Technische Universität München
Fakultät für Chemie
Werner Siemens-Lehrstuhl für Synthetische Biotechnologie

Upstream Bioprocess Development of Microbial lipids

Dania Awad

Vollständiger Abdruck der von der Fakultät für Chemie der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender		apl. Prof. Dr. Wolfgang Eisenreich
Prüfer der Dissertation	1.	Prof. Dr. Thomas Brück
	2.	Prof. Dr. Tom Nilges
	3.	Prof. Dr. Johannes Kabisch

Die Dissertation wurde am 29.07.2020 bei der Technischen Universität München eingereicht und durch die Fakultät für Chemie am 28.10.2020 angenommen

Abstract

With an estimated 50 years reserve of fossil fuels, the search for alternative energy sources and sustainable oleo-chemical building blocks is gaining momentum. To that end, Single Cell Oils (SCOs) offer a potentially sustainable solution. Since WW II, interest in SCOs has been tightly linked with fluctuating petroleum prices, lingering uncertainty in the realm of the geopolitical situation and allocation of resources. Commercialization of SCOs remains hindered by high production costs (e.g. feedstocks). Hence, any significant improvement in space-time yields is central to the accelerated industrial deployment for this promising technology. This work pertains to the upstream bioprocess development of microbial lipids from *Cutaneotrichosporon oleaginosus*. This prominent oleaginous yeast is industrially favorable due to its wide substrate spectrum, high lipid titers and resistance to inhibitory compounds. Under nitrogen-limiting and carbon excess conditions, this yeast can accumulate more than 60% ($g.g^{-1}$ DCW) lipids. In this work, we scrutinize the role of carbon and nitrogen in the fermentative potential of this yeast. We initially employed a computational Response Surface Methodology (RSM) to guide and streamline an experimental media optimization matrix, covering of a wide range of elemental carbon and nitrogen ratios and titers. Under optimal conditions (elemental C: N of 120:1), we subsequently provide new insight into *C. oleaginosus* physiology under variable nutritional states, including 12 nitrogen and 10 carbon sources. In accordance with literature, complex and organic nitrogen sources, such as yeast extract and urea, were favored by this yeast for biomass accumulation. Furthermore, this yeast performed well on all sugar sources (particularly lactose), except sugar alcohol, sorbitol. In this course of this work, we also developed a high-throughput, microtiter plate (MTP)-scale process analytical tool (PAT) for the absolute quantitation of intracellular lipids. This method adopts the inherent lipids of *C. oleaginosus* as a standard for Nile red analysis. With the appropriate correction factor, oleic acid was also proposed as readily available and easy-to-handle standard. This method was successfully employed in the course of strain development undertaken in this work. To that end, we opted for the classical approach given the limited genetic accessibility of this yeast. We report, for the first time, the irradiation of an oleaginous yeast via fast neutrons for the rapid generation of mutants. Isolation of enhanced variants was carried out via cerulenin-resistance screening coupled with the PAT, developed in this work. A mutant displaying enhanced growth and another displaying higher lipid content, in comparison with the wild type strain, were isolated via this approach. Subsequently, biofuel properties for the wild type strain and an enhanced mutant were addressed and found to fall within the range of international standards of biodiesel. The implementation of automatable high-throughput workflow in this work paves the way for the integration of robotic systems in

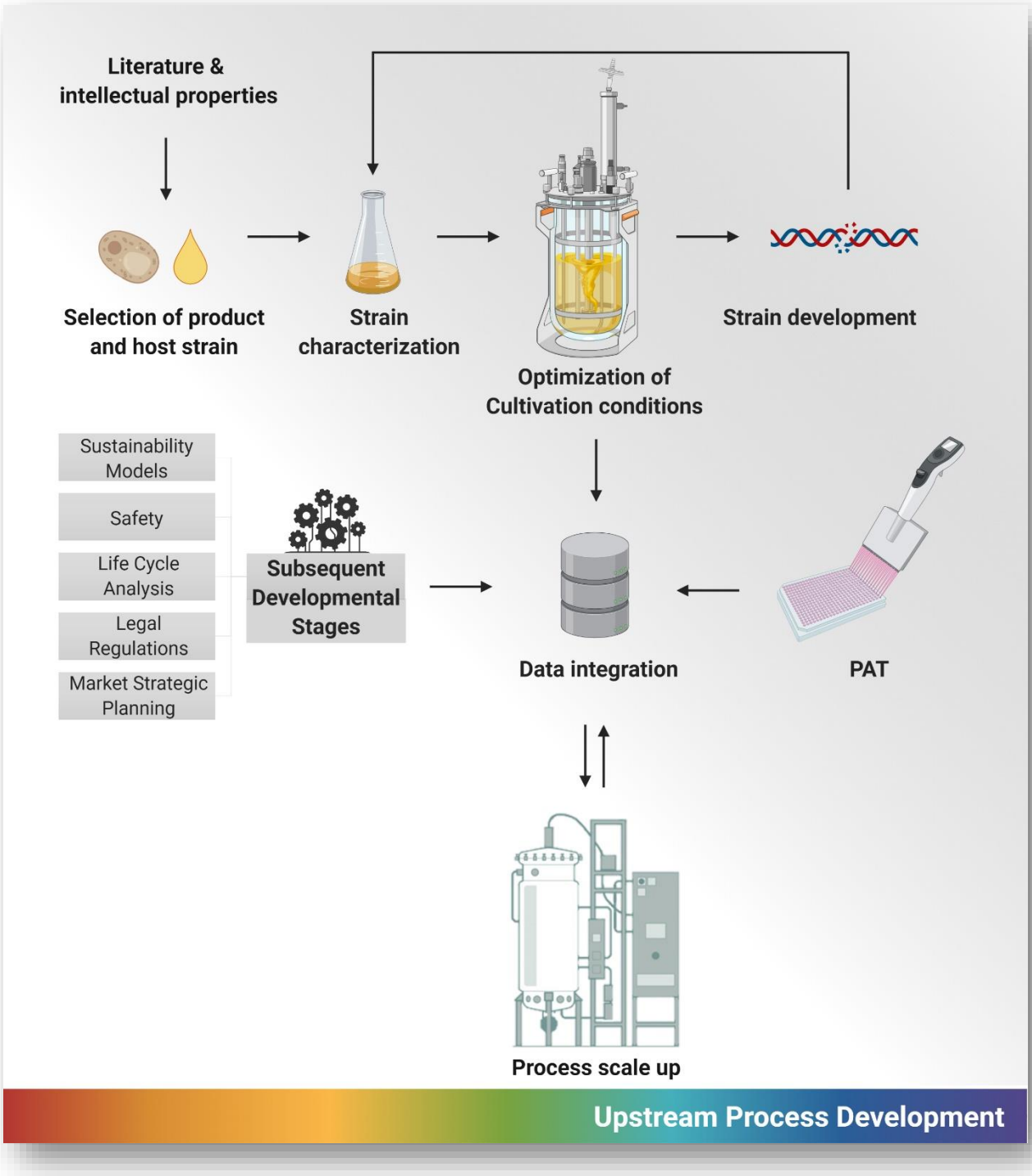
bioprocesses, which greatly reduces development efforts and improves market impact capacity.

Zumsammenfassungen

Mit einer Reserve an fossilen Brennstoffen von geschätzten 50 Jahren hat die Suche nach alternativen Energiequellen und nachhaltigen oleochemischen Bausteinen an Dynamik gewonnen. Zu diesem Zweck könnten mikrobielle Öle (SCO‘s) eine nachhaltige Lösung darstellen. Seit dem zweiten Weltkrieg war das Interesse an SCOs eng mit den fluktuierenden Ölpreisen sowie drohenden Unsicherheiten bezüglich der geopolitischen Situation und der Ressourcenverteilung verbunden. Zudem stehen die hohen Produktionskosten der Kommerzialisierung von SCOs weiterhin im Weg (z.B. Rohmaterialien). Daher ist jede signifikante Verbesserung der Raum-Zeit-Ausbeute von zentraler Bedeutung für das Vorantreiben der industriellen Anwendung dieser vielversprechenden Technologie. Die hier vorliegende Arbeit bezieht sich auf die intiale Bioprozess-Darstellung von mikrobiellen Lipiden aus *Cutaneotrichosporon oleaginosus*. Diese bedeutende oleogene Hefe eignet sich aufgrund ihres großen Substratspektrums, hohen Lipidtitern und Widerstandsfähigkeit gegenüber inhibitorischen Stoffen hervorragend für industrielle Anwendungen. Unter Stickstoff limitierenden Bedingungen, kann diese Hefe, gekoppelt an ein Kohlenstoff-Überangebot mehr als 60 % Lipide akkumulieren ($g.g^{-1}$ DCW). In dieser Arbeit beleuchten wir die Rolle von Stickstoff und Kohlenstoff im Hinblick auf das fermentative Potential dieser Hefe. Zunächst verwendeten wir die in-silico Response Surface Methode (RSM) an, um eine experimentelle Medien-Optimierungsmatrix anzuleiten und zu vereinheitlichen. Dadurch wurde eine große Bandbreite an elementaren Kohlenstoff zu Stickstoff-Verhältnissen und –Konzentrationen abgedeckt. Unter optimalen Bedingungen (120:1 von elementarem C:N) liefern wir anschließend neue Einblicke in die Physiologie von *C. oleaginosus* unter verschiedenen Nährstoffvorraussetzungen, darunter 12 Stickstoff- und 10 Kohlenstoffquellen. In Übereinstimmung mit der Literatur wurden von dieser Hefe komplexe und organische Stickstoffquellen wie Hefeextrakt und Harnstoff zur Akkumulation von Biomasse bevorzugt. Weiterhin konnte diese Hefe, mit Ausnahme des Zuckeralkohols Sorbitol, alle Zuckerquellen - insbesondere Laktose - metabolisieren. Im Verlauf dieser Arbeit entwickelten wir außerdem ein *High-Throughput* Prozessanalyse-Werkzeug (*process analysis tool*, PAT) im Mikrotiterplatten (MTP)-Maßstab zur absoluten Quantifizierung intrazellulärer Lipide. Diese Methode bedient sich der inherenten Lipide von *C. oleaginosus* als Standart für Nil-Rot-Analysen. Mit einem angemessenen Korrekturfaktor konnte Ölsäure ebenfalls als leicht erhältlicher und einfach zu handhabender Standart identifiziert werden.. Diese Methode wurde erfolgreich zur Stammentwicklung, welche im Verlauf dieser Studie durchgeführt wurde, eingesetzt. In Anbetracht der begrenzten genetischen Zugänglichkeit dieser Hefe, entschieden wir uns für die klassische Herangehensweise. Wir konnten erstmalig die Bestrahlung oleogener Hefen mit schnellen Neutronen zur rapiden Generierung von Mutanten

zeigen. Die Isolierung verbesserter Varianten wurde durch Cerulenin-Resistenz-Screening, gepaart mit dem PAT, welches in dieser Arbeit entwickelt wurde, erzielt. Durch diese Methode konnte im Vergleich zum Wildtypstamm eine Mutante mit verbessertem Wachstumsverhalten, sowie eine weiterere mit erhöhtem Lipidgehalt isoliert werden. Anschließend wurden die Biodiesel-Eigenschaften des Wildtypstamms sowie der verbesserten Mutante adressiert, welche sich als konform mit internationalen Standarts erwiesen. Die Implementierung eines automatisierbaren *High-Throughput* Arbeitsablaufs ebnet den Weg für die Integration von Robotiksystemen in Bioprozessen. Dies senkt den Entwicklungsaufwand drastisch und steigert somit den möglichen Markteinfluss.

Graphical Abstract



Bioprocess development strategies adopted in this work include strain characterization, optimization of cultivation condition and strain and process analytical Tools (PAT) development.

Acknowledgements

This work has been kept on track and seen through completion with the support and encouragement of many generous and kind well-wishers. Thank you does not seem sufficient, but it is said with appreciation and respect to my advisor Professor Dr. Thomas Brück for giving me the opportunity to pursue my doctoral degree in his research group. Prof. Dr. Brück provided an optimal environment to exercise and communicate science. Your mentorship, constant motivation, guidance and understanding are valued gifts.

I would like to cordially thank Prof. Dr. Wolfgang Eisenreich, Prof. Dr. Tom Nilges, Prof. Dr. Johannes Kabisch for their evaluation of my dissertation work.

I warmly thank my WSSB family, and in particular, the group leaders Dr. Norbert Mehlmer, Dr. Daniel Garbe and Dr. Farah Qoura for offering their valuable feedback and advice in the course of the many lively discussions, which contributed in many ways to the success of this study. I extend my thanks to Ms. Martina Haack, Ms. Guelnaz Celik, Ms. Veronika Redai and Ms. Petra Graban for their valuable analytical and technical support and warm friendship.

To my colleagues, Dirk, Elias, Felix B., Felix M., Jan, Johannes, Kati, Kevin, Mahmoud, Marion, Markus, Matthias, Max, Michael, Nate, Niko, Pariya, Samer, Sophia, Tobias, Wojtech, Wolfgang and Zora, thank you for making this journey an unforgettable experience. “Ich bin euch dankbar, dass Ihr mir geholfen habt, Deutsch zu lernen“.

Words are short to express my deep sense of gratitude towards my parents, Jamal and Bassam, for their unconditional love, support and the values they instilled in me. I am forever indebted to you for encouraging me to explore new directions in life and seek my own path. I would like to pay my high regards to Hania, who is nothing less than a mother to me. Thank you for being relentless in urging me to grow and guiding me through life. To my loving brother, Hadi, you might be younger, but I definitely look up to you. Thank you for being there for me, always.

To my loving family, your encouragement and constant motivation pushed me to heights I never thought possible, for that I dedicate this milestone to you.

Table of Contents

CHAPTER I SCIENTIFIC BACKGROUND

1 Introduction

1.1 Oleo-chemicals and Biofuels

Based on the current Reserves-to-Production (R/P) ratio, British Petroleum (BP) estimates that the world's fossil fuel reserves, would last: about 130 years of coal, and roughly 50 years of both oil and natural gas (*Statistical Review of World Energy 2020*) [1]. Ironically, former Saudi Arabian oil minister Sheikh Zaki Yamani told *The Economist*: "The Stone Age did not end for lack of stone, and the Oil Age will end long before the world runs out of oil" [2]. The projected decrease in fossil fuel reserves, the fluctuations in the price of crude oil, geopolitical turmoil, energy supply and energy security, in addition to escalating environmental pollution and climate change, have collectively driven the search for alternative energy sources. Recently, oleo-chemicals and biofuels are receiving considerable attention as potential alternatives to fossil fuel and petrochemicals [3,4].

Oleo-chemicals are bio-derived, renewable, biodegradable and have a low CO_2 footprint [4,5]. Derived from oils and fats, the basic oleo-chemicals are fatty acids, fatty alcohols, fatty amines and fatty acid methyl esters, with glycerol as a by-product [3]. These basic compounds can be converted by a variety of chemical, physical and/or biochemical methods to produce high-value end-products [4,5]. Oleo-chemicals are utilized in a wide range of applications including transportation fuels (e.g. biofuel, biodiesel), personal care (e.g. cosmetics, soap, shampoo) and industrial products (e.g. paints, lubricants, rubber, bioplastics). Worldwide, millions of tons of oleo-chemicals are produced each year, with vegetable oils representing the major feedstock for their production [3,4]. Among the popular vegetable crops for oil production, palm is currently the world's main contributor, followed by soybeans, rapeseed and sunflower oils. Of the 200 million metric tons (MMT) of vegetable oils produced worldwide in 2019, more than 20% were utilized for the production of biofuels and oleo-chemicals [5]. Although these first generation oleo-chemicals offer significant advantages over petroleum and petrochemicals, the overall carbon footprint, limited renewability, environmental damage, and above all, the food versus fuel competition, established its major drawbacks [6].

1.2 Single Cell Oils (SCOs)

Lipids and oils, naturally produced by all living macro- and microorganisms, play essential structural and functional roles such as the formation of the lipid-bilayer membranes of cells and organelles [7]. Oleaginous microorganisms can accumulate more than 20% ($g.g^{-1}$) lipids of their dry cell weight (DCW)[8,9]. These microbial lipids - termed single cell oils (SCOs) - are present almost invariably in the form of triacylglycerols (TAGs); esters derived from glycerol and three fatty acids [10]. TAGs serve as the major storage of energy and carbon in algae, yeast

and fungi [11,12]. Only select bacterial species accumulate lipids, which are then stored as biopolymers such as polyhydroxybutyrates (PHB) and alkanaoates, rather than TAGs [10]. The extent of lipid accumulation in oleaginous microorganisms is predetermined by the genetic constitution; maximal lipid contents vary greatly among various species and even among individual strains (**Table 1**) [13-15]. The terms microbial oils, lipids, TAGs and SCOs, will henceforth and throughout the dissertation, be considered as synonymous.

Table 1. SCOs accumulation in oleaginous microorganisms [13,14,16,17].

Microorganisms	**Lipid content** (% $g.g^{-1}$ DCW)	**Microorganisms**	**Lipid content** (% $g.g^{-1}$ DCW)
Algae		**Bacteria**	
Scenedesmus sp.	16-58	*Acinetobacter calcoaceticus*	27-38
Botryococcus braunii	25-75	*Arthrobacter AK 19*	78
Dunaliella sp.	37-58	*Arthrobacter sp.*	40
Nannochloropsis sp.	31-68	*Bacillus alcalophilus*	18-24
Nitzchia sp.	45-47	*Gordonia sp.*	13-52
Schizochytrium sp.	50-77	*Rhodococcus opacus*	24-66
Chlorella sp.	28-63		
Isochrysis sp.	22-53		
Neochloris oleoabundans	30-56		
Haematococcus pluvialis	30-40		
Fungi		**Yeast**	
Aspergillus oryzae	57	*Candida curvata*	58
Aspergillus terreus	57	*Cryptococcus sp.*	34-70
Entomopthora coronate	43	*Cunninghamella japonica*	60
Humicola lanuginose	75	*Debaryomyces etchellsii*	61-66
Morterella vinacea	66	*Lipomyces starkeyi*	30-60
Mortierella isabelline	86	*Rhizopus arrhizus*	57
Mortierella elongate	24	*Rhodosporidium toruloides*	56-72
Trichosporon cutaneum	39	*Trichosporon fermentans*	35
		Trichosporon pullulans	65
		Yarrowia lipolytica	38-67
		Cutaneotrichosporon oleaginosus	60-74

1.3 The History of SCOs

For decades, microorganisms have been exploited for the production of valuable compounds including enzymes, proteins, amino acids, antibiotics, and vitamins [18-20]. Remarkably, the idea of utilizing microorganisms for SCOs production dates back to the 19th century. Paul Linder,

professor at the Agricultural University of Berlin, was the first person to develop a small-scale process for fat production from the yeast *Endomyces vernalis*, currently known as *Trichosporon pullulans* [21,22]. Throughout WWI and WWII, German researchers began to explore oleaginous microorganisms as an alternative to vegetable oils, which were increasingly in short supply [23,24]. Notably, a factory producing yeast-derived SCOs from straw and sawdust as feedstock was constructed in Germany during WWII [23]. Research on oleaginous microorganisms was also conducted in Sweden in the 1940s [25]. The development of SCOs continued to grow during the first 4 decades of the last century. Unfortunately, progress was slow due to the lack of large-scale fermentation technology and the absence of genetic tools [22,26]. Nevertheless, great advances were made in the identification of oleaginous microorganisms, the evaluation of their oil production capacity and the determination of factors influencing its accumulation [22,26]. The development of fermentation technology in the mid-50s, the advancement of genetic engineering in the late-60s, and the oil crisis of the 70s, rekindled the interest in microbial-derived SCOs [26,27]. In the last 2 decades, uncertainties surrounding the availability of fossil fuels and the price of crude oil, in addition to mounting environmental concerns fueled a flurry of research on renewable energy sources, in particular, SCOs [28-31].

1.4 SCO Platforms for Oleo-chemicals Production

The use of oleaginous microorganisms as production platforms for oleo-chemicals offers a potentially sustainable alternative to plant-derived oils [17,32-34]. The cultivation of microorganisms is independent from seasonal or climatic constraints, requires no arable land nor precious resources (e.g. fresh water), does not increase deforestation practices and does not jeopardize food security. Microorganisms enjoy fast growth rates and consequently fast production rates, can utilize waste biomass as feedstock, have a small carbon footprint, and can be easily modified to produce tailored fatty acid profiles [9,17,32,35,36].

Production of oleo-chemicals and biofuels, such as biodiesel, requires fatty acids (FA) feedstocks possessing a very specific profile. SCOs, in particular, have attracted industrial interest due to their specific and precise biochemical and biophysical properties [34,35]. The major fatty acids of oleaginous microorganisms are oleic (C18:1), palmitic (C16:0), and stearic (C18:0), in addition to linoleic (C18:2), α-linolenic (C18:3) and palmitoleic (C16:1) [37-39]. This is also the dominant fatty acid profile of plant-oils used for biofuel and biodiesel production, including palm, canola and sunflower oil [9,40-42]. Fatty acid composition of microbial lipids is species- and strain-dependent (**Table 2**) [41,43]. Cultivation conditions (e.g. temperature, oxygen), substrate and media components, growth phase and age of culture greatly influence fatty acid profiles [43-45].

Table 2. Fatty acid composition of various oleaginous microorganisms in addition to plant-oils [16,17,38,46-50].

Lipid Source	Fatty acid composition (% g.g^{-1})				
Yeast	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**
Cutaneotrichosporon oleaginosus	32	-	8	57	8
Lipomyces starkeyi	34	6	5	51	3
Rhodosporidium toruloides	18	-	17	57	2
Yarrowia lipolytica	11	6	1	28	51
Rhodotorula glutinis	37	1	3	47	8
Trichosporon pullulans	15	-	2	57	24
Fungi					
Rhizopus arrhizus	18	-	6	22	9
Cunninghamella japonica	16	-	14	48	4
Algae					
Chlorella sp.	40	3	8	30	-
Nannochloropsis sp.	26	24	3	28	4
Scenedesmus obliquus	16	3	4	50	8
Vegetable oils					
Palm	32-60	-	1-8	27-52	5-14
Sunflower	3-10	-	1-10	14-65	20-75
Canola	1-5	-	1-3	55-60	18-21

1.4.1 Microalgae and Fungi

Commercial interest in SCOs focused primarily on oleaginous microalgae, including *Scenedesmus*, *Chlamydomonas*, *Chlorella* and *Nannochloropsis* species [51,52]. These microorganisms photosynthetically convert atmospheric CO_2 into intracellular lipids [53]. In addition to lipid production, microalgae enjoy favorable ecological advantages. Contrary to terrestrial crops, biomass generation from microalgae has high space and time yields [33]. Every year, around 280 tons/ha of algal dry biomass are produced worldwide in comparison to 3.9 tons/ha of plant biomass [54]. More interestingly, microalgae exhibit high carbon-fixation ability, sequestering 513 tons/ha of CO_2 per year [55,56]. However, drawbacks such as 1) inherently low biomass densities, 2) lengthy fermentation times, 3) difficulties in processing large volumes (dewatering) and harvesting (flocculation), 4) inconsistent growth due to seasonal variation and 5) predisposition to contamination by faster growing microorganisms, stalled the successful application of oleaginous microalgae at industrial-scale [15,36,51,57]. Although some species accumulate high lipid contents (>60% g.g^{-1} DCW), oleaginous fungi are mainly exploited for the production of specialty lipids including γ-linoleic acid (GLA ; 18:3), arachidonic acid (ARA; 20:4), eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). These fatty acids are of nutritional and pharmaceutical importance [17,58].

1.4.2 Yeasts

For millennia, yeasts have been harnessed for biotechnological applications in most societies around the world. The baker's yeast *Saccharomyces cerevisiae* has been utilized for the production of bread, beer, wine and other products [59]. Fermented beverages and foods have a unique economic and cultural importance, and the development of fermentation technologies is deeply rooted in their history. Accordingly, *S. cerevisiae* has been dubbed the "first domesticated microorganism" [60]. Yeasts are the preferred microorganisms for biotechnological applications because, in general, they are genetically accessible, easy to handle and have a long history of safe use as relatively few yeast species are known to be pathogenic. Pharmaceutical industries utilize yeasts, such as *S. cerevisiae* and *Pichia pastoris*, as hosts for production of recombinant proteins [59,60]. Oleaginous yeasts are particularly useful for sustainable production of SCOs for oleo-chemical and biofuel industries. Compared to microalgae and fungi, yeasts are easier to cultivate, have shorter duplication time, higher growth rates and production titers [46]. They can handle high sugar loads, tolerate growth inhibitors, and are viable for multiple generations [41]. Oleaginous yeasts can accumulate lipids from various low-value raw materials, including agricultural residues, waste streams and industrial by-products. They are capable of utilizing sugar carbon sources (pentoses and hexoses) as well as non-sugar carbon sources (e.g. ethanol, lactate, glycerol and acetate) [13,41]. Furthermore, during fermentative growth of yeasts, bacterial contamination can be controlled by decreasing the pH [59]. Oleaginous yeasts have thus emerged as a more robust platform for industrial production of oleo-chemicals and biofuels, outperforming both fungi and microalgae [41,59]

1.5 Lipogenesis in Yeasts

Approximately 5% of yeast species are considered oleaginous, with *Yarrowia lipolytica* [61,62], *Rhodosporidium toruloides* [63,64], *Lipomyces starkeyi* [65,66] and *Cutaneotrichosporon oleaginosus* [67,68] attracting the most attention within oleo-chemicals and biofuels research [69,70]. From an ecological perspective, oleaginous yeasts are generally found in relatively dry, nutrient-poor environments, where the storage of lipids is advantageous and essential for survival [71]. Lipids, being resistant to desiccating conditions due to their hydrophobic nature, would constitute a more robust nutrient storage than carbohydrates [59]. Lipid accumulation in oleaginous yeasts is not a constitutive function. Rather, it is an adaptive response to particular environmental factors, and a feature of unbalanced metabolism [33]. When essential nutrients are sufficiently present in the cultivation medium, growth and new cells synthesis occur, with minimal lipid accumulation. It is only when the cells run out of - or are deliberately deprived of

- a key nutrient (e.g. nitrogen, phosphorus, sulfur), along with excess carbon, that lipid accumulation begins [46]. Under these conditions, excess carbon is converted into fatty acids and TAGs, which are stored in specialized organelles termed lipid bodies or lipid droplets [59]. When oleaginous yeasts enter a phase of low carbon, the accumulated lipids are mobilized to generate energy and biomass. *R. toruloides*, *Y. lipolytica* and *C. oleaginosus* can accumulate more than 70% ($g.g^{-1}$ DCW) of lipids, whereas the non-oleaginous yeasts *S. cerevisiae*, *Candida utilis* and *Torulaspora delbrueckii* only accumulate 5–10% ($g.g^{-1}$ DCW) when grown under the same conditions [13,72]. For these non-oleaginous yeasts, excess carbon is diverted into polysaccharides such as glycogen and glucan, with minimal lipid production [41].

1.6 Factors Influencing Lipogenesis

Several factors have been reported to influence lipid accumulation and alter the fatty acids profiles in oleaginous yeasts. These include media composition, nutrients concentrations and cultivation conditions (e.g. temperature, pH) [38]. Carbon and nitrogen sources and titers are key aspects in lipogenesis.

1.6.1 Carbon source

Oleaginous yeasts can utilize various types of carbon sources for the production of biomass and lipids [9]. The suitability of a carbon source depends on the microorganism. *R. glutinis* accumulated maximal lipids content of 60% ($g.g^{-1}$ DCW) when cultivated on xylose [73], while both *Candida tropicalis* and *T. fermentans* accumulated more lipids from glucose rather than from xylose [74,75]. Conversion of lignocellulosic hydrolysates and waste products to lipids by oleaginous yeasts have been reported in many publications and summarized in reviews [59]. In addition to biomass concentration and lipid content, the carbon source was shown to influence lipid composition. From the accumulated lipids of *Rhodotorula garminis*, the proportion of monounsaturated fatty acids was much higher with mannose than with the other sugars (75% vs. 40% $g.g^{-1}$, respectively) [76].

1.6.2 Nitrogen source

Nitrogen limitation is generally assumed to induce TAGs production in oleaginous yeasts [38]. The effect of nitrogen source on lipid accumulation and fatty acid composition had varying results, depending on the yeast species. [59]. *R. toruloides* preferred organic glutamate over inorganic ammonium (NH_4^+), accumulating 50% ($g.g^{-1}$ DCW) compared to 18% ($g.g^{-1}$ DCW) of lipids, respectively [77]. Whereas *R. glutinis*, and *C. utilis* had similar lipid content when grown on ammonium chloride, asparagine and glutamate [78].

1.6.3 Carbon to Nitrogen ratio (C: N)

The most studied factor for modulating lipid accumulation and productivity is the carbon-to-nitrogen ratio (C: N, mol/mol) of the nutrient media [34,38,59]. As the ratio increases, excess carbon is redirected for SCOs production. Too high of a ratio (i.e. nitrogen exhaustion) limits cellular growth and biomass production, resulting in significant secondary metabolite formation and subsequently cell death [38]. Most studies reported optimal C: N ratios between from 50 to 100 depending on the species, but values can go much higher [8,41]. *L. starkeyi* accumulated 68% ($g.g^{-1}$ DCW) lipids when grown under a C: N of 150, compared with 40% ($g.g^{-1}$ DCW) at a C: N of 60 [79]. In *R. glutinis*, glucose to lipid conversion yield almost doubled as C: N increased from 150 to 350. However, at 350, the severe nitrogen depletion led to cell death [38]. In *R. toruloides*, increasing C: N from 115 to 140 indeed increased lipid content, however, due to the decrease in biomass formation, only a slight increase in the overall lipid productivity was reported [59]. Thus, a critical concentration of nitrogen is important, and choosing the optimal carbon to nitrogen ratio during fermentation is essential for maximal lipid titers.

1.7 Economic Boundaries and Bioprocess Development Strategies

Stand-alone production of SCOs for oleo-chemicals and biofuels applications remains economically unfeasible. The selling price of microbial-derived biodiesel is estimated at $20/gal, roughly five times more expensive than its plant-derived counterpart. Reducing costs entails minimizing raw material and feedstocks costs, enhancing product titers and improving the efficiency of the production process [34,59].

Designing a successful bioprocess for microbial lipids requires thorough analysis of numerous variables and their impact on the overall performance. Beyond that, process optimization constitutes an evolving network of strategic improvements and fine-tuning tactics, with a clear economic and quality imperative[80]. It is particularly favorable in realizing sustainable development[81,82]. Simply put, optimized processes require fewer resources. In that context, optimization efforts are carried out at bench-scale as the cost of commercial-scale optimization is substantially higher[83-85].

The operational units of bioprocess development are classified as “upstream” and “downstream”. Taking biopharmaceuticals as an example, process optimization has its greatest impact at the design stage (discovery and development)[86]. Upstream processes include operational unit that precedes the bioreaction step, addressing culture media and conditions, strain design and development, in addition to process scale up. Downstream processes include operational units that succeed the bioreaction step, addressing product

recovery and purification[87,88]. Strategically, upstream and downstream optimizations are conducted in parallel, which presents an added challenge[89].

1.7.1 Fermentation Parameters

One of the major hurdles preventing industrial-scale implementation of microbial lipids is the cost of nutrient media (carbon source and other media components) [34,68]. Synthetic nutrients, such as glucose, can account up to 60% of the fermentation total cost. Carbohydrate-dense lignocellulosic feedstock obtained from forestry waste, and agro-industrial residues, in addition to marine feedstocks (macroalgae, microalgae, and seagrass), have the potential to serve as low-cost sources of carbon for SCOs platforms. Zero-waste and by-product streamlines advances the implementation of integrated biorefineries. This approach allows SCOs to compete economically with other commodity type oils[34,59,68,90].

1.7.2 Strain Development

The goal of bioprocess development is to maximize product titers and minimize production costs[91]. However, as stated previously, maximal productivity titers are capped by the strain's genomic capacity. Similarly, substrate suitability is dictated by its metabolic efficiency. Taking this into account, designing robust production strains is at the core of bioprocess development [92]. Genomic improvement of microbial strains has been employed for numerous industrial applications, ranging from food to pharmaceutical products. The two strategies of strain development involve metabolic engineering and accelerated evolution. **Figure 1** presents the early stages of strain design efforts, which mark the birth of biotechnology[**93**].

Metabolic engineering employs DNA recombinant technologies in order to redirect the carbon flux toward SCOs production. The last century brought along ample of novel genetic tools (e.g. transformation, homologous recombination, TALENS, CRISPR) that serve this approach [7,85]. However, these tools require understanding of the genetic factors and the complex network of enzymatic reactions that contribute to the oleo-genic properties of microorganisms [94].

Long before the era of modern bioengineering and its fancy tools, strain development has been carried out via the classical approach (accelerated evolution) [93]. Adopting this approach requires little knowledge of the genetic/biochemical mechanisms involved in the synthesis of desired products. Due to its high-throughput generation of mutants, technical simplicity and low cost (no costly antibiotic selection), this approach directly found its way into industrial biotechnology[95]. The concept involves random mutagenesis for genetic diversification followed by functional screening of variants for desired traits. Genetic diversification is brought about by applying a mutagen, which incurs an alteration in the nucleotide sequence of the genome. These include physical (UV light), chemical (ethyl methane sulfonate; EMS and nitrosomethyl guanidine; NTG) mutagens in addition to ionizing irradiation (γ-rays, α-rays, X-

rays and neutron radiation) [96-100]. The main challenge associated with random mutagenesis lies in the large numbers of generated mutant libraries. Hence, efficient screening of these libraries constitutes the second portion of the classical method[101]. The standard in screening of oleaginous yeasts and other oleaginous microorganisms is cerulenin, a potent fatty acid synthase (FAS) inhibitor. Mutants that are able to overcome FAS inhibition display large colonies and are good candidates for improved growth or lipid production[97,98].

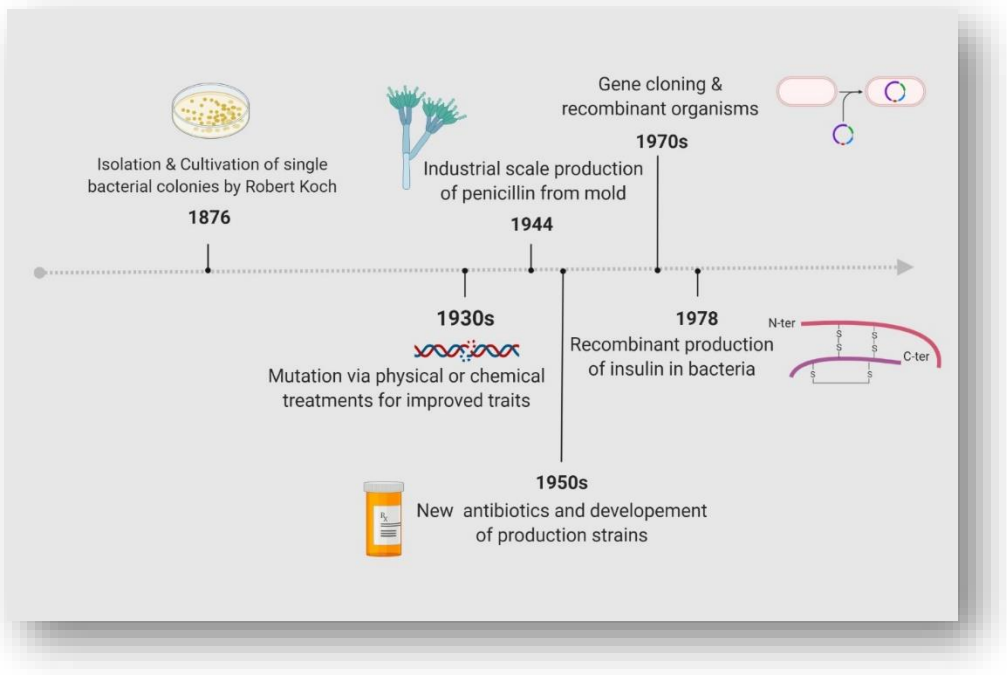


Figure 1. Early stages of strain design advances, which mark the birth of biotechnology.

The classical approach that gave us the first antibiotic continues to advance a multitude of fermentation products[93]. In the context of microbial lipids, advents in multidisciplinary biotechnological platforms have been shown to unleash the untapped potential of the classical approach for improving lipid titers and quality, expanding product range, and facilitating genetic accessibility[92,97]

1.7.3 Advances in Bioprocess Development

Innovation in bioprocess development has been accelerated by integrating state-of-the-art high-throughput (HTP) technologies, statistical optimization and modeling of variable parameters, process analytical tools (PAT), single-use devices, microfluidics tools and continuous downstream processing[83]. Statistical modeling and simulations, such as Response Surface Methodology (RSM) and Artificial neural networks (ANN), generate a surplus of

experimental information while minimizing the number of experiments[102-106]. In conjunction, cultivation in microtiter plates (MTP) and mini-bioreactors represents an optimal system of miniaturization for high-throughput screening[107,108]. Recent progress in single-use (SU) cultivation systems that incorporate single-use probes/sensors and fluidic components has additionally led to rapid development of upstream processes[109-111]. Equally, advancements in sensor technology (configuration and robustness) are vital for the automation of process monitoring[112]. The shift from traditional batch process to continuous downstream process has been also shown to reduce cost, time and labor[113-115]. Bio-pharmaceutical processes greatly benefitted from these tools for rapid strain and product development [87].

1.8 Cutaneotrichosporon oleaginosus

Cutaneotrichosporon oleaginosus (ATCC 20509), a Basidiomycota yeast, was originally isolated in 1978 from samples taken from factory drains of the Iowa State University Dairy Farm [116]. It was deposited under the name *Candida curvata D* at the American type culture collection, and since then was reclassified to *Apiotrichum curvatum* [117], *Cryotoccus curvatus* [118], and since 2011 as *Trichosporon oleaginosus* [119]. Liu et al. (2015) published a comprehensive phylogenetic study of the Tremellomycetes, placing this yeast in the genus of *Cutaneotrichosporon* while keeping its epithet [120]. Microboial lipids from *C. oleaginosus* were first explored in the 1980's for preparation of cocoa butter substitutes (CBS) as cocoa butter was in short supply. However, the process became uncompetitive when the world price of cocoa butter dropped from $8000/ton to < $2500/ton [32,39,121]. This yeast is strongly adapted to changing nutrient supply, and can uptake a broad spectrum of sugar and non-sugar carbon sources, as well as complex biomass feedstocks [33,41,119,122]. Furthermore, it exhibits fast growth rates, tolerance to fermentation inhibitors and a fatty acid profile similar to that of vegetable oils (**Table 2**) [41]. Depending on carbon and nitrogen sources, C: N, in addition to fermentation conditions, *C. oleaginosus* can accumulate up to 85 % ($g.g^{-1}$ DCW) of intracellular lipids. This yeast has been the focus of recent studies ranging from model-based culture media optimization [9], tailored fatty acid profiles [123], waste valorization and cyclic biorefinery platforms [33,70,90], in addition to techno-economic and socio-economic sustainability studies [68]. In the latter, oil from this wild-type yeast strain was estimated at US$1.6 kg^{-1}, cheaper than eco-certified palm oil (US$ 2.1kg^{-1}), with lower CO_2 emissions. In summary, *C. oleaginosus* displays industrially favorable characteristics for the production of biofuels and oleochemicals.

Study Objectives

To develop *C. oleaginosus* into a microbial cell factory for commercial lipid production, a multitude of bioprocess optimization strategies were combined in this work. Specifically, statistical modeling, high-throughput techniques, PAT and MTP-scale screening were employed for the efficient and rapid development. We present:

→ Multi-factorial-guided media optimization

→ Thorough characterization of *C. oleaginosus* nutritional requirements

→ Development of an Absolute Lipid Quantitation Method for High-throughput Process Monitoring

→ High-throughput genetic enhancement of *C. oleaginosus*

2 Materials and Methods

2.1 General Experimental Procedures

2.1.1 Yeast Strain and Maintenance

Cutaneotrichosporon oleaginosus ATCC 20509 was obtained from the culture collection of Werner Siemens Chair of Synthetic Biotechnology (WSSB), Technical University of Munich (TUM), Munich. *C. oleaginosus* wild-type was maintained on YPD (yeast extract peptone dextrose) agar plates (20 $g.L^{-1}$ agar, 20 $g.L^{-1}$ glucose, 20 $g.L^{-1}$ peptone/tryptone and 10 $g.L^{-1}$ yeast extract).

2.1.2 Inoculum Preparation and General Culture Media

For inoculum preparation, a single colony was inoculated in a 125 mL Erlenmeyer flask holding 50 mL YPD liquid medium (20 $g.L^{-1}$ glucose, 20 $g.L^{-1}$, tryptone/peptone (Carl Roth, Germany), 10 $g.L^{-1}$ yeast extract (Applichem, Germany) for 24 h at 28 ℃ and 120 rpm in a rotary incubator. YPD agar plates were prepared by adding an additional 20 $g.L^{-1}$ BD Difco™ Yeast Mold Agar (Thermo Fisher Scientific, Germany).

To evaluate the fermentative potential of *C. oleaginosus* strains, lipid accumulation was induced by subsequent inoculation in 125 mL Erlenmeyer flask holding 50 mL of Minimal-Nitrogen Media (MNM; 40 $g.L^{-1}$ glucose, 0.75 $g.L^{-1}$ yeast extract, 1.5 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.4 $g.L^{-1}$ KH_2PO_4, 0.22 $g.L^{-1}$ $CaCl_2 \cdot 2H_2O$ and trace elements: 1.2 $mg.L^{-1}$ $(NH_4)_2SO_4$, 0.55 $\mu g.L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 24.2 $\mu g.L^{-1}$ $MnCl_2 \cdot 4H_2O$, 25 $\mu g.L^{-1}$ $CuSO_4 \cdot 5H_2O$)[9]. MNM agar plates were prepared by adding an additional $g.L^{-1}$ BD Difco™ Yeast Mold Agar. With a starting optical density of 0.1, measured at 600 nm, fermentation was sustained for 96 h at 28 °C in a rotary incubator at 120 rpm.

2.1.3 Optimization of culture media

Optimization of culturing media was carried out at the level of (1) elemental carbon to nitrogen ratio (C: N), (2) concentrations and (3) sources. First, Response Surface Methodology (RSM) was utilized in the Design of Experiment (DoE) for defining optimal elemental carbon to nitrogen ratio and concentrations (**see section 2.4.1 Response Surface Methodology**). Subsequently, one-factor-at-a-time (OFAT) strategy was implemented to evaluate the fermentative potential of *C. oleaginosus* wild-type over a broad spectrum of carbon and nitrogen sources, listed in **Table 3**.

Table 3. Matrix of the assayed media components displaying the spectrum of nitrogen and carbon sources examined in this work as nutritional substrates for *C. oleaginosus* wild-type. Adapted Awad et al.[9].

	Nutrient Spectrum	Chemical Formula	Chemical Nature	Source
Nitrogen Source	Ammonium chloride	NH_4Cl	Defined, inorganic	VWR
	Ammonium sulfate	$(NH_4)_2SO_4$	Defined, inorganic	Applichem
	Ammonium phosphate	$(NH_4)_3PO_4$	Defined, inorganic	Carl Roth
	Calcium nitrate	$Ca(NO_3)_2$	Defined, inorganic	Alfa Aesar
	Potassium nitrate	KNO_3	Defined, inorganic	Applichem,
	Sodium nitrate	$NaNO_3$	Defined, inorganic	VWR
	Ammonium nitrate	NH_4NO_3	Defined, inorganic	Versorgung-TUM
	Ammonium chloride + sodium nitrate	NH_4Cl + $NaNO_3$	Defined, inorganic	VWR
	Tryptone/Peptone derived Yeast Extract	Protein and amino nitrogen content	Complex, organic	Carl Roth
	Yeast extract	Protein and amino nitrogen content	Complex, organic	Applichem
	Tryptone/Peptone	Protein and amino nitrogen content	Complex, organic	Carl Roth and Applichem
	Urea	CH_4N_2O	Defined, organic	Carl Roth
Carbon Source	Glucose	$C_6H_{12}O_6$	Monosaccharide, hexose	Versorgung-TUM
	Galactose	$C_6H_{12}O_6$	Monosaccharide, hexose	Applichem
	Mannose	$C_6H_{12}O_6$	Monosaccharide, hexose	Applichem
	Fructose	$C_6H_{12}O_6$	Monosaccharide, hexose	Sigma-Aldrich
	Xylose	$C_5H_{10}O_5$	Monosaccharide/ Pentose	Applichem
	Arabinose	$C_5H_{10}O_5$	Monosaccharide/ Pentose	Sigma-Aldrich
	Sorbitol	$C_6H_{14}O_6$	Sugar alcohol	Sigma-Aldrich
	Maltose	$C_{12}H_{22}O_{11}$	Disaccharide	Sigma-Aldrich
	Lactose	$C_{12}H_{22}O_{11}$	Disaccharide	Carl Roth
	Sucrose	$C_{12}H_{22}O_{11}$	Disaccharide	Applichem

Nitrogen Source

The chemical nature of the nitrogen examined here encompassed (1) defined and inorganic sources including: ammonium chloride, ammonium sulfate, ammonium phosphate, calcium nitrate, potassium nitrate, sodium nitrate, ammonium nitrate, 1:1 ammonium chloride and sodium nitrate, (2) defined and organic source: urea and (3) complex and organic sources including: yeast extract, tryptone/peptone and 1:1 yeast extract: tryptone/peptone. Based on supplier's information, the complex organic nitrogen sources, i.e. yeast extract and tryptone/peptone, contain 11.8% (w/w) and 10% (w/w) nitrogen, respectively.

The starting concentration for all nitrogen sources was 0.13 $g.L^{-1}$. The culturing media of *C. oleaginosus* wild-type subjected to the various nitrogen sources was supplemented with glucose at a concentration of 40 $g.L^{-1}$ (16 $g.L^{-1}$ carbon).

Carbon Source

A total of 10 different carbon sources were examined at an elemental concentration of 16 $g.L^{-1}$. Specifically, 4 hexose monosaccharides (glucose, galactose, mannose, and fructose), 2 pentose monosaccharides (xylose, arabinose), a sugar alcohol (sorbitol) and 3 disaccharides (maltose, lactose, sucrose) were examined. The culturing media of *C. oleaginosus* wild-type subjected to the various carbon sources was supplemented with yeast extract at a concentration of 1.10 $g.L^{-1}$ (0.13 $g.L^{-1}$ nitrogen). Besides the various carbon and nitrogen sources examined here, additional media components were adopted from Gorner et al. to include 0.22 $g.L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.4 $g.L^{-1}$ KH_2PO_4, 1.5 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$ and as trace elements: 25 µ $g.L^{-1}$ $CuSO_4 \cdot 5H_2O$, 24.2 µ $g.L^{-1}$ $MnCl_2 \cdot 4H_2O$, 1.2 $mg.L^{-1}$ $(NH_4)_2SO_4$, 0.55 $\mu g.L^{-1}$ $ZnSO_4 \cdot 7H_2O$ [123]. All experiments were carried out in shake flasks at the same liquid to air ratio as inoculum preparation (**see section 2.1.2 Inoculum Preparation and General Culture Media**). For every condition, identical culture conditions were maintained during a 120 h fermentation in a rotary incubator at 120 rpm and 28℃.

2.2 Molecular Techniques

2.2.1 Fast Neutron (FN) Irradiation

Preparation of *C. oleaginosus* Fast Neutron Pools

Exponentially-grown cultures of *C. oleaginosus* wild-type were harvested, washed twice and reconstituted in KCl-media (27 $g.L^{-1}$ NaCl, 6.6 $g.L^{-1}$ $MgSO_4.7H_2O$, 1.5 $g.L^{-1}$ $CaCl_2.2H_2O$, 5 $g.L^{-1}$ KNO_3, 0.07 $g.L^{-1}$ KH_2PO_4, 0.014 $g.L^{-1}$ $FeCl_3.6H_2O$ and 0.021 $g.L^{-1}$ $Na_2EDTA.2H_2O$). A total of 63 cell suspensions were prepared, labeled as triplicates of the 21 *C. oleaginosus* FN pools A – U and subjected to increasing irradiation dosage based on 50 Gy increments (FN Pool A:

non-irradiated *C. oleaginosus* suspension). The cell count of *C. oleaginosus* FN pools at maximum filling capacity of 2 mL Eppendorf tubes was set to 10^7 cells.mL^{-1} (**see section 2.3.1 Cell Count).**

Fast Neutron Irradiation Parameters

Irradiation of *C. oleaginosus* wild-type was conducted at the Research Neutron Source Heinz Maier-Leibnitz (FRM II) facility of the Technical University of Munich in Garching. The nuclear research reactor has achieved its first criticality in March 2004 and initiated operation in April 2005. At the reactor core, a chain reaction of the nuclear fission of uranium is initiated to produce free neutrons over 4 reactor cycles, of 60 days per year. The "Converter Facility" of FRM II contains two uranium plates situated 1 m from the reactor core as a secondary source. When bombarded by slow "thermal" neutrons, the converter plates deliver a thermal power of about 80 kW, releasing fast neutrons with an average energy of 1.9 MeV. The fission neutrons are then steered by geometric conditions into a horizontal beam tube (SR10), which ends at the instrument MEDAPP, specifically allocated for medical and industrial applications, physical and biological dosimetry as well as studies of material resistance to neutron irradiation [124,125]. The prepared cell suspensions of *C. oleaginosus* were placed horizontally within the beam area in custom-made 2 mL Eppendorf rack (3D-printed from poly-lactic acid (PLA)). The parameters of this experimental setup are displayed in **Table 4**. Following radiation clearance, *C. oleaginosus* FN mutant pools were stored in the dark at room temperature to prevent photo-repair events. Aliquots of the mutant library were stored at - 80 ℃ in 10% (w/w) glycerol.

Table 4. Experimental set up of FN irradiation of *C. oleaginosus* wild-type. Adapted from Awad et al.[35].

Parameters	Setup
Mass of uranium in converter plates	540 g
Degree of 235U enrichment	92.5 %
SR10 Irradiation	7 x10^8 fast neutrons.cm^{-2}s^{-1}
Irradiation area	40 x 30 cm²
Thermal power	80 kW
Reactor cycle length	60 days
Neutron flux density	3.2 x 10^8 cm^{-2}.s^{-1}
Distance to converter plate	5.33 m
Lead filter thickness	6 cm
D_n [Gy.min^{-1}]	0.4028
D_γ [Gy.min^{-1}]	0.1840
Cumulative dose [Gy.min^{-1}]	0.5868
Dosage Steps [Gy]	0-1000*
Ratio D_n D_γ^{-1}	2.189
Strain	*C. oleaginosus*

* *C. oleaginosus* FN pools A-U were irradiated with increasing increments of 50 Gy.

2.2.2 Mutant Selection via Cerulenin

To determine the working concentration of cerulenin for the selection of *C. oleaginosus*, approximately 2 × 10^6 *C. oleaginosus* wild-type were plated on YPD agar, supplemented with cerulenin (Applichem, Germany) at a concentration gradient of 2, 4, 6, 8, 10 and 12 µg.µL^{-1}. For this purpose, a cerulenin stock solution (1 mg.mL^{-1}) was prepared in DMSO (dimethyl sulfoxide).

Approximately 2 × 10^6 cells of *C. oleaginosus* FN mutant pool U were plated on YPD agar plates supplemented with 10 µg.µL^{-1} cerulenin. After a 7-day incubation, visibly large colonies were isolated and termed *C. oleaginosus* Fast Neutron Mutants (FN M) 1-9. The fermentative growth of these mutants were assessed over 96 h in liquid MNM (**see section 2.1.2 Inoculum Preparation and General Culture Media**).

2.3 Analytics

2.3.1 Cell Count

In order to prepare identical suspensions of *C. oleaginosus* wild-type for FN irradiation, suspension cell counts were set using S3 Cell Sorter (Bio-Rad, USA), equipped with 488 nm/ 100 mW laser beam. For this purpose, cell suspensions were passed through 40 µm nylon-mesh filters and counted at increment volumes of 100 µL. Cell counts, based on forward-scattered light (FSC) and side-scattered light (SSC), were acquired on a $\log_{10}$ scale using ProsSort™ software (Version 1.5). Automated alignment verification and drop delay determination was carried out using ProLine™ Universal Calibration Beads (Bio-Rad, USA). Optimal flow was determined at a drop delay of 33.16 and an event rate of 60,000. FSC and SSC trigger threshold was set to 0.05 with a voltage of 284 W for the former and 294 W for the latter.

2.3.2 Mutant Viability

The viability of the *C. oleaginosus* FN mutant pools A-U was assessed by plating a volume of 50 µL of irradiated suspensions on YPD agar plates. Following incubation at 28 °C for 96 h, colony counts were recorded and an exponential regression trend was fitted.

2.3.3 Mutant Stability

Following cerulenin selection, *C. oleaginosus* FN M1-9 were continuously sub-cultured over a period of 4 weeks on cerulenin-free MNM agar plate (**see section 2.1.1**) prior to the fermentative potential assessment of the mutants.

2.3.4 Growth Evaluation

Off-line Growth Monitoring

The growth of *C. oleaginosus* wild-type, subjected to various nutritional conditions, was monitored daily by light scattering measurements at 600 nm. For this purpose, a GENESYS ™ 10S UV/ VIS spectrophotometer (Thermo Fisher Scientific, Germany) was utilized with 1 mL cuvettes.

Gravimetric Biomass Analysis

The Dry Cell Weight (DCW) of *C. oleaginosus* wild-type, subjected to various nutritional conditions, was quantified gravimetrically for 2 mL culture volume following harvesting, washing and drying at 60℃ overnight.

