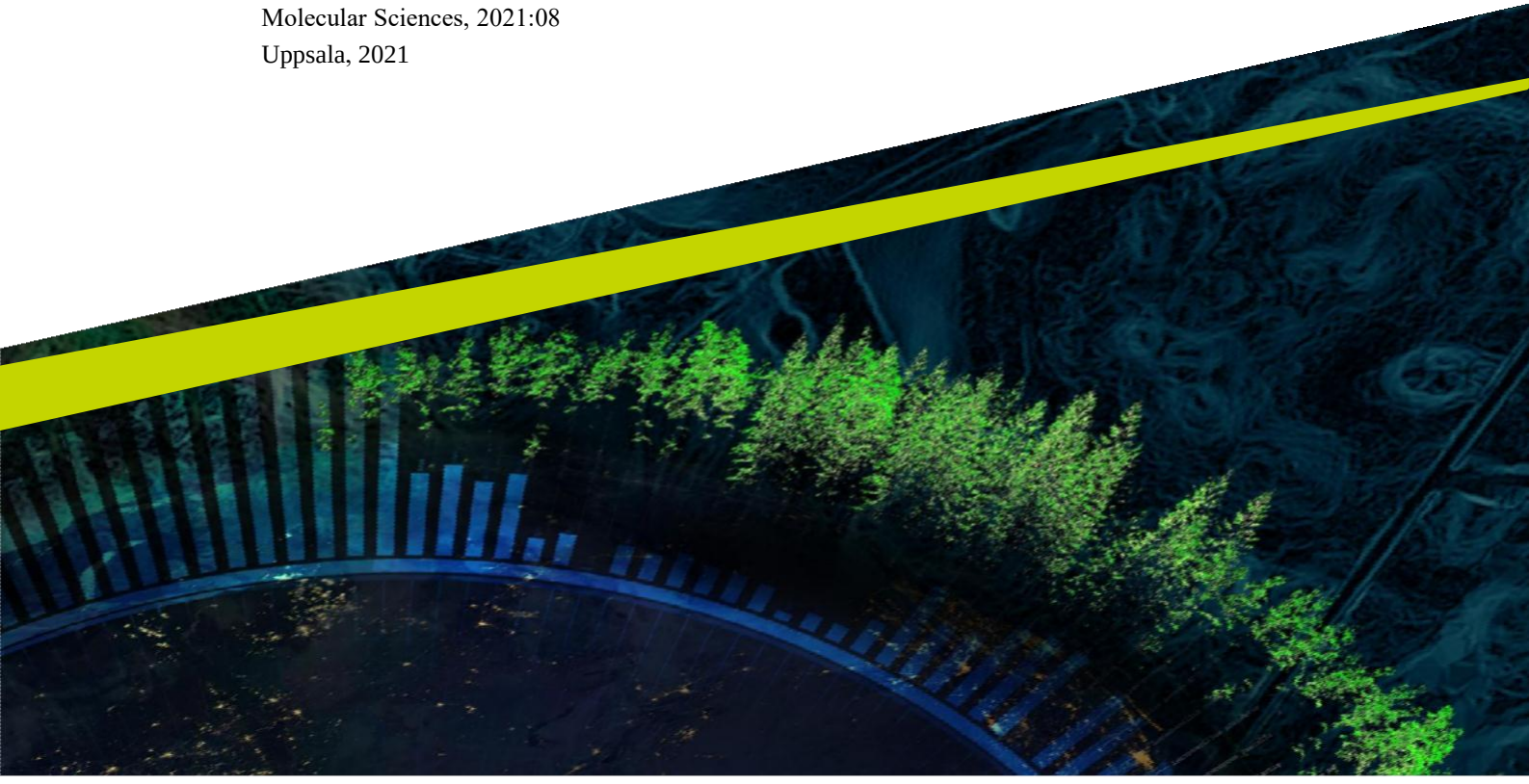
Lipid content and profile of *Rhodotorula toruloides* during fermentation

Khairul Kibria

Degree project/Independent project • (30 h)
Swedish University of Agricultural Sciences, SLU
Biology with specialization in Biotechnology- Masters Programme
Molecular Sciences, 2021:08
Uppsala, 2021



Lipid content and profile of *Rhodotorula toruloides* during fermentation

Author's name: Khairul Kibria

Supervisor: Jana Pickova, Swedish University of Agricultural Science, Department of Molecular Sciences

Examiner: Sabine Sampels, Swedish University of Agricultural Science, Department of Molecular Sciences

Credits: 30 hp
Level: Advanced A2E
Course title: Independent work in Biology
Course code: EX0895
Programme/education: Master's degree in Biology with specialization in Biotechnology
Course coordinating dept: Department of Molecular Sciences

Place of publication: Uppsala
Year of publication: 2021
Title of series: Molecular Sciences
Part number: 2021:08

Keywords: *Rhodotorula toruloides*, Fatty acids, Lipid class, Triglycerides, Phospholipid, GasChromatography.

Swedish University of Agricultural Sciences
Faculty of Natural Resources and Agricultural Sciences (NJ)
Department of Molecular Science

Publishing and archiving

Approved students' theses at SLU are published electronically. As a student, you have the copyright to your own work and need to approve the electronic publishing. If you check the box for **YES**, the full text (pdf file) and metadata will be visible and searchable online. If you check the box for **NO**, only the metadata and the abstract will be visible and searchable online. Nevertheless, when the document is uploaded it will still be archived as a digital file.

If you are more than one author you all need to agree on a decision. Read about SLU's publishing agreement here: https://www.slu.se/en/subweb/library/publish-and-analyse/register-and-publish/agreement-for-publishing/.

√ YES, I/we hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I/we do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.

Abstract

Oleaginous yeast such as *Rhodotorula toruloides* is a promising organism for production of lipids from lignocellulosic substrates. Microbial lipids have potential applications in energy, food, feed and pharmaceutical industry, because most of the lipids are triglycerides with long chain fatty acids that are comparable to typical vegetable oils and can be obtained without arable land requirement. This study investigated lipid production in the batch cultivation over time by the yeast *Rhodotorula toruloides*. Furthermore, the composition of fatty acids and lipid classes were analyzed by using gas chromatography (GS) and thin layer chromatography (TLC), respectively. Under the batch cultivation over time, maximum lipid content 34% of the cell dry weight was obtained on the day 5 and 6. However, interestingly polyunsaturated fatty acid (PUFA), omega-3 fatty acid (n-3) and omega-6 fatty acid (n-6) compositions on day 6 is higher than day 5. Through analyzing of total lipid compositions and TAG analysis, the most abundant fatty acids are oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2), which are useful precursors for conversion to biofuel production.
A higher n-6/n-3 ratio is observed during total lipid and triglyceride analysis, however, a lower ratio is advantageous in order to avoid negative impacts on human or animal health, if the oil is used for food or feed. The potentialities of lipids accumulation on shorter culture time and culture conditions are the key elements for the success of scale-up and profitability bioprocess.

Keywords: *Rhodotorula toruloides,* Fatty acids, Lipid class, Triglycerides, Phospholipid, Gas Chromatography.

Popular-scientific summary

Lipid content and profile of *Rhodotorula toruloides* during fermentation

When yeasts are able to accumulate lipids greater than 20 % of their cell dry weight then they are generally regarded as the oleaginous yeasts. The most of the interest of oleaginous yeast is that they often store gas prodcutionexcess carbon as triglycerides (TAGs). The main lipid classes are TAGs and sterol esters (SEs) which produced by the oleaginous yeasts. Triglycerides are the form of fat energy which is present as storage lipids in yeasts and Phospholipids (PL) are the key component of yeast cell membranes.

