Towards an optimized lipid production with unconventional oleaginous yeasts

- Process optimization and evaluation of lipid downstream methods

Zur Erlangung des akademischen Grades einer

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DISSERTATION

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Preamble

This doctoral thesis concerns about the single cell oil production optimization with unconventional oleaginous yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194. Parts of this work have been published as peer reviewed research articles or are submitted as such. The text of such paragraphs is partially identical to the content of the publications. Layout, citation style, figures and formatting have been modified analogue to the style of this thesis. All articles have been drafted during this work and describe the major results

The content of the chapter 3 is dealing with the upstream process optimization of *S. podzolica* and has been published in the journal *Biotechnology for Biofuels:*

Gorte, O., Kugel, M. & Ochsenreither, K. (2020). ‘’Optimization of carbon source efficiency for lipid production with the oleaginous yeast *Saitozyma podzolica* DSM 27192 applying automated continuous feeding. ‘’ *Biotechnol Biofuels* **13,** 181. doi.org/10.1186/s13068-020-01824-7.

Chapter 4 is about cell disruption for downstream processing of SCO of *S. podzolica* and *A. porosum* and is content of the publication:

Gorte, O., Hollenbach, R., Papachristou, I., Steinweg, C., Silve, A., Frey, W., Syldatk, C., Ochsenreither, K. (2020). ‘’Evaluation of Downstream Processing, Extraction, and Quantification Strategies for Single Cell Oil Produced by the Oleaginous Yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194. ‘’ *Front. Bioeng. Biotechnol.* 8, 1–15. doi:10.3389/fbioe.2020.00355.

Chapter 5 elaborates on the PEF pre-treatment of fresh *S. podzolica* biomass and is content of the publication:

Gorte, O., Nazarova, N., Papachristou, I., Wüstner, R., Leber, K., Syldatk, C., Ochsenreither, K., Frey, W., Silve, A. (2020). ‘’Pulsed Electric Field Treatment Promotes Lipid Extraction on Fresh Oleaginous Yeast *Saitozyma podzolica* DSM 27192. ‘’ *Front. Bioeng. Biotechnol.* 8, 1–14. doi:10.3389/fbioe.2020.575379.

List of publications

Peer reviewed original publications implied in this thesis

- **Optimization of carbon source efficiency for lipid production with the oleaginous yeast *Saitozyma podzolica* DSM 27192 applying automated continuous feeding**

  Gorte, Olga, Michaela Kugel and Katrin Ochsenreither (2020)

  Biotechnol Biofuels **13,** 181

  DOI: 10.1186/s13068-020-01824-7 (online 02. November 2020)

- **Pulsed electric field treatment promotes lipid extraction on fresh oleaginous yeast *Saitozyma podzolica* DSM 27192**

  Gorte, Olga, Natalja Nazarova, Ioannis Papachristou, Rüdiger Wüstner, Klaus Leber, Christoph Syldatk, Katrin Ochsenreither, Wolfgang Frey and Aude Silve (2020b)

  Frontiers in Bioengineering and Biotechnology. 8:575379

  DOI: 10.3389/fbioe.2020.575379 (online 11. September 2020)

- **Evaluation of downstream processing, extraction, and quantification strategies for single cell oil produced by the oleaginous yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194**

  Gorte, Olga*, Rebecca Hollenbach*, Ioannis Papachristou, Christian Steinweg, Aude Silve, Wolfgang Frey, Christoph Syldatk and Katrin Ochsenreither (2020a)

  Frontiers in Bioengineering and Biotechnology. 8:355

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  ***Co-first authorship**

Peer reviewed original publications <u>not</u> included in this thesis

- **Microwave-assisted one-pot lipid extraction and glycolipid production from oleaginous yeast *Saitozyma podzolica* in sugar alcohol-based media**

  André Delavault, Katarina Ochs, **Olga Gorte,** Christoph Syldatk, Erwann Durand and Katrin Ochsenreither (2021)

  Molecules 26, 470.

  DOI: 10.3390/molecules26020470 (online 18. January 2021)

- **In silico proteomic analysis provides insights into phylogenomics and plant biomass deconstruction potentials of the Tremelalles**

  Aliyu, Habibu*, **Olga Gorte***, Xinhai Zhou, Anke Neumann and Katrin Ochsenreither (2020b)

  Frontiers in Bioengineering and Biotechnology. 8:226

  DOI: 10.3389/fbioe.2020.00226 (online 03. April 2020)

  ***Co-first authorship**

- **Genomic insights into the lifestyles, functional capacities and oleagenicity of members of the fungal family *Trichosporonaceae***

  Aliyu, Habibu, **Olga Gorte**, Pieter De Maayer, Anke Neumann and Katrin Ochsenreither (2020a)

  Scientific Reports 10: 2780

  DOI: 10.1038/s41598-020-59672-2 (online 17. February 2020)

- **Continuous self-provided fermentation for microbial lipids production from acetate by using oleaginous yeasts *Cryptococcus podzolicus* and *Trichosporon porosum***

  Qian, Xiujuan, **Olga Gorte**, Lin Chen, Wenming Zhang, Weiliang Dong, Jiangfeng Ma, Fengxue Xin, Min Jiang and Katrin Ochsenreither (2020)

  Renewable Energy 146: 737–43

  DOI: 10.1016/j.renene.2019.07.013 (online 02. July 2019)

- **Co-production of single cell oil and gluconic acid using oleaginous *Cryptococcus podzolicus* DSM 27192**

  Qian, Xiujuan, **Olga Gorte**, Lin Chen, Wenming Zhang, Weiliang Dong, Jiangfeng Ma, Min Jiang, Fengxue Xin and Katrin Ochsenreither (2019)

  Biotechnology for Biofuels 12:127

  DOI: 10.1186/s13068-019-1469-9 (online 21. May 2019)

- **Draft genome sequence of the oleaginous yeast *Saitozyma podzolica* (syn. *Cryptococcus podzolicus*) DSM 27192**

  Aliyu, Habibu*, **Olga Gorte***, Anke Neumann, and Katrin Ochsenreither (2019) *Microbiology Resource Announcements* 8:e01676-18

  DOI: 10.1128/MRA.01676-18 (online 21. February 2019)

  ***Co-first authorship**

- **Draft genome sequence of the oleaginous yeast *Apiotrichum porosum* (syn. *Trichosporon porosum*) DSM 27194⁺**

  Gorte, Olga*, Habibu Aliyu*, Anke Neumann, and Katrin Ochsenreither (2019) *Journal of Genomics* 7: 11–13

  DOI: 10.7150/jgen.32210 (online 29. January 2019)

  ***Co-first authorship**

  ⁺Cover feature of *Journal of Genomics* Vol.7, 2019

- **Sustainable carbon sources for microbial organic acid production with filamentous fungi**

  Dörsam, Stefan, Jana Fesseler, **Olga Gorte**, Thomas Hahn, Susanne Zibek, Christoph Syldatk and Katrin Ochsenreither (2017)

  Biotechnology for Biofuels 10:242

  DOI: 10.1186/s13068-017-0930-x (online 23.Oktober 2017)

Conference Poster

- **Single cell oil downstream processing optimization of a newly isolated oleaginous yeast *Saitozyma podzolica* DSM 27192**

  Gorte, Olga, Rebecca Hollenbach and Katrin Ochsenreither

  VAAM Jahrestagung (2019), Mainz

Abstract

Increasing population growth as well as the associated energy and resources demand bear decisive ecological, economic and societal challenges. The foreseeable depletion of crude oil and the urgent need for the reduction of greenhouse gas emissions to prevent further climate change, highlight the need for competitive processing of renewable alternatives over fossil oil based or non-sustainable products. Using oleaginous plants as alternative source of oil raises the competition between food and raw materials production, since agricultural land is limited. Microbial oils, or single cell oil (SCO), produced by yeast, microalgae and fungi may overcome all these challenges and act as potential feedstock for crude and plant oil for various applications such as fuels, additives for food and cosmetics, and building blocks for oleochemicals. SCO produced by oleaginous yeasts, resembling plant oils, is especially of interest, since its production is independent of season, climate, location and high growth rate and oil productivity can be achieved due to short duplication time and the possibility to up-scale cultivation processes, which makes an industrial use realistic. Furthermore, sustainable carbon sources, e.g., lignocellulosic material and waste from food and other industries can be metabolized, enabling waste recycling and guaranteeing a sustainable process. Commercial microbial lipids production, however, is still not profitable and research on process optimization and cost reduction is required.

Therefore, this thesis provides a broad investigation on SCO production optimization and evaluation of process upstream and lipid downstream. For this purpose, the unconventional oleaginous yeast strains *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194 were examined, because of their interesting biotechnological potential to produce in addition to SCO further valuable product, i.e. organic acids or complex lignocellulosic plant biomass degrading enzymes.

Since carbon source accounts for the main costs in SCO production, for cost-efficiency efficient use of cheap materials is preferable and wastage of carbon source has to be avoided. Thus, this thesis reports in chapter 3 on the process optimization of *S. podzolica* using glucose or xylose aiming to reduce the applied carbon source amount without sacrificing lipid productivity. Firstly, by optimizing the process parameters, temperature and pH, lipid productivity was enhanced by 40%. Thereupon, by optimizing the two-phase strategy with an initial batch phase and a subsequent fed-batch phase for lipid production in which a constant carbon concentration of about 10 g/L was maintained, resulted in carbon saving of ~41% of total glucose and ~26% of total xylose. By performing the automated continuous carbon feed the total carbon uptake

was improved to 90.84 ± 3.78% for glucose and 92.30 ± 1.61 for xylose and thus prevented waste of unused carbon in the cultivation medium. In addition, on glucose the reduced sugar cultivation led to 28% higher biomass growth and 19% increase of lipid content. By using xylose, the by-product xylonic acid was identified for the first time as by-product of *S. podzolica*.

Besides the challenges in the upstream process, the downstream processing of SCO, as being an intracellular product, is also costly and prevents the broader application of SCO. Universal downstream strategies effective for all yeast species do not exist and methods have to be developed for each yeast species individually due to differences in cell wall composition. Direct transesterification of freeze-dried biomass is widely used for analytical purposes of biodiesel production at laboratory scale, but it is energy intensive and, therefore, expensive.

For these reasons this thesis further aimed in chapter 4 to evaluate three industrially relevant cell disruption methods combined with three extraction systems for the SCO extraction of by freezing or freeze-drying preserved biomass of the two unconventional yeasts based on cell disruption efficiency, lipid yield, and oil quality. Bead milling (BM) and high pressure homogenization (HPH) were effective cell disruption methods in contrast to ultrasound (U). By combining HPH (95% cell disruption efficiency) with ethanol-hexane-extraction highest lipid titer of *S. podzolica* was obtained, which was 2.7 times higher than with the least suitable combination (ultrasound + Folch). The most energy efficient method for lipid extraction of *S. podzolica* was as well HPH prior to EH extraction with 342 MJ/kg_{lipid}. *A. porosum* was less affected by cell disruption attempts. Here, the highest disruption efficiency was 74% after BM and the most efficient lipid recovery method was direct acidic transesterification after freeze drying resulting as to fatty acid methyl ester (FAME) derivatized lipids.

However, latter downstream methods consumed very high amounts of energy and would be consequently and too energy consuming for potential industrial scale application without further optimization. Therefore, a lipid recovery method, which is scalable and energy-efficient, was finally investigated in this thesis. Pulsed electric field (PEF) treatment is such an innovative alternative pre-treatment method, which relies on the application of short intense electric pulses, that when delivered to biological cells, induce an increase in transmembrane voltage and subsequently an increase in the permeability of the membrane.

This thesis indicates in chapter 5 the use of PEF to enhance lipid extraction yield using extraction with ethanol-hexane solvent system on fresh *S. podzolica* biomass. PEF-treatment was applied on the yeast suspension either directly after harvesting (unwashed route) or after a washing step (washed route), which induced a reduction of conductivity by a factor eight. The

washed route resulted in the most efficient lipid extraction yield enabled by PEF-treatment increasing from 26% (untreated) to 99% of total lipid. The energy input for the PEF-treatment never exceeded 150 kJ per liter of initial suspension. The best lipid recovery results were obtained using pulses of 1 µs, an electric field of 40 kV/cm and it required slightly less than 11 MJ/kg_{lipid}. Compared to lipid recovery using the best mechanical disruption method HPH prior the EH ~97% of energy was saved by using PEF. This amount of energy can be further reduced by optimizing the treatment and especially by increasing the concentration of the treated biomass. The process can be easily up-scaled and does not require any expensive handling of the biomass such as freezing or freeze-drying.

In conclusion all these findings provide a broad view of different cultivation process strategies with subsequent comparison and evaluation of the lipid production with *S. podzolica*. Additionally, new biotechnological characteristics of this yeast were highlighted regarding the ability to produce valuable organic acids from sustainable and renewable sugars. With respect to lipid downstream processing, this thesis clearly indicates cell disruption as the decisive step for SCO extraction. At disruption efficiencies of >90%, lipids can be extracted at high yields, whereas at lower cell disruption efficiencies, considerable amounts of lipids will not be accessible for extraction regardless of the solvents used. However, many combinations of biomass pre-treatments are costly and energy consuming, therefore this thesis demonstrates the high potential of PEF-treatment in the downstream processing of fresh oleaginous yeast biomass. Furthermore, it was shown that hexane-ethanol which is commonly used for extraction of algal lipids is also highly efficient for yeasts.

Zusammenfassung

Das kontinuierliche Bevölkerungswachstum sowie der damit verbundene erhöhte Energie- und Ressourcenbedarf birgt entscheidende ökologische, wirtschaftliche und gesellschaftliche Herausforderungen. Die absehbare Verknappung des Rohöls und die dringende Notwendigkeit der Reduzierung der Treibhausgasemissionen zur Verhinderung weiterer Klimaschäden verdeutlichen den Bedarf einer wettbewerbsfähigen Produktion erneuerbarer und nachhaltiger Alternativen. Die Erzeugung von Pflanzenölen als erneuerbare Alternative zu Rohöl ist aufgrund der Tank-oder-Teller-Diskussion negativ behaftet und nicht mit der limitierten Verfügbarkeit von landwirtschaftlicher Nutzfläche vereinbar.

Mikrobielle Öle oder Einzeller-Öle (Single Cell Oil, (SCO)), die von Hefen, Mikroalgen und Pilzen hergestellt werden, können all diese Herausforderungen überwinden und als potenzielle Ausgangsstoffe für Roh- und Pflanzenöl für verschiedene Anwendungen, wie Kraftstoffe, Zusatzstoffe für Lebensmittel und Kosmetika, sowie als Bausteine für Oleochemikalien, dienen. Das von oleogenen Hefen produzierte SCO hat ein Pflanzen-Öl ähnliches Profil und ist von besonderem Interesse, da seine Produktion unabhängig von Jahreszeit, Klima und Standort ist. Außerdem vermehren sich Hefen mit hohen Wachstumsraten und erreichen eine gute Ölproduktivität. Ferner ist die Möglichkeit der Skalierung der Hefe-Kultivierung in Bioreaktoren einfach zu verwirklichen, was eine industrielle Nutzung realistisch erscheinen lässt. Darüber hinaus können nachhaltige Kohlenstoffquellen, wie z.B. lignocellulose-haltiges Material und Abfälle aus der Lebensmittel- oder anderen Industrien verstoffwechselt werden, was ein Abfallrecycling ermöglicht und einen nachhaltigen Prozess garantiert. Die kommerzielle Herstellung von mikrobiellen Lipiden ist jedoch immer noch nicht rentabel und Forschung zur Prozessoptimierung und Kostenreduzierung ist erforderlich.

Daher bietet diese Dissertation eine umfassende Untersuchung zur Optimierung und Einschätzung der SCO-Produktion in der Upstream und Lipid-Downstream Phase. Zu diesem Zweck wurden unkonventionelle oleogene Hefestämme *Saitozyma podzolica* DSM 27192 und *Apiotrichum porosum* DSM 27194 wegen ihres interessanten biotechnologischen Potentials untersucht. Beide können neben SCO zusätzlich wertvolle Produkte erzeugen, wie z.B. organische Säuren oder Lignocellulose abbauende Enzyme.

Da die Kohlenstoffquelle der Hauptkostenfaktor der SCO-Produktion ist, sind für die Kosteneffizienz möglichst billige Materialien und geringe Mengen vorzuziehen. Daher wurde in dieser Arbeit im Kapitel 3 zunächst die Prozessoptimierung mit *S. podzolica* angestrebt. Dazu wurden

Glukose oder Xylose als Kohlenstoffquellen verwendet mit dem Ziel, die Menge der eingesetzten Zucker zu reduzieren, ohne jedoch die Lipidproduktivität zu beeinträchtigen. Zuerst wurde durch die Optimierung der Prozessparameter, Temperatur und pH, die Lipidproduktivität um 40% gesteigert. Daraufhin wurde die Zwei-Phasen-Strategie mittels einer anfänglichen Batch-Phase und einer anschließenden Fed-Batch-Phase optimiert, in der eine konstante Kohlenstoffkonzentration von etwa 10 g/L aufrechterhalten wurde. Dies führte zu einer Kohlenstoffeinsparung von ~41% der gesamten Glukose und ~26% der gesamten Xylose. Durch die Etablierung der automatisierten, kontinuierlichen Kohlenstoffzufuhr wurde die Verwertung des Gesamtkohlenstoffes auf 90,84 ± 3,78% für Glukose und 92,30 ± 1,61% für Xylose verbessert. Somit wurde die Verschwendung von ungenutztem Kohlenstoff im Kulturmedium minimiert. Darüber hinaus führte bei der Glukose Fermentation der reduzierte Zuckergehalt zu einem 28% höheren Wachstum der Biomasse und einer 19%igen Steigerung des Lipidgehalts. Bei der Verwendung von Xylose wurde zum ersten Mal Xylonsäure als Nebenprodukt von *S. podzolica* identifiziert.

Zusätzlich zu den Herausforderungen im Upstream der SCO-Produktion, ist auch die nachgeschaltete Lipidverarbeitung kostspielig und verhindert die breitere Anwendung von SCO, da es sich um ein intrazelluläres Produkt handelt. Es gibt keine universellen Downstream-Strategien, die für alle Hefespezies wirksam sind. Methoden müssen individuell entwickelt werden, aufgrund der Unterschiede in der Zellwand-Zusammensetzung. Die direkte Transesterifizierung von gefriergetrockneter Biomasse ist für analytische Zwecke für die Biodieselproduktion im Labor-Maßstab weit verbreitet, aber sie ist energieintensiv und daher teuer.

Aus diesen Gründen zielte Kapitel 4 dieser Arbeit darauf ab, drei industriell relevante Zellaufschlussmethoden in Kombination mit drei Extraktionssystemen für die SCO-Aufarbeitung von konservierter, gefrorener oder gefriergetrockneter, Biomasse der beiden unkonventionellen Hefen zu evaluieren. Dabei sollte die Zellaufschlusseffizienz, Lipidausbeute und Ölqualität im Fokus stehen. Kugelmühle (bead-milling (BM)) und Hochdruckhomogenisierung (high-pressure homogenization (HPH)) waren effektive Zellaufschlussmethoden im Gegensatz zum Ultraschall (ultrasound (U)). Die Kombination von HPH (95% Zellaufschlusseffizienz) mit Ethanol-Hexan-Extraktion (EH) wurde als effektivste Aufreinigungsmethode für Gesamtlipid von *S. podzolica* identifiziert, diese war 2,7-mal effizierter als bei der am wenigsten geeigneten Kombination (Ultraschall + Folch). Die energieeffizienteste Methode zur Lipidextraktion von *S. podzolica* war ebenfalls HPH vor der EH-Extraktion mit 342 MJ/kg_{Lipid}. *A. porosum* wurde

durch diese Zellaufschlussversuche weniger beeinträchtigt. Hier lag die höchste Zellausschlusseffizienz nach BM bei 74% und die effizienteste Methode zur Lipid-Rückgewinnung war die direkte saure Transesterifizierung in Form von Fettsäuremethylestern nach Gefriertrocknung.

Letztere Aufreinigungsmethoden verbrauchten jedoch sehr viel Energie und wären daher ohne weitere Optimierung für eine mögliche Anwendung im industriellen Maßstab zu energieaufwändig. Daher wurde in dieser Arbeit schließlich eine Methode untersucht, die skalierbar und energieeffizient ist. Die Behandlung mit gepulsten elektrischen Feldern (pulsed electric field (PEF)) ist solch eine innovative alternative Vorbehandlungsmethode. Sie beruht auf der Anwendung kurzer, intensiver elektrischer Impulse, die, wenn sie an biologische Zellen abgegeben werden, eine Erhöhung der Transmembranspannung und infolgedessen eine Erhöhung der Permeabilität der Membran bewirken.

Somit zeigt diese Dissertation in Kapitel 5 auf, dass durch die Verwendung von PEF eine Verbesserung der Lipid-Rückgewinnung aus frischer Biomasse von *S. podzolica* ermöglicht werden kann. Die Lipid-Rückgewinnung wurde durch Extraktion mit einem Ethanol-Hexan-Lösungsmittelsystem durchgeführt. Die PEF-Behandlung wurde auf die Hefesuspension entweder direkt nach der Ernte (ungewaschener Ansatz) oder nach einem Waschschritt (gewaschener Ansatz) angewendet, was eine Verringerung der Leitfähigkeit der Hefesuspension um den Faktor acht bewirkte. Die beste Ausbeute der Lipidextraktion lieferte der gewaschene Ansatz, hier stieg die Effizienz der Lipidextraktion von 26% (unbehandelt) auf 99% des gesamten Lipidgehalts. Der Energieaufwand für die PEF-Behandlung betrug nie mehr als 150 kJ pro Liter der Ausgangssuspension. Die besten Ergebnisse bei der Lipidrückgewinnung wurden mit Pulsen von 1 µs, einem elektrischen Feld von 40 kV/cm und einem Energiebedarf von etwas weniger als 11 MJ/kg_{Lipid} erzielt. Im Vergleich zur Lipid-Wiedergewinnung mit der besten mechanischen Aufschlussmethode HPH-EH wurden durch die Verwendung von PEF ~97% an Energie eingespart. Diese Energiemenge kann durch Optimierung der PEF-Behandlung und insbesondere durch Erhöhung der Konzentration der behandelten Biomasse weiter reduziert werden. Das Verfahren kann leicht hochskaliert werden und erfordert keine kostspielige Vorbehandlung der Biomasse, wie Gefrieren oder Gefriertrocknen.

Zusammenfassend lässt sich sagen, dass all diese Ergebnisse einen breiten Überblick über verschiedene Strategien der Kultivierungsprozesse mit *S. podzolica* bieten, sowie deren Vergleich und Evaluierung hinsichtlich der Lipidproduktion. Darüber hinaus wurden neue biotechnologische Eigenschaften dieser Hefe, wie die Fähigkeit wertvolle organische Säuren aus nachhalti-

gen und erneuerbaren Zuckern zu produzieren, hervorgehoben. In Bezug auf die Lipid-Rückgewinnung weist diese Arbeit eindeutig auf den Zellaufschluss, als den entscheidenden Schritt für die SCO-Extraktion, hin. Bei Aufschlusseffizienzen von > 90% kann SCO mit hoher Ausbeute extrahiert werden, während bei niedrigeren Zellaufschlusseffizienzen beträchtliche Mengen an Lipiden unabhängig von den verwendeten Lösungsmitteln nicht für die Extraktion zugänglich sind. Viele Kombinationen von Biomasse-Vorbehandlungen sind jedoch kostspielig und energieaufwendig, daher zeigt diese Arbeit das hohe Potenzial der PEF-Behandlung bei der SCO Aufarbeitung von frischer oleogener Hefebiomasse. Darüber hinaus wurde gezeigt, dass Ethanol-Hexan, das üblicherweise für die Extraktion von Algenlipiden verwendet wird, auch für Hefen sehr effizient ist.

Table of contents

1 Introduction

1 - Introduction

Our main sources for energy and material resources are fossil fuels and petrochemicals derived from crude oil and natural gas. The use of that has contributed enormously to the development of the prosperity of a substantial part of the world's population. However, the reserves of crude oil are not infinite. At the end of 2018 the proven crude oil reserves were stated at 1,498 billion barrels of oil equivalent (= 209,720 Mtoe (million tonnes of oil equivalent)) (OPEC, 2019). In comparison in 2018 the worlds' oil demand was 4,453 Mtoe (IEA, 2019). By the assumption of stable annual global oil demand, the reserves will last for approximately ± 50 more years, without taking into account the population growth and with that associated increased need for energy and resources. Indeed, it is well known that the excessive use and combustion of fossil material bears decisive ecological problems, such as destruction of land and habitats for drilling or mining, environmental pollution, increase of greenhouse gas emissions and consequential climate change. In addition it raises economic concerns about energy security as well as fossil fuel price volatility (Vasudevan and Briggs, 2008; Hochman et al., 2012). Therefore, the transition to renewable resources is urgently needed and bioenergy and bio-based materials gained in importance for industrial application in the last decades. Fats and oils of animal but mainly plant origin became essential raw materials (Metzger and Bornscheuer, 2006; Biermann et al., 2011). Soybean, palm, rapeseed and sunflower oil are the most prominent renewable oil sources for chemical industry (Metzger and Bornscheuer, 2006).

As a renewable alternative to fossil fuels biodiesel gained attention as an environmentally friendly fuel, since it is produced mainly from vegetable oil or to some extent of animal fats, and it is biodegradable (Vasudevan and Briggs, 2008). Moreover its production was of interest for the development of domestic and more secure fuel supplies (Hoekman et al., 2012) and to loosen the dependency on oil import. However, only first generation of biodiesel, produced of edible oils, was realized worldwide, which led to the expansion of food crops to produce energy. The competition between food and raw materials production was raised as ''food versus fuel'' debate, since agricultural land is limited (Lee and Lavoie, 2013). Into the bargain an expansion of oil seed cultures leads to forest land destruction (Escobar et al., 2009). Additionally, the exploitation of agricultural area for biofuel production contributed to price increase of e.g. soybean and corn (Hochman et al., 2012). By considering these detriments and taking into account that production costs of biodiesel are still high compared to fossil fuels, a larger commercial use of biodiesel and the substitution of petroleum based fuels is still hampered (Hoekman et al., 2012; Lee and Lavoie, 2013; Singh et al., 2019).

Microbial oils, known as single cell oils (SCO), produced by yeast, microalgae and fungi may overcome all these challenges and be potential substitutes for crude and plant oil for various applications, such as fuels, additives for the food and cosmetic industry and building blocks for oleochemicals (Ochsenreither et al., 2016; Probst et al., 2016; Vasconcelos et al., 2019). SCOs are intracellular storage lipids comprising triacylglycerols (TAGs) inhabiting a similar lipid profile as plant oils. Oleaginous yeasts are one of the most promising candidate for sustainable bio-based production, because of the short cell duplication time and resulting fast biomass formation and high productivities. The production can be realized in common biotechnological fermenters with minimal area demand and is therefore independent of season, climate and location (Ageitos et al., 2011; Ochsenreither et al., 2016). Full sustainability can be achieved by using a wide range of cheap and abundant carbon sources including waste streams from food and other agricultural based industries (Yousuf et al., 2010; Ling et al., 2013; Kot et al., 2017) or lignocellulosic carbon sources (Alvarez et al., 1992; Liu et al., 2015; Fei et al., 2016) with which the food or fuel concern can be omitted.

Despite all these advantages, commercial SCO production is restricted to high-value oils containing high amounts of polyunsaturated fatty acids (PUFAs) for nutritional purposes (Ratledge, 2004; Mendes et al., 2009; Ji et al., 2014). Production of SCO resembling plant oils, e.g. for biodiesel production, is currently too expensive to allow commercialization (Ratledge and Cohen, 2008; Leong et al., 2018). Costs arise from medium components and preparation, the cultivation process itself and lipid downstream processing as being an intracellular product (Probst et al., 2016; Vasconcelos et al., 2019).

To promote the commercial use of SCO, many challenges must be faced and more research and innovative concepts are highly in demand to reduce process cost. An interesting approach to enable economic feasibility is to extend the value-chain, i.e. to co-produce further valuable products such as biomass, organic acids or enzymes in addition to SCO. Therefore, the screening and isolation of new unconventional oleaginous yeast strains with the abilities to convert complex lignocellulosic waste biomass to access sugars for SCO production and additional by-products might expand the request for such co-productions and resolve the question of cost.

2 Theoretical background and research proposal

2.1 Lipids

Lipids are a diverse group of hydrophobic molecules, which have different function and chemistry. In general, these compounds appear as storage lipids as fats and oils (triacylglycerol and fatty acids), structural lipids in cell membranes such as phospholipids, sterols, glycolipids or sphingolipids and hormones as signal lipids. In smaller quantities other lipids act as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic hold for proteins, emulsifying agents and intracellular messengers (Nelson and Cox, 2008; Li-beisson, 2016; Berg et al., 2018). Taken together lipids are essential for any organism and belong together with proteins and carbohydrates to the principal organic structure components of a living cell. Apart from their biological importance, lipids are valuable compounds for chemical and biotechnological applications including food, chemical feedstocks, and fuel.

2.1.1 Triacylglycerols (TAGs) and fatty acids

The storage lipids, fats and oils, are chemically speaking TAGs. Generally, at room temperature at 20°C solid TAGs are defined as fats, whereas liquid TAGs at the same temperature are called oils (Sadava et al., 2008).

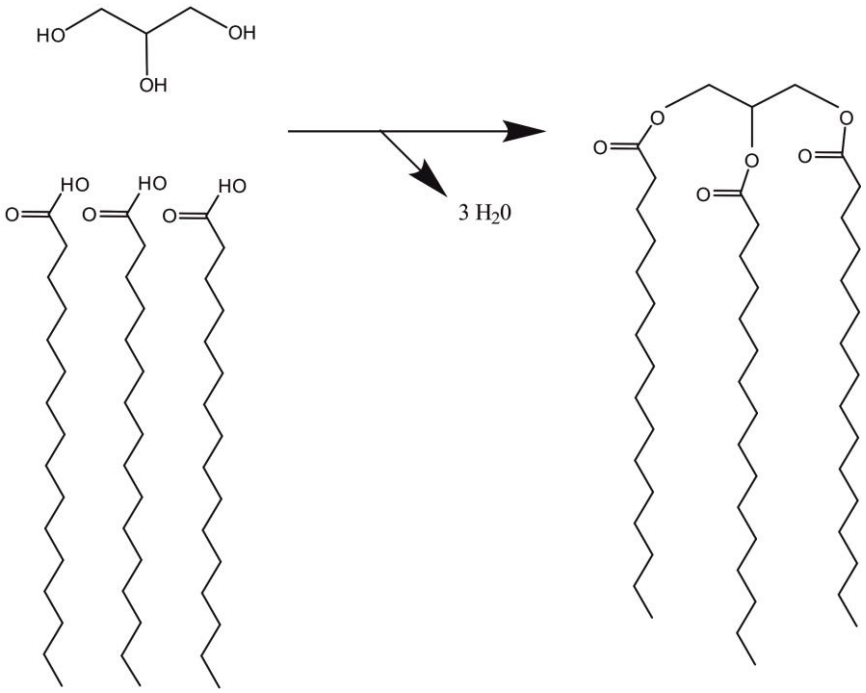

Figure 1: Illustration of the synthesis and structure of a TAG by ester linkage of three carboxyl groups of three fatty acids with each hydroxyl group of a glycerol under condensation of water.

All TAGs comprise a glycerol, a trivalent alcohol with three hydroxyl groups, and three fatty acids, which are composed of a nonpolar hydrocarbon chain and a functional, polar carboxyl group (Campbell et al., 2008; Nelson and Cox, 2008; Sadava et al., 2008) (Figure 1). A TAG is formed by three condensation reactions of each hydroxyl groups of the glycerol and one carboxyl group of each fatty acids resulting in three ester linkages and water (Campbell et al., 2008; Sadava et al., 2008)(Figure 1).

The fatty acid chain length and structure can vary. Usually in biological systems the amount of carbon atoms in a fatty acid chain is even, which is due to the biosynthesis process of the fatty acids, and ranging between 12 and 24 carbon atoms (C_{12} to C_{24}). Fatty acids with 16 and 18 carbon atoms are the most common (Berg et al., 2018), of which three are exemplified in Figure 2. Regarding the structure of fatty acids, the number and location of double bonds can be different. A fatty acid is saturated with hydrogen, when to every carbon atom as many as possible hydrogen atoms are bond and therefore the carbon skeleton comprises just single bonds and no double bonds (Campbell et al., 2008; Sadava et al., 2008). Saturated fatty acids are rigid and straight making fats, substances of such molecules, able to build a firm structure. Animal fat, such as lard or butter, mostly consists of saturated fatty acids. These fats are usually solid at room temperature and have a high melting point (Campbell et al., 2008). In Figure 2 the chemical structure of the fully saturated fatty acid palmitic acid is displayed.

