAs up to C:22 (Passoth 2017). Most notable examples of filamentous oleaginous fungi among zygomycetes are *Mortierella* sp and *Cunninghamella* sp. They were capable of accumulating between 24% and 50% lipids in DM, respectively, in which approximately 20% of FA were PUFA. Filamentous fungi like *Umbelopsis*

isabellina and *Thamnidium elegans* were found to produce lipids up to 71% of their biomass (Chatzifragkou *et al.* 2011; Bellou *et al.* 2016). Some other example of filamentous fungi capable of producing lipids are *Trichoderma reesei* and *Mucor circinelloides*, which were shown to reach lipid contents to 9.8% and 22.1% of their total biomass (dry weight), respectively, with wastewater as substrate (Bhanja *et al.* 2014).

1.3.1 Yeasts

Yeasts are eukaryotic single celled microorganisms in the fungi kingdom. They have been used by humans since ancient times. They can be found in a great variety of environments like soil, water, fruits, or flowers. They have had a significant impact, both positive and negative on our lives. First uses were alcoholic brewages, bread making and several other food fermentations, which are still of great importance in our days (Passoth 2017; Chmielarz *et al.* 2021). Some yeasts can also be cause of diseases (Kurtzman et al. 2011).

Nowadays, yeasts are used in more ways than for food fermentations. For example, *Saccharomyces cerevisiae* is used to produce bioethanol. Yeasts can also serve as important biomass producers - they could be utilised as a source of protein in the pharmaceutical industry or as animal feed. Yeasts producing microbial protein (called Single Cell Protein – SCP) can be grown on a variety of substrates with carbon sources ranging from pure sugars to waste products (Jay *et al.* 2008; Kurcz *et al.* 2016). Moreover, yeasts are also used for production of important proteins for pharmaceutical applications such as insulin and vaccines (Hou *et al.* 2012). Another, relatively new application of yeasts is the production of oil.

Oleaginous yeasts are capable of accumulating over 20% of their mass as lipids. They can be found among ascomycetes (e.g., *Lipomyces starkeyi*, *Yarrowia lipolytica*), and basidiomycetes (e.g., *Rhodotorula glutinis*, *Rhodotorula toruloides*, *Phaffia rhodozyma*) (Passoth 2017).

In most of our experiments we noticed that the way cells accumulate lipids were somewhat different – ascomycetous yeast produced a single, central lipid body, while basidiomycetous yeast formed several small lipid bodies in polar opposites of the cells – which usually merged if enough fat was accumulated (Figure 3).

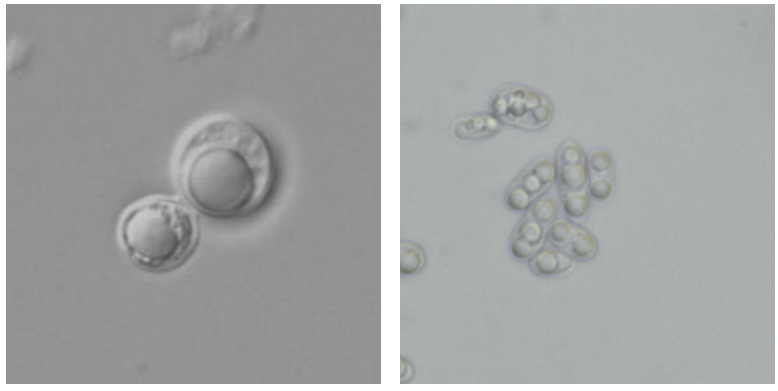

Figure 3. Micrograph of *Lipomyces starkeyi* CBS 1807 (left) and *Rhodotorula toruloides* CBS14 (right).

An interesting property of oleaginous yeasts is their ability to assimilate a great variety of carbon sources like glucose, xylose, glycerol, acetic acid and other organic acids (Paper II), polysaccharides like starch and even alkanes (Jin et al. 2015). These, and several other studies show the potential of these yeasts for conversion of low value substrates into high quality oil which could potentially replace vegetable oils in a more sustainable biodiesel production (Karlsson et al. 2016; Karlsson et al. 2017), or animal feed production (Blomqvist et al. 2018). A current obstacle is the still relatively high cost of microbial oil, when compared to vegetable oils or fossil fuels (Biddy et al. 2016; Sitepu et al. 2020).

1.3.2 Lipid accumulation in oleaginous yeasts

In oleaginous yeasts lipid accumulation follows traditional pathways of lipid metabolism (Figure 4). Carbon sources in forms of glucose, xylose and glycerol are transported inside the cell and through metabolic pathways converted down to pyruvate, which is subsequently transported via the monocarboxylic acid transporter into the mitochondrion to enter the tricarboxylic acid cycle. In oleaginous yeasts when there is nitrogen limitation, adenosine monophosphate (AMP) deaminase cleaves AMP into inosine monophosphate (IMO). This releases NH_4^+ that helps the cell in N-limitation. Because in oleaginous fungi isocitrate dehydrogenase (IDH) is dependent on activation by AMP this stops transformation of isocitrate into α-ketoglutarate. This leads to build-up of isocitrate and by extension citrate,

which is in equilibrium with it. After citrate levels reach a critical point, it is transported out by citrate-malate translocase in exchange with malate (Figure 5). Citrate, now present in cytoplasm, is then converted to acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACL). The accumulated Acetyl-CoA is then shuttled to fatty acid synthesis while oxaloacetate is converted into malate to counter the citrate efflux system (Passoth 2017). Besides N-limitation lipid accumulation can also be influenced by using sulphur limited (Wu *et al.* 2011) and phosphorus limited conditions (Wu *et al.* 2010).

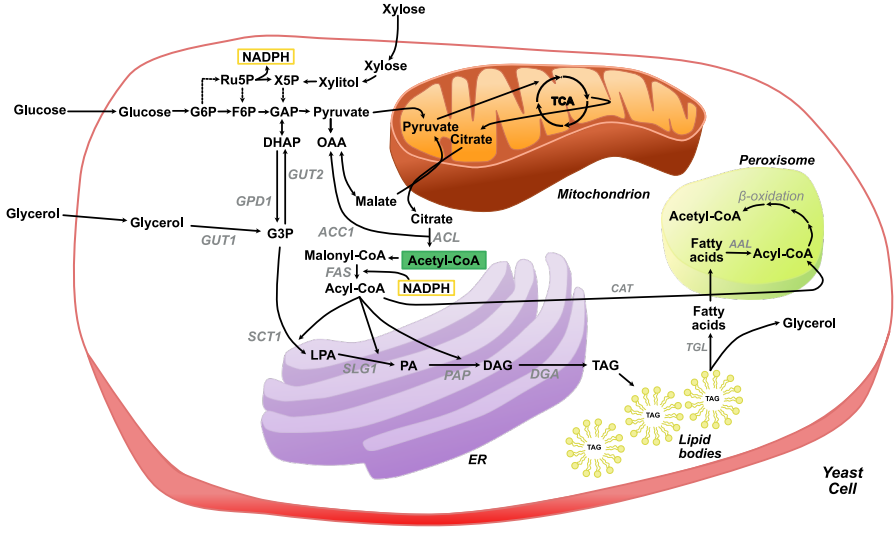

Figure 4. Lipid synthesis and degradation pathways in yeasts. Abbreviations: ACL: ATP-citrate lyase; ACC1: acetyl-CoA carboxylase; AAL: acyl/aryl-CoA ligase; CAT: carnitine acetyl-transferase; DAG: diacylglyceride; DGA: diacylglycerol acyltransferase; DHAP: dihydroxyacetone phosphate; ER: endoplasmic reticulum; F6P: fructose-6-phosphate; FAS: fatty acylsynthetase; G3P: glycerol-3-phosphate; G6P glucose-6-phosphate; GAP: glyceraldehyde phosphate; GUT1: glycerol kinase; GUT2: glycerol 3-phosphate-dehydrogenase; GPD: glycerol-3-phosphate dehydrogenase (NAD(þ)); LPA: lysophosphatidic acid; ME: malic enzyme; OAA: oxaloacetate; PA: phosphatidic acid; PAP: phosphatidic acid phosphatase; Ru5P: ribose-5-phosphate; SCT1: glycerol-3-phosphate acyl transferase; SLC1: 1-acyl-sn-glycerol-3-phosphate acyltransferase; TAG: triacylglyceride; TCA: tricarboxylic acid cycle; TGL: triacylglycerol lipase; X5P: xylulose-5-phosphate. Modified from Szczepańska *et al.* (2021).

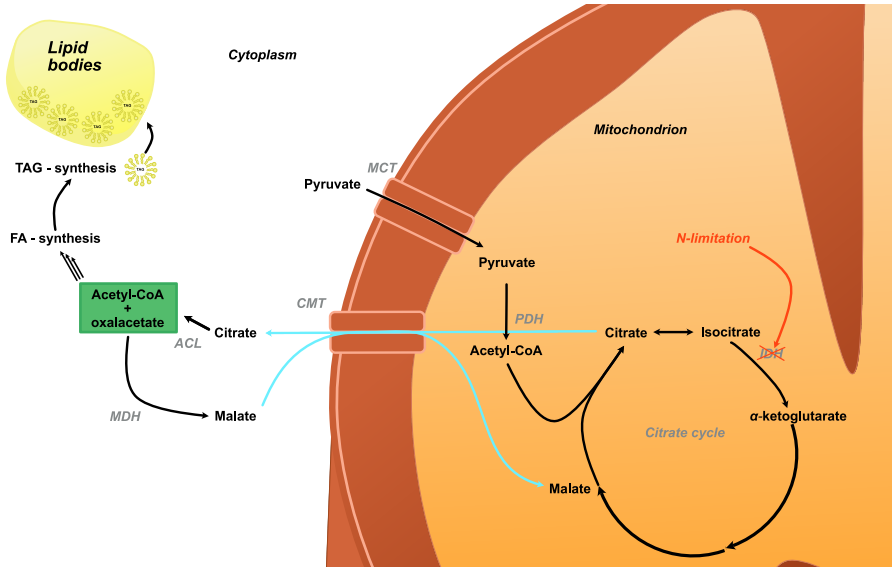

Figure 5. Simplified overview of lipid accumulation in oleaginous yeast during Nitrogen limitation. Abbreviations: monocarboxylic acid transporter (MCT), citrate-malate translocase (CMT), ATP-citrate-lyase (ACL), pyruvate dehydrogenase (PDH), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH).

1.4 Lipid analysis

The biggest obstacle in the extraction of the lipids from yeast is the cell wall. It is composed of several layers containing mannoproteins, beta glucans and chitin (Stewart 2017). Degrading the cell wall requires either physical destruction, for example by using French press or chemical means by using acids in a high temperature environment. This makes analysing lipid content and lipid composition a relatively difficult task. Over the years several analytical methods have been developed, of which some rely on solvent and chemical treatment, while the newer ones use less destructive methods like spectroscopy.

In past 50 years the most commonly used methods for lipid extraction were developed by Folch, Lees and Sloane Stanley (Folch *et al.* 1957), and Bligh and Dyer (Bligh & Dyer 1959). In the present study, the most commonly used technique in biological samples known as the Folch method was used for extracting yeast oil. This technique has been quoted over 45,000

times making it one of the most cited papers of all time. It's using a system of bi-phasic solvents made of chloroform/methanol/water in the volumetric ratio of 8:4:3 (Folch *et al.* 1957; Eggers & Schwudke 2016). However, in this project chloric acid was needed to destroy the yeast cell wall (Brandenburg *et al.* 2016). What differs from the original technique was that instead of traditional tissue sample a freeze-dried sample dissolved in hydrochloric acid was used. In the Folch- method two layers of liquid are separated – the upper face is consisting of water, methanol, hydrophilic components and salts, the lower phase is composed of chloroform and extracted lipids. Subsequently the lower face needs to be removed from the vessel and the solvent is evaporated to obtain the pure lipids.

Using this technique allowed us to quantify lipids in the cells during the cultivation. However, since it requires quite substantial amount of sample it was impractical to use this extraction method for lipid determination in earlier stages of cultivation. This illustrates the demand for a technique that doesn't require large amounts of sample, is preferably non-destructive and does not require environmentally troublesome solvents.

An example of such technique - based on infrared (IR) spectrum is Fourier transform Infra-red spectroscopy (FT-IR). It has been used for fish oil and olive oil analysis (Qu *et al.* 2015; Jiang & Chen 2019) and is also commonly used in industry to analyse the content of other compounds like sugar in grain (Sohn *et al.* 2007). The basic principle of infrared spectroscopy relies on measuring the molecular vibrations between carbon molecules bound to atoms like hydrogen after being excited by infrared light. In practice this allows to identify and quantify many biomolecules such as proteins, lipids, DNA, and other organic compounds. FT-IR was successfully used to predict lipid content in oleaginous yeast (Shapaval *et al.* 2019).

Another technique - Near Infrared (NIR) spectroscopy uses an electromagnetic wavelength spectrum that ranges from 780 nm to around 2500 nm. Above these wavelengths starts mid-IR region. The biggest downside of NIR spectroscopy is that absorption bands are broad and often overlapping, which results in complex spectra that are difficult to interpret (Stuart 2004).

Fourier transform near infrared spectroscopy (FT-NIR) differs from NIR spectroscopy by the method in which cell sample is scanned. In NIR spectroscopy a sequentially changing monochromatic light is used on the sample over the whole NIR wavelengths. In FT-NIR the principle is slightly

different - the sample is exposed to polychromatic light that is divided with a beam splitter which results in a so-called interferogram which shows the intensity of the light as a function of time. That interferogram is later transformed to frequency by Fourier transformation. The upside of is that it's much faster than traditional NIR spectroscopy and it allows simultaneous measurement over the whole wavelength range, on top of that it is more sensitive, has less background noise and is more precise (Stuart 2004). However, it's not without its downsides. The biggest downside of FT-NIR is water absorption potential. Water gives very strong signals that can overlap with regions of interest, thus the water in a sample has to be removed or at least reduced in content.

2. Aim

The goals of this thesis were to establish a rapid, spectrum- based analysis tool for lipid quantification (**I**), investigate the conversion potential of crude glycerol (CG) and hemicellulose hydrolysate (HH) to lipids by oleaginous yeasts (**II**) and to get an insight into physiology of conversion of CG and HH to yeast lipids (**III**). The relation between papers is presented in Figure 6.

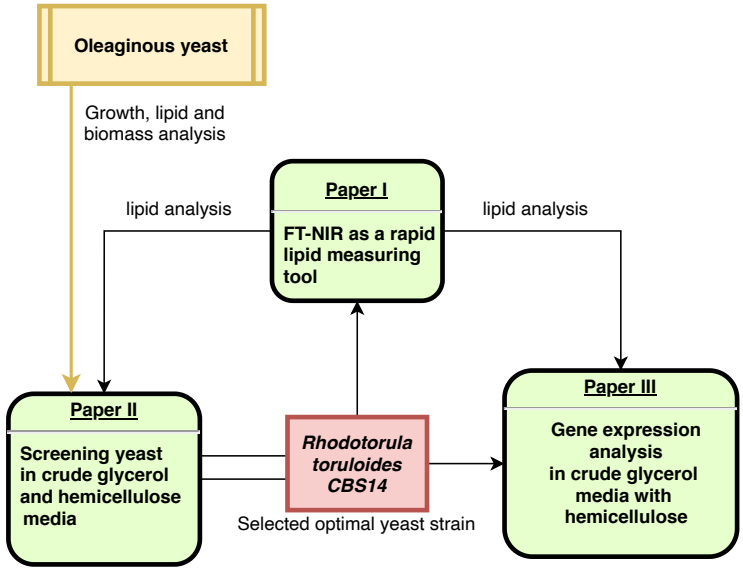

Figure 6. Relation and flow between each of the studies. Papers I and II were developed in parallel to each other. When Paper I was finished, the FT-NIR tools were applied in paper II, which was basis for paper III.

3. Tools for lipid quantification

As mentioned in introduction – lipid quantification is a time consuming and laborious process. Quite often it is difficult to follow kinetics of lipid accumulation, especially early in the culture (Shapaval *et al.* 2019; Brandenburg 2021). To address the issues of using harmful organic solvents, long processing time and need of relatively high sample size during lipid extraction we developed a faster, non-invasive method of analysis that requires less of sampling material compared to the established gravimetric method.

Currently there are several ways to utilise non-destructive IR light for analysis. All of the IR techniques rely on the measurement of light absorption by vibration of the molecules. The way measurement is done varies between methods. In typical IR - sometimes referred as mid infra-red (MIR) - wave numbers of 4000 – 400 cm^{-1} are used. In near infra-red (NIR) 12800 – 4000 cm^{-1} are used. MIR offers more defined peaks that can be easier assigned to functional groups in molecules. NIR is focused more on overtones and their combinations. A comparison between spectra can be seen in Figure 7.

FT-NIR uses additional equipment called interferogram which allows to plot intensity of light as function of time which is then Fourier transformed to frequency domain (paper **I)**. The Fourier transform (FT) is a mathematical transformation that decomposes functions of time into functions of frequency (Wikipedia 2020). Thanks to that, FT-NIR is a much faster technique allowing to measure the whole wavelength range simultaneously, is more sensitive and has less background interference when compared to IR (Stuart 2004).

FT-NIR spectroscopy has been used in many fields to analyse lipids. It was used to measure fat in the human body in a non-invasive way (Azizian *et al.* 2008), to quantify lipid content and its type in plants (Camps *et al.*

2014) and to determine olive oil quality and its authenticity (Mossoba *et al.* 2017). It also allowed to analyse maturity of cheese (Priyashantha *et al.* 2020), lipids in milk (Mlcek *et al.* 2016), yeasts (Ami *et al.* 2014; Laurens *et al.* 2019) and moulds (Kosa *et al.* 2017).

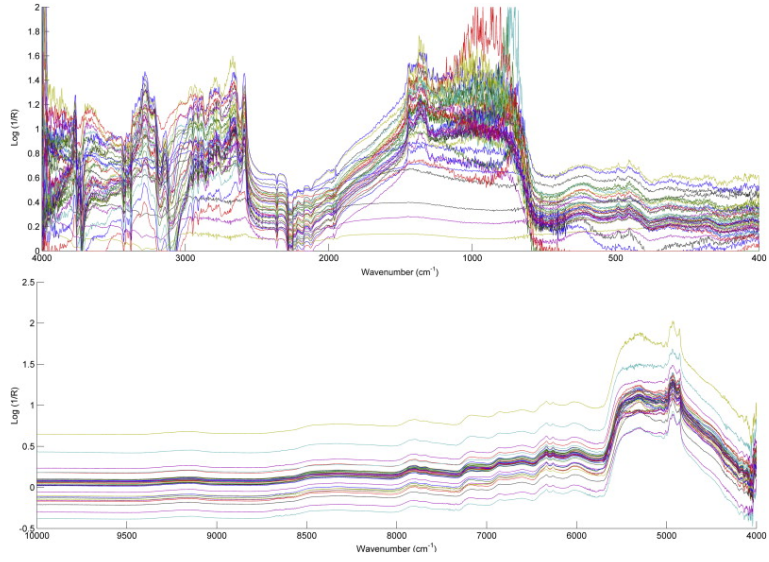

Figure 7. **Top -** an example of MIR spectra without pre-treatment (e.g., baseline adjustment), **Bottom** – an example of NIR spectra without pre-treatment (e.g., baseline adjustment). Reprinted from (Ferreira *et al.* 2014) with permission from Elsevier (2021).

3.1 FT-NIR as tool for lipid analysis in oleaginous yeasts

When compared with traditional lipid extraction methods, first steps of FT-NIR- based lipid quantification are identical – sample is collected, centrifuged, washed, frozen and then freeze-dried. But after this step FT-NIR differs from gravimetric lipid quantification. A small amount of the pellet is moved into a glass cylinder and put on top of a sphere chamber (in our case Multi-Purpose Analyser - MPA by Bruker was used) where it is exposed from below to NIR light. The instrument collects data and lipids in the resulting spectra of yeast can be quantified with a model that was established in Paper **I**.

3.1.1 *R. toruloides* and *L. starkeyi* models

To create a *R. toruloides* model, 179 spectra were collected out of unique 60 samples of *R. toruloides*. Samples after scanning then had to be analysed using our standard modified Folch method (Brandenburg *et al.* 2016). Results from lipid extraction were assigned to spectra in the OPUS software. The resulting model of *R. toruloides* had a cross-validation R^2 value of 98% and a RMSECV value of 1.53 at rank 9. For spectra pre-processing vector normalization was used. The FT-NIR regions used to produce the model were: 8562.9–8038.4 cm^{-1} and 4485.9–4069.3 cm^{-1}. The linear regression equation was: $y = 0.9726x + 0.9786$ (Figure 8). We compared the resulting model with standard lipid extraction (Figure 9). The average difference between real data and FT-NIR prediction was ~5.2%, which means that this method is suitable for single strain application. Unfortunately, the model cannot be used between species unless specifically designed for that. We tried using the *R. toruloides* model on *L. starkeyi*, however, results were unusable due to vast differences in spectra – the R^2 was 36% which is not better than a random guess by the software. To address that we made another model specifically aimed at *L. starkeyi*. We used 99 spectra and the resulting prediction model had a R^2 of 96% and a RMSECV of 2.4 at rank 2. Vector normalisation was used for spectra pre-processing, and FT-NIR regions used were: 8694.1–8061.5 cm^{-1}, 7151.2–6649.8 cm^{-1} and 4466.6–3849.5 cm^{-1}. The linear regression equation was: $y = 0.94x + 1.54$.

3.1.2 Combined model

A third model we prepared was combining all available spectra from *R. toruloides*, *L. starkeyi* and *Y. lipolytica*. The resulting prediction model was however, not as accurate as individual models. The resulting R^2 was 90.5% (Figure 8).