Among many oleaginous types of yeast, *Rhodotorula toruloides* may accumulate lipids to levels exceeding 70 % of their cell dry weight. *R. toruloides* yeast might be utilized in various industrial markets such as biofuels production, carotenoids, enzyme production and biosurfactants (Lyman *et al*., 2019). It is an important economic consideration of the ability to produce lipids from low cost substrates such as hemicellulosic hydrolysates and raw glycerol for successfully commercial production of lipids. This yeast can be cultivated efficiently on low cost carbon sources such as lignocellulose. Some vegetable oils (VO) such as palm and soybean oil have a high greenhouse effect with their production. Microbial oils or microbial lipids are mainly TAGs and they can replace traditional vegetable oils and can obtain without arable land requirement. Microbial lipids have potential applications in biodiesel production and even for food purposes, aside from oil; also proteins can be used in aquaculture and poultry industry. Moreover, *R. toruloides* is a natural producer of carotenoids and they are used as nutritional supplements, dye in dietary supplements, pharmaceuticals and cosmetic industries.

Yeasts are capable of synthesizing the fatty acids species which may be saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), depending on the presence and number of double bonds in hydrocarbonated chains. This study has to investigate lipids synthesized by using *Rhodotorula toruloides* yeast produced on lignocellulose medium in batch cultivation and fatty acids analysis and lipid class separation by using gas chromatography (GC) and thin layer chromatography (TLC), respectively. *R. toruloides* preculture has grown on to lignocellulose medium in batch cultivation in a fermenter and the cell has harvested at day0 up to day6 and freeze dried until lipid extraction for fatty acids analysis.

The present study showed that the lipids are increasing over time and lipid percentage reached 34 % on day, day5 and day6. Lipid composition has analyzed at over time between day0 to day6 using gas chromatography and the fatty acids proportions of oleic acid (18:1n-9), palmitic acid (16:0) and linoleic acid (18:2n-6) are the first, second and third highest respectively. In general, MUFA is decreasing over time and PUFA is increasing after day2 to day6. TAG and phospholipids (PL) have been separated and run through gas chromatography to understand the changes of fatty acids profiles and lipid classes. The degree of fatty acids proportions are observed the same as total lipid analysis and linolenic acid (C18:3n-3) is lower and ratio of linoleic acid (C18:2n-6)/linolenic acid (C18:3n-3) is higher in the triglyceride compare to phospholipid. A low n-6/n-3 (omega-6/omega-3 fatty acids) ratio is advantageous to avoid negative effects on health. Lipid classes have been evaluated through thin layer

chromatography to understand the fate of individual fatty acids. TAG is the highest portion and a high Tag/PL ratio is observed during the lipid class composition analysis, which suggested that the lipids are stored in giant lipid droplets rather than the cell membrane. To recognize yeast strain and optimization of fermentation conditions are the major topic of ongoing research that has a possibility to positively influence the fatty acids compositions and also the n-6/n-3 ratio.

Table of contents

List of tables……..	8
List of figures……	8
Abbreviations……	9
Introduction……...	10-12
Materials and Methods…….	13-14
Results.……...	15-18
Discussion...……...	19-20
Conclusion……...	21
References……	22-26
Acknowledgements……..	26

List of Tables

Table 1 Result overview of the *Rhodotorula toruloides* lipid weight (n=2) with lipid yield (mg) & lipid percentage from day 0 to day 6	15
Table 2. The proportion (%) of individual fatty acids (n=2) for *R. toruloides* during day 0 to day 6	16
Table 3. Fatty acid composition (%) of TAG (n=2) for *R. toruloides* during day 0 to day 6	16
Table 4. Fatty acids composition (%) of PL (n=2) for *R. toruloides* during day 0 to day 6	17

List of Figures

Figure 1. A general molecular structure of triacylglycerol (TAG) and sterol esters (SE)	10
Figure 2. Illustration of the intracellular lipid accumulation in *Rhodotorula toruloides* over time monitored using a microscope	11
Figure 3. Individual fatty acids composition in total lipids for *R. toruloides* during day 0 to day 6 with SFA, MUFA, PUFA, n-3, n-6 & n-6/n-3	15
Figure 4. Lipid class composition of total lipids from *R. toruloides* using TLC with PL, MAG, 1,2DAG, Sterols, FFA & TAG	18

Abbreviations

FA	Fatty acids
PL	Phospholipid
CDW	Cell dry weight
SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
SE	Sterol esters
TAG	Triglyceride
n-3	Omega-3
n-6	Omega-6
n-6/n-3	n-6/n-3 PUFA
MAG	Monoglyceride
1,2DAG	1,2-Diglyceride
C14:0	Myristic acid
C16:0	Palmitic acid
C16:1(n-7)	Palmitoleic acid
C17:1	Heptadecenoic acid
C18:0	Stearic acid
C18:1(n-9)	Oleic acid
C18:2(n-6)	Linoleic acid
C18:3(n-3)	Linolenic acid
C20:0	Arachidic acid
C22:0	Behenic acid
C24:1	Nervonic acid
C22:4(n-6)	Adrenic acid

Introduction

Many oleaginous microorganisms such as bacteria, fungi and microalgae are used as lipid producers, the oleaginous yeasts as lipid producers have potential advantages over many other microorganisms because of their more rapid than others growth, high cell biomass, high content of lipid and no endotoxin production (Wang et al., 2012). For instance, their duplication time is short (<1h), they can obtain high cell biomass in the culture (>20 g/l) and high lipid content (>50 % based on their cell dry weight), they can be easily modified by genetic engineering and use an extended range of substrates and a large-scale fermentation using them can be easily executed (Manuel et al., 2011 & Sitepu et al., 2014).

Lipids are dominant as the energy storage substance and prerequisite for cell integrity as the cell membrane and all membranes of organelles are mainly formed by lipids. Lipids contain many classes such as triglycerides, free fatty acids, hydrocarbons, sterols and phospholipids (Akpina-Bayizit, 2014). However, the main lipid classes produced by the oleaginous yeasts are triglycerides (TAGs) and sterol esters (SEs) (Figure 1) and all the TAGs and SEs are accumulated in the lipid particles in the oleaginous yeast cells (Figure 2) (Wang et al., 2015). Microbial lipids are also termed as microbial oils which are mainly made up of triglycerides and they have many potential biotechnological applications (Ochsenreither et al., 2016).

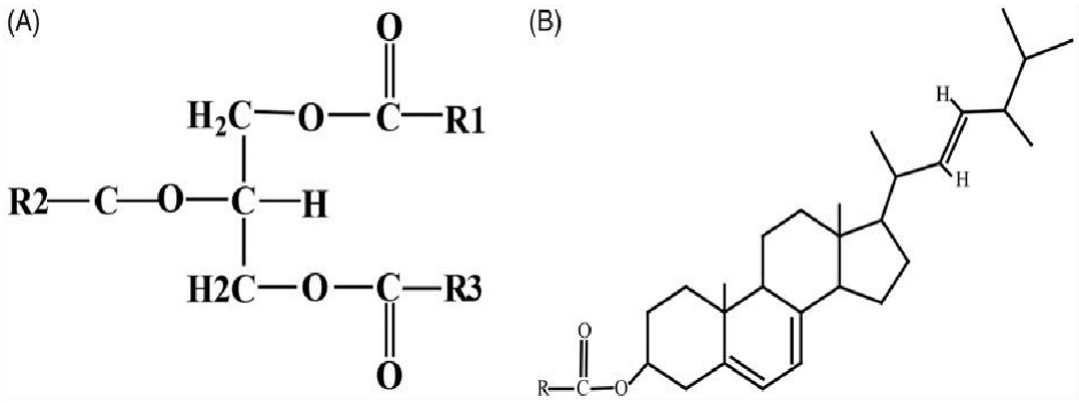


Fig 1. A general molecular structure of triglyceride (TAG) (R1, R2 & R3 are individual fatty acid moieties) (A) and a general molecular structure of sterol ester (SE) (R is a fatty acid moiety) (B).

