

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Towards Bioproduction of Yeast-Based Odd-Chain Fatty Acids

Screening, characterization and development of oleaginous yeasts for production of Odd-Chain
Fatty Acids

VERONICA BONZANINI

Department of Life Sciences
CHALMERS UNIVERSITY OF TECHNOLOGY

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VERONICA BONZANINI

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Division of Industrial Biotechnology

Department of Life Sciences

Chalmers University of Technology

SE-412 96 Gothenburg

Sweden

Telephone + 46 (0)31-772 1000

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny...'"

- Isaac Asimov

“Nessun luogo é lontano”

“*No place is far away*”

- Richard Bach

Dedicato a chi non c'è piú ma é sempre presente

Dedicated to those who are no longer here but are always present

Preface

This dissertation partially fulfils the requirements for obtaining the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The work was supported by the Swedish Foundation for Strategic Research (SSF) grant no. 21-0075 awarded to Prof. Lisbeth Olsson. The PhD studies were carried out between August 2022 and October 2026 under the supervision of Prof. Cecilia Geijer and the co-supervision of Prof. Lisbeth Olsson at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology; Kim Olofsson, PhD, and Ariana Causevic, PhD, in the R&D team at AAK AB; Majid Haddad Momeni, PhD, as external co-supervisor, and Prof. Carl Johan Franzén as examiner.

All the work in this thesis was carried out at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology. The analytical work has been carried out at Chalmers Mass Spectrometry Infrastructure (CMSI) with the help of Otto Savolainen. The characterization work has been done with the help of Sergio Morales Palomo, PhD, at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology. The metabolic engineering work has been done in collaboration with Hanna Alalam, PhD, Sergio Morales Palomo, PhD, and Manuel Francisco Blanco Roldán, PhD at the Division of Industrial Biotechnology at the Department of Life Sciences at Chalmers University of Technology.

Veronica Bonzanini,
October 2026

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Abstract

Odd-chain fatty acids (OCFAs) are high-value lipids with emerging medical and nutritional applications. However, their natural abundance is low, and conventional extraction or chemical synthesis methods are inefficient or unsustainable. Microbial fermentation using oleaginous yeasts offers a promising alternative, particularly when employing short-chain fatty acids digestates rich in propionic acid, the main precursor for OCFA synthesis.

In this thesis work yeast-based OCFA production has been explored by improving quantitative analytical methods, identifying suitable yeast cell factories, characterizing their performance, and initiating strain engineering efforts. First, a fatty acid methyl ester protocol was optimized for robust OCFA analysis. Second, eight strains capable of growth on 15 g/L propionic acid and production of OCFA were identified in a screening of nineteen oleaginous yeasts. Third, four yeast species were selected for further study: *Cutaneotrichosporon oleaginosus* for its high OCFA titers, *Rhodotorula toruloides* for its high OCFA yield, *Yarrowia lipolytica* for its high tolerance to propionic acid, and *Blastobotrys adeninivorans* for its ability to produce OCFAs, grow well in propionic acid and as a relatively underexplored yeast. Cultivation on mixtures of acetic and propionic acids at different carbon-to-nitrogen ratios revealed distinct and interesting performance characteristics for OCFA production in each yeast species. Fourth, a plasmid-based CRISPR-Cas9 metabolic engineering platform was established for *B. adeninivorans* through the identification of native autonomous replication systems, enabling the generation of mutants with enhanced OCFA content.

Overall, this work advances methodologies for fatty acid analysis, identifies promising oleaginous yeasts for OCFA production and characterize them, and establishes a new genetic engineering tool for strain development. These results provide a foundation for the development of efficient yeast cell factories and economically viable bioprocesses for sustainable OCFA production.

Keywords: odd-chain fatty acids, gas chromatography-mass spectrometry, fatty acid methyl ester, short chain fatty acids, oleaginous yeast, microbial oil, triacylglycerol, propionic acid

List of Publications

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

- I. Bonzanini Veronica, Savolainen Otto, Palomo Sergio Morales, Haddad Momeni Majid, Olsson Lisbeth and Geijer Cecilia. Optimization of a fatty acid methyl ester protocol for quantification of odd- and even-chain fatty acids in yeast. AMB Expr 16, 23 (2026). <https://doi.org/10.1186/s13568-026-02022-8>
- II. Bonzanini Veronica, Haddad Momeni Majid, Olofsson Kim, Olsson Lisbeth and Geijer Cecilia (2025). Impact of glucose and propionic acid on even and odd chain fatty acid profiles of oleaginous yeasts. BMC microbiology, 25(1), 79. <https://doi.org/10.1186/s12866-025-03788-w>
- III. Bonzanini Veronica*, Palomo Sergio Morales*, Causevic Ariana, Haddad Momeni Majid, Olsson Lisbeth and Cecilia Geijer. *Cutaneotrichosporon oleaginosus* outperforms other oleaginous yeasts in producing odd-chain fatty acids from acetic and propionic acid. (*Manuscript*)
- IV. Alalam Hanna*, Palomo Sergio Morales*, Bonzanini Veronica, Blanco Roldán Manuel Francisco, Causevic Ariana, Olsson Lisbeth, Haddad Momeni Majid and Cecilia Geijer. A plasmid-based CRISPR–Cas9 platform for metabolic engineering in the oleaginous yeast *Blastobotrys adeninivorans*. (*Manuscript*)

* The authors contributed equally

Contribution summary

- I. I designed and conducted the experiments, analyzed the data, and contributed to the interpretation of the results. I wrote the initial draft of the manuscript with support from CG and contributed to its revision with my co-authors.
- II. I planned and performed the experiments, analyzed the data, and contributed to the interpretation of the results. Together with CG and OS, I took a leading role in writing the first draft of the manuscript and participated in its revision alongside the other authors.
- III. I have planned, carried out the experiments and analyzed the data together with SMP. I have contributed to the interpretation of the results and took lead together with CG and SMP in writing the initial draft of the manuscript. I edited the manuscript together with the other authors.
- IV. I contributed to mutant generation, lipid extraction, FAME analysis, and metabolic map construction. I analyzed phenotyping data with SMP and HA, contributed to result interpretation, and supported manuscript editing with the other authors.

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* Reference to “Lord of The Rings” by J.R.R. Tolkien: “Three Rings for the Elven-kings under the sky, Seven for the Dwarf-lords in their halls of stone, Nine for Mortal Men doomed to die, One for the Dark Lord on his dark throne. In the Land of Mordor where the Shadows lie. *One Ring to rule them all*, One Ring to find them, One Ring to bring them all and in the darkness bind them. In the Land of Mordor where the Shadows lie.”

** Reference to a song named “Toxicity” by System of a Down, written by Daron Malakian, Shavo Odadjian and Serj Tankian: “...Eating seeds as a pastime activity, *The toxicity of our city, of our city...*”

Abbreviations

CDW	Cell Dry Weight
C/N	Carbon to Nitrogen ratio
dIS	deuterated Internal Standard
ECFA	Even Chain Fatty Acid
FA	Fatty Acid
FAME	Fatty Acid Methyl Ester
GC-MS	Gas Chromatography Mass Spectrometry
HAc	Acetic Acid
HPr	Propionic Acid
IS	Internal Standard
LCFA	Long Chain Fatty Acid
MCC	Methyl Citrate Cycle
MCFA	Medium Chain Fatty Acid
MUFA	Monounsaturated Fatty Acid
OCFA	Odd Chain Fatty Acid
OY	Oleaginous Yeast
PPP	Pentose Phosphate Pathway
PUFA	Polyunsaturated Fatty Acid
SCFA	Short Chain Fatty Acid
SCO	Single Cell Oil
TRY	Titer, Rate, Yield
VLCFA	Very Long Chain Fatty Acid

Chapter 1 - Introduction and Aim

1

Curiosity is defined by the Cambridge Dictionary as “an eager wish to know or learn about something”. In all its facets, curiosity is probably one of the fundamental characteristics of the animal kingdom and beyond. It’s an innate and powerful driver of discovery, closely linked to survival strategies and the acquisition of new knowledge, and therefore one of the catalysts of evolution. The current world has been built upon the accrued knowledge of countless generations of inquisitive and courageous individuals who dared to explore the unknown. For instance, our ancestors knew that leaving milled grains or smashed fruits in water for several hours could generate delicious bread or a tasty and inebriating drink, but they couldn’t explain how. Today we know that yeast is the main player of this ancient biotechnological process, which was named **fermentation** by Louis Pasteur. Fermentation has been utilized to enhance foods’ phytochemical compositions and functional benefits to humans since the dawn of civilization (1).

As time goes by, curiosity is more often accompanied by the need to search for alternatives. The modern world is largely built upon linear production systems that generate waste and place pressure on the environment, calling for sustainability and the transition toward a circular economy. Biotechnology, as an applied science, strives to develop and produce valuable bioproducts from raw material through sustainable processes that often harness microorganisms (2, 3). **Yeasts** have proven to be a mighty biotechnological platform with hundreds of assorted capabilities. However, to adequately exploit them as cell factories, deeper characterization is needed to expand the biological understanding, fill the knowledge gaps, and unlock their full potential (4, 5). Much of the research effort over the past century has focused on *Saccharomyces cerevisiae*, which is widely regarded as a model yeast. However, approximately another 2000 yeast species have been described to date, collectively referred to as “non-conventional yeasts” (4, 6). Just imagine how many hidden capabilities remain to be discovered! Thus, substantial academic and industrial research efforts, as well as this thesis, are directed towards non-conventional yeast screening, characterization and development to identify superior hosts for both classical and precision fermentation, to produce high value or commodity bioproducts. Among these non-conventional yeasts are the so-called **oleaginous yeasts (OYs)**, which are capable of accumulating large amounts of intracellular lipids under specific cultivation conditions. This characteristic makes them particularly attractive hosts for the production of a wide range of lipid-based compounds. OY definition, application and metabolism are further explored in *Chapter 2* and *Chapter 4*.

Lipids constitute a major target product in biotechnology, given their wide application in food and nutrition, pharma, chemical and fuel industries (7). The growing need for sustainable

alternatives for food and fuel has caused intensified interest in the exploration of OYs, which, beyond biofuel production, can produce a wide range of **high-value lipids** from diverse substrates. Their remarkable metabolic versatility makes OYs attractive platforms for the sustainable production of novel and high-value lipids with applications in medicine, nutrition, and cosmetics. These products may not be essential for human survival, but they can nevertheless contribute to improved health and quality of life.

This thesis is built around the concept of producing **odd-chain fatty acids (OCFAs)** using OYs. These relatively uncommon lipid compounds have recently attracted increasing attention as biologically active molecules with potential nutritional and health-promoting properties, including possible protective effects against the development of certain metabolic and cardiovascular diseases (8). However, OCFAs are relatively rare in nature, making their extraction from plant and animal sources challenging and potentially associated with high economic and environmental costs. Microbial production using OYs could therefore help overcome some of these limitations and provide a sustainable platform for producing nutritional or potentially medicinal oils enriched in beneficial OCFAs. Lipids and OCFAs are explored in *Chapter 2*.

One of the main constraints in fermentation processes is the availability and cost of the carbon substrate supplied to the microorganism (9), which is subsequently converted into the desired bioproduct. Since the carbon source often represents a major contributor to overall bioprocess costs, considerable research has focused on identifying alternative, low-cost substrates that are also compatible with circular economy principles. This includes the valorization of carbon-rich waste streams from other production processes, such as lignocellulosic biomass, sludges, and organic waste. Biobased **short-chain fatty acids (SCFAs)**, produced through the anaerobic fermentation of various waste biomasses, have been proposed as a promising substrate platform for OCFA production by OYs, as they can contain **propionic acid (HPr)**, an important precursor for OCFA biosynthesis (10). However, SCFAs represent challenging carbon sources because, as weak organic acids, they can inhibit yeast growth, and relatively little is known about how OYs metabolize these substrates for growth and lipid accumulation (11, 12). Therefore, a substantial part of this thesis was dedicated to understanding the utilization of SCFAs by OYs, as well as screening and characterizing yeast species capable of using these substrates for OCFA production. Characteristics of SCFAs and their effect on OYs are explored in *Chapter 2*, *Chapter 4* and *Chapter 5*.

To characterize the lipids produced from both qualitative and quantitative perspectives, a robust **analytical platform** and methodology is essential. Because OCFAs have historically been largely overlooked, limited analyses have been done on these FAs. OCFAs have often been used as internal standards (ISs), as they are usually present only at trace levels and seem to remain stable across different sample types (13). However, because OCFAs represent the main

analytes of interest in this thesis, it was essential to identify a suitable IS that would enable the simultaneous analysis of both OCFAs and ECFAs. Therefore, a substantial part of this work was dedicated to adapting and optimizing an existing **fatty acid (FA)** analytical method for the analysis of OCFAs and ECFAs.

1.1 Aim and overview: screening, characterization and development

Fine-tuning the production of a specific lipid requires microbial hosts harboring good potential to produce the targeted lipid and that can be engineered for further improvement. Furthermore, the selection and optimization of the growth conditions, and the bioprocess supported by a strong analytical platform are required to enhance productivity and improve economic feasibility.

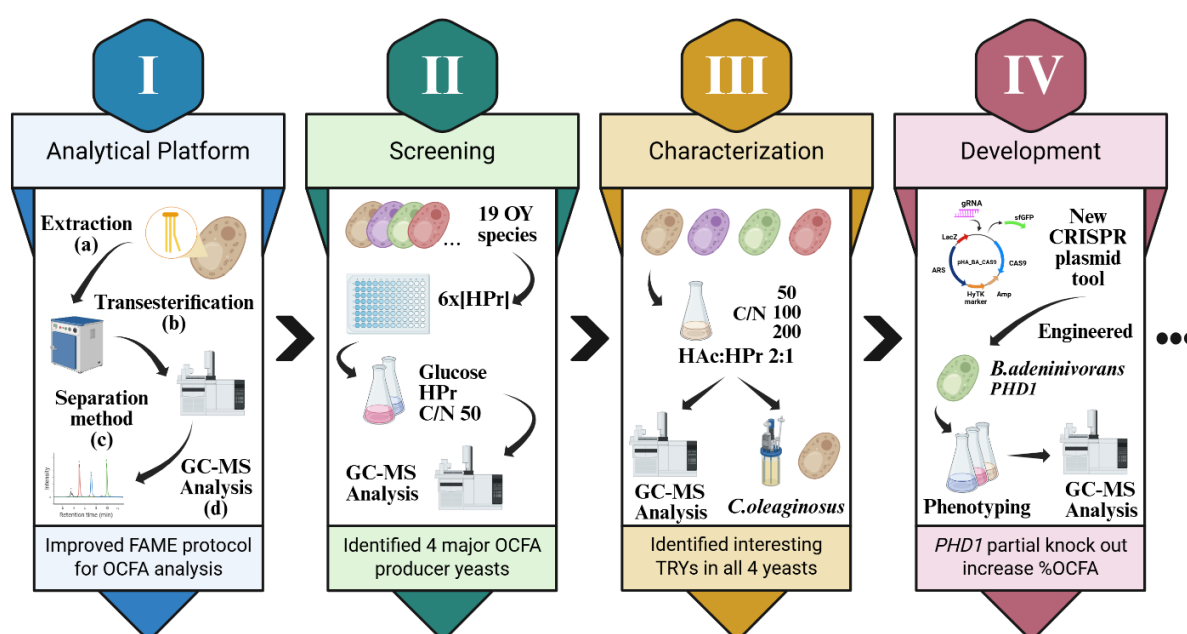


Figure 1. Summary of the work presented in this thesis. In paper **I**, an existing FAME protocol was improved for better TAG extraction and transesterification. In paper **II**, 19 different OY species were screened for growth under six different HPr concentrations and their FA profile in glucose and HPr has been analyzed. Four yeasts have been selected for further investigation. In paper **III**, these four yeasts were further characterized in HAC:HPr 2:1 at three different C/N ratios (50, 100, 200). *C. oleaginosus* was also cultivated in bioreactor batch mode for deeper characterization. In paper **IV**, a new CRISPR tool to engineer *B. adenivorans* was developed and PHD1 gene was successfully disrupted. The mutants were analyzed for growth and FA profiles in HAC:HPr medium.

Chapter 1 - Introduction and Aim

The overall **aim** of this thesis was to contribute to the growing knowledge on microbial OCFAs by *screening* and *characterizing* non-conventional OYs for their ability to grow on SCFAs under different carbon-to-nitrogen (C/N) ratio conditions and to synthesize OCFAs. This knowledge was then directly applied to the *development* of a proof-of-concept microbial cell factory for OCFA production. This thesis comprises four scientific original papers (summarized in Figure 1), and several research questions were proposed and addressed as follows:

How can an existing fatty acid methyl ester (FAME) protocol be adapted and optimized for OCFA analysis?

To establish a robust analytical platform for OCFA analysis and ensure reliable yeast characterization, an existing **FAME** protocol was identified. The protocol was adapted for OCFA analysis by selecting an appropriate IS, and the yeast lipid extraction and derivatization procedures were further optimized. Because the FA profiles of the selected OY were initially unknown, **gas chromatography–mass spectrometry (GC-MS)** was identified as the most suitable analytical technique. A GC oven temperature program was subsequently developed and optimized to ensure adequate separation of the target FA peaks. This work is presented in Paper **I** and *Chapter 3* of this thesis.

Are there any yeast species that naturally produce high levels of OCFAs when cultivated on glucose? Are there yeasts that can tolerate high concentrations of HPr and also produce OCFAs? How does the substrate influence the FA profile?

To uncover the hidden potential of emerging and less-characterized OYs, the scientific literature was thoroughly surveyed, leading to the identification of **19 OYs**, including both known and previously uncharacterized OCFA producers. Given the challenging nature of SCFAs as carbon sources, the selected OYs were first **screened** for growth performance across six different HPr concentrations. To further expand the screening, the OYs were cultivated in shake flasks using either glucose or HPr as the carbon source at a high C/N ratio, enabling comparison of their FA profiles by GC-MS. This screening identified four superior yeast species capable of growing on HPr while producing OCFAs: *Cutaneotrichosporon oleaginosus*, *Rhodotorula toruloides*, *Yarrowia lipolytica*, and *Blastobotrys adeninivorans*. This work is presented in Paper **II** and *Chapter 4* of this thesis.

Will a mixture of acetic acid (HAc) and HPr improve the OCFA titers and yields? Does the C/N ratio influence OCFA production?

To address these research questions, the four identified OYs were further **characterized** to gain a deeper understanding of their metabolism and production capabilities. The yeasts were cultivated in a mixture of HAc and HPr at a 2:1 ratio, while three different C/N ratios (50, 100, and 200) were evaluated to investigate the conditions supporting OCFA and biomass

production. All four OYs demonstrated interesting characteristics for OCFA production. *C. oleaginosus* showed to be the best yeast at consuming HPr and producing OCFA, hence has been cultivated in bioreactor batch mode for deeper characterization. This work is presented in Paper **III** and *Chapter 5* of this thesis.

Will disruption of PHD1 gene enhance OCFA accumulation in B. adenivorans?

Initially, a novel **CRISPR-Cas9 plasmid-based tool** containing a native autonomous replication sequence (ARS) was developed to enable genome editing in *B. adenivorans*. Using this tool, the *PHD1* gene, involved in the methyl citrate cycle (MCC), was successfully subjected to disruption (i.e. insertion/deletion of one or two nucleotides). The resulting mutants were subsequently phenotyped for total OCFA production during growth on HAc:HPr. This work is presented in Paper **IV** and *Chapter 6* of this thesis.

Chapter 1 - Introduction and Aim

Chapter 2 – Background Knowledge

As the final aim of this work is to produce nutritional FA from microbial sources, it is pertinent to give an overview of this type of lipid and the main correlated biological activities. Therefore, the aim of this introductory chapter is to provide the essential vocabulary and conceptual framework needed to understand the field of **microbial oil** and **odd-chain fatty acids**.

2.1 Fats: Structure, Classification and Health Implications

Lipids are a fundamental and structurally diverse class of macronutrients that serve as energy sources, structural components of cell membranes, and signaling molecules. In addition, certain lipids possess bioactive properties and may contribute to the prevention and management of chronic diseases by modulating processes such as inflammation, immune responses, and metabolism (14-17). Lipids are defined as small amphiphilic molecules that may originate entirely or in part through condensations of thioesters and/or isoprene units (18, 19). They are divided into eight main categories which are listed in Figure 2 (18, 20). Fatty acyls comprise FA and their functional derivatives which serve as one of the primary building blocks of glycerolipids as shown in Figure 2.

2.1.1 Fatty Acids

FAs are constituted by a hydrophobic fatty acyl group chain of different lengths, characterized by a repeating series of methylene groups $-(CH_2)_nCH_3$, and by a hydrophilic carboxylic group $(-COOH)$ at one end (Figure 2) (20). FAs can be classified according to the length of the acyl chain, the amount of double bonds on the acyl chain and the nature and positioning of the double bonds as shown in Figure 2 (21). For simplicity, throughout the text, the shorthand notation and the common (or trivial) name of FAs will be used (see Table 1).

The most common FAs in nature have an even number of carbons, representing about 95-100% of the total FA profiles, while OCFAs are present in small amounts, usually constituting 3-5% of total FAs (13, 22). The common chain lengths in animals and plants FAs profiles range from 12 to 22 carbons, but shorter or longer FAs may also occur naturally depending on the organism species (21). Most FAs naturally occur in the *cis* configuration (21). The structural differences illustrated in Figure 2 play a crucial role in determining the physical properties of the FAs, which in turn define biological activities, oxidative stability and, for example, also food physiochemical characteristics. Bioactive FAs possess numerous health benefits, like prevention of certain chronic diseases and improvement of metabolic health (15). Some FAs are considered essential FAs, i.e. nutrients which cannot be synthesized by the body or that are synthesized in too small amounts and must be obtained through the diet because of their fundamental role in human health (21).

Table 1. List of some relevant FAs.