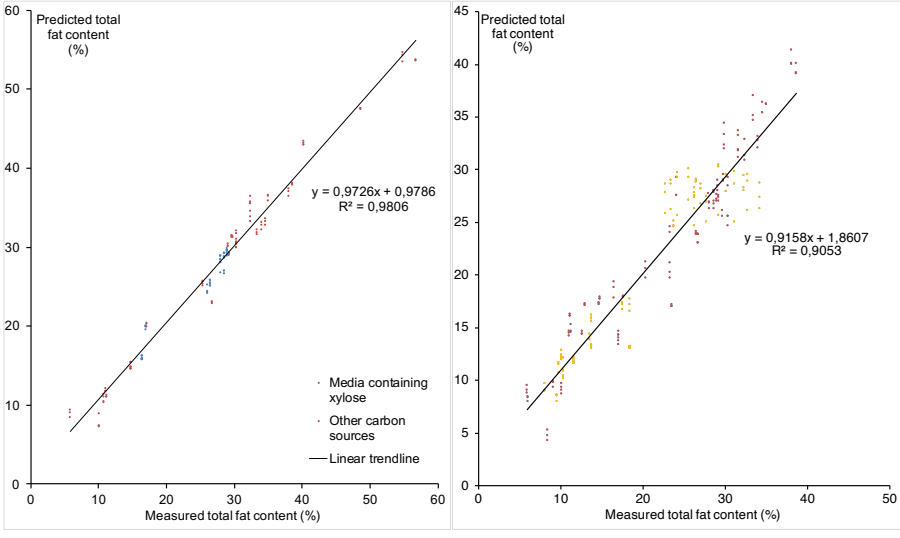

Figure 8. **Left** - Test set validation of the total fat content in the *R. toruloides* CBS14 model. Cells were grown using YNB media with glucose, xylose and glycerol. In total, 161 spectra were used for the calibration set of the model. Independent samples in form of 42 spectra were used as test set validation. **Right** - Cross-validation of the total fat content in the model of several combined yeast species (*R. toruloides, L. starkeyi, Y. lipolytica*), cultivations using hemicellulose hydrolysate, mixed hydrolysate, crude glycerol or glucose with YNB as cultivation media. Red dots represent red yeast, yellow dots represent *L. starkeyi* and *Y. lipolytica* samples. Figures reprinted from Chmielarz *et al.* (2019).

The combined model was not considered reliable enough to warrant usage. However, if provided with more samples and refinement it should in theory be possible to create a model that would be accurate enough for several strains of oleaginous yeast simultaneously.

In conclusion, we have established a non-invasive and rapid method for lipid quantification, which allows following the kinetics of lipid accumulation in *R. toruloides* and *L. starkeyi*. The *R. toruloides* model was used in (Paper II) and is currently being improved with an even more refined method that should allow an even faster sample analysis. In the new approach, samples do not need to be freeze-dried, but are instead dried on a glass slide and measured in a custom 3D printed holder. This potentially should allow to measure samples straight from bioreactor within 30 minutes instead of several hours (Brandenburg 2021).

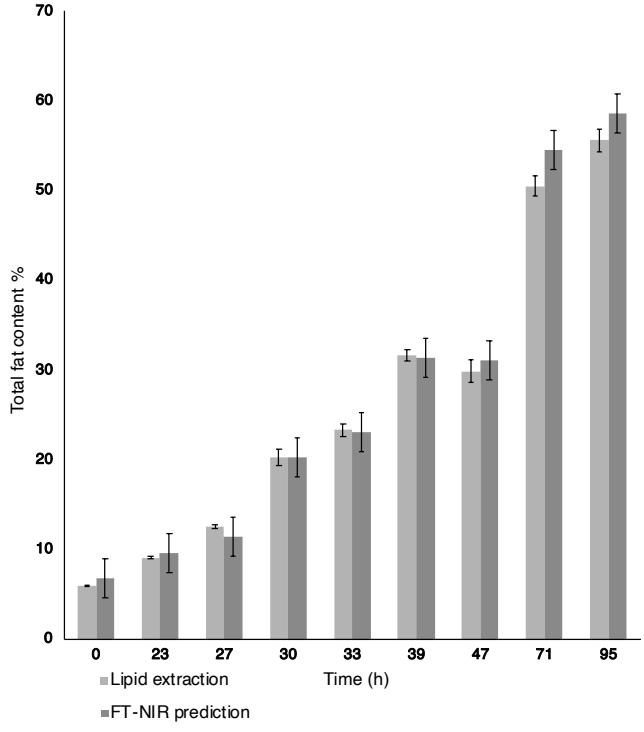

Figure 9. Comparison of the accuracy of FT-NIR lipid quantification based on the *Rhodotorula* (dark grey bar) and traditional lipid extraction (light grey). Values were obtained by analysing test culture samples using the Quant2 analysis package. The samples were obtained from the cultivation of *R. toruloides* cultivated in a lignocellulosic hydrolysate (n=27). The error in prediction was 2.17 for the FT-NIR model. Average difference between the lipid extraction results and the FT-NIR predictions was 5.2%. Figure reprinted from Chmielarz *et al.* (2019).

4. Conversion of CG and HH to yeast lipids

Crude glycerol is a problematic side product containing several toxic components like soaps, ash and methanol. These residues can be removed by several methods like distillation, ion exchange resin, membrane separation, acidification followed by neutralization and solvent extraction. All of the mentioned methods are resulting in loss of yield and some require high energy input (distillation – heating and high vacuum) (Abdul Raman *et al.* 2019). To address this there is a need to find ways to utilize CG by methods which function without expensive purification (Knothe 2010; Abbas *et al.* 2016; Okoye & Hameed 2016; Li *et al.* 2017). Using CG as a carbon source for microbial growth is not a novel concept and has been investigated previously (Papanikolaou & Aggelis 2009; Posada & Cardona 2010; Chatzifragkou *et al.* 2011; Diamantopoulou *et al.* 2020). In the past few years, it has been shown that glycerol can be converted to useable biomass, which then can be utilized as catalyst for fuel cells (Poladyan *et al.* 2020). CG can also be converted into fatty acids by *Y. lipolytica* (Gajdos *et al.* 2020) and to produce microbial oil and other valuable products alone or in combination with other waste substrates (Saenge *et al.* 2011; Yen *et al.* 2012; Shen *et al.* 2013; Spier *et al.* 2015; Xin *et al.* 2016; Diaz-Fernandez *et al.* 2019).

The second substrate used - HH is also toxic to most microorganisms due to a low pH (pH < 3), and the presence of inhibitors like furfural, hydroxymethylfurfural (HMF) and acetic acid. Moreover, not all microorganisms are capable of using xylose as carbon source, which is the dominant sugar present in most hemicelluloses (Girio *et al.* 2010; Biely *et al.* 2016; Passoth & Sandgren 2019; Brandenburg 2021). Our previous studies show that HH is a suitable carbon source for oleaginous yeasts

(Brandenburg *et al.* 2016; Karlsson *et al.* 2016; Brandenburg *et al.* 2021). Combining xylose and glycerol as carbon sources for microorganisms has been investigated before (Xin *et al.* 2016; Diaz-Fernandez *et al.* 2019; Diamantopoulou *et al.* 2020). However, this concept was not tested before using raw HH or crude forms of CG.

In this project we tested 27 strains for their potential of growth on CG as sole carbon source. In these growth tests on plates only 11 of the 27 strains showed significant growth. Most ascomycetes except for *L. starkeyi* CBS 7786 had little to no growth (Figure 10).

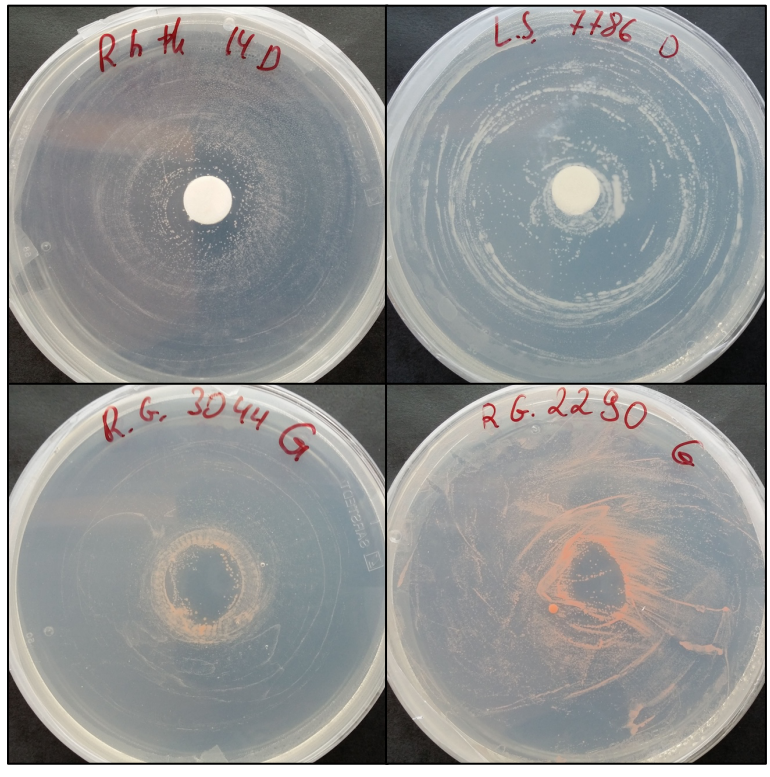

Figure 10. Example of four positive results in plate test. To the plates discs soaked in 50% crude glycerol (Top) or three drops of crude glycerol were added (bottom).

Additional tests were performed with 11 yeast strains that showed significant growth on CG. We evaluated the capacity of those 11 strains in media containing up to 120 g/L CG and if they could grow with addition of 20% HH (see sHH20 media in paper II). Differences in growth rates between

strains were observed. All strains showed relatively good growth, up to a CG concentration of 60 g/L, which was consistent with the results reported by Chatzifragkou *et al.* (2011).
The tested *Lipomyces* strain showed significant growth in crude glycerol, but significantly slower than the red yeasts. Indeed, its growth was already slowed down in a CG concentration of 60 g/L in contrast to reports in the literature, where glycerol was used effectively (Spier *et al.* 2015). The low tolerance was probably due to some inhibitors like soaps, free fatty acids and residual catalysts, which could be present in the sample due to differences in the purification process at each biodiesel plant.
Another observation was that mixing CG and HH negatively affected the growth and assimilation of glycerol and xylose in *L. starkeyi* CBS7786. The presence of xylose has earlier shown to have a somewhat negative effect on lipid accumulation in other yeasts or cause co-consumption of intracellular lipids (Diamantopoulou *et al.* 2020; Brandenburg 2021; Brandenburg *et al.* 2021).

After the series of initial screenings in shake flasks we focused on two strains of *Rhodotorula –R. toruloides* CBS14 and *R. glutinis* CBS3044. Of the tested strains, they performed best on both CG and HH. With these strains we performed series of cultivations in bioreactors with different ratios of mixed CG and HH and compared them with pure CG as sole carbon source. With those cultivations we found that we could obtain concentrations of lipids up to 10.6 g/L in 40% HH 60 g/L CG media. The same cultivation reached a lipid yield up to 0.23 g/g of carbon source.

During these cultivations we noticed an interesting effect on lipid production: adding as little as 10% HH (equivalent to total of ~3 g/L carbon in form of xylose, glucose and acetic acid) had a positive effect on lipid production rates in *Rhodotorula* species, in contrast to the *L. starkeyi* strain (see above). We investigated this effect in more detail in paper **III (**see following chapter).

5. Physiology of CG and HH conversion

Biology of the cell – for instance growth, responding to environment, metabolism, development and response to disease are regulated by various mechanisms like gene expression or enzyme activity. These mechanisms are very conserved and almost identical throughout the whole eucaryotic kingdom (Struhl 1995). In simple terms DNA is transcribed to mRNA which is then modified through splicing and then translated into proteins. These processes are heavily regulated inside the cell alongside all of the steps. Using that knowledge, we can identify bottlenecks in metabolism, enzyme activity and which genes are upregulated or downregulated (Struhl 1995; Hahn & Young 2011; Martín-Hernández *et al.* 2021).

During our cultivations we noticed an interesting effect in cultivations with mixed HH and CG. There seemed to be an increased glycerol uptake and lipids were synthesised faster when as little as 10% HH was present in media (II). We investigated this in more depth by analysing mRNA levels during various cultivation times (III). To decide which timepoints to use growth curves from Paper **II** were used. Based on how strains behaved in previous experiments the decided timepoints were 10, 30 and 60 h for CG cultivation and 10, 36 and 60 for a mix of CG and HH. 10 h CGHH still had a mixed substrate present, 36 h cell had only glycerol available and 60h had similar glycerol concentration in CG media as CGHH at 36 h.

To perform mRNA extraction from cells, a ready to use extraction kit is preferred. As ribonucleases (RNases) are present everywhere great care needs to be taken to not destroy the sample (Martín-Hernández *et al.* 2021). Extracted RNA was cleaned from ribosomal RNA and then processed by Ilumina and Nanopore sequencing. Results were analysed using bioinformatic tools and annotated using homology searches. KEGG orthology (KO) numbers were associated to genes probably encoding

enzymes. Using this data and KEGG mapper (*KEGG mapper* 1995-2021) it was possible to check and identify metabolic pathways, where genes were transcriptionally regulated. Although transcription is only one partial process in regulating metabolic activity, it is possible to generate sound hypotheses on regulation of certain metabolic pathways, when many genes of these pathways are regulated in the same way (Chubukov *et al.* 2012; Hara & Kondo 2015; Martín-Hernández *et al.* 2021).

Based on the results of mRNA sequencing, we concluded that most upregulated genes during addition of hemicelluloses were genes associated with energy metabolism, especially oxidative phosphorylation, other mitochondrial enzymes and gene expression, protein synthesis and – degradation. In most cases, the strongest upregulation was observed after 10 h of cultivation in CGHH (Paper **III**).

A number of upregulated genes were encoding components of the respiratory chain and other mitochondrial enzymes. Additionally, we observed an about two-fold transcriptional upregulation of ribosomal proteins suggesting increased protein production in cells growing in CGHH. Such upregulation was much later in CG cultures.

We decided to investigate glycerol-3-phosphate dehydrogenase (G3P) enzymes encoded by *GUT1* and *GUT2* (Sprague & Cronan 1977; Swinnen et al. 2013). We observed that glycerol transporters were more abundant in CG cultures from earlier timepoints but it was more enhanced in CGHH in later stages. The catabolic G3P- pathway was present in all conditions, but it was higher at later timepoints in CGHH culture. We tried to find a third, alternative pathway for glycerol assimilation through 3-P-D-glycerate, which can be found in *Neurospora crassa* (Klein *et al.* 2017). However, we did not observe enzymes necessary for this pathway meaning we could not prove this pathway exist in *R toruloides* CBS 14.

The transcriptional increase of genes involved in oxidative phosphorylation and many mitochondrial enzymes in CGHH at 10 h compared to CG at 10 h was one of the most striking findings. This effect was noticeable at later time points in the cultivation, after the increased carbon sources, in form of xylose, glucose and acetic acid had already been used, at least for some genes. This means that when the HH was added, the energy metabolism was engaged. A high ATP availability is essential for biomass and lipid production, and it may also allow for more effective uptake of scarce nutrients like nitrogen. The also increased transcription of genes

involved in protein synthesis was most likely related to coping with changing carbon source availabilities in CGHH (Hara & Kondo 2015).

The activation of numerous genes involved in the oxidative stress response has been linked to xylose growth. Our observations of transcriptional activity of those genes, particularly in CGHH, after 10 hours of cultivation validated this (Tiukova *et al.* 2019). The assimilation of xylose may cause a stress response in the cell, while it is unclear why this sugar causes this response.

In conclusion the addition of hemicellulose produces significant shifts in gene transcription in *R. toruloides*, which results in better crude glycerol to lipid conversion. Further research into the activating process could lead to the discovery of new targets for obtaining strains that rapidly accumulate lipids on low-value residual substrates.

6. Other findings and observations

In addition to the results related to the aims of this work, we also observed interesting behaviours in some yeasts that were not yet published but are worth mentioning and to consider for future research.

6.1 Biomass loss

Quite early in bioreactor cultivations we noticed that big chunks of yeast were stuck on the vessel walls forming “clumps”. These clumps were significant enough to impact dry weight and OD_{600} measurements and needed to be addressed (Figure 11). In beginning, we tried using detergents like Tween20 to see if it affects cell adhesion to bioreactor walls, which decreased cell attachment but only temporarily. Further testing showed that *R. toruloides* was able to consume Tween20 as carbon source thus making it unusable (Figure 12).

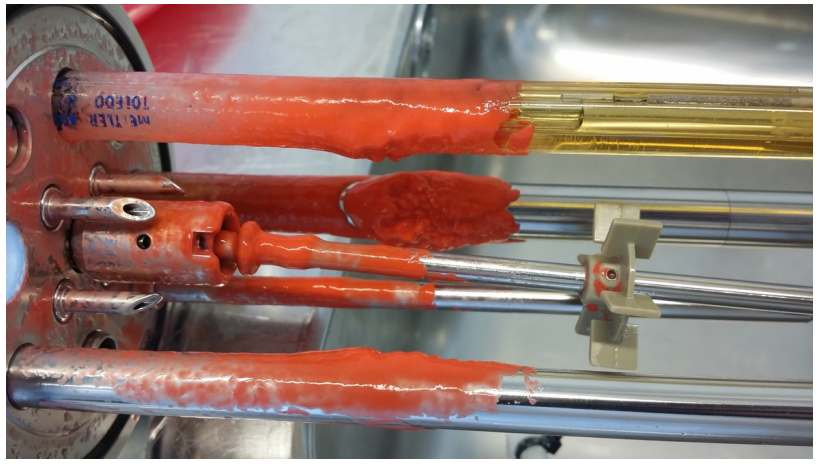

Figure 11. Example of cell adhesion in *R. toruloides*.

Figure 12. Cultivation of *R. toruloides* CBS14 with Tween detergent. In the second shake flask from the left labelled "Tween C" Tween was the sole carbon source.

During a shake flask experiment we decided to also test using the antifoaming agent polypropylene glycol (PPG) – which showed promising results – the cells had noticeably less adhesion. We also tested using higher stirring speed to see if it could wash away the cells but it had no effect. Finally – we found that using PPG in higher than standard concentrations (~0.05% of working volume) prevented the cells from adhering to the inside of the bioreactor vessel (Figure 13). Hence, it became standard practice to use that concentration of PPG when cultivating *Rhodotorula* species strains.

Figure 13. Cultivation of *R. toruloides* CBS14 using 0.1% PPG volume. Using PPG prevented yeast adhesion to vessel.

The yeast sticking to the vessel wall was most likely a stress response to nitrogen limitation in the media. We did not observe cell adhesion in YPD cultures. Cell adhesion seems to be a typical virulence factor in *Rhodotorula mucolaginosa* which secrete a mixture of proteins and polysaccharides to produce a biofilm (Jarros *et al.* 2020). In *R. glutinis* cell adhesion seems to serve as an competition prevention by inhibiting sporulation of other fungi (Li *et al.* 2016). It is also possible that cell adhesion in nature can serve as a beneficial survival mechanism or means of transport with help of insects. Another interesting observation was that during adhesion cultures had a pleasant floral like fragrance, especially when glycerol was used as carbon source. This makes it worth further to investigate the hypothesis of attracting insects.

6.2 *Rhodotorula babjevae* DVPG 8058

This strain was a surprisingly rapid producer of foaming agents resulting in overfilling fermenters with foam, causing spill (Figure 14). The culture also caused the supernatant to become highly viscous (viscosity was not measured) and we observed a yellowish-brown pellet formed in the bottom of the tubes after centrifugation. This fraction did not contain cells and the composition was determined to be glycolipids (Cajka *et al.* 2016) and we confirmed its structure (unpublished data).

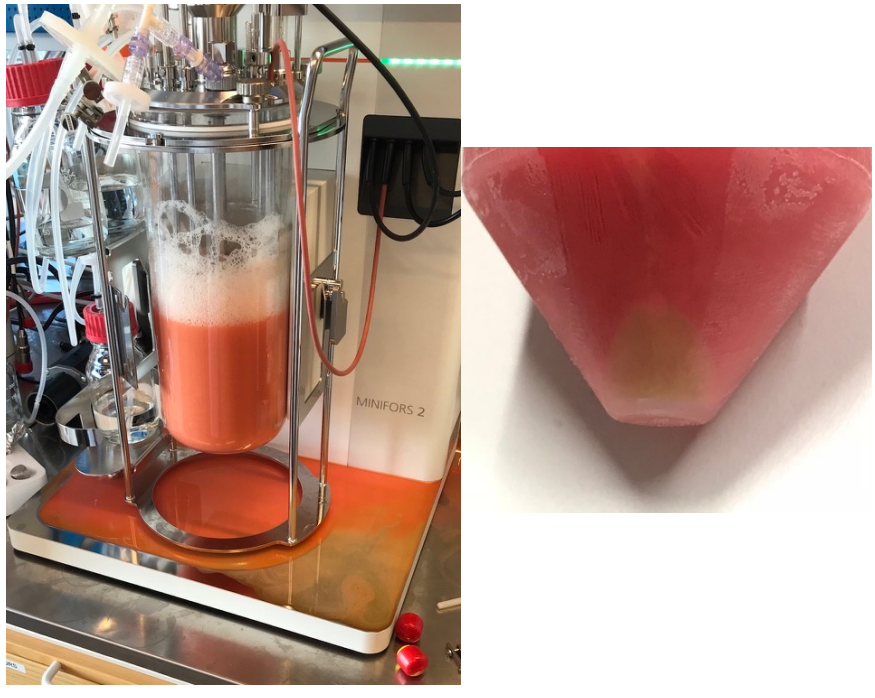

Figure 14. Overnight cultivation of *R. babjevae* DVPG 8058. Excessive foaming started within first 24h of cultivation and was not prevented by using starting amount of PPG. An interesting observation was a pleasant gently sweet smell coming from the spill (left). After centrifuging cultures, a yellowish sediment could be collected (right).

7. Conclusions

Oleaginous yeasts have great potential when grown on hemicellulose and crude glycerol. This thesis provided a new method to measure lipids in yeasts, identified potential strains of *R. toruloides* and *R. glutinis* capable of utilising HH and CG to produce lipids and investigated RNA expression of *R. toruloides* when xylose from hemicellulose hydrolysate was present.

The new established FT-NIR method (Paper I) was used in Paper II and is being improved with a new approach (Brandenburg 2021). This method considerably improves the possibility to follow the kinetics of lipid metabolism during cultivation and reduces usage of time and solvents, which should help with future optimisations of cultivation process. The precision of the used model can and will be improved over time by adding new data to the model.