Oleaginous microorganisms are defined as cells which can accumulate more than 20 % lipids of their dry biomass, are promising cell factories with great potential for biodiesel production (Passoth, 2014; Ratledge and Wynn, 2002). They are able to accumulate over 40 % and up to 70 % of its dry biomass in lipids and can be grown to high cell densities (Garay et al., 2016 & Li et al., 2007). The capability of accumulating lipids is attracting growing attention, as they can be used in a wide range of biotechnological applications, such as, to generate biofuels, chemicals or food and feed additives. Several important chemicals for instance, n-3 fatty acids, fatty alcohols, alkanes or carotenes can be derived from

the lipid metabolism (Sitepu et al., 2014; Mata-Gomez et al., 2014; Ochsenreither et al., 2016; Passoth, 2017).

Fig 2. Illustration of the intracellular lipid accumulation in *Rhodotorula toruloides* over time monitored using a microscope. Photo by Jule Brandeburg.

Microbial lipids are one fatty acid source that is also considered to be a potential feedstock for oleochemical production (Unrean et al., 2017). The most promising microbes for biofuel production are oleaginous yeast, characterized by their significant accumulation of fatty acid in the form of triglycerides and its useful precursors for conversion to biodiesel, green diesel and jet fuel (Kruger et al., 2018 and Nogue et al., 2018). The ability to produce lipids and high value co-products from a low cost substrate is an important economic consideration when producing biodiesel from microbial oils (Koutinas et al., 2014). The potential low-cost substrates for microbial oil production are hemicellulosic hydrolysates and raw glycerol. In aquaculture it is possible to replace vegetable oil by yeast oil produced from lignocellulose. Single cell oils or microbial oils have the capability as an inherent replacement for vegetable oil in biodiesel production and even for food purposes (Passoth, 2017; Ochsenreither et al., 2016; Sitepu et al., 2014).

The yeast *Rhodotorula toruloides*, formerly known as *Rhodosporidium toruloides* is an oleaginous microorganism that has recently been considered a workhorse for biotechnological applications. It has been found to be an exceptional storage lipid producer due to high cellular lipid content of over 70 % and high cell density of 100 g/L (Freitas et al., 2014 & Zhao et al., 2010). Also, *R. toruloides* is a natural producer of industrially high-value compounds for instance carotenoids (Park et al., 2017). These can be cultivated efficiently on low-cost carbon sources such as lignocellulose (Brandenburg et al., 2018; Passoth & sandgren, 2019). Interest in carotenoids has increased substantially due to the benefits to human health and also to animal feeds, caused by the growth of certain areas such as agriculture, especially aquaculture and poultry industry (Johnson & Schroeder, 1995; Britton & Hornero-Mendez, 1997). Moreover, they are used as nutritional supplements (Fraser & Bramley, 2004), dye and as an ingredient sun tanning creams in the pharmaceutical and cosmetic industries (Kort et al., 2016 & Jomova & valko, 2013). In addition, this strain is capable of metabolizing various substrates (Papanikolaou and Aggelis, 2011), including non-detoxified lignocellulosic hydrolysates (Bonturi et al., 2017).

Vegetable oils (VO) are known causing large environmental impacts and are also regarded as the fastest growing food commodities worldwide (Khatri & Jain, 2017). Some vegetable oils have a high greenhouse effect associated with their production, such as palm and soybean oil. They are assessed to emit more than 2000 kg CO_2 equivalents per ton produced and considerable areas of arable land are used for producing vegetable oils (Schmidt, 2015). It is possible to convert lignocellulose to a feed component, which can be utilised as a replacement of most vegetable oil as an energy source in e.g. aquaculture (Blomqvist et al.,

2018). Microbial lipids have potential applications in biodiesel production because most of those lipids are triglycerides with long chain fatty acids that can replace conventional vegetable oils and can be obtained without arable land requirement (Li et al. 2008; Adamczak et al. 2009 & Vicente et al. 2010). Furthermore, the yeast cells contain, aside from oil, also proteins and other components that can be made use of the fish, which was described by (Pettersson et al., 2009). Blomqvist et al., 2018 demonstrated that it is possible to replace VO and partially replace protein (casein in the control feed) with the yeast biomass, which has no significant effects on fish growth and final quality, as shown in other studies on utilizing yeasts as a partial protein source in fish feed (Huyben et al., 2017 & Nasseri et al., 2011). Also, several studies focused on the use of yeast proteins as an alternative for fish meal due to the high amount of crude protein and good production rate (Sanderson and Jolly, 1994; Tacon, 1994; Ferreira et al., 2010). However, some previous studies have shown that there is a limit to involve yeast based protein into fish diets (Huyben et al., 2017 & Hatlen et al., 2012).

Fatty acid composition is species as well as tissue specific. The most abundant fatty acids in yeast cells from different species are palmitoleic acid (16:1) and oleic acid (18:1) followed by palmitic acid (16:0), stearic acid (18:0) with minor amount of myristic acid (14:0) are also found in yeast (Tuller et al., 1999; Tehlivets et al., 2007). Besides they are capable of synthesizing vast majority of fatty acids from short hydrocarbonated chain (C6) to long hydrocarbonated chain (C36), which may be saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), depending on the presence and number of double bonds in hydrocarbonated chains.

This present study has investigated yeast oil extracted from *R. toruloides* to study variation of lipid content and lipid classes as well as fatty acids in the fermentation process during six days of fermentation. Typically, lipid class separation can be done by TLC, SPE or HPLC. Lipids have been analyzed by gas chromatography (GC) and this technique is used to identify and quantify the composition of fatty acids (Browning et al., 2012 & Cao et al., 2006). This usually requires fatty acids to be derivatized in order to get sufficiently volatile to be eluted at reasonable temperatures without thermal decomposition. GC involves the substitution of a functional group containing hydrogen to form esters or thioesters for analysis and methyl esters are produced by methylation. After methylation the ester bonds in complex lipids are hydrolyzed to release free fatty acids, which are becoming to form fatty acid methyl esters (FAME). FAME profile is recognized by GC and referred to as fatty acid composition also may be easily compared between different experimental groups (Burdge et al., 2000). GC allows both the proportions of individual fatty acids and their amounts to be measured. Moreover, the use of GC for analyzing fatty acids in nutrition studies within the food industry and also across a wide range of analytical fields.

The aim of the study is to investigate lipids synthesized by yeast produced in batch cultivation on lignocellulose hydrolysate from wheat straw using the *Rhodotorula toruloides*.

Materials and Methods

Strains and cell cultivation

Rhodotorula toruloides was maintained on Yeast Extract-Peptone-Dextrose (YPD) - agar plates (glucose 20 g/L, peptone 20 g/l, yeast extract 10 g/L and agar 15 g/L) at 25 °C for 48 to 72 hours. A 50 ml pre-culture was prepared in YPD medium and incubated at 25 °C for 48 to 72 hours with a shaking speed of 150 rpm. The pre-culture was transferred to lignocellulose medium at ratio of 1:9 (1part pre-culture: 9 part medium) and incubated at 25 °C for 6 days at 150 rpm with pH 6.0. The cells were harvested at day 0 up to day 6 and freeze dried until lipid extraction. To utilize the time harvested *R. toruloides* was provided for lipid extraction.