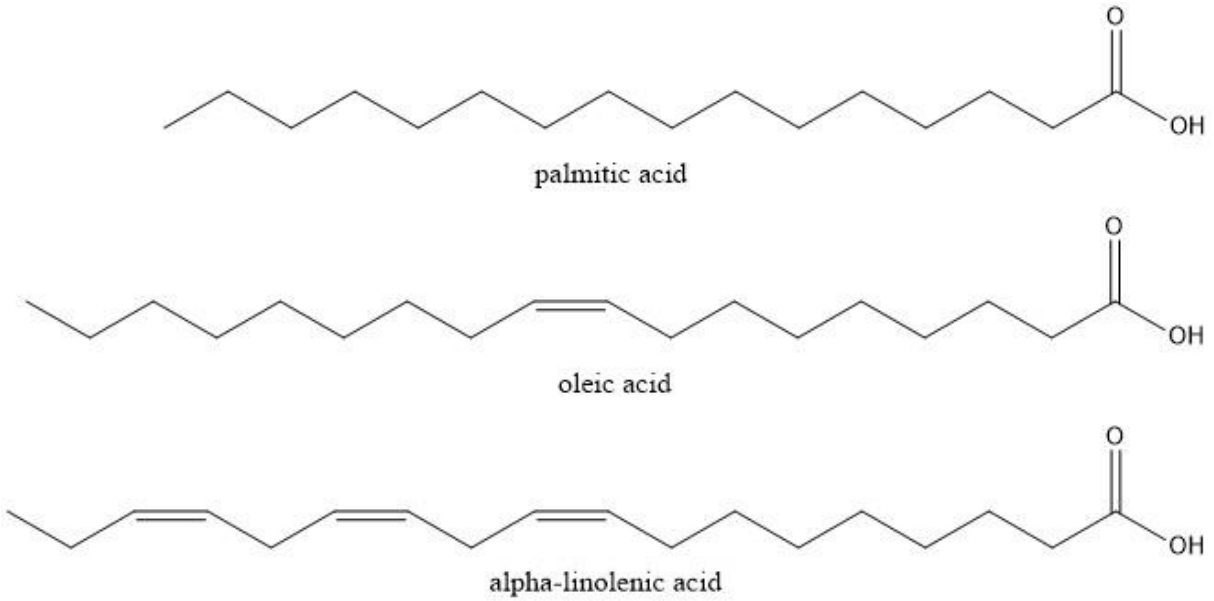

Figure 2: Examples of fatty acids with different length and saturation level: (top) fully saturated palmitic acid (16:0), (middle) monounsaturated oleic acid (18:1) and (bottom) polyunsaturated α-linolenic acid (18:3).

In contrast, fatty acids containing one or more double bonds in their chain are called unsaturated fatty acids. Every double bond causes a kink in the hydrocarbon skeleton, naturally occurring

in the *cis* configuration (Campbell et al., 2008). In Figure 2 (middle) oleic acid (*cis*-9-octadecenoic acid) exemplifies the structure of a monounsaturated fatty acid with one double bond between C-9 and C-10 (Δ^9). A polyunsaturated fatty acid (PUFA) comprises more than one double bonds, as can be seen in Figure 2 (bottom) in case of α-linolenic acid (*cis*-,*cis*- ,*cis*-9, 12, 15- octadecatrienoic acid). The single or multiple kinks of unsaturated fatty acids hinder these molecules to build tight structures, which is why oils containing unsaturated fatty acids are liquid at room temperature and have a low melting point. The TAG of plant oils, such as olive, corn or rapeseed oil are primarily composed of unsaturated fatty acids (Campbell et al., 2008; Sadava et al., 2008). In Table 1 most naturally occurring fatty acids are summarised.

Table 1: Most common naturally occurring fatty acids. Taken from Nelson and Cox (2008).

Carbon skeleton	**Common name**	**Systematic name**	**Structure**
12:0	Lauric acid	*n*-Dodecanoic acid	$CH_3(CH_2)_{10}COOH$
14:0	Myrictic acid	*n*-Tetradecanoic acid	$CH_3(CH_2)_{12}COOH$
16:0	Palmitic acid	*n*-Hexadecanoic acid	$CH_3(CH_2)_{14}COOH$
16:1 (Δ^9)	Palmitoleic acid	*cis*-9-Hexadecenoic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
18:0	Stearic acid	*n*-Octadecanoic acid	$CH_3(CH_2)_{16}COOH$
18:1 (Δ^9)	Oleic acid	*cis*-9-0ctadecenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
18:2 ($\Delta^{9, 12}$)	Linoleic acid	*cis*-, *cis*-9,12-Octadecadienoic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
18:3 ($\Delta^{9, 12, 15}$)	α-Linolenic acid	*cis*-, *cis*-, *cis*-9, 12, 15-Octadecatrienoic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$
20:0	Arachidic acid	*n*-Eicosanoic acid	$CH_3(CH_2)_{18}COOH$
20:4 ($\Delta^{5, 8, 11, 14}$)	Arachidonic acid	*cis*-,*cis*-,*cis*-,*cis*-5, 8,11 , 14- Icosatetraenoic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
24:0	Lignoceric acid	*n*-Tetracosanoic acid	$CH_3(CH_2)_{22}COOH$

2.2 Industrial applications and challenges of lipids derived oleochemicals

Focusing on renewability, fats and oils of plant and to a much lesser extend of animal origin are essential raw materials for chemical industry since decades and in present (Metzger and Bornscheuer, 2006; Biermann et al., 2011). Soybean, palm, rapeseed and sunflower oil are the most prominent renewable oil sources for chemical industry (Metzger and Bornscheuer, 2006). The most demanded derivatives of these oils are basic oleochemicals, i.e. free fatty acids, fatty acid methyl esters (FAMEs), fatty alcohols, fatty amines and glycerol as by-product (Gunstone, 2001). In Figure 3 the fields of application of these compounds are summarised. As illustrated, high demand of oleochemicals prevails in chemical, cosmetics, food and pharmaceutical industry as well as for biodiesel production (Biermann et al., 2011; Baerns et al., 2013). Regarding the importance of the different renewable basic oleochemicals for chemical industry, fatty acids are of highest demand (~ 52%) followed by fatty alcohols (~ 25%), FAMEs (~ 11%) and fatty amines (~ 9%) (Biermann et al., 2011).

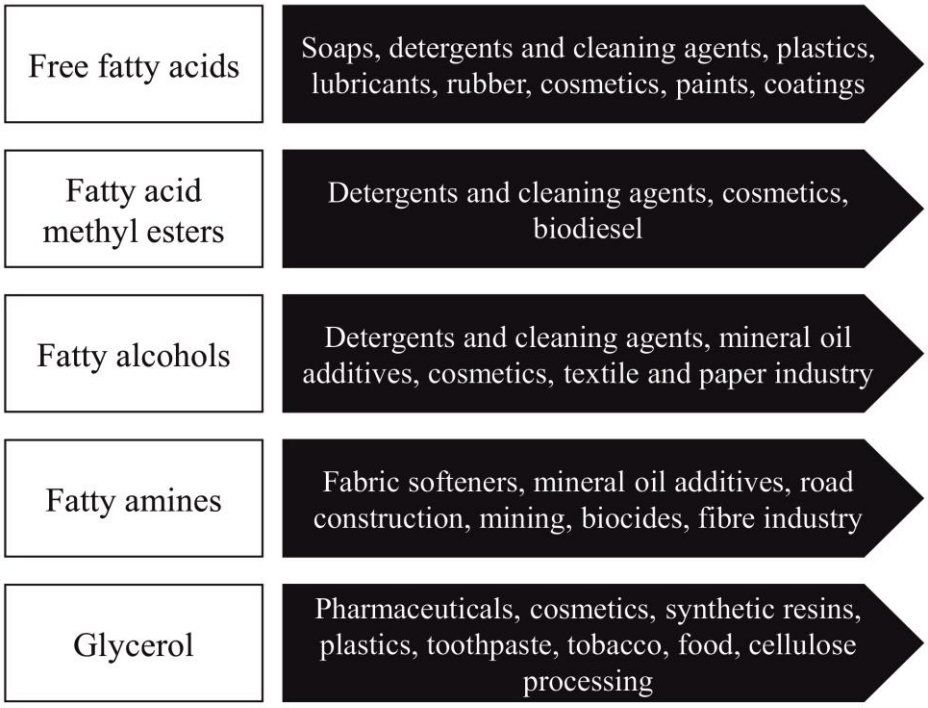

Figure 3: Industrial applications of basic oleochemicals. Adapted from Baerns et al. (2013).

2.2.1 Food industry

Primarily for food purposes the global production and consumption of major plant oils, such as palm, soybean, rapeseed, sunflower, olive and coconut, increased in the last decades. For sesame, linseed, castor and corn oils, as minor vegetable oils, a rising demand could also be observed, as well as for animal fats, like tallow, lard, butter and fish oil (Biermann et al., 2011).

For nutrition purposes fats and oils play a decisive role for the human body. Starting from breast feeding of infants, it provides energy and enables its storage, additionally fat-soluble vitamins and essential fatty acids are ingested with the fat supply. It is not possible to replace fats and oils with other substances, since they provide essential compounds, which the body is unable to produce by itself (Bockisch, 2015). For instance, mammalian cells lack the enzymes to synthesize the essential ω-3 PUFAs linoleic (LA; 18:2) and α-linolenic acid (ALA; 18:3) (Figure 2 (bottom); Table 1). However, ALA is the decisive precursor for mammalian cells to synthesize other important ω-3 PUFAs, such as eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) (Nelson and Cox, 2008). PUFAs are crucial structural compounds in all mammalian cell membranes and impact on the membrane fluidity and therefore on the cellular signalling with other cells or molecules (Bellou et al., 2016). EPA and the ω-6 PUFAs dihomo-γ-linolenic acid (DGLA; 20:3) and arachidonic acid (ARA; 20:4) form preliminary stages of the signalling molecules eicosanoids with functions in diverse physiological systems and pathological processes. For prevention or treatment of cardiovascular and inflammatory diseases, cancer, brain disorders, autoimmune diseases, obesity and diabetes PUFAs rich and balanced diets are recommended (Simopoulos, 2006). Furthermore, ARA and DHA play an important role in the brain formation, development of eyesight and the improvement of cognitive abilities of new-born and infants. Both are natural component of breast milk and should be ingested while pregnancy and be part of the new-born diet (Ratledge 2010a).

For that reasons PUFAs have a key role for nutrition and health and are of high importance for the food and dietary supplements industry. LA and ALA can be found in vegetable oils, whereas EPA and DHA mainly appear in fish and fish oils. However, the increasing human made pollution of the world's oceans with environmental toxins, such as heavy metals, dioxins and chemicals like polychlorobiphenyls (PCB), precludes the administration of dietary supplements of fish origin to pregnant women and infants (Ratledge 2010a). To substitute fish oil, new sources for PUFA rich additives for infant nutrition were and are highly demanded in the food industry.

The discovery of microorganisms as PUFA producers was a breakthrough and became increasingly important for the food industry. The production of DHA with the dinoflagellate microalgae *Crypthecodinium cohnii* was an industrially established microbial SCO process by the company Martek Corp and had a huge impact on the infant nutrition market (Ratledge 2010a). Another promising marine algal DHA producer was found to be *Ulkenia* sp. and Lonza Ltd., in Switzerland, developed a full-scale process for that purpose (Kiy, 2005). Lately, DSM announced an optimized DHA nutritional additive, named ‘’life’sDHA‘’, as a sustainable alternative to fish oil and produced by conventional (non-GM) *Schizochytrium* microalgae (DSM, 2020). Besides DHA, microbial ARA production is also possible and feasible in large scales of 50-100 t by using the fungus *Mortierella alpina* as production host (Yuan et al., 2002). The process was originally developed by Wuhan Alking Bioengineering Co., Ltd. (Ratledge 2010a). However, such valuable PUFAs are only produced by microalgae and filamentous fungi and not by oleaginous yeasts.

2.2.2 Biodiesel

Biodiesel consists of FAMEs of plant oils or animal fats and is a renewable transportation fuel. Since the combustion of fossil fuel leads to environmental pollution and by exhaust gases caused climate change, biodiesel became of high interest as environmentally friendly and biodegradable alternative (Vasudevan and Briggs, 2008; Hoekman et al., 2012). Biodiesel is defined as mono-alkyl esters of long-chain fatty acids derived from edible, non-edible, and waste oils (Hoekman et al., 2012; Singh et al., 2019). The production of biodiesel is achieved through an acidic or alkaline catalyst mediated transesterification of TAGs with methanol. The by-product of this reaction is glycerol. The FAME profile of different biofuels varies individually based on its substrate TAGs comprising fatty acid of C_{14}-C_{22} chain length (Demirbas, 2009). However, five of the most common naturally occurring fatty acids, as indicated in Table 1, are highly prominent and in their alkyl ester form typical biodiesel components, these include palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) (Hoekman et al., 2012).

Depending on the raw material, biodiesel is classified in generations. In the first biodiesel generation the feedstock was edible oils, i.e. from olive, palm, peanut, rapeseed, sunflower or soybean (Wan Ghazali et al., 2015; Li and Khanal, 2016). To the second generation, biodiesel of non-edible origins is accounted, including cottonseed, jatropha, linseed or neem (Wan Ghazali et al., 2015; Li and Khanal, 2016). The third generation comprises biodiesel production of renewable and local waste oil, for instance waste cooking oil, pork lard, tallow (Singh and Singh,

2010) and also oils from microalgae (Baskar et al. 2016). Depending on the country different feedstocks are used for biodiesel production of the well- established technologies of first generation biodiesel. In the USA it is soybean oil derived biodiesel, in Europe rapeseed oil is the most dominant raw material and palm oil in South Asia (Hoekman et al., 2012; Singh et al., 2019).

Biodiesel is environmental friendly (recycling carbon cycle, not increasing from fossil source), biodegradable and a sustainable alternative for consumption of fossil fuels. However, the production costs are still high compared to diesel, which prohibits a larger commercial use of biodiesel (Hoekman et al., 2012; Lee and Lavoie, 2013; Singh et al., 2019). Into the bargain, still only first generation biodiesel is mainly realized worldwide, which leads to the fuel versus food debate, by exploiting agricultural area for engine fuel and contributing to price increase of e. g. soybean and corn (Hochman et al., 2012). Single cell oil resembling plant oil might be a substitute feedstock for biodiesel production. However, an industrial realization was not possible yet due to high process cost.

2.3 Single cell oil (SCO)

Single cell oils (SCOs) are intracellular storage lipids comprising of TAGs produced by oleaginous microorganisms, such as algae, yeast, and moulds. Oleaginous organisms are defined as being able to accumulate between 20% and up to 80% lipid per dry biomass and might become a promising platform for renewable and sustainable chemicals (Ageitos et al., 2011; Ochsenreither et al., 2016). SCOs are chemically equivalent to plant oils or to some extent to animal or fish oils. However, SCOs can be produced independent of season, climate and location using a wide range of cheap and abundant carbon sources including waste streams from food and other agricultural based industries (Yousuf et al., 2010; Ling et al., 2013; Kot et al., 2017) or ligno-cellulosic carbon sources (Alvarez et al., 1992; Liu et al., 2015; Fei et al., 2016) and in case of photoautotrophic microalgae even CO_2. The highest theoretical yield of SCO is 33 g SCO per 100 g glucose. The highest practical yield was reported to be 20-27 g SCO per 100 g glucose (Ratledge and Cohen, 2008; Qiao et al., 2017). Considering the world's population increasing energy and resource demand, but serious concerns about depletion of crude oil, environmental pollution, climate change, overfishing of the oceans and food versus fuel debate, SCO has the potential to become the new renewable source for oleochemicals. As mentioned in section 2.2.1 for food industry the industrially produced SCO of oleaginous microalgae and the fungus *Mortierella alpina* is the source for highly valuable PUFAs including DHA and

ARA (Ratledge 2010a). However, for low value products as biodiesel the production of SCO is by far not economically competitive with plant or crude oil.

2.3.1 Oleoginous yeasts

One of the most promising oleaginous microorganisms are yeasts, mainly because of their fast cell growth, in contrast to microalgae, the high lipid productivity on waste carbon sources and the easy realization of process up-scaling.

Table 2: Overview of lipid yield of relevant oleaginous yeast on different carbon sources. Taken from Vasconcelos et al. (2019).

Yeasts	**Carbon source**	**$Y_{L/S}$ [g/g]**	**References**
Rhodotorula glutinis	Glycerol	0.101	(Karamerou et al. 2016)
	Corncob hydrolysate	0.159	(Liu et al., 2015)
	Sucrose	0.180	(Lorenz et al., 2017)
	Glucose	0.182	(Johnson et al., 1995)
Yarrowia lipolytica	Glycerol	0.140	(Sara et al. 2016)
	Acetic acid	0.160	(Xu et al., 2017)
	Glucose	0.270	(Qiao et al., 2017)
Rhodosporidium toruloides	Glycerol	0.220	(Yang et al., 2014)
	Glucose	0.260	(Li et al. 2007)
	Acetic acid	0.277	(Huang et al., 2016)
	Corn stove hydrolysate	0.290	(Fei et al., 2016)
Cryptococcus curvatus (syn. *Candida curvata*, *Trichosporon oleaginous*, *Cutaneotrichosporon oleaginosum*)	Corn stove hydrolysates	0.159	(Gong et al., 2014)
	Acetic acid	0.172	(Liu et al., 2017)
	Volatile fatty acids	0.187	(Liu et al., 2017)
	Glycerol	0.220	(Ryu et al., 2013)
	Cardboard hydrolysates	0.224	(Zhou et al., 2017)
	Glucose	0.246	(Zhang et al., 2011)
	Whey permeate	0.290	(Ykema et al., 1988)
Lipomyces starkeyi	Glycerol	0.150	(Wang et al., 2014)
	Sweet potato starch	0.160	(Wild et al., 2010)
	Glucose	0.180	(Gong et al., 2012)
	Xylose	0.180	(Gong et al., 2012)
	Cellobiose	0.200	(Gong et al., 2012)
	Hemicellulose hydrolysate	0.236	(Anschau et al., 2014)

It is hard to compare or to rank individual yeast species, since lipid productivities and yields vary greatly depending on the strain, carbon source, cultivation scale and conditions. The in literature most relevant representatives are quite diverse and belong to the phyla of asco- and basidiomycetes. These are summarized in Table 2 based on achieved lipid yield $Y_{L/S}$ ($g_{Lipid}/g_{Substrate}$), which means conversion yield of lipid formed per carbon source consumed. On glucose the highest yield of 0.27 g/g was reached with an engineered *Yarrowia lypolitica* strain (Qiao et al., 2017). Even higher yields of 0.29 g/g were achieved by *Rhodosporidium toruloides* (Fei et al., 2016) and *Cryptococcus curvatus* (Ykema et al., 1988) on sustainable carbon sources, like corn stover hydrolysate and whey permeate, respectively.

By phylogenetic comparisons of oleaginous yeasts, it becomes apparent that often the property of oleagenicity is not shared by distinct groups within their phyla (Aliyu et al. 2020a; Aliyu et al. 2020b). Thus, this characteristic cannot be identified by pedigree analyses. Neither is it known by which molecular and evolutionary mechanisms an organism becomes oleaginous, so it is subject of current research.

2.3.2 Biosynthesis of SCO

The biosynthesis and accumulation of large amounts of lipids in oleaginous yeasts is not due to different biochemical pathways compared to non-oleaginous yeast, such as *Saccharomyces cerevisiae* (Botham and Ratledge, 1979). However, the basic requirements for lipid accumulation are nutrient limitation, e.g. nitrogen, phosphate or sulphate, and carbon excess. Under these conditions continuous supply of acetyl-CoA and NADPH must be ensured for fatty acid production in oleaginous yeasts. In the TCA cycle a unique feature of oleaginous yeasts is the adenosine monophosphate (AMP) dependence of the enzyme isocitrate dehydrogenase (ICDH), which catalyses the oxidative decarboxylation of isocitrate to α-ketoglutarate. When nitrogen is limited, the concentration of AMP in the mitochondria decreases, which in turn significantly increases the activity of AMP-deaminase, which catalyses the cleavage of AMP to inosine monophosphate and ammonia. Due to AMP deficiency the activity of ICDH decreases. Consequently, isocitrate accumulates in the mitochondria. This is converted in an equilibrium reaction by the enzyme aconitase into citrate, which in turn accumulates. Citrate is transported into the cytosol and is cleaved by the enzyme ATP:citrate-lyase (ACL) to acetyl-CoA and oxaloacetate, which finally leads to the continuous supply of acetyl-CoA for the fatty acid synthesis. (Ratledge, 2002, 2004; Ratledge and Wynn, 2002). In the cytosol the enzyme fatty acid synthase (FAS) catalyses the fatty acid biosynthesis by a condensation reaction of acetyl-CoA and malonyl-CoA (prior converted from acetyl-CoA). However, every condensation reaction requires

2 mols of NADPH to reduce each 3-keto-fattyacyl group (Ratledge, 2004). The source of NADPH for SCO biosynthesis is not completely clarified yet, but in most yeasts the malic enzyme (ME) is considered to play a major role in this context. In the cytosol ME catalyses the decarboxylation of malate (reduced from oxaloacetate) to pyruvate, which is transported into the mitochondria. During the subsequent TAG synthesis, the synthesized fatty acids are esterified with glycerol and enclosed in lipid droplets via the endoplasmic reticulum (Ratledge, 2004).

2.3.3 Advantages and limitations of SCO production with yeasts

The production of SCO by yeasts is independent of season, climate, and location and requires limited amounts of area for cultivation and no agricultural land. Additionally, SCO production with yeasts is advantageous because of their fast growth rate, high oil productivity due to short duplication time and greater convenience to scale up cultivation than that of autotrophic microalgae since no light is needed (Li et al., 2008; Ageitos et al., 2011; Ochsenreither et al., 2016). Oleaginous yeast lipids can be used to produce biodiesel, and might find application in food industry and as building blocks for biopolymers (Vasconcelos et al., 2019). Pilot plant scale production of yeast SCO increases the potential of these microorganisms. Xue et al. (2010) cultivated *Rhodotorula glutinis* on starch wastewater in a 300 L scale and reached 35% lipid content. Moreover, Soccol et al. (2017) successfully processed a fed-batch strategy using sugarcane juice with *Rhodosporidium toruloides* in a 1000 L plant. Thereby, a 6.3-fold higher biodiesel yield was reached compared to standard biodiesel from soybean.

Despite all these advantages, commercial SCO production is restricted to high-value oils containing high amounts of PUFAs for nutritional purposes (Ratledge, 2004; Mendes et al., 2009; Ji et al., 2014). Production of SCO resembling plant oils, e.g. for biodiesel production, is currently too expensive to allow commercialization (Ratledge and Cohen, 2008; Leong et al., 2018).

Costs arise from medium components and preparation, the cultivation process itself and lipid downstream processing as being an intracellular product (Probst et al., 2016; Vasconcelos et al., 2019). More precisely, the carbon source is the main cost factor in the medium and for cost-efficient SCO production as low as possible carbon source amounts and cheap materials are desirable (Vasconcelos et al., 2019). Processes with high, unused sugar excess in the cultivation broth are uneconomical, unsustainable and need be optimized without sacrificing productivity. Sustainability and economic feasibility for microbial lipids produced by yeasts can be ensured

by using redundant and cheap ligno-cellulosic waste plant biomass or carbon rich waste products as substrate, such as ligno-cellulosic hydrolytes, crude glycerol or other waste products. However, these waste products often contain a considerable amount of toxic compounds (Cui et al., 2012; Ling et al., 2013; Dörsam et al., 2016; Poontawee and Limtong, 2020). Cell proliferation and product formation can be severely compromised upon high concentration of such substrates and therefore an individual adaption of process modi might be needed.

Further major challenges for the industrialization is the development of an efficient, cost-effective and low energy consuming method for the recovery of the intracellular product. The rigid and robust properties of the yeast cell wall significantly contribute to poor SCO accessibility and resistance against organic solvents (Jacob, 1992). For a successful oil extraction, cell pretreatment and/or cell disruption is pivotal. However, a universally accepted SCO recovery method does not exist and the most advantageous method needs to be identified individually for each species.

2.3.4 Unconventional yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194

Driven by urgent needs for sustainable alternatives, the transformation of the chemical industry from petrochemicals to greener alternatives is already ongoing for decades but is not completed yet. To promote this transformation, still many challenges must be faced and more research and innovative concepts are highly in demand. With regards to sustainable and non-food competitive biodiesel production with oleaginous yeasts, production costs needs to be reduced. Another interesting approach to enable economic feasibility is to extend the value-chain by co-production of SCO, organic acids and enzymes. Therefore, the screening and isolation of new unconventional oleaginous yeast strains with the abilities to convert complex lignocellulosic waste plant biomass via various carbohydrate-active enzymes (CAZymes) to accessible sugars, might be of interest for improvement of commercial available enzyme cocktail for plant biomass utilization. Additionally, the fermentative co-production of SCO, as intracellular product, and valuable extracellular by-products, such as organic acids, might enhance the feasibility of yeast cultivation processes and resolve the challenge of cost.

In previous work of Schulze et al. (2014) two novel oleaginous yeast *Saitozyma podzolica* (formerly *Cryptococcus podzolicus*) DSM 27192 and *Apiotrichum porosum* (formerly *Trichosporon porosum*) DSM 27194 were isolated from peat bog soil or grassland, respectively. These habitats offer a high excess of carbon in the form of ligno-cellulosic biomass with simultaneous

nitrogen limitation, which are ideal production conditions for SCO from sustainable carbon sources. Schulze et al. (2014) performed first investigations on lipid production and substrate spectrum. It could be confirmed that, both yeasts were able to convert glucose and xylose to SCO with comparable yields. Their lipid profile is reported in Table 3. As main by-product *S. podzolica* and *A. porosum* produced large amounts of gluconic acid (GA) when cultivated with glucose, which is a valuable sugar acid for, inter alia, food industry (Schulze et al., 2014).

Table 3: Lipid profile of *S. podzolica* and *A. porosum* characterised by Schulze et al. (2014).

Strain	Carbon source	Fatty acid profile [% total fatty acid]						
		16:0	16:1	18:0	18:1	18:2	18:3	Other
S. podzolica	Glucose	18.4	0.3	5.3	59.4	8.7	0.9	7.0
	Xylose	20.1	0.4	4.7	55.1	11.1	1.1	7.5
A. porosum	Glucose	19.5	0.3	17.0	40.4	17.8	1.3	3.7
	Xylose	21.1	0.3	15.5	39.6	18.7	1.0	3.8

The co-production of SCO and GA by *S. podzolica* was investigated more deeply by Qian et al. (2019), where it was shown that organic nitrogen sources enhanced SCO production but severely inhibited GA formation. High glucose concentration of up to 150 g/L, in turn, greatly stimulated GA production, but the combination of high glucose excess and high concentration of dissolved oxygen (DOC) deteriorated cell growth and therefore, had negative effects on SCO production. Thus, Qian et al. (2019) established a promising two-stage strategy of controlled DOC or glucose concentration for improved cell propagation and distribution of glucose between SCO and GA production. In addition, Qian et al. (2020) studied upon the enhancement of substrate spectrum of both yeasts and proved their ability to use acetic acid as carbon source for SCO production. Acetic acid is a low value, volatile fatty acid generated as by-product in industrial processes. The utilization of waste acetate is a promising strategy for sustainable and economic feasible production of SCO. By applying an automated fed-batch system, where acetic acid was used both as carbon source and as pH buffering agent, lipids contents of 38.8% and 39.7% were obtained by using *S. podzolica* and *A. porosum*, respectively (Qian et al., 2020).

Beyond the above mentioned investigations, genome sequencing and annotation was performed to better understand the metabolism and overall nature of these unconventional oleaginous yeasts (Aliyu et al. 2019; Gorte et al. 2019). Additionally, comparative genomic analysis was performed of thirty-three members of the family *Trichosporonaceae*, including *A. porosum*

(Aliyu et al. 2020a), and the thirty-five members of the order Tremelalles, along with *S. podzolica* (Aliyu et al. 2020b). The comparison of the proteomes of *Trichosporonaceae* signified abundant capacity of carbohydrate-active enzymes (CAZYmes) and peptidases (MEROPS) and suggest the potential of the yeasts to degrade a wide variety of biomass (Aliyu et al. 2020a). The comparative genomic analysis of the order Tremelalles was strengthened by growth experiments on various carbohydrates and revealed a broad substrate spectrum expansion of *S. podzolica*, comprising the ability to grow on complex carbohydrates, such as cellobiose, xylan, inulin, pectin etc. Furthermore, *S. podzolica* was proven to harbour higher numbers of CAZYmes (on average 317) compared to the rest of the investigated Tremelalles strains (on average 267–121) (Aliyu et al. 2020b). Hence, these findings reveal the high potential of *S. podzolica* to produce novel CAZYmes. These identifications are biotechnologically highly relevant for improvement of commercial available enzyme cocktails for pre-treatment and utilization of complex molecular structures of plant biomass (Aliyu et al. 2020b).

2.4 Process conditions for fermentative SCO production with yeasts

Usually SCO production is enhanced in the event of carbon excess and nutrient limitation, mostly nitrogen, as described in section 2.3.2. By coping with the nitrogen limitation stress biomass formation is interrupted and the excess carbon is channelled to TAG synthesis and accumulation for energy storage in form of intracellular lipid droplets (Ratledge, 2004). For that reason, SCO production is mostly performed in a two-stage process. A typical lipid accumulation process plot is illustrated in Figure 4.

In the first phase, the medium is rich in all required nutrients for optimal cell proliferation, protein synthesis and general cellular and metabolic maintenance. Then, the transition to SCO production phase is induced by nitrogen deficiency. Therefore, the medium formulation needs to offer all required nutrients in the beginning, but to reach the foreseeable nitrogen exhaustion and simultaneous excess of carbon source. Such conditions are reached by high C/N ratios (Ratledge, 1991, 2004). The optimum C/N ratio are reported to be close to 100 for most of the oleaginous yeasts (Li et al., 2008; Ageitos et al., 2011; Ochsenreither et al., 2016; Probst et al., 2016). However, the optimum C/N ratio needs to be determined and optimized individually for each oleaginous yeast strain.

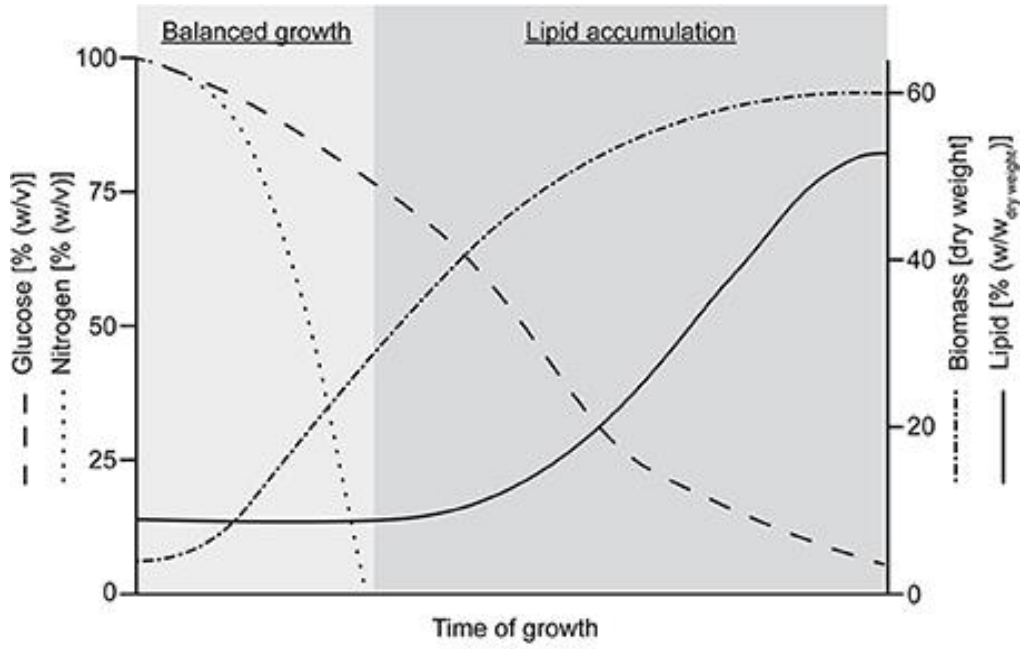

Figure 4: Idealized lipid accumulation profile of a SCO cultivation process with oleaginous yeasts. In the balanced growth phase the medium is initially formulated for optimal biomass formation but in the end of it nitrogen is exhausted and carbon is in excess. In the lipid accumulation phase biomass formation stagnates and the glucose is channelled to storage lipid synthesis. Taken from Ochsenreither et al. (2016).

Besides C/N ration, numerous parameters have a decisive impact on SCO production, including the type and concentration of carbon and nitrogen source, fermentation mode, pH, temperature and aeration. All of them must be specified for optimal SCO production under consideration of cost, energy supply and sustainability (Ageitos et al., 2011; Vasconcelos et al., 2019). For instance, main production costs are caused by medium components and preparation, cultivation and lipid downstream processing (Probst et al., 2016; Vasconcelos et al., 2019). The carbon source represents the highest cost factor in the medium (Vasconcelos et al., 2019). In literature a broad spectrum of investigation upon various carbon sources can be found, an insight is reviewed by Vasconcelos et al. (2019) and is adapted in Table 2.

Processes with high, unused carbon excess in the cultivation broth are uneconomical and should be optimized without sacrificing productivity. Therefore, process mode and feeding strategies have a significant impact on SCO production. The classical cultivation methods comprise either batch, fed-batch or continuous process mode. The optimal mode depends on the yeast strain and the combination of all the named parameters and therefore cannot be generalized. However, Ykema et al. (1988) compared all of the latter and additionally a partial biomass recycling culture strategy with *Cryptococcus curvatus* by using whey permeate as carbon source. The highest SCO productivities of 0.995 g/L/h were reached resulting from highest cell densities, which

was achieved upon the biomass recycling strategy. The second best lipid production rate was obtained by continuous process mode followed by fed-batch and batch. Regarding the cell densities, however, the fed-batch process reached nearly as high values as the biomass recycling strategy (Ykema et al., 1988). As reviewed by Vasconcelos et al. (2019) for the most relevant oleaginous yeasts (*Rhodotorula glutinis*, *Yarrowia lipolytica*, *Rhodosporidium toruloides*,and *Lipomyces starkeyi*) the fed-batch process mode attained the highest SCO productivities. The feeding strategy upon a fed-batch process can be performed periodically or continuously, resulting in a controlled supply of relevant nutrients, e.g. carbon source. Additionally, substrate inhibition, due to too high applied concentrations, can be excluded and a possible enhanced product concentration and yield can be achieved. Furthermore, the fed-batch mode provides a controlled dilution of the culture, which leads to reduced viscosity and counteract to the water loss due to aeration (Chmiel et al. 2018; Lim and Shin 2013; Poontawee and Limtong 2020).