Trivial or IUPAC name	Shorthand notation
OCFA	
Propionic Acid	C3:0
Pentadecanoic acid	C15:0
Pentadecenoic acid	C15:1 n-5
Margaric acid	C17:0
Heptadecenoic acid	C17:1 n-7
Nonadecanoic acid	C19:0
Tricosanoic acid	C23:0
Pentacosanoic acid	C25:0
ECFA	
Acetic Acid	C2:0
Butyric acid	C4:0
Lauric acid	C12:0
Myristic acid	C14:0
Palmitic acid	C16:0
Palmitoleic acid	C16:1 n-7
Stearic acid	C18:0
Oleic acid	C18:1 n-9
Linoleic acid	C18:2 n-6
α -Linolenic acid	C18:3 n-3
γ -linoleic acid	C18:3 n-6
Eicosanoic acid	C20:0
Arachidonic acid	C20:4 n-6
Eicosapentaenoic acid (EPA)	C20:5 n-3
Lignoceric acid	C24:0
Docosahexaenoic acid (DHA)	C22:6 n-3
Nervonic acid	C24:1 n-9

2.1.2 Odd-Chain Fatty Acids are “Odd” No More

Beyond the abundant and well-characterized even-chain fatty acids (ECFAs), OCFAs have recently attracted interest in the medical and nutritional fields as bioactive FAs whose physiological roles have long been overlooked. ECFAs represent >99% of the total FA plasma concentration in humans (13). The detectable OCFAs in human plasma or tissues, are predominantly represented by pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), heptadecenoic acid (C17:1), and tricosanoic acid (C23:0) (13).

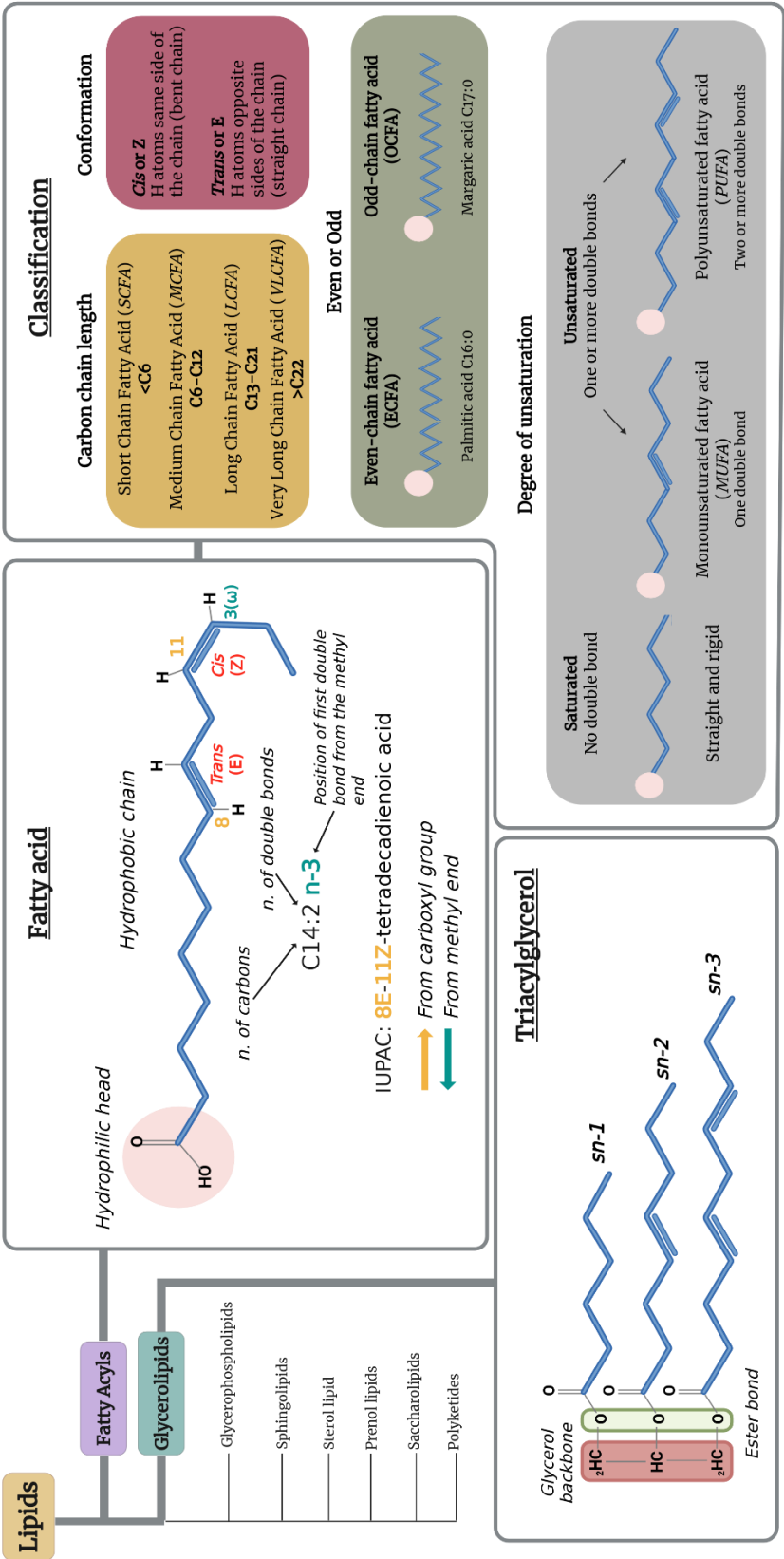


Figure 2. Comprehensive illustration of FA and TAG structures and different classification parameters of FAs. The FA in the “Fatty Acid” frame has exclusively educational purpose as this structure is invented. The figure has been generated using BioRender.com.

Given their low abundance, OCFAs have historically been considered biologically irrelevant FAs and were therefore commonly used as ISs for FA analysis, based on the assumption that their concentrations were consistently low and relatively uniform across biological samples (13). Moreover, they have incorrectly been regarded as exclusive biomarkers of dairy fat intake for the monitoring of cardiovascular diseases with the assumption that they were not endogenously produced or metabolized. In fact, the main diet source of OCFAs is ruminant milk (cow, sheep, goat, yak (23)) and its derivative products, with about 1-2% of C15:0 and 0.5-1% of C17:0 (typical 2:1 ratio) in the whole fat milk (13, 24). However, it is nowadays known that other diet sources and endogenous metabolism can mildly alter the OCFA circulating concentrations. OCFAs are also present in some types of meat and fish oils, and they can be produced endogenously from fermentable fibers, dietary propionate and branched-chain amino acids (e.g. methionine, valine, isoleucine, and threonine). These amino acids are absorbed and metabolized by the liver, and possibly also produced by α -oxidation and β -oxidation chain-shortening of 2-hydroxylated FAs or very long chain fatty acids (VLCFAs) present in the brain sphingolipids (8, 25, 26).

Given the high concentration of saturated ECFAs in whole fat milk (about 68%) and the rising evidence of the negative effect of saturated FAs on human health (reviewed in Bonus 1), dietary recommendations over the past 50 years have consistently advised reducing the intake of dairy and other general sources of saturated FAs. As a result, the average consumption of whole fat milk has decreased, which in turn might have led to a decrease in concentration of OCFAs in the human plasma (24, 27).

A growing body of evidence from observational studies and experimental research *in vitro* and *in vivo* indicates that OCFAs have biological relevance distinct from that of other saturated FAs. These FAs have likely been overlooked for decades due to their routine classification within the broader saturated FA group. In fact, saturated OCFAs (i.e. C15:0, C17:0), have been associated with a lower risk to develop adiposity, chronic inflammation, cardiovascular diseases, type 2 diabetes, non-alcoholic fatty liver disease, and metabolic syndrome (24, 28-33). Additionally, they seem to inhibit proliferation of certain cancers and have mitochondrial-protective effects among other potential benefits (see Table 2 for a summary of relevant studies) (24, 28-33). OCFAs have gained special interest when the evidence from different unrelated observational studies shared similar conclusive results on the correlation between a lower concentration of plasma OCFAs and a higher risk of developing type 2 diabetes and coronary heart disease, suggesting that **OCFAs might have protective effects** (30-35). In the past 5 years, some medical research, utilizing mammalian cell lines or mouse disease models, concluded that C15:0 has anti-inflammatory, metabolic, antifibrotic, mitochondrial-protective, immunomodulatory, anticancer and pro-longevity effects, and they propose this specific OCFA as a new essential FA (24, 29). However, little is known about the possible mechanisms underlying OCFAs bioactivity.

In conclusion, OCFAs are receiving increasing medical and nutritional attention because of their potential health benefits, highlighting the need for further clinical investigations. As interest in these FAs continues to grow, there may be an increasing demand for OCFA-rich oils with uses in nutrition and medical fields. Consequently, companies interested in producing OCFAs are beginning to explore alternative production methods, as extraction from natural sources is generally inefficient and costly. A promising biotechnological alternative is the production of OCFAs using microbes such as OYs, as further explored in *paragraph 1.2* of this chapter, *Chapter 4* and *5*.

Table 2. Summary of some of the relevant medical OCFA research.

Fatty acid	Beneficial effects	Type of study	Year	Ref.
C15:0	Anti-inflammatory, metabolic, antifibrotic, and mitochondrial-protective effects.	Research <i>in vivo</i> and <i>in vitro</i>	2020	(24)
	Anti-inflammatory, immunomodulatory, antifibrotic, and antiproliferative activities. Overlapping and even broader beneficial effects than EPA.	Research <i>in vitro</i>	2022	(29)
	Promote longevity, anti-inflammatory, anticancer, antifibrotic, antimicrobial and mTOR-inhibiting activity. Similarity to rapamycin and metformin.	Research <i>in vitro</i>	2023	(28)
	May enhance treatment of breast cancer cells.	Research <i>in vitro</i>	2022	(36)
	Strongly inhibit cancer cell proliferation.	Research <i>in vitro</i>	2021	(37)
	Inverse association with CVD and CHD.	Cohort	2013	(33)
	Inverse association with T2D.	Prospective and cross-sectional	2014	(34)
	Supplementation improved clinical indices in obese young adults.	RCT	2024	(38)
C17:0	Effective agent against non-small cell lung carcinomas.	Research <i>in vitro</i>	2019	(39)
Both saturated OCFAs	Protective against T2D (higher effect from C17:0).	Case-cohort	2021	(30)
	Inverse association with T2D.	Case-cohort	2014	(31)
	Associated with a favorable serum adipokine profile.	Cross-sectional	2017	(40)
	Inverse association with CHD.	Prospective cohort	2012	(32)
C17:1	Suggested for treating psoriasis and allergies.	Patent	1998	(41)

CVD, cardiovascular diseases; CHD, coronary heart disease; T2D, type 2 diabetes; RCT, randomized control trial.

BONUS 1 - The Story of the “Evil” Fats

In the late twentieth century, growing evidence linking diet and health, together with the recognition of obesity as a risk factor for mortality, led health institutions to recommend reducing dietary fat, particularly saturated FAs, while increasing carbohydrate intake (42-46). These recommendations emerged amid debate over the causes of coronary heart disease, with hypotheses focusing primarily on saturated FAs or refined carbohydrates and sugars (42). At the time neither of the two hypotheses were supported by sufficient evidence. Some randomized control trials and cohort studies pointed out that an increased intake of saturated FAs increases the blood total cholesterol level, thus, increasing the risk of developing coronary heart disease (42). However, subsequent evidence indicated that the relationship between saturated FAs and coronary heart disease is more complex. Replacing saturated FAs with refined carbohydrates had little benefit, whereas replacing them with whole grains or polyunsaturated FAs (PUFAs) and monounsaturated FAs (MUFAs) was associated with reduced coronary heart disease risk and mortality. Conversely, *trans* fats, initially considered a healthier alternative to saturated FAs, were later shown to have detrimental effects on health (15, 47-49). Notably, despite reductions in saturated FA consumption, the prevalence of obesity, type 2 diabetes, cardiovascular disease, metabolic syndrome, and non-alcoholic fatty liver disease has continued to increase (24).

These observations highlight the complexity of non-communicable diseases, which are influenced not only by individual nutrients but also by dietary patterns, food matrices, genetics, and lifestyle. Consequently, the health effects of individual FAs should be interpreted cautiously, particularly given the reductionist nature of many nutritional studies (50). Recent evidence further indicates that saturated FA chain length, *sn* position within TAGs, and dietary sources, can substantially influence their biological effects (14, 51, 52). Moreover, restricting dietary fat may compromise the intake of essential FAs and fat-soluble vitamins (53). Importantly, the potential beneficial effects of saturated OCFAs compared with the adverse effects associated with some saturated ECFAs further emphasize that saturated FAs should not necessarily be considered as a homogeneous group. Thus, it is fundamental to understand that it is too simplistic to look at the matter as a two-faced coin; it is rather a twenty-faced dice. Overall, dietary recommendations should therefore consider the quality, source, and structural characteristics of dietary fats rather than advocating a blind reduction of all saturated FAs.

2.1.3 Triacylglycerols

The glycerolipid category is constituted by glycerol-containing lipids. Glycerol (C₃H₈O₃) is an alkane triol made of three carbons with one hydroxyl group (-OH) each (Figure 2). Glycerol can be esterified with one, two, or three FAs, forming monoacylglycerols, diacylglycerols, and **triacylglycerols** (TAGs), respectively. Each carbon can have a stereochemical distinct FA bond, and the position is defined by a stereospecific numbering (*sn*) system as *sn*-1, *sn*-2 and *sn*-3.

In nature, TAGs are the most common while monoacylglycerols and diacylglycerols occur in minor amounts (21). TAGs are a class of neutral and non-polar lipids which can't dissolve in water and dissolve only in non-polar organic solvents like chloroform, hexane or acetone (54). TAGs are the primary form in which nature stores FAs, serving as the major energy reserve in many living organisms including yeasts, and they constitute the primary component of oils. The main dietary sources of TAGs are vegetables oils, fish oils, nuts, meats and processed sweets and cookies (15).

2.2 The Rise of Microbial Oils in Food Industry

The current food system is going through a severe crisis, with many sectors struggling with intensive exploitation of land, neglected animal welfare, massive water consumption, accelerating climate change and climate disasters, increase of demand, use of harmful chemicals (pesticides, antibiotics), challenges in sourcing of raw materials, and ethical concerns. Among these sectors, vegetable oil production systems, particularly those based on tropical oil crops, exemplify many of these challenges and have become a focal point of sustainability debates. Furthermore, the current unstable world order and the massive climate changes have made us realize that the world oil supply chain could be disrupted at any moment. This underscores the urgent need for a fundamental transformation of the oil supply chain and production system.

Microbes (such as bacteria, yeasts, filamentous fungi and microalgae) have already proved to be sustainable platforms for the production of novel oils. Microbes do not directly exploit land and are not directly impacted by the local climate; they have low greenhouse gas emissions and can use different biological waste feedstocks to improve resource efficiency. In addition, they promote biodiversity conservation and possess high nutritional profiles with similar FA compositions as vegetable oils (55). Microbial biotechnologists and visionary companies are increasingly leveraging advances in genetic engineering and precision fermentation to develop sustainable microbial oils, which can potentially revolutionize the current vegetable oil supply system. However, microbial oil fermentation has its own challenges to overcome. Public perception remains largely negative, competition with the conventional vegetable oil market is intense, strain improvement is slow and technically demanding, production costs remain high due to difficulties in industrial-scale upscaling, and regulatory requirements are stringent (1, 14). Therefore, further research is necessary to reduce production costs and some efforts are needed to shift public perception.

The historical interest in microbial oil, commonly referred to as **Single Cell Oil (SCO)**, is mirrored by the substantial number of scientific articles which doubled each decade from the 1960s to the 1990s (56). The first commercial SCO dates back to 1985. It was an oil rich in γ -linolenic acid (C18:3 ω 6) aimed to substitute for evening primrose oil, produced by the fungus

Mucor circinelloides. However, SCO became more successful on the market at the turn of the century for production of docosahexaenoic acid, supplemented into infant formula (57). Some SCO-producing microorganisms have been granted Qualified Presumption of Safety (QPS) status by EFSA (European Food Safety Authority), recognized as Generally Recognized as Safe (GRAS) by the FDA (Food and Drug Administration), and approved for use in novel foods within the European Union (56). Some companies currently produce SCOs for production of specialty lipids (mainly ω 3 FAs such as docosahexaenoic and eicosapentaenoic acid) for applications such as food supplements, functional food, feed, personal care and biodiesel. The SCO market size was valued at 79.26 million USD in 2025 and projected to grow in parallel with the exponential decrease of process costs, with DSM Firmenich, Cellana, and Corbion N.V. being the largest players (1, 58). Interestingly, Europe has been at the forefront of innovation, earning 44% of the global market in 2025 (58). Nevertheless, the SCO market is just at the start of its time and tremendous efforts are still needed to screen, characterize and develop new SCO factories to produce sustainable specialty lipids and supplant the old food system.

BONUS 2 – The Palm Oil Conundrum

In the past 20 years, given the population increase, the rise of the biofuel sector and the waves of new diet recommendations, vegetable oil demand increased tremendously. Vegetable oils are generally considered a healthier source of dietary fat than animal fats because of their richness in MUFAs and/or PUFAs, as well as other bioactive compounds (59). Among vegetable oils, palm oil accounts for more than 35% of global production and has the highest oil yield per hectare, making it economically attractive, more land-efficient than other oil crops and has brought millions of people out of poverty (60, 61). Nevertheless, its intensive cultivation is associated with deforestation, biodiversity loss, and also significant ethical concerns regarding labor conditions (62). Palm kernel oil also contains a high proportion of saturated FAs, although recent evidence suggests that the structural arrangement of these FAs in palm oil may mitigate some cardiovascular risks (52).

The palm oil debate therefore illustrates the complexity of balancing environmental, economic, and social considerations. Indeed, the International Union for Conservation of Nature (IUCN) has cautioned that simply boycotting palm oil could shift production to less land-efficient crops, potentially increasing overall land use and environmental pressure (62). This raises a broader question: can alternative production systems reduce the environmental and social burden of vegetable oil production without creating new problems elsewhere? Microbial single-cell oils (SCOs) could provide part of the solution, particularly if technological advances and appropriate investments enable their production at an industrial scale comparable to that of conventional oil crops.

2.2.1 Oleaginous Yeasts as Chassis for Lipid Production

For exclusive biotechnological classification, microbes capable of accumulating at least 20% (w/w) of their dry cell mass as intracellular lipids are defined as oleaginous (6, 63, 64). However, it is important to emphasize that this is merely a human-defined classification, as no other clear criteria have been established to define an oleaginous microbe. The classification therefore becomes ambiguous for yeasts that, for instance, accumulate 19% (w/w) lipids. Accordingly, this definition should be regarded as a general guideline rather than a strict boundary.

Among different oleaginous microbes that can be used to produce SCOs, OYs represent a substantial group. Of all the known yeast species, approximately 8.2% have been reported to contain oleaginous strains (data up to 2022) (65). The first OY, which appear to produce an oil droplet, was identified microscopically in 1895 and it is currently known as *Metschnikowia pulcherrima* (6). Interestingly, the oleaginous phenotype is not heritage of one species and one single evolution event. Instead, it is ubiquitous among clades and genera (e.g. *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon*, *Lipomyces*, etc. (66)), with members in both the Ascomycota and Basidiomycota phyla, suggesting that it is a relatively common evolutionary strategy in response to certain environmental conditions. However, OYs have been isolated from diverse types of locations like plants, soil, and marine environments (6). The common trait that triggers yeast lipid-accumulation is the response to the limitation of key elemental nutrients like nitrogen or phosphorus in the surrounding, thus driving excess carbon towards storage, as a form of chemical energy (66). The lipid biosynthetic pathway (explored in detail in *Chapter 4*) is generally conserved among the most well-characterized yeasts. However, certain key enzymes appear to play a more prominent role in OYs (see *Chapter 4*) and may be absent or differentially expressed in non-oleaginous yeasts such as *S. cerevisiae* (64).

OY lipid profiles mainly consist of storage lipids like TAGs and steryl esters (usually at ratio 10:1), which, given their hydrophobic nature, are contained in lipid bodies in the cytoplasm (62). Their FA composition, from storage and membrane lipids, range from medium chain FAs (MCFAs) to long chain FAs (LCFAs) and in some cases also VLCFAs. The most common yeast FAs are C18:0, C18:1, C18:2 and C16:0 with strong similarities with several vegetable fats (e.g. cocoa butter or palm oil), including several nutritional FAs (e.g. γ -linolenic acid and other PUFAs) displaying potential as nutritional oil (64). Nevertheless, the lipid profiles and contents vary a lot even between strains. Other than the valuable lipid profiles, OYs are perfect candidates for SCO production as they harbor some key industrial characteristics:

- i. relative fast doubling time (on average 1.5 - 4 hours, dependent on strain and condition);
- ii. high lipid content (up to 70-80% w/w depending on strain and condition) (6, 64);

- iii. unicellular morphology (easy to cultivate in large-scale bioreactors);
- iv. ability to metabolize a variety of low-cost substrates (e.g. single saccharides, hydrolysates, glycerol, SCFAs, waste waters, oils/fats, and molasses/syrups (6, 67));
- v. some can grow at rather low pH, high osmotic pressure and other relatively harsh fermentation conditions (68);

Furthermore, many of the declared OYs have been fully sequenced and the lipid-biosynthesis pathways are well characterized, making genetic manipulation approachable (6). Considering the substantial advances in lipid metabolic engineering in recent years, significant opportunities lie ahead (69). To date, thousands of research articles have been published on OYs with *Yarrowia lipolytica*, *Rhodotorula toruloides* and *Cutanetrichosporon oleaginosus* being the most researched so far (as detailed in Chapter 5). Some OYs have been successfully engineered to produce diverse bioproducts (e.g. fuels, lubricants, polymers, plastics, surfactants and nutritional fats (67)). However, most of these strains remain cultivated on laboratory scale due to scale-up constraints. Even so, given their remarkable metabolic capabilities and the wide range of bioproducts they can produce, it remains essential to identify novel OY strains and further characterize known species for the utilization of new substrates and the production of new and novel compounds, ultimately developing new bioprocesses.

2.3 Recycled Biobased Substrates for Microbial Oil Production

Given the current climate change problems, the waste disposal issues and the awareness that the present linear economic system is not sustainable, with enormous amounts of carbon and energy wasted, the concept of circular economy has emerged. This concept is shaped around a sustainability core promoting resources recycling, reuse and remanufacture. The circular bioeconomy branched from the original concept to cover the utilization of renewable carbon sources (e.g. wasted biomasses) to generate high-value bio-based products such as food, feed, biochemicals, and bioenergy (70, 71). One of the main tools of bioeconomy is the biorefinery, i.e. a facility which converts different biomasses and biogenic waste into final or intermediate products by means of thermochemical, biochemical and combustion technologies or using microbial production platforms (70). Some of these processes break down complex organic materials into simpler components that, among other things, can serve as suitable carbon substrates for OY growth and, consequently, SCO production.