Lipid extraction from *R. toruloides*

The total lipid analysis from yeast cells was performed according to Folch et al. 1957 with minor modifications. Lipids were extracted six samples from one fermenter were (six consecutive days day 0-day 6) used throughout this study. In this study, 25 mg freeze dried cells were used and soaked with 1 ml 1M HCL for 15 min. Afterwards, heating block was used for vortexing for 1 hr at 75 °C. Next, 1 ml KCL (0.8 %) and 3 ml Folch solution (2:1; chloroform: methanol) were added and vortexed, respectively. The sample was centrifuged at 2000 rpm for 5 min and supernatant was transferred to a fresh second tube. To ensure a high yield as possible, a second extraction was performed by adding additional 2 ml chloroform to the second tube. This preparation was vortexed for 5 min and centrifuged as mentioned above and the chloroform layer was again transferred to the first tube. Lipids were transferred to pre weight tube and evaporated under N2 gas and the lipids were weighed. The lipids were stored in 1ml hexane and frozen at -20 °C until further use.

Preparation of FAME

Fatty acid methyl esters (FAME) from total lipids in yeast were conducted with BF_3 methanol according to Appelqvist, 1968. Briefly, 2 mg lipid was used in 0.5 ml hexane, 2 ml dry methanol added with lipid and vortexed and incubated (60 °C) for 10min. Then, 3 ml BF_3 reagent was added and the samples were vortexed and incubated at 60 °C for a further 10 min. Afterwards, the samples were cooled to room temperature under running tap water and 2 ml 20 % NaCl and 2 ml hexane were added. The samples were vortexed and kept in the fridge +4 °C for separation for 20-30 minutes. The upper layer was transferred to a second small test tube and 1 ml hexane was added followed by repetition of the previous step and evaporated under N_2 gas. The dried FAMEs were dissolved in hexane (TL 300 µl, PL 50 µl, TAG 250 µl) and stored at -20 °C until analysis of fatty acid composition.

Lipid class composition

The desired lipid concentration was 1 µg/µl for the separation of lipid classes, which was performed according to Olsen and Henderson, 1989. To execute, 5 µg samples were applied with duplication and standard 18-5 and 18-6 (Nu-Chek) on TLC plate. Next, 10 ml mobile phase (85 ml hexane; 15 ml diethyl ether; 2 ml acetic acid) was used in automatic developing chamber and 25 ml for saturation.

Preparation of TAG and PL extraction

In this study, 2 mg lipid was used to extract triglyceride (TAG) and phospholipid (PL). For extraction, lipids were run on TLC plate and the plate was (covered with glass plate) developed in iodine vapour. Next, the PL and TAG area were scraped and put in a separate glass tube. Three type of solutions were prepared, solution 1- PL (methanol: chloroform-2:1), TAG (methanol: chloroform-1:2); solution 2- PL (methanol: chloroform-1:1), TAG (chloroform); solution 3 (chloroform). Next, 2 ml solution 1 was added with TAG and PL and kept it in the fridge for 1hr, after that transferred the solvent to second tube and followed the same procedure with solution 2 and 3 respectively. Finally, the sample was evaporated under N2 gas and hexane was added to dry samples and stored in a freezer at -20 °C and go for methylation further.

Check on TLC before GC

To check the FAME on TLC, 5 µl samples and standards 18-4A and 18-5 (Nu-Chek) were used on silica plate and the plate was placed in the TLC chamber (85 ml hexane; 15 ml diethyl ether; 2 ml acetic acid) for 1 hour and developed the silica plate by using iodine vapor. Then, the TLC plate was air dried and dipped into cupric acetate (3 % cupric acetate in 8 % phosphoric acid) solution and developed in the oven for 20 min at 140 °C. The detection of lipid classes in TLC plate was scanned by a TLC plate scanner (CAMAG TLC scanner 3, Switzerland), which was quantified by absorption or fluorescent signal.

Analysis using the Gas Chromatograph (GC)

FAME were analyzed by GC using a CP 3800 instrument (Varian AB, Stockholm, Sweden) equipped with a flame ionization detector, a split injector and separated on a 50 m fused silica capillary column BPX 70 (SGE, Austin, Tex) (0.22 mm i.d x 0.25 µm film thickness). The injector and detector temperature were 230 °C and 250 °C, respectively. Helium was the carrier gas (flow rate of 0.8 mL/min) and nitrogen was used as a make-up gas. Chromatograph peaks were identified by comparing their retention times with standard mixture GLC 68A (Nu-check Prep, Elysian, USA). Finally, peak areas were integrated using Galaxie chromatography data system software version 1.9 (Varian AB, Stockholm, Sweden).

Results

After harvesting *Rhodotorula toruloides* over time, lipids were extracted from the cells and taken for further analysis. All analyses were made in duplicate and then all the data presented are duplicate mean values of experiment.

According to Table 1, lipid weight is increasing over time but day 2 and 3 lipid percentage is stable as well as day 5 and 6.

Table 1. Result overview of the *Rhodotorula toruloides* lipid weight (n=2) with lipid yield (mg) & lipid percentage from day 0 to day 6.

	Yeast weight (mg)	Lipid yield (mg)	Percentage%
Day 0	25	1	4
Day 1	25	2	8
Day 2	25	7	28
Day 3	25	7	28
Day 4	25	8	32
Day 5	25	8.5	34
Day 6	25	8.5	34

This study aimed to investigate the development of fat metabolism over the time. One of the most investigated techniques for total lipid separation is GC. After methylation FAME were run through GC.

Figure 3, shows that SFA accumulation is the highest on day 4 and PUFA is the lowest on day 2 and again it is increasing from day 3. It is notable that MUFA is gradually decreasing over time. Omega 3 and omega 6 fatty acids are the lowest on day 2 and later it is increasing again from day 3.

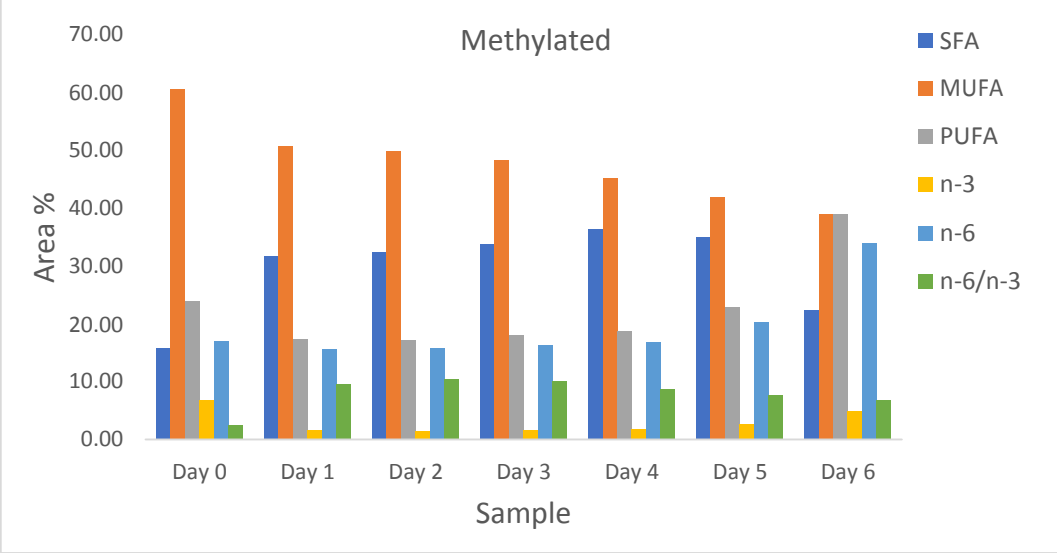


Fig 3. Individual fatty acids composition in total lipids for *R. toruloides* during day 0 to day 6 with SFA, MUFA, PUFA, n-3, n-6 & n-6/n-3.
Abbreviations: SFA- Saturated fatty acid, MUFA- Monounsaturated fatty acid, PUFA- Polyunsaturated fatty acid, n-3 - Omega-3, n-6 -Omega-6.