2.5 Lipid downstream processing

Since SCO is accumulated in form of intracellular lipid droplets, the downstream processing and lipid recovery is a decisive step for the microbial lipid production process. However, an efficient extraction and purification of SCO, which reaches all the requirements of sustainability, eco-friendliness, low energy and resource demand and economic feasibility, is hard to find and must be compromised. Therefore, it is also still subject of research. A major obstacle is the rigid and robust yeast cell wall, which is the main hindrance for lipid accessibility and responsible for the resistance to organic solvents (Ageitos et al., 2011; Ochsenreither et al., 2016; Probst et al., 2016). These circumstances have an evolutionary background to protect and adapt the cell to environmental conditions by building backbones of cross-linked glucan fibers, manno-protein and chitin (Phaff, 1971; Jacob, 1992). On this account, an efficient pre-treatment to weaken the cells is mandatory for successful lipid recovery and needs to be optimized for each yeast strain, since the cell wall composition may vary considerably between species. Unfortunately, universal methods for efficient SCO recovery from yeast biomass do not exist.

2.5.1 Biomass pre-treatments

The pre-treatment of oleaginous biomass for lipid downstream processing is a broad concept and can include everything from biomass pasteurisation for the inactivation of the yeast's lipases and esterases, to cell disruption for breakdown of the robust cell wall, to freeze- or spray drying for lipid quality stabilization. The requirement of pre-treatments is determined by oil

compositions and production purpose. For instance, the production of edible, PUFA rich microbial oils undergoes several operation steps to unsure oil quality and prevent PUFA oxidation (Ratledge et al. 2010b). However, only such high-value oils can compensate the production cost arising from many pre-treatment steps. For low-value microbial oil of biodiesel profile only the most efficient pre-treatments and as less as possible operations have the potential to be economical feasible. On that purpose, a number of different techniques have been applied successfully for the recovery of various yeasts' oil recovery in literature.

In order to release the SCO of the yeast's cells most promising methods are mechanical cell disruptions. Because of their great industrial potential and the use on large scale on microorganisms, including microalgae, and in other industries, bead-milling (BM) (mechanical disruption by grinding), high-pressure homogenization (HPH) (mechanical disruption by pressure) and ultrasonification (U) (mechano-physical disruption by cavitation) have been proved to be potent tools (Jacob 1992; Ochsenreither et al. 2016; Probst et al. 2016; Ratledge et al. 2010b). For instance, for purification of nutrition protein from bakers' yeast and brewers' yeast biomass, BM cell disruption is utilized in a continuous process (Chisti et al. 1986). The dairy industry is using HPH for fat globules size reduction, avoiding of creaming and stabilization of emulsions (Rodríguez-Alcalá at al. 2009). Additionally, HPH can be incorporated in a continuous process. U is industrially frequently used for emulsification, degassing or defoaming (Ochsenreither et al., 2016). On laboratory scale HPH was used after a fed-batch cultivation process on crude glycerol of *Cryptococcus curvatus* for biodiesel production (Thiru et al. 2011). BM was one of the most efficient methods on disruption of *Yarrowia lipolytica* (Meullemiestre et al., 2016) and the yeasts *Rhodotorula glutinis* and *Lipomyces kononenkoae* (Vasconcelos et al., 2018). U was used upon different parameters (520 kHz 40 W and 50 Hz 2800 W) on *Trichosporon oleaginosus* and a self-screened oleaginous fungal strain designated SKF-5 by Zhang et al. (2014).

Many other attempts on cell disruption or pre-treatment of oleaginous yeast biomass can be found in literature, e.g. physical disruption by microwave (Yu et al., 2015; Meullemiestre et al., 2016), chemical treatment with organic solvents (Breil et al., 2016) or acidic hydrolysis (Yu et al., 2015) and biological conversion of yeast biomass by enzymatic lysis (Jin et al., 2012). However, most of the latter stated methods lack an energy-efficiency balance and/or the possibility for up-scalability.

Another innovative cell pre-treatment method is pulsed electric field (PEF) treatment, which relies on the application of short intense electric pulses, that when delivered to biological cells, induce an increase in transmembrane voltage and subsequently an increase in the permeability

of the membrane (Pakhomov et al. 2010; Teissie et al. 2005). This process is effective both on animal and plant cells, which has resulted in applications in the medical domain as well as in the food industries. For instance, it is used in cancer electro-chemotherapy on tumour treatment (Gothelf et al. 2003; Mir et al. 2006) or for permeabilization of membranes in apple juice production or for processing of potatoes in the snack food industry (Barba et al. 2015; Toepfl et al. 2006). With regard to the extraction of oil from oleaginous biomass, trials have been reported on rapeseed, linseed, olive paste and black cumin seed with results indicating that pre-treatment with PEF can increase lipid extraction yields of traditional pressing methods without impacting on quality of the oil (Bakhshabadi et al. 2018; Guderjan et al. 2005; Puértolas and de Marañón 2015; Sarkis et al. 2015a; Sarkis et al. 2015b). On SCO of the microalgae, *Auxenochlorella protothecoides*, this method was broadly studied by Silve et al. (2018a; b). The PEF parameters must be determined for each microbial cell individually, based on their structure, size, and cell wall or membrane components. However, for future SCO industrial application purposes PEF might be highly potent for large scale production, since it can be applied in continuous flow, has reasonable energy consumptions and is adaptable to industrial equipment (Martínez et al., 2020).

2.5.2 Lipid extraction

Oleaginous yeasts mainly produce neutral lipids, such as TAGs as major component and di- and mono-acylglycerols, free fatty acids and sterols. These molecules are accumulated in lipid droplets surrounded by polar phospholipids and serve for energy storage. Polar lipids, like phospholipids, sphingolipids and glycolipids, are incorporated in the flexible cell membrane (Brown, 2001; Breil et al., 2017). To recover the whole lipid of microorganisms generally a system of polar and nonpolar solvents is applied. The polar co-solvent is needed to break up the protein layer surrounding the lipid droplets in order to make the lipids accessible to the non-polar solvent. The most famous and efficient laboratory-scale techniques are according to Folch (Folch et al. 1957) and Bligh and Dyer (Bligh and Dyer, 1959) using the solvents chloroform and methanol. In literature both are ‘’gold standard‘’ and known for their efficiency in lipid recovery (Schmid et al. 1973). However, chloroform is a highly toxic and carcinogenic solvent and its usage is therefore prohibited in the food industry (Grima et al., 1994; Breil et al., 2016). In the food industry, hexane, sometimes in combination with ethanol, is used as low-toxicity alternative on lipid extraction of oil seeds and canola (Grima et al., 1994; Biondo et al., 2015). These extraction solvents are also well-established for algal lipids (Silve et al. 2018a). For the extraction of microbially produced valuable PUFA-rich oils, hexane is also the solvent of choice

for large scale application. Here the extraction is performed by successive counter-current washes of pre-treated oleaginous biomass with hexane (Ratledge et al. 2010b). In contrast to chloroform, hexane is non-carcinogenic but still neurotoxic (Joshi and Adhikari, 2019). Nevertheless, there is barely an applied alternative to petroleum based hexane in lipid extraction of SCO on pilot scales or of industrial scale plant oil recovery (Xue et al., 2010; Breil et al., 2016; Soccol et al., 2017).

Potential green and bio-based alternative solvents for hexane in terms of solubilization of microbial oils on laboratory scale were reported to be solvents from agricultural sources such as ethyl acetate, cyclopentyl methyl ether and 2-methyltetrahydrofuran (Breil et al., 2016). In addition, a system of the green solvent ethyl acetate and ethanol was reported to be highly efficient with regards to extracted lipid yield of *Yarrowia lipolytica* biomass (Breil et al., 2017). However, more intense research effort will be needed to realize these green alternatives on pilot or industrial scale.

2.6 Research proposal

SCO can be produced independent of season, climate and location by using a wide range of cheap and abundant carbon sources, resulting in a green and sustainable substitute for commodity oils like crude, plant or fish oils. Considering the world's population increasing energy and resource demand, but serious concerns about depletion of crude oil, environmental pollution, climate change, overfishing of the oceans and food versus fuel debate, SCO has the potential to become the new renewable source for oleochemicals, food additives and biofuels. However, besides SCO containing highly valuable PUFAs, the production of SCO of plant oils profile is by far economically un-competitive with oilseeds or crude oil.

To overcome this, more research and innovative concepts are highly in demand. One possible way to succeed might be the use of new unconventional oleaginous yeast, such as *Saitozyma podzolica* and *Apiotrichum porosum*, with the abilities to co-produce valuable products, like SCO, organic acids and interesting enzymes for complex biomass pre-treatment.

To further understand the potential of these novel yeasts, the aim of this thesis was first to optimize the SCO process of *Saitozyma podzolica* with the goal to:

- improve SCO production.
- reduce the applied sugar amount without losing productivities to avoid sugar wastage.
- compare feeding strategies and establish an automated feeding process.
- investigate the potential of *Saitozyma podzolica* to produce xylonic acid.

Secondly, it was intended to fully characterize the lipid downstream process with both yeasts by using mechanical disruption systems in the aspects of:

- evaluation of the most efficient cell disruption method.
- assessment of solvent extraction systems for whole cell lipid extraction yield.
- comparison of biomass pre-treatments for biodiesel production by transesterification.
- rating the energy consumption of biomass pre-treatment and cell disruption.

Finally, the innovative pre-treatment pulsed electric field (PEF) was examined to enhance lipid extraction yield on fresh *Saitozyma podzolica* biomass. The following was examined:

- implication of applied PEF-treatment for subsequent lipid extraction.
- effect of biomass washing for the treatment.
- impact of electric field and specific treatment energy.
- level of energy consumption.

3 Optimization of carbon source efficiency for lipid production with *S. podzolica*

This chapter is mainly based on the submitted manuscript

Optimization of carbon source efficiency for lipid production with the oleaginous yeast *Saitozyma podzolica* DSM 27192 applying automated continuous feeding

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Authors' contribution to this manuscript

Olga Gorte designed and planed the study, performed the experiments, analyzed the data, drafted and revised the article.

Michaela Kugel generated the LabVIEW program for the continuous feed regulation and helped OG with continuous feed cultivations.

Katrin Ochsenreither supervised the project, constructively contributed to the content and critically revised the article.

3.1 Introduction

In this chapter the cultivation process optimization of the unconventional oleaginous yeast *S. podzolica*, screened by Schulze et al. (2014), is presented. Schulze et al. (2014) were able to establish a bioreactor cultivation process for lipid production as main product and gluconic acid (GA) as by-product, when cultivated on glucose. The process was realized by performing a manual pulsed restock of carbon source to 90 g/L daily, resulting in a steady sugar excess. However, process parameters and conditions were not optimized specifically for *S. podzolica.* The focus of this study, therefore, was first to improve the lipid production potential of the yeast by optimizing the process parameters temperature and pH. Secondly, precise adjustment of the overall applied sugar amount for optimal needed concentration was examined with the purpose to reduce sugar wastage, since main medium cost are derived from carbon source (Vasconcelos et al., 2019). In that course, in this study a two-phase process strategy was established comprising a first batch cultivation and later merging into a fed-batch. In the batch phase yeast growth and biomass production was enabled, whereas by shifting into the fed-batch phase with nitrogen limiting and sugar excess conditions lipid accumulation was stimulated. To provide further advance to the cultivation process, manual feeding was replaced by an automated continuous feed in the fed-batch phase. Manual procedures are costly on industrial scale and must be omitted to reduce production costs. All these cultivation proceedings with *S. podzolica* were optimized on glucose and xylose with the prospect to cultivate this yeast also on sustainable and renewable substrates, such as abundant plant waste material, since the main sugars of ligno-cellulosic biomass are composed of hexoses and pentoses, such as glucose and xylose (Kim et al., 2012).

3.2 Material and methods

3.2.1 Yeast strain

The yeast strain, which is subject of the study, was first described as *Cryptococcus podzolicus* DSM 27192 by Schulze et al., (2014) and deposited at the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig; Germany). After genome sequencing and annotation the strain was phylogenetically reclassified to *Saitozyma podzolica* DSM 27192 (Aliyu et al., 2019).

3.2.2 Chemicals and cultivation media

All utilized chemicals were obtained from Carl Roth GmbH & Co. KG (Karlsruhe; Germany) or Sigma-Aldrich Chemie GmbH (Taufkirchen; Germany) if not stated otherwise.
The yeast strain was reactivated from cryo preservation at -80°C on YM agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L agar, pH 7, sterile supplemented with 10 g/L glucose after autoclaving). Mineral salt media for cultivation and lipid production contained a phosphate buffer system (8.99 g/L KH_2PO_4 and 0.12 g/L $Na_2HPO_4 \times 2\ H_2O$), 0.1 g/L sodium citrate $\times 2\ H_2O$, 0.1 g/L yeast extract, 0.2 g/L $MgSO_4 \times 7\ H_2O$, 4.72 g/L $(NH_4)_2SO_4$. After autoclaving 2% (v/v) of sterile trace elements solution with 4 g/L $CaCl_2 \times 2\ H_2O$, 0.55 g/L $FeSO_4 \times 7\ H_2O$, 0.475 g/L citric acid, 0.1 g/L $ZnSO_4 \times 7\ H_2O$, 0.076 g/L $MnSO_4 \times H_2O$, 100 µl/L 18 M H_2SO_4 and 2% (v/v) of sterile salts solution comprising 20 g/L $MgSO_4 \times 7\ H_2O$ and 10 g/L yeast extract were added. Additionally, glucose or xylose was sterile supplemented.

3.2.3 Cultivation in shake flasks

For the first pre-culture the yeast cells were scratched from YM agar and transferred into 50 mL mineral salt medium in conical shake flasks and incubated at 130 rpm and 20°C for 24 h. Second pre-culture was inoculated from first pre-culture to an OD_{600nm} of ~ 1.0 in 200 mL medium and cultivated at the same parameters for 24 h. Main cultures were performed in 2 L conical shake flasks in 400 mL cultivation volume. The cultures were inoculated from second pre-culture to an OD_{600nm} of ~ 1.0 and cultivated at 130 rpm for 96 h. The amount of sugar was daily determined and restocked to 90 g/L. Additionally, a manual feed of 2% (v/v) sterile trace elements and 2% (v/v) of sterile salts solution were added daily. The cultivations were sampled and analysed for CDW, glucose consumption and lipid content. All experiment set-ups were performed in triplicates.

3.2.4 Cultivation in bioreactors

For bioreactor cultivations the pre-cultures were conducted in the same way as described for shake flask cultivation. Main culture was operated in duplicates in 2.5 L glass vessel bioreactors (Infors HT, Bottmingen, Switzerland; Minifors fermentor) with 1.2 L mineral salt medium with initial OD_{600nm} of ~ 1.0. The applied parameters were pH 5 (before optimization) or pH 4 (optimized), the temperature of 20°C (before optimization) or 22.5°C (optimized), 600 rpm and 1 vvm aeration rate for 96-144 h. The pH was automatically controlled by addition of 4 M H_3PO_4 and 4 M NaOH. Foam formation was detected by a foam probe (Infors HT, Bottmingen, Switzerland) and the anti-foaming agent Contraspum A 4050 HAC (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) was automatically supplied to prevent foaming. Exhaust gas was monitored with the online exhaust gas analyzers BlueVary controlled by the BlueVis 4.0 Software (BlueSens gas sensor GmbH, Herten, Germany). Yeasts growth and production was tracked by daily sampling and analysis of CDW, carbon source and ammonia consumption and lipid and organic acid content.

3.2.4.1 Pulsed feed in high sugar excess process

The starting carbon concentration was 50 g/L. Every 24 h a manual feed of 2 % (v/v) sterile trace elements, 2 % (v/v) sterile salts solution and carbon source supply to 90 g/L of glucose or 60 g/L of xylose was implemented after determining the consumed sugar amount.

3.2.4.2 Pulsed feed in sugar consumption adjusted process

To develop a sugar consumption adapted process data of five independent high sugar excess cultivations were used to calculate the average sugar consumption and establish a new feeding strategy.

Table 4: Sugar amount used for sugar consumption adapted process with pulsed daily feeding.

	Sugar applied in batch phase [g] (c_{end} [g/L])*)	**Sugar applied for pulsed fed-batch phase [g]**				**Total applied sugar [g]**
		1st day	**2nd day**	**3rd day**	**4th day**	
Glucose	71 (59.2)	31.5	24.5	21	21	169
Xylose	49 (40.8)	24.5	19	13	14.5	120

**initial cultivation volume was 1.2 L*

The cultivation method was adapted to the calculated consumption rates resulting in a two-phase process with initial batch fermentation for the first 48 h merging into a daily fed-batch strategy aiming a sugar excess of approximately 10 g/L at the lowest point for four more days. The daily manual feeds included 2 % (v/v) sterile trace elements, 2 % (v/v) sterile salts solution and the respective sugar amount, which is reported in Table 4.

3.2.4.3 Automated, continuous feed in adapted process

To establish an automated supply of carbon source, trace elements and salts solution during the fed-batch phase the feed solutions were prepared containing 50 % (v/v) of 500 g/L concentrated stock solution of the respective sugar, 25 % (v/v) of sterile trace elements solution and 25 % (v/v) of sterile salts solution. Operated sugar amount of the feed solutions are displayed in Table 5. The density ρ of glucose and xylose feed was determined and consequently daily feeding rates were calculated in g/h (Table 5.). The continuous feed was enabled by external peristaltic pumps (Watson-Marlow GmbH, Rommerskirchen, Germany), regulated by the weight reduction of the feed bottle located on a laboratory balance (OHAUS Adventurer Pro AV4102C, OHAUS Europe GmbH, Nänikon, Switzerland) and controlled by LabVIEW software (LabVIEW2016, National Instruments, Austin, TX, USA). The resulted daily dilution rates D are presented in Table 5.

Table 5: Sugar amount used for automated, continuous feeding and daily pumping rates of the feeding solutions.

	Sugar applied in batch phase [g] (c_{end} [g/L]*)	**Sugar applied for continuous fed-batch [g]**	**Daily feeding rates [g/h] (Dilution rate D [1/h])**				**Total applied sugar [g]**
			1st day	**2nd day**	**3rd day**	**4th day**	
Glucose	71 (59.2)	89	4.20 (0.0035)	4.19 (0.0035)	4.19 (0.0034)	3.45 (0.0027)	160
Xylose	49 (40.8)	101	5.40 (0.0044)	4.20 (0.0033)	4.19 (0.0032)	4.22 (0.0032)	150

**initial cultivation volume was 1.2 L*

3.2.5 Cell dry weight (CDW) determination

Cell dry weight (CDW) analysis was performed gravimetrically for each sample in duplicates. In pre-dried and pre-weighted 1.5 mL reaction tubes 1 mL cultivation broth was provided and centrifuged at 20.000 × *g* for 3 min. The supernatant was used for determination of carbon source, organic acid and ammonium concentration. The cell pellet was washed with 0.8 mL

physiological saline (0.9% w/v NaCl) at 20.000 × *g* for 3 min. The saline was withdrawn and the pellet was dried for 48 h at 60°C and weighed with a precision balance.

3.2.6 Quantification of carbon source concentration

Yeast cultivations were performed on glucose or xylose. Glucose consumption was determined enzymatically using the UV-method at 340 nm of the D-glucose test kit from R-Biopharm (Art. No. 10716251035, R-Biopharm AG, Darmstadt, Germany). All from manufacturer instructed volumes were minimized to one-third, beside that the manufacturer´s protocol was followed. Samples were diluted with 0.9% w/v NaCl to appropriate concentration before detection.
Xylose was measured with a standard HPLC apparatus (Agilent 1100 Series, Agilent Technologies Deutschland GmbH, Böblingen, Germany) equipped with a Rezex ROA organic acid H+ (8%) guard column (8 µm, 50 × 7.8 mm) (Phenomenex Inc., Aschaffenburg, Germany) followed by a Rezex ROA organic acid H+ (8%) column (8 µm, 300 × 7.8 mm) (Art. No. 00H-0138-K0, Phenomenex Inc., Aschaffenburg, Germany) under isocratic conditions at 50°C column temperature for 20 min with 5 mM H_2SO_4 as mobile phase at a constant flow rate of 0.5 mL/min. The injection volume was 10 µl. The detection was enabled via a refractive index detector (Agilent 1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). For detection, cultivation broth was diluted in 5 mM H_2SO_4 to appropriate concentrations. For xylose quantification and identification, calibration curves measurements of D-(+)-xylose (Art. No. 5537.3; Carl Roth GmbH & Co. KG; Karlsruhe; Germany) were performed.

3.2.7 Detection of organic acid formation

For sample preparation cultivation broth was diluted in 20 mM KH_2PO_4 pH 2.5 to appropriate concentrations for detection. Gluconic acid (GA) and xylonic acid (XA) were quantified with a standard HPLC device (Agilent 1100 Series, Agilent Technologies Deutschland GmbH, Böblingen, Germany) using the reversed phase column Synergi™ 4 µm Fusion-RP 80 Å (150 × 4.6 mm) (Art. No. 00F-4424-E0, Phenomenex Inc., Aschaffenburg, Germany). Compounds were separated in a gradient mobile phase system of 20 mM KH_2PO_4 pH 2.5 (A) and 100% methanol (B) with a flow rate of 1 mL/min. The elution conditions of the gradient were 0–0.5 min 100% eluent A, 0.5-10 min formation of the gradient of eluent B from 0% to 10%, 10–12 min decrease of eluent B back to 0%, 12–14 min reconditioning step of the column to 100% eluent A. The injection volume was 10 µL and the temperature of the column oven set to 30°C. The detection was performed with a UV detector at a wavelength of 220 nm. To quantify and identify the peaks of both acids, calibration curves of analytical standards were performed with

D-Xylonic acid (Art. No. 73671-100MG; Sigma Aldrich; Taufkirchen; Germany) and D-Gluconic acid (Art. No. 64188-100MG; Sigma Aldrich; Taufkirchen; Germany).

3.2.8 Ammonia quantification

Ammonium nitrogen was analyzed photometrically by use of the Spectroquant kit (1.14752.0001, Merck KGaA, Darmstadt, Germany). For the assay requested volumes were down scaled to 300 µL per sample and measured in microtiter plates in duplicates following the manufacturer´s instruction procedure.

3.2.9 Indirect lipid quantification via derivatization to FAME

Indirect quantification of produced lipids was conducted by direct transesterification of yeast biomass to fatty acid methyl esters (FAMEs). 15 ml of fresh biomass was collected daily from the cultivation broth and washed with 0.9% w/v NaCl at 4800 × *g* for 5 min. The pellet was freeze-dried for 24 h at −30°C and 0.370 mbar using the BETA 1-8 freeze dryer (Christ, Osterode am Harz, Germany). 20-30 mg of freeze dried biomass were applied in a glass tubes and a subsequent acidic transesterification was performed using a two phase system. The hexane phase contained 0.5 mL internal standard consisting of 2 mg/mL heptadecanoic acid in hexane and 1.5 mL pure hexane. The second phase had an equal volume of 2 mL 15% H_2SO_4 (reaction's catalyst) in methanol. The mixtures were incubated for 2 h at 100°C and 1000 rpm in a thermoshaker (Universal Labortechnik, Leipzig, Germany). All samples were additionally mixed every 30 min by vortexing. To stop the reaction, the tubes were placed on ice for 10 min. To improve phase separation, 1 mL distilled water was added. The upper hexane phase containing FAMEs was used for GC analysis.

3.2.10 GC analysis of fatty acid methyl esters (FAMEs)

The quantitative and qualitative analysis of FAMEs was performed via gas chromatography (GC) using the 6890 N Network GC-System (Agilent Technologies Deutschland GmbH; Waldbronn; Germany). The device was coupled with a DB- Wax column (30 m × 0.25 mm) (Art. No. 122–7032; Agilent Technologies Deutschland GmbH, Böblingen, Germany) and the detection was performed with a flame ionization detector under 1.083 bar working pressure. 1 µL of sample was injected at the initial temperature of 40°C. The separation was achieved by a temperature gradient from 40°C to 250°C with a rate of 8°C/min and was kept for 10 min at 250°C. To identify and quantify the FAMEs the RM3 FAME Mix standard (Art. No. 07256-1AMP; Sigma Aldrich; Taufkirchen; Germany) was used.

3.2.11 Approximation of carbon balance of cultivation process

To give an assumption about the carbon conversion in the process, absolute mass of applied sugar, by yeasts produced CO_2, cell mass (CDW), lipids, by-products (GA or XA, respectively) and excess sugar at process end was calculated and based on the molecular weight of carbon. To do so, in case of cell mass the empirical chemical formula $C_6H_{10}O_3N$ for baker's yeast cell mass (Rose and Harrison, 2012) was used after subtraction of the lipid content, since lipids are intracellular products and therefore also weighted with the CDW determination method. Thus the approximation of carbon converted to lipid free cell mass was calculated in case of CDW.

3.2.12 Statistical analysis

3.2.12.1 Mean of levels comparison

One-way ANOVA followed by *post hoc*-test Tukey using *p*-value <0.05were performed with the Origin Software [version 2019 (9.6)] to identify differences in means of values.

3.2.12.2 Fit of data

Sugar consumption, biomass production, lipid titer and organic acid concentration were fit using Origin Software [version 2019 (9.6)] performing the nonlinear curve fit with logistic model after Levenberg-Marquardt method with four parameters upon the equation 1. The response A_1 indicates the initial data value at x = 0. A_2 is x → ∞. x_0 is half way between the two limiting values A_1 and A_2 and indicates the mid-range concentration of 50 %. p is the slope factor. The Levenberg-Marquardt regression solves nonlinear least-squares problems by converging from a wide range of initial guess values of parameters (Davis, 1993). It is often used as logistic dose response in science and engineering.

$$y = \frac{A_1 - A_2}{1 + (\frac{x}{x_0})^p} + A_2 \qquad 1$$

3.3 Results

The data shown in this section were obtained from triplicates ($n = 3$) (shake flasks cultivations) or duplicates ($n = 2$) (bioreactor cultivations).

3.3.1 Process temperature and pH optimization

The first cultivation process using the oleaginous yeast *S. podzolica* for lipid production was reported by Schulze et al. (2014), however cultivation conditions were not optimized. In a shake flasks experiment five different temperatures ranging from 18 - 27°C were tested in a 96 h cultivation. In Appendix 1 the normalized lipid content per dry biomass is displayed, revealing 22°C as the most appropriate temperature at 96 h followed by 20°C with about 17% less lipid, 25 and 27°C were about 30% less efficient and 18°C was the least suitable condition with 40% less produced lipid compared to cultivation at 22°C.

In the next experimental set-up optimal pH for lipid production was investigated in bioreactors. Here, all cultivations were performed at 22°C while pH 4, 5 and 6 was tested. The normalized lipid content over 96 h is presented in Appendix 2 displaying pH 6 as least suitable condition with 50% less lipid at 96 h compared to cultivations with pH 4 or 5, which resulted both in a similar lipid formation.

To study the individual effects and interactions of influencing factors a response surface methodology aimed strategy was performed by testing temperatures 20, 22.5 and 25°C and pH 4; 4.5 and 5. Consequentially, the tendencies of optimal parameters were 22.5°C and pH 4. To confirm the optimized process parameters, the standard conditions from Schulze et al. (2014) (STD: 20°C; pH 5) were directly compared with the optimized process conditions (Opt: 22.5°C; pH 4). The normalized lipid content over 96 h of both set-ups is illustrated in Figure 5. A higher lipid content within the optimized conditions can be observed starting from 24 h, whereby the significance steadily increases over time. At 96 h *S. podzolica* produced 40 % more lipid in the optimized process compared to the standard conditions.

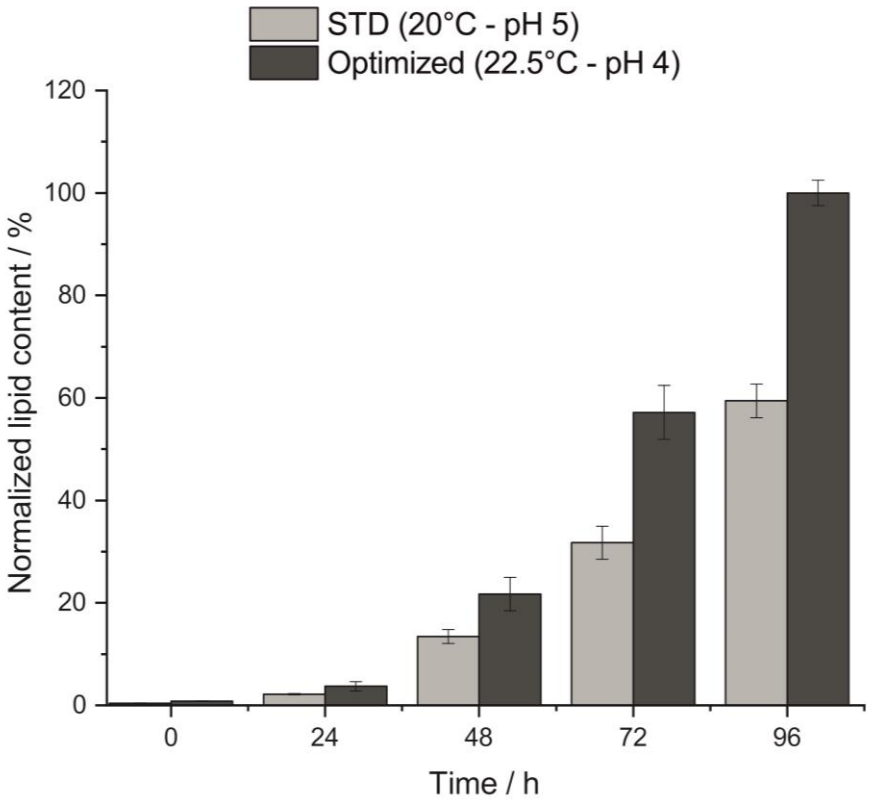

Figure 5: Comparison of produced lipid content by *S. podzolica* within the non-optimized standard process (STD: 20°C – pH 5) and under temperature and pH optimized conditions (Optimized: 22.5°C – pH 4) in bioreactors over 96 h process time. The data were normalized to the highest lipid titer. The error bars result from the standard deviation of two independent experiments.

3.3.2 Adjustment of applied sugar in a two-phase process

In the standard cultivation process, as illustrated in Figure 6 or Figure 7, a large amount of unused carbon source is still present at the end and is therefore wasted. After determining the actual sugar consumption of five independent standard cultivations a new two-phase process comprising an initial batch followed by a daily pulsed fed-batch was developed aiming for a reduced amount of applied carbon source.

Figure 6 and Figure 7 display the most important process features of the direct comparison of standard process and adjusted feeding strategy. Figure 6 illustrates the cultivations on glucose as carbon source comparing the daily feeding to 90 g/L (c(Glu) 90 g/L) and the calculated feed strategy (CFS Glu). In Figure 6a the two different glucose courses can be observed outlining the change in feeding strategy. The standard process (c(Glu) 90 g/L) starts with an initial glucose concentration of 55.4 ± 1.5 g/L. Every 24 h a manual glucose restock to ~ 90 g/L was performed after determining the amount of consumed sugar, except at 96 h where the feeding

procedure was skipped due to failure in glucose monitoring. The average daily glucose concentration before feeding was approximately 65 g/L, i.e. in this process strategy the yeasts were exposed to minimum ~ 65 g/L of sugar excess. The initial glucose concentration in the adjusted process is 63.6 ± 0.5 g/L, which is consumed in the first 46 h in a batch process to 9.6 ± 1.0 g/L. Subsequently, at 48, 72, 96 and 120 h a glucose supply was performed (as reported in Table 4). The average monitored lowest sugar concentration in that process was 12.5 g/L. In total 269 g glucose was used in the standard process, whereas it was 169 g in the adjusted process resulting in 37.17 % less applied sugar.