2.3.1 Short Chain Fatty Acids as Substrates for Microbial OCFA Production

The production of SCO using waste-derived substrates has attained much attention in the past years, given the possibility to reduce costs of industrial bioprocesses and align them in the circular economy strategy. A pertinent example of waste biorefinery is the conversion of complex organic substrates such as sludges, food waste, paper-mill effluents, animal manure

and agricultural residues into **SCFAs**, which in turn can be used as feedstock for OY-SCOs (72-74). Typically, SCFAs are produced synthetically from petrochemical derivatives (about 98% of global production) (9) while biobased SCFAs are an intermediate product of anaerobic digestion performed by a consortium of bacteria for the final production of biogas (CH₄ and CO₂) (72, 75). This process converts all biomasses components (carbohydrates, lipids, proteins) except lignin, into SCFAs (76). It is composed of four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, of which SCFAs are produced after the first two steps through anaerobic fermentation (74). Based on the type of converted biomass, different SCFAs profiles can be obtained. The common FAs in SCFAs range from two to six carbon atoms such as **HAc**, **HPr** and butyric acid, often at a ratio of 6:1:3 or 7:1:2, and iso-butyric, iso-valeric, valeric and caproic acids together with other macro and micronutrients and a high concentration of ammonia (73, 75, 76). The final ammonia concentration and pH of SCFA digestates vary based on the type of converted biomass and the anaerobic fermentation process parameters applied (74). Biobased SCFAs have, however, limited industrial application given some production constraints (e.g. low production efficiency, complications in purification and separation, postprocessing requirements, etc.) and competition with petroleum based SCFAs (USD 2.1 billion for HAc, 288 million for HPr, 97.5 million for butyric acid) (9). Although often considered a low-cost substrate, the production of biobased SCFAs can still be relatively expensive.

Nevertheless, SCFAs are valuable building blocks in the carboxylate platform to produce biofuels and several biochemicals. Additionally, some SCFAs represent a good substrate for OY-OCFA production. **HPr** acts as an **OCFA precursor**, which upon intracellular conversion to propionyl-CoA, can be used as carbon and energy source or feed the lipid pathway to generate mainly OCFA (OCFA production pathway is explained in detail in *Chapter 4*). However, as carboxylic acids, SCFAs are weak acids and some, like HPr, often affect OYs growth negatively (11, 77). Several studies have proved that low pH <4-5 strongly inhibits OYs growth, while close to neutral pH 6-7 allows OYs growth (75). Thus, cultivation pH is a determining factor for growth in SCFAs.

To date, only a few studies have reported the cultivation of OYs in real SCFA-based digestates to produce SCOs and OCFA, employing various cultivation strategies and OY species, as summarized in Table 3. These results highlight that it is possible to cultivate certain OYs in SCFA-based digestates and obtain high lipid yields. However, medium composition, pH, process mode and yeast species have huge impact on the final yields of total lipids and OCFA derived from real SCFA digestates, indicating that more research efforts are needed to be able to develop robust and scalable bioprocesses. Although cultivating yeasts on synthetic SCFAs as the sole carbon source can produce markedly different outcomes than cultivation on SCFA digestates, it provides a valuable baseline for understanding the effects of individual SCFAs before investigating more complex substrates. Therefore, it is useful to utilize these synthetic

substrates, to screen for OYs that are capable to grow and metabolize e.g. HPr other than be able to channel it towards OCFA production (HPr toxicity and OYs screening is explored in detail in *Chapter 4*).

Table 3. Some recent studies on the production of SCO and OCFAs from SCFA digestates.

Yeast	SCFAs source	Bioprocess	pH	Results	Year	Ref.*
Engineered <i>Y. lipolytica</i>	Protein rich – waste	Fed-batch with HAc	6.8	L: 1.1 g/L – 25% w/cdw OCFAs: 60% of tot. L	2025	(78)
<i>Y. lipolytica</i>	Microalgal biomass	Batch	6.5	L: 20% w/cdw – 0.08 g/g 6.4% C17:0 of tot. L	2020	(12)
<i>Cutaneotrichosporon curvatum</i>				L: 37% w/cdw – 0.13 g/g 6.8% C17:0 of tot. L		
<i>Lipomyces lipofer</i>				L: 17% w/cdw – 0.05 g/g 4.2% C17:0 of tot. L		
<i>R. toruloides</i>				L: 26% w/cdw – 0.06 g/g 3.3% C17:0 of tot. L		
<i>Cyberlindnera saturnus</i>				L: 34% w/cdw – 0.11 g/g 3% C17:0 of tot. L		
<i>Y. lipolytica</i>	Food Fruit/vegetable waste	Batch	8	L: 3.2 g/L – 22% w/cdw L: 3 g/L – 26% w/cdw	2020	(79)
<i>Y. lipolytica</i>	Food waste	Batch	6	L: 3.2 g/L – 24% w/cdw – 0.21 g/g	2022	(80)
<i>Apiotrichum brassicae</i>	Vegetable food waste	Batch	7	L: 3.40 g/L – 43% w/cdw 1.5% C15:0 – 5.2 C17:1	2020	(81)
<i>Candida tropicalis</i>				L: 1.38 g/L – 20% w/cdw 2.1 C15:0 – 15.5 C17:1		
<i>Metschnikowia pulcherrima</i>				L: 1.65 g/L – 24% w/cdw 2% C15:0 – 22% C17:1		
<i>Pichia kudriavzevii</i>				L: 3.33 g/L – 47% w/cdw 1.6% C15:0 – 4.5% C17:1		
<i>Y. lipolytica</i>	Food waste	Batch	6	L: 38% w/cdw – 0.25 g/g	2023	(82)

*Screening; only best data is reported.

Yields are all expressed as gram lipids on gram of substrate consumed.

L, lipids; tot, total; cdw, cell dry weight.

2.4 The TRYnity of Feasible Fermentation Processes

To develop a fermentation bioprocess that is economically feasible and competitive, several cost aspects need to be taken into consideration. Bioprocesses costs can vary depending on the type of process or product, as some products might require stringent and expensive quality control. However, bioprocesses often have generally high media expenses and operational costs (especially downstream processing), other than requiring a substantial initial investment for highly specialized infrastructures (83–84). There are several ways to

decrease process expenses including downstream processing optimization, implementation of renewable cheap feedstock sources, and improvement of microorganism performance (85). The latter one has a significant impact on the entire process viability. There are three important metrics that drastically influence a microbial fermentation process and its feasibility (86):

- **Titer**, the final concentration of the product at the end of the batch fermentation process (g/L, mol/L). This is an absolute metric only indirectly related to intrinsic strain performance because it is also affected by process conditions and cultivation duration. If the product titer is low, the downstream process often becomes costly (87). Titer can often be improved by optimizing the productivity and yield;
- **Rate**, the productivity of the cell factory, defined as volumetric productivity or q_p (g_p/L/h) or biomass-specific productivity or r_p (g_p/g biomass/h). The latter one defines the performance capabilities of the cell factory in certain process conditions, while the first one is more useful to evaluate the cost of a production plant (86);
- **Yield**, the ratio of substrate that is driven towards the product (g_p/g_s, mol_p/mol_s). This metric is very important in defining the substrate costs. The substrate is often partially consumed for biomass formation with an obvious trade-off with product formation. Increasing the product yield directly reduces the manufacturing costs (84), as a higher-yielding process requires less substrate to produce the same amount of product compared with a lower-yielding process.

Overall, process costs can vary considerably based on the type of product (e.g. cytotoxic product, intracellular or extracellular), final utilization (i.e. commodity chemical or specialty/fine chemical, food or pharma grade), the type of substrate (e.g. pure glucose or complex second-generation substrates) and the process yield (84, 85). In Bonus 3, two techno-economic assessment studies on the use of SCFAs for microbial biofuel production are critically reviewed, highlighting the importance of titer, rate, and yield (TRY) metrics for developing feasible bioprocesses.

This thesis stands at the starting point of a research line in which multiple OY species are investigated for their natural ability to produce the desired product in certain conditions. Despite being at the primordial stages, it's already fundamental to evaluate strain performances by analyzing the TRY metrics of the investigated hosts whenever possible, to better guide the identification of superior cell factories. Other than TRY, it is also important to holistically observe other variables like specific growth rate, substrate consumption rate, cultivation time and identify possible carbon sinks (i.e. pathways that sequester carbon and generate byproducts) which will help to guide future strain improvement.

BONUS 3 – Techno-economic Assessment of a Bioprocess

In 2014 a Korean group (87) led by professor Ho Nam Chang performed an interesting techno-economic simulation study of a Multi-Stage Continuous High Cell Density Culture (MSC-HCDC) to produce microbial biodiesel by *Cryptococcus albidus* using low-cost wood hydrolysates for cell mass generation in the first stage (at a low C/N ratio) and biobased SCFAs as substrate for lipid accumulation in the second stage (at a high C/N ratio). MSC-HCDC can lead to high productivity and titer for either extracellular or intracellular products given the high cell density of the system (88). The authors predicted for a process which produces 9 g/L/h of lipids with a plant capacity of 100.000 metric tonnes/year, 75% lipids content and 0.3 g/g lipid yield, a biodiesel cost of USD 0.951/L, making it at the time competitive with petroleum diesel (USD 0.914-1.039/L) but not cheaper. Raw materials represented 66% of operating costs with SCFAs production price being a very influential variable on the entire process together with lipid yield. Cheaper SCFAs (USD 0.00 to 0.10/kg) were foreseen to make microbial biodiesel even cheaper than petroleum diesel (0.526-0.809/L). The same authors in another study in 2017 (89) cultivated *Cryptococcus curvatus* in batch mode using SCFAs from rice straw hydrolysate and predicted a microbial lipid cost of USD 1.15/L considering a SCFAs cost of USD 0.15/kg and a lipid yield of 0.15 g/g.

Despite 9-12 years having passed from the publication of these studies to the best of this author knowledge, no viable bioprocess which utilizes biobased SCFA as substrates for microbial biodiesel production is currently on the market. Given the very high productivity required, perhaps what is really missing is a cell factory that can perform at the terms defined by the study. However, the modern advancement in strain-engineering technologies, the ongoing optimization of SCFAs biobased production processes (74), the persistent geopolitical instability and the resulting fluctuations in petroleum prices might help to bring microbial lipids closer to a competitive level. These studies provided valuable insights into the utilization of biobased SCFAs to produce a commodity lipid product, highlighting how much still needs to be done to develop a proper yeast cell factory. It would be interesting to translate the study for the current 2026 market prices and to perform a techno-economic assessment to produce high-value specialty lipids like OCFAs. Which minimum productivity would be required to make the fermentation process economically feasible?

Chapter 3 – One Method to Analyze Them All*

Screening multiple yeast species in parallel is inherently challenging because of the heterogeneous nature of their physiological capabilities, nutritional requirements, and the diversity of the sample matrices involved. The experimental design space is often limited by the screening strategy, the throughput requirements, the available instrumentation, time and budget. Consequently, standardized protocols within the framework of the experimental constraints may introduce intrinsic biases that can influence the observed performances and potentially favor one yeast species over another. It is therefore important to emphasize that the results presented in this thesis were obtained using standardized screening conditions and protocols that were applied uniformly to all yeast species. Thus, the yeast strains identified as the best OCFA producers should be considered within the context of the screening methodologies employed in this work.

To allow **screening** and **characterization** of several yeast species for OCFA production, there was a need for:

- i.* a screening platform to evaluate yeast growth in different media;
- ii.* a robust protocol for FA and total lipid extraction from yeast biomass;
- iii.* an analytical platform to analyze yeast substrate consumption and FA composition.

Furthermore, to **develop** a new OCFA cell factory, it was necessary to:

- iv.* generate a new genetic engineering tool customized to the identified yeast platform to perform strain improvement.

This chapter summarizes the principal technologies and experimental protocols employed throughout this work, highlighting the rationale behind their selection as well as the modifications introduced to improve and adapt the analysis.

3.1 Growth and Substrate Consumption

Many of the selected yeasts analyzed in this work have never been screened before for OCFA production from SCFAs. Therefore, one of the major screening goals was to identify yeasts that were able to grow on the challenging substrate HPr. To investigate the yeast growth capabilities (Paper II) in six different HPr concentrations (5, 10, 15, 19, 24 and 29 g/L), the yeasts were initially cultivated in 96-squared-well plates using the **Growth Profiler 960** (EnzyScreen). The instrument is a high-throughput shaking incubator equipped with cameras beneath each microplate. At predefined intervals, the wells are illuminated, and images of the transmitted light pattern are captured (90). Dedicated software then quantifies cell density and generates growth curves, typically expressed with arbitrary units serving as an indicator

of cell density to measure microbial growth. This approach permitted the identification of eight yeasts that were able to grow in 15 g/L of HPr at pH 6.8.

The identified yeasts have been subsequently cultivated in fermentation flasks for deeper physiological studies. However, cultivating different yeast species in parallel made it challenging to select a suitable sampling time, as their growth rates, lag phases, and transition to stationary phase varied markedly. To monitor the cultivations in real time, flasks were placed in a shaker incubator equipped with a **Cell Growth Quantifier** (sbi Scientific Bioprocessing), which periodically illuminated the cultures through the flask bottom and measured backscattered light as an indicator of cell density. The cultivations were sampled for cell dry weight (CDW), residual substrates and FA analysis at the end of cultivation. Four yeasts were selected for further characterization: *C. oleaginosus*, *R. toruloides*, *Y. lipolytica* and *B. adenivorans* (Paper II). For final characterization these yeasts have been tested in 15 g/L HPr and HAc:HPr 2:1 (10:5 g/L) at three different C/N ratios (50, 100, 200) to determine optimal substrates and nitrogen concentration for OCFA production (Paper III).

In the final stage of the thesis project (Paper IV), the cell growth quantifier was also used to phenotype multiple mutant strains in parallel. Growth on HPr and a mixture of HAc:HPr (2:1) was used to validate mutations affecting the MCC.

High Performance Liquid Chromatography (Jasco) has been used in all the stages of the research to quantify the residual substrates (Glucose, HPr, HAc) and metabolites (Citrate) in the supernatant, either at the end or throughout the cultivation, to be able to calculate yields and rates.

3.2 Fatty Acid Profile Determination

To evaluate the yeast ability to convert SCFAs into FAs and OCFAs, it was crucial to first establish a method for determining the FA profiles in quantitative and qualitative fashion. To initially analyze several unknown OY-FA profiles, a protocol was required that would enable simultaneous, unbiased analysis of both ECFAs and OCFAs. Consequently, a substantial initial effort has been dedicated to selecting proper IS, tailoring an extraction and derivatization protocol for yeast FAs and developing an efficient peak separation method for simultaneous analysis of ECFAs and OCFAs using GC-MS (Paper I). A typical procedure to analyze FAs in GC-MS is to convert them into FAMES, as summarized in Figure 3.

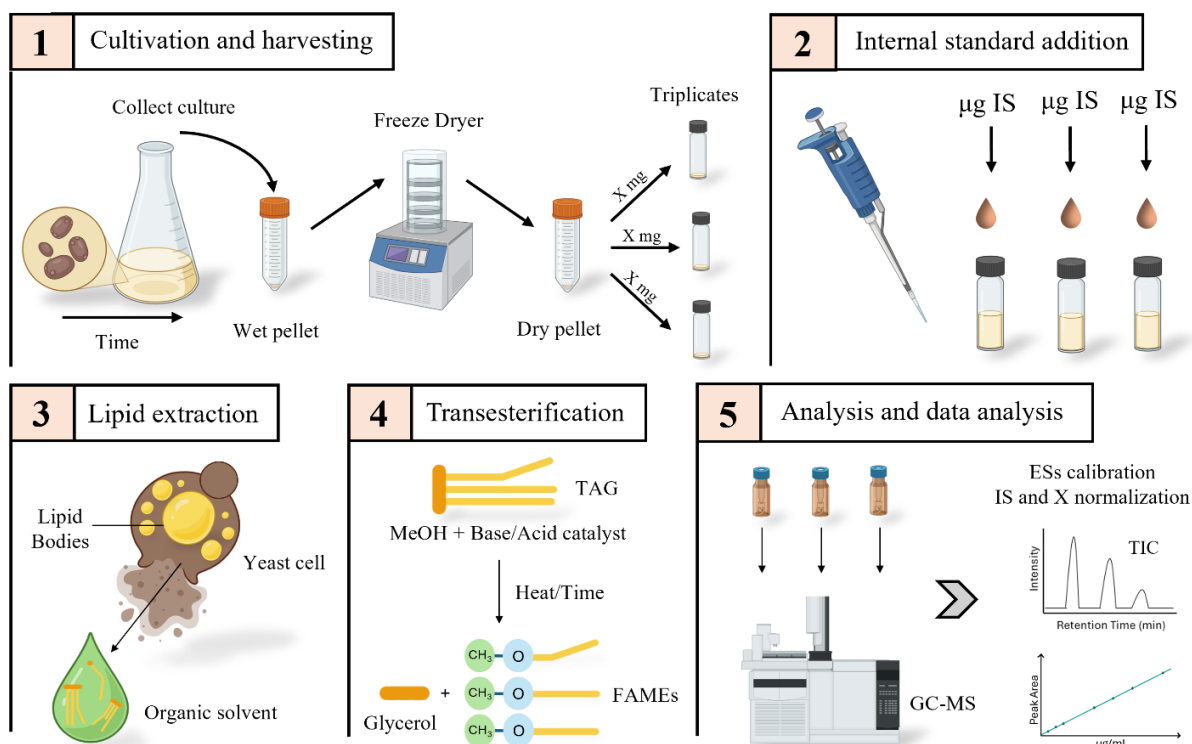


Figure 3. Visual summary of the procedure of a typical FAME protocol workflow. The workflow includes five steps: **1** Cultivation and harvesting of samples, including freeze drying of the biomass to remove residual water that can influence the FAME protocol, and weighing a defined amount of biomass in triplicates for subsequent lipid extraction. **2.** Addition of a defined amount of the selected IS, to track losses and for normalization. **3.** The lipid extraction process, which can be performed with different techniques and in the presence of an organic solvent to dissolve the TAGs and extract them from the complex biological matrix. **4.** Transesterification of the extracted triacylglycerols which represent the core reaction of the protocol to convert the FAs into FAMES, generating glycerol. **5.** The final analysis using GC-MS, followed by external standard calibration for quantification, data processing, and normalization. The figure has been generated using BioRender.com. Figure adapted from paper I under a CC BY 4.0 license. ES, external standards; IS, internal standard; TAG, triacylglycerol; TIC, total ion chromatogram.

3.2.1 Gas Chromatography - Mass Spectrometry

GC-MS is an analytical technique which combines a GC with an MS detector in series and is used to separate, identify, and quantify chemical compounds in complex mixtures. This technique is highly sensitive (i.e. accurately quantify trace compounds), highly specific (i.e. identify individual molecules) and precise (i.e. reproducible) (91). For these reasons it is widely used for analysis in many different fields like environmental, forensic, food and beverage, medical and chemical. GC-MS is also widely used for FAME analysis and allows for qualitative and quantitative profiling of FA composition of microbial lipids.

Chapter 3 – One Method to Analyze Them All

Gas Chromatography

In GC, the sample is injected into the injection port, vaporized and carried by a gas (e.g. He) through a capillary column coated with a stationary phase, where compounds are separated based on their volatility and polarity (92). The efficiency of a chromatographic column is influenced by several factors, including the type and flow rate of the carrier gas, the column dimensions, the thickness of the stationary phase, and the operating temperature (93). Careful selection and optimization of these factors can improve the resolution of the chromatograms.

Therefore, to ensure proper peak separation in a simultaneous analysis of several ECFAs and OCFAs, an existing temperature-programmed method for FA analysis of 12 minutes (established by the Chalmers Mass Spectrometry Infrastructure), was systematically optimized by adjusting the ramp rates, achieving high resolution of all the even and odd FAMES included in the external standards (from C6:0 to C24:1, including several MUFAs and PUFAs) in a time frame of 26 minutes (see M&M and Figure 3 in Paper I). The developed method is able to detect SCFAs (C6:0, C7:0, C8:0) and achieve sufficient separation between C18:2 and C19:0, and between C20:4 and C20:3n-6,9,12 (see Table 1 in Paper I for a detailed list of the FA analyzed in this work).

Mass Spectrometry

The eluted compounds are thereafter ionized in the MS, which detects their intensities and separates them in vacuum, based on their mass-to-charge ratios (m/z), generating a unique mass spectrum for each molecule allowing quantitative and qualitative analysis (93). This mass spectrum is a fingerprint of the molecule and the alignment with spectral libraries allows to identify unknown compounds in a sample. The final combined output of a GC-MS instrument is the *Total Ion Current* chromatogram which is the result of the sum of the intensities of all mass peaks belonging to the same scan against the *retention time* (i.e. the time at which they have been eluted from the GC column). Quantitative analysis of known FAs in yeast FA profiles can be achieved by analyzing in parallel a series of FAME external standards at known concentrations within the same chromatographic sequence of the samples, ensuring the construction of a reliable calibration curves. Furthermore, the addition of an IS to each sample normalize for experimental losses and together with a final normalization to initial sample biomass and the conversion of FAMES to the respective FAs is possible to quantify the yeast FA titers.

3.2.2 Key Improvements of a Microwave-Assisted FAME Protocol

To determine the efficiency of the method, two phylogenetically distinct OYs were compared in Paper I, however in this thesis only results from *Y. lipolytica* are presented. To ensure proper amounts of FAs the yeast have been cultivated in glucose at C/N 50. After harvesting and freeze-drying the yeast biomass, TAGs can be extracted with organic solvents (e.g.

hexane) and transesterified into volatile and thermally stable FAMES, typically using methanol and a catalyst (e.g. BF_3), prior to GC-MS analysis (Figure 3). In this work, a microwave-assisted FAME protocol proved to be ideal for parallel OCFA and ECFA analysis with the selected IS (Paper I).