2.3.5 Gravimetric Lipid Analysis

Extraction of lipids from *C. oleaginosus* wild-type that has been subjected to various nutritional conditions followed a modified procedure by Bligh and Dyer [126]. Briefly, a culture volume of 4 mL was washed and pelleted twice with 40% ethanol. This step is crucial in avoiding loss of "cellular floaters" that result from high lipid content[127]. Following high-pressure liquid homogenization via EmulsiFlex-B15 French Press (Avestin, Canada), 6 mL of Folch's reagent (2:1 chloroform: methanol) was added and shaken at 400 rpm for 2 h. The chloroform layer was then aspired and dried under nitrogen stream. The extracted lipids were eventually weighed gravimetrically. Lipid yield were then corrected by the biomass yield ($g.L^{-1}$, **see section 2.3.4 Growth Evaluation: Gravimetric Biomass Analysis)** to determine intracellular lipid content (%, $g.g^{-1}$).

2.3.6 Absolute High Through-put Nile Red spectrofluorometry

Relative quantitation of the intracellular lipid fraction of *C. oleaginosus* wild type and FN mutants was carried out according to a modified protocol of Nile Red analysis established by Sitepu et al. [44]. Briefly, a volume of 225 µL yeast culture, heretofore cultivated under the conditions detailed in **section 2.1.2 Inoculum Preparation and General Culture Media**, was diluted to an $OD_{600} < 1$ in a black Nunc ™ F96 MicroWell ™ Polystyrene Plate (Thermo Scientific Waltham, MA, USA). These plates ensure minimal fluorescence background and stray light. The addition of 50 µL of DMSO was followed by a 5 min incubation. Initial absorbance (growth monitoring at OD_{600}) and background fluorescence measurements were recorded using an EnSpire 2 microplate reader from Perkin Elmer (Waltham, MA, USA). Nile red (9-diethylamino-5H-benzo [alpha] phenoxazine-5-one; Sigma-Aldrich, Germany) was added to a final working concentration (WC) of 5 $µg.mL^{-1}$ from a stock solution (60 µg/mL), prepared in DMSO. Stock and working solutions of Nile Red were stored in the dark to avoid

photo-bleaching. Kinetic readings of fluorescence emission were measured at 590/35 nm with an excitation wavelength of 530/25 nm for 5 min with 30 s intervals. Lipid content (%, g.g of DCW) was calculated by norming maximal recorded fluorescence emissions (MFE) values with optical density measurements recorded at 600 nm, to correct for cell density and growth rate variation across cultures.

For the absolute quantitation of *C. oleaginosus* lipids, a calibration curve was generated based on purified microbial lipid (ML) extracted from *C. oleaginosus* wild-type. Specifically, Lipid yield ($g.L^{-1}$) was calculated by standardizing sample MFE against ML and maximal lipid productivity ($mg.L^{-1}.h^{-1}$) was calculated based on 96 h cultivation of *C. oleaginosus* wild-type. Standard preparation followed a modified protocol originally described by Priscu et al.[128]. Briefly, 1:1 ML: Chloroform was diluted in anhydrous ethanol to deliver intermediate ML concentrations of 2, 4, 8 and 10 $mg.mL^{-1}$. Chloroform was necessary for the solubilization of ML in ethanol. Following vigorous vortexing, the final dilution step was prepared so that the volumes of intermediate ML dilutions and the final diluent, here deionized water, are consistent across the working concentrations of ML: 20, 40, 80 and 100 $\mu g.mL^{-1}$. This procedure resulted in hydrophobic micelles of uniform size that mimic the nature of the *C. oleaginosus* samples' final solvent system, prepared according to Sitepu et al. Additionally, analytical grade trielein (TO; Sigma-Aldrich, Germany) and analytical grade oleic acid (Applichem, Germany) standard curves were prepared. Another oleic standard was also prepared following the described protocol, save for the initial chloroform dilution step.

2.3.7 Fatty Acid Profiling

To profile the fatty acid pool of *C. oleaginosus* wild-type and FN mutants, a methanol transesterification of lyophilized yeast biomass was carried out. Briefly, yeast cultures were harvested, washed twice and lyophilized. The transesterification protocol was originally adopted from Griffiths et al. and later modified in our lab by Gorner et al. [123,129]. FAMEs analysis was performed on a GC-2010 Plus Gas Chromatograph (Shimadzu, Japan) equipped with a Flame Ionization Detector (FID). A sample volume of 1 µL was applied via an AOC-20i Auto Injector (Shimadzu, Japan) onto a ZB-WAX column [30 m, 0.32 mm ID; 0.25 µm df; (Phenomenex, USA)]. The initial column temperature was set at 150℃ (maintained for 1 min). A temperature gradient was applied from150℃ to 240℃ ($5℃.min^{-1}$), followed by 6 min maintenance at 240℃. Hydrogen was used as carrier gas at a flow rate of 3 $mL.min^{-1}$ and constant flow compensation. Marine Oil FAME Mix (Restek, USA), which is composed of 20 fatty acids ranging in length from myristic acid (C14: 0) to nervonic acid (C24: 1), was used as a standard for retention time-based identification. Calculation of individual FAME concentrations was based on detected peak areas. Methyl Nonadecaanoate C19 (Sigma-Aldrich, Germany) was used as an internal standard to determine the esterification efficiency.

2.4 Bioinformatics

2.4.1 Response Surface Methodology

Optimization of carbon and nitrogen ratio and concentrations for *C. oleaginosus* wild-type was guided via RSM. Specifically, Box-Behnken design combined 12 carbon to nitrogen ratios in a combinatorial system, covering a range of 12: 1 to 240: 1. The examined elemental concentrations included 8, 16, 24, and 36 $g.L^{-1}$ carbon and 0.13, 0.26, and 0.67 $g.L^{-1}$ nitrogen (**Table 5**). Glucose and yeast extract served as carbon and nitrogen sources. Furthermore, analysis of variance (ANOVA) was implemented to evaluate the effect of nutritional variables, namely carbon and nitrogen levels, on the dependent values: Biomass ($g.L^{-1}$), Lipid weight ($g.L^{-1}$) and Lipid content % (g lipid weight.g^{-1} dry yeast cell weight). For this statistical approach, each of the dependent factors was individually fitted using a second-order polynomial equation. The general form of the second-order polynomial equation is shown below:

$$Y=\beta_o+\sum \beta_i x_i+\sum \beta_{ii} x2_i+\sum \beta_{ij} x_i x_j,$$

whereby, Y is the predicted response, βo is the interception coefficient, βi is the linear coefficient, βii is the quadratic coefficient and βij is the interaction coefficient. Then, multiple regression was carried out to obtain an empirical model that represent the most significant factors affecting the model. Given the availability of center-points replicates, and possibility of pure error calculation for this model, the quality of the regression equations was judged based on the coefficient of determination (R^2) and Lack of fit F-test. Consequently, optimal levels of carbon and nitrogen were inferred from the analysis of response surface plots. Model analysis and visualization was carried out via STATISTICA, version 7 (StatSoft Inc., Tulsa, USA). The model outcome was finally validated in a fermentation experiment.

Table 5. Box-Behnken Design of RSM for optimization of carbon and nitrogen concentration in cultivation media of *C. oleaginosus* wild-type. Adapted from Awad et al.[130].

Run	***C:N***	***X1*** *(C (g. L^{-1}))*	***X2*** *(N (g. L^{-1}))*
1	60	8	0.13
2	30	8	0.26
3	12	8	0.67
4	120	16	0.13
5	60	16	0.26
6	24	16	0.67
7	180	24	0.13
8	90	24	0.26
9	36	24	0.67
10	240	32	0.13
11	120	32	0.26
12	48	32	0.67

2.4.2 High-throughput Biomass Estimation

Gravimetric biomass measurements and offline growth monitoring measurements were correlated for *C. oleaginosus* wild-type, subjected to various nutritional conditions (**see sections 2.1.3 Optimization of culture media and 2.3.4 Growth Evaluation**). Based on the resulting linear regression, the DCW of *C. oleaginosus* FN mutants were subsequently calculated from the optical density measurements and validated.

2.4.3 Biofuel Properties

Based on FAMEs profiles of *C. oleaginosus* wild-type and FN M2, several physio-chemical properties of biofuels were determined using predictive models built for the trans-esterified SCOs [131]. The biofuel properties observed in this work and their corresponding mathematical equations are listed below:

Iodine Value (IV) $$IV = \sum_{i=1}^{n}(254Nw_i)/M_i,$$

whereby *N* is the number of double bonds in the acid molecule, w_i is the content of the individual fatty acid in the sum of total fatty acids, and M_i is the molecular weight of the fatty acid.

Cetane Number (CN) $$CN = \sum_{i=1}^{n} w_i\phi_i,$$

whereby ϕi is cetane number of the individual FAME calculated with the following equation:

$$\phi_i = -7.8 + 0.302M_i - 20N.$$

Higher Heating Value (HHV; MJ.kg^{-1}) $$HHV = \sum_{i=1}^{n} w_i\delta_i,$$

whereby δ_i is the higher heat value of the individual FAME calculated with the following equation:

$$\delta_1 = 46.19 - \frac{1794}{M_i} - 0.21N.$$

Kinematic Viscosity (KV; $mm^2.s^{-1}$) $\eta = \exp(\sum_{i=1}^{n} w_i \ln(\eta_i)),$

whereby $\ln(\eta_i) = -12.503 + 2.496\ln(M_i) - 0.178N.$

Density ($g.cm^{-3}$) $\rho_i = \sum_{i=1}^{n} w_i \rho_i,$

Whereby $\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118N.$

CHAPTER II RESEARCH

3 Overview of Included Publications

3.1 Multi-Factorial-Guided Media Optimization for Enhanced Biomass and Lipid Formation by the Oleaginous Yeast Cutaneotrichosporon oleaginosus

Dania Awad, Frank Bohnen, Norbert Mehlmer and Thomas Brueck

Published in *Bioprocess Engineering: A specialty section in Frontiers of Bioengineering and Biotechnology*, Volume 7 Article 54.

Specialty Chief Editor: Prof. Dr. Manfred Zinn (HES-SO Valais-Wallis, Sion, Switzerland)

DOI: 10.3389/fbioe.2019.00054

Date of Publication: 26 March 2019

3.1.1 Author Contributions

Conceptualization of the study and design of the methodological approach was jointly designed by all authors. Planning and execution of experiments was carried out by the author of this thesis, Dania Awad. Data validation was jointly carried out by all authors. Dania Awad prepared the original draft of the manuscript, which was jointly finalized and reviewed by all authors.

3.1.2 Summary

The inherent ability to metabolize a wide range of substrates is a coveted feature in microbial production strains[132]. This is particularly essential for realizing waste valorization and cyclic bio-refinery goals.[133]. In that respect, the industrial potential of the non-conventional oleaginous yeast *Cutaneotrichosporon oleaginosus* is rooted in its flexible substrate uptake and metabolism[67]. This physiological characteristic is thoroughly investigated and quantified in this study. The role of carbon and nitrogen in *C. oleaginosus* lipogenesis is especially scrutinized, as excess carbon and limited nitrogen supply constitute the biochemical basis of lipid accumulation.

For the first time, a multi-factorial guided Design of Experiment (DoE) strategy was employed to streamline the media optimization matrix for *C. oleaginosus*. Response Surface Methodology (RSM), utilizing a Box-Behnken Design, allowed for the concurrent optimization of distinct carbon and nitrogen concentrations and elemental weight ratios. Optimal conditions (40 $g.L^{-1}$ glucose, 1.10. $g.L^{-1}$ yeast extract; elemental C: N of 120:1) revealed by RSM were experimentally verified. The RSM model clearly illustrates that carbon and nitrogen concentrations highly influence biomass formation and lipid accumulation, respectively. In our experimental set up, the lipogenic potential of *C. oleaginosus* was assessed based on gravimetric biomass and lipid weight, in addition to the fatty acid profile.

Subsequently, classical one-factor-at-a-time (OFAT) method was employed to document the physiological response of this yeast to 10 carbon sources and 12 nitrogen sources. The various single carbon sources evaluated in this study include 4 hexose monosaccharides (glucose, galactose, mannose, and fructose), a sugar alcohol (sorbitol), 2 pentose monosaccharides (xylose, arabinose) and 3 disaccharides (maltose, lactose, sucrose). The chemical nature of the various nitrogen sources evaluated in this study include defined inorganic (ammonium chloride, ammonium sulfate, ammonium phosphate, calcium nitrate, potassium nitrate, sodium nitrate, ammonium nitrate, 1:1 ammonium chloride and sodium nitrate), complex organic (1:1 yeast extract: tryptone/peptone, yeast extract, tryptone/peptone) and defined organic (urea). The highest biomass (18.4 ± 2.20 $g.L^{-1}$) and lipid yields [9 ± 0.34 $g.L^{-1}$; 49.74 ± 5.16 % (g lipid weight.g^{-1} DCW)] were obtained with lactose and yeast extract

as carbon and nitrogen sources at an elemental weight ratio of 120:1, respectively. The physiological implications of these results are explained by the adaptive characteristics of *C. oleaginosus* to efficiently uptake and metabolize lactose and amino acids (abundant in yeast extract). Our data indicates that a metabolic ceiling for lipid accumulation in *C. oleaginosus* is obtained with the optimal carbon and nitrogen sources and their concentrations. Furthermore, fatty acid profiling revealed enhanced saturation concerted with inorganic nitrogen sources, sorbitol and arabinose as carbon sources.

The current work, focusing on the enhancement of *C. oleaginosus* fermentation process, provides insight into its molecular physiology. Additionally, the repertoire of this yeast's behavior over a wide range of carbon and nitrogen sources examined in this study, presents a valuable source for identifying adequate and cost-efficient feedstock.

3.1.3 Full-length Publication

frontiers in Bioengineering and Biotechnology

ORIGINAL RESEARCH
published: 26 March 2019
doi: 10.3389/fbioe.2019.00054

Multi-Factorial-Guided Media Optimization for Enhanced Biomass and Lipid Formation by the Oleaginous Yeast *Cutaneotrichosporon oleaginosus*

Dania Awad[1], *Frank Bohnen*[2], *Norbert Mehlmer*[1] *and Thomas Brueck*[1]*

[1] Werner Siemens-Lehrstuhl für Synthetische Biotechnologie, Technische Universität München, Garching, Germany, [2] BBSI GmbH, Moosinning, Germany

The non-conventional, oleaginous yeast *Cutaneotrichosporon oleaginosus* is flagged as an industrial cell factory for generation of oleochemicals and biofuels due to its substrate flexibility and high triglyceride yields. In this study, we employed a computational Response Surface Methodology to guide and streamline the experimental media optimization matrix with 12 nitrogen and 10 carbon sources in order to provide for high biomass and lipid accumulation toward an industrially relevant fermentation process. The resulting data provide new insights into *C. oleaginosus* physiology under variable nutritional states. Accordingly, the lipid content % (lipid weight/yeast dry weight) is controlled by a defined interplay between carbon and nitrogen. In our experimental setup, the highest biomass (18.4 ± 2.20 g/L) and lipid yield (9 ± 0.34 g/L; 49.74 ± 5.16% g lipid weight/g yeast dry cell weight) were obtained with lactose and yeast extract as carbon and nitrogen sources at an elemental weight ratio of 120:1, respectively. Interestingly, with ammonium salts as a N-source, the intracellularly accumulated triglycerides increasingly contain saturated fatty acids, which provides a new route to generate tailored fatty acid profiles for specific oleochemicals or food applications. Our data indicate that a metabolic ceiling for lipid accumulation in *C. oleaginosus* is obtained with the correct carbon and nitrogen source mixture.

Keywords: ***Cutaneotrichosporon oleaginosus*, Response Surface Methodology, media optimization, FAMEs, biofuel, lipids**

OPEN ACCESS

Edited by:
Vlada B. Urlacher, Heinrich Heine Universität Düsseldorf, Germany

Reviewed by:
Fu-Li Li, Qingdao Institute of Bioenergy and Bioprocess Technology (CAS), China
Seraphim Papanikolaou, Agricultural University of Athens, Greece

***Correspondence:**
Thomas Brueck
brueck@tum.de

Specialty section:
This article was submitted to Bioprocess Engineering, a section of the journal Frontiers in Bioengineering and Biotechnology

Received: *28 November 2018*
Accepted: *04 March 2019*
Published: *26 March 2019*

Citation:
Awad D, Bohnen F, Mehlmer N and Brueck T (2019) Multi-Factorial-Guided Media Optimization for Enhanced Biomass and Lipid Formation by the Oleaginous Yeast Cutaneotrichosporon oleaginosus. Front. Bioeng. Biotechnol. 7:54. doi: 10.3389/fbioe.2019.00054

INTRODUCTION

Dwindling fossil resources and climate change drive the development of sustainable bio-based processes for generation of chemicals, pharmaceuticals, and biofuels, such as biodiesel (Chisti, 2007; Tai and Stephanopoulos, 2013). With regard to biofuel products, current biodiesel is derived from plant-oils, which are associated with limited renewability due to utilization of agricultural land mass and fresh water resources as well as application of chemical fertilizers and pesticides. Therefore, this first-generation biofuel merely offers a partial solution with respect to sustainability and drives the socioeconomic food vs. fuel debate, which has limited the industrial roll out of biofuel processes (Hill et al., 2006). In that regard, current arable lands are not sufficient to meet the global-demand for plant derived biofuels (Smith et al., 2010). Furthermore, rising demands

for biodiesel lead to increasing prices of conventional plant oils. This situation lead to 2-fold price increase for rapeseed, peanut, and sunflower oils between 2007 and 2008 (Papanikolaou and Aggelis, 2011). Assuming a consistent pricing trend, 2020 price projection for the average vegetable oils are expected to exceed 2$.kg^{-1}, which does not alleviate current economic or ecological pressures for biodiesel production (Koutinas et al., 2014). While algae based oils (3rd generation biodiesel) present an ecological alternative to plant oils, their photoautotrophic production is still associated with significant cost (5.6–21.0 $.kg^{-1}) and technological barriers, including slow growth rate and difficulty in maintaining high-density cultures (Papanikolaou and Aggelis, 2011). Consequently, the development of new biofuel processes that utilize biomass waste without impacting agricultural activity are in demand to ensure global food security and address requirements for sustainable energy and oleochemical supply.

To that end, oleaginous yeasts offers a potentially sustainable alternative to meet the demand for bio-based energy and platform chemicals as they can fermentatively convert a broad spectrum of waste biomass substrates into triglycerides. Moreover, these organisms, can be cultivated in existing bioreactor systems without impacting agricultural activities (Li et al., 2008). Specifically, yeasts that intracellularly accumulate neutral lipids in excess of 20% w/cell dry weight when cultivated under nutrient (N, P limitation) restriction, are termed "oleaginous yeasts" (Ratledge and Wynn, 2002; Beopoulos et al., 2009). The majority of the generated triacylglycerols (TAGs) display a fatty acid profile similar to that of plant oils (Papanikolaou et al., 2013). Moreover, oleaginous yeasts, such as *Cutaneotrichosporon oleaginosus* (ATCC 20509) have a pronounced ability to utilize diverse carbon sources—such as glucose, galactose, xylose, n-acetylglucosamine, volatile fatty acids, cellobiose, sucrose, lactose, glycerol, and complex biomass waste materials as feedstock (Evans and Ratledge, 1983; Wu et al., 2010; Gujjari et al., 2011; Chi et al., 2012; Liang et al., 2014b; Willis et al., 2014; Liu et al., 2015; Rakicka et al., 2015). As this organism has the metabolic capacity to accumulate in excess of 50% w/cell dry weight as lipids when nutrient restriction is applied, it is in focus for industrial developments of sustainable oleochemical and biofuel production. Most interestingly, the fatty acid composition of *C. oleaginosus* is similar to that of plant-oils with C16 and C18 being the principal fatty acids (FAs) at 16–33 and 43–57% (g FAME/g dry yeast cell weight), respectively (Hassan et al., 1994a; Ageitos et al., 2011). In fact, B20 Biodiesel derived from *C. oleaginosus* microbial-oil meets the ASTM (D6751) certification (Wahlen et al., 2013; Willis et al., 2014). Being a suitable building block for biodiesel production, this microbial oil produced by oleaginous yeasts such as *C. oleaginosus* offers a sustainable alternative for plant-derived oil without competing with agricultural activities. Nonetheless, production cost of yeast SCO remains higher than that of 1st generation biofuel. While the former is estimated to 3 $.kg^{-1}, excluding feedstock cost, the latter ranges between 1.2 and 1.9 $.kg^{-1} for conventional vegetable oils such as rapeseed, soybean, and sunflower oil (Papanikolaou and Aggelis, 2011). Techno-economic evaluation of SCO processes has demonstrated that the main cost generators are associated with the fermenters required to maintain fed-batch conditions (Koutinas et al., 2014; Karlsson et al., 2016).

To meet economic boundary conditions for industrial implementation of the technology, process costs have to be significantly reduced. In that regard, innovative fermenter designs, recycling/valorization of process residues streams and low energy oil recovery and purification methods are key to the commercialization of microbial oil production technology (Koutinas et al., 2014; Karlsson et al., 2016). In the same manner, the feedstock materials represent a considerable fraction of the overall fermentation-based production costs (Singh et al., 2016). To that end, several strategies could be implemented to significantly improve the techno-economic boundary conditions of microbial oil production. These include the utilization of various biogenic waste materials as low or negative cost feedstocks, including as raw glycerol (biodiesel residue stream) and lignocellulosic hydrolysates (Zhao et al., 2016). Co-fermentation of lignocellulosic-derived carbohydrates containing a mixture of pentoses (e.g., xylose) and hexoses (e.g., glucose) could also alleviate process costs (Stephanopoulos, 2007). Beyond the use of low cost hydrolysates, the design of zero waste yeast oil processes, that valorize all process streams lead to a significant economic and ecological enhancement in the overall process viability. In that regard, our group has recently reported a zero waste yeast oil production process based on marine macroalgae hydrolysates, where feedstock residues have been utilized as biological absorbents for recovery/recycling of industrially relevant metals (Ce^{+3}, Cu^{+2}, and Ni^{+2}) from oil refining processes or for the removal of heavy metals (Pb^{+2}) from waste water streams. Additionally, due to its unique sugar composition, the spent *C. oleaginosus* cell wall after oil extraction was proposed as a performance animal feed additive to improve the techno-economic viability of the overall process (Masri et al., 2018). Another strategy to increase the economic viability of the yeast oil process, involves genetically engineering oleaginous yeast strains to improve substrate uptake and direct the metabolic flux into lipogenic pathways, thereby improving lipid productivity and tailor process specific FA profiles (Mlickova et al., 2004). Despite the high cost of manufacture (COM), microbial production of specialty lipids can achieve higher market prices and therefore rapid market viability. To that end, production of cocoa butter substitutes (CBS) from oleaginous yeast strains has been demonstrated at a technical scale since 1980s. However, recent records anticipate generalized market penetration of CBS in food industry following indications of future disappearance of cocoa plant (Davies and Holdsworth, 1992; Smit et al., 1992; Hassan et al., 1994b; Papanikolaou and Aggelis, 2011). In a specific genetic engineering approach, a Δ9 fatty acid desaturase from *C. oleaginosus* has been cloned and characterized for the production of CBS-SCOs (Papanikolaou and Aggelis, 2011). Beyond the production of cocoa butter, genetic engineering approaches to generate high value cosmetic fats, such as shea butter and sal fat has been explored as cocoa butter equivalents (CBE) (Koutinas et al., 2014). Prior to technical implementation of any of these strategies, assessment and optimization of cultivation media components is essential to

determine which factors influence biomass and lipid formation (Singh et al., 2016).

Identifying and designing optimal fermentation conditions plays a major role in the development of bioconversion systems, as the cultivation medium composition dictates product yield and volumetric productivity (Singh et al., 2016). In that respect, the carbon source is regarded as the most crucial element in the medium; as it represents the main energy source for growth, biomass formation, and subsequent lipid yield (Willis et al., 2014). In addition to the carbon source, the source of nitrogen is thought to be another essential factor determining formation of yeast biomass and lipids. In fact, selected nitrogen sources can elevate biomass productivity, while others may prevent the synthesis of certain metabolites (Singh et al., 2016). The biochemical basis of lipid accumulation in oleaginous yeasts is believed to be an adaptive response under a carbon-rich and nutrient-limited (mainly nitrogen) conditions, where excess carbon is incorporated into intracellular lipids (TAGs) as a form of energy storage (Willis et al., 2014). Furthermore, fatty acid composition differs greatly depending on the type and concentration of the carbon and nitrogen sources. Lipid yield and type, in addition to biomass formation, are likewise influenced by the carbon to nitrogen ratio (C: N) in addition to media components like carbon and nitrogen-sources as well as vitamins and minerals (Granger et al., 1993; Ratledge and Wynn, 2002). Enhancing strain genetics and improving media parameters are usually concomitant. *C. oleaginosus* ability to produce high amounts of intracellular lipids from a variety of substrates has previously been reported (Evans and Ratledge, 1983; Gujjari et al., 2011; Willis et al., 2014). However, genetically engineering this particular yeast remains a challenge. With many genes lacking functional annotations and absence of proteomic data, these engineering efforts only rely on recently reported transcriptomic sequences (Kourist et al., 2015). Recently methods that enable genetic accessibility of *C. oleaginosus* have been reported. These methods allowed an increase in total lipid yields as well as the generation of tailor-made, non-native fatty acid, such as polyunsaturated very long chain fatty acids eicosatrienoic and eicosadienoic acid and (E-10, Z-12) conjugated linoleic acid (Gorner et al., 2016). Based on the available data, this study will focus on a systematic, multi-factorial analysis of how carbon and nitrogen sources affect *C. oleaginosus* biomass and lipid formation.

To that end, conventional medium optimization studies are conducted following the classical one-factor-at-a-time (OFAT) method where, at a given time, only one factor is varied while all other variables remain constant. This strategy includes iterative removal, supplementation and replacement experiments of the various chemical and physical components of the medium, resulting in laborious protracted experiments (Singh et al., 2016). By contrast, design of experiment (DOE) guided studies, applied in this work, allow an accurate statistically validated, multi-factorial medium optimization strategy. This methodology reduces experimental time and labor compared to the OFAT method and generates statistically verified data. One approach to DOE is Response Surface Methodology (RSM), developed by Box and Wilson (Box and Wilson, 1951). RSM is a mathematical modeling algorithm that uses factorial designs to assess the correlation between the response and different variables either alone or in combination, eventually allowing the optimization of the production processes (Singh et al., 2016).

TABLE 1 | Matrix of assayed media components.

Compound number	Carbon source	Chemical nature	Nitrogen source	Chemical nature
1	Glucose	Monosaccharide/ Hexose	Ammonium chloride	Defined Inorganic
2	Galactose	Monosaccharide/ Hexose	Ammonium sulfate	Defined Inorganic
3	Mannose	Monosaccharide/ Hexose	Ammonium phosphate	Defined Inorganic
4	Fructose	Monosaccharide/ Hexose	Calcium nitrate	Defined Inorganic
5	Sorbitol	Sugar alcohol	Potassium nitrate	Defined Inorganic
6	Xylose	Monosaccharide/ Pentose	Sodium nitrate	Defined Inorganic
7	Arabinose	Monosaccharide/ Pentose	Ammonium nitrate	Defined inorganic
8	Maltose	Disaccharide	Ammonium chloride + sodium nitrate	Defined Inorganic
9	Lactose	Disaccharide	Tryptone/ Peptone derived Yeast Extract*	Complex Organic
10	Sucrose	Disaccharide	Yeast extract*	Complex Organic
11	–	–	Tryptone/ Peptone*	Complex Organic
12	–	–	Urea	Defined Organic

*Potential carbon and nitrogen sources.

The aim of this study is to use RSM as a computational guidance tool to simultaneously modulate and optimize multiple medium factors, in particular carbon and nitrogen, to ultimately improve biomass and lipid production of the oleaginous yeast *C. oleaginosus*. Initially, the response surface methodology, applying a Box-Behnken Design, is employed to evaluate the effect of nitrogen and carbon levels. Specifically, RSM assess the relative contribution of carbon and nitrogen sources on the *C. oleaginosus* growth and lipid production. Subsequently, 12 distinct nitrogen sources and 10 selected carbon sources, encompassing the most comprehensive media components involving complex, synthetic/inorganic and organic media composition for *C. oleaginosus*, are investigated (**Table 1**). This statistically verified, multi-factorial data interrogation provides insight into the physiology of *C. oleaginosus* (growth, lipid production and fatty acid profile) toward development of an industrial cultivation process.

MATERIALS AND METHODS

Yeast Strain and Inoculum Preparation

Cutaneotrichosporon oleaginosus ATCC 20509 (available in the laboratory culture collection of Werner Siemens Chair of Synthetic Biotechnology- WSSB, TU, Munich) was maintained on YPD (yeast extract peptone dextrose) agar plates (20 g/L

peptone, 20 g/L agar, 20 g/L glucose, 10 g/L yeast extract). Stock cultures were transferred to fresh agar plates and stored at 4°C weekly. A single colony was transferred to 50 mL YPD liquid medium in 125 mL Erlenmeyer flask and cultured at 28°C for 24 h in a rotary incubator at 120 rpm prior to inoculation into prospective optimization media.

Experimental Design

Media optimization can be divided into 3 stages, whereby the first stage assesses different carbon to nitrogen concentrations and ratios, the second and third stages compare the effect of different nitrogen and carbon sources on *C. oleaginosus* growth and lipid production, respectively. Aside from variation in carbon and nitrogen sources and concentrations, all cultures were comprised of 1.5 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L KH_2PO_4, 0.22 g/L $CaCl_2{\cdot}2H_2O$ in addition to trace elements: 1.2 mg/L $(NH_4)_2SO_4$, 0.55 μ g/L $ZnSO_4{\cdot}7H_2O$, 24.2 μg/L $MnCl_2{\cdot}4H_2O$, 25 μg/L $CuSO_4{\cdot}5H_2O$ (adapted from Gorner et al., 2016). All shake flask experiments were carried out in 125 mL Erlenmeyer flasks with a total media volume of 50 mL. Identical culture conditions were maintained during optimization at 28°C in a rotary incubator at 120 rpm for 5 days. Three biological replicates were prepared from varied conditions listed below.

A total of 12 carbon to nitrogen ratios were prepared in a combinatorial system based on 8, 16, 24, and 36 g/L carbon and 0.13, 0.26, and 0.67 g/L nitrogen, with glucose and yeast extract as carbon and nitrogen sources, respectively. Elemental carbon to nitrogen weight ratios were covered in this study in the range of 12: 1 to 240: 1. Based on supplier's information (Carl Roth, Germany), the complex media—yeast extract and tryptone/peptone—contain 11.8% (w/w) and 10% (w/w) nitrogen, respectively. Following RSM analysis, various nitrogen and carbon sources were investigated using one-factor-at-a-time (OFAT) strategy. The various nitrogen sources evaluated in this study encompass: ammonium chloride, ammonium sulfate, ammonium phosphate, calcium nitrate, potassium nitrate, sodium nitrate, ammonium nitrate, 1:1 ammonium chloride and sodium nitrate, 1:1 yeast extract: tryptone/peptone, yeast extract, tryptone/peptone and urea. To investigate the effect of the nitrogen source on yeast growth, media was supplemented with 40 g/L glucose (16 g/L carbon) and nitrogen concentration of 0.13 g/L. The last stage of optimization covered 10 different carbon sources at an elemental carbon concentration of 16 g/L comprising 4 hexose monosaccharides (glucose, galactose, mannose, and fructose), a sugar alcohol (sorbitol), 2 pentose monosaccharides (xylose, arabinose), and 3 disaccharides (maltose, lactose, sucrose). For the screening of carbon sources, cultivation was carried out in media supplemented with the optimal nitrogen source as observed in the previous stage of optimization; 1.10 g/L yeast extract (0.13 g/L nitrogen).

Analytical Methods

Gravimetric Method

Yeast growth was monitored daily by light scattering measurements at 600 nm. Dry Cell Weight (DCW) was quantified gravimetrically for 2 mL culture volume following harvesting, washing and lyophilization. Lipid extraction was conducted according to a modified procedure by Bligh and Dyer (1959) following yeast cells disruption using a high pressure homogenizer. Shortly, 6 mL of Folch's reagent (2:1 chloroform: methanol) was added to washed yeast cells. An incubation of 2 h was sufficient to transfer the cells' lipid content to the chloroform layer. The aspirated chloroform layer was later dried under nitrogen stream overnight and the extracted lipids were weighed.

Fatty Acid Profile Analysis

Fatty acid methyl esters (FAMEs) were obtained by methanol transesterification of lyophilized yeast biomass. The transesterification protocol was originally adopted from Griffiths et al. (2010) and modified in our lab by Gorner et al. (2016). FAME profiles were analyzed on a GC-2010 Plus gas chromatograph from Shimadzu (Nakagyo-ku, Kyoto, Japan) with flame ionization detector. One microliter sample was applied by AOC-20i auto injector (Shimadzu) onto a ZB-WAX column [30 m, 0.32 mm ID; 0.25 μm df; phenomenex (Torrance, CA, USA)]. The initial column temperature was set at 150°C (maintained for 1 min). A temperature gradient was applied from 150° to 240°C (5°C.min^{-1}), followed by 6 min maintenance at 240°C. Fatty acids were identified according to retention times of the authentic standard: Marine Oil FAME Mix (Restek, USA). Individual FAME concentrations were based on peak areas relative to Methyl Non-adecaanoate C19 (Sigma, Germany), which was incorporated as an internal standard in all samples.

Response Surface Methodology and Further Statistical Analysis

STATISTICA, version 7 (StatSoft Inc., Tulsa, USA) was adopted for design analysis of the first stage of media optimization. Analysis of Box-Behnken (response surface) design along with the analysis of variance (ANOVA) was used to estimate the appropriate statistical parameters. This design was selected for analyzing the effect of selected nutritional variables, namely carbon and nitrogen levels, present in growth media on the dependent variables: Biomass (g/L), Lipid weight (g/L) and Lipid content % (g lipid weight/ g dry yeast cell weight). The levels examined for each of the independent factors are displayed in **Table 2**. This statistical design involves interactions amongst the selected variables and follows a linear/quadratic approach for screening of factors. After discovering the significant factors, each of the dependent factors was individually fitted using a second-order polynomial equation and a multiple regression of the data was carried out for obtaining an empirical model to represent the most significant factors. The general form of the second-order polynomial equation is shown below:

$$Y = \beta_o + \sum .\beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j,$$

whereby, Y is the predicted response, $\beta_o$is the interception coefficient, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient. The quality of the regression equations were judged based on the coefficient of determination R^2 and Lack of fit F-test, given the availability of center points replicates, and possibility of pure error calculation. Optimal levels of carbon and nitrogen were inferred from analysis of response surface plots.

TABLE 2 | Box-Behnken Design of RSM for optimization of carbon and nitrogen concentration in cultivation media of *C. oleaginosus*.

Run	C:N	X1	X2	Biomass	Lipid weight	Lipid content	Lipid yield
		(C (g. L^{-1}))	(N (g. L^{-1}))	(g. L^{-1})	(g. L^{-1})	(% g. g^{-1})	(g lipid. g^{-1} C)
1	60	8	0.13	9.75 ± 1.09	3.25 ± 0.09	33.78 ± 4.68	0.04 ± 0.01
2	30	8	0.26	9.5 ± 0.11	1.82 ± 0.14	19.15 ± 1.53	0.23 ± 0.02
3	12	8	0.67	10.15 ± 0.13	1.39 ± 0.10	13.71 ± 1.10	0.17 ± 0.01
4	120	16	0.13	15.1 ± 0.60	6.69 ± 0.22	44.36 ± 2.56	0.42 ± 0.01
5	60	16	0.26	15.1 ± 0.41	3.18 ± 0.07	21.06 ± 0.48	0.20 ± 0.00
6	24	16	0.67	17.35 ± 1.10	2.33 ±0.29	13.48 ± 1.90	0.15 ± 0.02
7	180	24	0.13	16.85 ± 1.33	6.97 ± 0.76	41.66 ± 5.74	0.29 ± 0.03
8	90	24	0.26	19.7 ± 2.24	7.38 ± 1.01	38.09 ± 8.28	0.31 ± 0.04
9	36	24	0.67	23.95 ± 2.36	3.78 ± 0.61	15.98 ± 3.40	0.16 ± 0.02
10	240	32	0.13	15.4 ± 0.14	4.1 ± 0.47	26.64 ± 3.21	0.13 ± 0.01
11	120	32	0.26	22.9 ± 0.10	6.29 ± 0.73	27.48 ± 3.23	0.20 ± 0.02
12	48	32	0.67	24.3 ± 0.55	5.85 ± 0.14	24.09 ± 0.80	0.18 ± 0.00

Elemental weight ratio of carbon to nitrogen were delivered by glucose and yeast extract, respectively.

RESULTS AND DISCUSSION

In silico Guided Optimization of Carbon and Nitrogen Concentrations Using Response Surface Methods

To assess the effect of carbon and nitrogen concentrations in the cultivation media on growth and lipid production of *C. oleaginosus*, the composition was subjected to Box-Behnken design-based DOE analysis. This analysis was performed using glucose as carbon source, since yeasts are reported to harbor efficient glucose import systems. Similarly, yeast extract was used as a complex nitrogen source as it contains all essential amino acids as well as other nutritional factors such as fatty acids, vitamins and trace element, which in combination improve biomass and lipid yield [30, 31]. It was previously reported that an elemental carbon to nitrogen ratio of 12: 1 does not deliver a nitrogen-limited environment for *C. oleaginosus*, hence a range from 12: 1 to 240: 1 was analyzed in this study to cover the range between non-limiting and limiting cultivation conditions [32]. Initially, each concentration was carefully developed to fit one of three equally spaced values (−1, 0, and +1) to sufficiently generate a quadratic model. The model was later validated following an experimental set up of selected theoretical parameters. The Box-Behnken design matrix of the independent variables and their corresponding response on the dependent factors are displayed in **Table 2**. The fitted regression equations for each of the dependent factors are:

$$\begin{aligned} Y(\text{Biomass}) &= -2.40 + 8.16\text{X1} - 0.92\text{X12} + 28.79\text{X2} \\ &\quad - 32.40\text{X22} - 25.06\text{X1X2} + 28.26\text{X1X22} \\ &\quad + 4.22\text{X12X2} - 4.40\text{X12X22}, \\ Y(\text{Lipid Weight}) &= 7.11 + 0.13\text{X1} - 42.45\text{X2} + 50.45\text{X22} \\ &\quad + 4.70\text{X1X2} - 7.58\text{X1X22} + 0.35\text{X12X2} \\ &\quad - 0.32\text{X12X22}, \\ Y(\text{Lipid Content}) &= 69.71 - 2.76\text{X1} - 297.026\text{X2} + 328.79\text{X22} \\ &\quad + 18.97\text{X1X2} - 29.14\text{X1X22} + 3.13\text{X12X2} \\ &\quad - 3.64\text{X12X22}, \end{aligned}$$

TABLE 3 | Analysis of Variance (ANOVA).

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Dependent Variable = Biomass (g. L^{-1}); R^2 = 0.92592; Adj. R^2 = 0.92203; MS Pure Error = 1.705811					
X1 (L+Q)	1555.49	2.00	777.74	455.94	0.0000
X2	152.83	1.00	152.83	89.59	0.0000
X1X2	95.10	1.00	95.10	55.75	0.0000
Lack of Fit	14.17	4.00	3.54	2.08	0.0928
Pure Error	122.82	72.00	1.71	–	–
Total SS	1849.25	80.00	–	–	–
Dependent Variable = Lipid Weight (g. L^{-1}); R^2 = 0.95642; Adj. R^2 = 0.95224; MS Pure Error = 0.2361527					
X1 (L)	184.11	1.00	184.11	779.60	0.0000
X2 (L+Q)	132.88	2.00	66.44	281.35	0.0000
X1X2	49.61	4.00	12.40	52.52	0.0000
Lack of Fit	0.62	1.00	0.62	2.64	0.1085
Pure Error	17.00	72.00	0.24	–	–
Total SS	404.42	80.00	–	–	–
Dependent Variable = Lipid Content (w/w); R^2 = 0.90226; Adj. R^2 = 0.89288; MS Pure Error = 16.54639					
X1	1098.95	1.00	1098.95	66.42	0.0000
X2 (L+Q)	8828.59	2.00	4414.30	266.78	0.0000
X1X2	1230.42	4.00	307.61	18.59	0.0000
Lack of Fit	36.18	1.00	36.18	2.19	0.1436
Pure Error	1191.34	72.00	16.55	–	–
Total SS	12558.62	80.00	–	–	–

whereby, X1 and X2 represent carbon and nitrogen levels, respectively. **Table 3** summarizes the test for significance and adequacy of each of the regression models listed above. The optimal values for the investigated dependent factors were predicted from the 3D response surface plot. **Figure 1** illustrates the effects of the independent variables and their combined effect on biomass (g/L), lipid weight (g/L), and Lipid content % (g lipid weight/ g yeast dry cell weight). Accordingly, the

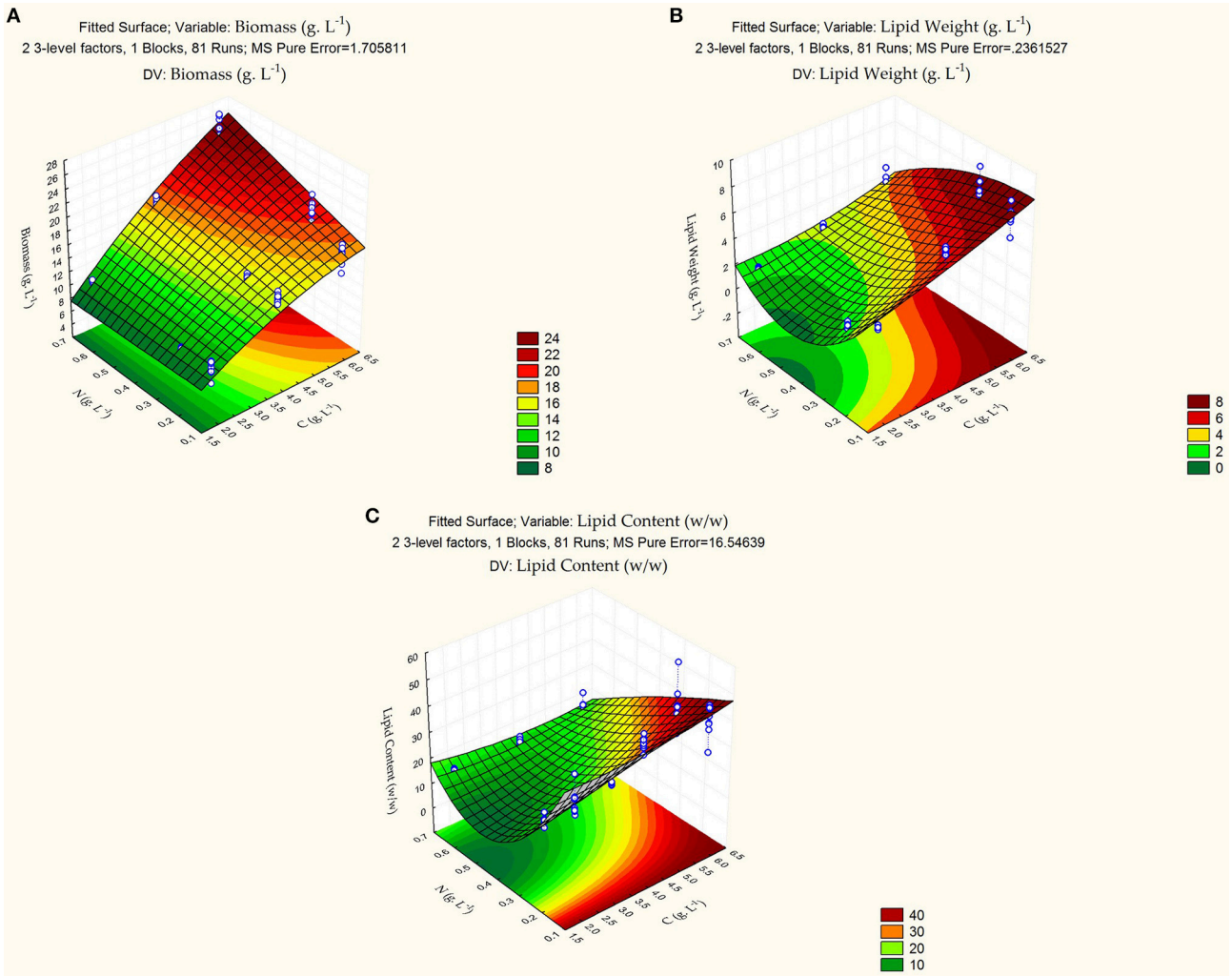


FIGURE 1 | 3D Response surface plot of the combined effects of carbon and nitrogen levels on **(A)** growth (g/L), **(B)** lipid weight (g/L), and **(C)** lipid content (g lipid weight/g dry yeast cell weight). Gradient legends display extent of measured factor. White points mark measured levels on which the Box-Behnken model was built.

optimal predicted values for the dependent factors are 15.1 ± 0.60 g/L, 6.69 ± 0.22 g/L, and 44.36 ± 2.56% (g lipid weight/g dry yeast cell weight), respectively. The determination confidence coefficient (R^2), which is conventionally applied to determine the model validity, implies that sample variation of 92.59, 95.64, and 90.23% for biomass, lipid weight, and lipid content, respectively, are attributed to the independent variables. Together with the significance of the Lack-of-fit test (p-values of 0.0928, 0.1085, and 0.1436), we determined the integrity of the model designed for this analysis (**Table 3**). As depicted in **Table 2**, the total growth increases with increasing carbon concentrations until a saturation level is obtained at 60 g/L glucose. Moreover, nitrogen limitation, which is essential for high lipid production, possess a strong negative influence on *C. oleaginosus* growth. For this reason, lipid content (g lipid weight/g dry yeast cell weight), as well as lipid weight (g/L), are equally scrutinized in this study. Furthermore, it is apparent from the three formulated regression equations that a combined effect of both independent factors significantly affects growth and lipid production. The second order polynomial equation was found to explain the optimal conditions by only considering the significant terms. Applying the RSM methodology allowed the role of carbon and nitrogen sources to be differentiated with respect to their importance and contribution to growth and lipogenesis potential. Those factors affecting Biomass are % C (linear and quadratic), % N (linear), and the linear interaction between % C and % N. In addition to those factors, the quadratic effect of % N highly influences lipid accumulation. Nitrogen-limitation has been previously assumed to be the strongest inducer of lipogenesis in *C. oleaginosus* [26]. The response surface plot (**Figure 1**) clearly depicts the influence and sensitivity of the respective factors on growth and lipid production. The currently incomplete comprehension of the metabolic network and regulatory mechanisms driving the lipogenic processes in *C. oleaginosus* might provide an explanation for our inability of attaining a higher determination coefficient [26]. Provided, that *C. oleaginosus* performs similarly at elemental carbon to nitrogen ratios of 120: 1 and 180: 1, further optimizations were carried out at C: N of 120:1 at

concentrations of 16 g/L carbon and 0.13 g/L nitrogen, to reduce optimization process cost. The validity of the designed Box-Behnken model is evident in reproducible measurements of *C. oleaginosus* growth and lipid yields [33]. This particularly holds true when the organism is cultivated (C: N 120: 1, 16 g/L carbon and 0.13 g/L nitrogen) with optimal nitrogen (see **Table 4**, yeast extract) and carbon (see **Table 5**, glucose) ratios. In addition to relative amounts of carbon and nitrogen, different elemental concentrations at same carbon to nitrogen ratios were examined. While runs 1(8 g/L carbon and 0.13 g/L nitrogen) and 5 (16 g/L carbon and 0.26 g/L nitrogen) maintain a carbon to nitrogen elemental weight ratio of 60: 1, their variation in carbon and nitrogen concentrations results in distinct biomass, lipid weight and lipid content. Similar observations were recorded for runs 4 and 11 at C: N of 120: 1.

TABLE 4 | Results of cumulative growth and lipid production by *C. oleaginosus* cultured in parallel with variable nitrogen sources at elemental nitrogen concentration of 0.13 g. L^{-1},holding all other conditions identical [C: N of 120: 1 and 40 g. L^{-1} glucose (16 g. L^{-1} carbon)].

Nitrogen source	Biomass(g. L^{-1})	Lipid weight(g. L^{-1})	%Lipid (g. g^{-1})
Ammonium chloride	1.93 ± 0.08	0.32 ± 0.03	16.35 ± 1.78
Ammonium sulfate	2.17 ± 0.20	0.35 ± 0.04	16.37 ± 1.97
Ammonium phosphate	2.12 ± 0.10	0.51 ± 0.06	24.27 ± 3.39
Calcium nitrate	2.24 ± 0.17	0.73 ± 0.02	32.72 ± 2.89
Potassium nitrate	2.21 ± 0.12	0.91 ± 0.09	41.28 ± 5.64
Sodium nitrate	2.46 ± 0.13	1.06 ± 0.10	43.3 ± 4.18
Ammonium nitrate	1.73 ± 0.08	0.39 ± 0.03	22.74 ± 2.62
Ammonium chloride + sodium nitrate	1.67 ± 0.12	0.3 ± 0.03	18.09 ± 2.17
Yeast extract: Tryptone/Peptone	13.17 ± 0.27	6.37 ± 0.22	48.44 ± 2.30
Yeast extract	13.29 ± 0.15	7.1 ± 0.50	53.41 ± 4.12
Tryptone/Peptone	11.18 ± 0.18	5.58 ± 0.32	49.94 ± 3.64
Urea	4.96 ± 0.07	1.82 ± 0.13	36.75 ± 2.82

TABLE 5 | Results of cumulative growth and lipid production by *C. oleaginosus* cultured in parallel with variable carbon sources at elemental carbon concentration of 16 g. L^{-1},holding all other conditions identical [C: N of 120 and 1.10 g. L^{-1} yeast extract (0.13 g. L^{-1} nitrogen)].

Carbon source	Biomass (g. L^{-1})	Lipid weight (g. L^{-1})	%Lipid (g. g^{-1})
Glucose	15.1 ± 0.60	6.62 ± 0.23	44.3 ± 2.53
Galactose	12.4 ± 1.22	4 ± 1.29	33.36 ± 11.98
Mannose	13.7 ± 1.82	7.03 ± 1.19	52.83 ± 13.82
Fructose	17 ± 0.77	7.25 ± 2.07	43.11 ± 11.63
Sorbitol	4.5 ± 0.45	0.6 ± 0.09	13.41 ± 2.52
Xylose	15.3 ± 0.43	5.63 ± 1.06	36.56 ± 6.57
Arabinose	8.7 ± 0.75	2.07 ± 0.50	23.91 ± 6.66
Maltose	14.5 ± 1.67	6.12 ± 1.11	41.38 ± 7.73
Lactose	18.4 ± 2.20	9 ± 0.34	49.74 ± 5.16
Sucrose	10.3 ± 1.14	2.79 ± 0.58	27.52 ± 6.60

Optimization of Carbon and Nitrogen Concentrations

Conventionally, laboratory medium compositions applied in initial research activities differ substantially from the medium in which the final production strain is expected to perform on a technical scale (Hahn-Hagerdal et al., 2005). Specifically, strain development is typically carried out in chemically-defined media to allow for strain-selection, easier metabolism definition, batch-to-batch variation exclusion and growth factor interference elimination (Zhang and Greasham, 1999). Conversely, complex and semi-defined media favored in industrial settings allow for greater biomass and metabolite yields, while complying with economic constraints (Cocaign-Bousquet et al., 1995). Complex media are composed of undefined components and quantities, such as biogenic wastes and hydrolysates. The complex nitrogen sources examined in this study include yeast extract and tryptone/peptone and their mixture. The multiplicity of carbon and nitrogen sources of synthetic, organic, and complex media presented in this study stands as future reference for strain improvements throughout developmental and industrial stages. The significance of the nitrogen source on *C. oleaginosus* growth and lipid formation is often neglected, placing more weight on carbon source performance (Swoboda, 1922; Evans and Ratledge, 1984; Godard et al., 2007; Gutierrez et al., 2013). Whereas, previous studies have focused on *C. oleaginosus* ability for sugars co-utilization (Yu et al., 2014; Gong et al., 2016; Meo et al., 2017), this work is directed toward equal scrutiny and comparison of the effect of distinct carbon and nitrogen sources on *C. oleaginosus* growth and lipid production. This comparison will aide in making an informed decision on the choice of genetic manipulations, that have to be implemented, to optimize sugar transport systems and streamline metabolic flux to enable efficient *C. oleaginosus* growth and lipid formation. Similar strategies have been adopted for increasing lipid yield and expanding the substrate utilization of *Yarrowia lipolytica* (Ryu et al., 2016; Spagnuolo et al., 2018).

Commonly, media supplemented with complex (undefined/multiple) nitrogen sources result in high biomass accumulation due to ample availability of readily metabolizable amino acids, polypeptides, nucleotides, and various vitamins and trace elements (such as Mg, Ca, K, and Fe). This is apparent in **Figure 2A** and **Table 4** with total biomass of 13.10 ± 0.27 g/L for yeast extract: tryptone/peptone, 13.29 ± 0.15 g/L for yeast extract and 11.18 ± 0.18 g/L for tryptone/peptone, after 120 h of cultivation. In contrast, low yeast biomass accumulation in media supplemented with synthetic/ inorganic nitrogen is attributed primarily to additional energy requirement for amino acid synthesis aside from energy requirement for self-propagation and growth. This is evident in media containing ammonium salts resulting in severely reduced growth compared to media containing complex nitrogen sources, followed by nitrates, with an average biomass of 2.1 ± 0.3 g/L. Additionally, ammonium uptake and growth is largely deterred by the acidification of the media in which excess protons, generated from amino acid biosynthetic pathway, are transported out of the cell to stabilize internal pH (Casey et al., 1983; Casey and Ingledew, 1986).