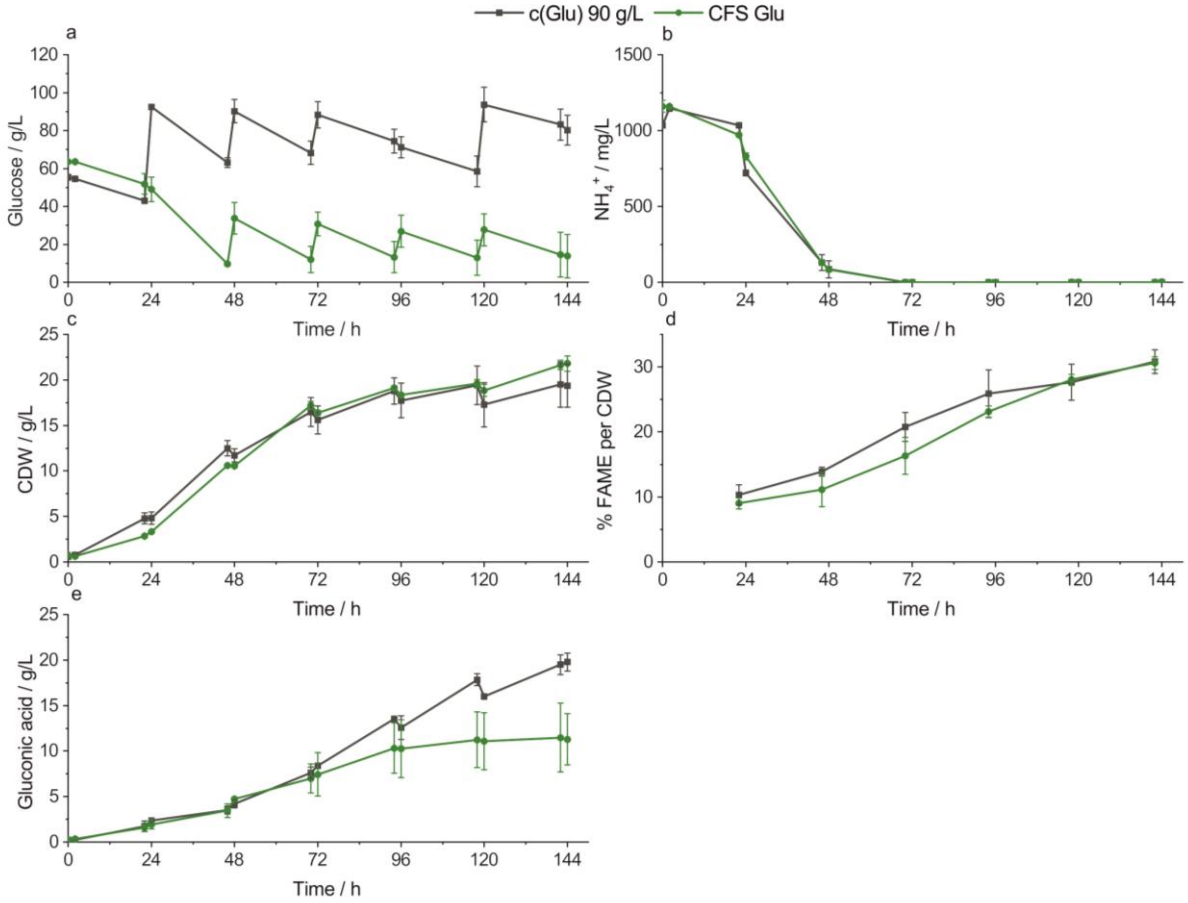

Figure 6: Direct confrontation of daily glucose pulsed feeding cultivation to high sugar excess (c(Glu) 90 g/L) and calculated lower sugar excess feed strategy (CFS Glu). Most important process conditions are illustrated against time. (a) tracked glucose concentration. (b) detected ammonia concentration. (c) biomass production. (d) analysed lipid titer. (e) gluconic acid production. The error bars result from the standard deviation of the experimental set-up in duplicates. c(Glu) 90 g/L, standard process with daily glucose restock to 90 g/L; CFS Glu, calculated feed strategy for glucose; CDW, cell dry weight; FAME, fatty acid methyl esters.

In most of the other process characteristics no significant difference can be observed between both feeding strategies. In Figure 6b the ammonia consumption is provided, showing similar consumption behavior in both cultivations. Within the first 48 h most of the ammonia is metabolized and after 70 h ammonia was not detected anymore. The produced biomass concentration

is displayed in Figure 6c. Both cultivation courses correspond to the ammonia consumption, since in the first 72 h up to 75 % of the biomass was produced. In the latter cultivation period the biomass concentration curves flatten. The produced lipids were indirectly analyzed as % FAME per CDW and are shown in Figure 6d. Here as well, no significant difference can be observed. In the standard process 30.8 ± 1.8 % FAME per CDW were produced in the end of the cultivation whereas, with the adjusted feeding strategy 30.6 ±1.0 % FAME per CDW were obtained, respectively. However, by looking at the produced by-product concentration of gluconic acid (Figure 6e) significantly more was produced at the end of the standard cultivation with 19.9 ± 1.0 g/L compared to 11.3 ± 2.8 g/L from the reduced sugar feeding.

In equivalent to Figure 6, Figure 7 presents the process on xylose as carbon source, whereas the daily feeding in the standard process was restocked to 60 g/L (c(Xyl) 60 g/L), which is documented in Figure 7a. The average daily xylose concentration before feeding was approximately 40 g/L in the standard process. However, the excess xylose concentration increases during the process from 26.4 ± 3.4 g/L at 46 h to 47.4 g/L at 142 h. In the adjusted xylose cultivation process 29.3 g/L xylose were consumed in the first batch phase and at 46 h the xylose concentration was determined to be 16.0 ± 1.7 g/L. A manual xylose supply was performed daily and the added amount is given in Table 4. However, the aimed xylose excess of 10 g/L could not be fully achieved. In the late fed-batch phase at 118 h and 142 h, xylose concentration was determined to be 1.9 ± 2.5 g/L and 0.4 ± 0.3 g/L, respectively. In comparison, 204 g xylose was used for the standard process and 120 g in the reduced sugar strategy, respectively. In Figure 7b and Figure 7c the ammonia consumption and biomass formation is presented, respectively. The ammonium consumption in the adjusted process was slower compared to the standard process, however, in both cases the ammonia was still exhausted after 70 h (monitored time point). Correspondingly, the biomass concentration is slightly lower in the adjusted process. Regarding the lipid content (Figure 7d) no significant difference can be observed. Though the produced lipid did not increase in the adjusted process in the last 24 h. In the standard process 26.9 ± 2.0 % FAME per CDW were produced in the end of the cultivation, and 23.2 ± 2.7 % FAME per CDW in the adjusted strategy, respectively. As a by-product of the xylose metabolism of *S. podzolica* xylonic acid was identified in the cultivation broth. Opposite to the cultivation on glucose, in both xylose process strategies a similar xylonic acid concentration was produced (Figure 7e). The maximum amount in the standard process was 0.8 ± 0.2 g/L. In the adjusted process the highest concentration was reached at 96 h with 0.9 ± 0.04 g/L, however, this concentration decreased slightly to 0.8 ± 0.3 g/L.

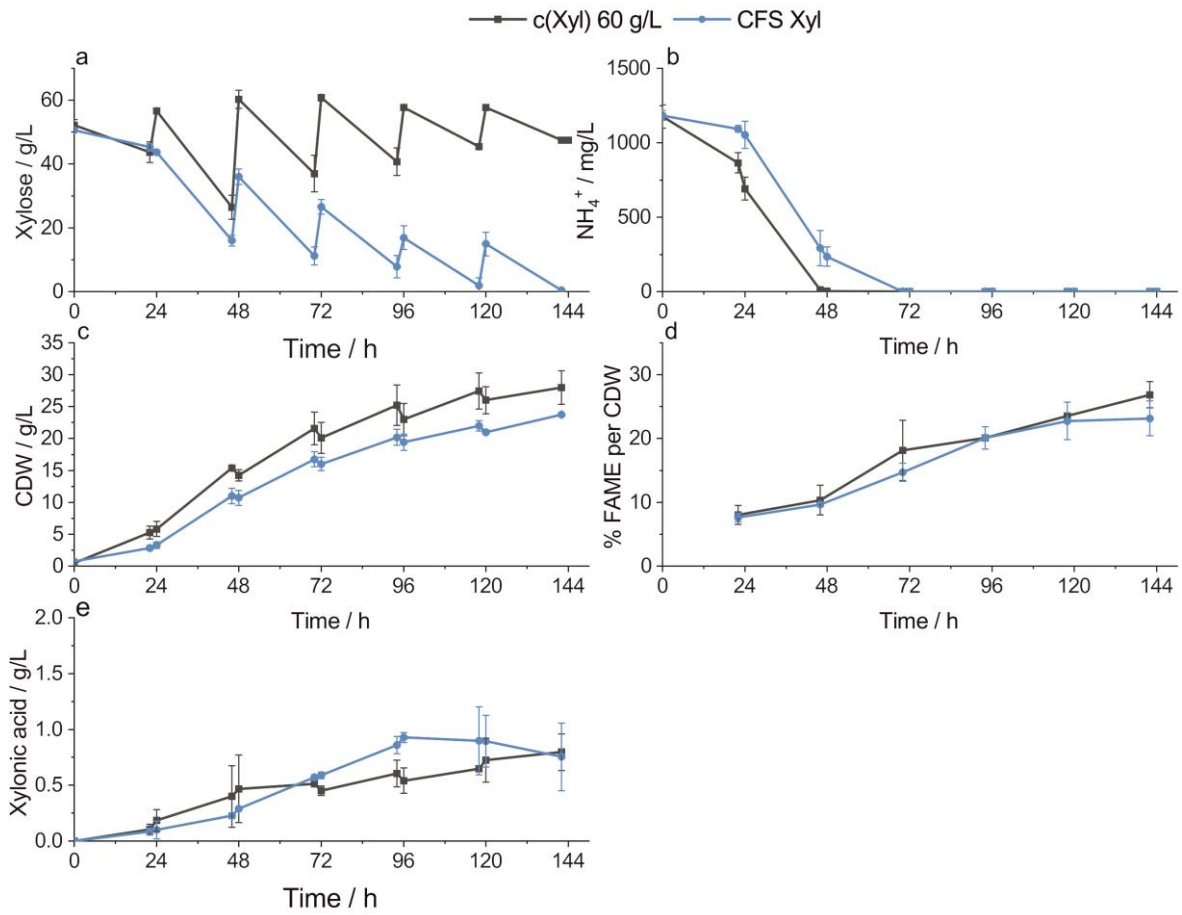

Figure 7: Comparison of cultivations on xylose with daily high xylose excess restock to 60 g/L (c(Xyl) 60 g/L) and and calculated lower xylose excess feed strategy (CFS Xyl). Process charasteristics like (a) xylose concentration (b) ammonia concentration (c) biomass production (d) lipid titer and (e) xylonic acid production, are presented. The standard deviation of the experimental set-up in duplicates is displayed with error bars. c(Xyl) 60 g/L, standard process with daily xylose restock to 60 g/L; CFS Xyl, calculated feed strategy for xylose; CDW, cell dry weight; FAME, fatty acid methyl esters.

In Table 6 the biomass yields ($Y_{x/s}$ [g/g]) and the lipid yields ($Y_{p/s}$ [g/g]) as well as the consumption (Q_s [g/L× h]) and production rates (Q_p [g/L× h]) are shown for the different cultivation phases. In general, the biomass yield is higher in the first three cultivation days compared to the latter periods in all monitored processes. This is especially underlined by calculating the yields within the nitrogen metabolism phase (0- 72 h) and the N-limitation phase (72-142 h).

The biomass yields in the latter period are about 50 % lower. For the lipid yields, however, the tendency is not as clear, but higher lipid yields are shifted to the nitrogen limitation phase.

The highest sugar consumption rates were detected on the second cultivation day in all processes with 1.33 ± 0.12 g/(L× h) in the standard process on glucose and 1.79 ± 0.25 g/(L× h) in the CFS on glucose. On xylose the rates were 1.37 ± 0.14 g/(L× h) in the c(Xyl) 60 g/L process and 1.26 ± 0.08 g/(L× h) in the xylose adjusted strategy. Similar to the lipid yields, the lipid production rates show no clear tendencies.

Table 6: Biomass yields ($Y_{x/s}$ [g/g]), lipid yields ($Y_{p/s}$ [g/g]), consumption (Q_s [g/L×h]) and production rates (Q_p [g/L×h]) in different cultivation periods in direct comparison between pulsed standard and adjusted pulsed process strategies.

	$Y_{x/s}$ [g/g]				$Y_{p/s}$ [g/g]				Q_s [g/L×h]				Q_p [g/L×h]			
Cultivation period [h]	c(Glu) 90 g/L	CFS Glu	c(Xyl) 60 g/L	CFS Xyl	c(Glu) 90 g/L	CFS Glu	c(Xyl) 60 g/L	CFS Xyl	c(Glu) 90 g/L	CFS Glu	c(Xyl) 60 g/L	CFS Xyl	c(Glu) 90 g/L	CFS Glu	c(Xyl) 60 g/L	CFS Xyl
0 - 24	0.34 ± 0.06	0.33 ± 0.11	0.56 ± 0.00*	0.39 ± 0.00*	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.57 ± 0.02	0.53 ± 0.22	0.39 ± 0.07	0.24 ± 0.04	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00
24 - 48	0.26 ± 0.02	0.23 ± 0.09	0.32 ± 0.02	0.28 ± 0.01	0.04 ± 0.00	0.04 ± 0.03	0.04 ± 0.01	0.03 ± 0.00	1.33 ± 0.12	1.79 ± 0.25	1.37 ± 0.14	1.26 ± 0.08	0.05 ± 0.00	0.06 ± 0.04	0.05 ± 0.01	0.04 ± 0.01
48 - 72	0.22 ± 0.04	0.31± 0.02	0.31 ± 0.03	0.24 ± 0.00	0.08 ± 0.02	0.09 ± 0.03	0.10 ± 0.02	0.06 ± 0.01	1.00 ± 0.01	0.99 ± 0.06	1.06 ± 0.13	1.13 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.09 ± 0.01	0.06 ± 0.01
72 - 96	0.23 ± 0.01	0.20 ± 0.04	0.26 ± 0.00*	0.22 ± 0.01*	0.10 ± 0.03	0.10 ± 0.02	0.06 ± 0.05	0.08 ± 0.00	0.63 ± 0.04	0.79 ± 0.09	0.91 ± 0.15	0.85 ± 0.06	0.06 ± 0.02	0.07 ± 0.01	0.05 ± 0.05	0.07 ± 0.01
96 -120	0.13 ± 0.01	0.15 ± 0.09	0.37 ± 0.03*	0.17 ± 0.02*	0.04 ± 0.00*	0.10 ± 0.00*	0.11 ± 0.01	0.06 ± 0.02	0.58 ± 0.12	0.63 ± 0.04	0.55 ± 0.01	0.68 ± 0.05	0.02 ± 0.00*	0.06 ± 0.01*	0.06 ± 0.00	0.04 ± 0.01
120 - 144	0.21 ± 0.02	0.22 ± 0.04	0.19 ± 0.05	0.19 ± 0.01	0.05 ± 0.06	0.09 ± 0.02	0.10 ± 0.06	0.03 ± 0.01	0.56 ± 0.05	0.58 ± 0.13	0.43 ± 0.00	0.62 ± 0.15	0.02 ± 0.03	0.05 ± 0.02	0.05 ± 0.03	0.02 ± 0.01
N-metabolization phase	0.25 ± 0.02	0.27 ± 0.07	0.34 ± 0.02	0.28± 0.01	0.05 ± 0.01	0.05 ± 0.03	0.06 ± 0.01	0.04 ± 0.00	0.91 ± 0.03	1.04 ± 0.03	0.89 ± 0.06	0.83 ± 0.04	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.01	0.04 ± 0.01
N-limitation phase	0.07 ± 0.02	0.12 ± 0.02	0.15 ± 0.01	0.14 ± 0.02	0.06 ± 0.00*	0.10 ± 0.00*	0.09 ± 0.04	0.06 ± 0.01	0.58 ± 0.00	0.61 ± 0.08	0.58 ± 0.04	0.66 ± 0.05	0.04 ± 0.00*	0.06 ± 0.01*	0.05 ± 0.03	0.04 ± 0.00
Total	0.19 ± 0.01	0.22 ± 0.05	0.26 ± 0.01*	0.22 ± 0.00*	0.06 ± 0.00	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	0.74 ± 0.01	0.83 ± 0.03	0.73 ± 0.05	0.74± 0.00	0.04 ± 0.00	0.06 ± 0.02	0.05 ± 0.01	0.04 ± 0.00

*$Y_{x/s}$ = biomass specific yield; $Y_{p/s}$ = product specific yield; Q_s = volumetric consumption rate; Q_p = volumetric production rate; c(Glu) 90 g/l = standard process with daily glucose restock to 90 g/L; CFS Glu = calculated feed strategy for glucose; c(Xyl) 60 g/l = standard process with daily xylose restock to 60 g/L; CFS Xyl = calculated feed strategy for xylose; * indicates statistical significant differences (p = 0.05)*

To illustrate, that despite the sugar reduction of the calculated feed strategy (CFS) process, productivities remained comparable to the standard process, of each phase a mean comparison analysis of standard process against the adjusted process was performed. No significant differences were observed in the majority of the cases. Just in a few periods statistical differences at level $p = 0.05$ were detected, which is within the biomass yield on xylose at the first, fourth, fifth cultivation day and for the total yield. The lipid yield on glucose is significantly different between 96 – 120 h and in the N-limitation phase. Correspondingly, in the same phases the production rates on glucose are significantly different.

3.3.3 Automated continuous sugar feed

In the next step, it was intended to omit the manual pulsed feeding in the fed-batch phase and to establish a continuous feeding with constant sugar excess of about 10 g/L. In Figure 8 the resulting cultivation plots on glucose (Figure 8a) and xylose (Figure 8b) are presented.

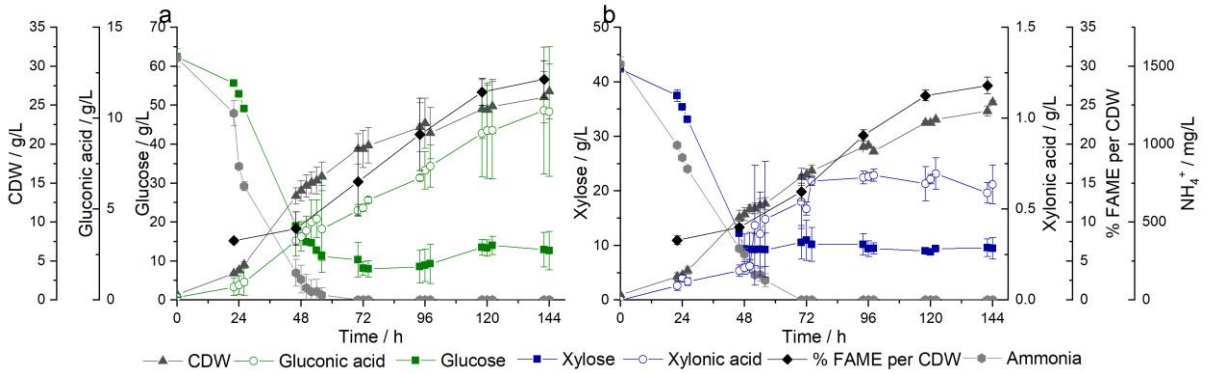

Figure 8: Process plots of established automated continuous feeding with constant sugar excess of about 10 g/L in the fed-batch phase on (a) glucose and (b) xylose. Error bars result from the standard deviation of the experimental set-up in duplicates. CDW, cell dry weight; FAME, fatty acid methyl esters.

In the first 48 h of batch process on glucose (Figure 8a) 46.21 ± 0.24 g/L glucose was consumed. In the following 96 h of continuous feed the average detected glucose concentration was 11.54 ± 2.28 g/L. As in the pulsed feeding processes, ammonia was completely metabolized after 70 h (monitored time point). At that time point the biomass concentration reached 19.38 ± 1.94 g/L and increased to 26.78 ± 3.50 g/L till the end of cultivation. As for the products, the maximum content of lipids was measured at the end of cultivation with 28.34 ± 2.38 % FAME per CDW, likewise, the maximum titer of gluconic acid was detected at 10.42 ± 3.50 g/L at 142 h. For the cultivation of xylose, the sugar consumption of 33.91 ± 2.64 g/L is illustrated in the first 48 h batch phase in Figure 8b. Subsequently, a constant xylose concentration of 9.59 ± 0.60 g/L (average of monitored sampling points) was ensured during continuous feeding. Ammonia was exhausted at 70 h. The highest biomass concentration and lipid content were reached at the end

of cultivation with 25.38 ± 0.25 g/L CDW and 27.53 ± 1.07 % FAME per CDW, respectively. The detected xylonic acid concentration was slightly lower compared to the manual fed-batch. As shown in Figure 8b the maximum concentration was reached at 122 h with 0.70 ± 0.09 g/L, but it decreased to 0.63 ± 0.11 g/L at the end of the cultivation.

3.3.4 Comparison of process characteristics on glucose and xylose

To highlight the differences of *S. podzolica*'s process behavior on continuous feed of glucose versus xylose, volumetric consumption and production rates (Q [g/L×h]) were calculated after logistic curve fits of carbon source, biomass, lipid and gluconic and xylonic acid, respectively. The comparison is presented in Figure 9. The volumetric sugar consumption rates are illustrated in Figure 9a. The peak of the curves reflects the highest rate reached and it can be clearly seen, that on glucose the productivity is more efficient with 2.02 g/(L×h) at 29.8 - 31 h compared to xylose with 1.44 g/(L×h) at 28.4 - 29.5 h. In addition, the parabolic curve of glucose consumption is wider and flattens only at 70.3 h below 0.1 g/(L×h), whereas the same occurs 10.9 h earlier at 59.4 h on xylose. The biomass production rates in Figure 9b differ greatly. The biomass production on glucose reaches higher productivity rates with 0.43 g/(L×h) at 36.0 – 37.3 h at its peak, and on xylose at maximum 0.28 g/(L×h) at 29.5 – 44.3 h. However, the biomass productivity on glucose drops faster compared to the biomass production on xylose, where the curve is at its peak for 14.8 h. At the end of the cultivation the biomass productivity rate on xylose (0.062 g/(L×h)) is still three fold higher compared to glucose (0.021 g/(L×h)). The volumetric productivity rates of lipids (Figure 9c) and gluconic or xylonic acid (Figure 9d), respectively, vary as well. The lipid productivity on glucose is higher at the peak (0.082 g/(L×h) between 56.9 – 65.6 h) than on xylose (0.075 g/(L×h) between 68.3 – 77.5 h), though from 87.5 h the lipid productivity on xylose is higher than on glucose. Most significantly the organic acid productivity curves differ between the two different sugars. Gluconic acid volumetric productivity reaches at maximum 0.092 g/(L×h) between 44.4 – 51.0 h, whereas xylonic acid productivity is maximally achieved between 61.7 – 68.0 h with 0.021 g/(L×h), which is 4.4 times lower compared to gluconic acid productivity.

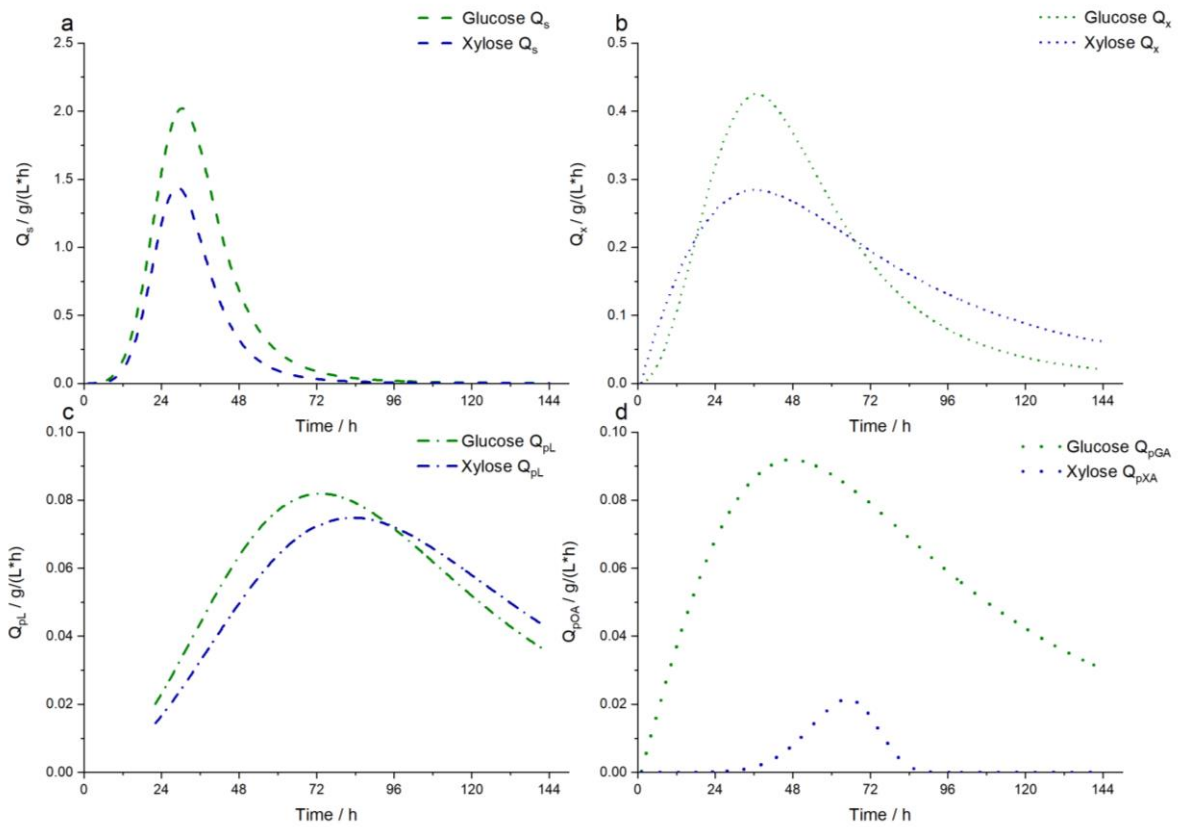

Figure 9: Volumetric consumption and production rates Q [g/L×h] of *S. podzolica* from automated continuous feed process. Q was calculated as derivation of logistic curve fits of carbon source, biomass, lipid and gluconic or xylonic acid, respectively, on glucose and xylose against time. (a) Q_s volumetric substrate consumption rate. (b) Q_x volumetric biomass production rate. (c) Q_{pL} volumetric lipid production rate. (d) Q_{pOA} volumetric organic acid production rate, in datail Q_{pGA}: volumetric gluconic acid production rate, Q_{pXA}: volumetric xylonic acid production rate.

Besides comparing the production rates, it was also aimed to approximate the overall carbon conversion of both sugar sources by assessing the exhaust gas and the analysed products during continuous feeding process. The plots of the CO_2 emission over time are provided in Appendix 3a for glucose and Appendix 3c for xylose. Here, a difference of CO_2 production curves on glucose or xylose can be observed. While on glucose a peak of produced CO_2 is reached during initial batch phase, which later decreases, on xylose beginning from the second cultivation day a nearly constant CO_2 production was detected.

The approximate carbon balance is shown in Figure 10. On glucose (Figure 10a) as well as on xylose (Figure 10b) a similar percentage of the overall carbon was converted into lipid-free cell mass (glucose: 18.55%; xylose: 18.87%) and lipids (glucose: 11.49%; xylose: 10.86%). However, on glucose clearly more carbon is channelled to produce the organic acid gluconic acid (8.38% of total carbon). Correspondingly, less CO_2 was produced on glucose with 46.8% of total carbon, on xylose, however, is was 54.08%. Regarding the lipids, on both carbon sources

a similar lipid profile ratio can be observed with oleic acid as main fatty acid followed by palmitic, linoleic and stearic acid. The amount of unconverted carbon, left in the cultivation broth, was comparable in both cases.

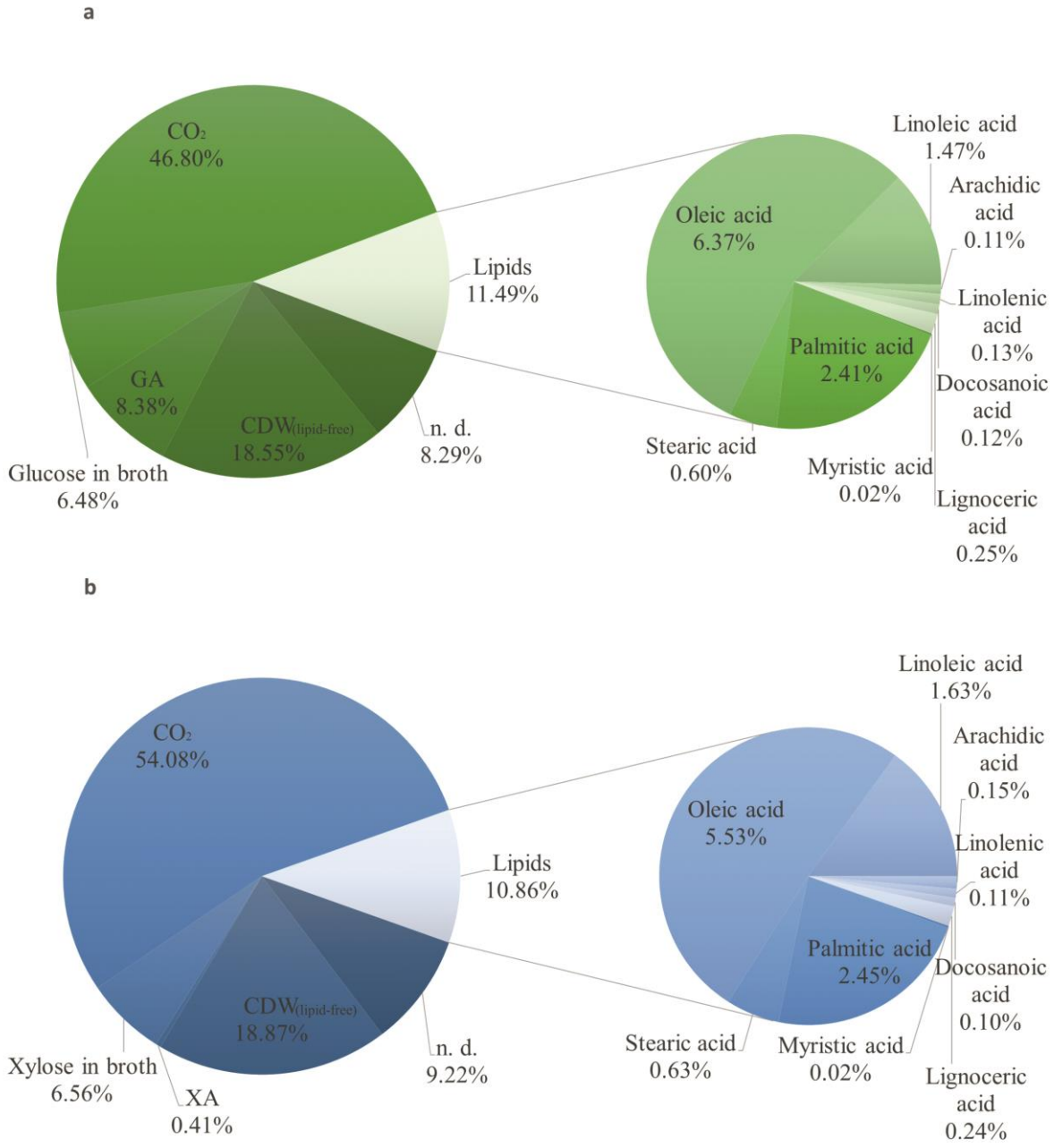

Figure 10: Approximative determination of the overall carbon distribution to process products including by exhaust gas assesed CO_2 emission from automated continuous feed process of *S. podzolica*. a) Approximative percentage distribution of glucose conversion. a) Approximative percentage distribution of xylose conversion. CDW, cell dry weight; n.d., not determined.

3.4 Discussion

3.4.1 Influence of temperature and pH on lipid production

The first cultivation process using the oleaginous yeast *S. podzolica* for lipid production was reported by Schulze et al., (2014), however cultivation conditions (Temperature: 20°C, pH 5) were not optimized. In order to reach the maximum potential of the production stain, in this study these process parameters were investigated to find the optimal conditions. According to literature, for oleaginous yeast temperature should generally be maintained between 25 – 30°C, while pH is to be regulated between 3 – 6 (Ageitos et al., 2011). However, an optimal lipid production temperature for the unconventional oleaginous yeast *S. podzolica* is barely known in literature. Here rather functional cultivation conditions at 25 or 30°C can be found, to obtain biomass for several characterization studies (Botes et al., 2005; Kunthiphun et al., 2018). As an isolate from peat bog (Schulze et al., 2014), known as acidic soil, acidic pH (4 - 6) and temperate temperatures between 18 – 27°C were considered to test. Interestingly, the warm-moderate temperatures (25 and 27°C) were unsuitable for the lipid productivity of *S. podzolica.* Still less suitable was the cold-moderate temperature of 18°C for lipid production (Appendix 1). As expected, a more acidic pH range turned out to be more suitable compared to pH 6 (Appendix 2). After a response surface aimed methodology (data not shown) the tendencies of optimal parameters were directed to 22.5°C and pH 4. The direct comparison of non-optimized standard process from Schulze et al. (2014) versus the optimized conditions revealed a 40% increase of produced lipids at 96 h, as shown in Figure 5. Thus, a remarkable impact of temperature and pH on the lipid content of *S. podzolica* can be concluded. In similar studies of other oleaginous yeasts the influence of temperature and pH, among other, was also reported (Angerbauer et al., 2008; Karatay and Dönmez, 2010; Li et al., 2010; Cui et al., 2012).

3.4.2 Reduction of excess sugar amount in a two-phase cultivation process

To optimize the cultivation process not only in the aspect of productivity but also in an economical context, a reduction of used sugar amount was sought while still ensuring a comparable lipid content. Since the accumulation of triacylglycerols (TAGs), as intracellular storage lipids in the yeast cell, is due to a stress response by lacking nutrients, e.g. nitrogen, with simultaneous excess of carbon source, high C/N ratios are required for lipid production (Ratledge, 2002, 2004). The optimum C/N ratio is reported to be close to 100 for most of the oleaginous yeasts (Li et al., 2008; Ageitos et al., 2011; Probst et al., 2016). However, too early or unregulated

nitrogen limitation will impact the yeasts growth and deteriorate the biomass production and consequently the SCO titer, as being an intracellular product (Beopoulos et al., 2009). For that reason, in this study a two-phase process was introduced, comprising an initial 48 h batch cultivation without nutrient limitation for the purpose of biomass production and merging into a pulsed (Figure 6 and Figure 7) or continuous (Figure 8) fed-batch process, respectively, with nitrogen limiting conditions. The initial C/N ratio in all in this study established cultivations was always kept high (glucose:130; xylose: 90). However, it was observed that in the fed-batch phase, where N is limited, the carbon excess can be reduced and does not need to be restocked to 90 g/L (glucose) or 60 g/L(xylose), respectively, as it was performed in the un-optimized standard process. As it is proven in Table 6, yields and productivities of *S. podzolica* on high or reduced carbon source cultivation were similar. On glucose even an improvement in biomass or product yields and productivities, respectively, can be observed. Too high sugar concentrations are not only un-economical but can rather lead to narrow productivity, due to substrate inhibition. It is known, however, that *S. podzolica* is able to tolerate high glucose concentrations of up to 150 g/L for lipid production, although inhibiting effects on cell propagation were observed, which consequently decreased the lipid titer (Qian et al. 2019). Therefore, resulting from this study, for lipid production with *S. podzolica* high C/N ratio in the batch phase is mandatory, however, once nitrogen is limited the excess sugar concentration of 10 g/L ensured stable productivities, as can be seen in Figure 8. Fei et al. (2016) were also able to establish an automated system, where the glucose concentration of a ligno-cellulosic hydrolysate was regulated around 10 ± 2 g/L, and led to a 52% higher lipid content and a 42% lipid productivity improvement compared to a batch cultivation.