ISs play a crucial role, as they account for sample preparation inconsistencies such as matrix differences, pipetting errors, variation in injection volume, and instrumental variations. Since the IS should be compatible with the protocol and must differ from the target analytes, OCFA-based ISs are unsuitable for OCFA analysis. Instead, deuterated internal standards (dISs) can be a valid alternative. dISs are stable isotope-labeled analogues that closely mimic the chemical and ionization behavior of their non-labeled counterparts but can be distinguished by their m/z in the MS (94, 95). The ISs are usually added before performing the lipid extraction and can be used to normalize for experimental losses and/or transesterification efficiency. If they are added as single FAs, they account for both normalizations, while if they are added as single FAMES they account only for experimental losses. To ensure accurate quantification, this work initially employed a non-methylated-fully deuterated myristic acid as IS (C14:0-d₂₇).

The original microwave-assisted protocol (96) was characterized by a simultaneous step of extraction and transesterification for which, after several trials, some issues were identified:

1. high risk of biomass burning and volume loss due to the intensive heat treatment that compromise sample integrity;
2. unknown non-methylated dIS recovery and esterification efficiency that cause inaccurate quantification.

To address the first issues and to determine TAG extraction efficiency of the protocol, a **pre-extraction step** was introduced to separate extraction from transesterification. This step involved bead beating with glass microbeads and mechanical disruption using Fast Prep, prior to microwave treatment. The addition of the pre-extraction step effectively eliminated sample burning, reducing sample loss and resulted in a substantial improvement in TAG recovery, with a total FAME recovery increased by an average of about 31% compared to the original microwave protocol (Figure 4A). The relative proportions of different FAMES were consistent between the tested extraction methods, indicating that no FA compositional biases were introduced (Figure 4B).

To address the second issue, FAME yields obtained from two different microwave methods were compared, calculating values with and without normalization of the non-methylated dIS. Microwave method 1 (MW1) represents a **less harsh microwave method** (slower temperature ramp compared to microwave method 2) whereas microwave method 2 represents a harsher microwave method (steeper temperature ramp and holding time at highest temperature, see M&M paper I). Under microwave method 1, only 40% of the added dIS was recovered (Figure

4C), while MW2 achieved about 70% recovery of the added dIS (Figure 4D). However, the raw concentration of the FAMES (in $\mu\text{g/mL}$) obtained after calibration, adjusted only for the dilution factor and initial biomass, without normalization to the non-methylated dIS found in the sample, showed no significant differences between the two microwave methods. This suggests that the non-methylated dIS undergoes esterification with different efficiencies in the two microwave protocols, whereas TAG conversion appears equally efficient in both. Thus, complete esterification of the non-methylated dIS requires either longer reaction times and/or elevated temperatures. Hence, further use of the **pre-methylated dIS** in combination with MW1 is suggested to avoid normalization uncertainties and too harsh microwave conditions. The FAME profiles remained consistent throughout all experiments, confirming the robustness and reproducibility of the qualitative profiling (Figure 4B).

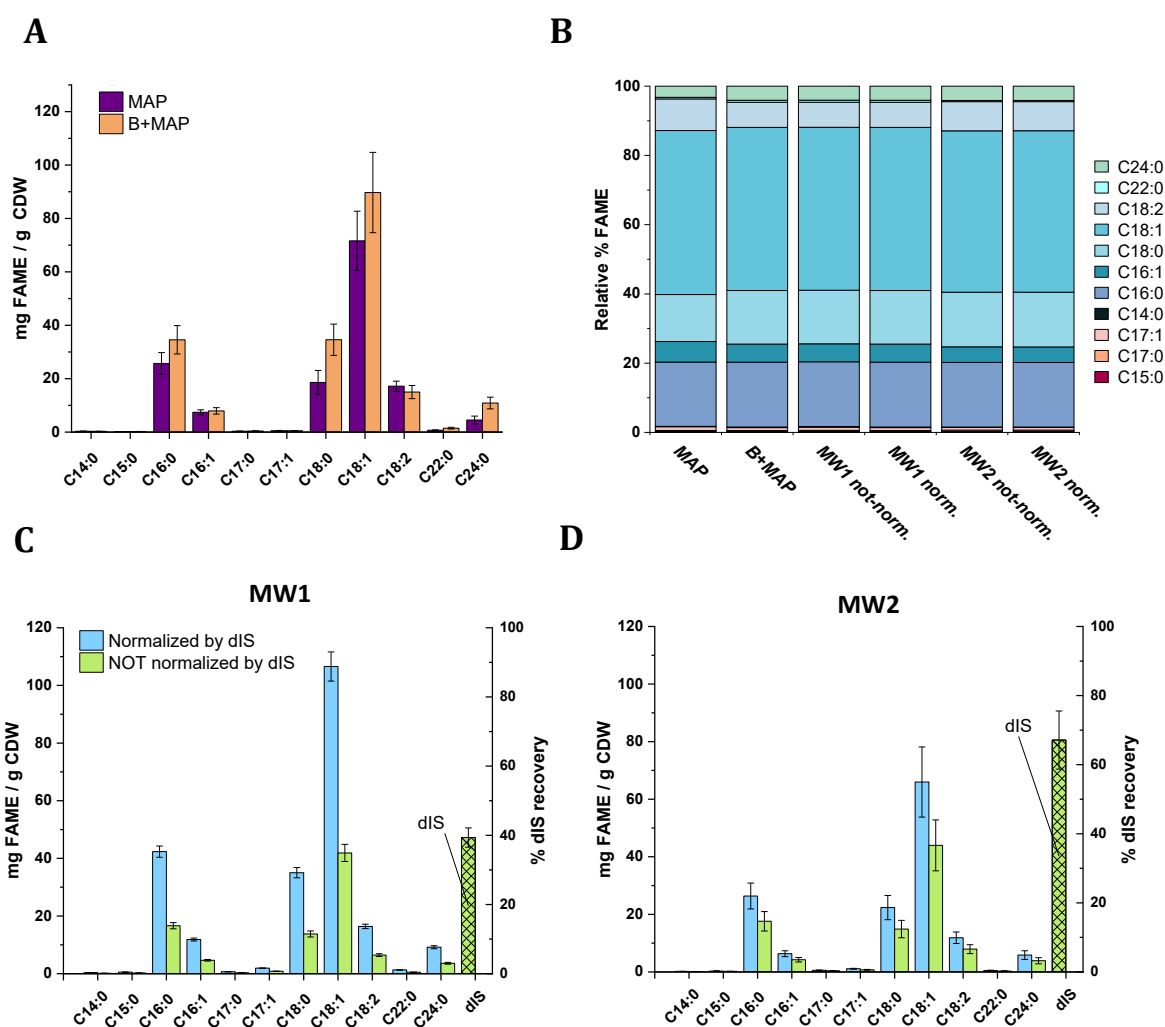


Figure 4. FAME protocol improvements in *Y. lipolytica*. Panel A shows results from pre-extraction step trial repeated with the methylated dIS. Panel B shows FA profile throughout the improvement steps. Panel C and D shows the FA and non-methylated dIS recovery in MW1 and MW2. Figure adapted and modified from paper I under a CC BY 4.0 license. MAP, microwave assisted protocol; B, beads; MW, microwave method.

3.3 Total Lipid Gravimetric Analysis

To rapidly assess the total lipid content of a yeast sample, gravimetric analysis can be used to determine the mass of lipids extracted from the biomass using organic solvents of different polarities. This approach enables determination of both the total lipid titer (g/L) and lipid content (% of CDW) (Paper III and IV). In this study, a modified version of the Bligh and Dyer lipid extraction method has been used (97). Typically, the extraction is performed in two steps with a combination of non-polar and polar organic solvents as chloroform and methanol (1:2 v/v), followed by an addition of an extra volume of chloroform and water or a salt solution (e.g. KCl) to remove the non-lipids components (98). In this work, the extraction procedure included a pre-treatment step in which yeast cells were disrupted at 90 °C for 1 h in 2 M HCl with vigorous vortexing to promote cell lysis and recovery of intracellular non-polar lipids (99). As final step, the non-polar lipids partition into the lower chloroform phase, were evaporated under a stream of nitrogen (N₂), leaving only the extracted oil which was subsequently weighed. This protocol does not ensure accuracy as it cannot be normalized to an internal standard and is influenced by the operator's experience and the balance accuracy. However, it permits obtaining a rough estimate of the total lipid amount in a sample and to compare total lipid yields and contents among different yeast species when all are treated in the same way.

3.4 New Tool for *B. adenivorans* Engineering

One of the goals of this research work was to develop a proof-of-concept OCFA cell factory. *B. adenivorans* represented a good candidate given its capability to grow on HPr and produce OCFA. Therefore, a **plasmid-based CRISPR-Cas9 genome-editing tool**, developed for this yeast (by a collaborator), was applied as a genome editing technology in this study (Paper IV).

The tool was developed by first identifying an endogenous autonomous replication sequence (ARS). Genomic DNA was sheared and ligated into an ARS-trap plasmid containing bacterial (ampicillin) and yeast (hygromycin B) selection markers (this latter one fused to the Herpes simplex virus thymidine kinase (HSV-TK)), an *E. coli* origin of replication, a *B. adenivorans* centromere with a *lacZα* fragment for blue–white screening of *E. coli*, and a guide RNA (gRNA) expression unit containing a superfolder green fluorescent protein (sfGFP) screening marker. The resulting library was amplified in *E. coli* and transformed into *B. adenivorans*. Yeast transformants able to grow on hygromycin B were considered to contain plasmids carrying a functional ARS. The identified ARS-containing plasmid was subsequently equipped with a Cas9 expression unit to generate a CRISPR-Cas9 editing tool.

This tool was successfully used to generate disruption of *TGL4* and *MFE2* genes, involved in β -oxidation and lipid turnover, and *PHD1* gene, involved in propionate metabolism. However,

in this thesis work only the phenotype obtained in *phd1* mutants is described. The methodology used to generate mutants is briefly summarized here and in Figure 5 and the phenotypic characterization of these mutants is presented in Chapter 6:

- i. *gRNA oligo hybridization*: the gRNA oligos were hybridized using a thermocycler program to generate the overhangs required for BsmBI cloning sites (restriction enzyme).
- ii. *Golden Gate assembly*: the hybridized gRNA was cloned into the Cas9 plasmid, replacing the superfolded GFP gene.
- iii. *Transformation of E. coli*: competent *E. coli* cells were transformed by heat shock method and recovered in Luria Broth at 30 °C for 1h. Cells were then concentrated and plated on Luria Broth + Ampicillin agar plates and incubated at 30 °C for 2 days to select transformants carrying the plasmid.
- iv. *Screening of positive colonies*: green fluorescent colonies indicated an empty plasmid, whereas white colonies indicated successful gRNA insertion. At least three positive colonies were confirmed by colony PCR, with the correct construct expected to produce a 280 bp band. Confirmed colonies were grown overnight in Luria Broth + Ampicillin at 30 °C and stored as glycerol stocks.
- v. *Plasmid preparation*: the selected *E. coli* cultures were subjected to lysis (MiniPrep) to isolate and amplify the CRISPR plasmid.
- vi. *Transformation of B. adenivorans*: competent *B. adenivorans* cells were transformed with the confirmed CRISPR plasmid by electroporation.
- vii. *Mutant screening and sequencing*: the plasmid was expected to replicate and express the CRISPR machinery, generating a double-strand break at the target locus. Transformants were selected on YPD (yeast, peptone, dextrose) + Hygromycin B, and six colonies were screened by colony PCR following cell lysis. Confirmed mutants were subsequently analyzed by Sanger sequencing to identify mutations at the target locus and verify the disruption of the gene.
- viii. *Plasmid curing and mutant preservation*: confirmed mutants were first cultivated in non-selective YPD medium and subsequently transferred to YPD + Brivudine. Brivudine activates the HSV-TK system in plasmid-containing cells, enabling counterselection against cells retaining the plasmid. Plasmid-free mutants were subsequently preserved as glycerol stocks for phenotypic characterization.

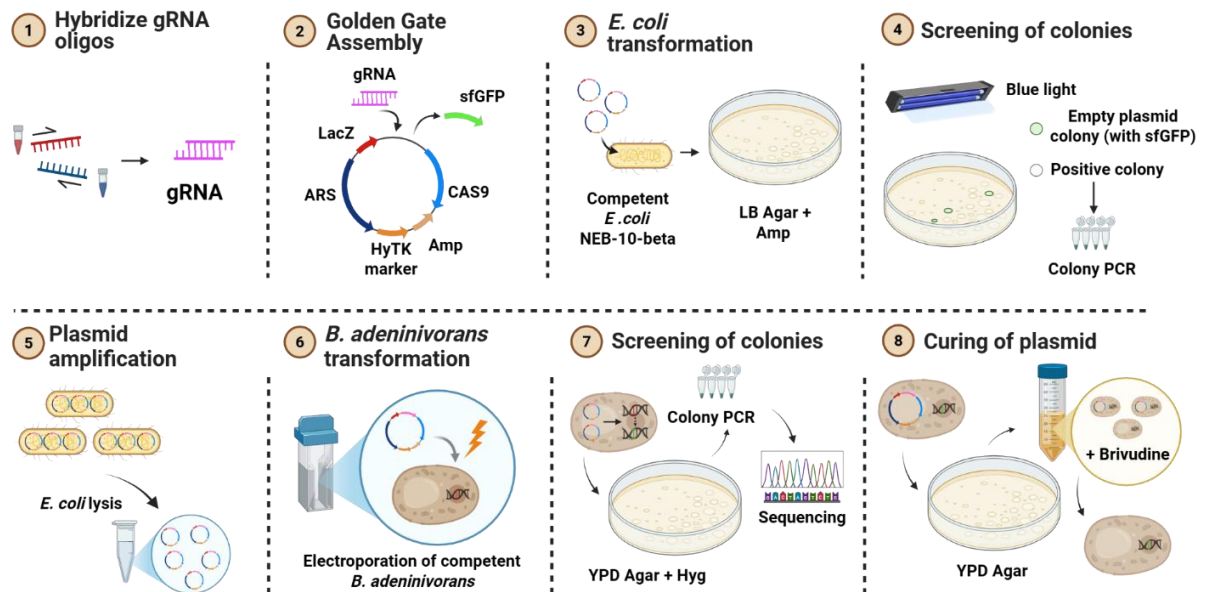


Figure 5. Workflow to generate *B. adenivorans* mutants with the newly developed CRISPR-Cas9 tool. Steps from 1 to 8 include insertion of gRNA in the plasmid, transformation and amplification in *E. coli*, transformation in *B. adenivorans*, screening and curing of the plasmid from the cells.

Chapter 3 – One Method to Analyze Them All

Chapter 4 – Oleaginous Yeasts at a Glance

To develop an efficient OCFA cell factory and gain a comprehensive understanding of the strategies employed by OYs to accumulate OCFA, it is essential to possess a solid understanding of OY lipid metabolism. However, as discussed in *Chapter 2*, the distinction between OYs and non-OYs is not clearly attainable, and the separating line between the two might be ambiguous to draw. Therefore, the information presented in this chapter should be regarded as a general synthesis of the knowledge available in literature, rather than as a strict definition of OY lipid metabolism. Nevertheless, this knowledge provides an essential basis for interpreting the results presented in this thesis. This chapter therefore explores OY lipid metabolism, including relevant terminology, enzymes and genes, and the main metabolic pathways, exploring different substrates in the context of this thesis. It also presents selected results from Papers **II** and **III**, focusing on FA profiles under different substrate conditions.

4.1 Lipid Metabolism in Oleaginous Yeasts

The established knowledge on OYs suggests that the intracellular accumulation of lipids is triggered when they are cultivated in presence of **excess carbon source** (usually glucose in laboratory condition) and a **limitation** of a specific nutrient like **nitrogen** (or phosphate, sulfate, magnesium), i.e. under **high C/N conditions** (100-102). The typical and generalized growth and metabolite profile in high C/N ratio includes two phases (Figure 6A):

- i. the *biomass formation phase*, where the cell initially consumes carbon together with nitrogen to support growth and cell division;
- ii. the *lipid accumulation phase*, where, on the onset of nitrogen depletion, the cell slows down the specific growth rate and drives the carbon in excess towards the storage of fats (i.e. TAGs), usually stored in lipid bodies (a monolayer of phospholipid with some embedded proteins) (64, 65, 100).

The lipid-accumulation phase in general starts towards the end of the exponential growth phase or at the onset of the stationary growth phase. However, the exact time at which the lipid accumulation phase starts is hard to pinpoint, and it might be influenced by the species and strain employed, the cultivation medium used (e.g. type of substrates, C/N ratio) and the applied process conditions. This makes selection of the correct sampling points important, but difficult to define (66, 103). Non-OYs cultivated under the same limiting conditions generally divert the excess carbon towards the accumulation of polysaccharides or cease carbon assimilation altogether (104).

In the presence of a low C/N ratio (i.e. excess nitrogen), biomass formation is favored over lipid accumulation (Figure 6B). Selecting a proper C/N ratio for lipid accumulation is strain and substrate specific and therefore needs to be carefully optimized to improve lipid TRYs and balance it with biomass formation. In fact, as lipids are intracellular products,

their titer is connected to biomass titer and partially to intracellular accumulation abilities; consequently, excessively high C/N ratios can often result in a lower lipid titer since biomass formation is highly affected by nitrogen availability.

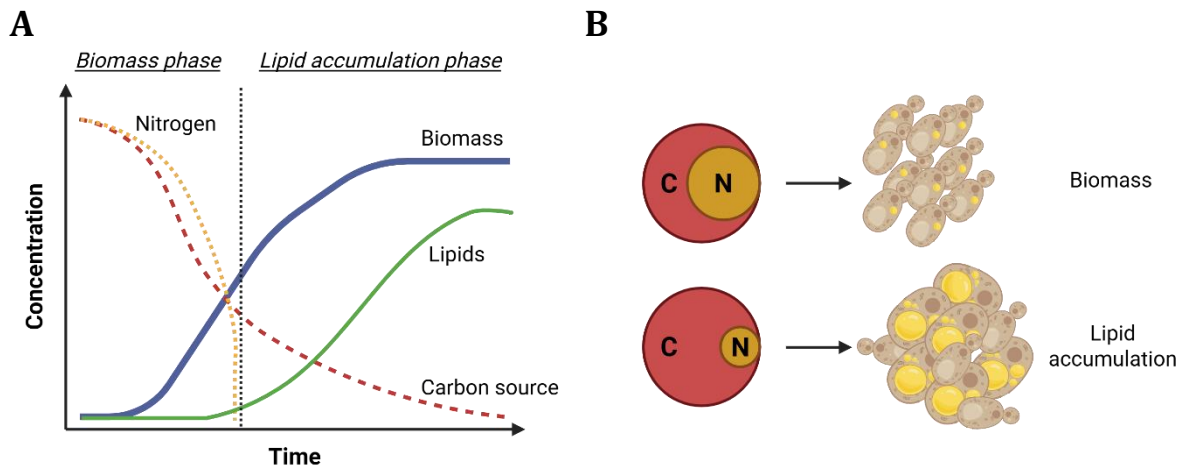


Figure 6. General traits of OYs growth and lipid accumulation. Panel A shows phases of growth and lipid accumulation in OYs cultivation and Panel B shows the C/N ratio concept. Figure inspired by (65). The figure has been generated using BioRender.com.

4.1.1 Common Lipid Synthesis Pathways in Oleaginous Yeasts

There are two lipid biosynthesis pathways majorly described in literature: the *de novo* pathway, activated when hydrophilic substrates like glucose or similar compounds (e.g. other mono- or disaccharides, starch, glycerol, etc. but also some SCFAs) are used, and the *ex novo* pathway when hydrophobic substrates like oil, fat and free FAs are used (64, 66).

De novo biosynthesis pathway

Generally, in the presence of hydrophilic substrates, upon nitrogen depletion, the cell-specific growth rate slows, while carbon assimilation can continue, causing carbon to accumulate in the cytoplasm (64). The excess carbon is consequently redirected toward lipid accumulation. Once extracellular nitrogen becomes limiting and then is depleted, OYs temporarily obtain NH_4^+ from AMP (adenosine monophosphate) thanks to the activity of an **AMP deaminase** (or dehydrogenase), generating IMP (inosine monophosphate) (Figure 7) (64). This directly causes an intracellular decrease of AMP which is an important allosteric activator of Icdh (NAD^+ -dependent isocitrate dehydrogenase), that converts isocitrate to α -ketoglutarate in the tricarboxylic acids cycle (TCA cycle) in the mitochondrion (100). In turn, isocitrate starts to get accumulated and is therefore converted back to **citrate** inside the mitochondria. When the concentration of citrate reaches a critical toxic value, it is exported to the cytoplasm through antiporters, generally in exchange with malate or α -ketoglutarate (63, 100). In the cytoplasm citrate is converted by **Acl (ATP-citrate lyase)**, a key lipogenic enzyme highly activated in OYs in nitrogen limiting conditions, into oxaloacetate and **acetyl-CoA** (Figure 7) (100). Oxaloacetate can be converted to malate, which serves as a counterion in the citrate efflux system from the

mitochondria (104). If the Acl-catalyzed flux is not high enough, citric acid gets accumulated in the cytoplasm lowering the intracellular pH, as a result is excreted in the extracellular environment, decreasing the flux towards lipids. The citrate excretion phenomenon has been observed in this thesis work in *Y. lipolytica* and *B. adeninivorans* as described in Chapter 5 and Paper III.

The formed acetyl-CoA is then carboxylated into malonyl-CoA, by the addition of a molecule of CO₂ from HCO₃⁻, thanks to **Acc (biotin dependent acetyl-CoA carboxylase)** (64). This irreversible reaction is considered a limiting step in the flux towards lipids (64). Acetyl-CoA and malonyl-CoA are then attached to an acyl-carrier protein (ACP) respectively and enter the **fatty acid synthase cycle (Fas cycle)** (64). In the FAS cycle these two molecules initially undergo a condensation reaction, giving rise to a 4-carbons molecule (ketoacyl-ACP) by losing a molecule of CO₂. Then follows a sequence of reduction, dehydration and a second reduction, consuming overall two NADPH (reducing power) and releasing H₂O, giving rise to a 4-carbon acyl-ACP chain, butyryl-ACP (Figure 7). Two-carbon units from malonyl-CoA are then sequentially added to the growing acyl chain during each cycle, leading to the formation of **even** saturated MCFAs and LCFAs (63, 64). The example formation of C16:0 is described by the following reaction (64):



Further elongation to VLCFAs and desaturation to MUFAs and PUFAs generally occur in the endoplasmic reticulum (ER) by Elo (**elongases**) and Ole and Fad (**desaturases**) (Figure 7) (100). The ER-based elongation requires the same substrates and cofactors as Fas cycle. Fas and Acl are generally inhibited by exogenous long aliphatic chains like FAs or n-alkanes (64). The combined regulation of FA elongation and desaturation (which depends on both FA chain length and enzyme substrate specificity) ultimately determines the cellular balance between saturated FAs, MUFAs and PUFAs and hence the specific FA profile of the OY (105, 106).