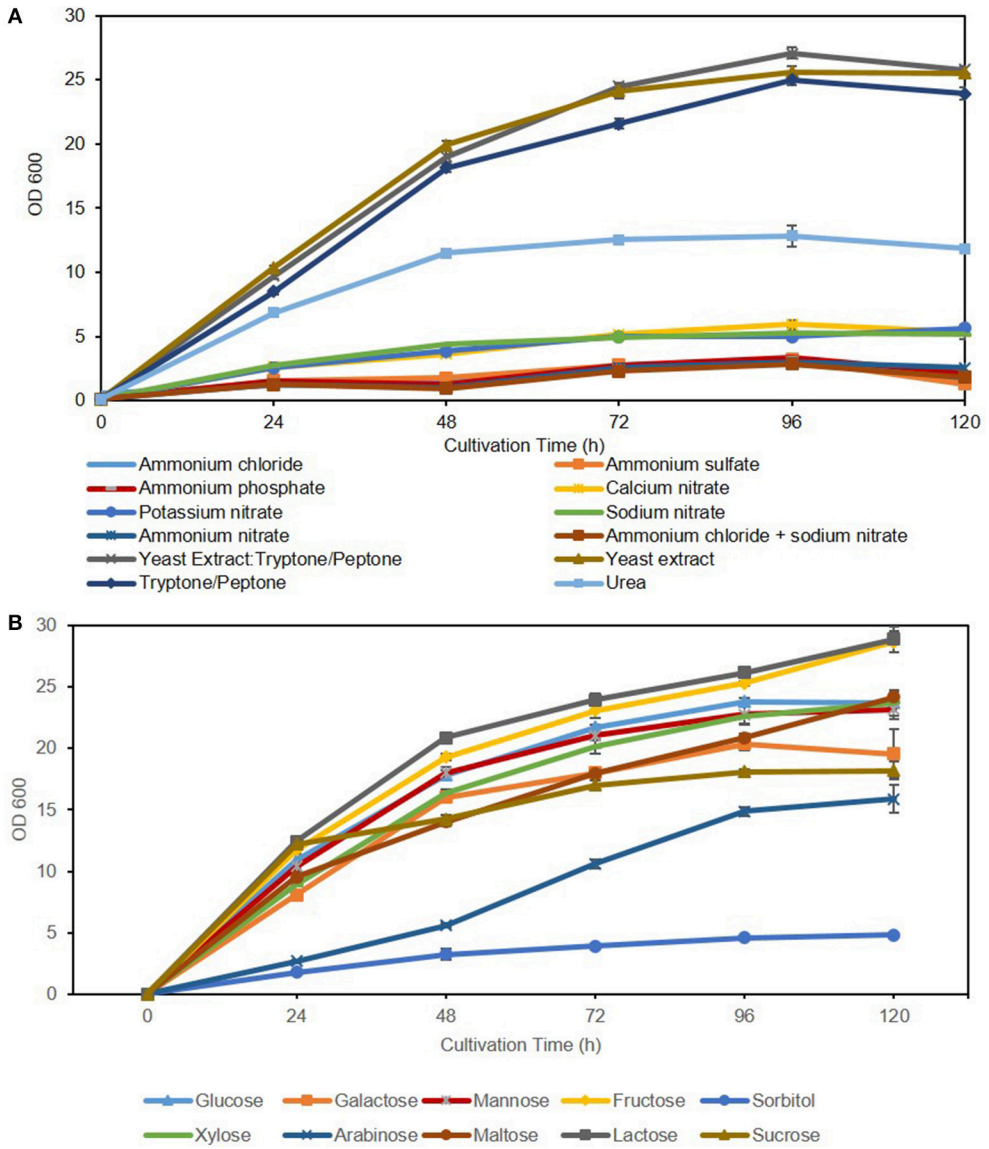


FIGURE 2 | Effect of nitrogen source **(A)** and carbon source **(B)** on *C. oleaginosus* growth.

Nonetheless, minimal potassium requirements for optimal yeast growth must be 100-fold higher than environmental concentrations (abundance of amino acids) when ammonium is used as the nitrogen source (Rodriguez-Navarro and Ramos, 1984; Hess et al., 2006). When synthetic nitrogen sources were utilized in this study, potassium levels were not adjusted accordingly, which could explain lower biomass measurements. Moreover, the target of rapamycin complex (TORC) associated signaling cascade is a key player in nutrient-starvation, which in oleaginous yeasts is linked to lipid accumulation and cell proliferation. In the model yeast *S. cerevisiae*, TORC is also linked to ammonium toxicity and chronological life span (CLS) shortening in absence of amino acids (Santos et al., 2013; Bracharz et al., 2017b). This correlation is yet to be established in oleaginous yeasts.

Our data indicates, that with respect to the lipid biosynthesis efficiency corrected for biomass yield, i.e., the lipid content % (g lipid weight/g dry yeast cell weight) is quantitatively comparable over various cultivation conditions. Conversely, the lipid weight (g/L) is directly proportional and influenced by the growth rate. With respect to the former, the lipid content of *C. oleaginosus* grown in nitrates surpasses those grown in ammonium salts. In that regard, a difference of 7.9% (g lipid weight/g dry yeast cell weight) in lipid content was found between *C. oleaginosus* cultivated in media supplemented with ammonium phosphate compared to equivalent cultivations with ammonium sulfate and ammonium chloride, elevated for the former. Additionally, no altered lipid formation response was detected in cultivation media containing different ammonium salts or different nitrates. Notably, coupling of ammonium salts

with nitrate by supplementing media with ammonium nitrate or 1:1 ammonium chloride: sodium nitrate resulted in a similar lipid content as yeast grown in ammonium salts alone. This data is an indication that *C. oleaginosus* may be sensitive to ammonium toxicity.

Organic nitrogen was also supplemented to cultivation media in the form of urea. In this cultivation scenario, an intermediate growth between synthetic (ammonium salts and nitrates) and complex media (yeast extract and tryptone/peptone), resulting in an average biomass of 5.0 ± 0.1 g/L and similar lipid content as yeast grown in nitrate [36.8 ± 2.8% (g lipid weight/g dry yeast cell weight)] was recorded. However, the final lipid weight collected after 120 h of cultivation was higher for *C. oleaginosus* grown in urea compared to synthetic nitrogen sources due to enhanced growth. Initial isolation of *C. oleaginosus* from Iowa State University Dairy Farm and its ability to metabolize urea triggered the annotation of its urate catabolic pathway (Bracharz et al., 2017a). Such studies have shown that nitrogen limitation results in upregulation of urea transporter gene DUR3, urate oxidase gene URO1, putative ammonia transporter genes and most allantoin permeases to facilitate the import of remaining nitrogen sources (Kourist et al., 2015). Whereas, glucose delivers 16 g/L of carbon concentration into cultivation media, urea merely delivers 0.055 g/L, indicating that the contribution of urea to the carbon concentration of the cultivation media is insignificant in comparison with glucose. In analogy, the contribution of yeast extract to the carbon concentration (0.44 g/L) of the cultivation is insignificant in comparison to the various sugars examined at 16 g/L, especially at the minute amount of yeast extract used to maintain nitrogen-limited conditions.

Growth and lipogenic potentials of *C. oleaginosus* on variable carbon sources are depicted in **Figure 2B** and **Table 5**. In addition to its high lipogenic potential, the relatively high growth rates observed for this strain on a wide variety of sugar sources makes it highly desirable for industrial purposes, as fermentation costs can be reduced with the use of complex waste materials and hydrolysates. In contrast, the model oleaginous yeast *Y. lipolytica*, which has been target of extensive metabolic engineering efforts can only grow on few select carbon sources (glucose, mannose and glycerol) (Sitepu et al., 2014; Shi and Zhao, 2017). Assimilation of pentose sugars, mainly xylose, has been heavily investigated over the last 30 years for its abundance in lignocellulosic (30%) and hemicellulosic (90%) biomasses, which in turn are cost effective and highly abundant in nature (Tanimura et al., 2018). Recent efforts enabling co-metabolism of hexose and pentose sugars by overcoming glucose repression in various oleaginous yeasts including *Rhodosporidium toruloides* and *Rhodotorula glutinis* has been reported (Yamada et al., 2017). Adversely, few oleaginous yeasts including *C. oleaginosus, L. starkeyi,* and *Geotrichum fermentans* exhibit the natural ability for sugar co-metabolism, which makes them directly applicable for growth on complex fermentation substrates (Tanimura et al., 2018). Furthermore, semi-defined media can be selected more carefully with the aid of the results presented in this study. With respect to biomass yield and lipid formation, it can be concluded that *C. oleaginosus* performs well on all tested carbon sources except the sugar alcohol, sorbitol. To this end, no record of *C. oleaginosus* efficient growth on sugar alcohols was evident in literature. Adaptation and remodeling of sugar transport system to accommodate for arabinose appears slower in *C. oleaginosus* compared to other sugar transporters (Brauer et al., 2005). This is evident in the slow initial growth of this yeast on arabinose following pre-culturing on favorable sugar—glucose. In contrast, the galactose-glucose disaccharide lactose recorded highest cumulative growth and subsequently ranked amongst the highest with respect to lipid production efficiency (lipid content, g lipid weight/g dry yeast cell weight). Interestingly, lactose serves as a superior carbon source in *C. oleaginosus* fermentations compared to either of its monomers galactose or glucose. As a result of the high growth rate of *C. oleaginosus* when cultured on lactose, two lactose hydrolases have been identified and thoroughly studied (West et al., 1990). Given that *C. oleaginosus* was originally isolated from dairy farm, it may be particularly adapted for efficient lactose metabolism (Bracharz et al., 2017a). Conversion of whey permeate to lipids by this strain is industrially favorable given the high cell density and lipid yield achieved due to high lactose content (Bracharz et al., 2017a). *C. oleaginosus*-based lipid production in pilot scale of 500 L bioreactors using lactose-rich deproteinised cheese whey has been reported in New Zealand since 1988 (Davies, 1988). Interestingly, lipid content (49.7% g/g) achieved in this study during lactose fermentation is consistent with recently reported lipid content (49.6% g/g) recorded for *C. oleaginosus* grown on lactose-rich deproteinised cheese whey and wine lees hydrolysate (Kopsahelis et al., 2018). However, fed-batch cultivation conditions employed by Kopsahelis et al. allowed for high lipid titers of 33.1 g/L, compared to 9 ± 0.34 g/L achieved in shake-flask fermentation in this study. Similarly, spent coffee hydrolysate offers another cost-efficient feedstock for *C. oleaginosus*, due to its high mannose content (**Table 5**) (Scully et al., 2016; Orrego et al., 2018). With respect to the assayed sugar monomers, galactose performs intermediately between glucose (C-4 epimer) and mannose (C-2 epimer). Sugar co-utilization studies have previously demonstrated a delayed uptake for galactose, which may be due to less efficient transport systems across the cell membrane compared to glucose (Meo et al., 2017). The ketonic hexose, fructose, was shown to enhance the total growth of *C. oleaginosus* by a minimum of 1.9 g/L in comparison to other hexoses, nevertheless its lipogenic potential was not enhanced. Whereas, growth of *C. oleaginosus* on media containing fructose as sole carbon source was not previously reported, Tchakouteu et al. described initial abundance of intracellular total sugars (ITS) composed of glucose and fructose upon growth on sucrose followed by 66% drop concurrent with lipid production (Tchakouteu et al., 2015). At variance with either fructose or glucose, sucrose resulted in much lower biomass (10.3 ± 1.14 g/L) and lipid yields (2.79 ± 0.58 g/L; 27.52 ± 6.60% g lipid weight/g dry yeast cell weight). We further confirm reported work by Bracharz et al. that arabinose cannot be metabolized by *C. oleaginosus* to generate biomass. However, most interestingly its aldopentose diastereomer, xylose, is a more favorable substrate for biomass generation than glucose under the evaluated growth conditions (Liang et al., 2014a; Bracharz et al., 2017a). In addition, maltose, a disaccharide of glucose,

mimicked glucose in both biomass and lipid weight, indicating that *C. oleaginosus* may harbor a very efficient extracellular maltose hydrolase activity or a very efficient maltose transporter in addition to an intracellular glycosidase shared with central metabolism, both of which have not been described to date.

Effect of Nitrogen and Carbon Source on Fames Profile

Industrial application of Single Cell Oils (SCO) is dependent on the fatty acid profile of the yeast in question, which in turn is influenced by cultivation conditions (Ochsenreither et al., 2016). Regardless of growth regime, the principal chemical composition of *C. oleaginosus* TAG mimics plant-derived lipids such as palm oil, with oleic acid [C18:1, 43–57% (g FAME/g dry yeast cell weight)] as main component succeeded by palmitic acid [C16:0, 16–33% (g FAME/g dry yeast cell weight)] (Hassan et al., 1994a). Increased concentrations of unsaturated fatty acids, as detected in *Rhodosporidium azoricum,* are essentially required to improve the cold-flowing properties of biodiesel (FAMEs). However, they are undesirable for Green-diesel production due to elevated hydrogen consumption. The maximal potential of *C. oleaginosus* is thus achieved in Green-diesel (HVO) processes, as it meets advanced feedstock specifications required by hydrotreatment process (Hydrogenated Vegetable Oil—HVO) due to its low degree of unsaturation (Capusoni et al., 2017). To achieve this level of saturation for medium chain fatty acids (MCFA), a recent study combines molecular design and TALENs in *Y. lipolytica* for the development of sustainable aviation fuels (Rigouin et al., 2017). The main influence exerted by media composition is most apparent in saturation degree perceived in **Figures 3A,B**. This is evident when ammonia is supplemented in the media, with a shift in the FA spectrum from palmitic acid [C16:0,

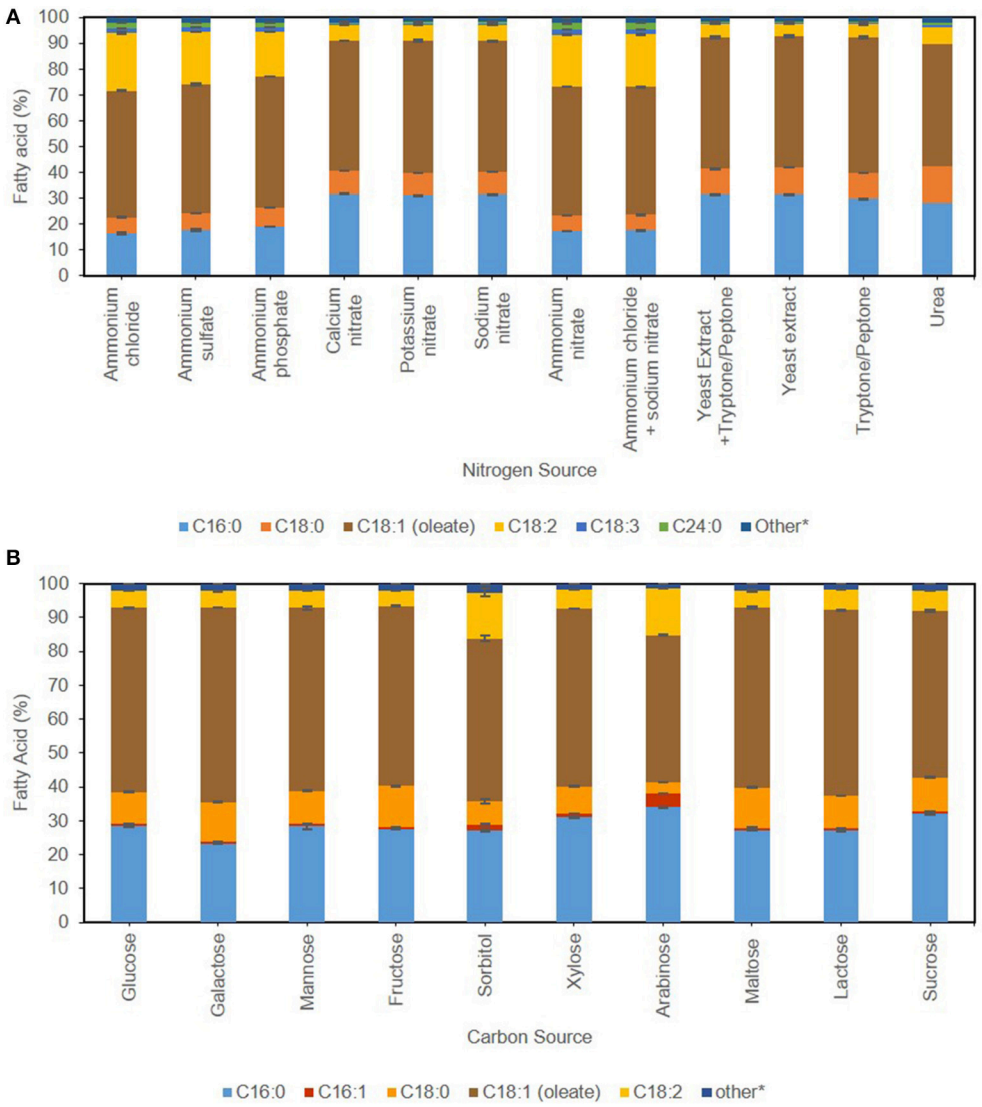


FIGURE 3 | Effect of nitrogen source **(A)** and carbon source **(B)** on FAMES profile of *C. oleaginosus*. Other constitute fatty acids present below 1% of total profile, including C12:0, C14:0, C18:3, C20:0, C20:1, C20:3, C22:0, C24:0 for **(A)** and C12:0, C14:0, C18:3, C20:0, C20:1, C20:3, C22:0, C24:0 for **(B)**.

13% (g FAME/g dry yeast cell weight)] and stearic acid [C18:0, 4% (g FAME/g dry yeast cell weight)] toward linoleic acid [C18:2,15% (g FAME/g dry yeast cell weight)], in addition to assumed elongation with emergence of 1.8% (g FAME/g dry yeast cell weight) lignoceric acid C24:0. The fatty acid profile of *C. oleaginosus* appears to be more susceptible to carbon source than nitrogen source, as visualized in **Figure 3B**. Whereas mannose, maltose, and lactose attain identical fatty acid profile as glucose, sorbitol and arabinose show maximal diversion. The latter show reduction in stearic [C18:0, 2–6% (g FAME/g dry yeast cell weight)] and oleic acid [C18:1, 6–8% (g FAME/g dry yeast cell weight)] in favor of linoleic acid [C18:2, 8% (g FAME/g dry yeast cell weight)] in comparison with glucose. Additionally, arabinose along with xylose and sucrose, show a slight decrease in oleic acid C18:1 in favor of palmitic acid [C16:0, 3% (g FAME/g dry yeast cell weight)]. When galactose is used as a carbon source, the result involves a decrease in palmitic acid (C16:0) consistent with parallel increase in stearic acid (C18:0) and recording highest fraction of oleic acid (C18:1) at 57% (g FAME/g dry yeast cell weight). The differentiation among carbon and nitrogen sources is more prominent with the fatty acid profiles. Specific fatty acid profiles associated with desired product application can be obtained with careful selection of media components.

CONCLUSION

Response Surface Methodology (Box-Behnken) allowed concurrent optimization of carbon and nitrogen concentrations and elemental ratios in cultivation media of *C. oleaginosus*, achieving a lipid yield of 0.42 ± 0.01 g lipid/g carbon, with 120:1 (16 g/L carbon and 0.13 g/L nitrogen). In our experimental setup, the highest biomass (18.4 ± 2.20 g/L) and lipid yield [9 ± 0.34 g/L; 49.74 ± 5.16% (g lipid weight/g dry yeast cell weight)] were obtained when lactose and yeast extract were used as carbon and nitrogen sources at an elemental weight ratio of 120:1, respectively. Conversely, the lowest biomass (1.93 ± 0.08 g/L) and lipid yield [0.32 ± 0.03 g/L, 16.35 ± 1.78% (g lipid weight/g dry yeast cell weight)] were obtained for glucose and ammonium chloride as carbon and nitrogen sources at an elemental weight ratio if 120:1, respectively. The physiological implications of these results are explained by the adaptive characteristics of *C. oleaginosus* to uptake and metabolize lactose and amino acids efficiently, in contrast to inorganic nitrogen entities. Furthermore, complex organic nitrogen sources such as yeast extract contain ample vitamins and trace minerals required for growth. Notably, the lowest lipid content [13.48 ± 1.90% (g lipid weight/g dry yeast cell weight)] was obtained with a C: N of 24:1 using glucose and yeast extract as a carbon and nitrogen sources, indicating that nutritional starvation was not reached. That the lowest biomass and lipid yields were obtained with ammonium chloride due to toxicity effects exerted by ammonium upon *C. oleaginosus* metabolism (see section Optimization of Carbon and Nitrogen Concentrations). These observations, in conjunction with the repertoire of this yeast's behavior in various carbon and nitrogen sources tested here, present a valuable source for identifying adequate and cost-efficient growth media (hydrolysates and waste materials). Comparison of fatty acid profiles of *C. oleaginosus*, when grown on different carbon and nitrogen sources, revealed enhanced saturation concerted with inorganic nitrogen sources, sorbitol and arabinose as carbon sources. Furthermore, it is pivotal to develop appropriate techniques for genetic manipulation to further improve the lipogenic potential and broaden the industrial application of *C. oleaginosus*. Future studies aimed at understanding *de novo* lipogenesis in *C. oleaginosus* from proteomic perspective will depend on optimal culture conditions developed in this study.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

Conceptualization of the study was conducted jointly by DA, NM, and TB. The methodological approach was designed and carried out by DA, FB, and NM. Data validation was jointly carried out by all authors. DA prepared the original draft of the manuscript. The manuscript was jointly finalized by all authors.

ACKNOWLEDGMENTS

TB gratefully acknowledges funding of the Werner Siemens foundation for establishing the research field of Synthetic Biotechnology at the Technical University of Munich. DA and NM gratefully acknowledge funding by the Bavarian State Ministry for Environmental and Consumer affairs for funding of the project Resource efficient PHB production processes within the project consortium BayBioTech (TP7, TLK01U-69045, http://www.baybiotech.de/startseite/). The authors gratefully acknowledge HPLC sample analysis conducted by Mrs. Martina Haack.

REFERENCES

Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P., and Villa, T. G. (2011). Oily yeasts as oleaginous cell factories. *Appl. Microbiol. Biotechnol.* 90, 1219–1227. doi: 10.1007/s00253-011-3200-z

Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J. L., Molina-Jouve, C., and Nicaud, J. M. (2009). *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res.* 48, 375–387. doi: 10.1016/j.plipres.2009.08.005

Bligh, E. G., and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917. doi: 10.1139/y59-099

Box, G. E. P., and Wilson, K. B. (1951). On the experimental attainment of optimum conditions. *J. R. Stat. Soc. Ser. B* 13, 1–45. doi: 10.1111/j.2517-6161.1951.tb00067.x

Bracharz, F., Beukhout, T., Mehlmer, N., and Bruck, T. (2017a). Opportunities and challenges in the development of *Cutaneotrichosporon oleaginosus* ATCC 20509 as a new cell factory for custom tailored microbial oils. *Microb. Cell Fact.* 16, 178. doi: 10.1186/s12934-017-0791-9

Bracharz, F., Redai, V., Bach, K., Qoura, F., and Bruck, T. (2017b). The effects of TORC signal interference on lipogenesis in the oleaginous yeast *Trichosporon oleaginosus*. *BMC Biotechnol.* 17:27. doi: 10.1186/s12896-017-0348-3

Brauer, M. J., Saldanha, A. J., Dolinski, K., and Botstein, D. (2005). Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. *Mol. Biol. Cell* 16, 2503–2517. doi: 10.1091/mbc.e04-11-0968

Capusoni, C., Rodighiero, V., Cucchetti, D., Galafassi, S., Bianchi, D., Franzosi, G., et al. (2017). Characterization of lipid accumulation and lipidome analysis in the oleaginous yeasts *Rhodosporidium azoricum* and *Trichosporon oleaginosus*. *Bioresour. Technol.* 238, 281–289. doi: 10.1016/j.biortech.2017.03.188

Casey, G. P., and Ingledew, W. M. (1986). Ethanol tolerance in yeasts. *Crit. Rev. Microbiol.* 13, 219–280. doi: 10.3109/10408418609108739

Casey, G. P., Magnus, C. A., and Ingledew, W. M. (1983). High gravity brewing: nutrient enhanced production of high concentrations of ethanol by brewing yeast. *Biotechnol. Lett.* 5, 429–434. doi: 10.1007/BF00131286

Chi, Z. M., Liu, T. T., Chi, Z., Liu, G. L., and Wang, Z. P. (2012). Occurrence and diversity of yeasts in the mangrove ecosystems in fujian, guangdong and hainan provinces of china. *Indian J. Microbiol.* 52, 346–353. doi: 10.1007/s12088-012-0251-5

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnol. Adv.* 25, 294–306. doi: 10.1016/j.biotechadv.2007.02.001

Cocaign-Bousquet, M., Garrigues, C., Novak, L., Lindley, N.D., and Loublere, P. (1995). Rational development of a simple synthetic medium for the sustained growth of *Lactococcus lactis*. *J. Appl. Microbiol.* 79, 108–116.

Davies, R., and Holdsworth, J. (1992). Synthesis of lipids in yeasts: biochemistry, physiology and production. *Adv. Appl. Lipid Res* 1, 119–159.

Davies, R. J. (1988). "Yeast oil from cheese whey; process development," in *Single Cell Oil*, ed R. S. Moreton (London), 99–145.

Evans, C. T., and Ratledge, C. (1983). A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon sources in continuous and batch culture. *Lipids* 18, 623–629. doi: 10.1007/BF02534673

Evans, C. T., and Ratledge, C. (1984). Effect of nitrogen source on lipid accumulation in oleaginous yeasts. *Microbiology* 130, 1693–1704. doi: 10.1099/00221287-130-7-1693

Godard, P., Urrestarazu, A., Vissers, S., Kontos, K., Bontempi, G., Van Helden, J., et al. (2007). Effect of 21 different nitrogen sources on global gene expression in the yeast Saccharomyces cerevisiae. *Mol. Cell. Biol.* 27, 3065–3086. doi: 10.1128/MCB.01084-06

Gong, Z., Zhou, W., Shen, H., Yang, Z., Wang, G., Zuo, Z., et al. (2016). Co-fermentation of acetate and sugars facilitating microbial lipid production on acetate-rich biomass hydrolysates. *Bioresour. Technol.* 207, 102–108. doi: 10.1016/j.biortech.2016.01.122

Gorner, C., Redai, V., Bracharz, F., Schrepfer, P., Garbe, D., and Bruck, T. (2016). Genetic engineering and production of modified fatty acids by the non-conventional oleaginous yeast *Trichosporon oleaginosus* ATCC 20509. *Green Chem.* 18, 2037–2046. doi: 10.1039/C5GC01767J

Granger, L. M., Perlot, P., Goma, G., and Pareilleux, A. (1993). Efficiency of fatty acid synthesis by oleaginous yeasts: prediction of yield and fatty acid cell content from consumed C/N ratio by a simple method. *Biotechnol. Bioeng.* 42, 1151–1156. doi: 10.1002/bit.260421004

Griffiths, M. J., Van Hille, R. P., and Harrison, S. T. (2010). Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. *Lipids* 45, 1053–1060. doi: 10.1007/s11745-010-3468-2

Gujjari, P., Suh, S. O., Coumes, K., and Zhou, J. J. (2011). Characterization of oleaginous yeasts revealed two novel species: *Trichosporon cacaoliposimilis* sp. nov. and *Trichosporon oleaginosus* sp. nov. *Mycologia* 103, 1110–1118. doi: 10.3852/10-403

Gutierrez, A., Beltran, G., Warringer, J., and Guillamon, J. M. (2013). Genetic basis of variations in nitrogen source utilization in four wine commercial yeast strains. *PLoS ONE* 8:e67166. doi: 10.1371/journal.pone.0067166

Hahn-Hagerdal, B., Karhumaa, K., Larsson, C. U., Gorwa-Grauslund, M., Gorgens, J., and Van Zyl, W. H. (2005). Role of cultivation media in the development of yeast strains for large scale industrial use. *Microb. Cell Fact.* 4:31. doi: 10.1186/1475-2859-4-31

Hassan, M., Blanc, P. J., Pareilleux, A., and Goma, G. (1994a). Production of single-cell oil from prickly-pear juice fermentation by *Cryptococcus curvatus* grown in batch culture. *World J. Microbiol. Biotechnol.* 10, 534–537. doi: 10.1007/BF00367661

Hassan, M., Blanc, P. J., Pareilleux, A., and Goma, G. (1994b). Selection of fatty acid auxotrophs from the oleaginous yeast *Cryptococcus curvatus* and production of cocoa butter equivalents in batch culture. *Biotechnol. Lett.* 16, 819–824. doi: 10.1007/BF00133960

Hess, D. C., Lu, W., Rabinowitz, J. D., and Botstein, D. (2006). Ammonium toxicity and potassium limitation in yeast. *PLoS Biol.* 4:e351. doi: 10.1371/journal.pbio.0040351

Hill, J., Nelson, E., Tilman, D., Polasky, S., and Tiffany, D. (2006). Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl. Acad. Sci. U.S.A.* 103, 11206–11210. doi: 10.1073/pnas.0604600103

Karlsson, H., Ahlgren, S., Sandgren, M., Passoth, V., Wallberg, O., and Hansson, P. A. (2016). A systems analysis of biodiesel production from wheat straw using oleaginous yeast: process design, mass and energy balances. *Biotechnol. Biofuels* 9, 229. doi: 10.1186/s13068-016-0640-9

Kopsahelis, N., Dimou, C., Papadaki, A., Xenopoulos, E., Kyraleou, M., Kallithraka, S., et al. (2018). Refining of wine lees and cheese whey for the production of microbial oil, polyphenol-rich extracts and value-added co-products. *J. Chem. Technol. Biotechnol.* 93, 257–268. doi: 10.1002/jctb.5348

Kourist, R., Bracharz, F., Lorenzen, J., Kracht, O. N., Chovatia, M., Daum, C., et al. (2015). Genomics and transcriptomics analyses of the oil-accumulating basidiomycete yeast *Trichosporon oleaginosus*: insights into substrate utilization and alternative evolutionary trajectories of fungal mating systems. *MBio* 6:e00918. doi: 10.1128/mBio.00918-15

Koutinas, A. A., Chatzifragkou, A., Kopsahelis, N., Papanikolaou, S., and Kookos, I. K. (2014). Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel* 116, 566–577. doi: 10.1016/j.fuel.2013.08.045

Li, Q., Du, W., and Liu, D. (2008). Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol.* 80, 749–756. doi: 10.1007/s00253-008-1625-9

Liang, Y., Jarosz, K., Wardlow, A. T., Zhang, J., and Cui, Y. (2014a). Lipid production by *Cryptococcus curvatus* on hydrolysates derived from corn fiber and sweet sorghum bagasse following dilute acid pretreatment. *Appl. Biochem. Biotechnol.* 173, 2086–2098. doi: 10.1007/s12010-014-1007-y

Liang, Y., Perez, I., Goetzelmann, K., and Trupia, S. (2014b). Microbial lipid production from pretreated and hydrolyzed corn fiber. *Biotechnol. Prog.* 30, 945–951. doi: 10.1002/btpr.1927

Liu, X. Z., Wang, Q. M., Goker, M., Groenewald, M., Kachalkin, A. V., Lumbsch, H. T., et al. (2015). Towards an integrated phylogenetic classification of the Tremellomycetes. *Stud. Mycol.* 81, 85–147. doi: 10.1016/j.simyco.2015.12.001

Masri, M. A., Jurkowski, W., Shaigani, P., Haack, M., Mehlmer, N., and Brück, T. (2018). A waste-free, microbial oil centered cyclic bio-refinery approach based on flexible macroalgae biomass. *Appl. Energy* 224, 1–12. doi: 10.1016/j.apenergy.2018.04.089

Meo, A., Priebe, X. L., and Weuster-Botz, D. (2017). Lipid production with Trichosporon oleaginosus in a membrane bioreactor using microalgae hydrolysate. *J. Biotechnol.* 241, 1–10. doi: 10.1016/j.jbiotec.2016.10.021

Mlickova, K., Roux, E., Athenstaedt, K., D'andrea, S., Daum, G., Chardot, T., et al. (2004). Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast *Yarrowia lipolytica*. *Appl. Environ. Microbiol.* 70, 3918–3924. doi: 10.1128/AEM.70.7.3918-3924.2004

Ochsenreither, K., Gluck, C., Stressler, T., Fischer, L., and Syldatk, C. (2016). Production strategies and applications of microbial single cell oils. *Front. Microbiol.* 7:1539. doi: 10.3389/fmicb.2016.01539

Orrego, D., Zapata-Zapata, A., and Kim, D. (2018). Optimization and scale-up of coffee mucilage fermentation for ethanol production. *Energies* 11:786. doi: 10.3390/en11040786

Papanikolaou, S., and Aggelis, G. (2011). Lipids of oleaginous yeasts. Part II: technology and potential applications. *Eur. J. Lipid Sci. Technol.* 113, 1052–1073. doi: 10.1002/ejlt.201100015

Papanikolaou, S., Beopoulos, A., Koletti, A., Thevenieau, F., Koutinas, A. A., Nicaud, J. M., et al. (2013). Importance of the methyl-citrate cycle on glycerol metabolism in the yeast *Yarrowia lipolytica*. *J. Biotechnol.* 168, 303–314. doi: 10.1016/j.jbiotec.2013.10.025

Rakicka, M., Lazar, Z., Dulermo, T., Fickers, P., and Nicaud, J. M. (2015). Lipid production by the oleaginous yeast *Yarrowia lipolytica* using industrial by-products under different culture conditions. *Biotechnol. Biofuels* 8, 104. doi: 10.1186/s13068-015-0286-z

Ratledge, C., and Wynn, J. P. (2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol.* 51, 1–51. doi: 10.1016/S0065-2164(02)51000-5

Rigouin, C., Gueroult, M., Croux, C., Dubois, G., Borsenberger, V., Barbe, S., et al. (2017). Production of medium chain fatty acids by *Yarrowia lipolytica*: combining molecular design and TALEN to engineer the fatty acid synthase. *ACS Synth. Biol.* 6, 1870–1879. doi: 10.1021/acssynbio.7b00034

Rodriguez-Navarro, A., and Ramos, J. (1984). Dual system for potassium transport in *Saccharomyces cerevisiae*. *J. Bacteriol.* 159, 940–945.

Ryu, S., Hipp, J., and Trinh, C. T. (2016). Activating and elucidating metabolism of complex sugars in *Yarrowia lipolytica*. *Appl. Environ. Microbiol.* 82, 1334–1345. doi: 10.1128/AEM.03582-15

Santos, J., Leao, C., and Sousa, M. J. (2013). Ammonium-dependent shortening of CLS in yeast cells starved for essential amino acids is determined by the specific amino acid deprived, through different signaling pathways. *Oxid. Med. Cell. Longev.* 2013:161986. doi: 10.1155/2013/161986

Scully, D., Jaiswal, A., and Abu-Ghannam, N. (2016). An investigation into spent coffee waste as a renewable source of bioactive compounds and industrially important sugars. *Bioengineering* 3:33. doi: 10.3390/bioengineering3040033

Shi, S., and Zhao, H. (2017). Metabolic engineering of oleaginous yeasts for production of fuels and chemicals. *Front. Microbiol.* 8:2185. doi: 10.3389/fmicb.2017.02185

Singh, V., Haque, S., Niwas, R., Srivastava, A., Pasupuleti, M., and Tripathi, C. K. (2016). Strategies for fermentation medium optimization: an in-depth review. *Front. Microbiol.* 7:2087. doi: 10.3389/fmicb.2016.02087

Sitepu, I., Selby, T., Lin, T., Zhu, S., and Boundy-Mills, K. (2014). Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. *J. Ind. Microbiol. Biotechnol.* 41, 1061–1070. doi: 10.1007/s10295-014-1447-y

Smit, H., Ykema, A., Verbree, E., Verwoert, I., and Kater, M. (1992). "Production of cocoa butter equivalents by yeast mutants," in *Industrial Applications of Single Cell Oils*, eds D. J. Kyle and C. Ratledge (Champaign, IL: AOCS Press), 185–195. doi: 10.1201/9781439821855.ch10

Smith, V. H., Sturm, B. S., Denoyelles, F. J., and Billings, S. A. (2010). The ecology of algal biodiesel production. *Trends Ecol. Evol.* 25, 301–309. doi: 10.1016/j.tree.2009.11.007

Spagnuolo, M., Shabbir Hussain, M., Gambill, L., and Blenner, M. (2018). Alternative substrate metabolism in *Yarrowia lipolytica*. *Front. Microbiol.* 9:1077. doi: 10.3389/fmicb.2018.01077

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science* 315, 801–804. doi: 10.1126/science.1139612

Swoboda, F. K. (1922). Nitrogen nutrition of yeast. *J. Biol. Chem.* 52, 91–109.

Tai, M., and Stephanopoulos, G. (2013). Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. *Metab. Eng.* 15, 1–9. doi: 10.1016/j.ymben.2012.08.007

Tanimura, A., Sugita, T., Endoh, R., Ohkuma, M., Kishino, S., Ogawa, J., et al. (2018). Lipid production via simultaneous conversion of glucose and xylose by a novel yeast, *Cystobasidium iriomotense*. *PLoS ONE.* 13:e0202164. doi: 10.1371/journal.pone.0202164

Tchakouteu, S. S., Chatzifragkou, A., Kalantzi, O., Koutinas, A. A., Aggelis, G., and Papanikolaou, S. (2015). Oleaginous yeast *Cryptococcus curvatus* exhibits interplay between biosynthesis of intracellular sugars and lipids. *Eur. J. Lipid Sci. Technol.* 117, 657–672. doi: 10.1002/ejlt.201400347

Wahlen, B. D., Morgan, M. R., Mccurdy, A. T., Willis, R. M., Morgan, M. D., Dye, D. J., et al. (2013). Biodiesel from microalgae, yeast, and bacteria: engine performance and exhaust emissions. *Energy Fuels* 27, 220–228. doi: 10.1021/ef3012382

West, M., Emerson, G. W., and Sullivan, P. A. (1990). Purification and properties of two lactose hydrolases from *Trichosporon cutaneum*. *J. Gen. Microbiol.* 136, 1483–1490. doi: 10.1099/00221287-136-8-1483

Willis, R. M., Mccurdy, A. T., Ogborn, M. K., Wahlen, B. D., Quinn, J. C., Pease, L. F. III., et al. (2014). Improving energetics of triacylglyceride extraction from wet oleaginous microbes. *Bioresour. Technol.* 167, 416–424. doi: 10.1016/j.biortech.2014.06.013

Wu, S., Hu, C., Jin, G., Zhao, X., and Zhao, Z. K. (2010). Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresour. Technol.* 101, 6124–6129. doi: 10.1016/j.biortech.2010.02.111

Yamada, R., Yamauchi, A., Kashihara, T., and Ogino, H. (2017). Evaluation of lipid production from xylose and glucose/xylose mixed sugar in various oleaginous yeasts and improvement of lipid production by UV mutagenesis. *Biochem. Eng. J.* 128, 76–82. doi: 10.1016/j.bej.2017.09.010

Yu, X., Zheng, Y., Xiong, X., and Chen, S. (2014). Co-utilization of glucose, xylose and cellobiose by the oleaginous yeast *Cryptococcus curvatus*. *Biomass Bioenergy* 71, 340–349. doi: 10.1016/j.biombioe.2014.09.023

Zhang, J., and Greasham, R. (1999). Chemically defined media for commercial fermentations. *Appl. Microbiol. Biotechnol.* 51, 407–421. doi: 10.1007/s002530051411

Zhao, Y., Cao, W., Wang, Z., Zhang, B., Chen, K., and Ouyang, P. (2016). Enhanced succinic acid production from corncob hydrolysate by microbial electrolysis cells. *Bioresour. Technol.* 202, 152–157. doi: 10.1016/j.biortech.2015.12.002

Conflict of Interest Statement: FB was employed by company BBSI GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

3.2 Towards High-Throughput Optimization of Microbial Lipid Production: From Strain Development to Process Monitoring

Dania Awad, Samer Younes, Matthias Glemser, Franz M. Wagner,

Gerhard Schenk, Norbert Mehlmer and Thomas Brueck

Featured on the Front cover of Sustainable Energy & Fuels: Issue 12 (December 2020)

Published in *Sustainable Energy and Fuels*

Date of Publication: 17 June 2020

DOI: 10.1039/D0SE00540A

3.2.1 Author Contributions

Conceptualization of the study and design of the methodological approach was jointly designed by all authors. Planning and execution of experiments was carried out by the author of this thesis, Dania Awad. Data validation was jointly carried out by all authors. Dania Awad and Samer Younes prepared the original draft of the manuscript, which was jointly finalized and reviewed by all authors.

3.2.2 Summary

In order to push the economic boundaries for industrial implementation of Single Cell Oils (SCOs), bioprocess costs have to be significantly reduced[134]. In that respect, any significant improvement in space-time yields of microbial lipids is central to the accelerated industrial deployment for this promising technology. One main strategy in addressing this bottleneck involves metabolic engineering of promising oleaginous yeasts (OY)[135]. *C. oleaginosus*, the focus of this and numerous strain development studies, display protective mechanisms preventing rapid and precise targeted genome manipulation (CRISPR)[67]. Genetic enhancement of this strain can be carried out by random mutagenesis coupled with an efficient screening method. This approach was adopted in this study for generating and isolating enhanced lipogenic variants of *C. oleaginosus*.

We describe, for the first time, the use of fast neutron (FN) irradiation for the rapid metabolic optimization of an oleaginous yeast. This is also the second report on fast neutron irradiation of yeast, with the earlier dating back to 1986 on *S. cerevisiae*[136]. The inherent stability of the mutations caused by this specific type of ionizing irradiation was further corroborated in our study. The generated library of mutants was screened for coveted traits - enhanced biomass and lipid accumulation - via cerulenin. Strains resistant to this fatty acid synthase (FAS) inhibitor were isolated and subsequently characterized and evaluated with respect to lipid titers and profiles.

In order to rapidly identify and efficiently monitor enhanced lipogenic mutants, we developed an absolute quantitative spectrofluorometric assay based on Nile Red analysis. Our approach implements the inherent microbial lipids (ML) of *C.* oleaginosus as a standard for the determination of intracellular lipid titers at a 96-wells MTP-scale. ML standard was further correlated with a previously reported standard, triolein, with the latter resulting in overestimation of lipid titers. The potential of oleic acid (C18:1) as a low-cost and easy to handle standard was also evaluated, given its prominence at 43–57% (w/w) in the fatty acid profile of this strain[137]. The developed method was successfully employed for the efficient screening and monitoring of fast neutron mutant library of *C. oleaginosus*.

Our experimental setup for strain development resulted in the isolation of 2 enhanced mutants: *C. oleaginosus* FN M2 and FN M8. FN M2 displayed a 21.67 % (w/v) increase in biomass formation, resulting in a 22.58 % (w/v) increase in total lipid yield, compared to the wild type strain. Biomass formation of FN M8 was substantially lower than that of the wild type strain. However, the lipid content of this mutant remarkably reached 73.16 % (w/v). These mutants are subject to further characterization of oleaginicity controls and upstream development. Industrial application of the microbial lipids is highly dependent on their fatty acid profiles [7]. The degree of fatty acid saturation dictates the physiochemical properties of biofuels [131]. These properties were investigated for *C. oleaginosus* wild type and FN M2, and compared with that of other prominent oleaginous yeasts and plants. The calculated values of these properties fall within the limits of internationally accepted requirements of biodiesel standards. The detected fatty acid profile shifts are valuable in expanding the industrial applications of this yeast beyond biofuels.

In summary, this study demonstrates that *C. oleaginosus* platform is amenable to implementation of high-throughput strategies for process design and development. To that end, the established approach provides a progressive path towards automatable strain development efforts in conjunction with high throughput analytical and process monitoring procedures.

3.2.3 Full-length Publication

Volume 4
Number 12
December 2020
Pages 5871–6332

Sustainable Energy & Fuels

Interdisciplinary research for the development of sustainable energy technologies

rsc.li/sustainable-energy

ISSN 2398-4902

ROYAL SOCIETY OF CHEMISTRY

PAPER

Thomas Brueck *et al.*

Towards high-throughput optimization of microbial lipid production: from strain development to process monitoring

Sustainable Energy & Fuels

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d0se00540a

Towards high-throughput optimization of microbial lipid production: from strain development to process monitoring†

Dania Awad, ‡[a] Samer Younes,‡[a] Matthias Glemser,[a] Franz M. Wagner,[b] Gerhard Schenk, [cd] Norbert Mehlmer [a] and Thomas Brueck *[a]

Digitalization drives accelerated process optimization by comprehensive automation. In the advanced biofuels sector this demands automatable high-throughput processes for production strain development and downstream process performance monitoring. In that context, the unit operations of oleaginous yeast-based biodiesel production are amenable to high-throughput process development. *Cutaneotrichosporon oleaginosus* is a leading production strain for high-energy biofuel options, that is capable of utilizing a broad range of substrates as carbon sources, thereby generating in excess of 60% (w/w) lipids under nutrient limiting conditions. For the first time, we report on the use of fast neutron (FN) irradiation for the rapid, high-throughput genetic enhancement of an oleaginous yeast in conjunction with high-throughput selection of enhanced lipid producing *C. oleaginosus* mutants by cultivation in the presence of the fatty acid biosynthesis inhibitor cerulenin. Performance monitoring of improved mutants was accomplished by development of a high-throughput lipid qualification methodology based on a miniaturized, low cost Nile red based spectrofluorimetric assay. From the FN mutant library, this high-throughput strain development approach allowed identification of a *C. oleaginosus* variant (FN M2) displaying a 21.67% (w/v) and 22.58% (w/v) increase in biomass formation and total lipid yield compared to wild-type strain, respectively. Mutant triglyceride characterization revealed a higher content of saturated fatty acids, which is favorable with respect to biofuels production standards, determined here for the first time. This study is an initial step towards an automatable, high-throughput yeast oil optimization process that facilitates accelerated industrial deployment.

Received 4th April 2020
Accepted 12th June 2020

DOI: 10.1039/d0se00540a

rsc.li/sustainable-energy

1. Introduction

Climate change and resulting environmental concerns drive the development of sustainable bioenergy and oleochemical solutions.[1–3] However, plant-derived biofuels and chemical entities only offer a partial solution as oil crops compete with agricultural crops over arable land, nutrients and water resources, ultimately affecting food prices.[1,3,4] Oleaginous microorganisms (OM), such as yeast, filamentous fungi and algae species can accumulate high amounts of intracellular lipids, which are commonly in excess of 20% (g g^{-1} of dry cell weight (DCW)).[1,2,5] Recently, utilization of single cell oils (SCOs) as a platform for biodiesel production has received considerable attention, since OM (a) do not require arable land, (b) can utilize waste material as feedstocks, (c) have short production-cycles and (d) are not affected by seasons or climate. Thereby, OM do not jeopardize food security, all the while providing triglyceride-based lipid products at a constant yield and quality independent of climate and land use change effectors.[1–3,6] The use of lipids, derived from OM and particularly oleaginous yeasts (OYs), for biofuel and bioenergy production has been reported previously.[7] However, a summary of Key Performance Indicators (KPIs) for yeast-derived fuel products has not yet been documented.

In the context of yeast-based biofuel generation, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Cutaneotrichosporon oleaginosus*, *Yarrowia lipolytica*, *Debaryomyces hansenii* and *Rhodotorula glutinis* are amongst the most promising yeast species for industrial applications of SCOs. Several studies reported lipid accumulation of 40–70% (g g^{-1} of DCW) in these wild-type strains when cultivated under nitrogen-limiting conditions with excess carbon (glucose).[1,2,5,8,9] *C. oleaginosus*, in particular, displays industrially favorable characteristics, such as the ability to accumulate more than 60% lipids (g g^{-1} of

[a]*Werner Siemens-Chair of Synthetic Biotechnology, Department of Chemistry, Technical University of Munich (TUM), Garching, Germany. E-mail: brueck@tum.de*
[b]*Forschungs-Neutronenquelle Heinz Maier-Leibnitz II (FRM II), Technische Universität München, Garching, Germany*
[c]*School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, QLD 4072, Australia*
[d]*Sustainable Minerals Institute, The University of Queensland, St. Lucia, QLD 4072, Australia*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0se00540a

‡ Both authors contributed equally to the work.

DCW), a high flexibility in carbon source utilization and a fatty acid composition similar to that of plant oils, specifically palm oil.[1,4,10–14] This yeast has been the focus of several strain development studies ranging from model-based (surface response methodology) culture media optimization, tailored fatty acid profile, development of sustainable and high performance waste-free biorefinery platforms, in addition to techno-economic studies.[1,10,13,15,16] However, yeast-derived lipids have not yet been commercialized due to technical (*e.g.* low space-time yields) and economic (*i.e.* cost of oil extraction) barriers. Moreover, high production costs, which mainly include expensive fermentation substrates (carbon sources) and complex lipid extraction protocols (cell rupture, oil extraction and purification), have prevented market entry of this technology.[3,6,17] To that end, rapid identification and optimization of OY production strains in synergy with appropriate cultivation and oil extraction conditions are required to develop a commercially viable process. Accordingly, any significant improvement in space-time yields of yeast lipids is central to the accelerated industrial deployment for this promising high-energy biofuel technology.[1,3,18]

In that context, digitalization accelerates and drives industrial process design, optimization and deployment through comprehensive automation. With respect to advanced bioprocesses, such as yeast-based biofuel production, this does not only demand specialized sensor and software development, but is also highly dependent on the availability of automatable, high-throughput processes, particularly in initial process development stages involving production strain generation, selection and performance monitoring. While this has been in part achieved for biogas and bioethanol processes, equivalent solutions are lacking for high-energy content biofuels processes.[19–22] To that end, the generation of microbial lipids for biodiesel production using yeast-based production platforms, such as *C. oleaginosus*, are amenable to implementation of high-throughput optimization of individual unit operations. Therefore, development of high-throughput methodologies for genetic production strain enhancement, rapid selection of process-relevant variants and downstream product-oriented performance monitoring are essential for automated oleaginous yeast biofuel process optimization scenarios.

