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# Holistic optimization of single cell oil processes: Creating and controlling the genetic toolbox as well as cultivation processes

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به آموختن گر بیندی میان  به دانش روی بر سپهر روان

شاهنامه فردوسی

*If learning is what you would hold most dear,*

*With wisdom you will stride the turning sphere*

*-The Shahnameh-*

*Ferdowsi*

# Abstracts

The ever-growing utilization of fossil fuels and their plant-based alternatives for oleochemicals and energy production have led to climate change and other environmental issues, due to carbon dioxide emissions and land use change. Further, the demand and supply imbalance of plant oils challenges the food security. Notably, studies have elucidated the oleaginous yeasts as promising renewable alternative feedstocks to replace plant sources and mineral oils in many applications provided that the cost of yeast oil production becomes competitive. Process optimizations, exploring new strains, and strain improvements are regarded as deciding factors in improving the economic feasibility of yeast bioprocesses. One of the key elements is the substrate cost. In this context, conversion of low-cost feedstocks, such as residual agricultural, industrial, and marine biomass to value-added products by microorganisms have become an essential part of circular bioeconomy. To that end, the strains that improve the hydrolysates specificity through broader substrate utilization, higher tolerance to inhibitors, and faster growth are favoured.

Therefore, five prominent oleaginous yeast strains; *Rhodotorula glutinis*, *Trichosporon asahii*, *Rhodotorula mucilaginosa*, *Rhodotorula toruloides*, and *Cutaneotrichosporon oleaginosus* were analysed in complex hydrolysates of marine and terrestrial origins. The systematic analysis of these strains provides new insights into their lipid and biomass yields, sugar utilization, fatty acid profile, and inhibitory effect of the hydrolysates on the growth. It also revealed that *C. oleaginosus* and *T. asahii* have a broader range of substrate uptake. In particular, *C. oleaginosus* showed to be the most efficient and versatile strain amongst all under tested conditions.

Moreover, genetic tractability allows for flexible strain optimization via metabolic engineering, which, in turn, unlocks the potential of promising strains for commercialization. Until now, random gene editing methods have been established for *C. oleaginosus*, which leads to unwanted genetic alterations. Therefore, *C. oleaginosus* was further studied to unravel its genetic tractability using CRIPSR/Cas system. We were able to develop an efficient, flexible targeted gene editing technique featuring a plasmid-free transient Cas9 delivery strategy. Consequently, metabolic engineering of *C. oleaginous* successfully led to strains with modified fatty acid profiles, such as those resembling sunflower and cocoa-butter compositions and a strain with 54% lipid yield enhancement. The developed technique paves the way towards building a robust platform for superior tailored oil production by *C. oleaginosus*, while it serves as a promising model for genetic engineering of other genetically inaccessible unconventional yeasts.

The data in this thesis have been published in two high-impact scientific publications and one patent.

# Zusammenfassung

Die steigende Nutzung fossiler Brennstoffe und ihrer pflanzlichen Alternativen für die Oleochemie und die Energieerzeugung hat aufgrund von Kohlendioxidemissionen und Landnutzungsänderungen zum Klimawandel und anderen Umweltproblemen geführt. Zudem gefährdet das Ungleichgewicht zwischen Angebot und Nachfrage bei Pflanzenölen die Ernährungssicherheit. Insbesondere haben Studien die ölhaltige Hefen als vielversprechende erneuerbare alternative Rohstoffe zum Ersatz von pflanzlichen Quellen und Mineralölen in vielen Anwendungen aufgezeigt. Um diese nachhaltigen Prozesse wettbewerbsfähig zu machen, müssen die Kosten der Hefeölproduktion signifikant reduziert werden. Dies erfordert weitreichende Prozessoptimierungen, bei der die Erforschung und Optimierung neuer Stämme ein entscheidender Faktor für die Verbesserung der wirtschaftlichen Machbarkeit von Hefe-Bioprozessen sind. In diesem Kontext, tragen auch die Substratkosten der Fermentation negativ zu den bisherigen Kostenstrukturen bei. In diesem Zusammenhang ist die Umwandlung von kostengünstigen biogenen Reststoffen wie landwirtschaftlicher, industrieller und mariner Biomasse in wertsteigerndes Hefeöl ein zielführender Ansatz zur Kostenreduktion und trägt durch Nutzung von Reststoffen zur Etablierung einerzirkulären Bioökonomie bei. Um mit diesen Reststoffen effiziente Hefeölproduktions Verfahren zu entwicklen, werden oleogene Hefestämme die sich durch ein schnelles Wachstum, eine breit Nutzung von verschiedenen Kohlenstoffquellen und einer hohen toleranz gegenüber Fermentationsinhibitoren auszeichnen klar bevorzugt.

Im ersten Teil dieser These wurden daher fünf gut charakterisierte, oleogene Hefestämme mit den oben genannten Eigenschaften - *Rhodotorula glutinis*, *Trichosporon asahii*, *Rhodotorula mucilaginosa*, *Rhodotorula toruloides* und *Cutaneotrichosporon oleaginosus* – auf Ihr Wachstum und die Felxibilität in Ihrer Nährstoffnutzung in komplexen Hydrolysaten marinen und terrestrischen Ursprungs systematisch evaluiert. DieseAnalyse lieferte neue Erkenntnisse über ihre Lipid- und Biomasse-Erträge, das Zuckerverwertungspotential, das Fettsäureprofil der verschienenden Hefestämme in komplexen Nährmedien. Darüberhinaus wurden Erkenntnisse über die wachstumshemmende Wirkung der Hydrolysate generiert. Hier zeigte sich, dass *C. oleaginosus* und *T. asahii* ein breiteres Spektrum der Substrataufnahme aufweisen. Insbesondere *C. oleaginosus* erwies sich unter den getesteten Bedingungen als der effizienteste und vielseitigster Stamm zur Herstellung von Hefeöl.

Die genetische Zugänglichkeit der jeweiligen olebildenden Hefestämme ermöglicht potentiall eine flexible Stammoptimierung durch metabolische Stoffwechseloptimierung. Dies Produktflexibilisierung, ist auch von kommeerzieller Bedeutung da so auch hochwertige

Ölprodukte, wie Kakaobutter dargestellt werden können. Bislang wurden für *C. oleaginosus* nur zufällige Genom-Editierverfahren eingesetzt. Diese führen zu eineem sher hohen Screeningaufwand und ungewollten Geninsertionen die zu unerwünschten phenotypischen Veränderungen führen können.

Im zweiten Teil dieser These wurde daher die gezielte, CRIPSR/Cas vermittelte Genom editoeirung für *C. oleaginosus* optimiert und zur generierung von neuen Fettsäureprofilen angewendet. Hier konnte eine effiziente, flexible und zielgerichtete Gene Editing-Verfahren entwicklet werden, was auf einer plasmidfreien, transienten Cas9-Dosierungsstrateige beruht. Die so vorangetriebene metabolische Optimierung von *C. oleaginous* führte zu rekombinanten Stämmen mit veränderten Fettsäureprofilen, die in Ihrer chemischen Zusammensetzung Sonnenblumen- und Kakaobutter ähneln. Weiterhin konnte einStamm mit einer um 54 % erhöhten Lipidausbeute generiert werden. Die entwickelte Technik ebnet den Weg zum Aufbau einer robusten Technologieplattform für eine kommerziell relevante, maßgeschneiderte Ölproduktion durch *C. oleaginosus*. Gleichzeitig dient dieses Verfahren als vielversprechendes Referenzmdell für die gentechnische Veränderung anderer bisher genetisch unzugänglicher Hefen.

Die Daten in dieser These wurden in zwei hochrangigen Fachpublikationen und einem Patent veröffentlicht.

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# Contents

# 1

# Introduction

## Emersion of circular bioeconomy

After the industrial revolution, the energy demand has been on an upward trend leading to a rapid draining of fossil fuel reserves. While the primary energy source is fossil fuels covering over 80% of the consumption, these reserves are limited in amount and accessibility.[1] Moreover, greenhouse gas emissions mainly carbon dioxide ($CO_2$) produced by burning these fuels result in severe problems in ecosystems and loss of biodiversity.[2-4]

Some examples of the climate change impact reported by the World Meteorological Organization (WMO) only for 2021 include a 1.1 °C increase in the average temperature compared to the pre-industrial mean; a new record of 4.5 mm rise in the global sea level; the Antarctic ozone hole expansion to 24.8 million km; heatwaves in North America and the Mediterranean reaching 54.4 °C in California and 48.8 °C in Sicily; hurricane (the most significant was Ida in North Atlantic); cyclones such as Seroja in Indonesia, Typhoon Raj which crossed the Philippines, Tauktae impacting India and Pakistan, and Gulab; flood in China and Western Europe; drought in Canada, US, Turkey, Iran, Turkmenistan, Afghanistan, and Pakistan; severe thunderstorms in Central and western Europe; wildfires in California, Canada, Mediterranean region e.g. Algeria, Greece and Turkey.[2]

The constant increase in the energy price and the environmental issues thereof has driven the scientific community to rapidly intensify the exploration of renewable and cleaner alternative sources.[5] Additionally, over-dependence on fossil resources, which are becoming scarce, has led to the use of plant oils for biofuel and oleochemical production. As a result, deforestation and converting land to produce food crops for non-food purposes (land-use change), and consequently biodiversity endangerment has increased. For example, palm oil demand causes 1.5% deforestation of tropical rainforests in Malaysia and Indonesia, which provide 86% of the global palm oil. These conversions, in turn, increase the $CO_2$ release up to 420 times more than the annual drop in greenhouse gas emissions that is achieved by replacing fossil fuels.[6,7] Further, rising demand for these feedstocks affects their prices and results in competition between food and non-food applications over land and water resources. Hence, food prices increase.[8-10] (Figure 1)

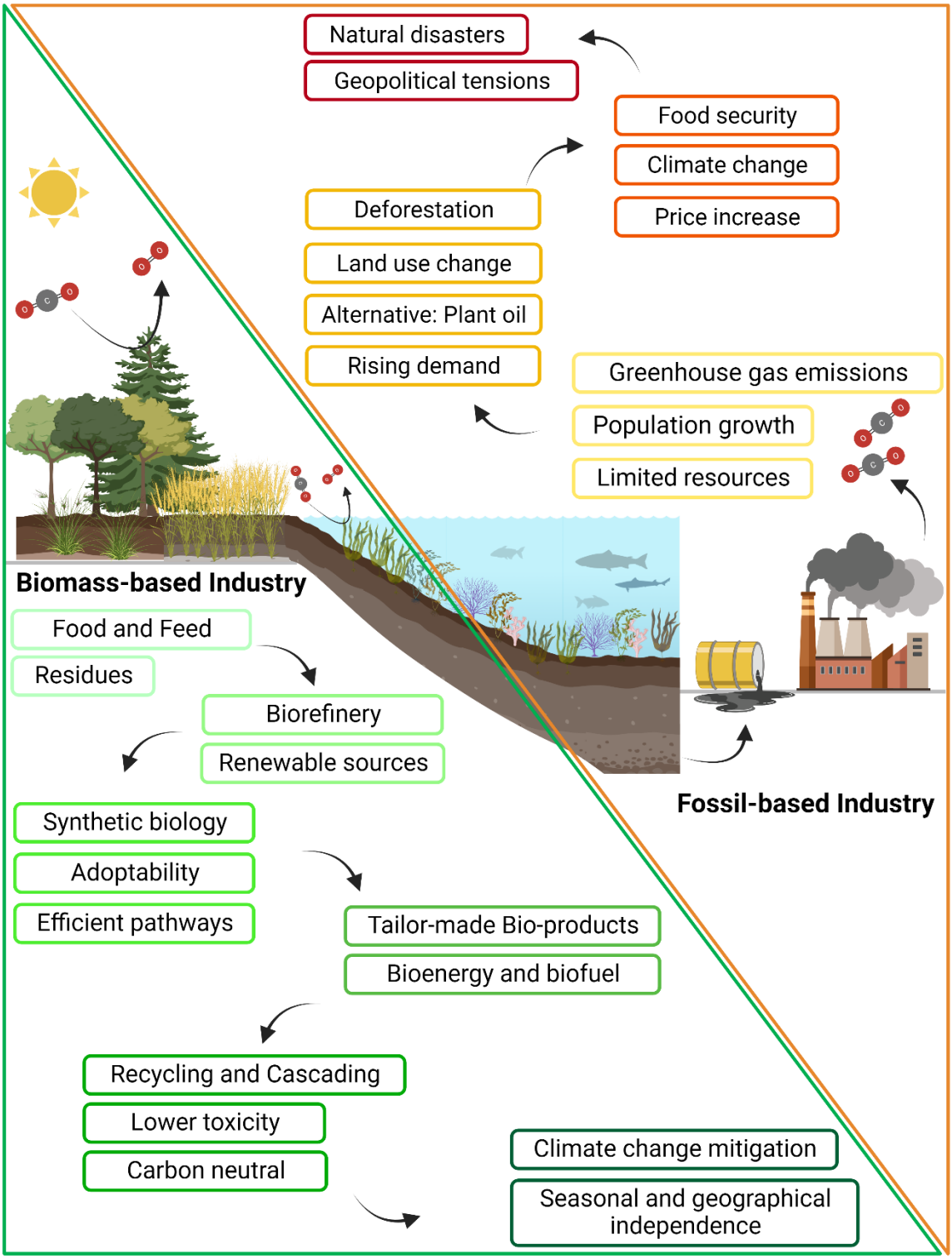


**Figure 1** *Fossil-based vs biomass-based industry.*

Moreover, the demand and consumption for vegetable oils increase remarkably every year as the applications for vegetable oils expand and the world population grows. Plants and animal

oils and fats are the major supply for the pharmaceutical, food, and cosmetic industries. Plant oils are still used worldwide mainly for human food purposes (65% in 2021).[11] The versatile application of these feedstocks has resulted in a yearly increase in their production, exceeding 200 Mt in 2021 [12,13], and it is projected to reach 249 Mt by 2031.[11] Therefore, the human population is intertwined with food security and environmental issues such as climate change, pollution, and high natural resources consumption rate.

Food security is as well constantly challenged by severe weather incidents, and unexpected events affecting the economy such as COVID-19 and the Russian-Ukrainian war.[2,14] Also, the Russian invasion of Ukraine contributed to the surges in food and energy prices that had been rising due to multiple former factors, namely global supply tightness, high demand, and yield reductions, for instance, reduced soybean output caused by drought in South America, palm oil diminution because of Typhoon Raj in Malaysia and COVID-19-associated circumscription, and rapeseed oil yield drop owing to drought in Canada. [14-16]

To that end, a circular and sustainable bioeconomy can play a significant role in overcoming these global problems.[17] By employing biological resources from agriculture, forestry, and marine origins efficiently, the “bioeconomy” aims at sustainable production of renewable carbon for many industrial applications. Biomass and waste transformation into chemicals and biofuels bring about carbon-neutral and renewable sources to mitigate crucial problems, such as global warming.[18] Further, the “circular economy” targets prolonging the value maintenance of resources and materials in the economy, and waste reduction through “cascading” which includes reuse, recycling, energy recovery, and remanufacturing (Figure 2).[19,20] These two concepts intersect to form the so called “circular bioeconomy”, where they share several goals: eco-efficiency and optimized resources; low GHG footprint; decreasing fossil carbon demand; enhanced valorisation of feedstocks as well as waste and side streams. Petroleum as the source of many commodities comes from biomass too. Biomass-based economy aims at replacing petroleum to achieve a bio-based industry featured by sustainable supply chains.[8] To that end, fungi, yeast, microalgae, and bacteria are capable of converting biomass, such as forestry- and agricultural residues and food waste to high-value products such as food, feed, biomaterials, and bioenergy.[8,19,21] Transformation of biomass provides renewable and carbon-neutral resources to produce commodities and alleviate land-use change, and negative impacts of fossil fuels, for instance, yeast cultivation to produce lipids for biodiesel production (Figure 1).[18,22]

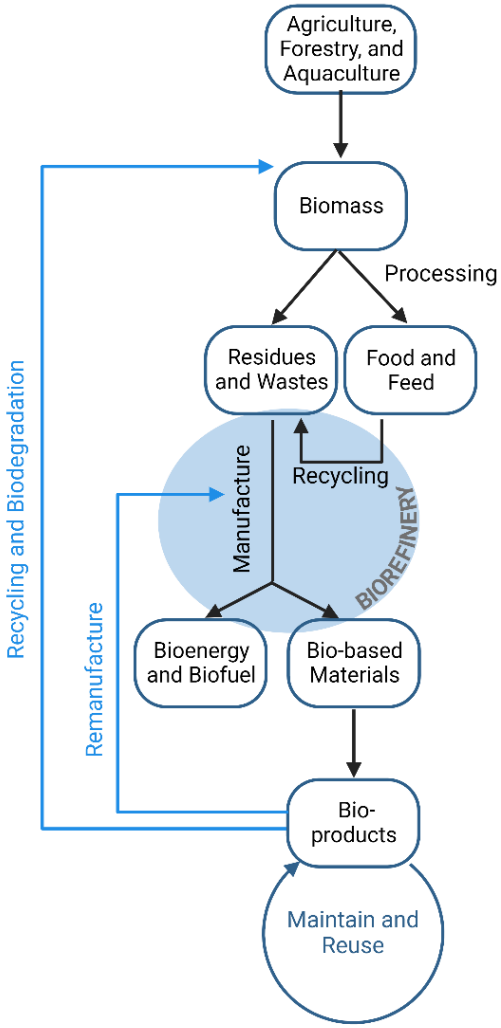


**Figure 2** *Circular bioeconomy Concept. Adopted from* [19].

## Oleaginous microorganisms

Therefore, third-generation feedstocks including oleaginous microorganisms have become the focus of alternative sources for oleochemicals and bioenergy rather than plant feedstocks.[23] Oleaginous microorganisms (e.g., bacteria, microalgae, yeast, and fungi) are capable of intracellular (or inside mycelia) lipid accumulation higher than 20% w/w of their biomass by substrate utilization. Microbial lipids or so-called single cell oils (SCOs) are mainly composed of triglycerides (TAGs). Also, there are glycolipids (in the membrane), phospholipids, and sterol esters present. In most cases, SCOs are chemically equivalent to several plant oils [24-26]. Compared with plant-based oils, these microorganisms have shown a high potential in terms of high oil content and high compositional quality (20% - 80% in microorganisms

compared with 5- 35 % in plants). Further, the need for water is reduced, and there is no need for fertile land, therefore they are not competing with agricultural activities. Moreover, the microorganisms can be easier genetically optimized, and they have better and stricter culture controllability e.g., pH, temperature, nutrient levels, and light intensity (for microalgae).[13,27]

Amongst the oleaginous microorganisms, oleaginous yeasts (OY) exhibit some advantages for lipid production, specifically fast growth rate, hence, short production cycles; ability to grow on a wide range of substrates including the renewable and cost-effective agricultural and industrial residues; growth independent of geographical, climatic, and seasonal conditions in contrast to plants and algae; high productivity; and no need for light and easier scale-up process in compared with algae.[17,21,27-29] The oleaginous yeast is the first microorganism to be observed for lipid accumulation and its exploration goes back to 1878. In 1895, *Metschnikowia pulcherrima* (previously known as *Torula pulcherrima*) was discovered. *Endomyces vernalis* was shown to produce 42% (w/w) lipid under nitrogen starvation conditions in 1915, which led to lipid productions for nutritional purposes in two factories in Germany during the next two years (world war I). The well-known oily yeast (OYs) strains namely, *Rhodotorula glutinis*, *Rhodotorula toruloides*, and *Lipomyces starkeyi* were discovered in the 1940s. Following nearly 100 years of OY investigations, the first biochemical study of yeast lipid accumulation was finally done in 1979, attributing the lipogenesis to a drop in intracellular AMP concentration and an increase in ATP level, as the nitrogen source is decreasing in the cultures. A series of reactions results in acetyl-CoA accumulation in the cytosol as the main precursor for lipid biosynthesis, where the ATP:citrate lyase has a crucial role. However, these events do not occur in non-oleaginous yeasts. Genetic optimization and modification of OYs surged in the 1990s, as genome sequencing and genetic engineering were advancing rapidly. For example, in 2004, *Yarrowia lipolytica* was fully sequenced, and genetically modified *Y. lipolytica* was reported to exhibit enhanced lipid production. The unprecedented increase in the number of publications on oleaginous yeasts during the last decade showcases the undeniable potential of these microorganisms in the emerging bioeconomic era.[17,29-31]

Until now, of the1600 yeast species that have been reported nearly 70 species have been observed to be oleaginous. The oil content varies depending on the strain, with TAGs reaching up to 90% of their DCW[13]. The well-characterized yeast genera mainly belong to Basidiomycota and Ascomycota, exemplary, *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, *Trichosporon*, *Cutaneotrichosporon*, *Lipomyces*, *Yarrowia*, as well as some *Candida* species.[13,21,24,25]

The lipid biosynthesis in OYs takes place through *de novo* and *ex novo* pathways. *Ex novo* pathway is activated in some yeasts for instance, *Y. lipolytica*, with the uptake and hydrolysis of hydrophobic substrates. The resultant free FAs are metabolized for cell growth or channelled into the lipid synthesis to generate a new fatty acid (FA) profile that varies from the initial substrate. This process takes place during cell growth and is independent of nutrient limitation. The Free FAs break further down into acetyl-CoA and acyl-CoA chains.[13,32] In *de novo* lipid accumulation, the sugar substrates are converted to pyruvate after glycolysis (Figure 3). The pyruvate is afterwards transferred to the mitochondria to form acetyl-CoA by pyruvate dehydrogenase (PDH) complex. At this stage, the generated acetyl-CoAs can be taken into the Krebs (TCA) cycle or transported to the cytoplasm. In non-oleaginous yeast, the acetyl-CoA is converted to acetyl-carnitine to be exported to the cytosol, since the mitochondrial membrane is not permeable to acetyl-CoA. However, in oleaginous strains, there is an additional source for acetyl-CoA generation. The adenosine monophosphate deaminase (AMPD) activity is enhanced due to nutrient (such as nitrogen) limitation in the media. Since this enzyme needs AMP, a sharp decrease in the AMP level occurs. This restricts $NAD^+$-dependent isocitrate dehydrogenase (IDH) activity, which requires AMP. Consequently, citrate accumulates in mitochondria, and is transferred to the cytosol via the citrate/malate shuttle, a process unique to oleaginous yeasts. In the cytosol, the citrate reservoir is converted to acetyl-CoA, the building block of FAs, and oxaloacetate by ATP:citrate lyase.[13,31-34]

Citrate + CoA + ATP → Acetyl- CoA + oxaloacetate + ADP + Pi

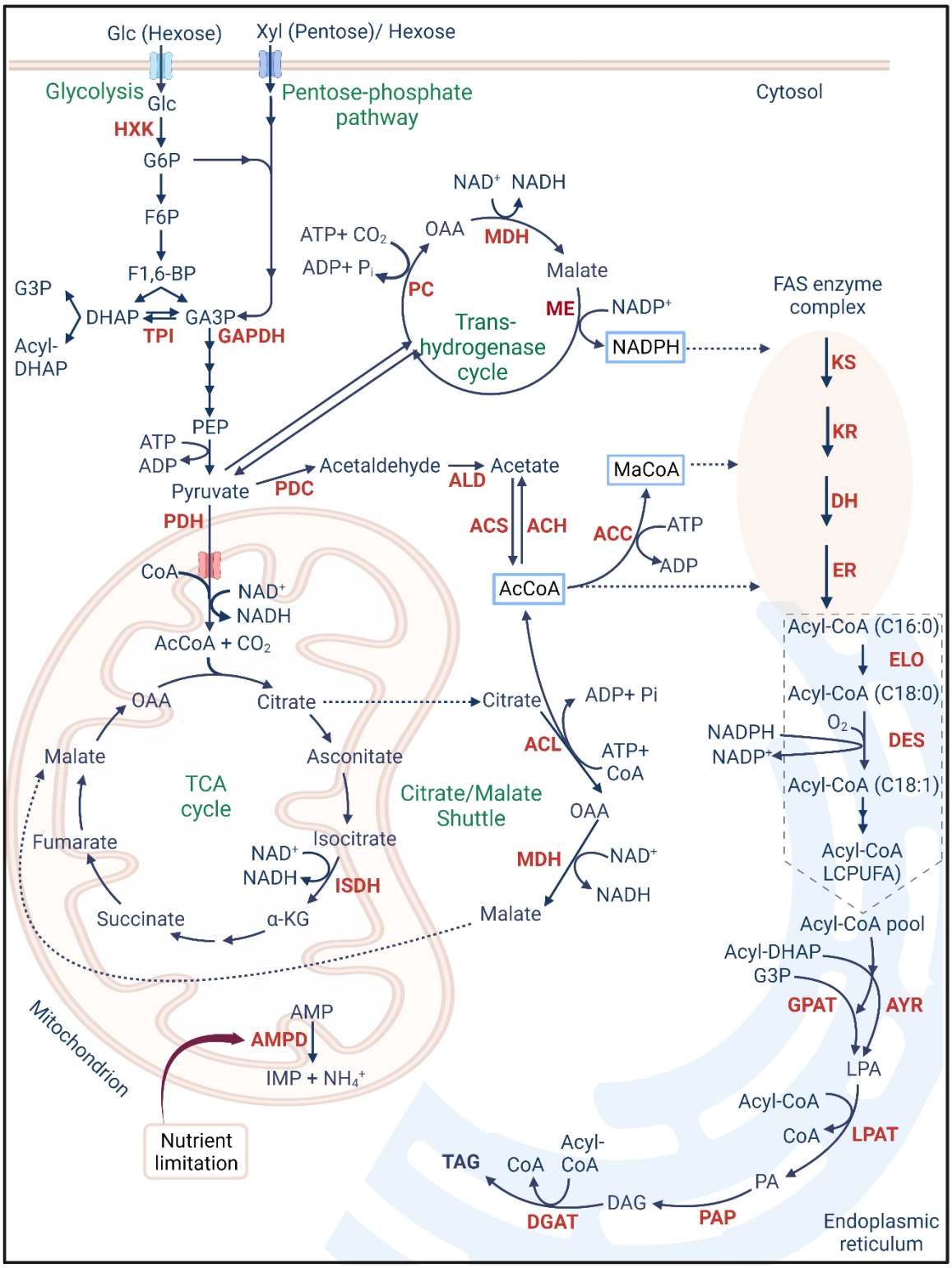


**Figure 3** *Lipid synthesis pathway in oleaginous yeast.*
*Glc, glucose; HXK, hexokinase; Xyl, xylose; G6P, glucose-6-phosphate; F6P: fructose-6-phosphate; TPI, triose phosphate isomerase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GA3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; G3P: glycerol-3-phosphate; PEP, phosphoenolpyruvate; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; AcCoA, acetyl-CoA, α-KG, α-Ketoglutaric acid; ISDH, isocitrate dehydrogenase; AMPD, adenosine monophosphate deaminase; IMP, inosine*

*monophosphate; PDC, pyruvate decarboxylase; ALD, acetaldehyde dehydrogenase; ACS, acetyl-CoA synthetase; ACH; acetyl-CoA hydrolase; ACL, ATP-citrate lyase; MDH, malate dehydrogenase; OAA: oxaloacetate; ACC, acetyl-CoA carboxylase; MaCoA, malonyl-CoA; PC, pyruvate carboxylase; ME, malic enzyme; FAS, fatty acid synthetase; KS, ketoacyl synthase; KR, ketoreductase; DH, dehydratase; ER, enoyl reductase; ELO, elongase; DES, desaturase; LCPUFA, long-chain polyunsaturated fatty acid; GPAT, glycerol 3-phosphate acyltransferase; AYR, acyl-DHAP reductase; LPA, lysophosphatidate; LPAT, lysophosphatidate transferase; PA, phosphatidate; PAP, phosphatidate phosphatase; DAG, diacylglyceride; DGAT, diacylglycerol acyltransferase; TAG, triglyceride. Adopted from [13,34-37].*

The acetyl-CoA carboxylase (ACC), an essential enzyme for the *de novo* lipid biosynthesis pathway, then catalyses the conversion of acetyl-CoA to malonyl-CoA. A sequence of condensation reactions, integrating acetyl-CoA as the initial building block and malonyl-CoA as the elongation unit along with NADPH, is catalysed by the fatty acid synthase (FAS) to synthesize fatty acyl-CoA.[38,39] The ability to provide the FAS with a steady cytoplasmic acetyl-CoA pool, as well as NADPH as a reductant, is shown to be of crucial importance for oleaginicity.[26] Depending on the species, the composition of fatty acyl-CoA can vary (14 or 16 saturated acyl-CoA molecules [C14:0-CoA, C16:0-CoA]). YO mainly consists of palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic acid (C18:2).[13] In addition, these oleaginous yeasts can produce rare and expensive lipids such as PUFAs, which could be of medical or nutritional importance.[24,25] Elongations by elongase enzymes to C18:0 or desaturation to C14:1-CoA, C16:1-CoA, or C18:1-CoA occur in the endoplasmic reticulum (ER). The common desaturases are Δ9 and Δ12 desaturase. The first double bond is inserted on a saturated fatty acid by Δ9 desaturase. The Δ12 desaturase catalyses the production of linoleic acid by inserting a second double bond.[38,39] All the synthesized fatty acyl-CoAs are pooled and diverted into lipid formation such as TAGs, the glycerol triesters with non-polar fatty acids that are accounted for the major component of the microbial oil. In the ER, a glycerol-3-phosphate (G3P) is combined with fatty acyl-CoAs, resulting in diacylglycerol (DAG), and further to TAG (Figure 3).[17,25]

## Optimization of yeast lipid production

For further advancement in bioeconomy, efficient, cost-effective, and sustainable microbial bioprocesses are crucial.[40,41] Process optimizations, exploring new strains, and strain enhancements can lead to developing bioprocesses favourable in cost.[23] The cost of substrates and the fermentation and downstream processing conditions such as oil recovery are key elements of the development of an economically feasible process. In fact, preparing the medium is the most expensive step.[5,42] To that end, researchers have evaluated and studied the utilization of municipal, industrial, forestry, marine, and agricultural residues as cost-effective substrates by microbes for lipid production[41,43,44], for instance, undetoxified corncob hydrolysate[45]; sugarcane bagasse hydrolysate[46]; potato wastewater[47]; bioethanol

wastewater[48]; corn stove hydrolysate[49]; cheese whey[50]; algae residues[51]. The valorisation of these streams in the bioprocesses producing high-value bio-based products brings about low-cost waste disposal as well as environmentally friendly end-products such as microbial oil as they exhibit properties such as biodegradability, biocompatibility, and non-toxicity.[41] This conversion of biomass into value-added materials is regarded as biorefinery, which also aims at making the process cost-competitive and closes the loop in the bio-based circular economy.[41,52] Cost, abundance, and fermentation productivity define the suitability of the biomass[5]. The productivity of oil production is affected by several factors, such as the selected strains, temperature, nutrient level in the media, pH, and the presence of inhibitors in the media.[42] Lignocellulosic biomass biorefinery, for instant, has been widely studied for several years as one of the most abundant and sustainable feedstocks for microbial oil production[35]. However, the utilization of pentose sugar, xylose the second-most abundant sugar in these residues, by microorganisms is considered challenging.[53] Moreover, growth inhibitors, such as furfural are the by-products of lignocellulosic biomass hydrolysis and pre-treatment.[54] To that end, the productivity of the bioprocess also depends upon the capabilities and features of the selected strain, e.g. substrate utilization, tolerance to stress and inhibiting factors and chemicals, fast growth, genetic tractability, and lipid yield. Therefore, finding a suitable yeast that can improve the hydrolysates specificity is an essential step of these bioprocesses, specifically to reduce the costs and improve the economic feasibility.[23,40,55] Consequently, many researchers have been exploring and screening new yeast strains for high productivity.[56-59] Moreover, the research for improving lipid accumulation with metabolic engineering attracted a lot of attention[34,60-62], for example, lipid production was enhanced by cytosolic redox engineering in *Yarrowia lipolytica*.[61] Furthermore, the production of high-value lipid products in yeasts was enabled by metabolic engineering[13], such as polyunsaturated fatty acids with a wide spectrum of health benefits[63], omega-3 fatty acids[64,65], conjugated linoleic acid with pharmaceutical effects[63], and hydroxy fatty acids with a wide range of applications in human health as well as in the production of cosmetics, surfactants, polymers, coatings, and lubricants.[66] Hence, strain selection as well as strain engineering would contribute greatly to enhancing the efficiency of the aforementioned bioprocesses.