However, as can be seen from this study's Figure 7a on the calculated feed strategy on xylose (CFS Xyl), the desired concentration of 10 g/L xylose could not be maintained constantly. In the last two cultivation days the lowest xylose concentration reached 1.9 ± 2.5 g/L at 118 h and 0.4 ± 0.3 g/L at 142 h, which led to a stagnation of lipid production (Figure 7d). Consequently, it can be concluded that at lower sugar concentrations the lipid production decreases and it can be assumed that a constant concentration of 1 g/L might be too low and is to avoid for stable productivities.

Thus, in the reduced sugar cultivation with constant feeding and regulated sugar excess of about 10 g/L 40.52% and 26.47% of glucose or xylose, respectively, were saved compared to the un-optimized standard process. By comparing the actual metabolized sugar with the applied sugar,

the difference between optimized and un-optimized feeding strategies is remarkable. The process of daily glucose restocking to 90 g/L led to a sugar utilization of just 56.01 ± 4.35%, in comparison to 90.84 ± 3.78% within the optimized constant feeding (Table 7). On xylose 72.49 ± 0.56% of carbon source were metabolized in the daily restocking to 60 g/L strategy, but 92.30 ± 1.61% in the constant feeding (Table 7). However, the total metabolized sugar amount does not differ significantly between the feeding strategies (Table 7). Hence, the constant feed reduced sugar strategy led to a more efficient carbon source uptake in the cultivation process and prevented wastage of unused sugar in the broth.

3.4.3 Comparison of feeding strategies

An overview and direct comparison of the process strategies in the high sugar manually pulsed feeding and the reduced sugar continuous feeding is provided in Table 7. As mentioned above, in the reduced sugar feed less carbon source is applied to the process in general and the sugar utilization is by far more efficient, as less sugar is left unused in the medium.

Regarding the product formation and productivities on glucose, the constant feeding turned out to be 28% more beneficial on biomass growth and 19% on lipid titer. The total glucose consumption (+ 19%) and biomass (+ 26%) and lipid (+ 20%) production rates increased compared to pulsed feeding, indicating more favorable production conditions for *S. podzolica* during constant feed. Similar findings on constant feed were observed with *Rhodosporidium toruloides* (Fei et al., 2016; Tsakona et al., 2016) and *Rhodosporidiobolus fluvialis* (Poontawee and Limtong, 2020).

However, the oxidation of glucose to the organic acid gluconic acid is more efficient during pulsed feeding, which is due to the higher glucose excess. As it was shown by Qian et al. (2019), *S. podzolica*'s GA production was greatly enhanced by high glucose concentration of 150 g/L. The same circumstances are reported in case of high sugar concentration stimulated citric acid production of *Yarrowia lipolytica* and *Rhodotorula glutinis* (Papanikolaou et al., 2006; Karamerou et al., 2017). In the reduced sugar process in this study 48% less GA was produced compared to the high sugar process (Table 7). Considering the improved lipid production in the reduced sugar process, clearly the carbon flux seems to be shifted to lipid accumulation in *S. podzolica,* when applying a lower sugar excess. If a co-production of intracellular lipids and extracellular GA is desired, a high sugar excess is recommended, which results, however, in a considerable wasting of carbon source in the cultivation broth (Qian et al. 2019). When aiming

for target-oriented lipid accumulation, a controlled feeding with constant sugar excess is more suitable, as shown in this study.

Table 7: Comparison of high sugar standard cultivation and reduced sugar cultivation with continuous feed.

	c(Glu) 90 g/L	cont. CFS Glu	c(Xyl) 60 g/L	cont. CFS Xyl
Feeding strategy	Daily manual restock	Batch/ continuous fed-batch	Daily manual restock	Batch/ continuous fed-batch
Feeding status	Un-optimized	Optimized	Un-optimized	Optimized
Total sugar amount applied [g]	269	160	204	150
Total sugar amount metabolised [g]	150.66 ± 11.71	145.35 ± 6.05	147.88 ± 1.17	138.46 ± 2.40
% sugar used by yeasts	56.01 ± 4.35	90.84 ± 3.78	72.49 ± 0.56	92.30 ± 1.61
Final CDW [g/L]	19.38 ± 2.37	26.78 ± 3.5	27.98 ± 2.65	25.38 ± 0.23
Final lipid content [% FAME/CDW]	30.82 ± 1.84	28.34 ± 2.38	26.87 ± 2.04	27.53 ± 1.07
Final lipid titer [g/L]	5.98 ± 0.40	7.41 ± 1.55	7.54 ± 1.28	6.68 ± 0.43
Final organic acid concentration [g/L]	GA: 19.78 ± 0.98	GA: 10.38 ± 3.57	XA: 0.90 ± 0.16	XA: 0.64 ± 0.11
Total consumption rate [g/(L × h)]	0.71 ± 0.06	0.88 ± 0.06	0.87 ± 0.03	0.78 ± 0.01
Total biomass production rate [g/(L× h)]	0.14 ± 0.02	0.19 ± 0.02	0.20 ± 0.02	0.18 ± 0.02
Total lipid production rate [g/(L× h)]	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00
Total organic acid production rate [g/(L× h)]	GA: 0.14 ± 0.01	GA: 0.07 ± 0.03	XA: 0.006 ± 0.001	XA: 0.0004 ± 0.0001
Total $Y_{x/s}$ [g/g]	0.19 ± 0.01	0.21 ± 0.01	0.26 ± 0.01	0.22 ± 0.00
Total $Y_{l/s}$ [g/g]	0.06 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.00
Total produced CO_2 [g]	84.62	109.79	84.96	118.90
Total produced CO_2 per CDW [g/g]	2.98 ± 0.36	3.58 ± 0.38	2.42 ± 0.05	3.83 ± 0.04

c(Glu) 90 g/l = standard process with daily glucose restock to 90 g/L; cont. CFS Glu = calculated feed strategy for glucose with continuous feed; c(Xyl) 60 g/l = standard process with daily xylose restock to 60 g/L; cont. CFS Xyl = calculated feed strategy for xylose with continuous feed, $Y_{x/s}$ = biomass specific yield; $Y_{l/s}$ = lipid specific yield, CDW = cell dry weight

The comparison of feeding strategies on xylose provides a different context. Here, product formation is more favored when higher sugar concentrations are pulsed-fed compared to constant feeding. Admittedly, the differences in productivities are rather low and the total lipid productivity rates are even equal (Table 7). By considering the notably reduced applied xylose amount and more efficiently metabolized xylose amount during constant feeding, still the latter should be chosen for a more economical lipid production. In a broader context, a continuous feed with low dilution rates is also more favorable regarding the use of sustainable carbon rich waste products, such as ligno-cellulosic hydrolytes, crude glycerol or other waste products, as substrate, since such waste products often contain a considerable amount of toxic compounds (Cui et al., 2012; Ling et al., 2013; Dörsam et al., 2016; Poontawee and Limtong, 2020). Cell proliferation and product formation can be severely compromised upon high concentration of such waste products in a batch or a high carbon source excess cultivation. By applying continuously low concentration in a constant feed, this can be overcome and the toxicity stress to the cells can be reduced (Yen et al., 2015; Poontawee and Limtong, 2020).

By looking at the produced CO_2 amount it is immediately apparent, that during continuous feeding with both sugars more CO_2 was emitted compared to pulsed feeding (Table 7). The same can be observed for the amount of CO_2 produced per dry biomass (Table 7). For glucose this might be explained due to the fact, that during pulsed feeding more GA is produced. The oxidation of glucose to GA is CO_2 neutral (Ramachandran et al. 2006). The by-product formation might be a stress coping strategy of the yeast to circumvent the high sugar excess, since a higher GA production is reported on higher glucose excess, which was already discussed above. For the process on xylose this argument is however weak, since just low concentrations of XA were produced. Here it is worthwhile to look at the CO_2 emission plots over time (Appendix 3). The CO_2 curves are smoother on continuous feeding of both sugars (Appendix 3a and c), compared to the pulsed fed sugar (Appendix 3b and d), where every 24 h the CO_2 production is interfered as a feed was applied. Consequently, during continuous feeding the respiration of the cells is less impaired by nutrient supply than during pulsed feeding.

3.4.4 Comparison of cultivation on glucose and xylose

To ensure sustainability and economic feasibility for SCO produced by yeasts, abundant and cheap ligno-cellulosic plant biomass became of interest as substrate. The main carbohydrates of hydrolysates of ligno-cellulosic biomass are composed of glucose and xylose (Kim et al., 2012). For that reason, the cultivation of *S. podzolica* was optimized on both sugars in this study. It was observed, that total lipid productivities and yields were similar on both carbon

sources (Table 7). The volumetric consumption and production rates differ, however, during the cultivation, as presented in Figure 9. The maximum consumption rate on glucose is higher and lasts longer compared to maximum xylose consumption rate, which indicates glucose as the more efficient and favorable carbon source, as it is the case for most yeasts compared to xylose (Gancedo, 1998). Interestingly, although the biomass production rate on xylose did not reach as a high value as on glucose, it stayed stable at maximum for 14.8 h during the batch phase. Additionally, in the later continuous fed- batch phase the volumetric biomass production rate on xylose was higher than on glucose. This indicates a similar ability of *S. podzolica* to produce biomass on both sugars. Also, volumetric lipid productions are highly comparable. On glucose higher values are reached, however, at the end more efficient productivities are achieved upon xylose. In other studies, with *Candida curvata* (syn. *Cryptococcus curvatus*, *Cutaneotrichosporon oleaginosum*) maximum lipid accumulation occurred in batch culture with xylose as carbon source (Evans and Ratledge, 1983). For *Lipomyces starkeyi* higher lipid contents were reached on xylose, yet on glucose a higher biomass concentration was produced (Juanssilfero et al., 2018).

Considering the CO_2 emission in Appendix 3a for glucose and Appendix 3c for xylose both during continuous feed, both plots differ remarkably. On glucose the CO_2 emission resembles a parabolic curve, on xylose, however, the CO_2 emission is more or less stable just below 1.5 mg from the second cultivation day until the end. This can be explained with the different applied glucose and xylose concentration as reported in (Table 5). For cultivation on glucose the starting concentration in the batch phase was higher than the initial xylose concentration, vice versa during fed batch less glucose and more xylose was added. In total a higher total CO_2 production was observed on xylose with 118.90 g compared to 109.79 g total CO_2 on glucose, which is 54.08% (Figure 10b) of total applied xylose channelled to CO_2 conversion and 46.8% (Figure 10a) of total glucose metabolized to CO_2, respectively. For instance, the overall lower carbon to CO_2 conversion on glucose in contrast to xylose may also be due to the high GA production. GA is the organic acid formed by the oxidation of the first carbon of glucose (Ramachandran et al. 2006). Hence, 8.38% (Figure 10a) of total glucose were channelled to GA production, whereas just 0.41% (Figure 10b) of total xylose were used to produced XA. Thus, the glucose oxidizing ability of *S. podzolica* is by far more efficient than the xylose oxidation, as can be witnessed by produced amount of and productivity rates (Table 7; Figure 10). This may suggest, that on xylose more carbon is converted to CO_2, since less by-product is produced compared to glucose.

Nevertheless, both organic acids are of high value. GA is a noncorrosive, nonvolatile, nontoxic, mild carboxylic acid (Ramachandran et al. 2006). It can be found abundantly in plants, honey and wine. It is categorized GRAS status and is listed as a generally permitted food additive (E 574) and therefore highly used for food manufacturing (Ramachandran et al. 2006). Apart from that, it is regarded as a bulk chemical in the textile, pharmaceutical, and construction industries (Singh and Kumar, 2007). Microbial conversion of glucose to GA has also been reported in by *Aspergillus niger*, *Penicillium*, and bacterial species such as *Pseudomonas ovalis*, *Acetobacter* and *Gluconobacter oxydans* (Ramachandran et al. 2006). Admittedly, GA production is just possible with glucose as substrate, which propagates the competition with food purposes. Therefore, the oxidation product of the non-edible xylose XA can substitute GA in some aspects. For instance, XA can be applied for food, pharmaceutical, and agricultural purposes (Liu et al., 2012), it can be used as sustainable solvent and biocatalyst (Ma et al., 2016) or as from waste biomass derived polymer precursor (Alonso et al. 2015). In literature microbial conversion of xylose to XA is reported by *Kluyveromyces lactis*, *Aspergillus niger, Gluconobacter oxydans*, or recombinant *Saccharomyces cerevisiae* and *Escherichia coli* (Liu et al., 2012; Alonso et al., 2015; Hahn et al., 2020). The XA production ability of *S. podzolica* was to the author's knowledge never described before. Despite the fact, that *S. podzolica*'s XA production rates are rather low, there is potential in this strain to optimize the cultivation process in the purpose to increase the XA production.

4 Evaluation of cell disruption for SCO downstream processing of *S. podzolica* and *A. porosum*

This chapter is mainly based on the publication

Evaluation of downstream processing, extraction, and quantification strategies for single cell oil produced by the oleaginous yeasts *Saitozyma podzolica* DSM 27192 and *Apiotrichum porosum* DSM 27194

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Authors' contribution to this publication

Olga Gorte designed and performed the experiments of *S. podzolica* DSM 27192 and *A. porosum* DSM 27194 cultivations in bioreactors, microscopical cell count, cell disruptions, oil recovery, transesterification to FAMEs, and GC analysis. Analysed the data and mainly drafted the manuscript.

Rebecca Hollenbach planned and conducted the enzymatic experiment part and HPLC analysis of this work and performed the statistical analysis. Drafted as co-first author the enzymatic hydrolysis and resulting free fatty acids section. This content of the publication was omitted in this dissertation.

Ioannis Papachristou performed together with Olga Gorte the nile red assay.

Christian Steinweg enabled the use of the bead mill.

Aude Silve constructively contributed to the content and revised the manuscript.

Wolfgang Frey revised the manuscript.

Christoph Syldatk contributed to the content and critically revised the manuscript.

Katrin Ochsenreither contributed to the design of the study, supervised the work and critically revised the article.

4.1 Introduction

In this section, combinations of cell disruption and extraction methods on *S. podzolica* and *A. porosum* are evaluated. Mechanical cell disruption methods, namely bead-milling (BM), high-pressure homogenization (HPH) and ultrasonification (U) were implemented because of their great industrial potential and the use at a large scale in other industries (Chisti and Moo-Young, 1986; Rodríguez-Alcalá et al., 2009; Ochsenreither et al., 2016). These disruption methods were applied on frozen, wet yeast biomass prior to chloroform methanol extraction according to Folch (F) (Folch et al., 1957) and Bligh and Dyer (BD) (Bligh and Dyer, 1959). Additionally, the ethanol and hexane (EH) system, which is commonly used in food industry (Grima et al., 1994; Cheng and Rosentrater, 2017), was tested in terms of whole lipid extraction yield. The extracted whole lipids, including storage and membrane lipids are applicable for oleochemical building blocks production. Further investigations were performed on lipid profiles after trans-esterification to FAME and analysis via GC-FID. All results were also compared with the lipid yield and profile from direct transesterification (DT) of freeze-dried biomass. The derivatization to FAME directly serves the purpose to produce biodiesel (Li et al., 2008; Sathish et al., 2014; Yousuf et al., 2017). Figure 11 outlines the experimental procedure.

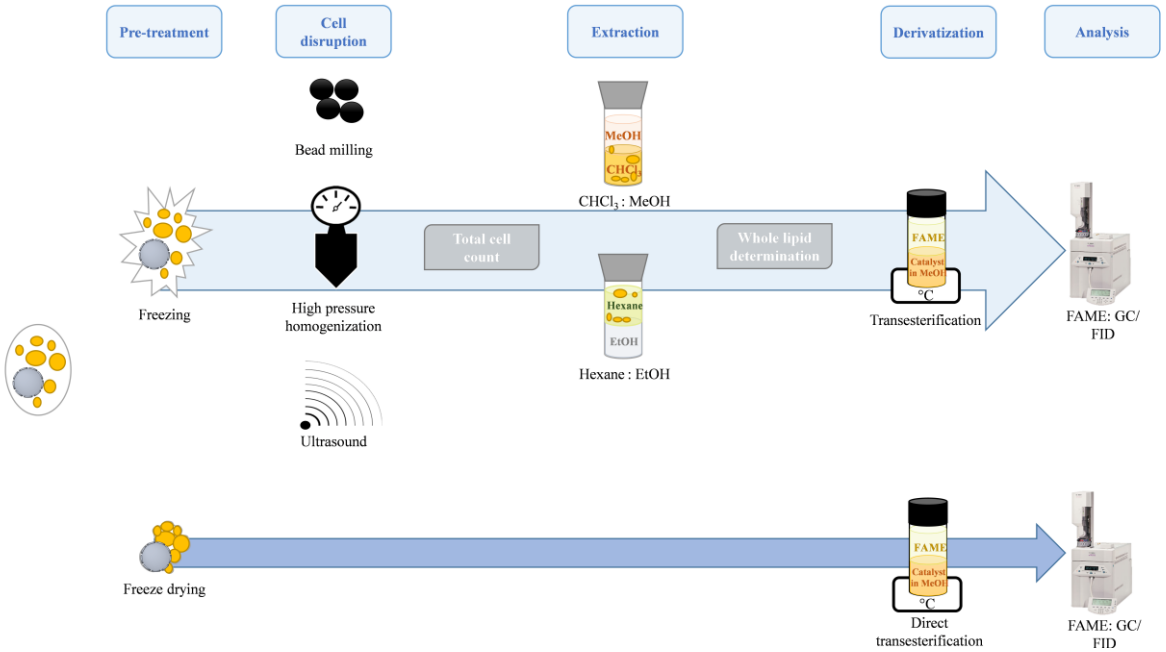

Figure 11: Overview of performed methods for lipid recovery in this paragraph.

4.2 Materials and methods

4.2.1 Microorganisms

The oleaginous basidiomycetes examined in this study were newly screened and deposited at the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig; Germany) as *Cryptococcus podzolicus* DSM 27192 and *Trichosporon porosum* DSM 27194 by Schulze et *al*. (2014). After genome sequencing and annotation both yeasts were phylogenetically reclassified to *S. podzolica* DSM 27192 (Aliyu et al., 2019) and *A. porosum* DSM 27194 (Gorte et al., 2019), respectively.

4.2.2 Chemicals

All utilized chemicals were purchased either from Carl Roth GmbH & Co. KG (Karlsruhe; Germany) or Sigma-Aldrich Chemie GmbH (Taufkirchen; Germany) if not stated otherwise.

4.2.3 SCO production

S. podzolica and *A. porosum* were cultivated in a 2.5 L Minifors bioreactor (Infors HT, Bottmingen, Switzerland) as described by Schulze et *al*. (2014). The cultivation of *S. podzolica* was performed at 22.5 °C and pH 4, while *A. porosum* was grown at 25 °C and pH 5. For SCO production, glucose was used as carbon source with initial concentration of 50 g/L. Each day, glucose was replenished manually to 90 g/L after determining the consumed carbon amount. After 96 h the cultivation broth was harvested in 50 mL aliquots by centrifugation at 4800 × *g* for 10 min. The supernatant was discarded and the biomass was preserved for one week at – 20°C for downstream processing using BM, HPH or U combined with chloroform-methanol extraction according to Bligh & Dyer (BD) and Folch (F) or by using ethanol-hexane (EH) as extractants.

4.2.4 Nile red staining and imagining

Nile red staining was applied according to the staining protocol of Silve et al. (2018a). Yeast cells concentration was adjusted to 0.1 g/L in 1 mL of 50 mM potassium phosphate buffer pH 7.4 and centrifuged for 5 min at 6000 × *g*. Afterwards, 200 µL of the supernatant was withdrawn and replenished with 200 µL of nile red stock solution (30 µg/mL in DMSO). The samples were mixed prior to a 10 min incubation period at 40°C. After that, the samples were washed with distilled water. For microscopic imaging the microscope Axioplan 2 (Zeiss; Jena; Germany) was used equipped with a × 63 LD Plan-Neofluar magnifying objective (Zeiss; Jena; Germany)

and Axiocam HRc (Zeiss; Jena; Germany). For fluorescence imaging the fluorescence filter set 09 from Zeiss was used, i.e. the following filters: excitation BP 450-490, beam splitter FT 510, emission LP 515.

4.2.5 Total cell count

The amount of total cells before and after disruption was visually counted under a microscope using Improved Neubauer (7178 05) counting chambers, consisting of large squares subdivided into 25 group squares of 0.04 mm^2 area. The chamber´s depth is 0.1 mm. Six group squares were counted per each 1:1000 diluted sample. For total cell count calculation, the average amount of cells per group square (N) was determined and used for equation 2.

$$Total\ cell\ count\ \left[\frac{cells}{mL}\right] = N\ \times\ Dilution\ (2.5\ \times\ 10^5)\ \frac{cells}{mL} \qquad 2$$

4.2.6 Cell disruption methods

For cell disruption the frozen biomass was thawed and washed twice with distilled water and resuspended in 50 mM potassium phosphate buffer pH 7.4 to a concentration of 100 g/L. After each disruption method the actual concentration of biomass was determined gravimetrically with a precision balance. In a pre-dried and pre-weighed 1.5 mL reaction tube 1 mL disrupted cells solution was provided and dried for 24 h at 100°C. Additionally, the same procedure was performed with 1 mL pure 50 mM potassium phosphate buffer. The weight of the buffer was subtracted from the weight of the biomass according to equation 3.

$$Weight\ (CDW)\ [g] = Disruption\ suspension\ [g] - Buffer\ [g] \qquad 3$$

4.2.6.1 High pressure homogenization (HPH)

The homogenizer EmulsiFlex-C3 (Avestin Europe GmbH; Mannheim; Germany) was used with a self-established continuous loop system by bridging the distance between the device outlet and the sample funnel with a 1 m silicone hose. During preliminary disruption experiments treatment time was optimized. High pressure homogenization was processed in a volume of 15 mL of cell suspension at 2000 bar for 5 min (20 s per loop), resulting in 15 passes per sample. Subsequently, the sample was collected in a reaction tube on ice.

4.2.6.2 Bead milling (BM)

All bead milling experiments were performed using the bead mill MM 300 (Retsch GmbH, Haan, Germany) in 1.5 mL superspin microtubes (20170-030; VWR International GmbH; Darmstadt; Germany) with 425-600 µm acid washed glass beads (G8772; Sigma-Aldrich Chemie GmbH; Taufkirchen; Germany). Sample and glass beads were distributed in 1:1 ratio (v/w) in the microtubes. In preliminary experiments disruption time and frequency was optimized: The milling process was set at the frequency of 30 Hz/s for 20 min. Thereafter, the suspensions were pooled before extraction.

4.2.6.3 Ultrasonification (U)

Cell disruption by ultrasonification was operated with the 20 kHz ultrasonic homogenizer Sonopuls HD 3100 equipped with the probe MS 72 (Bandelin electronic GmbH & Co. KG; Berlin; Germany). The optimal amplitude, number of cycles and sonication time were determined in preliminary experiments. For the presented experiments, 30 ml cell suspension cooled on ice was sonicated using the maximum amplitude of 97% in a cycle of 50 s pulsing and 10 s pause for 3 × 10 min.

4.2.7 Extraction methods and whole cell lipid determination

All extraction experiments were implemented in triplicates. 1 mL of disrupted cell suspension was used per extraction. As negative control, 1 mL of 50 mM potassium phosphate buffer pH 7.4 was processed.

4.2.7.1 Chloroform methanol extractions

The miniaturized version of the methods of Folch (F) (Folch et al., 1957) and Bligh & Dyer (BD) (Bligh and Dyer, 1959), as adapted by Vasconcelos et al. (2018), were slightly modified in this study. F was performed by combining 1.9 mL potassium phosphate buffer, 1 mL disrupted cells suspension, 9.66 mL chloroform and 4.83 mL methanol. For BD 5.525 mL potassium phosphate buffer were added to 1 mL sample, followed by 7.25 mL chloroform and 7.25 mL methanol. The reaction tubes were inverted 20 times, covered in aluminum foil and were shaken for 30 min. Afterwards, the phase separation was accelerated by centrifugation for 5 min and 1400 × *g*. To collect the entire lower chloroform phase, containing the lipids, a syringe and cannula was instrumentalised to puncture the interphase. The lipid phase was dispensed into pre-weighed glass tubes before complete evaporation of the chloroform in the vac-

uum concentrator Laborota 4000 (Heidolph Instruments GmbH & Co. KG; Schwabach; Germany) at 40°C, 700 × g and 10 mbar. For each method blank extractions containing buffer without biomass were performed to exclude artefactual results, as the used plastic consumables were slightly reactive to the solvents. To determine the whole cell lipid, the weight of the remaining lipids was detected gravimetrically and calculated according to equation 4 and 5.

$$Lipid\ extracted\ [g] = Lipid\ crude\ [g] - Blank\ [g] \quad (4)$$

$$\%\ Lipids\ per\ Weight\ (CDW) = \frac{Lipid\ extracted\ [g]}{Weight\ (CDW)\ [g]} \times 100\% \quad (5)$$

After weighting, the lipids were resuspended in 1 mL hexane and stored at - 20°C prior to sample preparations for further analytical purposes.

4.2.7.2 Ethanol hexane (EH) solvent system

Ethanol hexane (EH) extraction of S. *podzolica* biomass was conducted as described in Silve et al. (2018a) with slight modifications. 15.1 mL ethanol and 6.6 mL hexane were added to 1 mL disrupted cells suspension and mixed by inverting (20 times). The samples were wrapped in aluminum foil and shaken for 3 h at room temperature during which a monophasic suspension was formed. Cell debris were pelleted by centrifugation at 4800 × g for 10 min. Afterwards, 10 mL were transferred to new reaction tubes and 5 mL ddH_2O and 30 mL hexane were added to induce phase separation. After mixing for 3 min and centrifugation at 4800 × g for 10 min, 20 mL of the upper hexane phase was transferred to pre-weighed glass tubes before solvents were completely evaporated in the vacuum concentrator Laborota 4000 (Heidolph Instruments GmbH & Co. KG; Schwabach; Germany) at 40°C, 700 × g and 10 mbar. Similar to the other extraction methods, blank extractions without biomass were performed to exclude artefacts. The weight of the extracted lipids was determined with a precision balance and the whole cell lipid was calculated using equation 4 and 6.

$$\%\ Lipids\ per\ Weight\ (CDW) = \frac{Lipid\ extracted\ [g]}{Weight\ (CDW)[g]} \times \frac{22.7\ [mL]^{*}}{10\ [mL]} \times \frac{32.9\ [mL]^{**}}{20\ [mL]} \times 100\% \quad (6)$$

**Total volume of monophase*
*** 2.9 mL (ratio of hexane in 10 mL monophase) + 30 mL hexane (added for phase separation)*

The extracted lipids were resolved in 1 mL hexane and stored at - 20°C prior to sample preparations for further analytical purposes.

4.2.8 Lipids derivatization to FAME for analytical purposes

For gas chromatography (GC) analysis, extracted SCOs were transesterified to fatty acid methyl esters (FAME) in a two phase system using two different catalysts. All experiments were performed in triplicates. Transesterification efficiency of both catalysts was determined by using 25 mg trilinolein as control.

4.2.8.1 Acidic transesterification of extracted whole cell lipids

For acidic transesterification, 0.5 mL internal standard consisting of 2 mg/mL heptadecanoic acid in hexane and additional 0.5 mL hexane were added to 1 mL extracted lipids or 25 mg of standard trilinolein in hexane. An equal volume of 2 mL 15% H_2SO_4 in methanol was added as catalyst. The reaction mixtures were incubated for 2 h at 100°C and 1000 rpm in a thermoshaker (Universal Labortechnik, Leipzig; Germany). Samples were additionally mixed every 30 min by vortexing. To stop the reaction, the tubes were placed on ice for 10 min. To improve phase separation, 1 mL distilled water was added. The upper hexane phase containing FAMEs was transferred for GC analysis.

4.2.8.2 Alkaline transesterification of extracted whole cell lipids

Alkaline transesterification of extracted lipids and 25 mg standard trilinolein was done in respective to the acidic transesterification at 60°C and 1000 rpm for 20 min with 2 mL of 5% KOH in methanol as catalyst. 2 mg/ml of methyl benzoate in hexane was utilized as internal standard.

4.2.8.3 Direct transesterification of yeasts biomass

Indirect quantification of produced lipids was conducted by direct transesterification (DT) of yeast biomass to fatty acid methyl esters (FAMEs). 15 ml of fresh biomass was daily sampled from the cultivation broth and washed with 0.9% w/v NaCl at 4800 × *g* for 5 min. The pellets were freeze-dried for 24 h at −30°C and 0.370 mbar using the BETA 1-8 freeze dryer (Christ, Osterode am Harz, Germany). 20-30 mg of freeze dried biomass or 25 mg of the standard trilinolein were applied in a glass tubes and a subsequent acidic or alkaline transesterification was performed using a two phase system as described above. The hexane phase contained 0.5 mL

internal standard consisting of 2 mg/mL heptadecanoic acid in hexane and 1.5 mL pure hexane. The second phase had an equal volume of 2 mL 15% H_2SO_4 (reaction's catalyst) in methanol. The mixtures were incubated for 2 h at 100°C and 1000 rpm in a thermo-shaker (Universal Labortechnik, Leipzig, Germany). All samples were additionally mixed every 30 min by vortexing. To stop the reaction, the tubes were placed on ice for 10 min. To improve phase separation, 1 mL distilled water was added. The upper hexane phase containing FAMEs was used for GC analysis. This indirect lipid quantification system was used as standard method during all performed cultivations to track the produced lipid content.

4.2.9 GC analysis of FAMEs

The quantitative and qualitative analysis of FAMEs was performed via gas chromatography (GC) using the 6890 N Network GC-System (Agilent Technologies Deutschland GmbH; Waldbronn; Germany). The device was coupled with a DB- Wax column (30 m × 0.25 mm) (Art. No. 122–7032; Agilent Technologies Deutschland GmbH, Böblingen, Germany) and the detection was performed with a flame ionization detector (FID) under 1.083 bar working pressure. 1 µL of sample was injected at the initial temperature of 40°C. The separation was achieved by a temperature gradient from 40°C to 250°C with a rate of 8°C/min and was kept for 10 min at 250°C. To identify and quantify the FAMEs the RM3 FAME Mix standard (Art. No. 07256-1AMP; Sigma Aldrich; Taufkirchen; Germany) was used.

4.2.10 Statistical analysis

Origin Software (version 2019 (9.6)) was used for statistical analysis. One-Way Anova followed by post hoc-test Tukey were performed using p-value <0.05.

4.3 Results

4.3.1 Evaluation of mechanical cell disruption methods

For both yeasts, disruption efficiencies of the three applied methods were optically determined and compared via nile red fluorescence assay and light microscopy prior to whole lipid extraction. Figure 12A provides microscopic images of untreated and disrupted cells of *S. podzolica*.