With respect to rapid production strain enhancement, current methods of metabolic engineering encompass various targeted and random mutagenesis techniques.[23,24] Targeted mutagenesis, or directed evolution, *via* genetic engineering is conventionally a key technology in developing optimized strains with improved and tailored lipid production characteristics.[25] Although genomic and transcriptomic data of *C. oleaginosus* are available, this and other *de novo* OYs display protective mechanisms preventing rapid and precise genome manipulation (*via* mechanisms of homologous recombination/knockout). These organisms appear to be refractory to most targeted genetic engineering approaches, as observed by several studies performed in our group and others (*e.g.* CRISPR).[2,10,26–28] Specifically, the GC-rich genome (65%) of these organisms entails a strong bias in the codon usage, thus hindering the direct transfer of established site-directed genetic protocols from model yeasts like *Saccharomyces cerevisiae* to *C. oleaginosus* and other OYs.[10,26] However, random genetic manipulation of several OYs has been successful *via Agrobacterium tumefaciens*-mediated gene transfer (AGMT), but this method results in multiple, non-targeted insertions, is laborious and cannot be transferred into high-throughput automated formats.[2,10,26] Furthermore, with AGMT and other targeted mutagenesis techniques arises the need for selective agents (*e.g.* antibiotics) that incur additional costs and efforts, especially at industrial levels, in addition to legislative requirements involved in Genetically Modified Organism (GMO) control and containment.[29]

In contrast, strain development can also be carried out by random mutagenesis, also termed accelerated evolution. This method is (a) subject to no GMO controls, (b) requires minimal technical manipulation, (c) does not necessitate the integration of antibiotic resistance genes, (d) requires little knowledge of the genetic/biochemical mechanisms involved in the synthesis of desired products, and, most importantly, (e) can be transferred to automatable, high-throughput formats.[29–31] Generally, microbial strain improvement has previously been achieved *via* physical mutagens, such as UV light and ionizing radiation (IR, including γ-rays, α-rays, X-rays and neutron radiation), in addition to chemical mutagenic agents, such as ethyl methane sulfonate (EMS) and nitrosomethyl guanidine (NTG).[2,5,25,30,32] Chemically-induced mutations are genetically less stable and have a tendency to revert back to the wild-type form.[32,33] Similarly, UV-induced DNA damage (photo-lesions) is commonly repaired through photo-reactivation. Even in the absence of this process, photo-lesions can still be removed – albeit less efficiently – by DNA repair mechanisms.[34,35] The most commonly used IR are γ-rays, which have been implemented in the mutagenesis of diverse organisms (*e.g.* bacteria, yeasts, plants). The type of DNA damage caused by IR correlates with the relative biological effectiveness (RBE) of the specific radiation, which is a function of its linear energy transfer (LET). In that respect, fast neutron (FN) radiation shows greater LET (many folds higher) than those of low-LET radiation, such as γ- and X-rays. Amongst the various types of damage induced by mutagens the most biologically relevant are DNA double strand breaks (DSBs).[33,36] FN irradiation has been reported to cause a higher number of non-repairable DNA damage concurrent with highly delayed DNA repair mechanisms.[33,37] Thus, it is a more effective inducer of inherently stable DSBs, base substitutions or deletions/insertions compared to other mutagenic techniques (physical or chemical).[33,38,39] FN mutagenesis has been successfully and extensively employed for crop improvement in many plant species including soybean, rice, peanuts, tomato and peas.[33,40–42] However, FN irradiation is a less explored approach in the mutagenesis of yeast species, with the sole report of utilizing this specific IR in irradiating the non-oleaginous model yeast *S. cerevisiae* dating back to 1986.[43]

The main challenge associated with FN irradiation and any other random mutagenesis technique lies in the large numbers of generated mutant libraries. Thus, an efficient selection method commonly succeeds random mutagenic techniques.[5,18] With respect to OYs, selection methods should allow for the

isolation of mutants exhibiting enhanced lipid yields and enhanced biomass formation.[2,5,32] Interestingly, cerulenin, which was originally isolated from the fungus *Cephalosporium caerulens*, is a potent fatty acid synthase (FAS) inhibitor. Cerulenin results in the reduction of colony growth on culture plates.[44] This antifungal has become the standard for improved screening of OYs for lipid yield. The rational of this strategy resides in the fact that mutants displaying normal growth, in the presence of cerulenin, possess mutagenesis-induced metabolic alterations (*e.g.* enhanced FAS activity). By overcoming the inhibition, these mutants are good candidates for improved growth or lipid production.[2,5,25] When generating random mutagenesis libraries, a subsequent selective cultivation in the presence of cerulenin provides a targeted, fast, visible and labor-saving selection method for improved mutant strains. Improvements in lipid production following this combinatorial approach have been previously reported for *R. glutinis*, *R. toruloides*, *L. starkeyi* and *Y. lipolytica* but not *C. oleaginosus*.[5,18,25,45]

Generation and selection of mutant libraries still incur further qualification and characterization of fermentative growth potential. Conventional methods for the determination of OY lipid titers typically require cell disruption, solvent lipid extraction and weighing (gravimetric analysis), or derivatization of extracted fatty acids into fatty acid methyl esters (FAMES) followed by gas chromatography. These techniques are very tedious and time-consuming, especially when handling significant amounts of biological samples, such as mutant libraries.[2,5,18,46] In context of strain development for industrial commercialization of SCOs, process monitoring, which employs state-of-the-art analytical tools characterized by high-throughput and miniaturized scale, enables greater depth of strain characterization.[47] Recent advancements in efficient and fast quantification of lipids from OM have made use of several high-throughput colorimetric and spectrofluorimetric methods such as Nile red, Sudan black B and sulfo-phospho-vanillin. With the advantage of easy handling of small biological samples, these fast lipid quantitation methods can be paired with other cell component quantitation (*e.g.* protein, DNA) using the same sample for multi-analytical measurements. Specifically, Nile red analysis has been well optimized and validated. Lipid quantification *via* this assay strongly correlates with the gravimetric lipid content (%, g g^{-1} of DCW).[48–50] However, absolute quantification of lipid yields (g L^{-1}) *via* the Nile red method has been hindered by the high variability of the fluorescence measurements.[51,52] Glycerol triolein (TO) is a symmetrical triglyceride harboring one glycerol and three oleic acid (OA) units, which represent the most prominent fatty acid produced in *C. oleaginosus*. TO has previously been adopted as a standard for Nile red quantitation of lipids from OY.[53] However, the use of this standard for absolute quantitation comes with its own drawbacks. TO is expensive, difficult to handle, only linear in a narrow concentration range (2–100 μg mL^{-1}), and might result in over- or underestimation of the actual neutral lipid content in the cells.[3,54,55]

For the first time, this study employed random mutagenesis by FN irradiation for the rapid, high-throughput genetic enhancement of an OY. Following mutagenesis of *C. oleaginosus* ATCC 20509, potentially improved mutants displaying uninhibited growth on cerulenin-containing medium were rapidly selected for further qualification of their fermentative potential. Accordingly, evaluation of mutants displaying enhanced biomass and lipid formation entailed the development of a miniaturized, quantitative high-throughput assay (lipid yield, lipid content and maximal lipid productivity) based on Nile Red fluorospectrometry. This lipid detection methodology was based on a purified microbial lipid standard extracted from *C. oleaginosus*, which was further correlated with the previously reported model triglyceride, TO. Additionally, the potential of OA as a low-cost and easy-to-handle alternative standard was evaluated. Moreover, analysis of lipid profiles and biofuel KPIs of wild-type *C. oleaginosus* as well as improved mutant strains were conducted. This study provides initial processes for the development of a technology platform for automated high-throughput yeast lipid process optimization, that fosters accelerated industrial deployment of advanced high-energy biofuels, such as yeast-based biodiesel and sustainable aviation fuels. Our *C. oleaginosus* centered model study presents initial high-throughput solutions for essential unit operations covering rapid and automatable genetic strain enhancement, selection of improved variants in conjunction with a low cost, miniaturized yeast oil centered performance monitoring assay.

2. Materials and methods

2.1 Yeast strain and culture conditions

C. oleaginosus ATCC 20509 (from the culture collection of Werner Siemens Chair of Synthetic Biotechnology – WSSB, TU, Munich) was maintained on YPD (yeast extract peptone dextrose) agar plates (20 g L^{-1} peptone, 20 g L^{-1} agar, 20 g L^{-1} glucose, 10 g L^{-1} yeast extract). A single colony was initially cultured in 125 mL Erlenmeyer flask holding 50 mL YPD liquid medium at 28 °C and in a rotary incubator at 120 rpm for 24 h. Lipid accumulation was induced by subsequent inoculation in 125 mL Erlenmeyer flask holding 50 mL of minimal-mitrogen media MNM (40 g L^{-1} glucose, 0.75 g L^{-1} yeast extract, 1.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.4 g L^{-1} KH_2PO_4, 0.22 g L^{-1} $CaCl_2 \cdot 2H_2O$ and trace elements: 1.2 mg L^{-1} $(NH_4)_2SO_4$, 0.55 μg L^{-1} $ZnSO_4 \cdot 7H_2O$, 24.2 μg L^{-1} $MnCl_2 \cdot 4H_2O$, 25 μg L^{-1} $CuSO_4 \cdot 5H_2O$) prepared according to.[1] With a starting optical density of 0.1, measured at 600 nm, cultivation was sustained for 96 h at 28 °C in a rotary incubator at 120 rpm.

2.2 Experimental design

2.2.1 Fast neutron irradiation. FN mutagenesis was conducted at the Research Neutron Source Heinz Maier-Leibnitz (FRM II) facility of the Technical University of Munich in Garching, Germany. The nuclear research reactor produces free neutrons through uranium fission that is utilized for numerous scientific experiments (tumor treatment and physical and biological dosimetry). A secondary source, consisting of two uranium plates, stands one meter from the reactor core. This source produces fast fission neutrons that are guided through beam tube number 10 (SR-10) to the MEDAPP (medical

applications) instrument.[56] The MEDAPP set up displays a neutron flux density of approximately 3.2×10^8 cm^{-2}. s^{-1}. The experimental setup parameters are displayed in Table 1. Triplicate samples of exponentially grown cells of *C. oleaginosus* were set to 10^7 cells per mL^{-1} *via* fluorescence-activated cell sorting (FACS) counting in a KCl-media (27 g L^{-1} NaCl, 6.6 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1.5 g L^{-1} $CaCl_2 \cdot 2H_2O$, 5 g L^{-1} KNO_3, 0.07 g L^{-1} KH_2PO_4, 0.014 g L^{-1} $FeCl_3 \cdot 6H_2O$ and 0.021 g L^{-1} Na_2-EDTA$\cdot 2H_2O$). A total of 56 Eppendorf tubes (2 mL), which were completely filled with cell suspension, were placed horizontally within the beam area in a custom-made rack (3D-printed from polylactic acid (PLA)). Following radiation clearance, treated samples were stored in the dark at room temperature to prevent photorepair events. Aliquots of the mutant library were stored at −80 °C in 10% glycerol.

2.2.2 Cerulenin screening

A cerulenin stock solution (1 mg mL^{-1}) was prepared in DMSO (dimethyl sulfoxide). To estimate the proper working concentration of cerulenin for the selection of *C. oleaginosus* mutants, non-irradiated cells were plated on YPD agar, supplemented with cerulenin (Applichem, Germany) at a concentration gradient of 2, 4, 6, 8, 10 and 12 μg μL^{-1}. Based on the survival fraction, cerulenin selection took effect at a concentration of 10 μg μL^{-1}. Approximately 2×10^6 *C. oleaginosus* irradiated (mutants pool U) and non-irradiated (pool A) cells were plated on cerulenin-supplemented YPD agar plates. Following incubation for 7 days, large colonies were transferred to fresh cerulenin-free MNM agar plates, and sub-cultured over a period of 4 weeks. Subsequently, the individual colonies were cultivated in liquid MNM for 96 h to assess growth and lipid production (see section 2.1).

2.3 Analytical methods

2.3.1 High-throughput and absolute quantitation *via* Nile red analysis. Lipid titers of wild-type *C. oleaginosus* and its mutants were calculated based on a calibration curve generated using purified microbial oil (ML) extracted from wild-type *C. oleaginosus.* Additionally, an analytical grade TO (Sigma-Aldrich, Germany) standard curve was prepared. Also, a third standard curve was based on analytical grade OA (Applichem, Germany). All three standard curves were constructed following a modified protocol of Priscu *et al.*[54] Briefly, chloroform was chosen as a carrier for all standards (1 : 1 standard : chloroform) into anhydrous ethanol to make intermediate dilutions of 2.0, 4.0, 8.0 and 10.0 mg mL^{-1}. Additionally, another OA acid standard was prepared following the exact protocol, save for the initial chloroform dilution step. Following vigorous vortexing, the final dilution step was prepared in deionized (DI) water, specifically to realize an intermediate stock:final diluent (v/v) that is consistent across all prepared dilutions (20, 40, 80 and 100 μg mL^{-1}). This procedure resulted in hydrophobic micelles of uniform size that mimic the nature of the *C. oleaginosus* samples' final solvent system. In that respect, Nile red analysis of wild-type *C. oleaginosus*, mutants and prepared standards (ML, TO and ML) followed a modified protocol of Sitepu *et al.*[50] Briefly, triplicates of wild-type and mutant yeast cells were diluted with DI water to OD600 < 1 in black Nunc™ F96 MicroWell™ Polystyrene Plate (Thermo Scientific Waltham, MA, USA). These plates ensure minimal fluorescence background and stray light. Following the addition of 50 μL DMSO, initial readings were recorded for growth and background fluorescence. A Nile red (9-diethylamino-5H-benzo [alpha] phenoxazine-5-one) (Sigma-Aldrich, Germany) stock solution (60 μg mL^{-1}) was prepared freshly in DMSO. Additionally, the stock and staining solutions were protected from light to avoid photo-bleaching. Nile red was then added to a working concentration (WC) of 5 μg per mL per well. Kinetic reading of fluorescence emission was measured at 590/35 nm with an excitation wavelength of 530/25 nm for 5 min with 30 s intervals using an EnSpire 2 microplate reader from Perkin Elmer (Waltham, MA, USA). Maximal fluorescence emission (MFE) values were recorded to calculate lipid yields (g L^{-1}) and maximal lipid productivity (mg L^{-1} h^{-1}). MFE values were further corrected for cell density variations by norming with optical density measurements at 600 nm to obtain normalized fluorescence and subsequently calculate the lipid content (%, g g^{-1}) of DCW.

2.3.2 Fatty acid profile analysis. Fatty acid methyl esters (FAMEs) were obtained by methanol transesterification of lyophilized yeast biomass. The transesterification protocol was originally adopted from Griffiths *et al.* and modified in our lab by Gorner *et al.*[10,57] Briefly, pre-weighed lyophilized cells were loosened in toluene and sodium methoxide in methanol and incubated at 80 °C and 600 rpm for 20 min. Following a sequential incubation step at room temperature, HCl/methanol was added and the incubation was repeated. FAME extraction followed shaking of samples in hexane and water and a quick centrifugation at 4000 rpm. The upper phase was aspired and transferred into GC vials. FAME profiles were analyzed on a GC-2010 Plus gas chromatograph from Shimadzu (Nakagyo-ku, Kyōto, Japan) equipped with a flame ionization detector. A sample of 1 μL was applied by AOC-20i auto injector (Shimadzu) onto a ZB-WAX column (30 m, 0.32 mm ID; 0.25 μm df; phenomenex (Torrance, CA, USA). The initial column temperature was 150 °C (maintained for 1 min). A temperature gradient was applied from 150 °C–240 °C (5 °C min^{-1}), followed by 6 min maintenance at 240 °C. Fatty acids were identified according to retention times of the authentic standard: Marine

Table 1 Experimental setup for FN irradiation of *C. oleaginosus*

Parameters	Setup
Distance to converter plate	5.33 m
Lead filter thickness	6 cm
D_n [Gy min^{-1}]	0.4028
D_γ [Gy min^{-1}]	0.1840
Cumulative dose [Gy min^{-1}]	0.5868
Dosage steps [Gy]	0–1000, increment of 50[a]
Ratio $D_n D_\gamma^{-1}$	2.189
Strain	*C. oleaginosus*

[a] Pools of *C. oleaginosus* subjected to increasing irradiation dosage based on 50 Gy increments were labeled A–U (Pool A: non-irradiated cells).

Oil FAME Mix (Restek, USA). Individual FAME concentrations were based on peak areas relative to Methyl Nonadecaanoate C19 (Sigma-Aldrich, Germany), which was incorporated in all samples as an internal standard. Biological triplicate measurements were recorded for each of the screened yeast mutants.

2.4 Statistical analysis

2.4.1 Survival rate. To estimate cellular concentration and viability, a volume of 50 μL of each of the irradiated and non-irradiated suspensions (Samples A–U) was plated on YPD agar plates in triplicates. Following incubation for 96 h at 28 °C, triplicate colony counts were recorded and an exponential regression trend was fitted.

2.4.2 Calculations of biofuel properties. Various physiochemical properties of biofuels based on FAMEs profiles of the wild-type and improved mutants of *C. oleaginosus* were determined using predictive models and mathematical equations for the transesterified SCOs.[58] These properties include iodine value (IV), cetane number (CN), higher heating value (HHV), kinematic viscosity (KV) and density.

3. Results and discussion

3.1 Accelerated *C. oleaginosus* evolution by fast neutron irradiation

In this study, our efforts to rapidly generate improved *C. oleaginosus* variants are based on random mutagenesis by FN irradiation. This is the first report on the application of FN irradiation to generate improved OY mutants.[33,43] The experimental set up for the nuclear research reactor of this study is displayed in Fig. 1A. The high source strength allowed for high irradiation dosage of 0.5868 Gy min^{-1} by maintaining high neutron fluxes. The lead filter was used to reduce gamma radiation and obtain a neutron-to-gamma ratio of 2–2.7. This ratio was dependent on the installed converter plate and collimator that were changed between reactor cycles. Exponentially grown cells of *C. oleaginosus* were irradiated in KCl media to reduce the formation of longer-lasting radioactive isotopes such as sodium isotopes (half-life: $t_{1/2}$ $^{24}Na = 14.96$ h).[59] Accordingly, decay times of 24 hours were sufficient to obtain non-critical residual activation levels as the decay of radioactive isotopes was ensured. A dose/response assay was performed to determine the optimal irradiation exposure period required to realize the highest accumulation of DNA mutations in *C. oleaginosus* cells, as indicated by low survival rates.[44] The relationship between the irradiation dose and survival rate was fitted by least squares to an exponential equation ($y = 73.098e^{-0.003x}$) with a correlation coefficient of 0.9366 (Fig. 1B). Determination of the survival fraction was based on the colony formation assay. *C. oleaginosus* mutant pool U, which was subjected to highest irradiation dosage (1000 Gy), had a 4.17% survival rate. The resulting mutants underwent subsequent high-throughput selective cultivation using a cerulenin-containing screening medium to identify *C. oleaginosus* variants with improved growth and/or lipid yield (Fig. 1C).

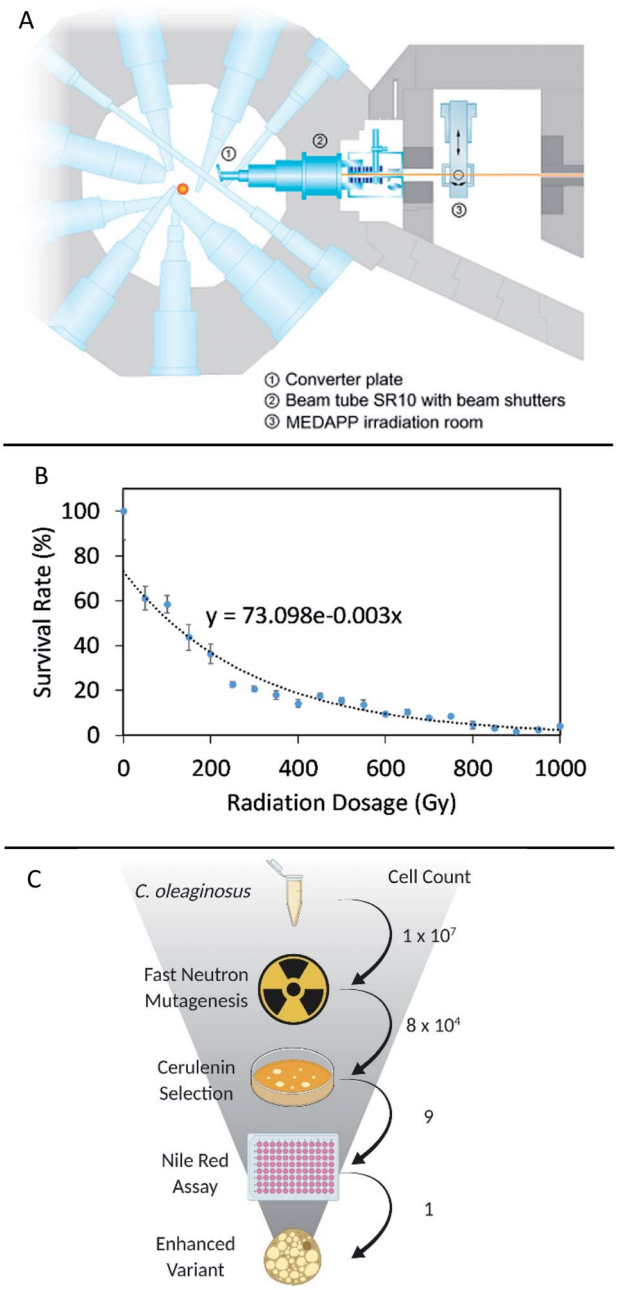


Fig. 1 (A) Experimental set up for the nuclear research reactor of this study. (B) Survival rate of FN-irradiated *C. oleaginosus* cells subjected to increasing irradiation dosage based on 50 Gy increments. The data were fitted to an exponential regression function with a coefficient of determination of 0.9366. (C) Schematic of strain development design and methods employed in this study.

3.2 Cerulenin screening

Cerulenin screening has been successfully employed for the generation of high lipid-producing mutants of *R. glutinis*, *R. toruloides, L. starkeyi* and *Y. lipolytica*.[18,44,60–62] According to the original work presenting this methodology, the optimal working concentration (WC) of cerulenin was measured at 10 $\mu g.\mu L^{-1}$ for *R. glutinis*.[18] This concentration was also determined to be optimal in the screening of *R. toruloides* and *L.*

starkeyi mutants.[44,61] Screening of wild-type *C. oleaginosus* on a concentration gradient ranging from 2 to 12 μg μL^{-1} revealed an equivalent WC (10 μg μL^{-1}). Screening of approximately 2×10^6 *C. oleaginosus* mutants on cerulenin resulted in the isolation of 9 mutants with potentially enhanced lipid biosynthesis capacity. The reduction in mutant library complexity is displayed in Fig. 1C. These isolated variants, which displayed visibly large colonies on cerulenin-containing YPD media, were termed *C. oleaginosus* Fast Neutron Mutants (FNM) 1–9.

Previous studies have shown that FN irradiation results in a higher number of non-repairable DNA damage compared to other physical or chemical mutagenesis methods.[32,33,36,37] The inherent stability of the mutations caused by this specific type of IR was further corroborated in our study. Cerulenin-selected FN mutants were initially sub-cultured on cerulenin-free YPD media over a period of 4 weeks. Subsequently, the fermentative potential of the mutants was evaluated following a 96 h batch cultivation without cerulenin selection pressure. For this purpose, a high-throughput spectrofluorimetric method was developed.

3.3 High-throughput variant qualification *via* Nile red spectrofluorimetry

3.3.1 Method development: absolute Nile red quantitation. The traditional gravimetric analysis of neutral lipids is laborious, requires the use of toxic organic solvents and necessitates considerable amounts of biological material. In the search for high-lipid producers, this method is not suitable for large-scale screening of randomly genetically modified yeast strains.[51] Accordingly, evaluation of the fermentative performance of *C. oleaginosus* mutants was carried out by spectrofluorimetry. In that context, a Nile red-based analysis was developed in the last decade into a reliable quantitative method. This method has been extensively employed for the relative quantitation (%, g g^{-1}) of lipids from several OYs.[53,62–64] The major drawback of high-throughput fluorescent techniques (*e.g.* Nile red) for microbial triglycerides (TAGs) assessment is the difficulty in absolute quantification. In that regard, relative quantitative measurements are conventionally conducted by correlating normalized Nile red fluorescence readings to gravimetric measurements.[48–50] This, however, only allows for an estimation of lipid content (%, g g^{-1}). To circumvent this issue, various researchers opted for the utilization of the model triglyceride, TO as a standard to generate a calibration curve, referring to the absolute measurements as TO-equivalents.[53,65–67] TO was also chosen in this study given the prominence of OA (C18 : 1) in the native fatty acid profile of *C. oleaginosus* at about 43–57% lipids (g g^{-1} of DCW).[1] Nevertheless, the use of this particular standard results in various inaccuracies as the Nile red assay depends on the even distribution of lipids within the solution. Exceeding a concentration threshold, the TO emulsion becomes heterogeneous and lipid droplets form, consequently leading to erratic and erroneous fluorescence measurements. Therefore, the droplets would float in or out of the spectrophotometer detector range, resulting in non-reproducible measurements. Hence, the use of TO leads to over or underestimation of the actual neutral lipid content in the cells. This is due to differences in the relative fluorescence of Nile red among the various lipid classes.[52,54,68]

In our study, purified ML extracted from *C. oleaginosus* has been favored for absolute lipid quantification, as it is the most authentic control of *C. oleaginosus* lipids formed. Linear regression of ML was calculated with an R^2 of 0.9921 over a concentration range of 20–100 μg mL^{-1} (Fig. 2). Based on our experimental setup, TO lead to an insignificant overestimation of wild-type *C. oleaginosus* lipid yields (0.67 g L^{-1}) compared to ML. The linear correlation between ML and TO trend-lines, depicted in Fig. 2, is represented by function (1) as:

$$y = 84.72x - 1096.70\ . \quad (1)$$

The spectrofluorimetry method utilized in this study was originally developed in 1990 by Priscu *et al.* and later utilized by Massart *et al.* in 2010 for lipid estimation from microalgae strains.[54,66] These studies all reported the use of large volumes (10 mL) for standard preparation. In this study, miniaturization of sample volume (300 μL) allowed the use of a 96-well plate, which is a crucial step for high-throughput sample handling, automation and the development of an absolute lipid quantitation method, especially in industrial settings. In an attempt to further reduce cost and develop this analytical method, OA was tested for its potential as a standard for absolute quantitation of *C. oleaginosus* lipid titers. Both ML from *C. oleaginosus* and TO have OA as the most prominent fatty acid and basic component, respectively. Compared to TO, OA is inexpensive, easy to handle and does not require a toxic organic solvent carrier, such as chloroform, to deliver the hydrophobic molecules into aqueous solution (Fig. S1 in ESI†). Additionally, OA was found to maintain a stable emulsion in water at higher lipid concentrations than TO (data not shown). In our study, OA resulted in a significant overestimation of wild-type *C. oleaginosus* lipid yields (3.70×) compared to ML, as depicted in a linear correlation with a function (2) represented by:

$$y = 652.56x - 2354.58. \quad (2)$$

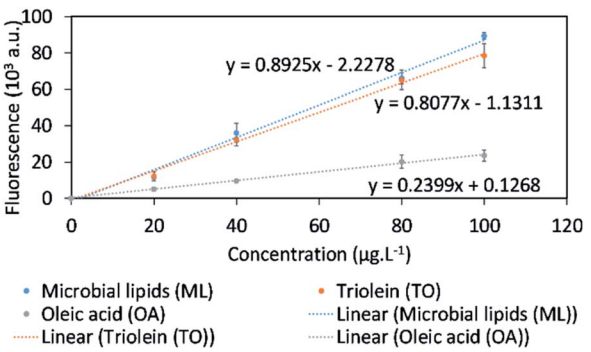


Fig. 2 Nile red spectrofluorimetry standard curves generated from purified microbial lipids from *C. oleaginosus* (R^2 = 0.9921), TO (R^2 = 0.9965) and OA (R^2 = 0.9974).

Yet, in the absence of purified ML from *C. oleaginosus*, OA can be used as a standard for absolute lipid quantitation by correcting the fluorescence readings of the samples by the function displayed above (2). For other OYs, OA can also be utilized as a standard in high-throughput lipid quantitation, following a similar correlation between OA and the respective purified ML. This general lipid assay can therefore be utilized for screening of other OYs, including *R. glutanis*, *Y. lipolytica* and *L. starkeyi*.

3.3.2 Absolute and relative quantitation of *C. oleaginosus* lipids *via* Nile red. By applying the quantitative Nile red method coupled with the ML standard detailed above (Fig. 2), lipid yields (g L^{-1}) of wild-type *C. oleaginosus* and mutants were calculated from their respective detected fluorescence readings (Fig. 3). Total fluorescence measurements and their respective calculated lipid yields (g L^{-1}) are displayed in Fig. 2 and Table 2. In one of our recent studies, maximal lipid productivity for *C. oleaginosus* was determined at 96 h of cultivation. Accordingly, this time point was used in the calculation of lipid productivity (mg L^{-1}. h^{-1}) in this study (Table 2). Additionally, biomass estimation was based on a linear correlation (R^2 = 0.9237) between DCW (g L^{-1}) and optical density (OD_{600}; a.u.), which were both previously determined for the wild-type *C. oleaginosus* strain under various culture conditions (Fig. S2 in ESI†).[1] The calculated biomass yields (g L^{-1}) were employed, along with lipid yields (g L^{-1}) to calculate the intracellular lipid content (%, g g^{-1}) (Table 2). In parallel, the relative quantitation of lipid content (%, g g^{-1}) is also reflected in the normalized fluorescence measurements displayed in Fig. 2.

Notably, *C. oleaginosus* FN M2 recorded a higher total fluorescence measurement than the wild-type *C. oleaginosus* strain, corresponding to lipid yields of 8.05 g L^{-1} and 6.56 g L^{-1}, respectively. This 22.58% (g L^{-1}) increase in lipid yield was due to enhanced growth and biomass yield (13.42 g L^{-1}) rather than an improved intracellular lipid content (%, g g^{-1}). The increase in lipid yield of *C. oleaginosus* FN M2 resulted in a maximal lipid productivity of 83.81 mg L^{-1} h^{-1}. This enhanced variant was attained following the strain development design shown in Fig. 1C, which was aimed at greatly reducing the complexity of the mutant library. By contrast, *C. oleaginosus* FN M8 displayed a higher normalized fluorescence reading compared to the wild-type control. Consequently, this strain had a calculated lipid content of 73.16% (g g^{-1} of DCW). In comparison, under the same conditions, the intracellular lipid content of the wild-type *C. oleaginosus* strain was determined to be 59.45% (g g^{-1} DCW). Quantitation of the lipid content (%, g g^{-1}) of wild-type *C. oleaginosus* in this study was found to be consistent with the gravimetric quantitation of the lipid fraction previously reported by our group.[1] However, the increase in lipid content of *C. oleaginosus* FN M8 was concurrent with a 37.90% (g L^{-1}) decrease in DCW (6.85 *versus* 11.03 g $L^{-1}$for the wild-type control). These data translates to a 16.70% (g L^{-1}) decrease in total lipid yield and maximal lipid productivity of this mutant.

The high-throughput random mutagenesis technique employed in this study, coupled with rapid cerulenin-selection singled out *C. oleaginosus* FN M2 as a candidate strain for industrial applications due to its enhanced overall lipid yields, fast growth rate and maximal productivity. Further, the enhanced intracellular lipid content of *C. oleaginosus* FN M8 renders it a candidate for studying the tight regulatory controls of lipogenesis, specifically with respect to comparative proteomics. Similar strain improvements *via* random mutagenesis for other OY strains have been reported in the last decade. To that end, UV mutagenesis triggered a 43.2% increase in lipid productivity of a mutant strain of *R. toruloides*.[6] Similarly, a 20% (g L^{-1}) increase in lipid production was observed for the green microalgae *Chlorella sp.* following FN irradiation.[69] Further, a lipid yield increase of 55% (g L^{-1}) for *Y. lipolytica* following EMS mutagenesis were reported in an extensive bioreactor optimization study,[70] and in another controlled fermentation a 30.7% increase in lipid productivity by *L. starkeyi* was achieved by random mutagenesis coupled to cerulenin screening.[5]

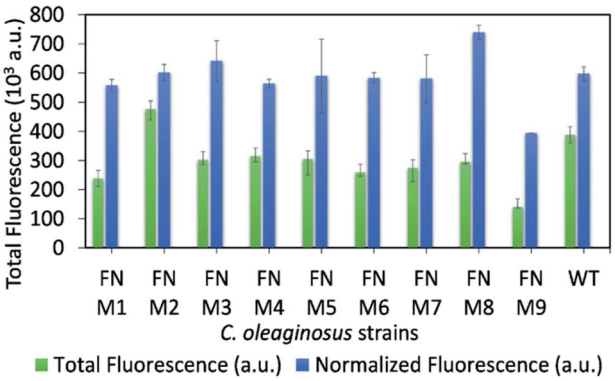


Fig. 3 Total and normalized fluorescence (a.u.) measured for wild-type *C. oleaginosus* and its FN mutants, which were selected on cerulenin.

3.4 Fatty acid profile and biofuel properties

Although the lipid profiles of the *C. oleaginosus* FN mutants diverged slightly from the wild-type strain, the main fatty acid components remained OA (C18 : 1), palmitic acid (C16 : 0) and stearic acid (C18 : 0). The effect of our strain improvement on the fatty acid profile of *C. oleaginosus* is most apparent in the increased degree of the fatty acids saturation (Fig. 4). This shift in saturation level is observed as an increase in stearic acid (C18 : 0) concurrent with a decrease in OA (C18 : 1) and linoleic acid (C18 : 2). This effect was most evident for *C. oleaginosus* FN M2, M5, M7 and M9. Except for *C. oleaginosus* FN M3, M4 and M8, a minor, yet significant decrease in palmitic acid was also recorded for all mutants. Notably, several FN mutants have an up to two-fold increase in long-chain fatty acids concentrations (Table S1 in ESI†), *i.e.* FN M1 (C20 : 2), FN M2, FN M5 and FN M9 (C22 : 0 and C24 : 0), FN M7 (C20 : 3) and FN M10 (C22 : 0).

From a biofuel production perspective, these fatty acid profiles are interesting. In fact, their degree of saturation determines the physiochemical properties of biofuels such as iodine value (IV), cetane number (CN), higher heating value (HHV), kinematic viscosity (KV) and density.[44] These biofuel properties were estimated for several OYs based on rigid

Table 2 Fermentative performance of wild-type *C. oleaginosus* and its FN mutants. Yeast DCW (g L^{-1}) and lipid yield (g L^{-1}) are calculated based on a correlation between optical density measure at 600 nm and the DCW and ML standard curves, respectively

C. oleaginosus strains	DCW (g L^{-1})	Lipid yield (g L^{-1})	Lipid content (%, g g^{-1} DCW)	Maximal lipid productivity (mg L^{-1} h^{-1})
FN M1	7.3 ± 0.76	4.04 ± 0.45	55.29 ± 2.08	42.06 ± 4.7
FN M2	13.42 ± 0.55	8.05 ± 0.63	59.92 ± 2.77	83.81 ± 6.56
FN M3	8.07 ± 0.45	5.11 ± 0.26	63.63 ± 6.80	53.27 ± 2.72
FN M4	9.51 ± 0.68	5.33 ± 0.33	56.04 ± 1.42	55.49 ± 3.39
FN M5	8.86 ± 0.35	5.16 ± 0.92	58.61 ± 12.45	53.78 ± 9.62
FN M6	7.59 ± 0.56	4.39 ± 0.22	57.83 ± 1.71	45.69 ± 2.29
FN M7	8.05 ± 0.57	4.64 ± 0.78	57.64 ± 8.05	48.36 ± 8.12
FN M8	6.85 ± 0.32	5.01 ± 0.11	73.16 ± 2.30	52.16 ± 1.15
FN M9	6.1 ± 0.13	2.39 ± 0.05	39.23 ± 0.06	24.91 ± 0.53
WT	11.03 ± 0.45	6.56 ± 0.47	59.45 ± 2.38	68.37 ± 4.91

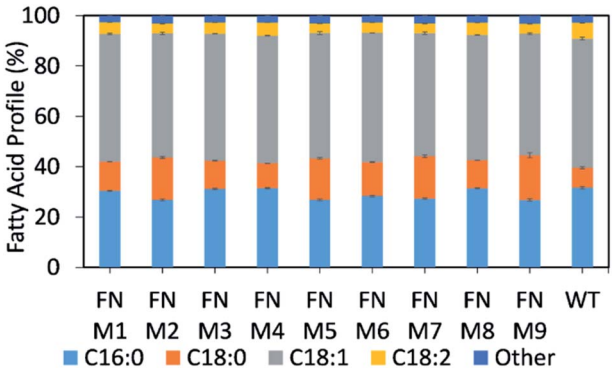


Fig. 4 Fatty acid profiles of wild-type *C. oleaginosus* and its FN mutants, which were selected on cerulenin. "Other" constitutes fatty acids with a presentation below 1%, including C12 : 0, C14 : 0, C16 : 1, C18 : 3, C20 : 0, C20 : 1, C20 : 2, C20 : 4, C20 : 3, C20 : 5, C22 : 0, C22 : 1 and C24 : 0.

calculations by Sergeeva *et al.*[58] These calculations were adopted in this study for wild-type *C. oleaginosus* and its FN M2 mutant, given the high lipid titers of the latter (Table 3).[61] The determined properties were found to be positioned within the limits specified by internationally accepted biofuel standards (US biodiesel ASTM D6751 and EU biodiesel standard EN 14214). A comparison with other prominent OY lipid profiles, including those of *Y. lipolytica, L. starkeyi and R. toruloides,* is presented in Table 3. Interestingly, the *C. oleaginosus* lipid profile shows significant similarities to that of palm oil with respect to the prominence of oleic and palmitic acids in its fatty acid distribution. Hence, he biofuel properties of palm oil were also listed in Table 3.[58] The calculated density values were applied in this study to deliver appropriate concentrations in the preparation of ML standard. The degree of saturation indicated by IV was lower in *C. oleaginosus* FN M2, in comparison to the wild-type strain, yet remained within the limits of the internationally accepted IV range. Notably, lipids derived from the *C. oleaginosus* FN M2 variant demonstrated an improved CN compared to the wild-type strain. Higher CN values are associated with improved cold-start properties and reduced smoke formation.[62] Similar improvements in biofuel properties were also reported for *Y. lipolytica* following chemical mutagenesis coupled with cerulenin selection.[62]

4. Conclusion

Microbial-derived high-energy biofuels, *i.e.* microbial biodiesel, enjoy a worldwide research focus as an alternative energy source for personal mobility concepts. However, commercialization has been hampered by the relatively high production cost. A major challenge for SCOs in achieving economic feasibility for large-scale industrial applications lies in the isolation of microbial strains displaying high lipid titers.[51] In this study, FN irradiation was employed for the first time in the mutagenesis of an OY strain. The application of cerulenin selection pressure

Table 3 Calculated and reported biodiesel characteristics obtained from prospective OYs, palm oil and corresponding requirements of biodiesel standards according to United Stated and European regulations

Biodiesel Source	Iodine value	Cetane number	Higher heat value (MJ kg^{-1})	Kinematic viscosity (mm^2 s^{-1})	Density (g cm^{-3})	Reference
C. oleaginosus wt	57.56	60.06	38.38	3.85	0.91	This study
C. oleaginosus FN M2	51.39	61.62	38.30	3.93	0.90	This study
Y. lipolytica	80.95	60.11	39.13	3.25	0.86	58
L. starkeyi	77.07	59.80	38.83	3.20	0.85	58
R. toruloides	74.13	62.19	39.14	3.43	0.86	58
Palm oil	57.95	65.19	39.46		0.87	58
US biodiesel ASTM D6751	≤120	≥47	—	1.9–6.0	0.86–0.91	58 and 61
EU biodiesel standard EN 14214	≤120	≥51	—	3.5–5.0	0.86–0.90	58 and 61

on mutated *C. oleaginosus* cells (subjected to 1000 Gy) allowed for the isolation of two strains with improved growth and lipid characteristics. High-throughput Nile red analysis revealed one mutant (FN M2) displaying a 21.67% increase in biomass formation (g L^{-1}), which in turn resulted in a 22.58% increase in total lipid yield (g L^{-1}) compared to the wild-type strain. Further characterization of this mutant showed a higher degree of fatty acid saturation, with biofuels properties that meet international standards. Another mutant (FN M8) displayed an improved lipid content (73.16%, g g^{-1}). However, as biomass formation of this *C. oleaginosus* mutant was substantially lower than that of the wild-type strain, total lipid yield (g L^{-1}) was not enhanced. This work demonstrates that the FN mutagenesis technique can be successfully applied to manipulate biomass formation, lipid productivity and fatty acid composition of OYs. Furthermore, our study clearly indicates that the enhanced phenotypes (improved lipid and biomass accumulation) of mutants - FN M2 and M8-were stable over the observed growth cycles/generations, even in the absence of selection pressure. Specifically, these enhanced mutants did not require the continuous presence of cerulenin pressure to deliver higher lipid titers. Hence, this approach has subsequent industrial applications (*e.g.* biodiesel) without the need for targeted genetic engineering.

Adopting "zero-concepts" with respect to emissions and excess resources in bioprocess engineering is expected to drive industrial biotechnology to become highly integrated into sustainable technology systems.[71] *C. oleaginosus* has been previously adapted to various cost-effective fermentative conditions (*e.g.* lignocellulose and hemicelluloses hydrolysates, waste materials) while maintaining elevated lipid titers. Specifically, brown algae and seagrass have been applied as feed-stock in a well-designed waste-free cyclic bio-refinery approach for biofuels and the oleochemical industry.[4] The fermentative growth of *C. oleaginosus* FN M2 should be assessed under these conditions. Most interestingly, growth and lipid production capabilities of this mutant should be assessed, when cultivated in a nitrogen-rich medium with glucose and acetic acid as carbon sources, which allows concomitant growth and lipid formation without the need for N-limiting cultivation conditions. In a recent study performed by our group, wild-type *C. oleaginosus* was capable of accumulating 85% (g g^{-1} DCW) lipids when grown under these specific conditions.[16] Following high-yield fermentation in aerated stirred tanks, techno-economic analyses estimated that oil from this wild-type yeast strain (US$1.6 kg^{-1}) would be cheaper than eco-certified palm oil (US$ 2.1 kg^{-1}) and would also result in lower CO_2 emissions.[16] *C. oleaginosus* FN M2 with its inherent enhanced growth and lipid yields, and FN M8, with its inherent higher lipid content, can potentially achieve even greater biomass and lipid productivity under these desirable high-monoauxic growth conditions, thereby significantly lowering the cost of *C. oleaginosus* oil production.

The analytical approach established in this study links current efforts in SCO advancements to an accelerated process development by implementing high-throughput selection. In fact, cost-efficient, rapid and automatable Nile red analysis coupled to our high-fidelity correlation system alleviated the need for time-consuming laborious analytical techniques (*e.g.* gravimetric analysis, growth rate analysis, biomass analysis). The use of purified ML from *C. oleaginosus* delivered a more accurate representation of intracellular lipid concentrations than the previously reported TO standard. Additionally, our correlation study demonstrates that OA is a low-cost, easy to handle and accurate standard for absolute lipid quantification, when harmonized with the determined correlation factor in this study.

The high-throughput technologies for genetic yeast strain enhancement, production variant selection and performance monitoring presented in this study are each amenable to be transferred into a consolidated, miniaturized and automatable processing format. These consolidated processes have the capacity to provide a technology platform for a completely automated yeast lipid and biofuel production optimization process, where artificial intelligence-based software solutions guide and accelerate developments with a minimized need for human intervention. This scenario allows for accelerated and more cost efficient process optimization that ultimately allow fast track industrial deployment of oleaginous yeast-based high-energy biofuel production processes. Realizing such a scenario requires consequent miniaturization and validation of all processes presented in this study and their integration in a consecutive work flow on an automated robotic workstation. To that end, integrated robotic systems have been previously employed for high-throughput screening in various cell culture conditions.[72,73] Moreover, several strategies have successfully shortened process development timelines from large to micro-scale processes by employing high-throughput technology platforms such as microtiter plate (MTP) culture, micro-scale bioreactors, and parallel fermentation systems.[74,75] Accordingly, the synergistically integrated high-throughput methods for strain mutagenesis, selection and characterization developed in this study can also be coupled with FACS sorting using 96 deep well MTP formats for quantitative evaluation of the fermentative potential of all generated mutants, even in the absence of cerulenin. Hence, future studies in our group will employ an automated high-throughput process development (HTPD) approach, which employs robotic platforms. This would incorporate necessary tools for MTP culture studies, automated fast screening, as well as advanced artificial intelligence (AI)-guided analytical capabilities (assessment of cell growth, lipid content) without operator intervention.[76,77] With the advantage of high sensitivity and reproducibility, correlating batch data would reliably predict culture performance. Additionally, benchtop parallel bioreactor systems can establish robust scalable processes and enable reliable Design of Experiment (DoE) studies for mutants with metabolic altered pathways. Employing this HTPD approach will lead to an accelerated manufacturing process, with significantly reduced risk of project failure, decreased costs and higher market penetration capability. Finally, investigation of the metabolic alterations using genomic and proteomic tools (sequencing, transcriptomic analysis) should be performed to identify key genes/enzymes that were modified during mutagenesis.[78] These

identified genes can be target of genetic engineering of this yeast in future studies for elevated lipid titers.

Author contributions

Conceptualization of the study was conducted jointly by DA and TB. The methodological approach was designed and carried out by DA and SY. Data validation was jointly carried out by all authors. DA and SY prepared the original draft of the manuscript. The manuscript was jointly finalized by all authors.

Funding

The authors gratefully acknowledge funding of the Werner Siemens foundation for establishing the research field of Synthetic Biotechnology at the Technical University of Munich. SY, DA and NM gratefully acknowledge funding by the Bavarian State Ministry for Environmental and Consumer affairs for funding of the project Resource efficient PHB production processes within the project consortium BayBioTech (TP7, TLK01U-69045, http://www.baybiotech.de/startseite/).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

List of Abbreviations

AGMT	Agrobacterium tumefaciens-mediated gene transfer
CN	Cetane number
CRISPR	Clustered regularly interspaced short palindromic repeats
DI	Deionized
DCW	Dry cell weight
DMSO	Dimethyl sulfoxide
EMS	Ethyl methane sulfonate
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FN	Fast neutron
GMO	Genetically modified organism
HHV	Higher heating value
IR	Ionizing radiation
IV	Iodine value
KPI	Key Performance Indicator
KV	Kinematic viscosity
LET	Linear energy transfer
MEDAPP	Medical applications
MFE	Maximal fluorescence emission
ML	Microbial lipid
MNM	Minimal-nitrogen media
NTG	Nitrosomethyl guanidine
OA	Oleic acid
OM	Oleaginous microorganism
OY	Oleaginous yeast
PLA	Polylactic acid
SCO	Single-cell Oil
TAG	Triglycerides
TO	Triolein
YPD	Yeast peptone dextrose

Acknowledgements

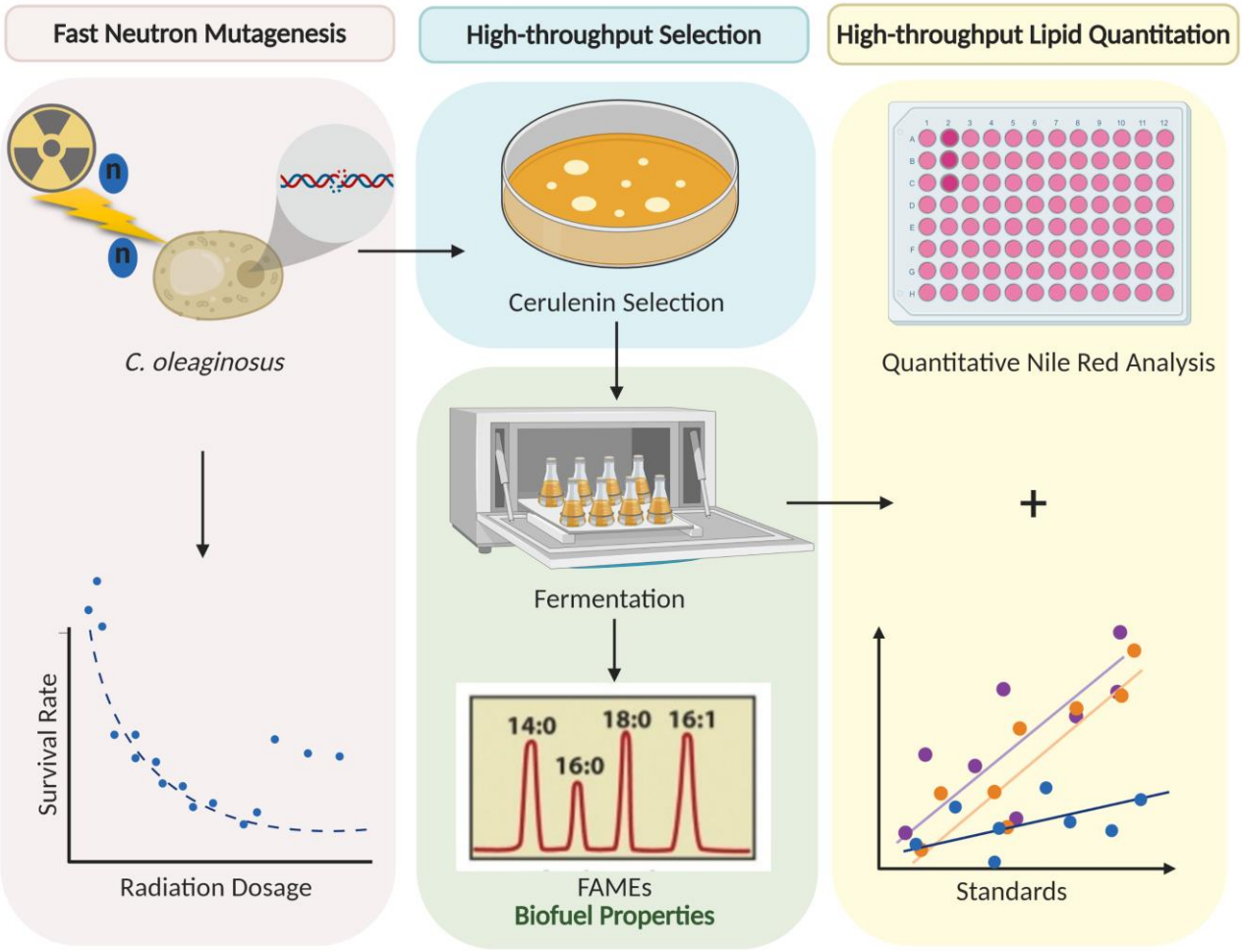
The authors gratefully acknowledge the valuable input offered by Elias Kassab with regard to GC-MS sample analysis. The authors also acknowledge Mahmoud Masri for providing a purified extract of ML.

References

1 D. Awad, F. Bohnen, N. Mehlmer and T. Brueck, *Frontiers in bioengineering and biotechnology*, 2019, **7**, 54.
2 S. Bruder, S. Hackenschmidt, E. J. Moldenhauer and J. Kabisch, in *Lipid Modification by Enzymes and Engineered Microbes*, Elsevier, 2018, pp. 257–292.
3 C. Huang, X.-f. Chen, L. Xiong, L.-l. Ma and Y. Chen, *Biotechnol. Adv.*, 2013, **31**, 129–139.
4 M. A. Masri, S. Younes, M. Haack, F. Qoura, N. Mehlmer and T. Brück, *Energy Technol.*, 2018, **6**, 1026–1038.
5 E. Tapia, A. Anschau, A. L. Coradini, T. T. Franco and A. C. Deckmann, *AMB Express*, 2012, **2**, 64.
6 M. Guo, S. Cheng, G. Chen and J. Chen, *Eng. Life Sci.*, 2019, **19**, 548–556.
7 K. W. Chew, S. R. Chia, P. L. Show, T. C. Ling and J.-s. Chang, in *Bioreactors for Microbial Biomass and Energy Conversion*, ed. Q. Liao, J.-s. Chang, C. Herrmann and A. Xia, Springer Singapore, Singapore, 2018, pp. 359–388, DOI: 10.1007/978-981-10-7677-0_9.
8 X. Meng, J. Yang, X. Xu, L. Zhang, Q. Nie and M. Xian, *Renewable energy*, 2009, **34**, 1–5.
9 S. Papanikolaou and G. Aggelis, *Eur. J. Lipid Sci. Technol.*, 2011, **113**, 1052–1073.
10 C. Gorner, V. Redai, F. Bracharz, P. Schrepfer, D. Garbe and T. Bruck, *Green Chem.*, 2016, **18**, 2037–2046.
11 P. Gujjari, S.-O. Suh, K. Coumes and J. J. Zhou, *Mycologia*, 2011, **103**, 1110–1118.
12 A. Yaguchi, D. Rives and M. Blenner, *AIMS Microbiol.*, 2017, **3**, 227–247.
13 S. Younes, F. Bracharz, D. Awad, F. Qoura, N. Mehlmer and T. Brueck, *Bioprocess Biosyst. Eng.*, 2020, DOI: 10.1007/s00449-020-02354-0.
14 F. Bracharz, T. Beukhout, N. Mehlmer and T. Bruck, *Microb. Cell Fact.*, 2017, **16**, 178.
15 M. A. Masri, W. Jurkowski, P. Shaigani, M. Haack, N. Mehlmer and T. Brück, *Appl. Energy*, 2018, **224**, 1–12.
16 M. A. Masri, D. Garbe, N. Mehlmer and T. B. Brück, *Energy Environ. Sci.*, 2019, **12**, 2717–2732.
17 Y. Zhang, M. He, S. Zou, C. Fei, Y. Yan, S. Zheng, A. A. Rajper and C. Wang, *Bioresour. Technol.*, 2016, **207**, 268–275.
18 J. Wang, R. Li, D. Lu, S. Ma, Y. Yan and W. Li, *World J. Microbiol. Biotechnol.*, 2009, **25**, 921–925.