As a final step in the lipid biosynthesis pathway, the newly formed FAs are esterified onto a glycerol backbone (originating from a glycerol-3-phosphate) through the Kennedy pathway. At the start, LPA (lysophosphatidic acid, with a FA in *sn*-1) is formed and then converted to PA (phosphatidic acid, with a FA in *sn*-1 and *sn*-2) which is dephosphorylated to produce a diacylglycerol (64, 100). Finally, another FA is esterified in *sn*-3 position and a TAG is formed which is then stored in lipid bodies (Figure 7).

FAs are highly reduced molecules which require a continuous supply of the reductant NADPH to be synthesized. Therefore, the OY cells must maintain a functional regeneration pathway of the NADPH consumed to allow FAs biosynthesis. Two major NADPH-regenerating pathways are described in OYs: the conversion of malate to pyruvate (releasing a molecule of CO₂) in the cytoplasm thanks to ME (malic enzyme), and the pentose phosphate pathway (PPP) (107), but other regeneration pathways are available.

The preference of one or the other pathway might be strain specific, and it may be influenced by the substrates used, highlighting those different strategies in distinct conditions evolved to allow OYs to accumulate lipids. For instance, a *in silico* analysis, has reported that in *R. toruloides* cultivated in glucose medium, the PPP should produce 63% of the required NADPH, with a smaller contribution from ME (108). While in glycerol medium, the NADPH demand is mainly met by the ME (108). In another flux analyses study (supported by proteomic data), in acetate medium the cytosolic ME was the sole supplier of NADPH in the nitrogen limiting phase (109). Another significative example is the lack of the cytoplasmic ME activity in *Y. lipolytica*, which then relies only on PPP as main donor of reducing power (110, 111).

Ex novo synthesis pathway

Some OYs can grow on fats and oils and accumulate lipids by secreting lipases that release FAs from TAGs (64). The free FAs are internalized and activated as acyl-CoA in the cytoplasm. They can then be elongated in the ER (or stay as they are) and stored in TAGs within lipid bodies or oxidized via β -oxidation to generate acetyl-CoA for cell growth and maintenance (75). Importantly, it appears that when the *ex novo* pathway is activated, lipid accumulation seems to be uncorrelated to the concentration of assimilable nitrogen, thus it appears to be a growth-coupled process (75).

β -oxidation and lipid turnover

In case of carbon starvation, some OYs can very rapidly mobilize the stored lipids as energy and carbon sources. This process is called lipid turnover. It has been found that the ability to activate lipid turnover following *de novo* lipid accumulation may depend on the strain and on nitrogen availability in the medium (112). During lipid turnover, lipid bodies are degraded using **Tgl (TAG lipases)** and steryl ester hydrolases, liberating free FAs which are transported to the peroxisome where the **β -oxidation** takes place (Figure 7) (66, 100). Through the β -oxidation pathway FAs are catabolized to shorter acyl-CoA chains till acetyl-CoA or propionyl-CoA are formed (64, 66, 100). The β -oxidation consists of four key steps: dehydrogenation, hydration, oxidation and final addition of a thiol group from CoA-SH. The hydration and oxidation steps are performed by **Mfe (multifunctional enzyme)**. Other than the function to produce energy, the β -oxidation process may also serve as lipid remodeling pathway.

4.1.2 OCFAs from Glucose

During growth on glucose- or HAc-based media, OYs mainly produce ECFAs; however, OCFAs have been detected in trace amounts in certain OYs, accounting for up to 5% of total FAs (Figure 9A, Paper II). To produce OCFAs, acetyl-CoA is replaced by propionyl-CoA as the main precursor. In nature, several pathways can in fact generate intracellular propionyl-CoA from intermediates of sugar catabolism: the 2-oxobutyrate pathway (from threonine or citramalate), the methyl malonyl-CoA pathway (from succinyl-CoA), the acrylate pathway (from acryloyl-CoA coming from lactate), the 3-hydroxypropionate pathway, the 1,2-propanediol pathway (from fucose and rhamnose) and the amino acid degradation pathway (from branched chain amino acids like isoleucine or valine) (10, 113). These pathways can be expressed, combined, or optimized in a production host to enhance propionyl-CoA intracellular supply.

4.2 Metabolism of Propionic Acid and OCFA Synthesis

In contrast to the well-studied glucose catabolism, which is believed to be highly conserved between yeast species (114), the metabolic pathways involved in SCFAs uptake and assimilation in yeasts remain largely unexplored. It is reasonable to believe that SCFAs are metabolized through part of the *de novo* biosynthesis pathway and used as building blocks for LCFAs. It is known that when HAc and HPr are supplemented in the cultivation medium, they are internalized and activated in the cytoplasm into acetyl-CoA and propionyl-CoA, respectively, by Acs (Acetyl-CoA synthetase) (109, 115). Acetyl-CoA readily enters the central carbon metabolism to be used as carbon and energy source. In contrast, **propionyl-CoA** is thought to primarily enter the **methyl citrate cycle (MCC)**, to be utilized as energy source, or to be channeled towards OCFA production following the *de novo* synthesis pathway (Figure 8) (115). In the MCC, propionyl-CoA is condensed with oxaloacetate to form 2-methyl citrate, catalyzed by methyl citrate synthase. Secondly, **Phd (2-methylcitrate dehydratase)** removes a water molecule to form 2-methyl-*cis*-aconitate. The latter one is rehydrated by an aconitase to form 2-methyl isocitrate, which is subsequently cleaved by a lyase to yield succinate and pyruvate that can enter the TCA cycle, the glyoxylate cycle, gluconeogenesis and other metabolic pathways to support the central carbon metabolism. Based on the OY strain, some of the MCC enzymes are sequestered in the mitochondria and/or in the cytoplasm, therefore this pathway is often split between cellular compartments (116, 117). Alternatively, propionyl-CoA can enter the FA biosynthesis pathway, where it is condensed with malonyl-CoA to form a five-carbon intermediate (3-oxovaleryl) that enters the FAS cycle, generating OCFAs (22, 113).

The effect of the C/N ratio in HPr-only cultures remains unclear; however, it is reasonable to hypothesize that a high C/N ratio would indirectly limit the channeling of HPr-derived carbon towards central metabolism, thereby making it more available for lipid accumulation. In a recent study on *Y. lipolytica* and *Cutaneotrichosporon curvatum*, different C/N ratios and synthetic SCFA concentrations have been analyzed, revealing that the influence of C/N ratio on biomass and lipid content depends on SCFA concentration

(118). Moreover, a too high C/N ratio reduced biomass formation, promoted citric acid excretion (wasted carbon) and aggravated SCFA toxicity (118). The response to different C/N ratios and lipid accumulation was highly strain-specific, highlighting the need to tailor the optimal C/N ratio to the OY used and the process being developed. Similar results were observed when cultivating the selected OYs in HPr medium in this research work (Figure 3D in Paper III). The C/N ratio affected the final FA titers, although the magnitude of this effect was species-specific. Moreover, excessively high C/N ratios appeared detrimental, and promoted citric acid excretion in *Y. lipolytica* (Figure 3B in Paper III).

4.2.1 The Toxicity of our Precursor, of our Precursor**

SCFAs are ideal substrates for OCFA production, but they come with an intrinsic challenge, they are often toxic and can inhibit microbial growth. Their **toxicity** is determined by different factors such as the physiological characteristic of OY strain, the medium composition, the extracellular pH and acid concentration. HPr is, in fact, a common food preservative, mainly used in bread and bakery products, developed to hinder the growth of mold and delay spoilage (119).

It has been shown that a methyl citrate synthase deletion mutant of *Aspergillus nidulans*, when grown on glucose and HPr, accumulates toxic amounts of propionyl-CoA which in turn inhibits the activity of CoA dependent enzymes such as pyruvate dehydrogenase, succinyl-CoA synthetase, and ATP citrate lyase (120). Furthermore, in *S. cerevisiae*, exposure to sub-inhibitory concentrations of HPr can lead to enhanced endocytosis, cell cycle arrest, and disruption of cellular respiration (121). In *Candida albicans*, it has been proven that HPr promotes the accumulation of reactive oxygen species, which in turn promote apoptosis (122). Thus, the MCC and the lipid accumulation pathways might be strategies to detoxify high concentration of propionyl-CoA, especially if no alternative metabolic route exists. However, the literature clearly indicates that one of the main reasons for SCFAs toxicity is the acidification of the intracellular environment, a phenomenon that is indirectly controlled by extracellular pH and concentration of the carboxylic acid (123, 124).

Besides being a potential carbon source, SCFAs are weak acids that at pH values below their pKa (4.87 in case of HPr and HAc) are predominantly undissociated and can enter the plasma membrane through simple diffusion and/or facilitated diffusion (124). Once inside the neutral cell environment, they dissociate into their negatively charged anion and H⁺ which acidifies the cytoplasm, disrupting metabolic processes, and consuming cellular energy to mitigate the acid stress, which diminish growth capabilities (123-125). Intracellular acidification results also in other deleterious effects including oxidative stress, protein aggregation, disruption of the cell membranes, inhibition of membrane trafficking, and of the PPP (126). The majority of these studies on yeast SCFA toxicity have been carried out using HAc, as one of the main inhibitors in lignocellulosic hydrolysates, and *S. cerevisiae*, as the model yeast. Nevertheless, these findings can be considered reasonably transferable to HPr as a weak carboxylic acid (with only one more carbon

atom) and to OYs. However, it is worth remembering that acetate and propionate anions follow distinct downstream pathways, which can lead to quite different outcomes.

When the external pH is above the pKa, the SCFAs are mainly in their dissociated form allowing slower and more controlled import and reducing cellular stress, as shown for HAc (124, 127). Much less is known how exactly propionate is transported inside the cell, however, as a small anion of a carboxylic acid it is likely transported by the carboxylic acid transport mechanism i.e. actively transported through permeases with the import of H⁺ (125). A recent transcriptomic study in *Y. lipolytica* suggests that propionate and acetate may be transported by Jen (carboxylate transporters) and/or Gpr (G-protein-coupled receptor) transport systems (115). Although protons are still transported into the cell, the resulting flux may be more tightly regulated than when HPr freely diffuses across the membrane and dissociates intracellularly, allowing the cell to allocate its resources more efficiently without facing the severe stress associated with low intracellular pH.

4.2.2 Growth Screening of Several Oleaginous Yeasts Species on Propionic Acid

The final product titer depends on the amount of carbon available in the medium, the yeast's ability to internalize and convert it into the desired product as well as the process conditions. However, high HPr concentrations can be deleterious; therefore, **yeasts capable of tolerating high HPr concentrations have the potential to become new workhorses for OCFA production.**

Considering that only a few OYs have been tested to grow in SCFAs so far, it is of outmost importance to explore the OY collections available in the scientific community to identify new potential OCFA cell factories. Therefore, in this thesis work and in Paper II, nineteen OY species from both the Ascomycota and Basidiomycota phyla were selected. The list included species previously tested on SCFAs and shown to produce OCFAs, as well as species that had not previously been tested under these conditions (Table 1 in Paper II summarizes selection procedure). These nineteen OYs were screened for their ability to grow at pH 6.8 and different concentrations of HPr (5, 10, 15, 19, 24 and 29 g/L) (Figure 8) (Paper II). The cultivation C/N ratio (g/g) was set to about 8, to favor growth and biomass formation. Despite the high pH, eleven out of nineteen strains were still unable to grow on HPr. However, different outcomes might have been obtained with longer cultivation periods and/or higher initial ODs (Figure 8A). Nonetheless, these results correlate well with the findings in a recent publication, where only a small subset of the >1400 yeast strains tested could grow on mixtures of SCFAs, including HPr (128). This highlights how stressful this carbon source is for the majority of yeasts, while at the same time, emphasizing the remarkable metabolic potential of some non-conventional yeasts and their enormous diversity.

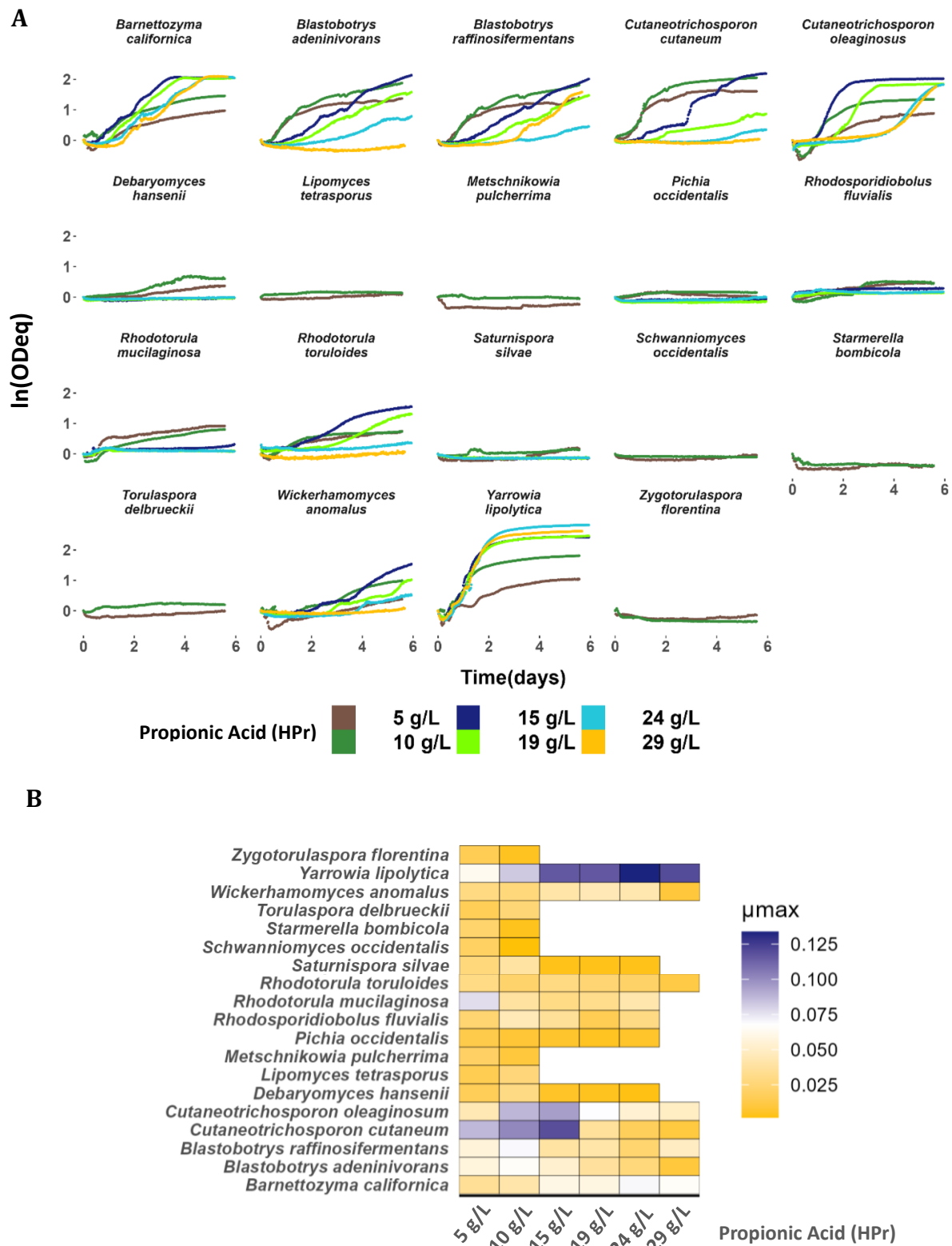


Figure 8. Growth curves of 19 OYs grown in different concentrations of HPr. Panel A shows the growth curve of the OYs tested in different concentrations of HPr. On the y-axis is represented the natural logarithm of the OD equivalent. On the x-axis is represented the time in days. The curves at different concentrations are represented by different colors as the figure legend clarifies. Panel B shows a heatmap of the μ_{max} (h^{-1}) of the different OYs in the different concentrations of HPr. Figure adapted and modified from paper II under a CC BY 4.0 license.

The eight strains that could clearly grow on HPr concentrations up to 29 g/L were: *B. adenivorans*, *Blastobotrys raffinifermentans*, *Barnettozyma californica*, *Cutaneotrichosporon cutaneum*, *C. oleaginosus*, *R. toruloides*, *Wickerhamomyces anomalus* and *Y. lipolytica*. At the highest HPr concentration, *Y. lipolytica*, *B. californica*, and *C. oleaginosus* exhibited the best maximum specific growth rates (0.12, 0.065 and 0.049 h⁻¹ respectively) (Figure 8B), whereas *R. toruloides* and *W. anomalus* struggled the most. Furthermore, increasing the concentration of HPr increased the lag phases of most of the yeasts in the span of days (from 1 to 3 days) (Figure 8A), suggesting that they go through an adaptation phase. Overall, the generally preferred HPr concentration was around 15 g/L, with lag phases ranging from 13-45 hours and maximum specific growth rates ranging from 0.028 to 0.12 h⁻¹. Overall, these data are consistent with most of the literature. Different OYs screened for growth on synthetic SCFAs or biobased digestates have shown growth inhibition directly correlated with external SCFA concentrations above 5–10 g/L, as reported for *R. toruloides* and other yeasts (12, 129). In contrast, *Y. lipolytica*, *C. curvatum*, and *C. oleaginosus* have been shown to thrive at high HPr concentrations, up to 40 g/L of pure HPr (12, 118, 129, 130). Relating back to the mechanisms of SCFA toxicity described in this section, these observed differences in the ability to grow on HPr, even at high pHs, may be related to differences in the rate of influx of the acid and/or limitations in the downstream metabolic pathways. This could be caused by the absence or low expression of key enzymes of the MCC detoxification pathway, unfavorable enzyme kinetics, or other unknown cellular stresses.

In conclusion, the ability to grow and metabolize HPr seems strictly species-specific even at relatively high pH, and the growth performances are influenced by HPr concentration in the extracellular environment.

4.2.3 Typical Fatty Acids Profile of Oleaginous Yeasts Grown on Glucose or SCFAs

As mentioned, acetyl-CoA predominantly generates ECFAs, whereas propionyl-CoA predominantly generates OCFAs. In Paper II, FA profiles were analyzed and compared for nineteen OYs grown on glucose (20 g/L, C/N 43 g/g, pH 6.8) and for seven OYs able to grow on HPr (15 g/L, C/N 43 g/g, pH 6.8) (Figure 9).

The FA analysis revealed that **ECFAs are the predominant FAs** produced under **glucose-based conditions**, accounting for 95.0-99.5% of the total FAs across all studied strains (Figure 9A). The percentage of specific FAs relative to total FAs varied between the different yeasts, although C16:0, C16:1, C18:0, C18:1 and C18:2 were among the most abundant FAs for all strains. Most yeasts produced more C16:0 than C16:1, but on the contrary, the unsaturated FAs C18:1 and C18:2 were generally favored over the saturated C18:0 while only 5 yeasts produced also C18:3. VLCFAs such as behenic acid (C22:0), C24:0 and cerotic acid (C26:0) were mainly produced by *Rhodotorula spp.*, highlighting the capability of this yeast species to synthesize VLCFAs (131). In terms of OCFAs, C15:0, C17:0 and C17:1 were the main FAs produced, although as a minimal percentage of the total FAs overall (0.5-5%). These results agree well with most reported studies so far (132, 133). It

should be reminded that OCFAs often are excluded from the FA analysis using GC-MS of glucose-grown cells, and thus there is a lack of information about potential OCFA production for many strains. Interestingly, *M. pulcherrima* was reported here for the first time to be able to produce a relatively high percentage of OCFAs, almost 5% of the total FAs, followed by the two *Blastobotrys* strains (equally 3.4%) and *B. californica* (3.1%). Olstorpe and colleagues reported up to 30% of C17:1 on total FAs in rich yeast medium (YPD) at 25-30 °C for *B. adenivorans* CBS 7377 and CBS 8244, highlighting that the growth conditions matter for final OCFA content (134).

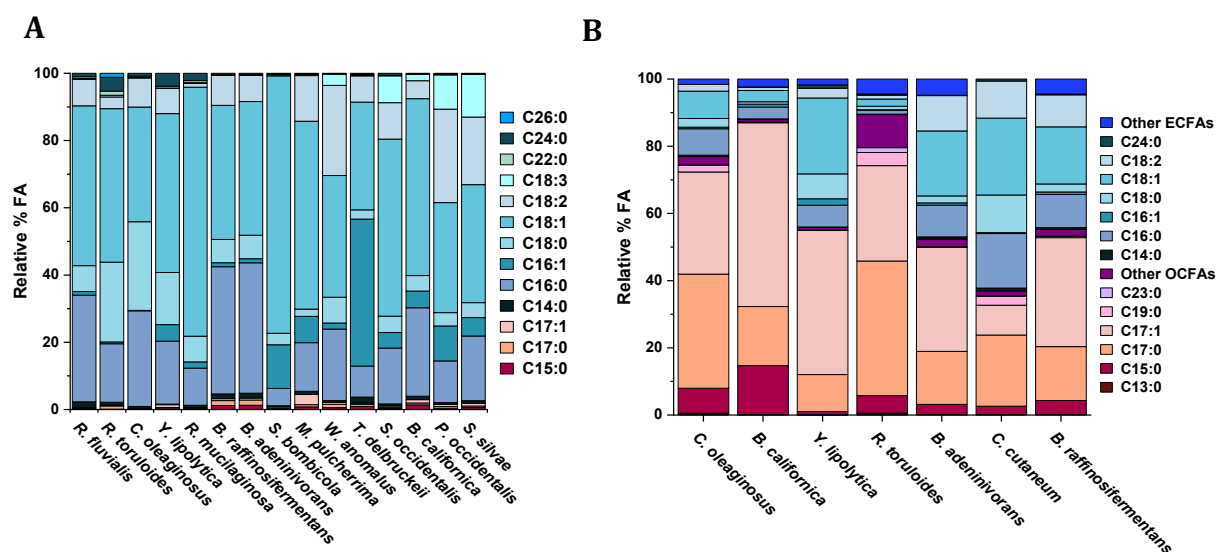


Figure 9. FA profiles of OYs cultivated on different substrates. Panel A shows FA profile analyzed in glucose medium. Panel B shows FA profiles analyzed in HPr medium. On the y-axis the relative percentage of the single FA is represented on the total FAs. On the x-axis the yeast species analyzed are represented. The different colors indicate different FA species. Figure adapted and modified from paper II under a CC BY 4.0 license.