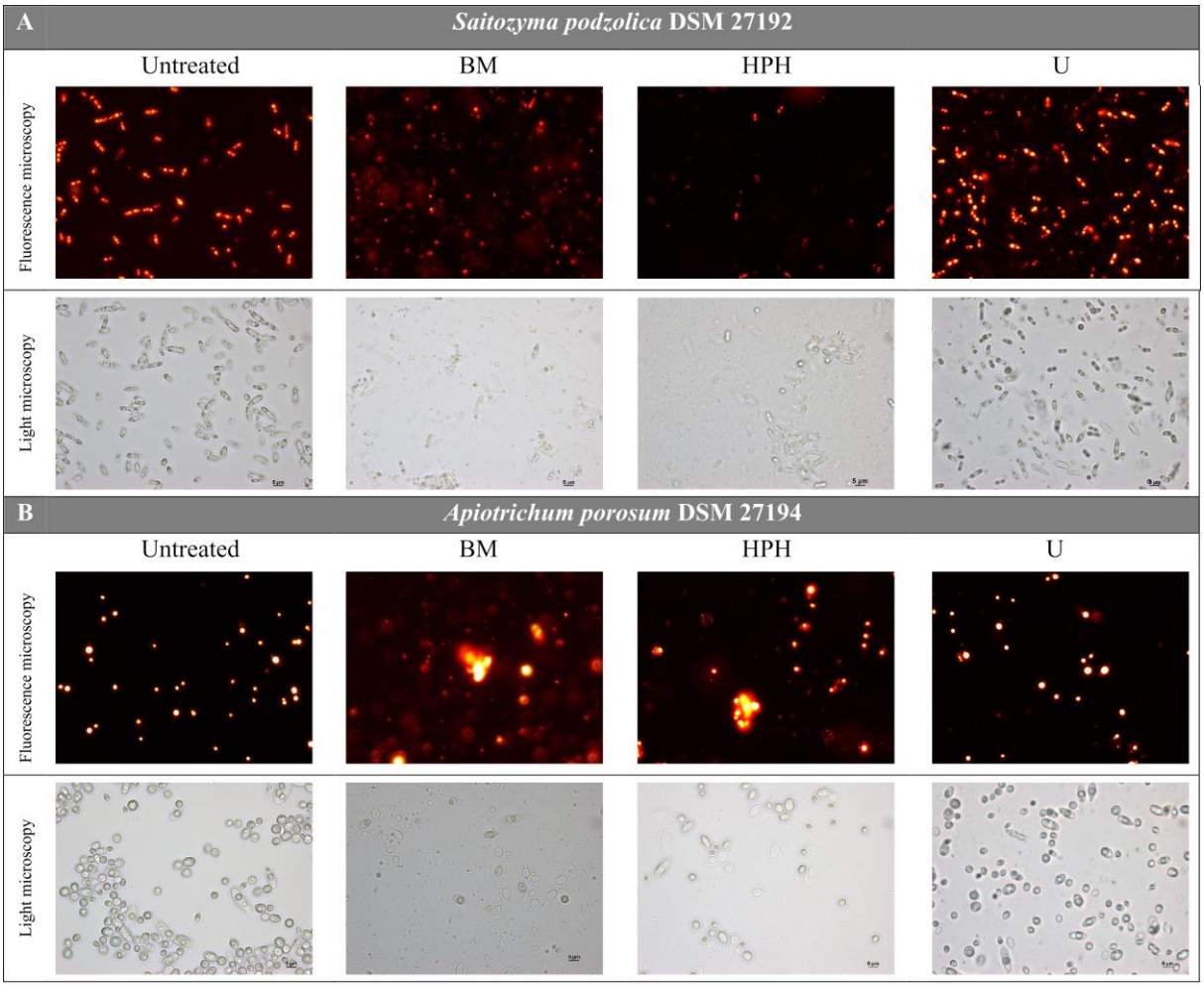

Figure 12: Light microscopy and nile red stained pictures of untreated and via BM, HPH and U mechanically disrupted yeast cells. (A) *S. podzolica*, (B) *A. porosum*.

After bead-milling (BM) and high pressure homogenization (HPH) mainly cell debris were observed, whereas after treatment with ultrasound (U) most of the cells seemed still to be intact. Total cell count confirmed the microscopical observation: after BM treatment 93 % of the cells were found to be disrupted, 95 % after HPH, but only 27 % after treatment with U, although maximal amplitude was applied. Disruption of the cells probably enables lipid bodies to escape and to aggregate in aqueous buffer solution, which can be observed in the nile red images.

Furthermore, HPH treatment might lead to emulsification by reduction of lipid droplet size as it is used for this purpose in the dairy industry. Therefore, fluorescence intensity of *S. podzolica* seems to be reduced after HPH treatment. The effect of the cell disruption approaches on *A. porosum* is shown in Figure 12B. Similar to *S. podzolica*, BM and HPH also seemed to be more efficient compared to U. However, all three treatments appeared to be less destructive to *A. porosum* than to *S. podzolica*, which was also confirmed by total cell count. After BM, HPH and U only 74, 53, and 8 % of the cells were disrupted, respectively.

4.3.2 Comparison of whole cell lipid extractions

After cell disruption whole lipid was extracted either by chloroform and methanol using two different protocols, according to Folch (F) and Bligh and Dyer (BD), or by ethanol and hexane (EH). Latter extraction was only performed for *S. podzolica*. Whole cell lipid extraction yields per CDW are illustrated in Table 8. For *S. podzolica*, whole lipid yield and cell disruption efficiency are clearly correlated. Therefore, highest amounts of whole cell lipid were obtained after HPH, which was the most efficient cell disruption method, followed by BM and U regardless of the solvent system used for extraction. Consistently, extraction efficiency was always superior with EH, followed by BD and F for all cell disruption methods. The best method for whole cell lipid extraction of *S. podzolica* is therefore the combination of HPH and EH (46.9 ± 4.4 %) which is 2.7 times higher than with the least favourable combination (U and F: 17.3 ± 3.1 %). Observed differences are in most cases statistically significant, however, no significant difference was detected between HPH-EH and HPH-BD. Hence, both methods, HPH-BD and HPH-EH, are equally well-suited to achieve the best whole cell lipid yield for *S. podzolica*.

As expected from low disruption efficiencies, extracted whole cell lipid yields of *A. porosum* are also generally low (Table 8), i.e., lower than published lipid contents extracted by direct transesterification. The best combination was BM and BD with 20.0 ± 3.2 %. Although highest whole lipid yields were also achieved from the more efficient cell disruption method (BM), trends are not as clear as for *S. podzolica* and observed differences are not statistically significant. The data for extracted whole cell lipid after U are not shown for *A. porosum* since cell disruption was not sufficient as already mentioned above.

Table 8: Extracted whole cell lipid yields per cell dry weight (CDW) of *S. podzolica* and *A. porosum* after different cell disruption methods and solvent extraction.

Cell disruption method	Extraction method	*S. podzolica*		*A. porosum*	
		Disruption efficiency	Whole lipid per CDW [%]	Disruption efficiency	Whole lipid per CDW [%]
BM	F	93%	27.9 ± 2.0[a]	74%	16.8 ± 2.7[a]
	BD		32.3 ± 2.5[b]		20.0 ± 3.2[a]
	EH		37.3 ± 3.4[c]		n.d.
HPH	F	95%	37.8 ± 2.3[c]	53%	15.0 ± 2.8[a]
	BD		43.4 ± 1.2[d]		14.1 ± 0.9[ab]
	EH		46.9 ± 4.4[d]		n.d.
U	F	27%	17.3 ± 3.1[e]	8%	n.d.
	BD		20.7 ± 3.0[e]		n.d.
	EH		n.d.		n.d.

All values are given as the mean ± standard deviation of three independent experiments. a, b, c, d, e indicates statistical differences (p=0.05). ab indicates statistical difference between the disruption methods without changing the extraction method. Statistical analysis was performed separately for each yeast species. BM, bead mill; HPH, high pressure homogenization; U, ultrasonic treatment; F, extraction according to Folch; BD, extraction according to Bligh and Dyer; EH, ethanol- hexane extraction; n.d., not determined.

4.3.3 Determination of optimal fatty acid methyl ester (FAME) production

Quantification of lipids can be achieved indirectly via FAMEs by GC. To do so, sample preparation is necessary and might have an impact on (calculated) amount and fatty acid composition leading to underestimation of both lipid yield and quality. Transesterification of fatty acids derived from triacylglycerols/SCOs to FAMEs can be achieved by both acidic and alkaline methylation with H_2SO_4 or KOH as catalyst, respectively. For both methods transesterification efficiency and impact on polyunsaturated fatty acids were investigated by using the standard triacylglycerol trilinolein. FAME yield with KOH was 92.3 ± 2.8 %, whereas, H_2SO_4 derivatization resulted in a FAME yield of 97.1 ± 3.1 %. The results for transesterification of extracted

lipids from biomass are shown in Appendix 4. No significant difference was observed between F and BD extractions for both yeasts after transesterification. On that account, Figure 13 exemplifies the comparison with KOH and H_2SO_4 produced FAMEs after HPH-BD treatment and direct transesterification of yeast biomass.

For *S. podzolica* (Figure 13A) both catalysts achieved an equal yield for prior disrupted and extracted lipids. For direct transesterified biomass the KOH technique is four times less efficient compared to acidic transesterification. Statistically, all three successful methods (disrupted and extracted lipids treated with KOH and H_2SO_4 and DT-H_2SO_4) are equally potent to achieve high FAME yields. Figure 13B likewise indicates no difference for *A. porosum* in transesterification catalyst for recovered lipids. Similar to *S. podzolica*, alkaline direct transesterification is not convenient and nine times less potent compared to acidic technique for mechanically untreated biomass. Therefore, DT-H_2SO_4 is the most effective method to produce FAMEs from *A. porosum* biomass.

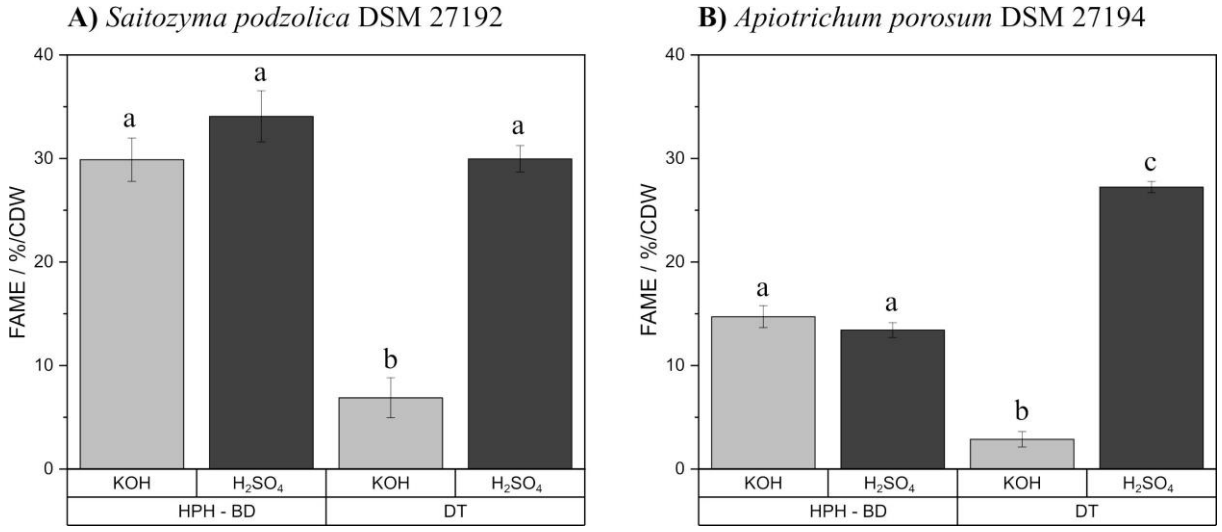

Figure 13: Comparison of KOH and H_2SO_4 catalysts for FAME production after HPH-BD treatment and direct transesterification (DT) of yeast biomass. % FAME per CDW from *S. podzolica* biomass is demonstrated in (A), while (B) presents the same for *A. porosum*. The standard deviation of three independent experiments are indicated by the error bars. a, b, c illustrate statistical differences ($p = 0.05$).

Since for processed lipids both catalysts are appropriate and for DT the acidic method is more adequate, Figure 14 contrasts the comparison of all disruption methods using BD extraction and DT after acidic transesterification. Figure 14A elucidates for *S. podzolica* with BM, HPH, and DT a statistically comparable FAME yield at approximately 30 % per CDW. On the contrary, U is less efficient with 23.8 ± 2.3 % FAME/CDW. However, EH after HPH is significantly the optimal method with the highest lipid yield (Appendix 4). Different findings were observed for *A. porosum* (Figure 14B). By performing direct transesterification, the highest yield of 27.2 ±

0.5 % FAME/CDW was detected. BM with 20.2 ± 1.2 and HPH with 13.4 ± 0.7 % FAME/CDW are significant less sufficient methods to extract the lipids from this yeast.

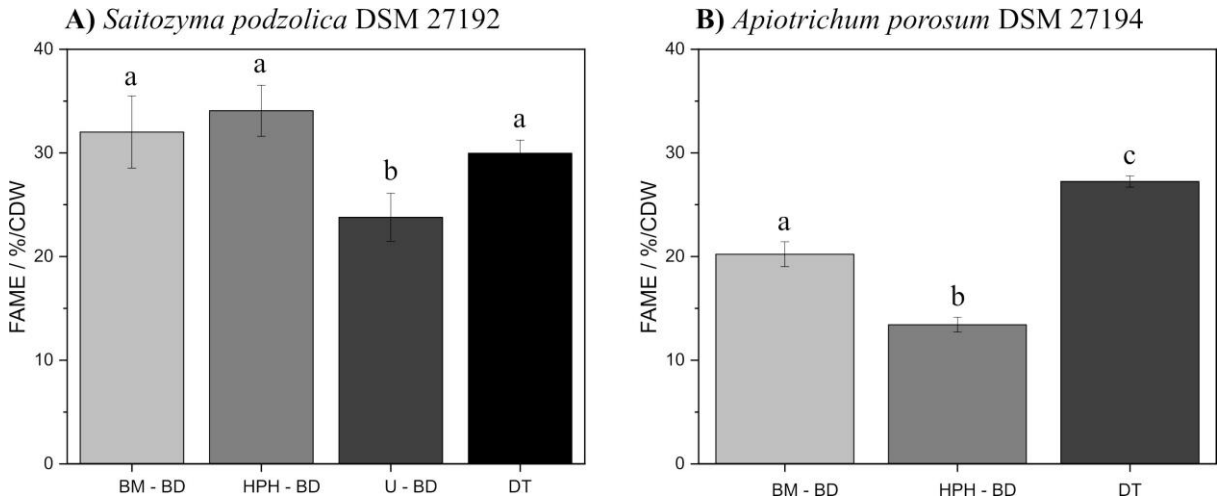

Figure 14: Comparison of all processed lipid recovery methods, using BD extraction and acidic transesterification, and acidic DT method. (A) % FAME per CDW from *S. podzolica* biomass. (B) % FAME per CDW from *A. porosum* biomass. The error bars result from the standard deviation of three independent experiments. a, b, c reveal statistical differences ($p = 0.05$).

4.3.4 Impact of performed treatments on lipid profiles

In order to judge which method is most suitable for extraction of oxidation sensitive polyunsaturated fatty acids, lipid profiles were determined. In terms of extraction systems, differences in lipid profiles were not observed when comparing the extraction methods F and BD (Appendix 5 and 6). In contrast, extraction with EH resulted in significantly higher amounts of stearic acid and significantly lower amounts of linoleic acid.

With regard to cell disruption, no or only minor differences in lipid profile for *S. podzolica* biomass were detected when using HPH and BM (Appendix 5). Analysis of the lipid profile of *A. porosum*, however, revealed significantly more linoleic acid and significantly less stearic acid for BM compared to HPH (Appendix 6).

Additionally, the investigated transesterification techniques did influence the lipid profiles for both yeasts. Figure 15 compares fatty acid profiles of both yeasts obtained by BM - BD and acidic direct transesterified biomass. Extracted and with KOH transesterified lipids of *S. podzolica* resulted in higher amounts of oleic acid and linolenic acid compared to lipids transesterified with H_2SO_4 (Figure 15A). However, lowest linoleic acid yields were detected with KOH transesterification of extracted lipids. No significant difference on lipid profile of DT and disrupted and H_2SO_4 treated lipids were observed for *S. podzolica*.

Similar to *S. podzolica*, *A. porosum* (Figure 15B) showed lower oleic acid yields for disrupted and H_2SO_4 handled lipids compared to DT or extracted and KOH treated lipids. For *A. porosum* linoleic acid yield is significant higher after DT than for the other disruption methods.

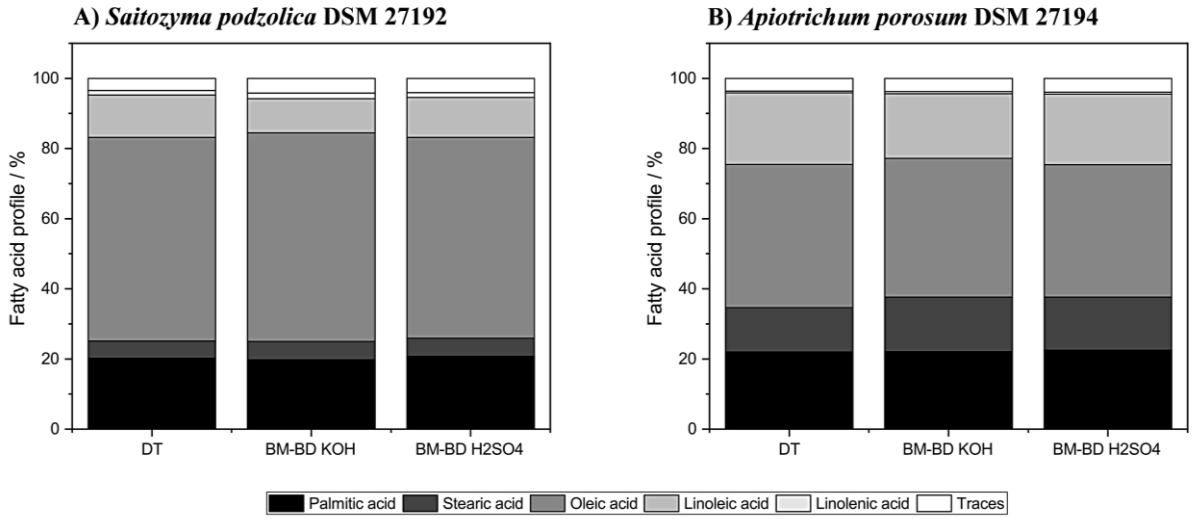

Figure 15: Fatty acid distribution of *in situ* transesterified and BM prior to BD processed biomass analyzed by GC. (A) Fatty acid profiles in % per CDW of *S. podzolica*. (B) Fatty acid distribution in % per CDW of *A. porosum*.

4.4 Discussion

4.4.1 Comparison of cell disruption efficiencies

Mechanical cell disruption methods ensure cell wall disruption by forces of shear, abrasion and cavitation due to high pressure, velocity, heat, or sonication (Jacob, 1992; Ochsenreither et al., 2016; Probst et al., 2016). In this study the tendencies of all three cell disruption methods on wet, but frozen and thawed, biomass was similar for both yeasts, indicating HPH and BM as potent disruption methods, whereas U resulted to be least effective regardless of the fact, that the maximum amplitude was used. In literature sonication is known to be unsuitable for microorganisms inhabiting rigid and tough cell walls (Jacob, 1992; Yu et al., 2015; Probst et al., 2016). However, due to the different definitions and measurements a comparison of cell disruption methods in literature is difficult. For the ascomycete *Yarrowia lipolytica*, e.g., ultrasound turned out to be one of the most efficient techniques for lipid recovery (Zhang et al., 2014; Meullemiestre et al., 2016).

The cell wall structure of ascomycetous yeasts usually consists of two layers including an inner layer with polysaccharides and an outer layer bearing glycoproteins covalently bound to the inner layer. In contrast, for some basidiomycetes multi-layered cell walls were observed (Van Der Klei et al., 2011). The difference in cell layer quantity may contribute to the higher cell wall rigidity of the basidiomycetes presented in this study compared to ascomycetous yeasts. HPH and BM enforce high shear stress to the cells, either by pressurizing cells through a small valve followed by striking a wall at high velocity in case of HPH or by impact with agitated grinding beads in case of bead-milling (Chisti, Yusuf and Moo-Young, 1986; Middelberg, 1995; Probst et al., 2016). The SCO of the oleaginous yeast *Cryptococcus curvatus* (Thiru et al., 2011) and the microalgae *Nannochloropsis* sp. (Halim et al., 2016) was recovered after HPH treatment, though yields of other disruption strategies were not compared. Whereas for *Y. lipolytica* (Meullemiestre et al., 2016) and for the yeast *C. curvatus*, the fungus *Mortierella isabellina* and the microalga *Chlorella sorokiniana* (Yu et al., 2015) among other methods, BM was compared with U, resulting, similarly to this study, in higher effective cell disruption after BM.

Considering the total cell count and the microscopic images after disruption (Figure 12) *A. porosum* was less affected by mechanical disruption than *S. podzolica*. This might be due to differences in cell wall compositions of both yeasts. Since both yeasts are newly screened structural analysis of the cell walls does not exist in literature. In general, the yeast cell wall consists

of heterogeneous and complex cross-linked polymers of oligosaccharides (glucan, mannoprotein, chitin). The highly elastic β-(1,3)-glucan chains and rigid β-(1,6)-glucan crosslinks contribute to the stability and firmness (Phaff, 1971; Jacob, 1992). In addition, a cell wall is reinforced in stationary growth phase by chitin crosslinking and increased mannoprotein binding (De Nobel et al., 1990; Touhami et al., 2003). Comparisons of cell wall composition and organization indicated high variabilities between different fungal organisms (Free, 2013). Especially remarkable is the ability of some species to form an exterior capsule of carbohydrate polymers (Doering, 2009), which are known to be produced by the members of the family *Trichosporonaceae* (Duarte-Oliveira et al., 2017) to which *A. porosum* belongs (Aliyu et al., 2020a) and which might also contribute to the challenge to efficiently disrupt the cell.

The most effective lipid recovery method for *A. porosum* is the acid catalyzed direct transesterification of freeze-dried biomass, in which cell disruption, lipid extraction, and transesterification to FAMEs is combined in a single reaction under harsh conditions at 100°C for 2 h. In contrast, the direct transesterification with KOH using milder conditions (60°C for 20 min) turned out to be insufficient for direct transesterification, which proves the necessity of high temperature for direct FAME production for both tested yeasts. However, freeze drying as pretreatment of the biomass is critical for this method. Moisture considerably decreases the yield of the direct transesterification (Sathish et al., 2014; Yousuf et al., 2017). The moisture content of wet oleaginous microorganisms is over 80% and therefore, higher than in oilseeds and needs to be removed (Ehimen et al., 2010; Jin et al., 2012). Consequently, freeze drying is absolutely necessary though it is the most energy consuming technique. For biodiesel, a low value product, this strategy contributes mainly to production costs.

In comparison, *S. podzolica* is easier to break with equal yields after derivatization of both wet biomass, disrupted using HPH and BM, and dry biomass using DT (Figure 14). In fact, this yeast is a potential candidate for biodiesel or oleochemical production. Cell disruption is the decisive step for SCO extraction in yeasts. At an efficiency of over 90% of disrupted cells according to total cell count (*S. podzolica*: 93 and 95% after BM and HPH, respectively) the conditions are suitable for subsequent extraction, at lower cell disruption efficiencies (*A. porosum*: 74% by BM and 53% after HPH), only a limited part of the SCO can be extracted.

4.4.2 Energy consumption of biomass pre-treatment and cell disruption

Depending on the purpose of the extracted lipid a suitable method for downstream processing can be chosen by taking the energy costs and labor into account. In Table 9 the amount of required energy for 1 kg of extracted SCO is presented for each performed downstream method and both yeasts. The energy consumption to extract whole lipid of *S. podzolica* is about 100 kWh/kg for all tested methods. This is derived from the extraction efficiency, disruption time and the method's device power consumption, e.g. U is the least efficient but also the least power consuming method, therefore more biomass must be treated to reach 1 kg whole lipid resulting in comparable energy consumption in kWh/kg as with the other methods.

Table 9: Comparison of energy consumption of extracted whole cell lipid and FAME production.

				S. podzolica		*A. porosum*	
	Pre-treatment parameters	Disruption [FAME production] parameters		Whole lipid extraction	FAME production	Whole lipid extraction	FAME production
				kWh/ kg product			
BM	-20°C for 168 h	20 min [60°C; 20 min]	BD	110	274	161	321
			EH	96	251	n.d.	n.d.
HPH		5 min [60°C; 20 min]	BD	102	305	283	569
			EH	95	200	n.d.	n.d.
U		25 min [60°C; 20 min]	BD	107	351	n.d.	n.d.
DT	-30°C at 0.370 mbar for 24 h	[100°C; 2 h]	-	-	5588	-	6144

Calculated data are extrapolations from the laboratory scale and energy consumption of laboratory devices. n.d., not determined.

However, the most energy efficient methods are BM and HPH prior the EH extraction with 96 and 95 kWh/kg, respectively. For whole lipid extraction from *A. porosum* nearly twice as much

energy is required compared to *S. podzolica*, which is due to the low achieved cell disruption efficiency of this strain. On this account, *A. porosum* is less appropriate for potential industrial use.

Regarding the energy consumption for FAME production for application as biodiesel, the most energy saving method is HPH-EH-KOH for *S. podzolica* with 200 kWh/kg. For *A. porosum*, 60 % more energy is needed for 1 kg FAME even when the most efficient method (BM-BD-KOH) for this yeast is used.

Comparing the energy consumption for FAME production with DT and mechanically treated biomass, DT is clearly the most energy consuming method. By performing HPH-EH-KOH with *S. podzolica* biomass just 3.6 % of energy percentage is needed for one kg FAME compared to DT, for *A. porosum* biomass treated with BM-BD-KOH just 5.2 % of DT's energy amount is required. This fact results from the different required pre-treatment methods. Although the mechanically treated biomass was frozen for 168 h at –20°C, still less energy was consumed than for 24 h of vacuum freeze-drying. Consequently, freeze-drying is the most energy consuming technique, which is also confirmed by other studies (Meullemiestre et al., 2016). By considering the extrapolated data from laboratory scale in this study, *S. podzolica* is more suitable for potential industrial application for both, whole lipid recovery for oleochemicals as well as for transesterification for biodiesel production.

Within this study a higher SCO production potential of the yeast *S. podzolica* is proven, compared to the previous studies performing only DT with this yeasts' biomass for analytical purposes (Qian et al. 2020; Qian et al. 2019; Schulze et al. 2014). Moreover, the downstream process using HPH and ethanol-hexane extraction prior to transesterification is not only more efficient but also more energy saving than DT. However, there is still optimization potential regarding energy efficiency, e.g., shorter pre-treatment times and higher biomass scales need to be investigated.

4.4.3 Influence of solvents on extraction yields

Analyzing the extraction techniques on whole lipid extraction, BD was more effective compared to F for *S. podzolica* probably because the ratio of the polar methanol is higher in BD leading to a more efficient extraction of polar lipids (Dong et al., 2016; Vasconcelos et al., 2018). For *A. porosum* both extraction methods showed no significant differences, presumably due to the insufficient cell disruption. After transesterification, however, F und BD extraction

revealed the same yield of transesterifiable lipids for both yeasts proving an equal ability of the methods to recover neutral lipids.

The less harmful EH solvent mixture turned out to be highly suitable for SCO purification of *S. podzolica*. After HPH treatment EH extraction proved to be similarly potent as BD extraction and the highest whole cell lipid yields were achieved. Remarkably, after derivatization of the HPH treated biomass the highest FAME yields resulted from EH extracted lipids with 46.0 ± 6.8% FAME/CDW. Compared to the former standard analysis DT (30.0 ± 1.3% FAME/CDW) with this new approach 53% increase of production potential of *S. podzolica* was reached. Since HPH is the most effective disruption method for *S. podzolica*, here the highest amount of lipids was accessible for extraction. Hexane has a lower polarity than chloroform, consequently neutral lipids have a higher affinity to non-polar solvents (Cooney et al., 2009). Thus, more transesterifiable acylglycerols and FFAs should be accessible for EH extraction. Additionally, the EH method was completed in 3 h, whereas BD was carried out in 30 min. The longer extraction time might also contribute to this result. In literature ethanol-hexane extractions are commonly used for microalgae lipid recovery with likewise high extraction effectiveness (Grima et al. 1994; Silve et al. 2018a). For the yeast *Lipomyces kononenkoae*, also the less polar solvent toluene revealed higher extraction yields over chloroform (Vasconcelos et al., 2018). Hence, hazardous chloroform methanol mixtures can be dispensed with less harmful ethanol hexane system without sacrifying oil recovery for *S. podzolica*.

4.4.4 Impact of cell disruption and transesterification on lipid profile

Alkaline transesterification methods are recommended for lipid analysis as they are faster, more efficient and the reaction is in general more complete than acidic transesterification (Christie, 1993; Liu, 1994; Aldai et al., 2005; Carlini et al., 2014). Another limitation of sulphuric acid catalyzed transesterification, especially for up-scaling, is possible corrosion of reaction vessels due to salt interactions (Carlini et al., 2014). However, with the alkaline methods only transesterification is possible but not the esterification of FFAs. Instead, the acidic catalysis is suitable for both esterification and transesterification (Liu, 1994; Aldai et al., 2005). Another difference between alkaline and acidic transesterification is that *N*-acyl lipids are not transesterified by alkaline methods but only by acid catalysis (Mayberry, 1981; Galbraith and Wilkinson, 1991; Christie, 1993). A disadvantage of acidic transesterification is that this method could cause isomerization and methoxy artifacts in conjugated fatty acids (Aldai et al., 2005). However, Yamasaki et al., (1999) showed sulphuric acid catalyzed transesterification is a relatively

mild method as 71% of conjugated linoleic acid are not isomerized after 120 h of methylation. Therefore, differences in lipid profiles between alkaline catalysis and acid catalysis might be more likely due to esterification of FFAs and *N*-acyl lipids with the acidic method, whereas these are not quantified with alkaline transesterification. However, the total amount of FAME is the same for both methods, as alkaline triglyceride transesterification might be more complete and therefore compensate the non-methylated FFAs and *N*-acyl lipids in regards to total FAME amount.

Significant differences in lipid profile of *A. porosum* treated with BM and HPH were observed. After HPH linoleic acid content was significantly lower than after BM. To the author's knowledge, there are no studies existing addressing the lipid profiles of yeasts in relation to cell disruption methods. The influence of BM on fatty acid composition was addressed only in a single publication where no differences in lipid composition of *Chlorella vulgaris* after BM compared to grinding, ultrasonication, enzymatic lysis, and microwaves was observed (Chi et al., 2011). Studies on the influence of HPH on the fatty acid composition of various foods provide different results. Thus, Rodríguez-Alcalá et al., (2009) reported no influence of HPH on fatty acid composition of milk up to a pressure of 3500 bar. In tomato juice an increase in the oxidation product of unsaturated fatty acids, *n*-hexanal, was reported after HPH treatment at a pressure of 5000 bar (Porretta et al., 1995). While Kuhn and Cunha, (2012) observed a significant increase in primary oxidation products due to HPH treatment of whey protein isolate stabilized oil in water emulsions of flaxseed oil already at a pressure of 800 bar. Therefore, the differences in linoleic acid content after BM and HPH treatment might be due to oxidation of linoleic acid during HPH treatment

5 Pulsed electric field (PEF) treatment on *S. podzolica*

This chapter is mainly based on the publication

Pulsed electric field treatment promotes lipid extraction on oleaginous yeasts *Saitozyma podzolica* DSM 27192

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Authors' contribution to this publication

Olga Gorte performed yeast cultivations, the PEF experiments and lipid extractions, designed and planned the study, analyzed the data and drafted the article.

Natalja Nazarova performed the PEF experiments and lipid extractions, designed and planned the study and analyzed the data.

Ioannis Papachristou performed the PEF experiments and lipid extractions.

Rüdiger Wüstner performed the PEF treatment by operating PEF devices.

Klaus Leber performed the PEF treatment by operating PEF devices.

Christoph Syldatk constructively contributed to the content.

Katrin Ochsenreither constructively contributed to the content.

Wolfgang Frey constructively contributed to the content.

Aude Silve supervised the project, performed the PEF experiments and lipid extractions, designed and planned the study and analyzed the data, drafted the article.

5.1 Introduction

This section describes the possibility to use PEF-treatment as a pre-treatment method to weaken oleaginous yeast cells prior to solvent extraction. The study was performed on the fresh biomass of the yeast *S. podzolica*. In order to evaluate the benefit of PEF-pre-treatment for lipid extraction, yeast suspension was either processed directly after harvesting (unwashed route) or after a washing step aiming at reducing the conductivity (washed route).

Experiments were performed in *n* independent experiments (*n*=2 or 3) with internal duplicates in each experiment. Duplicate values were averaged to calculate one output value per experiment. The experimental set-up is graphically visualised in Figure 16.

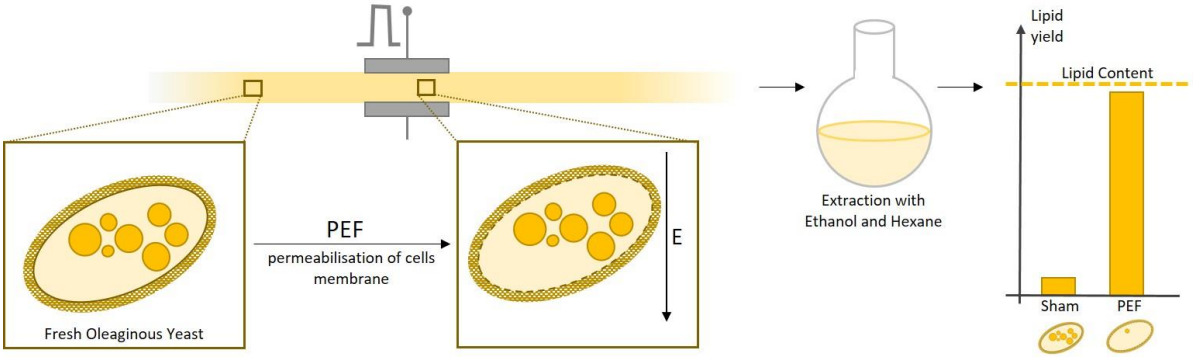

Figure 16: Pulsed electric field treatment performed on fresh yeast biomass induced permeabilisation of cells and greatly improved the yields of subsequent lipid extraction using ethanol and hexane.

5.2 Material and methods

5.2.1 Microorganisms

All experiments were performed with the oleaginous yeasts *Saitozyma podzolica* DSM 27192, which was newly screened from peat bog soil and deposited at the DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig; Germany) as *Cryptococcus podzolicus* DSM 27192 by Schulze et al., (2014). After genome sequencing and annotation, the strain was phylogenetically reclassified as *S. podzolica* DSM 27192 (Aliyu et al., 2019).