In Paper II we identified strains that are good producers of lipids from crude glycerol and hemicellulose hydrolysate. This became subsequently the main focus for further studies (Martín-Hernández *et al.* 2021). Utilising these strains in larger scale like biorefinery for lipid production could increase sustainability – we proved that utilising waste substrates like crude glycerol and hemicelluloses should be possible. Yields showed that there is huge potential in these yeasts for further development and optimisation of processes. We also discovered an activating effect of addition of hemicelluloses, which increased *R. toruloides* lipid production and glycerol uptake.

The final partial study of this PhD- project (Paper III) evaluated and revealed some of the mechanisms responsible for the increase in lipid production and glycerol consumption with added hemicellulose hydrolysate. The identified genes might be interesting targets for manipulation in order to increase lipid production in future experiments.

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Popular science summary

Currently most of the world is dependent on a linear approach to economy which is based on using non-renewable fossil raw materials. This approach has negative effects on climate and environment. Humanity's goal should be to adopt sustainable circular approaches which are not reliant on resources that will be exhausted one day. One possible approach is a bio-based economy in which products and waste can be re-used in a sustainable way. Utilisation of green energy like solar and wind is a first step towards a sustainable economy. In terms of bioeconomy, we have been using biodiesel and bioethanol for several years. When it comes to these biofuels unfortunately, they come at a cost of rainforests. So called "first generation" biofuels rely on growing crops in very unsustainable way and rainforest are being cut down to make space for plantation of for example palm oil. To address this, next generations of biofuels were created.

In this thesis we used a microorganism called oleaginous yeast. Yeast are fungi most known for being used in baking bread and brewing beer. Oleaginous is term describing organisms that can accumulate over 20% of their dry mass as oil (lipids). Yeasts that were used belong to *Rhodotorula* genus, most recognized for their red/pink colour and *Lipomyces starkeyi*.

To grow those yeasts, we used a waste product form biodiesel industry – crude glycerol. Another source of carbon for the yeasts was hemicellulose hydrolysate which is one of the fractions formed after digesting plant residues during a process called steam explosion. Both waste products are quite toxic in their raw form to most microorganisms so it was important to find yeast strains that can grow well on these substrates. During this project we found that the red yeast *Rhodotorula toruloides* is most versatile when using these two substrates to make lipids.

Because yeasts have resistant cell walls their lipids are not easy to access. Usually harsh treatment, long extraction process and organic solvents are necessary. This difficulty makes lipid quantification challenging, but lipids need to be quantified throughout the fermentation to be able to understand the physiology of oleaginous yeasts and to optimise lipid production. To solve that problem, we developed a method based on near infra-red light called Fourier transform – near infra-red spectroscopy (FT-NIR). It works by beaming infra-red light on a dried yeast sample and the machine measures how much light was absorbed by the molecules. Then the resulting spectra can be associated with lipid concentrations determined by an extraction method and create a prediction model for further samples. Using this method, we created prediction models that allowed us to measure how much lipids are inside cultivated yeast with 98% accuracy in red *Rhodotorula toruloides* yeast and 96% accuracy in *Lipomyces starkeyi* yeast.

During the growth we also noticed that when hemicellulose hydrolysate was added, yeasts were consuming glycerol and producing lipids faster. To find out why, we looked at gene transcription by sequencing RNA, which is the template to make the proteins of the cells. We compared the expressed RNA and how its levels in cells differed in cultivations with and without hemicellulose hydrolysate at different timepoints. The biggest difference was an increase of enzymes that are related to mitochondria, which are producers of energy in cell. We concluded that adding hemicellulose causes a major shift in expression levels which caused increased consumption of glycerol and lipid production.

Populärvetenskaplig sammanfattning

För närvarande är större delen av världen baserad på linjära ekonomier som bygger på användning av icke förnybara fossila råvaror. Dessa system har negativa effekter på klimat och miljö. Ett övergripande mål för våra samhällsystem bör vara att få på plats hållbara cirkulära system som inte är beroende av ändliga resurser som kommer att ta slut en dag om vi inte förvaltar dessa på ett bra sätt. En del av ett framtida cirkulärt samhälle är en biobaserad ekonomi där produkter och avfall kan återanvändas på ett hållbart sätt. Användning av grön energi som sol och vind är ett första steg mot en hållbar ekonomi. När det gäller bioekonomi har vi redan använt biodiesel och bioetanol under flera decennier. Produktion av dessa biodrivmedel orsakar ibland skövling av regnskogar. Så kallade ”första generationens” biobränslen förlitar sig på att odla grödor på ett mycket ohållbart sätt och regnskogen skövlas för att skapa plats för plantering av till exempel träd för palmolja. För att ta itu med denna problematik måste vi utveckla nästa generation av biodrivmedel.

I denna avhandling använde vi mikroorganismer som kallas lipidackumulerande jäst. Jäst är svampar som är mest kända för att användas vid bakning av bröd och bryggning av öl. Lipidackumulerande är en term som vi använder för att beskriva organismer som kan samla över 20% av sin torra massa som olja (lipider). Jäst som används tillhör *Rhodotorula*-släktet, mest känt för sin röda eller rosa färg och *Lipomyces starkeyi*.

För att odla jästen använde vi i detta arbete en avfallsprodukt från biodieselindustrin – råglycerol. En annan kolkälla som vi har använt för att odla jästen är hemicellulosahydrolysat, vilket är en av de fraktioner som bildas efter att ha behandlat växtrester under en process som kallas ångexplosion. Båda avfallsprodukterna är ganska giftiga i sin råa form för de flesta mikroorganismer, så det är viktigt att hitta jäststammar som kan växa

bra på dessa odlingssubstrat. Under detta projekt fann vi att den röda jästen *Rhodotorula toruloides* var mest framgångsrik för lipidproduktion på dessa två substrat.

Eftersom jäst har resistenta cellväggar är det inte lätt att komma åt lipiderna som finns inuti jästen. Vanligtvis är hård behandling, lång extraktionsprocess och organiska lösningsmedel nödvändigt för att få ut lipiderna. Denna svårighet gör lipidkvantifiering utmanande, men lipider måste kvantifieras under odling av jästen för att kunna förstå fysiologin hos jästen och för att optimera lipidproduktionen. För att uppnå detta har vi utvecklat en metod baserad på nära infrarött ljus som heter Fouriertransform – nära infraröd spektroskopi (FT-NIR). Denna metod fungerar genom att stråla infrarött ljus på ett torkat jästprov och en maskin mäter hur mycket ljus som absorberades av molekylerna. Sedan kan det reflekterande ljuset sammankopplas med kända lipidkoncentrationer från extraktion och på så sätt skapas en modell för haltbestämning av framtida jästprover. Den nya FT-NIR-metoden som vi har tagit fram har gjort det möjligt att enkelt mäta hur mycket lipider som finns i odlad jäst med 98% noggrannhet i röd *Rhodotorula toruloides*-jäst och med 96% noggrannhet i *Lipomyces starkeyi*-jäst.

När vi gjorde mätningar av våra jäststammar under odling märkte vi också att när hemicellulosahydrolysat tillsattes konsumerade jästen glycerol och producerade lipider snabbare. För att ta reda på varför detta skedde tittade vi på uttryck av jästens gener genom att sekvensera dess RNA, vilket är mallen för att tillverka proteiner i cellerna. Vi jämförde det uttryckta RNA:t och hur dess nivåer i celler skilde sig åt i odlingar med och utan hemicellulosahydrolysat vid olika tidpunkter. Den största skillnaden var en ökning av uttryck av enzymer som är relaterade till mitokondrier, som producerar energi i celler. Vi drog slutsatsen att tillsats av hemicellulosa orsakar en stor förändring i uttrycksnivåer som kan leda till ökad konsumtion av glycerol och produktion av lipider.

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Tack så mycket!

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METHODOLOGY

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FT-NIR: a tool for rapid intracellular lipid quantification in oleaginous yeasts

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Abstract

Background: Lipid extraction for quantification of fat content in oleaginous yeasts often requires strong acids and harmful organic solvents; it is laborious and time-consuming. Therefore, in most cases just endpoint measurements of lipid accumulation are performed and kinetics of intracellular lipid accumulation is difficult to follow. To address this, we created a prediction model using Fourier-transform near-infrared (FT-NIR) spectroscopy. This method allows to measure lipid content in yeast.

Methods: The FT-NIR calibration sets were constructed from spectra of freeze-dried cells of the oleaginous yeasts *Rhodotorula toruloides* CBS 14, *Lipomyces starkeyi* CBS 1807 and *Yarrowia lipolytica* CBS 6114. The yeast cells were obtained from different cultivation conditions. Freeze-dried cell pellets were scanned using FT-NIR in the Multi Purpose Analyser (MPA) from Bruker. The obtained spectra were assigned corresponding to total fat content, obtained from lipid extraction using a modified Folch method. Quantification models using partial least squares (PLS) regression were built, and the calibration sets were validated on independently cultivated samples. The *R. toruloides* model was additionally tested on *Rhodotorula babjevae* DBVPG 8058 and *Rhodotorula glutinis* CBS 2387.

Results: The R^2 of the FT-NIR model for *R. toruloides* was 98%, and the root mean square error of cross-validation (RMSECV) was 1.53. The model was validated using a separate set of *R. toruloides* samples with a root mean square error of prediction (RMSEP) of 3.21. The R^2 of the *Lipomyces* model was 96%, with RMSECV 2.4 and RMSEP 3.8. The R^2 of the mixed model, including all tested yeast strains, was 90.5%, with RMSECV 2.76 and RMSEP 3.22, respectively. The models were verified by predicting the total fat content in newly cultivated and freeze-dried samples. Additionally, the kinetics of lipid accumulation of a culture were followed and compared with standard lipid extraction methods.

Conclusions: Using FT-NIR spectroscopy, we have developed a faster, less laborious and non-destructive quantification of yeast intracellular lipid content compared to methods using lipid extraction.

Keywords: FT-NIR, Lipid quantification, *Rhodotorula toruloides*, *Lipomyces starkeyi*, *Yarrowia lipolytica*

Background

Lipids from renewable sources are of particular interest due to their potential use as a sustainable feedstock for biodiesel and chemicals production, and as ingredients in food or animal feed [1–6]. Oleaginous yeasts can accumulate lipids to more than 20% of their dry weight. The basidiomycetous red yeast *Rhodotorula toruloides* has the ability to utilise and accumulate lipids using glycerol, glucose and xylose as sole carbon sources. Thus, *R. toruloides* has the potential to convert side products such as lignocellulose from forest and agricultural production systems, or crude glycerol from biodiesel transesterification, into lipids of higher value. There are also ascomycetous oleaginous yeasts of interest, e.g. *Lipomyces starkeyi* that is known for its high lipid content and potential for fuel production [2], and the nonpathogenic dimorphic aerobic yeast *Yarrowia lipolytica,* for its highly developed tool box for genetic modification, for instance, to yield

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high lipid content or the production of long-chain Ω-3 fatty acids [3].

One of the biggest challenges in optimising yeast-based lipid production systems is the ability to monitor lipid accumulation in yeast cells during their cultivation. Lipid extraction from microbes usually relies on organic solvents, often in the presence of strong acids and at elevated temperatures to break the cell walls [5–8]. Organic solvents are hazardous for the environment and to human health. Moreover, because extraction is performed in a biphasic system, errors can be introduced to lipid quantification due to pipetting errors. These errors can, however, be kept small by extracting lipids from large sample volumes. This can, however, affect experimental fermentations, since the total fermentation volume can be significantly changed during the time course of the cultivation by the removal of large sample volumes. Apart from this, lipid extraction is also labour-intensive and time-consuming. For these reasons, lipid production is in most cases monitored by endpoint measurement methods [7, 8], and the kinetics of lipid accumulation has rarely been investigated.

Infrared spectroscopy (IR) measures the absorption of light by fundamental molecular vibrations. It can be used to identify biomolecules such as proteins, lipids, DNA and other organic compounds [9]. Near-infrared (NIR) spectroscopy measures absorption of light vibrational overtones and their combinations. The NIR region of the electromagnetic wavelength spectrum ranges from 780 nm (wave number 12,800 cm^{-1}) to about 2500 nm (4000 cm^{-1}). At wavelengths above 2500 nm, the mid-IR region starts. Both IR and NIR spectroscopy have a number of useful applications, in industry and within research, for the identification and quantification of different organic compounds, product quality control and authentication [10–12]. These spectroscopic methods have also been applied for yeast species identification and strain differentiation [13, 14]. NIR spectroscopy among other methods is used to analyse sugar and lipid content in the bioethanol industry [15]. The absorption bands in NIR spectroscopy are broad and often overlapping, resulting in complex spectra that are difficult to interpret [16]. In classical NIR spectroscopy, the sample is scanned by a monochromatic light which is sequentially changed to different wavelengths over the whole NIR wavelength bandwidth. The principle of Fourier-transform (FT)-NIR is slightly different: a sample is exposed to polychromatic light, which is then divided at the beam splitter resulting in a so-called interferogram showing the intensity of the light as a function of time. The interferogram is then transformed to the frequency domain by Fourier transformation. The advantage of FT-NIR compared to classical NIR spectroscopy is that it is faster, as it allows simultaneous measurement over the whole wavelength range, is more sensitive as there is a lower background noise, and has a higher precision [16]. Both IR and NIR spectroscopy techniques have been used before, for example, to analyse lipids in the human body in a non-invasive way [17], lipid content and type in plants [18], to determine olive oil quality [19], or to analyse lipids in milk [20], yeasts [21, 22] and moulds [23]. FT-NIR could hence also be a possible method to analyse yeast cell total fat content in a non-destructive way without the need for prior lipid extraction and the use of organic solvents.

The spectra produced by NIR spectroscopy contain a lot of information. It is sensitive to compound concentrations, physical structure, water content, etc. Since the NIR absorption bands are so broad and overlapping, the whole spectra or selected regions are selected for analysis. Multivariate analysis such as partial least squares (PLS) regression is often used to produce predictive models. A set of NIR calibration spectra are assigned to reference values obtained from available analytical methods [24]. The aim of this study was to establish a rapid non-destructive FT-NIR method for lipid quantification in oleaginous yeasts and additionally to minimise the usage of organic solvents used in conventional lipid concentration determination methods.

Results

Pellets of yeast cells grown on different carbon sources at different cultivation times (see "Materials and methods") were collected and analysed further. From the obtained spectra, a lipid prediction model was generated for *R. toruloides*.

For the FT-NIR calibration set, 60 unique samples of *R. toruloides* were analysed in triplicates, from which 179 spectra were used, followed by lipid extraction to provide a reference value. As an external validation set, 18 samples were collected and analysed in the same way. The FT-NIR wavenumber regions of interests for lipid concentration measurements were the following: 4167–4545 cm^{-1}, 5600–6150 cm^{-1}, 6900–7300 cm^{-1} and 8000–9000 cm^{-1}. At these regions, C–H bonds are absorbing infrared light [25].

The determined fat content in the analysed *R. toruloides* samples varied between 5.9 and 59% of the cell dry weight (Figs. 1, 2).

An FT-NIR quantification model for *R. toruloides* total fat content was generated using the OPUS software included in the FT-NIR software package provided by the instrument manufacturer, and this FT-NIR quantification model was compared with the fat content data that were obtained from lipid extraction (Fig. 1). The calibration set produced for the *R. toruloides* cells with different lipid concentrations resulted in an FT-NIR quantification

model with a cross-validation R^2 value of 98% and a RMSECV value of 1.53 at rank 9. Vector normalisation was used for spectra pre-processing. The FT-NIR regions used to produce the model were: 8562.9–8038.4 cm^{-1} and 4485.9–4069.3 cm^{-1}. The linear regression equation was: $y = 0.9726x + 0.9786$. Model accuracy was confirmed by comparing the lipid content of the samples predicted by the model with results obtained from the lipid extractions (Figs. 3 and 4). Additionally, separate test samples were used for external validation of the model showing that the produced model had an RMSEP value of 3.21. Samples were obtained from cultures grown in hemicellulose hydrolysate, mixed hydrolysate, crude glycerol or glucose with an addition of yeast nitrogen base (YNB). Figure 1 shows that the FT-NIR lipid predictions were not influenced by the medium composition. The yeast lipid accumulation data were compared with the prediction model data (see Fig. 4). In this comparison, the average difference between real data and predicted lipid content was 5.2%. To see whether the functioning *R. toruloides* model works on different yeast species, we tested the model on *Lipomyces starkeyi* samples, which resulted in large prediction errors (Fig. 5).

In a similar fashion as for the model for *R. toruloides*, spectra and analytical data for *L. starkeyi* were used to create a *Lipomyces* lipid FT-NIR quantification model. The *Lipomyces* model consisted of 99 calibration spectra, with a R^2 of 96% (Fig. 6) and a RMSECV of 2.4 at rank 2. Vector normalisation was used for spectra pre-processing, and FT-NIR regions used were: 8694.1–8061.5 cm^{-1}, 7151.2–6649.8 cm^{-1} and 4466.6–3849.5 cm^{-1}. The linear regression equation was: $y = 0.94x + 1.54$. To test the model, 98 spectra from *L. starkeyi* strains (CBS 1807, CBS 7544), and *Yarrowia lipolytica* CBS 6114 were used with a resulting validation RMSEP of 3.8 (Fig. 7).

Spectra and analytical data of *R. toruloides, L. starkeyi* and *Y. lipolytica* were used to create a combined lipid FT-NIR quantification model. The combined model consisted of 238 calibration spectra, with a R^2 of 90.5% (Fig. 2) and a RMSECV of 2.76. The second derivative was used for spectra pre-processing, and FT-NIR regions used were: 8775.1–8034.5 cm^{-1} and 6001.8–5554.3 cm^{-1}. The linear regression equation was: $y = 0.9158x + 1.8607$. To test the combined model, 55 spectra, including additionally *R. glutinis* CBS 2387 and *R. babjevae* DBVPG 8058, were used to test elasticity of the FT-NIR model with a resulting validation RMSEP of 3.22.

We compared predictions of lipid concentrations based on the combined model and the *Lipomyces* model with values determined by lipid extraction on four independent *L. starkeyi* CBS 1807 samples. The *Lipomyces* model

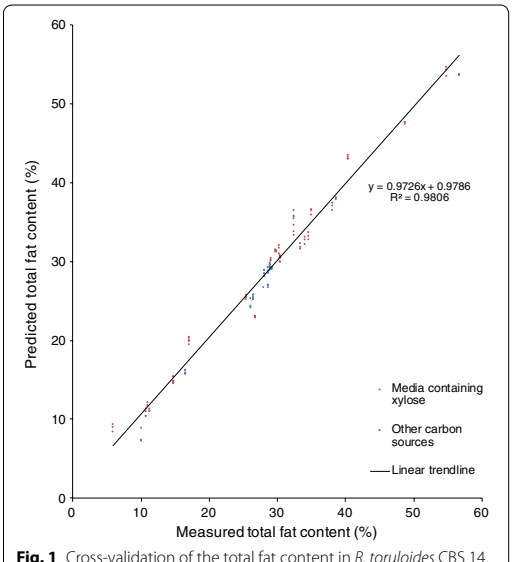

Fig. 1 Cross-validation of the total fat content in *R. toruloides* CBS 14. From cultures grown in hemicellulose hydrolysate, mixed hydrolysate, crude glycerol or glucose with YNB. Blue dots represent cultures grown in media containing xylose as the main carbon source; red dots represent cultures grown in media containing other carbon sources (glycerol and glucose)

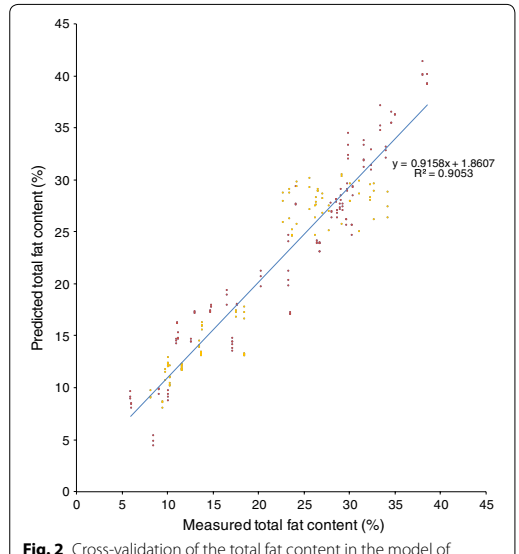

Fig. 2 Cross-validation of the total fat content in the model of several combined yeast species (*R. toruloides*, *L. starkeyi*, *Y. lipolytica*), cultivations using hemicellulose hydrolysate, mixed hydrolysate, crude glycerol or glucose with YNB as cultivation media. Red dots represent red yeast; yellow dots represent *L. starkeyi* and *Y. lipolytica* samples

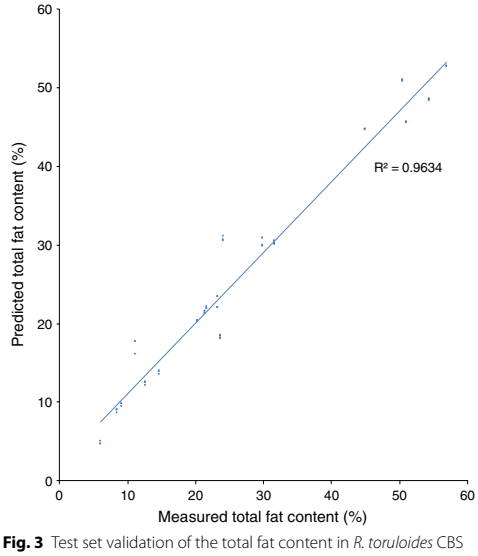

Fig. 3 Test set validation of the total fat content in *R. toruloides* CBS 14 model. Cells were grown using YNB media with glucose, xylose and glycerol. A total 161 spectra were used for the calibration set of the model. Independent samples in form of 42 spectra were used as test set validation

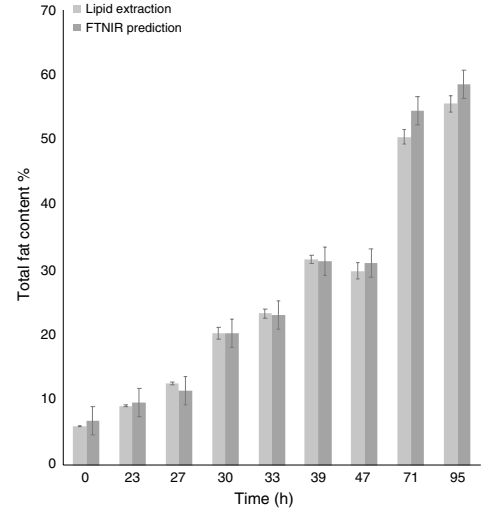

Fig. 4 Comparison of *Rhodotorula* accuracy (dark grey bar) with traditional lipid extraction (light grey). Values were obtained by analysing test culture samples using the Quant2 analysis package. The samples were obtained from the cultivation of *R. toruloides* cultivated in a lignocellulosic hydrolysate ($n = 27$). The error in prediction was 2.17 for the FT-NIR model. Average difference between the lipid extraction results and the FT-NIR predictions was 5.2%

values were more similar to the lipid extraction values than the combined model predictions (Fig. 8).