### $CO_2$-Lubricant project

To that end, the $CO_2$-Lubricant project was funded by the German Federal Ministry of Education and Research, in order to bring the yeast oil (YO) one step closer to industrial application. This project consisted of industrial and scientific partners: Audi, Klüber Lubrication, Subitec GmbH, and the Technical University of Munich.

The $CO_2$ from air is sequestered by Algae and plants and is fixed via a reductive photosynthetic pathway, efficiently producing biomass, lipids, and other high-value compounds.[67,68] Therefore, for biorefinery purposes the residual agricultural and marine biomass were hydrolysed to free monomeric sugar, which can be heterotrophically assimilated by yeast.

The project aimed to convert the carbon sources resulting from photosynthesis to yeast lipids, which in turn would be used for bio-lubricant production. Therefore, various well-known oleaginous yeasts were analysed in terms of lipid production and growth efficiencies for biorefinery of biomass with marine as well as terrestrial origins. Further, the genetic tractability of the selected yeast was studied to ultimately enable the strain optimizations required to provide the YO with a proper fatty acid profile for future applications in bio-lubricant industries.

# 2

# Methods

This chapter includes the materials and methods used in the thesis. Detailed information is described and reported in the method and supplementary sections of manuscripts included in this thesis.

## 2.1 Strains, and media

*Cutaneotrichosporon oleaginosus* ATCC 20509 (DSM-11815) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) (Braunschweig, Germany), and *Rhodosporidium toruloides* CBS 14 was obtained from Centraalbureau voor Schimmelcultures (CBS). *Rhodotorula glutinis* (IBY050), *Trichosporon asahii* (IBY051), and *Rhodotorula mucilaginosa* (IBY052) were obtained from the Werner Siemens-Chair of Synthetic Biotechnology (WSSB) culture collection.

The wild-type strains were transferred and maintained on YPD- agar plates (glucose, 20 g/L; tryptone, 20 g/L; yeast extract, 10 g/L; agar-agar, 20 g/L). The inoculum media was YPD as well. For screening different Yeast wild type strains generic synthetic media for lipid accumulation include nitrogen limitation medium (carbon source: glucose, xylose, or mannitol 30 g/L; yeast extract, 0.75 g/L; $(NH_4)_2SO_4$, 0.0012 g/L; $MgSO_4 \cdot 7H_2O$, 1.5 g/L; $KH_2PO_4$, 0.4 g/L; $CaCl_2 \cdot H_2O$, 0.22 g/L; $ZnSO_4 \cdot 7H_2O$, 0.55 µg/L; $MnCl_2 \cdot 4H_2O$, 24.2 µg/L; $CuSO_4 \cdot 5H_2O$, 25 µg/L; $FeSO_4 \cdot 7H_2O$, 25 µg/L), and phosphate limitation medium (N-acetyl glucosamine 30 g/L; yeast extract, 0.75 g/L; $NH_4Cl$ 0.5 g/L; $MgSO_4.7H_2O$ 1.5 g/L; $KH_2PO_4$ 0.11 g/L; $Na_2HPO_4$ 0.387 g/L; $CaCl_2.2H_2O$ 0.22 g/L; $ZnSO_4 \cdot 7H_2O$, 0.55 µg/L; $MnCl_2 \cdot 4H_2O$, 24.2 µg/L; $CuSO_4 \cdot 5H_2O$, 25 µg/L)[63] were used. The pH of all synthetic media was adjusted to 6.5 prior to sterilization. Three complex media were used for screening of the wild-type oleaginous yeast strains, including wheat straw hydrolysate (WSH), corn steep liquor (CSL), and hydrolysate of brown algae *Laminaria digitata* (BAH). The *Laminaria digitata* biomass was hydrolyzed enzymatically at a 2 L scale according to our previous study.[69] The wheat straw hydrolysate was obtained from Clariant (Germany), diluted, and subjected to crossflow filtration using a 10 kDa polyether sulfone filter (Pall Corporation, US) to remove all proteins. The pH in both complex media was also set at 6.5 prior to sterile filtration. The corn steep liquor powder was commercially available and obtained from the TT baits (Germany). It was autoclaved at 134 ºC for 20 minutes and added to WSH at the final concentration of 5 g/L under sterile conditions before the shake flask experiments.

The *C. oleaginosus* auxotroph mutants were maintained on $YNB^{+}5FOA$ agar plates (yeast nitrogen base (YNB), 1.7 g/l; $NH_4SO_4$, 5 g/l; uracil, 50 mg/l; glucose, 20 g/l; agar, 20 g/l; 5-Fluoroorotic acid (5FOA, Fluorochem, Germany), 1 g/l), by initially resuspended in agar medium below 50 ˚C as top agar (containing 0.1 to 1 g/l 5FOA and YNB, 1.7 g/l; $NH_4SO_4$, 5

g/l; glucose, 20 g/l; agar, 20 g/l). The mutants harbouring an auxotrophy-complementing marker gene (selection marker *URA*5) were maintained on YNB$^-$ agar plates (without 5FOA), within an upper layer of agar (without 5FOA). The inoculum media was YPD. For cultivations of *C. oleaginosus* mutants, two media were used, minimal nitrogen media containing glucose (MNM+Glu)[63], and a nitrogen-rich media containing glucose and acetic acid (RM+AA+Glu) (glucose 30 g/L; yeast extract, 3 g/L; peptone 1.5 g/L; $(NH_4)_2SO_4$, 0.3 g/L; $MgSO_4 \cdot 7H_2O$, 1.5 g/L; $KH_2PO_4$, 2.4 g/L; $Na_2HPO_4$ 0.91 g/L; $CaCl_2 \cdot 2H_2O$, 0.22 g/L; $ZnSO_4 \cdot 7H_2O$, 0.55 µg/L; $MnCl_2 \cdot 4H_2O$, 22.4 µg/L; $CuSO_4 \cdot 5H_2O$, 25 µg/L; $FeSO_4 \cdot 7H_2O$, 25 µg/L), in both media pH was set at 6.5. *Trichoderma reesei* RUT-C30 (ATCC 56765) was obtained from The American Type Culture Collection. The cultivation medium for *T. reesei* consisted of yeast extract, 10 g/l; glucose, 10 g/l; $NH_4SO_4$, 1.4 g/l; $KH_2PO_4$, 2 g/l; $CaCl_2 \cdot 2H_2O$, 0.4 g/l; $MgSO_4 \cdot 7H_2O$, 0.3 g/l; NaCl; 1 g/l; $FeSO_4 \cdot 7H_2O$, 5 mg/l; $CoCl_2.6H_2O$, 3.7 mg/l; $MnSO_4.H_2O$, 1.6 mg/l; $ZnSO_4 \cdot 7H_2O$, 1.4 mg/l. For cloning and plasmid amplification, the *E. coli* DH5 alpha strain from Merck Millipore was used. *E. coli* DH5α was cultivated at 37 °C in Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone/ peptone, 10 g/L NaCl). Transformed *E. coli* was selected on LB-agar plates supplemented with kanamycin sulphate at a final concentration of 50 µg/mL (Roth, Germany).

## 2.2 Cultivation conditions

### 2.2.1 Screening oleaginous yeast wild-type strains

Inoculation cultures were made by taking single colonies of the yeast strains from YPD-agar plates into 20 mL of YPD and incubated overnight (120 rpm shaking, 28 °C). The cultivations were started by inoculating 100 mL of each medium to a start $OD_{600\ nm}$ of 0.1 in a 500 mL baffled shaking flask. All experiments were done in triplicates. The shaking flasks were incubated at 28 ºC and 120 rpm for 96 h. Samples were taken every 24 h and processed further for biomass, lipid, and fatty acid analysis. Error bars represent the standard deviation.

## 2.3 Cultivation of *C. oleaginous* mutants

### 2.3.1 Screening the engineered C. oleaginous strains

Screening experiments were started using an overnight culture for inoculation. The inoculations were conducted in 100 mL of liquid minimal nitrogen media (MNM+Glu) to an $OD_{600\ nm}$ of 0.1 in 500 mL baffled shake flasks (120 rpm shaking, 28 °C). The pH was set at

6.5 prior to sterilization. Samples were taken every 24 h for biomass, lipid, and fatty acid analysis. All experiments were done in triplicates. Error bars represent the standard deviation.

### 2.3.2 High cell density cultivation of the engineered *C. oleaginous* strains

The high cell density experiments were performed in DASbox four parallel bioreactor system (Eppendorf, Germany). The inoculation cultures were made as described above. The experiment started by inoculating 150 mL media in each bioreactor to a starting $OD_{600\ nm}$ of 0.5. The pH was adjusted at 6.5 with 3 M NaOH or 70–100% (w/w) acetic acid according to Masri et al.[70] The temperature was set to 28˚C, and dissolved oxygen was maintained at 50% by Stirring (350–800 rpm), aeration (8.0– 1.5 vessel volume/minute [vvm]), oxygen ratio (21–100%), and pressure (1.25–1.5 bar). In order to prevent foaming, an antifoam agent (Antifoam 204; Merck) was used (5% (v/v) solution). All cultivations were done in triplicates. Samples were taken every 24 hours. Error bars represent the standard deviation.

## 2.4 Generation of targeting and editing constructs

The construction and preparation of all nucleic acids and proteins used in targeted genome engineering of *C. oleaginosus* is reported previously. [71]

### 2.4.1 sgRNAs

Initially, all published scaffolds of the whole genome sequence of *C. oleaginosus* [72] were thoroughly analysed in smaller fragments (by Geneious Prime ® 2022.0.1 https://www.geneious.com), and an *in-silico* sgRNA library was constructed. All possible PAMs for Cas9 and its variants were determined via Geneious and corresponding sgRNAs were added to the library. The off-targets were analysed against the whole *C. oleaginous* genome in Geneious and on-target activities (using the target scoring algorithm by Doench et al. [73]) were determined. The sgRNAs with zero off-targets and possibly high target scores were selected for genetic engineering. The selected sgRNAs were ordered from Synthego (USA), and Eurofins Genomics (Germany).

Table 1 Single guide RNAs used in the study.

| crRNA sequence | Number |
|---|---|
| GCACCAACCAGAGCUGACGU | sgRNA1 |
| UCUUCCCAUCAUCUCCAUCG | sgRNA2 |
| GCUGUAGUCGACACACCCAG | sgRNA3 |
| GAACACCAUACUGGGCGCUG | sgRNA4 |
| AAGAAACCCAAGUGGCACAU | sgRNA5 |
| GUGACCGUAUGACCGUGGCA | sgRNA6 |
| AGGGUGGCGGCAGACAUUGU | sgRNA7 |
| CAACAUGACUCCAUGACCCA | sgRNA8 |
| UGGACUCGGAGUAGGCCUGG | sgRNA9 |
| UGGAUGCUCAUCAAGCCUCG | sgRNA10 |

### 2.4.2 Repair DNAs

In order to introduce the desired sequences, repair DNA sequences were applied. These repair DNAs were constructed by amplifying from genomic DNA or obtained from Twist biosciences (USA), and further assembled and cloned into a pUC vector harbouring the kanamycin resistance gene. The plasmid assembly was done in *E. coli* DH5α.

The plasmid constructions were done using restriction digestion enzymes according to the manufacturer's instructions (Thermo fisher Scientific) followed by ligation using T4 DNA ligase (Thermo Fisher scientific). The DH5α competent cells were transformed via the heat shock method, plated on LB-Kan agar plates, and incubated at 37 ˚C overnight.

To identify the correct sequences in the colonies, colony PCR was performed. Afterwards, single colonies were obtained for positive transformants and inoculated into liquid LB medium. The overnight cultures were then applied to plasmid isolation using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Ultimately, the complete repair DNA sequences were linearized by fast digest enzymes or PCR amplification, followed by electrophoresis on 1% agarose gel stained with MIDORI Green Xtra (NIPPON genetics). The fast digest reactions were performed according to the manufacturer's instructions (Thermo Fisher Scientific, Germany). The PCR reactions were performed using the Thermo Fisher Scientific GC buffer and Phusion High-Fidelity DNA-Polymerase according to the manufacturer's instructions. In order to recover the PCR products or digested gene fragments from the gel, the Monarch DNA Gel Extraction Kit (NEB) was used. Lastly, all repair DNA sequences were confirmed by Sanger sequencing (Eurofins Genomics, Germany) before yeast transformations. All repair DNAs constructed in this study were validated by sequencing (summarized in the supplementary tables included in the manuscript [71]).

### 2.4.3 CRISPR-Cas enzymes

Both Cas9 enzyme (EnGen Spy Cas9 NLS) and EnGen Spy Cas9 Nickase were obtained from New England BioLabs GmbH (NEB, Germany). The Cas9 mRNA was synthesized in our laboratory. Initially, a T7 promoter was introduced by the forward primer in front of the Cas9 gene (including NLS). The Q5 High-Fidelity DNA Polymerase (NEB) was utilized for the amplification. The PCR product was purified after the quality of the PCR reaction was evaluated by agarose gel electrophoresis. Subsequently, it was applied to *in vitro* transcription using HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB). In order to remove the DNA template, the synthesized RNA was treated with DNase I, followed by a spin column purification step (NEB Monarch RNA Cleanup Kit). Next, capping and poly A tailing were conducted using NEB Vaccinia Capping System and NEB E. coli Poly(A) polymerase, respectively. Finally, the mRNA was purified using Monarch RNA Cleanup Kit (NEB) and stored at − 80 °C until use.

## 2.5 Strain construction

### 2.5.1 Spheroplasting.

A tailored spheroplasting procedure was developed for *C. oleaginosus* based on a previously described method with some modifications.[74] The spheroplasting procedure was done with two different lysis enzyme mixtures, a commercial lysing enzyme from *Trichoderma harzianum* (Glucanex obtained from Sigma-Aldrich), and a tailored enzyme mixture isolated from a hydrolase-producing organism, *Trichoderma reesei*.[71] In order to generate the enzyme system from *T. reesei* (HEST), the filamentous fungi were cultivated in the cultivation medium supplemented with *C. oleaginous* biomass at 28 ˚C for 48 hours. After separating the supernatant by centrifugation at 2000 g for 10 min, it was filtered through a 0.45 µm filter, concentrated 30-fold, and stored at 4 ˚C. For the spheroplasting procedure, 50 mL YPD medium was inoculated with a single yeast colony. The cells were harvested by centrifugation once the density was at 2 x $10^7$ cells/mL. The pellet was washed once with sterile water followed by 1 M sorbitol. Each lysis enzyme (Lysis enzyme from *T. reesei* (HEST) and Glucanex) was separately added to the spheroplasting buffer (1M sorbitol, 0.1 M sodium citrate, pH 5.8, 10 mM EDTA, 30 mM ß-mercaptoethanol, final concentration for HEST 10% v/v and for Glucanex 2% w/v) and mixed. An equal amount of yeast cells was resuspended in each spheroplasting buffer and incubated at 30˚C. The cell wall removal progress was analysed at 15-minute intervals, by adding 400 µl of the cells to 100 µl of sodium dodecyl sulphate (final concentration of 1%), followed by measuring the decrease in turbidity ($OD_{600}$

nm). Once more than 90% of the cells were spheroplasted, the mixture was centrifuged at 300 g for 2 minutes, and the pellets were washed multiple times to fully remove the lysis enzymes. Afterwards, the cells were resuspended in 2 mL CaST solution (10 mM $CaCl_2$, 1 M sorbitol, 10 mM Tris-HCl, pH 7.5) and 2 mL Frozen cell storage solution (40% glycerol, 14% dimethylsulfoxide, 0.2 M mannitol, 0.32 M sucrose, 0.1 M sorbitol, 0.2 M trehalose), and stored at − 80 °C until transformation. The efficiency of the procedure was further confirmed by plating 50 µl of the yeast spheroplasts onto non-isotonic plates. Also, the spheroplasts were tested for viability by plating them (50 µl) within a layer of top agar containing 1 M sorbitol.

### 2.5.2 Yeast transformation

Yeast transformations were performed as described previously.[71] The spheroplasts were washed with 1 M sorbitol (three times at 300 g) after thawing at 37 ˚C. Next, the pellet was resuspended in sorbitol containing 5 % (v/v) electroporation buffer (0.3 mM $Na_2HP0_4$, 0.02 mM $KH_2PO_4$, 10% glycerol), and incubated on ice for 5 minutes. Finally, the spheroplasts were pelleted and resuspended in 2 mL sorbitol containing 0.5 % (v/v) electroporation buffer.[74] Before electroporation, 50 µl of the spheroplasts were mixed with the CRISPR/Cas elements, and the repair DNA. The mixture was transferred to a pre-cooled electroporation cuvette with a 2 mm gap (Biolab, Germany), and pulsed once at 1500 voltage (MicroPulser Electroporator, BioRad, Germany). The cells were subsequently mixed with 5 ml liquid top agar (below 50˚ C) and poured onto selective agar plates. The plates were incubated at 28˚ C until single colonies were visible. In the Δ-9 desaturase gene knockout experiment, the plates were further supplemented with Oleic acid (AppliChem, Germany) for selection. Two *D9FAD* knockout plates, with and without C18:1 supplement, were incubated at each temperature, 28˚ C and 37 ˚C.

## 2.6 Analysis

### 2.6.1 Analysis of genomic DNA editing

Genomic DNA editing was performed as described before.[71] For all experiments, transformants were streaked out on new plates to obtain single colonies. Afterwards, single colonies were inoculated into a selection medium to grow overnight. The cells were harvested after 12- 48 hours of cultivation and used for genomic DNA extraction using Yeast DNA Extraction Kit (Thermo Fisher Scientific). In order to analyse the genetic modifications, PCR was performed on genomic DNA using genome-specific primers on the upstream and downstream of the targeted locus. The PCR products were resolved by electrophoresis and

purified using Monarch DNA Gel Extraction Kit. The DNA fragments were submitted to sanger sequencing, and the sequences were analysed using SnapGene software (from Insightful Science; available at snapgene.com).

### 2.6.2 Gravimetric analysis of biomass and lipids

In order to quantify yeast growth, dry cell weight (DCW) was determined by pelleting 2 mL culture (10000 g, 10 min). The pellets were washed with a 2 mL $ddH_2O$, followed by lyophilization in pre-weighed microtubes for 2 days at − 80 °C and 0.04 mbar (VaCo 5, Zirbus Technology, Germany). The Biomass yield (carbon: carbon efficiency), and biomass productivity were calculated based on equations 1 and 2, respectively:

1

$$\text{Biomass yield } (Growth\ efficiency)\ g/g = \frac{w\ biomass\ synthesized\ (g)}{w\ sugar\ consumed\ (g)}$$

2

$$Biomass\ productivity\ \frac{g}{Lh} = \frac{w\ biomass\ obtained\ (g)}{V\ of\ culture\ extracted\ (L) \times\ incubation\ time(h)} \times 1000$$

Intracellular total lipid weights were determined by extraction using chloroform and methanol (Folch solution) according to the protocol of Bligh–Dyer.[75] After washing the harvested cells were with $ddH_2O$, they were destructed by a high-pressure homogenizer (Mulsiflex C3, Avestine, Canada). Two times sequential solvent extraction using Folch solution was carried out on the destructed yeast cells, incubated in glass tubes for 2 h and 1 h, respectively. The chloroform layer containing yeast lipids was separated by centrifugation at 1000 rpm and aspirated under a nitrogen stream in pre-weighed glass vials. Finally, the extracted lipids, remaining in the vials were weighed, and the percentage of lipid content and total lipid (lipid titer) were calculated based on equations 3 and 4, respectively:

3

$$Lipid\ content\ \%\ w/w = \frac{w\ obtained\ lipid(g)}{w\ obtained\ dried\ biomass\ (g)} \times 100$$

4

$$Total\ lipid\ \left(lipid\ titre\ or\ concentration\ \frac{g}{L}\right) = \frac{w\ lipid\ obtained\ (g)}{V\ of\ culture\ extracted\ (L)}\ \times 1000$$

The lipid yield (substrate to lipid conversion rate), and lipid productivity were calculated based on equations 6 and 6 respectively.

5

$$Lipid\ yield\ g/g = \frac{w\ lipid\ obtained\ (g)}{w\ substrate\ consumed\ (g)}$$

6

$$Lipid\ productivity\ \frac{g}{Lh} = \frac{w\ lipid\ obtained\ (g)}{V\ culture\ extracted\ (L) \times\ incubation\ time(h)} \times 1000$$

### 2.6.3 Sugar analysis

Sugar analysis was performed by an Agilent 1260 infinity II LC system with a quarntenary pump and equipped with Diode Array (DA) and Refractive Index (RI) detectors. In this method, a Rezex ROA-organic H+ 8% column from Phenomenex was used (300x 7.8 mm). The isocratic mobile phase (5 mM H2SO4) was pumped at a flow rate of 0.5 mL/min. The run time was 60 minutes. The oven temperature was set at 70 ˚C, and the measurement was done by RID at 40 ˚C without cooling. The injection volume was 10 µl. All the sugars used for calibrations were obtained from Sigma, Germany.

The percentage of substrate consumed was calculated according to equation 7:

7

$$Substrate\ consumed\ \%\ \mathrm{w/w}\ at\ time\ point\ x = \frac{w\ substrate\ consumed\ at\ time\ point\ x\ (g)}{w\ total\ available\ substrate\ at\ the\ start\ point\ (g)} \times 100$$

### 2.6.4 Fatty Acid Determination

Fatty acid analysis was done according to the modified protocol of Griffiths et al [76]. The lyophilized pellets were weighed into glass tubes (between 2 and 10 mg). For quantification, an internal standard was added to each tube prior to the reaction (500 µl glyceryl trinonadecanoate C19:0-TAG, 0.2 mg/mL in GC grade toluol). The resulting solution was mixed at 1000 rpm for 1 minute. Afterwards, 1 mL of 0.5 M sodium methoxide solution in methanol was added to each tube, followed by heating up to 80 °C and mixing (750 rpm) for 20 min, subsequently cooling down to 5°C. Next, 1 mL of hydrogen chloride-methanol solution

(Sigma, Germany) was pipetted to each tube, followed by heating up to 80 °C and mixing (750 rpm) for 20 min, subsequently cooled down to 5°C. Finally, 400 µL dd$H_2O$ (1000 rpm, 30 s), and 1 mL hexane (2000 rpm, 3x 20s) were mixed with the samples, respectively (in the automated procedure, the mixing was performed in the quickMix device). The tubes were centrifuged for 3 min at 1000 rpm and cooled down to 5°C. 200 µL sample of the organic phase was transferred to a 1.5 mL vial for measurements by GC-2025 Plus gas chromatograph flame ionization detection (GC-FID) (Shimadzu, Japan) as previously reported.[63] Standardization was done with the Fatty Acid Methyl Ester (FAME) Marine Oil Standard. The fatty acid analysis was done manually for yeast wild-type screenings.[77] For the *C. oleaginous* experiments after genetic engineering, the weighed lyophilized pellets were applied to an automated fatty acid methyl esterification following the steps described above, by the Multi-Purpose Sampler MPS robotic from Gerstel.[71]

### 2.6.5 Visualization

Figures 1, 2, 3, and 4 were created with BioRender.com.

# 3
# Publications

## 3.1 Summary and Author Contribution

### Chapter I

**Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers**

The article “Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers” has been published in Microbial Cell Factories in December 2021 (DOI: 10.1186/s12934-021-01710-3). The author of this thesis, Pariya Shaigani, conceived the study, designed, and carried out the experimental work, performed data collection and evaluation, and wrote the manuscript.

Dependence on fossil fuels and population growth are associated with global warming and negative environmental impacts. The third-generation lipid feedstocks regarded as oleaginous microorganisms have the potential to meet future global demand in a sustainable manner. More generally, oleaginous yeasts are of great importance owing to high productivity, fast growth rate, a wide range of substrate utilization, and climate- and season-independent growth.

The yeast lipids are chemically equivalent to plant oils, as they are mostly composed of triglycerides. Therefore, they are promising sources for oleochemical and biofuel production as well as pharmaceuticals, food, and feed. Nonetheless, the production cost of yeast oil is higher than plant-based oils. Therefore, cost-effective options for fermentation medium, which makes for the majority of yeast oil production, should be explored. To that end, lignocellulosic residues as well as marine biomass have been reported for great sugar content, while they alleviate the need for land use change. Furthermore, yeast strains should be screened in corresponding substrates for higher lipid productivities and growth rates. Also, the pre-treatment steps in biomass hydrolysis result in liberating growth inhibitors, such as phenolic compounds. However, the cost of detoxification is not in favour of the economic feasibility of yeast oil production, hence, the capability to tolerate the growth inhibitors play an important role in the respective bioprocess.

The presented study compares five well-known oleaginous yeast strains in hydrolysates of terrestrial lignocellulosic residues and marine biomass, wheat straw, and brown algae, respectively. Owing to the abundance of agricultural residues and the area productivity of algae, these feedstocks are considered promising sustainable carbon sources for microbial

fermentation. The data collected on these cultivations were correlated and compared with control experiments, where each monomeric sugar available in hydrolysates was used as a sole carbon source in simple synthetic media under nutrient limitation conditions.