All seven yeasts grown in **HPr** showed **FA profiles enriched in OCFAs**, although the ratio of OCFAs relative to total FAs varied significantly (Figure 9B). These results agree well with literature, where upon utilization of synthetic or biobased SCFAs, an increase in OCFAs was reported in all tested yeasts (11, 12, 77, 129, 135, 136). *R. toruloides* and *B. californica* produced nearly 90% of their total FAs as OCFAs, while *C. oleaginosus* reached about 80%. *Y. lipolytica* and the two *Blastobotrys* strains produced about 55% OCFAs, and *C. cutaneum* reached 37%. Interestingly, *C. cutaneum* produced more ECFAs than OCFAs even on HPr, perhaps suggesting a strong drive to channel HPr towards formation of ECFAs precursors through the MCC and/or a low affinity of the Fas for propionyl-CoA. The percentage of individual FAs on total FAs varied between yeasts, although all preferentially accumulated C15:0, C17:0 and C17:1 and some also small amounts of C19:0, C23:0, nonadecenoic acid (C19:1) and C25:0. The primary ECFAs accumulated were C16:0, C18:0, C18:1 and C18:2.

These results confirm that the capability to produce ECFAs or OCFAs is strongly influenced by the carbon source present in the medium and it is yeast specie specific. Moreover, the distinct chain-length and unsaturation degrees indicate strain-specific preferences, which in turn suggest different characteristics of the elongase and desaturase machinery. Although odd-chain precursors favor OCFA formation, all OYs still produce ECFAs in HPr medium, highlighting that even-chain precursors are indeed formed and equally channeled towards FA biosynthesis.

Chapter 5 – “Odd” Oleaginous Yeasts Cell Factories

To develop an efficient OCFA cell factory and gain a comprehensive understanding of the strategies employed by OYs to accumulate lipids and OCFAs in SCFA-based media, an important first step is to elucidate the capabilities of different strains under relevant cultivation conditions, such as a blend of synthetic HPr and HAc. In this chapter, the performance of four OYs identified in Paper II and further characterized in Paper III is explored under different cultivation conditions, with a particular focus on their ability to utilize SCFAs and produce OCFAs in different C/N ratios.

5.1 Exploring Performance of Four Oleaginous Yeasts Grown on Synthetic SCFAs

The four yeasts that were selected for further characterization based on their distinct traits are (data available in paper II, Figure 3 and 5):

- i. ***C. oleaginosus*** ATCC 20.509, a Basidiomycota yeast belonging to the Trichosporonaceae family, proved to be the most efficient OCFA producer in HPr (0.07 g/g, 80% on total FAs) under the conditions tested. It also showed the ability to fully metabolize HPr within 188h, which is an important parameter for the selection of a promising cell factory. These data agree well with most of the available literature which highlights the ability of this yeast to grow in rather harsh environments such as SCFA digestates (89, 130, 136);
- ii. ***R. toruloides*** CBS 14, a Basidiomycota yeast belonging to the Sporidiobolaceae family, proved to have an interesting ability to drive the little HPr consumed towards OCFA synthesis (0.05 g/g, 90% on total FAs) despite showing difficulties to grow on HPr. These data agree well with literature which describes that this yeast struggle to grow on SCFAs concentrations higher than 10 g/L but seems to possess a Fas with high affinity for propionyl-CoA (12, 105);
- iii. ***Y. lipolytica*** W29, an Ascomycota yeast belonging to the Dipodascaceae family, proved to have high HPr tolerance and ability to grow and consume HPr even at very high concentrations (up to 29 g/L with a maximum specific growth rate of 0.12 h⁻¹) other than being able to produce OCFAs, as proved already in literature (0.01 g/g, 55% on total FAs) (12, 118). As one of the most extensively studied OYs, this yeast benefits from the availability of several engineering tools, making it a convenient and relatively easy-to-engineer cell factory. It's, in fact, the most engineered strain to produce OCFAs from SCFAs but also from glucose (22, 137-141);
- iv. ***B. adeninivorans*** Y-17692, an Ascomycota yeast belonging to the Trichomonascaceae family, it's a relatively underexplored OY which has been tested for the first time in this thesis for growth ability in SCFAs. This yeast proved to efficiently tolerate and metabolize HPr, showing high biomass yield on HPr (0.42

g/g) and demonstrate to be able to produce OCFA (0.02 g/g, 55% on total FAs). These results highlight its potential as an OCFA-producing cell factory. Furthermore, several research studies describe this yeast as able to grow in different carbon sources and at high temperatures, such as 42 °C, making it an interesting strain with industrial characteristic worth exploring (134, 142, 143).

Successful lipid production depends on the yeast's ability to efficiently assimilate the carbon source while balancing biomass generation and lipid accumulation to achieve high titers. As previously mentioned in *paragraph 4.2*, has been seen in several studies that the effect of the C/N ratio on SCFA media is dependent on SCFA concentration and on the yeast species (80, 118, 144). Thus, OCFA yields and titers could be further improved by fine-tuning the C/N ratio in a strain-specific manner.

To assess how the selected OYs navigate this balance, they were further characterized in batch mode at flask scale, evaluating their performances to grow and produce OCFA on a mixture of HAc:HPr at ratio of 2:1 (10:5 g/L), at pH 6.8, while testing different C/N ratios (50, 100, 200 g/g) (Paper III). Ideally, HAc in the medium, which is primarily converted to acetyl-CoA upon uptake, can serve as both a carbon and energy source and support FA synthesis, including OCFA synthesis, as acetyl-CoA is converted to malonyl-CoA, a key building block for FA elongation. In contrast, HPr could support OCFA formation as the main precursor, although it can also be used as a carbon and energy source metabolized through the MCC pathway.

The results of paper III have been revisited, and some performance parameters are presented in this thesis chapter (Table 4). The TRY values were calculated at the end of each cultivation period; thus, they represent the overall average performance of the OYs over a certain time period. It is important to keep in mind that in HAc:HPr medium the relative flux contribution of each substrate into lipid or OCFA metabolism cannot be clearly distinguished. However, since one molecule of HPr should mechanistically serve as the starting building block for one molecule of OCFA, calculating the OCFA yield in mol/mol based on consumed HPr can be useful for assessing how efficiently different yeasts incorporate HPr into OCFA. Since OCFA are also formed through the incorporation of carbon from malonyl-CoA (which come mainly from HAc) during the elongation process, it's appropriate to calculate the OCFA yield in mol-C/mol-C based on the total substrate consumed. A complete carbon balance is also difficult to obtain because no metabolite or CO₂ analysis were performed. Furthermore, the biomass composition is affected by lipid accumulation and may therefore vary between cultivation conditions, requiring empirical determination for each condition. Consequently, the mol-C biomass yield was not included in Table 4, and performance is instead discussed in terms of titer and yield (g/g).

Table 4. Comparison of different performance parameters at final cultivation point in HAC:HP_r 2:1 (10:5 g/L) cultivation medium.

Yeast	HAC:HP _r											
	<i>C. oleaginosus</i>				<i>R. toruloides</i>				<i>Y. lipolytica</i>			
C/N	50	100	200	Unit	50	100	200		50	100	200	
Time	118	118	118	Hours	118	118	118		118	118	118	
HAc*	10.3	9.8	9.8	g/L	10.3	9.7	9.5		10.3	9.6	8.7	
HP_r*	5.1	5.0	5.0	g/L	2.8	1.3	0.9		4.6	3.8	2.5	
CDW - L (x-L)	2.3	1.8	1.1	g/L	1.9	1.9	1.7		3.0	3.0	2.1	
Y_{x-L/S}*	0.15	0.12	0.08	g/g	0.15	0.17	0.16		0.20	0.22	0.19	
Tot Lipids	43 ± 3.47	54 ± 0.85	59 ± 1.09	% w/w	36 ± 1.29	38 ± 0.95	46 ± 2.31		12 ± 0.71	14 ± 0.36	21 ± 0.52	
Tot C in FAs	54.2	80.2	49.9	mmol-C/L	28.3	32.3	35.9		17.1	22.8	30.8	
q_{FA}	7.26	10.74	6.67	mg/L/h	3.80	4.33	4.81		2.29	3.05	4.11	
Γ_{FA}	3.17	5.97	5.90	mg/g _{x-L} /h	1.97	2.30	2.90		0.76	1.01	1.96	
Y_{FA/S}*	0.098	0.15	0.094	mol-C/mol-C	0.062	0.086	0.10		0.032	0.048	0.079	
Y_{FA/S}*	0.06	0.09	0.05	g/g	0.03	0.05	0.05		0.02	0.03	0.04	
Tot OCFAs	0.36	0.92	0.48	mmol/L	0.63	0.60	0.64		0.091	0.095	0.095	
Tot C in OCFAs	6.1	15.8	8.3	mmol-C/L	11.4	10.6	11.1		1.5	1.6	1.6	
q_{OCFA}	0.82	2.12	1.11	mg/L/h	1.54	1.44	1.50		0.21	0.21	0.22	
Γ_{OCFA}	0.36	1.18	0.98	mg/g _{x-L} /h	0.80	0.76	0.90		0.07	0.07	0.10	
Y_{OCFA/S}*	0.011	0.030	0.016	mol-C/mol-C	0.025	0.028	0.031		0.003	0.003	0.004	
Y_{OCFA/HP_r}*	0.51	1.37	0.72	% mol/mol	1.65	3.33	5.20		0.15	0.19	0.28	
Citrate	3	4	4	mmol-C/L	4	5	4		17	16	42	
Y_{cit/S}*	0.01	0.01	0.01	g/g	0.01	0.01	0.01		0.04	0.04	0.12	
Y_{cit/S}*	0.006	0.008	0.007	mol-C/mol-C	0.009	0.013	0.011		0.032	0.034	0.11	

* consumed substrate

x, biomass; cit, citrate; C, carbon; S, substrate; L, lipid titer

5.1.1 *Cutaneotrichosporon oleaginosus*

C. oleaginosus proved to be the best performing yeast across all TRYs and in all C/N ratios (Table 4) compared to other yeasts. The highest FA production was obtained at **C/N 100**, reaching 1.01 g/L ECFAs and **0.25 g/L OCFAs** (Figure 10A), with OCFAs representing 20% of total FA profile (Figure 10B). The highest total lipid content is, however, at C/N 200 reaching approximately 60% (w/w) with a evident decrease in final biomass titer and yield (g/g). These relatively high titers are supported by the yeast's strong ability to fully consume all available substrates within 118 h in all C/N ratios. These results are consistent with previous literature studies. The cultivation of this strain in a mixture of 30 g/L (C/N ratio 62) HAc:HPr:butyric acid (6:3:1) showed full HAc and HPr consumption (130). Similarly, the cultivation of this strain with HAc:HPr 1:1 (15 g/L each) at C/N 100 resulted in 91% of substrate utilization (136). Interestingly, the cultivation of the closely related *Cutaneotrichosporon curvatum* yeast in 15 g/L SCFA (from C2 to C6) at C/N ratio of 80, 200 and 232 revealed that the C/N ratio did not influence the SCFA consumption and the biomass titer was significantly decreased at C/N 232 (118).

However, only 15% of the total carbon consumed was directed toward FA biosynthesis with only 3% incorporated in OCFAs, in line with the high ECFA titers observed (Figure 10C). However, only 1.37% of the consumed moles of HPr were directed toward OCFA production, indicating that the majority of HPr was utilized by the MCC pathway or other cellular processes. The relatively high OCFA titers are more reasonably attributed to the strain's strong overall FA production capacity rather than a specific efficiency in OCFA synthesis. The high OCFA titers are likely explained by its ability to completely consume the HPr available in the medium. This highlights the need for further medium optimization in terms of substrate concentration ratios, diversification of SCFA mixtures, and targeted strain engineering to redirect more carbon towards OCFA synthesis. For instance, another study achieved 1.37 g/L OCFAs using a 5:15:10 HAc:HPr:butyric acid at a C/N ratio of 100, where HAc was suggested to promote lipid production, while butyric acid promoted both OCFA and other FA accumulation, thereby supporting biomass formation and lipid accumulation (136).

In conclusion, this yeast demonstrated a remarkable ability to utilize SCFA substrates and accumulate high amounts of intracellular lipids, resulting in relatively high OCFA titers and highlighting its potential as a chassis for industrial lipid production. However, further improvements are needed to establish it as an efficient chassis for OCFA production.

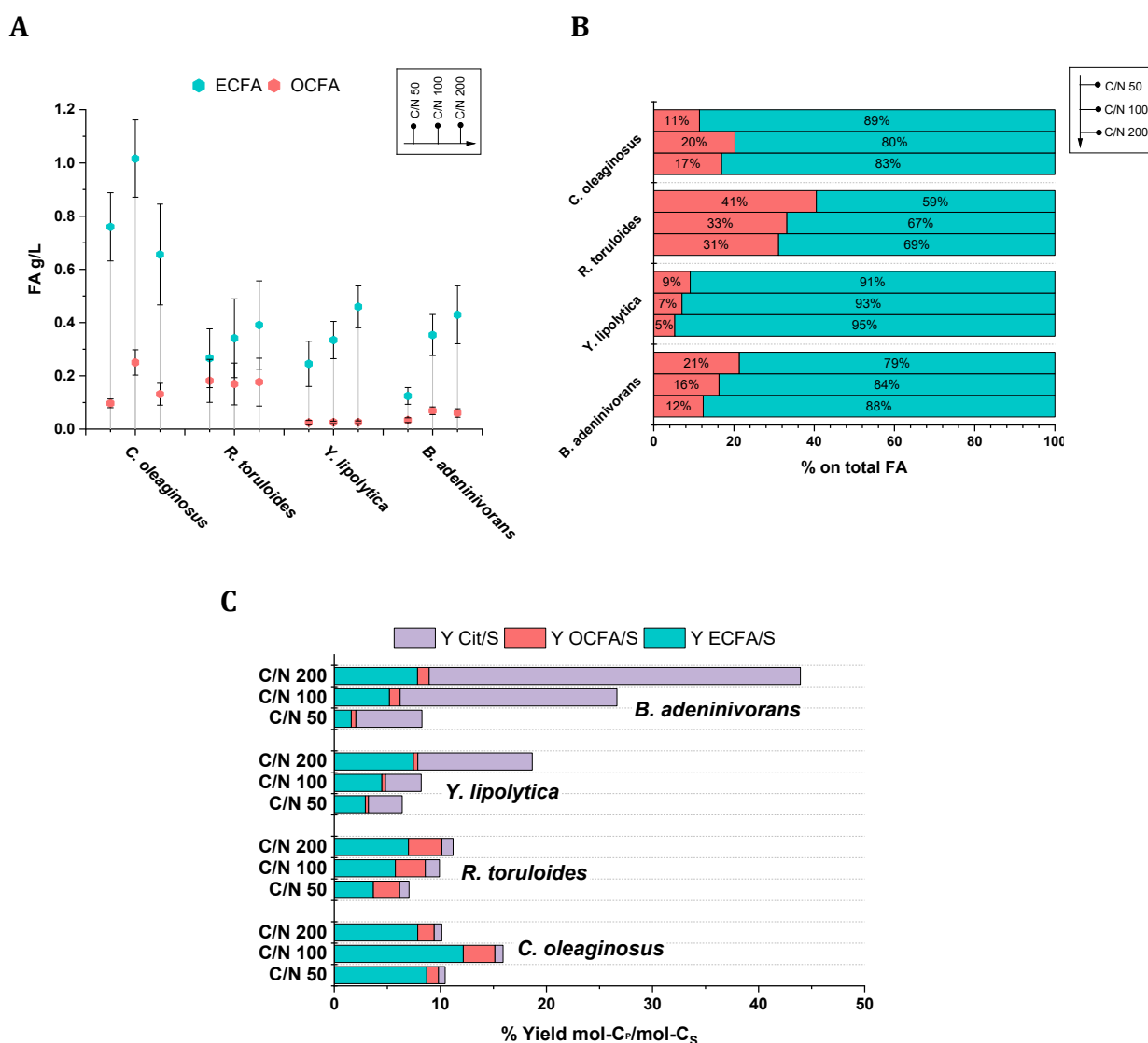


Figure 10. Titrers, yields and FA percentage in HAC:HPr condition. Panel A shows OCFA and ECFA titers in g/L in HAC:HPr. The C/N ratios are ordered ascending from left to right. Panel B shows the % of OCFAs and ECFAs on total FAs in the same conditions. The C/N ratios are ordered ascending from top to bottom. Panel C shows the percentage (%) of the mol-C_p/mol-C_s yields of citrate, OCFAs and ECFAs. Figure modified from Paper III. Cit, citric acid; P, product; S, substrate consumed

5.1.2 *Rhodotorula toruloides*

R. toruloides proved to fully consume all HAC at any given C/N ratios but struggle to consume HPr, more evident by the increase of the C/N ratio (Table 4) in accordance with previous results obtained in Paper II and from other literature studies (12, 129, 144). However, the yeast demonstrated the highest drive to direct HPr towards OCFA synthesis and the highest relative content of OCFAs in the total FA profile compared to other yeasts. The highest FA production was obtained at C/N 200, reaching 0.4 g/L ECFAs and 0.18 g/L OCFAs (Figure 10A), with OCFAs accounting for 31% of total FA profile (Figure 10B), and a total lipid content of approximately 46% (w/w).

However, it seems that the C/N ratios do not have much influence on the OCFA titers, but the yeast is able to drive a higher fraction of HPr consumed into OCFA synthesis in highly

nitrogen limited conditions (5.2% mol/mol, Table 4). These results suggest that, at low C/N ratios, a larger fraction of HPr internalized is used as an energy source through the MCC supporting biomass formation or other cellular processes. As the C/N ratio increases, less carbon needs to be assimilated due to the lower nitrogen availability, and a greater proportion of the HPr consumed is directed towards OCFA synthesis. However, beyond a certain threshold, increasing HPr consumption does not further increase OCFA synthesis, suggesting that the cells may already receive sufficient propionyl-CoA under all conditions and perhaps a stricter control on the FA profile is exerted or other enzymatic or cellular constraints are in play. The high OCFA yield may be explained by a recent study in which the Fas complex from *R. toruloides* was heterologously expressed in *S. cerevisiae*, demonstrating that RtFas has a high affinity for propionyl-CoA, with a higher binding energy than for acetyl-CoA (105). These findings, together with those from this thesis, suggest that Fas plays a crucial role in determining which precursor is preferentially used for FA production and that this preference is influenced by the intracellular availability of propionyl-CoA.

Overall, only 10% of the total carbon consumed is directed towards FA production with only 3% incorporated in OCFAs (Figure 10C), indicating that, other than the biomass, there are other carbon sinks that need to be addressed. In a recent study, a flux balance analysis of *R. toruloides* CCT 0783 (using data from turbidostat experiment) revealed that the highest theoretical lipid yields using HAc as the carbon source is 50% of the carbon consumed directed into lipid synthesis (145). Interestingly, since HAc is directly converted into acetyl-CoA via Acs, Acl seemed to be used minimally and about 51% of the HAc-derived carbon have been simulated to enter the glyoxylate shunt (109, 145). The glyoxylate shunt bypasses the Icdh step in the TCA cycle and might serve to provide reduced cofactors for respiration and ATP production during the nitrogen limiting phase. However, in light of the results obtained in this thesis, the need for both process optimization and strain improvement to channel more carbon towards FA synthesis is evident.

All together, these results suggest that *R. toruloides* possesses an interesting metabolic driving force to channel propionyl-CoA towards OCFAs in nitrogen-limiting conditions, highlighting the remarkable yet largely untapped potential of this red yeast when cultivated under appropriate conditions.

5.1.3 *Yarrowia lipolytica*

Y. lipolytica, contrary to its extensive history as a model OY, was one of the lowest producers of lipids and OCFAs under all conditions tested. The highest FA production was obtained at C/N 200, reaching 0.46 g/L ECFAs and **0.03 g/L OCFAs** (Figure 10A), with OCFAs accounting for **5% of total FA profile** (Figure 10B) and a total lipid content of approximately 21% (w/w).

The OCFA TRYs seem unaffected by the C/N ratio, however they are the lowest among all the OYs tested, with a maximum of 0.28% of consumed moles of HPr directed towards OCFA synthesis (Table 4). Overall, 7.9% of the total carbon consumed is directed towards FA production with barely 0.4% incorporated in OCFAs (Figure 10C). These results strongly suggest that HPr is preferentially utilized as an energy source through the MCC (or other cellular processes), particularly given that a large fraction of the HPr present in the medium is readily consumed, and perhaps a higher affinity of the Fas complex towards acetyl-CoA as a primer for FAs in line with the relatively high ECFA titers.

Interestingly, this yeast **excreted** a large amount of **citric acid**, with 1.4 g/L at C/N 200, representing 11% of the total carbon being re-directed into this byproduct (Figure 10C). The release of citric acid increased under nitrogen-limiting conditions, suggesting that citric acid formation may become uncoupled from the lipid production machinery when carbon availability exceeds the capacity for lipid synthesis. This may reflect unbalanced carbon fluxes and indicate a threshold in the amount of carbon that the lipid production machinery can efficiently channel towards lipid accumulation in these yeasts. This could partially explain the lower lipid, FA, and OCFA titers, as citric acid is a fundamental precursor for FA synthesis. This phenomenon has also been seen in another study, where independently of the SCFA concentration, *Y. lipolytica* excreted 1.5 g/L citric acid at the end of fermentation with C/N 80 and 6 g/L at C/N 200–230 (118). Notably, similar behavior has been observed in nitrogen-limited media in the presence of excess glucose, glycerol or similar (which could hold for HAc as well), where a sequential production of intracellular lipid and then increased extracellular citric acid has been registered (146, 147). It has been seen that, during the initial stages of nitrogen limitation, lipids are first accumulated and then sequentially degraded, albeit some substrate remained into the medium, and this coincided with the secretion of citric acid (146, 147). This phenomenon has been reduced by cultivating the yeast in fed-batch fermentation mode with pulsed glucose (148). Another study suggested that the problem arises from a bottleneck at the Acl level. The K_m value for the endogenous Acl is very high (3.6 mM) (i.e. low affinity for citrate and only works at a high citrate concentration) and the heterologous expression of a high affinity Acl from *Mus musculus* ($K_m = 0.05$ mM) increased lipid production up to 200% in *Y. lipolytica* accompanied by a decrease of extracellular citrate by 30% (149).