Discussion

In the present study, we prove that it is possible to rapidly measure lipid content in red (*Rhodotorula* sp.) and other (*Lipomyces starkeyi*, *Yarrowia lipolytica*) yeast cells using FT-NIR spectroscopy with a high accuracy. It was only necessary to use organic solvent for lipid extractions during the establishment of the FT-NIR model, and afterwards they were only needed when clear outliers were present, or to further refine the FT-NIR model with new model data points. The verification of the produced FT-NIR lipid model on samples collected during cultivation of *R. toruloides* CBS 14 on different growth media, especially those taken during cultivation on lignocellulosic hydrolysate, further indicates that different cultivation methods do not substantially influence the FT-NIR-based lipid concentration predictions, as long as the analysed yeast cells are washed prior to the FT-NIR measurements. The FT-NIR model can also be used for analysing samples taken during cultivations using "difficult" cultivation media, to follow the lipid accumulation in the culture over time. In the previous studies, Laurens et al. established a lipid content model by using NIR measurements [21]. However, they only tested standard cultivation media, and used nitrogen limitation to get higher variation in lipid accumulation. In our study, we used different types of media, including lignocellulose hydrolysate, which, due to its complexity, could create problems if resulting in a variation in the lipid composition of the cells. In food science, it has been shown, for example, that it was possible to detect differences in the lipid composition due to variation of the cultivation conditions (e.g. region or temperature) [26, 27]. In contrast to this, our results clearly show that the cultivation media did not cause a significant variation in the lipid detection in yeast samples.

Laurens et al. [21] used a similar approach as in our study, but applied a different apparatus and processing of spectra. In particular, they used fatty acid methyl esters (FAME) instead of total fat. Unfortunately, both methods cannot directly be compared, as raw measuring data have to be processed by the according transformations.

It was not possible to use our existing red yeast model for the prediction of lipid content in non-pigmented yeasts. Similarly, the *Lipomyces* model could not be used for determining lipids in red yeasts. However, it was

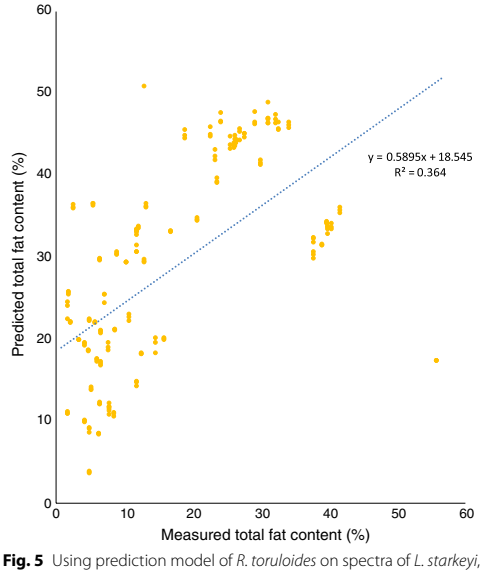

Fig. 5 Using prediction model of *R. toruloides* on spectra of *L. starkeyi*, cultured in YNB with the addition of glycerol or glucose

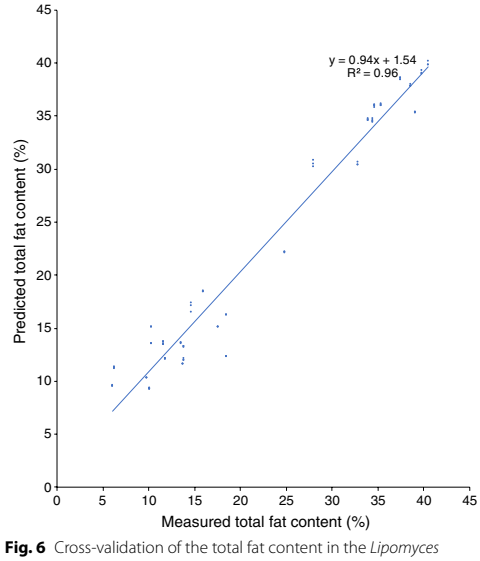

Fig. 6 Cross-validation of the total fat content in the *Lipomyces* model of *L. starkeyi*, CBS 1807, cultivations using glycerol or glucose with YNB as cultivation media

possible to use the *Lipomyces* model on *Y. lipolytica*. It needs to be tested on a per-case basis whether a strain belonging to another species can be analysed using a specific model. However, our results show that it is possible to combine species from different phyla of fungi when creating a model (Figs. 2, 8), in accordance with the results by Laurens et al. [21]. Compared to their model, our combined model was of acceptable quality. However, the model itself and the accuracy of it can be improved further by continuously adding additional samples [12, 25].

An advantage of using an FT-NIR-based lipid concentration determination method is that it is possible to share the model and data between different groups and users, thereby making it possible to expand the produced models by adding more samples from various sources. The data used to produce the prediction model are not restricted to one type of FT-NIR machine. This is already being done in, e.g., the bioethanol industry when measuring starch content in grains from different farms and harvest years, and at different production facilities. The models used by the industries are based on results from research activities on FT-NIR analysis of starch content in grains [28]. A future goal could be to establish an open access FT-NIR database used by academic groups carrying out research on lipid accumulating yeasts and industries focusing on yeast lipid production systems.

It has previously been pointed out and discussed that it can be difficult to create reliable and robust FT-NIR calibration models due to necessary knowledge of chemometrics [12]. In case of the OPUS software, which was used in our study, establishing and expanding models requires only basic knowledge and can be relatively rapidly performed. This simplifies the use of the model, and it is also possible to improve model accuracy by adding additional data points in the future. It should be noted that it is not required to use FT-NIR hardware for data processing (only for spectra collection), so the model itself can be analysed offline.

In the present study, the sample volumes used for classical lipid extraction and the measurement carried out by FT-NIR were approximately the same. However, it is possible to use smaller FT-NIR probes and thereby reduce the sample volume used [21, 29]. This is another advantage, especially when following lipid accumulation during the whole fermentation process when cell density is low, e.g. at the beginning of the cultivation, or during small-scale testing or screening.

We have created FT-NIR models to determine lipid content that is easy to implement for end user and that can be easily shared thanks to the possibility of exporting the model and sample values to other types of FT-NIR equipments with compatible software. As mentioned earlier, other studies tried different approaches with FT-IR

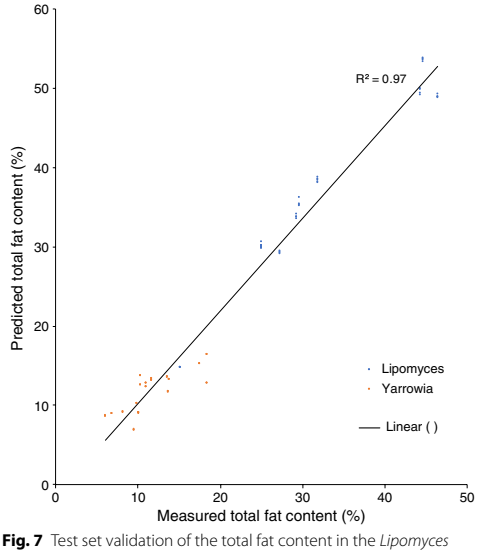

Fig. 7 Test set validation of the total fat content in the *Lipomyces* model. Cells were grown using YNB media with glucose, xylose and glycerol. In total, 98 spectra from *L. starkeyi* and *Y. lipolytica* were used for the test set validation

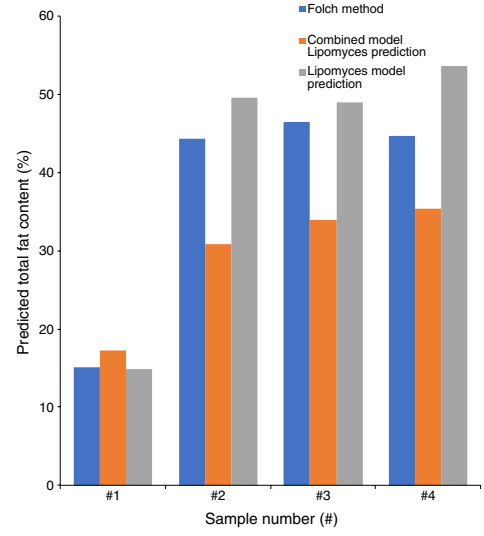

Fig. 8 Comparison between combined model and *Lipomyces* model prediction to values obtained with Folch extraction on four samples of *Lipomyces starkeyi* CBS 1807

and NIR for lipid detection [21, 22]. Both solutions are viable alternatives to our approach. With the FT-NIR method in combination with a dedicated software, a narrower spectra band than IR is used and most of the statistical operation is automated and performed without the user input. In all methods, freeze-dried sample is preferred, but wet yeast cells should be possible to be analysed, too, because there are examples of measuring fat in water solution like milk [20]. In the future, the technique could be also used to evaluate lipid classes and/or, fatty acid composition and level of oxidation in addition to total fat content similarly as described in [12, 21].

Conclusions

To the best of our knowledge, we present for the first time working FT-NIR models for quantification of total fat content in oleaginous yeasts as fast and non-destructive alternatives for lipid extraction. These new models drastically reduce the time needed to obtain total fat content values, from few hours to a matter of minutes per sample analysed. Although gravimetric lipid determination is still more precise than prediction of the lipid content by the model, FT-NIR analyses will enable high-throughput measurements of lipid formation and kinetics studies of intracellular lipid accumulation. The precision of the model prediction can be increased further by including a growing number of new calibration values into the model.

Materials and methods

Yeast strains and cultivation conditions

The yeast strains used were *R. toruloides* CBS 14, *L. starkeyi* CBS 1807, *L. starkeyi* CBS 7544, *Y. lipolytica* CBS 6114, *R. glutinis* CBS 2387 (obtained from Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) and *R. babjevae* DBVPG 8058 (Industrial Yeast Collection, Perugia, Italy originally strain number J195, strain collection of the Department of Molecular Sciences, Swedish University of Agricultural Sciences, isolated from apple). They were stored at − 80 °C in 50% glycerol stock media. Before usage, the strains were transferred onto Yeast extract–Malt extract (YM) plates—10 g/L glucose (≥ 99%, Fluka Analytical, France), 5 g/L peptone (from casein, Merck KGaA, Germany), 3 g/L Yeast extract (Bacto™ Yeast Extract, BD, France), 3 g/L malt extract (Merck KGaA, Germany), 16 g/L agar (VWR Chemicals, Sweden). After 3 days of growth at 25 °C, the agar plates were stored at 4 °C. Pre-cultures were grown in YPD medium, in shake flasks at 25 °C for 72 h.

All media components except for yeast nitrogen base (YNB) and lignocellulose hydrolysates were autoclaved at 121 °C for 20 min. YNB and hydrolysate were sterile-filtered with a 0.2-µm filter (VWR International, LLC, USA).

All experimental media were based on YNB medium with additional salts: 1.7 g/L YNB (Difco™ yeast nitrogen

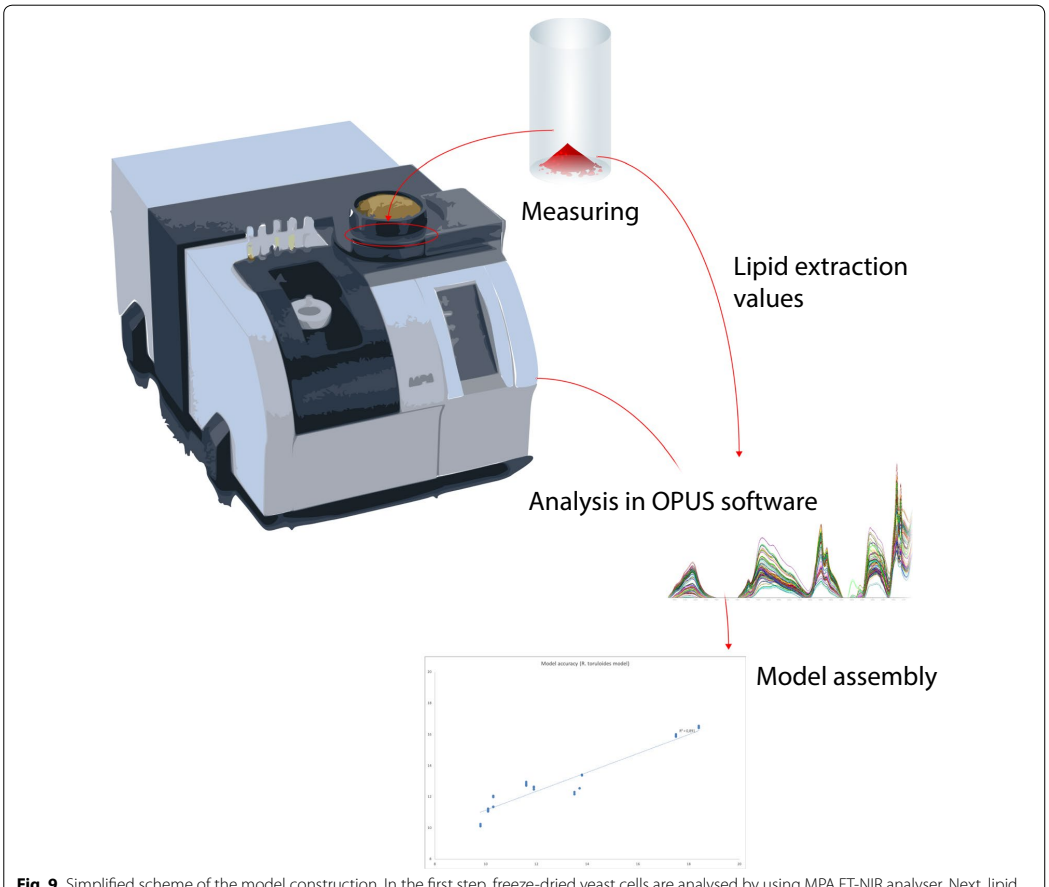

Fig. 9 Simplified scheme of the model construction. In the first step, freeze-dried yeast cells are analysed by using MPA FT-NIR analyser. Next, lipid extraction is performed on the same cells and values are assigned to the respective spectra. Finally, when at least 50 samples are scanned and assigned to the analytical values, a model can be created with the help of Quant2 included in the OPUS software

base w/o Amino acids and Ammonium Sulphate, BD, France), 0.75 g/L Yeast extract (Bacto™ Yeast Extract, BD, France), 2 g/L $(NH_4)_2HPO_4$ ($\geq$ 98%, Sigma-Aldrich, USA), $MgCL_2$ 1 g/L (Merck KGaA, Germany). The following carbon sources and additives were used: crude glycerol from biodiesel production (850 g/L glycerol (85% purity), provided by Perstorp AB), glucose (10–30 g/L), xylose (10–30 g/L), acetic acid (0.5–3 g/L, 95–97%, Sigma-Aldrich, USA), hemicellulose hydrolysate (from wheat straw—up to 40% v/v of hydrolysate used in experiments), cellulose hydrolysate (from wheat straw—up to 60% v/v of hydrolysate used in experiments). The hemicellulose/cellulose hydrolysate mixture carbon sources were determined by HPLC (Agilent 1100 Series, Germany) 50.8 g/L glucose, 29.3 g/L xylose and 5.2 g/L acetic acid. Wheat straw hydrolysate was obtained by steam explosion as described in Blomqvist et al. [2].

Yeast cultivations were performed in 2-L bioreactors (Minifors, Infors HT, Switzerland), 500 mL (Multifors, Infors HT, Switzerland) and in 100-mL baffled shake flasks (Werner-Glas, Sweden). The cultivations in bioreactors were performed with the following parameters: pH = 6 (acid 3 M H_3PO_4, base 5 M NaOH), DO = 21% O_2, and temperature = 25 °C. Cultivations in shake flasks conditions: initial pH = 6, cultivation time 72 h, shaking at 130 rpm, 25 °C (Ecotron, Infors HT, Switzerland).

Each of the collected cultivation samples was washed with water two times and freeze-dried for 72 h in

−100 °C (CoolSafe Scanvac, LaboGene ApS, Denmark) to remove water and other possible compounds that can interfere with lipids in the spectra. After the collection of spectra, lipids from cells were extracted and quantified using a modified Folch method as described earlier [8].

A multi-purpose analyser (MPA) FT-NIR spectrometer from Bruker equipped with sample compartment and integrating sphere, combined with OPUS [OPUS ver. 7.5 build: 7.5.18 (20140810)], was used to obtain and analyse all FT-NIR spectra. All measurements were done in triplicates by using the integrated sphere compartment with the following parameters: Quartz beam splitter, RT_PbS detector, resolution of scan 16 cm^{-1}, background measured internally. Glass vials containing freeze-dried cell pellets were scanned 32 times. Spectra were collected in the wavelength range from 4000 to 10,000 cm^{-1}. A prediction model was generated with PLS regression using OPUS software Quant2 tool.

To ensure accuracy of the FT-NIR model, a smaller independent test set of samples, separate from calibration, were cultivated and analysed in the same manner. These samples were used to compare model prediction with actual extraction results.

The measuring procedure is illustrated in Fig. 9.

Abbreviations

IR: infrared; NIR: near infrared; FAME: fatty acid methyl esters; FT-IR: Fourier-transform infrared; FT-NIR: Fourier-transform near infrared; RMSECV: root mean square error of cross-validation; RMSEP: root mean square error of prediction; PLS: partial least square; MPA: multi-purpose analyser; YNB: yeast nitrogen base; YM: yeast extract–malt extract; YPD: yeast peptone dextrose; HIP: hexane isopropanol.

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Authors' contributions

MC was involved in designing the study, performing the major part of the laboratory work, model creation and writing the first draft of the manuscript. SS was involved in laboratory work, evaluation of the results and performed a major contribution to writing. JBl contributed to laboratory work, evaluation of the results and writing. JBr and FW contributed in evaluating the results and writing the manuscript. MS and VP provided a major contribution to design the study, to evaluate the results and to write the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agreed to publish this article.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH

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Microbial lipid production from crude glycerol and hemicellulosic hydrolysate with oleaginous yeasts

Mikolaj Chmielarz, Johanna Blomqvist, Sabine Sampels, Mats Sandgren and Volkmar Passoth*

Abstract

Background: Crude glycerol (CG) and hemicellulose hydrolysate (HH) are low—value side-products of biodiesel transesterification and pulp—and paper industry or lignocellulosic ethanol production, respectively, which can be converted to microbial lipids by oleaginous yeasts. This study aimed to test the ability of oleaginous yeasts to utilise CG and HH and mixtures of them as carbon source.

Results: Eleven out of 27 tested strains of oleaginous yeast species were able to grow in plate tests on CG as sole carbon source. Among them, only one ascomycetous strain, belonging to *Lipomyces starkeyi*, was identified, the other 10 strains were *Rhodotorula* spec. When yeasts were cultivated in mixed CG/ HH medium, we observed an activation of glycerol conversion in the *Rhodotorula* strains, but not in *L. starkeyi*. Two strains—*Rhodotorula toruloides* CBS 14 and *Rhodotorula glutinis* CBS 3044 were further tested in controlled fermentations in bioreactors in different mixtures of CG and HH. The highest measured average biomass and lipid concentration were achieved with *R. toruloides* in 10% HH medium mixed with 55 g/L CG—19.4 g/L and 10.6 g/L, respectively, with a lipid yield of 0.25 g lipids per consumed g of carbon source. Fatty acid composition was similar to other *R. toruloides* strains and comparable to that of vegetable oils.

Conclusions: There were big strain differences in the ability to convert CG to lipids, as only few of the tested strains were able to grow. Lipid production rates and yields showed that mixing GC and HH have a stimulating effect on lipid accumulation in *R. toruloides* and *R. glutinis* resulting in shortened fermentation time to reach maximum lipid concentration, which provides a new perspective on converting these low-value compounds to microbial lipids.

Keywords: *Oleaginous yeast*, *Hemicelluloses*, *Crude glycerol*, *Lipids*, *R. torouloides*

Background

There is increased need for recycling of waste products from food, wood industry and agriculture in recent years. Vast quantities of organic residues are a challenge that is being addressed by converting waste and low purity sugars into higher quality products [1].

Crude glycerol (CG) is a side product of biodiesel production, which is released during transesterification of vegetable oils. CG is a highly problematic side product; it contains methanol, soap and ash. Industrial application of CG requires extensive purification, making its application quite expensive [2–5]. In recent years there have been developments to utilse glycerol and convert it to useable biomass and for example turn it into microbial fuel cells [6], or to convert glycerol into valuable fatty acids using *Yarrowia lipolytica* [7]. It was also tested as an additive with other waste substrates to make microbial oil and other valuable products [8–13].

Hemicellulose is, besides of cellulose and lignin, one of the major polymers of the plant cell wall. It is a

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heteropolysaccharide, which composition differs between different groups of plants. Hardwood hemicellulose consists mainly of xylans, while softwood is mainly built of glucomannans. Hemicellulose of lignocellulosic materials of monocoyledons like wheat straw consist of a xylan backbone with side chains of arabinose and glucuronic acid [14, 15]. In pulp and paper industry, hemicellulose is a side product and used for steam generation. However, it is rather inefficient for this application, due to its low heating value (13.5 MJ/ kg) [5]. In lignocellulose-based bioethanol production, separating hemicellulose from the cellulose fraction can be advantageous, since conversion of the pentoses, which are the major sugar monomers of hemicellulose to ethanol is still a challenge [7, 8]. When lignocellulose is thermochemically pretreated by acid hydrolysis, hemicellulose is solubilised and hydrolysed, resulting in the release of monosaccharides [16]. Since the major parts of cellulose and lignin are still in the solid phase, it is easy to separate the hemicellulose hydrolysate from the other components of lignocellulose hydrolysate. Hemicellulose hydrolysates contain, in addition to released sugars, a variety of inhibitors such as furaldehydes, weak organic acids and phenolic compounds [17], and frequently it is necessary to remove those inhibitors or to dilute hemicellulose hydrolysate to enable bioconversion of hemicellulose hydrolysates [13, 18, 19].

