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Abstract

Oleaginous yeasts, with their high lipid accumulation, offer potential as sustainable alternatives to vegetable oils. They also have a unique capability for accumulating carotenoids together with lipids. Also, they have the ability to thrive on various waste and low-cost carbon sources. This thesis examined ways of converting lignocellulosic biomass into fatty acids and carotenoids using oleaginous yeasts and potential methods for extracting these valuable compounds efficiently, with the ultimate aim of establishing a foundation for their future incorporation as essential ingredients in the food and feed industries.

Comprehensive investigations into the impact of conventional and supercritical carbon dioxide (SC-CO_2) extraction methods on the composition of lipid and carotenoid extracts derived from the yeast species *Rhodotorula toruloides* revealed that SC-CO_2 has promising capability for isolating these compounds, particularly carotenoids, unlike conventional extraction methods. A previously undetected adverse influence of including saponification in conventional methods on yield and composition of carotenoids was observed. This novel insight highlights the need for re-evaluating and refining existing lipid and carotenoid extraction methods. Further analysis of the lipid and carotenoid profiles of various *R. toruloides* strains yielded valuable insights, particularly concerning the hybrid strain CBS 6016^T, for which predominant inheritance and expression of genetic traits from one parental strain (CBS 14), but not the other (CBS 349), was revealed. This research offers significant biotechnological potential. SC-CO_2 extraction enhances eco-friendly lipid and carotenoid production in *R. toruloides*, while insights into hybrid strain CBS 6016^T can aid genetic engineering for higher yields. Overall, this thesis opens the way toward greener, more efficient processes in the food, feed, and biotech sectors.

Keywords: *Rhodotorula toruloides*, lignocellulose, supercritical carbon dioxide extraction, saponification, lipid, carotenoids, *R. toruloides* strains

Uthålliga biokemikalier-extraktion av lipider och karotenoider från *Rhodotorula toruloides*, en oleaginös jäst

Sammanfattning

Oleaginösa jästsvampar med sin stora förmåga att lagra lipider ger oss hållbara alternativ till vegetabiliska oljor. Dessa mikroorganismer kan också syntetisera karotenoider tillsammans med lipider. De har förmågan att trivas på olika avfalls- och lågkostnadskolhydratkällor. I denna avhandling har vi undersökt olika sätt att omvandla lignocellulosa biomassa till fettsyror och karotenoider med hjälp av oleaginösa jästsvampar och potentiella nya metoder för att effektivt extrahera dessa värdefulla föreningar, med det övergripande målet att etablera en plattform för deras framtida användning som viktiga ingredienser inom livsmedels- och foderindustrin.

Omfattande undersökningar av konventionella och superkritisk koldioxid (SC-CO_2) extraktionsmetoders påverkan på sammansättningen av lipid- och karotenoidextrakt som härstammar från jästsvamparten *Rhodotorula toruloides* visar att SC-CO_2 är en lovande extraktionsmetod för att isolera dessa föreningar, särskilt karotenoider, till skillnad från konventionella extraktionsmetoder. Inverkan av förtvålning i konventionella extraktionsmetoder på utbyte av enskilda karotenoider upptäcktes. Ett behov att ompröva och förbättra befintliga metoder för extraktion av lipider och karotenoider, finns. Analys av lipid- och karotenoidprofiler hos olika *R. toruloides*-stammar gav värdefulla insikter, särskilt i hybridstammen CBS 6016^T, för vilken en övervägande ärftlig och uttrycksrelaterad genetiska egenskaper från en föräldrastam (CBS 14), men inte den andra (CBS 349). Dessa forskningsresultat har en betydande bioteknologisk potential. SC-CO_2-extraktion ökar möjligheten till miljövänlig produktion av lipider och karotenoider från *R. toruloides*, medan kunskapen om hybridstammen CBS 6016^T kan hjälpa till med genetiskt ingenjörskap för att uppnå högre avkastning. Sammanfattningsvis ger resultaten i denna avhandling grönare och mer effektiva processer inom livsmedel, foder och bioteknik.

Keywords: *Rhodotorula toruloides*, lignocellulosa, superkritisk koldioxidextraktion, saponifikation, lipid, karotenoider, *R. toruloides*-stammar

Dedication

To my family and friends

Contents

List of publications

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

I. Nagaraj, YN., Burkina, V., Okmane, L., Blomqvist, J., Rapoport, A., Sandgren, M., Pickova, J., Sampels, S., Passoth, V. (2022). Identification, quantification and kinetic study of carotenoids and lipids in *Rhodotorula toruloides* CBS 14 cultivated on wheat straw hydrolysate. *Fermentation,* 8(7), 300.

II. Nagaraj YN, Blomqvist J, Sampels S, Pickova J, Sandgren M, Passoth V. Supercritical carbon-dioxide (SC-CO_2) extraction of lipids and carotenoids from *Rhodotorula toruloides* CBS 14 in comparison with conventional extraction method (manuscript).

III. Nagaraj YN, Blomqvist J, Sampels S, Pickova J, Sandgren M, Passoth V. Profile of lipids and carotenoids from different strains of *Rhodotorula toruloides* extracted with supercritical carbon-dioxide (SC-CO_2) (manuscript).

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The contribution of Yashaswini Nagavara Nagaraj to the papers included in this thesis was as follows:

I. Took part in planning the project. Performed all laboratory work. Took part in writing—original draft preparation.

II. Took part in planning the project. Performed all laboratory work. Took part in writing—original draft preparation.

III. Took part in planning the project. Performed all laboratory work. Took part in writing—original draft preparation.

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Abbreviations

AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
CoA	Coenzyme A
DMAPP	Dimethylallyl pyrophosphate
FFAs	Free fatty acids
G-3-P	Glycerol-3-phosphate
GC	Gas chromatography
GGPP	Geranylgeranyl pyrophosphate
GRAS	Generally Recognized as Safe
HPLC	High pressure liquid chromatography
ICDH	Isocitrate dehydrogenase
IMP	Inosine monophosphate
LC	Liquid chromatography
NADPH	Nicotinamide adenine dinucleotide phosphate
OCC	Open column chromatography
PDA	Photodiode array
PUFAs	Polyunsaturated fatty acids
SC-CO_2	Supercritical carbon-dioxide
SCOs	Single cell oils

SFE	Supercritical fluid extraction
TAGs	Triacylglycerols
TCA	Tricarboxylic acid
TLC	Thin-layer chromatography
UHPLC	Ultra-high pressure liquid chromatography

1. Introduction

1.1 Research background

The current situation with fossil resource depletion and harmful environmental consequences of emissions of greenhouse gases has prompted researchers to actively seek sustainable alternatives to replace fossil resources in production of fuels, food, feed, biochemicals and materials. As the demand for fossil resources, particularly fossil-derived fuels in transportation, continues to rise and depletion of these resources becomes imminent, there is an urgent need to explore alternative methods for producing fuels, chemicals, food and feed.

In work to develop alternative production methods for critical resources, various microorganisms with high lipid content, such as yeasts, bacteria and micro-algae, have been attracting attention. These microorganisms possess valuable attributes such as posing minimal competition for arable land and food resources and good capacity to produce long-chain lipids, which can be converted to a variety of useful chemicals, including biofuels (Steen et al., 2010; Beopoulos & Nicaud, 2012; Spagnuolo et al., 2019; Ali et al., 2022).

Applications involving lipid-accumulating microorganisms are currently a main focus of researchers, due to their ability to produce lipids at levels often exceeding 20% of dry biomass (Meng et al., 2009; Ageitos et al., 2011; Nagaraj et al., 2022). Among these microorganisms, *Rhodotorula toruloides*, an oleaginous yeast, has emerged as a promising candidate, since it can accumulate lipids to over 75% of its dry biomass when cultivated in conditions with limited nitrogen and surplus carbon (Ratledge & Wynn, 2002; Saini et al., 2020). *Rhodotorula toruloides* also possesses the capacity to produce carotenoids, secondary metabolites that belong to a group of

natural pigments synthesised by plants and microorganisms. Carotenoids possess inherent antioxidant properties and are of great significance in various industries within e.g., the pharmaceutical, chemical, food and animal feed production sectors.

Rhodotorula toruloides, a basidiomycetous yeast, can grow effectively on cost-effective carbon sources such as lignocellulose hydrolysates and can efficiently convert the carbon present in lignocellulosic materials into lipids and carotenoids. Thus, it is a promising candidate for generating resources for the production of fuels, chemicals and even foods or feeds, particularly since the lipids and carotenoids it produces bear a close resemblance to conventional vegetable oils (Christophe et al., 2012; Ma et al., 2018; Bharathiraja et al., 2017; Blomqvist et al., 2018). Use of hydrolysed lignocellulosic material as a feedstock for microbial lipid and carotenoid production represents a sustainable approach that is urgently needed to help overcome the problems of fossil resource depletion and environmental damage (Vignesh et al., 2020; Valdés et al., 2020; Alexandri et al., 2022).

The work presented in this thesis forms part of global efforts to create a circular system where pre-treated lignocellulose hydrolysate is transformed, through fermentation by oleaginous yeasts, into valuable food components such as oils and carotenoids. The residual materials after extraction can then be re-purposed as a source of nitrogen in plant fertilisers, completing the cycle (Figure 1).

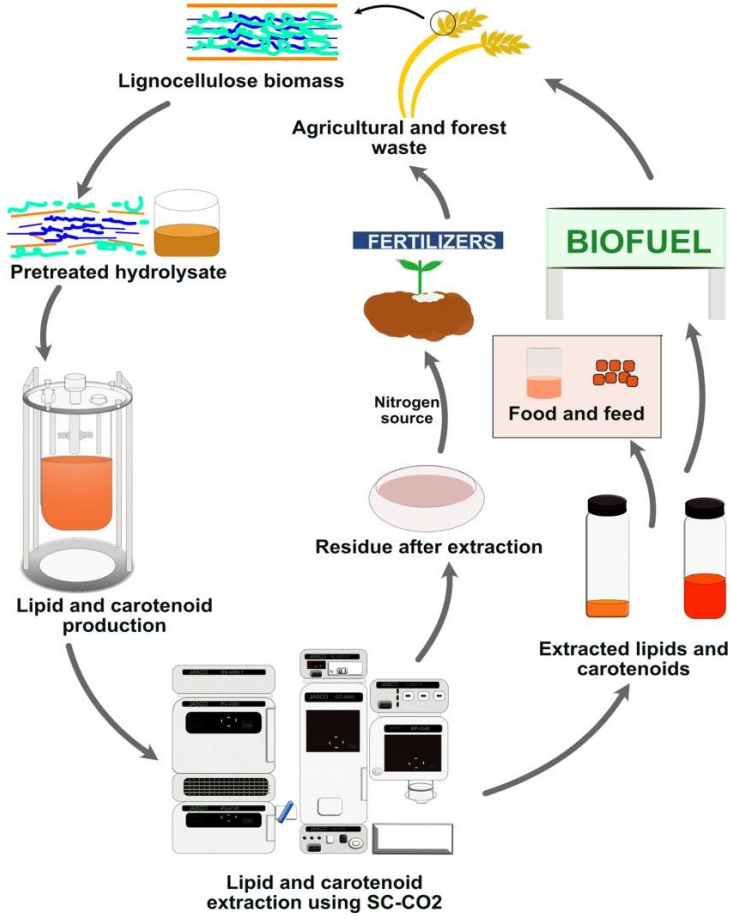

Figure 1. Schematic representation of pathways for converting low-cost lignocellulose substrate into high-value compounds using oleaginous yeasts.

To maximise the potential of *Rhodotorula toruloides* in producing lipids and carotenoids, it is crucial to develop efficient techniques for harvesting these substances from the yeast cells. Over the past few decades, much effort has been dedicated to developing extraction methods for this purpose. However, the presence in yeast cells of diverse types of lipids and carotenoids, each with a different level of polarity, presents a challenge in extracting these compounds separately. Moreover, their sensitivity to oxidation imposes constraints on use of process factors such as excessive heat, light, acids and prolonged extraction duration.

Given their hydrophobic nature, i.e., natural reluctance to mix with water, lipids and carotenoids are typically extracted using organic solvents. These include non-polar solvents, such as hexane, petroleum ether or tetrahydrofuran (THF), and polar solvents, such as acetone, ethanol and ethyl acetate (Saini & Keum, 2018; Khot et al., 2020).

In efforts to enhance the sustainability of yeast cell processing, work is underway to identify more environmentally friendly solvents, known as green solvents, for extracting lipids and carotenoids. In particular, supercritical carbon dioxide (SC-CO_2) has been suggested as a suitable green solvent, especially for harvesting compounds such as lipids and carotenoids (Sahena et al., 2009; Saini & Keum, 2018). However, the performance of SC-CO_2 first needs to be compared with that of conventional extraction methods.

The main aim of this thesis was therefore to investigate the effects of conventional and SC-CO_2 extraction techniques on the characteristics of lipids and carotenoids derived from *Rhodotorula toruloides*. An additional aim was to assess and compare the lipid and carotenoid composition of three primary strains of *Rhodotorula toruloides* (CBS 14, CBS 349, CBS 6016^T) (Figure 2).

1.2 Oleaginous microorganisms

Oleaginous microorganisms possess a unique capability to accumulate lipids to levels exceeding 20% of their total dry biomass (Ratledge & Wynn, 2002; Ageitos et al., 2011). The type and yield of lipids accumulated in oleaginous microorganisms depend on factors such as their origin, i.e., type of organism, culture conditions and the carbon source provided (Balan, 2019). The accumulated lipids that are typically present in oleaginous microorganisms serve either as integral components of cell membranes, for storage purposes, or perform regulatory functions (Eisenberg & Büttner, 2014; Sandager et al., 2002). The group of oleaginous microorganisms includes certain species of bacteria, yeasts, moulds and algae (Zhao et al., 2011). These microorganisms have been found to have similar potential in terms of acting as a lipid source as conventional energy crops such as jatropha, sunflower or palm oil (Vicente et al., 2010). However, accurate knowledge of the lipid profile of specific microorganisms within this group is critical for assessing their suitability as alternatives for various industrial applications.

1.2.1 Oleaginous yeasts

Oleaginous yeasts are a group of microorganisms that have the ability to accumulate large quantities of lipids, primarily in the form of triacylglycerols (TAGs), within their cells (Ageitos et al., 2011; Passoth et al., 2023). These lipids can account for more than 20% of total dry cell weight of oleaginous yeasts, making these microorganisms promising candidates for lipid production in various industrial applications (Ratledge, 2013). Oleaginous yeasts constitute about 11% of all yeast species (Abeln & Chuck, 2021) and can be found in different clades within the phyla Ascomycota and Basidiomycota. Their ability to accumulate lipids depends on an environmental trigger, typically limitation of nitrogen, phosphorus or other environmental factors, and on carbon availability (Passoth et al., 2023; Brandenburg et al., 2021; Chmielarz et al., 2021; Ratledge & Wynn, 2002).
An intriguing suggestion is that oleaginous yeasts could offer an alternative to oil crops due to their similar lipid profile, efficient land use and lack of dependence on weather conditions for cultivation (Whiffin et al., 2016). The number of known oleaginous yeast genera currently exceeds 160, while ongoing research is expected to identify more in the future (Abeln & Chuck, 2021). Some of the genera containing oleaginous representatives are: *Yarrowia, Lipomyces, Rhodotorula, Myxozyma, Sporidiobolus, Sporobolomyces, Apiotrichum, Cutaneotrichosporon, Filobasidium* and *Moesziomyces* (Passoth et al., 2023; Sitepu et al., 2014).
The oleaginous yeasts exhibit versatility in utilising various carbon sources, including glucose, xylose, cellobiose, glycerol, fats and organic acids. Previous studies have explored their growth on diverse substrates, including hydrolysates of agricultural residues (e.g., straw, stover), industrial wastes (e.g., molasses, whey) and municipal wastes (e.g., sewage sludge) (Brandenburg et al., 2021; Qin et al., 2017; Valdés et al., 2020; Passoth et al., 2023).

1.3 *Rhodotorula toruloides* – a yeast of interest

Rhodotorula toruloides is a non-conventional oleaginous yeast belonging to the family Sporidiobolaceae within the phylum Basidiomycetes (Spagnuolo et al., 2019); (Ageitos et al., 2011). While it is sometimes referred to as *Rhodosporidium toruloides* in the literature, the taxonomic name *Rhodotorula* takes precedence (Wang et al., 2015). This yeast species has

attracted significant attention in research and has been extensively studied as an oleaginous microorganism, second only to the model yeast *Yarrowia lipolytica* (Chattopadhyay & Maiti, 2021).
Under nutrient limitation, *R. toruloides* exhibits a remarkable ability to accumulate a very high quantity of lipids (Zhao et al., 2011; Passoth et al., 2023; Chmielarz et al., 2021; Huang et al., 2018; Wang et al., 2018). *Rhodotorula toruloides* also exhibits broad substrate utilisation capability for lipid production, encompassing various carbon sources such as sugarcane juice, crude glycerol, lignocellulosic hydrolysates and vegetable market waste (Fei et al., 2016; Singh et al., 2018; Brandenburg et al., 2018; Passoth & Sandgren, 2019; Chmielarz et al., 2021; Passoth et al., 2023). Notably, the yeast can achieve very high lipid content, of up to 76% of dry cell weight (Xue et al., 2018). Due to this exceptional lipid production capacity, it is an attractive candidate for lipid metabolic engineering (Xue et al., 2018; Chattopadhyay et al., 2021).
The ability of *R. toruloides* to tolerate the inhibitory effects of different compounds in biomass hydrolysates adds to its appeal as a candidate for industrial applications. Thus, the species has been explored for its potential in producing valuable biomolecules apart from lipids, including carotenoids, enzymes and polysaccharides (Rapoport et al., 2021; Passoth et al., 2023; Vinals et al., 2023; Mussagy et al., 2023). This broad biosynthetic capacity makes it a versatile and promising candidate for various biotechnological applications.

1.3.1 Strains of *Rhodotorula toruloides*

Rhodotorula toruloides has a unique reproductive system, with two opposite mating types, A1 and A2. Strain CBS 14 belongs to mating type A1, while strain CBS 349 belongs to mating type A2 (Sambles et al., 2017; Sampaio, 2011). During its life cycle, *R. toruloides* utilises both conjugation between A1 and A2 strains and cell budding as common methods of reproduction (Sampaio, 2011).
One property of *R. toruloides* is variation between the strains in terms of their ability to utilise different carbon sources and convert them into lipids (Brandenburg et al., 2021; Chmielarz et al., 2021). The species spans a broad geographical range and thrives in many different habitats due to its ubiquitous saprophytic nature, which enables *R. toruloides* to grow in

environments ranging from decomposing conifer wood in Sweden (*R. toruloides* CBS 14) to the soil of Japan (*R. toruloides* CBS 349).
Through hyphal conjugation, the mating strains CBS 14 (A1) (Rennerfelt, 1937) and CBS 349 (A2) (Okunuki, 1931) can engage in a reproductive process, which was exploited to obtain the hybrid strain CBS 6016^{T} (Banno, 1967). The intricate hyphal conjugation mechanism contributes significantly to the genetic diversity and adaptability exhibited by *R. toruloides*.

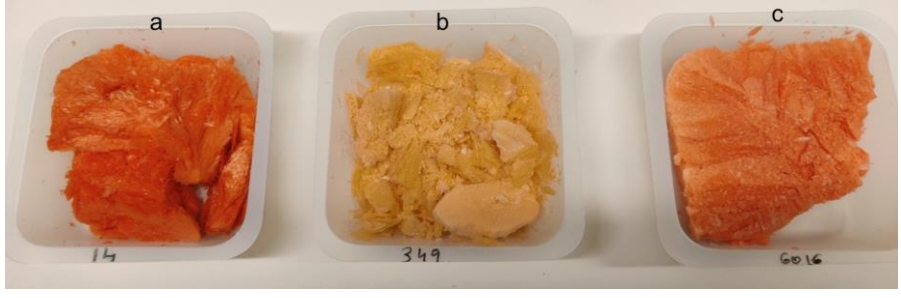

Figure 2. Freeze-dried cells of three important strains of *Rhodotorula toruloides*. (a) CBS 14, (b) CBS 349 and (c) CBS 6016^{T}. CBS14 and CBS 349 are the parental strains of the type strain CBS6016.

1.4 Lignocellulose as carbon source

1.4.1 Structure

Lignocellulose is an intricate and readily available biomass compound that constitutes the basic framework in plant components such as stems, leaves and other plant structures (Yadav et al., 2020; Kumar et al., 2018; Maitan-Alfenas et al., 2015). This composite material is composed of three primary constituents, cellulose, hemicellulose and lignin, that intertwine densely, creating a multifaceted framework that confers robustness upon plant cell walls (Yadav, Paritosh, et al., 2019).
Cellulose, a linear polysaccharide composed of glucose units connected by β-1,4-glycosidic bonds, arranges into microfibril bundles. These microfibrils further organise into larger structures called cellulose fibre that endow plant cell walls with primary strength and integrity. Enzymatic degradation of cellulose releases glucose units, making them accessible for fermentation by microorganisms (Isikgor & Becer, 2015; Passoth et al., 2023).

Hemicellulose is made up of diverse and branched polysaccharides and displays varying composition and structure depending on the plant of origin (Biely et al., 2016; Gírio et al., 2010). Unlike cellulose, hemicellulose features an array of sugar units, including glucose, xylose and mannose. It envelops cellulose microfibrils, enhancing cohesion, and contributes to both structural support and cellulose fibre protection. Its relative heterogeneity permits easier breakdown into constituent sugars than cellulose (Agbor et al., 2011; Scheller & Ulvskov, 2010; Passoth et al., 2023).

Lignin, a complex polymer with strong cross-linkages, provides rigidity and impermeability to plant cell walls. It comprises phenolic compounds such as coniferyl, sinapyl and p-coumaryl alcohols that envelop and embed the cellulose-hemicellulose matrix, creating a robust, resilient framework (Yadav et al., 2018). While essential for plant integrity, the intricate structure of lignin poses challenges in industrial applications due to its resistance to enzymatic degradation (Jönsson & Martín, 2016; Jørgensen et al., 2019; Passoth et al., 2023).

The close interactions between cellulose, hemicellulose and lignin are responsible for the characteristic strength and durability of lignocellulose. However, these interactions also hinder efficient access and breakdown of components for industrial processes such as biofuel production. Therefore, pre-treatment methods are essential to disrupt these interactions and render cellulose and hemicellulose accessible for enzymatic hydrolysis and subsequent conversion into fermentable sugars (Yadav, Singh, et al., 2019).

1.4.2 Pre-treatment of lignocellulose

Pre-treating lignocellulosic biomass is a pivotal initial phase in its transformation into valuable resources (Figure 3). This preparatory stage aims to dismantle structural obstacles, rendering the biomass more amenable to subsequent processes such as enzymatic hydrolysis and fermentation. Common methods for pre-treatment can be subdivided into mechanical, chemical and physico-chemical approaches.

Mechanical methods apply physical force, such milling, grinding or shredding, to disrupt the intricate lignocellulosic structure (Silva et al., 2012; Zhao et al., 2006). By fragmenting the biomass into smaller particles, these methods amplify surface area and facilitate enzyme access during hydrolysis.

Chemical pre-treatment methods employ acids, bases or solvents to cleave lignin and hemicellulose, thus exposing the cellulose for further processing

(Yadav et al., 2020). Physico-chemical methods, such as steam explosion, combine mechanical and chemical actions, contributing to structural disintegration and enhanced enzyme interactions (Huang et al., 2015; Vivekanand et al., 2013; Bensah & Mensah, 2018; Baksi et al., 2023).

After pre-treatment, the lignocellulosic biomass is more receptive to enzymatic hydrolysis due to the dismantling of its complex structure. Enzymatic hydrolysis involves breaking the β-1,4-glycosidic bonds between sugar molecules in cellulose and hemicellulose. Enzymes such as cellulases and hemicellulases are used in this process (Maitan-Alfenas et al., 2015). Cellulases include endoglucanases, which sever internal cellulose bonds, exoglucanases, which remove glucose units from cellulose ends, and β-glucosidases, which convert cellobiose into glucose. Hemicellulases target diverse hemicellulose sugars, e.g., xylanases target xylan and mannanases target mannans (Van Dyk & Pletschke, 2012; Houfani et al., 2020). Good synergy of these enzyme types within cellulase and hemicellulase mixtures is essential for efficient hydrolysis, enabling comprehensive breakdown of intricate polysaccharides into simple sugars such as glucose, xylose, mannose and others (Hu et al., 2013; Andlar et al., 2018; Galbe & Wallberg, 2019; Passoth et al., 2023).

