HIGH-PRESSURE METHODS FOR EXTRACTION OF LIPIDS FROM *RHODOSPORIDIUM TORULOIDES*

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MELIS ERDIR KOLCU

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

JULY 2023

Approval of the thesis:

HIGH-PRESSURE METHODS FOR EXTRACTION OF LIPIDS FROM *RHODOSPORIDIUM TORULOIDES*

submitted by **MELİS ERDİR KOLCU** in partial fulfillment of the requirements for the degree of **Master of Science** i**n Food Engineering, Middle East Technical University** by,

Prof. Dr. Halil Kalıpçılar
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Hami Alpas
Head of the Department, **Food Engineering** _____

Prof. Dr. Hami Alpas
Supervisor, **Food Engineering, METU** _____

Prof. Dr. Deniz Çekmecelioğlu
Co-Supervisor, **Food Engineering, METU** _____

Examining Committee Members:

Prof. Dr. Alev Bayındırlı
Food Engineering, METU _____

Prof. Dr. Hami Alpas
Food Engineering, METU _____

Prof. Dr. Mecit Halil Öztop
Food Engineering, METU _____

Prof. Dr. Deniz Çekmecelioğlu
Food Engineering, METU _____

Prof. Dr. Faruk Tahsin Bozoğlu
Academy of Health Sciences, KTO Karatay University _____

Date: 24.07.2023

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name Last name : Melis Erdir Kolcu

Signature :

ABSTRACT

HIGH-PRESSURE METHODS FOR EXTRACTION OF LIPIDS FROM *RHODOSPORIDIUM TORULOIDES*

Erdir Kolcu, Melis
Master of Science, Food Engineering
Supervisor: Prof. Dr. Hami Alpas
Co-Supervisor: Prof. Dr. Deniz Çekmecelioğlu

July 2023, 143 pages

Oleaginous microorganisms are known to accumulate lipids ranging from 20 to 80%. In this study, the lipid producing yeast was *Rhodosporidium toruloides* DSM 4444 was grown in enzymatically hydrolyzed cheese whey was used for 8 days and obtained biomass was disrupted by high pressure homogenizer (HPH) and high hydrostatic pressure (HHP) methods. For HPH, spent fermentation medium was directly used, applying pressures between 50-200 MPa with 3-13 passes. Response surface methodology (RSM) was used to design experimental conditions. For HHP, two different procedures were followed pressurizing dried biomass slurry and direct pressurization of the fermentation medium. High-speed homogenization (HSH) was chosen as the control for comparative evaluation.

The optimal conditions for HPH were determined as 125 MPa pressure and 5 passes, resulting in a lipid yield of 32.4%. In experiments conducted with the HHP method,

the optimum conditions were 230 MPa pressure, 15 minutes duration, and 1 g biomass/25 mL organic solvent. For experiments conducted with direct pressurization of the fermentation medium, the optimum conditions were determined as 300 MPa pressure, 5 minutes duration, and 30°C. In repeated experiments for the HHP method, lipid yields of 20% and 27.6% were obtained for different experimental procedures.

Fatty acid methyl ester analysis demonstrated that microbial oils derived from *R. toruloides* contained significant amounts of palmitic, stearic, oleic, and linoleic acids. Based on the results, it can be inferred that the HPH method provided more efficient outcomes for cell disruption and subsequent lipid extraction compared to the HHP method.

Keywords: yeast lipid, *Rhodosporidium toruloides*, high pressure homogenizer, high hydrostatic pressure, whey

ÖZ

RHODOSPORIDIUM TORULOIDES'TEN LİPİTLERİN EKSTRAKSİYONUNA YÖNELİK YÜKSEK BASINÇ YÖNTEMLERİ

Erdir Kolcu, Melis
Yüksek Lisans, Gıda Mühendisliği
Tez Yöneticisi: Prof. Dr. Hami Alpas
Ortak Tez Yöneticisi: Prof. Dr. Deniz Çekmecelioğlu

Temmuz 2023, 143 sayfa

Yağlı mikroorganizmaların %20 ile 80 arasında yağ biriktirebildiği bilinmektedir. Bu çalışmada, yağ üreten bir maya türü olan *Rhodosporidium toruloides* DSM 4444, enzimatik olarak hidrolize edilmiş peynir altı suyunda 8 gün boyunca yetiştirildi ve elde edilen biyokütle yüksek basınçlı homojenizatör (YBH) ve yüksek hidrostatik basınç (YHB) yöntemleri ile parçalandı. YBH yönteminde, harcanmış fermentasyon ortamı doğrudan kullanılarak, 50-200 MPa basınç aralığında ve 3-13 geçiş sayısıyla çalışılmıştır. YHB yöntemi için ise kurutulmuş biyokütle organik çözücü ile süspansiyon şeklinde basınçlandırma veya fermentasyon ortamının doğrudan basınçlandırılması kullanılmıştır. Yöntemlerin göreceli başarısını değerlendirebilmek için kontrol olarak yüksek hızlı homojenizatör yöntemi seçilmiştir.