Many oleaginous yeasts, i.e., yeasts that can accumulate more than 20% of their biomass as lipids, can convert both glycerol as well as sugars and organic acids derived from hemicellulose to oil. This oil has a similar composition as some vegetable oils [20]. Production of vegetable oil can have considerable greenhouse gas potential [21] and thus, replacement of vegetable oil by yeast oil may result in more sustainable biodiesel [22, 23] or animal feed production [24]. However, production costs of microbial oil are still too high to be competitive with oil from fossil resources or vegetable oils [25, 26]. Therefore, it is important to explore the conversion of low-value side products such as lignocellulose hydrolysate, hemicellulose hydrolysate, or crude glycerol to microbial oils. Another approach to improve cost efficiency of microbial lipid production is to identify strains with high productivity when converting the mentioned residual products to lipids and to understand their physiology, to identify targets for strain optimisation [27, 28].

Converting a mixture of CG and HH to yeast oil may provide a possibility of simultaneously converting these side streams to a high-value products. At the same time, inhibitors present in only one of these side streams might be diluted and thus less inhibitory. It was our aim to get a survey about the diversity of strains when converting CG and HH to lipids. We also intended to investigate effects of mixing these two problematic substrates. We tested several of strains belonging to the genera *Lipomyces* (ascomyceteous oleaginous yeasts) and *Rhodotorula* (basidiomycetous oleaginous yeasts) for their ability to grow and synthetise lipids on a mixture of CG and HH.

Results

Crude glycerol and hemicellulose growth tests

The ability of 27 yeast strains to utilise CG as sole carbon source and their resistance against inhibitors in CG was tested on solid media. A droplet of undiluted crude glycerol or a filter soaked with CG was placed at the centre of YNB plates inoculated with the tested strains. Out of the 27 tested strains, 11 strains showed visible growth close to the droplet/filter and thus were able to tolerate CG, and to utilise it as carbon source (experiments were performed in duplicates). Those strains were: *Rhodotorula glutinis* CBS 2203, *R. glutinis* CBS 2889, *R. glutinis* CBS 2890, *R. glutinis* CBS 3044, *R. glutinis* CBS 5182, *R. glutinis* CBS 7538, *Rhodotorula minuta* CBS 8013, *Rhodotorula mucolaginosa* CFSQE 63, *Rhodotorula babjevae* CBS 7809, *Rhodotorula toruloides* CBS 14 and *Lipomyces starkeyi* CBS 7786. Interestingly, only one ascomyceteous strain (*L. starkeyi* 7786) showed substantial growth on the plates with crude glycerol. These strains were also tested in liquid shake flask cultures with 40% hemicellulose hydrolysate as sole carbon source, and in cultures with different concentrations of crude glycerol (up to 120 g/l) as sole carbon source. After 96 h of cultivation almost all red yeast strains grew well in hemicellulose hydrolysate (OD 20–40) and in 120 g/L CG (OD > 40). *R. minuta* CBS 8013 grew slower than other *Rhodotorula* strains in CG concentrations higher than 60 g/L. *L. starkeyi* CBS 7786 grew comparatively well in hemicellulose hydrolysate and 30 g/L CG medium, but had lower growth compared to the tested red yeast strains in higher concentrations of CG.

Testing of growth in a mixture of crude glycerol and hemicellulose

To evaluate the effect of mixing CG and HH in the cultivation media we tested five strains, *L. starkeyi* CBS 7786, *R. toruloides* CBS 14, *R. glutinis* CBS 3044, *R. glutinis* CBS 2889 and *R. mucolaginosa* CFSQE 63, for their growth on these mixed substrates and compared it to cultivations with either glucose (positive control) or crude glycerol as sole carbon source (Figs. 1–4). The strains were chosen among those that showed good growth on crude glycerol and hemicellulose hydrolysate media. There were varying responses of the strains to the different carbon sources. In the glucose control medium (Control media), *R. toruloides* CBS 14 had the lowest OD_{600} after 96 h of cultivation (21), while *R.*

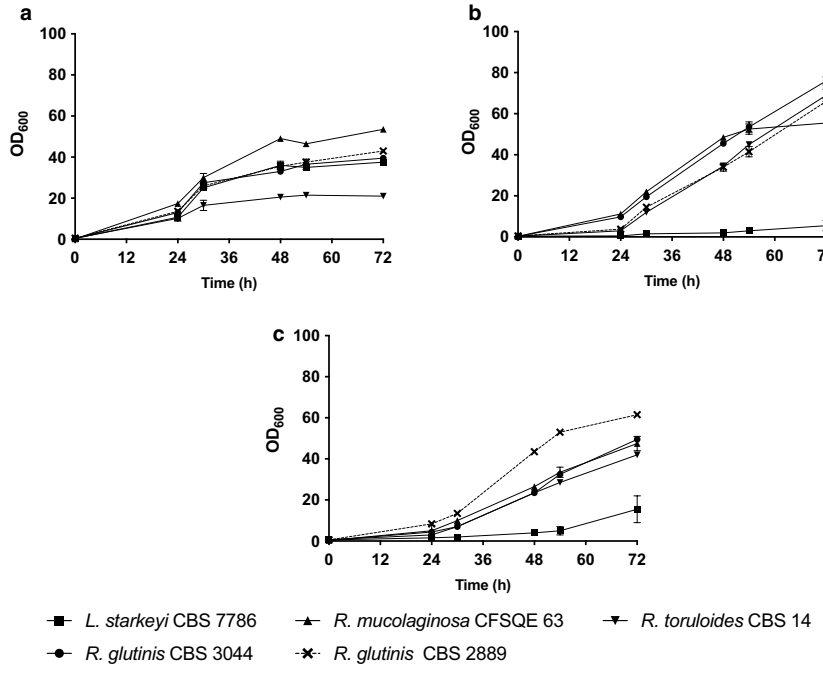

Fig. 1 Growth of five strains tested in different media. **a** control medium, **b** hemicellulose hydrolysate mixed with crude glycerol and **c** crude glycerol + YNB media in shake flask cultures. 60 g/L Crude Glycerol, and 40% Hemicellulose hydrolysate. Strains were grown in duplicates

mucolaginosa CFSQE 63 reached an OD_{600} more than twice as high as *R. toruloides* after 96 h. In mixed media (sHH40CG60 media), results were almost the opposite. *R. toruloides* had the second highest OD_{600} after *R. glutinis* CBS 3044. Out of the red yeasts, *R. mucolaginosa* CFSQE 63 had the lowest OD_{600}. In media with crude glycerol (sCG60 media) only, *R. glutinis* CBS 2889 had the highest OD_{600} and *R. glutinis* CBS 3044 reached the second highest OD_{600}. Interestingly, faster growth was observed for the red yeasts when cultivated using a mixed medium, compared to cultivations using pure CG.

L. starkeyi CBS 7786 showed slow growth on both CG alone and the mixture of CG and HH (Fig. 1). In contrast to the red yeasts, mixing CG with HH had no stimulating effect but was rather inhibiting the growth. In sHH40CG60 media, from starting glycerol concentration of approximately 50 g/L, *Lipomyces starkeyi* CBS 7786 consumed only around 25% of this, with 38.2 g/L glycerol still remaining after 72 h cultivation (Fig. 2). It was also only able to assimilate a small proportion of xylose, which was consumed by the other tested strains within 30 h.

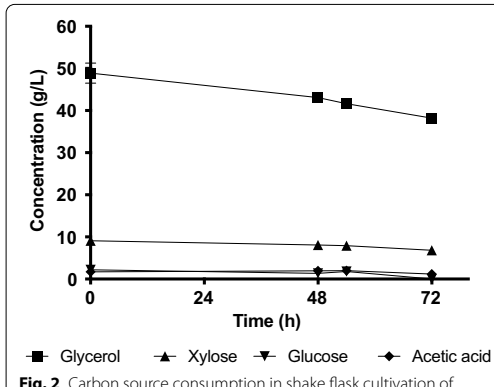

Fig. 2 Carbon source consumption in shake flask cultivation of *L. starkeyi* CBS 7786 in sHH40CG60 medium. Xylose had a final concentration of 6.8 g/L; final concentration of glycerol was 38.2 g/L. The values were obtained from duplicate cultivations

In *R. toruloides* CBS 14 cultivations, the remaining glycerol concentration was 26 g/L after 70 h of cultivation. Xylose consumption started after 30 h (Fig. 3). *R.*

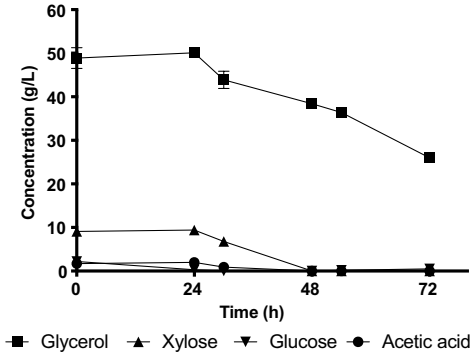

Fig. 3 Carbon source consumption in shake flask culture of *R. toruloides* CBS 14 grown in sHH40CG60. Xylose was consumed after 30 h of cultivation, glycerol levels were dropping to a final concentration of 26.0 g/L after 70 h of cultivation. Cultivations were grown in duplicates

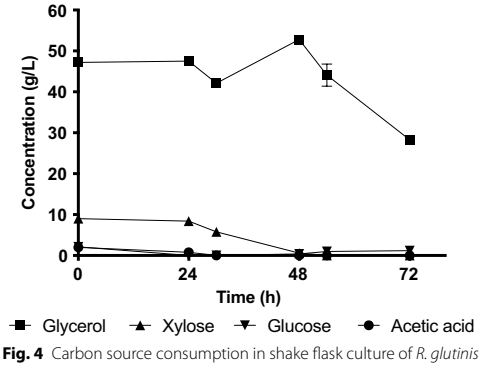

Fig. 4 Carbon source consumption in shake flask culture of *R. glutinis* CBS 3044 grown in sHH40CG60. Xylose was consumed after 30 h of cultivation, glycerol levels dropped to a final concentration of 28.3 g/L after 70 h. Cultivations were grown in duplicates

glutinis CBS 3044 (Fig. 4), also consumed xylose between the 30 and 48-h measurement. The remaining concentration of glycerol was 28.3 g/L.

For *R. mucolaginosa* CFSQE 63 and *R. glutinis* CBS 2889 it turned out to be difficult to measure the concentration of carbon sources in the medium, because during filtration of all samples taken after inoculation, filter membranes rapidly got clogged. Although the supernatants were centrifuged before filtration as described before [19], it was in principle not possible to filter the supernatant and for the little amount of liquid that passed through the filter, results were not reproducible (results not shown). Production of exopolysaccharides has been shown in several strains of *Rhodotorula* spec. [29–31], and the clogging of the filters may be due to production of those polysaccharides in these two strains. Due to the difficulties in analysing the metabolites in the fermentations, we did not further investigate these strains in this study.

Fermentation in bioreactors

Two of the investigated strains, *R. toruloides* CBS 14 and *R. glutinis* CBS 3044, were tested under controlled conditions in 500 ml fermenters for their abilities to utilise crude glycerol and hemicellulose hydrolysate and mixtures of them.

Cultivations were performed in 55 g/L of CG (CG55) as sole carbon source for the control and in a mixture of 55 g/l glycerol with 10% HH (HH10CG55). The C/N ratio of HH10CG55 mixture was 53.6, and CG50 C/N ratio was 48 which are slightly below the reported optimum for lipid production of 60–100 [32]. To obtain an impression on the lipid accumulation kinetics, the intracellular lipid content during fermentation was checked over the whole fermentation time using our recently established FT-NIR-based method for intracellular lipid determination [33].

In the cultivation of *R. toruloides* CBS 14, glycerol was faster consumed in the HHCG mixture, compared to the CG control. After 72 h, more than 75% of all glycerol was consumed in the mixed medium, and after 96 h no glycerol was detectable any more. In the control with only CG as carbon source, even after 120 h of cultivation 4.7 g/L glycerol was left in the culture and complete consumption was only found after 144 h. Moreover, the highest cell dry weight (21.2 g/L) and lipid concentration (12.5 g/L) were reached in the HHGC mixture after 96 h cultivation (Fig. 5b). In the culture with CG as sole carbon source, the maximum dry weight (19.1 g/L) and lipid concentration (8.81 g/L) were detected after 140 h cultivation (Fig. 5a).

Similarly, an activating effect was observed for *R. glutinis* CBS 3044 when cultivated in HHGC compared to CG as sole carbon source (Fig. 5c, d). The glycerol was almost finished after 72 h, while in the control with only CG, more than 40% of the initial carbon source was present at this time. *R. glutinis* CBS 3044 in CG consumed glycerol slightly faster than *R toruloides* CBS 14 – all of it was consumed by 120 h. In the culture with CG as sole carbon source, the maximum dry weight (20.3 g/L) and lipid concentration (7.6 g/L) were detected after 120 h cultivation (Fig. 5c). Highest lipid concentration (7.6 g/L) was reached in the HHGC mixture after 96 h cultivation (Fig. 5d). The highest cell dry

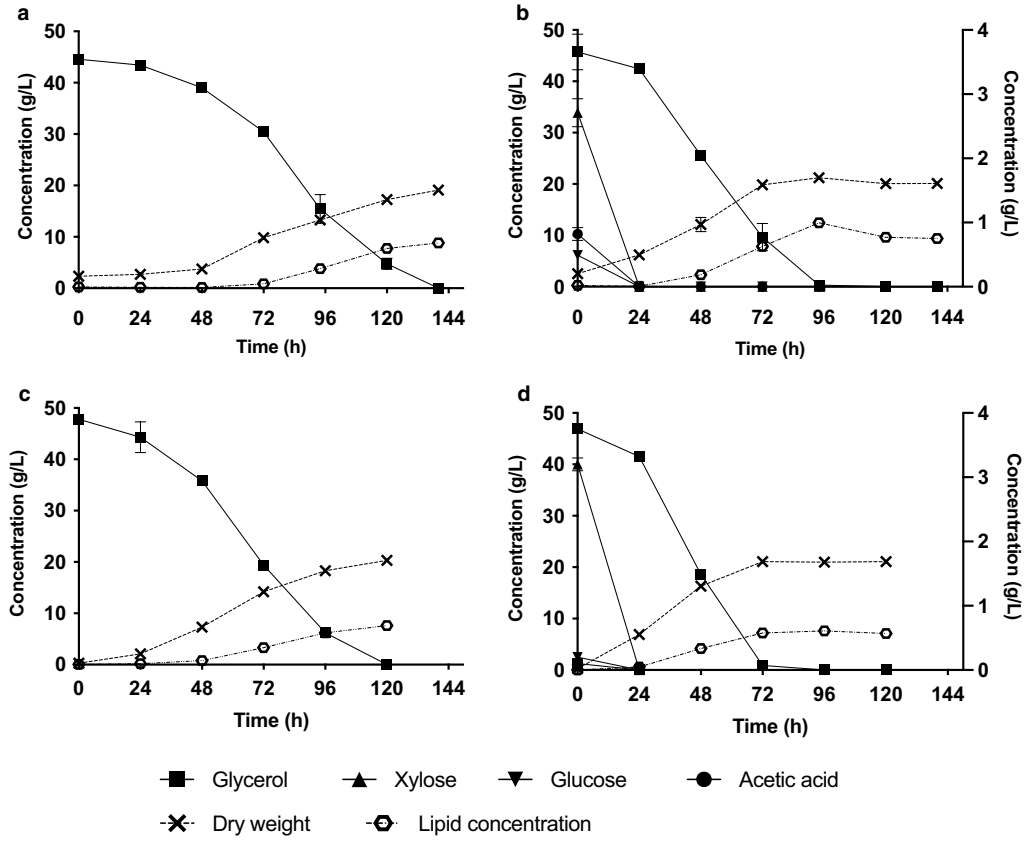

Fig. 5 Dry weight, lipid concentration and carbon source consumption over time in media with CG as sole carbon source (CG55) or mixed carbon source (HH10CG55). **a** *R. toruloides* CBS 14 bioreactor cultivation CG55. **b** *R. toruloides* CBS 14 bioreactor cultivation in HH10CG55; **c** *R. glutinis* CBS 3044 bioreactor cultivation in CG55. **d** *R. glutinis* CBS 3044 bioreactor cultivation in HH10CG55; Glucose, xylose and acetic acid are shown on the right y axis. All cultures were done in triplicates

weight (21.0 g/L) was achieved after 72 h and it only changed by 0.1 g/L during the next 48 h.

In addition, we analysed the specific lipid production rates in 55 g/L CG media (Additional file 1: Fig. S1). The lipid accumulation was highest in both red yeast strains in media containing HH. *R. toruloides* had similar specific lipid production rates compared to *R. glutinis* (maximum specific rates of 0.014 vs 0.013 g/g/h), but growth was delayed by 24 h. According to a fixed effect linear model the hemicellulose hydrolysate additive had a significant impact on *R. toruloides* lipid accumulation ($p < 0.001$) but not on *R. glutinis* ($p = 0.0625$). The volumetric production rates followed the same trend as the specific rates. *R. toruloides* CBS14 in mixed medium had its maximum production rate (0.23 g/L lipid per hour) between 48 and 72 h of cultivation, in CG medium *R. toruloides* CBS14 had the highest rate (0.16 g/L lipid per hour) between 96 and 120 h. *R. glutinis* CBS 3044 in mixed medium had its maximum production rate (0.15 g/L lipid per hour) between 24 and 48 h of cultivation, in CG medium *R. glutinis* CBS 3044 had the highest rate (0.12 g/L lipid per hour) between 72 and 96 h.

These results confirmed under controlled conditions the activating effect of mixing HH and CG, which we already observed for red yeasts in shake flasks. To further verify this effect, we performed a variety of further controlled fermentations with varying concentrations of CG and HH (Additional file 2: Fig. 2 and Additional file 3:

Fig. 3). For all tested concentrations (with a C/N ratio from 38.3 to 72) we found a more rapid glycerol consumption and lipid formation in cultures with a mixed carbon source.

Lipid content, concentration, yield and biomass at the end of fermentation times are presented in Table 1. In most cases, the final lipid yields per g consumed carbon source were not significantly different between the cultivations with mixed carbon source and CG alone, the major effect was due to more rapid initial glycerol consumption. The highest lipid content was measured in *R. toruloides* CBS 14 grown in HH40CG60 medium (C/N ratio 72.2), which was probably due to the higher concentration of carbon source and higher C/N ratio. In addition, a comparatively high lipid yield (0.23 g lipids per g consumed carbon source) was observed. However, in this cultivation, not all glycerol was consumed and it turned out that the glycerol consumption rate decreased towards the end of the culture (Additional file 3: Fig. 3). In the cultivation with HH10CG55, all carbon source was consumed and the yield was even higher (0.25). The lipid yields of *R. glutinis* CBS 3044 were slightly lower compared to *R. toruloides* CBS 14.

Fatty acid analysis

The average fatty acid composition for *R. toruloides* CBS 14 is presented in Table 2. In all cases, there were four dominant fatty acids; oleic acid, palmitic acid, linoleic acid and stearic acid. Oleic acid (C18:1) was the most common fatty acid with the highest proportion of 46.0% of total identified fatty acids. The second major compound was palmitic acid (C16:0) with its highest proportion of 28.0%. The third major fatty acid was linoleic acid (C18:2), the highest proportion of which was 16.3% of total identified fatty acids. The last major fatty acid was stearic acid (C18:0). Its highest proportion was 12.6% of total identified fatty acids. Proportions of the remaining fatty acids can be seen in Table 2. The difference in proportion of the fatty acid concentration in samples that were taken from same cultures at 96 h and 144 h was not larger than four percent. The biggest changes were oleic acid for palmitic acid in HH10CG50 media and the opposite for CG50 after 48 h. It should also be noted that behenic acid (C22:0) was above detectable levels only

Table 2 Average fatty acid composition of *R. toruloides* CBS 14 ($n=2$) in different media (percent (%) of total identified ± mean absolute deviation)

Fatty acid	HH40CG60	CG60	HH10CG50	CG50
C14:0	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.0
C16:0	25.6 ± 0.0	22.2 ± 0.1	26.0 ± 0.4	28.0 ± 0.6
C16:1	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.0
C18:0	11.3 ± 0.3	12.6 ± 0.1	12.1 ± 1.5	12.2 ± 0.0
C18:1	45.6 ± 0.0	46.0 ± 0.1	40.7 ± 0.3	39.6 ± 0.5
C18:2	12.0 ± 0.0	12.1 ± 0.1	14.0 ± 1.2	13.8 ± 0.0
C18:3	2.1 ± 0.1	3.2 ± 0.1	2.9 ± 0.3	2.5 ± 0.0
C22:0	N/D	0.6 ± 0.0	0.5 ± 0.0	N/D
C24:0	0.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.3	0.8 ± 0.0

Table 1 Endpoint lipid content, concentration, yield and specific lipid production in *Rhodotorula toruloides* CBS 14 and *Rhodotorula glutinis* CBS 3044 at the end of fermentation on different media

Strain	Media carbon source	Cell lipid content (%)	Max. lipid concentration (g/L)	Lipid yield (g lipid/ g carbon source)	Final biomass (g/L)
R. toruloides CBS 14	10% HH 55 g/L CG	46.8 ± 1.0	12.5 ± 0.5	0.25 ± 0.02	20.1 ± 0.7
	55 g/L CG	46.2 ± 5.6	8.8 ± 0.1	0.20 ± 0.01	19.1 ± 0.3
	10% HH 50 g/L CG	45.8 ± 3.5	6.5 ± 0.5	0.18 ± 0.02	13.5 ± 0.01
	50 g/L CG	46.3 ± 1.7	6.3 ± 0.2	0.19 ± 0.01	13.5 ± 0.01
	40% HH 60 g/l CG	54.6 ± 1.5*	10.6 ± 0.3*	0.23 ± 0.01*	19.4 ± 0.0*
	60 g/L CG	40.5 ± 2.7*	5.7 ± 0.4*	0.17 ± 0.00*	28.0 ± 0.5*
R. glutinis CBS 3044	10% HH 55 g/L CG	36.4 ± 0.6	7.6 ± 0.5	0.15 ± 0.01	20.3 ± 0.3
	55 g/L CG	37.3 ± 1.9	7.1 ± 0.1	0.16 ± 0.01	21.1 ± 0.01

Averages of triplicate cultivations with standard deviation, *average of duplicate cultivations with average deviation. lipid yield = (Total lipid concentration) / (Total carbon consumed)

in cultivations using CG60 or HH10CG50, after 90 h cultivation.