5.2.2 Yeast cultivation

The oleaginous yeast *S. podzolica* was cultivated in a mineral salt medium, as described by Schulze et al., (2014), containing a phosphate buffer system (8.99 g/L KH_2PO_4 and 0.12 g/L $Na_2HPO_4 \times 2\ H_2O$), 0.1 g/L sodium citrate × 2 H_2O, 0.1 g/L yeast extract, 0.2 g/L $MgSO_4 \times 7\ H_2O$, 4.72 g/L $(NH_4)_2SO_4$. After autoclaving 2% (v/v) of sterile trace elements solution with 4 g/L $CaCl_2 \times 2\ H_2O$, 0.55 g/L $FeSO_4 \times 7\ H_2O$, 0.475 g/L citric acid, 0.1 g/L $ZnSO_4 \times 7\ H_2O$, 0.076 g/L $MnSO_4 \times H_2O$, 100 µl/L 18 M H_2SO_4 and 2% (v/v) of sterile salts solution comprising 20 g/L $MgSO_4 \times 7\ H_2O$, 10 g/L yeast extract were added. Additionally, glucose was supplemented aseptically at concentration of 50 g/L. The yeast cells were activated in pre-cultures in conical shake flasks by scratching yeast cells from YM agar (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L agar, pH 7, sterily supplemented with 10 g/L glucose after autoclaving). The first pre-culture was performed in 50 mL medium at 130 rpm and 20°C for 24 h. Second pre-culture was inoculated from first pre-culture to an OD_{600nm} of 1.0 in 200 mL medium and cultivated at the same parameters for 24 h. Lipid production was conducted in duplicates in a 2.5 L bioreactor (Infors HT, Bottmingen, Switzerland; Minifors fermentor) with 1.2 L mineral salt medium with initial OD_{600nm} of 1.0. The cultivation was performed at pH 4 (automatically controlled by addition of 4 M H_3PO_4 and 4 M NaOH), 22.5 °C, 600 rpm and with 1 vvm aeration rate. To prevent foam formation, the bioreactors were equipped with a foam probe (Infors HT, Bottmingen, Switzerland) using Contraspum A 4050 HAC (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) as an anti-foaming agent. Every 24 h a manual feed of 2% (v/v) sterile trace elements solution, 2% (v/v) sterile salts solution and glucose supply to 55 g/L was implemented after determining the consumed carbon amount. The yeasts growth was monitored by daily sampling and analysis of OD_{600nm}, cell dry weight (CDW), glu-

cose and ammonium consumption. SCO production was indirectly analyzed by gas chromatography via acidic direct transesterification. After six days the biomass was harvested and directly further processed.

5.2.3 Quantification of cell dry weight (CDW)

Before and after PEF treatment the determination of CDW was performed in triplicates. For each sample, 1 mL of culture broth was added in a reaction tube and centrifuged at 6000 × g for 5 min. The cell pellet was washed with sterile physiological saline (0.9% w/v NaCl) and resuspended in 1 mL saline. The yeast suspension was poured into a pre-weighted (DW_{empty} [g]) aluminum cap. The exact masses of 1 mL yeast suspension ($w_{suspension}$ [g]) and, separately, of 1 mL pure saline (w_{saline} [g]) were determined using a precision balance. All caps were dried in a drying oven at 90°C for about 24 h. Afterwards, the weight of the dry biomass and saline was measured again (DW_{full}[g]). CDW was calculated using equation 7.

$$CDW\ \left[\frac{g}{kg_{sus}}\right] = \left(\frac{(DW_{full} - DW_{empty})_{suspension}}{w_{suspension}} - \frac{(DW_{full} - DW_{empty})_{saline}}{w_{saline}}\right) \times 1000 \qquad 7$$

5.2.4 Yo-pro staining

Yo-pro staining is generally used as a marker of membrane integrity. In particular, it was used in this study to detect how washing could render cells permeable. Permeabilized cells will appear positive to Yo-Pro staining. The initial yeast samples at approximately 20 g/L were diluted to 1:300 with their own medium filtered at 0.2 µm. 1 mL of the sample was supplemented with 10 µL of Yo-Pro (YO-PRO™-1 Iodide (491/509), Invitrogen™, Thermo Fisher Scientific) at 0.1 mM. The sample was left 10 min at room-temperature for staining and then diluted 1:5 before input in the flow cytometer. Flow cytometer measurements were conducted on an Attune NxT (Thermo Fisher Scientific) with a 488 nm laser as excitation source. Emission fluorescence signal was collected with the green filter of the device (530/30).

5.2.5 Conductivity measurement

The conductivity σ [µS/cm] of the yeast suspensions was measured using a conductivity meter (Endress + Hauser, CLM 381). No automatic temperature compensation was used, but temperature T [°C] was recorded in parallel with conductivity. The equivalent conductivity at 20°C,

σ_{20} [µS/cm], was calculated using equation 8, where α_{20} is the temperature coefficient of variation at 20°C (Grimnes and Martinsen, 2008). The coefficient α_{20} was obtained experimentally by measuring conductivity of a yeast suspension at different temperatures (data not shown) and had a value of 2.58%/°C.

$$\sigma_{20} = \sigma_T \frac{1}{1 + \alpha_{20}(T - 20)} \qquad 8$$

5.2.6 PEF treatment

The yeasts were treated either directly after harvesting or after a washing step, i.e. in suspension with a conductivity at 20°C of either 10.14 ± 0.63 mS/cm or 1.27 ± 0.09 mS/cm. In both cases, PEF-treatment was performed in continuous flow. Two treatment chambers were used, one for each of the two conductivities mentioned above. They consisted of two parallel circular stainless steel electrodes separated by a polycarbonate housing (Figure 17) imposing a distance between the electrodes of 4 mm. In order to deliver square shaped pulses, the setup needs to ensure impedance matching, i.e. the output impedance of the generator (50 Ω in our case) needs to match the impedance of the load, which consists in the treatment chamber filled with the suspension to be treated. Therefore, the dimensions of the volume between the electrodes filled with suspension to be treated were chosen to ensure that the impedance of the chambers is equal to 50 Ω when chambers were filled with suspensions with a conductivity of 1.5 and 12 mS/cm, respectively.

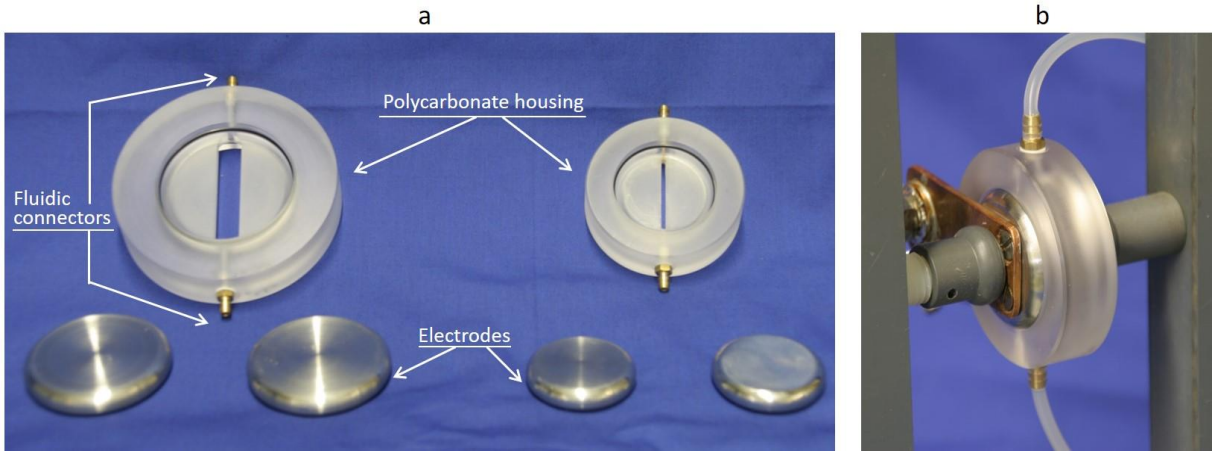

Figure 17: Illustration of the PEF-treatment chambers. (a) Disassembled treatment chambers. The chambers are designed for treating suspensions with conductivities of 1.5 mS/cm (left) and 12 mS/cm (right) with a 50 Ohm transmission-line generator. (b) Illustration of an assembled treatment chamber with the tubes used for the continuous flow of the yeast suspension.

Considering the increase in conductivity caused by the temperature increase that occurs during the treatment inside the chamber, the treatment chambers are ideal to treat suspension with initial conductivities of 1–1.2 and 8–10 mS/cm, respectively, and therefore suitable for processing washed and unwashed yeast suspensions. Please note, that more detailed explanations on impedance matching or impedance computation in general, can be found in the appropriate textbooks (Smith, 2002) or publications (Silve et al. 2012). PEF treatment was performed with a custom-made transmission-line generator with an output impedance of 50 Ω. Pulse duration, was fixed at Δt = 1 µs and electric field intensity was varied between E = 1.4 and E = 4 MV/m. The electric pulses were applied continuously with a pulsing repetition rate f_{rep} adjusted between 0.1 and 12 Hz, in order to adjust the specific treatment energy. The flow rate of the yeast suspension inside the treatment chambers was fixed at 0.1 mL/s except in some specific cases (mentioned along in the sections) for which it was reduced to 0.05 mL/s in order to apply the required specific energies.

5.2.7 Lipid extraction of fresh or treated samples

The lipid extraction protocol was adapted from the one developed for microalgae and fully detailed in Silve et al. (2018b). In brief, 15 mL of the yeast suspension at approximately 20 g_{DW}/L were centrifuged, supernatant was discarded and the wet biomass pellet was resuspended in 16 mL of 100% ethanol and 6,5 mL of hexane in order to reach a final extraction system of water/ethanol/hexane, 1:18:7.3 vol/vol/vol. Note that the water in the extraction system corresponds to the remaining water in the yeast pellet, i.e. no water was intentionally added. Extraction took place in Teflon tubes (Nalgene® Oak Ridge Centrifuge Tubes, Teflon® FEP, 50mL Thermo Scientific), overnight, with agitation and in the dark. After 20 h, the Teflon tubes were centrifuged and 10 mL of the solvent containing the raw extract were pipetted and complemented with 30 mL of hexane and 5 mL of water in order to accomplish phase separation. The upper hexane phase was collected, and evaporated under nitrogen flow. Extraction yields were determined gravimetrically with a precision balance.

5.2.8 Yeast biomass composition analysis

5.2.8.1 Biomass preparation

Composition analysis was performed with a 0.02% (w/v) saline washed yeast suspension. The washed yeast suspension was centrifuged at 10,000 × g for 10 min and supernatant was discarded. The pellet was frozen at -20°C before subsequent freeze-drying for 24 h in a laboratory

freeze-drier (Alpha 1-4 LDplus, Christ). The freeze-dried pellet was then kept at -20°C before further analysis of content.

5.2.8.2 Total lipid extraction

Total lipid extraction was performed with a commercial Soxhlet apparatus (behrotest® Kompakt-Apparatur KEX 30 from Behr Labor-Technik). Approximately 0.5 g of the washed and freeze-dried yeast biomass was precisely weighted and bead-milled for 5 min at 30 Hz in stainless steel cups (Grinding jar for MM 400, 50mL, 01.462.0216, Retsch, Haan, Germany) using nine 12 mm stainless steel beads (05.368.0037, Retsch) and a commercial bead-miller (Mixer mill, MM400, Retsch). The biomass was placed inside a permeable paper thimble (Extraction Thimbles Cellulose, 90022080, Albet LabScience, Dassel, Germany) and deposited inside the Soxhlet chamber. Approximately 50 mL of hexane was used with a heating temperature of 170-200°C. The extraction was run for at least 3 hours, which corresponded to at least 20 extraction cycles. At the end of the extraction, the solvent was siphoned out of the apparatus and the boiling flask along with extracted lipids was removed, let to cool down under nitrogen atmosphere, and the total lipid content was determined gravimetrically.

5.2.8.3 Total protein determination

Total protein content of the yeast biomass was evaluated using sodium hydroxide extraction at high temperature. Approximately 10 mg of washed and freeze-dried yeast powder was resuspended in 2 ml of sodium hydroxide (1 M) and incubated at 95°C for 1 h. After this incubation, samples were cooled to ambient temperature, centrifuged at 4500 × g for 10 min, and the supernatant was processed for protein determination applying a modified Lowry method (DC™ Protein Assay, BioRad), using bovine serum albumin as standard.

5.2.8.4 Total carbohydrates quantification

Determination of carbohydrate was performed using the Anthrone Sulfuric Acid assay. Fresh starch aqueous solutions with concentrations ranging from 0.02 g/L to 0.2 g/L were prepared from starch powder (Merck 1.01257). They were used as standards and processed like the samples. Approximately 10 mg of the washed and freeze-dried yeast biomass was precisely weighted and diluted in distilled water to an exact concentration of 1 mg/mL. All samples were processed in duplicates. The anthrone reagent was prepared on the day of the experiment by dissolving anthrone (Merk 1.01468) in 95% sulfuric acid (AnalaR NORMAPUR: VWR Chemicals 20700) at a final concentration of 0.1% (w/v). The solution was well mixed and kept on

ice for at least 5 minutes. Afterwards 400 µL of diluted sample or standard were transferred into 1.5 mL Eppendorf Safe Lock tube. 800 µL of anthrone reagent were added and homogenized with the sample solution through inversion. After 5 min of incubation on ice, the mixed solution was transferred into a thermo-incubator, pre-heated at 95°C and shaken at 300 rpm for 16 minutes, and then cooled down on ice. Optical density of the cooled samples was measured in triplicate at 625 nm and carbohydrate concentration was calculated using the standard curve and by considering the dilution factors.

5.2.8.5 Total inorganic content investigation

Approximately 200 mg of washed and freeze-dried yeast biomass was measured in a precision balance and heated for 20 h at 650°C in a high temperature furnace (Hochtemperaturofen Supertherm HT04/17, Nabertherm, Germany). After removal from the furnace, the samples were let to cool down to room temperature, whereupon they were measured again with precision balance allowing for the calculation of the inorganic solid content designated as ashes content.

5.3 Results

5.3.1 Determination of yeast biomass composition

Composition of the yeast biomass on the harvesting day was analysed and is reported in Figure 18. Biomass composition showed good reproducibility and in total more than 86% of the biomass could be attributed to the following fractions: lipids 26.4 ± 4.6%, proteins 15.2 ± 1.9%, carbohydrates 39.3 ± 0.7% and ashes 3.1 ± 0.1%. A systematic fraction of 16% could not be identified and was missing to close the mass balance. This non- identified fraction is suspected to be some complex molecules of the resistant cell wall but further investigations were not carried out in the course of this study.

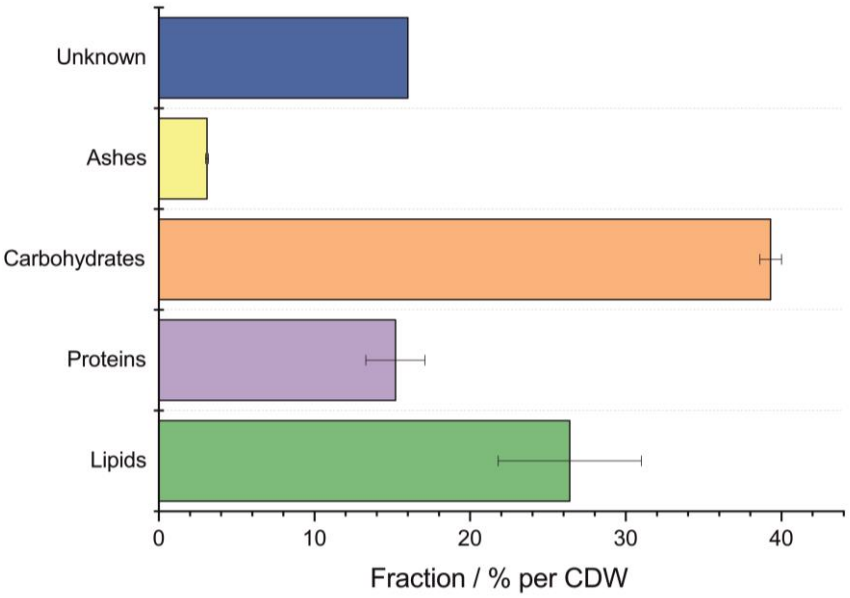

Figure 18: Composition of yeast biomass on harvesting day. Error bars result from the standard deviation of four independent experiments.

5.3.2 Strategies for biomass washing treatments

When performing PEF-treatment in a continuous flow manner, the specific energy of the treatment W [J/kg$_{DW}$] can be expressed using equation 9, where E [V/m] is the electric field, Δt [s] is the duration of the pulses, f_{rep} [Hz] the pulse repetition rate, t_{res} [s] the residency time of suspension inside the treatment chamber, C [kg/m^3] the biomass concentration and σ [S/m] the conductivity of the suspension inside the treatment chamber. Treatment specific energy therefore depends on the treatment parameters, which should be chosen based on their efficiency

(Escoffre et al., 2009), but also on the properties of the suspension to handle, especially its concentration and conductivity.

$$W = E^2 \times \sigma \times t_{res} \times \frac{\Delta t \times f_{rep}}{C} \qquad 9$$

Yeast suspension collected from the bioreactor at the end of a cultivation cycle had an average cell dry weight of 20.2 ± 2.9 g/L (i.e. a concentration of 20.2 ± 2.9 kg/m^3). This was kept as a working concentration for all experiments in this study. Regarding the conductivity of the suspension, the value of unwashed suspension directly harvested from the reactor was σ_U = 10.14 ± 0.63 mS/cm at 20°C. A suspension with such a conductivity can in principle be directly treated but at the expense of a high-energy consumption. To avoid the high-energy cost of PEF, an alternative is to wash the yeast suspension although this also represents energy cost, as well as fresh water consumption. The present study therefore investigated two different processing routes as described in Figure 19: the "unwashed route" in which PEF-treatment is applied on the yeast suspension directly after harvesting and the "washed route" in which the yeast suspension is washed before the PEF-treatment. In both cases, the impact of a supplementary washing step after the PEF-treatment and before the subsequent lipid extraction, was tested.

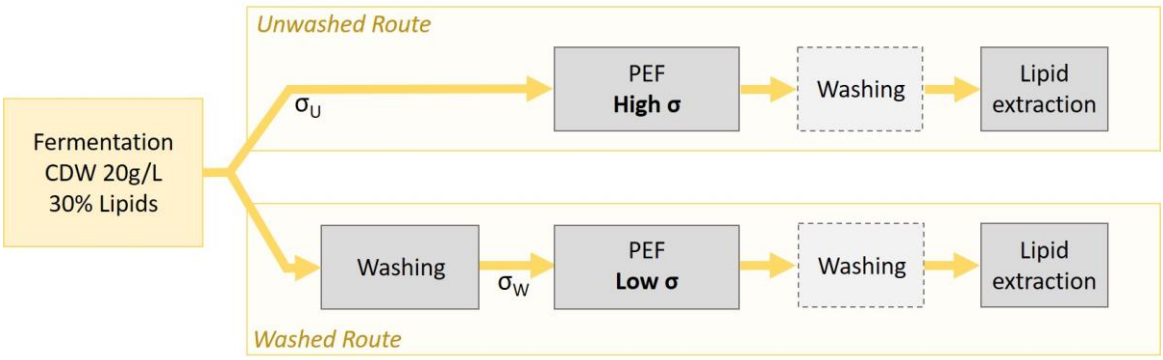

Figure 19: Illustration of the two washing treatment strategies "Unwashed" and "Washed".

The washing procedure was performed in order to reduce the initial conductivity of the suspension σ_U by a factor 8, in order to reach a value σ_W close to 1.2 mS/cm. The two following washing procedures were tested for their impact on cell permeabilization and on further lipid extraction.

A) Add distilled water to the yeast suspension until the desired conductivity (i.e. 8 times lower than unwashed suspension) is reached. Centrifuge and remove the excess supernatant to recover the initial cell concentration

B) Centrifuge the suspension and remove the supernatant. Add then the same amount of distilled water and enough NaCl to reach the desired conductivity (i.e. 8 times lower than unwashed suspension).

Results of Yo-Pro staining and lipid extraction yield obtained after washing using the two above-mentioned techniques, are displayed on Figure 20. With washing technique A, the percentage of Yo-pro positive cells, i.e. permeable cells, remained low, i.e. only 5 % compared to 2 % for the absolute unwashed control. This percentage reached 18 % using washing technique B. Regarding lipid extraction using ethanol-hexane blend, the yields normalized to the CDW were 2.41 % for the unwashed control and 6.0 % and 12.9 %, for the yeasts washed with technique A and technique B, respectively.

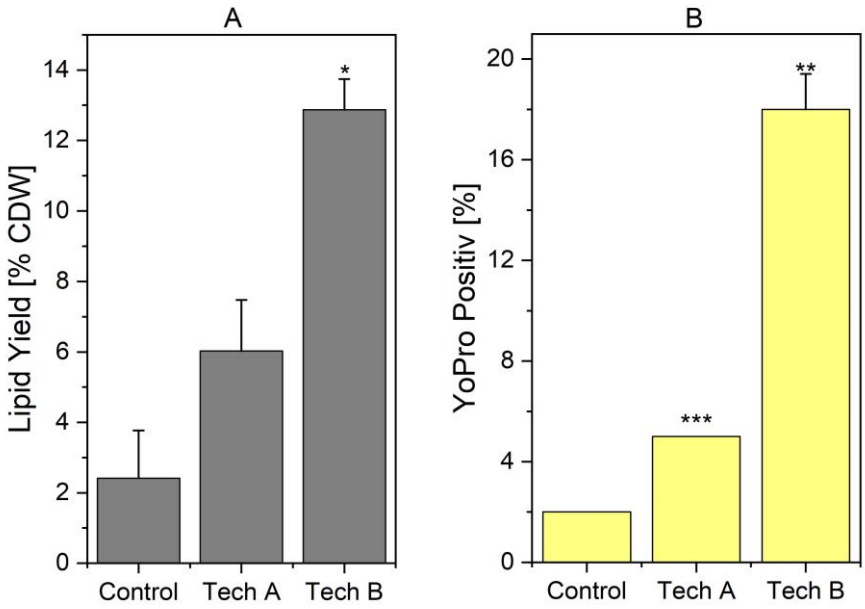

Figure 20: Impact of washing on lipid extraction (A) and Yo-Pro uptake (B). Two different washing techniques were implemented. In technique A, distilled water was added to reduce conductivity in yeast broth prior to centrifugation and in technique B, the yeast suspension was centrifuged beforehand, subsequently the biomass was resuspended to the same concentration as before with distilled water supplemented with NaCl to reach the targeted conductivity. Results are average ± standard deviation of two independent experiments (n = 2) with duplicates in each. Control refer to yeast processed directly after harvest, i.e. not submitted to any washing treatment. Statistical significances are indicated in comparison with control. P-values less than 0.05 are indicated with *, less than 0.01 with ** and less than 0.001 with ***.

Based on those experiments, the washing technique A appeared as less harmful to the yeast cells. Washing technique B induced more damage, but this method required less water and the volumes to be centrifuged were almost ten times lower. For those practical reasons, which are crucial at an industrial level, washing technique B was chosen for the rest of the study. The

average conductivity obtained after the washing procedure in the rest of the study, i.e. during the lipid extraction experiments, was $\sigma_W = 1.27 \pm 0.09$ mS/cm at 20°C.

5.3.3 Lipid extraction after PEF treatment in the unwashed route

First experiments conducted on unwashed yeast suspension intended to screen pulse parameters. Pulse duration was not varied in this study and was kept at 1 µs. The electric field magnitude was varied from 14 to 40 kV/cm and the repetition rate was adjusted in order to keep an applied energy of 150 kJ per liter of suspension in all cases. Results are displayed on Figure 21a . As can be seen, the lipid extraction yield obtained after the solvent extraction was 15% when electric field magnitude was fixed at 40 kV/cm. By decreasing the electric field magnitude at constant specific energy, the extraction efficiencies were slightly reduced, reaching 10% at 14kV/cm. For a few conditions, a washing step was added after the PEF treatment just before the lipid extraction. This extra washing step increased all yields, enabling to reach up to 22% of lipids of CDW. Nevertheless, in the unwashed route, none of the tested PEF-treatment conditions enabled to extract more than 54% of the evaluated lipid content when no washing was added after PEF-treatment. This value increased to 81% in case a washing step was added, leaving therefore 19% of lipids unextracted.

In a next step, the impact of energy input was tested, keeping the electric field value at 14 kV/cm. For that matter the repetition rate and therefore the average number of pulses applied, was varied in order to test specific applied energies ranging between 6 kJ/L and 150 kJ/L. Results are presented in Figure 21b and show that already the lowest tested energy, i.e. 6 kJ/L positively impacts the lipid extraction yield, although with too low extraction efficiency. Extraction yield then gradually increased with the applied energy, with the maximum extraction efficiency obtained for the maximum tested energy, i.e. 150 kJ/L. For all tested energies, extraction yields significantly increased when yeast suspension was washed after the PEF-treatment, before proceeding with the lipid extraction.

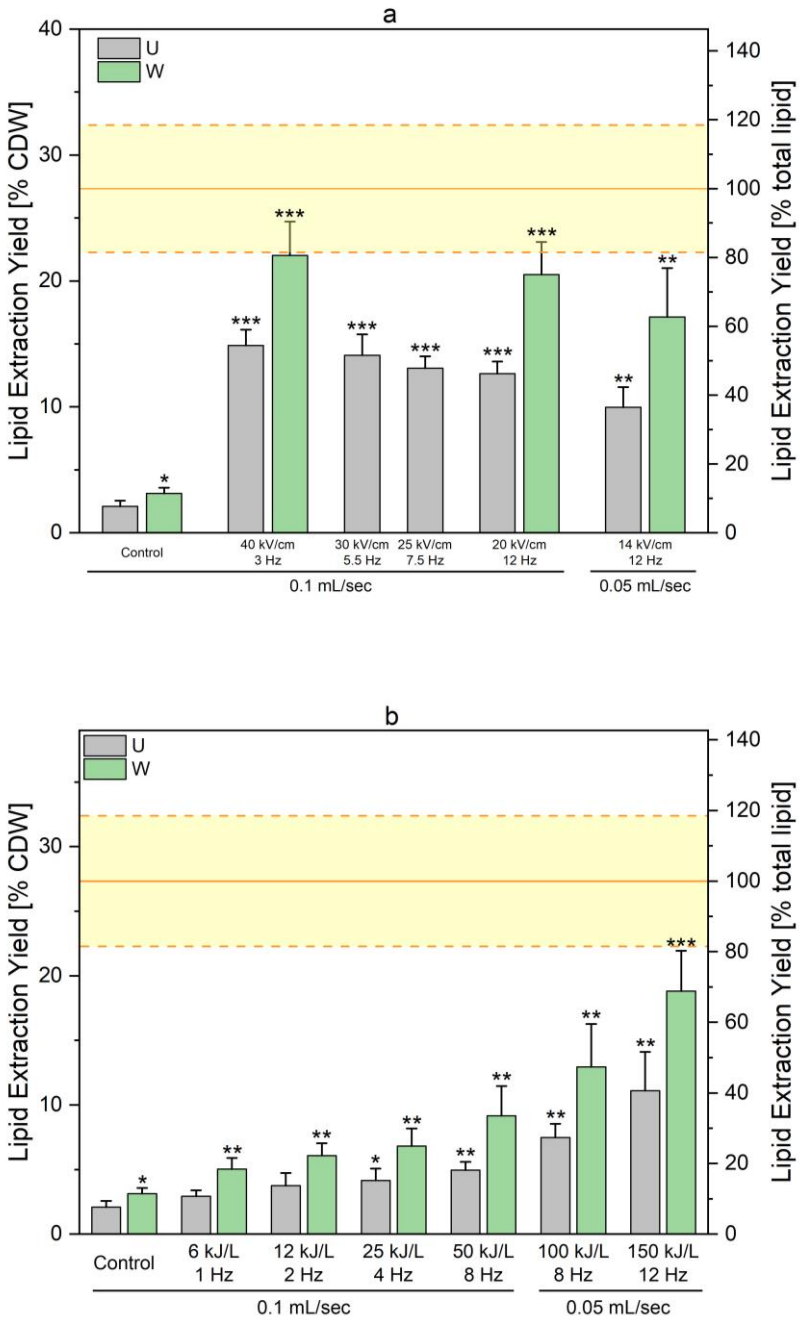

Figure 21: Lipid yields obtained after processing along the unwashed route. PEF treatment was applied directly after harvesting. Pulse duration was kept constant at Δt=1µs. (A) Impact of the electric field value at a constant specific treatment energy of 150 kJ/L. The x-axis indicates for each condition the applied electric field in kV/cm (upper values), the repetition rate in Hz at which pulses were applied (middle values) and the working flow rate of the suspension in the treatment chambers (lower values). (B) Impact of the specific treatment energy at a constant electric field value of 14 kV/cm. The applied specific energies, the repetition rate in Hz at which pulses were applied as well as the working flow rates have been indicated on the x-axis. For both graphics, the grey bars are the results of lipid extraction performed directly after the PEF-treatment (U) and the green bars are the extraction yield obtained when a washing step is added after PEF-treatment before addition of extraction solvent (W). The yellow lines indicate the average ± standard deviation of Soxhlet extractions, as absolute control. Results are reported as the average + standard deviation of three independent experiments (n = 3) with samples processed in duplicates. Statistical significances are indicated in comparison with the unwashed control. P-values less than 0.05 are indicated with *, less than 0.01 with ** and less than 0.001 with ***.

5.3.4 Lipid extraction after PEF treatment in the washed route

In order to compare the washed route with the previously tested unwashed route, two experiments were designed according as sketched in Figure 22.

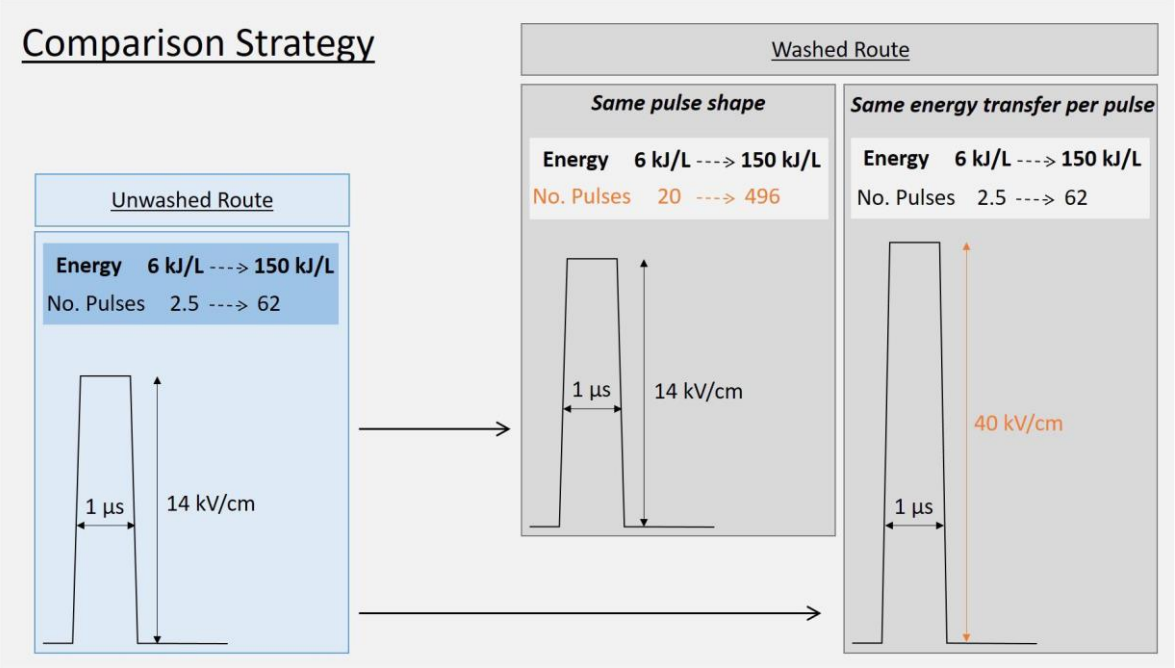

Figure 22: Strategy to compare washed route with the unwashed route. In the washed route the conductivity of the yeast suspension is 8 times lower than in the unwashed route.

The strategy consisted in mirroring the experiment described above (Figure 21b), i.e. testing the efficiency of specific energies ranging from 6 kJ/L to 150 kJ/L either by:

(I) exact application of the same pulse shape, i.e. E=14 kV/cm and Δt=1 µs. This implied the application of more pulses in order to compensate for the lower conductivity of the washed yeast suspension and to achieve the targeted specific energies,

or by

(II) transfer the same energy per pulse into the suspension, which implies increasing the electric field strength to 40 kV/cm to compensate energy transfer for the lower conductivity of the washed yeast suspension.