Elde edilen optimum koşullar, YBH için 125 MPa basınç ve 5 geçiş sayısıdır ve bu koşulda yağ verimi %32.4 olarak ölçülmüştür. YHB yöntemiyle yapılan deneylerde, kuru biyokütle kullanılarak gerçekleştirilen deneylerde optimum koşullar 230 MPa basınç, 15 dakika süre ve 1 g biyokütle/25 mL organik çözücü olarak belirlenirken, fermentasyon ortamının doğrudan basınçlandırılmasıyla yapılan deneylerde optimum koşullar 300 MPa basınç, 5 dakika süre ve 30 °C olarak bulunmuştur. YHB için tekrarlanan deneylerde, farklı deney prosedürleri için sırasıyla %20 ve %27.6 yağ verimi elde edilmiştir.

Elde edilen yag asidi metil ester analiz sonuçlarına gore, *R. toruloides*'ten elde edilen mikrobiyal yağların yüksek miktarda palmitik, stearik, oleik ve linoleik asit içerdiğini göstermiştir. Elde edilen sonuçlara göre, hücre parçalanması ve sonraki yağ ekstraksiyonu için YBH yönteminin YHB yöntemine göre daha verimli sonuçlar verdiği görülmektedir.

Anahtar Kelimeler: Maya Yağı, *Rhodosporidium toruloides*, yüksek basınçlı homojenizatör, yüksek hidrostatik basınç, peynir altı suyu

To my precious family and those who meant a lot to me,

ACKNOWLEDGMENTS

First and foremost, I would like to express my gratitude to my supervisor Prof. Dr. Hami Alpas, and co-supervisor Prof. Dr. Deniz Çekmecelioğlu. Their support, understanding, patience, and guidance have been priceless during this journey. They always supported me to do my best.

I would like to thank the Scientific and Technological Research Council of Turkey (TÜBİTAK) for funding this study.

In particular, I want to give a special thanks to my parents Nesrin and Recai Erdir and my little lovely sister Melda Erdir for their unconditional love, sacrifices and unwavering belief in me. Your wisdom, support, and unwavering belief in me have shaped me into the person I am today.

I wish to extend my thanks to my friends, Ayşe Sultan Kurt, Berfin Özışık, Deniz Günalan, Deniz Tunalıer, Ummulkhairi Tukur, and my dear teammate Ayşe Alara Batıgün, for providing invaluable support and being an incredible source of friendship, and motivation. Also, I am deeply grateful to Sinem and Buruç Kolcu for their unwavering support, which has made them feel like a family member.

Last but not least, I want to express my deepest appreciation to Hürkan Kolcu, whose constant love and unwavering support has meant the world to me and has significantly contributed to making this adventure more meaningful.

TABLE OF CONTENTS

APPENDICES

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

In current years, due to on-going environmental concerns, generation of biofuels from recyclable sources has been still an issue. For biofuel production, microbial oils were found to be a good alternative as some microorganisms such as yeasts, bacteria, fungi, and microalgae can produce oils in particular growing conditions (Patel et al., 2018). These microorganisms that can produce microbial oil are called oleaginous microorganisms and they can produce up to 80 % lipids based on their dry biomass (Dias et al., 2022). The oleaginous microorganisms accumulate single cell oil (SCO) in media, containing low amount of nitrogen sources and high amount of carbon sources (Alakraa et al., 2020). These edible oils can be a good option to commercial palm oil, cocoa butter, and soybean oil due to their similar fatty acid profile (Tuhanioglu, 2021).

There are more than 70 oleaginous yeast species, but the most described oleaginous yeast genera are *Yarrowia, Candida*, *Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon,* and *Lipomyces* (Bettencourt et al., 2020). *Rhodosporidium toruloides* can produce lipids at almost 60 % (w/w) among these microorganisms. It can use different carbon sources such as glucose, fructose, xylose, and glycerol (Bommareddy et al., 2015).

To extract lipids from oleaginous yeasts, conventional and novel methods are being used. In conventional method, lipid extractions are conducted by using organic solvents such as hexane, methanol, and chloroform. Because using conventional solvent extraction methods need relatively cheap equipment, they are mostly preferred. Soxhlet apparatus extraction, Folch method, and Blight & Dyer method

are some of these conventional methods. Nevertheless, cell wall destruction forms the main idea of novel extraction methods (Patel et al., 2018). Applying these methods with solvent extraction methods gives higher lipid yield. Some mostly known novel extraction methods are high-pressure homogenizer (HPH), microwave irradiation, enzymatic lysis, ultrasonication, and pulsed electric field (Tuhanioglu, 2021).

High pressure homogenization (HPH) relies on the reduction of particle size and mechanical disruption of the microorganisms by using high pressure (50-400 MPa) (Kruszewski et al., 2021). HPH can easily be adjustable and accepted as one of the most environmentally safe methods. After harvesting the biomass, HPH can be carried out instantaneously and this method does not need any chemical use. By using different solvents that are capable of penetrating the cell, lipids and other cellular components can be extracted through the process of HPH (Dias et al., 2022).