Discussion

Crude glycerol proved to be a problematic substrate for oleaginous yeasts. Only one third of all tested strains showed substantial growth in plate-cultivation tests with CG as sole carbon source, all except one being basidiomycetes. All positively tested yeast strains were able to grow in medium containing up to 120 g/L CG, although there were differences in growth rates between the strains. All strains showed comparatively good growth up to CG concentrations of 60 g/L, which is in accordance with results presented by Chatzifragou et al. [34].

Only one of the tested *Lipomyces* strains showed substantial growth on crude glycerol, but considerably slower than the tested red yeasts, in fact, its growth was already retarded at CG concentrations of 60 g/L. The reason for low tolerance of *Lipomyces* strains to crude glycerol in all cases is unknown as the literature clearly shows that they can utilise glycerol effectively [9]. Most likely some inhibitors from ash were the cause of low tolerance. Other inhibitors in trace amount like soaps, free fatty acids and residual catalysts might also be present in our samples due to differences in purification processes of each biodiesel production plant. Another observation was that mixing both CG and HH had a negative effect on both growth and assimilation of glycerol and xylose for *L. starkeyi*. The presence of xylose had a slightly negative effect on lipid accumulation in other oleaginous yeasts [35]. However, from the current data we cannot draw any valid conclusion about the physiological background of the different behaviour of the red yeasts and *L. starkeyi* in mixtures of CG and HH.

In contrast, for the two in detail tested red yeast strains, we found a considerable activating effect of mixing HH with CG. Cells started consuming glycerol and producing lipids much earlier than in culture without HH. At least for *R. toruloides* CBS 14 there was a clear effect on specific lipid production after the additional carbon sources were consumed. Thus, the higher glycerol conversion was not only due to an increased biomass because of the additional carbon sources but to an increased metabolic activity. The impact on specific lipid production was less pronounced in *R. glutinis* CBS 3044, still, the activating effect of adding HH was clearly visible for this strain as well.

From the results it can be concluded, that an addition of HH to the culture media has a positive effect on lipid production rate for *Rhodotorula* species. The addition of HH also had an improving effect on biomass production. An addition of just 10% of HH (approximately 2.6 g/L xylose, 0.6 g/L glucose, 0.8 g/L acetic acid) resulted in consumption of all available carbon sources within 96 h of cultivation. Cultures without HH needed at least 24 h more to consume all glycerol in the media. The addition of HH did not impact specific glycerol uptake significantly but it accelerated reaching maximum lipid production rate in *R. toruloides.* This increased rate of lipid production by supplementing crude glycerol media with hemicellulose hydrolysate has not been reported before for yeast, and this is worth further investigations. In previous studies mixed substrates of lignocellulosic hydrolysates supplemented with glycerol have been performed. Shen et al. found a similar positive effect in *Trichosporon fermentans* of faster glycerol metabolism when adding sweet potato waste, which contained 30 g/L of reducing sugars. Gong et al. described increased lipid yield and productivity in *Cutaneotrichosporon curvatum* (synonym *Cryptococcus curvatus*) when cultivating it in a mixture of corn stover hydrolysate and glycerol [10, 36]. However, in another study with the oleaginous zygomycete *Cunninghamella echinulata*, where tomato hydrolysate was used with glycerol no such effect has been observed [37]. Our results showed higher total lipid concentrations when compared to recently published studies, which investigated mixtures with xylose and crude glycerol of higher quality (90–95% vs our 80%) as growth substrates [35]. This may be due to better controlled conditions in bioreactors compared to shake flask cultures.

The 40% HH supplement to the culture media used to grow *R. toruloides* also resulted in the highest average lipid content. Even the 10% HH supplement apparently decreased the time in which lipid contents of > 40% were achieved. Without HH addition, lipid content in CG media (CG50 and CG60) was similar to that in experiments with other *R. toruloides* strains grown using glycerol as carbon source [38]. It is possible that with proper adjustment of the ratio between HH and CG an even higher lipid concentrations could be achieved in less than 96 h. Shortening the fermentation time has been described as a crucial aspect of an efficient process, as aerobic fermentations, in contrast to anaerobic processes, require substantial energy input for aeration [22]. The calculated yields of lipids per consumed substrate in *R. toruloides* in mixed media (0.18–0.25) were higher or comparable to previously reported ones using glucose, which were 0.21 g/g [39] or pure glycerol with *R. toruloides* 0.16 g/g [35]. In *C. curvatum* in cornstover hydrolysate, lipid yield was 0.18 g/g [36].

In our hands, it was necessary to add additional nitrogen to the hydrolysates when cultivating red yeasts, as the cells stopped growth and lipid accumulation during cultivation, probably due to too low nitrogen contents. For industrial processes, cheap and abundant nitrogen sources have to be identified. Food wastes are examples of

such nitrogen sources, and recent results demonstrated that for instance waste milk or protein hydrolysates from chicken byproducts can be used for the production of yeast biomass [40, 41].

The activating effect by the addition of HH seems to be specific for red yeasts, as in *L. starkeyi* CBS 7786 the addition of HH had a rather inhibiting effect. The metabolism of xylose and glycerol are both generating glyceraldehyde-3-phosphate (G-3-P). In red yeasts, glycerol assimilation is possibly activated due to an increased amount of G-3-P. Glycerol is also required to form triacylglycerol (TAG), the major storage lipid in oleaginous yeasts. It is possible that the cell activates glycerol uptake to produce TAG from the additional carbon sources. Acetic acid could also play an important role in this process, as it is converted to acetyl-CoA, the precursor of fatty acid production [42]. Nevertheless, for the moment we can only speculate on the mechanism of the activation of the lipid metabolism by HH in *Rhodotorula* species. Further investigations are required to understand this phenomenon, for instance studies of the transcriptome of the yeasts.

The fatty acid profiles achieved for *R. toruloides* in this study are similar to those of vegetable oils like palm oil, and match biodiesel profile requirements [20, 43, 44]. Moreover, the yeast oil can also be used in other applications, such as for the replacement of vegetable oil in fish feed [24]. Interestingly, the fatty acid profile did not vary much despite that different cultivation media were used and the time of cultivations had varied.

Conclusions

This study proved that the low-value side products CG and hemicellulose can be used for production of lipids. Addition of HH to CG considerably increased lipid production rate in *R. toruloides* and to some extent in *R. glutinis*. Based on the results of our study it seems to be possible to add value to side streams of biodiesel production and pulp and paper industry. Glycerol utilisation and shorter cultivation times are crucial factors to improve the efficiency of biodiesel production based on oleaginous yeasts [22]. In addition, this study has proven that *R. toruloides* CBS 14 has a good potential for microbial lipid production utilising CG and HH as carbon sources during cultivation.

Materials and methods

Yeast strains

Strains used are presented in Table 3.

All strains were stored at − 80 °C in 50% v/v glycerol and pre-grown on YPD plates (glucose 20 g/L (≥ 99%, Fluka Analytical, France), yeast extract 10 g/L (Bacto™ Yeast Extract, BD, France), peptone 20 g/L (from casein, Merck KGaA, Germany), agar powder 20 g/L (VWR chemicals, Belgium, pH = 6,) in 4 °C and were re-streaked every 4 weeks.

Table 3 List of oleaginous yeast strains tested for the capability to utilise crude glycerol and hemicellulose hydrolysate as carbon source

Species	Strain
Lipomyces	
lipofer	CBS 944
lipofer	CBS 5842
starkeyi	CBS 1807
starkeyi	CBS 2512
starkeyi	CBS 6047
starkeyi	CBS 7536
starkeyi	CBS 7544
starkeyi	CBS 7545
starkeyi	CBS 7786
starkeyi	CBS 7851
starkeyi	CBS 7852
Rhodotorula	
babjevae	CBS 7808
babjevae	CBS 7809
glutinis	CBS 20
glutinis	CBS 2203
glutinis	CBS 2367
glutinis	CBS 2889
glutinis	CBS 2890
glutinis	CBS 3044
glutinis	CBS 5182
glutinis	CBS 5805
glutinis	CBS 7538
glutinis	CBS 9477
graminis	CBS 3043
minuta	CBS 8013
mucolaginosa	CFSQE 63
toruloides	CBS 14

CBS—Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands, CFSQE—The Center for Food Safety and Quality Enhancement, Griffin, Georgia, USA

All cultivation media were sterilised by autoclaving for 20 min at 121 °C and 4 bars unless stated otherwise. Fragile media components were sterile filtered using 0.2 µm syringe filters (Sarstedt, Germany). Hemicellulose hydrolysate was filtered using 0.45 µm bottle top unit filters followed by 0.2 µm bottle top unit filters (VWR, Belgium).

To prepare a pre-culture, three types of media were used: (a) YPD—Glucose 20 g/L (≥ 99%, Fluka Analytical, France), Yeast extract 10 g/L (Bacto™ Yeast Extract, BD, France), Peptone 20 g/L (from casein, Merck

KGaA, Germany), pH = 6; (b) YPG—Glycerol 30 g/L, Yeast extract 10 g/L, Peptone 20 g/L, pH = 6; and (c) YPXG – Xylose 10 g/L, Glycerol 20 g/L, Yeast extract 10 g/L, Peptone 20 g/L, pH = 6.

All pre—cultures were started from yeast colonies grown on YPD plates and run in 500 mL baffled flasks in 100 mL of media, except the pre-cultures for the plate screening tests, which were done in 100 mL shake flasks with 10 mL of media. All starter cultures were incubated at 25 °C, for 72 h.

For preparation of inoculum, cultures were collected into sterile 50 mL falcon tubes and centrifuged at 3000 × *g* for 5 min. The supernatant was discarded and the pellet washed with a solution of sterile filtered NaCl (9 g/L). This process was repeated once. After refilling with the NaCl solution, OD_{600} was measured to calculate inoculation volume to reach a starting OD_{600} of ~ 1.

Media used in growth tests contained—1.7 g/L Yeast Nitrogen Base (YNB) w/o amino acids and 2 g/L ammonium sulphate (DifcoTM, Becton–Dickinson and Company, USA), with different concentrations of crude glycerol, hemicellulose hydrolysate or combinations of both, together with 0.1 M Potassium phosphate buffer pH 6. Tests were performed in 100 mL Erlenmeyer flasks, incubated at 25 °C on a rotary shaker at 120 rpm. In all shake flask tests, OD_{600} measurement was used to quantify cell growth. All media used are presented in Table 4.

C/N ratios were determined as described in [36]. For yeast extract a C/N ratio of 3.6:1 was assumed as described in [45], which also was confirmed by own analyses (unpublished results).

Hemicellulose hydrolysate was based on wheat-straw subjected to acid-based steam explosion which was processed by the Department of Biochemical Process Engineering Luleå University of Technology, Sweden, as described previously [46]. According to HPLC-measurements, HH contained 26.2 g/L xylose, 7 g/L glucose, 6.6 g/L acetic acid and trace amounts of arabinose (< 0.5 g/L). The pH of the HH was set to 6 by addition of 5 M NaOH. In the experiments, the HH was diluted with water.

Crude glycerol was provided by Perstorp Holding AB. The composition was 80% glycerol, 5% ash, 15% water, 0.5% methanol and trace amounts of feedstock—vegetable oil.

Growth experiments

Crude glycerol and hemicellulose growth test

To test strains for the ability to grow on CG, plates with YNB and agar (16 g/L) were used without carbon source. The inoculation cultures were diluted to reach a density of ~ 0.5 McFarland and plated on the testing plates. Testing plates were (I) a CG drop was placed in the centre of the plate, (II) a sterile disk soaked in 50% CG was placed in the centre of the plate and (III) negative control without any carbon source. All the plates were incubated for 72 h in 25 °C.

For confirming the growth on mixed CG and HH, six liquid media were used: Control, sCG3, sCG6, sCG9, sCG12 and sHemi (Table 4). 20 ml of media was used in 100 ml Erlenmeyer flasks. Cultures were grown for 96 h at 25 °C on a rotary shaker at 120 rpm, with OD_{600} measurements after 72 h and 96 h.

A mixture of CG and HH (sHH40CG60, C/N ratio ~ 78) was compared to CG alone (sCG60, C/N ratio ~ 58) or a control (Table 4). All samples were set up in duplicates. Cultures were grown for 72 h with OD_{600} measurements each 24 h and subsequently every 6 h.

Table 4 Media used in this study; HH—hemicellulose hydrolysate, CG—Crude glycerol, s—shake flask culture

Name	Media components	
Control	20 g/L glucose, YNB 1.7 g/L, 2 g/L NH_4SO_4, 0.1 M Potassium phosphate buffer pH 6, YE 0.75 g/L	
sHemi	40% HH	+ YNB 1.7 g/L, 2 g/L NH_4SO_4, 0.1 M Potassium phosphate buffer pH 6, YE 0.75 g/L
sCG30	30 g/L CG	
sCG60	60 g/L CG	
sCG90	90 g/L CG	
sCG120	120 g/L CG	
sHH40CG60	40% HH + 60 g/L CG	
HH40CG60	40% HH + 60 g/L CG	+ YNB 1.7 g/L, 2 g/L NH_4SO_4, YE 0.75 g/L, 1 g/L $MgCl_2$
HH10CG50	10% HH + 60 g/L CG	
CG50	50 g/L CG	
CG60	60 g/L CG	
HH10CG55	10% HH + 55 g/L CG	
CG55	55 g/L CG	

Bioreactor cultivations

Fermentations were performed in 500 mL bioreactors Multifors 2 (Infors HT, Switzerland) containing 350 ml medium. The fermentation conditions were 25 °C, pH = 6 (acid 3 M H_3PO_4, base 5 M NaOH), stirrer starting at 200 rpm with maximum speed 800 rpm, aeration 0.3 L/min (0.9 vvm) and DOT set to 20%. Polypropylene glycol 2000 was added as antifoaming agent, when needed. Unless stated otherwise, each tested strain was cultivated in triplicate. The media tested are stated in Table 4. Fermentations were performed for 120 or 144 h depending on strain and carbon consumption. Biomass concentrations were determined by cell dry weight determination.

Specific lipid production was calculated for the midpoint of an according measuring interval:

$$\textit{Specific lipid production} = (\Delta\, \textit{Lipid concentration})/(\textit{Average}\, X * \Delta t)$$

Δ Lipids were determined as difference between the measured lipid concentration values after the respective midpoints and before. Average X were calculated from the mean of biomasses determined after and before the respective midpoints. Δt was the time interval between the two measuring points.

Analytical methods

The optical density (OD_{600}) was measured at a wavelength of λ = 600 nm using CO8000 Cell density meter (Nordic Biolabs). HPLC and cell dry weight measurements were performed as described before [19]. To prepare samples for lipid extraction, harvested cells were freeze dried in vacuum for 72 h with condenser set to − 100 °C (CoolSafe Scanvac, LaboGene ApS, Denmark). Lipid concentration and fatty acid profile were determined as described earlier [19]. For rapid determination of lipid content we used our recently described FT-NIR method [33].

Abbreviations

CG: Crude Glycerol; FAME: Fatty Acid Methyl Esters; FT-NIR: Fourier Transform Near-Infra-red; HH: Hemicellulose Hydrolysate; YNB: Yeast Nitrogen Base; YM: Yeast Extract–Malt Extract; YPD: Yeast Peptone Dextrose; HIP: Hexane: Isopropanol.

Supplementary Information

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Additional file 1. Figure 1. Specific lipid production rates of *R. toruloides* CBS 14 and R. glutinis CBS 3044 in 55 g/L crude glycerol media and 55 g/L crude glycerol media with 10% hemicellulose hydrolysate. Negative values are due to a decrase of lipid concentrations during the measuring interval.

Additional file 2. Figure 2. *R. toruloides* CBS 14, HH40CG60 media grown in duplicates, dry weight and change of compounds concentration in media over time, average lipid concentration was 10.57 g/L after 70h. Glucose, xylose and acetic acid are presented on secondary y axis.

Additional file 3. Figure 3. Bioreactor cultivation of *R. toruloides* CBS 14 in triplicates, (A) CG50 medium, (B) HH10CG50 medium. Glucose, xylose and acetic acid are presented on secondary Y axis.

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Authors' contributions

MC was involved in designing the study, performed the major part of the lab work, and wrote the first draft of the manuscript. SaS was involved in laboratory work, evaluation of the results and performed a major contribution to writing. JB contributed to laboratory work, evaluation of the results and perfromed a major contribution to writing. MS and VP provided a major contribution to designing the study, to evaluate the results and to write the manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication.

All authors agreed to publish this article.

Competing interests

The authors declare that they have no competing interests.

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III

Differential gene transcription of *Rhodotorula toruloides* CBS14 during growth in crude glycerol and a mixture of crude glycerol and hemicellulose hydrolysate

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(Manuscript)

Abstract

This study aimed to get an insight into the physiological basis of a recently observed metabolic activation of growth, substrate consumption and lipid accumulation by the oleaginous yeast *Rhodotorula toruloides* CBS14, when cultivated on a mixture of crude glycerol and hemicellulose hydrolysate (GCHH). rRNA- depleted total RNA isolated from yeast cells grown on either crude glycerol (CG) or CGHH was sequenced and the abundance of transcripts was compared.

We observed enhanced transcription of genes involved in oxidative phosphorylation and in general of genes encoding enzymes localised in mitochondria. This was especially the case after 10 h in CGHH, compared to all other measuring points in CGHH and CG. Genes involved in protein synthesis- ribosomal proteins, translation elongation factors and RNA- polymerases also showed an enhanced transcription in CGHH compared to CG. Another group of activated genes is involved in handling oxidative stress and degradation of aromatic compounds.

The initial activation of both energy metabolism and protein synthesis may be the key to the activation of the metabolism. The additional carbon sources of the hemicellulose hydrolysate are rapidly consumed by the cells, which then have induced the biochemical machinery to rapidly convert the glycerol to biomass and lipids.

Keywords: Oleaginous yeasts, *Rhodotorula toruloides*, transcriptome, lipids, lignocellulose, xylose, biofuels

Background

Vegetable oils (VO) are regarded as the fastest growing food commodities worldwide (Khatri & Jain, 2017). Many VO have a high greenhouse gas potential during their production, for instance for palm- and soybean oil emissions of more than 2000 kg CO_2 equivalents per produced ton have been determined, and there is considerable land use for producing VO (Schmidt, 2015). Even the production of rapeseed oil can cause a higher CO_2 release as palm- or soybean oil (Uusitalo et al., 2014). Since also biodiesel is produced from VO, their consumption in Sweden (and the EU) is much higher than the own production, and thus, a major proportion of the utilised VO is

imported (Harnesk & Brogaard, 2017). There is substantial rainforest clearing due to palm- and soya oil production (Hoang & Kanemoto, 2021).

Microbial oils have the potential to replace vegetable oils in biodiesel-, feed and food production (Blomqvist et al., 2018; Karlsson et al., 2016; Lundin, 1950). Especially oleaginous yeasts are known as the most rapid lipid producers on earth, which can convert a variety of residues such as lignocellulose hydrolysates, including hemicellulose hydrolysate, or crude glycerol (CG), a residue of biodiesel production, to lipids (Balan, 2019; Brandenburg et al., 2016; Chmielarz, Blomqvist, Sampels, Sandgren, & Passoth, 2021; Papanikolaou & Aggelis, 2020).

In a recent study, we discovered that lipid formation from glycerol was activated in *Rhodotorula* species, when the yeast was cultivated in a mixture of CG and hemicellulose hydrolysate (CGHH) (Chmielarz et al., 2021). The physiological basis of this phenomenon is not clear yet. The recently assembled high quality, chromosome- level genome of *Rhodotorula toruloides* CBS14 (Martín-Hernández et al., 2021) allowed to study differential gene expression under different cultivation conditions. It was the aim of this study to investigate gene transcription during cultivation on CGHH, compared to the cultivation on CG alone, to get an insight in metabolic pathways that are activated under the respective conditions.

Materials and Methods

RNA was isolated from fermentations described in Chmielarz et al. 2021, i.e. *R. toruloides* CBS14 was cultivated in 500 mL bioreactors (Multifors, Infors HT, Bottmingen, Switzerland) with either a mixture of hemicellulose hydrolysate and crude glycerol or only crude glycerol as carbon source. Additionally, the media contained 0.75 g/L yeast extract (BactoTM Yeast Extract, BD, France), 1 g/L $MgCl_2$ $6xH_2O$ (Merck KGaA, Germany), 2 g/L $(NH_4)_2HPO_4$ (≥98%, Sigma-Aldrich, USA), and 1.7 g/L YNB without amino acids and ammonium sulphate (DifcoTM, Becton Dickinson, France). Samples were taken for the cultures on CG after 10 h, 30 h, and 60 h and on CGHH after 10 h, 36 h, and 60 h. Cultivations were performed in triplicates, at 25°C, pH 6 and oxygen tension of 21%. The RNA extraction followed a protocol as described before (Martín-Hernández et al., 2021).

Transcriptome analysis of the obtained RNA-Seq samples was performed using the RNAflow differential gene expression pipeline v1.2.0 (Lataretu & Holzer, 2020) comprising all steps starting from read quality control, reference-based mapping, gene quantification, count normalization, differential expression calling, and result visualization. RNAflow uses the workflow system Nextflow and Conda or Docker to ensure a robust detection of differentially expressed features with a high level of reproducibility. In short, samples were quality checked with FastQC and raw reads were trimmed using fastp to remove low-quality bases and adaptor contaminants (Chen, Zhou, Chen, & Gu, 2018). Remaining rRNA was detected and removed using SortMeRNA (Kopylova, Noe, & Touzet, 2012). Then, reads were splice-aware aligned with HISAT2 (Kim, Langmead, & Salzberg, 2015) to the *R. toruloides* assembly as previously reported (Martín-Hernández et al., 2021). Gene-level expression quantification was performed with featureCounts (Liao, Smyth, & Shi, 2014) only considering uniquely mapped reads und using the annotation file from (Martín-Hernández et al., 2021). TPM (transcripts per million kilobases) values were calculated by RNAflow to identify and filter out lowly expressed genes in further downstream analyses. For each gene and sample, a TPM value was calculated, followed by the calculation of the mean TPM value in each condition. A gene needs to have at least a certain mean TPM greater than a defined threshold to be considered in downstream analyses. We performed all calculations with a default TPM threshold of 5. Finally, a differential gene expression analysis was performed with DESeq2 (Love, Huber, & Anders, 2014) to identify significantly (adjusted p-value <0.05) differentially expressed genes. Principal component analysis, expression heatmaps and box plots were generated by respective R packages implemented in the RNAflow pipeline. For details about the used tool versions, R packages, and custom scripts please refer to https://github.com/hoelzer-lab/rnaflow.