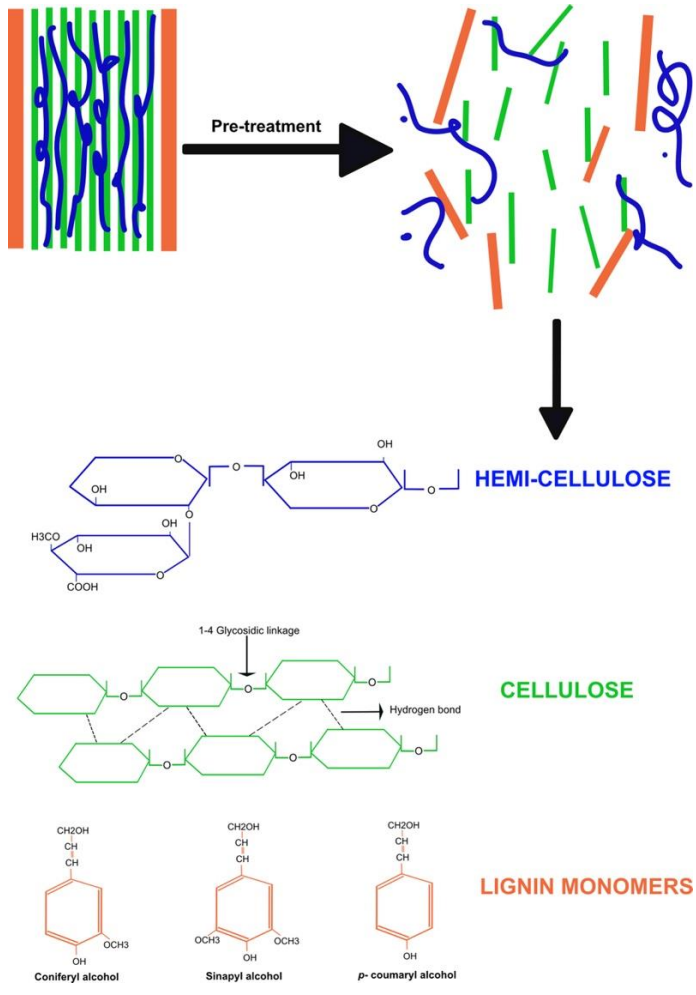

Figure 3. Schematic presentation of pre-treatment of lignocellulosic biomass.

1.5 Thesis aims and objectives

The techniques available for extracting lipids and carotenoids have undergone rapid advances in recent years, involving e.g., improved solvents and methods that prioritise swiftness, cost-effectiveness and efficiency. In particular, utilisation of environmentally friendly solvents such as SC-CO_2 instead of conventional organic solvents has attracted substantial interest. Thus, the work in this thesis primarily sought to enhance understanding of the impacts of conventional and SC-CO_2 extraction methods on the

composition of lipids and carotenoids extracted from *Rhodotorula toruloides*. A further aim was to address an existing knowledge gap concerning the variations in lipid and carotenoid production in different strains of *R. toruloides* by analysing lipid and carotenoid production in three main strains of *Rhodotorula toruloides*.

Specific objectives were to:

1. Determine the lipid and carotenoid profile of *Rhodotorula toruloides* strain CBS 14 cultivated on wheat straw hydrolysate using conventional extraction methods (Paper I).
2. Evaluate and compare the effects of conventional extraction and supercritical carbon-dioxide (SC-CO_2) extraction on the lipid and carotenoid profile of *Rhodotorula toruloides* strain CBS 14 cultivated on wheat straw hydrolysate (Paper I and Paper II).
3. Assess the lipid and carotenoid profile of different strains of *Rhodotorula toruloides* following extraction with SC-CO_2 (Paper III).

2. Lipids and carotenoid production by *Rhodotorula toruloides*

2.1 Lipid metabolism

Lipid accumulation in oleaginous yeasts is influenced by various factors and the level of lipid accumulation can vary even under constant physiological conditions. For cells to store lipids, there must be an excess carbon source, accompanied by limited availability of a specific nutrient, e.g., nitrogen, phosphorus or sulphur (Figure 4). Lipid production in oleaginous yeasts also requires adequate levels of key cellular metabolites such as citric acid and malic acid and available energy in the form of adenosine triphosphate (ATP), which is essential for fatty acid synthesis (Granger et al., 1993; Ratledge & Wynn, 2002; Beopoulos et al., 2009). This process occurs in both the cytoplasm and mitochondria of the cell.

To ensure cellular maintenance upon nitrogen starvation, oleaginous yeasts produce adenosine monophosphate (AMP) deaminase, which facilitates breakdown of AMP into inosine monophosphate (IMP) and ammonium ions ($NH4^+$) (Figure 4). This process helps overcome limitations in external nutrient availability. The consequential sharp reduction in intracellular AMP levels significantly affects specific segments of the tricarboxylic acid (TCA) cycle. Specifically, in oleaginous yeasts, it leads to deactivation of isocitrate dehydrogenase (ICDH), an enzyme typically activated through allosteric interactions with AMP. ICDH, operating within the mitochondria, plays a crucial role in converting isocitrate into α-ketoglutarate. This deactivation causes accumulation of isocitrate, which is then converted into citrate in the mitochondria by the enzyme aconitase. As a result, the TCA cycle is interrupted at this stage, leading to an increase in citrate levels within the

cell. The excess citrate is transported to the cytoplasm and converted into acetyl-CoA by ATP citrate lyase (ACL) (Ageitos et al., 2011).
Acetyl-CoA is further transformed into malonyl-CoA by acetyl-CoA carboxylase. The fatty acid synthase complex in oleaginous yeast cells utilises both acetyl-CoA and malonyl-CoA, along with nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, to synthesise acyl-CoA. NADPH is typically generated by enzymes such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of the pentose phosphate pathway, or maleic enzyme (ME) (Ratledge, 2014). The resulting acyl-CoA chains are then transported to the endoplasmic reticulum, where they undergo esterification with glycerol-3-phosphate (G-3-P). This process leads to the production of structural lipids, including phospholipids and glycolipids, as well as storage lipids in the form of TAGs through the Kennedy pathway (Fakas, 2017; Passoth et al., 2023). In the Kennedy pathway, glycerol-3-phosphate is first acylated by glycerol-3-phosphate acyltransferase (Sct1) to form lysophosphatidic acid. Subsequently, lysophosphatidic acid acyltransferase (Slc1) and acyltransferase for lysophosphatidylethanolamine (Ale1) catalyse acetylation of lysophosphatidic acid, resulting in the formation of phosphatidic acid. The final steps involve dephosphorylation of phosphatidic acid to form diacylglycerol by phosphatidic acid phosphatase and the subsequent transfer of an acyl group from acyl-CoA to the third carbon of diacylglycerol by diacylglycerol-acyltransferases (Dga1, Dga2) to form TAG (Kennedy, 1961; Tkachenko et al., 2013).

2.2 Carotenoid formation in *Rhodotorula toruloides*

Carotenoids are naturally synthesised in various organisms, including plants, algae and fungi. Fungal carotenoids exhibit unique structural diversity compared with those found in other sources. Within fungi, the carotenoid synthesis pathway is responsible for generating pigments such as β-carotene (Goodwin, 2012; Sandmann, 2022). Notably, this biosynthesis pathway shares acetyl-CoA as a common precursor molecule with lipid metabolism (Fakankun & Levin, 2023). This connection gains significance in the context of oleaginous yeasts, particularly as regards the species that was the main study object in this thesis (*R. toruloides*).

The biosynthetic pathway of carotenoids in different *Rhodotorula* species has been extensively studied. One such study found that *R. minuta, R. glutinis, R. graminis, R. mucilaginosa* and *Rhodotorula* CRUB 1032 (belonging to a hitherto unknown species) have similar carotenoid profiles, with β-carotene, γ-carotene, torulene and torularhodin being the main carotenoids present in all these species (Buzzini et al., 2007). An earlier study (Villoutreix, 1960) reported a similar pigment profile in *R. mucilaginosa*, further supporting the idea of a conserved carotenoid biosynthetic pathway within the genus *Rhodotorula*. General pathways for carotenoid biosynthesis have been reviewed previously (Simpson et al., 1964; Goodwin & Goodwin, 1980).

The biosynthetic pathway of carotenoids starts with acetyl-CoA (Figure 4), which is converted to 3-hydroxy-3-methylglutaryl-CoA by the enzyme hydroxymethylglutaryl-CoA synthase. Subsequently, 3-hydroxy-3-methylglutaryl-CoA is transformed into mevalonic acid through the action of hydroxymethylglutaryl-CoA reductase. Mevalonic acid is then converted into isopentenyl pyrophosphate (IPP) through a series of reactions involving specific kinases and diphosphomevalonate decarboxylase. Isopentenyl pyrophosphate undergoes isomerisation to form dimethylallyl pyrophosphate (DMAPP). Through an addition reaction involving three IPP molecules, geranylgeranyl pyrophosphate (GGPP) is synthesised (Kot et al., 2018).

Condensation of two molecules of GGPP leads to the formation of phytoene, a reaction which is catalysed by phytoene synthase. Subsequent reactions are catalysed by phytoene desaturase, an enzyme that plays a crucial role in the formation of neurosporene by catalysing up to three desaturation steps. Neurosporene may undergo further transformations to produce lycopene or β-zeacarotene (Goodwin & Goodwin, 1980; Hayman et al., 1974; Simpson et al., 1964). Lycopene cyclisation or β-zeacarotene dehydrogenation results in the formation of γ-carotene (Hayman et al., 1974; Igreja et al., 2021).

The γ-carotene molecule serves as a precursor for the biosynthesis of other carotenoids, such as β-carotene and torulene (Figure 4). Torularhodin is obtained through further transformations of torulene, including hydroxylation and oxidation (Kot et al., 2016). Torulene and torularhodin have been identified as potent antioxidants with greater peroxyl-radical quenching ability than β-carotene (Buzzini et al., 2007). In order to protect themselves from oxidative damage caused by elevated lipid concentrations,

yeast cells undergo a transition in the γ-carotene flux towards torulene, which possesses stronger antioxidant properties (Han et al., 2016). It has been reported that under increasing stress conditions, *R. mucilaginosa* cells exhibit an increase in torulene and torularhodin concentrations, while β-carotene concentration remains constant (Ghilardi et al., 2020).

Production of total carotenoids by oleaginous yeasts is influenced by various factors such as the carbon/nitrogen ratio, aeration, pH, temperature, light, presence of metal ions and type of sugar present (Aksu & Eren, 2005; Tang et al., 2019; Pinheiro et al., 2020). The type of carotenoid synthesised by oleaginous red yeasts can also be influenced by culture conditions and the growth phase of the cells.

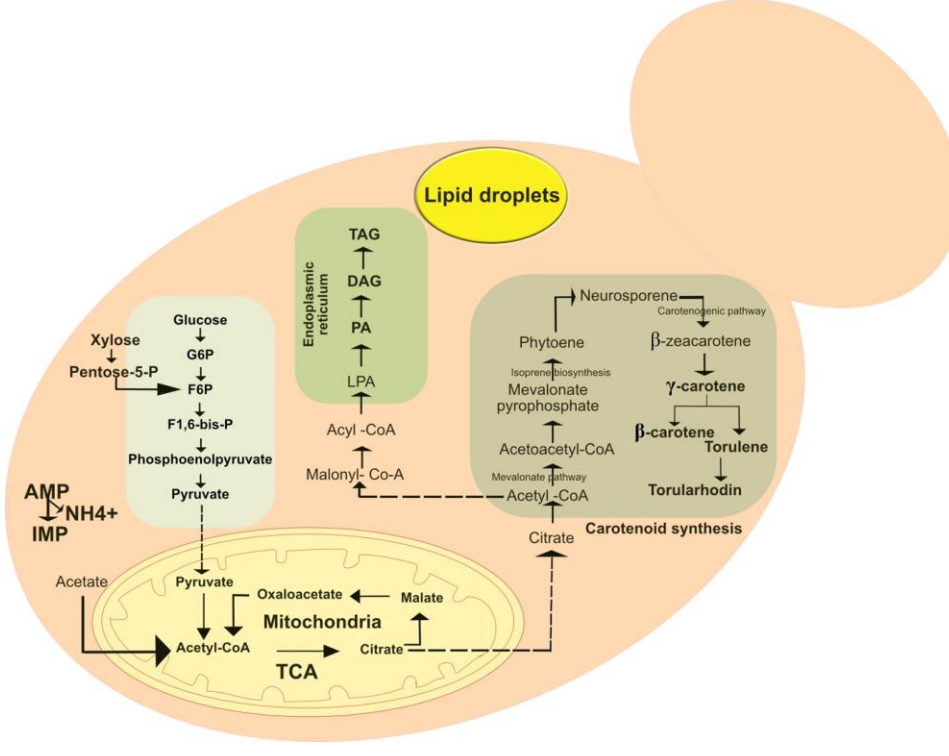

Figure 4. Simplified schematic diagram of lipid and carotenoid accumulation in *Rhodotorula toruloides* under nitrogen limitation.

3. Industrial applications of microbial oils and carotenoids

3.1 Properties of microbial oils compared with vegetable oils

3.1.1 Vegetable oils

Vegetable oil serves a diverse range of purposes beyond its use as a food product. There is growing evidence of the vital role that fatty acids (FAs) play in human nutrition, encompassing both therapeutic and preventive effects on various diseases. The significance of fats and fatty acids in human health means that these compounds are the subject of many ongoing research endeavours. Beyond being a dietary staple, fatty acids have gained prominence in different industrial sectors, e.g., in production of items such as soaps, detergents, cosmetics, lubricants, inks, varnishes and paints, and are notably relevant in the expanding energy market, particularly for biodiesel production (Kumar et al., 2016). Consequently, there is growing demand for oilseed crops to cater to both nutritional needs and industrial requirements. Plants produce a diverse array of fatty acids, each possessing distinct structural attributes that confer unique physicochemical properties, thereby extending their range of applications (Table 1). Soybean, rapeseed, palm, sunflower and peanut crops in particular are major contributors to global edible vegetable oil production (Zhou et al., 2020).

Table 1. Main fatty acid composition of different plant oils

Oil type	Fatty acid types (% of total fatty acids)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Sunflower	5.2	0.1	3.7	33.7	56.5	0.0
Palm	39.3	0.2	4.4	42.5	11.4	0.0
Soybean	10.1	0.0	4.3	22.3	53.7	8.1
Rapeseed	3.0	0.0	1.0	64.4	23.3	8.0
Cocoa butter	23.3	0.9	24.5	28.7	3.9	0.0

Data summarised from (Caporusso et al., 2021)

However, the surge in demand for vegetable oil comes with various adverse repercussions. The expansion of oilseed crop plantations, particularly in tropical regions, has triggered deforestation and degradation of habitats (Oosterveer, 2015; Danielsen et al., 2009). This in turn leads to biodiversity loss, disruption of ecosystems and the uprooting of indigenous communities. Furthermore, conversion of natural ecosystems to oilseed cultivation sites results in depletion of vital carbon sinks (Tilman et al., 2009; Dornburg et al., 2010). This contributes to heightened emissions of greenhouse gases, exacerbating the issue of climate change.

In response to these challenges, there are growing calls to replace vegetable oil production, which competes with tropical forests, with other forms of oil production. Several potential alternatives to vegetable oil are being explored, and one promising option is the use of single-cell oils derived from oleaginous microorganisms.

3.1.2 Microbial oils

Microbial oils, often referred to as single-cell oils (SCOs), are category of edible oils produced by oleaginous microorganisms (Cohen & Ratledge, 2005). A feature of interest in the context of this thesis work is that oleaginous yeasts have the capacity to generate oils that are similar in composition to those found in vegetable oils. These oils predominantly consist of palmitic, stearic, oleic and linoleic acids (Parsons et al., 2020; Shapaval et al., 2019) (Table 2). This similarity in lipid composition makes microbial oil one of natural oil feedstocks with the highest potential for

biodiesel production (Ma, 2006; Patel et al., 2017; Ma et al., 2018; Liu & Zhao, 2007; Li et al., 2008).

Table 2. Fatty acid composition of different oleaginous yeasts cultivated on glucose medium.

	Fatty acids (% of total fatty acids)					
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Lipomyces starkeyi	31.7	4.1	5.8	53.2	2.8	0.3
Rhodotorula babjevae	22.4	2.6	1.6	54.5	11.5	4.1
R. toruloides	26.6	2.1	3.1	46.2	16.4	2.2
R. graminis	20.3	1.6	2.5	57.4	11.4	3.5

Data summarised from (Shapaval et al., 2019)

An intriguing aspect is that the lipid profile of oleaginous microorganisms can be fine-tuned by adjusting the culture conditions. This means that, to a certain extent, it is possible to achieve a desirable fatty acid composition by cultivating yeast or algal strains under specific culture conditions (Parsons et al., 2020).

A significant advantage of SCOs is the possibility for the yeasts that produce these to be cultivated using various waste streams and with less land demand (Stellner et al., 2023). This reduces competition with traditional agriculture for vital resources such as water and fertile soil (Caporusso et al., 2021; Galán et al., 2020). Consequently, SCOs are emerging as a promising sustainable solution to address the increasing demand for edible oils and animal feeds.

3.2 Microbial oils in the food and feed industry

In recent times, there has been significant focus on investigating the feasibility of using microbial oils as potential sources for producing biodiesel. This interest stems from the need to find sustainable and renewable alternatives to conventional fossil fuels (Li et al., 2008). Apart from applications in biodiesel production, microbial oils are also the focus of attention as regards potential applications in the food and feed industries, demonstrating their versatile utility (Bellou et al., 2016; Galán et al., 2020).

One of the initial applications of microbial lipids was as a substitute for cocoa butter, which has diverse uses in the food and cosmetic industries. Various attempts have been made to replicate the composition of cocoa butter using mixtures of different fats from exotic plants and palm oils (Caporusso et al., 2021). However, these efforts have been hampered by the high cost of exotic fats and unsatisfactory results (Ward & Singh, 2005). As an alternative, lipids produced by oleaginous yeasts have emerged as promising candidates for cocoa butter substitutes, since these lipids closely resemble cocoa butter in terms of their fatty acid profile. However, a key challenge is their lower production of C18:0 fatty acid. Numerous strategies have been devised to enhance the levels of C18:0 in microbial lipids, as comprehensively discussed in the literature (Zhu et al., 2010; Uemura, 2012; Ji & Huang, 2019; Kothri et al., 2020). Likewise, considerable research has been dedicated to increasing production of polyunsaturated fatty acids (PUFAs) in oleaginous microorganisms (Bellou et al., 2016). PUFAs play vital roles in various physiological functions within the human body. They serve as crucial components of membrane phospholipids and act as precursors to eicosanoids, hormone-like molecules that influence the cardiovascular, immune and central nervous system and even inflammatory responses (Laoteng et al., 2011). The demand for PUFAs in the food industry is substantial, especially as additives in infant foods, a continuously expanding market.

Scientists in Germany conducted experiments during World War I to explore commercial production of microbial oils as a means to address food shortages (Yamauchi et al., 1983). Since then, there have been ongoing efforts to identify species of oleaginous microorganisms that could potentially serve as novel sources of edible oils, replacing traditional vegetable oils and animal fats. The initial introduction of microbial oils as a dietary supplement took place in 1985, when a linolenic acid-rich oil was developed as a substitute for evening primrose oil (Ratledge, 2013). This product, named "Oil of Javanicus," was derived from the fungus *Mucor javanicus*. However, it was eventually replaced by borage oil (*Borago officinalis*), which has a higher linolenic acid content (Shahidi, 2005). The breakthrough for microbial oils in the infant formula market occurred in 2001, when the US Food and Drug Administration granted Generally Recognized as Safe (GRAS) status to SCOs. By February 2002, over half of the infant formula market in the USA was fortified with SCOs (Fichtali &

Senanayake, 2010). Today, numerous industrial players such as DuPont, DSM, Cargill Alking Bioengineering (CABIO), Nestle and Nutricia are actively engaged in industrial-scale production of microbial oils (Xue et al., 2013; Galán et al., 2020; Caporusso et al., 2021).
In addition to their applications in the food industry, microbial lipids have found a place in the field of animal feed. Notably, Alltech, a prominent global company specialising in animal nutrition, has ventured into producing microbial (algal) docosahexaenoic acid (DHA) specifically targeted for the animal feed market (Galán et al., 2020). Microbial oils have also been used as a replacement for vegetable oil in fish feed production (Blomqvist et al., 2018).

3.3 Microbial carotenoids in the food industry

In recent years, there has been a noticeable decline in enthusiasm for artificially created pigments, as most of these have been found to be toxic, carcinogenic and capable of causing birth defects. Consequently, there is an increasing shift towards microbial sources as a safe and viable alternative (Babu & Shenolikar, 1995; Cañizares-Villanueva & Rios-Leal, 1998; Johnson & Schroeder, 2006; Babitha, 2009; Kirti et al., 2014; Tuli et al., 2015; Papapostolou et al., 2023). Microbial pigments have distinct advantages over their synthetic and inorganic counterparts, with the process of fermentation, which is swifter and more efficient than conventional chemical methods, being a key factor. Microorganisms also offer the advantage of possessing sizable and easily modifiable gene structures. Additionally, the production of pigments by microorganisms remains unaffected by weather conditions, preventing variations in colour shades, and these microorganisms can be cultivated on low-cost substrates (Joshi et al., 2003; Petrik et al., 2013; Paul et al., 2023).
Carotenoids, a specific class of natural pigments, are of significant importance within this context. They are synthesised by both plants and microorganisms in response to various environmental stresses (Kirti et al., 2014). Carotenoids serve various purposes, functioning as colorants, dietary supplements and nutraceuticals (Mussagy et al., 2019). Among the microbial sources of carotenoids, particular attention has been directed towards yeast species, particularly those belonging to the genera *Rhodotorula* and *Phaffia* (Martin et al., 1993; Meyer & Du Preez, 1994; Frengova & Beshkova, 2009;

Marova et al., 2010; Allahkarami et al., 2021; Li et al., 2022). These pigmented yeasts possess advantages over algae, filamentous fungi and bacteria in terms of their rapid growth rate and adaptability to cost-effective fermentation conditions (Malisorn & Suntornsuk, 2008; Panesar et al., 2015; Sen et al., 2019).

Recognition of the significance of carotenoids in healthcare, as well as in the food and nutraceutical industries, continues to grow. The escalating demand for carotenoids in those markets is fuelled by emerging clinical studies highlighting their diverse health benefits. Among the numerous naturally occurring carotenoids, only a few have demonstrated value in industries related to both humans and animals, primarily due to their antioxidant and light-absorbing properties (Bendich, 1994; King, 1995; Krinsky, 2001; Perera & Yen, 2007; Barnett & Chandi & Gill, 2011; Berman et al., 2015; Bogacz-Radomska et al., 2020; Rapoport et al., 2021; Paul et al., 2023).

Carotenoids derived from microorganisms are preferable to synthetic food colourants owing to their consistent availability, year-round production feasibility, scalability and higher yield (Mussagy et al., 2019). While a wide spectrum of carotenoids exists in nature, only a select few, including β-carotene, lycopene, astaxanthin, canthaxanthin and lutein, are commercially accessible (Johnson & Schroeder, 2006). These carotenoids are used in various food products such as juices, baked goods, dairy items, canned goods, confectionery and health-focused condiments. Interestingly, animals such as birds, fish and crustaceans benefit from β-carotene supplementation for colour enhancement, which also increases the visual appeal of pet food (Cantrell et al., 2003; Sandmann, 2015).

The carotenoids that have been least explored to date are torulene and torularhodin, which are primarily synthesised by yeast species within the genera *Rhodotorula*, *Sporobolomyces* and *Sporidiobolus* (Kot et al., 2018; Mussagy et al., 2019). These compounds take on an attractive rosy-red shade at sufficient concentrations (Zoz et al., 2014). Although currently limited to research applications, their distinctive chemical structure and inherent properties hint at potential utility across diverse industries. Their potential extends to sectors such as food production, where they could serve as valuable additives, as well as roles in animal feed, cosmetics and pharmaceutical formulations. Their versatility arises from their dual attributes as antioxidants and colorants, coupled with potent antimicrobial capabilities (Zoz et al., 2014; Kot et al., 2018).