The most versatile substrate utilization was observed in the two taxonomically related basidiomycetes; *C. oleaginous* and *T. asahii*. The highest sugar uptake and growth efficiency in synthetic media were measured for *C. oleaginosus*. In complex hydrolysates, *C. oleaginosus* and *R. toruloides* were able to co-utilize glucose, xylose, and mannitol in brown algae hydrolysate. However, *R. toruloides* did not exhibit growth in the wheat straw hydrolysate. *C. oleaginosus* achieved a higher lipid content and productivity in wheat straw hydrolysate than in brown algae hydrolysate. The presented study showed that the addition of corn steep liquor powder to the cultivation media boost the growth and despite an increase in the nitrogen content, it does not suppress lipid accumulation.

## Chapter II

### Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector

The article “Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector” has been published in Microbial Cell Factories in February 2023 (DOI: 10.1186/s12934-023-02033-1). The author of this thesis, Pariya Shaigani, conceived the study, designed and carried out the experiments, performed data collection and analysis, and wrote the manuscript.

The unconventional oleaginous yeast *Cutaneotrichosporon oleaginosus (formerly known as Apiotrichum curvatum; Trichosporon oleaginosus)* has been studied since the 1980s, as it resembled a cocoa butter fatty acid profile. Until now, this strain has shown superiority in terms of lipid yield, range of substrate metabolism, and tolerance to growth inhibitors. In fact, it is the first yeast reported to channel lignin-derived aromatics through lipogenesis.

However, the lack of A stable, autonomously replicating plasmid, and dominance of non-homologous end joining over homologous recombination the genetic manipulation of *C. oleaginosus* has been only accomplished through random insertion of T-DNA containing the gene of interest and a selection marker (Hygromycin B resistance gene) into the genome by *Agrobacterium tumefaciens*. This method, however, is reported to lead to the insertion of several T-DNA copies in various loci, resulting in undesired genetic and phenotypic alterations.

In order to bypass these limitations, a plasmid-free transient Cas9-mediated gene editing technique was developed. In contrast to the agrobacterium-mediated transformation, the revolutionary CRISPR/ Cas method has many advantages. Importantly, it is target specific and accurate, while it is favoured to other nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) in terms of cost and simplicity.

In order for effective delivery of CRISPR elements including protein and nucleic acids through the cell wall, viable spheroplasts were successfully isolated using a tailored hydrolytic enzymatic mixture. Also, the functionality of Cas protein and mRNA in inducing targeted gene alterations was tested. The off-target effects and toxicity of Cas have been reported to be lower with a transient expression rather than stable. Further, the sgRNA design plays an important role in decreasing the off-target and increasing the specificity. The high GC content of *C. oleaginosus* and the presence of repetitive sequences in its genome challenges the sgRNA design. Also, the lack data base and online tools to identify the off-target activity of

sgRNAs for *C. oleaginosus* hinders the effective and straightforward employment of the CRISPR/Cas system in this strain. Therefore, an in-silico library of sgRNAs was built and used in the design of experiments and sgRNAs.

This study describes the performance of the newly Cas-mediated constructed strains in both minimal media and rich media under lipid accumulating conditions. One strain with overexpressed ∆9-desaturase depicted a 54% increase in the lipid yield. Further, the ∆D12-desaturase gene knockout resulted in increased oleic acid content and the absence of PUFAs in the yeast oil. While this technique permits flexible strain improvements to turn *C. oleaginosus* into a robust platform, it is also a promising tool to unravel the genetic accessibility in other unconventional yeasts.

## 3.2

# Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers

Microbial Cell Factories

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# Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers

Pariya Shaigani, Dania Awad, Veronika Redai, Monika Fuchs, Martina Haack, Norbert Mehlmer* and Thomas Brueck*

## Abstract

**Background:** Oleaginous yeasts are promising microbial platforms for sustainable, bio-based production of biofuels and oleochemical building blocks. Bio-based residues provide sustainable and cost-effective carbon sources for fermentative yeast oil production without land-use change. Considering the regional abundancy of different waste streams, we chose complex biomass residue streams of marine origin; macroalgae hydrolysate, and terrestrial origin; wheat straw hydrolysate in the presence, and absence of corn steep liquor as a complex nitrogen source. We investigated the biomass and lipid yields of an array of well-described oleaginous yeasts; *R. glutinis*, *T. asahii*, *R. mucilaginosa*, *R. toruloides, C. oleaginosus* growing on these hydrolysates. Furthermore, their sugar utilization, fatty acid profile, and inhibitory effect of the hydrolysates on yeast growth were compared. For correlative reference, we initially performed comparative growth experiments for the strains on individual monomeric sugars separately. Each of these monomeric sugars was a dominant carbon source in the complex biomass hydrolysates evaluated in this study. In addition, we evaluated N-acetylglucosamine, the monomeric building block of chitin, as a low-cost nitrogen and carbon source in yeast fermentation.

**Results:** *C. oleaginosus* provided the highest biomass and lipid yields. In the wheat straw and brown algae hydrolysates, this yeast strain gained 7.5 g/L and 3.8 g/L lipids, respectively. Cultivation in algae hydrolysate resulted in a higher level of unsaturated fatty acids in the lipids accumulated by all yeast strains. *R. toruloides* and *C. oleaginosus* were able to effectively co-utilize mannitol, glucose, and xylose. Growth rates on wheat straw hydrolysate were enhanced in presence of corn steep liquor.

**Conclusions:** Among the yeast strains investigated in this study, *C. oleaginosus* proved to be the most versatile strain in terms of substrate utilization, productivity, and tolerance in the complex media. Various fatty acid profiles obtained on each substrate encourage the manipulation of culture conditions to achieve the desired fatty acid composition for each application. This could be accomplished by combining the element of carbon source with other formerly studied factors such as temperature and oxygen. Moreover, corn steep liquor showed promise for enhancement of growth in the oleaginous strains provided that carbon substrate is available.

**Keywords:** Oleaginous yeasts, Carbon substrate preference, Fermentation inhibitor tolerance, Biomass yield, Lipid yield, Complex lignocellulosic/marine biomass hydrolysate

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## Background

Growing world population and climate change, combined with overused fossil resources are driving the development of sustainable bio-based processes. First generation

bioprocesses propagated the use of edible plant oils and starch for the generation of biofuels, chemical building blocks, cosmetics, and pharmaceuticals. However, the increasing use of edible oils for non-food applications has led to significant land use change and an associated loss of biodiversity, exemplified by the ever-growing demand for palm oil [1–5].

Oleaginous yeasts are promising microbial platforms for sustainable, bio-based production of oleochemical building blocks and biofuels [6, 7]. To that end, oleaginous yeasts allow oil production with short production cycles independent of geographical, seasonal, and climate limitations [8, 9]. Furthermore, they can utilize low-value substrates and turn them into valuable triglycerides (TAGs) [10]. Yeast triglyceride oil product is chemically equivalent to plant oil resources, while its production does not induce land use change or compete with any agricultural activity [11].

Nevertheless, it is inefficient to implement the industrial use of yeast oils (YOs) without a sustainable and cost-effective yeast bioprocess. The cost of the fermentation medium and a high oil yield are two of the main challenges in the techno-economic feasibility of YO production [11, 12]. Global regions (for instance Ukraine, US, Argentina, and Russia) with access to large agricultural lands generate large quantities of terrestrial lignocellulosic residues, and on the coast, marine biomass could be considered as the renewable and cheap feedstocks. Advanced bioprocesses enable the use of bio-based residues without land use change to generate commodity products and fine chemicals. For oleochemicals, this pertains particularly to the use of complex biomass residues, such as cereal straw, wood waste, or algae as a fermentation feedstock for oleaginous yeasts [11]. Therefore, the mild enzymatic hydrolysis of forestry- and agro-industrial residues, to liberate fermentable sugars, has attracted a lot of attention in that regard [4]. These waste biomass hydrolysates have been flagged as sustainable and low-cost carbon sources for fermentative YO production. Using these hydrolysates would enhance the economic and ecological efficiency of the process and would eliminate further deforestation and biodiversity loss by expanding plant oil production [13].

A promising sustainable feedstock for biorefinery purposes is algae, highly available as marine biomass. The benefits of this type of raw material are fast growth, high availability of alluvial biomass, and no competition with agricultural land [14]. Compared to the terrestrial plant biomass, marine residues have a 6–10 times higher area productivity and lack lignin, which eliminates the need for pre-treatments and provides for simplified enzymatic hydrolysis, both aspects increasing the energy, economic and ecological efficacy of the entire bioprocess [10, 11].

In addition, an abundant and sustainable lignocellulosic agricultural residue of terrestrial origin is wheat straw, which has also been established as straw hydrolysates for the production of biofuels, such as bioethanol or yeast-derived biodiesel [15, 16]. The worldwide estimation of wheat residues production in the year 2012 was 887 million tons, from which 400 million tons of wheat straw remained unused after all other applications [17]. A further important agro-industrial feedstock is corn steep liquor, a by-product of the corn wet-milling industry. Since 1909 it has attracted attention as an inexpensive alternative source of organic nitrogen and vitamins to supplement the fermentation medium [18–20]. This feedstock contains a considerable amount of water-soluble vitamins, polypeptides, and amino acids, which are great sources of organic nitrogen, as well as minerals cumulatively acting as growth stimulants [21].

In this study, five wellknown oleaginous yeast strains *Rhodotorula glutinis*, *Rhodotorula mucilaginosa*, *Rhodotorula toruloides* CBS14 (Synonym: *Rhodosporidium toruloides*), *Trichosporon asahii*, and *Cutaneotrichosporon oleaginosus* were compared in terms of growth and lipid accumulation, as well as fatty acid profiles. All strains are fromm Basidiomycota phylum. *R. glutinis*, *R. mucilaginosa*, and *R. toruloides* are close strains from genera *Rhodotorula* (class Microbotryomycetes, order Sporidiobolales). Lipid accumulation for these strains on glucose can reach 72, 15, and 58% (w/w) on a dry weight basis, respectively [22, 23]. In addition to lipid accumulation, these three yeast strains have attracted attention due to their natural ability to produce carotenoids, both being industrially relevant compounds [24, 25]. Furthermore, they can grow on a wide range of carbon sources [25–27]. For example, *R. mucilaginosa* has been grown on sugarcane bagasse, wheat straw, and wheat bran hydrolysate as well as Durian peel hydrolysates [22]. It is worth mentioning that the improvements in the genetic engineering tools and multi-omics data availability of *R. toruloides* have led to increased interest and application in both academy and industry [25, 28]. *T. asahii* and *C. oleaginosus* are from genera *Cutaneotrichosporon* and *Trichosporon* (class Tremellomycetes, order Trichosporonales), which were reported to be close relatives in this class [29]. Lipid accumulation for these strains on glucose has reached 33% and 53% on a dry weigh basis [30, 31]. *C. oleaginosus* yeast can grow and accumulate lipids up to 63.2%, 45%, 39.6%, 69.5% on a wide range of substrates, including volatile fatty acids, seagrass waste hydrolysate, waste-activated sludge, and aromatics, respectively [32–34]. *Cutaneotrichosporon oleaginosus* is a promising organism in this regard as a potential source of fatty alcohols and TAGs and lipids from *Cutaneotrichosporon oleaginosus*, resembles a cocoa butter-like fatty

acid composition [35]. In a recent study, *C. oleaginosus* yielded the highest intracellular lipid amongst oleaginous yeasts through a new process by converting acetic acid and sugar into lipid [11]. Strains were selected that showed process flexibility and robustness in terms of sugar utilization and tolerance to fermentation inhibitors [36].

For correlative reference, we initially carried out comparative growth experiments for the strains on four monomeric sugars, separately: glucose, xylose, mannitol, and N-acetylglucosamine. Next, the strains were cultivated and analyzed on complex biomass hydrolysates derived from terrestrial and marine biomass. In the fermentations performed, different substrate preferences and associated growth efficiencies of the selected yeasts could be determined. Finding the yeast strains which are capable of tolerating the by-products of hydrolysis and pre-treatment processes that potentially have inhibitory effects is an essential step for the industrialization of yeast oil production using complex biomass hydrolysates as a fermentation feedstock [27].

To diversify the application of yeast oils, modulation of the fatty acid distribution of generated triglyceride oils is required. This can be achieved by altering the fermentation conditions [37]. In our study, we demonstrate that metabolizing different carbon sources can significantly change the fatty acid distributions. This is the first systematic study comparing the sugar utilization and inhibitory effects of the hydrolysates for an array of well-described oleaginous yeasts using both defined and complex fermentation media.

## Results

### Sugar uptake, and growth efficiencies

*Synthetic media containing sole carbon sources* Four different monomeric sugars, including glucose, xylose, mannitol, and N-acetylglucosamine, were used as sole carbon source in the cultivation media of all five investigated yeast strains (Table 1). The choice of sugars in synthetic media was based on the monomeric content of the hydrolysates tested in this work. In order to induce lipogenesis, nutrient limitations were applied in each medium: nitrogen limitation in media containing glucose (MNM-Glu), xylose (MNM-Xyl), and mannitol (MNM-Man), and phosphate limitation in the medium containing N-acetylglucosamine (MPM-GlcNAc). *C. oleaginosus* and *T. asahii* were able to metabolize all four types of monosaccharides, while *R. glutinis* and *R. mucilaginosa* did not grow in the MPM-GlcNAc and MNM-Man, and *R. toruloides* did not show growth in MPM-GlcNAc (Additional file 1: Fig. S1 compares the growth of all strains in each medium). In the synthetic media containing glucose, xylose, and N-acetylglucosamine, the maximum dry cell weight (DCW $g_{dried\ biomass}/L_{culture}$) was reached by *C. oleaginosus* (p-value $\leq 0.05$), while in the synthetic media containing mannitol the maximum DCW was reached by *R. toruloides* (p-value $\leq 0.05$). The data showed that the final DCW in the MNM-Glu was slightly higher than in the MNM-Xyl. The determined sugar uptake rates in each cultivation are in line with the DCWs (Fig. 1). In the synthetic minimal nitrogen media containing glucose (MNM-Glu), the sugar content was exhausted by *C. oleaginosus* during the cultivation in the MNM-Glu, resulting in the maximum biomass concentration ($9.6 \pm 0.1$ g/L after 96 h of cultivation), while in the medium containing xylose (MNM-Xyl) 80% (w/w) of the available sugar was consumed. However, the other strains utilized glucose and xylose up to 55% (w/w) (Fig. 1 and Table 2). *C. oleaginosus* metabolized 66% (w/w) (20 g/L) of available GlcNAc, while *T. asahii* metabolized 30% (w/w) (9 g/L) GlcNAc over the cultivation period. Notably, by utilizing GlcNAc, *C. oleaginosus* reached its maximum DCW ($7.61 \pm 0.17$ g/L) within 48 h, whereas the same biomass concentration in the MNM-Xyl was measured after 96 h ($7.65 \pm 0.25$ g/L) (Fig. 1). In the medium containing mannitol, *R. toruloides* was the most efficient yeast strain amongst all tested in terms of sugar consumption (46% w/w of the available sugar).

**Table 1** Sugar content of the synthetic media and complex hydrolysates

| | Media | [Glucose] g/L | [Xylose] g/L | [Mannitol] g/L | [GlcNAc] g/L | [CSL] g/L |
|---|---|---|---|---|---|---|
| Synthetic media | MNM-Glu | 30 | – | – | – | – |
| | MNM-Xyl | – | 30 | – | – | – |
| | MNM-Man | – | – | 30 | – | – |
| | MPM-GlcNAc | – | – | – | 30 | – |
| Complex hydrolysates | *Laminaria digitata* hydrolysate | $19.0 \pm 0.1$ | $4.4 \pm 0.02$ | $9.2 \pm 0.04$ | nd | – |
| | Wheat straw hydrolysate | $27.5 \pm 0.3$ | $14.0 \pm 0.2$ | nd | nd | – |
| | Wheat straw hydrolysate + corn steep liquor | $27.5 \pm 0.3$ | $14.0 \pm 0.2$ | nd | nd | 5.0 |

*nd* not detected

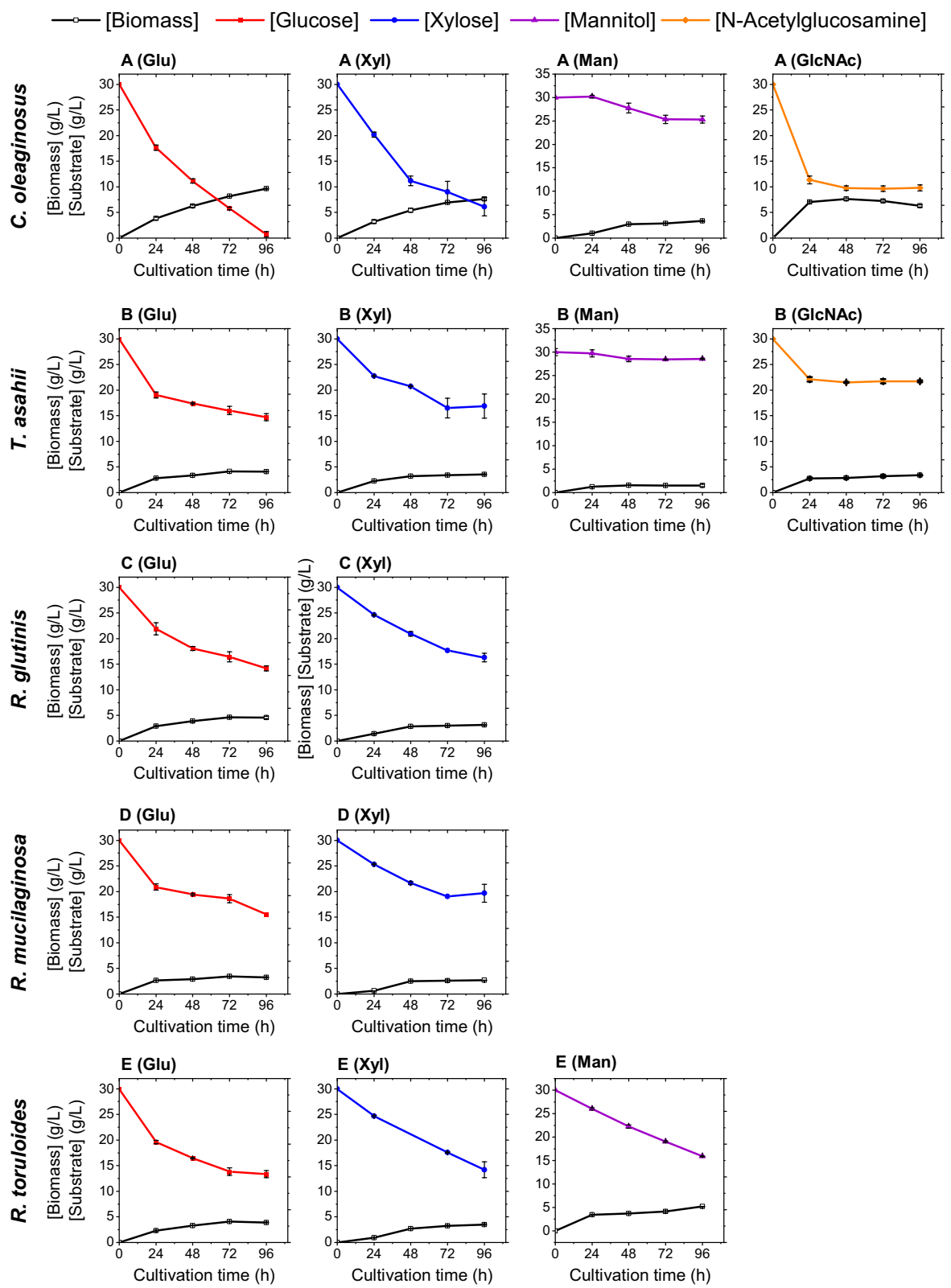


**Fig. 1** Growth rates and substrate consumption of each yeast strain in the synthetic media: **A** *C. oleaginosus* growth rates and substrate consumption on each monomeric sugar as the only carbon source; **B** *T. asahii*. **C** *R. glutinis*. **D** *R. mucilaginosa*. **E** *R. toruloides*

**Table 2** Measurement of lipid productivity and yield, biomass yield, and total utilized sugar in all media

| Strain | Media | Incubation time (h) | Lipid productivity (g/Lh) | Lipid yield $g_{Lipid}/g_{Sugar}$ | Biomass yield $g_{Biomass}/g_{Sugar}$ | [Total utilized sugar] g/L | Total sugar consumption % (w/w) |
|---|---|---|---|---|---|---|---|
| *C. oleaginosus* | MNM-Glu | 72 | 0.066 | 0.19 | 0.337 | 24.2 | 80.8 |
| | | 96 | 0.062 | 0.17 | 0.329 | 29.3 | 97.6 |
| | MNM-Xyl | 72 | 0.057 | 0.20 | 0.326 | 21.0 | 70.0 |
| | | 96 | 0.036 | 0.15 | 0.306 | 23.9 | 79.7 |
| | MNM-Man | 72 | 0.011 | 0.177 | 0.679 | 4.7 | 15.5 |
| | | 96 | 0.009 | 0.183 | 0.770 | 4.7 | 15.7 |
| | MPM-GlcNAc | 48 | 0.042 | 0.10 | 0.376 | 20.2 | 67.5 |
| | | 72 | 0.027 | 0.10 | 0.357 | 20.4 | 67.8 |
| | | 96 | 0.018 | 0.09 | 0.312 | 20.2 | 67.3 |
| | BAH | 72 | 0.045 | 0.13 | 0.675 | 24.6 | 73.8 |
| | | 96 | 0.040 | 0.16 | 0.697 | 24.4 | 73.2 |
| | WSH | 72 | 0.091 | 0.18 | 0.421 | 34.9 | 83.2 |
| | | 96 | 0.079 | 0.19 | 0.394 | 40.3 | 96.0 |
| | WSH + CSL | 72 | 0.097 | 0.19 | 0.373 | 40.2 | 95.8 |
| | | 96 | 0.072 | 0.17 | 0.419 | 40.2 | 95.8 |
| *T. asahii* | MNM-Glu | 72 | 0.005 | 0.03 | 0.295 | 14.0 | 46.6 |
| | | 96 | 0.011 | 0.07 | 0.269 | 15.3 | 51.0 |
| | MNM-Xyl | 72 | 0.005 | 0.03 | 0.258 | 13.5 | 45.0 |
| | | 96 | 0.009 | 0.06 | 0.277 | 13.1 | 43.8 |
| | MNM-Man | 72 | 0.003 | 0.14 | 0.939 | 1.6 | 5.3 |
| | | 96 | 0.001 | 0.07 | 1.058 | 1.4 | 4.8 |
| | MPM-GlcNAc | 48 | 0.004 | 0.02 | 0.333 | 8.5 | 27.6 |
| | | 72 | 0.003 | 0.03 | 0.389 | 8.3 | 27.6 |
| | | 96 | 0.003 | 0.03 | 0.407 | 8.3 | 27.6 |
| | BAH | 72 | 0.020 | 0.08 | 0.780 | 18.1 | 54.5 |
| | | 96 | 0.019 | 0.10 | 0.804 | 18.5 | 55.5 |
| | WSH | 72 | 0.007 | 0.04 | 0.401 | 13.1 | 31.3 |
| | | 96 | 0.009 | 0.06 | 0.460 | 13.8 | 32.8 |
| | WSH + CSL | 72 | 0.008 | 0.03 | 0.266 | 18.5 | 44.0 |
| | | 96 | 0.009 | 0.04 | 0.239 | 23.6 | 56.2 |
| *R. glutinis* | MNM-Glu | 72 | 0.009 | 0.05 | 0.341 | 13.5 | 45.2 |
| | | 96 | 0.011 | 0.06 | 0.291 | 15.8 | 61.8 |
| | MNM-Xyl | 72 | 0.012 | 0.07 | 0.243 | 7.3 | 41.1 |
| | | 96 | 0.007 | 0.05 | 0.230 | 13.7 | 45.7 |
| | BAH | 72 | 0.035 | 0.11 | 0.713 | 23.3 | 69.9 |
| | | 96 | 0.028 | 0.12 | 0.723 | 23.5 | 70.5 |
| | WSH | 72 | 0.020 | 0.08 | 0.549 | 18.7 | 44.5 |
| | | 96 | 0.019 | 0.09 | 0.488 | 19.7 | 46.8 |
| | WSH + CSL | 72 | 0.027 | 0.08 | 0.427 | 23.9 | 57.0 |
| | | 96 | 0.023 | 0.08 | 0.449 | 27.0 | 64.3 |

**Table 2** (continued)

| Strain | Media | Incubation time (h) | Lipid productivity (g/Lh) | Lipid yield $g_{Lipid}/g_{Sugar}$ | Biomass yield $g_{Biomass}/g_{Sugar}$ | [Total utilized sugar] g/L | Total sugar consumption % (w/w) |
|---|---|---|---|---|---|---|---|
| *R. mucilaginosa* | MNM-Glu | 72 | 0.004 | 0.03 | 0.303 | 11.4 | 37.9 |
| | | 96 | 0.007 | 0.04 | 0.225 | 14.4 | 48.1 |
| | MNM-Xyl | 72 | 0.005 | 0.03 | 0.246 | 11.0 | 36.6 |
| | | 96 | 0.003 | 0.03 | 0.268 | 10.3 | 34.4 |
| | BAH | 72 | 0.021 | 0.07 | 0.704 | 20.6 | 62.0 |
| | | 96 | 0.014 | 0.06 | 0.709 | 20.6 | 62.0 |
| | WSH | 72 | 0.010 | 0.05 | 0.553 | 13.3 | 31.6 |
| | | 96 | 0.007 | 0.05 | 0.518 | 14.1 | 33.7 |
| | WSH + CSL | 72 | 0.010 | 0.04 | 0.468 | 19.2 | 45.7 |
| | | 96 | 0.009 | 0.04 | 0.474 | 21.5 | 51.2 |
| *R. toruloides* | MNM-Glu | 72 | 0.019 | 0.09 | 0.253 | 16.2 | 53.9 |
| | | 96 | 0.018 | 0.10 | 0.242 | 16.7 | 55.6 |
| | MNM-Xyl | 72 | 0.013 | 0.07 | 0.260 | 12.4 | 41.4 |
| | | 96 | 0.011 | 0.07 | 0.219 | 15.8 | 52.7 |
| | MNM-Man | 72 | 0.022 | 0.14 | 0.375 | 11.0 | 36.6 |
| | | 96 | 0.021 | 0.14 | 0.367 | 14.1 | 47.0 |
| | BAH | 72 | 0.038 | 0.11 | 0.614 | 25.6 | 76.8 |
| | | 96 | 0.015 | 0.05 | 0.593 | 26.3 | 78.9 |

The stationary phases were determined according to the DCWs, and afterward time points 72 h and 96 h were selected to analyze the lipid accumulations

*Complex biomass hydrolysates* To determine the growth of the yeasts on terrestrial lignocellulosic and marine biomass hydrolysates, we chose two complex media for further analysis; brown algae hydrolysate (BAH) from *Laminaria digitata* and wheat straw hydrolysate (WSH). Additionally, the wheat straw hydrolysate was then supplemented with corn steep liquor as a nitrogen source (WSH + CSL) (Table 1). The biomass yield per gram substrate consumed in Table 2 indicates that, relative to synthetic media, cultivations in the hydrolysates enhanced the growth efficiency ($g_{biomass}/g_{utilized\ sugar}$) of all yeast strains considerably (p-value $\leq 0.05$).

The BAH was composed of three carbon sources: glucose, xylose, and mannitol, and it allowed the growth of all five yeast strains (Table 1). In particular, *C. oleaginosus* and *R. toruloides* utilized all three sugars simultaneously. In addition, an improved mannitol consumption rate by *C. oleaginosus* could be determined. Over 80% (w/w) of the glucose content, 54% (w/w) xylose, and 62% (w/w) mannitol were consumed by *C. oleaginosus*, and over 90% of xylose and glucose, and 30% (w/w) of mannitol were consumed by *R. toruloides* (Fig. 2 and Additional file 2: Table S1). An efficient co-utilization of glucose and xylose (97% and 86% w/w, respectively) was seen in *R. glutinis* as well. An uptake of less than 10% (w/w) of the mannitol source could be determined in *R. glutinis.* Interestingly, the growth trend, the final DCW, and the biomass yield of *R. glutinis* matched those of *C. oleaginosus*, both showing the highest biomass growth in the BAH (DCW after 96 h; $16.95 \pm 0.21$ g/L and $16.97 \pm 0.25$ g/L, corresponding to 0.72 $g_{biomass}/g_{sugar}$ and 0.69 $g_{biomass}/g_{sugar}$, respectively (Table 2). Moreover, *T. asahii* and *R. mucilaginosa* consumed glucose preferentially to xylose and mannitol, indicating the glucose carbon catabolite repression. Even though *T. asahii* is taxonomically closer than *R. glutinis* to *C. oleaginosus*, it did not perform well in the BAH in the current study [29].