19 S. Ochoa, G. Wozny and J.-U. Repke, *J. Process Control*, 2010, **20**, 983–998.
20 E. G. Kana, J. Oloke, A. Lateef and M. Adesiyan, *Renewable energy*, 2012, **46**, 276–281.
21 E. C. Rivera, S. C. Rabelo, D. dos Reis Garcia, R. M. Filho and A. C. da Costa, *J. Chem. Technol. Biotechnol.*, 2010, **85**, 983–992.
22 S. Hansen and A. Mirkouei, 2018.
23 S. Y. Lee and H. U. Kim, *Nat. Biotechnol.*, 2015, **33**, 1061–1072.
24 S. d'Oelsnitz and A. Ellington, *Curr. Opin. Biotechnol.*, 2018, **53**, 158–163.
25 G. Katre, N. Ajmera, S. Zinjarde and A. RaviKumar, *Microb. Cell Fact.*, 2017, **16**, 176.
26 R. Kourist, F. Bracharz, J. Lorenzen, O. N. Kracht, M. Chovatia, C. Daum, S. Deshpande, A. Lipzen, M. Nolan and R. A. Ohm, *mBio*, 2015, **6**, e00918-00915.
27 P. B. Otoupal, M. Ito, A. P. Arkin, J. K. Magnuson, J. M. Gladden and J. M. Skerker, *mSphere*, 2019, **4**(2), DOI: 10.1128/msphere.00099-19.
28 Y. K. Park, J. M. Nicaud and R. Ledesma-Amaro, *Trends Biotechnol.*, 2018, **36**, 304–317.
29 T. Beacham, V. M. Macia, P. Rooks, D. White and S. Ali, *J. Appl. Biotechnol. Rep.*, 2015, **7**, 87–94.
30 T. T. Y. Doan and J. P. Obbard, *Algal Res.*, 2012, **1**, 17–21.
31 D. R. Ort, X. Zhu and A. Melis, *Plant Physiol.*, 2011, **155**, 79–85.
32 Y.-r. Cheng, Z.-j. Sun, G.-z. Cui, X. Song and Q. Cui, *Enzyme Microb. Technol.*, 2016, **93**, 182–190.
33 S. Kumawat, N. Rana, R. Bansal, G. Vishwakarma, S. T. Mehetre, B. K. Das, M. Kumar, S. Kumar Yadav, H. Sonah and T. R. Sharma, *Plants*, 2019, **8**, 164.
34 J. I. Lucas-Lledó and M. Lynch, *Mol. Biol. Evol.*, 2009, **26**, 1143–1153.
35 U. M. Tillich, S. Lehmann, K. Schulze, U. Dühring and M. Frohme, *PLoS One*, 2012, **7**, e49467.
36 C. B. Bennett, L. K. Lewis, G. Karthikeyan, K. S. Lobachev, Y. H. Jin, J. F. Sterling, J. R. Snipe and M. A. Resnick, *Nat. Genet.*, 2001, **29**, 426.
37 J. H. Hendry, *Radiat. Res.*, 1991, **128**, S111–S113.
38 M. Frankenberg-Schwager, D. Frankenberg, R. Harbich and C. Adamczyk, *Int. J. Radiat. Biol.*, 1990, **57**, 1151–1168.
39 E. Schmid, F. Wagner, H. Romm, L. Walsh and H. Roos, *Radiat. Environ. Biophys.*, 2009, **48**(1), 67–75.
40 J. M. Al-Khayri, S. M. Jain and D. V. Johnson, *Advances in plant breeding strategies: Breeding, biotechnology and molecular tools*, Springer, 2015.
41 N. Gajendiran, K. Tanaka, T. S. Kumaravel and N. Kamada, *J. Radiat. Res.*, 2001, **42**, 91–101.
42 K. Sakai, S. Suzuki, N. Nakamura and S. Okada, *Radiat. Res.*, 1987, **110**, 311–320.
43 P. Unrau, *Radiat. Res.*, 1986, **107**, 39–48.
44 V. E. Tapia, A. Anschau, A. L. Coradini, T. F. T and A. C. Deckmann, *AMB Express*, 2012, **2**, 64.
45 R. Yamada, T. Kashihara and H. Ogino, *World J. Microbiol. Biotechnol.*, 2017, **33**, 99.
46 E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.*, 1959, **37**, 911–917.
47 E. Marcellin and L. K. Nielsen, *Curr. Opin. Biotechnol.*, 2018, **54**, 33–40.
48 H. Morschett, W. Wiechert and M. Oldiges, *Microb. Cell Fact.*, 2016, **15**, 34.
49 L. Balduyck, C. Veryser, K. Goiris, C. Bruneel, K. Muylaert and I. Foubert, *J. Microbiol. Methods*, 2015, **118**, 152–158.
50 I. Sitepu, L. Ignatia, A. Franz, D. Wong, S. Faulina, M. Tsui, A. Kanti and K. Boundy-Mills, *J. Microbiol. Methods*, 2012, **91**, 321–328.
51 W. Chen, C. Zhang, L. Song, M. Sommerfeld and Q. Hu, *J. Microbiol. Methods*, 2009, **77**, 41–47.
52 H. De la Hoz Siegler, W. Ayidzoe, A. Ben-Zvi, R. Burrell and W. McCaffrey, *Algal Res.*, 2012, **1**, 176–184.
53 Y. Liang, T. Tang, A. L. Umagiliyage, T. Siddaramu, M. McCarroll and R. Choudhary, *Appl. Energy*, 2012, **91**, 451–458.
54 J. C. Priscu, L. R. Priscu, A. C. Palmisano and C. W. Sullivan, *Antarct. Sci.*, 1990, **2**, 149–155.
55 V. Orr and L. Rehmann, *J. Appl. Phycol.*, 2015, **27**, 2181–2189.
56 F. M. Wagner, P. Kneschaurek, A. Kastenmüller, B. Loeper-Kabasakal, S. Kampfer, H. Breitkreutz, W. Waschkowski, M. Molls and W. Petry, *Strahlenther. Onkol.*, 2008, **184**(12), 643–646.
57 R. Morales-Rayas, P. F. Wolffs and M. W. Griffiths, *Int. J. Food Microbiol.*, 2010, **139**, 48–55.
58 Y. E. Sergeeva, E. B. Mostova, K. V. Gorin, A. V. Komova, I. A. Konova, V. M. Pojidaev, P. M. Gotovtsev, R. G. Vasilov and S. P. Sineoky, *Appl. Biochem. Microbiol.*, 2017, **53**, 807–813.
59 D. Ekendahl, P. Rubovic, P. Zlebcik, O. Huml and H. Mala, *Radiat. Prot. Dosim.*, 2018, **178**, 329–332.
60 Y. Liu, C. M. J. Koh, S. A. Yap, M. Du, M. M. Hlaing and L. Ji, *BMC Microbiol.*, 2018, **18**, 14.
61 R. Yamada, T. Kashihara and H. Ogino, *World J. Microbiol. Biotechnol.*, 2017, **33**, 99.
62 G. Katre, N. Ajmera, S. Zinjarde and A. RaviKumar, *Microb. Cell Fact.*, 2017, **16**, 176.
63 I. R. Sitepu, L. Ignatia, A. K. Franz, D. M. Wong, S. A. Faulina, M. Tsui, A. Kanti and K. Boundy-Mills, *J. Microbiol. Methods*, 2012, **91**, 321–328.
64 I. R. Sitepu, M. Jin, J. E. Fernandez, L. da Costa Sousa, V. Balan and K. L. Boundy-Mills, *Appl. Microbiol. Biotechnol.*, 2014, **98**, 7645–7657.
65 M. Isleten-Hosoglu, I. Gultepe and M. Elibol, *Biochem. Eng. J.*, 2012, **61**, 11–19.
66 A. Massart, E. Aubry and A.-L. Hantson, *Biotechnol., Agron., Soc. Environ.*, 2010, **14**, 567–572.
67 E. Bertozzini, L. Galluzzi, A. Penna and M. Magnani, *J. Microbiol. Methods*, 2011, **87**, 17–23.
68 W. Chen, M. Sommerfeld and Q. Hu, *Bioresour. Technol.*, 2011, **102**, 135–141.
69 S. Liu, J. Xu, W. Chen, H. Fu, L. Y. Ma, H. Xu, L. Xinnian, M. Wu and F. Ma, *Biomass Bioenergy*, 2016, **91**, 196–203.
70 L. Liu, A. Pan, C. Spofford, N. Zhou and H. S. Alper, *Metab. Eng.*, 2015, **29**, 36–45.

71 A. J. J. Straathof, S. A. Wahl, K. R. Benjamin, R. Takors, N. Wierckx and H. J. Noorman, *Trends Biotechnol.*, 2019, **37**, 1042–1050.
72 C. Daniels, J. Rodriguez, E. Lim and M. Wenger, *Eng. Life Sci.*, 2016, **16**, 202–209.
73 S. R. Decker, R. Brunecky, M. P. Tucker, M. E. Himmel and M. J. Selig, *BioEnergy Res.*, 2009, **2**, 179.
74 Q. Long, X. Liu, Y. Yang, L. Li, L. Harvey, B. McNeil and Z. Bai, *J. Biotechnol.*, 2014, **192**, 323–338.
75 F. L. Gonzalez, S. R. Wisniewski, K. Katipally, J. M. Stevens, V. Rosso, B. Mack and T. M. Razler, *Org. Process Res. Dev.*, 2019, **23**, 1143–1151.
76 L. A. d. C. Meleiro, F. J. V. Zuben and R. M. Filho, *Eng. Appl. Artif. Intell.*, 2009, **22**, 201–215.
77 A. L. Oliveira, *Biotechnol. J.*, 2019, **14**, e1800613.
78 D. Awad and T. Brueck, *Anal. Bioanal. Chem.*, 2020, **412**, 449–462.



Graphical Summary

4 Discussion & Outlook

4.1 Multi-Factorial-Guided Media Optimization

One of the most challenging aspects of biomanufacturing involves providing for the diverse nutritional requirements that are unique to every production strain[138]. It is estimated that 30-40% of bioprocess production costs are allocated for culturing media[139]. For this reason, fermentation media formulation and optimization remains a hot topic in biotechnology.

Although the fundamentals of statistical modeling have been uncovered since early 1900s, their integration into biotechnological platforms is rather recent. Statistical experimental planning, factorial design, and design-of-experiments (DoE), are synonymous concepts for investigating the mathematical interactions between input and output variables of a defined system [139,140]. In this study, RSM enabled simultaneous evaluation of a number of independent (elemental carbon and nitrogen ratios and concentrations) and dependent variables (biomass and lipid yields and lipid content (%, w/w) in *C. oleaginosus* fermentation at laboratory bench-scale (shake-flask). The integration of statistical modeling into bioprocess development drastically reduced the time required for identification of optimal cultivation conditions [141]. In our setup, based on Box-Behnken design, 12 experimental conditions were sufficient to cover a wide range of carbon (glucose) to nitrogen (yeast extract) ratios (12:1 - 240:1). The model was validated, with 92.59, 95.64, and 90.23% of variations in dependent factors attributed directly to the independent variables. The optimal carbon to nitrogen ratio (120: 1) attained for *C. oleaginosus* is comparable with other prominent oleaginous yeasts [142-144].

Induction of lipogenesis by nutritional limitation, through high carbon to nitrogen ratios, results in lower fermentation rates, compared to enriched and complex media[145]. For this reason, evaluating the lipogenic potential based on lipid content (%, w/w) alone, via spectrofluorometric methods (relative quantitation), is insufficient [44,146]. In order to adequately asses the lipogenic potential of *C. oleaginosus*, biomass and lipid accumulation were equally scrutinized. Applying RSM methodology also allowed for the role of carbon and nitrogen to be differentiated with respect to their contribution to growth and lipogenesis. In accordance with literature of other oleaginous yeasts, the quadratic effect of nitrogen concentration in our model was found to highly influence lipid accumulation[147].

Variations within dependent factors were detected for conditions with matching carbon to nitrogen ratios yet differing in the total concentrations of carbon and nitrogen. The data generated by such a matrix serves as an indication of the nutritional saturation levels in our experimental set-up (40 $g.L^{-1}$ glucose and 1.10 $g.L^{-1}$ yeast extract).

The power of prediction tools in streamlining the development of microbial lipid processes has been well documented [148-150]. For example, the pretreatment conditions of corn bran have been optimized via RSM for microbial lipid production from *C. oleaginosus*[106]. Likewise, co-

production of lipids and carotenoids from *R. toruloides* was rapidly improved via RSM[151]. Besides media composition, statistical modeling was also adopted for the optimization of cultivation conditions (pH, temperature and duration of fermentation) of microbial platforms[150]. Moreover, statistical modelling has found its way into downstream process development. For instance, lipid extraction from several oleaginous species has been optimized via RSM[152-155].

Applying statistical modeling to address vitamin and mineral requirements of *C. oleaginosus* can provide detailed insight on the metabolic performance of this yeast. In the same way, this strategy can serve fermentation scale up experiments (bioreactor, pilot and production scale), whereby an increasing number of input parameters (independent factors) can continuously improve the robustness and integrity of the model[156]. Furthermore, statistical modeling of other - or a combination of - nutritional limitations, such as phosphorus, sulfur and iron, can also contribute to our understanding of microbial lipogenesis. In order to increase the throughput and information derived from these statistical methods, automation of process analytical tools (PAT), such as lipid quantitation techniques, is needed.

4.2 Thorough Characterization of *C. oleaginosus* Nutritional Requirements

The role of carbon and nitrogen in fermentative platforms is well established since early twentieth century [23]. Carbon, in particular, has been the subject of extensive investigations. Microbial lipid production utilizing a plethora of carbon sources, such as glucose, xylose, lactose, molasses, glycerol, N-acetylglucosamine, have been reported [13,41]. It is noteworthy that nitrogen, the most potent inducer of lipogenesis, remains relatively unexplored in terms of sources, concentrations and mixtures [78,157,158]. We present, for the first time, the most thorough nutritional study for *C. oleaginosus*. Specifically, we compare the efficiency of this strain to uptake and metabolize an array of 10 carbon and 13 nitrogen sources of distinct chemical natures.

Fermentative conditions of SCO platforms differ drastically in design and feedstock based on the purpose and scale [159,160]. At laboratory bench-scale, semi- and defined media is preferred as it facilitates strain-selection, defining metabolic routes, minimizing batch-to-batch variation and eliminating interference of growth factors [161]. In contrast, complex and semi-defined media, rich in peptides, lipids, vitamins and minerals, are favored at industrial scale in order to achieve greater biomass and product yields [162]. In this study, *C. oleaginosus* exhibited 6.5x more biomass accumulation when cultured in complex organic nitrogen sources, such as yeast extract and tryptone/peptone, compared to defined inorganic nitrogen sources. As a by-product of various processes, inorganic nitrogen (e.g. NH_4Cl, KNO_3) is often the preferred source for SCOs industrial production. However, various studies confirmed that organic

nitrogen sources (e.g. yeast extract, peptone) are more suitable despite the higher cost [34]. Interestingly, organic nitrogen sources influence both biomass production and lipid accumulation, while inorganic nitrogen influences only biomass production [163]. Complex organic nitrogen sources, such as yeast extract and peptone, have been supplemented to various crude feedstocks to help increase SCOs production [164,165].

C. oleaginosus performed well on all carbon sources except for sorbitol, making it highly desirable for industrial purposes. Notably, this is the first report on the performance of *C. oleaginosus* on sugar alcohols. This class of organic compounds does not constitute a favorable feedstock for microbial lipid production processes. As means to reduce the overall cost of manufacture (COM), biogenic waste hydrolysates and industrial and agricultural effluents and residues, rich in multiple sugars, became the standard feedstocks at production scale [166-170]. In that context, *C. oleaginosus* is industrially favorable because it can inherently co-uptake and metabolize hexose and pentose sugars [171]. The lipogenic potential of *C. oleaginosus* has been evaluated and optimized on seagrass, brown algae and microalgae hydrolysates [68,70,90]. Such practices directly address the requirements for sustainable energy and oleo-chemicals supply, in addition to waste management [172]. Furthermore, valorization of the bioprocess waste-streams has been recently achieved for *C. oleaginosus* in a cyclic-biorefinary [90]. In our work, this yeast performed remarkably on lactose and urea, which are abundant in the dairy-sector. Since *C. oleaginosus* was originally isolated from Iowa State University Dairy Farm, it is particularly adapted for the efficient metabolism of these nutrients [137]. Hence, a cost-efficient feedstock for *C. oleaginosus* can be sourced from dairy farms, production plants and markets. This has been demonstrated with lactose-rich deproteinised cheese whey as quality feedstock for *C. oleaginosus* at pilot scale (500 L) in New Zealand in 1988 [173].

Industrial applications of the microbial lipid is highly dependent on the fatty acid profile of *C. oleaginosus*, which in turn is influenced by cultivation parameters[174]. In our study, lipid composition (fatty acids obtained from the transesterification of the extracted TAGs) was determined for all examined conditions. We observed that media composition has its strongest impact on the saturation levels of fatty acids. In this context, the inherently low degree of unsaturation, observed for this strain is of particular interest for Green-diesel processes and aviation fuels[175,176]. Our results demonstrate that specific fatty acid profiles, associated with desired product applications, can be obtained with careful selection of media components.

The presented repertoire of this yeast's behavior can further advance upstream development of *C. oleaginosus* by supporting the decision-making process at laboratory bench-scale. Specifically, the multiplicity of carbon and nitrogen sources of synthetic, organic, defined and complex media presented here stands as a future reference for media formulation, genetic

manipulations, optimization of sugar transport systems and streamlining of the metabolic flux. The fatty acid profiles presented in this work can serve as a blueprint for the fine-tuning (lipid physiochemical properties) of the end-product. The findings of our study can also support the decision-making process for the most appropriate feedstock in industrial settings. It should be taken into consideration that the quality of the feedstock is also dictated by the respective industrial application. For instance, biogenic residues (e.g. microalgae biomass) are preferred over industrial and agricultural waste-streams for the production of high-end products in food, pharmaceuticals and skin care industries [177]. Lastly, optimization of *C. oleaginosus* cultivation conditions is a subject matter that should be revisited throughout upstream development processes. Specifically, the nutritional requirements of newly developed strains and cultivation parameters during fermentation scale up should be addressed.

4.3 Development of an Absolute Lipid Quantitation Method for High-throughput Process Monitoring

Process analytical tools (PAT) are considered an integral part of upstream and downstream development processes. These tools are essential for monitoring product titers and ensuring the stability of genetically improved strains and the quality of the product throughout the development process [87,113]. For this reason, the development of cost-effective and high-throughput PAT that properly qualify lipids is key for rapid implementation of SCO platforms.

Lipid quantitation techniques have seen little improvements throughout the years. With a citation record of 53863, Bligh and Dyer's lipid extraction protocol developed in 1959 remains the standard in the gravimetric lipid quantitation. Although this method is well developed, it necessitates the use of toxic and carcinogenic solvents (chloroform and methanol) [126]. The lipophilic nature of certain fluorescent dyes also allows for the direct quantitation of intracellular lipids in-vivo (bypassing lipid extraction) [178]. In 2012, Sitepu et al. extensively developed a spectrofluorometric method based on Nile red. With the advantage of easy sample handling and micro-scale (300 µl in a 96-well microplate), this method has high throughput capacity. Lipid quantitation by this method strongly correlates with the lipid content, calculated by the equation:

$$Lipid\ Content\ (g.g^{-1}DCW) = \frac{Lipid\ yield\ (g.L^{-1})}{Biomass\ yield\ (g.L^{-1})}$$

whereby lipid and biomass yields are determined gravimetrically (Bligh & Dyer's method)[44]. Although Nile red analysis constitutes a powerful comparative tool, absolute quantitation remains a challenge. Given the prominence of oleic acid (C18:1) in the fatty acid profile of oleaginous yeasts (**Table 2**), triolein has been repeatedly adopted as a standard for

quantitation of microbial lipids. This strategy suffers from several drawback; triolein is expensive, difficult to handle and is only linear in a narrow concentration range (2 – 100 µg.mL^{-1}). Furthermore, as a symmetrical triglyceride harboring one glycerol and three oleic acid units, triolein does not fully represent the entire microbial lipid profile [179].

In this work, the inherent lipids of *C. oleaginosus* - termed ML - was employed as a standard for Nile red analysis. Based on the degree of fatty acid saturation, the density of ML was calculated according to Sergeeva et al. (**see Section 4.4: High-throughput Genetic Development of *C. oleaginosus***) [131]. Following extraction and purification, precise ML concentration gradients were devised, based on a method originally described for the preparation of triolein standard. Initially developed by Prisco and utilized by Massart and Huang, this method brings about the dilution of triolein in aqueous solution. Briefly, 1:1 triolein: chloroform is further diluted with isopropanol to realize intermediate solutions. Finally, dilutions in 10 mL water gave a working concentration range of 1-10 µg.mL^{-1} [128,180,181]. Nile Red assay depends on the even distribution of lipids in the solution. Exceeding a concentration threshold, triolein-water emulsion becomes heterogeneous and lipid droplets form, consequently leading to erroneous fluorescence readings [182,183]. We were able to successfully scale down Prisco's method from 10 mL to 225 uL, in order to carry out Nile red analysis in MTP (based on Sitepu et al). Most notably, our miniaturized method retained the homogeneity of the ML solutions over a concentration range of 1-100 µg.mL^{-1}, resulting in reproducible measurements. **Figure 2** illustrates the main steps of ML standard preparation developed in this study.

Additionally, triolein and oleic acid standards were prepared in the same manner. Compared to ML, both standards resulted in the overestimation of lipid titers. However, in contrast to triolein, oleic acid, is readily available and easier to handle. Remarkably, the first step of standard preparation (dilution 1:1 in chloroform), which is essential for micelles formation, was successfully bypassed for oleic acid. Eliminating the use of chloroform reduces the health risk on the operating personnel. Oleic acid is applicable as a reliable standard if a correction factor is applied. To obtain this (divergence) factor, we correlated the standard curves of oleic acid and ML, represented in the equation:

$$y = 652.56\,\mathrm{x} - 2354.58$$

The absolute quantitative method developed in this study was successfully employed for the screening of genetically improved strains of *C. oleaginosus* (**see Section 4.4: High-throughput Genetic Development of *C. oleaginosus***). It can also be applied for other oleaginous yeasts, taking the respective microbial lipid as a standard. The method can also be easily integrated in a multi-analytical measurement system, whereby multiple factors are

quantified from the same sample. Such systems are particularly important for monitoring of bioprocesses that employ co-production strains.

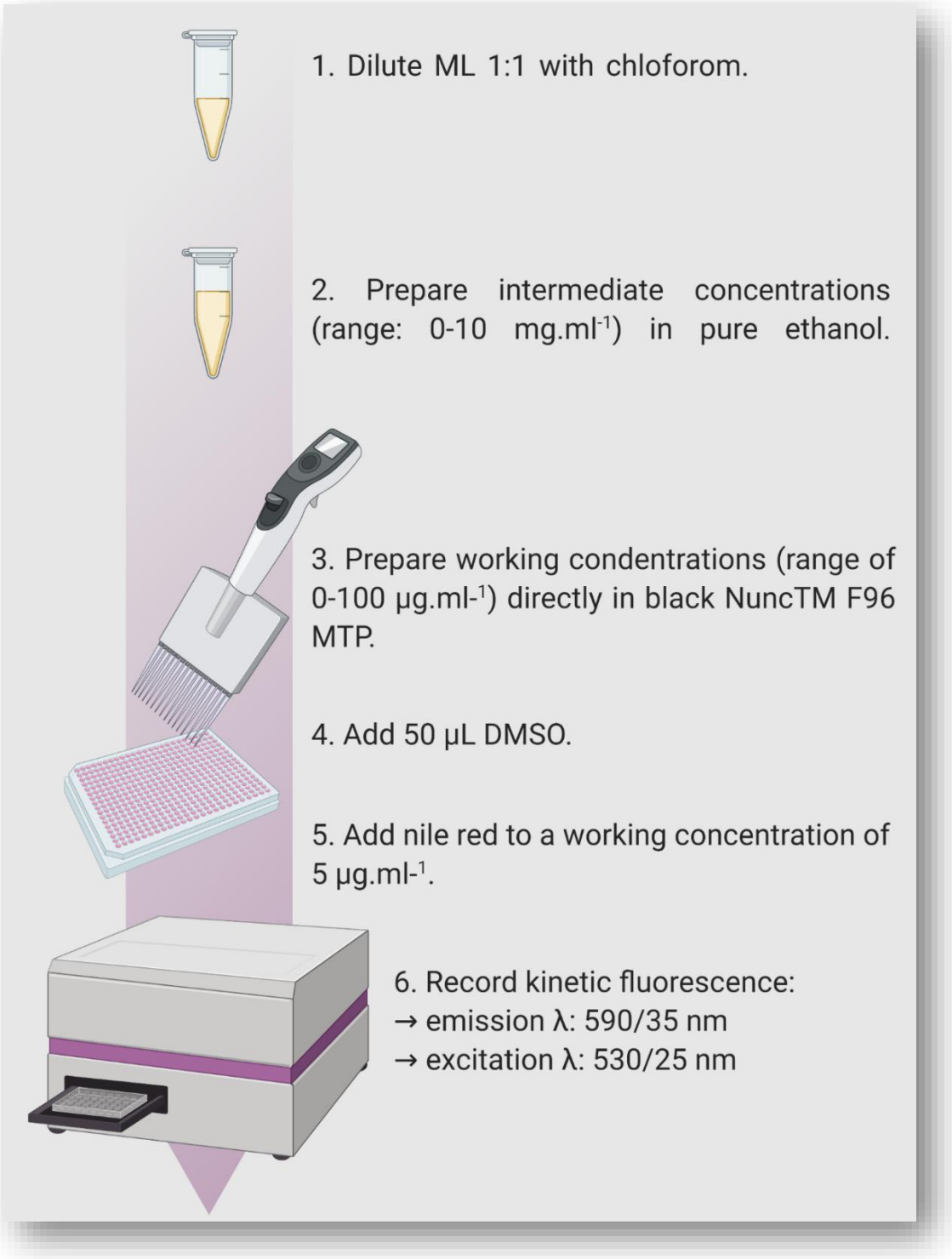


Figure 2. Simplified schematics for standard implementation in Nile Red Analysis.

In the context of strain development for industrial commercialization of SCOs, process monitoring that employs such state-of-the-art, high-throughput and miniaturized analytical tools is of special interest [87]. The method developed in this study is directly applicable for automation of PAT. Moreover, PAT that pertain to the quality of the microbial lipids can also see development efforts. In that context, automation of lipid extraction and esterification methods can further increase the data output of PAT.

4.4 High-throughput Genetic Development of *C. oleaginosus*

Literature and intellectual properties guide researchers in selecting a valuable microbial product and an appropriate host strain, which in turn dictates the best-fit approach for strain development [184,185]. One can utilize a model organism with genetic accessibility and robust genetic tools or work with an unconventional organism evolved for targeted products, but typically lacking in genomic data and tools for engineering [35]. In both approaches, several factors must be considered for SCOs production: genetic accessibility, substrate flexibility, biomass and lipid accumulation, and facility under industrial settings [186].

Metabolic engineering strategies that aim to redirect the carbon flux towards TAGs accumulation have been demonstrated to enhance lipid titers and quality [135]. Comprehensive metabolic knowledge and well-developed genetic tools are readily available for ascomycetous yeast species, such as *S. cerevise* and *Y. lipolytica* [187-191]. Over that last 2 decades, *Y. lipolytica* has been extensively engineered for boosting lipid titers and signature profiles [59,132,175]. As a Basidiomycota, *C. oleaginosus* is not as well characterized, and is refractive to most genetic engineering approaches [137]. Although basidiomycetes enjoy a superior baseline (broad substrate range and high tolerance to growth inhibitors), their characterization and genetic tools remain underdeveloped [59,71]. Specifically, the GC-rich genome (65%) of *C. oleaginosus* entails a strong bias in the codon usage, thus hindering the direct transfer of established site-directed genetic tools. Genetic engineering of this strain has only been carried out via multiple random genomic insertions e.g. *Agrobacterium tumefaciens*-mediated gene transfer (AGMT) [123,192]. Targeted-gene approaches, such as CRISPR or TALENS, require further development.

For *C. oleaginosus* and other genetically inaccessible strains, the classical approach holds potential. Below is an excerpt from the book “Concepts in Biotechnology: History, Science and Business for Buchholz K. and Collins J. (2014)[193]:

In 1977, at the fifth Workshop Conference Hoechst: Pancreatic beta cell culture. Professor E. von Wasielewski, then Head of the board of Directors of Hoechst AG, Frankfurt am Main, was asked by Walter Gilbert: "What would you pay for an E. coli clone producing human insulin, and what would you then do with it?". von Wasielewski replied: "I would pay 5$ million and then irradiate it to produce variants that made more insulin!".

Several strains of *L. starkeyi, R. toruloides* and *R. glutinis* have been optimized via UV irradiation coupled with cerulenin screening [97,194,195]. Recently, Liu et al. established a trackable and reliable method for genome-wide insertional mutagenesis of *R. toruloides* [196]. These random mutagenic treatments do not incur EU legislative requirements involved in Genetically Modified Organism (GMO) labeling, control and containment. Microbial lipids sourced from *C. oleaginosus* mutants, developed in this study via the classical approach, are hence applicable in food and cosmetic industry.

We describe, for the first time, the use of fast neutron irradiation for the rapid metabolic optimization of an oleaginous yeast, *C. oleaginosus*. This ionizing radiation is an established method in plant breeding (soybean, rice, peanuts, tomato and peas), where it is reported to generate stable mutations (double-stranded breaks; DSBs) [197-201]. Yet, it remains a less explored mutagenic technique in yeasts. Only *S. cervisiae* has been subjected to this type of irradiation in a study dating back to 1986, with the sole purpose of evaluating the nature of genomic disruptions [136]. As such, our study is the first to report on the enhanced production of microbial products via fast neutron irradiation. With a total of 12 neutron sources in Europe with direct allocation for research, it is noteworthy that centralized facilities is the norm due to expense and safety aspects. Nevertheless, regional availability does not limit the research output of radiation-related facilities [202]. FRM II neutron source in Garching is currently the most powerful neutron source in Germany. Relative to its thermal power (20 MW), it also produces the highest neutron flux (8 × 1014 neutrons $cm^{-2}s^{-1}$) in the world.

The design of strain development approach adopted in this work and its efficiency are displayed is **Figure 3**. Based on a dose/response assay, mutant pool U, which was subjected to the highest irradiation dosage (1000 Gy), recorded a 4.17% survival response. This pool of mutants was selected for subsequent processing. Primary screening for cerulenin resistant mutants from this pool resulted in 9 possibly enhanced variants. Subsequently, secondary screening via absolute Nile red analysis (**see Section 4.3: Development of an Absolute Lipid Quantitation Method for High-throughput Process Monitoring**) confirmed 2 enhanced mutants. One mutant, *C. oleaginosus* FN M2, was characterized with 21.67 % and

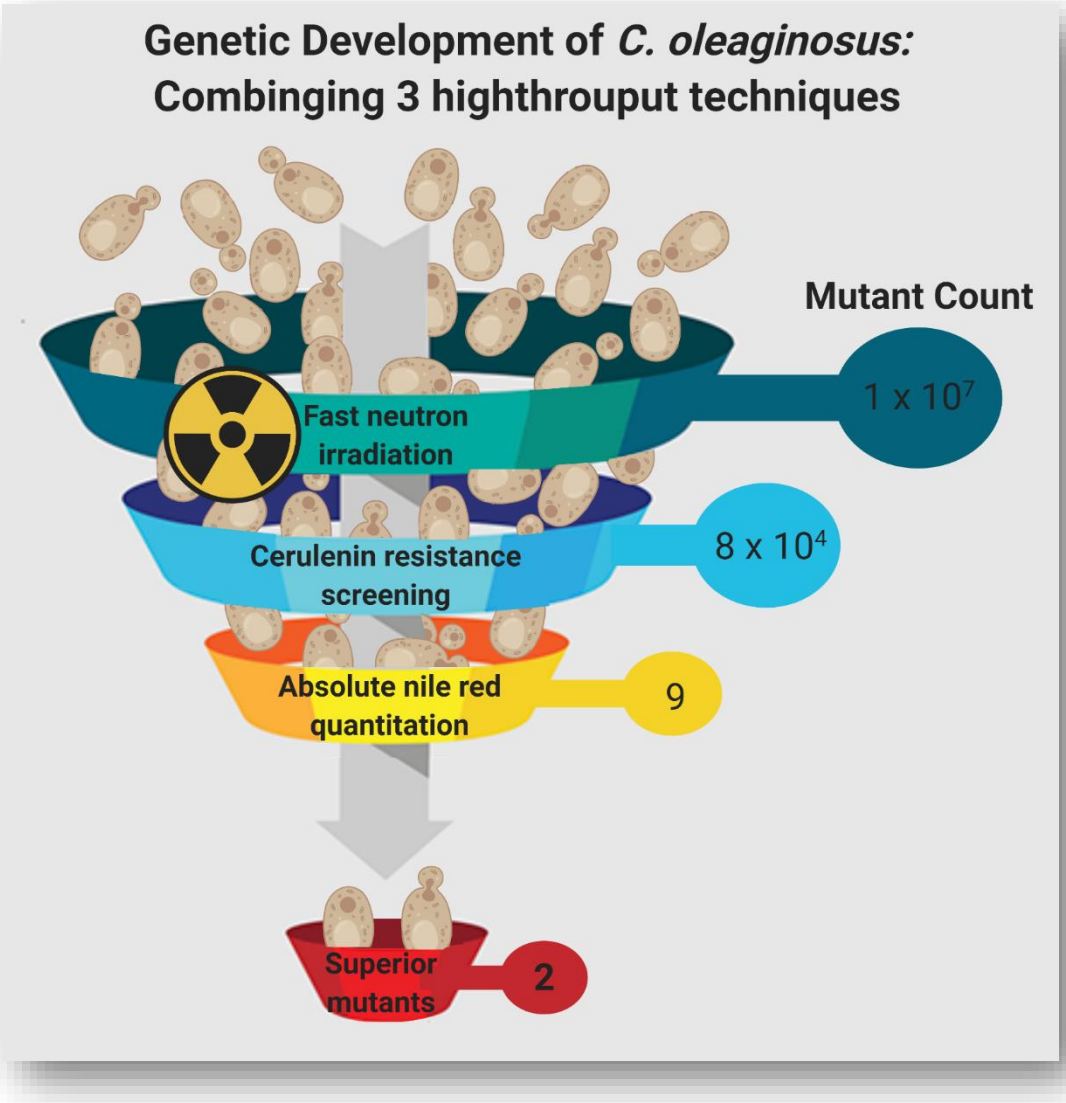


Figure 3. Design of the strain development approach adopted in this work for *C. oleaginosus* microbial lipid production.

22.58 % (w/v) improvement in growth and lipid productivity titers, respectively, in comparison with the wild type strain. This mutant is industrially favorable and prospective research involves characterization of this mutant and optimization of its cultivation conditions. Another mutant, *C. oleaginosus* FN M8, displayed higher intracellular lipid content (73.16 %; w/w), in comparison with the wild type strain. However, this mutant displayed a 37.90 % decrease in biomass accumulation, hence it does not qualify as a good production strain due to its low lipid productivity titers. This mutant, however, is particularly interesting for elucidating the metabolic drivers and regulators of lipogenesis in *C. oleaginosus* via Systems Biology approaches. Stability of the fast neutron mutants was also corroborated in the absence of selection pressure, via multiple subsequent cultivation and monitoring of lipid titers [125,197,200]. It is

particularly instrumental to screen fast neutron mutant pools for mutants that are more genetically accessible, as these can accelerate genetic manipulation of this strain [203,204]. As the application of antibiotics-treated organisms for food and beverage industries are problematic due to issues of public concerns, auxotrophic mutants, particularly valuable in cloning and genome editing, could alleviate the need for costly antibiotics-resistance selection methods[205,206]. These mutants can be screened for via dropout media.

In order to identify lipid profile shifts of high-value, the fatty acid profiles of cerulenin-screened mutants were examined. The effect of our strain improvement on *C. oleaginosus* lipids is most apparent in the increased degree of saturation. Furthermore, the biofuel properties of *C. oleaginosus* wild type and FN M2 lipids were investigated based on the degree of saturation with respect to: Iodine Value (IV), Cetane Number (CN), Higher Heating Value (HHV), Kinematic Viscosity (KV) and Density [131]. These properties were found to be well positioned within the limits of internationally accepted requirements of biodiesel standards (EU biodiesel standard EN14214 and US biodiesel ASTM D6751) [207,208]. Hence, *C. oleaginosus* lipids could directly substitute palm or rapeseed oil as feedstock for biofuel production. It is noteworthy that the calculated density values were valuable in delivering appropriate concentrations in the preparation of ML standard (**see Section 4.3: Development of an Absolute Lipid Quantitation Method for High-throughput Process Monitoring**).

The described strategy for *C. oleaginosus* development can be applied to other oleaginous yeasts. The consolidated operational units of this design are high-throughput, miniaturized and transferable to automatable platforms. Although the screening method (cerulenin resistance) constitutes a bottleneck in this approach, the advent of automation by means of robotics with minimal operator interference, high-throughput PAT, continuous cultivation systems, and microprocessor control would allow the numbers of isolates that can be processed per unit time to be increased by several orders of magnitude[209,210] [211,212]. In these settings, PAT that employ absolute Nile red quantitation with FACS for screening of the entire mutant library could alleviate the need for primary screening by cerulenin [213,214]. Finally, unravelling the mechanisms of gene expression and biochemical regulation of lipogenesis would facilitate the design of specialized screening procedures that can maximize the expression of the desirable mutations. Recent technological advancements in state-of-the-art Machine Learning, the Big Data and the Artificial Intelligence can also provide insight into linking of desirable phenotypes and selectable traits in producer strains [215]. All these factors significantly contribute to the probability of attaining improved strains, by making the classical approach less random.

4.5 Closing Remarks: Prospective Development and Industrial Applications

In summary, we present a concerted design for upstream development processes of *C. oleaginosus* [130]. This work can guide researchers throughout the developmental and industrial stages of lipid bioprocesses from this yeast. We also demonstrate the power of statistical modeling in providing a continuous learning potential for the improvement of bioprocess parameters [9]. The high-throughput and miniaturized techniques implemented in our approach are key for automation, which can accelerate commercial deployment of the technology [156]. In that respect, the employment of robotic systems in bioprocesses greatly reduces developmental efforts [216].

The fast neutron mutant library, generated for *C. oleaginosus* in the course of this work, opens the door for a myriad of genetic developmental possibilities. Moreover, System Biology and multi-omics investigations of oleaginicity can support genome-wide metabolic engineering of this yeast, speeding up the entire developmental process [102]. Specifically, proteomics and metabolomics can complement available transcriptomic and lipidomic data [176,217]. In order to facilitate upcoming proteomics exploration, we optimized in another study the method of protein extraction from this highly oleaginous yeast[127].

Beyond upstream processes, a holistic assessment of the developmental design includes downstream processes, which are oriented towards industrial scale and subsequent applications of SCOs. In that context, enrichment, secretion and further modification and derivatization of *C. oleaginosus* fatty acids can expand its industrial application [132,174,218]. Besides biofuels, commercial efforts in the next 5–10 years will likely focus on high-value specialty oleochemicals, such as hydroxylated fatty acids for food and feed applications, waxes for cosmetics and lactone fragrances [219]. Finally, adopting a cyclic biorefinary design and valorization of low-value feedstocks is expected to drive this sustainable biotechnology towards commercialization [90].

5 List of Publications

5.1 Multi-Factorial-Guided Media Optimization for Enhanced Biomass and Lipid Formation by the Oleaginous Yeast *Cutaneotrichosporon oleaginosus*

Dania Awad, Frank Bohnen, Norbert Mehlmer and Thomas Brueck

Frontiers in Bioengineering and Biotechnology **2019** March 26

DOI: 10.3389/fbioe.2019.00054

Page 39: CHAPTER II: Research, section 3: Overview of Included Publication

5.2 Optimization of Protein Isolation by Proteomic Qualification from *Cutaneotrichosporon oleaginosus*

Dania Awad and Thomas Brueck

Anal Bioanal Chem **2019** December 04

DOI: 10.1007/s00216-019-02254-7

Page 125: CHAPTER III: Index, section 10: Additional Full-length publications

5.3 Microbial lipid production by oleaginous yeasts grown on *Scenedesmus obtusiusculus* microalgae biomass hydrolysate

Samer Younes, Felix Bracharz, **Dania Awad**, Farah Qoura, Norbert Mehlmer and Thomas Brueck

Bioprocess and Biosystems Engineering **2020** April 28

DOI: 10.1007/s00449-020-02354-0

Page 143: CHAPTER III: Index, section 10: Additional Full-length publications

5.4 Towards High-Throughput Optimization of Microbial Lipid Production: From Strain Development to Process Monitoring

Dania Awad, Samer Younes, Matthias Glemser, Franz Wagner, Gerhard Schenk, Norbert Mehlmer and Thomas Brueck

Sustainable Energy and Fuels **2020** June 17

DOI: 10.139/D0SE00540A

Page 59: CHAPTER II: Research, section 3: Overview of Included Publication

5.5 Systems biology engineering of the Pantothenate pathway to enhance 3HB productivity in *Escherichia coli*

Samer Younes, **Dania Awad**, Elias Kassab, Claudia Schuler, Norbert Mehlmer and Thomas Brueck

Bioprocess and Biosystems Engineering

In review

5.6 Differential Proteome Analysis of the Oleaginous Yeast Cutaneotrichosporon oleaginosus in Relation to Carbohydrate Utilization

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

EMBO

In review

5.7 Elucidation of *Cutaneotrichosporon oleaginosus* Lipogenesis via Time-resolved Proteomics and Metabolomics Approach

Manuscript Preparation

6 Literature

1 Petroleum, B. Statistical Review of World Energy (London, UK, 2020).
2 in *The Economist* (2003).
3 Biermann, U., Bornscheuer, U., Meier, M. A., Metzger, J. O. & Schäfer, H. J. Oils and fats as renewable raw materials in chemistry. *Angewandte Chemie International Edition* **50**, 3854-3871 (2011).
4 Gunstone, F. D. Basic oleochemicals, oleochemical products and new industrial oils. *Oleochemical manufacture and application. Sheffield Academic Press, Sheffield*, 1-22 (2001).
5 Pfleger, B. F., Gossing, M. & Nielsen, J. Metabolic engineering strategies for microbial synthesis of oleochemicals. *Metabolic engineering* **29**, 1-11 (2015).
6 Smith, V. H., Sturm, B. S., Denoyelles, F. J. & Billings, S. A. The ecology of algal biodiesel production. *Trends in ecology & evolution* **25**, 301-309 (2010).
7 Ochsenreither, K., Glück, C., Stressler, T., Fischer, L. & Syldatk, C. Production strategies and applications of microbial single cell oils. *Frontiers in microbiology* **7**, 1539 (2016).
8 Ratledge, C. in *Single cell oils* 3-26 (Elsevier, 2010).
9 Awad, D., Bohnen, F., Mehlmer, N. & Brueck, T. Multi-factorial-guided media optimization for enhanced biomass and lipid formation by the oleaginous yeast Cutaneotrichosporon oleaginosus. *Frontiers in bioengineering and biotechnology* **7**, 54 (2019).
10 Ratledge, C. Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie* **86**, 807-815 (2004).
11 Koch, B., Schmidt, C. & Daum, G. Storage lipids of yeasts: a survey of nonpolar lipid metabolism in Saccharomyces cerevisiae, Pichia pastoris, and Yarrowia lipolytica. *FEMS microbiology reviews* **38**, 892-915 (2014).
12 Alvarez, H. M. Triacylglycerol and wax ester-accumulating machinery in prokaryotes. *Biochimie* **120**, 28-39 (2016).
13 Meng, X. *et al.* Biodiesel production from oleaginous microorganisms. *Renewable energy* **34**, 1-5 (2009).
14 Patel, A. *et al.* An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3 fatty acid-based industries. *Microorganisms* **8**, 434 (2020).
15 Mallick, N., Bagchi, S. K., Koley, S. & Singh, A. K. Progress and challenges in microalgal biodiesel production. *Frontiers in microbiology* **7**, 1019 (2016).
16 Babau, M. *et al.* Towards a microbial production of fatty acids as precursors of biokerosene from glucose and xylose. *Oil & Gas Science and Technology–Revue d'IFP Energies nouvelles* **68**, 899-911 (2013).

17 Li, Q., Du, W. & Liu, D. Perspectives of microbial oils for biodiesel production. *Applied microbiology and biotechnology* **80**, 749-756 (2008).
18 Singh, R., Kumar, M., Mittal, A. & Mehta, P. K. Microbial metabolites in nutrition, healthcare and agriculture. *3 Biotech* **7**, 15 (2017).
19 Diez, B., Mellado, E., Rodriguez, M., Fouces, R. & Barredo, J. L. Recombinant microorganisms for industrial production of antibiotics. *Biotechnology and bioengineering* **55**, 216-226 (1997).
20 Ledesma-Amaro, R., Santos, M., Jiménez, A. & Revuelta, J. in *Microbial production of food ingredients, enzymes and nutraceuticals* 571-594 (Elsevier, 2013).
21 Lindner, P. Das problem der biologischen fettbildung und fettgewinnung. *Angewandte Chemie* **35**, 110-114 (1922).
22 Woodbine, M. Microbial fat: Microorganisms as potential fat producers. *Progress in industrial microbiology* **1**, 181-245 (1959).
23 Lundin, H. Fat synthesis by micro-organisms and its possible applications in industry. *Journal of the Institute of Brewing* **56**, 17-28 (1950).
24 Stanier, R. Some aspects of microbiological research in Germany BIOS Final Report No 691, Item No 24. *British Intelligence Objectives Sub-Committee, London* (1946).
25 Nilsson, R., Enebo, L., Karsvik, E. & Lundin, H. Improved yield of yeast when straw extract is added to the culture solution. *Lantbrukshogskolans Annaler* **11**, 190-200 (1943).
26 Wynn, J. P. & Ratledge, C. Oils from microorganisms. *Bailey's Industrial Oil and Fat Products* (2005).
27 Sheehan, J., Dunahay, T., Benemann, J. & Roessler, P. Look back at the US department of energy's aquatic species program: biodiesel from algae; close-out report. (National Renewable Energy Lab., Golden, CO.(US), 1998).
28 Bharathiraja, B., Sridharan, S., Sowmya, V., Yuvaraj, D. & Praveenkumar, R. Microbial oil–a plausible alternate resource for food and fuel application. *Bioresource technology* **233**, 423-432 (2017).
29 Bonatsos, N. *et al.* Techno-economic analysis and life cycle assessment of heterotrophic yeast-derived single cell oil production process. *Fuel* **264**, 116839 (2020).
30 Donot, F., Fontana, A., Baccou, J., Strub, C. & Schorr-Galindo, S. Single cell oils (SCOs) from oleaginous yeasts and moulds: production and genetics. *Biomass and Bioenergy* **68**, 135-150 (2014).
31 Ma, Y., Gao, Z., Wang, Q. & Liu, Y. Biodiesels from microbial oils: opportunity and challenges. *Bioresource technology* **263**, 631-641 (2018).
32 Ward, O. P. & Singh, A. Omega-3/6 fatty acids: alternative sources of production. *Process biochemistry* **40**, 3627-3652 (2005).

33 Younes, S. *et al.* Microbial lipid production by oleaginous yeasts grown on Scenedesmus obtusiusculus microalgae biomass hydrolysate. *Bioprocess and biosystems engineering* (2020).
34 Probst, K. V., Schulte, L. R., Durrett, T. P., Rezac, M. E. & Vadlani, P. V. Oleaginous yeast: a value-added platform for renewable oils. *Critical reviews in biotechnology* **36**, 942-955 (2016).
35 Awad, D. *et al.* Towards High-Throughput Optimization of Microbial Lipid Production: From Strain Development to Process Monitoring. *Sustainable Energy & Fuels*, doi:10.1039/D0SE00540A (2020).
36 Zhang, X., Rong, J., Chen, H., He, C. & Wang, Q. Current status and outlook in the application of microalgae in biodiesel production and environmental protection. *Frontiers in Energy Research* **2**, 32 (2014).
37 Meesters, P., Huijberts, G. & Eggink, G. High-cell-density cultivation of the lipid accumulating yeast Cryptococcus curvatus using glycerol as a carbon source. *Applied microbiology and biotechnology* **45**, 575-579 (1996).
38 Beopoulos, A. *et al.* Yarrowia lipolytica as a model for bio-oil production. *Progress in lipid research* **48**, 375-387 (2009).
39 Ratledge, C. & Wynn, J. P. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Advances in applied microbiology* **51**, 1-52 (2002).
40 Papanikolaou, S. *et al.* Importance of the methyl-citrate cycle on glycerol metabolism in the yeast Yarrowia lipolytica. *Journal of biotechnology* **168**, 303-314 (2013).
41 Ageitos, J. M., Vallejo, J. A., Veiga-Crespo, P. & Villa, T. G. Oily yeasts as oleaginous cell factories. *Applied microbiology and biotechnology* **90**, 1219-1227 (2011).
42 Rattray, J. Yeasts. *Microbial lipids* **1**, 555-697 (1988).
43 Ratledge, C. & Wilkinson, S. G. *Microbial lipids*. Vol. 2 (Academic Pr, 1988).
44 Sitepu, I. *et al.* An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. *Journal of microbiological methods* **91**, 321-328 (2012).
45 Sitepu, I. R. *et al.* Manipulation of culture conditions alters lipid content and fatty acid profiles of a wide variety of known and new oleaginous yeast species. *Bioresource technology* **144**, 360-369 (2013).
46 Ratledge, C. & Cohen, Z. Microbial and algal oils: do they have a future for biodiesel or as commodity oils? *Lipid Technology* **20**, 155-160 (2008).
47 Thangavel, K. *et al.* Growth and metabolic characteristics of oleaginous microalgal isolates from Nilgiri biosphere Reserve of India. *BMC microbiology* **18**, 1 (2018).
48 Volkman, J., Jeffrey, S., Nichols, P., Rogers, G. & Garland, C. Fatty acid and lipid composition of 10 species of microalgae used in mariculture.

Journal of Experimental Marine Biology and Ecology **128**, 219-240 (1989).
49 Breuer, G. *et al.* Analysis of fatty acid content and composition in microalgae. *JoVE (Journal of Visualized Experiments)*, e50628 (2013).
50 Pinzi, S., Mata-Granados, J., Lopez-Gimenez, F., de Castro, M. L. & Dorado, M. Influence of vegetable oils fatty-acid composition on biodiesel optimization. *Bioresource technology* **102**, 1059-1065 (2011).
51 Tibocha-Bonilla, J. D., Zuñiga, C., Godoy-Silva, R. D. & Zengler, K. Advances in metabolic modeling of oleaginous microalgae. *Biotechnology for biofuels* **11**, 241 (2018).
52 Sun, Z., Fang, X.-p., Li, X.-y. & Zhou, Z.-g. Oleaginous microalgae from dairy farm wastewater for biodiesel production: Isolation, characterization and mass cultivation. *Applied biochemistry and biotechnology* **184**, 524-537 (2018).
53 Dragone, G., Fernandes, B. D., Vicente, A. A. & Teixeira, J. A. Third generation biofuels from microalgae. (2010).
54 Bai, A. *et al.* The significance of forests and algae in CO2 balance: a hungarian case study. *Sustainability* **9**, 857 (2017).
55 Bhola, V., Swalaha, F., Kumar, R. R., Singh, M. & Bux, F. Overview of the potential of microalgae for CO 2 sequestration. *International Journal of Environmental Science and Technology* **11**, 2103-2118 (2014).
56 Sayre, R. Microalgae: the potential for carbon capture. *Bioscience* **60**, 722-727 (2010).
57 Yoshida, M., Tanabe, Y., Yonezawa, N. & Watanabe, M. M. Energy innovation potential of oleaginous microalgae. *Biofuels* **3**, 761-781 (2012).
58 Du, J., Wang, H., Jin, H., Yang, K. & Zhang, X. Fatty acids production by fungi growing in sweet potato starch processing waste water. *Chin J Bioprocess Eng* **5**, 33-36 (2007).
59 Sitepu, I. R. *et al.* Oleaginous yeasts for biodiesel: current and future trends in biology and production. *Biotechnology advances* **32**, 1336-1360 (2014).
60 Vaughan-Martini, A. & Martini, A. Facts, myths and legends on the prime industrial microorganism. *Journal of industrial microbiology* **14**, 514-522 (1995).
61 Lazar, Z., Liu, N. & Stephanopoulos, G. Holistic approaches in lipid production by Yarrowia lipolytica. *Trends in biotechnology* **36**, 1157-1170 (2018).
62 Zeng, S. Y. *et al.* Recent advances in metabolic engineering of Yarrowia lipolytica for lipid overproduction. *European Journal of Lipid Science and Technology* **120**, 1700352 (2018).
63 Park, Y.-K., Nicaud, J.-M. & Ledesma-Amaro, R. The engineering potential of Rhodosporidium toruloides as a workhorse for

biotechnological applications. *Trends in biotechnology* **36**, 304-317 (2018).
64 Zhu, Z. *et al.* A multi-omic map of the lipid-producing yeast Rhodosporidium toruloides. *Nature communications* **3**, 1-12 (2012).
65 McNeil, B. A. & Stuart, D. T. Lipomyces starkeyi: an emerging cell factory for production of lipids, oleochemicals and biotechnology applications. *World Journal of Microbiology and Biotechnology* **34**, 147 (2018).
66 Sutanto, S., Zullaikah, S., Tran-Nguyen, P. L., Ismadji, S. & Ju, Y.-H. Lipomyces starkeyi: its current status as a potential oil producer. *Fuel processing technology* **177**, 39-55 (2018).
67 Bracharz, F., Beukhout, T., Mehlmer, N. & Brück, T. Opportunities and challenges in the development of Cutaneotrichosporon oleaginosus ATCC 20509 as a new cell factory for custom tailored microbial oils. *Microbial Cell Factories* **16**, 178, doi:10.1186/s12934-017-0791-9 (2017).
68 Masri, M. A., Garbe, D., Mehlmer, N. & Brück, T. B. A sustainable, high-performance process for the economic production of waste-free microbial oils that can replace plant-based equivalents. *Energy & Environmental Science* **12**, 2717-2732 (2019).
69 Kamineni, A. & Shaw, J. Engineering triacylglycerol production from sugars in oleaginous yeasts. *Current Opinion in Biotechnology* **62**, 239-247 (2020).
70 Masri, M. A. *et al.* A Seagrass-Based Biorefinery for Generation of Single-Cell Oils for Biofuel and Oleochemical Production. *Energy Technology* **6**, 1026-1038 (2018).
71 Kurtzman, C., Fell, J. W. & Boekhout, T. *The yeasts: a taxonomic study*. (Elsevier, 2011).
72 Kolouchová, I., Maťátková, O., Sigler, K., Masák, J. & Řezanka, T. Lipid accumulation by oleaginous and non-oleaginous yeast strains in nitrogen and phosphate limitation. *Folia microbiologica* **61**, 431-438 (2016).
73 Dai, C.-c., Tao, J., Xie, F., Dai, Y.-j. & Zhao, M. Biodiesel generation from oleaginous yeast Rhodotorula glutinis with xylose assimilating capacity. *African Journal of Biotechnology* **6** (2007).
74 Dey, P. & Maiti, M. Molecular characterization of a novel isolate of C andida tropicalis for enhanced lipid production. *Journal of Applied Microbiology* **114**, 1357-1368 (2013).
75 Zhu, L., Zong, M. & Wu, H. Efficient lipid production with Trichosporonfermentans and its use for biodiesel preparation. *Bioresource Technology* **99**, 7881-7885 (2008).
76 Galafassi, S. *et al.* Lipid production for second generation biodiesel by the oleaginous yeast Rhodotorula graminis. *Bioresource Technology* **111**, 398-403 (2012).