The current interest in carotenoids has been spurred by epidemiological investigations clearly suggesting that integrating carotenoid-rich foods into diets could notably reduce the risk of various diseases. These include health conditions such as cancers, cardiovascular disorders, age-related macular degeneration, cataracts, immune-related issues and other degenerative ailments (Krinsky, 2001; Perera & Yen, 2007).

4. Extraction methods for lipids and carotenoids

In order to accurately assess yeast production by oleaginous microorganisms, it is imperative to employ an extraction method that offers both high efficiency and reproducibility. Standardising the extraction process is essential to facilitate precise comparisons of results. However, it is important to recognise that there is no universally efficient extraction method that is applicable to all oleaginous microorganisms (Yu et al., 2015).
Studies involving various species of microalgae or yeasts have found that the most effective extraction method can vary between species (Fang & Wang, 2002; Lee et al., 2010; Prabakaran & Ravindran, 2011; Bonturi et al., 2015). This variation arises from the fact that different microorganisms possess unique physical characteristics, such as varying cell shapes, cell wall structure and lipid compositions. Furthermore, even within the same yeast species, variations can occur as cell walls become thicker with growth and with the appearance of scars resulting from cell division (Jacob, 1992). The development of thicker walls renders cells resistant to specific solvents, adding complexity to the extraction process (Ageitos et al., 2011). Effectively disrupting the cells of oleaginous microorganisms is thus a pivotal step in achieving successful extraction. To accommodate the diverse composition of different species of microalgae or yeasts, it is generally advantageous to employ a combination of polar and non-polar solvents during the extraction process (Li et al., 2014).

4.1 Traditional extraction methods

Historically, lipids and carotenoids from oleaginous yeasts have been extracted using organic solvents. These techniques have a long-standing

tradition, have been extensively documented in the scientific literature and have seen widespread application in both research and laboratory environments.

4.1.1 Conventional lipid extraction

Numerous techniques have been developed over time to optimise recovery of lipids from different oleaginous yeast species. The majority of previous studies investigating lipids in oleaginous yeasts have employed lipid extraction based on a modified version of the Folch extraction method (Folch et al., 1957). The fundamental principle of the Folch method is partitioning of lipids within a biphasic mixture of chloroform and methanol. Methanol plays a crucial role in breaking hydrogen bonds between lipids and proteins once an organic solvent such as chloroform is introduced. The total fat content is then determined through gravimetric measurements.

However, when extracting lipids from oleaginous yeasts, it is essential to pre-treat the biomass to disrupt the intricate and resilient cell wall structure and membrane system within yeast cells. Such pre-treatment is necessary to enable extraction of the majority of intracellular lipids. A range of mechanical, chemical and enzymatic pre-treatment methods can be employed to effectively break down the cells and make most of the intracellular lipids accessible for extraction (Jacob, 1992).

In Paper I, a physical in-house technique involving a French press was used to disrupt the cell wall of the oleaginous yeast prior to commencing the lipid extraction process. Inside the French press, the liquid sample is pressurised by a piston and forced through a small valve at high pressure. As the sample passes through this valve, the cells experience shear stress, leading to the disruption of cellular structures. The pre-treated cell biomass is then subjected to lipid extraction. This specific procedural approach, which incorporates certain modifications to the original Folch method for lipid extraction, is described in detail in Paper I. In brief, the freeze-dried, French-pressed biomass undergoes a multi-step treatment process involving heating and separation using 1 M HCl, 0.8% KCl, Folch solution (consisting of chloroform and methanol in a 2:1 v/v ratio) and pure chloroform. Separation of components is carried out using separating funnel and the lower phase rich in lipids is collected in a pre-weighed glass tube (Figure 5).

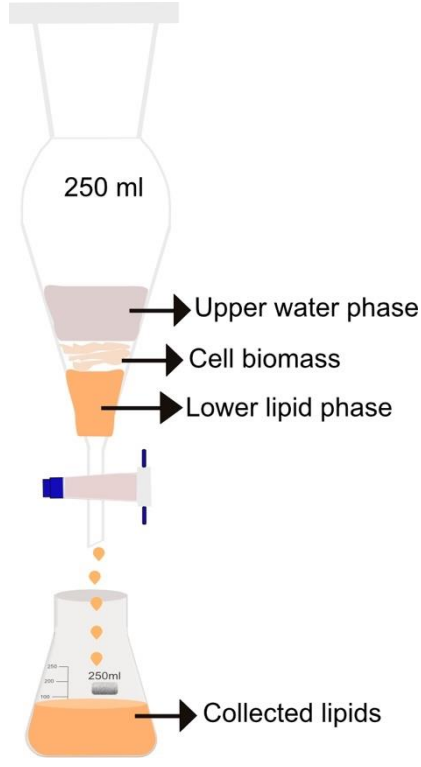

Figure 5. Schematic representation of lipid extraction from oleaginous yeast using a separating funnel.

4.1.2 Conventional carotenoid extraction

Over the past few decades, there have been focused efforts to develop improved methods for extracting carotenoids. The main challenge arises from the presence of a diverse range of carotenoids with varying degree of polarity, making extraction of single compounds a complex task. Furthermore, carotenoids are susceptible to oxidation, necessitating precautions against prolonged exposure to excessive heat, light, acids and extended extraction duration (Moliné et al., 2010).

Due to the hydrophobic properties of carotenoids, conventional extraction methods rely on organic solvents. Typically, a non-polar solvent such as hexane, petroleum ether or tetrahydrofuran is ideal for extracting non-polar carotenes or esterified xanthophylls, while a polar solvent such as acetone, ethanol or ethyl acetate is better suited for extracting polar carotenoids.

In the study described in detail in Paper I, the acetone extraction method originally outlined by Reif et al., (Reif et al., 2013) with some modifications, was used to extract carotenoids from oleaginous yeast. In brief, yeast biomass which had undergone the French-press treatment was subjected to an extraction process involving acetone and cyclohexane. At the end of the extraction, the acetone collected in the glass tube was evaporated under nitrogen atmosphere, leaving behind the carotenoid extract (Paper I).
The primary aim of using this extraction method for analysing carotenoid compounds in samples was to maximise the recovery of a wide spectrum of analytes. Among the various stages involved in carotenoid extraction, it is often considered essential to conduct saponification of co-extracted lipids prior to quantification. The saponification step serves the purpose of simplifying the separation process by eliminating substances, such as lipids, that could potentially interfere with chromatographic detection of carotenoids. Consequently, use of methanolic potassium hydroxide for saponification has long been a fundamental step in carotenoid analysis. The effect of saponification was examined in detail in Paper I.

4.2 Supercritical carbon-dioxide (SC-CO_2) extraction

Over the past decade, there has been growing interest in eco-friendly extraction techniques that can lower energy consumption and avoid the use of conventional solvents. Ideally, these methods prioritise the use of non-toxic, non-flammable solvents derived from biomass sources (Chemat et al., 2012). One noteworthy approach in this regard is supercritical fluid extraction (SFE), a widely adopted method for retrieving valuable substances from natural source materials.

4.2.1 Supercritical carbon dioxide (SC-CO_2)

The supercritical state is attained when both the temperature and pressure of a substance exceed their respective critical value. In this state, the traditional boundaries between the liquid and gas phases cease to exist, and the substance cannot be converted into a liquid by increasing the pressure or into a gas by raising the temperature (Figure 6). Consequently, physical and chemical characteristics of the substance, such as density, diffusivity, dielectric constant and viscosity, can be readily manipulated by adjusting

either the pressure or the temperature, all without undergoing phase transitions (Sihvonen et al., 1999).

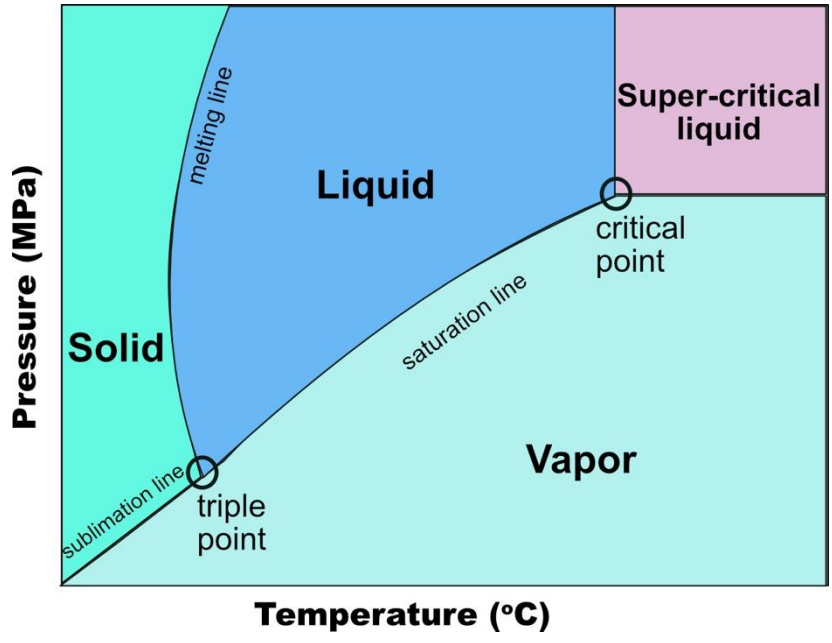

Figure 6. Carbon-dioxide phase diagram modified from (Marini, 2006).

Supercritical carbon dioxide exhibits unique properties, including low viscosity, gas-like diffusion, liquid-like density and almost negligible surface tension (Hitchen & Dean, 1993). Additionally, the carbon dioxide (CO_2) is non-toxic, readily available, volatile, non-flammable, has low critical temperature and can be recycled during the process to mitigate greenhouse effects (Ghasemi et al., 2011; Uwineza & Waśkiewicz, 2020). Consequently, SC-CO_2 prevents thermal and chemical degradation of extracts, as CO_2 can be easily removed from the extract as a gas through simple decompression (Picot-Allain et al., 2021). Moreover, the selectivity of SC-CO_2 can be readily adjusted by modifying extraction conditions, such as pressure and temperature, or by incorporating a co-solvent (Picot-Allain et al., 2021; Uwineza & Waśkiewicz, 2020; Cardoso et al., 2012).

4.2.2 SC-CO_2 apparatus

The process begins with withdrawal of liquid CO_2 from a cylinder and subjecting it to compression using a pump (Paper I). Before reaching the extraction cells, this compressed CO_2 is directed through a pre-heated column. The extraction vessel itself is maintained at a specific temperature using an oven, ensuring precise temperature control. To control the pressure within the system, a back-pressure regulator is employed to maintain the desired pressure conditions (Figure 7). One notable characteristic of CO_2 is its non-polar nature, which means that it is not particularly effective as a solvent for polar compounds. To enhance its ability to dissolve polar substances, a modifier is introduced into the system. This modifier is combined with the CO_2 in a dedicated modifier pump before being introduced into the extraction vessel.

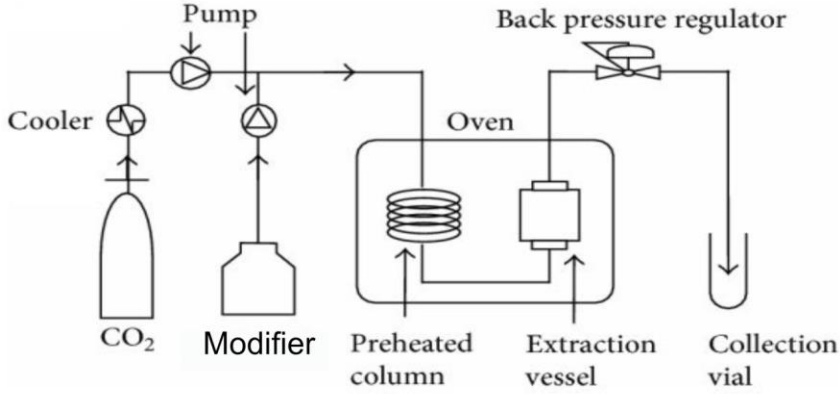

Figure 7. Schematic diagram of an idealised supercritical carbon dioxide extraction process.

The process begins with placing a freeze-dried yeast sample (see Figure 8) in the extraction vessel, where it is mixed with a layer of silica beads (Papers II and III). These beads serve as distributors for the flow of solvent. Extraction of compounds from the yeast sample relies on the density of the supercritical fluid, which in turn depends on its pressure when the temperature is held constant. Once the extraction of compounds from the yeast sample is complete, the CO_2 is depressurised to atmospheric pressure. At this reduced pressure, the extracted compounds become practically insoluble in CO_2 and are collected in designated collection bottles.

4.2.3 Advantages of the supercritical fluid extraction (SFE) technique over conventional methods

Supercritical fluid extraction has several unique properties that make it a promising alternative to conventional solvent extraction methods. Some of its key advantages are summarised below.

1. Supercritical fluids (SFs) have relatively lower viscosity and higher diffusivity than organic solvents. Therefore, they can penetrate into porous solid materials more effectively than liquid solvents and can achieve much faster mass transfer, resulting in faster extraction (Wheeler & McNally, 1989; Marsili & Callahan, 1993; Brühl & Matthäus, 1999; Díaz-Reinoso et al., 2006; Dumont & Narine, 2007).
2. In SFE, the solvation power of the fluid can be manipulated by changing the pressure and/or temperature, so remarkably high selectivity can be achieved. The adjustable solvation power of SFs is particularly useful for extraction of complex samples (Reverchon et al., 1993; Abbas et al., 2008).
3. Solutes dissolved in SC-CO_2 can be easily separated from the solvent by depressurisation. Therefore, SFE can eliminate the sample concentration process, which usually is time-consuming and often results in loss of volatile components (Henning et al., 1994; Dron et al., 1997; Lang & Wai, 2001).
4. Unlike most conventional extraction methods, SFE usually is performed at low temperature (from around 35 °C to up to 80°C), so it is an ideal technique for studying thermally labile compounds and may lead to the discovery of new natural compounds (Dron et al., 1997; Lang & Wai, 2001; Lopes et al., 2012; Sahena et al., 2009; Viguera et al., 2016).
5. Significantly less or no environmentally hostile organic solvent is used in SFE, which may require at most a few mL of organic solvent (Wheeler & McNally, 1989; Otterbach & Wenclawiak, 1999; Rozzi & Singh, 2002; Ramsey et al., 2009).
6. SFE may allow direct coupling with a chromatographic method, which can be a useful means to extract and directly quantify highly volatile compounds (Huston & Ji, 1991; Nielsen et al., 1991; Smith & Burford, 1992; Modey et al., 1996).
7. In large-scale SFE processes, the fluid (usually CO_2) can be recycled or reused, thus minimising waste generation.

8. SFE can be applied to systems of different scales, e.g., analytical, preparative, pilot plant or large industrial scale (Reverchon et al., 1993; Fuh et al., 1995; Lang & Wai, 2001; Luu, 2010).

4.2.4 Utilisation of SC-CO_2 residues

Enhancing the economic sustainability of yeast production involves optimising the utilisation of both the extracted components and the by-products of extraction. The residual materials from SC-CO_2 extraction include valuable compounds such as proteins, polysaccharides, starch and other substances that are insoluble in CO_2 (Gao et al., 2012). These residual resources have the potential to serve various purposes, such as supplementing human nutrition, applications in economically viable sectors such as animal feed production or as a substrate for biogas generation (Gao et al., 2012; Grimm et al., 2015; Tibbetts et al., 2017). It has been shown that the protein content of *Spirulina* biomass remains largely unaffected following SC-CO_2 extraction, experiencing only a marginal decrease of approximately 1% in essential amino acids (Qiuhui, 1999). This finding confirms that the nutritional value of oleaginous yeast biomass is preserved during the extraction process. It has also been demonstrated that, following SC-CO_2 extraction, biomass of the microalga *Nannochloropsis granulata* can serve as a valuable protein source with minimal amino acid degradation (Tibbetts et al., 2015).

In a sustainability perspective, utilisation of the entire volume of yeast biomass produced minimises waste generation and associated management costs. It is crucial to note that the choice of extraction method significantly influences the quality and potential for re-utilisation of both extracts and extraction residues, as well as the environmental impacts of the overall process.

Figure 8. Freeze-dried biomass of *Rhodotorula toruloides* CBS14 (a) before and (b) after supercritical extraction of lipids and carotenoids (Paper II).

5. Analysis of lipid and carotenoid extracts

5.1 Lipid analysis

Conventional approaches for lipid quantification involve measurement through gravimetric assays. The lipid composition is analysed by chromatographic techniques such as gas chromatography (GC) or thin-layer chromatography (TLC), methods characterised by time-consuming and multi-step procedures (Mubarak et al., 2015).

5.1.1 Fatty acid profile

Liquid chromatography methods (LC, HPLC, ultra-high-performance liquid chromatography (UHPLC) and GC are extensively used in lipid profiling and are frequently combined with mass spectrometry (-MS) to enable comprehensive analysis (Patel et al., 2019). Following the lipid extraction process, a commonly employed step prior to GC involves conversion of saponifiable lipids into acyl esters of fatty acids through a transesterification reaction, typically accomplished using methylation (Hounslow et al., 2017). This reaction entails addition of a catalyst, often an acid or a base, together with an alcohol, with methanol being most frequently used type (Cavonius et al., 2014) (Papers I-III).

The main fatty acids identified in oleaginous yeasts, particularly *R. toruloides*, in this thesis were oleic acid, palmitic acid, linoleic acid, linolenic acid and stearic acid. The proportions of these fatty acid vary with the cell growth period, as demonstrated in Paper I, where there was an increase in the percentage of saturated fatty acids and a decrease in unsaturated fatty acids during the course of cell cultivation (Figure 9).

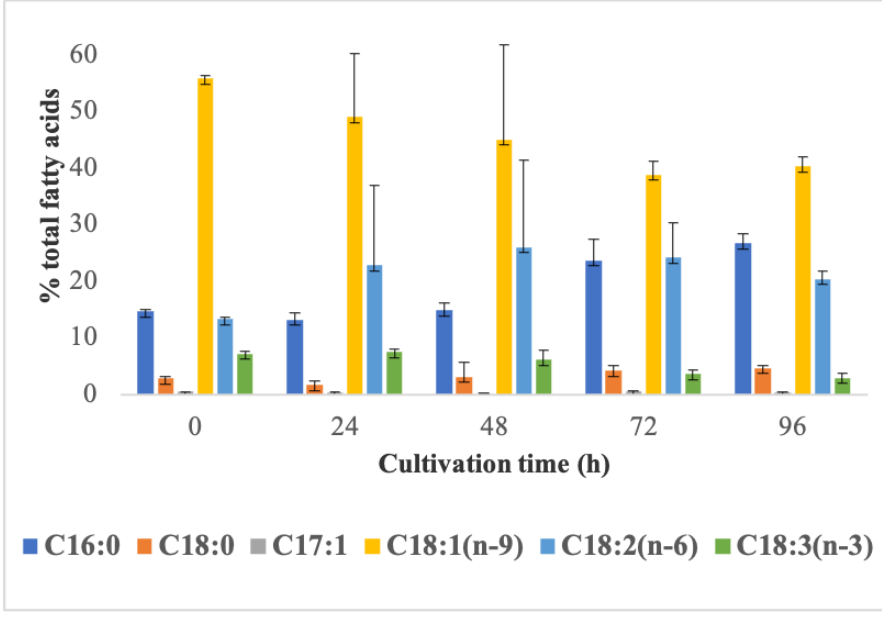

Figure 9. Fatty acid composition of *Rhodotorula toruloides* strain CBS 14 over time during cultivation on wheat straw hydrolysate (data from Paper I).

The relative ratios of these fatty acids may also undergo changes due to different conditions introduced by various extraction techniques. In the case of SC-CO_2 extraction, the process occurs in an oxygen-free environment and at lower temperatures, resulting in an extract with a higher concentration of unsaturated fatty acids (Paper II) compared with conventional extraction methods (Han et al., 2020; Kayathi et al., 2020) (Figure 10).

In Paper III in this thesis, the main strains of *Rhodotorula toruloides* were examined to investigate variations in lipid production between the strains. An intriguing finding was that no notable distinctions were detected in the major fatty acid profile of the strains.

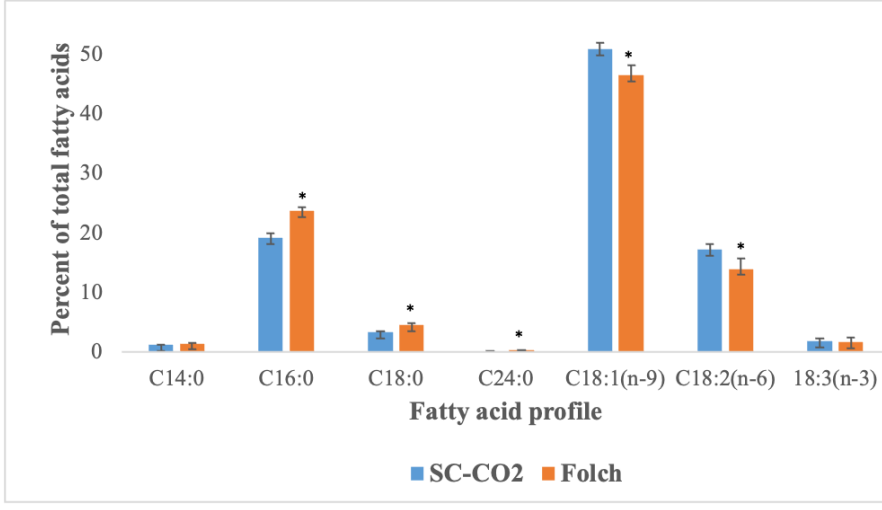

Figure 10. Fatty acid composition of *Rhodotorula toruloides* strain CBS 14 samples extracted using the Folch and supercritical carbon dioxide (SC-CO_2) methods. Asterisks indicate statistically significant differences ($p<0.05$) (data from Paper II).

5.1.2 Lipid classes

Thin-layer chromatography (TLC) is a commonly employed method for separating, identifying and quantifying individual lipids, owing to its cost-effectiveness, straightforward approach and user-friendly benefits. This chromatographic technique uses silica gel coated on a thin plate as the stationary phase and a blend of organic solvents as the mobile phase. Separation is achieved based on differences in the affinity of compounds for both phases. Compounds in the mobile phase migrate upward over the surface of the stationary phase due to capillary forces (Lade et al., 2014; Cai, 2014).

Triacylglycerols are the most abundant lipid class in oleaginous yeasts, followed by free fatty acids (FFAs) (Shapaval et al., 2019; Caporusso et al., 2021; Nagaraj et al., 2022). The relative proportions of these lipid classes undergo changes in conjunction with the different growth stages of the yeast cells (Paper I). The analyses in this thesis showed that FFAs were prominently present in *R. toruloides* strain CBS 14, as the dominant lipid class in early growth phases and as the second most abundant lipid class (after TAGs) towards the end of the fermentation process (Paper I).

5.2 Carotenoid analysis

Carotenoids, which are typically analysed using chromatography and spectrophotometry (UV/Visible), require an initial extraction step from the sample. Since carotenoids are lipophilic, a blend of organic solvents is commonly employed for this purpose (de Quirós & Costa, 2006; Kopec et al., 2012). Maintaining the stability of carotenoids during extraction is crucial to ensure accurate analytical results, because carotenoids are prone to isomerisation, oxidation and degradation (Kopec et al., 2012). Carotenoids have a highly conjugated structure, making them susceptible to deterioration when exposed to heat, light, oxygen and metal ions (Philip & Francis, 1971; Scita, 1992; Henry et al., 1998; Moliné et al., 2010). Therefore, it is essential to handle samples in low or red-light conditions, evaporate solvents in an atmosphere of inert gas (e.g., nitrogen) and store samples in darkness at temperatures below -20 °C.

Before carotenoid extracts are subjected to chromatographic analysis, saponification has to be performed in a critical preliminary step. In essence, saponification is a chemical process initiated by interactions between free hydroxide ions and triglycerides which results in cleavage of ester bonds that link the fatty acids and glycerol components. The main aim in including a saponification step is primarily to break down lipids, substances that typically do not readily dissolve in water. This particular step has significant importance in eliminating undesired lipids, which have the potential to disrupt chromatographic separation and reduce the lifespan of the chromatographic column. Saponification is typically carried out using a solution containing either potassium or sodium hydroxide in methanol, ethanol or water. It is important to note that while saponification effectively removes unwanted lipids, it may also result in some loss of carotenoids during the process (Paper I; Khachik et al., 1986; Kimura et al., 1990; Nierenberg & Nann, 1992; Lietz & Henry, 1997).