According to the annotation, KEGG orthology (KO) numbers were added to each gene identified to encode an enzyme. Then it was checked in which metabolic pathways or cellular processes the regulated genes are involved, using the KEGG mapper (https://www.kegg.jp/kegg/tool/map_pathway.html).

Results

RNA was isolated after 10, 30 and 60 h of cultivation in CG and after 10, 36 and 60 cultivation in CGHH mixture (Chmielarz et al., 2021) (Figure 1), and rRNA- depleted RNA was sequenced in triplicates. Derived transcriptome reads were quality- controlled, mapped against the annotated genome of *R. toruloides* CBS 14 (Martín-Hernández et al., 2021) and quantified on gene level for subsequent differential expression analysis with DESeq2. In addition, the number of TPM was calculated per gene and sample to compare robust expression values normalised by gene length and sequencing depth between conditions and to filter out lowly expressed genes). Differences in global gene expression between the samples and clustering of biological replicates were analysed by PCA. The analysis showed that gene transcription significantly differed at all investigated time points.

Transcription levels of genes were compared between the different measuring points. More than 200 genes were significantly higher transcribed ($p<0.05$) in CGHH10 h compared to CG10 h. In most cases, these genes were also higher expressed compared to the other measuring points, both in CGHH and CG. Moreover, most of these genes were still higher transcribed in CGHH 36 h compared to CG 60 h. After 36 h in CGHH, the only detectable carbon source was glycerol, in a similar concentration as in CG after 60 h (Figure 1).

Most of these upregulated genes could be associated to energy metabolism, especially oxidative phosphorylation and other mitochondrial enzymes, as well as gene expression/ protein synthesis, protein degradation (proteasome) (Tables 1, 2, supplementary Tables 1-3. Supplementary tables are provided under https://biodisk.molbio.slu.se:5001/sharing/5B0odKLYB, user name and password will be provided by the corresponding author on request).

Seventeen genes involved in the mitochondrial electron transport chain- complex 1 (NADH ubiquinone oxidoreductase), 3 (cytochrome c reductase), 4 (cytochrome c oxidase) and F-type ATPase were significantly regulated (Figure 2).

Highest transcription levels were observed in the 10 h samples in both media. After 36 h in CGHH, the TPM levels were frequently similar to those of CG 10 h, TPM- values at the later time points were lower than at the beginning of the cultures (Table 1, 2, supplementary Table 1). Two prohibitin genes, involved in the formation of respiratory supercomplexes (Azuma, Ikeda, & Inoue, 2020) were regulated in a similar way as genes of complexes of the respiratory chain.

A variety of genes encoding mitochondrial enzymes or components of them followed a similar pattern as the genes of the respiratory chain, i.e. their TPM were highest in CGHH 10 h, lower at CGHH 36 h but still at a similar level as CG 10 h. This includes for instance genes of enzymes involved in the tricarboxylic acid cycle (isocitrate dehydrogenase, succinate- CoA ligase and fumarate hydratase, NANOZOOG 5845, 7009 and 5604, respectively) and other genes. Genes encoding enzymes involved in synthesis of cofactors of mitochondrial enzymes followed a similar pattern, including three enzymes of riboflavin- synthesis (NANOZOOG9252, 2556, and 2045), lipoyl synthase (cofactor of pyruvate dehydrogenase, PDH and α-keto glutarate dehydrogenase, α-KGDH (NANOZOOG5711) and thiamine pyrophosphate (TPP, cofactor among others in PDH and α-KGDH; NANOZOOG9040, thiamine pyrophosphokinase). Frequently, transcription of these genes was also higher in CGHH after 30 h compared to CG 60 h, where glycerol was the only carbon source and in similar concentrations. This, however, does not fit to the mentioned enzymes of the tricarboxylic acid cycle (Table 1).

Almost 30 ribosomal proteins were significantly upregulated (about two fold), including both cytoplasmic and mitochondrial ribosomes (Figure 3, supplementary Tables 1-3). In CGHH, for most of the genes the transcript numbers (TPM) decreased in the later stages of cultivation. In contrast, in CG TPM increased in later stages of cultivation, reaching the same or

even higher transcription level compared to the sample at 10 h. Other genes involved in gene expression, including components of all three DNA- dependent RNA-polymerases, translation initiation factors and ribosome biogenesis were 1.5- 4 fold higher expressed on CGHH 10 h compared to CG 10 h and the later time points on CGHH. These genes were even at the later time points in CGHH transcribed to the same level or even higher than in CG (supplementary Table 1-3).

Apart from genes involved in protein synthesis, also transcription of genes involved in protein turnover were activated. Transcription of 12 genes involved in building the proteasome was upregulated at CGHH at 10 h of about 2-3 fold compared to all other measuring points, both on CGHH and CG (Figure 4, supplementary Tables 1-3).

One ammonium transporter (NANOZOOG1250) was very lowly transcribed in CGHH at 10 h (0 TPM), but then upregulated at later time points (44 and 42 TPM). In CG the transcription level reached 16 TPM after 10 h, 29 after 30 and 101 after 60 h (significant change between all measuring times and substrates except CG 10 h vs. CG 30h and 60 h, CGHH 36 h vs. CG 30 and 60 h, and CGHH 36 h vs. CGHH 60 h).

Two genes involved in fatty acid biosynthesis were significantly upregulated in CGHH 10h. NANOZOOG8056, annotated as 3-oxoacyl-[acyl carrier protein] reductase, was more than 30 times upregulated at CGHH 10 h compared to CG 10 h, and similarly compared to all other time points. Another gene, NANOZOOG8822, annotated as enoyl carrier protein reductase, was more than two-fold upregulated compared to CG 10 h. At the other time points the transcription levels were similar to CG 10 h. However, there was a significant upregulation of five genes involved in fatty acid degradation in CGHH 10h, three of located in the mitochondria, the other peroxisomal. Transcription levels in CGHH 10 h were 3- 16 times higher than in CG 10 h. At later time points, transcription of these genes decreased in CGHH to levels comparable in CG (Table 2). The fatty acid synthase genes *RtFAS1* and *RtFAS2* were lowly transcribed after 10 h cultivation in both substrates, but actually lower in CGHH than in CG. After 36/ 30 h cultivation, transcription was upregulated, and it was about double as high in CGHH compared to CG. For both *FAS*- genes, the highest TPM was observed in CGHH 30, however, significance of the difference between CGHH and CG could not be proven, due to high standard deviations between the three parallel cultivations. At 60 h, transcription on both substrates was similar. Interestingly, *RtFAS21*, which is transcribed from the opposite strand as *RtFAS2*, and thus forms an antisense RNA (Martín-Hernández et al., 2021) was expressed in CGHH after 36 and 60 h cultivation. The gene of the acetyl-CoA forming enzyme acetyl-CoA synthetase ACS was highly transcribed in CGHH after 10 h cultivation. This enzyme converts acetate to acetyl-CoA, and it is probably activated as a part of the acetate assimilation pathway, as the cells are consuming the acetic acid present in the hemicellulose hydrolysate (Brandenburg et al., 2016; Chmielarz et al., 2021). Interestingly, the TPM in CG 10 h was relatively high, about 50% of that in CGHH 10 h. At later time points, transcription of this gene was down regulated. ATP-dependent citrate- lyase (ACL) is supposed to be the main producer of acetyl-CoA in fatty acid synthesis (Athenaki et al., 2018). In CGHH it was lowly transcribed after 10 h, however, at later time points higher TPM were found in CGHH compared to CG, although significance could not be proven.

Transcription of genes, which encode for enzymes involved in NADPH- generation, Glucose- 6-P dehydrogenase (G6PDH), 6-P-gluconate dehydrogenase (6PGDH) did not differ significantly over the measured time period and between the substrates (Table 3).

NADPH- generation

As in CGHH the glycerol was much faster consumed than in CG alone (Chmielarz et al., 2021), Figure 1), we also checked regulation of genes involved in glycerol metabolism. In *Saccharomyces cerevisiae*, glycerol assimilation has been demonstrated to be performed by the catabolic G3P pathway and involves glycerol kinase (EC 2.7.1.30) and FAD-dependent glycerol-3-phosphate dehydrogenase (EC 1.1.5.3) encoded by *GUT1* and *GUT2*. Another suggested pathway, the catabolic DHA pathway, is performed by a NAD^+-glycerol dehydrogenase (GDH, EC 1.1.1.6) and dihydroxyacetone kinase (DAK, EC 2.7.1.29)

(Klein, Swinnen, Thevelein, & Nevoigt, 2017). Deletion studies of *GUT1* and *GUT2* demonstrated that the catabolic G3P pathway is the main pathway of glycerol utilisation in *S. cerevisiae* (Sprague & Cronan, 1977; Swinnen et al., 2013). For the DHA pathway, there is only one study in *Schizosaccharomyces pombe* demonstrating its utilisation in glycerol assimilation in yeasts (Matsuzawa et al., 2010).

A third pathway of glycerol assimilation has been suggested in *Neurospora crassa*, the catabolic GA-pathway. In this pathway, glycerol is first oxidised to glyceraldehyde by a $NADP^+$-dependent GDH (EC 1.1.1.372 or 1.1.1.72). Then it can be either phosphorylated to glyceraldehyde-3-P by a glyceraldehyde kinase (2.7.1.28) or reduced to D-glycerate by an aldehyde dehydrogenase (EC 1.2.1.3). D-glycerate is then converted to 3-P-D-glycerate by glycerate-3-kinase (2.7.1.31) (Klein et al., 2017; Tom, Viswanath-Reddy, & Howe, 1978; Viswanath-Reddy, Bennett, & Howe, 1977).

After 10 h of cultivation, transcripts of two potential glycerol transporters were more abundant under on CG compared to CGHH. For all later time points, transcription of these genes was slightly enhanced in CGHH (Table 4), however, significance of these differences could not be proven.

The catabolic G3P- pathway was transcribed under all conditions. For the *R. toruloides GUT1* homologue (*RTGUT1*, NANOZOOG4714) was higher transcribed in CG after 10 h of cultivation, compared to 10 h in CGHH. After 36 h of cultivation, a time point where the additional carbon sources from the hemicellulose hydrolysate were finished and glycerol was the sole carbon source, no significant differences in transcription were observed compared to CG. After 60 h on CGHH, *RtGUT1* transcription was lower than in CG. For the transcription of *RtGUT2* no significant differences could be identified (Table 4).

We found indications for the presence and expression of alternative pathways of glycerol assimilation. Two potential glycerol dehydrogenases (*GCY1*- homologues) were identified, NANOZOOG370 and NANOZOOG 4361. According to homology analyses, these genes encode for NAD'/$NADP^+$ - dependent glycerol dehydrogenases (EC1.1.1.72 and 1.1.1.156), converting glycerol to glyceraldehyde or DHA, respectively. NANOZOOG4361 was highest expressed at 10 h in both CGHH and CG, about double as high in CGHH compared to CG. At the later time points, transcription level decreased in both substrates, with slightly higher transcription levels in CG. NANOZOOG370 was more than 30-fold higher expressed in CGHH 10 h compared to CG 10 h, where transcription level was 0 TPM. TPM at 36 h in CGHH was only 50% of that after 10 h, however, it was still more than 3 fold higher than in CG after 30 h. After 60 h, transcription was low in CGHH, while it reached 17 TPM in CG. We also identified a potential gene encoding a glyceraldehyde/ DHA- kinase (NANOZOOG2321, EC2.7.1.28/29, homolog to *DAK2*). However, DHA/ Glyceraldehyde- kinase was transcribed on a low level (Table 4).

Moreover, a variety of aldehyde dehydrogenases was transcribed, including NANOZOO8569, which was highly transcribed under all conditions (101, 113, 31 TPM on CGHH 10 h, 36 h, and 60 h, respectively and 413, 169, and 42 TPM at CG 10 h, 30 h and 60 h, respectively). However, we were not able to identify a glycerate-3-kinase gene, thus, glycerol assimilation via 3-P-D-glycerate cannot be proven in *R. toruloides*.

Transcription of most glycolytic genes was not upregulated in CGHH at 10 h, when the cells still had glucose as carbon source (except a phospho-glucomutase, PGM, NANOZOO6772, which was also higher expressed in CHHH 30 h compared to CG 60 h). Probably, those genes are more or less constitutively expressed and were not regulated at the measured time points. Of the genes of xylose metabolism, obviously only xylitol dehydrogenase was correctly annotated. This gene (NANOZOOG9007) was highest transcribed in GCHH at 10 h at 105 TPM, at 30 h 4 TPM and at 60 h 7 TPM. In CG it reached 16 TPM at 10 h, 19 TPM at 36 h and 12 at 60 h. One gene (NANOZOOG909) was identified as xylose reductase, however, its transcription level was 0 at all time points and on both substrates. No xylulose kinase was identified among the annotated genes (Table 5).

Some genes involved in handling oxidative stress were also upregulated in the CGHH substrate. This includes three thioredoxin genes (NANOZOOG3685, 7078, and 3176), a glutathione - peroxidase gene (NANOZOOG2851), and a glutathione-S transferase (NANOZOOG9349). However, there were also several genes upregulated in CG at least after 10 h cultivation. A catalase gene was upregulated in CG compared to CGHH (Table 6).

Five genes potentially included in degradation of aromatic compounds were also regulated, and specifically highly expressed in CGHH after 10 h (Table 7).

Discussion

In an earlier study, more rapid growth, substrate consumption, and lipid formation has been observed in crude glycerol mixed with rather little amounts of hemicellulose hydrolysate (Chmielarz et al., 2021). We investigated total gene transcription in such cultures to obtain insights into the mechanism of this activation.

We observed a considerable transcriptional upregulation in CGHH at 10 h compared to CG at 10 h of genes involved in oxidative phosphorylation and of several mitochondrial enzymes. This effect was at least for some genes even visible at later time points in the cultivation, when the additional carbon sources were already consumed, especially for CGHH after 36 h compared to CG after 60 h, where the remaining glycerol concentrations were similar. This indicates that the energy metabolism was activated in response to adding the hemicellulose hydrolysate. A high availability of ATP is central for both biomass- and lipid formation, a more efficient uptake of limiting resources, such as nitrogen, and inhibitor tolerance (Hara & Kondo, 2015). Among the mitochondrial enzymes, a rather lower transcription was observed in CGHH 36 h compared to CG 60 h for isocitrate dehydrogenase (IDH) and succinate- CoA ligase, although significance could only be proven for succinate- CoA ligase. Nevertheless, this observation would fit to the observed specific increase in lipid production during growth on glycerol in CGHH compared to CG (Chmielarz et al., 2021), because inhibition of IDH is a central element in lipid accumulation (Garay, Boundy-Mills, & German, 2014) in oleaginous yeasts and succinate- CoA ligase is a reaction behind IDH in the TCA- cycle.

Transcription of genes involved in protein synthesis was largely upregulated in CGHH, especially at the beginning of the culture. This might partially be due to the availability of additional nitrogen in the hemicellulose hydrolysate. After 10 h in CGHH, an ammonium transporter was significantly downregulated. This argues for the presence of sufficient nitrogen, as the cells were obviously not needing this transport capacity. Since the amount of added HH was rather small, the additional nitrogen was probably already consumed after 36 h.

The upregulation of protein metabolism - both synthesis and turnover, was most probably required to cope with the different carbon sources and other compounds that were present in the HH.

Complete glycerol consumption took about 24 h less in the CGHH cultures. However, at least after 10 h in CGHH, almost no upregulation of genes specifically involved in glycerol assimilation was observed. However, a potential bypass pathway of the standard *GUT1* and *GUT2*- pathway (Klein et al., 2017) was expressed, similar to the DHA- pathway in *S. pombe* (Matsuzawa et al., 2010). However, in this case the glycerol dehydrogenases were annotated as NAD^+/$NADP^+$- dependent and there were genes encoding two enzymes, generating either glyceraldehyde or dihydroxy acetone. These were at least initially higher transcribed in CGHH- cultivated cells. They might represent additional pathways of channeling glycerol into the main metabolic pathways and at the same time provide NADPH for biosynthetic pathways including fatty acid synthesis. When the cells are growing on CGHH, they are assimilating acetic acid at the same time as glucose. This provides additional amounts of acetyl- CoA, which is the main precursor of fatty acid synthesis (Passoth, 2017). Cytoplasmic acetyl-CoA synthetase (ACS,

NANOZOOG8257) was highly transcribed after 10h cultivation in CGHH (103 TPM) compared to CGHH 60 h (4 TPM) and CG 60 h (14 TPM), indicating high activity of acetyl-CoA production. The alternative glycerol degradation pathway and acetate assimilation may deliver the NADPH and additional acetyl-Co required for the observed enhanced lipid synthesis in CGHH (Chmielarz et al., 2021). Genes encoding glycolytic enzymes were largely not regulated in the presence of HH, except one PGM. This might be due to the fact that glycolytic genes are anyway expressed, and that xylose was much more abundant than glucose in the substrate. Interestingly, overexpression of a *PGM*- gene improved xylose fermentation in a recombinant *Saccharomyces cerevisiae*- strain (Garcia Sanchez, Hahn-Hagerdal, & Gorwa-Grauslund, 2010).

However, after 10 h cultivation in CGHH, transcription of genes involved in fatty acid production was rather low, in contrast, genes involved in lipid degradation were higher expressed compared to later time points in the cultivation and to cultivation in CG. On one hand, this is again indicating the addition of nitrogen with the HH. On the other hand, this behavior fits to previous studies, where cultivation on xylose as carbon source activated genes involved in β- oxidation, although the cells were under nitrogen limitation and accumulating lipids (Tiukova et al., 2019). In a recent study, we even observed parallel xylose assimilation and degradation of intracellular lipids in a strain of the closely related species *Rhodotorula glutinis* (Brandenburg et al., 2021).

Growth on xylose has been described with correlation to an induction of several genes involved in oxidative stress response (Tiukova et al., 2019). This was confirmed by our observation of transcriptional activation of those genes especially in CGHH after 10 h of cultivation. Assimilation of xylose may induce a stress response in the cell, although it is not completely clear, why this sugar is inducing such a response. In our cultivation, also the presence of acetic acid may have contributed to the activation of an oxidative stress response, as in *S. cerevisiae*, exposure of cells to acetic acid resulted in the production of reactive oxygen species (ROS) (Ludovico et al., 2002).

We suggest that the physiological reason for activation of the metabolism, which was the basis for accelerated glycerol assimilation and more rapid lipid production was in the first line the activation of enzymes providing energy, i.e., enzymes involved in oxidative phosphorylation and mitochondrial function. The presence of HH provided additional carbon sources, but obviously also induced some stress responses. Induction of respiratory genes might be also a response to stress provided by the substrate. In this connection it is also interesting that transcription of genes involved in degradation of aromatic compounds was activated. It is possible that some soluble aromatic compounds were released during pretreatment of lignocellulose and present in the HH (Jonsson & Martin, 2016). Those compounds can be used as carbon source by *Rhodotorula* yeasts (Middelhoven, 1993) , but they are also quite toxic to the cells. Thus, also these compounds might have been involved in the cellular stress response. Activation of β-oxidation may also indicate an increased demand of energy of the cells in the presence of the HH. After detoxification of the substrate, which probably occurred quite rapidly due to the comparable low concentration of hydrolysate, cells still had a high level of ATP and thus enough energy to shift the metabolism to glycerol metabolism and lipid accumulation. Moreover, protein synthesis and turnover were activated in the presence of HH. This may have enabled the cells to rapidly respond to the changing conditions when the additional carbon sources were finally consumed. The additional nitrogen in HH may also have stimulated enzyme production and a more rapid growth at the beginning of the fermentation.

Changes in the transcriptome cannot simply be transferred to enzyme activities or metabolic fluxes, it is one partial process in the chain of information transfer in a cell, and there is no direct proportionality between level of transcription and enzyme activity (Fredlund, Beerlage, Melin, Schnurer, & Passoth, 2006). There are even processes, which importance has yet not been understood, such as the generation of an antisense transcript of the *FAS2*-gene. In

so far, our results can only provide hypotheses about mechanisms that are causing the activation of metabolism and lipid accumulation on CG when a small proportion of HH is added to the medium. Nevertheless, conclusions can be drawn from observation of transcription of a large number of genes of certain pathways. Obviously, addition of HH causes substantial changes of gene transcription in *R. toruloides*, which then, in turn, induces improved conversion of crude glycerol to lipids. Thus, our transcriptome analyses point the way for further investigations of the underlying mechanism and help to identify novel targets to obtain strains that rapidly accumulate lipids on residual, low value substrates.

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Tables and Figures

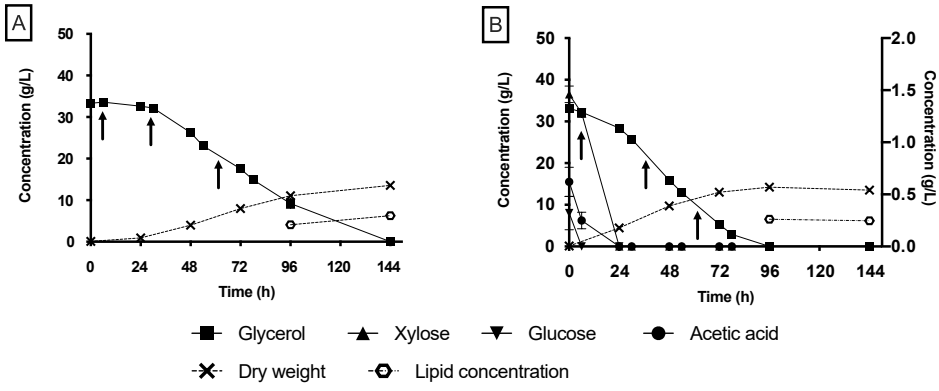


Figure 1; Bioreactor cultivation of R. toruloides CBS 14 in triplicates, (A) CG50 medium, (B) HH10CG50 medium. Arrows indicate sampling points for RNA extraction.