The WSH contained glucose and xylose (Table 1) and our data demonstrated that all strains tested are able to utilize both monosaccharides as sole carbon sources in the synthetic media, resulting in biomass and lipid formation (Fig. 3). However, *R. toruloides* did not exhibit any growth when cultivated in the WSH. In contrast, the final biomass accumulation, and consumed sugar of *C. oleaginosus* in this hydrolysate (sugar exhausted, DCW at $15.86 \pm 0.28$ g/L) was remarkably higher compared to the other strains. Other yeast strains utilized only up to 50% of available, fermentable sugars (Fig. 2). In general, in BAH, better performance in terms of cell mass production by *R. glutinis*, *T. asahii*, *R. mucilaginosa* and

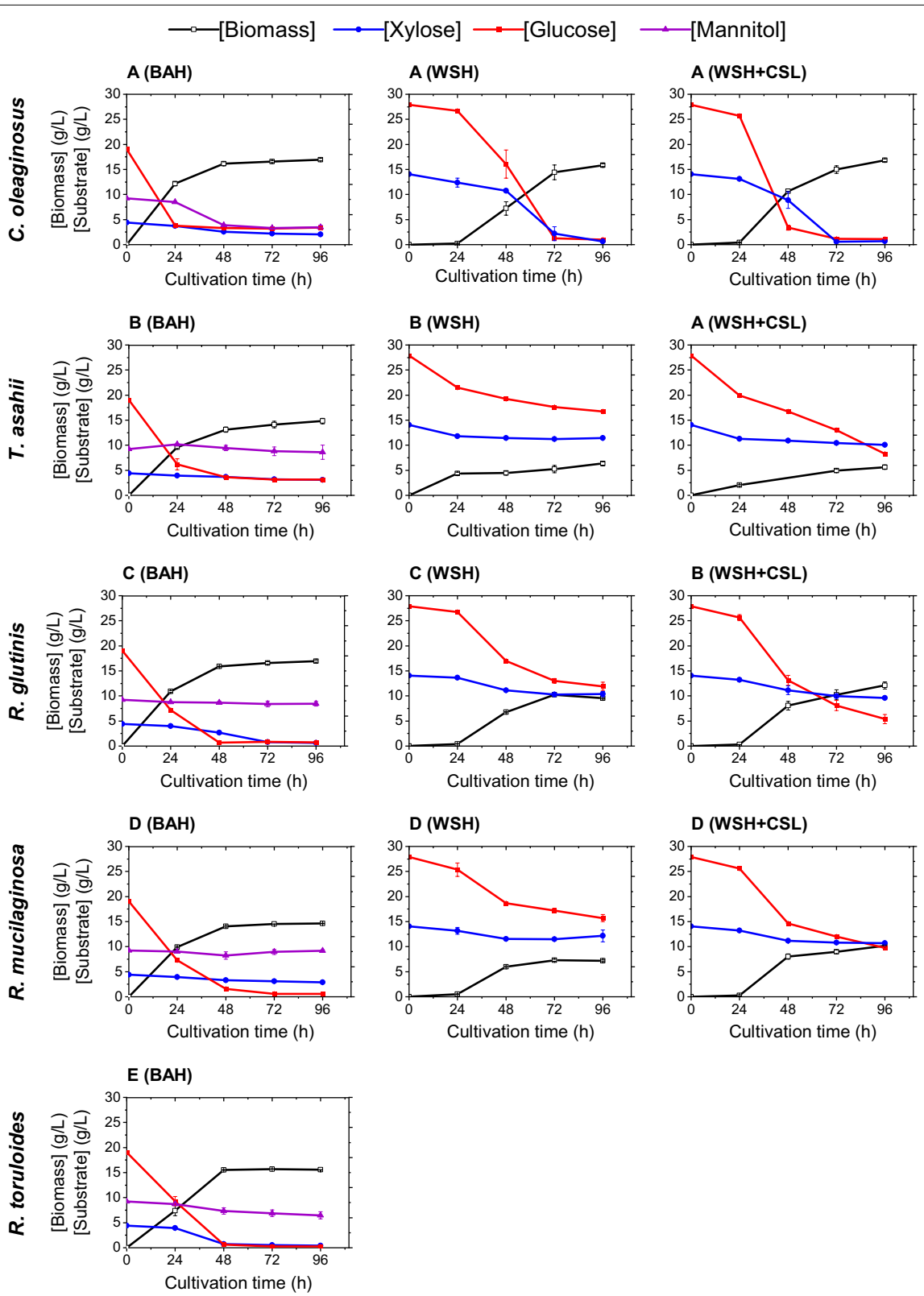


**Fig. 2** Growth rates and substrate consumption of each yeast strain in the complex media: **A** *C. oleaginosus* growth rates and substrate consumption **B** *T. asahii*. **C** *R. glutinis*. **D** *R. mucilaginosa*. **E** *R. toruloides*

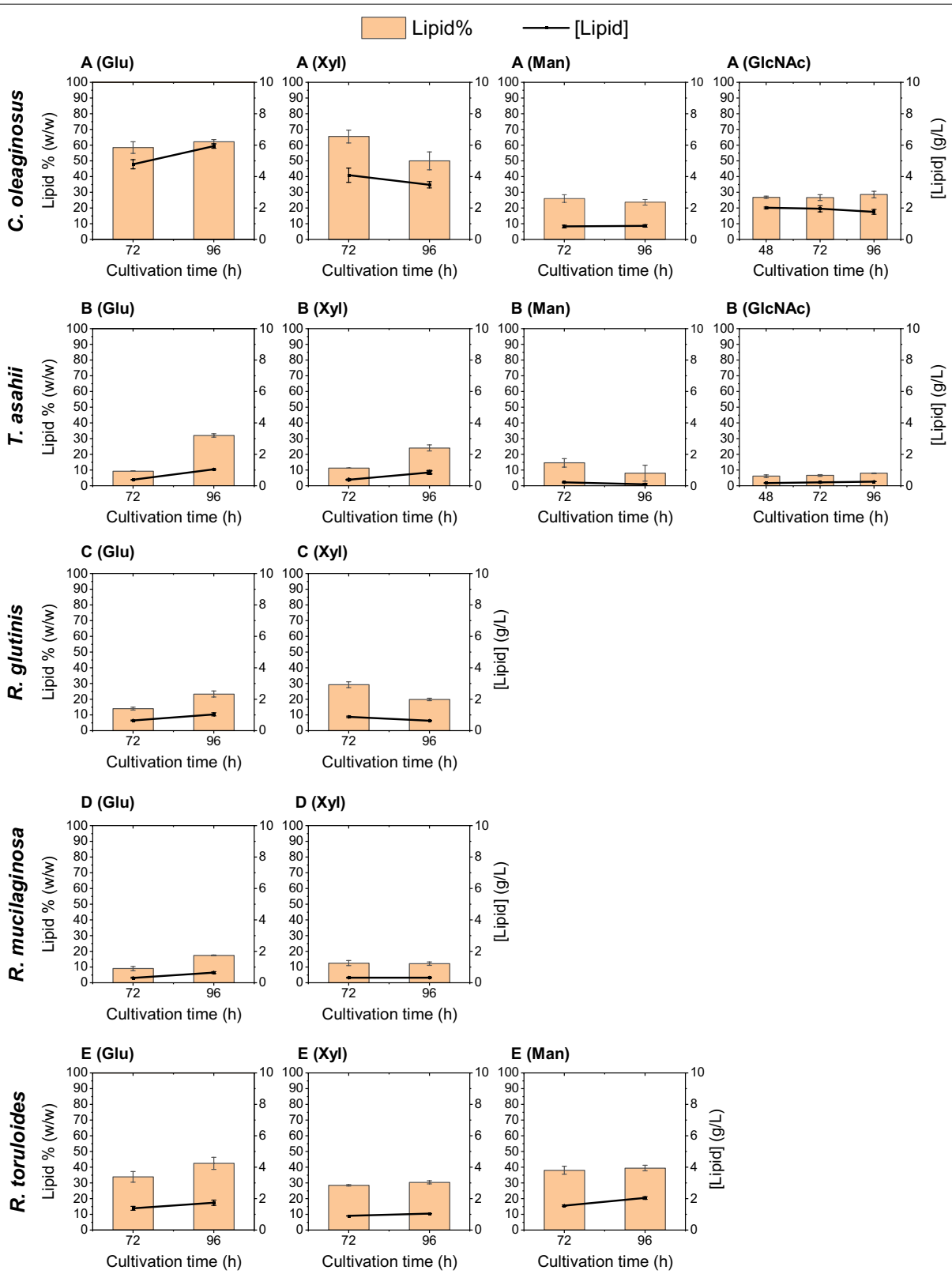


**Fig. 3** Lipid contents and total lipid of each yeast strain in the synthetic media: **A** *C. oleaginosus* lipid content and lipid concentrations in synthetic medium containing each monomeric sugar as the only carbon source; **B** *T. asahii*. **C** *R. glutinis*. **D** *R. mucilaginosa*. **E** *R. toruloides*

*R. toruloides* could be observed when compared to WSH (Fig. 2).

Therefore, CSL was added to WSH as a source of nutrients to boost growth. Adding CSL to the WSH led to improvements in the DCW for *C. oleaginosus*, *R. glutinis* and *R. mucilaginosa* by 6%, 24%, and 41% (w/w) at the end of the cultivation time, respectively. This enhancement of growth was mirrored in the sugar consumption rates. *R. glutinis,* and *R. mucilaginosa* increased their monosaccharide uptake in the wheat straw hydrolysate supplied with CSL, compared to WSH with no extra nitrogen source (Table 2). *T. asahii* consumed more sugar in the presence of CSL as well, however, no difference in its biomass and lipid yield could be measured due to cell-aggregation of *T. asahii* in the WSH cultivation after 72 h.

#### Lipid yield and productivity

*Synthetic media containing sole carbon sources* The total intracellular lipid contents (gram lipid per gram biomass) varied with the examined yeast strains and the choice of culture medium. Except for MNM-Man, *C. oleaginosus* demonstrated the highest lipid contents in the tested media (Fig. 3). In MNM-Man, *R. toruloides* yielded the highest lipid content (39 ± 1.7% w/w of dry weight in 96 h). This yeast strain achieved a higher lipid content in MNM-Glu (42.4 ± 3.8% (w/w) of dry weight in 96 h) compared to MNM-Xyl (30.4 ± 0.9% w/w in 96 h). Notably, *R. mucilaginosa* and *T. asahii* also showed better performance on glucose than on xylose. The lipid content in *T. asahii* reached its maximum after 96 h cultivation in both MNM-Glu and MNM-Xyl, measuring at 31% (w/w) and 24% (w/w) on a dry weight basis, respectively. By contrast, cultivation of *C. oleaginosus* in xylose-containing synthetic medium led to an accelerated, and higher lipid accumulation (65% w/w within 72 h), compared to glucose medium (58% and 62% w/w in 72 h and 96 h, respectively). This is consistent with previous literature data, which showed that *C. oleaginosus* yielded a higher lipid content in medium containing xylose (57% w/w in 72 h), compared to medium containing glucose (48% w/w in 72 h) [35]. However, it is notable that 20% (w/w) of the xylose remained unconsumed, while glucose was metabolized quantitatively. In this study, the same behavior was observed in *R. glutinis* where it reached a higher lipid content in a shorter time by utilizing xylose in comparison to glucose (29 ± 1.8% w/w with a substrate to lipid conversion rate of 7 $mg_{lipid}/g_{sugar}$ in 72 h in MNM-Xyl and, 23 ± 1.9% w/w with 6 $mg_{lipid}/g_{sugar}$ in 96 h in MNM-Glu, Table 2). In the MPM-GlcNAc *T. asahii* and *C. oleaginosus* were the only strains that could utilize the N-acetyl glucosamine, the latter was able to accumulate 28 ± 2.1% of its biomass as lipid in this medium, while *T. asahii* did not produce high amounts of oil under these conditions (7.9 ± 0.2% w/w). In addition, our data indicated that the lipid content obtained by *C. oleaginosus* did not increase significantly between the time points of 48 h and 96 h of cultivation in MPM-GlcNAc (p-value > 0.05).

*Complex biomass hydrolysates* The final total lipid (g/l) were generally elevated on the complex carbon sources (Fig. 4), compared to synthetic media containing sole carbon sources (Fig. 3). For *C. oleaginosus*, *R. glutinis*, and *T. asahii*, the lipid contents were higher in WSH than in BAH (Fig. 4). *C. oleaginosus* built up biomass equally in the WSH and BAH (Fig. 2). However, cultivation in WSH yielded higher lipid productivity than BAH after 96 h, providing 0.078 g/Lh (15.86 g/L biomass, 47% w/w lipid) and 0.04 g/Lh (16.9 g/L biomass, 22% w/w lipid), respectively (Table 2). The concentration of fermentable sugars in WSH was higher compared to BAH. Notably, all available sugars were exhausted in the WSH, and the final lipid yield (per carbon unit consumed) was higher in WSH cultivations (Table 2). *C. oleaginosus* accumulated 0.19 g lipid per gram of carbon substrate consumed in WSH within 96 h, which is higher than that of BAH (0.16 $g_{Lipid}/g_{Sugar}$). This correlation in MNM-Glu and MNM-Xyl was calculated at 0.17 and 0.2 $g_{Lipid}/g_{Sugar}$ after 96 h and 72 h, at the time point where the maximum lipid content was reached, respectively. The other strains, however, performed best on BAH, which resulted in a higher total lipid and productivity compared to WSH (Table 2). Exemplary, *R. glutinis* had lipid productivity of 0.028 g/Lh (16.95 g/L biomass, 15% w/w lipid) in BAH while this value in WSH was 0.019 g/Lh (9.5 g/L biomass, 18% w/w lipid). Adding CSL as a nitrogen source to WSH did not show any negative impact on the maximum lipid yield per gram sugar consumed ($g_{Lipid}/g_{Sugar}$) (Table 2). Furthermore, the addition of CSL to WSH did not improve the lipid content (% w/w on a dry weight basis) of *C. oleaginosus* and *R. glutinis* but the maximum lipid content in WSH + CSL was achieved in a shorter time (72 h), resulting in higher lipid productivity (Table 2). In the complex BAH, *R. toruloides* performed as well as *R. glutinis* in terms of total lipid and biomass formation (Figs. 2, 4).

#### Fatty acid profile variations associated with media choice and composition

*Synthetic media containing sole carbon sources* An overview of the fatty acid profiles is summarized in Fig. 5 (and Additional file 2: Table S2). In general, the fatty acid profiles showed variations in different media and strains, while oleic acid (C18:1) remained the predominant fatty acid throughout all tested strains. The cultivations in the two different MNM, supplemented with xylose or glucose resulted in comparable fatty acid profiles for each strain. In these synthetic media, *T. asahii* reached the highest

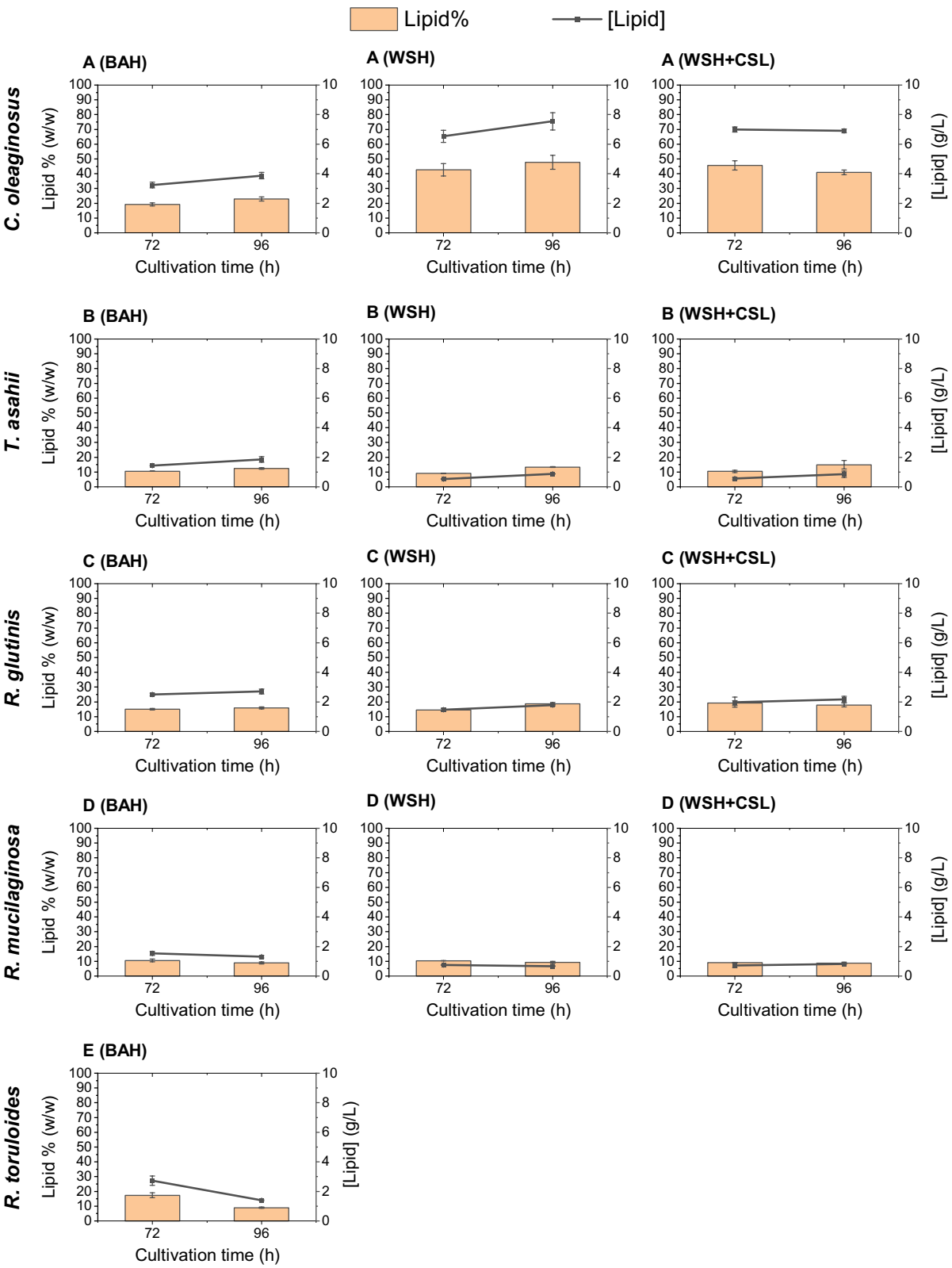


**Fig. 4** Lipid contents and total lipid of each yeast strain in the complex media: **A** *C. oleaginosus* lipid content and lipid concentrations in each complex medium **B** *T. asahii*. **C** *R. glutinis*. **D** *R. mucilaginosa*. **E** *R. toruloides*

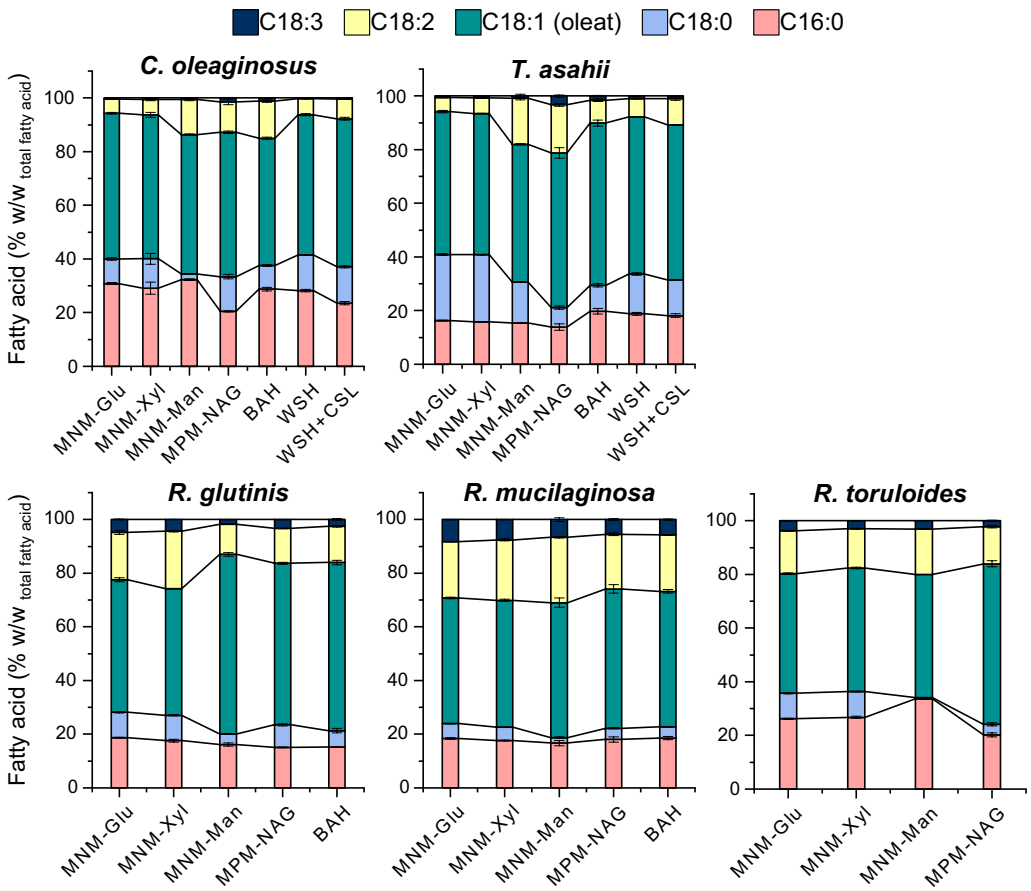


**Fig. 5** Fatty acid profile of each strain in different media after 96 h incubation

percentage of Stearic acid C18:0 amongst all strains analyzed (25% w/$w_{total\ fatty\ acid}$) which was decreased significantly in MPM-GlcNAc (p-value $\leq 0.05$). *T. asahii* and *C. oleaginosus* were the only strains that exhibited growth on N-acetylglucosamine. Notably, the resulting fatty acid profile depicted a higher level of unsaturated FAs in this medium when compared to synthetic Glu/Xyl-containing media. To that end, *C. oleaginosus* synthesized twice the concentration of C18:2, and a lower ratio of C16:0 in MPM-GlcNAc. Interestingly, MNM-Man resulted in an increase in unsaturated fatty acids of *C. oleaginosus* and *T. asahii* as well, while the C18:0 percentages were lower in this media (p-value $\leq 0.05$). This fatty acid was measured lower than 1% in MNM-Man in *R. toruloides*.

*Complex biomass hydrolysates* The cultivation of all strains in BAH could increase the total unsaturated fatty acids contents. *C. oleaginosus* showed a higher percentage of C18:2 resulting in a decrease of oleic acid and a final increase of PUFAs by 8% (w/$w_{total\ FAs}$). While all strains yielded lower saturated FAs and in turn higher unsaturated FAs, in BAH. In addition, *R. toruloides* and *R. glutinis* had a notable surge in the C18:1 concentration when cultivated in BAH. *T. asahii* and *R. mucilaginosa* in general produced a higher amount of unsaturated FAs. *R. mucilaginosa* reached the highest percentage of unsaturated FAs (81% w/$w_{total\ FAs}$) as well as PUFAs (30% w/$w_{total\ FAs}$) amongst all strains tested in BAH. Cultivations in WSH (± CSL) had slighter effects on the fatty acid profiles. By comparison to the synthetic media, this hydrolysate resulted in a 10% (w/$w_{total\ FAs}$) and 7% (w/$w_{total\ FAs}$) increase in the C18:1 ratio of *R. glutinis*, and unsaturated FAs obtained from *T. asahii,* respectively.

## Discussion

Five well-established oleaginous yeast strains were studied for their carbon source preference, fermentation inhibitor tolerance, and lipid yields on complex biomass hydrolysates derived from wheat straw and brown algae (*Laminaria digitate*). For control and calibration purposes strains were also cultivated in defined, synthetic minimal media containing the main component sugars of both complex media. Moreover, the yeast's capacity to metabolize N-acetylglucosamine, the monomeric component of chitin, which is the second most abundant

biopolymer after cellulose in nature, was examined systematically for the first time. Additionally, the use of corn steep liquor as a cost-efficient nitrogen source was examined in combination with complex biomass hydrolysates. All strains tested in this study were able to channel the extra-cellular carbon towards lipid neo-synthesis under nutrient limiting conditions (nitrogen limitation in MNM-Glu, MNM-Xyl, and MNM-Man, and phosphate limitation in MPM- GlcNAc) which indicates the lipid accumulation through the "de novo" lipid biosynthesis pathway [7, 29, 38, 39]. In this study, we utilized the two-phase cultivation protocol involving an initial growth period, followed by nutrient limitation in each defined synthetic media [40].

The growing interest in YOs during the past decades shows that YO will not only remain a significant part of the biochemical research but also turn into a bigger platform in the respective industries. In order to fully recover the resources, it is preferred that the oleaginous strain consumes all types of carbon substrates in the hydrolysate. For the feasibility of such a process, it is necessary to identify the yeast strains that can tolerate the hydrolysis by-products of the local abundant feedstocks. Several studies investigated *Laminaria* hydrolysates for lipid accumulation in different yeasts. The wheat straw hydrolysate was investigated as well for microbial oil production [33, 41, 42]. This is the first systematic study comparing differential substrate preferences, growth efficiencies as well as tolerance against the potential inhibitory effects of the hydrolysates from both terrestrial and marine origin, as well as their respective synthetic control medium, for an described prominent oleaginous yeasts.

A number of previous studies have investigated oleaginous yeasts in terms of utilization of pentose sugars, while this study represents the first comparative investigation on growth and lipid production using a pentose sugar both as a sole carbon source and as a part of a complex hydrolysate. Pentose sugars like xylose, generated through the hydrolysis of the lignocellulosic material, are challenging substrates since most microorganisms cannot utilize them [43]. In a study, it has been shown that most of the strains of *Yarrowia* clade were able to utilize xylose in switchgrass hydrolysate [44]. Furthermore, lignin makes up 10–30% of the lignocellulosic biomass by weight [45] and is depolymerized by the pre-treatment of wheat straw. Its degradation products such as vanillin, 4-hydroxybenzaldehyde, and syringaldehyde are potential growth inhibitors for most microorganisms. *C. oleaginosus* is reported to have a high tolerance towards a wide range of lignin-derived aromatic compounds (e.g., resorcinol and protocatechuate) as well as acetic acid, the by-products of wheat straw hydrolysis. In fact, *C. oleaginosus* can utilize these harmful compounds as substrates to accumulate lipids [11, 34]. In the wheat straw hydrolysate, glucose and xylose are co-utilized by *C. oleaginosus*. In a previous study, it is reported that a co-utilization of glucose and xylose by *C. oleaginosus* in a defined synthetic media (glu:xyl mass ratio at 45:25 g/g equal to 1.8:1 g/g) resulted in the substrate to lipid conversion of 16.4% $g_{lipid}/g_{sugar}$ [46]. By comparison, a higher conversion rate (18.7% $g_{lipid}/g_{sugar}$) was achieved in the current study, when this strain was cultivated in the WSH (with a similar glu:xyl mass ratio at 27:14 g/g equal to 1.9:1 g/g). The higher rate in *C. oleaginosus* in our study can be explained by its potential for utilization of lignin-derived aromatic compounds [34]. This data demonstrates that this strain is capable of co-utilizing the aromatics and both glucose and xylose towards lipid production. According to our data, the highest growth on WSH was achieved by *C. oleaginosus*. The WSH used in our study was not detoxified. Therefore, it could be concluded that the other strains examined in this study are not as tolerant to the phenolic compounds derived from lignin hydrolysis or other growth inhibitors resulting from pretreatment steps as *C. oleaginosus*. *R. toruloides* CBS14 performed as second-best in terms of growth as well as lipid on glucose and xylose in synthetic media (Fig. 3, and Additional file 1: Fig. S1). However, it did not exhibit any growth in WSH, indicating that the hydrolysate components inhibited the growth. Similar behavior of *R. toruloides* was reported by Yu et al., whereby, unlike the non-detoxified hydrolysate, the detoxified WSH allowed growth and lipid accumulation. Furthermore, the types of growth inhibitors generated by mild acidic pretreatment of WSH were identified [33]. By comparison, *Y. lipolytica* accumulated lipid up to 4.6% of its dry cell weight in this hydrolysate [33]. The tolerance of yeast strains to these inhibitors could be improved using genetic engineering tools. Further research is needed to determine the mechanisms underlying the different tolerance degrees of yeast strains to the inhibitors by metabolome analysis.