5.2.1 Chromatographic analysis

Chromatography using descending gravity-flow columns, often with slight pressure assistance from a water aspirator, is referred to as open column chromatography (OCC). This classical method is employed for the separation of carotenoids in quantitative analysis and also for isolating and purifying carotenoids to serve as standards in HPLC. The initial techniques for separating carotenoids and chlorophylls through OCC date back to 1906

(Tswett, 1906). A photodiode array (PDA) detector is the most commonly used detector in HPLC carotenoid analysis (Sedmak et al., 1990). However, alternatives such as electrochemical detectors (ECD), fluorescence, mass spectrometry (MS), and nuclear magnetic resonance (NMR) can also be applied.

In recent years, reversed-phase HPLC using C18 columns has become the preferred approach for carotenoid separation in quantitative analysis. The popularity of C18 columns stems from their weaker hydrophobic interactions with analytes (which tend to be less destructive than the polar forces in normal-phase OCC), and their compatibility with most carotenoid solvents, adaptability to the polarity range of carotenoids and widespread commercial availability (Rodriguez-Amaya & Kimura, 2004). More recently, UHPLC technology has begun to be employed to analyse carotenoids in various samples (Li et al., 2012). The UHPLC approach offers several advantages over HPLC, including higher peak capacity, narrower peak width, increased sensitivity and enhanced chromatographic resolution (Gupta et al., 2015).

In this thesis, a combined UHPLC-PDA method was employed to identify and quantify the carotenoids produced by *R. toruloides*. The carotenoids identified included β-carotene, γ-carotene, torulene and torularhodin, as described in detail in Papers I-III. The impact of saponification on quantification of these individual carotenoids was extensively investigated in Paper I (Figure 11). We observed a positive correlation between the accumulation of lipids and the production of carotenoids. This suggests that these metabolic pathways, despite utilizing the same precursor, do not compete with each other (Paper I).

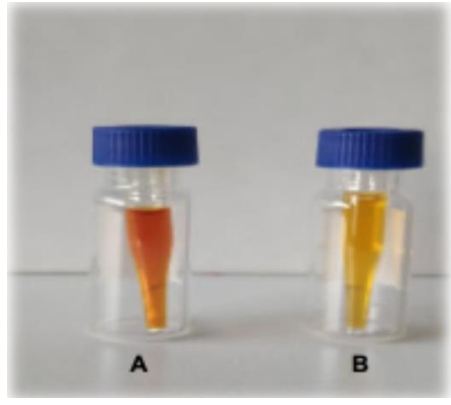

Figure 11. Effect of saponification on carotenoid extract from *Rhodotorula toruloides* cultivated on wheat straw hydrolysate. (A) Unsaponified extract and (B) saponified extract.

The quantity of these extracted carotenoids was found to vary depending on the extraction technique used. Notably, in the case of SC-CO_2 extraction, where no saponification was applied, torularhodin and torulene were observed in higher concentrations compared with β-carotene obtained through the acetone extraction method (Paper I). This finding supported the hypothesis tested in Paper II that saponification impairs accurate quantification of carotenoids (Figure 12).

On examining the carotenoid profile of the parental strains *R. toruloides* CBS 14 and CBS 349 in comparison with that of the hybrid strain *R. toruloides* 6016^T, it was found that CBS 6016^T had a carotenoid profile and a total carotenoid content similar to CBS 14, but not CBS 349 (Paper III). This indicates that the genetic traits responsible for efficient carotenoid synthesis in CBS 14 are inherited and actively expressed in the hybrid strain CBS 6016^T. This in turn implies that the genetic makeup of the hybrid strain is primarily, if not exclusively, derived from CBS 14, particularly representing the A1 mating type (Sambles et al., 2017). These findings align with prior observations made within our research group (Pappas, 2023). Visual confirmation of these similarities in carotenoid concentration was obtained by examining the appearance of freeze-dried cells of the three strains (see Figure 2).

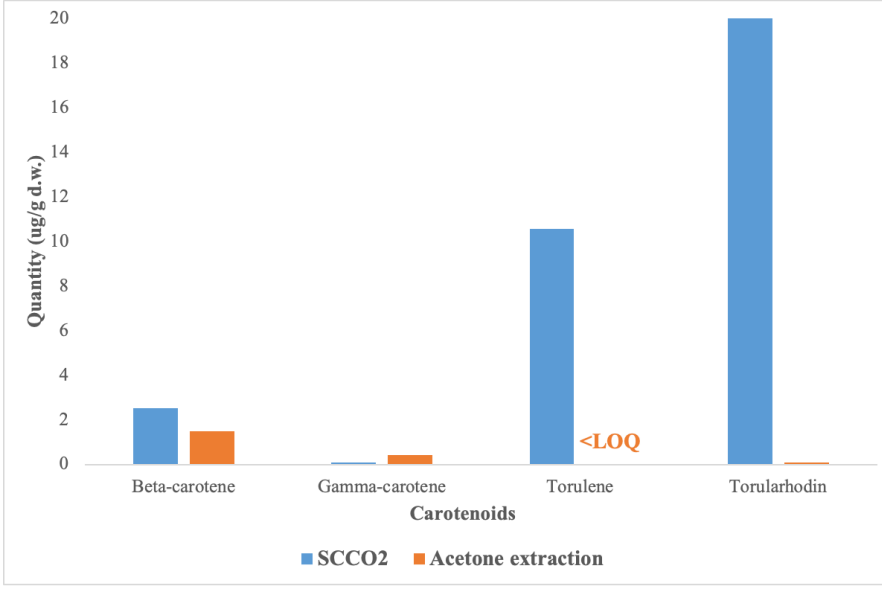

Figure 12. Quantities of different carotenoids extracted from *Rhodotorula toruloides* strain CBS 14 using supercritical carbon dioxide (SC-CO_2) and acetone extraction techniques (modified from Papers I and II).

5.2.2 UV/Visible spectrophotometric analysis

Carotenoids are primarily characterised by a series of conjugated double bonds (Maoka, 2020), which is why they typically exhibit absorption within the 400-500 nm range (Udensi et al., 2022). Spectral data are invaluable for distinguishing and identifying various carotenoid species (Scott, 2001). In the case of common carotenoids, UV/Visible spectra offer additional insights, primarily into the chromophore of the carotenoid (Kopec et al., 2012). However, the spectra of carotenoids can be influenced by different solvents and carotenoids may interact with proteins and lipids, leading to alterations in their spectral characteristics (Mendes-Pinto et al., 2012; Gong et al., 2018; Meinhardt-Wollweber et al., 2018).

Most carotenoids display maximal absorption at three distinct wavelengths, resulting in a tri-peak spectrum. As the number of conjugated double bonds in a carotenoid increases, the wavelength of maximum absorption (λ_{max}) shifts toward longer values (Rodriguez-Amaya & Kimura, 2004). Individual standard curves, such as those for β-carotene, are typically employed to quantify the total carotenoid content in a sample (Paper I).

6. Conclusions and future perspectives

Oleaginous yeasts possess a remarkable capacity to produce and accumulate triacylglycerides (TAGs), to levels exceeding 20% of their dry cell weight. Microbial lipids closely resemble vegetable oils, so these yeasts have significant potential for biodiesel production from their TAGs but also for creating substitutes for cocoa butter and generating polyunsaturated fatty acids (PUFAs), with applications in both nutrition and medicine. Yeast oil may also find uses as a substitute for vegetable oil in production of animal feed and fish feed. Oleaginous *Rhodotorula* yeasts are various shades of orange, red and pink in colour, due to the presence of diverse carotenoids. These carotenoids are important as valuable bioproducts in the nutraceutical and food industries. Oleaginous yeasts offer several additional advantages over alternative oil sources, including ease of cultivation, rapid growth rate and the ability to thrive on low-cost carbon sources.

To unlock the biotechnological capabilities of oleaginous yeasts for lipid and carotenoid production, highly efficient extraction methods are needed. Optimisation of the extraction process is essential to ensure maximum yield and purity of lipids and carotenoids. This involves careful selection of appropriate solvents, extraction techniques and conditions that can effectively break down the cell structure of the yeasts to release the desired compounds. The extraction method used must also be designed to minimise losses and preserve the quality of lipids and carotenoids.

This thesis provided valuable new insights into how different extraction techniques affect the composition of lipids and carotenoids extracted from the oleaginous yeast species *Rhodotorula toruloides*. An investigation into the growth kinetics of *R. toruloides* strain CBS 14 revealed a correlation between yeast growth stage and the relative quantities of different lipid fractions, individual lipid classes and their fatty acid composition. A positive

correlation between accumulation of lipids and production of carotenoids was observed, suggesting that these metabolic pathways, which use the same precursor, do not affect each other. Further analyses revealed a substantial impact of saponification on the concentrations of carotenoids, particularly torulene and torularhodin. This highlights the need to develop an extraction method that can quantify carotenoids from yeasts more accurately without the need for a saponification step. Tests on the supercritical carbon dioxide (SC-CO_2) method, as an alternative to conventional extraction, revealed that lipids were extracted before carotenoids, eliminating the need for saponification. The findings also highlighted the SC-CO_2 method's effectiveness in maintaining the integrity of unsaturated lipids and the abundance of carotenoids, leading to the generation of high-quality extracts. Further research is needed to refine and optimise the SC-CO_2 extraction method in order to improve efficiency, reduce energy consumption and make the process more cost-effective.

Analyses of the lipid and carotenoid profiles of the three main *R. toruloides* strains revealed similar fatty acid profile in all strains. The results also revealed that the hybrid strain *R. toruloides* CBS 6016^T predominantly exhibited genetic traits governing carotenoid profile from one of its parental strains (CBS 14), but not the other (CBS 349). These findings strongly indicate that CBS 6016^T primarily acquired and expresses the genetic characteristics associated with CBS 14, suggesting a dominant influence of this specific parental strain.

Future research should explore the possibility of genetically engineering oleaginous yeasts to simultaneously produce a broader spectrum of valuable compounds, alongside lipids and carotenoids, and thus diversify the range of bioproducts derived from these yeasts. Parallel scrutiny of the regulatory conditions and market dynamics pertaining to products derived from oleaginous yeasts, such as biodiesel, would ensure that this research aligns seamlessly with industry standards and complies with relevant regulations.

References

Abbas, K., Saeed, M., Abdulamir, A., & Abas, H. (2008). A review on supercritical fluid extraction as new analytical method. *American Journal of Biochemistry and Biotechnology*, 4(4), 345-353.

Abeln, F., & Chuck, C. J. (2021). The history, state of the art and future prospects for oleaginous yeast research. *Microbial Cell Factories*, 20(1), 1-31.

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., & Levin, D. B. (2011). Biomass pretreatment: fundamentals toward application. *Biotechnology Advances*, 29(6), 675-685.

Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P., & Villa, T. G. (2011). Oily yeasts as oleaginous cell factories. *Applied Microbiology and Biotechnology*, 90, 1219-1227.

Aksu, Z., & Eren, A. T. (2005). Carotenoids production by the yeast *Rhodotorula mucilaginosa*: use of agricultural wastes as a carbon source. *Process Biochemistry*, 40(9), 2985-2991.

Alexandri, M., Kachrimanidou, V., Papapostolou, H., Papadaki, A., & Kopsahelis, N. (2022). Sustainable Food Systems: The Case of Functional Compounds towards the Development of Clean Label Food Products. *Foods*, 11(18), 2796.

Ali, S. S., Mastropetros, S. G., Schagerl, M., Sakarika, M., Elsamahy, T., El-Sheekh, M., Sun, J., & Kornaros, M. (2022). Recent advances in wastewater microalgae-based biofuels production: A state-of-the-art review. *Energy Reports*, 8, 13253-13280.

Allahkarami, S., Sepahi, A. A., Hosseini, H., & Razavi, M. R. (2021). Isolation and identification of carotenoid-producing *Rhodotorula* sp. from Pinaceae forest ecosystems and optimization of in vitro carotenoid production. *Biotechnology Reports*, 32, e00687.

Andlar, M., Rezić, T., Marđetko, N., Kracher, D., Ludwig, R., & Šantek, B. (2018). Lignocellulose degradation: An overview of fungi and fungal enzymes involved in lignocellulose degradation. *Engineering in Life Sciences*, 18(11), 768-778.

Babitha, S. (2009). Microbial pigments. Biotechnology for Agro-Industrial Residues Utilisation: *Utilisation of Agro-Residues*, 147-162.

Babu, S., & Shenolikar, I. (1995). Health & nutritional implications of food colours. *The Indian Journal of Medical Research*, 102, 245-249.

Baksi, S., Saha, D., Saha, S., Sarkar, U., Basu, D., & Kuniyal, J. (2023). Pre-treatment of lignocellulosic biomass: review of various physico-chemical

and biological methods influencing the extent of biomass depolymerization. *International Journal of Environmental Science and Technology*, 1-28.

Balan, V. (2019). Microbial lipid production: methods and protocols. Springer.

Banno, I. (1967). Studies on the sexuality of *Rhodotorula*. *The Journal of General and Applied Microbiology*, 13(2), 167-196.

Barnett, Y., & King, C. (1995). An investigation of antioxidant status, DNA repair capacity and mutation as a function of age in humans. *Mutation Research/DNAging*, 338(1-6), 115-128.

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

Bendich, A. (1994). Recent advances in clinical research involving carotenoids. *Pure and Applied Chemistry*, 66(5), 1017-1024.

Bensah, E. C., & Mensah, M. Y. (2018). Emerging physico-chemical methods for biomass pretreatment. Fuel Ethanol Production from Sugarcane, 41-62.

Beopoulos, A., Chardot, T., & Nicaud, J.-M. (2009). *Yarrowia lipolytica*: A model and a tool to understand the mechanisms implicated in lipid accumulation. *Biochimie*, 91(6), 692-696.

Beopoulos, A., & Nicaud, J.-M. (2012). Yeast: A new oil producer? Oléagineux, Corps gras, *Lipides*, 19(1), 22-28.

Berman, J., Zorrilla-López, U., Farré, G., Zhu, C., Sandmann, G., Twyman, R. M., Capell, T., & Christou, P. (2015). Nutritionally important carotenoids as consumer products. *Phytochemistry Reviews*, 14, 727-743.

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

Biely, P., Singh, S., & Puchart, V. (2016). Towards enzymatic breakdown of complex plant xylan structures: state of the art. *Biotechnology Advances*, 34(7), 1260-1274.

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

Bogacz-Radomska, L., Harasym, J., & Piwowar, A. (2020). Commercialization aspects of carotenoids. In Carotenoids: Properties, Processing and Applications (pp. 327-357). *Elsevier*.

Bonturi, N., Matsakas, L., Nilsson, R., Christakopoulos, P., Miranda, E. A., Berglund, K. A., & Rova, U. (2015). Single cell oil producing yeasts *Lipomyces starkeyi* and *Rhodosporidium toruloides*: selection of extraction strategies and biodiesel property prediction. *Energies*, 8(6), 5040-5052.

Brandenburg, J., Blomqvist, J., Shapaval, V., Kohler, A., Sampels, S., Sandgren, M., & Passoth, V. (2021). Oleaginous yeasts respond differently to carbon

sources present in lignocellulose hydrolysate. *Biotechnology for Biofuels*, 14(1), 124.

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

Brühl, L., & Matthäus, B. (1999). Extraction of oilseeds by SFE–a comparison with other methods for the determination of the oil content. *Fresenius' Journal of Analytical Chemistry*, 364, 631-634.

Buzzini, P., Innocenti, M., Turchetti, B., Libkind, D., van Broock, M., & Mulinacci, N. (2007). Carotenoid profiles of yeasts belonging to the genera *Rhodotorula*, *Rhodosporidium, Sporobolomyces,* and *Sporidiobolus*. *Canadian Journal of Microbiology*, 53(8), 1024-1031.

Cai, L. (2014). Thin layer chromatography. Current Protocols Essential Laboratory Techniques, 8(1), 6.3. 1-6.3. 18.

Cañizares-Villanueva, R., & Rios-Leal, E. (1998). Microbial sources of pigments. *Revista Latinoamericana de Microbiologia*, 40(1-2), 87-107.

Cantrell, A., McGarvey, D., Truscott, T. G., Rancan, F., & Böhm, F. (2003). Singlet oxygen quenching by dietary carotenoids in a model membrane environment. *Archives of Biochemistry and Biophysics*, 412(1), 47-54.

Caporusso, A., Capece, A., & De Bari, I. (2021). Oleaginous yeasts as cell factories for the sustainable production of microbial lipids by the valorization of agri-food wastes. *Fermentation*, 7(2), 50.

Cardoso, L. C., Serrano, C. M., Rodríguez, M. R., de la Ossa, E. J. M., & Lubián, L. M. (2012). Extraction of carotenoids and fatty acids from microalgae using supercritical technology.

Cavonius, L. R., Carlsson, N.-G., & Undeland, I. (2014). Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. *Analytical and Bioanalytical Chemistry*, 406, 7313-7322.

Chandi, G. K., & Gill, B. S. (2011). Production and characterization of microbial carotenoids as an alternative to synthetic colors: a review. *International Journal of Food Properties*, 14(3), 503-513.

Chattopadhyay, A., & Maiti, M. K. (2021). Lipid production by oleaginous yeasts. *Advances in Applied Microbiology*, 116, 1-98.

Chattopadhyay, A., Mitra, M., & Maiti, M. K. (2021). Recent advances in lipid metabolic engineering of oleaginous yeasts. *Biotechnology Advances*, 53, 107722.

Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences*, 13(7), 8615-8627.

Chmielarz, M., Blomqvist, J., Sampels, S., Sandgren, M., & Passoth, V. (2021). Microbial lipid production from crude glycerol and hemicellulosic hydrolysate with oleaginous yeasts. *Biotechnology for Biofuels*, 14(1), 1-11.

Christophe, G., Kumar, V., Nouaille, R., Gaudet, G., Fontanille, P., Pandey, A., Soccol, C. R., & Larroche, C. (2012). Recent developments in microbial oils production: a possible alternative to vegetable oils for biodiesel without competition with human food? *Brazilian Archives of Biology and Technology*, 55, 29-46.

Cohen, Z., & Ratledge, C. (2005). Single Cell Oils: Microbial and Algal Oils.

Danielsen, F., Beukema, H., Burgess, N. D., Parish, F., Brühl, C. A., Donald, P. F., Murdiyarso, D., Phalan, B., Reijnders, L., & Struebig, M. (2009). Biofuel plantations on forested lands: double jeopardy for biodiversity and climate. *Conservation Biology*, 23(2), 348-358.

de Quirós, A. R.-B., & Costa, H. S. (2006). Analysis of carotenoids in vegetable and plasma samples: A review. *Journal of Food Composition and Analysis*, 19(2-3), 97-111.

Díaz-Reinoso, B., Moure, A., Domínguez, H., & Parajó, J. C. (2006). Supercritical CO_2 extraction and purification of compounds with antioxidant activity. *Journal of Agricultural and Food Chemistry*, 54(7), 2441-2469.

Dornburg, V., van Vuuren, D., van de Ven, G., Langeveld, H., Meeusen, M., Banse, M., van Oorschot, M., Ros, J., van den Born, G. J., & Aiking, H. (2010). Bioenergy revisited: key factors in global potentials of bioenergy. *Energy & Environmental Science*, 3(3), 258-267.

Dron, A., GUYERU, D. E., GAGE, D. A., & LIRA, C. T. (1997). Yield and quality of onion flavor oil obtained by supercritical fluid extraction and other methods. *Journal of Food Process Engineering*, 20(2), 107-124.

Dumont, M.-J., & Narine, S. S. (2007). Soapstock and deodorizer distillates from North American vegetable oils: Review on their characterization, extraction and utilization. *Food Research International*, 40(8), 957-974.

Eisenberg, T., & Büttner, S. (2014). Lipids and cell death in yeast. *FEMS yeast research*, 14(1), 179-197.

Fakankun, I., & Levin, D. B. (2023). Oleaginous Red Yeasts: Concomitant Producers of Triacylglycerides and Carotenoids. *Encyclopedia*, 3(2), 490-500.

Fakas, S. (2017). Lipid biosynthesis in yeasts: A comparison of the lipid biosynthetic pathway between the model nonoleaginous yeast *Saccharomyces cerevisiae* and the model oleaginous yeast *Yarrowia lipolytica*. *Engineering in Life Sciences*, 17(3), 292-302.

Fang, T. J., & Wang, J.-M. (2002). Extractability of astaxanthin in a mixed culture of a carotenoid over-producing mutant of *Xanthophyllomyces dendrorhous* and *Bacillus circulans* in two-stage batch fermentation. *Process Biochemistry*, 37(11), 1235-1245.

Fei, Q., O'Brien, M., Nelson, R., Chen, X., Lowell, A., & Dowe, N. (2016). Enhanced lipid production by *Rhodosporidium toruloides* using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. *Biotechnology for Biofuels*, 9, 1-12.

Fichtali, J., & Senanayake, S. N. (2010). Development and Commercialization of Microalgae-Based Functional Lipids. *Functional Food Product Development*, 206-225.

Folch, J., Lees, M., & Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J biol Chem*, 226(1), 497-509.

Frengova, G. I., & Beshkova, D. M. (2009). Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. *Journal of Industrial Microbiology and Biotechnology*, 36(2), 163.

Fuh, M.-R., Pan, W., Hsieh, I., & CHUO, C.-M. (1995). Preparative-scale supercritical fluid extraction of essential oils from *Syzygium aromaticum* (clove bud). *American laboratory* (Fairfield), 27(18), 36-41.

Galán, B., Santos-Merino, M., Nogales, J., De la Cruz, F., & García, J. L. (2020). Microbial oils as nutraceuticals and animal feeds. Health consequences of microbial interactions with hydrocarbons, oils, and lipids, 401-445.

Galbe, M., & Wallberg, O. (2019). Pretreatment for biorefineries: a review of common methods for efficient utilisation of lignocellulosic materials. *Biotechnology for Biofuels*, 12, 1-26.

Gao, M.-T., Shimamura, T., Ishida, N., & Takahashi, H. (2012). Investigation of utilization of the algal biomass residue after oil extraction to lower the total production cost of biodiesel. *Journal of Bioscience and Bioengineering*, 114(3), 330-333.

Ghasemi, E., Raofie, F., & Najafi, N. M. (2011). Application of response surface methodology and central composite design for the optimisation of supercritical fluid extraction of essential oils from Myrtus communis L. leaves. *Food Chemistry*, 126(3), 1449-1453.

Ghilardi, C., Sanmartin Negrete, P., Carelli, A. A., & Borroni, V. (2020). Evaluation of olive mill waste as substrate for carotenoid production by *Rhodotorula mucilaginosa*. *Bioresources and Bioprocessing*, 7(1), 1-11.

Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., & Bogel-Łukasik, R. (2010). Hemicelluloses for fuel ethanol: a review. *Bioresource Technology*, 101(13), 4775-4800.

Gong, N., Fu, H., Wang, S., Cao, X., Li, Z., Sun, C., & Men, Z. (2018). All-trans-β-carotene absorption shift and electron-phonon coupling modulated by solvent polarizability. *Journal of Molecular Liquids*, 251, 417-422.

Goodwin, T. (2012). The biochemistry of the carotenoids: volume I plants. *Springer Science & Business Media*.

Goodwin, T., & Goodwin, T. (1980). Biosynthesis of carotenoids. The Biochemistry of the Carotenoids: Volume I *Plants*, 33-76.

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

Grimm, P., Risse, J. M., Cholewa, D., Müller, J. M., Beshay, U., Friehs, K., & Flaschel, E. (2015). Applicability of *Euglena gracilis* for biorefineries demonstrated by the production of α-tocopherol and paramylon followed by anaerobic digestion. *Journal of Biotechnology*, 215, 72-79.

Gupta, P., Sreelakshmi, Y., & Sharma, R. (2015). A rapid and sensitive method for determination of carotenoids in plant tissues by high performance liquid chromatography. *Plant Methods*, 11(1), 1-12.

Han, L., Han, Q., Yang, Y., Wang, H., Wang, S., & Li, G. (2020). Characterization and biological activities of seed oil extracted from *Berberis dasystachya* Maxim. by the supercritical carbon dioxide extraction method. *Molecules*, 25(8), 1836.