Lipid composition was analyzed at over time between day 0 to day 6. Among all the fatty acids the proportion of oleic acid (C18:1n-9) is the highest and the palmitic acid (C16:0) and linoleic acid (C18:2n-6) is the second and third highest, respectively.

Table 2. The proportion (%) of individual fatty acids (n=2) for *R. toruloides* during day 0 to day 6.

	Day 0 mean	Day 1 mean	Day 2 mean	Day 3 Mean	Day 4 mean	Day 5 Mean	Day 6 Mean
C14:0	0.35	1.09	1.07	1.16	1.28	1.35	1.00
C16:0	12.9	24.6	25.0	25.7	27.0	25.7	17.9
C16:1(n-7)	1.78	0.95	0.98	1.07	0.86	0.94	1.26
C17:1	1.64	0.00	0.00	0.00	0.00	0.00	0.00
C18:0	2.47	5.86	6.24	6.84	7.97	7.88	3.45
C18:1(n-9)	56.9	49.6	48.7	47.0	44.2	40.9	37.5
C18:2(n-6)	15.9	14.6	14.4	15.3	15.7	18.9	32.6
C18:3(n-3)	6.85	1.64	1.50	1.63	1.92	2.66	4.98
C22:4(n-6)	0.97	1.03	1.25	0.94	1.01	1.32	1.15

Triglyceride and phospholipid classes were separated to understand the changes of fatty acids profiles and lipid classes. Triglycerides are storage fat, according to table 3, in TAG, MUFA accumulation is higher than PUFA, during the fermentation process. It also shows that MUFA is higher concentration during beginning of the culture and day 2 MUFA shows the lowest and again increasing and almost stable on day 5 and day 6. On the other hand, PUFA is decreasing over time.

Table 3. Fatty acid composition (%) of TAG (n=2) for *R. toruloides* during day 0 to day 6.

FA	Day0	Day1	Day2	Day3	Day4	Day5	Day6
C14:0			1.57	1.46	1.21	1.25	1.16
C16:0	21.9	27.6	29.0	28.0	26.3	25.2	24.7
C16:1(n-7)			0.92	0.54	0.98	0.98	0.98
C18:0		8.1	8.98	8.53	7.02	6.24	5.89
C18:1(n-9)	89.0	44.0	42.37	44.8	48.4	50.3	51.1
C18:2(n-6)		19.6	14.4	13.0	12.8	12.9	12.6
C18:3(n-3)			1.55	1.44	1.23	0.68	1.22
C22:0				0.51	0.52	0.54	0.41
C22:4(n-6)			1.45	1.09	1.14	1.14	1
SFA	21.9	35.7	39.6	38.5	35.0	33.2	32.4
MUFA	89.0	44.6	43.2	45.4	49.3	51.3	52.1
PUFA		19.6	17.4	15.6	15.2	14.7	14.8
n-3			1.54	1.44	1.23	0.68	1.22
n-6		19.6	15.9	14.1	13.9	14.0	13.6
n-6/n-3			10.3	9.84	11.3	20.8	11.2

Abbreviations: See under figure 3, TAG- Triglyceride.

Based on the phospholipids analysis, table 4 shows the MUFA and PUFA proportions are similar over time. Here, the fatty acids concentrations were too low for day 0, day 1 and day 2 and to recover these individual day fatty acids incorporated together to run for GC. Besides, day 5 fatty acids compositions were excluded because of non-reliable data compare to other days fatty acids compositions.

Table 4. Fatty acid composition (%) of PL (n=2) for *R. toruloides* during day 0 to day 6.

FA	Day 0+1+2	Day 3	Day 4	Day 6
C16:0	16.6	17.2	9.79	15.9
C18:0	7.54	7.32	3.34	9.26
C18:1(n-9)	39.4	35.7	21.4	37.1
C18:2(n-6)	31.4	33.1	13.7	34.0
C18:3(n-3)	4.99	5.74	1.9	3.57
C24:1			5.25	
SFA	24.1	24.5	13.1	25.2
MUFA	39.4	35.7	26.7	37.1
PUFA	36.4	38.8	15.6	37.6
n-6/n-3	6.30	5.77	7.21	9.54

Abbreviations: See under figure 3, PL- Phospholipid.

By using thin layer chromatography (TLC), the lipid classes were looked on after extraction. Lipid classes graph shows that phospholipid (PL) and free fatty acid (FFA) volume is decreasing over time except day 4 for PL. It also shows that monoglyceride (MAG), 1,2 diglyceride (1,2 DAG) and sterols are decreasing compare to day 1 to day 6, however they are slightly fluctuated over time. The storage fat, TAG is the highest portion of the lipid class composition. In addition, a high TAG/PL ratio was observed in the lipid class analysis.

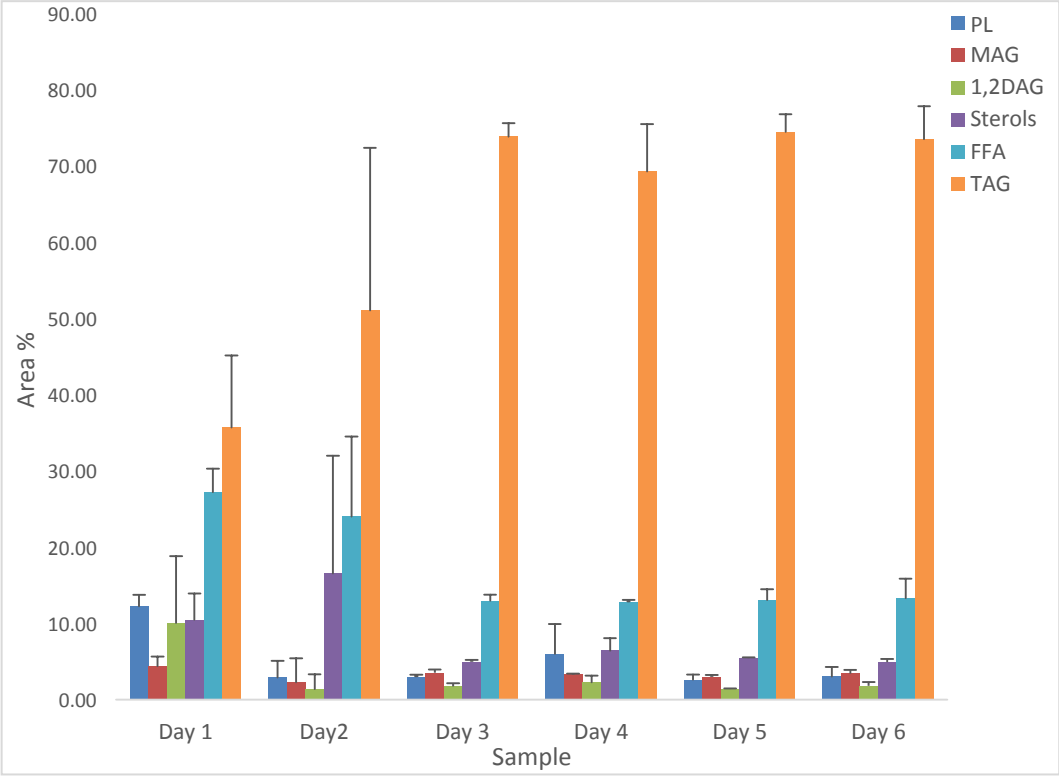


Fig 4. Lipid class composition of total lipids from *R. toruloides* using TLC with PL, MAG, 1,2DAG, Sterols, FFA & TAG.