Overall, these studies may reflect strain-dependent responses and/or a strong influence of cultivation conditions, highlighting the multifactorial regulation of lipid metabolism in this yeast and the resulting complexity of its metabolism when grown on SCFAs. Strain improvement will therefore be necessary to enhance carbon flux towards lipid production and, subsequently, improve OCFA synthesis.

5.1.4 *Blastobotrys adeninivorans*

B. adeninivorans produced the highest amount of biomass among the OYs tested at low C/N ratio but a relatively low amount of OCFAs. The highest FA production was obtained at C/N 200, reaching 0.43 g/L ECFAs and **0.06 g/L OCFAs** (Figure 10A), with OCFAs

accounting for **12% of total FA profile** (Figure 10B) and a total lipid content of approximately 25% (w/w). At low C/N ratio the total FA titers were very low while 3.8 g/L of lipid free biomass were produced, highlighting a strong preference to direct the consumed SCFAs towards biomass production when nitrogen is available.

Similarly to *Y. lipolytica*, this yeast also excreted a large amount of citric acid, 3.9 g/L at C/N 200, representing 35% of the total carbon being re-directed into this byproduct (Figure 10C). Furthermore, less than 9% of the total carbon consumed was directed towards FA production with about 1.1% incorporated in OCFAs (Figure 10C). However, it channeled about 1.78% of the HPr moles consumed towards OCFAs at C/N 200, which is the highest amount after *R. toruloides*. Likely due to this yeast's strong tendency to channel carbon towards biomass production, highly nitrogen-limiting conditions promote the redirection of carbon towards FA and OCFA production despite lower HPr consumption, thereby increasing their yields. Under less nitrogen-limiting conditions, HPr may instead be preferentially channeled towards detoxification through the MCC or other unknown pathways.

Overall, *B. adenivorans* demonstrated promising performance as an OCFA producer in SCFAs. Together with its favorable industrial characteristics, this makes it an attractive target for metabolic engineering to increase OCFA yields by improving carbon flux distribution, minimizing carbon loss, and reducing MCC activity to redirect more HPr towards OCFA production.

5.1.5 General Trends and Conclusions

It is difficult to generalize the results, as the performances were highly yeast specific, such as the metabolic responses determining how assimilated carbon was partitioned between OCFA and ECFA synthesis across the tested C/N ratios. However, some general considerations can be highlighted:

- i. *C. oleaginosus* consumed all HPr across all C/N ratios, whereas HPr consumption by the other yeasts decreased with increasing C/N ratio. This suggests that substrate internalization may be influenced by nitrogen availability in these yeasts, while *C. oleaginosus* appears to utilize HPr as readily as HAc, indicating that both substrates may be similarly valuable carbon sources. On the contrary, HAc was fully consumed under all conditions by all OYs, indicating that HAc is generally a more readily utilized and less toxic substrate than HPr, likely due to its direct entry into central carbon metabolism;
- ii. High C/N ratios generally increase total lipid content while decreasing biomass titer. However, the response of OCFA titers remains yeast-specific, whereas increasing the C/N ratio generally appears to promote greater incorporation of HPr into OCFAs;
- iii. The substantial diversion of carbon toward citrate may explain, at least in part, the lower lipid titers observed in the Ascomycota yeasts compared to the

- Basidiomycota yeasts and suggests an imbalance in central lipid carbon fluxes that leads to citrate overflow;
- iv. The fraction of consumed HPr diverted towards OCFA synthesis was relatively low across all yeasts, suggesting that most HPr was instead directed towards other metabolic pathways, most likely the MCC. This may indicate that routing HPr through the MCC is a preferred strategy for mitigating propionate toxicity and that the MCC may accommodate a greater carbon flux than the lipid biosynthetic machinery.
 - v. The limited FA titers might also be given by other unknown carbon sinks and the availability of NADPH and ATP in the respective yeasts.

5.2 Batch bioreactor cultivation of *C. oleaginosus*

Paper **II** and **III**, together with other research studies, proved *C. oleaginosus* to be a promising chassis for lipid and OCFA production in SCFAs medium. To better understand the physiology of this yeast, including the timing of nitrogen depletion and the onset of lipid accumulation, *C. oleaginosus* was cultivated in 600 mL **batch bioreactors** at the optimal C/N ratio identified previously (100), at pH 6.8, using HAc:HPr 2:1 (10:5 g/L) as cultivation medium (Figure 11) (Paper **III**).

The trends were similar to those obtained in flask cultivation. However, it was revealed that nitrogen was rapidly consumed and became fully depleted after 35-45 h of cultivation, corresponding to the middle of the exponential growth phase (Figure 11A). The total lipid concentration increased substantially from the middle-exponential phase (ME) to the late exponential/early stationary phase (EE), rising from 0.7 to 3.0 g/L. Similarly, the intracellular lipid content increased from 52 to 79% (w/w) of CDW (Figure 11B). However, no further significant increase in either lipid titers or intracellular lipid percentages was observed from the onset of stationary phase until the end of the cultivation (ES), likely due to HAc limitation occurring after approximately 100 h. High CO₂ production (~0.38% of the off-gas, Figure 11C) coincided with ammonia availability, indicating high respiratory activity. Following ammonia depletion, CO₂ evolution progressively decreased, suggesting a progressively lower biomass formation, respiratory activity and a metabolic shift towards lipid production which also produce CO₂.

Taken together, these observations suggest that the major phase of lipid accumulation begins after nitrogen depletion, despite cells still being in the exponential growth phase. During this phase, nitrogen is likely supplied by internal reserves (66), which triggers the metabolic shift toward lipid accumulation. Importantly, although HPr was also completely depleted after approximately 120 h, no substantial lipid turnover was observed, suggesting that it might be influenced by the limited availability of nitrogen. Furthermore, the data indicates that additional carbon availability during stationary phase could potentially have supported further lipid accumulation. It is worth noting that, although the final CDW is the same between the bioreactor and flask cultures, the yeast was able to

produce more lipids and accumulate a higher proportion of lipids within the cells at the end of cultivation (76% vs 54% w/w) under the more controlled bioreactor conditions. This lipid content is higher to the maximum lipid content reported in a pH-stat fed-batch cultivation in HAc as a sole carbon source which was 65% w/w at pH 8 (150). These results indicate that more controlled cultivation conditions, such as those provided by bioreactors, where oxygenation, pH, and agitation can be closely regulated, positively influence lipid accumulation.

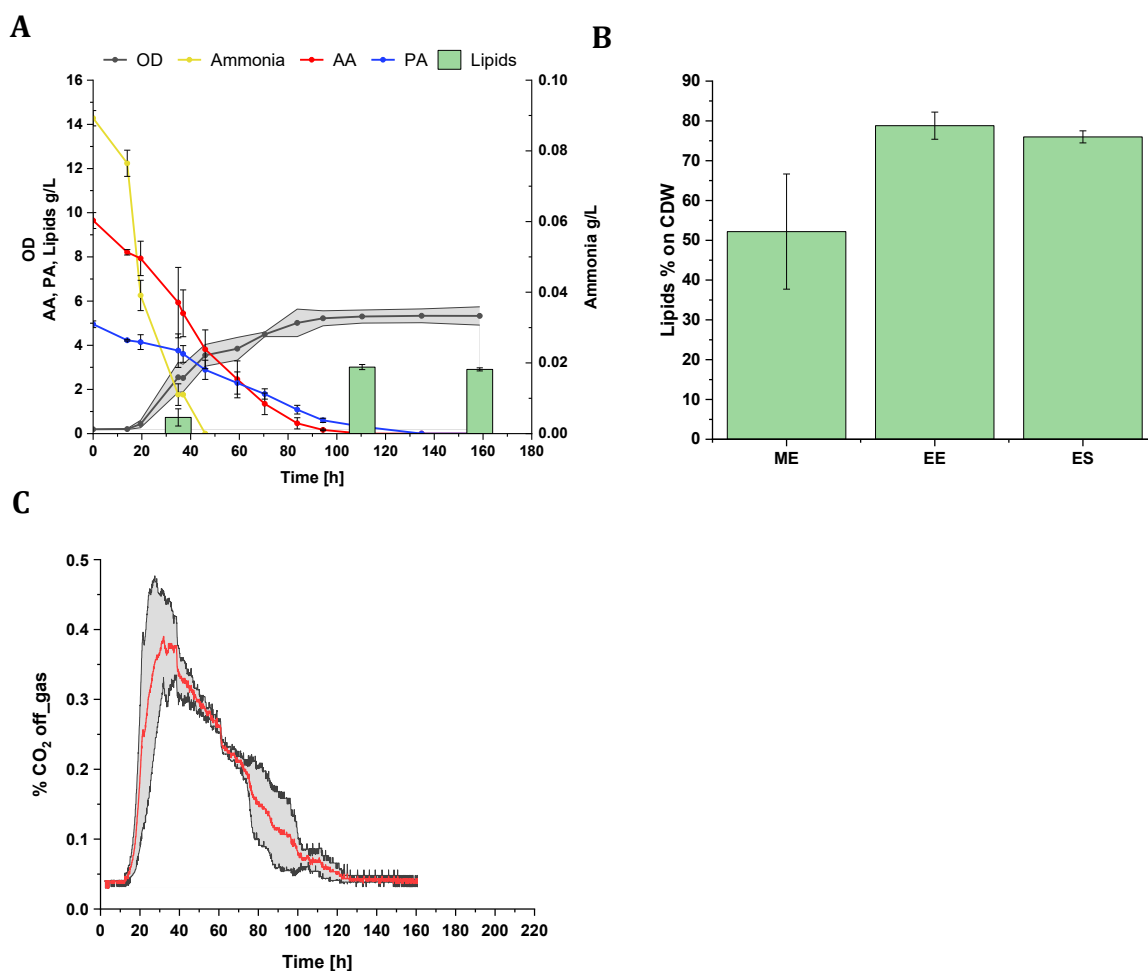


Figure 11. Batch bioreactor results from *C. oleaginosus* grown in HAc:HP. Panel A shows growth curve, substrates consumption curves and lipid titers. Panel B shows total lipid content (% w/w) in different phases of the yeast growth. Panel C shows the percentage of CO₂ in the off gas. Figure modified from Paper III.

ME, middle of exponential; EE, end of exponential; ES, end of stationary phase.

Chapter 6 – Paving the Way for Future “Odd” Cell Factories

So far, screening and characterization have been at the core of the research, making strain improvement the final step towards completing this work (Paper IV). Based on the physiological characterization, *B. adenivorans* emerged as a promising candidate for strain improvement toward producing OCFAs. Therefore, in the following chapter, the application of the novel plasmid-based CRISPR-Cas9 genome-editing tool is explored by performing disruption of a selected gene involved in propionyl-CoA metabolism.

6.1 Strain Improvement Through Metabolic Engineering

Traditional genetic modification primarily focuses on establishing genotype-phenotype relationships, whereas metabolic engineering aims to deliberately introduce or modify metabolic pathways to redirect cellular carbon flux and enhance the production of a specific compound, or improve a desired cellular trait (151, 152). For industrial purposes, the main concept is to **build a functional and high-performing cell factory with improved TRYs** of the desired product by increasing the metabolic flux towards the product, while reducing fluxes toward competing pathways.

Identifying yeasts that naturally possess the desired pathways and phenotypes is highly valuable, as metabolic engineering is challenging due to the interconnected and often unpredictable nature of cellular metabolism. Furthermore, strain engineering can cause metabolic imbalances, substrate and cofactor competition, toxic metabolite accumulation, reduced fitness, and increased metabolic burden (151). Nevertheless, iterative design-build-test-learn cycles can enable the development of high-performing cell factories with improved TRYs and industrial relevance.

6.1.1 Typical Target Genes to Improve Lipid Production

Metabolic engineering of OYs can generate high lipid-producing, or obese, strains by targeting lipid-related genes. As discussed in *Chapter 4*, lipid accumulation is supported by distinctive metabolic features of OYs, including high Acl activity and Icdh inactivation under nitrogen limitation. Accordingly, heterologous expression of a high affinity Acl in *Y. lipolytica* increased lipid accumulation (149). However, lipid biosynthesis involves multiple enzymes, sequential reactions, and cellular compartments, making the pathway highly interconnected. Consequently, manipulating a single gene often provides limited improvements, as additional metabolic bottlenecks may remain elsewhere in the pathway.

Among the enzymes involved in *de novo* neutral lipid biosynthesis, Acc, Fas, and diacylglycerol acyltransferase (Dgat) are considered key regulatory enzymes (69). In *Y. lipolytica*, lipid accumulation has been enhanced by overexpressing genes involved in central lipid metabolism, including *ACL1*, *ACL2*, *MAE*, and *DGAT*, combined with deletion of

β -oxidation genes (*PEX10* and *MFE2*), resulting in lipid contents above 90% of CDW (153). Similarly, overexpression of *DGA1* and *DGA2* combined with deletion of the lipase gene *TGL3* increased lipid yield (154). In *R. toruloides*, overexpression of *ACC1*, *DGA1*, and *SCD* increased lipid titers to 89.4 g/L (155). Numerous additional engineering targets have been reviewed elsewhere (69), reflecting extensive efforts to improve lipid TRYs in OYs over the past two decades.

In conclusion, the most common strategies to increase carbon flux towards lipid biosynthesis combine **overexpression of rate-limiting enzymes with deletion of competing pathways** involved in lipid turnover, such as lipases and β -oxidation.

6.1.2 Strategies to Improve OCFA Production

OCFA production is a relatively recent research area, driven by growing interest in these FAs for nutritional and medical applications. Consequently, only a limited number of studies have investigated strategies to enhance OCFA production in OYs, with most focusing on *Y. lipolytica*. These strategies are briefly discussed in this chapter.

One of the pioneering studies on OCFA overproduction in *Y. lipolytica* was conducted by Y. K. Park *et al.*, who investigated several engineering strategies targeting HPr or glucose utilization (139). In 2018, **deletion of *PHD1*** gene, which encodes the 2-methylcitrate dehydratase of the MCC pathway, was shown to enhance OCFA production, reaching 47% of OCFAs on the total FA profile in strains grown on glucose and HPr (139). Combining this modification with an **enhanced lipid-producing background** (deletion of *MFE2* and *TGL4*, and overexpression of *DGA2* and *GPD1* (glycerol-3-phosphate dehydrogenase)) further increased OCFA production to 0.57 g/L, with C17:1 as the predominant OCFA (139).

In a 2020 study, Y. K. Park *et al.* aimed to **increase the intracellular pool of odd-chain precursors**, including propionyl-CoA and β -ketovaleryl-CoA. They heterologously expressed propionyl-CoA transferase (Pct) and β -ketothiolase (BktB) from *Ralstonia eutropha* in the engineered *Y. lipolytica* strain developed in 2018, additionally overexpressing *LDP1*, which encodes a lipid droplet protein (138). Pct transfers CoA from a donor, typically acetyl-CoA, to an acceptor, while BktB condenses acetyl-CoA and propionyl-CoA to form β -ketovaleryl-CoA. These modifications increased OCFA accumulation by 33% in glucose:HPr:HAc medium compared with the control, reaching 1.32 g/L (138).

Finally, in the same year, Y. K. Park *et al.* investigated ***de novo* OCFA production** in *Y. lipolytica* **from glucose** without HPr supplementation by exploiting pathways that generate intracellular propionyl-CoA from glucose-derived metabolites (137). A modular pathway comprising three modules and seven genes was constructed to enhance the α -ketobutyrate pathway, which converts oxaloacetate to α -ketobutyrate via threonine and subsequently to propionyl-CoA through the native pyruvate dehydrogenase complex. The resulting strain accumulated OCFAs to 3.86% of the total FA profile. Introducing the

pathway into an obese strain further increased OCFA production 7.2-fold compared with the control, reaching 0.36 g/L (137).

Another strategy was explored by Tabaa Chalabi *et al.* in 2024, who **overexpressed the $\Delta 9$ FA desaturase (*OLE1*) and *DGA2*** in *Y. lipolytica* while optimizing sodium propionate and sodium acetate concentrations in a sugar beet molasses + crude glycerol cultivation medium (156). The optimal sodium propionate to acetate ratio for cell density and C17:1 production was approximately 1:7.8. This significantly increased OCFA content, with C17:1 reaching ~46% of the total FA profile but did not increase total lipid content (156).

A further engineering approach was reported by Messina *et al.* in 2023. **Deletion of *CRC1***, encoding a mitochondrial carrier involved in the carnitine/acetyl-carnitine shuttle, was combined with **heterologous expression of Pct** from *R. eutropha* (157). This strategy enhanced HPr utilization as a co-substrate to glucose and increased HPr conversion into OCFAs, reaching ~5.5% on dry biomass and 0.5 g/L (157).

Finally, Meng *et al.* in 2025 engineered *S. cerevisiae* for *de novo* OCFA production by **enhancing threonine biosynthesis** through heterologous enzyme expression, silencing the native Fas, and **introducing the Fas from *R. toruloides***, which showed higher affinity for propionyl-CoA (105). These modifications increased OCFAs to ~52% of total FAs. Further engineering of lipid metabolism enabled the production of odd-chain FFAs at up to 0.18 g/L (105).

6.2 CRISPRoZoic Era

CRISPR, which stands for Clustered Regularly Interspaced Short Palindromic Repeats, is a versatile genome-editing technology derived from the adaptive immune systems of bacteria and archaea. The CRISPR system was first identified in 1987 as a mechanism protecting bacteria against invading genetic elements such as viruses and bacteriophages (158). In 2012, Jennifer Doudna and Emmanuelle Charpentier demonstrated its potential as a programmable gene-editing tool, for which they were awarded the Nobel Prize in Chemistry in 2020. Owing to its high efficiency, simplicity, and programmability, CRISPR has revolutionized genetic engineering and is now widely used in medicine, agriculture, and biotechnology.

The CRISPR–Cas9 system primarily consists of the Cas9 (CRISPR-associated protein 9) endonuclease and a guide RNA (gRNA). Cas9 cleaves target DNA, while the sgRNA contains a sequence complementary to the target and directs Cas9 to the corresponding genomic locus through base pairing. Upon target recognition, Cas9 introduces a site-specific double-strand break, although off-target cleavage can also occur. The resulting DNA break is repaired by the host cell through either non-homologous end joining (NHEJ) or homologous recombination (HR). NHEJ is active throughout the cell cycle and does not require an exogenous DNA template. It is error-prone and typically introduces small insertions or deletions at the cleavage site, which can cause frameshift mutations or

premature stop codons and thereby disrupt gene function (158). The HR is a very precise repair mechanism, most active in the late S and G2 phases, and it needs a donor DNA template to repair the double-strand break (158). These DNA repair pathways are utilized in many genome-engineering strategies. HR is the dominant DNA repair pathway in *S. cerevisiae*, while in most other yeasts NHEJ is the favored one. This influences the feasibility and efficiency of classical genetic modification and genome-editing approaches (159, 160).

The mutants generated in this thesis were mutants which carried disrupted *PHD1* gene with mainly insertion of one or two nucleotides which causes shifts of the open reading frame resulting in truncated proteins and affecting protein function (see Paper III, Figure 6A). The mutants were designed both to improve the understanding of *B. adenivorans* metabolism and to develop a higher-performing OCFA cell factory. Thus, genome editing to decipher genotype-phenotype connections and metabolic engineering represent overlapping objectives in this work.

6.3 Initial Steps to make *B. adenivorans* an OCFA Cell Factory

In mixed HAc:HPr media, HAc is assumed to primarily support carbon and energy metabolism, whereas HPr may be directed towards OCFA synthesis. However, as clarified by the results presented in Chapter 5, HPr is preferentially metabolized through the MCC, likely to mitigate HPr toxicity. A disruption of *PHD1* gene, may, hypothetically, reduce the MCC activity and redirect propionyl-CoA towards OCFA synthesis. This approach was evaluated in Paper IV.

6.3.1 Conservation and Divergence of Lipid Synthesis Pathways

To be able to perform genome editing, it is essential to identify target genes and their sequences to design the gRNAs for the Cas9. Due to the lack of a well-annotated genome for *B. adenivorans* NRRL Y-17692, the annotated genome of *B. adenivorans* LS3 was used to identify putative orthologs in pathways for HPr metabolism and FA synthesis and degradation, using protein sequences from the well-characterized *Y. lipolytica* CLIB89 (W29) strain as queries (Table 5). *B. adenivorans* LS3 nucleotide sequences were used in BLASTn searches against the *B. adenivorans* NRRL Y-17692 genome. The sequence identity was high in all cases (data not shown), suggesting a strong conservation of core metabolic functions between the two *Blastobotrys* strains.

The analysis indicated that **metabolic pathways** involved in carbon assimilation and FA synthesis are **largely conserved** in *B. adenivorans*, at least at the protein sequence level (Table 5). The MCC was identified in *B. adenivorans*, with proteins showing 67.1-83.1% sequence identity to their *Y. lipolytica* counterparts. Similarly, high sequence conservation was observed for most proteins of the malate-citrate cycle and the FA synthesis pathway, including enzymes involved in ER-associated elongation and desaturation.

Table 5. Details of aligned protein sequences from *Y. lipolytica CLIB89 (W29)* to *LS3 strain*.