Han, M., Xu, Z.-y., Du, C., Qian, H., & Zhang, W.-G. (2016). Effects of nitrogen on the lipid and carotenoid accumulation of oleaginous yeast *Sporidiobolus pararoseus*. *Bioprocess and Biosystems Engineering*, 39, 1425-1433.

Hayman, E. P., Yokoyama, H., Chichester, C. O., & Simpson, K. L. (1974). Carotenoid biosynthesis in *Rhodotorula glutinis*. *Journal of Bacteriology*, 120(3), 1339-1343.

Henning, J. A., Core, R. J., & Gardea-Torresdey, J. L. (1994). Extracting volatile compounds from single plants using supercritical fluid extraction. *Crop Science,* 34(4), 1120-1122.

Henry, L., Catignani, G., & Schwartz, S. (1998). Oxidative degradation kinetics of lycopene, lutein, and 9-cis and all-trans β-carotene. *Journal of the American Oil Chemists' Society*, 75(7), 823-829.

Hitchen, S., & Dean, J. (1993). Properties of supercritical fluids. In Applications of supercritical fluids in industrial analysis (pp. 1-11). *Springer*.

Houfani, A. A., Anders, N., Spiess, A. C., Baldrian, P., & Benallaoua, S. (2020). Insights from enzymatic degradation of cellulose and hemicellulose to fermentable sugars–a review. *Biomass and Bioenergy*, 134, 105481.

Hounslow, E., Noirel, J., Gilmour, D. J., & Wright, P. C. (2017). Lipid quantification techniques for screening oleaginous species of microalgae for biofuel production. *European Journal of Lipid Science and Technology*, 119(2), 1500469.

Hu, J., Arantes, V., Pribowo, A., & Saddler, J. N. (2013). The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. *Biotechnology for Biofuels*, 6(1), 1-12.

Huang, X., Luo, H., Mu, T., Shen, Y., Yuan, M., & Liu, J. (2018). Enhancement of lipid accumulation by oleaginous yeast through phosphorus limitation under high content of ammonia. *Bioresource Technology*, 262, 9-14.

Huang, Y., Wei, X., Zhou, S., Liu, M., Tu, Y., Li, A., Chen, P., Wang, Y., Zhang, X., & Tai, H. (2015). Steam explosion distinctively enhances biomass enzymatic saccharification of cotton stalks by largely reducing cellulose polymerization degree in G. barbadense and G. hirsutum. *Bioresource Technology*, 181, 224-230.

Huston, C. K., & Ji, H. (1991). Optimization of the analytical supercritical fluid extraction of cloves via an on-column interface to an ion trap GC/MS system. *Journal of Agricultural and Food Chemistry*, 39(7), 1229-1233.

Igreja, W. S., Maia, F. d. A., Lopes, A. S., & Chisté, R. C. (2021). Biotechnological production of carotenoids using low cost-substrates is influenced by cultivation parameters: A review. *International Journal of Molecular Sciences*, 22(16), 8819.

Isikgor, F. H., & Becer, C. R. (2015). Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. *Polymer Chemistry*, 6(25), 4497-4559.

Jacob, Z. (1992). Yeast lipids: extraction, quality analysis, and acceptability. *Critical Reviews in Biotechnology*, 12(5-6), 463-491.

Ji, X.-J., & Huang, H. (2019). Engineering microbes to produce polyunsaturated fatty acids. *Trends in Biotechnology*, 37(4), 344-346.

Johnson, E. A., & Schroeder, W. A. (2006). Microbial carotenoids. Downstream processing biosurfactants carotenoids, 119-178.

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

Jørgensen, H., van Hecke, J., Zhang, H., Malik, P. L., Felby, C., & Schjoerring, J. K. (2019). Wheat as a dual crop for biorefining: straw quality parameters and their interactions with nitrogen supply in modern elite cultivars. *Gcb Bioenergy*, 11(2), 400-415.

Joshi, V., Attri, D., Bala, A., & Bhushan, S. (2003). Microbial pigments.

Kayathi, A., Chakrabarti, P. P., Bonfim-Rocha, L., Cardozo-Filho, L., & Jegatheesan, V. (2020). Selective extraction of polar lipids of mango kernel using supercritical carbon dioxide ($SC–CO_2$) extraction: Process optimization of extract yield/phosphorous content and economic evaluation. *Chemosphere*, 260, 127639.

Kennedy, E. P. (1961). Biosynthesis of complex lipids. Fed. Proc.,

Khachik, F., Beecher, G. R., & Whittaker, N. F. (1986). Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *Journal of Agricultural and Food Chemistry*, 34(4), 603-616.

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

Kimura, M., Rodriguez-Amaya, D. B., & Godoy, H. T. (1990). Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. *Food Chemistry*, 35(3), 187-195.

Kirti, K., Amita, S., Priti, S., Mukesh Kumar, A., & Jyoti, S. (2014). Colorful world of microbes: carotenoids and their applications. *Advances in Biology*, 2014, 1-13.

Kopec, R. E., Cooperstone, J. L., Cichon, M. J., & Schwartz, S. J. (2012). Analysis methods of carotenoids. Analysis of Antioxidant-Rich Phytochemicals, 105-148.

Kot, A. M., Błażejak, S., Gientka, I., Kieliszek, M., & Bryś, J. (2018). Torulene and torularhodin:"new" fungal carotenoids for industry? Microbial cell factories, 17(1), 1-14.

Kot, A. M., Błażejak, S., Kurcz, A., Gientka, I., & Kieliszek, M. (2016). *Rhodotorula glutinis*—potential source of lipids, carotenoids, and enzymes for use in industries. *Applied Microbiology and Biotechnology*, 100, 6103-6117.

Kothri, M., Mavrommati, M., Elazzazy, A. M., Baeshen, M. N., Moussa, T. A., & Aggelis, G. (2020). Microbial sources of polyunsaturated fatty acids (PUFAs) and the prospect of organic residues and wastes as growth media for PUFA-producing microorganisms. *FEMS Microbiology Letters*, 367(5), fnaa028.

Krinsky, N. I. (2001). Carotenoids as antioxidants. *Nutrition*, 17(10), 815-817.

Kumar, A., Sharma, A., & C Upadhyaya, K. (2016). Vegetable oil: nutritional and industrial perspective. *Current Genomics*, 17(3), 230-240.

Kumar, S., Paritosh, K., Pareek, N., Chawade, A., & Vivekanand, V. (2018). De-construction of major Indian cereal crop residues through chemical pretreatment for improved biogas production: An overview. *Renewable and Sustainable Energy Reviews*, 90, 160-170.

Lade, B. D., Patil, A. S., Paikrao, H. M., Kale, A. S., & Hire, K. K. (2014). A comprehensive working, principles and applications of thin layer chromatography. Res. *J. Pharm. Bio. Chem. Sci*, 5(4), 486-503.

Lang, Q., & Wai, C. M. (2001). Supercritical fluid extraction in herbal and natural product studies—a practical review. *Talanta*, 53(4), 771-782.

Laoteng, K., Čertík, M., & Cheevadhanark, S. (2011). Mechanisms controlling lipid accumulation and polyunsaturated fatty acid synthesis in oleaginous fungi. *Chemical Papers*, 65, 97-103.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., & Oh, H.-M. (2010). Comparison of several methods for effective lipid extraction from microalgae. *Bioresource Technology*, 101(1), S75-S77.

Li, H., Deng, Z., Wu, T., Liu, R., Loewen, S., & Tsao, R. (2012). Microwave-assisted extraction of phenolics with maximal antioxidant activities in tomatoes. *Food Chemistry*, 130(4), 928-936.

Li, Q., Du, W., & Liu, D. (2008). Perspectives of microbial oils for biodiesel production. *Applied Microbiology and Biotechnology*, 80, 749-756.

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., Tannock, S., & Schenk, P. M. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. *Microbial Cell Factories*, 13(1), 1-9.

Li, Z., Li, C., Cheng, P., & Yu, G. (2022). *Rhodotorula mucilaginosa*—alternative sources of natural carotenoids, lipids, and enzymes for industrial use. *Heliyon*, e11505.

Lietz, G., & Henry, C. (1997). A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. *Food Chemistry*, 60(1), 109-117.

Liu, B., & Zhao, Z. (2007). Biodiesel production by direct methanolysis of oleaginous microbial biomass. Journal of Chemical Technology & Biotechnology: *International Research in Process, Environmental & Clean Technology*, 82(8), 775-780.

Lopes, B. L., Sánchez-Camargo, A. P., Ferreira, A. L., Grimaldi, R., Paviani, L. C., & Cabral, F. A. (2012). Selectivity of supercritical carbon dioxide in the fractionation of fish oil with a lower content of EPA+ DHA. *The Journal of Supercritical Fluids*, 61, 78-85.

Luu, T. D. (2010). Supercritical CO_2 extraction of vetiver essential oil & economic incentive for use of vetiver grass in phytoremediation UNSW Sydney].

Ma, Y. (2006). Microbial oils and its research advance. *Chin J Bioprocess Eng*, 4(4), 7-11.

Ma, Y., Gao, Z., Wang, Q., & Liu, Y. (2018). Biodiesels from microbial oils: opportunity and challenges. *Bioresource Technology*, 263, 631-641.

Maitan-Alfenas, G. P., Visser, E. M., & Guimarães, V. M. (2015). Enzymatic hydrolysis of lignocellulosic biomass: converting food waste in valuable products. *Current Opinion in Food Science*, 1, 44-49.

Malisorn, C., & Suntornsuk, W. (2008). Optimization of β-carotene production by *Rhodotorula glutinis* DM28 in fermented radish brine. *Bioresource Technology*, 99(7), 2281-2287.

Maoka, T. (2020). Carotenoids as natural functional pigments. *Journal of Natural Medicines,* 74(1), 1-16.

Marini, L. (2006). Geological sequestration of carbon dioxide: thermodynamics, kinetics, and reaction path modeling. *Elsevier*.

Marova, I., Carnecka, M., Halienova, A., Breierova, E., & Koci, R. (2010). Production of carotenoid-/ergosterol-supplemented biomass by red yeast

Rhodotorula glutinis grown under external stress. *Food Technology and Biotechnology*, 48(1), 56-61.

Marsili, R., & Callahan, D. (1993). Comparison of a liquid solvent extraction technique and supercritical fluid extraction for the determination of α-and β-carotene in vegetables. *Journal of Chromatographic Science*, 31(10), 422-428.

Martin, A. M., Lu, C., & Patel, T. R. (1993). Growth parameters for the yeast *Rhodotorula rubra* grown in peat extracts. *Journal of Fermentation and Bioengineering*, 76(4), 321-325.

Meinhardt-Wollweber, M., Suhr, C., Kniggendorf, A.-K., & Roth, B. (2018). Absorption and resonance Raman characteristics of β-carotene in water-ethanol mixtures, emulsion and hydrogel. *AIP Advances*, 8(5).

Mendes-Pinto, M. M., LaFountain, A. M., Stoddard, M. C., Prum, R. O., Frank, H. A., & Robert, B. (2012). Variation in carotenoid–protein interaction in bird feathers produces novel plumage coloration. *Journal of the Royal Society Interface*, 9(77), 3338-3350.

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

Meyer, P., & Du Preez, J. (1994). Astaxanthin production by a *Phaffia rhodozyma* mutant on grape juice. *World Journal of Microbiology and Biotechnology*, 10, 178-183.

Modey, W. K., Mulholland, D. A., Mahomed, H., & Raynor, M. W. (1996). Analysis of extracts from cedrela toona (meliaceae) by on-line and off-line supercritical fluid extraction-capillary gas chromatography. *Journal of Microcolumn Separations*, 8(1), 67-74.

Moliné, M., Flores, M. R., Libkind, D., Carmen Diéguez, M. d. C., Farías, M. E., & van Broock, M. (2010). Photoprotection by carotenoid pigments in the yeast *Rhodotorula mucilaginosa*: the role of torularhodin. *Photochemical & Photobiological Sciences*, 9(8), 1145-1151.

Mubarak, M., Shaija, A., & Suchithra, T. (2015). A review on the extraction of lipid from microalgae for biodiesel production. *Algal Research*, 7, 117-123.

Mussagy, C. U., Ribeiro, H. F., & Pereira, J. F. (2023). *Rhodotorula* sp. as a cell factory for production of valuable biomolecules. *Advances in Applied Microbiology*, 123, 133-156.

Mussagy, C. U., Santos-Ebinuma, V. C., Gonzalez-Miquel, M., Coutinho, J. A., & Pereira, J. F. (2019). Protic ionic liquids as cell-disrupting agents for the recovery of intracellular carotenoids from yeast *Rhodotorula glutinis* CCT-2186. *ACS Sustainable Chemistry & Engineering*, 7(19), 16765-16776.

Nagaraj, Y. N., Burkina, V., Okmane, L., Blomqvist, J., Rapoport, A., Sandgren, M., Pickova, J., Sampels, S., & Passoth, V. (2022). Identification, quantification

and kinetic study of carotenoids and lipids in *Rhodotorula toruloides* CBS 14 cultivated on wheat straw hydrolysate. *Fermentation*, 8(7), 300.

Nielsen, T. J., Jaegerstad, I. M., Oeste, R. E., & Sivik, B. T. (1991). Supercritical fluid extraction coupled with gas chromatography for the analysis of aroma compounds absorbed by low-density polyethylene. *Journal of Agricultural and Food Chemistry*, 39(7), 1234-1237.

Nierenberg, D. W., & Nann, S. L. (1992). A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. *The American Journal of Clinical Nutrition*, 56(2), 417-426.

Okunuki, K. (1931). Beitrage zur Kenntnis der rosafarbigen Sprosspilze. Japanese *Journal of Botany*, 5, 285-322.

Oosterveer, P. (2015). Promoting sustainable palm oil: viewed from a global networks and flows perspective. *Journal of Cleaner Production*, 107, 146-153.

Otterbach, A., & Wenclawiak, B. (1999). Ultrasonic/Soxhlet/supercritical fluid extraction kinetics of pyrethrins from flowers and allethrin from paper strips. *Fresenius' Journal of Analytical Chemistry*, 365, 472-474.

Panesar, R., Kaur, S., & Panesar, P. S. (2015). Production of microbial pigments utilizing agro-industrial waste: a review. *Current Opinion in Food Science*, 1, 70-76.

Papapostolou, H., Kachrimanidou, V., Alexandri, M., Plessas, S., Papadaki, A., & Kopsahelis, N. (2023). Natural Carotenoids: Recent Advances on Separation from Microbial Biomass and Methods of Analysis. *Antioxidants*, 12(5), 1030.

Pappas, F. (2023). Genomic scaffolding and comparative genomics analysis of 23 oleaginous yeast strains belonging to the *Rhodotorula* genus. *Master's dissertation*, Swedish university of agricultural sciences, Uppsala, Sweden.

Parsons, S., Raikova, S., & Chuck, C. J. (2020). The viability and desirability of replacing palm oil. *Nature Sustainability*, 3(6), 412-418.

Passoth, V., Brandenburg, J., Chmielarz, M., Martín-Hernández, G. C., Nagaraj, Y., Müller, B., & Blomqvist, J. (2023). Oleaginous yeasts for biochemicals, biofuels and food from lignocellulose-hydrolysate and crude glycerol. *Yeast*.

Passoth, V., & Sandgren, M. (2019). Biofuel production from straw hydrolysates: current achievements and perspectives. *Applied Microbiology and Biotechnology*, 103, 5105-5116.

Patel, A., Antonopoulou, I., Enman, J., Rova, U., Christakopoulos, P., & Matsakas, L. (2019). Lipids detection and quantification in oleaginous microorganisms: an overview of the current state of the art. *BMC Chemical Engineering*, 1, 1-25.

Patel, A., Arora, N., Mehtani, J., Pruthi, V., & Pruthi, P. A. (2017). Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for

potential biodiesel production. *Renewable and Sustainable Energy Reviews*, 77, 604-616.

Paul, D., Kumari, P. K., & Siddiqui, N. (2023). Yeast carotenoids: Cost-effective fermentation strategies for health care applications. *Fermentation*, 9(2), 147.

Perera, C. O., & Yen, G. M. (2007). Functional properties of carotenoids in human health. *International Journal of Food Properties*, 10(2), 201-230.

Petrik, S., Kádár, Z., & Márová, I. (2013). Utilization of hydrothermally pretreated wheat straw for production of bioethanol and carotene-enriched biomass. *Bioresource Technology*, 133, 370-377.

Philip, T., & Francis, F. (1971). Oxidation of capsanthin. *Journal of Food Science*, 36(1), 96-97.

Picot-Allain, C., Mahomoodally, M. F., Ak, G., & Zengin, G. (2021). Conventional versus green extraction techniques—A comparative perspective. *Current Opinion in Food Science*, 40, 144-156.

Pinheiro, M. J., Bonturi, N., Belouah, I., Miranda, E. A., & Lahtvee, P.-J. (2020). Xylose metabolism and the effect of oxidative stress on lipid and carotenoid production in *Rhodotorula toruloides*: insights for future biorefinery. *Frontiers in Bioengineering and Biotechnology*, 8, 1008.

Prabakaran, P., & Ravindran, A. D. (2011). A comparative study on effective cell disruption methods for lipid extraction from microalgae. *Letters in applied microbiology*, 53(2), 150-154.

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

Qiuhui, H. (1999). Supercritical carbon dioxide extraction of *Spirulina platensis* component and removing the stench. *Journal of Agricultural and Food Chemistry*, 47(7), 2705-2706.

Ramsey, E., Qiubai, S., Zhang, Z., Zhang, C., & Wei, G. (2009). Mini-Review: Green sustainable processes using supercritical fluid carbon dioxide. *Journal of Environmental Sciences*, 21(6), 720-726.

Rapoport, A., Guzhova, I., Bernetti, L., Buzzini, P., Kieliszek, M., & Kot, A. M. (2021). Carotenoids and some other pigments from fungi and yeasts. *Metabolites*, 11(2), 92.

Ratledge, C. (2013). Microbial oils: an introductory overview of current status and future prospects. *Ocl*, 20(6), D602.

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

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

Reif, C., Arrigoni, E., Schärer, H., Nyström, L., & Hurrell, R. F. (2013). Carotenoid database of commonly eaten Swiss vegetables and their estimated contribution to carotenoid intake. *Journal of Food Composition and Analysis*, 29(1), 64-72.

Rennerfelt, E. (1937). Studies on the fungal infection of ground wood pulp and its development therein. *Svenska Skogsvardsforeningens Tidskrift*, 35(1).

Reverchon, E., Donsi, G., & Sesti Osseo, L. (1993). Modeling of supercritical fluid extraction from herbaceous matrices. *Industrial & Engineering Chemistry Research*, 32(11), 2721-2726.

Rodriguez-Amaya, D. B., & Kimura, M. (2004). HarvestPlus handbook for carotenoid analysis (Vol. 2). *International Food Policy Research Institute (IFPRI) Washington*.

Rozzi, N., & Singh, R. (2002). Supercritical fluids and the food industry. *Comprehensive Reviews in Food Science and Food Safety*, 1(1), 33-44.

Sahena, F., Zaidul, I., Jinap, S., Karim, A., Abbas, K., Norulaini, N., & Omar, A. (2009). Application of supercritical CO_2 in lipid extraction–A review. *Journal of Food Engineering*, 95(2), 240-253.

Saini, R., Hegde, K., Brar, S. K., & Vezina, P. (2020). Advanced biofuel production and road to commercialization: An insight into bioconversion potential of *Rhodosporidium* sp. *Biomass and Bioenergy*, 132, 105439.

Saini, R. K., & Keum, Y.-S. (2018). Carotenoid extraction methods: A review of recent developments. *Food Chemistry*, 240, 90-103.

Sambles, C., Middelhaufe, S., Soanes, D., Kolak, D., Lux, T., Moore, K., Matoušková, P., Parker, D., Lee, R., & Love, J. (2017). Genome sequence of the oleaginous yeast *Rhodotorula toruloides* strain CGMCC 2.1609. *Genomics Data*, 13, 1-2.

Sandager, L., Gustavsson, M. H., Ståhl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H., & Stymne, S. (2002). Storage lipid synthesis is non-essential in yeast. *Journal of biological chemistry*, 277(8), 6478-6482.

Sandmann, G. (2015). Carotenoids of biotechnological importance. *Biotechnology of Isoprenoids*, 449-467.

Sandmann, G. (2022). Carotenoids and their biosynthesis in fungi. *Molecules*, 27(4), 1431.

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual review of Plant Biology*, 61, 263-289.

Scita, G. (1992). [16] Stability of β-carotene under different laboratory conditions. In Methods in enzymology (Vol. 213, pp. 175-185). *Elsevier*.

Scott, K. J. (2001). Detection and measurement of carotenoids by UV/VIS spectrophotometry. *Current Protocols in Food Analytical Chemistry* (1), F2. 2.1-F2. 2.10.

Sedmak, J. J., Weerasinghe, D. K., & Jolly, S. O. (1990). Extraction and quantitation of astaxanthin from *Phaffia rhodozyma*. *Biotechnology Techniques*, 4, 107-112.

Sen, T., Barrow, C. J., & Deshmukh, S. K. (2019). Microbial pigments in the food industry—challenges and the way forward. *Frontiers in Nutrition*, 6, 7.

Shahidi, F. (2005). Bailey's Industrial Oil and Fat Products, Industrial and Nonedible Products from Oils and Fats (Vol. 6). *John Wiley & Sons*.

Shapaval, V., Brandenburg, J., Blomqvist, J., Tafintseva, V., Passoth, V., Sandgren, M., & Kohler, A. (2019). Biochemical profiling, prediction of total lipid content and fatty acid profile in oleaginous yeasts by FTIR spectroscopy. *Biotechnology for Biofuels*, 12(1), 1-12.

Sihvonen, M., Järvenpää, E., Hietaniemi, V., & Huopalahti, R. (1999). Advances in supercritical carbon dioxide technologies. *Trends in Food Science & Technology*, 10(6-7), 217-222.

Silva, G. G. D., Couturier, M., Berrin, J.-G., Buléon, A., & Rouau, X. (2012). Effects of grinding processes on enzymatic degradation of wheat straw. *Bioresource Technology*, 103(1), 192-200.

Simpson, K. L., Nakayama, T., & Chichester, C. (1964). Biosynthesis of yeast carotenoids. *Journal of Bacteriology*, 88(6), 1688-1694.

Singh, G., Sinha, S., Bandyopadhyay, K., Lawrence, M., & Paul, D. (2018). Triauxic growth of an oleaginous red yeast *Rhodosporidium toruloides* on waste 'extract'for enhanced and concomitant lipid and β-carotene production. *Microbial Cell Factories*, 17, 1-10.

Sitepu, I. R., Garay, L. A., Sestric, R., Levin, D., Block, D. E., German, J. B., & Boundy-Mills, K. L. (2014). Oleaginous yeasts for biodiesel: current and future trends in biology and production. *Biotechnology advances*, 32(7), 1336-1360.

Smith, R. M., & Burford, M. D. (1992). Optimization of supercritical fluid extraction of volatile constituents from a model plant matrix. *Journal of Chromatography A*, 600(2), 175-181.

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

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., & Keasling, J. D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*, 463(7280), 559-562.

Stellner, N., Rerop, Z., Kyselka, J., Alishevich, K., Beneš, R., Filip, V., Celik, G. l., Haack, M., Ringel, M., & Masri, M. (2023). Value-Added Squalene in Single-Cell Oil Produced with *Cutaneotrichosporon* oleaginosus for Food Applications. *Journal of Agricultural and Food Chemistry*.

Tang, W., Wang, Y., Zhang, J., Cai, Y., & He, Z. (2019). Biosynthetic pathway of carotenoids in *Rhodotorula* and strategies for enhanced their production.

Tibbetts, S. M., Bjornsson, W. J., & McGinn, P. J. (2015). Biochemical composition and amino acid profiles of *Nannochloropsis granulata* algal biomass before and after supercritical fluid CO_2 extraction at two processing temperatures. *Animal Feed Science and Technology*, 204, 62-71.

Tibbetts, S. M., Yasumaru, F., & Lemos, D. (2017). In vitro prediction of digestible protein content of marine microalgae (*Nannochloropsis granulata*) meals for Pacific white shrimp (*Litopenaeus vannamei*) and rainbow trout (*Oncorhynchus mykiss*). *Algal Research*, 21, 76-80.

Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., & Somerville, C. (2009). Beneficial biofuels—the food, energy, and environment trilemma. *Science*, 325(5938), 270-271.