Abbreviations: PL- Phospholipid, MAG- Monoglyceride, 1,2DAG- 1,2-Diglyceride, FFA- Free fatty acids, TAG- Triglyceride.

Discussion

Yeast *R. toruloides* was grown on wheat straw hydrolysate and the lipid accumulation, fatty acid composition and lipid classes were analyzed during cultivation time.

This present study shows that the lipid accumulation is increasing and reaching 34 % on the day 5 and day 6 over time. This result is similar to the data obtained from another fermentation run, where the lipid content was estimated to be 42.7 % after 96 hours of inoculation (Unpublished data, Nagaraj Y.N., 2021). According to Brandenburg et al. (2016), during cultivation time with *Lipomyces starkeyi* the lipid content of the cell dry weight (CDW) increased, reaching 51.3 % in the culture fed with hydrolysate. In addition, rapid and efficient lipid production from the substrate can greatly improve the overall energy output and greenhouse gas impacts of any single cell oil production process (Karlsson et al., 2016 & Karlsson et al., 2017), and therefore, optimization of fermentation conditions and strains for lipid production are major topics of ongoing research.

Lipid accumulation reached the highest during the batch cultivation phase on day 5 and 6 but interestingly, PUFA, n-3 fatty acid and n-6 fatty acid compositions on day 6 is higher than day 5 (Figure 3). PUFA is increasing over time to day 6, the reason behind it might be the yeasts were in aerobic stress in fermenter during cultivation and after 96 hours the sugars were still left in the medium, a reason to why they were not using TAGs as nutrients for growth. The n-6/n-3 ratio reached 8.73 % after 96 hours of inoculation, as similar data obtained from another study where n-6/n-3 ratio reached 5.96 % after 96 hours inoculation of *R. toruloides* (Unpublished data, Nagaraj Y.N., 2021). The major fatty acids are oleic acid (C18:1n-9), palmitic acid (C16:0) and linoleic acid (C18:2n-6), which together represent 70-90 % of all fatty acids (Table 2). Similar result was observed under lipid extracted from *R. toruloides* and the predominant fatty acids were palmitic (C16:0), oleic (C18:1n-9) and linoleic acid (C18:2n-6), with over 90 % of detected fatty acids by Guerreiro et al., 2018. In addition, the fatty acid compositions of yeast species are very similar (Li et al., 2007; Yen et al., 2015; Wang et al., 2014 & Sitepu et al., 2013), except for *R. toruloides* NCYC 921 (Freitas et al., 2014), that presents a high value (71.3 % w/w) of stearic acid. Brandenburg et al., 2016 carried out the experiment by using *Lipomyces starkeyi* and found that the palmitic acid (C16:0) and oleic acid (C18:1n-9), which comprised 70-85 % of all fatty acids. In this study, the composition of fatty acids implies to be promising for biodiesel production, since the most suitable fatty acids for biodiesel production are palmitic (C16:0), oleic (C18:1n-9) and linoleic acid (C18:2n-6), as also mentioned by (Guerreiro et al., 2018). In general, the degree of saturation of the fatty acids increased during batch cultivation phase, which is beneficial for using the yeast oil for biodiesel production (Knothe, 2005). In addition, the individual fatty acid composition of various yeasts varies with both strain and cultivation conditions (Olstorpe et al., 2014; Brandenburg et al., 2016 and Brandenburg et al., 2018).

Triglycerides are the form in which fat energy is stored in adipose tissue and they are present as storage lipids in fungi and yeast (Sandhir R. 2014). In general, the degree of saturated fatty acids (SFA) and unsaturated fatty acids (MUFA,

PUFA) reaches the highest and almost steady phase on day 5 and day 6. The fatty acids proportions are oleic acid (C18:1n-9), palmitic acid (C16:0) and linoleic acid (C18:2n-6) are the first, second and third highest respectively, observed same as total lipid analysis. Moreover, during the batch phase a higher ratio of linoleic acid (C18:2) and linolenic acid (C18:3) was also observed. Some of studies have shown that there should be a balanced relation between n-6 and n-3 fatty acids in healthy diets. It has been established that the n-6 fatty acid and the n-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) collectively protect against coronary heart disease and the balance n-6/n-3 ratio should be ~6:1 (Wijendran and Hayes 2004). They have been described to have positive characteristics for human nutrition and it would be interesting to identify appropriate cultivation conditions over time under *R. toruloides* accumulates these fatty acids. According to (Olstorpe et al., 2014 & Rossi et al., 2009), formation of linoleic and linolenic acid has been mentioned in mesophilic yeasts at low temperature, however is rather low or absent at this cultivation temperature. Selecting appropriate yeast strains and culture conditions may thus represent a possibility to positively influence the fatty acid composition and thereby the n-6/n-3 ratio (Blomqvist et al., 2018).

Among the comparison between TAG and PL composition based on the table 3 and 4 it shows that linolenic acid (C18:3n-3) is lower and fraction of linoleic acid (C18:2n-6)/linolenic acid (C18:3n-3) is higher in the storage lipid compare to phospholipid. A low n-6/n-3 ratio is advantageous but in the most modern diet, this ratio is too high, leading to a variety of diseases (Simopoulos 2006). Besides, the n-6/n-3 ratio is usually very high in modern diets and this should be reduced to avoid negative effects on health (Simopoulos 1999 & Bonacic et al., 2016). This study had limitations in amount of lipids produced in the fermentation process. Therefore more trials have to be performed. Especially the difference in fatty acid composition of lipid classes depending on fermentation time is of interest. We are therefore placing more research based on the results obtained in the present study.

Lipid classes are very important to understand the fate of individual fatty acid. In this study, yeast was taken for lipid class profiling to investigate whether the distribution of lipid classes changed during the cultivation. Free fatty acid (FFA) observed as the highest proportion in the beginning of batch culture and they are downloaded somewhere and make new energy, which is going to be stored as a storage fat. Consequently, the storage fat TAG is significantly increasing however there are large standard deviations of some individual lipid classes. Phospholipid proportions decreased after day 1 and remained almost unchanged during day 2 to day 6, except day 4, which was reflected by a pronounced increase in TAG/PL ratio compared to *S. cerevisiae* (Ejsing et al., 2009) or *Y. lipolytica* (Kerkhoven et al., 2016). According to Tiukova et al., 2019, the high TAG/PL ratio in *R. toruloides* suggested that the accumulated neutral lipids are stored in giant lipid droplets rather than the cell membrane.