The results of the two experiments are displayed in Figure 23. In both cases an increase of the lipid extraction yield is observed with increasing PEF treatment energy input. In case the electric field is fixed at 14 kV/cm (Figure 23a), 95% of the lipid content (25% of CDW) can be extracted with a specific energy of 100 kJ/L. Further increasing the energy to 150 kJ/L did not further improve the extraction yield. In case the electric field was increased to 40 kV/cm

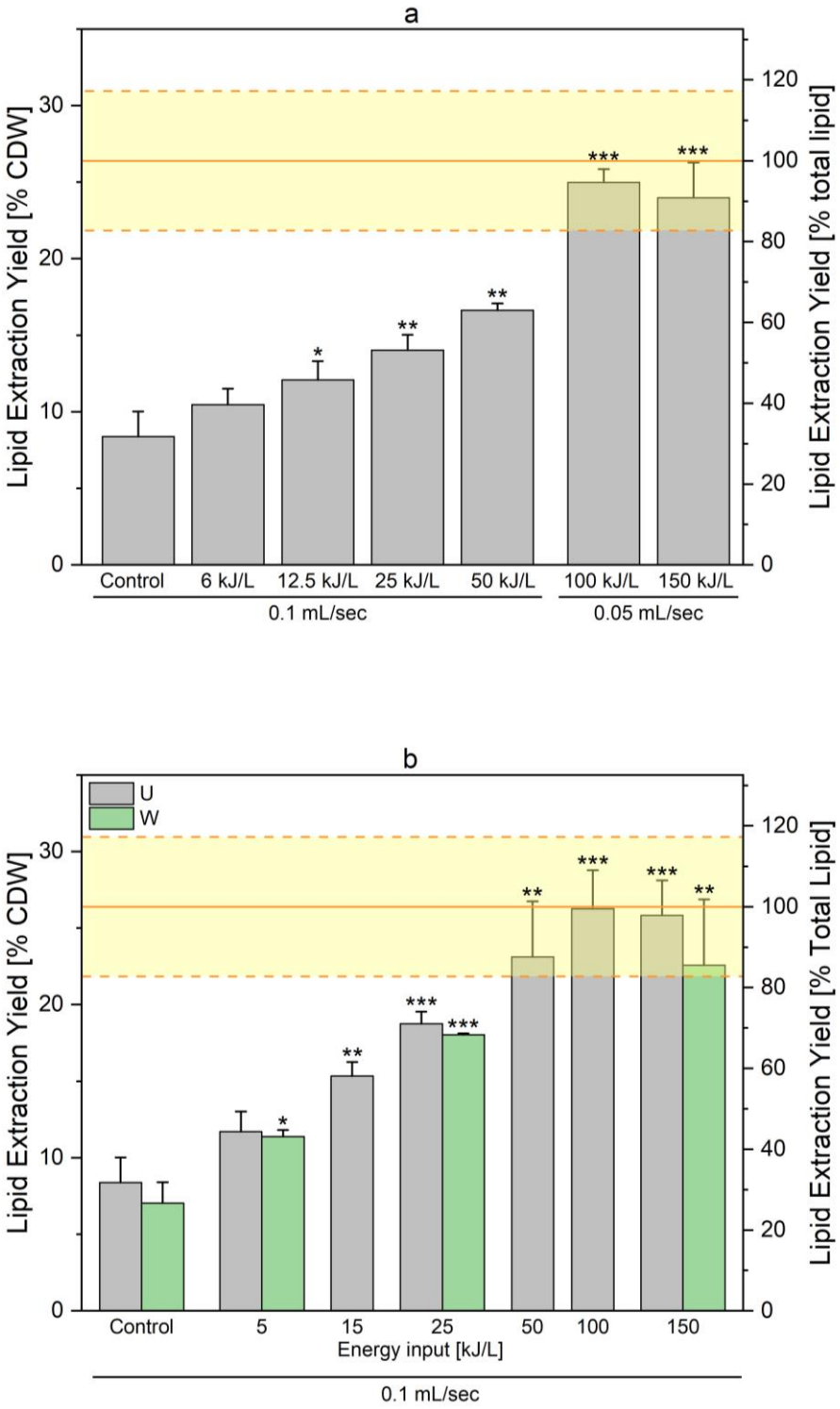

Figure 23: Extraction yields obtained after processing along the washed route. PEF treatment was applied on the washed yeast biomass after harvesting. Impact of the specific treatment energy at a constant electric field value of (A) 14 kV/cm and (B) 40 kV/cm. For the bottom graph, the grey bars are the results of lipid extraction performed directly after the PEF-treatment and the green bars are the lipid extraction yields obtained when a washing step is added after PEF-treatment before addition of extraction solvents. The yellow lines illustrate the average ± standard deviation of Soxhlet extractions, as absolute control. For both experiments, the results are reported as the average + standard deviation of three independent experiments (n = 3) with samples processed in duplicates. Statistical significances are indicated in comparison with the unwashed control. P-values less than 0.05 are indicated with *, less than 0.01 with ** and less than 0.001 with ***.

(Figure 23b), even higher yields could be obtained, with already 87 % of the lipid recovered at a specific energy of 50 kJ/L and up to 99 % at 100 kJ/L. Note that in that case, the impact of an additional washing step between the PEF-treatment and the lipid extraction was tested, and that it appeared as not providing any significant improvement of the lipid recovery.

5.4 Discussion

5.4.1 Comparison of extraction efficiency in washed and unwashed route

In the unwashed route, the best extraction yields were obtained using pulses of 1 µs with an electric field strength of 40 kV/cm and an energy of 150 kJ/L of suspension (Table 10). The extraction efficiency was nevertheless only 15% reported to cell dry weight, i.e. about 50% of the total lipid content evaluated by Soxhlet. In case a washing step was added after the PEF-treatment, just before the lipid-extraction step, the final yield slightly increases and up to 81% of the total lipid were recovered. Much better results could be obtained in the washed route, i.e. when treating the lower-conductivity yeast suspension. In that case, 87% and 99% of the evaluated lipid content could be recovered with energy input of 50 and 100 kJ/L, respectively (Table 10). The reason for a higher efficiency of the washed route has not be further investigated but several hypotheses can be proposed. First, it might be that the whole procedure, i.e. PEF-treatment and lipid extraction, is more efficient on washed yeasts since cells are already weakened by the washing (Figure 20). However, since a washing step added in the "unwashed-route" immediately after PEF-treatment (i.e. before the lipid extraction) helps to recover higher yields, a second hypothesis is that the washing is not required for the PEF treatment itself but more for the extraction. Some constituents of the medium such as the high glucose content might indeed interact with the solvents and reduce the extraction efficiency. In order to test this hypothesis, a possibility would be to reduce the number of possible elements disturbing the extraction at the end of cultivation by improving the medium composition and especially by stopping the sugar feeding at the end of cultivation in order not to have any sugar in excess. Since this optimization of medium is required in any case, to reduce the costs of up-stream related to nutrients, it appears as an obvious next step to this work. Finally, the higher efficiency of the "washed-route" might be simply explained by the fact that at identical energy input, the PEF-parameters used in the washed-route are harsher either in terms of number of pulses or of field intensity. Indeed, the fact that conductivity is 8 times lower in the washed route enables for given pulse parameters to apply 8 times more pulses or for a given number of pulses to apply an electric field $\sqrt{8}$ (i.e ~ 2.8) times higher. In any case, despite the fact that this study demonstrates that PEF-treatment can be applied directly after the harvest of the yeast, it appears that in practice a washing step will still be required for the current way of cultivation. Future cultivation optimization efforts should go for reducing the nutrients content in suspension at the time of harvest.

Table 10: Energies involved for different treatment conditions chosen based on their efficiency.

Route	PEF parameters	Energy [kJ/L]	Energy [kJ/kg_{DW}]	Extraction yield [% CDW]	Extraction yield [% Lipid content]	Energy [kJ/kg lipid]	Energy [kWh/kg lipid]
Unwashed route CDW = 20.21±2.87	1 µs 40 kV/cm	150	7422	15	54	49480	13.74
				22	81	33737	9.37
Washed route CDW = 19.96±3.22	1 µs 14 kV/cm	100	5010	25	95	20040	5.57
	1 µs 40 kV/cm	50	**2505**	**23**	**87**	**10891**	**3.03**
	1 µs 40 kV/cm	100	5010	26	99	19269	5.35

The values underlined correspond to values obtained after addition of a washing step after PEF-treatment before lipid extraction. Bold values indicate the best obtained results for energy efficiency.

5.4.2 Evaluation of energy consumption for PEF aided SCO extraction

Regarding the absolute energy consumption, the best parameters of the study enabled to recover lipid using 10,891 kJ/kg_{LIPID} (Table 10), i.e. 3 kWh/kg_{LIPID}. To classify this energy balance with studies of other oleaginous yeasts, it is evident that our method is similarly competitive in terms of energy consumption compared to reported low energy demanding methods. As reviewed by Dong et al., (2016) for the hydrochloric acid digestion under heat of *Cryptococcus curvatus* biomass 9.3 - 18.6 MJ/kg_{LIPID} (2.58 - 5.17 kWh/kg_{LIPID}) is required (Yu et al., 2015), for the enzymatic digestion of *Rhodosporidium toruloides* biomass the energy consumption of 13.3 MJ/kg_{LIPID} (3.67 kWh/kg_{LIPID}) is reported (Jin et al., 2012). However, cell lysis by acid hydrolysis is disadvantageous due to formation of corrosion in the process equipment and the use of enzymes for cell wall digestion causes high costs on large scale. In contrast mechanical methods, such as bead milling, are considered to be highly scalable but are more energy demanding. For the oleaginous yeasts *Yarrowia lypolytica* bead milling, as one of the most energy efficient pre-treatment methods for lipid extraction, consumed 115.2 MJ/kg_{LIPID} (32 kWh/kg_{LIPID}) of energy (Meullemiestre et al., 2016), which is about 10-fold higher than the other discussed methods including this presented PEF method.

For the PEF method the amount of energy in this current study can most probably be further reduced using standard strategies related to PEF-treatment. First of all, PEF-parameters can be improved since they have crucial role in PEF efficiency (Escoffre et al., 2009). The importance of the parameters can be seen already on the fact that in the washed route, for a constant energy

input of 100 kJ/L, pulses of 40 kV/cm were more efficient than pulses of 14 kV/cm (Table 10). Further improvements should therefore be made for example by optimising pulse duration. Additionally, it is possible to reduce the energy consumption by treating a yeast suspension with higher cell concentration since PEF-treatment efficiency does not decrease for denser cell suspension (Goettel et al., 2013). Required PEF-treatment energy linearly decreases with increasing biomass density in the suspension to be treated (Goettel et al., 2013). Only limitation for this approach will be the viscosity of the suspension which should not increase to much, in order not to increase energy requirement for pumping during the continuous flow process. Finally, an incubation period could be added after PEF-treatment since it was shown in the past on microalgae that this strategy allowed reducing energy input up to 6 times (Silve et al. 2018b).

5.4.3 Choice of solvents

The approach presented in this study was developed sticking as much as possible to the requirements of industry in order to offer a downstream approach that can be up-scaled. In particular, no freezing or freeze drying was used since those two processes are known to be highly energy-demanding (Willis et al., 2014). Apart from the possible improvements regarding the energy demand of the PEF-treatment, additional efforts in the future should be focused on reducing the amount of solvent and also switching to more sustainable ones. Currently, most existing studies agree that a system of polar and non-polar solvents is the most efficient approach to recover all lipids. For analytical purposes the most popular and effective methods at laboratory-scale are systems using chloroform and methanol according to Folch (Folch et al., 1957) or Bligh & Dyer (Bligh and Dyer, 1959). However, chloroform is highly toxic and carcinogenic and its usage should be avoided even in the laboratory (Prat et al., 2015). The ethanol-hexane blend used in this study is for that matter more suitable. Indeed for potential industrial scale application hexane is considered as a less toxic alternative for chloroform and it is already commonly used in food industry (Biondo et al., 2015). However, hexane is still a petro-based solvent and therefore from environmental point of view not reasonable.

Further work should therefore focus specifically on solvent in order to propose a solvent system both economically viable and sustainable. Not only the type of solvent but also the volumes at play and the easiness to recycle will be determinant for the implementation at larger scale.

6 Conclusions

To satisfy the increasing energy and resources demand for our growing population and to circumvent with attendant ecological and economic challenges in the next decades, research and finding of sustainable realization procedures are urgently needed. Biotechnologically produced microbial oils could play an important role in this respect and be potential alternatives for crude and plant oils. The research and investigation on novel potent yeast production strains, like *Saitozyma podzolica* and *Apiotrichum porosum*, can contribute to find innovative concepts for process cost reduction or value enhancement and ensuring an economic feasibility in future.

The aim of this study was first, to optimize the upstream SCO process of *S. podzolica* with respect to enhance SCO productivity and prevent carbon source wasting. By adapting the process parameters. Furthermore, the intended reduction of applied sugar amount was achieved by establishing a new two-phase process comprising a batch and automated continuous fed-batch strategy with about 10 g/L excess sugar in the latter phase. Compared to the former pulsed feeding strategy with high carbon source excess, ~41% of total glucose and ~26% of total xylose could be saved. Using glucose, the reduced sugar cultivation with continuous carbon source supply led to 28% higher biomass growth and 19% increase of lipid titer. Using xylose, the sustainable and valuable side-product xylonic acid was detected and identified for the first time as being produced by *S. podzolica*. Consequently, it was possible to optimize the cultivation process for *S. podzolica* with regards to sustainable and economical aspects, since the applied sugar amount was reduced and high sugar wastage during cultivation was prevented without losing productivities. On that account, an automated feeding strategy was established, which on industrial level could save costs.

To further promote to sustainability of the SCO production with *S. podzolica* future research should be focused on sustainable carbon sources. Since renewable lignocellulosic biomass is composed of mixed complex carbohydrates, in future studies, the efficiency of mixed sugars and lignocellulosic hydrolysates is to be addressed.

For the lipid downstream processing it was intended to investigate the impact of cell disruption, extraction, and transesterification methods on fatty acid extraction yields and their lipid profile of SCO of the both unconventional yeasts, *S. podzolica* and *A. porosum*, to assess their potential economic profitability. It was shown that BM and HPH were the most appropriate cell disruption methods for *S. podzolica* whereas DT was best for *A. porosum* followed by BM. No differences could be observed between F and BD as extraction methods for derivatized lipids, while the EH system was superior for *S. podzolica*, which is less harmful in comparison to the

highly toxic chloroform-methanol mixture. With regard to the lipid profiles, differences between BM and HPH were observed, which might be due to oxidation of linoleic acid while HPH processing. Comparison of alkaline and acidic transesterification for biodiesel production of recovered oil revealed higher linoleic acid yield for H_2SO_4 catalysis. Both are similarly potent transesterification methods for recovered oil, whereas direct transesterification needs to undergo harsh conditions provided by this study's acidic transesterification. By rating the energy consumption, the most energy efficient methods are BM and HPH prior the EH extraction with 96 and 95 kWh/kg lipid, respectively. For whole lipid extraction from *A. porosum* nearly twice as much energy was required compared to *S. podzolica*, which is due to the low achieved cell disruption efficiency of this strain. On this account, *A. porosum* is less appropriate for potential industrial use. However, for *S. podzolica* a 53% higher production potential was shown using HPH-EH prior to transesterification, compared to the former standard analysis DT. Moreover, just 3.6% of energy percentage is needed for one kg FAME compared to DT, which is due to the omission of biomass freeze-drying

Since these examined yeasts are promising SCO hosts, but the lipid processing was proved to be challenging, a further innovative idea is the deep eutectic solvent (DES) and lipase assisted ''one-pot'' production of glycolipids from these yeasts' biomass by using microwaves. Therefore, in such ''one-pot'' reactions the yeast cells will be disrupted by microwave treatment and release the lipids. The lipase will then catalyse the glycolipid synthesis by using fatty acids and the DES sugar-alcohols, e.g. sorbitol or xylitol, as substrates.

Finally, it was aimed to examine the innovative pre-treatment pulsed electric field to enhance lipid extraction yield on fresh *S. podzolica* biomass. It was demonstrated, that lipid extraction efficiencies were remarkably enhanced by PEF-treatment of the *S. podzolica* biomass prior to organic solvent lipid recovery. Moreover, by applying a washing step before PEF- treatment the extraction yield increased to 99% of total lipid. In comparison in the unwashed route, the best extraction efficiency was only 15% reported to CDW, i.e. 54% of the total lipid content. Since the conductivity was 8 times lower in the washed-route, the higher efficiency might be due to the fact that at identical energy input, the PEF- parameters used in the washed-route are harsher either in terms of number of pulses or of field intensity. The influence of conductivity on PEF energy requirements and extraction yields was indicated, and results suggest a higher efficiency of PEF-treatment in terms of energy when treatment is performed at lower conductivity. In addition, it was emphasized, that the amount of required energy for PEF treatment was reasonable, with 3.03 kWh/kg_{lipid} using the best obtained parameters, and competitive with other low-

energy consuming yeast pre- treatment methods, such as enzymatic digestion. This study was the first insight in the PEF technique of oleaginous yeasts.

In future studies, it will be interesting to examine in detail the energy and economic costs associated with the whole PEF process, from cultivation of the yeast until lipid extraction. Such an analysis will enable to propose a realistic evaluation of the potential applications for the lipids extracted using this processing route.

Taken all these finding together, the potential of the unconventional oleaginous yeasts *S. podzolica* and *A. porosum* were affirmed as interesting production strain for biotechnological purposes. Especially, for *S. podzolica* the improvement of the SCO productivity and the expansion of the ability to produce valuable organic acids indicated high capabilities for a broad range of biotechnological application, particularly, because the carbon amount for production could be reduced. Regarding downstream processing, this study is the first to evaluate the properties of these novel SCO producing yeasts in this respect for potential industrial use like biodiesel or oleochemical production. Additionally, it was signified how different yeast species may be in their characteristics towards biomass pre-treatments, as the oil of *A. porosum* was more difficult to recover, compared to *S. podzolica,* and more efficient with high energy consuming pre-treatment processes. In respect to energy consumption, this study provided the highly energy-efficient pre-treatment method for SCO recovery, namely pulsed electric field, which showed remarkable extraction efficiencies on fresh *S. podzolica* biomass. Compared to lipid recovery using the best mechanical disruption method HPH 91.97 kWh/kg_{lipid} can be saved by using PEF, which represents an energy saving of ~ 97%. Moreover, for such good PEF results just washing of the biomass was performed in contrast to costly friezing or freeze-drying. Omitting of many costly and elaborate pre-treatments is highly relevant for industrial processes and for reduction of cost. Therefore, this study provides a broad understanding for processing and treatment of SCO produced by novel oleaginous yeasts and the development of an innovative and efficient SCO recovery method using PEF, which was examined on microalgae but not on oleaginous yeasts before.

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List of figures

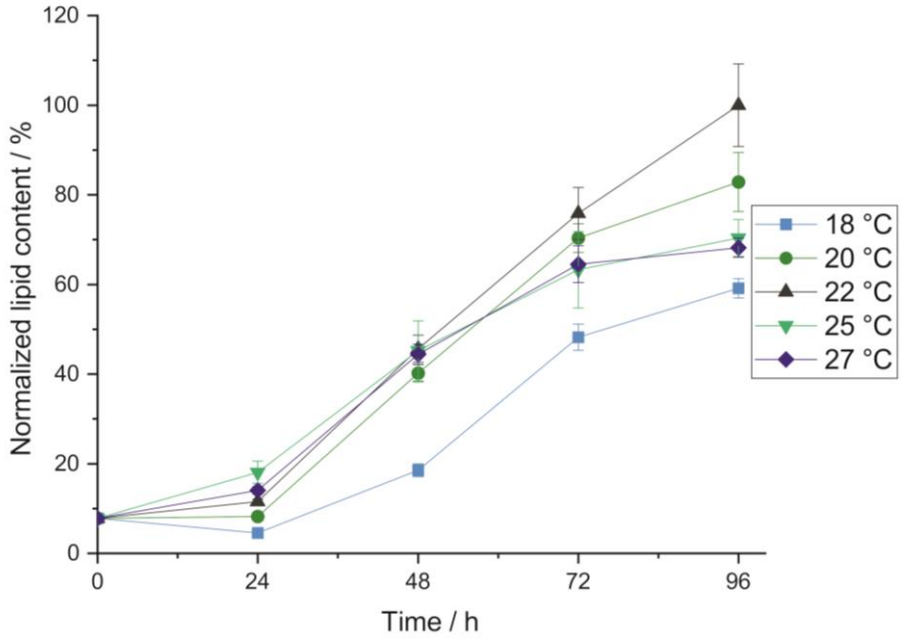
List of tables

List of abbreviations

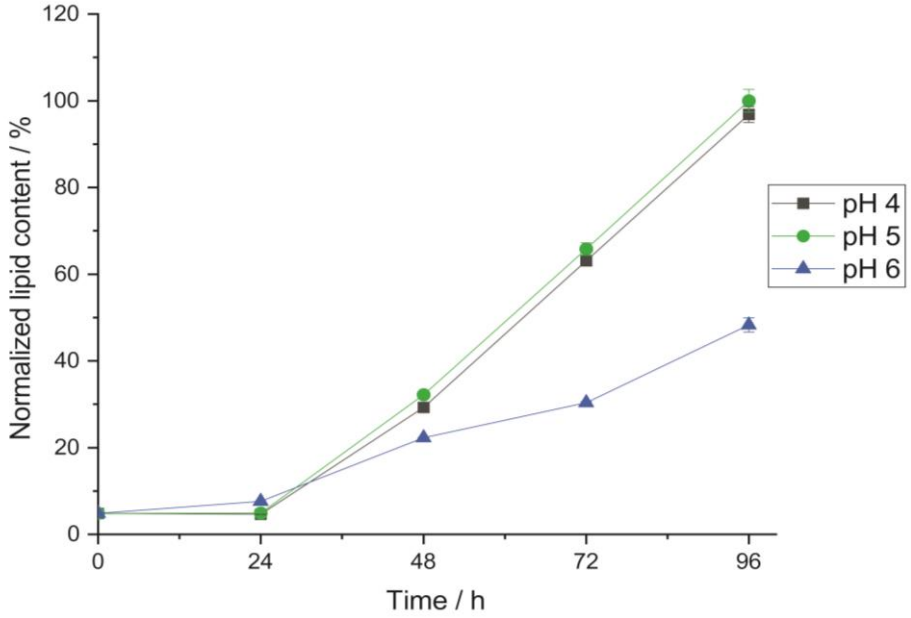
ACL	ATP:citrate lyase
ALA	α-Linolenic acid
AMP	Adenosine monophosphate
ARA	Arachidonic acid
BD	Bligh and Dyer
BM	Bead milling
CAZYmes	Carbohydrate-active enzymes
CDW	Cell dry weight
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DT	Direct transesterification
EH	Ethanol hexane extraction system
EPA	Eicasopentaenoic acid
F	Folch extraction protocol
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FID	Flame ionization detector
GA	Gluconic acid
GC	Gas chromatography
HPLC	High performance liquid chromatography
HPH	High pressure homogenization
ICDH	Isocitrate dehydrogenase
LA	Linoleic acid
ME	Malic enzyme
NADPH	Nicotinamide adenine dinucleotide phosphate
PEF	Pulsed electric field
PUFA	Polyunsaturated fatty acid
SCO	Single cell oil
U	Ultrasonification
TAG	Triacylglycerol
XA	Xylonic acid

Appendix

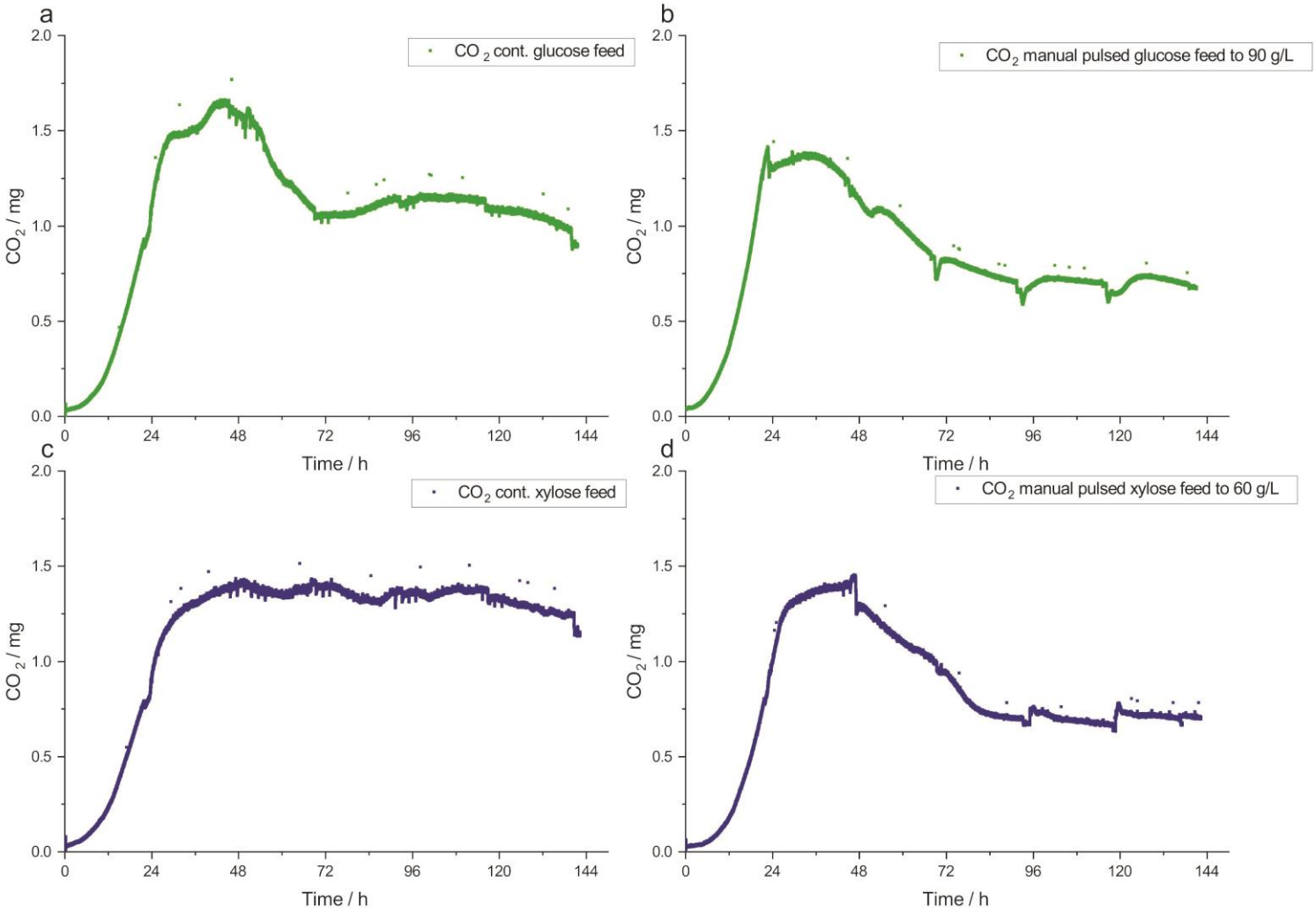
Appendix 1: Investigation of the optimal temperature for lipid production of *S. podzolica* in shake flasks. 18, 20, 22, 25 and 27°C were tested over a 96 h cultivation. The data were normalized to the highest lipid concentration [g/L] value. The error bars result from the standard deviation of experimental set-ups in triplicates each.



Appendix 2: Illustration of produced lipids by *S. podzolica* in bioreactors at 22°C at three different pH of 4, 5 and 6 in 96 h process time. The data were normalized to the highest lipid concentration [g/L] value. The error bars result from the standard deviation of duplicate experimental set-ups.



Appendix 3: CO_2 emission of *S. podzolica* at different cultivation modes over time. a) automated continuous feed process on glucose. b) daily pulsed glucose restock to 90 g/L. c) automated continuous feed process on xylose. d) daily pulsed xylose restock to 60 g/L.



Appendix 4: Recovered lipid yields of methylated fatty acids (FAME) from *S. podzolica* and *A. porosum*.

		% FAME/CDW			
		S. podzolica		***A. porosum***	
		KOH	**H_2SO_4**	**KOH**	**H_2SO_4**
BM	**F**	30.7 ± 0.6 [a]	30.1 ± 1.5 [a]	23.4 ± 0.5 [a]	19.6 ± 1.0 [b]
	BD	30.1 ± 1.5 [a]	32.0 ± 3.5 [a]	23.8 ± 1.1 [a]	20.2 ± 1.2 [b]
	EH	32.9 ± 1.7 [a]	28.9 ± 1.0 [a]	n.d.	n.d.
HPH	**F**	30.4 ± 0.9 [a]	29.1 ± 1.3 [a]	13.9 ± 1.5 [c]	14.8 ± 1.2 [c]
	BD	29.9 ± 2.1 [a]	34.1 ± 0.1 [a]	14.7 ± 1.1 [c]	13.4 ± 0.7 [c]
	EH	46.0 ± 6.8 [b]	44.2 ± 4.0 [b]	n.d.	n.d.
U	**F**	19.0 ± 0.4 [c]	25.7 ± 1.8 [d]	n.d.	n.d.
	BD	19.8 ± 0.8 [c]	23.8 ± 2.3 [c]	n.d.	n.d.
DT	**-**	6.9 ± 1.9 [e]	30.0 ± 1.3 [a]	2.9 ± 0.8 [d]	27.2 ± 0.5 [e]

The standard deviation of three independent experiments is indicated. FAME: fatty acid methyl ester; CDW: cell dry weight; BM: bead mill; HPH: high pressure homogenization; U: ultrasonic treatment; DT: direct transesterification; F: extraction according to Folch; BD: extraction according to Bligh and Dyer; EH: ethanol-hexane extraction. a, b, c, d, e indicate statistical differences (p=0.05). ab indicates statistical difference between the disruption methods without changing the extraction method. Statistical analysis was performed separately for each yeast species and separately.

Appendix 5: Lipid profiles of *S. podzolica* biomass after derivatisation of each downstream processing method. The indicated standard deviation comprises of three independent analyses.

	% FAME/CDW																	
	BM						HPH						U				DT	
	F		BD		EH		F		BD		EH		F		BD		-	
	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4
Palmitic acid	19.8 ±0.0	20.8 ±0.0	19.8 ±0.1	20.8 ±0.0	20.4 ±0.1	21.5 ±0.1	20.0 ±0.1	20.5 ±0.1	19.9 ±0.0	20.6 ±0.0	20.5 ±0.1	21.3 ±0.1	20.0 ±0.1	20.5 ±0.1	19.9 ±0.1	20.6 ±0.1	19.7 ±0.1	20.3 ±0.2
Stearic acid	5.3 ±0.1	5.2 ±0.1	5.2 ±0.1	5.2 ±0.2	5.4 ±0.0	5.7 ±0.0	5.2 ±0.0	4.8 ±0.0	5.16 ±0.0	5.0 ±0.1	5.6 ±0.1	5.4 ±0.0	5.0 ±0.0	3.4 ±2.2	5.0 ±0.0	4.7 ±0.1	4.7 ±0.2	4.9 ±0.0
Oleic acid	59.4 ±0.0	57.0 ±0.4	59.5 ±0.2	57.2 ±0.2	60.6 ±0.1	57.5 ±0.2	59.3 ±0.1	57.0 ±0.3	59.5 ±0.0	57.4 ±0.2	60.8 ±0.2	58.6 ±0.3	59.2 ±0.3	58.1 ±2.4	59.0 ±0.1	56.5 ±0.3	63.2 ±0.6	58.0 ±0.1
Linoleic acid	9.8 ±0.0	11.9 ±0.2	9.8 ±0.0	11.4 ±0.2	8.2 ±0.2	9.4 ±0.2	9.9 ±0.0	12.0 ±0.2	9.9 ±0.0	11.5 ±0.2	7.6 ±0.2	8.7 ±0.1	10.8 ±0.1	12.5 ±0.2	10.6 ±0.0	12.6 ±0.2	10.6 ±0.1	12.1 ±0.1
Linolenic acid	1.6 ±0.0	1.3 ±0.0	1.6 ±0.0	1.4 ±0.0	1.6 ±0.0	1.5 ±0.0	1.6 ±0.0	1.4 ±0.0	1.6 ±0.0	1.4 ±0.0	1.7 ±0.0	1.5 ±0.0	1.6 ±0.0	1.3 ±0.0	1.5 ±0.0	1.3 ±0.0	1.3 ±0.0	1.4 ±0.0
Traces	4.1	3.8	4.2	4.1	3.8	4.5	4.0	4.4	4.0	4.2	3.7	4.3	3.6	4.2	4.0	4.4	0.5	3.4

FAME: fatty acid methyl ester; CDW: cell dry weight; BM: bead mill; HPH: high pressure homogenization; U: ultrasonic treatment; DT: direct transesterification; F: extraction according to Folch; BD: extraction according to Bligh and Dyer; EH: ethanol-hexane extraction; n. d.: not determined

Appendix 6: Lipid profiles of recovered and transesterified SCO of *A. porosum*. The indicated standard deviation comprises of three independent analyses.

	% FAME/CDW									
	BM				**HPH**				**DT**	
	F		**BD**		**F**		**BD**		**-**	
	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4	KOH	H_2SO_4
Palmitic acid	22.4 ±0.5	22.5 ±0.1	22.1 ±0.0	22.5 ±0.1	22.1 ±0.2	23.2 ±0.1	22.0 ±0.1	23.2 ±0.1	22.7 ±0.6	22.0 ±0.5
Stearic acid	15.7 ±0.3	15.2 ±0.0	15.5 ±0.1	15.2 ±0.1	17.6 ±0.0	15.9 ±0.1	17.5 ±0.1	15.9 ±0.1	14.4 ±2.3	12.6 ±1.6
Oleic acid	40.01±0.7	37.6 ±0.2	39.6 ±0.1	37.7 ±0.2	39.0 ±0.2	37.2 ±0.2	39.2 ±0.0	37.2 ±0.2	43.3 ±1.3	40.8 ±1.7
Linoleic acid	18.8 ±0.3	20.3 ±0.1	18.5 ±0.1	20.1 ±0.2	17.0 ±0.1	19.3 ±0.1	17.0 ±0.1	19.4 ±0.2	19.7 ±0.5	20.4 ±0.3
Linolenic acid	0.6 ±0.01	0.6 ±0.0	0.6 ±0.0	0.6 ±0.0	0.7 ±0.0	0.6 ±0.0	0.6 ±0.0	0.6 ±0.0	n.d.	0.5 ±0.1
Traces	3.5	3.9	3.8	3.9	3.7	3.8	3.7	3.8	n.d.	3.6

FAME: fatty acid methyl ester; CDW: cell dry weight; BM: bead mill; HPH: high pressure homogenization; U: ultrasonic treatment; DT: direct transesterification; F: extraction according to Folch; BD: extraction according to Bligh and Dyer; EH: ethanol-hexane extraction; n. d.: not determined.