77 EVANS, C. T. & RATLEDGE, C. Influence of nitrogen metabolism on lipid accumulation by Rhodosporidium toruloides CBS 14. *Microbiology* **130**, 1705-1710 (1984).
78 Evans, C. T. & Ratledge, C. Effect of nitrogen source on lipid accumulation in oleaginous yeasts. *Microbiology* **130**, 1693-1704 (1984).
79 Angerbauer, C., Siebenhofer, M., Mittelbach, M. & Guebitz, G. Conversion of sewage sludge into lipids by Lipomyces starkeyi for biodiesel production. *Bioresource technology* **99**, 3051-3056 (2008).
80 Kennedy, M., Reader, S., Krouse, D. & Hinkley, S. Process optimization strategies for biotechnology products: from discovery to production. *BIOTECHNOLOGY-Volume IV: Fundamentals in Biotechnology* **4**, 131 (2009).
81 Salcedo, M. F. C., Valencia-Ochoa, G. & Jhan, P. R. Five Steps for Process Development under the Energy Sustainability Criteria. *Chemical Engineering Transactions* **67**, 625-630 (2018).
82 Parameswaran, K. in *Innovative Process Development in Metallurgical Industry* 257-280 (Springer, 2016).
83 Bjork, S. M. & Joensson, H. N. Microfluidics for cell factory and bioprocess development. *Current opinion in biotechnology* **55**, 95-102 (2019).
84 Wyman, C. *Handbook on bioethanol: production and utilization*. (CRC press, 1996).
85 Moo-Young, M. *Comprehensive biotechnology*. (Elsevier, 2019).
86 Doelle, H. W., Rokem, J. S. & Berovic, M. *BIOTECHNOLOGY-Volume VIII: Fundamentals in Biotechnology*. Vol. 8 (EOLSS Publications, 2009).
87 Gronemeyer, P., Ditz, R. & Strube, J. Trends in upstream and downstream process development for antibody manufacturing. *Bioengineering* **1**, 188-212 (2014).
88 Tripathi, N. K. & Shrivastava, A. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. *Frontiers in Bioengineering and Biotechnology* **7**, doi:10.3389/fbioe.2019.00420 (2019).
89 Liu, S., Farid, S. S. & Papageorgiou, L. G. Integrated optimization of upstream and downstream processing in biopharmaceutical manufacturing under uncertainty: a chance constrained programming approach. *Industrial & Engineering Chemistry Research* **55**, 4599-4612 (2016).
90 Masri, M. A. *et al.* A waste-free, microbial oil centered cyclic bio-refinery approach based on flexible macroalgae biomass. *Applied Energy* **224**, 1-12 (2018).
91 Council, N. R. *Putting biotechnology to work: bioprocess engineering*. (National Academies Press, 1992).

92 Selifonova, O., Valle, F. & Schellenberger, V. Rapid evolution of novel traits in microorganisms. *Applied and environmental microbiology* **67**, 3645-3649, doi:10.1128/AEM.67.8.3645-3649.2001 (2001).

93 Wackett, L. Biotechnology. Edited by Ulrich Kuck and Nicole Frankenberg-Dinkel. De Gruyter, 2015. Pp. XV+443. Price 49.95 EUR/70.00 USD/37.99 GBP (Paperback). ISBN 978-3-11-034110-2. *Acta Crystallographica Section D* **73**, 465-466, doi:doi:10.1107/S2059798317004557 (2017).

94 Mahajan, D., Sengupta, S. & Sen, S. Strategies to improve microbial lipid production: Optimization techniques. *Biocatalysis and Agricultural Biotechnology* **22**, 101321, doi:https://doi.org/10.1016/j.bcab.2019.101321 (2019).

95 Winkler, J. D. & Kao, K. C. Recent advances in the evolutionary engineering of industrial biocatalysts. *Genomics* **104**, 406-411 (2014).

96 Bruder, S., Hackenschmidt, S., Moldenhauer, E. J. & Kabisch, J. in *Lipid Modification by Enzymes and Engineered Microbes* 257-292 (Elsevier, 2018).

97 Tapia, V. E., Anschau, A., Coradini, A. L., T, T. F. & Deckmann, A. C. Optimization of lipid production by the oleaginous yeast Lipomyces starkeyi by random mutagenesis coupled to cerulenin screening. *AMB Express* **2**, 64, doi:10.1186/2191-0855-2-64 (2012).

98 Katre, G., Ajmera, N., Zinjarde, S. & RaviKumar, A. Mutants of Yarrowia lipolytica NCIM 3589 grown on waste cooking oil as a biofactory for biodiesel production. *Microb Cell Fact* **16**, 176, doi:10.1186/s12934-017-0790-x (2017).

99 Doan, T. T. Y. & Obbard, J. P. Enhanced intracellular lipid in Nannochloropsis sp. via random mutagenesis and flow cytometric cell sorting. *Algal Research* **1**, 17-21 (2012).

100 Cheng, Y.-r., Sun, Z.-j., Cui, G.-z., Song, X. & Cui, Q. A new strategy for strain improvement of Aurantiochytrium sp. based on heavy-ions mutagenesis and synergistic effects of cold stress and inhibitors of enoyl-ACP reductase. *Enzyme and microbial technology* **93**, 182-190 (2016).

101 Wang, J. *et al.* A quick isolation method for mutants with high lipid yield in oleaginous yeast. *World Journal of Microbiology and Biotechnology* **25**, 921-925 (2009).

102 Pham, N. T. *et al.* Genome-scale metabolic modelling underscores the potential of Cutaneotrichosporon oleaginosus ATCC 20509 as a cell factory for biofuel production. (2020).

103 Sewsynker-Sukai, Y., Faloye, F. & Kana, E. B. G. Artificial neural networks: an efficient tool for modelling and optimization of biofuel production (a mini review). *Biotechnology & Biotechnological Equipment* **31**, 221-235 (2017).

104 Takahashi, M. B., Coelho de Oliveira, H., Fernández Núñez, E. G. & Rocha, J. C. Brewing process optimization by artificial neural network and evolutionary algorithm approach. *Journal of Food Process Engineering* **42**, e13103 (2019).
105 Bhushanam, V. & Malothu, R. Bioprocess Optimization of L-Lysine Production by Using RSM and Artificial Neural Networks from Corynebacterium glutamicum ATCC13032. *Chemical Product and Process Modeling* **1** (2020).
106 Lee, J.-E., Vadlani, P. V. & Faubion, J. Corn bran bioprocessing: Development of an integrated process for microbial lipids production. *Bioresource technology* **243**, 196-203 (2017).
107 Tan, J. S. *et al.* Microtiter miniature shaken bioreactor system as a scale-down model for process development of production of therapeutic alpha-interferon2b by recombinant Escherichia coli. *BMC microbiology* **18**, 1-8 (2018).
108 Huth, I., Schrader, J. & Holtmann, D. Microtiter plate-based cultivation to investigate the growth of filamentous fungi. *Engineering in Life Sciences* **17**, 1064-1070 (2017).
109 Ralf, H., Christine, G. & Josefine, H. A. *Advances in pharmaceutical cell therapy: principles of cell-based biopharmaceuticals*. (World Scientific, 2015).
110 Santra, T. S. & Tseng, F. G. (Multidisciplinary Digital Publishing Institute, 2014).
111 Jiang, C.-Y. *et al.* High-throughput single-cell cultivation on microfluidic streak plates. *Applied and environmental microbiology* **82**, 2210-2218 (2016).
112 Demuth, C. SENSORS FOR SINGLE-USE BIOREACTORS–A REVIEW OF PERSPECTIVES AND CHALLENGES. (2017).
113 Rathore, A. S., Kateja, N. & Agarwal, H. Continuous downstream processing for production of biotech therapeutics. *Continuous Biomanufacturing: Innovative Technologies and Methods*, 259-288 (2017).
114 Jungbauer, A. Continuous downstream processing of biopharmaceuticals. *Trends in biotechnology* **31**, 479-492 (2013).
115 Somasundaram, B., Pleitt, K., Shave, E., Baker, K. & Lua, L. H. Progression of continuous downstream processing of monoclonal antibodies: Current trends and challenges. *Biotechnology and Bioengineering* **115**, 2893-2907 (2018).
116 Moon, N. J., Hammond, E. & Glatz, B. A. Conversion of cheese whey and whey permeate to oil and single-cell protein. *Journal of Dairy Science* **61**, 1537-1547 (1978).

117 Ykema, A., Verbree, E., Van Verseveld, H. & Smit, H. Mathematical modelling of lipid production by oleaginous yeasts in continuous cultures. *Antonie Van Leeuwenhoek* **52**, 491-506 (1986).
118 Ratledge, C. Single cell oils—have they a biotechnological future? *Trends in biotechnology* **11**, 278-284 (1993).
119 Gujjari, P., Suh, S.-O., Coumes, K. & Zhou, J. J. Characterization of oleaginous yeasts revealed two novel species: Trichosporon cacaoliposimilis sp. nov. and Trichosporon oleaginosus sp. nov. *Mycologia* **103**, 1110-1118 (2011).
120 Liu, X.-Z. *et al.* Towards an integrated phylogenetic classification of the Tremellomycetes. *Studies in Mycology* **81**, 85-147 (2015).
121 Ratledge, C. Microorganisms as sources of polyunsaturated fatty acids. *Structured and modified lipids*, 351-399 (2001).
122 Liang, Y., Jarosz, K., Wardlow, A. T., Zhang, J. & Cui, Y. Lipid production by Cryptococcus curvatus on hydrolysates derived from corn fiber and sweet sorghum bagasse following dilute acid pretreatment. *Applied biochemistry and biotechnology* **173**, 2086-2098 (2014).
123 Gorner, C. *et al.* Genetic engineering and production of modified fatty acids by the non-conventional oleaginous yeast Trichosporon oleaginosus ATCC 20509. *Green Chemistry* **18**, 2037-2046, doi:10.1039/C5GC01767J (2016).
124 FRMII. <http://www.frm2.tum.de/en/the-neutronsource/irradiation-facilities/irradiation-with-fast-neutrons/ > (2017).
125 Wagner, F. M. *et al.* The munich fission neutron therapy facility MEDAPP at the research reactor FRM II. *Strahlentherapie und Onkologie* **184**, 643-646 (2008).
126 Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917, doi:10.1139/o59-099 (1959).
127 Awad, D. & Brueck, T. Optimization of protein isolation by proteomic qualification from Cutaneotrichosporon oleaginosus. *Anal Bioanal Chem* **412**, 449-462, doi:10.1007/s00216-019-02254-7 (2020).
128 Priscu, J. C., Priscu, L. R., Palmisano, A. C. & Sullivan, C. W. Estimation of neutral lipid levels in Antarctic sea ice microalgae by Nile red fluorescence. *Antarctic Science* **2**, 149-155 (1990).
129 Griffiths, M. J., van Hille, R. P. & Harrison, S. T. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. *Lipids* **45**, 1053-1060, doi:10.1007/s11745-010-3468-2 (2010).
130 Awad, D. *et al.* Towards high-throughput optimization of microbial lipid production: from strain development to process monitoring. *Sustainable Energy & Fuels*, doi:10.1039/D0SE00540A (2020).

131 Sergeeva, Y. E. *et al.* Calculation of Biodiesel Fuel Characteristics Based on the Fatty Acid Composition of the Lipids of Some Biotechnologically Important Microorganisms. *Applied Biochemistry and Microbiology* **53**, 807-813, doi:10.1134/s0003683817080063 (2017).
132 Ledesma-Amaro, R. & Nicaud, J.-M. Metabolic Engineering for Expanding the Substrate Range of Yarrowia lipolytica. *Trends in Biotechnology* **34**, 798-809, doi:https://doi.org/10.1016/j.tibtech.2016.04.010 (2016).
133 Vasconcelos, B., Teixeira, J. C., Dragone, G. & Teixeira, J. A. Oleaginous yeasts for sustainable lipid production—from biodiesel to surf boards, a wide range of "green" applications. *Applied Microbiology and Biotechnology* **103**, 3651-3667, doi:10.1007/s00253-019-09742-x (2019).
134 Huang, C., Chen, X.-f., Xiong, L., Ma, L.-l. & Chen, Y. Single cell oil production from low-cost substrates: the possibility and potential of its industrialization. *Biotechnology advances* **31**, 129-139 (2013).
135 Shi, S. & Zhao, H. Metabolic Engineering of Oleaginous Yeasts for Production of Fuels and Chemicals. *Front Microbiol* **8**, 2185, doi:10.3389/fmicb.2017.02185 (2017).
136 Unrau, P. The Relative Biological Effectiveness of 14.5-MeV Neutrons for the Induction of Gene Conversion and Mutation in Yeast. *Radiation Research* **107**, 39-48, doi:10.2307/3576848 (1986).
137 Bracharz, F., Beukhout, T., Mehlmer, N. & Bruck, T. Opportunities and challenges in the development of Cutaneotrichosporon oleaginosus ATCC 20509 as a new cell factory for custom tailored microbial oils. *Microb Cell Fact* **16**, 178, doi:10.1186/s12934-017-0791-9 (2017).
138 Gifford, J. *et al.* in *Animal Cell Technology Meets Genomics* 549-553 (Springer, 2005).
139 Batista, K. A. & Fernandes, K. F. Development and optimization of a new culture media using extruded bean as nitrogen source. *MethodsX* **2**, 154-158, doi:10.1016/j.mex.2015.03.001 (2015).
140 Breiman, L. Statistical Modeling: The Two Cultures (with comments and a rejoinder by the author). *Statist. Sci.* **16**, 199-231, doi:10.1214/ss/1009213726 (2001).
141 Neubauer, P., Glauche, F. & Cruz-Bournazou, M. N. Editorial: Bioprocess Development in the era of digitalization. *Engineering in Life Sciences* **17**, 1140-1141, doi:10.1002/elsc.201770113 (2017).
142 Lopes, H. J. S., Bonturi, N., Kerkhoven, E. J., Miranda, E. A. & Lahtvee, P.-J. C/N ratio and carbon source-dependent lipid production profiling in Rhodotorula toruloides. *Applied Microbiology and Biotechnology* **104**, 2639-2649, doi:10.1007/s00253-020-10386-5 (2020).
143 Kuttiraja, M., Douha, A., Valéro, J. R. & Tyagi, R. D. Elucidating the Effect of Glycerol Concentration and C/N Ratio on Lipid Production

Using Yarrowia lipolytica SKY7. *Appl Biochem Biotechnol* **180**, 1586-1600, doi:10.1007/s12010-016-2189-2 (2016).

144 Akoh, C. C. *Food Lipids: Chemistry, Nutrition, and Biotechnology, Fourth Edition*. (CRC Press, 2017).

145 Das, S., Zarur Coelho, M. A., Amaral, P. F. F. & Sil, J. Development of nutrient media to increase the accumulation of lipids without genetic modification of a lipogenic microorganism. *RSC Advances* **7**, 38149-38154, doi:10.1039/C7RA04026A (2017).

146 Miranda, C. *et al.* Modified high-throughput Nile red fluorescence assay for the rapid screening of oleaginous yeasts using acetic acid as carbon source. *BMC Microbiology* **20**, 60, doi:10.1186/s12866-020-01742-6 (2020).

147 Sargeant, L. A., Chuck, C. J., Donnelly, J., Bannister, C. D. & Scott, R. J. Optimizing the lipid profile, to produce either a palm oil or biodiesel substitute, by manipulation of the culture conditions for Rhodotorula glutinis. *Biofuels* **5**, 33-43, doi:10.4155/bfs.13.64 (2014).

148 Kim, M. *et al.* Increased biomass and lipid production of Ettlia sp. YC001 by optimized C and N sources in heterotrophic culture. *Scientific Reports* **9**, 6830, doi:10.1038/s41598-019-43366-5 (2019).

149 Nazir, Y., Shuib, S., Kalil, M. S., Song, Y. & Hamid, A. A. Optimization of Culture Conditions for Enhanced Growth, Lipid and Docosahexaenoic Acid (DHA) Production of Aurantiochytrium SW1 by Response Surface Methodology. *Scientific Reports* **8**, 8909, doi:10.1038/s41598-018-27309-0 (2018).

150 Bandhu, S. *et al.* Statistical design and optimization of single cell oil production from sugarcane bagasse hydrolysate by an oleaginous yeast Rhodotorula sp. IIP-33 using response surface methodology. *Springerplus* **3**, 691, doi:10.1186/2193-1801-3-691 (2014).

151 Singh, G. *et al.* Concomitant Production of Lipids and Carotenoids in Rhodosporidium toruloides under Osmotic Stress Using Response Surface Methodology. *Frontiers in Microbiology* **7**, doi:10.3389/fmicb.2016.01686 (2016).

152 Yellapu, S. K., Bezawada, J., Kaur, R., Kuttiraja, M. & Tyagi, R. D. Detergent assisted lipid extraction from wet yeast biomass for biodiesel: A response surface methodology approach. *Bioresource Technology* **218**, 667-673, doi:https://doi.org/10.1016/j.biortech.2016.07.011 (2016).

153 Hadrich, B. *et al.* Optimization of lipids' ultrasonic extraction and production from Chlorella sp. using response-surface methodology. *Lipids in Health and Disease* **17**, 87, doi:10.1186/s12944-018-0702-z (2018).

154 Mubarak, M., Shaija, A. & Suchithra, T. V. Optimization of lipid extraction from Salvinia molesta for biodiesel production using RSM and

its FAME analysis. *Environmental science and pollution research international* **23**, 14047-14055, doi:10.1007/s11356-016-6343-8 (2016).
155 Cescut, J., Severac, E., Molina-Jouve, C. & Uribelarrea, J. L. Optimizing pressurized liquid extraction of microbial lipids using the response surface method. *Journal of chromatography. A* **1218**, 373-379, doi:10.1016/j.chroma.2010.12.003 (2011).
156 Predictive Modeling With Big Data: Is Bigger Really Better? *Big Data* **1**, 215-226, doi:10.1089/big.2013.0037 (2013).
157 Swoboda, F. K. NITROGEN NUTRITION OF YEAST. *Journal of Biological Chemistry* **52**, 91-109 (1922).
158 Gutierrez, A., Beltran, G., Warringer, J. & Guillamon, J. M. Genetic basis of variations in nitrogen source utilization in four wine commercial yeast strains. *PLoS One* **8**, e67166, doi:10.1371/journal.pone.0067166 (2013).
159 McNeil, B. & Harvey, L. *Practical fermentation technology*. (John Wiley & Sons, 2008).
160 Hahn-Hägerdal, B. *et al.* Role of cultivation media in the development of yeast strains for large scale industrial use. *Microbial Cell Factories* **4**, 31, doi:10.1186/1475-2859-4-31 (2005).
161 Zhang, J. & Greasham, R. Chemically defined media for commercial fermentations. *Applied Microbiology and Biotechnology* **51**, 407-421, doi:10.1007/s002530051411 (1999).
162 Loublere, M. C. B. C. G. L. N. N. D. L. P. Rational development of a simple synthetic medium for the sustained growth of Lactococcus lactis. *Journal of Applied Microbiology* **79**, 108-116 (1995).
163 Zhang, J. *et al.* Microbial lipid production by the oleaginous yeast Cryptococcus curvatus O3 grown in fed-batch culture. *Biomass and Bioenergy* **35**, 1906-1911 (2011).
164 Thiru, M., Sankh, S. & Rangaswamy, V. Process for biodiesel production from Cryptococcus curvatus. *Bioresource technology* **102**, 10436-10440 (2011).
165 Yen, H.-W., Yang, Y.-C. & Yu, Y.-H. Using crude glycerol and thin stillage for the production of microbial lipids through the cultivation of Rhodotorula glutinis. *Journal of bioscience and bioengineering* **114**, 453-456 (2012).
166 Kawaguchi, H., Hasunuma, T., Ogino, C. & Kondo, A. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. *Current opinion in biotechnology* **42**, 30-39 (2016).
167 Yang, S.-T., El-Ensashy, H. & Thongchul, N. *Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers*. (John Wiley & Sons, 2013).
168 Maiti, S. *et al.* Agro-industrial wastes as feedstock for sustainable bio-production of butanol by Clostridium beijerinckii. *Food and Bioproducts Processing* **98**, 217-226 (2016).

169 Dahiya, S. *et al.* Food waste biorefinery: Sustainable strategy for circular bioeconomy. *Bioresource technology* **248**, 2-12 (2018).
170 Koutinas, A., Wang, R. & Webb, C. Restructuring upstream bioprocessing: technological and economical aspects for production of a generic microbial feedstock from wheat. *Biotechnology and bioengineering* **85**, 524-538 (2004).
171 Meo, A., Priebe, X. L. & Weuster-Botz, D. Lipid production with Trichosporon oleaginosus in a membrane bioreactor using microalgae hydrolysate. *Journal of Biotechnology* **241**, 1-10 (2017).
172 Hatti-Kaul, R., Törnvall, U., Gustafsson, L. & Börjesson, P. Industrial biotechnology for the production of bio-based chemicals–a cradle-to-grave perspective. *Trends in biotechnology* **25**, 119-124 (2007).
173 Davies, R. J. Yeast oil from cheese whey -process development. *Single cell oil*, 99-145 (1988).
174 Ochsenreither, K., Gluck, C., Stressler, T., Fischer, L. & Syldatk, C. Production Strategies and Applications of Microbial Single Cell Oils. *Front Microbiol* **7**, 1539, doi:10.3389/fmicb.2016.01539 (2016).
175 Rigouin, C. *et al.* Production of Medium Chain Fatty Acids by Yarrowia lipolytica: Combining Molecular Design and TALEN to Engineer the Fatty Acid Synthase. *ACS Synth Biol* **6**, 1870-1879, doi:10.1021/acssynbio.7b00034 (2017).
176 Capusoni, C. *et al.* Characterization of lipid accumulation and lipidome analysis in the oleaginous yeasts Rhodosporidium azoricum and Trichosporon oleaginosus. *Bioresour Technol* **238**, 281-289, doi:10.1016/j.biortech.2017.03.188 (2017).
177 Kyle, D. J. Arachidonic acid and methods for the production and use thereof. USA patent (1991).
178 Greenspan, P., Mayer, E. P. & Fowler, S. D. Nile red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol* **100**, 965-973, doi:10.1083/jcb.100.3.965 (1985).
179 Liang, Y. *et al.* Utilization of sorghum bagasse hydrolysates for producing microbial lipids. *Applied Energy* **91**, 451-458 (2012).
180 Huang, G.-H., Chen, G. & Chen, F. Rapid screening method for lipid production in alga based on Nile red fluorescence. *Biomass and bioenergy* **33**, 1386-1392 (2009).
181 Massart, A., Aubry, É. & Hantson, A.-L. Étude de stratégies de culture de Dunaliella tertiolecta combinant haute densité cellulaire et accumulation de lipides en vue de produire du biodiesel. *BASE* (2010).
182 De la Hoz Siegler, H., Ayidzoe, W., Ben-Zvi, A., Burrell, R. & McCaffrey, W. Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. *Algal research* **1**, 176-184 (2012).

183 Chen, W., Sommerfeld, M. & Hu, Q. Microwave-assisted Nile red method for in vivo quantification of neutral lipids in microalgae. *Bioresource technology* **102**, 135-141 (2011).
184 Pham, J. V. *et al.* A Review of the Microbial Production of Bioactive Natural Products and Biologics. *Frontiers in Microbiology* **10**, doi:10.3389/fmicb.2019.01404 (2019).
185 Chen, W., Zhang, C., Song, L., Sommerfeld, M. & Hu, Q. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of microbiological methods* **77**, 41-47 (2009).
186 Chisti, Y. Biodiesel from microalgae. *Biotechnology advances* **25**, 294-306 (2007).
187 d'Espaux, L. *et al.* Engineering high-level production of fatty alcohols by Saccharomyces cerevisiae from lignocellulosic feedstocks. *Metab Eng* **42**, 115-125, doi:10.1016/j.ymben.2017.06.004 (2017).
188 Van Rossum, H. M., Kozak, B. U., Pronk, J. T. & van Maris, A. J. Engineering cytosolic acetyl-coenzyme A supply in Saccharomyces cerevisiae: pathway stoichiometry, free-energy conservation and redox-cofactor balancing. *Metabolic engineering* **36**, 99-115 (2016).
189 Kwak, S. & Jin, Y.-S. Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: a review and perspective. *Microbial Cell Factories* **16**, 82 (2017).
190 Borodina, I. & Nielsen, J. Advances in metabolic engineering of yeast Saccharomyces cerevisiae for production of chemicals. *Biotechnology journal* **9**, 609-620 (2014).
191 Zhu, Q. & Jackson, E. N. Metabolic engineering of Yarrowia lipolytica for industrial applications. *Current opinion in biotechnology* **36**, 65-72 (2015).
192 Koivuranta, K. *et al.* Enhanced Triacylglycerol Production With Genetically Modified Trichosporon oleaginosus. *Frontiers in Microbiology* **9**, doi:10.3389/fmicb.2018.01337 (2018).
193 Buchholz, K. & Collins, J. *Concepts in biotechnology: history, science and business*. (John Wiley & Sons, 2014).
194 Yamada, R., Kashihara, T. & Ogino, H. Improvement of lipid production by the oleaginous yeast Rhodosporidium toruloides through UV mutagenesis. *World J Microbiol Biotechnol* **33**, 99, doi:10.1007/s11274-017-2269-7 (2017).
195 Guo, M., Cheng, S., Chen, G. & Chen, J. Improvement of lipid production in oleaginous yeast Rhodosporidium toruloides by ultraviolet mutagenesis. *Engineering in Life Sciences* **19**, 548-556 (2019).
196 Liu, Y. *et al.* Identification of novel genes in the carotenogenic and oleaginous yeast Rhodotorula toruloides through genome-wide insertional

mutagenesis. *BMC Microbiology* **18**, 14, doi:10.1186/s12866-018-1151-6 (2018).
197 Kumawat, S. *et al.* Expanding Avenue of Fast Neutron Mediated Mutagenesis for Crop Improvement. *Plants* **8**, 164 (2019).
198 Al-Khayri, J. M., Jain, S. M. & Johnson, D. V. *Advances in plant breeding strategies: Breeding, biotechnology and molecular tools*. (Springer, 2015).
199 Sakai, K., Suzuki, S., Nakamura, N. & Okada, S. Induction and subsequent repair of DNA damage by fast neutrons in cultured mammalian cells. *Radiation research* **110**, 311-320 (1987).
200 Hendry, J. H. The slower cellular recovery after higher-LET irradiations, including neutrons, focuses on the quality of DNA breaks. *Radiation research* **128**, S111-S113 (1991).
201 Gajendiran, N., Tanaka, K., Kumaravel, T. S. & Kamada, N. Neutron-induced adaptive response studied in go human lymphocytes using the comet assay. *Journal of radiation research* **42**, 91-101 (2001).
202 John Womersley, A. S. NEUTRON USERS IN EUROPE: Facility-Based Insights and Scientific Trends. (Sweden, 2018).
203 Hu, J., Vanderstraeten, S. & Foury, F. Isolation and characterization of ten mutator alleles of the mitochondrial DNA polymerase-encoding MIP1 gene from Saccharomyces cerevisiae. *Gene* **160**, 105-110 (1995).
204 Muteeb, G. & Sen, R. in *In vitro mutagenesis protocols* 411-419 (Springer, 2010).
205 Akada, R. Genetically modified industrial yeast ready for application. *Journal of Bioscience and Bioengineering* **94**, 536-544, doi:https://doi.org/10.1016/S1389-1723(02)80192-X (2002).
206 Mülleder, M., Campbell, K., Matsarskaia, O., Eckerstorfer, F. & Ralser, M. Saccharomyces cerevisiae single-copy plasmids for auxotrophy compensation, multiple marker selection, and for designing metabolically cooperating communities. *F1000Research* **5**, 2351-2351, doi:10.12688/f1000research.9606.1 (2016).
207 Willis, R. M. *et al.* Improving energetics of triacylglyceride extraction from wet oleaginous microbes. *Bioresour Technol* **167**, 416-424, doi:10.1016/j.biortech.2014.06.013 (2014).
208 Wahlen, B. D. *et al.* Biodiesel from Microalgae, Yeast, and Bacteria: Engine Performance and Exhaust Emissions. *Energy & Fuels* **27**, 220-228, doi:10.1021/ef3012382 (2013).
209 Park, J. H., Lee, S. Y., Kim, T. Y. & Kim, H. U. Application of systems biology for bioprocess development. *Trends in biotechnology* **26**, 404-412 (2008).
210 Medema, M. H., Alam, M. T., Breitling, R. & Takano, E. The future of industrial antibiotic production: from random mutagenesis to synthetic biology. *Bioengineered bugs* **2**, 230-233 (2011).

211 Janzen, N. H. *et al.* Implementation of a Fully Automated Microbial Cultivation Platform for Strain and Process Screening. *Biotechnology Journal* **14**, 1800625, doi:10.1002/biot.201800625 (2019).
212 Haby, B. *et al.* Integrated robotic mini bioreactor platform for automated, parallel microbial cultivation with online data handling and process control. *SLAS TECHNOLOGY: Translating Life Sciences Innovation* **24**, 569-582 (2019).
213 Rumin, J. *et al.* The use of fluorescent Nile red and BODIPY for lipid measurement in microalgae. *Biotechnology for biofuels* **8**, 42-42, doi:10.1186/s13068-015-0220-4 (2015).
214 Satpati, G. G. & Pal, R. Rapid detection of neutral lipid in green microalgae by flow cytometry in combination with Nile red staining—an improved technique. *Annals of Microbiology* **65**, 937-949, doi:10.1007/s13213-014-0937-5 (2015).
215 Schmidt, J., Marques, M. R. G., Botti, S. & Marques, M. A. L. Recent advances and applications of machine learning in solid-state materials science. *npj Computational Materials* **5**, 83, doi:10.1038/s41524-019-0221-0 (2019).
216 Daniels, C., Rodriguez, J., Lim, E. & Wenger, M. An integrated robotic system for high-throughput process development of cell and virus culture conditions: Application to biosafety level 2 live virus vaccines. *Engineering in Life Sciences* **16**, 202-209 (2016).
217 Kourist, R. *et al.* Genomics and transcriptomics analyses of the oil-accumulating basidiomycete yeast Trichosporon oleaginosus: insights into substrate utilization and alternative evolutionary trajectories of fungal mating systems. *MBio* **6**, e00918-00915 (2015).
218 Ledesma-Amaro, R., Dulermo, R., Niehus, X. & Nicaud, J.-M. Combining metabolic engineering and process optimization to improve production and secretion of fatty acids. *Metabolic Engineering* **38**, 38-46, doi:https://doi.org/10.1016/j.ymben.2016.06.004 (2016).
219 Marella, E. R., Holkenbrink, C., Siewers, V. & Borodina, I. Engineering microbial fatty acid metabolism for biofuels and biochemicals. *Current opinion in biotechnology* **50**, 39-46 (2018).

CHAPTER III APPENDIX

7 List of Abbreviations

ANN	Artificial neural networks
ANOVA	Analysis of Variance
ARA	arachidonic acid
BP	British Petroleum
C	Carbon
C: N	Carbon to nitrogen ratio
CN	Cetane number
CRISPR repeats	Clustered regularly interspaced short palindromic
DCW	Dry cell weight
DHA	docosahexaenoic acid
DoE	Design of Experiment
EPA	eicosapentaenoic acid
EMS	ethyl methane sulfonate
FA	Fatty acid
FAME	Fatty acid methyl ester
FN	Fast neutron
FN M	Fast neutron mutant
FRM II	Forschungsreaktor München II
FSC	Forward-scattered light
GLA	γ-linoleic acid
Gy	Gray
HHV	Higher Heating Value
HTP	High-throughput
IV	Iodine Value
KV	Kinematic Viscosity
MEDAPP	Medical Applications

MFE	Maximal recorded fluorescence emissions
MMT	Million Metric Tons
MNM	Minimal-nitrogen media
ML	microbial lipids
MTP	Micro-titer plates
N	Nitrogen
NTG	nitrosomethyl guanidine
OFAT	One-factor-at-a-time
PAT	Process analytical tools
PHB	polyhydroxybutyrates
PLA	poly-lactic acid
R/P	Reserves-to-Production ratio
RSM	Response Surface Methodology
SCOs	Single cell oils
SSC	Secondary scattered light
SU	Single use
TAG	triacylglyceride
TALENS	Transcription activator-like effector nucleases
UV	Ultraviolet
WC	Working concentrations
YPD	Yeast Peptone Dextrose

8 List of Tables and Figures

8.1 Figures

8.2 Tables

9 Preprint Permissions

9.1 Frontiers Copyright Statement

This means that you must obtain any necessary third-party consents and permissions to reproduce third-party materials in your articles submitted to Frontiers.

E-books are subject to the same licensing conditions as the articles within them.

Articles published prior to the effective date of this notice: Please note that reproduction of third-party graphics and other third-party materials contained in articles published prior to the effective date of this notice may be subject to third-party notices prohibiting their reproduction without permission. You must comply with those notices.

Articles published prior to July 2012: The licence granted for these articles may be different and you should check the pdf version of any article to establish what licence was granted. If an article, dating from before July 2012, carries only a non-commercial licence and you wish to obtain a commercial licence, please contact Frontiers at editorial.office@frontiersin.org.

All software used on this website, and the copyright in the code constituting such software, is the property of or is licensed to Frontiers and its use is restricted in accordance with the Frontiers Terms and Conditions. All copyright, and all rights therein, are protected by national and international copyright laws.

This Copyright Statement comes into effect on 25th May, 2018.

Source: https://www.frontiersin.org/legal/copyright-statement

Copyright Clearance Center | Marketplace™

Royal Society of Chemistry - License Terms and Conditions

This is a License Agreement between Dania Awad ("You") and Royal Society of Chemistry ("Publisher") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Royal Society of Chemistry, and the CCC terms and conditions.

All payments must be made in full to CCC.

Order Date	31-Jul-2020	**Type of Use**	Republish in a thesis/dissertation
Order license ID	1052277-1		
ISSN	2398-4902	**Publisher**	Royal Society of Chemistry
		Portion	Chapter/article

LICENSED

Publication Title	Sustainable Energy & Fuels	**Country**	United Kingdom of Great Britain and Northern Ireland
Author/Editor	Royal Society of Chemistry (Great Britain)	**Rightsholder**	Royal Society of Chemistry
Date	01/01/2016	**Publication Type**	e-Journal
Language	English		

REQUEST DETAILS

Portion Type	Chapter/article	**Rights Requested**	Main product
Page range(s)	1-14	**Distribution**	Worldwide
Total number of pages	14	**Translation**	Original language of publication
Format (select all that apply)	Print, Electronic	**Copies for the disabled?**	No
Who will republish the content?	Academic institution	**Minor editing privileges?**	No
Duration of Use	Life of current edition	**Incidental promotional use?**	No
Lifetime Unit Quantity	Up to 499	**Currency**	EUR

CONTENT

NEW WORK DETAILS

Title	Upstream Bioprocess Development of Microbial	**Institution name**	Technischen Universität München lipids
		Expected presentation	2020-09-15
Instructor name	Prof. Dr. Thomas Brück	**date**	

ADDITIONAL DETAILS

Order reference number	N/A	**The requesting person / organization to appear on the license**	Dania Awad

REUSE CONTENT DETAILS

Title, description or numeric reference of the portion(s)	Paper #2	**Title of the article/chapter the portion is from**	3.2.3 Full-length Publication
Editor of portion(s)	N/A	**Author of portion(s)**	Royal Society of Chemistry (Great Britain)
Volume of serial or monograph	N/A	**Issue, if republishing an article from a serial**	N/A
Page or page range of portion	61-74	**Publication date of portion**	2016-01-01

PUBLISHER SPECIAL TERMS AND CONDITIONS

Permission is granted as long as the article is fully acknowledged and a link is given back to the article on our Platform. Please go to rsc.li/permissions for details. Please note that if the material speci ed above or any part of it appears with credit or acknowledgement to a third party then you must also secure permission from that third party before reproducing that material.

CCC Republication Terms and Conditions

1. Description of Service; De ned Terms. This Republication License enables the User to obtain licenses for republication of one or more copyrighted works as described in detail on the relevant Order Con rmation (the "Work(s)"). Copyright Clearance Center, Inc. ("CCC") grants licenses through the Service on behalf of the rightsholder identi ed on the Order Con rmation (the "Rightsholder"). "Republication", as used herein, generally means the inclusion of a Work, in whole or in part, in a new work or works, also as described on the Order Con rmation. "User", as used herein, means the person or entity making such republication.

2. The terms set forth in the relevant Order Con rmation, and any terms set by the Rightsholder with respect to a particular Work, govern the terms of use of Works in connection with the Service. By using the Service, the person transacting for a republication license on behalf of the User represents and warrants that he/she/it (a) has been duly authorized by the User to accept, and hereby does accept, all such terms and conditions on behalf of User, and (b) shall inform User of all such terms and conditions. In the event such person is a "freelancer" or other third party independent of User and CCC, such party shall be deemed jointly a "User" for purposes of these terms and conditions. In any event, User shall be deemed to have accepted and agreed to all such terms and conditions if User republishes the Work in any fashion.

3. Scope of License; Limitations and Obligations.

    3.1. All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The license created by the exchange of an Order Con rmation (and/or any invoice) and payment by User of the full amount set forth on that document includes only those rights expressly set forth in the Order Con rmation and in these terms and conditions, and conveys no other rights in the Work(s) to User. All rights not expressly granted are hereby reserved.

    3.2. General Payment Terms: You may pay by credit card or through an account with us payable at the end of the month. If you and we agree that you may establish a standing account with CCC, then the following terms apply: Remit Payment to: Copyright Clearance Center, 29118 Network Place, Chicago, IL 60673-1291. Payments Due: Invoices are payable upon their delivery to you (or upon our notice to you that they are available to you for downloading). After 30 days, outstanding amounts will be subject to a service charge of 1-1/2% per month or, if less, the maximum rate allowed by applicable law. Unless otherwise speci cally set forth in the Order Con rmation or in a separate written agreement signed by CCC, invoices are due and payable on "net 30" terms. While User may exercise the rights licensed immediately upon issuance of the Order Con rmation, the license is automatically revoked and is null and void, as if it had never been issued, if complete payment for the license is not received on a timely basis either from User directly or through a payment agent, such as a credit card company.

    3.3.

Unless otherwise provided in the Order Con rmation, any grant of rights to User (i) is "one-time" (including

the editions and product family speci ed in the license), (ii) is non-exclusive and non-transferable and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Order Con rmation or invoice and/or in these terms and conditions. Upon completion of the licensed use, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period).

3.4. In the event that the material for which a republication license is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) which are identi ed in such material as having been used by permission, User is responsible for identifying, and seeking separate licenses (under this Service or otherwise) for, any of such third party materials; without a separate license, such third party materials may not be used.

3.5. Use of proper copyright notice for a Work is required as a condition of any license granted under the Service. Unless otherwise provided in the Order Con rmation, a proper copyright notice will read substantially as follows: "Republished with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc. " Such notice must be provided in a reasonably legible font size and must be placed either immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee speci ed in the Order Con rmation, in addition to the use fee itself and any other fees and charges speci ed.

3.6. User may only make alterations to the Work if and as expressly set forth in the Order Con rmation. No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

4. Indemnity. User hereby indemni es and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

5. Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF
BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, a liates, successors and assigns.

6. Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER
THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED,

INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.
ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS
OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

7. E ect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the license set forth in the Order Con rmation and/or these terms and conditions, shall be a material breach of the license created by the Order Con rmation and these terms and conditions. Any breach not cured within 30 days of written notice thereof shall result in immediate termination of such license without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

8. Miscellaneous.

    8.1. User acknowledges that CCC may, from time to time, make changes or additions to the Service or to these terms and conditions, and CCC reserves the right to send notice to the User by electronic mail or otherwise for the purposes of notifying User of such changes or additions; provided that any such changes or additions shall not apply to permissions already secured and paid for.

    8.2. Use of User-related information collected through the Service is governed by CCC's privacy policy, available online here:https://marketplace.copyright.com/rs-ui-web/mp/privacy-policy

    8.3. The licensing transaction described in the Order Con rmation is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the license created by the Order Con rmation and these terms and conditions or any rights granted hereunder; provided, however, that User may assign such license in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in the new material which includes the Work(s) licensed under this Service.

    8.4. No amendment or waiver of any terms is binding unless set forth in writing and signed by the parties. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by the User or its principals, employees, agents or a liates and purporting to govern or otherwise relate to the licensing transaction described in the Order Con rmation, which terms are in any way inconsistent with any terms set forth in the Order Con rmation and/or in these terms and conditions or CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Con rmation, and whether such writing appears on a copy of the Order Con rmation or in a separate instrument.

    8.5. The licensing transaction described in the Order Con rmation document shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of con icts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such licensing transaction shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Con rmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.If you have any comments or questions about the Service or Copyright Clearance Center, please contact us at 978-7508400 or send an e-mail to support@copyright.com.

9.3 Springer Nature

Optimization of protein isolation by proteomic qualification from Cutaneotrichosporon oleaginosus
Author: Dania Awad et al
Publication: Analytical and Bioanalytical Chemistry
Publisher: Springer Nature
Date: Dec 4, 2019
Copyright © 2019, Springer Nature

Rights and permissions

Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

Microbial lipid production by oleaginous yeasts grown on Scenedesmus obtusiusculus microalgae biomass hydrolysate
Author: Samer Younes et al
Publication: Bioprocess and Biosystems Engineering
Publisher: Springer Nature
Date: Apr 28, 2020
Copyright © 2020, Springer Nature

Rights and permissions

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

Creative Commons

This is an open access article distributed under the terms of the Creative Commons CC BY license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

You are not required to obtain permission to reuse this article.

To request permission for a type of use not listed, please contact Springer Nature

10 Additional Full Length Publications

10.1 Optimization of Protein Isolation by Proteomic Qualification from *Cutaneotrichosporon oleaginosus*

Dania Awad and Thomas Brueck

Published in *Analytical and Bioanalytical Chemistry*, Volume 412, Pages 449–462

DOI: 10.1007/s00216-019-02254-7

Date of Publication: 04 December 2019

Analytical and Bioanalytical Chemistry (2020) 412:449–462
https://doi.org/10.1007/s00216-019-02254-7

RESEARCH PAPER

Optimization of protein isolation by proteomic qualification from *Cutaneotrichosporon oleaginosus*

Dania Awad[1] · Thomas Brueck[1]

Received: 12 September 2019 / Revised: 23 October 2019 / Accepted: 30 October 2019 / Published online: 4 December 2019

Abstract

In the last decades, microbial oils have been extensively investigated as a renewable platform for biofuel and oleochemical production. Offering a potent alternative to plant-based oils, oleaginous microorganisms have been the target of ongoing metabolic engineering aimed at increasing growth and lipid yields, in addition to specialty fatty acids. Discovery proteomics is an attractive tool for elucidating lipogenesis and identifying metabolic bottlenecks, feedback regulation, and competing biosynthetic pathways. One prominent microbial oil producer is *Cutaneotrichosporon oleaginosus*, due to its broad feedstock catabolism and high lipid yield. However, this yeast has a recalcitrant cell wall and high cell lipid content, which complicates efficient and unbiased protein extraction for downstream proteomic analysis. Optimization efforts of protein sample preparation from *C. oleaginosus* in the present study encompasses the comparison of 8 lysis methods, 13 extraction buffers, and 17 purification methods with respect to protein abundance, proteome coverage, applicability, and physiochemical properties (pI, MW, hydrophobicity in addition to COG, and GO analysis). The optimized protocol presented in this work entails a one-step extraction method utilizing an optimal lysis method (liquid homogenization), which is augmented with a superior extraction buffer (50 mM Tris, 8/2 M Urea/Thiourea, and 1% C7BzO), followed by either of 2 advantageous purification methods (hexane/ethanol or TCA/acetone), depending on subsequent applications and target studies. This work presents a significant step forward towards implementation of efficient *C. oleaginosus* proteome mining for the identification of potential targets for genetic optimization of this yeast to improve lipogenesis and production of specialty lipids.

Keywords Proteomics · *C. oleaginosus* · Oleaginous · Yeast · Method optimization · Protein purification

Introduction

Climate change drives the development of sustainable bioprocesses in the chemical and pharmaceutical industry. To that end, microbial oils have been identified as a renewable alternative to petroleum-based chemical entities [1]. Leading strains in development are *Cryptococcus sp.*, *Lipomyces sp.*, *Rhodotorula sp.*, *Rhodosporidium sp.* and *Trichosporon sp.* [2–4], and *Cutaneotrichosporon oleaginosus* (ATCC 20509) [5]. The latter yeast species, *C. oleaginosus* is metabolically capable of converting a wide range of carbohydrates (glucose, galactose, cellobiose, xylose, sucrose, and lactose) and complex biomass-derived residual substrates (whey, glycerol, volatile fatty acids, ethanol, and N-acetylglucosamine) into lipids (60% w/w)[1, 6, 7]. The fatty acid profile of *C. oleaginosus* mimics that of plant-oils with 16–33% C16:0 and 43–57% C18:1 and therefore can be used to generate biodiesel and oleochemical specialty products [8].

Development of an industrially relevant strain requires a comprehensive understanding of the complex genomic, proteomic, and metabolic system networks that determine and control microbial oleaginicity [3]. Finding novel genes and pathways committed to oleaginicity should facilitate strain engineering for improved lipid titers, robustness, and techno-economics of the microbial production of fatty acid derivatives [3]. Synergistically, the study of oleaginous yeast proteome is an increasingly attractive method for dissecting the molecular basis of lipogenesis [9]. For discovery proteomics,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-019-02254-7) contains supplementary material, which is available to authorized users.

✉ Thomas Brueck
brueck@tum.de

[1] Werner Siemens-Lehrstuhl für Synthetische Biotechnologie, Technische Universität München, Lichtenbergstrasse 4, 85748 Garching, Germany

sample preparation procedures should comprehensively and reproducibly capture the protein repertoire with minimal artifactual modification, degradation, and contamination [10].

Most oleaginous yeast species possess robust cell walls composed mainly of polysaccharides (up to 90% w/w). These recalcitrant complex of sugar matrices maintain the mechanical strength, which conventionally require harsh physiochemical lysis systems. Hence, disruption methods must take into consideration the origin of the cells, physical strength of the cell wall, processing volume, and compatibility with downstream processes [11]. Several disruption methods have been developed including mechanical, chemical, and enzymatic treatments [12] . Mechanical methods entail harsh destruction of cell walls in a non-specific manner [11]. Industrial grinders include Waring® Blender and Playtron Mixer [13, 14]. On a smaller scale, pulverizing cells in liquid nitrogen, liquid homogenization, and ultrasonication has shown prospect for yeast cells [9, 11]. Alternatively, physical disruption of bonds within cell walls can be achieved by high temperatures and/or repeated cycles of freezing and thawing [12]. In a more specific manner, enzymatic digestion is a gentle method of disintegration [15, 16]. Nevertheless, the high cost and restricted availability of enzymes limit their utilization in large-scale processes [11]. Furthermore, these enzymes are commonly unfavorable, especially in discovery proteomics, due to complications arising from interference with downstream processes [17]. In addition to enhanced permeability, chemical treatments offer an increased value to protein extraction–protein solubilization [18]. Simultaneous cell lysis and protein solubilization is thus achieved by combining physical and chemical (detergents) methods [11]. Unfortunately, reconstituting membrane proteins into these solvent systems has been shown to be a tedious and challenging task. This is reflected in the under-representation of structural and mechanistic records for membrane proteins in Protein Data Bank (less than 1%) [18]. Given that severe solubility problems hamper the analysis of membrane proteins, successful discovery proteomics is thus reliant on careful selection of solubilizing detergents [19, 20].

As new detergents are continually being developed, choosing an appropriate detergent for discovery proteomic studies is hampered by the vast selection of detergents available today. Unfortunately, there is no ideal detergent for all applications and results often vary for the same application. The efficacy of detergents in extracting and solubilizing proteins can be further refined by additives such as chaotropes (urea and thiourea) [20]. Trial and error is the best strategy to find the optimal extraction buffer and the use of a mixture of detergents should also be considered.

The high concentrations of detergents (0.5–4%) and chaotropes (5–8 M), typically required for efficient solubilization of elusive proteins, are incompatible with downstream processes, as they inhibit trypsin activity, suppress LC-ESI-MS ionization, compromise chromatographic separation, and generate high-abundance ions that interfere with MS analysis [21]. Hence, their elimination is as crucial for subsequent analytical manipulations as the removal of inherent interfering compounds (lipids, nucleic acids, phenolic compounds, carbohydrates, proteolytic and oxidative enzymes, and pigments). To minimize protein modifications and proteolysis arising from these compounds, purification method should also be optimized [22]. In addition to typical challenges of protein sample preparation arising from inherent protein heterogenecity, structural complexity, and instability, the high lipid content of *C. oleaginosus* presents an added challenge. Specifically, lipids and phospholipids have been associated with Matrix Effects (MEs). This phenomenon is commonly associated with Electrospray ionization (ESI) and is characterized by analyte signal suppression (false negative), enhancement (false positive), or mass deviation arising from matrix components. Accordingly, ME can dramatically influence the identification and quantification of proteins [23, 24]. Particularly as lipids co-precipitate with proteins, a high protein purity is difficult to achieve without extensive protocol optimization [9, 25].

Although purification of native proteins is a challenging exercise, several reliable approaches, such as salting out and precipitation by non-ionic hydrophilic polymers (PEG), have been established in protein biochemistry [26–28]. Purification by aqueous alcohols (methanol, ethanol, isopropyl alcohol, and butanol) and organic solvents such as acetone has been carried out for over a hundred years on commercial and industrial scale [22, 26, 29]. Trichloroacetic acid (TCA), nonetheless, is more effective at lower concentrations (15% for TCA, 75% for acetone and 90% for ethanol), yet requires a consequential step of removal by acetone [30]. The aggressive removal of non-protein compounds by TCA has been clearly demonstrated in diverse and complex biological samples such as soil [9, 31]. Protein extracts from various resistant plant tissues such as wood, olive leaves, maize, and hemp roots are also efficiently generated by phenol-based purification [9, 25]. Common protocols of lipid extraction have also been applied reversibly for delipidation of protein extracts [32]. Rigorous testing demonstrated that replacing chloroform with methyl-tert-butylether (MTBE), which is non-toxic and non-carcinogenic, delivers similar or better recovery of lipids from human blood and brain samples [33–35]. Further attempts aimed at reducing dangers of chloroform toxicity adopt hexane as a lipid carrier. The efficiency of this method has been demonstrated in the recovery of several lipid classes from leaf tissues of Arabidopsis, tomato, soybean, and sunflower cake [36–38]. Despite the availability of diverse purification and delipidation methods for attaining adequate protein quality, finding the optimal method is a laborious comparative task, as different methods may result in depletion of particular protein species and relative enrichment of others [39].

In spite of the increased number of studies aimed at understanding lipogenesis in various oleaginous yeast species, scarce records for the optimization of protein purification and delipidation methods are deposited in literature. Thick floating lipid pads are often observed during the extraction of proteins from highly oleaginous yeasts. Nonetheless, partial loss of hydrophobic and membrane proteins is thus assumed when this lipid pad is scooped out and discarded prior to purification [40]. Proteomic studies of *Rodosporidium toruloides*, *Yarrowia lipolytica*, and *Mucor circinelloides* adopt common purification systems, which are optimized for reluctant plant tissues such as TCA/acetone and biphasic chloroform/methanol method without prior method optimization [2, 3, 41–44].

As it is difficult to predict which protocol could result in optimal proteome coverage of the non-model oleaginous yeast *C. oleaginosus*, we provide a comprehensive study that qualifies protein preparation methods and their downstream applicability based on qualitative and quantitative methods of proteins and lipids. This study addresses the three most challenging aspects of protein sample preparation by examining 7 methods of disintegration methods, 13 extraction buffers for protein solubilization, and 17 methods of purification/delipidation for optimal protein sample preparation from the oleaginous yeast *C. oleaginosus.*

Materials and methods

Yeast strain and cultivation media

Cutaneotrichosporon oleaginosus ATCC 20509 (from the culture collection of Werner Siemens Chair of Synthetic Biotechnology–WSSB, TU, Munich) was maintained on YPD (yeast extract peptone dextrose) agar plates (20 g L^{-1} peptone, 20 g L^{-1} agar, 20 g L^{-1} glucose, 10 g L^{-1} yeast extract). A single colony was initially cultured in 125 mL Erlenmeyer flask holding 50 mL YPD liquid medium at 28 °C and in a rotary incubator at 120 rpm for 24 h. Lipid accumulation was induced by subsequent inoculation in 125 mL Erlenmeyer flask holding 50 mL of Minimal-Nitrogen Media MNM (40 g L^{-1} glucose, 0.75 g L^{-1} yeast extract, 1.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.4 g L^{-1} KH_2PO_4, 0.22 g L^{-1} $CaCl_2 \cdot 2H_2O$, and trace elements 1.2 mg L^{-1} $(NH_4)_2SO_4$, 0.55 μg L^{-1} $ZnSO_4 \cdot 7H_2O$, 24.2 μg L^{-1} $MnCl_2 \cdot 4H_2O$, 25 μg L^{-1} $CuSO_4 \cdot 5H_2O$) prepared according to [1].With a starting optical density of 0.1, measured at 600 nm, cultivation was sustained for 96 h at 28 °C in a rotary incubator at 120 rpm.

Experimental design

Sample preparation

Yeast cells from 10 mL cultures were pelleted and washed twice with 40% ethanol. Processing conditions included the addition of a volume of 3:1 extraction buffer: pellet of the buffer (25 mM Tris-HCl, 5 mM 2-Mercaptoethanol, 5 mM EDTA, and 100 μM PMSF, pH 8.0) for all samples subjected to the varied attempted lysis methods. To ensure minimal proteolysis and protein modification, operating conditions were restricted to low temperatures. Optimization of protein extraction was portrayed at two levels: lysis method and extraction buffer.

Disintegration methods

Cell lysis was attempted in 8 different methods including (1) thermolysis at 121 °C for 15 min using autoclave (Systec VE-150, Germany), with an estimated processing time of 80 min, (2) 7 cycles of freezing at − 20 °C and thawing at 4 °C, and (3) lyophilization using Alpha 2-4 LD plus Lyophilizer (Martin Christ, Germany) following sample freezing at − 80 °C. Mechanical disruption covered (4) liquid homogenization via EmulsiFlex-B15 French Press (Avestin, Canada) with 4 consecutive passes at 8 bar, (5) pulverizing with Mortar and Pestle in liquid nitrogen, (6, 7) sonication of iced-bathed samples using (Bandelin Sonopuls, Germany) for 5 intermittent cycles of 30 s acoustic waves delivered at 90% power (in the presence and absence of glass beads, probe height 1 cm from container base), and (8) 5 expedited autolysis by intermittent cycles of 30 s vortexing in the presence of glass beads.

The homogenates were then centrifuged at 12,000 rpm for 60 min at 4 °C and supernatants were precipitated by TCA/acetone according to [45]. The efficiency of cell lysis was monitored by 4 parameters: visualizing pelleted cell debris under light microscopy and attending to their granularity, measuring the concentration of extracted soluble proteins and assessing their quality by SDS-PAGE.