Interestingly, the addition of CSL, a rich source of nitrogen and nutrients, to the WSH enhanced the growth and accelerated the lipid accumulations of all strains tested. Although the limited growth of *T. asahii*, *R. glutinis*, *R. mucilaginosa*, and *R. toruloides* was thought to be due to their lower tolerance to the lignin-derived aromatics, it appeared that their performance was improved by adding CSL as a source of nitrogen and nutrients. *C. oleaginous* was able to consume all available sugars in the WSH (42 g/L sugar in 96 h). Therefore the addition of CSL did not affect its final yield in comparison to the other strains in WSH + CSL *C. oleaginosus* remained oleaginous despite a higher nitrogen concentration. This study demonstrates that cultivation with the wheat straw hydrolysate resulted in 7.5 g/L lipids, 47% of its

dry weight by *C. oleaginosus*. A continuous or fed-batch fermentation could result in even a higher final lipid and biomass, where the required carbon source is provided in the presence of CSL.

According to Papanikolaou et al. as far as glucose metabolism is concerned, 100 g of glucose (equal to 0.56 mol) could yield 1.1 mol of Acetyl-CoA. Xylose however can be catabolized by the phosphoketolase reaction as well as the pentose phosphate pathway, resulting in 1.2 mol and 1.0 mol Acetyl-CoA from 100 g xylose (0.66 mol), respectively. Hence, under lipid accumulating conditions, where all the Acetyl-CoA is utilized to form lipids, the theoretical cellular lipid yield is 0.32 g per gram consumed glucose. When xylose is utilized under the involvement of the phosphoketolase pathway, the value slightly increases to 0.34 g/g [39]. The maximum carbon:carbon efficiency for *C. oleaginosus* and *R. glutinis* in MNM-Xyl was also calculated to be higher than that in MNM-Glu (20 $mg_{lipid}/g_{sugar}$ and 17 $mg_{lipid}/g_{sugar}$ for *C. oleaginosus* and 7 $mg_{lipid}/g_{sugar}$ and 6 $mg_{lipid}/g_{sugar}$ for *R. glutinis* at their highest lipid accumulation points, respectively (Table 2). Based on the stoichiometry of sugar metabolism, these findings could suggest, that *C. oleaginosus* and *R. glutinis* use the phosphoketolase pathway for the xylose metabolism, under the evaluated cultivation conditions. Phosphoketolase(s) have been found formerly in *R. glutinis* and *R. toruloides* as well [47, 48]. The exhaustion of xylose by these two strains as well as their higher carbon:carbon efficiencies in the BAH by comparison to synthetic media (biomass and lipid yields reported in Table 2) could be an indication of a relatively higher phosphoketolase activity under the tested conditions in comparison to the other strains. The better performance of *C. oleaginosus* on WSH than BAH in terms of both lipid yield per gram sugar consumed and lipid content can again be explained by the involvement of the phosphoketolase pathway. Especially when considering the fact that the pentose content is much higher in the WSH in contrast to the BAH. A further investigation is needed to pinpoint the pathway preferences in oleaginous yeast, especially the effect of the C/N ratio and co-consumption of different sugars on the sugar assimilation pathways.

In addition to glucose and xylose, BAH contains mannitol. This sugar alcohol is one of the major carbon sources of brown algae. Co-utilization of all three sugars was observed for *R. toruloides* and *C. oleaginosus.* Until now, there are only a few studies reporting yeast strains that are naturally able to absorb mannitol and channel it into lipids biosynthesis [49]. The mannitol uptake mechanism, as well as the consumption effect of mannitol on fatty acid profiles and lipid accumulation in oleaginous yeasts, are not yet well studied. It was shown that in an engineered *S. cerevisiae*, the mannitol assimilation is conducted through mannitol transporters into the cytoplasmic space and subsequently metabolized to D-fructose by mannitol-2-dehydrogenase consuming $NAD^+$ [14, 50]. Interestingly, a study on the marine yeast *Rhodosporidiobolus fluvialis* Y2 demonstrated that mannitol uptake resulted in higher levels of PUFAs, suggesting the NADH required for desaturases is provided by mannitol-2-dehydrogenase, therefore the fatty acid desaturases become more active in presence of mannitol [49]. A similar effect was seen in our study. As a result of mannitol uptake as a sole carbon source in the MNM-Man, the amount of PUFAs (C18:2) produced by *C. oleaginosus* and *T. asahii* was increased and a considerable decrease in C18:0 was determined, which could demonstrate a mannitol-dependent activation of Δ12 and Δ9 desaturases. These changes were also detected in the BAH in the presence of mannitol, nevertheless, *T. asahii* also showed a higher amount of C18:1 in BAH which could be due to a higher Δ9 desaturase activity and slightly lower Δ12 desaturase activity when utilizing a mixture of monomers compared with utilizing mannitol as a sole carbon source. In contrast, in *R. toruloides*, the mannitol utilization resulted in an elevated level of palmitic acid (C16:0).

When using N-acetylglucosamine as a sole carbon source, only *C. oleaginous* and *T. asahii*, were able to utilize this sugar. This observation is consistent with genome and transcriptome analysis of *C. oleaginosus* which predicted the genes involved in GlcNAc utilization including GlcNAc kinase with homology to the *C. albicans* NAG kinase, as well as N-acetyl-glucosamine-6-phosphate deacetylase [Triol1|281629] and glucosamine-6-phosphate deaminase [Triol1|281628]) [29]. Notably, *C. oleaginosus* was far more efficient than its closest sequenced relative, *T. asahii*, in terms of GlcNAc uptake as well as growth and lipid accumulation in this media. The metabolism of GlcNAc is not well characterized in the oleaginous yeasts. An N-acetylglucosamine kinase (Hxk1) in *C. albicans* was first reported in 1974 [51]. It has been shown that the GlcNAc uptake capacity of the cells is proportional to the level of a specific binding protein expressed by the cells [52]. In *C. albicans*, the N-acetylglucosamine transporter (Ngt1) mediates the entry of GlcNAc into the cells, which represented the first eukaryotic GlcNAc transporter. In addition, the *Saccharomyces cerevisiae* expressing Ngt1 exhibited GlcNAc uptake ability. This showed that the Ngt1 in a direct way functions as a GlcNAc transporter [53]. Inside the cells, the binding of free GlcNAc to the Ngs1 protein (a GlcNAc sensor and transducer) is required for GlcNAc signaling. It induces other transcriptional responses to this amino sugar in the nucleus, and it is conserved in various fungi. The mutations in the Ngs1, the binding site of

N-acetylglucosamine, abolished growth on GlcNAc in *C. albicans*. [54, 55]. Until now, no transporter and sensor-transducer for GlcNAc is identified in *C. oleaginosus* or other oleaginous yeasts. However, the high efficiency of *C. oleaginosus* to utilize this sugar compared to *T. asahii* (63% w/w (19 $g_{GlcNAc}$/ L) and 26% w/w (8 $g_{GlcNAc}$/ L) uptake in 24 h) indicates a more efficient GlcNAc metabolism in this yeast, most likely including highly effective transporter and sensor-transducer. It is noteworthy that GlcNAc metabolism is reported to release $NH4^+$ and acetate leading to a higher of extracellular pH [56, 57]. *C. oleaginosus* in general is able to consume and channel acetate to lipid biosynthesis [58]. However, the accumulation of ammonia and high pH could interfere with further growth due to their cytotoxicity effects [59]. The extreme changes of pH could be avoided under controlled conditions in a continuous or batch fermentation mode. Therefore, the tolerability of oleaginous yeasts to ammonia and acetate plays an important role in efficient lipid production from GlcNAc. In order to further identify the respective system in *C. oleaginosus*, proteomic analysis is required. A better valorization of chitin-based feedstocks can be accomplished through modification of yeast strains, therefore a better understanding of GlcNAc metabolism is necessary.

## Conclusion

Among the yeast strains investigated in this study, *C. oleaginosus* performed as the most versatile strain in terms of substrate utilization, productivity, and tolerance towards fermentation inhibitors in the complex media. It yielded 7.5 g/L lipids in the wheat straw hydrolysate. Commercially available lignocellulosic residues, like WSH, are currently mainly used for the production of bioethanol [60]. A better energetic and economic valorization of lignocellulosic waste could be accomplished by just switching this process to lipid production, as a product of higher energy density is formed. To that end, CSL was also shown as a promising inexpensive source of nitrogen to improve the growth to produce YOs. In the current study, CSL enhanced the growth in the oleaginous strains such as *R. glutinis*, *C. oleaginosus*, and *R. mucilaginosa*. In this work, *C. oleaginosus*, *R. glutinis*, and *R. toruloides* showed a high potential for lipid production on the marine biomass hydrolysate with efficient pentose utilization. Furthermore, this study has demonstrated that the fatty acid profile varies when cultivation is carried out on different feedstocks, especially on BAH due to the presence of mannitol. Combining the element of carbon source with other formerly-studied factors such as temperature [61–63], nitrogen source [31], and oxygen [37, 64] will enable us to achieve the desired fatty acid composition for diverse applications. Moreover, the degree of fatty acid saturation determines the physiochemical properties of resulting biofuels. These properties, which include Iodine Value (IV), Cetane Number (CN), Higher Heating Value (HHV), Kinematic Viscosity (KV), and Density, have been qualified for *C. oleaginosus*, *Y. lipolytica*, *R. turoloides*, and *L. starkeyi* and found to be comparable with palm oil. These oils are also positioned within internationally accepted biofuel standard limits for US biodiesel: ASTM D6751 and for EU biodiesel: EN 14214 [65–68]. Another viable application for *C. oleaginosus* targets the food sector. Solvent-free lipid extraction from *C. oleaginosus*, grown at a technical scale, in addition to life cycle analysis, showcased the economic feasibility of using this microbial oil in the food industry.

## Methods and materials

### Strains, environmental samples and media

Five prominent oleaginous yeast strains were screened in this study. *Cutaneotrichosporon oleaginosus* ATCC 20,509 (DSM-11815) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) (Braunschweig, Germany), and *Rhodosporidium toruloides* CBS 14 was obtained from Centraalbureau voor Schimmelcultures (CBS). *Rhodotorula glutinis* (IBY050), *Trichosporon asahii* (IBY051), and *Rhodotorula mucilaginosa* (IBY052) were obtained from the Werner Siemens-Chair of Synthetic Biotechnology (WSSB) culture collection. The strains were transferred and maintained on YPD- agar plates. The inoculum media was YPD as well. Generic synthetic media for lipid accumulation include nitrogen limitation medium (carbon source: glucose, xylose, or mannitol 30 g/L); and phosphate limitation medium (carbon source: N-acetyl glucosamine 30 g/L); [35]. The pH of all synthetic media was adjusted to 6.5 prior to sterilization. The complex medium used in this screening were wheat straw hydrolysate, corn steep liquor, and hydrolysate of brown algae *Laminaria digitata*. The brown algae were hydrolyzed enzymatically at a 2 L scale according to our previous study [10]. The wheat straw hydrolysate was obtained from Clariant (Germany). The hydrolysate was diluted and subjected to crossflow filtration (10 kDa polyethersulfone filter; Pall Corporation, US) to remove all proteins. The pH of both hydrolysates was set at 6.5 prior to sterile filtration. The corn steep liquor was commercially available and obtained from the TT baits (Germany) in a powder form. It was autoclaved at 134 °C for 20 min and added to WSH at the final concentration of 5 g/L under sterile conditions before the inoculation.

### Cultivation conditions

Single colonies of the yeast strains from YPD-agar plates were inoculated into 20 mL of YPD medium overnight as

the pre-cultures. The cultivations were started by inoculating 100 mL of each medium to an $OD_{600\,nm}$ of 0.1 in a 500 mL baffled shaking flask. All experiments were done in triplicates. The shaking flasks were incubated at 28 °C and 120 rpm for 96 h. Samples were taken each 24 h and stored at – 20 °C.

#### Fatty acid determination

Two ml cultivation medium was pelleted, washed with $ddH_2O$, and lyophilized. Lyophilization was carried out for 2 days at − 80 °C and 0.04 mbar (VaCo 5, Zirbus Technology, Germany). Fatty acid analysis was done according to the modified protocol of Griffiths et al. [69]. Glyceryl trinonadecanoate (C19:0-TAG, 0.2 mg/mL in GC grade toluol) was added prior to the reaction as an internal standard. The dried biomass was directly converted into fatty acid methyl esters (FAME) by simultaneous extraction and transesterification of yeast lipids using 0.5 M Sodium methoxide solution in GC grade methanol and hydrogen chloride-methanol solution (Sigma, Germany) the fatty acid profiles were measured by a GC-2025 Plus gas chromatograph (Shimadzu, Japan) according to Woortman et al. [70].

#### Gravimetric analysis of biomass and lipids

Dry cell weight (DCW) was determined by pelleting 2 mL samples (10,000 g, 10 min), washing cells once with 2 mL $ddH_2O$, and lyophilized in pre-weighed microtubes. Intracellular total lipid weight was obtained by extraction using chloroform and methanol according to the protocol of Bligh–Dyer [71]. The harvested cells were washed with $ddH_2O$ and destructed by a high-pressure homogenizer (Mulsiflex C3, Avestine, Canada), followed by two times sequential solvent extraction using Folch solution incubated for 2 h and 1 h, respectively. The chloroform layer containing yeast lipids was aspirated under a nitrogen stream and the lipids were weighed. The percentage of lipid content and total lipid was calculated based on Eq. 1 and Eq. 2

$$Lipid\ content\ \%\ w/w = \frac{w\ obtained\ lipid\ (g)}{w\ obtained\ dried\ biomass\ (g)} \times 100 \quad (1)$$

$$Total\ lipid\ (lipid\ concentration\ g/L) = \frac{w\ lipid\ obtained\ (g)}{Volume\ culture\ (L)} \quad (2)$$

The Biomass yield carbon: carbon efficiency) was calculated based on Eq. 3

$$Biomass\ yield\ (Growth\ efficiency)\ g/g = \frac{w\ biomass\ synthesized\ (g)}{w\ sugar\ consumed\ (g)} \quad (3)$$

The lipid yield (substrate to lipid conversion rate), and lipid productivity were calculated based on Eqs. 4, and 5 respectively.

$$Lipid\ yield\ g/g = \frac{w\ lipid\ obtained\ (g)}{w\ substrate\ consumed\ (g)} \quad (4)$$

$$Lipid\ productivity\ g/Lh = \frac{w\ lipid\ obtained\ (g)}{V\ culture(L) \times\ incubation\ time\ (h)} \quad (5)$$

#### Sugar analysis

Sugar analysis was carried out using an Agilent 1260 Infinity II LC system with quintenary pump and equipped with Diode Array (DA) and Refractive Index (RI) detectors. In this method, a Rezex ROA-organic H + 8% column from Phenomenex was used (300 × 7.8 mm). The isocratic mobile phase (5 mM H2SO4) was pumped at a flow rate of 0.5 mL/min. The run time was 60 min. The oven temperature was set at 70 °C, and the measurement was done by RID at 40 °C without cooling. The injection volume was 10 µl. All the sugars used for calibrations were obtained from Sigma, Germany.

The percentage of substrate consumed was calculated according to Eq. 6.

$$Substrate\ consumed\ \%\ w/w\ at\ time\ point\ x = \frac{w\ substrate\ consumed\ at\ time\ point\ x\ (g)}{w\ total\ available\ substrate\ at\ the\ start\ point\ (g)} \times 100 \quad (6)$$

#### Element analysis

Elemental analysis (C, H, N, S) was done using a Euro EA CHNS elemental analyzer (HEKAtech Ltd.), based on dynamic spontaneous combustion in the Sn boat at approximately 1800 °C with subsequent gas chromatographic separation and detection using a thermal conductivity detector (TCD).

**Abbreviations**
SCO: Single cell oil; WSH: Wheat straw hydrolysate; BAH: Brown algae hydrolysate; CSL: Corn steep liquor; MNM: Minimal nitrogen medium; MNM-Glu: Minimal nitrogen medium containing glucose; MNM-Xyl: Minimal nitrogen medium containing xylose; MNM-Man: Minimal nitrogen medium containing mannitol; MPM-GlcNAc: Minimal phosphate medium containing N-acetylglucosamine; GC-FID: Gas chromatography-flame ionization detection; DCW: Dry cell weight; YO: Yeast oil; h: Hours; FA: Fatty acid; w: Weight; V: Volume; TAGs: Triglycerides.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01710-3.

**Additional file 1: Figure S1.** Growth curves of all strains in each medium.

**Additional file 2: Table S1.** Specific sugar consumption in complex media. **Table S2**. The fatty acid profile of each strain in different media. **Table S3** Measurement of dry cell weights for each experiment throughout the cultivation. **Table S4** Lipid contents determined on a dry weight basis for each yeast strain. **Table S5** The compositional analysis of corn steep liquor powder. **Table S6** Overview of lipid contents and lipid concentrations measured in previous studies compared with the current study.

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**Authors' contributions**
PS and TB conceived the study. PS, DA, MF, and VR designed the experimental approach. PS, DA, MF, and VR performed data collection on MNM-Glu, MNM-Xyl, and MPM-GlcNAc. PS performed data collection on MNM-Man, BAH, WSH, and WSH + CSL. MH assisted in sample analysis and data collection. PS drafted the manuscript. TB, NM, and DA edited and revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
The datasets supporting the conclusions of this article are included within the article [and its Additional information files].

## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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## Publisher's Note

## 3.3

# Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector

Microbial Cell Factories

RESEARCH

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# Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector

Pariya Shaigani, Tobias Fuchs, Petra Graban, Sophia Prem, Martina Haack, Mahmoud Masri, Norbert Mehlmer* and Thomas Brueck*

## Abstract

**Background** Sustainable production of triglycerides for various applications is a major focus of microbial factories. Oleaginous yeast species have been targeted for commercial production of microbial oils. Among all the oleaginous yeasts examined in a previous comparative study, *Cutaneotrichosporon oleaginosus* showed the highest lipid productivity. Moreover, a new lipid production process for *C. oleaginosus* with minimal waste generation and energy consumption resulted in the highest lipid productivity in the history of oleaginous yeasts. However, productivity and product diversity are restricted because of the genetic intractability of this yeast. To date, successful targeted genetic engineering of *C. oleaginosus* has not yet been reported.

**Results** The targeted gene editing was successfully carried out in *C. oleaginosus* using CRISPR/Cas system. A tailored enzyme system isolated to degrade the *C. oleaginosus* cell wall enabled the isolation of viable spheroplasts that are amenable to in-cell delivery of nucleic acids and proteins. The employment of both Cas9 protein and Cas mRNA was effective in obtaining strains with *URA5* knockout that did not exhibit growth in the absence of uracil. Subsequently, we successfully created several strains with enhanced lipid yield (54% increase compared to that in wild type) or modified fatty acid profiles comparable with those of cocoa butter or sunflower oil compositions.

**Conclusion** This study establishes the first targeted engineering technique for *C. oleaginosus* using the CRISPR/Cas system. The current study creates the foundation for flexible and targeted strain optimizations towards building a robust platform for sustainable microbial lipid production. Moreover, the genetic transformation of eukaryotic microbial cells using Cas9 mRNA was successfully achieved.

**Keywords** Oleaginous yeast, Yeast oil, Genome engineering, CRISPR/Cas, Tailored plant oil alternatives, *Cutaneotrichosporon oleaginosus*, High-oleic sunflower oil, Cocoa butter, Fatty acid biosynthesis

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## Background

The increasing global demand for plant- and animal-based lipids in the biofuel, pharmaceutical, and oleochemical industries has negatively impacted biodiversity and caused land-use changes. Microbial oils have shown significant improvements in yield and sustainability compared to vegetable oils [1–4]. The oleaginous yeast *Cutaneotrichosporon oleaginosus* can accumulate lipids via the *de-novo* lipid biosynthesis pathway. Its superiority over other oleaginous microorganisms in having high

triglyceride (TAG) content, substrate flexibility, and rapid growth rate has made this yeast a prime candidate for oleochemical production [5–7]. To date, *C. oleaginosus* has displayed the highest carbon source flexibility linked with high *de-novo* oil productivity and yield of any oleaginous yeast strain [7–13]. Moreover, compared to other yeasts, *C. oleaginosus* is highly resistant to fermentation inhibitors (including weak organic acids e.g. acetic acid [8], sugar-derived furans, and phenolic compounds [14]) formed during hydrolysis and pre-treatment processes. This enables the cultivation of *C. oleaginosus* in cost-efficient, undetoxified, and complex waste biomass streams. This economic potential of *C. oleaginosus* has led to a great interest in generating tailor-made lipids for the large-scale production of various high-value fats [15]. The cost of microbial oil production is higher than that of the common plant oils [8, 15]; however, in a previous study, a highly efficient lipid production process by *C. oleaginosus* using a new, residue-free, circular production process with minimal energy consumption was established that reached a maximum of 2.4 g/Lh lipids.The cost was estimated $1.6/kg lipid, which is more cost-efficient than the eco-certified palm oil. Various parameters were demonstrated that affected the process economics, such as a short fermentation time, solvent-free lipid recovery, and the low cost of acetic acid [8, 16].

Genetic engineering has a key role in flexible optimization of high-value compounds, such as triglycerides, terpenoids, and other metabolically derived value-added compounds, for diverse industrial applications. To date, untargeted genetic manipulation of *C. oleaginosus* has been established using *Agrobacterium*-mediated transformation (AMT) with stable integration of expression cassettes into the genome [17]. However, productivity and product diversity are restricted because of a lack of targeted genome editing. Moreover, the dominance of non-homologous end joining (NHEJ) to homologous recombination (HDR) in *C. oleaginosus* and the lack of an artificial plasmid for this yeast have impeded targeted and efficient genetic engineering [18]. Therefore, the establishment of an efficient and targeted method is necessary for the genetic tractability of *C. oleaginosus* as the most efficient yeast for oil production.

Here, we aim to demonstrate the first targeted genetic engineering method for *C. oleaginosus* using the clustered regularly interspaced short palindromic repeats associated nucleases (CRISPR/Cas) technology. We have established a novel, tailored spheroplasting strategy and transformation method that allows the delivery of all forms of nucleic acids and proteins. Finally, *C. oleaginous* was metabolically engineered to generate various fatty acid (FA) profiles. For this purpose, we selected the Δ-9 and Δ-12 desaturases of *C. oleaginosus* to target the fatty acid metabolism, as they are the key enzymes to synthesize unsaturated fatty acids. Δ-9 desaturase is reported to insert a double bond into saturated fatty acids (such as stearic acid to produce oleic acid). Further, Δ-12 desaturase incorporates another double bond into oleic acid to produce linoleic acid [19, 20].

## Results

### Establishing a method for targeted genetic engineering of *C. oleaginosus*

#### *Building a library of guide RNAs (gRNA) for the whole genome of C. oleaginosus*

For building an *in-silico* library, the whole genome sequence of *C. oleaginosus* was analysed thoroughly. All possible protospacer adjacent motifs (PAMs) for Cas9 and its variants were determined, and the corresponding sgRNAs were added to the library. This enabled the analysis of the off-target activity of each sgRNA against the whole genome of *C. oleaginosus*, which is an essential step for removing and minimising off-target genome integration. However, at present, there are no online tools for the *in-silico* analysis of off-target activities dedicated to *C. oleaginosus*. Therefore, we identified and curated the off-target sites manually.

#### *Spheroplast preparation*

The self-generated hydrolase enzyme system from *Trichoderma reesei* (HEST) and the commercial Glucanex enzyme resulted in more than 95% of the cells to form spheroplasts after incubation for 15 and 45 min, respectively. This indicates a higher efficiency of HEST owing to the specificity of this enzyme system for *C. oleaginosus* cells (Fig. 1a).

#### *Delivery of CRISPR/Cas components into the nucleus*

The strategies adopted to implement the Cas platform in *C. oleaginosus* are shown in Fig. 2. The delivery of CRISPR/Cas elements into spheroplasts prepared using Glucanex did not generate colonies on the yeast nitrogen base plates containing 5-fluoroorotic acid ($YNB^{+}5FOA$) plates. However, spheroplasts prepared with HEST were successfully transformed. This indicated that the custom-tailored HEST permitted efficient cell wall removal, which allowed for the effective transfer of nucleic acids and proteins into the yeast spheroplasts. Positive clones showed uracil auxotrophy and could not grow in absence of uracil on $YNB^{-}$ plates. Positive transformants were obtained using both the Cas protein and mRNA delivery strategies. The editing efficiency was 100%, 75%, and 20% for Cas9 mRNA, Cas9n D10A protein, and Cas9 protein, respectively. Wild-type spheroplasts were transformed with Cas protein/mRNA and template ssDNA ensured the lack of Cas activity in the absence of sgRNAs.

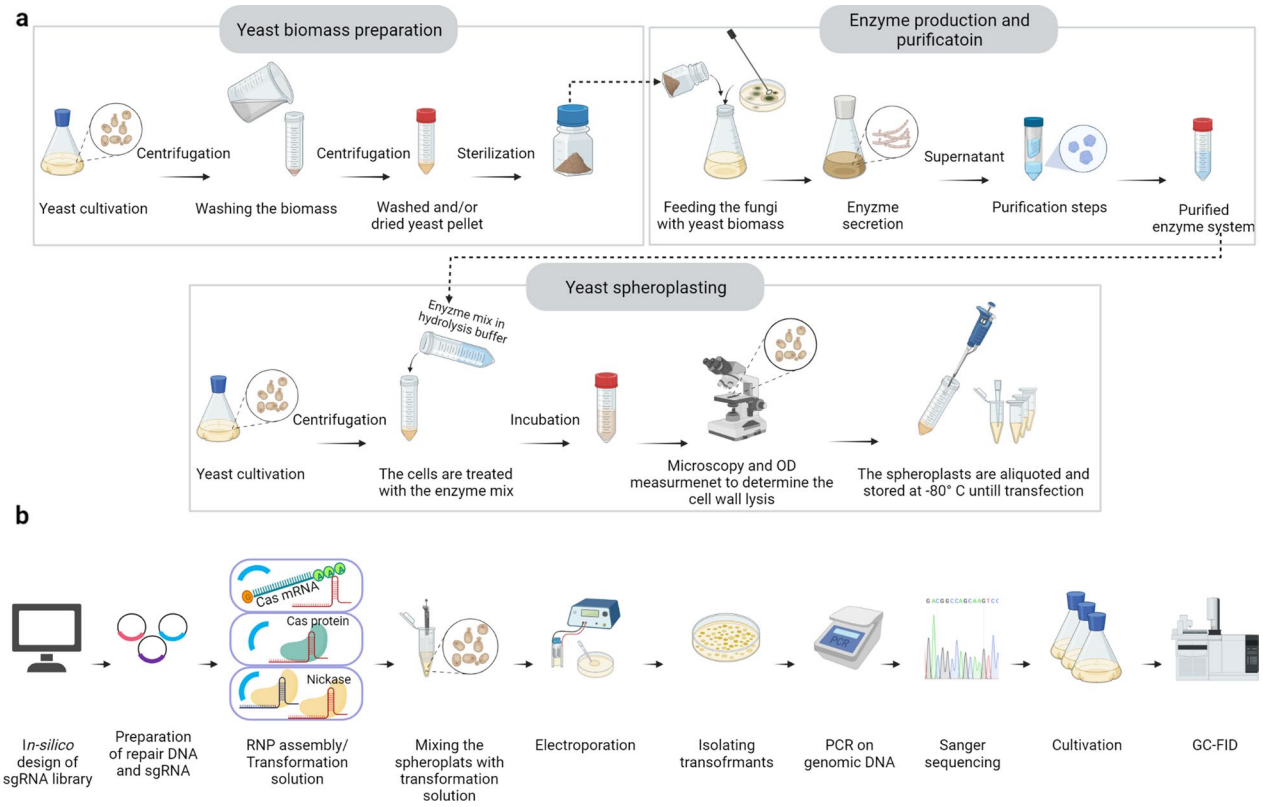


**Fig. 1** Targeted genetic engineering of *Cutaneotrichosporon oleaginosus* using CRISPR/Cas technology. **a** Schematic illustration of the spheroplasting procedure of *C. oleaginosus* and custom enzyme isolation. **b** Schematic illustration of the genetic engineering of *C. oleaginosus* using CRISPR/CAS system

Transformation of spheroplasts with template DNA alone did not produce any colonies.

### Metabolic engineering of *C. oleaginosus* to generate tailored lipid profiles

Three approaches were designed for upregulation, downregulation, and knockout of Δ9- and Δ12-fatty acid desaturase genes (*D9FAD* and *D12FAD*) in a uracil-auxotrophic strain (Fig. 3). Targeting *D9FAD* for a full knockout did not result in any transformants with or without oleic acid supply in the $YNB^{-}$ plates.

All strains were analysed under nitrogen limited conditions (MNM + Glu media, Fig. 3). At this stage, the WT FA composition (FAC) was 30% ($w/w_{\text{total FA (TFA)}}$) palmitic acid (C16:0), 8% stearic acid (C18:0), 52% oleic acid (C18:1), 9% linoleic acid (C18:2), and 1% linolenic acid (C18:3). Strain D9OE exhibited an increase in C18:1 content (55%) and a decrease in C18:0 content ($p < 0.001$). Strain D12OE also showed a minimal increase in C18:2 content ($p < 0.0001$).