Tkachenko, A., Tigunova, O., & Shulga, S. (2013). Microbial lipids as a source of biofuel. *Cytology and genetics*, 47, 343-348.

Tswett, M. (1906). Physikalisch-chemische Studien über das Chlorophyll. Die Adsorptionen. Berichte der deutschen botanischen Gesellschaft, 24(316-323), 20.

Tuli, H. S., Chaudhary, P., Beniwal, V., & Sharma, A. K. (2015). Microbial pigments as natural color sources: current trends and future perspectives. *Journal of Food Science and Technology*, 52, 4669-4678.

Udensi, J., Loskutova, E., Loughman, J., & Byrne, H. J. (2022). Quantitative Raman analysis of carotenoid protein complexes in aqueous solution. *Molecules*, 27(15), 4724.

Uemura, H. (2012). Synthesis and production of unsaturated and polyunsaturated fatty acids in yeast: current state and perspectives. *Applied Microbiology and Biotechnology*, 95, 1-12.

Uwineza, P. A., & Waśkiewicz, A. (2020). Recent advances in supercritical fluid extraction of natural bioactive compounds from natural plant materials. *Molecules*, 25(17), 3847.

Valdés, G., Mendonça, R. T., & Aggelis, G. (2020). Lignocellulosic biomass as a substrate for oleaginous microorganisms: a review. *Applied Sciences*, 10(21), 7698.

Van Dyk, J., & Pletschke, B. (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—factors affecting enzymes, conversion and synergy. *Biotechnology Advances*, 30(6), 1458-1480.

Vicente, G., Bautista, L. F., Gutiérrez, F. J., Rodríguez, R. a., Martínez, V., Rodríguez-Frómeta, R. A., Ruiz-Vázquez, R. M., Torres-Martínez, S., & Garre, V. (2010). Direct transformation of fungal biomass from submerged cultures into biodiesel. *Energy & Fuels*, 24(5), 3173-3178.

Vignesh, N. S., Vimali, E., Sangeetha, R., Arumugam, M., Ashokkumar, B., Ganeshmoorthy, I., & Varalakshmi, P. (2020). Sustainable biofuel from

microalgae: application of lignocellulosic wastes and bio-iron nanoparticle for biodiesel production. *Fuel*, 278, 118326.

Viguera, M., Marti, A., Masca, F., Prieto, C., & Calvo, L. (2016). The process parameters and solid conditions that affect the supercritical CO_2 extraction of the lipids produced by microalgae. *The Journal of Supercritical Fluids*, 113, 16-22.

Villoutreix, J. (1960). Carotenoids of *Rhodotorula mucilaginosa*. Study of their biosynthesis with the aid of analysis of mutants and the use of an inhibitor of carotenogenesis. *Biochimica et Biophysica Acta*, 40, 442-457.

Viñals, N. O., Estrada, D. A., Pérez, E. F., Álvarez, l. G. M., Martínez-hernández, J. L., Valdés, R. A., Caraballo, A. N., & Iliná, A. (2023). *Rhodotorula toruloides* as a biofactory of carotenoids, lipids, and enzymes.

Vivekanand, V., Olsen, E. F., Eijsink, V. G., & Horn, S. J. (2013). Effect of different steam explosion conditions on methane potential and enzymatic saccharification of birch. *Bioresource Technology*, 127, 343-349.

Wang, Q.-M., Yurkov, A., Göker, M., Lumbsch, H., Leavitt, S., Groenewald, M., Theelen, B., Liu, X.-Z., Boekhout, T., & Bai, F.-Y. (2015). Phylogenetic classification of yeasts and related taxa within Pucciniomycotina. *Studies in Mycology*, 81(1), 149-189.

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

Ward, O. P., & Singh, A. (2005). Omega-3/6 fatty acids: alternative sources of production. *Process Biochemistry*, 40(12), 3627-3652.

Wheeler, J., & McNally, M. (1989). Supercritical fluid extraction and chromatography of representative agricultural products with capillary and microbore columns. *Journal of Chromatographic Science*, 27(9), 534-539.

Whiffin, F., Santomauro, F., & Chuck, C. J. (2016). Toward a microbial palm oil substitute: oleaginous yeasts cultured on lignocellulose. Biofuels, *Bioproducts and Biorefining*, 10(3), 316-334.

Xue, S.-J., Chi, Z., Zhang, Y., Li, Y.-F., Liu, G.-L., Jiang, H., Hu, Z., & Chi, Z.-M. (2018). Fatty acids from oleaginous yeasts and yeast-like fungi and their potential applications. *Critical Reviews in Biotechnology*, 38(7), 1049-1060.

Xue, Z., Sharpe, P. L., Hong, S.-P., Yadav, N. S., Xie, D., Short, D. R., Damude, H. G., Rupert, R. A., Seip, J. E., & Wang, J. (2013). Production of omega-3 eicosapentaenoic acid by metabolic engineering of *Yarrowia lipolytica*. *Nature Biotechnology*, 31(8), 734-740.

Yadav, M., Paritosh, K., Chawade, A., Pareek, N., & Vivekanand, V. (2018). Genetic engineering of energy crops to reduce recalcitrance and enhance biomass digestibility. *Agriculture*, 8(6), 76.

Yadav, M., Paritosh, K., Pareek, N., & Vivekanand, V. (2019). Coupled treatment of lignocellulosic agricultural residues for augmented biomethanation. *Journal of Cleaner Production*, 213, 75-88.

Yadav, M., Paritosh, K., & Vivekanand, V. (2020). Lignocellulose to bio-hydrogen: an overview on recent developments. *International Journal of Hydrogen Energy*, 45(36), 18195-18210.

Yadav, M., Singh, A., Balan, V., Pareek, N., & Vivekanand, V. (2019). Biological treatment of lignocellulosic biomass by *Chaetomium globosporum*: process derivation and improved biogas production. *International Journal of Biological Macromolecules*, 128, 176-183.

YAMAUCHI, H., MORI, H., KOBAYASHI, T., & SHIMIZU, S. (1983). Mass production of lipids by *Lipomyces starkeyi* in microcomputer-aided fed-batch culture. *Journal of Fermentation Technology*, 61(3), 275-280.

Yu, X., Dong, T., Zheng, Y., Miao, C., & Chen, S. (2015). Investigations on cell disruption of oleaginous microorganisms: hydrochloric acid digestion is an effective method for lipid extraction. *European Journal of Lipid Science and Technology*, 117(5), 730-737.

Zhao, H., Kwak, J. H., Wang, Y., Franz, J. A., White, J. M., & Holladay, J. E. (2006). Effects of crystallinity on dilute acid hydrolysis of cellulose by cellulose-ball-milling study. *Energy & Fuels*, 20(2), 807-811.

Zhao, X., Hu, C., Wu, S., Shen, H., & Zhao, Z. K. (2011). Lipid production by *Rhodosporidium toruloides* Y4 using different substrate feeding strategies. *Journal of Industrial Microbiology and Biotechnology*, 38(5), 627-632.

Zhou, Y., Zhao, W., Lai, Y., Zhang, B., & Zhang, D. (2020). Edible plant oil: global status, health issues, and perspectives. *Frontiers in Plant Science*, 11, 1315.

Zhu, Q., Xue, Z., Yadav, N., Damude, H., Pollak, D. W., Rupert, R., Seip, J., Hollerbach, D., Macool, D., & Zhang, H. (2010). Metabolic engineering of an oleaginous yeast for the production of omega-3 fatty acids. In Single cell oils (pp. 51-73). *Elsevier.*

Zoz, L., Carvalho, J. C., Soccol, V. T., Casagrande, T. C., & Cardoso, L. (2014). Torularhodin and torulene: bioproduction, properties and prospective applications in food and cosmetics-a review. *Brazilian Archives of Biology and Technology*, 58, 278-288.

Popular science summary

Oleaginous yeasts, these small but incredible microbes, are silently leading a remarkable transformation in sustainable food production and environmental protection. They possess a unique talent for prolific production of lipids and carotenoids, while their ability to thrive on low-cost carbon sources makes them environmentally friendly. What truly sets them apart is their role in closing the sustainability loop. Through fermentation of pre-treated lignocellulose hydrolysate, oleaginous yeasts transform seemingly useless biomass into valuable food components such as oils and carotenoids. Once these valuable compounds have been extracted, the fermentation residues have a new purpose as a source of nitrogen fertiliser, completing a virtuous cycle as in figure 1.

Lipid and carotenoid production by oleaginous yeasts is quite remarkable. These microorganisms efficiently convert carbon sources into lipids, primarily triglycerides, which can serve as a basis for sustainable food production and biofuels. They are also prolific carotenoid producers, contributing to the vibrant colours and nutritional value of various processed foods. Extraction methods involving solvents and mechanical processes are currently employed to harvest these valuable compounds, but scientists are constantly working to improve extraction methods in order to make them function more efficiently and create less waste.

One of the key advances in this thesis was the development of a superior extraction method, called supercritical carbon dioxide (SC-CO_2), for isolating lipids and carotenoids from the oleaginous yeast species *Rhodotorula toruloides*. Tests showed that SC-CO_2 outperformed conventional extraction methods. This finding has the potential to revolutionise extraction of these precious compounds, making the process more efficient and environmentally friendly. Further investigations revealed

a hidden challenge in carotenoid quantification by showing that the saponification process, a crucial step for separating lipids from carotenoids before measuring their content, can actually have an adverse effect on carotenoid levels. This unexpected finding indicates a need to re-think and improve existing extraction methods, which generally involve a saponification step.

Another part of the research in this thesis was to analyse different strains of *Rhodotorula toruloides*, with particular focus on the hybrid strain CBS 6016. Surprisingly, the results showed that this hybrid strain predominantly inherits genetic traits from one parent (CBS 14) and not the other (CBS 349). This insight could assist in future genetic engineering efforts aimed at boosting compound yields from strains of *Rhodotorula toruloides*.

Populärvetenskaplig sammanfattning

Oljeackumulerande jästsvampar, eller oleaginösa jästsvampar som dessa också kallas för, är små men otroligt fascinerande mikroorganismer. Dessa oljeackumulerande jästsvampar är i dag inblandade i forskning som har som mål ett hållbart och miljövänligt livsmedelsproduktionssystem. Några av dessa svampar, t.ex. jästen *Rhodotorula toruloides*, har en unik förmåga till relativt stor produktion av både lipider och karotenoider. *R. toruloides* och andra oljeackumulerande jästsvampar har också en unik förmåga att trivas och växa på lågkostnadskolhydratkällor, som ofta är restprodukter inom skog och jordbruksproduktion. Denna förmågan ger oss nya verktyg för att sluta de ekologiska kretsloppen inom bioteknologiska processystem på ett bra sätt. Genom odling på av på olika sätt förbehandlad lignocellulosahydrolysat omvandlar oljeackumulerande jästsvampar lågvärdig biomassa i naturen, eller restprodukter inom olika typer av skogs eller jordbruksindustri, till värdefulla föreningar. Men det slutar inte där – den överblivna jästbiomassan, när de huvudsakliga produkterna har extraherats från svampen, kan få ytterligare en användning som en kvävekälla vid produktion av biogödselmedel, som på ett bra sätt sluter det kretslopp som tidigare har visats i figur 1.

Jästlipiderna kan exempelvis användas som förnyelsebar råvara inom livs- och fodermedelsproduktion eller produktion av biobränslen. Karotenoider är antioxidanter och kan ha hälsofrämjande effekt i livsmedel eller kosmetika och kan användas som färgämne. För att ta fram lipider och karotenoider från jästsvamparna används i dag extraktionsmetoder som ofta involverar organiska lösningsmedel samt mekaniska processer. Forskare försöker hela tiden förbättra dessa extraktionsmetoder för att få dem att fungera bättre och producera mindre avfall och därmed orsaka mindre miljöpåverkan.

En av de viktigare upptäckterna som presenteras i denna avhandling är att en extraktionsmetod kallad superkritisk koldioxidextraktion (SC-CO_2) är fördelaktig jämfört med extraktionsmetoder med organiska lösningsmedel för att extrahera lipider och karotenoider från oleaginösa jästsvampar. Speciellt är det så att det är nu bättre möjlig att separera lipider och karotenoider och att omättade fettsyror är skyddat mot oxidering. Denna upptäckt har en potential att kunna revolutionera hur vi i framtiden extraherar lipider och karotenoider på och därmed gör dessa extraktionsprocesser mer effektiva och miljövänliga. Etablering av SC-CO_2-metoden är också viktigt för karotenoidkvantifiering. Om lipider ko-extraheras, så måste man ta bort fetterna, för att de kan skada de vanligaste analysequipment. Fetterna tas bort med saponifiering, eller förtvålningsprocess som det också kallas. Denna förtvålningsprocess har visat sig kan ha en stor påverkan på karotenoidnivåerna själva. Denna oväntade upptäckt som vi har gjort visar på att det finns ett stort behov av att ompröva och förbättra våra i dag befintliga lipid- och karotenoid- extraktions- och analysmetoder.

Inom ramen för detta avhandlingsarbete har vi även karaktäriserat olika stammar av den oljeackumulerande jästsvampen *Rhodotorula toruloides*, med ett särskilt fokus på en hybridstam av denna svamp som kallas CBS 6016. Det som förvånade oss mest när vi har undersökt denna hybridstam är att den verkar ha ärvt mer genetiska egenskaper från den en av sina två föräldrastammar, CBS 14, än från den andra, CBS 349. Denna insikt kan öppna dörrar i framtiden för ett genetiskt ingenjörsarbete som syftar till att öka förekomsten av viktiga och värdefulla kemiska föreningar i dessa jästsvampar.

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Identification, Quantification and Kinetic Study of Carotenoids and Lipids in *Rhodotorula toruloides* CBS 14 Cultivated on Wheat Straw Hydrolysate

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Abstract: Production of carotenoids and lipids by *Rhodotorula toruloides* CBS 14 cultivated on wheat straw hydrolysate was investigated. An ultra-high-performance liquid chromatography (UHPLC) method for carotenoid quantification was developed and validated. Saponification effects on individual carotenoid quantification were identified, and lipid and carotenoid kinetics during cultivation were determined. The carotenoids β-carotene, γ-carotene, torularhodin, and torulene were identified; β-carotene was the major carotenoid, reaching a maximum of 1.48 mg/100 g dry weight. Recoveries of the carotenoids were between 66 and 76%, except torulene and torularhodin, which had lower recoveries due to saponification effects. Total carotenoid content in saponified and unsaponified yeast extract, respectively, determined by UHPLC or photometer, respectively, was 1.99 mg/100 g and 4.02 mg β-EQ/100 g dry weight. Growth kinetics showed a positive correlation between carotenoid content and lipid accumulation. β-carotene was the major carotenoid at all time points. At the end of the cultivation, triacylglycerols (TAGs) were the major lipid class, with 58.1 ± 3.32% of total lipids. There was also a high proportion of free fatty acids, reaching from 20.5 to 41.8% of total lipids. Oleic acid (C18:1) was the major fatty acid. The lipid yield at the end of the cultivation was 0.13 g/g of sugar consumed.

Keywords: *Rhodotorula toruloides* CBS 14; carotenoids; spectrophotometer; UHPLC-PDA analysis; saponification effect; lipids; gas chromatography; kinetic study

1. Introduction

Oleaginous yeasts have the ability to accumulate lipids to more than 20% of their total dry biomass weight [1]. The lipids produced are referred to as single-cell oil (SCO) and are dominated by triacylglycerols (TAGs). The oil mainly includes oleic (18:1), linoleic (18:2), palmitic (16:0), and palmitoleic (16:1) acids [2]. This fatty acid profile is comparable to that of vegetable oils such as palm or sunflower oil [3]. Thus, microbial oil has the potential to be utilized in similar applications as vegetable oil, e.g., the production of biodiesel [4,5], or the production of pharmaceuticals and cosmetics, as well as in the nutritional sector [6,7].

In oleaginous yeasts, lipid synthesis and storage are initiated at a surplus of carbon sources coupled with the limitation of nutrients such as nitrogen, sulfur, etc. [8]. Most of the formed lipids are TAGs, which are synthesized via the Kennedy pathway [9,10].

Carotenoids are a group of natural pigments synthesized by plants and microbes. They are tetraterpenoids, which are composed of eight isoprenoid units joined together in a polyene chain [11] and belong to the class of organic, lipid-soluble compounds. They exhibit natural antioxidant properties and are important components for the pharmaceutical, chemical, food, and feed industries [12]. These commercially important pigments are produced naturally by a variety of yeasts, including those belonging to the genus *Rhodotorula*. Therefore, these microorganisms have been regarded as potential pigment sources [13]. The basidiomycetous yeast genus *Rhodotorula* comprises a variety of oleaginous, pigmented species that can be cultivated efficiently on low-cost carbon sources such as lignocellulose [14–16].

In *Rhodotorula* species, carotenoids are synthesized via biosynthetic pathways that include the mevalonate pathway, isoprene biosynthesis, and the carotenogenic pathway. The proportion of each carotenoid produced depends on the strain and culture conditions [17,18].

The major carotenoid pigments in *Rhodotorula* species are γ-carotene, β-carotene, torularhodin, and torulene [19]. Perrier et al. [19] showed that the amounts of torulene and torularhodin pigments produced in *Rhodotorula* species vary by strain.

For identification and quantification of the carotenoids produced in specific *Rhodotorula* species, an accurate analytical method is required. Usually, a spectrophotometric technique, based on a standard curve with different concentrations of one individual carotenoid, is used to identify the total carotenoid content. In one study, torulene and torularhodin were quantified based on a β-carotene standard curve and related to the specific pigment extinction coefficients [20]. In another study, torulene was prepared as a crude pigment, isolated and purified from a torulene-producing yeast, and used as a standard for quantification in yeast samples [21]. Apart from these methods, due to unavailability of commercial standards, other methods such as thin-layer chromatography, liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry (LC-APCI-MS), and flow cytometry have been used for separation and identification of torulene and torularhodin in yeast samples [19,22,23].

Carotenoid extraction is one of the most crucial steps in a successful analytical procedure when determining carotenoid production by yeasts. The classical acetone extraction of carotenoids from yeast samples includes freeze-drying, homogenization, centrifugation, heating, and saponification. Applying these pretreatment steps to yeast samples allows preconcentration of all carotenoids in the final extract [24]. The main purpose of adopting an extraction procedure for analyzing a multicomponent carotenoid composition in a sample is to obtain the highest possible recovery of a broad range of analytes. Among the different steps of carotenoid extraction, saponification of the coextracted lipids is often considered an essential step prior to quantification [25]. Previously, the effect of saponification on the total carotenoid content obtained from plant-derived sources has been studied [26], but there is a lack of information about the extent of the saponification effect on individual carotenoids produced by yeasts.

Currently, most of the commercially available carotenoids are chemically synthesized. However, in modern society, the growing interest in "natural" ingredients among consumers and their increased concern about synthetic pigments and their detrimental effects on health and the environment has reawakened interest in the use of natural colorants [27]. In this context, yeast-based carotenoids may be used to replace chemically synthesized carotenoids. Costs of production may be reduced by using improved strains and inexpensive (often byproduct) carbon and nitrogen sources in culture media [28].

Rhodotorula strains have the potential to play an important role in the production of SCOs, which have been proven to be a highly interesting class of biotechnological products ranging from bulk chemicals to high-value products [29].

This study aimed to monitor the formation of carotenoids and lipids in *R. toruloides* CBS 14, grown on wheat straw hydrolysate. To reach this aim, we developed a UHPLC-based quantification method for carotenoids, using commercial standards, and characterized the effect of saponification on individual carotenoids. Our results show dynamic changes in carotenoid and lipid composition during the course of cultivation.

2. Materials and Methods

2.1. Hydrolysate Preparation

Lignocellulose hydrolysate was prepared at the Department of Chemical Engineering, Lund University, Sweden, as described previously [6,30], i.e., in brief, the acid-soaked (1% acetic acid) biomass was steam exploded at 190 °C for 10 min in a 10 L steam pretreatment reactor. The liquid fraction was further separated from the solid fraction, and the latter was enzymatically hydrolyzed using a mixture of enzymes that includes cellulases, exoglucanases, β-glucosidases, and hemicellulases at 45 °C and pH 4.8.

2.2. Rhodotorula toruloides Cultivation

2.2.1. Inoculum Preparation

R. toruloides CBS 14 was obtained from the Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Cells were stored in frozen stocks at −80 °C. *R. toruloides* CBS 14 inoculum was prepared as described before by Shapaval et al. [2], with slight modifications. For bioreactor cultivations, cells from YPD-agar plates (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L, agar 15 g/L) were inoculated into 300 mL of YPD (glucose 20 g/L, peptone 20 g/L, yeast extract 10 g/L) in a 3 L Erlenmeyer flask and incubated at 25 °C for 48–72 h at 150 rpm. The cells were harvested by centrifugation (4000 g, 10 min), washed twice with sterile saline solution (NaCl, 9 g/L), resuspended in saline, and inoculated into the fermenters. Similarly, for shake-flask cultivations, 50 mL of preculture was prepared in 250 mL Erlenmeyer flasks, and the cells were washed, resuspended in saline, and inoculated into the shake-flask cultivation.

2.2.2. Fermentation Experiments

*S*hake-flask cultures were performed in 250 mL sterile baffle shake flasks with 50 mL of growth medium. The bioreactor cultures were run in Minifors 2, Bench-Top bioreactors (INFORS HT, Switzerland, working volume 2 L) with 1.5 L of growth medium. The growth medium in both the cultures included filter-sterilized lignocellulose hydrolysate and filter-sterilized 10× YNB (yeast nitrogen base) solution without amino acids and ammonium sulfate mixed with $(NH_4)_2SO_4$ 20 g/L, KH_2PO_4 70 g/L, NaH_2PO_4 20 g/L, $MgSO_4.7H_2O$ 15 g/L, and yeast extract 10 g/L [31]. The cultures were inoculated with cells prepared as described above (Section 2.2.1). The shake-flask cultures were incubated at 25 °C for 4 days at 150 rpm, with an initial pH set to 6.0. After cultivation, the cells were washed with Milli-Q water, harvested, freeze-dried, and stored at −20 °C until carotenoid extraction.

In bioreactors, the pH was adjusted to 6.0 and automatically controlled by addition of NaOH (5M) or H_3PO_4 (3M). The aeration was initially set to 0.7 L/min, and during the experiment, it was increased up to 1 L/min. Dissolved oxygen tension (pO2) was set to 20% and was maintained by changing the stirring speed. A 1 mL volume of polypropylene glycol 2000 (Alfa Aesar, Karlsruhe, Germany) was added to minimize foaming. Immediately after the inoculation, 100 mL of culture broth was collected from each fermenter, and similarly, for the next 4 days, samples were collected every 24 h and then harvested (4000 g, 10 min), washed, freeze-dried, and stored at −20 °C until lipid and carotenoid extraction. At each time point, samples were collected for determining optical density, cell dry weight, and sugar content.

2.3. Analytical Techniques

2.3.1. Optical Density (OD_{600})

The optical density of the culture broth at each time point was monitored using a Biochrom WPA CO8000 cell density meter (UK) at a wavelength of 600 nm with distilled water as a blank.

2.3.2. Cell Dry Weight Determination

For dry weight determination, 2 mL of the broth culture was centrifuged at 15,000 g for 2 min. The cell pellets were washed thrice with deionized water and transferred onto a preweighed aluminum plate, which was then dried at 105 °C for 24 h, followed by weight measurement. All samples were analyzed in triplicate.

2.3.3. Sugars and Acetic acid Analysis

From the supernatant preserved after cell harvesting, the sugar and acetic acid content was measured as described by Brandenburg et al. [30]. A 1 mL volume of the supernatant was filtered using a sterile syringe filter with 0.2 µm polyether sulfone membrane (VWR International, Europe) and analyzed for sugars (glucose and xylose) and acetic acid in an Agilent Technologies high-performance liquid chromatography (HPLC) (California, United States), 1100 Series system containing refraction index detector, and a Rezex-ROA-Organic Acid H + 300 × 7.80 mm column (Skandinaviska Genetec AB). The column was eluted at 60 °C with 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

2.3.4. Lipid Extraction

Lipids were extracted by using a modified Folch method [32]. Freeze-dried cells (100 mg) were soaked in 2 mL of 1 M HCl for 15 min in a glass tube and then heated at 75 °C for 1 h on a heating block. The mixture was then transferred to a separation funnel, and 2 mL of 0.8% KCl was added, followed by 6 mL of Folch solution (chloroform–methanol, 2:1 v/v). The mixture in the funnel was thoroughly mixed and then left to stand for 15 min to facilitate phase separation. The lower lipid phase was collected into a preweighed glass tube. Subsequently, 4 mL of chloroform was added into the funnel, mixed well with the remaining phase, and left to stand until phase separation occurred. Then, the lower lipid phase was again transferred to the same glass tube. The extraction with chloroform was repeated until a clear lipid phase was obtained. Finally, the tube with the lipid phase was dried under nitrogen to evaporate the chloroform. The tube containing lipids was weighed. The dried lipid samples were resuspended in 1 mL of hexane and stored at −20 °C until methylation.