Conclusion

The fatty acid compositions from the total lipid analysis over time (day 0 to day 6) depend on the *Rhodotorula* strain and culture conditions. Lipid accumulation from low cost substrates in batch cultivation can be used for the microbial oil production. This study showed that the fatty acid compositions using *R. toruloides* over time, the dominating fatty acids are oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2) in total lipid and TAG, which showed a great potential as raw material for biodiesel production. In this study during cultivation of *R. touloides* the fatty acid compositions in total lipid analysis found that the saturated fatty acids gradually decreased after 96 hours and polyunsaturated fatty acids gradually increased after 48 hours over time. Time may influence the production of long chain monounsaturated fatty acids and polyunsaturated fatty acids in *R. toruloides* which was observed during fatty acid compositions in total lipid, TAG and lipid class analysis. However, the experiments have to be redone to confirm this. Different conditions in the fermentation process can cause alterations of the lipid and fatty acid profiles. Lipids produced from this type of lignocellulose have a potential as oils in several applications in industry, fuels, chemicals, foods and feeds. It seems as a possibility of decreasing CO_2 emissions in cultures of oil crops globally. Further research should be done to examine the culture conditions that might represent a possibility to positively influence the fatty acid composition and evaluate accumulation of storage fat, linoleic and linolenic acid over time.

References

Adamczak, M., Bornscheuer, U. T., Bednarski, W. (2009). The application of biotechnological methods for the synthesis of biodiesel. *Eur. J. Lipid Sci. Technol.* 111: 800-813.

Akpina-Bayizit, A. (2014). Fungal lipids: the biochemistry of lipid accumulation. Int J Chem Eng Appl 5:409–414.

Blomqvist, J., Pickova, J., Tilami, S. K., Samples, S., Mikkelsen, N., Brandenburg, J., Sandgren, M., Passoth, V. (2018). Oleaginous yeast as a component in fish feed. Sci Rep 8, 15945.

Bonacic, K., Estévez, A., Bellot, O. & Conde-Sieira, M. (2016). Dietary Fatty Acid Metabolism is Affected More by Lipid Level than Source in Senegalese Sole Juveniles: Interactions for Optimal Dietary Formulation. *Lipids,* 51(1), pp. 105-122.

Bonturi N, Crucello A, Viana AJC, Miranda EA (2017) Microbial oil production in sugarcane bagasse hemicellulosic hydrolysate without nutrient supplementation by a Rhodosporidium toruloides adapted strain. Process Biochem 57:16–25.

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

Brandenburg, J., Blomqvist J., Pickova J., Bonturi N., Sandgren M., Passoth V. (2016). Lipid production from hemicellulose with Lipomyces starkeyi in a pH regulated fed-batch cultivation. Yeast (Chichester, England). 33(8): 451-462.

Britton G, Hornero-Méndez D (1997). Carotenoids and colour in fruit and vegetables. In Phytochemistry of fruit and vegetables. Edited by Tomás-Barberán FA, Robins RJ. Oxford: Clarendon Press: 11–27.

Browning, L.M. et al., (2012). Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. Am. J. Clin. Nutr. 96(4), 748-758.

Burdge, G.C., Wright, P., Jones, A.E. & Wootton, S.A. (2000). A method for separation of phosphatidylcholine, triacyglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. Br. J. Nutr. 84, (5) 781-787.

Cao, J., Schwichtenberg, K.A., Hanson, N.Q. & Tsai, M.Y. (2006). Incorporation and clearance of omega-3 fatty acids in erythrocyte membranes and plasma phospholipids. Clin. Chem. 52(12), 2265-2272.

Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, et al. (2009). Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. Proc Natl Acad Sci USA.106(7):2136–41.

Ferreira, I.M.P.L.V.O., Pinho, O., Vieira, E. Tavarela J.G., (2010). Brewer's Saccharomyces yeast biomass: char-acteristics and potential applications. Trends Food Sci Techno. 21: 77–84.

Folch J., Less M., Stanley G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226: 497-509.

Fraser PF, Bramley PM (2004). The biosynthesis and nutritional uses of carotenoids. Prog Lipid Res. 43:228–265.

Freitas, C., Nobre, B., Gouveia, L., Roseiro, J. et al., (2014). New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth. *Process Biochem*. 49: 554-562.

Freitas, C., Parreira, T. M., Roseiro, J., Reis, A. et al., (2014). Selecting low-cost carbon sources for carotenoid and lipid production by the pink yeast *Rhodosporidium toruloides* NCYC 921 using flow cytometry. *Bioresour. Technol.*158: 355-359.

Garay LA, Sitepu IR, Cajka T, et al., (2016). Eighteen new oleaginous yeast species. J Ind Microbiol Biotechnol. 43:887–900.

Guerreiro, F., Constantino, A., Lima-Costa, E., Raposo, S. (2018). A new combined approach to improved lipid production using a strictly aerobic and oleaginous yeast. Eng Life Sci. 19: 47-56.

Hatlen, B., Berge, G. M., Odom, J. M., Mundheim, H. & Ruyter, B. (2012). Growth performance, feed utilisation and fatty acid deposition in Atlantic salmon, *Salmo salar* L., fed graded levels of high-lipid/high-EPA *Yarrowia lipolytica* biomass. Aquaculture. 364: 39–47.

Huyben, D. et al. (2017). Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. Aquaculture. 473: 528–537.

Iassonova DR, Hammond EG, Beattie SE (2008). Oxidative stability of polyunsaturated triacylglycerols encapsulated in oleaginous yeast. J Am Oil Chem Soc 85:711–716.

Johnson EA, Schroeder WA (1995). Microbial carotenoids. In A Fiecher. Volume 53. Edited by Springer-Verlage. Heidelberg: Adv Biochl Eng Biotech: 119–178.

Jomova, K. & Valko, M. (2013). Health protective effects of carotenoids and their iteractions with other biological antioxidants. European journal of medicinal chemistry. 70: 102-110.

Karlsson, H. et al. (2016). A systems analysis of biodiesel production from wheat straw using oleaginous yeast: process design, mass and energy balances. Biotechnol Biofuels 9, 229.

Karlsson, H. et al. (2017). Greenhouse gas performance of biochemical biodiesel production from straw: soil organic carbon changes and time-dependent climate impact. Biotechnol Biofuels 10, 217.

Kerkhoven EJ, Pomraning KR, Baker SE, Nielsen J. (2016). Regulation of amino-acid metabolism controls flux to lipid accumulation in *Yarrowia lipolytica*. NPJ Syst Biol Appl. 2:16005.

Khatri, P & jain, S. (2017). Environmental life cycle assessment of edible oils: A review of current knowledge and future research challenges. J Cleaner Prod 152: 63-76.

Knothe G. (2005). Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86: 1059-1070.

Kot, A. M., Błażejak, S., Kurcz, A., Gientka, I., & Kieliszek, M. (2016). *Rhodotorula glutinis* - potential source of lipids, carotenoids, and enzymes for use in industries. *Applied Microbiology and Biotechnology*, 100(14): 6103-6117.

Koutinas AA, Chatzifragkou A, Kopsahelis N, Papanikolaou S, Kookos IK (2014). Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. Fuel 116:566–577.

Kruger JS, Cleveland NS, Yeap RY, Dong T, Ramirez KJ, Nagle NJ, Lowell AC, Beckham GT, McMillan JD, Biddy MJ, (2018). Recovery of fuel-precursor lipids from oleaginous yeast. ACS Sustain Chem Eng, 6:2921-2931.

Li, Q., Du, W., Liu, D. (2008). Perspectives of microbial oils for biodiesel production. *Appl. Microbiol. Biotechnol.* 80: 749-756.