Metabolic pathway	Yl protein	Function	Yl Gene ID	LS3 Gene ID	Score	E-value	% identity	% Subj.Cov.
Methyl citrate cycle	Cit1	Mitochondrial 2-methyl citrate synthase	YAL1E01019	ARAD1D42504	1.845	0.00E+00	80	95
	Phd1	2-methylcitrate dehydratase	YAL1F03803	ARAD1D07942	2.116	0.00E+00	81.6	93.9
	Aco1	Mitochondrial aconitate hydratase	YAL1D11984	ARAD1D26070	3.329	0.00E+00	83.1	99.9
	Aco2	Mitochondrial aconitate hydratase	YAL1E17935	ARAD1C43692	2.861	7.69E-134	72.5	92.3
Malate citrate cycle	Icd2	Methylisocitrate lyase	YAL1F39620	ARAD1B15796	2.027	0.00E+00	67.1	98.2
	Mdh2	Cytoplasmic malate dehydrogenase	YAL1E17214	ARAD1D04884	1.381	0.00E+00	80.4	67.3
	Mae1	Malic enzyme	YAL1E22303	ARAD1D04114	1.475	9.37E-72	55.6	86.2
	Pyc1	Pyruvate carboxylase	YAL1C33152	ARAD1D32912	4.959	0.00E+00	81.1	96.9
Intermediate pathway enzymes	Acl2	ATP citrate lyase	YAL1D32268	ARAD1D32164	2.174	1.97E-157	81.9	101.2
	Acl1	ATP citrate lyase	YAL1E41315	ARAD1B07414	2.955	0.00E+00	87.2	99.8
	Pdc1	Pyruvate decarboxylase	YAL1D12832	ARAD1D26004	1.894	5.14E-94	66	94.1
	Acs1	Acetyl-CoA synthetase	YAL1F08845	ARAD1D12826	2.677	0.00E+00	75.6	99.2
FAS cycle	Acc1	Acetyl-CoA-carboxylase	YAL1C15991	ARAD1D05412	8.175	0.00E+00	75.1	92.3
	Fas1	Fatty acid synthase subunit β	YAL1B19844	ARAD1A16258	7.591	0.00E+00	69.7	97
	Fas2	Fatty acid synthase subunit α	YAL1B25427	ARAD1A16676	6.852	0.00E+00	69.8	100.6
ER FA elongation	Elo1	Fatty acyl-CoA elongase (LCFA)	YAL1B26350	ARAD1C21098	1.072	2.34E-90	70.7	83.8
	Elo2	Fatty acyl-CoA elongase (VLCFA)	YAL1A05116	ARAD1D30294	712	3.39E-37	54.3	68.4
ER FA desaturation	Ole1	Delta-9 fatty acid desaturase	YAL1C07638	ARAD1D01914	1.663	0.00E+00	66.9	90.3
	Fad2	Delta-12 fatty acid desaturase	YAL1B13699	ARAD1D11396	1.433	6.67E-108	64.5	93.5
TAG mobilization	Tgl4	Principal TAG-degrading lipase	YAL1F13550	ARAD1C00528	1.349	8.61E-60	52.6	74.7
	Tgl3	Supporting TAG-degrading lipase	YAL1D21511	ARAD1D06072	1.018	8.32E-42	45.6	70.5
β -oxidation	Fat1	LCFA acyl-CoA synthetase and LCFA transporter	YAL1E19172	ARAD1D34980	1.505	2.87E-80	56.3	76.7
	Pox2	Fatty-acyl-CoA oxidase	YAL1F14495	ARAD1C08998	2.363	0.00E+00	63.5	97.3
	Mfe2	Multifunctional β -oxidation enzyme	YAL1E18441	ARAD1C36784	4.53	6.11E-152	62.5	140.6
	Pat1	Acetyl-CoA C-acetyltransferase	YAL1B11532	ARAD1D47278	1.394	2.58E-52	65.3	102
	Pot1	3-ketoacyl-CoA thiolase	YAL1E22338	ARAD1D09306	1.507	1.23E-100	69.6	101.2

ATP, adenosine triphosphate; CoA, coenzyme A; ER, endoplasmic reticulum; Subj. Cov., subject coverage

In contrast, lower sequence identities were observed for enzymes involved in TAG mobilization, including Tgl3 (45.6%) and Tgl4 (52.6%), and for several β -oxidation enzymes (56.3-69.6%) (Table 5). The comparison also revealed lineage-specific changes in gene family size. For example, whereas *Y. lipolytica* contains six fatty acyl-CoA oxidases (Pox1-6), only a single homolog was identified in *B. adeninivorans*, suggesting gene expansion occurred after species divergence. Conversely, the single copy thiolase Pot1 of *Y. lipolytica* corresponded to two homologs in *B. adeninivorans*, indicating gene duplication in the latter species. These differences, together with the lower conservation of TAG mobilization and β -oxidation pathways, suggest an evolutionary divergence in FA turnover and utilization. However, sequence comparisons alone are insufficient to infer functional differences, which require biochemical and physiological validation.

6.3.2 Disruption of *PHD1* Gene Increase OCFA Content

Based on the metabolic reconstruction of FA biosynthesis pathways in *B. adeninivorans*, a single homolog of *PHD1* gene was identified, sharing 81.6% protein sequence identity with the corresponding enzyme in *Y. lipolytica* CLIB89. Disruption of *PHD1* gene abolished growth on HPr as the sole carbon source for up to 96 h, after which growth resumed in some strains, suggesting genetic reversion (data not shown). This phenotype indicates that *PHD1* is likely present as a single-copy gene and confirms its essential role in HPr catabolism through the MCC in *B. adeninivorans*.

To investigate the metabolic consequences of the MCC disruption, the mutant was cultivated in HAC:HPr medium at a C/N ratio of 100. The wild type strain reached an OD of 12 within 72 h, completely consumed HAC, and utilized 51.7% of the supplied HPr (Figure 12A). In contrast, the ***phd1* mutant showed impaired growth**, reaching a final OD of only 6.6 after 118 h. HAC consumption was slower, and HPr utilization decreased to 24.6%. These results suggest that disruption of the MCC strongly affects carbon metabolism and may lead to intracellular accumulation of propionyl-CoA and related toxic intermediates. Similar effects have been reported in *Y. lipolytica* cultivated in glycerol under nitrogen or carbon limiting conditions, where a *PHD1* gene knockout impaired TCA cycle activity, reduced biomass formation, and increased lipid accumulation through the accumulation of 2-methylcitrate (146). The 2-methylcitrate synthase, at the first step of the MCC, accepts both propionyl-CoA and acetyl-CoA, thereby contributing to both the MCC and citrate formation in the TCA cycle. Inactivation of *PHD1* gene causes 2-methylcitrate accumulation, which inhibits aconitase and may consequently impair TCA cycle activity (146). Thus, *PHD1* gene inactivation could mimic nitrogen limitation by promoting citrate accumulation and its export from mitochondria for secretion and/or lipid synthesis. The observed growth defects in *B. adeninivorans* may also be consistent with perturbation of TCA cycle function, an effect that could be even more pronounced because the yeast were cultivated under nitrogen-limiting conditions. Therefore, the cultivation of this mutant in excess nitrogen might lead to higher lipid or OCFA accumulation.

To verify that the internalized HPr was partially sent towards OCFA synthesis, the FA profile of the wild type strain and the *phd1* mutant was analyzed. The wild type strain FA profile was dominated by ECFAs, like C18:1 and C18:2, while OCFA accounted for 14%. Remarkably, the ***phd1* mutant accumulated 44% OCFA**s, primarily C17:1, C17:0 and C19:1. This shift was accompanied by a pronounced reduction in C18:1 suggesting that the increased propionyl-CoA pool competed with acetyl-CoA as the primer for FA synthesis. These observations are consistent with studies in *Y. lipolytica*, where deletion of the homologous *PHD1* gene blocked MCC activity, increased intracellular propionyl-CoA availability, and elevated OCFA accumulation [138, 139].

Together, these results demonstrate that MCC disruption is an effective strategy for redirecting carbon flux toward OCFA synthesis in *B. adeninivorans*.

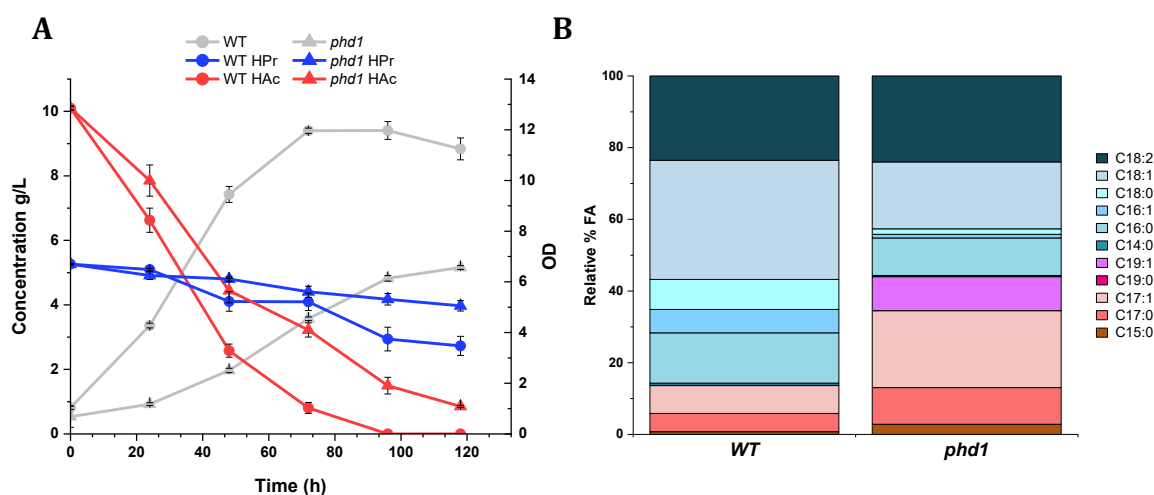


Figure 12. Phenotyping of *phd1* mutants. Panel A shows growth and substrate consumption curves of WT and *phd1* mutant grown in HAc:HPr medium. Panel B shows the FA profile of *phd1* mutant. Figure modified from Paper IV.

6.3.3 Main Conclusions

Despite the successful disruption of *PHD1* gene and the resulting increase in OCFA content, it is important to consider that the genetic modification consisted only of small insertions or deletions (indels), involving the gain or loss of a few nucleotides. These mutations alter the open reading frame and are expected to produce a non-functional protein. Although such modifications are generally sufficient for rapid phenotype assessment, they may be susceptible to genetic reversion. In other words, over multiple generations of growth, the mutation may be lost, through naturally occurring DNA replication errors and/or as a consequence of selective pressures that favor cells in which *PHD1* gene function has been restored. The resulting revertant subpopulation could eventually outcompete the mutant cells and dominate the culture. Therefore, the next step will be to generate complete gene knockouts by introducing a larger DNA cassette flanked by homology regions at the Cas9-

induced double-strand break site. This strategy would ensure permanent gene disruption and substantially reduce the likelihood of genetic reversion.

Further enhancement of OCFA production in *B. adenivorans* likely requires the development of an *obese* strain with an increased capacity for lipid accumulation and production. This could be achieved through the combinatorial engineering of key metabolic pathways, including the overexpression of genes involved in precursor supply of FA biosynthesis, FA elongation and TAG synthesis in parallel with the disruption of competing pathways, such as the β -oxidation pathway, in the *phd1* mutant background. Collectively, these modifications may improve FA synthesis and consequently OCFA titers and ultimately enable the development of *B. adenivorans* as an efficient microbial cell factory for OCFA production.

Chapter 7 - Conclusions and Outlook

This chapter brings together the main findings, key scientific contributions, and insights gained throughout the research. It critically discusses the significance of the work, identifies its main limitations and potential areas for improvement, and provides an outlook on future research directions to advance the field of OCFA production.

7.1 Key insights

In this thesis, an exploratory research effort provides insights into OCFA synthesis in several OY species under different growth conditions. Four promising candidate OYs were selected for further characterization and development toward industrially relevant performance. Although much remains to be explored and understood, the research presented across the four scientific papers establishes fundamental knowledge about OCFA analysis, SCFA utilization for OCFA biosynthesis, the characteristics of well-known and unconventional OYs, and OCFA metabolic engineering. Together, these findings provide important building blocks for advancing the field of OCFA production. The main insights are summarized as follows, divided by the research questions posted in the introduction section.

How can an existing fatty acid methyl ester (FAME) protocol be adapted and optimized for OCFA analysis?

OCFA analysis has been widely overlooked in analytical chemistry since they have often been considered being irrelevant. In Paper I, it was proven that an existing FAME protocol can be adapted for OCFA analysis. The most important insights are:

- i. Separation of TAG extraction from the transesterification step proved to be a successful strategy for improving TAG recovery and sample stability in the microwave-assisted protocol. The **introduction of a bead-based extraction step** enhanced TAG recovery compared with microwave-assisted extraction alone and eliminated the problem of microwave-induced biomass burning.
- ii. A fully deuterated IS (C14:0-d₂₇) initially proved challenging to implement but ultimately provided a suitable means of normalizing data for the quantification of OCFAs and ECFAs. Higher accuracy was achieved with the **pre-methylated internal standard** since the esterification efficiency of non-methylated dIS has high variation.
- iii. Importantly, the **qualitative FA profiles** were highly reproducible and remained consistent across all protocol modifications, underscoring the **robustness** of both the FA profiles and the relative FA composition .

Conclusions and Outlooks

Overall, this work highlights the importance of carefully adapting and re-evaluating the FAME protocol whenever a new IS is introduced or any parameter or procedural step is modified, to ensure the highest possible analytical quality, accuracy, and precision.

Are there any yeast species that naturally produce high levels of OCFAs when cultivated on glucose? Are there yeasts that can tolerate high concentrations of HPr and also produce OCFAs? How does the substrate influence the FA profile?

Very little is known in the scientific literature about OCFA synthesis in OYs, as interest in the production of these uncommon FAs has increased only in recent years. Therefore, in Paper II, the screening of multiple OYs represented a valuable starting point for uncovering their unexplored capabilities and identifying potential OCFA-producing cell factories, while also providing insights into the influence of substrate type on the resulting FA profile. The most important insights are:

- i. Glucose supplementation generally resulted in FA profiles enriched in ECFAs while **HPr supplementation resulted in FA profiles enriched in OCFAs**. Only minor amounts of OCFAs were detected in glucose-based media, with their abundance varying among species. This finding suggests that the conversion of glucose into propionyl-CoA is not a prioritized metabolic pathway in most OYs.
- ii. Eight of the nineteen OYs were able to grow on HPr at near-neutral pH, indicating that they possess both tolerance mechanisms and the metabolic capacity to utilize this substrate. All eight yeasts could produce and accumulate OCFAs during growth on HPr; however, their **FA profiles differed markedly among species**. These results highlight the strong species-specific nature of OCFA biosynthesis and accumulation.

Overall, this screening highlighted eight OYs that can grow and accumulate ECFAs and OCFAs in pure HPr, some of which were never tested before. These results expand the portfolio of potential OY hosts for OCFA production and highlight the strong influence of the substrate choice on the FA profiles.

Will a mixture of HAc and HPr improve the OCFA titers and yields? Does the C/N ratio influence OCFA production?

To better explore the utilization of SCFAs, it is important to understand how the combination of SCFAs might affect OY metabolism. The characterization carried out in Paper III resulted in valuable insight into OCFA TRYs and FA profile ratios (compared to FA profiles obtained in Paper II in glucose and HPr media). The most important insights are:

- i. HAc has similar metabolic effects as glucose, as both HAc and glucose are metabolized via acetyl-CoA. As a result, **FA profiles in HAc:HPr were enriched in ECFAs** compared to those obtained in HPr medium, as HPr leads mainly to propionyl-CoA.
- ii. A **low OCFA yield in mol/mol on consumed HPr** was evident, suggesting that the majority of the HPr consumed was still metabolized as carbon and energy source in the MCC or directed towards other unidentified carbon sinks. Overall, performance analysis pinpointed that only a small fraction of the total carbon consumed was incorporated into FA and OCFAs, highlighting that all the wild type strains analyzed require substantial strain improvement to increase FA synthesis.
- iii. Nitrogen-limited conditions resulted in greater total lipid and FA accumulation, indicating that lipid accumulation in HAc:HPr medium may be regulated by mechanisms similar to those observed during growth on glucose. However, strong differences were observed among yeast species and across C/N ratios related to HPr consumption. **Increasing the C/N ratio** generally appears to **promote greater incorporation of HPr into OCFAs**.

All OY species tested demonstrated, to varying degrees, the potential to serve as OCFA-producing cell factories. The most important insights are:

- i. *C. oleaginosus*, stood out as the **best lipid producer**, capable of fully consuming HPr and producing the highest OCFA titer among the tested OYs. This yeast also proved to be the best in terms of volumetric productivity, highlighting the industrial relevance of this OY.
- ii. *R. toruloides*, possessed the capability to drive the **highest fraction of the consumed HPr towards OCFAs**, despite showing low HPr consumption. If the ability to take up HPr could be improved, the OCFA titers on HPr consumed might increase substantially.
- iii. *Y. lipolytica*, was the least promising strain in this screening, with very low OCFA yields and a tremendous carbon loss. However, being an OY model, many tools are available to engineer it, and many studies have already proved that deletion and overexpression of certain genes improve OCFA titers.
- iv. *B. adenivorans* is a rather underexplored yeast, which demonstrated robust growth on HPr, produced good biomass titers and could drive HPr towards OCFA synthesis. However, its strong overflow of citric acid suggests that redirecting its carbon fluxes toward FA synthesis could improve its general performance.

Overall, this characterization work highlighted the distinct intrinsic performances of the analyzed OYs, identifying optimal C/N ratios and potential bottlenecks in the utilization of synthetic SCFA mixtures. These findings provide a foundation for further optimization of individual bioprocesses and may help identify promising targets for future metabolic engineering strategies.

Will disruption of PHD1 gene enhance OCFA accumulation in B. adenivorans?

Strain improvement is often a critical step toward further enhancing performance and developing industrially relevant strains. In Paper **IV**, the development of a novel plasmid-based CRISPR-Cas9 tool enabled the first stages of strain improvement in the promising species *B. adenivorans*, with the goal of developing an OCFA cell factory. The most important insights are:

- i. **Disruption of *PHD1* gene resulted in increased OCFA accumulation** compared with the wild type, as hypothesized and consistent with observations reported in other yeast species. These results suggest that disruption of the MCC may redirect a greater proportion of propionyl-derived carbon toward OCFA synthesis, indicating that the MCC represents a significant competing pathway for HPr utilization and OCFA production.

Overall, this work demonstrated that the newly developed CRISPR-Cas9 tool is functional and provides a valuable platform for future genetic engineering of *B. adenivorans*.

7.2 Ideas to drive OCFA research

The work presented in this thesis provides an initial springboard for further exploration of OCFA metabolism in the identified yeast species. Considerable work remains to be investigated before a yeast strain and bioprocess capable of achieving industrially relevant OCFA production can be developed. Based on the findings, several promising directions for future research can be identified.

- i. **CARBON SOURCE**
 - a. Different HAc:HPr ratios could be investigated, as the optimal ratio for maximizing OCFA production may be species-specific. Other SCFA mixtures could also be explored; in particular, valeric acid may serve as an alternative precursor for OCFA synthesis, while butyric acid has been associated with indirect contribution in OCFA production, by promoting biomass and lipid accumulation (136).
 - b. Once the metabolism of different synthetic SCFA mixtures is better understood, the next step should be to evaluate real SCFA digestates. These complex carbon sources may require dilution or compositional adjustment to optimize yeast performance (80).

- ii. ADAPTIVE LABORATORY EVOLUTION (ALE)
 - a. *R. toruloides* showed promising OCFA yields but relatively low HPr consumption. Using ALE to increase SCFAs or HPr tolerance could enhance carbon utilization in *R. toruloides* but also in the other identified OYs, although increased HPr consumption may instead favor competing pathways such as the MCC rather than OCFA production. Nevertheless, ALE could provide insights into HPr utilization and potentially yield strains with improved production phenotypes.
- iii. METABOLIC ENGINEERING
 - a. The development of double and triple mutants targeting *TGL4*, *MFE2*, and *PHD1* genes in *B. adenivorans* or other identified OYs, combined with the overexpression of key lipid biosynthetic genes such as *ACC*, *ACL*, or *FAS*, could be explored to enhance lipid accumulation and redirect propionyl-CoA toward OCFA biosynthesis (22).
 - b. Introducing a high-affinity FAS complex, such as that identified in *R. toruloides*, into other OYs could represent a promising strategy for improving OCFA production, as demonstrated in *S. cerevisiae* (105).
 - c. The OYs identified as high OCFA producers on glucose warrant further investigation, as they may possess metabolic pathways enabling OCFA production from this readily available carbon source (138). In particular, introducing strategies to increase the intracellular propionyl-CoA pool could enhance OCFA production by *B. adenivorans* during growth on glucose. This may also enable the use of lignocellulosic hydrolysate as a low-cost, sustainable glucose source, as the carbon source cost is a major contributor to process feasibility (87). Using complex lignocellulosic substrates may require additional metabolic engineering to enhance the tolerance to inhibitors or favor the use of the sugars present in the biomass such as mannose or xylose.
- iv. METABOLIC FLUX ANALYSIS
 - a. A substantial proportion of the HPr consumed by the investigated yeasts did not appear to be recovered as OCFA. Metabolic flux analysis could therefore provide valuable insights into the fate of HPr-derived carbon and identify the major competing carbon sinks (161). Such information would enable a more precise reconstruction of propionate metabolism and reveal metabolic bottlenecks and strain engineering targets for OCFA production.
- v. BIOPROCESS DEVELOPMENT
 - a. Importantly, before developing a yeast cell factory and bioprocess, it would be insightful to perform a techno-economic assessment and life cycle assessment to evaluate the feasibility of the proposed fermentation process based on the selected carbon source and determine the minimum target TRYs required for

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economic viability. This analysis should also consider scale-up costs and capital expenditure (CAPEX).

- b. The final step in this journey would be the development of a fully optimized scaled up bioprocess capable of maximizing carbon conversion toward the desired OCFA-rich oil product. Determining optimal pH, temperature, medium composition, oxygenation and agitation are important parameters to ensure sustainable fermentation processes.

Overall, this work advances methodologies for FA analysis, identifies promising OYs for OCFA production and characterizes them, and establishes a new genetic engineering tool for strain development. These results provide a foundation for the development of efficient yeast cell factories and economically viable bioprocesses for sustainable OCFA production.

THE ROAD IS STILL LONG, BUT WE MUST BELIEVE IN IT!

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