Notably, the strains AKRp-D9 and TEFp-D9 displayed significant enhancement of the saturated FAs (S-FAs) (Additional file 2: Table S1; 64% and 62% w/w $_{\text{TFA}}$, respectively) at the expense of C18:1 (25% and 29% w/w $_{\text{TFA}}$, respectively). This suggests a higher gene expression rate under D9FADp than that under both AKRp and TEFp under the experimental conditions.

Interestingly, the D12FADp replacement caused a slight increase in C18:2 content ($p < 0.05$), indicating a lower expression rate under the native promoter than both AKRp and TEFp. Furthermore, the knockout of *D12FAD* (ΔD12 strain) resulted in the absence of C18:2 and C18:3; consequently, C18:1 content was enhanced by up to 64% w/w $_{\text{TFA}}$.

### Tailored lipid production by *C. oleaginosus* using advanced fermentation technologies

Selected transformants were subjected to high cell density cultivations using rich medium containing acetic acid and glucose (RM + AA + Glu) and minimal medium (MNM + Glu) (Fig. 4). For the WT, C18:1 content continues to increase slightly throughout the 96 h cultivation (C18:1 content: 57% and 54% w/w $_{\text{TFA}}$ in RM + AA + Glu and MNM + Glu, respectively). The final C18:0 yield was higher in RM + AA + Glu than in MNM + Glu (23% and 10% w/w $_{\text{TFA}}$, respectively).

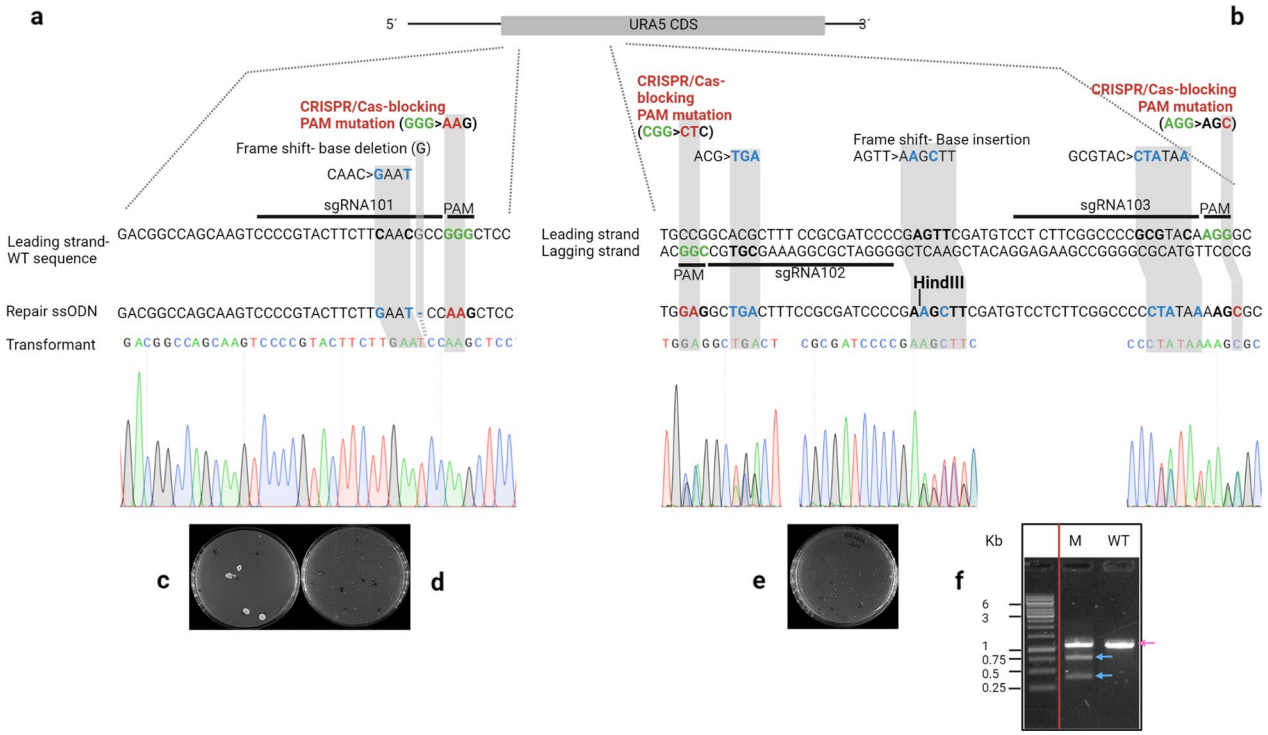


**Fig. 2** Overview of the *URA5* knockout strategies. As a proof of principle for CRISPR/Cas-mediated genetic modifications, we attempted to knockout the orotate phosphoribosyltransferase gene (*URA5*) to counter select 5-fluoroorotic acid (5FOA). We selected three sgRNAs from the library, which showed no off-target activities within the *C. oleaginosus* genome through *in-silico* prediction, and selectively targeted *URA5*. We then followed three parallel strategies to implement the Cas9 platform in *C. oleaginosus*. Both spheroplast batches prepared by Glucanex and HEST were used to test all strategies. **a** Strategy one and two: genome editing by Cas nuclease delivered into spheroplasts by electroporation in two forms separately: protein (Cas:sgRNA ribonucleoprotein [RNP]) and mRNA. In both strategies one single guide RNA (sgRNA) was used to target *URA5*. A single stranded DNA (ssDNA) was simultaneously transferred to introduce the repair sequences, including base deletions and base substitutions. **b** Strategy three: genome editing using Cas nickase as an RNP. Here, two sgRNAs targeting the leading and lagging strands were delivered to create the double-strand break (DSB). The repair ssDNA included base insertions and substitutions. A non-cutting restriction site (HindIII) was also introduced in the *URA5* loci of mutants. The protospacer adjacent motifs (PAMs) were mutated in all strategies to prevent further DNA cleavage after the repair. **c**–**e** Colonies on selection agar plates with *URA5* knockout using Cas mRNA, nuclease protein, and nickase protein, respectively. **f** The agarose gel electrophoresis of digested *URA5* gene from WT and Δura5 strains. The *URA5* locus was PCR amplified from the genomic DNA of mutants and WT and subjected to fast digestion by HindIII restriction enzyme. The digestion resulted in appearance of two smaller bands in the gene isolated from the Δura5 strain, indicating the integration of repair DNAs by Cas nickase. The WT *URA5* gene was not digested

Moreover, C16:0 was produced in lower ratios in the RM + AA + Glu medium than in the MNM + Glu medium (28% and 18% w/w $_{TFA}$, respectively). The data show that *D9FAD* overexpression has a pulling effect on the elongation of C16:0 towards the production of a higher ratio of C18:1 during fermentation (61% and 59% w/w $_{TFA}$ in RM + AA + Glu and MNM + Glu, respectively). Interestingly, strain D9OE displayed enhanced growth (DCW: 49.2 ± 5 g/L), which in turn resulted in a 54% boost in total lipid titre (38.8 ± 0.8 g/L, $p < 0.005$) compared to that in the wild-type strain (DCW: 31.3 ± 1.8 g/L; total lipid: 25.1 ± 2 g/L), despite the equal lipid content (Fig. 4 and Table 1). Similar to D9OE, the cultivation of strain ΔD12 in RM + AA + Glu also resulted in a significantly higher total lipid titre (33.3 ± 1.5 g/L, $p < 0.05$). These data indicate that C18:1 as the main FA in both mutants may enhance yeast biomass production. Similar effects have been reported in the model oleaginous yeast *Yarrowia lipolytica* [21].

Interestingly, the promoter substitution of *D9FAD* profoundly affected the FA profile. The comparison of FAC during cultivation highlighted the changes in *D9FAD* regulation. The expression level of *D9FAD* was lower under the control of AKRp and TEFp, resulting in a higher accumulation of C18:0 (TEFp-D9: 50%, 31%; AKRp-D9: 52%, 28% w/w $_{TFA}$) compared with that in the WT (10% and 17% w/w $_{TFA}$) after 24 h in minimal and rich media, respectively. While expression levels under D9FADp varied slightly with the growth phase and media, expression under AKRp and TEFp

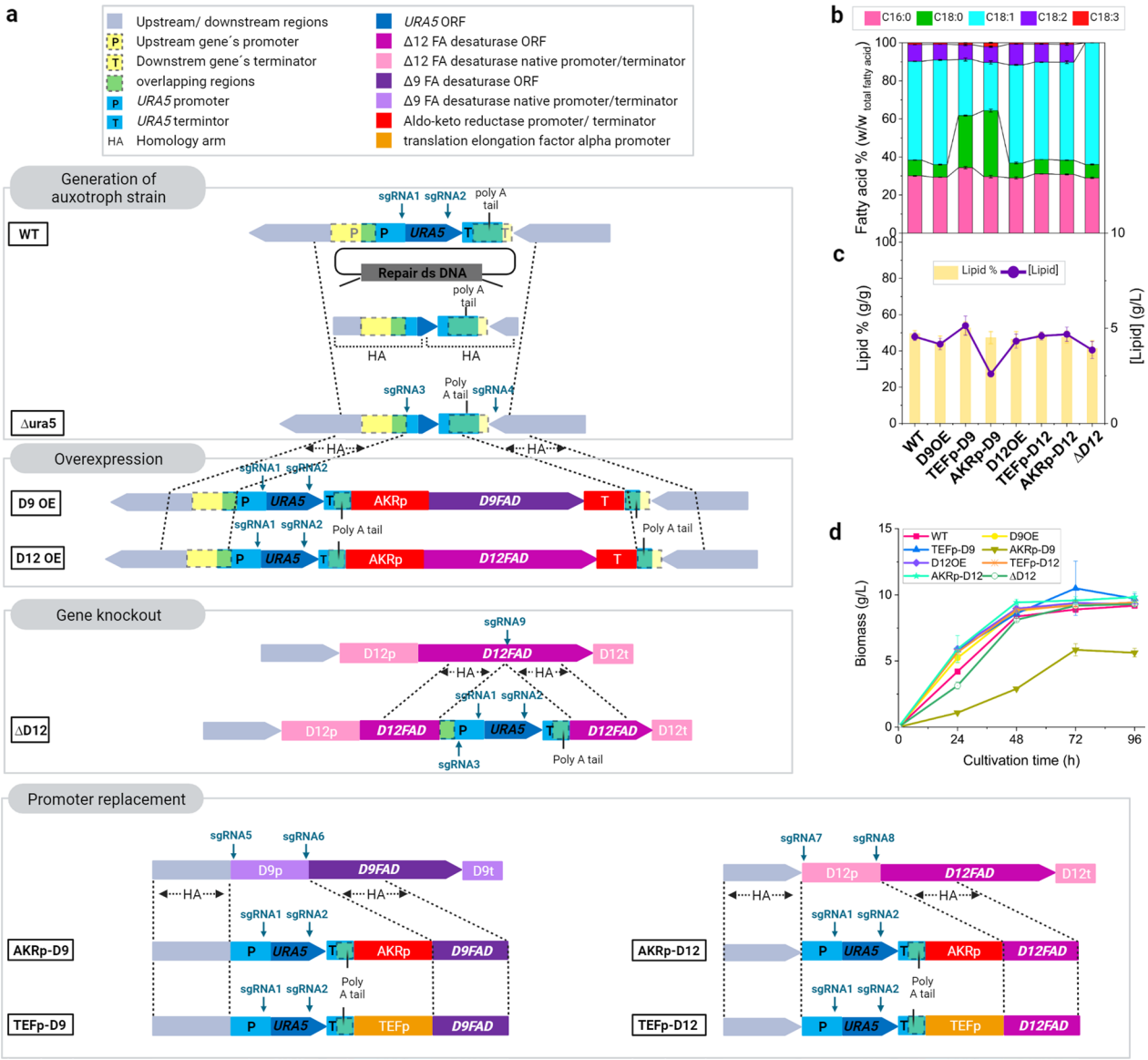


**Fig. 3** Metabolic engineering. **a** The *URA5* gene, including its promoter, was deleted to generate the Δura5 strain (using single guide RNAs (sgRNAs 1 and 2). The 3′ end of the coding sequence (90 bp) and terminator were not deleted, as they contained the terminator elements of the downstream gene. The complete *URA5* coding sequence, with its native promoter and terminator, was used as a selection marker. The *D9FAD* and *D12FAD* overexpression was accomplished by inserting a second copy fused to AKRp and AKRt, and the selection marker into the upstream region of *URA5* locus in Δ*ura5* strain (sgRNAs 3 and 4), thus generating the strains D9OE and D12OE, respectively. The *D12FAD* knockout was carried out by inserting the *URA5* in the Δ12 desaturase locus (sgRNA9). The *D9FAD* promoter exchange was performed by separate insertion of AKRp or TEFp, and simultaneous deletion of the native promoter to modify their transcriptional regulation (sgRNAs 5 and 6), generating the AKRp-D9 and TEFp-D9 strains, respectively. The same strategy was used for *D12FAD* (sgRNAs 7 and 8), resulting in AKRp-D12 and TEFp-D12, respectively. **b** Fatty acid profile, **c** lipid contents and titres, and **d** growth obtained with the WT and engineered *C. oleaginous* strains in MNM + Glu in shake flasks after 96 h cultivation. All data and error bars represent average and standard deviation of biological triplicates. The WT yielded 9.2 ± 0.2 g/L biomass and 50 ± 1.5% [$w_{lipid}/dw_{biomass}$] lipids. The biomass and lipid accumulated by D9OE, D12OE, and TEFp-D9 are comparable to the WT ($p > 0.05$). In Contrast, the AKRp-D9 exhibited lower growth rate (DCW at 5.6 ± 0.3 g/L) but maintained the cellular lipid accumulation levels after 96 h (47 ± 3% [$w_{lipid}/dw_{biomass}$] ($p > 0.05$)). The *D12FAD* knockout and promoter exchange did not affect the ability of the strains to grow and accumulate lipid

was strongly correlated with the growth phase and medium. However, the C18:0 content of the TEFp-D9 and AKRp-D9 mutants remained higher than that of the WT throughout cultivation. Specifically, C18:0 content in TEFp-D9 reached 34% and 26% after 96 h in RM + AA + Glu and MNM + Glu, respectively. This

implies a lower gene expression level under TEFp control in RM + AA + Glu than in MNM + Glu. Contrary to WT, TEFp-D9 with 24% C16:0, 34% C18:0, and 34% C18:1 yielded sufficient abbre to mimic cocoa butter (CB) FAC, while maintaining a high growth rate and total lipid content. Interestingly, the resulting FAC of AKRp-D9 in RM + AA + Glu showed a notable increase in palmitic acid content (up to 39% w/w $_{TFA}$) after 96 h. Consequently, the unsaturated fatty acids (U-FAs) comprised only 28% of the TFAs, including 21% w/w $_{TFA}$ C18:1 and 7% w/w $_{TFA}$ C18:2, similar to the palm oil FAC. This suggests a lower expression rate under AKRp than under TEFp in the RM + AA + Glu. Compared to WT, the AKRp-D9 strain showed a lower growth rate and total lipid content (Fig. 4), possibly because of higher level of S-FAs [21].

## Discussion

The application of a targeted engineering method without decreasing lipid productivity would improve the significance of *C. oleaginosus* as a flexible source of industrial lipid production. Concerning lipid supply chains, this would allow the transition from an ecologically sensitive, horizontal plant-based agricultural system to a sustainable, vertical cellular agriculture system [22]. Moreover, a precise targeted engineering method helps build a robust platform by enabling an in-depth study of metabolism and gene regulation.

To date, engineering of *C. oleaginosus* has been achieved through untargeted methods, such as untargeted mutagenesis and AMT [17, 23–25], which result in unpredictable gene expression levels, which mainly depend on the variable loci and the number of insertion events. Furthermore, random stable insertion of the *Cas* gene using AMT results in various unwanted genetic alterations. Several reports have demonstrated that *Cas* overexpression results in toxic effects, while continuous and stable expression increases the risk of accumulating off-target effects [26–29]. Further, off-target activity of CRISPR/Cas is reduced when delivered in the form of ribonucleoprotein (RNP) compared with that in case of Cas-expressing gene.

To avoid the aforementioned limitations, we successfully developed an efficient Cas-mediated genome editing technique by employing Cas mRNA and RNP. These two elements permitted circumventing the challenges of promoter efficiency and expression levels of Cas genes. Additionally, it offers DNA-free genome editing. The RNPs are degraded within hours, while the Cas mRNA results in a transient expression of Cas protein, which provides effortless cloning and alleviates off-target effects in eukaryotic cells [30, 31].

Electroporation of *C. oleaginosus* spheroplasts provides a rapid procedure for the delivery of all types of gene elements and proteins. In this study, we established a method for targeted *C. oleaginosus* engineering. Efficient spheroplast isolation was achieved by treating yeast cells with an enzyme mixture isolated from the hydrolase-producing fungus *T. reesei*. Using this tailored enzyme system for *C. oleaginosus*, we successfully removed the cell wall and generated spheroplasts amenable to the transfer of all types of genetic elements and proteins. This was not possible using previously available commercial enzymes. Moreover, spheroplasts can be stored at − 80 °C for extended duration to allow staggered transformations. Cell wall removal is a vital step in this process, where the effectiveness and specificity of the enzyme system plays an essential role. Glucanex, a commercial enzyme commonly used for yeast cell wall removal [32], was ineffective against *C. oleaginosus*, and the transfer of all types of nucleotides and proteins was unsuccessful in the resulting spheroplasts. To date, genetic modifications by transferring Cas9 mRNA for genetic modifications have only been performed in higher eukaryotic cells. Our study, for the first time, shows that genome engineering of eukaryotic microbial cells can be accomplished using Cas9 mRNA.

The GC (61%) rich genome of *C. oleaginous* complicates sgRNA design [18]. While the 20 bp sgRNA is believed to specify the Cas9 for targeted sequence, it was previously observed that three to five mismatches in the PAM-distal part can be tolerated [33]. In addition to PAM, the 10 to 12 bp seed sequence of sgRNA located adjacent to PAM, was suggested to generally plays a more important role in on-target binding of the Cas9:sgRNA complex than the rest of gRNA [34]. Therefore, the existence of similar repetitive sequences in the genome of *C. oleaginosus* contributes to the off-target effects. The Cas9 nickases (Cas9n), which require two adjacent sgRNAs for double strand break

(See figure on next page.)

**Fig. 4** Fed-batch fermentation of *C. oleaginosus* strains. Left column: Fermentation using MNM + Glu. Right column: Fermentation using RM + AA + Glu. **a**, **b** Time course of fatty acid composition (FAC) in WT in MNM + Glu and RM + AA + Glu, respectively. **c**, **d** FAC in D9OE strain. **e**, **f** FAC in TEFp-D9. **g**, **h** FAC in AKRp-D9. **i**, **j** FAC in Δd12. **k**, **l** Lipid contents (yellow bars) and lipid titres (purple line), and **m**, **n** growth (dry cell weights) obtained with the WT and engineered *C. oleaginous* strains in MNM + Glu after 96 h of cultivation and in RM + AA + Glu after 72 h of cultivation, respectively. All data and error bars represent average ± standard deviation of biological triplicates. Statistically significant differences between the WT and each engineered *C. oleaginosus* strain were defined using the two-tailed Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

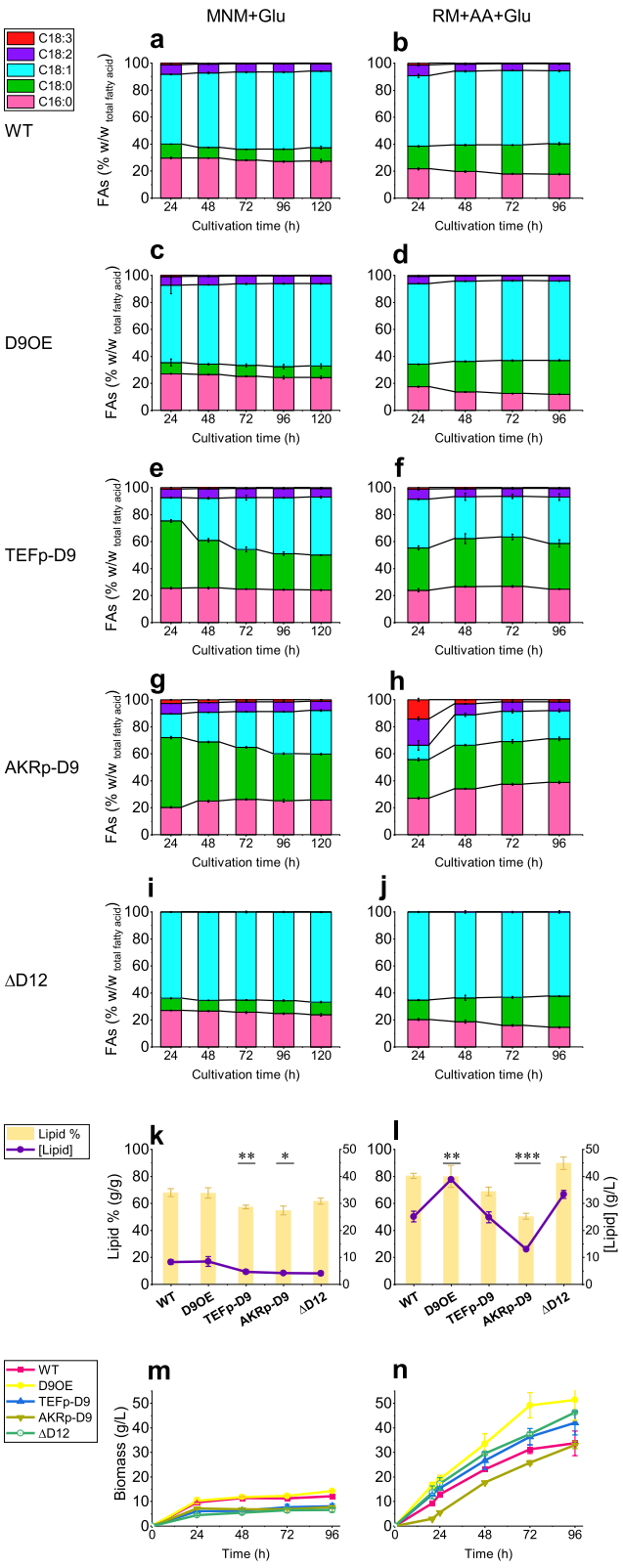


**Fig. 4** (See legend on previous page.)

**Table 1** Measurement of lipid content, titre, and biomass productivity

| | | Lipid | | | | | | Biomass productivity g/Lh | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Content % w/w | SD | Titre g/l | SD | Productivity g/Lh | SD | After 96 h | SD | After 72 h | SD |
| MNM + Glu | WT | 67.7 | 2.84 | 8.26 | 0.85 | 0.11 | 0.01 | 0.13 | 0.01 | 0.12 | 0.01 |
| | D9OE | 67.5 | 3.93 | 8.48 | 1.79 | 0.12 | 0.02 | 0.15 | 0.00 | 0.13 | 0.00 |
| | TEFp-D9 | 57.2** | 1.36 | 4.64** | 0.62 | 0.06* | 0.01 | 0.08** | 0.01 | 0.08* | 0.01 |
| | AKRp-D9 | 54.6* | 3.26 | 4.20** | 0.12 | 0.06* | 0.00 | 0.08** | 0.00 | 0.07* | 0.00 |
| | ΔD12 | 61.3 | 2.20 | 4.03** | 0.65 | 0.06* | 0.01 | 0.07** | 0.01 | 0.07** | 0.01 |
| RM + AA + Glu | WT | 80.1 | 1.82 | 25.1 | 1.96 | 0.35 | 0.03 | 0.47 | 0.07 | 0.43 | 0.03 |
| | D9OE | 79.6 | 8.11 | 38.8** | 0.09 | 0.54** | 0.00 | 0.79* | 0.06 | 0.68* | 0.07 |
| | TEFp-D9 | 68.6* | 3.15 | 24.9 | 2.04 | 0.35 | 0.03 | 0.58 | 0.07 | 0.50 | 0.05 |
| | AKRp-D9 | 50.2*** | 2.04 | 13.0** | 0.14 | 0.18** | 0.00 | 0.46 | 0.02 | 0.36* | 0.01 |
| | ΔD12 | 89.6 | 4.66 | 33.3* | 1.49 | 0.41 | 0.08 | 0.64* | 0.00 | 0.52 | 0.01 |

Lipid analysis was performed after reaching the stationary phase in each medium. In the RM + AA + Glu medium, the analysis was performed after 72 h of cultivation, and in the MNM + Glu medium after 96 h of cultivation in the fed-batch fermentations. The biomass productivity was determined in both media after 72 h and 96 h of cultivation. All data represent averages of biological replicates, and significant differences between the WT strain and each engineered C. oleaginosus strain were defined using the two-tailed Student's t-test; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

*SD* standard deviation

generation, decrease off-target effects [35], therefore, facilitate sgRNA design in *C. oleaginosus*. For the first time, nuclease Cas and nickase Cas, which require one and two guide RNAs, respectively, have been successfully incorporated in *C. oleaginosus* by our strategy. A gRNA library specific for *C. oleaginosus* enabled us to eliminate off-targets prior to transformation. The current library has been designed for Cas9 and its variants that require an NGG PAM. However, this can be extended to a variety of PAM sequences targeted by other Cas variants to further increase the availability of PAM options for any target locus.

This study demonstrates the construction of a series of strains with tailored FAC while maintaining a high lipid content, which can be useful for various industrial applications. Our analysis revealed that FAC with a higher oleic acid content favours higher lipid productivity. Compared to that in WT, lipid productivity increased by 54% and 16% in D9OE and ΔD12, respectively. Therefore, applying the D9OE strain to the high-performance process of lipid production reported for *C. oleaginosus* [8] would yield 3.6 g/Lh lipids. In addition to total lipid yield, lipid composition is of industrial relevance. High-oleic oils can be used in the development of bio-lubricants, hydraulic fluids, and oils for electrical transformers because of their excellent oxidative and thermal stability [4]. Moreover, a high monosaturated FA content without PUFAs accumulation (similar to ΔD12) results in an even higher oxidative stability, which can offer a wider temperature range for various applications [36, 37]. Accordingly, the absence of the two PUFAs (C18:2 and C18:3) in ΔD12 resulted in higher oxidative stability of the oil (Additional File 2: Table S1). Furthermore, C18:1 can be channelled into the production of other valuable compounds such as 10-(R)-hydroxy stearic acid [38, 39] by expressing additional enzymes in the respective strains.

Since 1980, *C. oleaginosus* is being extensively investigated by researchers, namely, in an attempt to increase the C18:0 content in yeast oil to produce CB equivalents by decreasing the oxygen supply [40], random mutagenesis [25], or addition of Δ9-desaturase inhibitor [41]. It was reported that *C. oleaginosus* produces higher amounts of TAGs and POP (C16:0–C18:1–C16:0) than that produced by the other oleaginous yeast strains, which makes this strain a potential source for CB-like lipid (CBL) production, when the C18:0 content is increased [42]. We compared the effects of the *D9FAD* native promoter with those of endogenous TEFp and AKRp. The high levels of C18:1 in *C. oleaginosus* WT due to the high expression levels of *D9FAD* were fine-tuned in the TEFp-D9 strain to resemble the CB profile (iodine value: 44, Additional File 2: Table S1,). Currently, stearic acid is the only material used to produce safe solid fats owing to its neutral effect on serum lipoprotein cholesterol. Additionally, highly saturated oil decreases the need for hydrogenation in food and cosmetic products. Genetically improved sunflowers, for instance, produce elevated amounts of stearic and oleic acid. In contrast, WT sunflower oil is mainly composed of U-FAs, which are liquid at room temperature. Therefore, hydrogenation or transesterification is required for solidification [43, 44].

The saturation was further increased in AKRp-D9 cultivated in RM + AA + Glu, similar to the palm oil composition. It implies that *C. oleaginosus* can also accumulate high-saturation oils (72% S-FAs in AKRp-D9) during growth. Moreover, further optimisation would lead to the creation of a strain which can produce FAs with higher contents of both palmitic and oleic acids, equivalent to those in palm oil.

Demand for plant oils in the food, chemical, and pharmaceutical industries is increasing. However, use of tropical plant oils, such as monocrop palm and cocoa, result in tropical forest destruction and adversely affect biodiversity [43, 45]. Among plant oils, palm oil has the highest market volume (market volume: 73 MT in 2020/21 [46]); January 2022: U.S. $1344/ton [47]), and CB is the most highly priced raw material (more than $5000/ton in 2021 [48]; 0.172 MT CB is produced mainly by three countries [49] with wide applications in the food, chemical, and cosmetic industries. Moreover, high-oleic sunflower oil accounts for 32.94% of the global sunflower oil market [50] market volume: 18.46 MT in 2020/21; $2361/ton in March 2022). The supply and climate impact concerns have increased a customer-driven demand for alternative sources, such as yeast oil, which demonstrate an improved ecological profile. The mutants developed in our study demonstrate potential to address the industry demands and concerns.