Li, Y., Zhao, Z. (Kent), Bai, F (2007). High-density cultivation of oleaginous yeast Rhodosporidium toruloides Y4 in fed-batch culture. Enzym Microb Technol. 41: 312–317.

Lyman, M., Urbin, S., Strout, C., Rubinfeld, B. (2019). The Oleaginous Red Yeast Rhodotorula/Rhodosporidium: A Factory for Industrial Bioproducts. Yeasts in Biotechnology. (DOI: 10.5772/intechopen.84129).

Manuel Ageitos J, Andres Vallejo J, Veiga-Crespo P, et al. (2011). Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol. 90:1219–1227.

Mata-Gomez LC, Montanez JC, Mendez-Zavala A, Aguilar CN (2014). Biotechnological production of carotenoids by yeasts: an overview. Microb Cell Fact 13:12.

Nagaraj Y.N., (2021). Determination of total lipid and carotenoid content and their composition in *Rhodotorula toruloides* (Unpublished).

Nasseri, A. T., Rasoul-Amini, S., Morowvat, M. H. & Ghasemi, Y. (2011). Single cell protein: Production and process. American Journal of Food Technology 6: 103-116.

Nogue VSI, Black BA, Kruger JS, Singer CA, Ramirez KJ, Reed ML, Cleveland NS, Singer ER, Yi XN, Yeap RY et al. (2018). Integrated diesel production from lignocellulosic sugars via oleaginous yeast. Green Chem, 20:4349-4365.

Ochsenreither, K., Gluck, C., Stressler, T, Fischer, L. & Syldatk, C., (2016). Production strategies and applications of microbial single cell oils. Frontiers in Microbiology 7: 1539.

Olsen, R.E. & Henderson, R.J. (1989). The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. Journal of Experimental Marine Biology and Ecology. 129(2): 189-197.

Olstorpe, M., Pickova, j., Kisseling, A., Passoth, V. (2014). Strain and temperature dependent changes of fatty acid composition in *Wickerhamomyces anomalus* and *Blastobotrys adeninivorans.* Biotechnol Appl Biochem. 61: 45-50.

Papanikolaou S, Aggelis G (2011) Review article lipids of oleaginous yeasts. Part II Technology and potential applications: 1052–1073.

Park YK, Nicaud JM, Ledesma-Amaro R (2017). The engineering potential of Rhodosporidium toruloides as a workhorse for biotechnological

applications. Trends Biotechnol 36:304–317.

Passoth, V. (2014). Molecular mechanisms in yeast carbon metabolism: bioethanol and other biofuels. In molecular Mechanisms in Yeast Carbon Metabolism, J Piskur, C Compagno (eds). Springer: Berlin, Heidelberg; 217-259.

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

Passoth, V. (2017). In biotechnology of Yeasts and Filamentous Fungi (ed. Andriy A. Sibirny) 149-204 (Springer International Publishing).

Passoth, V., (2017). Conventional and non-conventional yeasts for the production of biofuels. In: Satyanarayana T, Kunze G (eds) Yeast diversity in human welfare. Springer, Singapore. pp 385-416.

Pettersson, A., Johnsson, L., Brännäs, E. & Pickova, J. (2009). Effects of rapeseed oil replacement in fish feed on lipid composition and selfselection by rainbow trout (*Oncorhynchus mykiss*). Aquaculture Nutrition. 15: 577–586.

Ratledge, C.,Wynn, J., (2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbiol. 51*, 1–51.

Rossi, M., Buzzini, P., Cordisco, L. et al., (2009). Growth, lipid accumulation and fatty acid composition in obligate psychrophilic, facultative psychrophilic and mesophilic yeasts. FEMS Microbial Ecol. 69: 363-372.

Sanderson, G.W., Jolly, S.O., (1994). The value of Phaffia yeast as a feed ingredient for salmonid fish. Aquaculture 124: 193–200.

Sandhir, R. (2014). Encyclopedia of Food Microbiology (Second Edition).

Schmidt, J. H. (2015). Life cycle assessment of five vegetable oils. J Cleaner Prod 87: 130-138.

Simopoulos, A.P. (1999). Evolutionary aspects of omega-3 fatty acids in the food supply. *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 60(5–6), pp. 421-429.

Simopoulos, A.P. (2006). Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. Biomed Pharmacother. 60: 502-507.

Sitepu, I. R., Sestric, R., Ignatia, L., Levin, D. et al., (2013). Manipulation of culture conditions alters lipid content and fatty acid pro[illegible]les of a wide variety of known and new oleaginous yeast species. Bioresour Technol. 144: 360– 369.

Sitepu, I.R., Garay, L.A., Sestric, R., (2014). Oleaginous yeasts for biodiesel: Current and future trends in biology and production. Biotechnology Advances 32, 1336-1360.

Tacon, A.G.J., (1994). Feed ingredients for carnivorous fishspecies: alternatives to fishmeal and other dietaryresources. FAO Fisheries Circulation. 881, 835.

Tiukova, L.A., Brandenburg, J., Blomqvist, J., Sampels, S., Mikkelsen, N., Skaugen, M., Arntzen, M.m Nielsen, J., Sandgren, M., Kerkhoven, E.J. (2019). Proteome analysis of xylose metabolism in *Rhodotorula toruloides* during lipid production. Biotechnol Biofuels. 12: 137.

Unrean P, Khajeeram S, Champreda V (2017). Combining metabolic evolution and systematic fed-batch optimization for efficient single-cell oil production from sugarcane bagasse. Renew Energy 111:295–306.

Vicente, G., Bautista, L. F., Gutiérrez, F. J., Rodríguez, R., et al. (2010). Direct transformation of fungal biomass from submerged cultures into biodiesel. *Energy Fuels* 24: 3173-3178

Wang GY, Zhang Y, Chi Z, et al. (2015). Role of pyruvate carboxylase in accumulation of intracellular lipid of the oleaginous yeast Yarrowia lipolytica ACA-DC 50109. Appl Microbiol Biotechnol. 99:1637–1645.

Wang, C. L., Li, Y., Xin, F. H., Liu, Y. Y. et al., (2014). Evaluation of single cell oil from *Aureobasidium pullulans* var. *melanogenum* P10 isolated from mangrove ecosystems for biodiesel production. Process Biochem. 49: 725– 731.

Wang, GY., Chi, Z., Song, B., Wang, Z.-P. et al., (2012). High level lipid production by a novel inulinaseproducing yeast *Pichia guilliermondii* Pcla22. *Bioresour. Technol.*124: 77-82.

Wijendran, V. and Hayes, K.C. (2004). Dietary n-6 and n-3 fatty acid balance and cardiovascular health. PubMed. Annu. Rev. Nutr. 24:597–615.

Yen, H.-W., Chen, P.-W., Chen, L.-J., (2015). The synergistic effects for the co-cultivation of oleaginous yeast-*Rhodotorula glutinis* and microalgae-*Scenedesmus obliquus* on the biomass and total lipids accumulation. Bioresour. Technol. 184: 148– 152.

Zhao, X., Wu, S., Hu, C., Wang, Q. et al., (2010). Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *J. Ind. Microbiol. Biotechnol.* 37: 581-585.

Acknowledgements

I would like to sincerely thank my supervisor, Professor Jana Pickova for giving me the opportunity to participate in this project with her enthusiasm and guidance which has an enormous impact on me to think critically, plan and work independently in the lab. The author is immensely grateful to Volkmar Passoth for helping with both questions and inspirations. Furthermore, I would like to express my gratitude to Yashaswini Nagavara Nagaraj and Jule Brandenburg for providing yeast sample with valuable information.