2.3.5. Fatty Acid Profile

The extracted lipid samples were methylated, and the resulting fatty acid methyl esters (FAME) were analyzed using a gas chromatography (GC) system. The methylation was carried out using the procedure with boron trifluoride reagent described by Appelqvist [33]. Briefly, 0.5 mL of the extracted lipid in hexane (with 2 mg/mL concentration) was mixed with 2 mL of dry methanol in a new glass tube, which was then incubated at 60 °C for 10 min. Then, 3 mL of boron trifluoride reagent was added. After thorough mixing, the tube was incubated for another 10 min at 60 °C. The mixture was then cooled to room temperature, and subsequently, 2 mL of 20% NaCl and 2 mL of hexane were added, and the mixture was vortexed well and then left to stand to facilitate phase separation.

The upper hexane phase with the FAME was transferred into a new glass tube. A 10 µL volume of the sample was used to check for complete derivatization to FAME on a thin-layer chromatography (TLC) silica-coated plate. Finally, the fatty acids were analyzed in a GC system (CP-3800, CTC Analytics AG, Switzerland) equipped with a flame ionization detector and a split injector and fitted with a 50 m long × 22 mm i.d., 0.25 µm film thickness, BPX 70 fused-silica capillary column. The GC was programmed to start at 158 °C, with temperature increasing at a rate of 2 °C min^{-1} until 220 °C and a final constant

time of 13 min at 220 °C. The peaks were identified by comparing their retention times with those of the standard mixture GLC-68D (Nu-Chek Prep, Elysian, MN, USA) and other authentic standards. The response factors were also evaluated by comparison with the GLC-68D standard.

2.3.6. Lipid Profile Analysis

Lipid classes were analyzed using the method of Olsen and Henderson [34] with some modifications. The extracted lipid samples were diluted to a concentration of 1 g/L in hexane and were separated by TLC. In brief, 5 µL of each sample was applied in 2 mm bands with a CAMAG TLC Sampler ATS4 (CAMAG, Switzerland) on a TLC plate (pre-coated with silica gel TLC plates, 20 × 10 cm; Silicagel 60; 0.20 mm layer, Merck, Darmstadt, Germany). All samples were applied in duplicate.

Separation of the lipid classes was carried out using hexane–diethyl ether–acetic acid (85:15:2; v/v/v) as the mobile phase in a CAMAG Automatic Developing Chamber 2 (ADC 2) (CAMAG, Switzerland). After separation, the TLC plates were dipped into a solution of 3% cupric acetate in 8% phosphoric acid and incubated for 20 min at 120 °C. Quantitative analysis was performed with a CAMAG TLC Scanner 3 (Camag, Switzerland). The scanning was performed at a speed of 20 mm/s and a data resolution of 100 µm/step, with a slit dimension of 6.00 × 0.45 mm at a wavelength of 350 nm. Finally, the separated lipid classes were identified by comparing with external standards (TLC 18-4A, Nu-Chek Prep, Elysian, USA; Ergosterol, PHR1512, Sigma-Aldrich, Sweden). VisionCATS Ultimate software was used for data analysis using Savitsky-Golay 7 mode. Manual baseline and peak correction were used if necessary.

2.4. Carotenoid Extraction

The extraction procedure was carried out in dark conditions to minimize carotenoid degradation. All the chemicals for extraction were obtained from Sigma-Aldrich (Europe). Freeze-dried cells (100 mg) were disrupted using a French press (Constant Systems LTD, Daventry, UK) at 276 mPa after addition of 45 mL of ultrapure water to the freeze-dried cells. An acetone extraction method was applied to extract carotenoids from yeast as described by Reif et al. [35], with minor modifications. Briefly, 4.5 mL of French-pressed yeast biomass (10 mg dry weight) was transferred to a glass tube containing 2 mL of acetone. This mixture was vortexed for 10 s and centrifuged at 450 g for 10 min. The upper phase was collected into a Teflon tube, and the extraction procedure with acetone for the lower phase in the glass tube was repeated until the supernatant was clear. For the final acetone extraction step, cyclohexane was added to the collected upper acetone phase in the Teflon tube (*v*/*v*, 1:1), which was vortexed for 10 s and centrifuged at 1890 g for 5 min. The upper acetone phase from the Teflon tube was then transferred into a new glass tube. This final extraction with acetone and cyclohexane was repeated until the supernatant in the Teflon tube was clear. At the end of extraction, the acetone collected in the new glass tube was evaporated under nitrogen and the remaining carotenoid extract was subjected to saponification.

A saponification step was applied to remove lipids from the extracts, which may interfere with the UHPLC analysis. For this, 2 mL of ethanol (96%), 1.2 mL of butylated hydroxytoluene (BHT, 0.2 mg/mL in methanol), 0.6 mL of methanol, and 1.2 mL of methanolic potassium hydroxide (2M KOH, in methanol) were added to the carotenoid extract. Saponification was performed at 56 °C for 20 min. After saponification, 3 mL of hexane was added at room temperature, and the sample was vortexed for 10 s and centrifuged at 700 g for 10 min. The upper phase was transferred to a new tube, and the remaining solvent was evaporated under nitrogen. Final extracts were diluted with methanol–acetone (1:1, *v*/*v*).

2.5. Total Carotenoid Quantification Using Spectrophotometry

Total carotenoid content in the yeast extracts was measured using the method described by Davey et al. [36]. A six-point β-carotene calibration curve was used to determine the total carotenoid content. For this, six standard solutions with different concentrations of β-carotene (0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 μg/mL) were prepared using hexane as solvent. The absorbances of unsaponified and saponified standard samples of β-carotene were measured in a 1 cm cuvette at 450 nm using a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) and calibrated against the blank containing only hexane. The total carotenoid content was expressed as mg of β-carotene equivalent (β-EQ)/100 g of dried yeast weight.

The molar extinction coefficient (ε_{max}) for the β-carotene absorbance at 450 nm was calculated as follows:

$$\varepsilon_{max} = A/CL \quad (1)$$

where 'A' is the absorbance of β-carotene at maximum wavelength (λ_{max}), 'C' is concentration of β-carotene, and 'L' is the width of the cuvette (1 cm) [37].

2.6. Carotenoid Identification and Quantification Using UHPLC-PDA

The analysis was carried out using a Shimadzu UHPLC-Nexera instrument (Kyoto, Japan) equipped with an autosampler (SIL-20AC) and quaternary pumps (LC-20AD). The equipment included a column oven (CTO-20AC) and a PDA detector (Shimadzu, model SPD-M20A) connected in series. LabSolutions software was used to control the instruments, data acquisition, and processing. The UV−visible spectra were acquired between 250 and 600 nm, and the peaks of β-carotene and torularhodin were acquired at 450 nm, ɣ-carotene at 462 nm, and torulene at 478 nm, respectively.

2.6.1. Chromatographic Method Conditions

Carotenoid separation was performed using an analytical RP C18 Kinetex 100 Å column (100 mm length, 4.6 mm internal diameter, 2.6 μm particle size; Phenomenex) and binary gradient system with the mobile phases A, consisting of acetonitrile–methanol (7:3, *v*/*v*), and B, consisting of ultrapure water with 0.1% formic acid. The gradient was set as follows: 0–3 min 60% B; 3–7 min 100% B; 7–30 min 100% B, and 30–35 min 60% B. The flow rate was 0.3 mL/min, the column temperature was 40 °C, and the sample volume was 20 μl.

2.6.2. Instrument Performance

The performance of the chromatographic method was assessed with regard to its linearity, limit of detection (LOD), limit of quantification (LOQ), precision, recovery, and matrix effect. Four analytes were quantified using a twelve-point calibration curve. The analytical standards of β- and ɣ-carotenes used for the analysis were purchased from Sigma-Aldrich (Sweden), whereas torularhodin and torulene were purchased from CaroteNature (Switzerland). The concentration range used was 20-0.05 μg/mL for torularhodin, 140-0.1 μg/mL for torulene, 12.5-0.001 μg/mL for β-carotene, and 50-0.005 μg/mL for γ-carotene. Analytical standards were used for preparation of standard mixtures to determine the linear range of the tested carotenoid. For all carotenoids, the analyzed concentration range covered four orders of magnitude, from ng/mL to μg/mL, which corresponds to the carotenoid content in the samples.

All analyses were performed in triplicate. Precision was calculated as the relative standard deviation (RSD) of the replicates. For evaluation of the matrix effects on the signals of the target analytes, the response factors delivered from the calibration curve were compared to the matrix-matched standard response. Matrix-matched standard was prepared from the sample extract by spiking with the standards of the carotenoids at concentration levels of 2 μg/mL torularhodin, 36 μg/mL torulene, 1.7 μg/mL β-carotene, and 25.5 μg/mL γ-carotene. The relative recoveries of four carotenoids were obtained by

preparation of fortified samples containing 1 μg/mL torularhodin, 0.2 μg/mL torulene, 5 μg/mL β-carotene, and 10 μg/mL γ-carotene. All the samples were extracted as per the procedure stated in Section 2.4.

Quality control samples were analyzed before and after each quantification run at three different concentrations along with procedural blanks to monitor the method and instrumental performance and to check for contamination of the equipment.

The LOD and LOQ were determined based on the residual standard deviation of calibration curve:

$$LOD = 3.3 \times (s/b) \quad (2)$$

$$LOQ = 10 \times (s/b) \quad (3)$$

where 's' is the residual standard deviation of the calibration line in the LOD region, and 'b' is the slope of the calibration curve [38].

2.7. Effect of Saponification on Total Carotenoids

To analyze the effect of saponification on carotenoids, individual carotenoid standards were subjected to extraction procedures similar to that of sample extraction described earlier (see Section 2.4). To compare the saponification effect, all extractions were made in triplicate with and without including the saponification step. These samples were analyzed by UHPLC-PDA, using the same chromatographic conditions as described above (Section 2.6.1).

2.8. Statistical Analysis

The statistical analysis was performed using R (v. 1.2.5001, 2009–2019 RStudio, Inc., Boston, MA, USA) software package. The data are expressed as the mean ± standard deviation. Total carotenoid content was compared and evaluated using one-way analysis of variance (ANOVA), followed by the Tukey test for pairwise comparisons among experimental conditions. The results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Establishing Carotenoid Analysis Method

3.1.1. Characterization of Calibration Curves of the Two Carotenoid Analysis Methods

Linear regression characteristics of carotenoid standard curves, as measured by UHPLC and spectrophotometer methods, are presented in Table 1.

Determination of the carotenoid content using UHPLC-PDA: Carotenoids were eluted as governed by their retention factors and the hydrophobicity of the stationary phase [39]. Accordingly, torularhodin appeared first in the chromatogram, followed by torulene, γ-carotene, and β-carotene. An unknown peak was identified at a retention time of 28.89 min. We have currently no explanation as to which compound this peak could represent; this is a topic for future investigation. The coefficient of linearity (R2) of the calibration curve was found to be more than 0.99 for all identified carotenoids. The repeatability of the proposed method was performed at one concentration level with satisfactory relative standard deviation (RSD) for all carotenoids. RSD ranged from 2 to 15%. The developed method shows that LOD and LOQ for each respective carotenoid differed by two orders of magnitude; they were in the range of 0.26–14.5 ng/mL for LOD and 0.72–44.1 ng/mL for LOQ (Table 1).

Table 1. Characterization of carotenoid standard curves for UHPLC-PDA and spectrophotometric methods.

Carotenoid	RT, min	λ_{max}, nm	Slope	Intercept	R2	RSD, %	LOD, ng/mL	LOQ, ng/mL
UHPLC-PDA-method								
Torularhodin	16.41	450	400.01	64556	0.999	2	0.97	2.95
(E/Z) -Torulene	23.49	478	223.29	177705	0.999	15	14.5	44.1
β-carotene	32.16	450	2331.4	877044	0.993	12	0.26	0.72
γ-carotene	25.75	462	216.81	92861	0.999	14	6.86	20.8
Spectrophotometric method								
β-carotene	–	450	1.23	0.03	0.999	n.d	n.d	n.d

Abbreviations: RT—retention time; RSD—relative standard deviation; LOD—limit of detection; LOQ—limit of quantification; n.d—not determined.

Determination of the carotenoid content using spectrophotometric method: The determined absorbance of the β-carotene standard was linear between 0.25 and 2 mg/mL. With a calibration curve slope of 1.234, the molar extinction coefficient was 126,833 $mM^{-1}cm^{-1}$ at 450 nm, which is similar to the values determined by Biehler et al. [37].

3.1.2. Relative Recovery and Matrix Effect

The relative recovery in saponified samples was detected in procedural blanks. The blanks were prepared and extracted in the same way as the samples. The relative recovery rates were obtained for four carotenoid compounds (Table 2). The highest recovery rate was found for γ-carotene (76%), followed by β-carotene. Torularhodin was only recovered to 4%, whereas torulene was below the limit of quantification.

The change in the ionization efficiency of target analytes in the presence of coeluting compounds in the same matrix often leads to a matrix effect. This might lead to either loss in response (ion suppression) or an increase in response (ion enhancement), which would in turn affect the analytical performance of a method [40]. The results of the evaluation of these matrix effects in our yeast sample are summarized in Table 2. The lowest matrix effect was observed for γ-carotene (12%), followed by torularhodin (18%) and β-carotene (31%) from *R. toruloides* yeast extracts. The matrix effect could not be determined for torulene since its concentration was below the LOQ.

Table 2. Evaluation of relative recovery and matrix effect in saponified samples as a percentage of compounds quantified based on response factors from calibration curves or matrix-matched standards (n = 3). Data are presented as mean ± standard deviation.

Carotenoids	Relative Recovery, %	Matrix Effect, %
Torularhodin	4.2 ± 0.04	−18 ± 0.15
(E/Z) -Torulene	<LOQ	n.a.
β-carotene	65 ± 0.04	−31 ± 0.16
γ-carotene	72 ± 0.06	12 ± 0.07

Abbreviations: LOQ—limit of quantification; n.a.—not applicable.

3.1.3. Saponification Effect

To evaluate the effect of saponification on carotenoids, we performed additional analyses with individual carotenoid standards. Torulene was excluded from analysis, as it was below the LOQ, which is most likely attributed to the saponification effect. As demonstrated in Figure 1, saponification reduced the concentrations of β-carotene and γ-carotene by 14% and 7%, respectively.

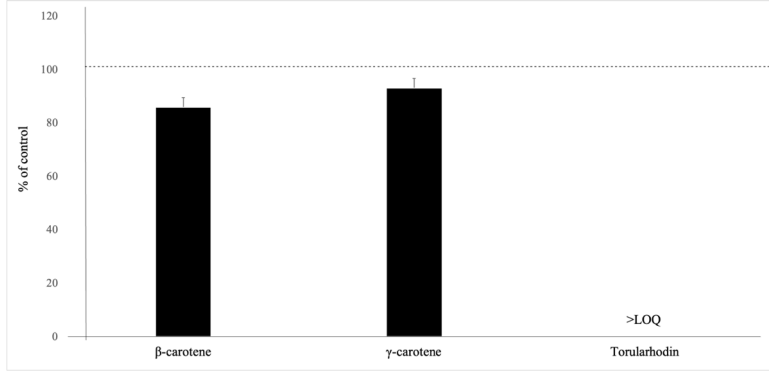

Figure 1. Effect of saponification on the carotenoids tested by UHPLC-PDA, n = 3. Data presented as percent of control values (mean ± standard deviation). The dashed line represents the levels of carotenoids in unsaponified samples.

Torularhodin was below LOQ after saponification. The results of this study show that quantification of torularhodin and torulene are affected considerably by saponification and the yeast matrix. This effect of saponification on individual carotenoids produced by *R. toruloides* has not been studied before. A previous report on carotenoids in broccoli suggests that saponification with 30% methanolic KOH at room temperature for 3 h under nitrogen atmosphere reduces β-carotene and 15,15′-cis-β-carotene by 6% and 17–84%, respectively [41]. Additionally, Nierenberg and Nann [42] reported a significant reduction in the recoveries of five carotenoids (lutein, cryptoxanthin, lycopene, α-carotene, and β-carotene) in human tissues when saponified using 5% methanolic KOH. In addition, Kimura et al. [43] also observed that even with mild saponification using 10% KOH, lutein, zeaxanthin, and violaxanthin degraded significantly in tomato, kale, and papaya samples. Lietz and Henry [44] also reported significant losses of α-carotene (19%), β-carotene (28%), and α-tocopherol (18%), as well as isomerization of α-carotene and β-carotene in a standard mixture during HPLC analysis of red palm oil after the saponification of the extract. Our report demonstrates for the first time that saponification has a similar effect on yeast carotenoids.

3.1.4. Carotenoid Content in *R. toruloides*

Total carotenoid content in *R. toruloides* was quantified using two methods, spectrophotometry and UHPLC-PDA (Table 3). Only saponified samples were analyzed by UHPLC-PDA to avoid unwanted interference of the lipids with the analytical column, whereas saponified and unsaponified extracts were analyzed by the spectrophotometric method. The total carotenoid content in saponified extracts measured by the two methods did not differ significantly (p = 0.23), and similarly, there was no significant difference observed between the total carotenoid content in saponified and unsaponified extracts from spectrophotometer results (p = 0.11). The spectrophotometric method showed a much higher standard deviation than chromatography, and this could probably be due to the presence of several compounds in the sample mixture that affect total absorbance. The accuracy of the spectrophotometric method is poor in sample mixtures with different carotenoids because absorbance is measured only at 450 nm, which is equivalent to beta carotene absorbance [45]. A significant difference in total carotenoid content was observed only comparing saponified (UHPLC-PDA analysis) and unsaponified extracts (spectrophotometer analysis) (p = 0.05). Our results strongly indicate that this difference was mainly due to the saponification effect on torulene and torularhodin.

Table 3. Quantification of individual carotenoids, averaged to total in saponified samples, as determined by UHPLC-PDA, and total carotenoids in both saponified and unsaponified samples, as determined spectrophotometrically. Data are presented as mean ± standard deviation (n = 3) and different superscript letters represent significant differences ($p \leq 0.05$).

	UHPLC-PDA Saponified mg/100 g d.w.	Spectrophotometer Saponified mg β-EQ/100 g d.w.	Spectrophotometer Unsaponified mg β-EQ/100 g d.w.
Torularhodin	0.09 ± 0.028	n.a.	n.a.
(E/Z) -Torulene	<LOQ	–	–
β-carotene	1.48 ± 0.18	–	–
γ-carotene	0.42 ± 0.06	–	–
Total carotenoids	1.99 ± 0.27 [a]	3.09 ± 0.78 [ab]	4.02 ± 0.53 [b]

Abbreviations: LOQ—limit of quantification; n.a.—not applicable; d.w.—dry weight; β-EQ—β-carotene equivalent.

The total carotenoid content from *R. toruloides* CBS 14 was further characterized by detecting individual carotenoids by UHPLC-PDA. Overall, three out of four identified carotenoids were above the LOQ (Table 3). Levels of torulene were below the LOQ. Torulene and torularhodin compounds produced by *R. toruloides* were identified and quantified using their respective commercial reference standards, unlike some previous studies, where they were quantified using either β- or γ-carotene as the reference standards [20,46].

The most abundant carotenoid observed in *R. toruloides* CBS 14 was β-carotene (74%), followed by γ-carotene (21%) and torularhodin (4.5%). The total carotenoid concentration in the saponified yeast extract was 1.99 ± 0.27 mg/100 g dry weight. According to Perrier et al. [19], β-carotene (70%) was the main carotenoid produced by 13 different *Rhodotorula* strains, whereas only eight *Rhodotorula* strains were observed to produce torulene. The concentration of β-carotene varied within the yeast strains by 0.5–7.5 mg/100 g dry weight [19,47]. Nam et al. [47] reported 3.6 mg/100 g dry weight total carotenoids in *Rhodotorula glutinis* extract (excluding the concentration of torulene), which was similar to our results.

3.2. Kinetics of Carotenoid and Lipid Formation in Bioreactor Cultivation

3.2.1. Cell Growth, Cell Dry Weight, Sugar, and Lipid Content Analysis

The cell growth, biomass, sugar consumption, and lipid production of *R. toruloides* in the hydrolysate media were monitored at time intervals of 24 h. Cell growth was monitored by measuring the optical density (OD) and cell dry weight. There was a considerable increase in the cell dry weight between 24 and 72 h of the growth phase (Figure 2). All the sugars were consumed within 96 h of cultivation. Significant lipid accumulation was mainly observed at the two last measuring time points, 72 h and 96 h, and the onset of lipid accumulation was most probably correlated to the depletion of nitrogen from the culture medium [48]. Moreover, between these time points, it was observed that the lipid yield per g consumed sugar was 0.5. This is by far above the theoretical maximum, which is 0.31 for glucose [48] and 0.34 for xylose [49]. It has been observed in other oleaginous yeasts that they accumulate endopolysaccharides in earlier stages of the fermentation [50,51]. It is possible that this strain was also accumulating endopolysaccharides in the earlier stages of fermentation and that it partially converted these endopolysaccharides to lipids towards the end of the fermentation. However, this hypothesis needs further verification. At the end of the cultivation period, the final lipid content in the yeast cells from all three fermenters was 41 ± 1%. The lipid yield was 0.13 g/g of sugar consumed.

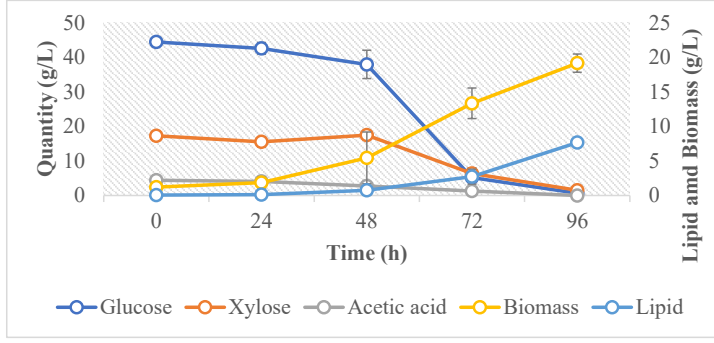

Figure 2. Average cell dry weight, lipid concentration, and sugar content during the fermentation of *R. toruloides*.

3.2.2. Lipid Composition and Classes

During cultivation, considerable changes were observed in the fatty acid profile of the yeast (Table 4). Oleic acid (C18:1(n-9)) was the major fatty acid. Initially, it comprised 55.8% of the total fatty acids, which decreased to 40.3% at the end of the cultivation. Palmitic acid (C16:0) comprised about 13–15% of total fatty acids until 48 h of cultivation, but its proportion increased to more than 20% during the last two measuring points. Linoleic acid (C18:2 (n-6)) was the second most abundant fatty acid at most of the measuring points, typically comprising more than 20% of the total fatty acids (apart from at t_0). The cells also formed considerable amounts of alpha-linolenic acid (C18:3(n-3)), more than 7% of the total fatty acids at t_0 and after 24 h of cultivation. This proportion decreased in the further course of cultivation to 2.9% at 96 h. Formation of alpha-linolenic acid (C18:3 (n-3)) has previously been observed as a response to low cultivation temperatures in other yeasts [52]. The production of polyunsaturated fatty acids such as alpha-linolenic acid makes this lipid more suitable for production of food and feed additives [53]. In general, there was an increase in the percentage of saturated fatty acids and a decrease in unsaturated fatty acids during the course of cultivation. This should be due to the increasing amount of storage lipids (Figure 2). The proportion of saturated fatty acids in lipid bodies is usually higher than in the membrane systems in the cell [54,55].

Additionally, a detectable amount of heptadecenoic acid (C17:1) was identified in our yeast sample. This fatty acid has been rarely identified and reported in yeasts [56] but it was identified in *Rhodotorula babjevae* when cultivated on wheat straw hydrolysate [14].