Extraction buffer

Following the determination of the optimal lysis method, identical sample handling proceeded for the optimization of extraction buffer. A list of all examined detergents and their general properties is presented in Table 1. Briefly, ionic detergent (2% SDS), anionic detergents (1% Triton X-100, 2% Tween® 20, and 2% Tween® 80), and zwitterionic detergents (CHAPS, 1% C 7BzO, and 1% SB3-10) were compared for their extraction and solubilization potential. Molar equivalence of the aforementioned detergents amounting to 2% w/w was also compared, labeled here as "2% Mixture." The concentrations of detergents used in this study were selected based on manufacture recommendation; Triton X-100, Tween® 20, and CHAPS (Carl Roth, Germany); SDS (SERVA, Germany); and Tween® 80 (AppliChem, Germany). Additionally, the solubilization prospective of urea was assessed single-handedly and concurrently with thiourea (Carl Roth, Germany). The combined effect of detergents and chaotropic agents was also considered with a mixture of 2%

Table 1 Detergents examined in this study with their structural categories, properties, and target applications

Detergents	Structural category	Properties	Target
SDS	Ionic	Charged head group	Protein–protein interactions
Tween 20 Tween 80	Non-ionic	Uncharged head group	Lipid–lipid and lipid–protein interactions
Triton X-100			
CHAPS C7BzO	Zwitterionic	Net neutral charge head group	Combined properties of ionic and non-ionic
SB3-10			

SDS and 8 M urea. SB3-10 and C7BzO were examined as constituent of Extraction Reagents Type 2 (ERT2) and 4 (ERT4), respectively (Sigma Aldrich, USA). Furthermore, ERT4 was freshly reproduced in the lab with and without 50 mM Tris-HCl. Restriction of protein oxidation and proteolysis was attained by addition of 5 mM 2-Mercaptoethanol and 100 μM PMSF to all compared buffers. All reagents used in this study are detergent-grade.

The efficiency of extraction buffer to solubilize whole proteome was assessed by measuring the concentration of protein extracts by Bradford assay and visual inspection of corresponding bands on SDS-PAGE, following TCA/acetone precipitation according to [45]. Additional evaluation criteria accounted for the count of spectra, unique peptides, and proteins following in-solution tryptic digestion and mass spectrometry.

Purification protocols

Protein extracts of equal volumes and concentrations, prepared via found optimal disintegration method and extraction buffer, were subjected to 17 purification methods. These methods, in addition to any deviations from adopted protocols are recorded in Table 2. All reagents used are of high purity/HPLC quality when applicable. The quantity and quality of recovered proteins was attended to by Bradford quantitation and SDS-PAGE. Furthermore, purification methods were compared based on the number of identified proteins following in-solution digestion and LC MS/MS analysis, GRAVY scores, pI, and MW distributions of identified proteins, in addition to Clusters of orthologous groups (COGs) and Gene Ontology (GO) analyses. The extent of delipidation of each method was also assessed by measuring the FAME content in purified dry protein sample.

Analytical methods

Microscopy

The extent of cell ruptures during optimization of lysis method was evaluated by visualizing pelleted cell debris under oil immersion light microscope (Motic, China) equipped with Moticam 5.0 MP. The thickness of *C. oleaginosus* cell wall was investigated using a JSM-7500F scanning electron microscope (SEM) (JEOL, Japan). SEM was equipped with an accelerating voltage of 1, 2, or 5 kV and a secondary detector.

Flow cytometry

Lysis efficiency was estimated based on granularity of cell fragments following lysis methods described above. Initially, lysates were passed through a 40 μm nylon mesh prior analysis on S3 Cell Sorter (Bio-Rad, USA), equipped with 488 nm/100 mW laser beam. Automated alignment verification and drop delay determination was carried out using ProLine™ Universal Calibration Beads (Bio-Rad, USA). A drop delay of 33.16 was found to provide optimal flow with an event rate of 60,000. Forward-scattered light (FSC) and side-scattered light (SSC) trigger threshold was set to 0.05 with a voltage of 284 W for the former and 294 W for the latter. Sheath fluid (2.978 g L^{-1} disodium EDTA, 2.069 g L^{-1} potassium phosphate, 2.266 g L^{-1} Potassium chloride, 18.852 g L^{-1} sodium phosphate, and 64.985 g L^{-1} sodium chloride) carried 100 μL of each of 8 generated lysates, in addition to a sample of intact *C. oleaginosus* cells, through the cytometer. FSC AND SSC were acquired on a $\log_{10}$ scale using ProsSort™ software (Version 1.5). The region occupied by intact pool of *C. oleaginosus* cells dictated the quadrant reserved for cells, which withstood disintegration forces, and served as basis in the calculation of lysis efficiency.

Lipid quantitation

The extent of lipid contamination in protein pellets, following various purification methods, was measured by accounting for the sum of fatty acid methyl esters (FAMEs), obtained by methanol transesterification. The transesterification protocol was originally adopted from [46]. and modified in our lab by [47]. FAME profiles were analyzed on a GC-2025 gas chromatograph from Shimadzu (Nakagyo-ku, Kyōto, Japan) with flame ionization detector. One microliter sample was applied by AOC-20i auto injector (Shimadzu) onto a ZB-

Table 2 Protein purification methods attempted in this study

Purification method	Protocol modifications[c]	Source
Ethanol	9 volumes of ethanol	[46]
100% acetone	–	[47]
80% acetone	–	[48]
TCA/acetone	13.3% TCA	[45]
Optimized TCA/acetone	Glass beads	[10]
Phenol:methanol:ammonium acetate	–	[49]
Chloroform:methanol	–	[50]
PCI[a]	Collect organic phase	[51]
Methanol:MTBE:water	–	[35]
Butanol/di-isopropyl ether	Collect aqueous phase	[52]
Sequential[b]	–	[9]
PEG 6000	–	[27]
Hexane:ethanol	Ethanol replaced isopropanol, 60 °C incubation for 30 min	[38]
Size exclusion	Modified PES 10 kDa, 500 μL	VWR, Germany
Ammonium sulfate	–	[53]
DISSOLVAN® 7:ethanol	Adapted protocol from (38)	Clariant
DISSOLVAN® 5:ethanol	Adapted protocol from (38)	Clariant

[a] *PCI* phenol/chloroform:isoamyl alcohol

[b] Sequential (TCA/acetone + phenol)

[c] Modifications made to adopted protocols

WAX column (30 m, 0.32 mm ID; 0.25 μm df; phenomenex (Torrance, CA, USA)). The initial column temperature was 150 °C (maintained for 1 min). A temperature gradient was applied from 150–240 °C (5 °C min^{-1}), followed by 6 min maintenance at 240 °C. Fatty acids were identified according to retention times of the authentic standard: Marine Oil FAME Mix (Restek, USA). Individual FAME concentrations were based on peak areas relative to Methyl Nonadecaanoate C19 (Sigma, Germany), which was incorporated as an internal standard in all samples. Percent lipid was calculated from the sum of individually identified FAMEs with respect to pellet dry weight.

Protein quantification and SDS-PAGE

Protein concentrations were quantified using Bradford protein assay (Carl Roth, Germany) following TCA/acetone precipitation. Bovine serum albumin (BSA) was chosen for modeling standard curves and measurements were recorded in triplicates in 96-well plates on EnSpire® Multimode Plate Reader (PerkinElmer, USA). Protein extracts were conveyed on 12% one-dimensional SDS polyacrylamide gel electrophoresis, using Bio-Rad Mini-Protean II Equipment and PageRuler ™ Unstained Protein Ladder (ThermoFischer Scientific, USA), to assess the gross qualitative variances in protein profiles. After electrophoresis, gels were stained with Coomassie brilliant blue (CBB) R-250.

Shotgun proteomics

In-solution tryptic digestion was carried out on 1 mg of purified protein pellet resolved in 50 mM ammonium bicarbonate. Digestion proceeded with Sequencing Grade Modified Trypsin (Promega, USA) at a ratio of 1:20 trypsin:protein w/w at 37 °C overnight following sequential reduction and alkylation in 10 mM DTT (95 C for 5 min then 60 °C for 30 min) and 20 mM iodoacetamide (at room temperature in the dark for 20 min). Termination of tryptic treatment was assumed by incubation on ice for 5 min. Peptides were then vacuum dried and reconstituted in 1% formic acid. Trypsin and other contaminants were eliminated from peptides by centrifugal filtration using low protein binding, modified PES centrifugal filters with 10 kDa cutoff (VWR, USA).

LC MS/MS was performed on filtrates using an Ultimate 3000 RSLCnano system (Dionex/Thermofischer) coupled online to LTQ Orbitrap XL mass spectrometer. Tryptic digests of 100 ng were loaded onto Acclaim™ PepMap™ trap column (100 C18; 3 μm, 75 μm × 20 mm) at a flow rate of 5 μL min^{-1} prior to reverse-phase separation on Acclaim™ PepMap™ column (100 C18; 2 μm, 75 μm × 500 mm) at a flow rate of 200 nl min^{-1}. Reverse-phased buffer system combined 0.1%

trifluoroacetic acid aqueous solution (buffer A) and acetonitrile with 0.1% trifluoroacetic acid (buffer B). A separation cycle of 150 min gradient (0–4% buffer B for 7 min, 4–35% buffer B for 102 min, 35–65% buffer B for 3 min, 65–90% buffer B for 2 min; after maintaining buffer B at 90% for 10 min, the entire system was then re-equilibrated by 4% buffer B for 26 min) and an inter-sample 60 min blank delivered peptides to the Nanospray Flex Ion Source. MS parameters allowed a scan range of 350–1400 Da with resolution of 60,000. The mass spectrometer was set such that one MS scan was followed by 6 MS/MS scan events; opt for most intense ion signal. MS/MS parameters limited minimum signal intensity to 1000, isolation width to 2 Da and allowed for dynamic exclusion.

Bioinformatics

Raw MS/MS files were searched in Proteome Discoverer 2.2 software (Thermo Fisher Scientific, Germany) against *C. oleaginosus* database, downloaded from UniProt (https://www.uniprot.org/proteomes/, 8317 proteins) using SEQUEST. Search parameters allowed 10 ppm and 0.02 Da tolerance for the precursor and fragment, respectively. Semi-cleavage was tolerated up to 2 missed cleavages for trypsin with cysteine residues (57.0215 Da) and methionine residues (+ 15.9949 Da) as constant and variable modifications, respectively. Cross correction values (Xcorr) of at least 1.2 (+ 1), 1.9 (+ 2), 2.3 (+ 3), and 2.6 (> + 4), ΔC_n cutoff value of 0.05 and high confidence peptide filters with a minimum length of 6 amino acids were applied to ensure less than 1% peptide level FDR. The hydrophobicity of proteins was based on the Grand Average of Hydropathy (GRAVY) scores, whereby protein sequences from MS/MS data of identified proteins were imported into GRAVY web-based tool (http://www.gravy-calculator.de). Clusters of orthologous groups (COGs) were created with the aid of WebMGA, a web-based tool for fast metagenomic analysis [48]. The biological processes and molecular functions of identified proteins for Gene Ontology (GO) analysis were assigned using WEGO with baker's yeast as reference strain, following blast against *C. oleaginosus* database, interpro, mapping, and annotation in Blast2Go [49, 50].

Results and discussion

Disintegration methods

Lysis efficiency

The extent of cell lysis has direct consequences on the overall quality of protein isolation process and is approached as the first bottleneck in proteomic analysis of cell wall–enclosed species [11]. The highest degree of fragmentation in this study was achieved by liquid homogenization with 75.2% efficiency, based on cell granularity measurements (Fig. 1a, d). Mechanical disruption is generally regarded as random and non-uniformal, yet it delivers maximal destructive impact applicable for many yeast and plant species to impair their robust cell walls [11]. In fact, the overall SDS-PAGE band intensities associated with French press disruption is exceptionally distinguishable from remaining methods that resulted in incomplete protein liberation (see Electronic Supplementary Material (ESM) Fig. S1b). Physical impairment and release of cellular components of yeast cells has been predominantly aided by glass beads [51]. In this study, coupling of sonication with glass beads generated lysis efficiency of 44.7%, compared to 30.5% for standalone sonication (Fig. 1a). The level of proteins released in this method amounts to 7 μg μL^{-1} and their validation by SDS-PAGE revealed faint band pattern inferior only to liquid homogenization (ESM Fig. S1). This method of disintegration is hence recommended in absence of costly homogenization instruments. However, temperature control is crucial and obligatory as most of the ultrasound energy, absorbed by the suspension, is translated to heat [11].

Manual grinding of crop in liquid nitrogen is regarded as the conventional disruptive method to combat the tensile strength of cellulose, chitin, and other polysaccharides constituting the plant cell wall [9]. This method of disintegration has been adopted protein sample preparation from *R. toruloides* for comparative proteomics [2]. With mere 13.3% overthrow success, minimal concentration of soluble proteins (1.8 μg μL^{-1}), and scant protein representation on SDS-PAGE, this method is considered incompetent in accessing *C. oleaginosus* (Fig. 1 and ESM Fig. S1). Lyophilization, freeze/thawing cycles, vortexing in presence of beads, sonication, and thermolysis also failed to achieve their purpose in this analysis (Fig. 1 and ESM Fig. S1). The unreasonably high protein concentrations measured for samples that were subjected to lyophilization and thermolysis can be attributed to protein profile shifts through induction of heat and cold shock proteins (ESM Fig. S1a) [52]. Furthermore, formation of odorant compounds in reaction to thermal damage has been correlated with significant increase in the respective precursor amino acids leucine (3-methylbutanal), ornithine, and proline (2-acetyl-1-pyrroline) (Münch & Schieberle, 1998).

While lytic enzymes infer a more gentle and effective disintegration alternative, they were eliminated from this study as they impose additional downstream processing in proteomic exploration (removal of lytic enzymes and/or preparation of laborious MS exclusion lists) [53, 54]. Notably, pelleting of *C. oleaginosus*, for media elimination and washing, necessitated the use of 40% v/v ethanol to avoid loss of "cellular floaters" that result from high lipid content. This step of sample preparation bears no destructive impact on this yeast. Uncoupling the optimization of disintegration method from solubilization method, allowed for objective evaluation of lysis method independently from extraction buffer. The development of an efficient lysis

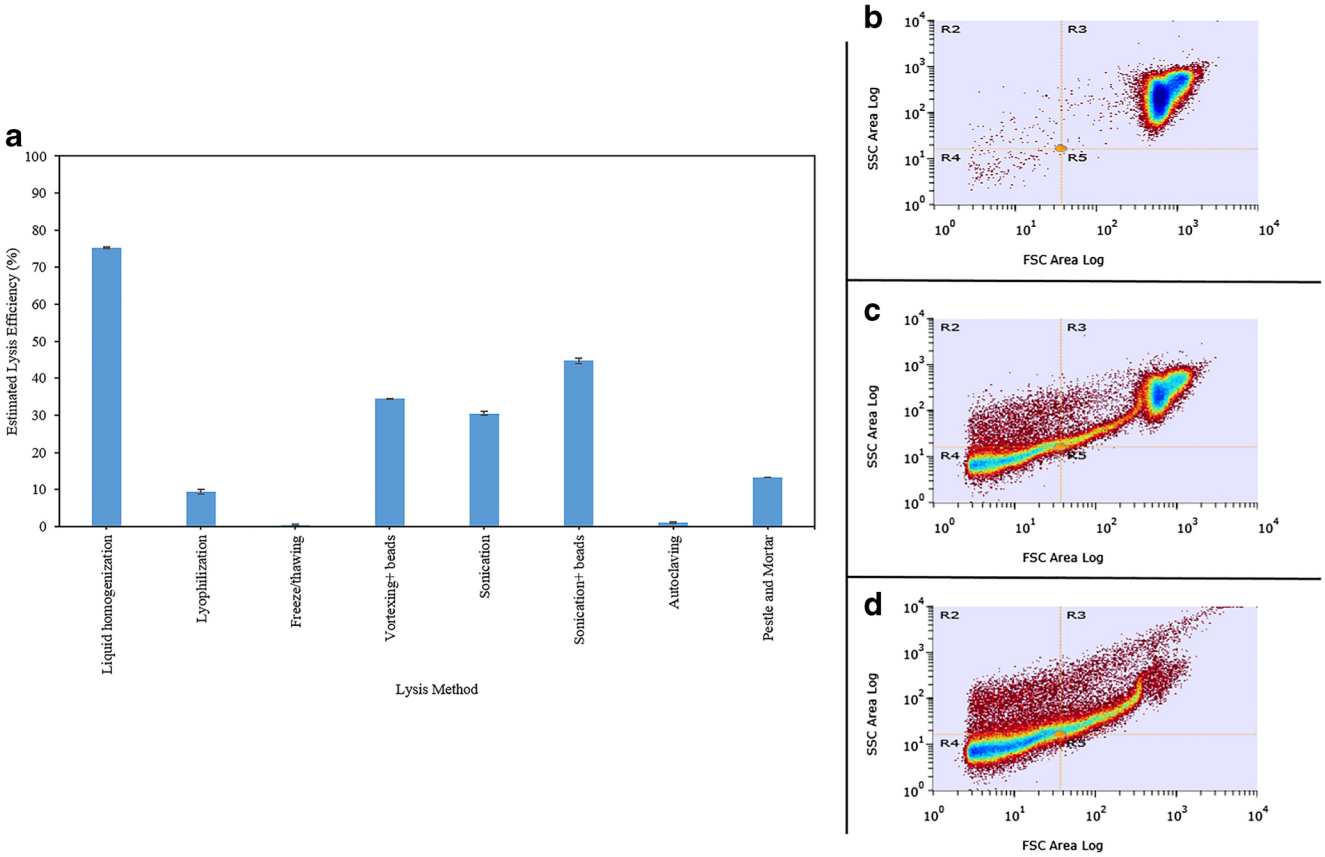


Fig. 1 Estimated lysis efficiency plot (**a**) based on cell granularity measurements for untreated cells (**b**) and cells broken by sonication (**c**) and liquid homogenization (French Press) (**d**). Calculations are based on forward and secondary scatter plot division into 4 quadrants with quadrant (R3) reserved for unbroken cells. Scatter plots of remaining lysis methods are available in Fig. S2 of Supplementary Material

method tailored for *C. oleaginosus* is also applicable to recover the yeast oil from cellular debris.

Cell wall thickness

The strength of this yeast cell wall is undoubtedly demonstrated in ESM Fig. S3 with numerous "ghosts" visible under direct microscopy for attempted lysis methods. This is indicative of incomplete cell wall destruction and intracellular components retention. This prompted the measuring of this yeast cell wall thickness. *C. oleaginosus* cells are assumed to be elliptical spheroids with an average diameter ranging between 3.5 and 6.3 μm (Fig. 2a, b). The thickness of this yeast cell wall is threefold greater than the laboratory strain *S. cerevisiae* BY4741, measuring at 0.5–0.6 μm by scanning electron microscopy (Fig. 2c, d) [55]. Further studies are requisite for understanding the specific composition, mechanical properties, and molecular forces behind the strength of *C. oleaginosus* cell wall.

Extraction buffer

In order to accent the degree of cell lysis and efficiently solubilize this oily yeast proteome, the use of detergents in extraction buffer is necessary. These two vital roles of detergents cannot be individually investigated, but their pooled outcome is marked by the refined quantity and quality of extracted proteins. While assessment of the former involves quantifying the concentration of protein extracts, the latter requires further downstream processes, including SDS-PAGE, in-solution digestion, and mass spectrometry. One-step extraction in lysis buffer was adopted in this study as it is considered the simplest and most useful straightforward procedure [10].

In contrast to the reported inefficiency of standalone chaotropes for handling complex membrane samples [20], urea extracted double the amount of proteins, in comparison with standalone detergents, with a concentration of 7.5 μg μL^{-1}(Fig. 3b). Moreover, recorded increase in the number of protein bands for urea on SDS-PAGE (ESM Fig. S4e and f) implies the extraction of additional proteins. This was confirmed by LC MS/MS with the identification of 599 unique proteins (Fig. 3a). However, samples containing urea should not be exposed to temperatures higher than 37 °C as urea establishes equilibrium with cyanate in solution, which covalently modifies amino acid side chains in a reaction that is greatly accelerated by heat and alkaline conditions [56].

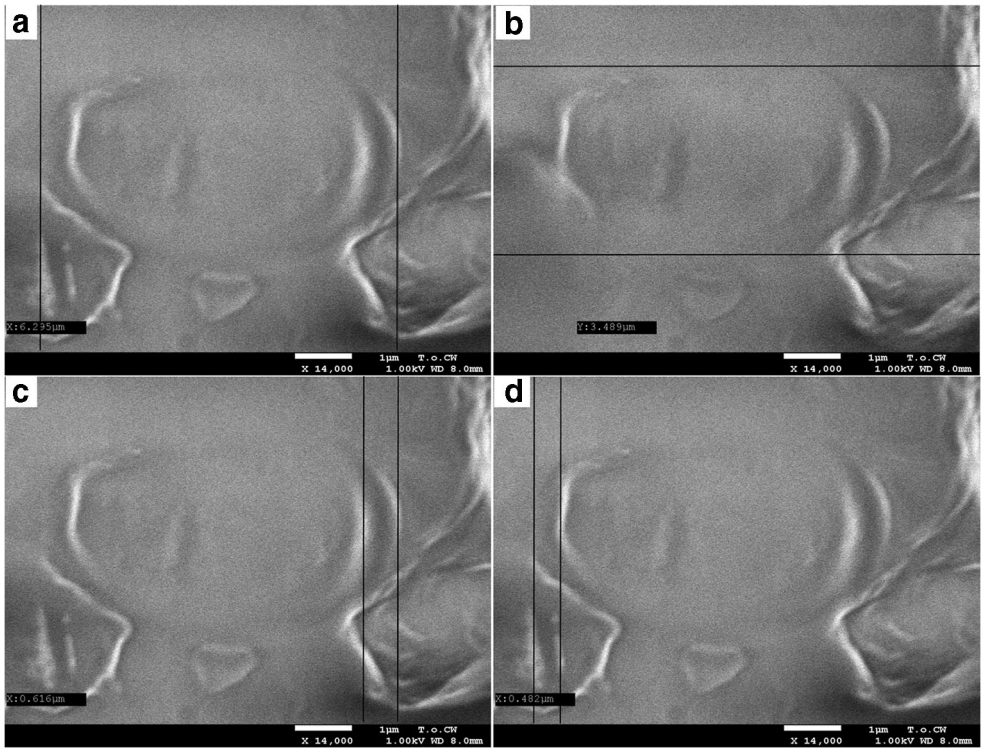


Fig. 2 Morphology of *C. oleagino*sus visualized by a scanning electron microscope (SEM). Applied energy, 1.00 kV, LEI; detector, SEM/LM. Vertical and horizontal rulers mark cell borders in estimating cell diameter (**a**, **b**) and cell wall thickness (**c**, **d**). Scale bar = 1 µm at 14,000 x magnification

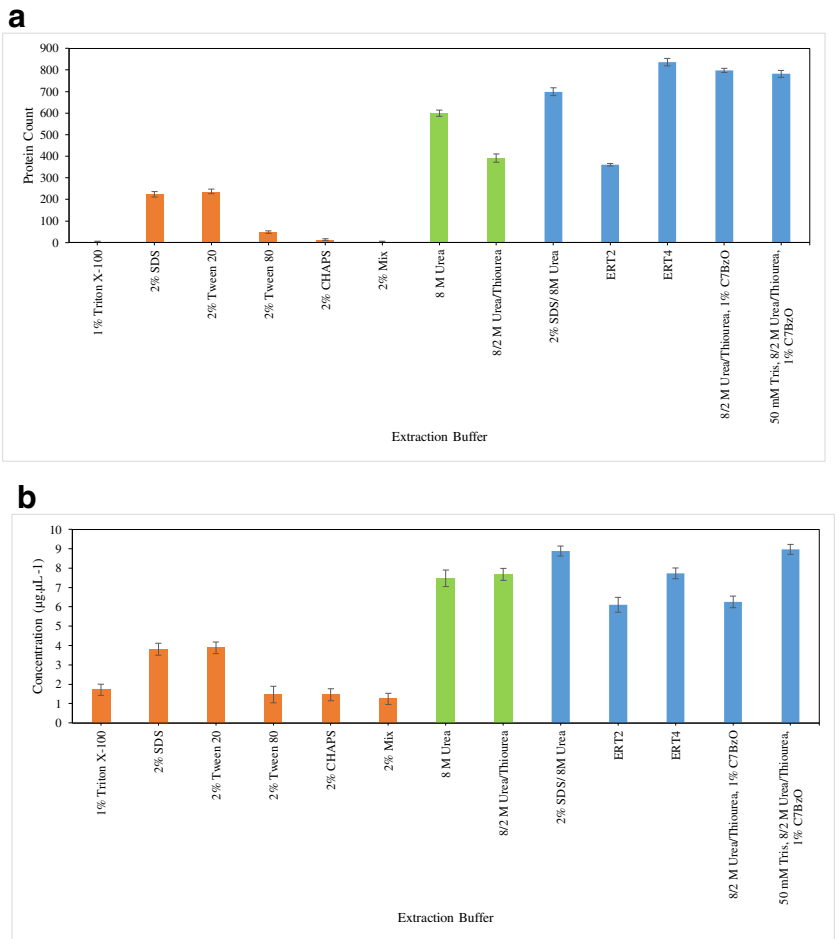


Fig. 3 The count of total uniquely identified proteins (**a**) and the quantitation of whole proteome (**b**) extracted from *C. oleaginosus* with examined detergents. Calculations are based on Bradford assay per BSA standards curve (R^2 value of 0.9914)

Thiourea does not appear to enhance the extraction potential of the buffer as it resulted with the extraction of 392 unique proteins only (Fig. 2a). The use of a chaotropic agents in the extraction of *C. oleaginosus* proteins has shown to be crucial with a 2.3-fold increase in protein concentrations resulting from coupling of urea with SDS (8.87 μg μL^{-1}), compared to SDS treatment solely (3.81 μg μL^{-1}) (Fig. 3b). This was concurrent with identification 699 and 224 unique proteins, respectively (Fig. 3a). Albeit the proven competence of this buffer, we recommend against it when downstream processes such as 2D-PAGE are in order, on the account of irreversible aggregation and precipitation of proteins brought about by SDS removal [19].

Unlike ionic and non-ionic detergents, zwitterionic detergents have been demonstrated to have additional value in downstream processes. Henceforth, the combined power of chaotropic agents and zwitterionic detergents, SB3-10 and C7BzO, was attended to by evaluating two extraction buffers offered by Sigma Aldrich with claims of increasing efficiency, ERT2 and ERT4, respectively. ERT4 performed superiorly on LC MS/MS with 839 identified proteins, compared to 360 proteins for ERT2 (Fig. 3b). C7BzO, the detergent constituent of ERT4, is named amongst best candidates for protein solubilization [20]. Its extraction and solubilization power and compatibility with IEF and 2D electrophoresis make it a promising detergent for proteomic analysis of *C. oleaginous* [57]. Further evaluations of freshly prepared in-house replica of ERT4 were aimed towards cost reduction of the extraction protocol. Accordingly, the significance of Tris at 50 mM in the extraction buffer was upheld with 2.7 μg μL^{-1} increase in protein concentration and the identification of 16 additional unique proteins (Fig. 3). In the light of the presented results, subsequent optimization of protein sample preparation from *C. oleaginosus* proceeded in a one-step extraction method via liquid homogenization in an extraction buffer containing 50 mM Tris, 8/2 M Urea/Thiourea, and 1% C7BzO.

Purification method

Purification efficiency

Protein purification, often achieved by precipitation, is the final and fairly demanding step in protein isolation. This work compares the purification efficiency of 17 methods in (1) the elimination of contaminants and secondary metabolites, namely, lipids for this oleaginous strain, and (2) delivering a protein sample with highest protein coverage and minimal loss. In addition to widely applied purification methods including sequential purification, our optimization efforts comprise methods commonly used for lipid, DNA, and RNA extractions—modified to retain protein fractions and efficiently eliminate the otherwise collected fraction. These methods are listed in Table 2. It must be noted that interfering substances constitute one of the major problems in performing a Bradford assay [58]. For this reason, when purity of protein sample is in question, the intensity of band patterns of resolved proteins on SDS-PAGE provide more meaningful information on the quantity and quality of protein sample. For this reason, an SDS-PAGE is presented in Fig. 3c to evaluate the suitability of the purification methods for gel applications.

Precipitation by 100% acetone resulted in one of the highest number of identified unique proteins (805 unique proteins) (Fig. 4a). However, acetone tends to co-precipitate different types of lipids with proteins [59]. This is clearly depicted in Fig. 4b with 20–50% (g FAMEs/g dry protein pellet weight) of precipitate content detected as lipids for acetone precipitations. As lipids are highly associated with ME, which severely compromises the analysis performance, their elimination is at the core of method development and validation [23]. Precipitation by 80% acetone was aimed at simplifying the final re-dissolution step; however, this method does not achieve improved results, neither in protein identification (737 unique proteins) nor in delipidation (48.1% (g FAMEs/g dry protein pellet weight)). Similarly, ethanol precipitation, best known in fractionating human serum, delivered intermediate results with 662 uniquely identified proteins and fade SDS-PAGE bands (Fig. 4a, c) [26]. Resembling acetone precipitation, high lipid content (37.9% g FAMEs/g dry yeast weight) was detected in the precipitate of ethanol precipitation (Fig. 4b).

With the advantage of reduced solvent volume, TCA/acetone resulted in the second highest record of identification with 770 unique proteins (Fig. 4a). In comparison with other organic solvents and aqueous alcohols, this solvent system efficiently rids the protein precipitate from lipids with less than 1% of FAMEs (g FAMEs/g dry protein pellet weight) detected, as shown in Fig. 4b. However, this method irreversibly incorporates DNA, yielding DNA-protein aggregates, which cause precipitation, bad focusing, and protein streaking [59]. For this reason, when downstream processes include gel applications, this purification method is not recommended. In an attempt to overcome the laborious re-dissolution step, Hao et al. optimized TCA/acetone method by incorporating a subsequent step: the hard precipitate was ground into fine powder to enlarge the contact area between the precipitate and the washing solvent [10]. When applied to *C. oleagino*sus protein extract, this method delivered inferior results compared to standard TCA/acetone precipitation (Fig. 4) and protein loss was attributed to non-specific protein adsorption to glass beads.

Salting out by ammonium sulfate and PEG resulted with faint and highly distorted band patterns on SDS-PAGE; 637 and 688 identified unique proteins, respectively (Fig. 4a, c). One common drawback of these common methods is the difficulty of salt and PEG removal, with the former entailing laborious dialysis in a cold room against several volumes of

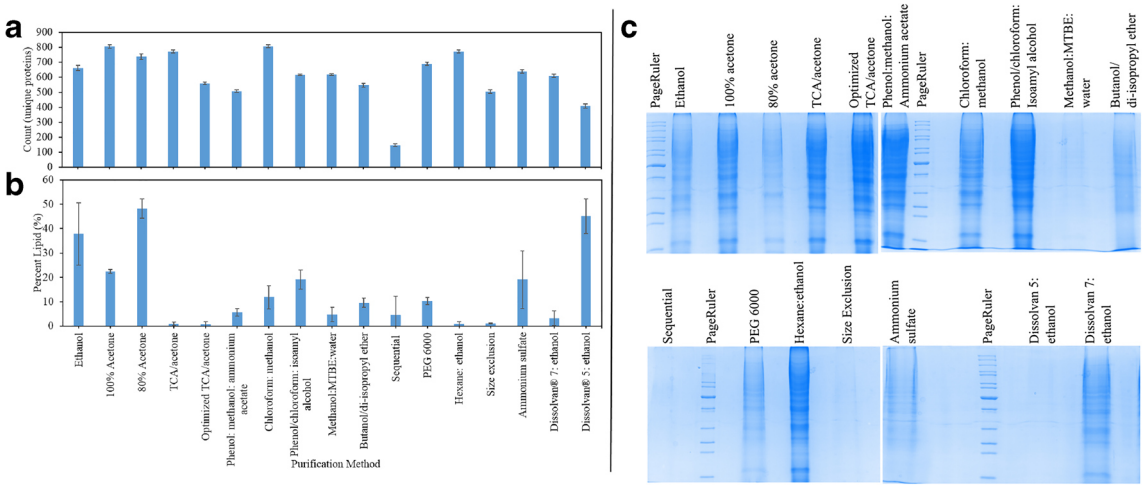


Fig. 4 The count of total unique proteins identified following purifications (**a**). Lipid content (%) of purified protein samples, calculated by summation of FAMEs to biomass (g FAMEs/g dry yeast weight) (**b**). Composite of 1D SDS-PAGE analyses for proteins purified by ethanol (a), 100% acetone (b), 80% acetone (c), TCA/acetone (d), optimized TCA/acetone (e), phenol:methanol:ammonium acetate (f), chloroform:methanol (g), phenol/chloroform:isoamyl alcohol (h), methanol:MTBE:water (i), butanol/di-isopropyl ether (j), sequential (k) PEG 6000 (l), hexane:ethanol (m), size exclusion (n), ammonium sulfate (o), Dissolvan 7:ethanol (p), and Dissolvan 5:ethanol (q), whereby L represents the protein ladder (**c**)

buffer over long period of time and the latter requiring chromatographic steps on ion-exchange or affinity columns, ultrafiltration or salt-induced phase separation (Burgess 2009) [27]. Furthermore, the density of high concentrations of ammonium sulfate approaches that of protein aggregates resulting in floating of precipitate. Since this problem is further exacerbated in presence of high lipid content, this method should also be avoided when dealing with protein samples from oleaginous yeasts [26]. PEG 6000 was chosen for this study as larger polymers offer no advantage yet further increase the solution viscosity [27]. Purification by size exclusion using 10 kDa centrifugal filters, although gentle and straightforward, delivered subpar results with 505 identified unique proteins and undetected protein bands on SDS-PAGE (Fig. 4a, c). This method suffered from technical difficulties due to the presence of high lipid content as a lipid pad formed, blocking the filter.

Liquid-liquid extraction systems, aimed at DNA, RNA, and lipid isolation are highly exploited to efficiently eliminate these biomolecules in preparation of high-quality protein samples [21, 60]. These methods are appealing in preparation of protein samples from oleaginous yeasts. As such, the most common delipidation technique—chloroform:methanol—delivered one of the highest protein coverage (comparable with precipitation by 100% acetone) with 806 uniquely identified proteins and decent resolution of separated protein bands on SDS-PAGE (Fig. 4a, c). Nevertheless, this method is superior to precipitation by 100% acetone as it rids the protein sample from 88.2% (g FAMEs/g dry protein pellet weight) of the lipid content. In fact, this method has also been shown to improve the resolution of 2D-PAGE protein spots, when applied to low-density lipoproteins [61]. However, chloroform carcinogenicity poses considerable health risks for laboratory personnel [35]. Replacement of chloroform with MTBE promises more contact between solvents and biomolecules in a one-phase system [35]. However, MTBE is less efficient than the chloroform in the purification of the *C. oleaginosus* proteins. Similarly, the most common procedure used for delipidation of plasma, protein solutions, or cell culture medium makes use of butanol/di-isopropyl ether solvent system. Although this solvent system have been successfully employed in the defatting of whole buckwheat seeds, it has failed to improve protein yield of *C. oleaginosus* (546 unique proteins) and does not appear compatible with gel application (Fig. 4a, c) (Ma et al. 2006) [22]. Hexane/ethanol was reported to extract lipids with high efficiency from numerous plant species [37, 38]. This method delivered similar results as TCA/acetone in the number of uniquely identified proteins (770 proteins) and delipidation efficiency (less than 1% FAMEs (g FAMEs/g dry protein pellet weight)) (Fig. 4a, b). However, this method is more applicable than TCA/acetone in subsequent gel applications (Fig. 4c). High PSM counts were observed for hexane:ethanol (and ethanol) and these denote peptides identified repeatedly [62]. Demulsifier base chemistry technology was also attempted given its renowned application in petroleum and gas industry for separation of crude oil refining [63]. Furthermore, protein purification with the use of demulsifiers has been under-reported in literature [64]. Two demulsifying agents offered by Clariant, DISSOLVAN® 5 and 7, were compared in this study. These surfactants identified 407 and 608 unique proteins, respectively (Fig. 4a). While DISSOLVAN® 7 eliminated 96.7% (g FAMEs/g dry protein pellet weight) of lipids from protein sample, DISSOLVAN® 5 showed minimal delipidation efficiency,

comparable with precipitation by ethanol and 80% acetone. Although protein purification by DISSOLVAN® 7 suffers from considerable protein loss, it can be attempted when preservation of protein conformation is required [64].

Phenol extraction methods, standalone or in combination with chloroform, have high cleanup capacity—especially for nucleic acids—given their original purpose. This is evident is the quality of SDS-PAGE protein bands delivered by phenol:methanol:ammonium acetate and phenol/chloroform:isoamyl alcohol purifications (Fig. 4c) [65]. However, the count of uniquely identified proteins for these methods (508 and 615, respectively) suggests that proteins were not properly retained (Fig. 4a). Phenol extraction also suffers from drawback of handling of toxic solvents (phenol and methanol) in addition to lengthy processing time (16 h). Lowest protein coverage in this study was associated with sequential purification, TCA/acetone followed by phenol:methanol:ammonium acetate. With the identification of 147 unique proteins and lack of SDS-PAGE protein representation, protein loss was owed to poor dissolution of the hard precipitate, resulting from TCA/acetone purification, in phenol (Fig. 4a, c).

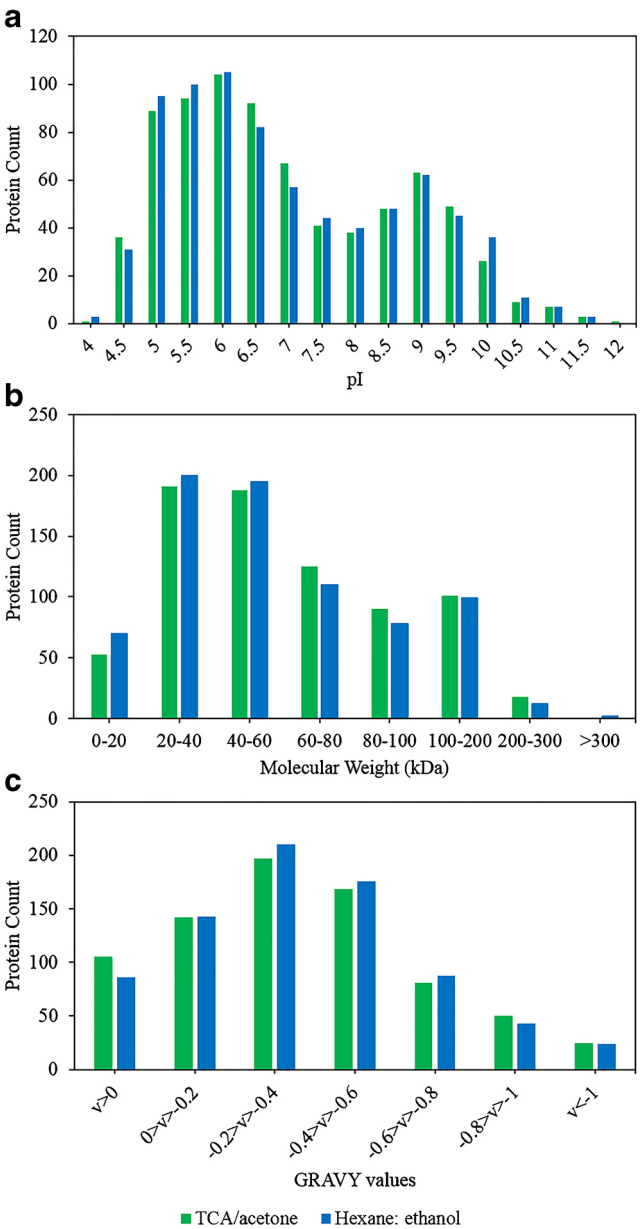


Fig. 5 PI (**a**), molecular weight (**b**), and GRAVY score (**c**) distributions for whole *C. oleaginosus* proteome extracts obtained following purification by TCA/acetone and hexane:ethanol

High-performance purification methods

Protein physiochemical heterogeneity is at the core of substantial differences in protein extracts amongst different methods [10]. For this reason, the hydropathicity, MW and pI distribution, Clusters of Orthologous Groups (COGs), and Gene Ontology (GO) analyses were performed to compare purification methods with highest protein coverage and delipidation efficiency—TCA/acetone and hexane:ethanol. These analyses did not reveal any clear patterns that might account for protein difference between methods, implying the complexity of protein extraction. As such, proteins purified by these methods equally fell in two pI ranges 4.5–6.5 and 8.5–10 (Fig. 5a). Yet, in accordance with literature, enrichment of basic proteins by this method in the pH range of 8.5–9.5 is detected [10]. The molecular weight of proteins detected for these methods fall in the range of 10–130 kDa, with proteins up to 300 kDa identified (Fig. 5b). Furthermore, GO analysis did not reveal any differences amongst these purification methods due to the limited choice of reference organisms (baker's yeast) (ESM Fig. S5). Yet, this comparison can help make an informed choice of method based on the application and downstream processing. In that perspective, hydropathicity analysis revealed that TCA/acetone has retained the highest number of hydrophobic proteins (GRAVY > 0) (105 proteins) (Fig. 5a). Hence, this method is recommended when membrane proteins of *C. oleaginosus* are studied. Alternatively, COGs analysis performed in this study can help choose optimal method for targeted proteomic analysis of *C. oleaginosus* depending on number of identified genes associated with each method for the functional categories. Accordingly, hexane:ethanol method is recommended when energy production and conversion or carbohydrate transport and metabolism of *C. oleaginosus* are studied, as they achieved highest identification by this method, at 10.90 and 7.17%, respectively (ESM Fig. S6). Given that lipogenesis is triggered by nutrient starvation and represents a state energy preservation, this purification method is preferred when lipogenesis is target of proteomic studies. Furthermore, this method is also recommended for lipogenesis studies as the importance of the carbon source and concentrations for this yeast is upheld in nutritional starvation state [1]. Moreover, this method is more compatible with

subsequent gel applications than TCA/acetone. This method is also recommended for proteomic studies of other oleaginous yeasts.

Conclusion

This work comprises the first large-scale comparative study of extraction (8 lysis methods and 13 extraction buffers) and purification (17 methods) approaches of the non-model oleaginous yeast *C. oleaginosus*, with an emphasis on delipidation efficacy and method-specific differential proteomic profiles. The rigid cell wall (0.5–0.6 μm) of this yeast necessitated the use of liquid homogenization (French Press) to achieve a lysis efficiency of 75.2%. One-step extraction in lysis buffer was adopted to further augment the lysis efficiency. MS-based proteomic analysis revealed that Extraction Reagent Type 4 (50 mM Tris, 8/2 M Urea/Thiourea and 1% C7BzO) is superior in terms of proteome coverage, abundance, and subsequent gel application compatibility. MS qualification and delipidation efficiency of the large repertoire of purification methods revealed the superiority of TCA/acetone and hexane:ethanol. Further analysis pertaining to physiochemical heterogeneity revealed suitability of hexane:ethanol purification method for lipogenesis studies and TCA/acetone for membrane proteins. This paper marks the first attempt of method development for proteomic analysis of oleaginous yeasts, which is crucial for elucidating *de novo* lipogenesis and future genomic engineering aimed towards diverse applications in biofuel and oleochemicals.

Acknowledgments The authors gratefully acknowledge MS/MS sample analysis conducted by Mrs. Katja Bäuml.

Author contributions Conceptualization of the study was conducted jointly by DA and TB. The methodological approach was designed and carried out by DA. Data validation was jointly carried out by all authors. DA prepared the original draft of the manuscript. The manuscript was jointly finalized by all authors.

Funding information The authors received funding from the Werner Siemens Foundation for establishing the research field of Synthetic Biotechnology at the Technical University of Munich.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

References

1. Awad D, Bohnen F, Mehlmer N, Brueck T. Multi-factorial-guided media optimization for enhanced biomass and lipid formation by the oleaginous yeast Cutaneotrichosporon oleaginosus. Front Bioeng Biotechnol. 2019;7(54). https://doi.org/10.3389/fbioe.2019.00054.
2. Liu H, Zhao X, Wang F, Li Y, Jiang X, Ye M, et al. Comparative proteomic analysis of Rhodosporidium toruloides during lipid accumulation. Yeast. 2009;26(10):553–66. https://doi.org/10.1002/yea.1706.
3. Zhu Z, Zhang S, Liu H, Shen H, Lin X, Yang F, et al. A multi-omic map of the lipid-producing yeast Rhodosporidium toruloides. Nat Commun. 2012;3:1112. https://doi.org/10.1038/ncomms2112.
4. Ratledge C, Wynn JP. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol. 2002;51:1–51.
5. Bracharz F, Beukhout T, Mehlmer N, Bruck T. Opportunities and challenges in the development of Cutaneotrichosporon oleaginosus ATCC 20509 as a new cell factory for custom tailored microbial oils. Microb Cell Factories. 2017;16(1):178. https://doi.org/10.1186/s12934-017-0791-9.
6. Chi ZM, Liu TT, Chi Z, Liu GL, Wang ZP. Occurrence and diversity of yeasts in the mangrove ecosystems in Fujian, Guangdong and Hainan provinces of china. Indian J Microbiol. 2012;52(3):346–53. https://doi.org/10.1007/s12088-012-0251-5.
7. Wu S, Hu C, Jin G, Zhao X, Zhao ZK. Phosphate-limitation mediated lipid production by Rhodosporidium toruloides. Bioresour Technol. 2010;101(15):6124–9. https://doi.org/10.1016/j.biortech.2010.02.111.
8. Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG. Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol. 2011;90(4):1219–27. https://doi.org/10.1007/s00253-011-3200-z.
9. Wu X, Xiong E, Wang W, Scali M, Cresti M. Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. Nat Protoc. 2014;9(2):362–74. https://doi.org/10.1038/nprot.2014.022.
10. Hao R, Adoligbe C, Jiang B, Zhao X, Gui L, Qu K, et al. An optimized trichloroacetic acid/acetone precipitation method for two-dimensional gel electrophoresis analysis of Qinchuan cattle longissimus dorsi muscle containing high proportion of marbling. PLoS One. 2015;10(4):e0124723. https://doi.org/10.1371/journal.pone.0124723.
11. Klimek-Ochab M, Brzezinska-Rodak M, Zymanczyk-Duda E, Lejczak B, Kafarski P. Comparative study of fungal cell disruption–scope and limitations of the methods. Folia Microbiol (Praha). 2011;56(5):469–75. https://doi.org/10.1007/s12223-011-0069-2.
12. Ren X, Yu D, Yu L, Gao G, Han S, Feng Y. A new study of cell disruption to release recombinant thermostable enzyme from Escherichia coli by thermolysis. J Biotechnol. 2007;129(4):668–73. https://doi.org/10.1016/j.jbiotec.2007.01.038.
13. Limonlason J, Hoare M, Orsborn CB, Doyle DJ, Dunnill P. Reactor properties of a high-speed bead mill for microbial cell rupture. Biotechnol Bioeng. 1979;21(5):745–74. https://doi.org/10.1002/bit.260210503.
14. Agerkvist I, Enfors SO. Characterization of E. coli cell disintegrates from a bead mill and high pressure homogenizers. Biotechnol Bioeng. 1990;36(11):1083–9. https://doi.org/10.1002/bit.260361102.
15. Moo-Young M. Comprehensive biotechnology: Elsevier Science; 2011.
16. Ibrahim HR, Yamada M, Matsushita K, Kobayashi K, Kato A. Enhanced bactericidal action of lysozyme to Escherichia coli by

inserting a hydrophobic pentapeptide into its C terminus. J Biol Chem. 1994;269(7):5059–63.
17. Crapisi A, Lante A, Pasini G, Spettoli P. Enhanced microbial cell lysis by the use of lysozyme immobilized on different carriers. Process Biochem. 1993;28(1):17–21. https://doi.org/10.1016/0032-9592(94)80031-6.
18. Arnold T, Linke D. Phase separation in the isolation and purification of membrane proteins. Biotechniques. 2007;43(4):427–30, 432, 434 passim. https://doi.org/10.2144/000112566.
19. Seddon AM, Curnow P, Booth PJ. Membrane proteins, lipids and detergents: not just a soap opera. Biochim Biophys Acta. 2004;1666(1-2):105–17. https://doi.org/10.1016/j.bbamem.2004.04.011.
20. Rabilloud T, Luche S, Santoni V, Chevallet M. Detergents and chaotropes for protein solubilization before two-dimensional electrophoresis. Methods Mol Biol. 2007;355:111–9. https://doi.org/10.1385/1-59745-227-0:111.
21. Vaisar T. Thematic review series: proteomics. Proteomic analysis of lipid-protein complexes. J Lipid Res. 2009;50(5):781–6. https://doi.org/10.1194/jlr.R900005-JLR200.
22. Martínez-Maqueda D, Hernández-Ledesma B, Amigo L, Miralles B, Gómez-Ruiz JÁ. Extraction/fractionation techniques for proteins and peptides and protein digestion. In: Toldrá F, Nollet LML, editors. Proteomics in Foods: principles and applications. Boston, MA: Springer US; 2013. p. 21–50. https://doi.org/10.1007/978-1-4614-5626-1_2.
23. Cappiello A, Famiglini G, Palma P, Pierini E, Termopoli V, Trufelli H. Overcoming matrix effects in liquid chromatography-mass spectrometry. Anal Chem. 2008;80(23):9343–8. https://doi.org/10.1021/ac8018312.
24. Zhou W, Yang S, Wang PG. Matrix effects and application of matrix effect factor. Bioanalysis. 2017;9(23):1839–44. https://doi.org/10.4155/bio-2017-0214.
25. Chatterjee M, Gupta S, Bhar A, Das S. Optimization of an efficient protein extraction protocol compatible with two-dimensional electrophoresis and mass spectrometry from recalcitrant phenolic rich roots of chickpea (Cicer arietinum L.). Int. J Proteome. 2012;2012:536963. https://doi.org/10.1155/2012/536963.
26. Burgess R (2009) Chapter 20 Protein precipitation techniques, vol 463.
27. Ingham KC. Precipitation of proteins with polyethylene glycol. Methods Enzymol. 1990;182:301–6.
28. Harrison RG. Protein purification process engineering. Organization and strategy. Bioprocess Technol. 1994;18:1–9.
29. Moure A, Sineiro J, Domínguez H, Carlos Parajó J. Functionality of oilseed protein products: a review. 2006;39. https://doi.org/10.1016/j.foodres.2006.07.002.
30. Jiang L, He L, Fountoulakis M. Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. J Chromatogr A. 2004;1023(2):317–20.
31. Chourey K, Jansson J, VerBerkmoes N, Shah M, Chavarria KL, Tom LM, et al. Direct cellular lysis/protein extraction protocol for soil metaproteomics. J Proteome Res. 2010;9(12):6615–22. https://doi.org/10.1021/pr100787q.
32. Wessel D, Flugge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem. 1984;138(1):141–3.
33. Ichihara K, Yoneda K, Takahashi A, Hoshino N, Matsuda M. Improved methods for the fatty acid analysis of blood lipid classes. Lipids. 2011;46(3):297–306. https://doi.org/10.1007/s11745-011-3531-7.
34. Abbott SK, Jenner AM, Mitchell TW, Brown SH, Halliday GM, Garner B. An improved high-throughput lipid extraction method for the analysis of human brain lipids. Lipids. 2013;48(3):307–18. https://doi.org/10.1007/s11745-013-3760-z.
35. Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res. 2008;49(5):1137–46. https://doi.org/10.1194/jlr.D700041-JLR200.
36. Hara A, Radin NS. Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem. 1978;90(1):420–6.
37. Markham JE, Li J, Cahoon EB, Jaworski JG. Separation and identification of major plant sphingolipid classes from leaves. J Biol Chem. 2006;281(32):22684–94. https://doi.org/10.1074/jbc.M604050200.
38. Baümler ER, Crapiste GH, Carelli AA. Solvent extraction: kinetic study of major and minor compounds. J Am Oil Chem Soc. 2010;87(12):1489–95. https://doi.org/10.1007/s11746-010-1637-3.
39. Zellner M, Winkler W, Hayden H, Diestinger M, Eliasen M, Gesslbauer B, et al. Quantitative validation of different protein precipitation methods in proteome analysis of blood platelets. Electrophoresis. 2005;26(12):2481–9. https://doi.org/10.1002/elps.200410262.
40. Liu H, Zhao X, Wang F, Jiang X, Zhang S, Ye M, et al. The proteome analysis of oleaginous yeast Lipomyces starkeyi. FEMS Yeast Res. 2011;11(1):42–51. https://doi.org/10.1111/j.1567-1364.2010.00687.x.
41. Wang W, Vignani R, Scali M, Sensi E, Tiberi P, Cresti M. Removal of lipid contaminants by organic solvents from oilseed protein extract prior to electrophoresis. Anal Biochem. 2004;329(1):139–41. https://doi.org/10.1016/j.ab.2004.02.044.
42. Shi J, Feng H, Lee J, Ning Chen W. Comparative proteomics profile of lipid-cumulating oleaginous yeast: an iTRAQ-coupled 2-D LC-MS/MS analysis. PLoS One. 2013;8(12):e85532. https://doi.org/10.1371/journal.pone.0085532.
43. Pomraning KR, Kim Y-M, Nicora CD, Chu RK, Bredeweg EL, Purvine SO, et al. Multi-omics analysis reveals regulators of the response to nitrogen limitation in Yarrowia lipolytica. BMC Genomics. 2016;17(1):138. https://doi.org/10.1186/s12864-016-2471-2.
44. Tang X, Chen H, Gu Z, Zhang H, Chen YQ, Song Y, et al. Comparative proteome analysis between high lipid-producing strain Mucor circinelloides WJ11 and Low Lipid-Producing Strain CBS 277.49. J Agric Food Chem. 2017;65(24):5074–82. https://doi.org/10.1021/acs.jafc.7b00935.
45. Gorg A, Obermaier C, Boguth G, Csordas A, Diaz JJ, Madjar JJ. Very alkaline immobilized pH gradients for two-dimensional electrophoresis of ribosomal and nuclear proteins. Electrophoresis. 1997;18(3-4):328–37. https://doi.org/10.1002/elps.1150180306.
46. Griffiths MJ, van Hille RP, Harrison ST. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids. 2010;45(11):1053–60. https://doi.org/10.1007/s11745-010-3468-2.
47. Gorner C, Redai V, Bracharz F, Schrepfer P, Garbe D, Bruck T. Genetic engineering and production of modified fatty acids by the non-conventional oleaginous yeast Trichosporon oleaginosus ATCC 20509. Green Chem. 2016;18(7):2037–46. https://doi.org/10.1039/C5GC01767J.
48. Wu S, Zhu Z, Fu L, Niu B, Li W. WebMGA: a customizable web server for fast metagenomic sequence analysis. BMC Genomics. 2011;12:444. https://doi.org/10.1186/1471-2164-12-444.
49. Ye J, Zhang Y, Cui H, Liu J, Wu Y, Cheng Y, et al. WEGO 2.0: a web tool for analyzing and plotting GO annotations, 2018 update. Nucleic Acids Res. 2018;46(W1):W71–5. https://doi.org/10.1093/nar/gky400.
50. Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 2005;21(18):3674–6. https://doi.org/10.1093/bioinformatics/bti610.
51. Tingting Z, Jie L, Hanjiang Y, Kun X, Rui W, Zhiying Z. An improved method for whole protein extraction from yeast

Saccharomyces cerevisiae. Yeast. 2011;28(11):795–8. https://doi.org/10.1002/yea.1905.
52. Pozo-Bayón MÁ, Andujar-Ortiz I, Alcaide-Hidalgo JM, Martín-Álvarez PJ, Moreno-Arribas MV. Characterization of commercial inactive dry yeast preparations for enological use based on their ability to release soluble compounds and their behavior toward aroma compounds in model wines. J Agric Food Chem. 2009;57(22):10784–92. https://doi.org/10.1021/jf900904x.
53. Ren X, Yu D, Han S, Feng Y. Thermolysis of recombinant Escherichia coli for recovering a thermostable enzyme. Biochem Eng J. 2007;33(1):94–8. https://doi.org/10.1016/j.bej.2006.09.017.
54. Burden DW, Whitney DB. Biotechnology proteins to PCR: a course in strategies and lab techniques. Boston: Birkhäuser; 2012.
55. Molon M, Woznicka O, Zebrowski J. Cell wall biosynthesis impairment affects the budding lifespan of the Saccharomyces cerevisiae yeast. Biogerontology. 2018;19(1):67–79. https://doi.org/10.1007/s10522-017-9740-6.
56. von der Haar T. Optimized protein extraction for quantitative proteomics of yeasts. PLoS One. 2007;2(10):e1078. https://doi.org/10.1371/journal.pone.0001078.
57. Luche S, Santoni V, Rabilloud T. Evaluation of nonionic and zwitterionic detergents as membrane protein solubilizers in two-dimensional electrophoresis. Proteomics. 2003;3(3):249–53. https://doi.org/10.1002/pmic.200390037.
58. Pollard HB, Menard R, Brandt HA, Pazoles CJ, Creutz CE, Ramu A. Application of Bradford's protein assay to adrenal gland subcellular fractions. Anal Biochem. 1978;86(2):761–3.
59. Pavokovic D, Križnik B, Krsnik-Rasol M. Evaluation of protein extraction methods for proteomic analysis of non-model recalcitrant plant tissues. 2012;85. https://doi.org/10.5562/cca1804.
60. Sharma R, Dill BD, Chourey K, Shah M, VerBerkmoes NC, Hettich RL. Coupling a detergent lysis/cleanup methodology with intact protein fractionation for enhanced proteome characterization. J Proteome Res. 2012;11(12):6008–18. https://doi.org/10.1021/pr300709k.
61. Karlsson H, Leanderson P, Tagesson C, Lindahl M. Lipoproteomics I: mapping of proteins in low-density lipoprotein using two-dimensional gel electrophoresis and mass spectrometry. Proteomics. 2005;5(2):551–65.
62. Frank AM. A ranking-based scoring function for peptide-spectrum matches. J Proteome Res. 2009;8(5):2241–52. https://doi.org/10.1021/pr800678b.
63. Obasi TC, Moldovan R, Toiu A, Braicu C, Bodoki E, Berindan-Neagoe I, et al. Molecular-trapping in emulsion's monolayer: a new strategy for production and purification of bioactive saponins. Sci Rep. 2017;7(1):14511. https://doi.org/10.1038/s41598-017-15067-4.
64. Jarudilokkul S, Paulsen E, Stuckey DC. The effect of demulsifiers on lysozyme extraction from hen egg white using reverse micelles. Bioseparation. 2000;9(2):81–91.
65. Faurobert M, Pelpoir E, Chaïb J. Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. In: Thiellement H, Zivy M, Damerval C, Méchin V, editors. Plant proteomics: methods and protocols. Totowa: Humana Press; 2007. p. 9–14. https://doi.org/10.1385/1-59745-227-0:9.