## Conclusions

In summary, this is the first report demonstrating the targeted engineering of *C. oleaginosus*. The current study unlocks the significant potential for harnessing *C. oleaginosus* for the production of a wide range of oleochemicals for industrial and academic purposes. Optimised pathways lead to a superior cell factory capable of both high productivity and product diversity. Notably, a series of strains with tailored FAC were constructed. Interestingly, the D9OE strain with overexpressed Δ9-desaturase showed elevated C18:1 content and 54% increase in the lipid yield. Further, the ΔD12-desaturase gene knockout resulted in increased C18:1 content and absence of PUFAs in the yeast oil. Furthermore, the CRISPR-mediated genetic engineering using Cas mRNA delivered to yeast spheroplasts were reported for the first time.

The genetic engineering technique developed in our study offers the opportunity to target further strain improvements, including broader substrate utilisation capability, high tolerance to toxic compounds, tailor-made lipids with desired composition or function. Moreover, this novel approach applied to *C. oleaginosus*, coupled with recent advancements, such as the biorefinery process chain including recycling steps [8] and automatable Nile Red analysis [23], can accelerate the creation of a commercially viable, flexible and robust platform. Furthermore, the procedure of the tailored enzyme system isolation for spheroplasting and delivery of Cas elements by electroporation were crucial steps of this flexible technique, which also helps unravelling the genetic accessibility in other unconventional yeasts.

## Methods

### Strain, media, and chemicals

The *C. oleaginosus* ATCC 20509 (DSM-11815) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DMSZ) (Braunschweig, Germany). The wild-type strain was inoculated and maintained on yeast extract peptone dextrose (YPD) agar plates. The auxotroph mutants were maintained on $YNB^{+}5FOA$ agar plates (yeast nitrogen base [YNB], 1.7 g/L; $NH_4SO_4$, 5 g/L; uracil, 50 mg/L; glucose, 20 g/L; agar, 20 g/L; 5-fluoroorotic acid [Fluorochem, Germany], 1 g/L), and the top agar contained 0.1–1 g/L 5FOA. Mutants harbouring an auxotrophy-complementing marker gene (selection marker *URA5*) were transferred and maintained on $YNB^{-}$ agar plates (YNB, 1.7 g/L; $NH_4SO_4$, 5 g/L; glucose, 20 g/L; agar, 20 g/L). The top agar plate contained YNB (1.7 g/L), $NH_4SO_4$ (5 g/L), glucose (20 g/L), and agarose (5 g/L). YPD was used as the inoculum medium. For cultivation, two media were used: a minimal nitrogen medium containing glucose (MNM + Glu) [17] and nitrogen rich medium containing glucose and acetic acid (RM + AA + Glu). The latter consisted of the following: glucose, 30 g/L; yeast extract, 3 g/L; peptone, 1.5 g/L; $(NH_4)_2SO_4$, 0.3 g/L; $MgSO_4{\cdot}7H_2O$, 1.5 g/L; $KH_2PO_4$, 2.4 g/L; $Na_2HPO_4$, 0.91 g/L; $CaCl_2{\cdot}2H_2O$, 0.22 g/L; $ZnSO_4{\cdot}7H_2O$, 0.55 µg/L; $MnCl_2{\cdot}4H_2O$, 22.4 µg/L; $CuSO_4{\cdot}5H_2O$, 25 µg/L; $FeSO_4{\cdot}7H_2O$, 25 µg/L; pH 6.5. *Trichoderma reesei* RUT-C30 (ATCC 56,765) was obtained from the American Type Culture Collection. The cultivation medium for *T. reesei* contained 10 g/L yeast extract, 10 g/L glucose, 1.4 g/L $NH_4SO_4$, 2 g/L $KH_2PO_4$, 0.4 g/L $CaCl_2{\cdot}2H_2O$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 1 g/L NaCl, 5 mg/L $FeSO_4{\cdot}7H_2O$, 3.7 mg/L $CoCl_2.6H_2O$, 1.6 mg/L $MnSO_4.H_2O$, 1.4 mg/L $ZnSO_4{\cdot}7H_2O$. The *Escherichia coli* DH5α strain was obtained from Merck Millipore and used for cloning and plasmid amplification. DH5α was grown at 37 °C in Luria–Bertani (LB) medium. Transformed *E. coli* was selected on LB agar plates supplemented with kanamycin sulfate at a final concentration of 50 µg /mL (Roth, Germany).

## Generation of targeting and editing constructs

### *Single guide RNAs*

An in-silico sgRNA library was constructed by analysing all scaffolds of the whole genome sequence of *C. oleaginosus* [5] in smaller fragments (using Geneious Prime® 2022.0.1 https://www.geneious.com). All possible PAMs for Cas9 and its variants were determined using Geneious and the corresponding sgRNAs were added to the library (Additional File 1: Data File 1). The off-targets against the whole *C. oleaginous* genome were analysed and on-target activities were determined using the target scoring algorithm reported by Doench et al. [51]. The sgRNAs with zero off-targets and possibly high target scores were selected for genetic engineering and ordered from Synthego (USA) and Eurofins Genomics (Germany) (Additional File 2: Table S2).

### *Repair DNAs*

The repair DNA sequences were partially amplified from genomic DNA and cloned into pUC vectors harbouring the kanamycin resistance gene or ordered from Twist Biosciences (USA), and further assembled into plasmids for each experiment. Plasmid construction was performed in DH5α cells using restriction digestion (Thermo Fisher Scientific) and ligation with T4 DNA ligase (Thermo Fisher Scientific), following the manufacturer's instructions. *E. coli* competent cells were transformed by the heat shock method, plated on LB agar plates containing kanamycin, and incubated overnight at 37 °C. Positive colonies were identified using colony polymerase chain reaction (PCR), inoculated into liquid LB medium, and plasmids were isolated using a GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). Complete repair DNA sequences were linearised by PCR amplification or using fast digest enzymes and resolved by electrophoresis on a 1% agarose gel stained with MIDORI Green Xtra (NIPPON Genetics). Fast digestion reactions were performed according to the manufacturer's instructions (Thermo Fisher Scientific, Germany). The PCR reactions were performed using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) and Phusion GC-Buffer (Thermo Fisher Scientific) according to the manufacturer's instructions. The PCR products or digested gene fragments were recovered from the gel using a Monarch DNA Gel Extraction Kit (NEB) and confirmed by Sanger sequencing (Eurofins Genomics, Germany). All the repair DNAs constructed in this study were validated by sequencing and are summarised in Additional File 3: Table S3.

### *CRISPR-Cas enzymes*

For synthesising Cas9 mRNA, the *Cas9* gene (including nuclear localisation signal [NLS]) was amplified using Q5 high-fidelity DNA polymerase (NEB) to introduce the T7 promoter upstream of the gene. The quality of the PCR was analysed by agarose gel electrophoresis. The PCR product was purified and used for in vitro transcription using a HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB). RNA was treated with DNase I to remove the DNA template, followed by a spin column purification step (NEB Monarch RNA Cleanup kit). Subsequently, capping (NEB Vaccinia Capping System) and poly A tailing were performed using NEB *E. coli* poly A polymerase. Finally, the mRNA was purified using Monarch RNA Cleanup kit (NEB) and stored at − 80 °C until use. Cas9 enzyme (EnGen Spy Cas9 NLS) and EnGen Spy Cas9 Nickase were purchased from New England BioLabs GmbH (NEB, Germany).

## Strain construction

### *Spheroplasting*

A tailored spheroplasting procedure was developed using a previously described method with some modifications [52]. Optimised and efficient spheroplast isolation was achieved by treating the yeast cells with an enzyme mixture isolated from a hydrolase-producing organism, *T. reesei*. This filamentous fungus was cultivated in a cultivation medium supplemented with *C. oleaginous* biomass at 28 °C for 48 h. The supernatant was separated by centrifugation at 2,000 g for 10 min, filtered through a 0.45 µm filter, concentrated 30-fold, and stored at 4 °C.

A single yeast colony was inoculated into 50 mL of YPD medium. The cells were harvested by centrifugation (1,000 g for 5 min) at a density of $2 \times 10^7$ cells/mL and washed once with sterile water and once with 1 M sorbitol. Each lysis enzyme (lysis enzyme from *T. reesei* [HEST] and Glucanex) was mixed separately with the spheroplasting buffer (pH 5.8, 1 M sorbitol, 0.1 M sodium citrate, 10 mM EDTA, 30 mM β-mercaptoethanol), to obtain final concentration of 10% v/v for HEST and 2% w/v for Glucanex. The yeast cells were resuspended in the spheroplast buffer and incubated at 30 °C. The spheroplasting progress was monitored at 15 min intervals, by adding 400 µL of the cells to 100 µL of sodium dodecyl sulfate (final concentration of 1% w/v), followed by measuring the decrease in turbidity ($OD_{600\ nm}$). Cells were harvested after more than 90% of the cells were converted to spheroplasts. The pellets were washed multiple times using 1 M sorbitol to completely remove the lysis enzymes. The cells were resuspended in 2 mL STC (1 M sorbitol, 10 mM Tris–HCl [pH 7.5], and 10 mM $CaCl_2$) and 2 mL frozen cell storage solution (40% glycerol, 14% dimethyl sulfoxide, 0.2 M mannitol, 0.32 M sucrose, 0.1 M sorbitol,

0.2 M trehalose), and stored at − 80 °C until further use for transformation. Cell viability was tested by plating 50 µL of the cells on agar plates containing 1 M sorbitol. For plating, the spheroplasts were added to 5 mL top agar (containing 1 M sorbitol) and plated on agar plates containing 1 M sorbitol. Spheroplasting efficiency was tested by plating 50 µL of yeast spheroplasts onto non-isotonic plates.

#### *Yeast transformation*

The spheroplasts were thawed and washed thrice with 1 M sorbitol. Then, they were resuspended in sorbitol containing 5% electroporation buffer (0.3 mM $Na_2HP0_4$, 0.02 mM $KH_2PO_4$, 10% glycerol), and incubated on ice for 5 min. Finally, the cells were pelleted and resuspended in 2 mL of 1 M sorbitol mixed with 10 µL electroporation buffer. For electroporation, 50 µL of the spheroplast suspension was mixed with the CRISPR/Cas elements and repair DNA; further, this mixture was transferred to an electroporation cuvette with a 2 mm gap (Biolab, Germany) and pulsed once at 1500 V (MicroPulser Electroporator, Bio-Rad, Germany) according to Bio-Rad's instructions The cells were resuspended in 5 mL liquid top agar (below 50 °C), poured onto selective agar plates, and incubated at 28 °C until single colonies were visible. The Δ-9 desaturase gene knockout experiments were performed under four conditions; selection plates supplemented with oleic acid (C18:1) (AppliChem, Germany) incubated at 28 °C; selection plates without C18:1 incubated at 28 °C; selection plates supplemented with C18:1 incubated at 37 °C; selection plates without C18:1 incubated at 37 °C. For all experiments, transformants were streaked on new plates and single colonies were inoculated into the selection medium to grow overnight. The editing efficiency was calculated by dividing the number of the positively confirmed transformants by the total number of clones analysed by PCR. The cultures were centrifuged, and genomic DNA was extracted using a yeast DNA extraction kit (Thermo Fisher Scientific). To analyse genetic modifications, PCR was performed on genomic DNA using genome-specific primers upstream and downstream of the targeted locus and subjected to Sanger sequencing.

### Cultivation

Screening experiments were conducted using 100 mL of liquid MNM in 500 mL baffled shake flasks (120 rpm at 28 °C). Cultivation was initiated by inoculating 100 mL of each medium to an $OD_{600\ nm}$ of 0.1. The transformants with significant FA change, exhibiting high-value lipid composition, were subjected to high cell density cultivation in bioreactors under controlled conditions. High cell density cultivation was performed in a DASbox four parallel bioreactor system (Eppendorf, Germany) with a working volume (V) of 150 mL each (starting $OD_{600\ nm} = 0.5$) using RM + AA + Glu. The pH was adjusted to 6.5 using 3 M NaOH or acetic acid (70%–100% (w/w)), according to Masri et al. [8]. For control, the MNM with glucose (MNM + Glu) was used as the sole carbon source, and the pH was adjusted to 6.5 using 3 M NaOH or 3 M HCL. In all bioreactors, the temperature was set to 28 °C. Dissolved oxygen was maintained at 50% by stirring at 350–800 rpm; controlled aeration (8.0–1.5 vvm), oxygen ratio (21%–100%), and pressure (1.25–1.5 bar) were maintained during the experiment. An antifoam agent (Antifoam 204, Merck) was used to prevent foaming (5% v/v solution). All cultivations were performed in triplicate. The samples were collected every 24 h.

### Analysis

#### *Gravimetric analysis of biomass and lipids*

The dry cell weight (DCW) was analysed by pelleting 2 mL culture and washing the pellets with double distilled water ($ddH_2O$), followed by lyophilisation in preweighed microtubes for 2 days at − 80 °C and 0.04 mbar (VaCo 5, Zirbus Technology, Germany). The intracellular total lipid was measured by extraction using chloroform methanol mixture according to the protocol described by Bligh and Dyer.[53] The harvested cells were washed with $ddH_2O$ and destructed and homogenised using a high-pressure homogeniser (Mulsiflex C3, Avestine, Canada). Lipids from the homogenates were extracted using two sequential solvent extractions with Folch solution (2:1 chloroform: methanol mixture) for 2 h and 1 h, respectively. After extraction, the chloroform layer containing yeast lipids was aspirated under a nitrogen stream and the lipid content was weighed. The lipid content percentage, total lipid, and lipid and biomass productivities were calculated according to equations Eqs. 1, 2, 3, and 4 respectively:

$$Lipid\ content\%w/w = \frac{w\ obtained\ lipid(g)}{w\ obtained\ dried\ biomass(g)} \times 100 \quad (1)$$

$$Total\ lipid(lipid\ titer\ g/L) = \frac{w\ lipid\ obtained(g)}{V\ of\ culture\ extracted(L)} \quad (2)$$

$$Lipid\ productivityg/Lh = \frac{w\ lipid\ obtained(g)}{V\ of\ culture\ extracted(L) \times incubation\ time(h)} \quad (3)$$

$$Biomass\ productivityg/Lh = \frac{w\ biomass\ obtained(g)}{V\ of\ culture\ extracted(L) \times incubation\ time(h)} \quad (4)$$

### *Fatty acid analysis*

Fatty acids composition of the lipids was analysed according to the modified protocol reported by Griffiths et al. [54]. The lyophilised pellets (2–10 mg) were taken in glass tubes and subjected to automated fatty acid methyl esterification using the Multi-Purpose Sampler MPS robotic from Gerstel. For quantification, 500 µL glyceryl trinonadecanoate (C19:0-TAG, 0.2 mg/mL in GC grade toluol) was added to the tubes prior to esterification as an internal standard, followed by mixing at 1000 rpm for 1 min. Next, 1 mL of 0.5 M sodium methoxide dissolved in methanol was added to each tube, heated up to 80 °C, mixed at 750 rpm for 20 min, and then cooled down to 5 °C. Then, 1 mL of hydrogen chloride-methanol solution (Sigma, Germany) was added to each tube, and heated up to 80 °C, mixed at 750 rpm for 20 min, and then cooled down to 5 °C. Finally, 400 µL dd$H_2O$ was mixed with the samples at 1000 rpm for 30 s, followed by mixing with 1 mL hexane with shaking three times at 2000 rpm for 20 s in a quickMix device. The tubes were centrifuged for 3 min at 1000 rpm and cooled down to 5 °C; 200 µL sample of the organic phase was transferred to a 1.5 mL vial for analysis using GC-2025 Plus gas chromatograph with flame ionisation detection (GC-FID) (Shimadzu, Japan) [55]. Standardisation was performed using the fatty acid methyl ester marine oil standard.

**Abbreviations**

| | |
|---|---|
| AMT | Agrobacterium-mediated transformation |
| NHEJ | Non-homologous end joining |
| HDR | Homologous recombination |
| FA | Fatty acid |
| TFA | Total fatty acid |
| gRNA | Guide RNA |
| sgRNAs | Single guide RNAs |
| PAM | Protospacer adjacent motif |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| Cas | CRISPR-associated proteins |
| HEST | Hydrolase enzyme system from *T. reesei* |
| 5FOA | 5-Fluoroorotic acid |
| RNP | Ribonucleoprotein |
| DSB | Double-strand break |
| Cas9n | Cas9 nickases |
| YNB | Yeast nitrogen base |
| YNB + 5FOA | Yeast nitrogen base media containing uracil and 5FOA |
| ssDNA | Single stranded DNA |
| dsDNA | Double stranded DNA |
| YNB − | YNB media lacking amino acids |
| YPD | Yeast extract peptone dextrose |
| WT | Wild-type |
| FAC | Fatty acid composition |
| MNM + Glu | Minimal nitrogen medium containing glucose |
| RM + AA + Glu | Rich media containing acetic acid and glucose |
| DCW | Dry cell weight |
| LB | Luria–Bertani |
| CBL | CB-like lipid |
| GC-FID | Gas chromatography-flame ionization detection |
| HA | Homology arm |
| TAGs | Triglycerides |
| ddH2O | Double distilled water |
| S-FAs | Saturated FAs |
| U-FAs | Unsaturated fatty acids |
| DMSZ | Deutsche Sammlung von Mikroorganismen und Zellkulturen |
| PCR | Polymerase chain reaction |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-023-02033-1.

**Additional file 1.** Data File1.gff

**Additional file 2: Table S1.** The fatty acids content and estimated oil properties based on fatty acid profiles. S-FAs = saturated fatty acids (% w/w total fatty acid), U-FAs = unsaturated fatty acids (% w/w total fatty acid), CN = cetane number, IV = iodine value, HHV = higher heating value (MJ/kg), KV = kinematic viscosity (mm2/s), D = density (g/cm3), SV = saponification value, OS = oxidative stability. The S-FAs and U-FAs contents were determined by gas chromatography described in the methods section. Empirical formulas were used to estimate the lipid properties. All data represent averages. **Table S2.** Single guide RNAs used in the study.

**Additional file 3: Table S3.** Repair DNAs used in the study.

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**Author contributions**

PS, NM, and TB conceived the project. PS designed and carried out experiments, analysed the data, and prepared the manuscript. NM contributed with scientific expertise in molecular biology. TF contributed to data collection on the cultivations. MH, PG, SP, and MM supported the analytical work. All authors read and approved the final manuscript. TB, NM, and PS finalized manuscript preparation.

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**Availability of data and materials**
All data supporting the conclusions of this study are included in this published article (and its Additional files).

## Declarations

**Ethics approval and consent to participate**
Not applicable.

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## Publisher's Note

# 4

# Discussion and Outlook

The emergence of the oil and energy crisis in the 1970s led to an acceleration in research to develop sustainable and renewable alternatives, such as SCOs for chemical and fuel production.[17,78,79] Consequently, the political and scientific interest in biofuel was increased, also due to the limited oil reserves.[21,80] On the grounds that oleaginous yeasts can potentially assist in satisfying the global oil and energy demand, and mitigate climate change, the development of cost-competitive bioprocesses through both cost reduction and productivity enhancement has been the focus of many studies in this field.[5,78] Currently, the cost of YO production is higher than that of the plant oils. The major cost is the media preparation due to several factors such as the cost of the carbon and nitrogen sources. For instance, one effective carbon source is glucose, which accounts for 80% of the total medium cost and approximately 60% of the total production cost. Therefore, exploring low-cost alternative substrates that are abundant and renewable is of great importance towards optimizing the economic process of YOs production for their commercialization.[5,81,82] In addition, due to the transportation expenses, the feedstock supply can also be considered a crucial factor in the final production costs.[44] High feedstock transportation cost is one of the challenges of the supply chain in the emerging bioeconomy era. Thus, exploring locally and regionally available feedstocks would be preferred.[44,83] Moreover, employing various substrates would help alleviate the risk of supply disruptions, including sudden changes in the supply due to unexpected events, and seasonal availability. Securing the feedstock supply would lead to full capacity operations, cost-effective storage, and transportation, hence the economic feasibility of the biorefineries.[83] Alternatively, the production sites could be built in regions with renewable feedstock accessibility, for instance by integrating biorefinery technologies into the existing facilities where residues and side-streams are attainable[44] or by the transformation of existing facilities into biorefineries.[84]

Other variables of the production cost are the fermentation process, downstream processing, and the selected strains.[5,13] The economic feasibility of TAGs microbial production depends upon the biomass and lipid productivity and yield of the strain. In addition to an efficient conversion system, applying low-cost substrates would contribute to profitability.[35] Subsequently, strains that have higher productivity on the low-cost feedstocks are favoured.[40] The high yield and rapid growth of yeast and bacteria favour them in these bioprocesses as opposed to algae, which have slow growth rates .[35] To that end, the productivity of five prominent oleaginous strains on cost-effective feedstocks with marine and terrestrial origins was evaluated in the study entitled ‘Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers’ presented here.[77] In this study, wheat straw and brown algae hydrolysates (WSH and BAH, respectively) were used as

sustainable and renewable carbon sources. Additionally, powdered corn steep liquor (CSL), a by-product of the corn wet-milling industry, as a low-priced source of nutrients and nitrogen from terrestrial origin was tested in combination with the WSH. We also performed control cultivations using synthetic media containing the sugar components of hydrolysates as sole carbon sources. We were able to present a systematic comparison between these well-known strains firstly in their substrate preferences, growth efficiencies, and tolerance against the inhibitory effects specific to each hydrolysate. Secondly, we determined the lipid productivity and yield of each strain growing on both complex hydrolysates. Thirdly, the resulting fatty acid profile for each experiment was detected. The fatty acid profile of the accumulated oil has a major role in its industrial application.[79]

The valorisation of chemically complex, cost efficient lignocellulosic biomass feedstocks, such as straw are crucial in the bio-economy era[85]. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. Agricultural wastes are the major source of hexose and pentose that can be used in the production of microbial-based products.[86] These feedstocks have been used and scaled up in numerous microbial bioprocesses like ethanol production.[86,87] For instance, straw hydrolysates as a low-cost substrate is studied vastly throughout various bioprocesses, such as co-generation of bioethanol and electricity by yeast[88], yeast lipid and carotenoid production[89-91] , biobutanol[92], biohydrogen, and biogas production[93]. The conversion of lignocellulosic biomass to YOs comprises two steps: biomass hydrolysis and pre-treatment which is a mature industry and started ca 30 years ago; and conversion of fermentable sugars to TAGs by oleaginous yeasts, where utilizing the liberated pentose (mainly xylose), and tolerating inhibitors are the associated challenges[78]. Various detoxification methods have been used for lignocellulosic-based hydrolysates, including physical (such as neutralization, activated charcoal treatment[89], and calcium hydroxide over-liming[94] [also in combination with ion exchange or ethyl acetate]), and biological methods for instance enzymatic removal using laccase.[95] The detoxification of such hydrolysates results in sugar loss, high cost, and complication of the process.[45] For example, in ethanol production using willow hydrolysate, the detoxification step accounted for 22% of the final production cost.[96] Therefore, screening suitable strains can help unlock the vast utilization of these feedstocks in microbial TAGs production. Exemplary, strains tolerant to the inhibitory effects of the lignin-derived aromatic compounds liberated during the pretreatment process, as well as strains that can utilize both glucose and xylose, are preferred. By comparing the growth and biomass yield ($g_{biomass}/g_{sugar}$) of each strain in MNM-Glu, MNM-Xyl media to that in the WSH, we demonstrated that *C. oleaginous*, *T. asahii*, *R. glutinis*, and *R. mucilaginosa* (all studied strains except *R. toruloides*) are tolerant to the potential inhibitory effects of lignin-

derived compounds in this hydrolysate, and in fact, they exhibited a higher biomass yield (per each gram sugar utilized) in WSH compared to the synthetic control media. The maximum lipid content, total lipid, and DCW in WSH were achieved by *C. oleaginosus*. Further, *T. asahii*, *R. glutinis*, and *R. mucilaginosa* consumed a lower percentage of glucose and xylose in WSH compared to BAH. These could indicate a better growth ability of *C. oleaginosus* in presence of the growth inhibitors in comparison with the other strains tested, i.e., a higher tolerance to these compounds. Importantly, also in another comparative study, *C. oleaginosus* yielded a higher lipid concentration than *Y. lipolytica* (ATCC 20460) and *L. starkeyi* (ATCC 12659) using both detoxified and non-detoxified wheat straw hydrolysate [97]. In a recent study, the evaluation of thirty-six yeast species for aromatic metabolism showed that aromatic metabolism is mostly seen in basidiomycetes, and mostly in *Rhodotorula*, *Rhodosporidiobolus*, *Cutaneotrichosporon*, and *Trichosporon* genera.[98] Moreover, *C. oleaginosus* has been shown to metabolize aromatics (phenol, 4-hydroxybenzoic acid (pHBA), and resorcinol as sole carbon sources), hence, suggested to be advantageous in industrial phenolic wastewater and lignin valorisation.[99]

Furthermore, we showed that by adding CSL, despite a decrease in C/N ratios in the complex medium, the yeast strains remained oleaginous, and in fact, an accelerated lipid accumulation process was observed in these strains. Further, *T. asahii*, *R. glutinis*, and *R. mucilaginosa* also exhibited enhanced growth and higher sugar uptake in the presence of CSL. In a previous study, *Cutaneotrichosporon oleaginosum* ATCC 20508 (identical to *C. oleaginosus* ATCC 20509 [100]) reached 0.25 g/Lh lipid productivity in fed-batch cultivation using crude glycerol supplemented with CSL and deoiled recycled *C. oleaginosus* lysate to reduce the nutrient cost.[101] Our data show that CSL can be used as a cost-effective nutrient source in combination with other feedstocks to ensure enhanced growth. In particular, considering the recent advancements in *C. oleaginous* cultivation using acetic acid in combination with other feedstock such as recycled yeast biomass or brown algae hydrolysate as substrates[70], CSL can play an effective role to decrease nitrogen costs, especially under nitrogen-rich conditions, and boost the growth efficiencies.

Unlike terrestrial biomass, macroalgae benefit from a lack of lignin and low hemicellulose content, which competitively lowers the costs of the required pretreatment. Further, they can grow under poor environmental conditions due to their strong adaptabilities, such as in seawater and saline-alkali land, hence fresh water, and costly nutrients and fertilizers are not needed for their cultivation. Importantly, they reach a much higher biomass productivity per unit area compared to terrestrial lignocellulosic resources due to their fast growth rate and high photosynthetic efficiency. In particular, brown algae have higher areal productivity

compared to red and green algae and lower protein content (3- 15 % by weight [102]). Therefore, these feedstocks can as well be considered sustainable and renewable substrates with high sugar contents for the industrial production of value-added bioproducts.[103-106] However, there are several challenges associated with seaweed valorisation. For example, the high water content increases transportation costs and results in high energy consumption during the dehydration process.[107] Moreover, a life cycle assessment (LCA) of YOs production using the oleaginous yeast *Metschnikowia pulcherrima* from macroalgae estimated between 2.5 and 9.9 (kg $CO_2$ eq. $kg^{-1}$ refined oil) climate change impact, while the seaweed cultivation is accounted for 39% of the final impact. However, this process yielded lower climate change impact compared to microalgae and terrestrial plant oil productions.[17,103] To that end, several studies demonstrated a synergic effect in an algae and yeast co-culture. The $CO_2$ produced by yeast can be captured by algae, and the generated oxygen via photosynthesis is used by yeast for growth and lipid production, which in turn leads to a reduction in aeration cost. In fact, intensified lipid productivity is expected when both algae (mainly microalgae) and yeast were oleaginosus.[17,108-112]. Having said that, one of the most efficient oleaginous yeast strains, *C. oleaginosus*, yet encounters a knowledge gap in regard to algal co-cultivations and thereof potential synergistic effects. In other terms, there have been no publications so far illustrating the ability of this particular strain under co-culturing conditions. Therefore, screening different oleaginous microalgae strains, such as *Chlorella* sp., individually under co-culturing conditions with *C. oleaginosus* and determining and analysing the resulting growth efficiency and lipid yield would be the first step for evaluating *C. oleaginosus* potential for such a bioprocess. It is noteworthy that some organic acids released by yeast during growth, which decrease the pH, can be taken up by microalgae for its growth.[113] Hence, the off-gas, dissolved oxygen, pH, and extracellular metabolites should be analysed to determine the synergistic effects.[114] Moreover, the nutritional mode is one of the key technological aspects of co-cultivation.[113,115] Thus, comparing photoautotrophic, heterotrophic, and mixotrophic conditions for microalgae in these co-cultivations would give further information for optimizing such a bioprocess for *C. oleaginosus*.