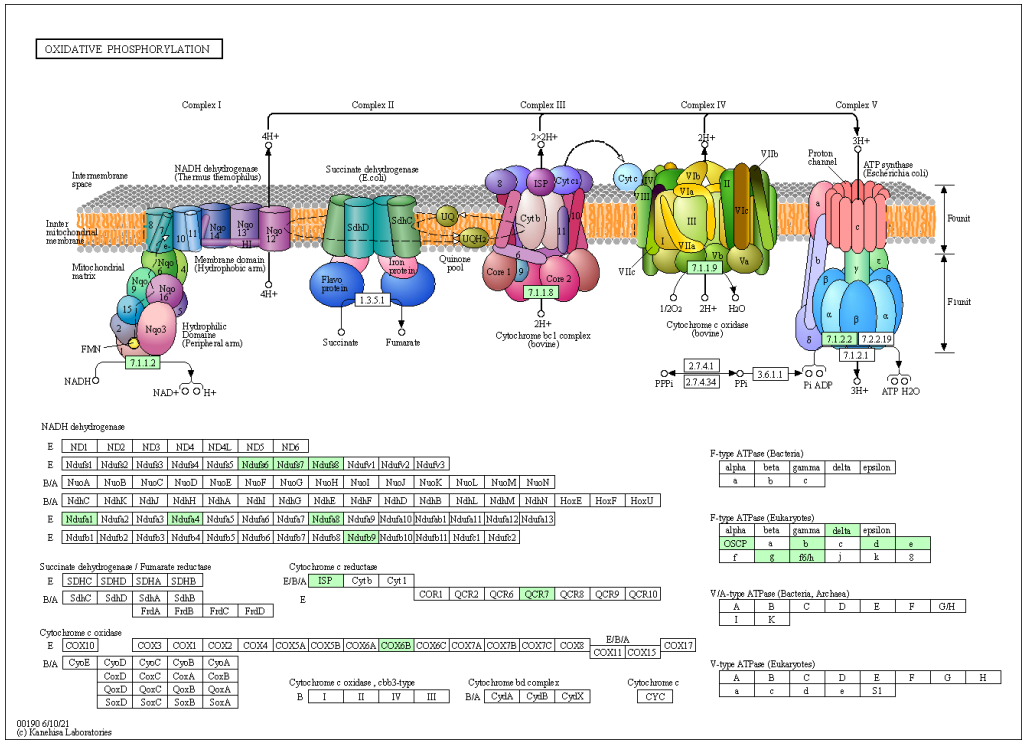


Figure 2. Upregulated genes involved in the respiratory chain. Affected enzymes are highlighted in green. Generated using the KEGG mapper (https://www.kegg.jp/kegg/tool/map_pathway.html).

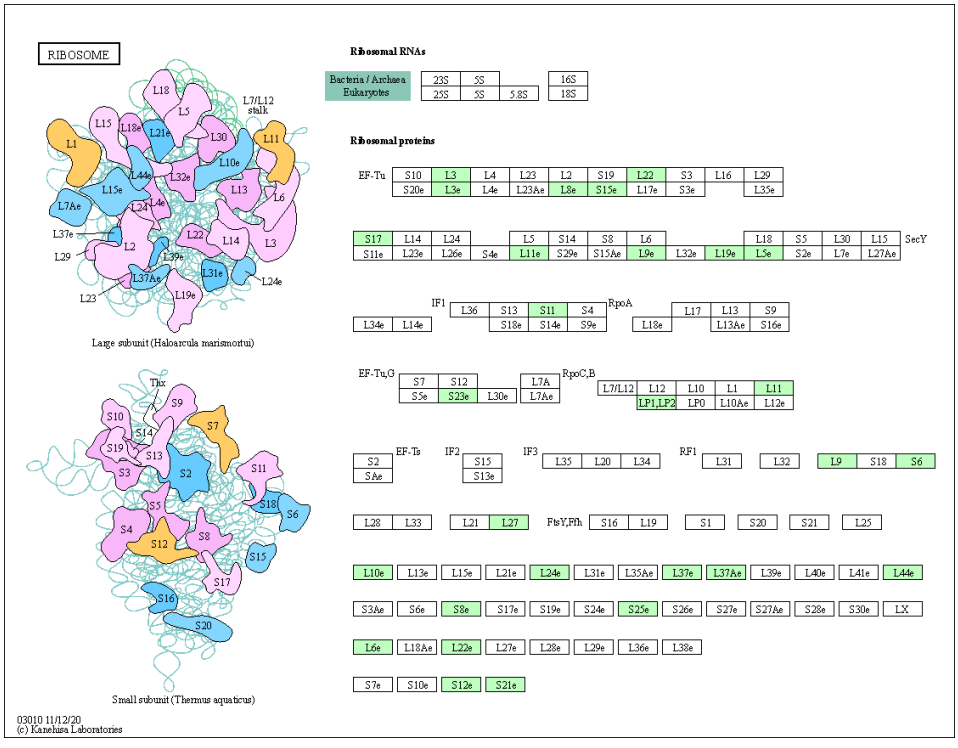


Figure 3. Upregulated genes involved in ribosomal proteins. Affected enzymes are highlighted in green. Generated using the KEGG mapper (https://www.kegg.jp/kegg/tool/map_pathway.html).

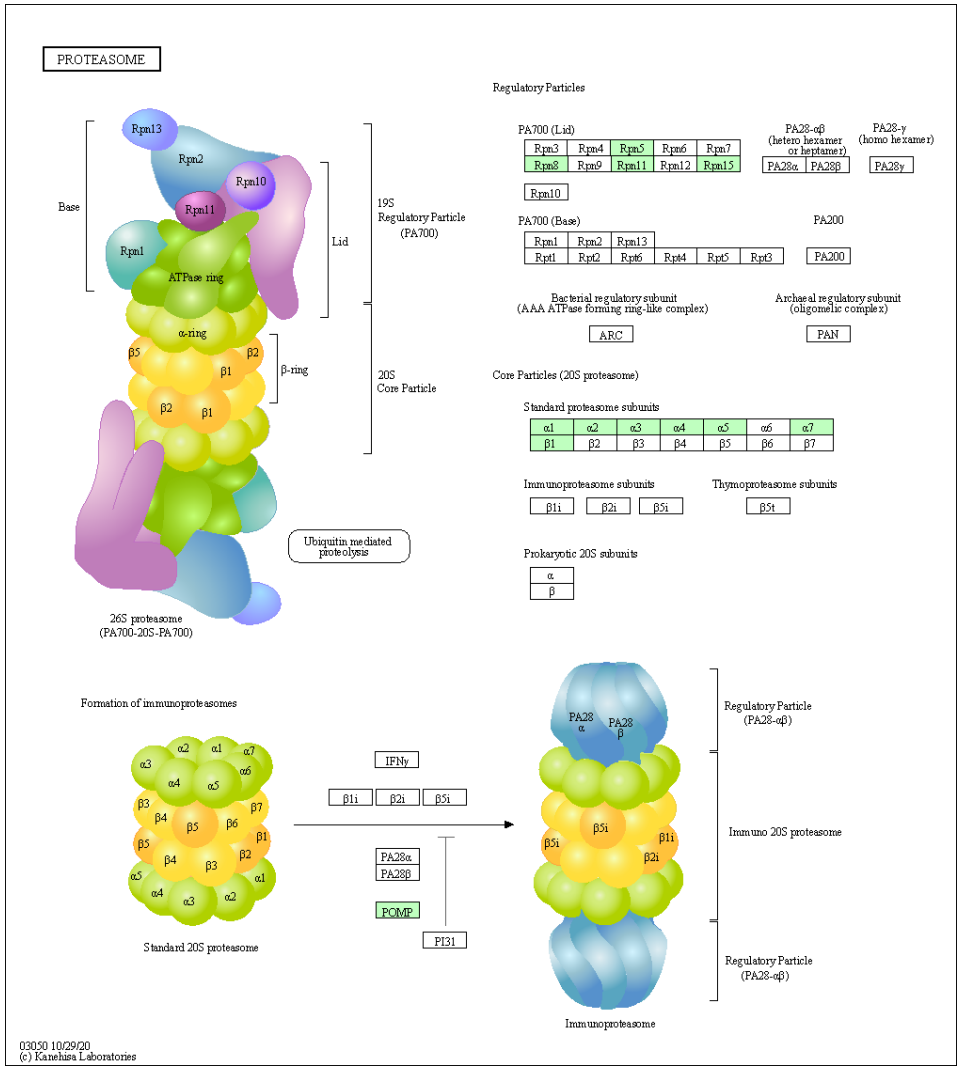


Figure 4. Upregulated genes involved in building of proteasome. Affected enzymes are highlighted in green. Generated using the KEGG mapper (https://www.kegg.jp/kegg/tool/map_pathway.html).

Table 1. Transcription of genes encoding enzymes located in mitochondria with enhanced transcription ($p<0.05$) in CGHH 10 h compared to CG 10 h. Values are given in TPM.

Enzyme, KO- Nr. / Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
Complex I (NADH ubiquinone oxidoreductase)			
Lactobacillus shifted protein, 3939/ 1991 defghk	347 141	132 108	76 56
NADH- Ubi OR[1] 19.3 kD- subunit, 3940/ 6298 defghk	283 134	127 147	55 95
NADH-Ubi OR[1] 20.8 kD- subunit, 3952/6990 defghkm	766 327	199 191	121 144
NADH- Ubi OR[1] 23 kD- subunit, 3941/ 7015 defhk	84 39	55 34	27 19
NADH-Ubi OR[1], MWFE subunit, 3945/ 7030 defghkm	290 223	137 117	72 74
Pro- rich prot. 36, 3948/ 2057 dio	18 0	29 14	4 28
LYR- protein, 3965/ 4848 defghk	542 209	287 222	164 191
Complex 3 (Cytochrome c reductase)			
Rieske subunit, 0411/ 1224 defghkl	173 87	121 114	44 77
Cytochr b-c1 Subunit 7, 0417/ 3327 bcdefghikmo	1845 1204	539 830	390 424
Complex IV (Cytochrome c oxidase)			
Subunit 12, 2267/ 6045 defghm	1606 682	301 491	218 300
F- type ATPase			
ATP-Syn[2], subunit delta, 2134/ 7299 cdefghklmn	1775 561	481 546	169 237
ATP-Syn[2], subunit 4, 2127/ 8484 cdefghkm	500 288	209 262	99 149
ATP-Syn[2], subunit 5, 2137/ 7743 defgh	376 223	152 168	82 123
ATP-Syn[2], subunit d, 2138/ 5110 defghk	540 149	133 93	54 61
ATP-Syn[2], subunit h, 2141/ 7884 bdefghkm	326 167	98 84	29 50
Other mitochondrial enzymes and cofactors			
Prohibitin 1, 17080/ 8333 defgh	243 92	84 78	64 83
Prohibitin 2, 17081/ 7096 defk	69 44	69 40	36 35
ADP/ ATP translocase 2, 5863/ 1808 defgh	511 337	373 294	250 429
DHBP synthase, 2858/ 9252 defghk	151 97	78 14	38 17
DMRL- synthase 0794/ 2556 defgh	293 127	127 151	73 175
Riboflavin synthase, 0793/ 2045 defgh	85 40	31 28	24 26
Thiamine-pyrophospho	98 15	12 2	2 8

kinase, 0949/ 9040 [defgh]			
Lipoyl synthase, 3644/ 5711 [defgko]	63 31	52 26	39 35
Cyclophilin D, 5864/ 5522 [defgh]	72 23	18 38	15 47
Isocitrate DH, 0031/ 5845 [defgh]	46 19	19 19	13 30
Succinate- CoA ligase, subunit beta, 1900/ 7009[defhk]	65 42	57 60	34 68
Methylmalonate-semialdehyde dehydrogenase, 0140/ 3041[degk]	36 1	5 10	12 39
Enoyl-ACP reductase, 7512/ 8822 [defgh]	76 32	16 22	23 19
Glutaryl-CoA dehydrogenase, 0252/ 7323 [defgh]	114 7	4 8	8 11
Carnitine-O-acetyltransferase, 0624/ 1185[defghkim]	60 18	3 3	4 8
Zn- maintenance protein 1, 18170/ 3643 [defgh]	111 34	47 25	18 31
Short/ branched chain acyl-CoA dehydrogenase, 9478/ 5684 [defgh]	76 7	5 0	3 8
3-Ketoacyl-CoA thiolase, 0626/ 3149 [dem]	20 8	17 13	22 31
Mitochondrial carrier, 15100/ 8109 [defg]	71 38	41 47	34 46

1- NADH ubiquinone oxidoreductase

2- ATP- synthase

Superscript letters indicate significant differences ($p<0.05$): a CG 10 h vs CG 30 h; b CG 10 h vs CG 60 h; c CG 30 h vs CG 60 h; d CGHH 10 h vs CG 10 h; e CGHH 10 h vs CG 30 h; f CGHH 10 h vs CG 60 h; g CGHH 10 h vs CGHH 36 h; h CGHH 10 h vs CGHH 60 h; i CGHH 36 h vs CG 10 h; j CGHH 36 h vs CG 30 h; k CGHH 36 h vs CG 60 h; l CGHH 36 h vs CGHH 60 h; m CGHH 60 h vs CG 10 h; n CGHH 60 h vs CG 30 h; o CGHH 60 h vs CG 60 h

Table 2. Transcription of genes involved in fatty acid synthesis or degradation. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
Fatty acid synthesis			
ACL/ 7175[defgh]	2 44	170 81	64 42
ACS/ 8257[abcdefghimn]	103 56	9 24	10 4
ACC/ 8968	8 28	88 48	30 38
FA oxidation complex, subunit α/ 8056[defgh]	363 11	9 6	5 12
Enoyl carrier protein reductase (mitochondrial)/ 8822[defgh]	76 32	16 22	23 19
FAS1/ 8939[g]	3 12	58 39	26 20
FAS2/ 6383[g]	6 18	80 48	32 36

FAS21/ 6386	0 0	12 0	5 0
Fatty acid degradation			
Acyl-CoA dehydrogenase/ 4755[bdefghi]	153 29	8 23	15 11
Short/ branched chain acyl-CoA dehydrogenase, 9478/ 5684[defgh]	76 7	5 0	3 8
Glutaryl-CoA dehydrogenase, 0252/ 7323[defgh]	114 7	4 8	8 11
3-Ketoacyl-CoA thiolase (peroxisomal)/ 52[defgh]	169 17	15 9	13 49
3-Ketoacyl-CoA thiolase, 0626/ 3149[dem]	20 8	17 13	22 31

Superscript letters indicate significant differences ($p<0.05$), see Table 1

Table 3. Transcription of genes involved in NADPH- generation. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
G6PDH/ 6681[k]	79 115	134 139	79 126
PGDH/ 3886	26 88	140 101	28 71
ME/ 4381	8 5	10 12	4 11

Superscript letters indicate significant differences ($p<0.05$), see Table 1

Table 4. Transcription of genes involved in glycerol assimilation. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
Major facility symporter/ 7915 (*STL1*)	8 51	48 22	8 69
Glycerol transporter/ 4353 (*GPU1*)	0 6	4 0	4 6
GK/ 4714[cfhmn] (*GUT1*)	8 51	48 22	8 69
G3PDH/ 5741 (*GUT2*)	9 9	12 11	7 20
Glycerol DH[1]/ 4361 (*GCY1*)[efgh]	90 52	12 23	7 18
Glycerol DH[2]/ 370 [defh]	32 0	15 4	2 17
Triokinase[3]/ 2321	3 4	3 12	1 10
TIM1/ 1080[cdefhko]	205 70	98 97	46 47
TIM2/ 7548	37 55	32 31	31 65

1. EC1.1.1.156

2. EC1.1.1.72

3. EC2.7.1.28/29

Superscript letters indicate significant differences ($p<0.05$), see Table 1

Table 5. Transcription of genes involved in glycolysis. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)			
Hexokinase/ 4916[gh]	17 46	60 45	45 64
Hexokinase/ 7316	12 44	27 15	16 27
G-6P isomerase/ 771	20 54	47 38	14 32
PFK/ 3173[defgh]	6 41	40 46	41 71
F-1,6 aldolase/ 3590[bfm]	70 204	69 78	39 40
GAPDH/ 7990[bk]	58 222	215 118	52 54
GAPDH/ 1838	1 3	2 3	1 3
GAPDH/ 4829	6 1	10 13	5 15
PGK/ 2622[bm]	18 50	32 38	9 15
PGM/ 6772[bcdefghko]	400 182	191 166	104 50
PGM/ 2179	4 30	30 6	13 25
Enolase/ 2969[kl]	34 56	135 62	19 25
PK/ 4157[g]	4 35	94 27	15 46

Superscript letters indicate significant differences ($p<0.05$), see Table 1

Table 6. Transcription of genes potentially involved in handling oxidative stress. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
Thioredoxin (short)/ 3685[defg]	147 45	52 23	60 39
Thioredoxin-1/ 7078[defgh]	93 45	35 54	17 29
Thioredoxin-like protein/ 3176[defghko]	143 57	69 63	57 41
Oxidative stress protein/ 5522[defgh]	72 23	18 38	15 47
Glutathione- S-transferase/ 3574[defgh]	62 20	24 13	7 11
Glutathione- S-transferase/ 4618[efgh]	57 2	0 0	1 0
Glutathione- S-transferase/ 9349[aefgm]	33 40	8 0	3 12
Glutathione-peroxidase/ 2851[f]	24 12	20 5	9 8
Glutathione dep. formaldehyde dehydrogenase/ 5726[efghi]	86 50	16 35	30 47
Catalase/ 1031[dfm]	0 29	3 2	0 16

Superscript letters indicate significant differences ($p<0.05$), see Table 1

Table 7. Transcription of genes potentially involved in degradation of aromatic compounds. Values are given in TPM.

Enzyme/ Gene (NANOZOOG)	CGHH CG 10 h	CGHH CG 36/ 30h	CGHH CG 60 h
Hydroxyquinol 1,2 dioxygenase/ 2281^{defgh}	217 12	4 10	9 6
Aryl alcohol dehydrogenase/ 7746^{defgh}	50 9	11 2	11 15
Salicylate hydroxylase/ 4610^{defgh}	84 9	5 19	9 20
4-Nitrophenyl phosphatase/ 8643^{defg}	104 37	37 32	51 47
Glutaryl- CoA dehydrogenase/ 7323^{defgh}	114 7	4 8	8 11

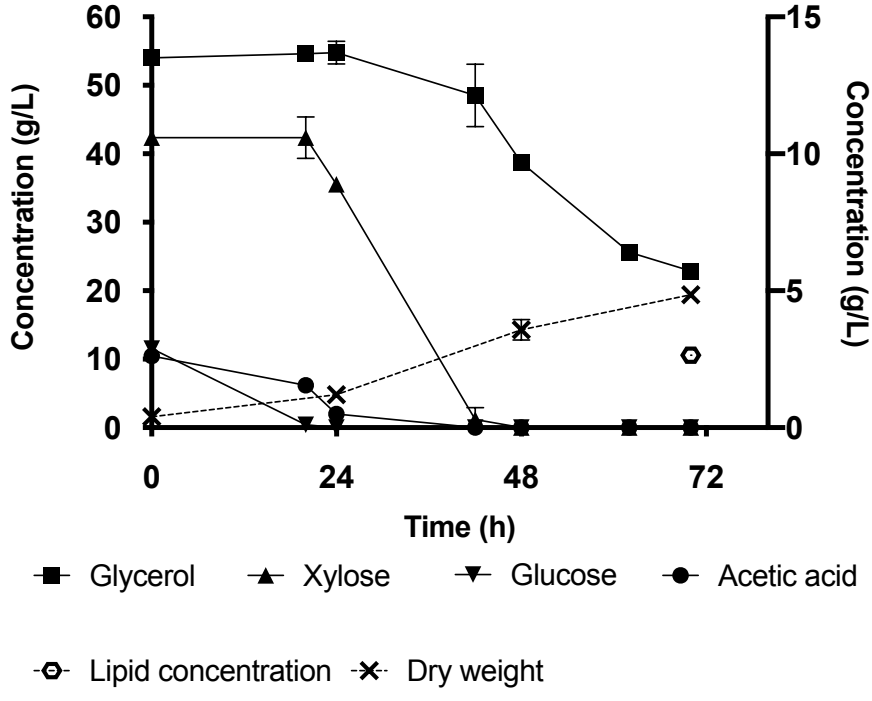
Superscript letters indicate significant differences ($p<0.05$), see Table 1

S.I.

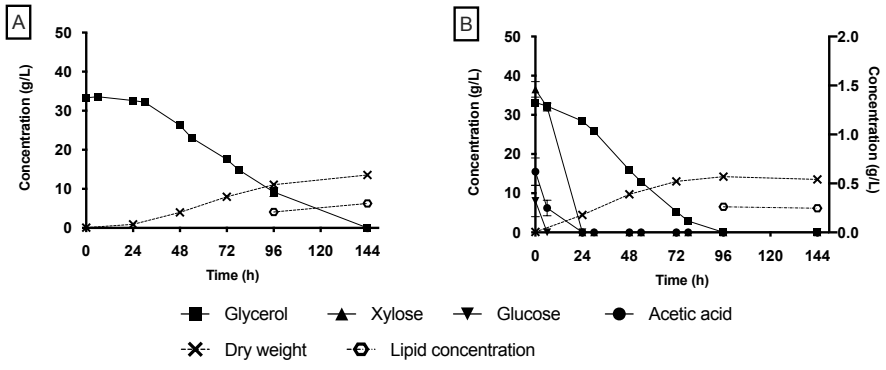
Supporting information

To paper II

Additional file 1: Specific lipid production rates of *R. toruloides* CBS 14 and *R. glutinis* CBS 3044 in 55 g/L crude glycerol media and 55 g/L crude glycerol media with 10% hemicellulose hydrolysate. Negative values are due to a decrease of lipid concentrations during the measuring interval.



Additional file 2: *R. toruloides* CBS 14, HH40CG60 media grown in duplicates, dry weight and change of compounds concentration in media over time, average lipid concentration was 10.57 g/L after 70h. Glucose, xylose and acetic acid are presented on secondary y axis.



Additional file 3: Bioreactor cultivation of *R. toruloides CBS* 14 in triplicates, (A) CG50 medium, (B) HH10CG50 medium. Glucose, xylose and acetic acid are presented on secondary Y axis.