Table 4. Quantification of different fatty acids in the yeast sample at different time points during cultivation. Data are presented as mean % ± standard deviation of fatty acids in the samples from three fermenters.

Cultivation Time (h)	Fatty Acid Profile (%) of the Total Fatty Acids					
	C16:0	C18:0	C17:1	C18:1(n-9)	C18:2(n-6)	C18:3(n-3)
0	14.7 ± 0.40	2.79 ± 0.27	0.47 ± 0.04	55.8 ± 0.54	13.3 ± 0.35	7.22 ± 0.36
24	13.2 ± 1.18	1.68 ± 0.67	0.30 ± 0.10	49.1 ± 11.23	22.9 ± 14.00	7.53 ± 0.45
48	14.8 ± 1.42	3.13 ± 2.60	0.17 ± 0.06	45.0 ± 16.82	26.0 ± 15.30	6.18 ± 1.59
72	23.7 ± 3.83	4.24 ± 0.84	0.47 ± 0.06	38.8 ± 2.31	24.2 ± 6.09	3.55 ± 0.78
96	26.6 ± 1.66	4.70 ± 0.44	0.36 ± 0.03	40.3 ± 1.73	20.4 ± 1.31	2.93 ± 0.87

Neutral lipids in the lipid bodies of oleaginous yeast are stored as monoacylglycerols (MAG), diacylglycerols (DAG), and triacylglycerols (TAG) [57]. In this study, during the cultivation period, as the carbon source was consumed, the percentage of TAG increased;

indeed, TAG was observed to be the major lipid class by the end of cultivation, comprising 58% of all lipids (Table 5).

Table 5. Lipid class quantification in the yeast sample at different time points during cultivation. Data are presented as mean % ± standard deviation of lipid classes in the samples from three fermenters.

Cultivation Time (h)	Lipid Class Expressed as % of Total Lipid Classes					
	Phospholipids	**MAG**	**DAG**	**Sterols**	**FFAs**	**TAG**
0	12.6 ± 0.47	5.09 ± 0.24	3.60 ± 0.71	8.54 ± 0.51	37.2 ± 0.32	33.5 ± 0.60
24	12.1 ± 0.92	4.99 ± 1.39	3.90 ± 0.94	9.12 ± 0.64	41.8 ± 4.42	28.2 ± 3.37
48	13.8 ± 4.72	4.90 ± 1.00	4.02 ± 0.75	8.68 ± 0.93	38.3 ± 6.14	30.3 ± 13.2
72	7.40 ± 0.34	4.42 ± 0.26	3.95 ± 0.77	6.92 ± 0.38	27.9 ± 5.01	49.4 ± 6.66
96	8.05 ± 1.20	4.02 ± 0.63	2.54 ± 0.42	6.80 ± 0.42	20.5 ± 1.58	58.1 ± 3.32

Abbreviations: MAG—monoacylglycerol; DAG—diacylglycerol; FFAs—free fatty acids; TAG—triacylglycerol.

Free fatty acids (FFAs) were very abundant during cultivation, comprising around 20–40% of the total lipid content. FFAs were the most abundant lipids during the first three measuring points, with TAG becoming more abundant at the last two sampling points. Still, even at these later measuring points, FFAs were the second most abundant lipid class (Table 5). In *Saccharomyces cerevisiae*, an extensive concentration of FFAs was toxic and resulted in mainly necrotic cell death [58]. However, our findings are in line with previous results, and we also found a substantial amount of FFAs in a variety of oleaginous yeast strains [2]. Obviously, oleaginous yeasts have a higher tolerance to FFAs, and *R. toruloides* has evolved mechanisms to handle the toxicity of FFAs. Towards the end of the cultivation, in a situation with enhanced lipid accumulation, production of TAG was obviously enhanced, resulting in a decrease in the proportion of FFAs. FFAs in yeasts are in equilibrium with acyl-CoA, which is used for TAG production [59], so it is possible that the pool of FFAs is used for the production of TAG. However, since the absolute proportion of FFAs relative to the total biomass was still increasing towards the end of fermentation, the cells were obviously still producing considerable amounts of FFAs. We have currently no explanation for the biological function of FFAs in *R. toruloides*.

In yeast cells, phospholipids and sterols are mainly found in the plasma membrane. Some sterols are also present in secretory vesicles and lipid particles [60]. In this study, the proportion of both phospholipids and sterols decreased over the cultivation period. The proportion of phospholipids decreased from 12% to about 8% at the end of the growth phase due to an increase in triacylglycerols, which is in line with the increase in total lipid content. Storage lipids are usually stored in lipid bodies, which mainly contain TAGs [55]. Thus, an increase in storage lipids would be reflected by an increase in the proportion of TAGs, which is in line with our results.

3.2.3. Carotenoid Kinetics and Quantification

Carotenoid content in the saponified extracts of *R. toruloides* analyzed at different time points during the cultivation is summarized in Table 6. β-carotene constituted the majority of total carotenoids produced throughout the growth phase, followed by γ-carotene. As described in Section 3.1.2 above, due to the effect of saponification, torulene was observed to be below the limit of quantification (LOQ) at all time points, and torularhodine could not be determined. The proportion of γ-carotene increased during cultivation, nearly reaching concentrations as high as that of β-carotene towards the end of cultivation. Similar observations have been made by Tkacova et al. [61]. The reason for the increase in γ-carotene is unclear. This carotenoid is formed from lycopene by the enzyme encoded by *crtYB*. However, it is converted to β-carotene by the same enzyme. The activity of this enzyme might in general be increased, which would explain the increase in both

γ-and β-carotene concentrations, but not the relative increase in γ-carotene. However, γ-carotene can also be formed by dehydrogenation of β –zeacarotene, which might be enhanced as well [62].

Table 6. Quantification of individual carotenoids in saponified samples at different growth time points. Data are presented as mean ± standard deviation of carotenoid content in the samples from three fermenters.

Cultivation Time (h)	Quantity of Carotenoids (mg/100 g d.w.)			
	β-Carotene	γ-Carotene	Torularhodin	Total
0	0.15 ± 0.002	0.01 ± 0.0005	0.0003 ± 0.0001	0.16 ± 0.001
24	0.22 ± 0.02	0.03 ± 0.03	0.004 ± 0.004	0.25 ± 0.03
48	0.45 ± 0.17	0.12 ± 0.11	0.01 ± 0.01	0.58 ± 0.24
72	0.85 ± 0.16	0.46 ± 0.16	0.03 ± 0.01	1.33 ± 0.27
96	1.47 ± 0.14	1.32 ± 0.14	0.06 ± 0.003	2.85 ± 0.26

Abbreviations: d.w.—dry weight.

From this kinetic study, a correlation between the metabolism of lipids and the carotenoid pigments was also observed. With increased lipid accumulation, an increase in the total carotenoid content was observed during the growth period, as also reported in some studies [46,63] but somewhat contradictory to another study [61]. In this latter study, lipid and carotenoid formation of *R. glutinis* was investigated in glucose media with different C/N ratios, with the finding that there are differences in lipid and carotenoid production, depending on strain, type of carbon and nitrogen source, and other factors. In our study, when *R. toruloides* CBS 14 was grown on wheat straw hydrolysate, the yeast first consumed glucose and acetate and then assimilated xylose. Since both lipid content and carotenoid content increased during the last two sampling points, there was obviously no limitation of acetyl-CoA [64]. By the end of the cultivation period, the total carotenoid content in the saponified yeast extract was 2.85 ± 0.26 mg/100 g dry weight. Carotenoids act as antioxidants and protect the cell from oxidative stress, including lipid oxidation [63]. Thus, a synchronized increase in the cellular lipid and carotenoid contents is a logical consequence and is in keeping with our results.

4. Conclusions

In this study, we have shown that four carotenoids (torularhodin, torulene, β-carotene, and γ-carotene) are produced by *R. toruloides*, and β-carotene was identified as the major carotenoid. Torulene was below the limit of quantification when analyzed by the current method, due to matrix effects and saponification. Removal of the lipids coeluted with the carotenoid extracts is required before running the samples in the UHPLC system, as the presence of lipids will lead to inaccurate quantification of carotenoids, i.e., lower values, and it might destroy the analytical column as well. Therefore, when using standard methods for extraction of carotenoids, saponification cannot be avoided. In this study, we also confirmed a significant effect of saponification on the concentration of torulene and torularhodin. This impact of saponification makes it necessary to develop a lipid-free extraction method for better quantification of carotenoids from yeasts, without the need for saponification. We have shown that yeast growth is accompanied by proportional changes in amounts of lipid fractions and individual lipid classes, as well as in their fatty acid composition. There was a positive correlation between lipid accumulation and carotenoid formation, indicating that these metabolic pathways do not interfere with each other, although using the same precursor.

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References

1. Ageitos, J.M.; Vallejo, J.A.; Veiga-Crespo, P.; Villa, T.G. Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol.* **2011**, *90*, 1219–1227.
2. Shapaval, V.; Brandenburg, J.; Blomqvist, J.; Tafintseva, V.; Passoth, V.; Sandgren, M.; Kohler, A. Biochemical profiling, prediction of total lipid content and fatty acid profile in oleaginous yeasts by FTIR spectroscopy. *Biotechnol. Biofuels* **2019**, *12*, 1–12.
3. Vicente, G.; Bautista, L.F.; Gutiérrez, F.J.; Rodríguez, R.A.; Martínez, V.; Rodríguez-Frómeta, R.A.; Ruiz-Vázquez, R.M.; Torres-Martínez, S.; Garre, V. Direct transformation of fungal biomass from submerged cultures into biodiesel. *Energy Fuels* **2010**, *24*, 3173–3178.
4. Abeln, F.; Chuck, C.J. The history, state of the art and future prospects for oleaginous yeast research. *Microb. Cell Factories* **2021**, *20*, 221.
5. Dai, C.-c.; Tao, J.; Xie, F.; Dai, Y.-j.; Zhao, M. Biodiesel generation from oleaginous yeast *Rhodotorula glutinis* with xylose assimilating capacity. *Afr. J. Biotechnol.* **2007**, *6*, 2130–2134.
6. Blomqvist, J.; Pickova, J.; Tilami, S.K.; Sampels, S.; Mikkelsen, N.; Brandenburg, J.; Sandgren, M.; Passoth, V. Oleaginous yeast as a component in fish feed. *Sci. Rep.* **2018**, *8*, 15945.
7. Schulze, I.; Hansen, S.; Großhans, S.; Rudszuck, T.; Ochsenreither, K.; Syldatk, C.; Neumann, A. Characterization of newly isolated oleaginous yeasts-*Cryptococcus podzolicus, Trichosporon porosum* and *Pichia segobiensis*. *AMB Express* **2014**, *4*, 24.
8. Granger, L.-M.; Perlot, P.; Goma, G.; Pareilleux, A. Effect of various nutrient limitations on fatty acid production by *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* **1993**, *38*, 784–789.
9. Beopoulos, A.; Cescut, J.; Haddouche, R.; Uribelarrea, J.-L.; Molina-Jouve, C.; Nicaud, J.-M. *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res.* **2009**, *48*, 375–387.
10. Takaku, H.; Matsuzawa, T.; Yaoi, K.; Yamazaki, H. Lipid metabolism of the oleaginous yeast *Lipomyces starkeyi*. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 6141–6148.
11. Kaczor, A.; Baranska, M. *Carotenoids: Nutrition, Analysis and Technology*; John Wiley & Sons: Hoboken, NJ, USA, 2016.
12. Rapoport, A.; Guzhova, I.; Bernetti, L.; Buzzini, P.; Kieliszek, M.; Kot, A.M. Carotenoids and some other pigments from fungi and yeasts. *Metabolites* **2021**, *11*, 92.
13. Ungureanu, C.; Marchal, L.; Chirvase, A.A.; Foucault, A. Centrifugal partition extraction, a new method for direct metabolites recovery from culture broth: Case study of torularhodin recovery from *Rhodotorula rubra*. *Bioresour. Technol.* **2013**, *132*, 406–409.
14. Brandenburg, J.; Poppele, I.; Blomqvist, J.; Puke, M.; Pickova, J.; Sandgren, M.; Rapoport, A.; Vedernikovs, N.; Passoth, V. Bioethanol and lipid production from the enzymatic hydrolysate of wheat straw after furfural extraction. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6269–6277.
15. Passoth, V.; Sandgren, M. Biofuel production from straw hydrolysates: Current achievements and perspectives. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 5105–5116.
16. Chmielarz, M.; Blomqvist, J.; Sampels, S.; Sandgren, M.; Passoth, V. Microbial lipid production from crude glycerol and hemicellulosic hydrolysate with oleaginous yeasts. *Biotechnol. Biofuels* **2021**, *14*, 65.
17. Frengova, G.I.; Beshkova, D.M. Carotenoids from *Rhodotorula* and *Phaffia*: Yeasts of biotechnological importance. *J. Ind. Microbiol. Biotechnol.* **2009**, *36*, 163.
18. Buzzini, P.; Innocenti, M.; Turchetti, B.; Libkind, D.; van Broock, M.; Mulinacci, N. Carotenoid profiles of yeasts belonging to the genera *Rhodotorula, Rhodosporidium, Sporobolomyces,* and *Sporidiobolus*. *Can. J. Microbiol.* **2007**, *53*, 1024–1031.
19. Perrier, V.; Dubreucq, E.; Galzy, P. Fatty acid and carotenoid composition of *Rhodotorula* strains. *Arch. Microbiol.* **1995**, *164*, 173–179.

20. Flieger, K.; Knabe, N.; Toepel, J. Development of an improved carotenoid extraction method to characterize the carotenoid composition under oxidative stress and cold temperature in the rock inhabiting fungus *Knufia petricola* A95. *J. Fungi* **2018**, *4*, 124.
21. Shi, Q.; Wang, H.; Du, C.; Zhang, W.; Qian, H. Tentative Identification of Torulene Cis/trans Geometrical Isomers Isolated from *Sporidiobolus pararoseus* by High-Performance Liquid Chromatography–Diode Array Detection–Mass Spectrometry and Preparation by Column Chromatography. *Anal. Sci.* **2013**, *29*, 997–1002.
22. Weber, R.W.; Anke, H.; Davoli, P. Simple method for the extraction and reversed-phase high-performance liquid chromatographic analysis of carotenoid pigments from red yeasts (Basidiomycota, Fungi). *J. Chromatogr. A* **2007**, *1145*, 118–122.
23. Freitas, C.; Nobre, B.; Gouveia, L.; Roseiro, J.; Reis, A.; da Silva, T.L. New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth. *Process Biochem.* **2014**, *49*, 554–562.
24. Schüler, L.M.; Gangadhar, K.N.; Duarte, P.; Placines, C.; Molina-Márquez, A.M.; Léon-Bañares, R.; Sousa, V.S.; Varela, J.; Barreira, L. Improvement of carotenoid extraction from a recently isolated, robust microalga, *Tetraselmis* sp. CTP4 (chlorophyta). *Bioprocess Biosyst. Eng.* **2020**, *43*, 785–796.
25. de Quirós, A.R.-B.; Costa, H.S. Analysis of carotenoids in vegetable and plasma samples: A review. *J. Food Compos. Anal.* **2006**, *19*, 97–111.
26. Granado, F.; Olmedilla, B.; Blanco, I.; Rojas-Hidalgo, E. Carotenoid composition in raw and cooked Spanish vegetables. *J. Agric. Food Chem.* **1992**, *40*, 2135–2140.
27. Kalra, R.; Conlan, X.A.; Goel, M. Fungi as a potential source of pigments: Harnessing filamentous fungi. *Front. Chem.* **2020**, *8*, 369.
28. Buzzini, P. An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source. *J. Ind. Microbiol. Biotechnol.* **2000**, *24*, 41–45.
29. Ochsenreither, K.; Glück, C.; Stressler, T.; Fischer, L.; Syldatk, C. Production strategies and applications of microbial single cell oils. *Front. Microbiol.* **2016**, *7*, 1539.
30. Brandenburg, J.; Blomqvist, J.; Pickova, J.; Bonturi, N.; Sandgren, M.; Passoth, V. Lipid production from hemicellulose with *Lipomyces starkeyi* in a pH regulated fed-batch cultivation. *Yeast* **2016**, *33*, 451–462.
31. Enshaeieh, M.; Abdoli, A.; Madani, M.; Bayat, M. Recycling of lignocellulosic waste materials to produce high-value products: Single cell oil and xylitol. *Int. J. Environ. Sci. Technol.* **2015**, *12*, 837–846.
32. Folch, J.; Lees, M.; Stanley, G.S. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509.
33. Appelqvist, L.-Å. Rapid methods of lipid extraction and fatty acid methyl ester preparation for seed and leaf tissue with special remarks on preventing the accumulation of lipid contaminants. *Ark. Kemi* **1968**, *28*, 551–570.
34. Olsen, R.; Henderson, R. The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. *J. Exp. Mar. Biol. Ecol.* **1989**, *129*, 189–197.
35. Reif, C.; Arrigoni, E.; Schärer, H.; Nyström, L.; Hurrell, R.F. Carotenoid database of commonly eaten Swiss vegetables and their estimated contribution to carotenoid intake. *J. Food Compos. Anal.* **2013**, *29*, 64–72.
36. Davey, M.W.; Keulemans, J.; Swennen, R. Methods for the efficient quantification of fruit provitamin A contents. *J. Chromatogr. A* **2006**, *1136*, 176–184.
37. Biehler, E.; Mayer, F.; Hoffmann, L.; Krause, E.; Bohn, T. Comparison of 3 spectrophotometric methods for carotenoid determination in frequently consumed fruits and vegetables. *J. Food Sci.* **2010**, *75*, C55–C61.
38. Vilaro, F.; Perez-Hedo, M.; Eras, J.; Canela, R.; Eizaguirre, M. UHPLC-MS Analysis of Juvenile Hormone II in Mediterranean Corn Borer (*Sesamia nonagrioides)* Hemolymph Using Various Ionization Techniques. *J. Agric. Food Chem.* **2012**, *60*, 3020–3025. https://doi.org/10.1021/jf204621h.
39. Ligor, M.; Kováčová, J.; Gadzała-Kopciuch, R.; Studzińska, S.; Bocian, S.; Lehotay, J.; Buszewski, B. Study of RP HPLC retention behaviours in analysis of carotenoids. *Chromatographia* **2014**, *77*, 1047–1057.
40. Gosetti, F.; Mazzucco, E.; Zampieri, D.; Gennaro, M.C. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. A* **2010**, *1217*, 3929–3937.
41. Khachik, F.; Beecher, G.R.; Whittaker, N.F. Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography. *J. Agric. Food Chem.* **1986**, *34*, 603–616.
42. Nierenberg, D.W.; Nann, S.L. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. *Am. J. Clin. Nutr.* **1992**, *56*, 417–426.
43. Kimura, M.; Rodriguez-Amaya, D.B.; Godoy, H.T. Assessment of the saponification step in the quantitative determination of carotenoids and provitamins A. *Food Chem.* **1990**, *35*, 187–195.
44. Lietz, G.; Henry, C. A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. *Food Chem.* **1997**, *60*, 109–117.
45. Barba, A.O.; Hurtado, M.C.; Mata, M.S.; Ruiz, V.F.; De Tejada, M.L.S. Application of a UV–vis detection-HPLC method for a rapid determination of lycopene and β-carotene in vegetables. *Food Chem.* **2006**, *95*, 328–336.
46. Pham, K.D.; Shida, Y.; Miyata, A.; Takamizawa, T.; Suzuki, Y.; Ara, S.; Yamazaki, H.; Masaki, K.; Mori, K.; Aburatani, S. Effect of light on carotenoid and lipid production in the oleaginous yeast *Rhodosporidium toruloides*. *Biosci. Biotechnol. Biochem.* **2020**, *84*, 1501–1512.

47. Nam, H.; Cho, S.; Rhee, J.S. High-performance liquid chromatographic analysis of major carotenoids from *Rhodotorula glutinis*. *J. Chromatogr. A* **1988**, *448*, 445–447.
48. Ratledge, C.; Wynn, J.P. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. In *Advances in Applied Microbiology*; Academic Press: Cambridge, MA, USA, 2002; Volume 51, pp. 1–52.
49. Zhou, W.; Wang, Y.; Zhang, J.; Zhao, M.; Tang, M.; Zhou, W.; Gong, Z. A metabolic model of *Lipomyces starkeyi* for predicting lipogenesis potential from diverse low-cost substrates. *Biotechnol. Biofuels* **2021**, *14*, 148.
50. Cescut, J.; Fillaudeau, L.; Molina-Jouve, C.; Uribelarrea, J.-L. Carbon accumulation in *Rhodotorula glutinis* induced by nitrogen limitation. *Biotechnol. Biofuels* **2014**, *7*, 164.
51. Maina, S.; Pateraki, C.; Kopsahelis, N.; Paramithiotis, S.; Drosinos, E.H.; Papanikolaou, S.; Koutinas, A. Microbial oil production from various carbon sources by newly isolated oleaginous yeasts. *Eng. Life Sci.* **2017**, *17*, 333–344.
52. Suutari, M.; Rintamäki, A.; Laakso, S. The effect of temperature on lipid classes and their fatty acid profiles in *Lipomyces starkeyi*. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1071–1073.
53. Das, U.N. Essential fatty acids: Biochemistry, physiology and pathology. *Biotechnol. J. Healthc. Nutr. Technol.* **2006**, *1*, 420–439.
54. Wood, J.; Enser, M.; Fisher, A.; Nute, G.; Sheard, P.; Richardson, R.; Hughes, S.; Whittington, F. Fat deposition, fatty acid composition and meat quality: A review. *Meat Sci.* **2008**, *78*, 343–358.
55. Sandager, L.; Gustavsson, M.H.; Ståhl, U.; Dahlqvist, A.; Wiberg, E.; Banas, A.; Lenman, M.; Ronne, H.; Stymne, S. Storage lipid synthesis is non-essential in yeast. *J. Biol. Chem.* **2002**, *277*, 6478–6482.
56. Tronchoni, J.; Rozès, N.; Querol, A.; Guillamón, J.M. Lipid composition of wine strains of *Saccharomyces kudriavzevii* and *Saccharomyces cerevisiae* grown at low temperature. *Int. J. Food Microbiol.* **2012**, *155*, 191–198.
57. Patel, A.; Arora, N.; Mehtani, J.; Pruthi, V.; Pruthi, P.A. Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production. *Renew. Sustain. Energy Rev.* **2017**, *77*, 604–616.
58. Eisenberg, T.; Büttner, S. Lipids and cell death in yeast. *FEMS Yeast Res.* **2014**, *14*, 179–197.
59. Tehlivets, O.; Scheuringer, K.; Kohlwein, S.D. Fatty acid synthesis and elongation in yeast. *Biochim. Et Biophys. Acta (BBA)-Mol. Cell Biol. Lipids* **2007**, *1771*, 255–270.
60. Zinser, E.; Paltauf, F.; Daum, G. Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. *J. Bacteriol.* **1993**, *175*, 2853–2858.
61. Tkacova, J.; Caplova, J.; Klempova, T.; Certik, M. Correlation between lipid and carotenoid synthesis in torularhodin-producing *Rhodotorula glutinis*. *Ann. Microbiol.* **2017**, *67*, 541–551.
62. Kot, A.M.; Błażejak, S.; Gientka, I.; Kieliszek, M.; Bryś, J. Torulene and torularhodin: "New" fungal carotenoids for industry? *Microb. Cell Factories* **2018**, *17*, 49.
63. Kot, A.M.; Błażejak, S.; Kieliszek, M.; Gientka, I.; Bryś, J. Simultaneous production of lipids and carotenoids by the red yeast *Rhodotorula* from waste glycerol fraction and potato wastewater. *Appl. Biochem. Biotechnol.* **2019**, *189*, 589–607.
64. Aksu, Z.; Eren, A.T. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: Use of agricultural wastes as a carbon source. *Process Biochem.* **2005**, *40*, 2985–2991.

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Oleaginous yeasts, with the ability to thrive on low-cost carbon sources, can accumulate lipids and carotenoids, offering potential applications in food and feed industries. The research identifies issues with conventional extraction methods, highlighting the need for refinement. Supercritical carbon-dioxide (SC-CO_2) extraction emerged as a promising approach for isolating lipids and carotenoids from *Rhodotorula toruloides*. The research also reveals insights about the hybrid strain, CBS 6016, showing a dominant inheritance of genetic traits from one parental strain (CBS 14).

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