Importantly, *C. oleaginosus* was recently reported to obtain 0.59 g/Lh using *L. digitata* hydrolysate and acetic acid, and 2.4 g/Lh using acetic acid and yeast hydrolysate (recycled resources) as the substrates in a high-performance process, estimating a climate impact at 3.56 kg $CO_2$ eq. $kg^{-1}$ produced oil.[70] In contrast, the lipid productivity in the aforementioned LCA study for *M. pulcherrima* was considered 0.52 g/Lh. Also, the lipid productivity of this yeast strain was measured in different cultivation strategies. Abeln et al. reported that *M. pulcherrima* obtained a maximum 0.37 g/Lh lipid productivity in semi-continuous cultivation

mode where the culture medium contained a high glucose concentration and was fed with recycled cells.[116] Later, the effects of volatile fatty acids (VFAs) on the growth of *M. pulcherrima* were investigated. Li and colleagues demonstrated that this strain yields the highest lipid content (13.8%) when cultivated on acetic acid compared to other VFAs, however, its growth was inhibited at higher acetic acid concentrations (10 g/L and higher).[117] To that end, analysing the effect of various VFAs with different concentrations on the growth and lipid production of *C. oleaginosus*, and in particular in combination with CSL, would provide useful information for further enhancement of the existing processes, as this strain exhibited a relatively high potential in valorisation of acetic acid. Notably, the application of macroalgae (such as *L. digitata*) hydrolysates as substrates is also reported for microalgae.[118] Subsequently, such a use of combined feedstocks (e.g. VFAs, recycled cells, and macroalgae hydrolysates) could be expanded to co-cultures of oleaginous *C. oleaginosus* and microalgae.

There is vast literature addressing the valorisation of algal biomass into valuable bioproducts; including bioenergy (such as ethanol production using wastewater algal biomass and biohydrogen using *A. platensis* and *L. digitata* hydrolysates); organic acids and biochemicals (such as carotenoid using Laminaria japonica hydrolysates)[119]; Polyhydroxyalkanoates (PHAs); biochar; fatty acids; and biodiesel.[104] The selection of algae strain depends on the resultant substrate availability after the hydrolysis step.[104] To date, there are several pretreatment methods, such as chemical (e.g. acidic pretreatment that leads to inhibitor formation), physical (that increases the energy cost, yet is not fully effective), and biological methods. The latter provides a more advantageous, green, and mild biomass processing using enzymes.[119] An optimized, energy-effective enzymatic hydrolysis of *L. digitata* was presented in a previous study, which resulted in 62.5% biomass conversion into soluble fermentable sugars.[69] We used this method to ensure a high hydrolytic rate of BAH and maximize the resource utilization in yeast cultivations. Interestingly, the biomass yield ($g_{Biomass}/g_{Sugar}$) of *C. oleaginosus* in BAH was measured higher than that in WSH, despite the higher lipid productivity in WSH. Further, looking at the biomass yield of *C. oleaginosus*, *T. asahii*, and *R. toruloides* in MNM-Man, which is much higher compared to the yield in glucose- and xylose-containing media, it could be concluded that mannitol, a unique compound in brown macroalgae hydrolysate[104], contributes to biomass build-up significantly. The effect of sugar alcohols, such as mannitol and sorbitol on yeast (*M. pulcherrima*) growth was previously studied and showed that these sugar alcohols can contribute to growth.[120]

Altogether, based on this systematic comparison, we were able to demonstrate that *C. oleaginosus* is best performing in terms of substrate utilization, tolerance to the potential inhibitory effects of the lignin-derived compounds, simultaneous sugar uptake, growth, and

lipid productivity on cost-effective substrates with both terrestrial and marine origins. Needless to say, *C. oleaginosus* is also reported to have the most diverse sugar metabolism amongst the well-known oleaginous yeasts.[91] Moreover, it is the first yeast known to utilize lignin-derived aromatics and channel them through lipogenesis.[99] Further, It depicts the highest lipid yield in the history of oleaginous yeast.[70]

While research on strain exploration is ongoing, investigations have been in progress as well to genetically modify the strains to introduce the desirable traits and outcomes, such as enhanced inhibitor tolerance, altered fatty acid profile, improved growth rate, and elevated lipid yield. Modification and adjustment of fatty acids profile, for instance, has been an essential step on the way to enabling industrial use of YOs. For this reason and also due to restrictions associated with random gene editing methods, environmental factors such as oxygen[121], temperature[122], and media[77] have been investigated to alter the fatty acid profile.

However, the genetic tractability of *C. oleaginosus* has been challenging since the first attempt for its commercialization in 1988 to produce microbial equivalents of cocoa butter (CB), an important ingredient of chocolate. Interestingly, Ykema et al. reported 1 g/ Lh lipid productivity by *C. oleaginosus* (formerly known as *Apiotrichum curvatum*) on whey-permeate as the substrate with partial recycling. However, a drop in CB price at that time resulted in ceasing YO production.[123] In the following years, Ykema et al. created random mutations in *C. oleaginosus* using N -methyl-N'-nitro- N'-nitrosoguanidine (MNNG) to optimize the strain for a more economically viable microbial CB equivalent (CBE) production. Because *C. oleaginous* lipid profile provides a higher C18:1 and a lower C18:0 percentage compared to the range required for CBE.[124] Having screened 200.000 colonies, they were able to isolate 6 mutants that were stable unsaturated fatty acid auxotrophs (Ufa) with a defective Δ9-desaturase. Subsequently, many researchers carried out various random mutagenesis methods for *C. oleaginosus* to improve its genetic accessibility, for instance, mutagenic treatments with EMS[125], UV irradiation[126], and fast neutron irradiation.[127] Nevertheless, random mutagenesis methods established until now are limited to bringing about base-pairing errors, transversions, frameshift mutations, base transition and deamination, and small insertions/deletions. In addition, since the identification and isolation of all DNA changes are not possible, screenings for narrowing down to the best-fitting strain are time-consuming and challenging.[128-130]

Importantly, in 2016, the complete genome sequences of *C. oleaginosus* have been determined, and its transcriptomics analyses under different growth conditions identified up- and down-regulation of genes under limiting and non-limiting conditions and different carbon source availability.[72,131,132] Next, *Agrobacterium tumefaciens* mediated transformation (AMT)

of *C. oleaginosus* was established, which enabled stable and efficient insertion of genes of interest in the genomic DNA for the first time.[63] However, AMT leads to the occurrence of multiple insertions in various random loci, accompanied by gene inactivation, due to considerable genomic alterations such as deletions, inversions, or translocations.[133] In addition, this method did not yet facilitate targeted gene knockouts or site-specific gene insertions.

Recently, a proteomic study on carbohydrate oligomer utilisation of *C. oleaginosus* elucidated the abundance of proteins involved and their expression patterns. Also, a potential signal peptide was determined by investigating secreted enzymes.[134] Moreover, a metabolic model was constructed including 1553 reactions in 11 compartments, to understand its lipid accumulation on glycerol as an inexpensive carbon source.[132,135]

Although *C. oleaginous* was investigated for over 100 years and has shown numerous advantages, its limited genetic tractability has been a barrier to harnessing its full potential and further strain developments and characterizations.[131] To this end, we investigated the employment of the CRISPR/Cas system in *C. oleaginosus*.

The CRISPR/Cas system, which is divided into two classes, was discovered as adaptable for gene engineering in 2012. The CRISPR/Cas9 in type II (most widely used) consists of three components: an endonuclease (Cas9), and guide RNA (gRNA) including the CRISPR RNA (crRNA), and trans-activating crRNA (tracrRNA). The crRNA is a 20-nucleotide sequence that recognizes and pairs with the targeted site within the genome, while tracrRNA links the crRNA to the Cas9 enzyme. Subsequently, the Cas9 is able to bind to that specific locus on the genome, provided that a Protospacer adjacent motif (PAM) is recognized upstream (5' end) of the target.[136-139]

A double-strand break (DSB) is created 3 bp upstream of the PAM motif by one of the two distinct lobes of Cas9, the nuclease (NUC) lobe, which has two endonuclease domains, RuvC- and HNH-like nuclease domains. Both domains are needed to cut both DNA strands.[136] In Cas9 derivatives (dCas9), the catalytic residues in these two domains (H840A in HNH and D10A in RuvC) are mutated to convert the nuclease activity of Cas9 to nickase activity, as only one strand can be cut.[140] Hence, these Cas9 derivatives require two adjacent sgRNAs that target opposite DNA strands to generate the required DSB, consequently, decreasing off-target effects [140], therefore, facilitating sgRNA design in *C. oleaginosus* with a high GC content.

The gRNA-base functionality of CRISPR/Cas allows for simpler, faster, and more cost-effective genetic engineering in comparison with previously reported methods such as zinc

finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) that rely on producing a site-specific nuclease.[136,137,141]

During the last decade, the CRISPR/Cas system has been established in various yeast strains. The most studied yeast for CRISPR/Cas system is *S. cerevisiae*, owing to its wide employment in bioprocesses. However, due to limitations that *S. cerevisiae* encounters, nonconventional yeasts have been investigated as potential platforms with unique characteristics in areas that are not industrially feasible with *S. cerevisiae*. Therefore, some nonconventional yeasts have been also the target of gene engineering using Cas9.[141,142] Naturally, the development of the CRISPR/Cas system in other yeasts was mostly found to be more challenging compared to that in *S. cerevisiae*.[143] For instance, the lack of a portable promoter for sgRNA expression in *Schizosaccharomyces pombe* (used for heterologous production of mammalian proteins) hindered the development of CRISPR/Cas genome editing in this yeast. Subsequently, a combined expression vector harbouring Cas9 and sgRNA sequences using promoter/leader RNA of K RNA (*rrk1*) was suggested for Cas9-mediated genetic engineering.[144] A more laborious example is the nonconventional yeast, *Pichia pastoris*, which is used for the production of pharmaceutical proteins and industrial enzymes. The development of the CRISPR/Cas system in this strain was accomplished after testing 95 combinations of various Cas9 DNA and gRNAs sequences expressed under different promoters, out of which 6 constructs were reported functional.[145,146]

Amongst 1500 unconventional yeasts identified until today [147], only a few have been reported for the availability of CRISPR/Cas genetic engineering tools. Other examples include *Kluyveromyces marxianus* (Ethanol and volatile acetate esters production) [141,148], *Hansenula polymorpha* -previously *Pichia angusta* or *Ogataea polymorpha*- (protein production such as hepatitis B vaccine and biosynthesis of chemicals, e.g. ethanol).[145,149,150] The CRISPR/Cas system used in *S. cerevisiae* was adopted for the industrially relevant yeast *Kluyveromyces lactis* [151], nonetheless, this system was not functional in the well-known unconventional oleaginous ascomycete *Yarrowia lipolytica*. In *Y. lipolytica* expression cassette is transferred by lithium acetate transformation or electroporation using an auto-replicating plasmid.[152] A two-plasmid CRISPR system was first developed for this strain in which a Cas9 and sgRNA expression plasmid along with a donor DNA harbouring plasmid was transferred by lithium acetate transformation.[153] Gao et al. has subsequently reported a single-plasmid CRISPR/Cas system in *Y. lipolytica*.[154]

In the herein presented study entitled ‘Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector’ [71], we were

able to establish the CRISPR/Cas system as the first targeted genetic engineering method for *C. oleaginosus*. The established process alleviates the barriers towards the genetic tractability of this promising strain by enabling the successful delivery of Cas elements both in protein and nucleic acid form. The CRISPR/Cas-based gene engineering is carried out by transferring (1) genes coding Cas9 and gRNA into the genome, (2) Cas9 enzyme and gRNA in the form of ribonucleoprotein (RNP) to the nucleus, and (3) Cas9-coding mRNA and gRNA into the nucleus (Figure 4). In our current technique, both Cas RNP and mRNA could be transferred into the nuclei, resulting in precise and targeted gene editing. The implementation of Cas9 in *S. pombe* showed that the overexpression of Cas protein in cells results in toxic effects, thereby eliminating the Cas9 function and its associated genome manipulation.[144] Therefore, it is suggested to use a moderate-strength promoter for Cas expression or to adjust the plasmid copy number.[155] However, the promoters of *C. oleaginosus* have not been fully studied. In addition, there are no autonomously replicating sequences (ARS) identified in *C. oleaginosus* in order to construct stable plasmids for transformations. The development of the CRISPR/Cas system in oleaginous basidiomycete *R. toruloides* also faced a similar challenge. Therefore, the cas9 expression cassette was integrated into the genome via AMT [156] and via lithium acetate transformation [157] due to the lack of autonomously replicating sequences (ARS). This may be associated with a constitutive sgRNA and Cas9 expression that lead to toxicity and off-target activities.[158]

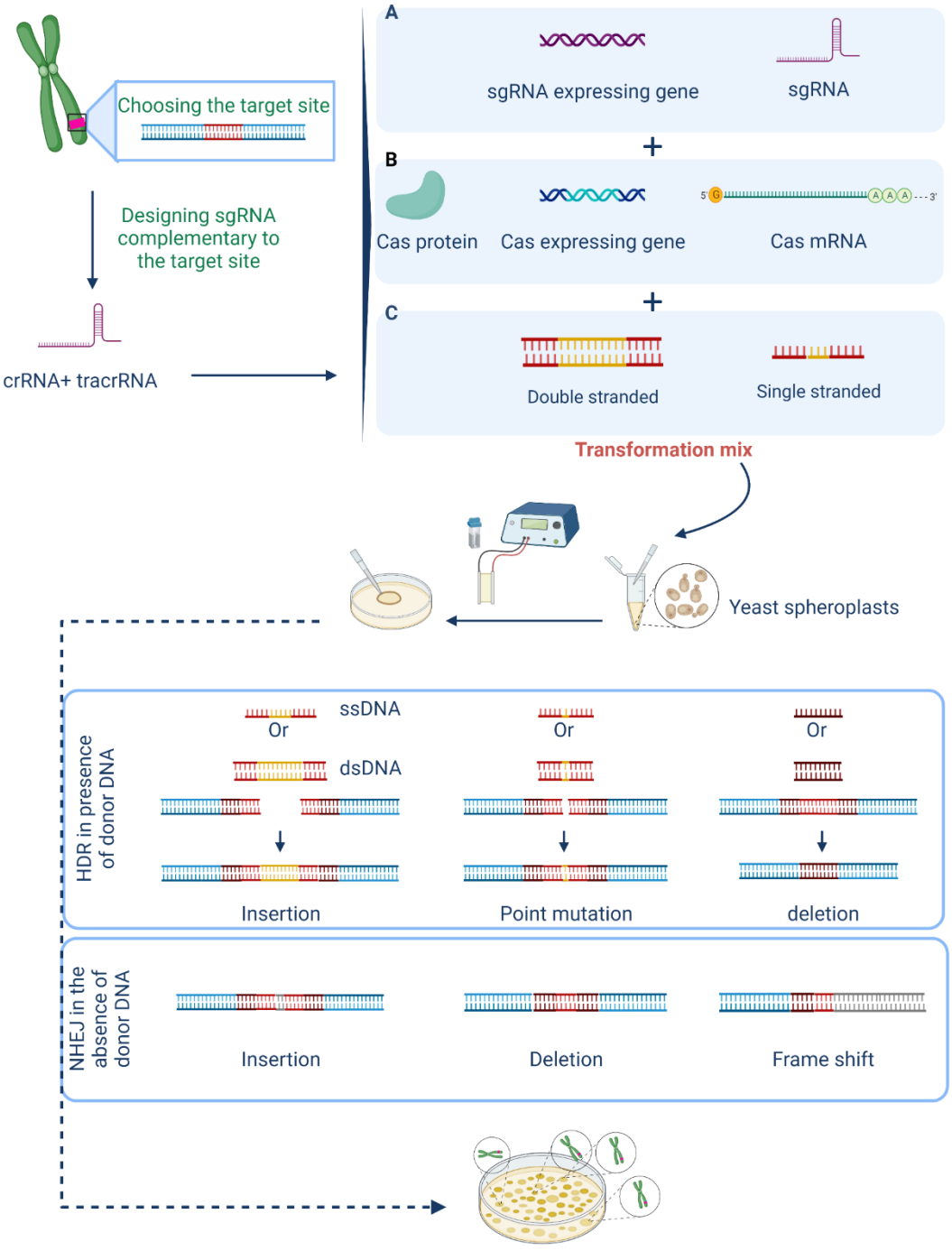


**Figure 4** *Overview of CRISPR/Cas-mediated genetic engineering.*
*The transformation mix consists of three components: A) The guide RNA can be introduced in the form of sgRNA or guide RNA-expressing gene. B) Cas can be delivered in protein, mRNA, and Cas-expressing gene forms. C) When needed, ssDNA and dsDNA can be used as the donor sequence.*
*In our current technique, chimeric sgRNA, Cas protein and mRNA, and ssDNA and dsDNA were delivered via electroporation to yeast spheroplasts.*

Notably, the AMT method was reported to result in the insertion of different numbers of T-DNA (single or multiple copies) in random positions in other yeasts and fungi.[133,159] Therefore, achieving an optimized number of RNP units using the AMT-based insertion of Cas9- and gRNA-coding genes into the genome within the nucleus is challenging. Furthermore, studies have shown that the off-targeting activity of CRISPR/Cas is reduced when delivered in the form of ribonucleoprotein (RNP) compared with that in the case of the Cas-expressing gene. However, the existence of a cell wall in yeasts hinders the delivery of proteins and nucleic acid by electroporation. To unravel their delivery to the nucleus through the robust cell wall of *C. oleaginosus,* we were able to isolate a customized enzyme mixture that allows for the efficient isolation of viable spheroplasts. Moreover, in the current Cas-mediated genome editing technique, we were able to circumvent the time-consuming plasmid construction, lack of stable plasmids with optimized copy numbers and steady expression across the population, and laborious optimization of the expression level of Cas9 and sgRNA under various promoters, by using a plasmid-free transient Cas9 delivery strategy.

To date, web-based computational tools have been developed to facilitate the gRNA design with minimized off-target effects for several yeast strains such as *S. cerevisiae*, *C. albicans*, *P. pastoris*, *K. lactis*, and *Y. lipolytica*.[160] However, the lack of such a tool for *C. oleaginous* prevents the successful development of the CRISPR/Cas system in this yeast. In particular, due to the high GC content and repetitive sequences of this strain, the design of such genetic constructs is challenging and complicated.[131] Hence, the *in-silico* library built in the study entitled 'Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector' permitted the selection and design sgRNAs for recognition of wild-type SpCas9 PAM (5′-NGG-3′) that do not exhibit any off-target sites on the whole *C. oleaginosus* genome. This library can further be widened to include other PAM sequences in engineered Cas9 variations, to target previously inaccessible loci.

Targeted genetic engineering is essential to unlock the potential and improve the capacity of *C. oleaginosus* for diverse biotechnological applications. For example, several factors challenge the profitability of a bioprocess like the wheat straw conversion to YOs, namely lipid yield, tolerance to growth inhibitors, and lack of co-products of industrial and economical importance. Metabolic engineering to introduce ethanol production by fermenting hexose sugar present in these hydrolysates could enhance the mass and energy balance. Ethanol could be used as opposed to methanol in the transesterification step to produce biodiesel (FAME).[161,162] To this category also belongs other examples such as lipases[163], carotenoids[164-166], gluconic acid[167], citric acid[168], itaconic acid, triacetic acid lactone[169], eicosapentaenoic acid[164], and exopolysaccharides.[170] To that end, genetic engineering can contribute to the

economic viability of CSO production. Moreover, different applications require different FA profiles. Any alteration in the fatty acid profile changes the lipid properties. For example, highly saturated oils are in favour of the cetane number, cold flow behaviour, and kinematic viscosity of biodiesel, while lipids containing unsaturated fatty acids support the desired density and high heating value for biodiesel.[22] Therefore, complete genetic tractability creates the foundation for strain optimization via metabolic engineering, which is required for commercial applications.

The lipid composition and fatty acid profile depend on the yeast species, but also environmental conditions, such as the carbon source [77], oxygen [121], and temperature [122]. We were able to induce various stable FA profile alterations in *C. oleaginosus* using the established CRISPR/Cas technique in our study including high oleic oils and highly saturated oils mimicking the FA profile of CB which retails between $5000 and 8000 per tonne.[103] High oleic oils were obtained by deleting the ∆12-desaturase gene (89.5% w/w lipid content leading to 21 g/L oleic acid titer, and 61% w/w lipid content leading to 22.9 g/L oleic acid titer), as well as overexpressing ∆9-desaturase (and 61% w/w lipid content leading to 22.9 g/L oleic acid titer). High oleic acid content results in high thermal stability and a low pour point, which are desired properties for lubricants.[171,172] The current sources for high oleic oils are plant oils, such as sunflower, soybean, palm, and peanut oils, with limited global supply and associated with environmental issues. In this context, metabolic engineering of the well-known *Y. lipolytica* via the implementation of a push-pull-block strategy was recently carried out to develop strains for the production of high oleic acid oils.[173] The developed CRISPR/Cas technique in *C. oleaginosus* creates the foundation for implementing metabolic engineering strategies, such as push and pull, or push-pull-block strategies for further strain improvements, namely, activated and improved substrate uptake, resistance to growth inhibitors in complex and cost-effective feedstock, lipid yield enhancement and composition optimizations, and deleting the competing pathways. In addition to metabolic engineering, genome engineering, such as yeast chromosome construction, and transcriptional regulation have been accomplished using CRISPR systems.[138] Further, the CRISPR-mediated gene disruption permits a profound characterization of genes and promoters and their roles in each pathway.[148]

Importantly, the created knowledge in the *C. oleaginous* performance and behaviour on various substrates.[69,77,99,174,175] carbohydrate-active enzyme expressions[134], tolerance to effects of growth inhibitors[77,99], growth conditions and media[176,177], transcriptome data, high-performance fermentations processes[70], and high-throughput automatable Nile Red analysis[127] can lead to efficient strain optimizations and Omics-informed genetic engineering via CRISPR/Cas technique. The genetic tractability of *C. oleaginosus* coupled with its high

performance and lipid yield paves the way towards a robust platform for superior tailored oil production.

## 4.1 Concluding remarks

The oleaginous yeasts have shown promise as the third-generation feedstocks for oleochemicals and bioenergy rather than plant feedstocks. With over-dependence on fossil resources and imbalance in plant oils demand and supply, the yeasts as an emerging microbial call factory can play a vital role in many industries to overcome environmental issues such as climate change, deforestation, land conversion, and biodiversity endangerment.

The unconventional yeast, *Cutaneotrichosporon oleaginosus*, in particular, showed high potential in comparison with other yeasts throughout the history of oleaginous yeasts. The presented study here provides further insights into the substrate utilization, tolerance to the potential inhibitory effects of the lignin-derived compounds, simultaneous sugar uptake, growth, lipid productivity on cost-effective substrates with both terrestrial and marine origins, and targeted engineering of *C. oleaginosus*. We were able to develop an efficient, flexible targeted gene editing technique which is a crucial element in building a robust platform for the biotechnological and industrial application of this promising strain. Further system biology studies including multi-omics analysis can in the future guide metabolic engineering design and strain improvements toward economically feasible tailored oil productions.

This genetically modified oleaginous yeast successfully produced equivalents of cocoa butter and sunflower oil at a high yield. Hence, this can serve as highly valuable raw material in the food, chemical, and cosmetic industries to stabilize the supply chain, especially in light of global supply chain insecurities invoked by the COVID pandemic and the Ukraine war.

In conclusion, the developed technique in the herein presented work shows a high potential for the genetic engineering of unconventional yeasts in the future, to maximize the conversion efficiency of biomass such as forestry- and agricultural residues and food waste to high-value products such as food, feed, biomaterials, and bioenergy in the bioeconomic era.

# 5
# List of Publications

**A waste-free, microbial oil centered cyclic bio-refinery approach based on flexible macroalgae biomass**

Mahmoud A. Masri, Wojciech Jurkowski, **Pariya Shaigani,** Martina Haack, Norbert Mehlmer, Thomas Brück

Original research article, Applied Energy, Masri et.al. 2018

DOI: 10.1016/j.apenergy.2018.04.089

**Identifying carbohydrate-active enzymes of Cutaneotrichosporon oleaginosus using systems biology**

Tobias Fuchs, Felix Melcher, Zora Rerop, Jan Lorenzen, **Pariya Shaigani**, Dania Awad, Martina Haack, Sophia Prem, Mahmoud Masri, Norbert Mehlmer, and Thomas B. Brueck

Original research article, Microbial Cell Factories, Fuchs et.al. 2021

DOI: 10.1186/s12934-021-01692-2

**Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers**

**Pariya Shaigani**, Dania Awad, Veronika Redai, Monika Fuchs, Martina Haack, Norbert Mehlmer, Thomas Brueck

Original research article, Microbial Cell Factories, Shaigani et.al. 2021

DOI: 10.1186/s12934-021-01710-3

**Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector**

**Pariya Shaigani**, Tobias Fuchs, Petra Graban, Sophia Prem, Martina Haack, Mahmoud Masri, Norbert Mehlmer, and Thomas Brueck

Original research article, Microbial Cell Factories, Shaigani et.al. 2023

DOI: 10.1186/s12934-023-02033-1

**Patent: A method for genetic modification for high CG content microorganisms**

**Pariya Shaigani**, Thomas Brück, and Norbert Mehlmer

Pending, European patent register Nr. 21 217 144.1

# 6

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**Oleaginous yeasts- substrate preference and lipid productivity: a view on the performance of microbial lipid producers**

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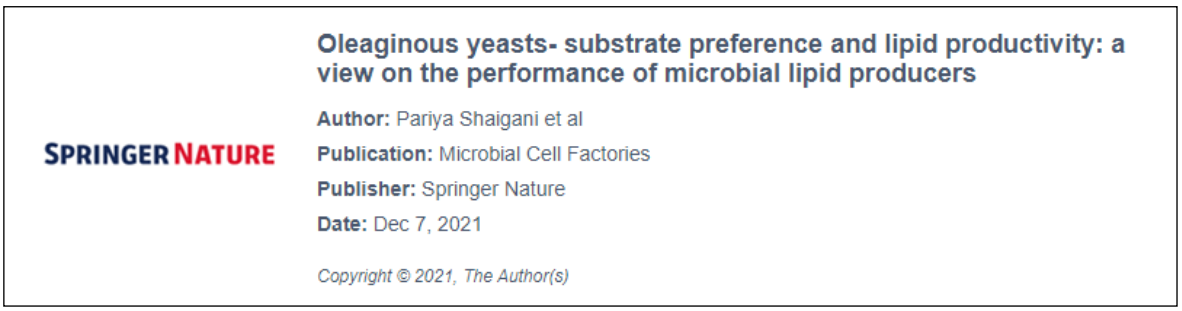




**Mastering targeted genome engineering of GC-rich oleaginous yeast for tailored plant oil alternatives for the food and chemical sector**

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# 7
# Appendix

## 7.1 Abbreviation

SCO: single cell oil; WSH: wheat straw hydrolysate; BAH: brown algae hydrolysate; CSL: corn steep liquor; LB, Luria–Bertani; YPD, yeast extract peptone dextrose; MNM: Minimal nitrogen medium; MNM-Glu (same as “MNM+Glu”): minimal nitrogen medium containing glucose; MNM-Xyl: minimal nitrogen medium containing xylose; MNM-Man: minimal nitrogen medium containing mannitol; MPM-GlcNAc: minimal phosphate medium containing N-acetylglucosamine; RM+AA+Glu: Rich media containing acetic acid and glucose; GC-FID: gas chromatography-flame ionization detection; DCW: Dry cell weight; YO: yeast oil; h: hours; FA: fatty acid; w: weight; AMT: Agrobacterium mediated transformation; NHEJ: Non-homologous end joining; HDR: Homologous recombination; FA: fatty acid; gRNA: guide RNA; sgRNAs: single guide RNAs; PAM: protospacer adjacent motif; CRISPR: Clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated proteins; Cas9n, Cas9 nickases; HEST: Hydrolase enzyme system from *T. reesei*; 5FOA: 5-Fluoroorotic acid; RNP: Ribonucleoprotein; DSB: double strand break; YNB: yeast nitrogen base; $YNB^{+}$5FOA: YNB media containing uracil and 5FOA; ssDNA: single stranded DNA; dsDNA: double stranded DNA; $YNB^{-}$: YNB media lacking amino acids; WT: wild type; FAC: fatty acid composition; TFA: total fatty acid; CB: Cocoa butter; CBL : CB-like lipid; CBE: CB equivalent; V: volume; HA: Homology arm, TAGs: triglycerides, $ddH_2O$: double distilled, , S-FAs: saturated FAs, U-FAs: unsaturated fatty acids; GC-FID: gas chromatography-flame ionization detection; DMSZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; PCR: polymerase chain reaction

## 7.2 Figures and Tables

# 8

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