10.2 Microbial lipid production by oleaginous yeasts grown on *Scenedesmus obtusiusculus* microalgae biomass hydrolysate

Samer Younes, Felix Bracharz, Dania Awad, Farah Qoura, Norbert Mehlmer, Thomas Brueck

Published in *Bioprocess and Biosystems Engineering*

DOI: 10.1007/s00449-020-02354-0

Date of Publication: 28 April 2020

Bioprocess and Biosystems Engineering
https://doi.org/10.1007/s00449-020-02354-0

RESEARCH PAPER

Microbial lipid production by oleaginous yeasts grown on *Scenedesmus obtusiusculus* microalgae biomass hydrolysate

Samer Younes[1] · Felix Bracharz[1] · Dania Awad[1] · Farah Qoura[1] · Norbert Mehlmer[1] · Thomas Brueck[1]

Received: 18 February 2020 / Accepted: 13 April 2020

Abstract

Due to increasing oil prices and climate change concerns, biofuels have become increasingly important as potential alternative energy sources. However, the use of arable lands and valuable resources for the production of biofuel feedstock compromises food security and negatively affect the environment. Single cell oils (SCOs), accumulated by oleaginous yeasts, show great promise for efficient production of biofuels. However, the high production costs attributed to feedstocks or raw materials present a major limiting factor. The fermentative conversion of abundant, low-value biomass into microbial oil would alleviate this limitation. Here, we explore the feasibility of utilizing microalgae-based cell residues as feedstock for yeast oil production. We developed an efficient, single-step enzymatic hydrolysis to generate *Scenedesmus obtusiusculus* hydrolysate (SH) without thermo-chemical pretreatment. With this eco-friendly process, glucose conversion efficiencies reached 90–100%. *Cutaneotrichosporon oleaginosus*, *Cryptococcus curvatus* and *Rhodosporidium toruloides* were cultivated on SH as sole nutrients source. Only *C. oleaginosus* was able to accumulate intracellular lipids, with a 35% (g lipid/g DCW) content and a yield of 3.6 g/L. Our results demonstrate the potential valorization of algal biomass into desired end-products such as biofuels.

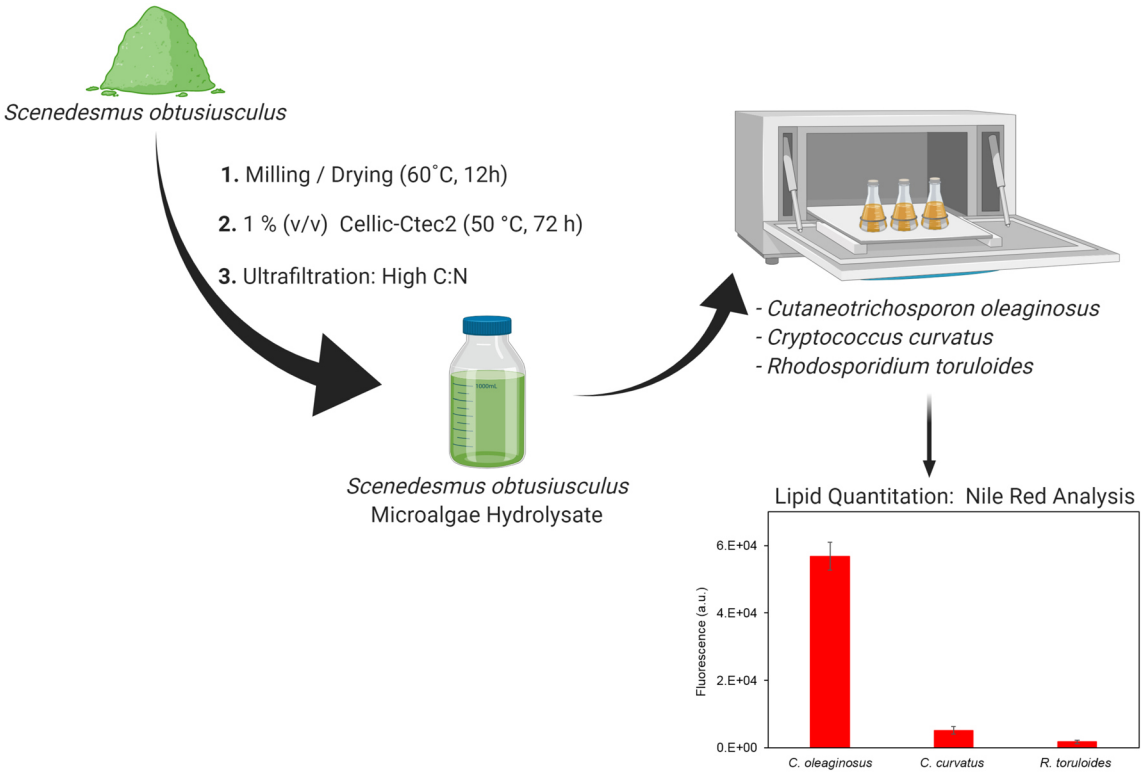
Samer Younes and Felix Bracharz contributed equally to the work.

✉ Thomas Brueck
brueck@tum.de

[1] Werner Siemens-Lehrstuhl für Synthetische Biotechnologie, Technische Universität München, Garching, Germany

Published online: 28 April 2020

Graphic Abstract



Keywords *Scenedesmus obtusiusculu* · *Cutaneotrichosporon oleaginosus* · Enzymatic hydrolysis · Microalgae biomass · Lipid production

Introduction

The ever-increasing energy demand in today's industrial world led to the widespread use of non-renewable fossil fuels such as petroleum. The transition from a society with waste generating, linear production routes to one cyclic valorization path in conjunction with renewable resource management is one of the most demanding technological goals for establishing sustainable bioeconomy [1, 2]. This scenario particularly applied renewable energy supply routes that demand a switch from finite fossilto sustainable platform solutions. Moreover, dwindling of fossil resources, escalating environmental pollution, surging CO_2 and greenhouse gas emissions, in addition to climate change have collectively driven the search for alternative energy sources [3]. Accordingly, technological innovations that enable a more sustainable lifestyle are coveted [4].

Biofuels have garnered great interest in recent years as alternatives for fossil fuel. In fact, plant-derived biofuel offers a partial solution to the ever-increasing energy demand, due to their renewability. However, this first-generation of biofuels, generated from edible crops, impacts agricultural activity and jeopardizes food security [1] To meet the current annual global-demand of biodiesel, more than double of the currently arable land would be required to grow crops that are explicitly grown for fuel production [5]. Consequently, alternative sources for biofuel production that do not affect food security are in high demand [1]. One of those alternatives is the use of oleaginous microorganisms such as algae and yeast [6].

Oleaginous microorganisms accumulate lipid at a minimum of 20% (g lipids/g dry cell weight (DCW)) [7]. However, lipid accumulation in oleaginous microorganisms, such as yeast, fungi and microalgae, is not a constitutive feature, but rather an adaptive response to particular environmental factors [8]. In environmental conditions abundant in carbon source and deficient in specific nutrients such as nitrogen, phosphorus or sulfur, oleaginous microorganisms convert excess carbon into fatty acids and incorporate them into triglycerides (TAGs) as a form of energy storage [9]. TAGs are stored in specialized organelles called lipid bodies (also known as lipid droplets) [10]. Single Cell Oils (SCOs) can be efficiently converted into biodiesel and biofuel [11, 12]. Various oleaginous yeasts have been subject to extensive

investigations such as *Yarrowia lipolytica*, *Rhodosporidium toruloids* and *Lipomyces starkeyi*, with reports of lipid accumulation in excess of 70% (g lipid/g DCW) [13, 14]. The potential biotechnological applications of these oleaginous yeasts, utilizing various carbon sources have been previously reported [9, 15, 16].

Moon et al. first isolated *Cutaneotrichosporon oleaginosus* (ATCC 20509) in 1978 from factory drain samples of the Iowa State University Dairy Farm. *C. oleaginosus* readily utilizes glucose, galactose, cellobiose, xylose, sucrose, and lactose as carbon source [1, 17–19]. Furthermore, this yeast is able to metabolize glycerol, *N*-acetylglucosamine, volatile fatty acids and ethanol and 4-hydroxymethylfurfural [20–22]. To improve the sustainability of SCOs from socio-economic aspects, *Y. lipolytica* has undergone extensive genetic engineering aimed at simultaneous sugar uptake (hexoses and pentoses) from complex and wastewater hydrolysates, which is an inherent ability in *C. oleaginosus* [8]. Depending on carbon, nitrogen sources and stress conditions (nitrogen, phosphate or sulfate limitation), cellular lipid accumulation in *C. oleaginosus* can reach up to 85% (g lipid/g DCW) [23, 24]. In addition to fast growth rate, this oleaginous yeast exhibits a fatty acid profile that mimics that of vegetable oils, specifically palm oil, with palmitic, stearic and oleic acid as dominant fatty acids [25]. McCurdy et al. reported that biodiesel B20 derived from *C. oleaginosus* TAGs meet the ASTM (D6751) certification [1, 9]. Through recycling and finding appropriate industrial sink for bio-compounds, a recent study touching on the socio-economic sustainability of *C. oleaginosus* SCOs has been recently prepared in our group [4].

Other oleaginous species that can be exploited for the biofuel sector include microalgae. In contrast to industrial crops, such as palm or canola plants, biomass generation from microalgae has high space and time yields. Globally, around 280 tons/ha of algae dry biomass and 3.9 tons/ha of forest biomass are produced every year [26]. Additionally, microalgae display high CO_2 fixation ability (513 tons of sequestered CO_2 per hectare per year). Specifically, 1.6-2 grams of CO_2 is captured for every gram of algal biomass produced, at an efficiency of 80-99% [27, 28]. While microalgae can provide renewable oils by photosynthetically converting atmospheric CO_2 to lipids, yields are conventionally lower compared to oleaginous yeast species [29–31]. In most algae oil production processes, the extracted cell residue is not contributing to the overall process economy [29, 31].

Yet recently, several value-adding outlets for this residue have been achieved, either by feeding it back into renewable production (oil, food, feed, etc.) or by recycling of resources [4, 32]. Similar waste-free biorefinery approaches have been considered in the design and optimization of biogas production processes [33]. In that respect, the residual biomass, which is rich in fermentable sugars, can be used as feedstock for oleaginous yeasts cultivation [34]. Specifically, *Scenedesmus* spp. belong to the most common freshwater green algae. *Scenedesmus obtusiusculus* A189, a newly isolated member of the *Chlorophyta* genus, is characterized by high growth rates in fresh and saline media, in addition to high inherent carbohydrate content [35]. Fermentative conversion of various biomass sources into SCOs and subsequent biofuel via oleaginous yeasts has recently received an increasing interest in the scientific and industrial community [2, 4]. Specifically, microalgae biomass does not contain recalcitrant lignin, making it suitable for eco-friendly and cost-effective hydrolysis methods [35]. To that end, efficient hydrolysis of algae biomass could provide a sustainable stream of monomeric hexose and pentose sugars for fermentative growth. However, chemical biomass hydrolysis, which is most commonly applied in industry, tarnishes the eco-friendly aspect of biofuel production [2]. Alternatively, enzymatic hydrolysis efficiently generates sugar-rich fermentation media, yet necessitates thermo-chemical pretreatment steps to break down the lignocellulosic biomass components into monomeric hexose and pentose sugars [36]. These pretreatment steps release inhibitory compounds that hinder growth of subsequent oleaginous yeast inoculum in the prospective hydrolysate. Removal of these inhibitory compounds imposes additional costs, time and effort for the production process [37].

In this study, we examine the enzymatic hydrolysis of microalgae-based cell residues and the subsequent use of its hydrolysate (SH) for the cultivation of oleaginous yeasts (*C. oleaginosus*). A single-step enzymatic hydrolysis process was devised and optimized, allowing efficient hydrolysis and saccharification of microalgae biomass, without thermo-chemical pretreatment. The resulting *S. obtusiusculus* hydrolysate (SH), barring expensive additives, was utilized as the sole fermentative media for *C. oleaginosus*, *Cryptococcus curvatus* and *Rhodosporidium toruloides*. The accumulated lipids, deposited as intracellular lipid bodies (LBs), were relatively quantified by Nile red analysis. Moreover, *C. oleaginosus* growth, dry biomass and lipid weight were evaluated.

Materials and methods

Algae strains and biomass determination

Scenedesmus obtusiusculus (A189) residues were obtained from Pharmaceutical Biology Group, Ernst Moritz Arndt University (EMAU), Griefswald, Germany. Water content was determined following the milling and drying of the algal samples at 60 °C overnight. Total carbohydrate concentration was determined by the thymol-sulfuric acid method [38]. The standard Kjeldahl procedure was utilized to determine the amount of protein in the algae biomass

[39]. Total lipids were extracted according to Folch et al., and determined gravimetrically after solvent evaporation [40]. Biomass ash content was determined by following the AOAC procedure [41]. Biological replicates ensured reproducible measurements.

Enzymatic hydrolysis and preparation of SH

Scenedesmus obtusiusculus Hydrolysate (SH) was prepared by hydrolyzing autoclaved algae biomass. Briefly, dry biomass samples weighing 50 g were transferred to 2 L glass bottles containing 1 L of 50.0 mM sodium acetate buffer, pH 5.0. Different hydrolytic enzyme mixtures were examined, including Cellic-Ctec2 (Novozymes, Denmark), Celli-Htec2 (Novozymes, Denmark), Pectinex (Novozymes, Denmark) and Fungamyl (Novozymes, Denmark). Hydrolytic Reactions were initiated by adding the enzyme solution and incubating the mixture at 50 °C for 72 h. Buffer and enzymes were sterile-filtered prior to hydrolysis. Samples were then spun down for 30 min. Cross-filtration using a 10 kDa membrane made from regenerated cellulose was completed under the following parameters: Inlet-Pressure (P1) of 2 bar, Repentant-Pressure (P2) of 0.3–0.5 bar and permeate was open to atmospheric pressure. Flow-Rates of retentate and permeate were adjusted to 2 L/min and 0.1 L/min respectively. A 0.2 µm filter capsules were installed at the outlet to sterilize the resulted hydrolysate. Biological triplicates of the SH were prepared.

Sugar analysis

Sugar composition of the hydrolysate was determined by an Agilent 1100 series HPLC with a Refractive Index (RI) detector (Shodex, RI101) and Ultraviolet Index (Sedere-France, Sedex75). Following cross-filtration, 5 µL sample was injected on an Aminex HPX-87P column (8% cross-linked resin, lead ionic, Bio-Rad) and separated at 70 °C with double-distilled water as mobile phase. Run parameters were set to a duration of 30 min, a flow rate of 0.4 mL/min and detection at 50 °C. Samples' RI signal was aligned with that of internal standard curves.

Yeast strains and culture conditions

Yeast strains *Cutaneotrichosporon oleaginosus* (ATCC 20509), *Cryptococcus curvatus* (CBS 5324) and *Rhodosporidium toruloides* (NP11) were maintained on yeast peptone dextrose (YPD) agar (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose, 20 g/L agar) at 4 °C for short-term storage. Minimal nitrogen media (MNM) (30 g/L glucose, 1.5 g/L yeast extract, 0.5 g/L NH_4Cl, 7.0 g/L KH_2PO_4, 5.0 g/L $Na_2HPO_4{\cdot}12H_2O$, 1.5 g/L $MgSO_4{\cdot}7H_2O$, 0.08 g/L $FeCl_3{\cdot}6H_2O$, 0.01 g/L $ZnSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}2H_2O$, 0.1 mg/L $MnSO_4{\cdot}5H_2O$, 0.1 mg/L $CuSO_4{\cdot}5H_2O$, 0.1 mg/L $Co(NO_3)_2{\cdot}6H_2O$; pH 5.5) was adopted from Suutari et al. for induction of lipogenesis [42].

Following pre-culturing in YPD broth for 24 h, yeast cells were centrifuged, washed with PBS buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4; pH 7.4) and inoculated in 250 mL baffled flasks, containing 50 mL of MNM, at an initial seeding OD_{600} of 0.5. Incubation lasted for 4 days in a rotary shaker at 120 rpm and 28 °C. To evaluate the capability of utilizing hydrolysates for yeast growth and lipid accumulation, the selected oleaginous strains were cultured solely on SH lacking any additives or carbon supplementation. Biological cultures of the oleaginous yeasts were carried out in triplicates.

Nile red staining

Samples were analyzed in technical triplicates using a modified protocol from Sitepu et al. [43]. Briefly, 225 µL of each yeast culture was transferred to a 96-well black microtiter plate. Serial dilutions were performed in triplicates to ensure an optical density < 1 before 50 µl DMSO was added to each well. Initial absorbance readings were taken at 600 nm and for growth monitoring and correction of fluorescence readings for growth variation. A volume of 25 µL Nile red was then added to each well (final concentration of 50 µg/mL). Fluorescence measurements (recorded before and after Nile red addition) at excitation at 530/25 nm; emission at 590/35 nm; and kinetic reading for 5 min with 30 s interval were taken. Maximal emission values were determined and fluorescence measurements were corrected for variation in cell density by dividing the fluorescence unit by background optical density OD_{600} values.

Gravimetric analysis

Technical triplicates of the total lipid content of the oleaginous yeasts were determined by a modified method by Folch et al. [40]. Briefly, 15 mL of yeast cultures were washed and homogenized using an Avestin Emulsiflex at a sample port pressure of 1200 bar and a chamber pressure of 8 bar. Lipids from the homogenate were extracted with 6 mL of Folch solution (2:1 chloroform: methanol). Lipid extraction continued overnight at room temperature and shaking speed of 120 rpm. Subsequently, 1.2 mL of 0.9% NaCl were added to aid phase separation. The lower phase was aspirated using a syringe and added to pre-weighed glass vessels. The chloroform was fully evaporated under a nitrogen stream and glass vials were weighed again. The extracted lipid samples were used to calculate lipid content as total lipid weight and as percent of dry yeast weight.

Dry biomass determination

A volume of 2 mL of each yeast culture was transferred in triplicates to pre-weighed 2 mL Eppendorf tubes. The tubes were weighed again the following centrifugation at 14,000 g for 5 min, washing and drying at 60 °C overnight. Measurements were recorded in triplicates by subtracting the weight of the sample tubes from their respective pre-weights.

Results

Algae biomass analysis

In this study, the biomass composition of *S. obtusiusculus* was determined (Table 1). This green algae displays high amounts of carbohydrates and crude proteins comprising 34% and 49% (g/g DCW) respectively. Other components encompassed water, lipids and ash measured at 3.7, 8.3 and 1.9% (g/g DCW), respectively. The acquired biomass data suggest that *S. obtusiusculus* can be quantitatively hydrolyzed by chemical and enzymatic systems to release monomeric pentose and hexose sugars, which could serve as a carbon source for microbial cultivation.

Hydrolysis of *S. obtusiusculus* dry biomass

Various commercial hydrolase enzyme mixtures were tested for their biomass liquefaction efficiencies including a cellulase mix (Cellic-Ctec2, Novozymes), a hemicellulose-mix (Cellic-Htec2, Novozymes), a pectinase mix (Pectinex, Novozymes), and an amylase mix (Fungamyl, Novozymes). Biomass to glucose conversion ratios from the various enzyme mixtures is presented in Fig. 1a. The cellulase mix (Cellic CTec 2) exhibited the best activity. Glucose monomerization reached a saturation level between 12 and 14 g/L starting from a 50 g algae biomass, which translates to a glucose yield of 0.24-0.28 g/g of DCW. Furthermore, a 1% (v/v) of cellulase mixture Cellic-Ctec2 was combined with varying concentrations of the remaining enzyme mixtures, none of which yielded a significantly better conversion ratio (Fig. 1b).

Table 1 Biochemical composition of *S. obtusiusculus*, calculated as percent of total dry weight

Biomass component	Content % % (g/g dry biomass weight)
Water	3.7
Carbohydrates	33.8
Proteins	48.7
Lipids	8.3
Pigments, secondary metabolites	3.6
Ash	1.9

Relative standard deviation for all given numbers is ≤ ± 2%

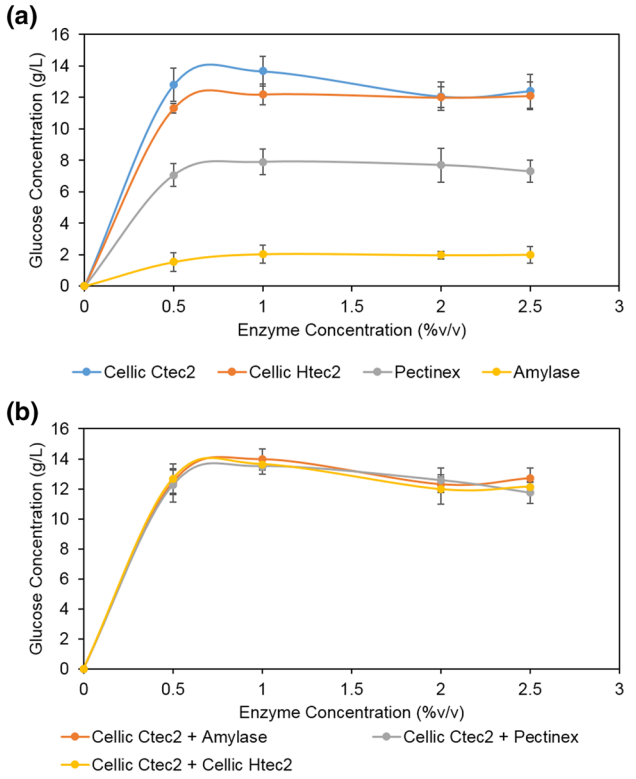


Fig. 1 Glucose concentration of SH displayed as a factor of various enzymes mixes and concentrations (**a**). Glucose concentration of SH displayed as a factor of the combination of enzyme mixtures with Cellic CTec 2 (**b**)

To assess the efficiency of glucose liberation from the algae biomass, measurements of enzymatic hydrolysis were compared with those of acidic hydrolysis. Table 2 confirms that glucose conversion efficiencies reached (90–100%), with glucose constituting up to two-thirds (g/g of DCW) of the total carbohydrate content of SH.

The combined enzymatic conversion rate of mannose and galactose, which were not distinguished by HPLC, was limited to only 20–25% (g/g DCW).

However, total sugar concentration in the hydrolysate was still relatively low (12–14 g/L glucose) given a carbohydrate content of 34% (g/g DCW). Accordingly, hydrolysis was repeated with higher amounts of biomass retaining a 1% (v/v) concentration of the cellulase mixture. As a result, glucose concentration in SH reached 48 g/L starting with a 200 g *Scenedesmus* dry biomass. This accounts fora glucose yield of 0.24 g/g of DCW, maintaining the high conversion efficiencies of (90–100%). Subsequent experiments were conducted with SH comprising this high glucose concentration (48 g/L).

Table 2 A comparison of monosaccharide content % (g/g dry biomass weight) resulting from acidic and enzymatic hydrolysis

Sugar	Acidic hydrolysis % (g/g dry biomass weight)	Enzymatic hydrolysis % (g/g dry biomass weight)	Conversion Efficiency (%)
Glucose	22	20–22	90–100
Mannose		–	–
Galactose	10	2–2.5	20–25
Rhamnose	~1	0	0
Fucose	~1	0	0
Ribose	~1	0	0

Relative standard deviation for all given numbers is ≤ ± 2%

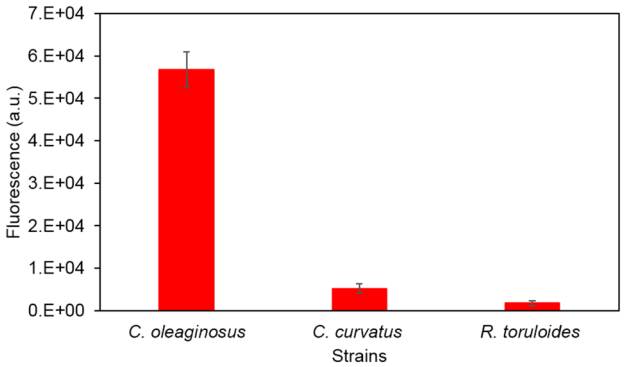


Fig. 2 Rapid estimation of lipid contents in *C. oleaginosus*, *C. curvatus* and *R. toruloides* following 4 days cultivation on SH, determined by Nile red assay

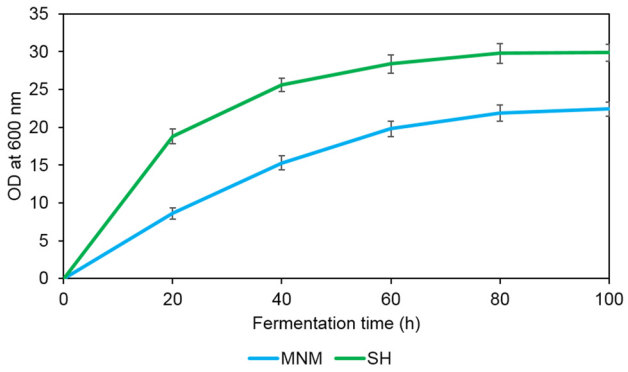


Fig. 3 *C. oleaginous* growth trend when grown on MNM and SH over 4 days in a shake flask fermentation

Yeast growth and lipid production

High throughput Nile red screening was employed to determine the lipid yield of three oleaginous yeast strains *C. oleaginosus*, *C. curvatus* and *R. toruloides*. Following a 4-day fermentative growth on SH as the sole carbon source, Nile red analysis revealed low lipid content in *C. curvatus* and *R. toruloides*. Contrarily, *C. oleaginosus* was able to accumulate a considerable amount of lipid bodies when cultivated on the same media (Fig. 2).

Thus, *C. oleaginosus* underwent subsequent experiments to determine growth rate and lipid absolute quantitation when cultivated on MNM and SH. To evaluate *C. oleaginosus* growth-rate in MNM and SH media, optical density at 600 nm was measured over 4 days (Fig. 3). SH media resulted in highest final growth for *C. oleaginosus* measured at OD_{600} of about 30, compared to OD_{600} of about 22 in MNM.

Gravimetric analysis was performed to determine total lipid content in *C. oleaginosus* following fermentation on MNM and SH in shake flasks (Fig. 4). The yeast accumulated nearly 61% and 35% (g lipid/g DCW) lipids when grown on MNM and SH, respectively. After 4 days fermentation, lipid yield in *C. oleaginosus* reached about 5.3 g/L of culture when cultivated on MNM, and 3.6 g/L of culture when cultivated on SH media.

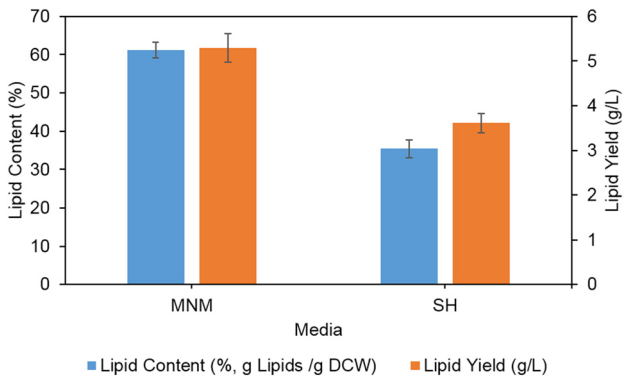


Fig. 4 Lipid content (%, g lipid/g DCW) and lipid yield (g/L) of *C. oleaginous* cultivated in MNM and SH media for 4 days in shake flask fermentation

Discussion

For years, microalgae have been exploited as a source for value-added products, with numerous commercial applications that include enhancing the nutritional value of food and animal feed, as well as being incorporated into cosmetics [44]. The significant properties of microalgae biomass as raw material for microbial cultivation include high carbohydrates contents and lack of recalcitrant lignin [45]. The use of microbes as a platform for lipid and subsequent biofuel and biodiesel production offers: (1) renewability and potential sustainability, (2) requires less labor and fewer raw materials, (3) is easier to scale up, (4) does not compete with edible-plants for land, (5) generates less waste and (6) is not affected by season or climate [46]. Recently, the valorization of seagrass and brown macroalgae biomass as feedstock for *C. oleaginosus* lipid production, in addition to the techno-economic feasibility of the bioprocesses have been conducted in our group [2, 4]. In this study, *S. obtusiusculus* biomass was chosen as feedstock for oily yeast growth, due to its high carbohydrates content 34% (g/g dry biomass weight). In comparison, *Scenedesmus obliquus*, *Chlorella vulgaris*, *Chlamydomonas rheinhardii*, and *Dunaliella salina* algae species exhibit sugar content per dry biomass weight of 10%, 12%, 17% and 32% respectively [44].

Complete chemical hydrolysis (H_2SO_4) have been regularly implemented for the production of hydrolysate from lignocellulosic biomass [47, 48]. Lately, two-stage hydrolysis processes starting with mild chemical treatment (dilute sulfuric acid) and followed by enzymatic hydrolysis have gained popularity amongst industrial applications [49]. Corn-stover biomass hydrolysis required a pretreatment of the biomass with 0.5 M NaOH at 80 °C for 75 min [50]. However, these methods often generate inhibitory substances that might hinder or completely abolish the growth of microorganisms cultivated in the resulting hydrolysates. Furfural was found to elongate the lag-phase; while benzoic acid reduced growth rate and biomass yield [51]. Thus, complex detoxification step would be necessary prior to the fermentation, ensuing additional costs and tarnishing the eco-friendly aspect of the biofuel production process [36]. Accordingly, we opted to use a single-step enzymatic approach in this study that would allow efficient hydrolysis of algae biomass without the need for any pretreatment steps. Sterilization of *S. obtusiusculus* biomass was performed in a laboratory-scale autoclave at 120 °C for 15 min to eliminate the microbial contaminants present within the microalgae residue. This not considered as a pretreatment step since hydrolysis of hemicellulose and cellulose only starts at temperatures greater than 150 °C [37]. Efficient saccharification of *S. obtusiusculus* biomass by single-step enzymatic hydrolysis using a cellulase mix was possible. In fact, the Cellic CTec 2 combines a number of different enzymatic activities (exo-, endo-glucanase activity and proteinase activity). The optimal activity was obtained at an industrially relevant concentration of 1% (v/v) at 50.0 °C and pH 5.0 in 50 mM, sodium acetate buffer for 72 h. Quantitative biomass to glucose conversion ratio remained high, even when raising substrate amounts up to 200 g/L. Beyond this point, viscosity was too high for effective hydrolysis. Notably, the diverse heteropolymeric structure of algal cell wall might account for the low conversion efficiency of mannose and galactose [52]. Commonly available enzyme mixtures including Celli-Htec2 (Novozymes), Pectinex (Novozymes) and Fungamyl (Novozymes) failed to liberate total monosaccharides from these structures.

Lipid accumulation in oleaginous yeasts is usually triggered upon excess carbon and nutrient deficiency (e.g., nitrogen phosphate or sulfur). Lipid yields and fatty acid profile vary depending on the type and concentration of the carbon and nitrogen source [1, 7, 53]. Single-step enzymatic hydrolysis generated glucose-rich hydrolysate, the preferred monomeric sugar for microbial fermentation. A 10 KDa cross-filtration was subsequently implemented, and the permeate product (SH), exhibiting now nitrogen limitation and high C/N ratio, was used as fermentation media for high lipid accumulation in yeast.

The oleaginous yeast *Cutaneotrichosporon oleaginous* is able to metabolize a broad monosaccharide spectrum including hexoses and pentoses into intracellular TAGs [23]. This yeast was also able to grow well in a model medium with a carbohydrate mixture that resembled a typical microalgae derived-hydrolysate [54]. In this work, *C. oleaginosus*, as well as *C. curvatus* and *R. toruloides* were cultivated in *S. obtusiusculus* hydrolysate. Most interestingly, in contrast to the other two oleaginous yeasts, high-throughput Nile red analysis indicated that only *C. oleaginosus* was able to accumulate significant amounts of intracellular lipids when grown in SH.

Without any nutritional addition to the hydrolysate (biotin, yeast extract, pure glucose…), SH was utilized as the sole carbon source for lipid production in *C. oleaginosus.* The assessment was conducted on the basis of lipid accumulation. The results were evaluated along with the data from cultivation in the synthetic MNM—a medium known to induce lipid biosynthesis in oleaginous yeasts [55]. *C. oleaginosus* grew faster in SH media, in comparison with MNM. The yeast yielded 61% (g lipid/g DCW) of intracellular lipid when grown on MNM, and about 35% (g lipid/g DCW) when grown on SH. Following fermentation, *C. oleaginosus* achieved total lipid yield of 3.6 g/L when cultivated on SH media. In scaled-up experiments, SH

would prove a cost-effective alternative for the relatively expensive synthetic MNM media.

To establish nutrient limitation, the microalgae hydrolysate underwent ultrafiltration thus eliminating proteins and peptides and establishing a high C/N ratio. However, other factors besides nitrogen-limitation could induce lipogenesis in *C. oleaginosus*. Effect of phosphate and sulfur limitation on lipid accumulation in oleaginous yeasts have been previously reported [56]. High C/P ratio prompted high lipid yield in *R. toruloides* even in the presence of excess nitrogen [22]. For future work, soluble phosphates could be precipitated and removed by interaction with metal ions, such as Ca^{2+}, Mg^{2+}, or Fe^{3+} [57], and the resulting hydrolysate—now exhibiting high C/N and C/P ratios—could allow for even higher for lipid accumulation by *C. oleaginosus.*

Cutaneotrichosporon oleaginosus cultivated in *S. obtusiusculus* hydrolysate achieved a high growth rate and accumulated substantial amount of intracellular lipids. Previous research showed that this yeast accumulates lipid in the form of triacylglycerides, with a fatty acid (FA) profile consisting mainly of C16 and C18 FA [58]. Palmitic acid, stearic acid and oleic acid constitute the major raw material for downstream processing and subsequent conversion into green biofuels [59]. Furthermore, chemo-catalytic conversion of lipids produced by *C. oleaginosus* into biodiesel was achieved with a 98.9% w/w recovery [9]. The physical properties of resulting B20 - comparable to Soybean B20 - meet the ASTM requirements [9]. This FA profile makes SCOs from *C. oleaginosus* a suitable alternative for plant and vegetable oils.

Conclusion

This study demonstrated that *Scenedesmus obtusiusculus* biomass could be valorized as a substrate for microbial lipid production. A single-step enzymatic hydrolysis was implemented that efficiently released monomeric sugars from the biomass without the need for any pretreatment. This approach alleviated the need for detoxification steps, reduced upstream processing costs and maintained the eco-friendly aspect of biofuel production. The oleaginous yeast *C. oleaginosus* was able to grow fast and accumulate 3.6 g/L of lipids when cultivated on the microalgae hydrolysate, and the resulting microbial oil could be converted to high-grade biodiesel. Microalgae biomass offer value-added biofuel yield potential as compared to terrestrial plantation; biomass-to-fuel conversion processes are improved by necessitating no agricultural land, alleviating direct competition with food security and requiring low water and resource demand. Furthermore, the integration of yeast and algae species in a single SCO platform towards "zero concepts" with respect to emission and excess resources has recently been reported. In one study, the oleaginous microalgae *Phaeodactylum tricornutum* was supplemented with CO_2 supplied from the oleaginous yeast *C. curvatus* in a co-fermentation approach [32]. In another study focused on the holistic valorization of unexploited marine biomass, a waste-free, microbial oil-centered cyclic bio-refinery approach integrated the production of yeast lipids and animal feed with precious metal biosorbents [4]. In that respect, the algal effluent resulting from the ultra-filtration of the algae hydrolysate should be further characterized and profiled for possible added-value in the process described in this study.

Acknowledgement Open Access funding provided by Projekt DEAL. The authors acknowledge Prof. Dr. Sabine Mundt for providing *Scenedesmus obtusiusculus* (A189) residues. TB gratefully acknowledges funding of the Werner Siemens foundation for establishing the research field of Synthetic Biotechnology at the Technical University of Munich. This work was supported by the BMBF (German Federal Ministry of Education and Research, 03SF0446A). SY, DA and NM gratefully acknowledge funding by the Bavarian State Ministry for Environmental and Consumer affairs for funding of the project Resource-efficient PHB production processes within the project consortium BayBioTech (TP7, TLK01U-69045, http://www.baybiotech.de/startseite/). The authors gratefully acknowledge HPLC sample analysis conducted by Ms. Martina Haack.

Author contributions Conceptualization of the study was conducted jointly by FB, NM, and TB. The methodological approach was designed and carried out by FB and NM. Data validation was jointly carried out by SY, DA and NM. SY and DA prepared the original draft of the manuscript. The manuscript was jointly finalized by all authors.

Data availability All datasets generated for this study are included in the manuscript.

Compliance with ethical standards

Conflict of interest statement The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Awad D, Bohnen F, Mehlmer N, Brueck T (2019) Multi-factorial-guided media optimization for enhanced biomass and lipid

formation by the oleaginous yeast *Cutaneotrichosporon oleaginosus*. Front Bioeng Biotechnol 7:54
2. Masri MA, Younes S, Haack M, Qoura F, Mehlmer N, Brück T (2018) A seagrass-based biorefinery for generation of single-cell oils for biofuel and oleochemical production. Energy Technol 6(6):1026–1038
3. Knothe G (2008) "Designer" biodiesel: optimizing fatty ester composition to improve fuel properties. Energy Fuels 22(2):1358–1364
4. Masri MA, Jurkowski W, Shaigani P, Haack M, Mehlmer N, Brück T (2018) A waste-free, microbial oil centered cyclic bio-refinery approach based on flexible macroalgae biomass. Appl Energy 224:1–12
5. Smith VH, Sturm BS, Billings SA (2010) The ecology of algal biodiesel production. Trends Ecol Evol 25(5):301–309
6. Kyle DJ (2010) Future development of single cell oils. Single cell oils. Elsevier, New York, pp 439–451
7. Ratledge C, Wynn JP (2002) The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol 51:1–52
8. Bracharz F, Beukhout T, Mehlmer N, Bruck T (2017) Opportunities and challenges in the development of Cutaneotrichosporon oleaginosus ATCC 20509 as a new cell factory for custom tailored microbial oils. Microb Cell Fact 16(1):178. https://doi.org/10.1186/s12934-017-0791-9
9. McCurdy AT, Higham AJ, Morgan MR, Quinn JC, Seefeldt LC (2014) Two-step process for production of biodiesel blends from oleaginous yeast and microalgae. Fuel 137:269–276
10. Coradetti ST, Pinel D, Geiselman GM, Ito M, Mondo SJ, Reilly MC, Cheng Y-F, Bauer S, Grigoriev IV, Gladden JM, Simmons BA, Brem RB, Arkin AP, Skerker JM (2018) Functional genomics of lipid metabolism in the oleaginous yeast Rhodosporidium toruloides. eLife 7:e32110. https://doi.org/10.7554/elife.32110
11. Kosa M, Ragauskas AJ (2011) Lipids from heterotrophic microbes: advances in metabolism research. Trends Biotechnol 29(2):53–61
12. Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34(1):1–5
13. Rakicka M, Lazar Z, Dulermo T, Fickers P, Nicaud JM (2015) Lipid production by the oleaginous yeast Yarrowia lipolytica using industrial by-products under different culture conditions. Biotechnol Biofuels 8:104. https://doi.org/10.1186/s13068-015-0286-z
14. Azambuja SPH, Bonturi N, Miranda EA, Gombert AK (2018) Physiology and lipid accumulation capacity of different *Yarrowia lipolytica* and *Rhodosporidium toruloides* strains on glycerol. bioRxiv. https://doi.org/10.1101/278523
15. Rakicka M, Lazar Z, Dulermo T, Fickers P, Nicaud JM (2015) Lipid production by the oleaginous yeast Yarrowia lipolytica using industrial by-products under different culture conditions. Biotechnol Biofuels 8(1):104
16. Wang R, Wang J, Xu R, Fang Z, Liu A (2014) Oil production by the oleaginous yeast *Lipomyces starkeyi* using diverse carbon sources. BioResources 9(4):7027–7040
17. Moon NJ, Hammond E, Glatz BA (1978) Conversion of cheese whey and whey permeate to oil and single-cell protein1. J Dairy Sci 61(11):1537–1547
18. Evans CT, Ratledge C (1983) A comparison of the oleaginous yeast, *Candida curvata*, grown on different carbon sources in continuous and batch culture. Lipids 18(9):623–629
19. Liang Y, Jarosz K, Wardlow AT, Zhang J, Cui Y (2014) Lipid production by *Cryptococcus curvatus* on hydrolysates derived from corn fiber and sweet sorghum bagasse following dilute acid pretreatment. Appl Biochem Biotechnol 173(8):2086–2098
20. Chi Z, Ahring BK, Chen S (2012) Oleaginous yeast *Cryptococcus curvatus* for biofuel production: ammonia's effect. Biomass Bioenerg 37:114–121
21. Iassonova DR (2009) Lipid synthesis and encapsulation by *Cryptococcus curvatus*. Iowa State University, Ames
22. Wu S, Hu C, Zhao X, Zhao ZK (2010) Production of lipid from *N*-acetylglucosamine by *Cryptococcus curvatus*. Eur J Lipid Sci Technol 112(7):727–733
23. Gujjari P, Suh S-O, Coumes K, Zhou JJ (2011) Characterization of oleaginous yeasts revealed two novel species: trichosporon *Cacaoliposimilis* sp. nov. and *Trichosporon oleaginosus* sp. nov. Mycologia 103(5):1110–1118
24. Masri MA, Garbe D, Mehlmer N, Brück TB (2019) A sustainable, high-performance process for the economic production of waste-free microbial oils that can replace plant-based equivalents. Energy Environ Sci 12(9):2717–2732
25. Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG (2011) Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol 90(4):1219–1227
26. Bai A, Popp J, Pető K, Szőke I, Harangi-Rákos M, Gabnai Z (2017) The significance of forests and algae in CO_2 balance: a hungarian case study. Sustainability 9(5):857
27. Bhola V, Swalaha F, Ranjith Kumar R, Singh M, Bux F (2014) Overview of the potential of microalgae for CO_2 sequestration. Int J Environ Sci Technol 11(7):2103–2118. https://doi.org/10.1007/s13762-013-0487-6
28. Sayre R (2010) Microalgae: the potential for carbon capture. Bioscience 60(9):722–727. https://doi.org/10.1525/bio.2010.60.9.9
29. Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK (2014) Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 116:566–577
30. Guo M, Cheng S, Chen G, Chen J (2019) Improvement of lipid production in oleaginous yeast *Rhodosporidium toruloides* by ultraviolet mutagenesis. Eng Life Sci 19(8):548–556
31. André A, Chatzifragkou A, Diamantopoulou P, Sarris D, Philippoussis A, Galiotou-Panayotou M, Komaitis M, Papanikolaou S (2009) Biotechnological conversions of bio-diesel-derived crude glycerol by Yarrowia lipolytica strains. Eng Life Sci 9(6):468–478
32. Dillschneider R, Schulze I, Neumann A, Posten C, Syldatk C (2014) Combination of algae and yeast fermentation for an integrated process to produce single cell oils. Appl Microbiol Biotechnol 98(18):7793–7802. https://doi.org/10.1007/s00253-014-5867-4
33. Achinas S, Achinas V, Euverink GJW (2017) A technological overview of biogas production from biowaste. Engineering 3(3):299–307. https://doi.org/10.1016/J.ENG.2017.03.002
34. Htet AN, Noguchi M, Ninomiya K, Tsuge Y, Kuroda K, Kajita S, Masai E, Katayama Y, Shikinaka K, Otsuka Y, Nakamura M, Honda R, Takahashi K (2018) Application of microalgae hydrolysate as a fermentation medium for microbial production of 2-pyrone 4,6-dicarboxylic acid. J Biosci Bioeng 125(6):717–722. https://doi.org/10.1016/j.jbiosc.2017.12.026
35. Wirth R, Lakatos G, Böjti T, Maróti G, Bagi Z, Kis M, Kovács A, Ács N, Rákhely G, Kovács KL (2015) Metagenome changes in the mesophilic biogas-producing community during fermentation of the green alga *Scenedesmus obliquus*. J Biotechnol 215:52–61
36. Huang H, Guo X, Li D, Liu M, Wu J, Ren H (2011) Identification of crucial yeast inhibitors in bio-ethanol and improvement of fermentation at high pH and high total solids. Bioresour Technol 102(16):7486–7493
37. Silveira MHL, Morais ARC, da Costa Lopes AM, Olekszyszen DN, Bogel-Łukasik R, Andreaus J, Pereira Ramos L (2015) Current pretreatment technologies for the development of cellulosic ethanol and biorefineries. Chemsuschem 8(20):3366–3390

38. Shetlar M, Masters YF (1957) Use of thymol-sulfuric acid reaction for determination of carbohydrates in biological material. Anal Chem 29(3):402–405
39. Kjeldhal J (1883) A new method for estimation of nitrogen in organic compounds. Z Anal Chem 22:366
40. Folch J, Lees M, Sloane-Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226(1):497–509
41. Chemists AoOA (1990) Official methods of analysis: changes in official methods of analysis made at the annual meeting. Supplement, vol 15. Association of Official Analytical Chemists,
42. Suutari T, Priha P, Laakso S (1993) Temprature shifts in regulation of lipids accumulated by *Lippmyces starkeyi*, p. 891–894
43. Sitepu I, Ignatia L, Franz A, Wong D, Faulina S, Tsui M, Kanti A, Boundy-Mills K (2012) An improved high-throughput Nile red fluorescence assay for estimating intracellular lipids in a variety of yeast species. J Microbiol Methods 91(2):321–328
44. Spolaore P, Joannis-Cassan C, Duran E, Isambert A (2006) Commercial applications of microalgae. J Biosci Bioeng 101(2):87–96
45. Posten C (2009) Design principles of photo-bioreactors for cultivation of microalgae. Eng Life Sci 9(3):165–177
46. Li Q, Du W, Liu D (2008) Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol 80(5):749–756
47. Morales-delaRosa S, Campos-Martin JM, Fierro JL (2014) Optimization of the process of chemical hydrolysis of cellulose to glucose. Cellulose 21(4):2397–2407
48. Tsigie YA, Wang C-Y, Kasim NS, Diem Q-D, Huynh L-H, Ho Q-P, Truong C-T, Ju Y-H (2012) Oil production from *Yarrowia lipolytica* Po1g using rice bran hydrolysate. BioMed Research International, Lonon
49. Zhang Y, Zhang M, Reese RA, Zhang H, Xu B (2016) Real-time single molecular study of a pretreated cellulose hydrolysis mode and individual enzyme movement. Biotechnol Biofuels 9(1):85
50. Gong Z, Shen H, Yang X, Wang Q, Xie H, Zhao ZK (2014) Lipid production from corn stover by the oleaginous yeast *Cryptococcus curvatus*. Biotechnol Biofuels 7(1):158
51. Zha Y, Muilwijk B, Coulier L, Punt PJ (2012) Inhibitory compounds in lignocellulosic biomass hydrolysates during hydrolysate fermentation processes. J Bioprocess Biotechniq 2(1):112–122
52. Takeda H (1988) Classification of Chlorella strains by cell wall sugar composition. Phytochemistry 27(12):3823–3826
53. Granger LM, Perlot P, Goma G, Pareilleux A (1993) Efficiency of fatty acid synthesis by oleaginous yeasts: prediction of yield and fatty acid cell content from consumed C/N ratio by a simple method. Biotechnol Bioeng 42(10):1151–1156
54. Meo A, Priebe XL, Weuster-Botz D (2017) Lipid production with Trichosporon oleaginosus in a membrane bioreactor using microalgae hydrolysate. J Biotechnol 241:1–10
55. L-j MA, H-m WANG, Y-f WEN, H-l JIANG, D-h XUE (2008) Optimized culture medium and fermentation conditions for lipid production with filamentous fungus. J Changchun Univ Technol 4:023
56. Zhang G, French WT, Re Hernandez, Hall J, Sparks D, Holmes WE (2011) Microbial lipid production as biodiesel feedstock from *N*-acetylglucosamine by oleaginous microorganisms. J Chem Technol Biotechnol 86(5):642–650
57. Jenkins D, Ferguson JF, Menar AB (1971) Chemical processes for phosphate removal. Water Res 5(7):369–389
58. Kourist R, Bracharz F, Lorenzen J, Kracht ON, Chovatia M, Daum C, Deshpande S, Lipzen A, Nolan M, Ohm RA (2015) Genomics and transcriptomics analyses of the oil-accumulating basidiomycete yeast Trichosporon oleaginosus: insights into substrate utilization and alternative evolutionary trajectories of fungal mating systems. MBio 6(4):e00918–e01015
59. Zhao C, Brück T, Lercher JA (2013) Catalytic deoxygenation of microalgae oil to green hydrocarbons. Green Chem 15(7):1720–1739