High hydrostatic pressure (HHP) is a non-thermal technique employed to enhance food safety and extend shelf life through the disruption of microorganisms. Thus, HHP can be used for cell component extraction. Its pressure range changes between 100 to 800 MPa and process temperature changes up to 50 °C, depending on the targeted aim (Rendueles et al., 2011).

In this study, hydrolyzed whey was chosen as the fermentation medium, while *R. toruloides* was selected as the oleaginous yeast for producing microbial oil in hydrolyzed whey. Before initiating the fermentation process, the composition of whey was analyzed using HPLC, followed by hydrolysis of whey using the beta-galactosidase enzyme.

The main objective of this study was to compare the efficiency of two novel methods for extracting microbial oil from *R. toruloides*. For this purpose, HPH and HHP methods were employed. Different parameters were employed to determine the

highest lipid yield and identify the optimum conditions for HPH and HHP methods. Besides, viable cell count, SEM analysis, fatty acid methyl ester, and peroxide value analysis were performed.

Within this scope, a literature review was conducted to provide brief summaries on microbial oil and its synthesis mechanism, factors influencing microbial oil production, oleaginous microorganisms, as well as HPH and HHP methods (Chapter 2). Subsequently, the materials and methods used in this study were briefly described (Chapter 3). The results obtained from this study are presented in detail and explicitly, analyzing the effects of HPH and HHP methods on microbial oil production by *R. toruloides* in hydrolyzed whey (Chapter 4). Additionally, recommendations are provided for the outcomes of this study and future experiments (Chapter 5).

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Oil

In recent years, the rise in energy expenses and increasing environmental awareness have sparked significant interest in the search for renewable biofuels as a substitute for petroleum-derived fuels (Meng et al., 2009). Their significant renewable and clean energy alternative is biodiesel. Biodiesel can be derived from plant and animal sources, however there are some challenges such as rising prices of key feedstocks and need for large lands for diesel plants. On the other hand, one other biodiesel source is microorganisms (Ye et al., 2021). On the contrary of animal and plant sources, microbial sources do not need large spaces, have shorter cultivation time than plant sources (Bonturi et al., 2015) and they can be more productive by using some genetic techniques to produce the desired compounds (Lin et al., 2013). Also, obtaining microbial oil from microorganisms is not dependent on geographical or climatic changes, and various substrates such as industrial wastes and by-products from several industries can be utilized for production (Ochsenreither et al., 2016).

In the late 19th century and throughout the mid-20th century, German scientists conducted extensive pilot-scale studies about microbial oil production, specifically focusing on *Trichosporon pulluland* and *Torula utilis*. However, the difficulties faced in scaling up production and extracting the lipid led to the failure of commercial microbial oil projects. In the early 1940s, Harder and Witsch reported the production of microalgal oil by *Pennales* spp., which exhibited the capability to accumulate up to 50% lipid (w/w dry basis) in their cells (Ghazani & Marangoni,

2022). During the 1940s to 1950s, numerous countries carried out additional studies to explore the potential commercial applications of microbial oils. However, the large-scale production of agricultural products, such as plant seed oils, and the widespread availability of inexpensive vegetable oils like soybean oil, caused the edible oil market to become oversaturated and prices to decline, making microbial oils economically unviable (Ratledge, 2010). In 1964, Professor F. Lynen from Munich University in Germany received the Nobel Prize in Chemistry for his groundbreaking research on fatty acid biosynthesis in yeast, which marked a significant milestone in the field of microbial oils (Ghazani & Marangoni, 2022).

Microbial oils, also known as single cell oils (SCOs) which refer to edible oils that can be obtained from single-celled microorganisms, with yeasts, fungi, and algae being the primary sources (Ratledge, 2010). These microorganisms, known as oleaginous microorganisms, have the ability to accumulate lipids up to 80% of their dry biomass (Dias et al., 2022). Microbial oils have emerged as a promising alternative in the biofuel industry due to their similar compositions to traditional vegetable oils (Gurel, 2019). Single cell oils reserves consist of various components including triacylglycerols (TAGs), free fatty acids, polar lipids, hydrocarbons, and pigments (Mhlongo et al., 2021) Additionally, their composition makes microbial oils suitable for various applications in the food and chemistry industries (Darcan & Sarıgül, 2016). Microbial oils can be used to enrich infant foods (Ratledge, 2013), and meat products and it can be used as milk emulsions (Ochsenreither et al. 2016).

Mostly available fatty acids in microbial oil are omega-3 (alpha linolenic acid, ALA (C18:3); eicosatetraenoic acid, EPA (C20:5); docosahexaenoic acid, DHA (C22:6)), omega-6 (linoleic acid, LA (C18:2); gamma linoleic acid, GLA (C18:3); arachidonic acid, ARA (C20:4)) and omega-9 (oleic acid (C18:1), OA) (Kannan et al., 2021). These are unsaturated fatty acids and have numerous benefits for human health (Farag & Gad, 2022).

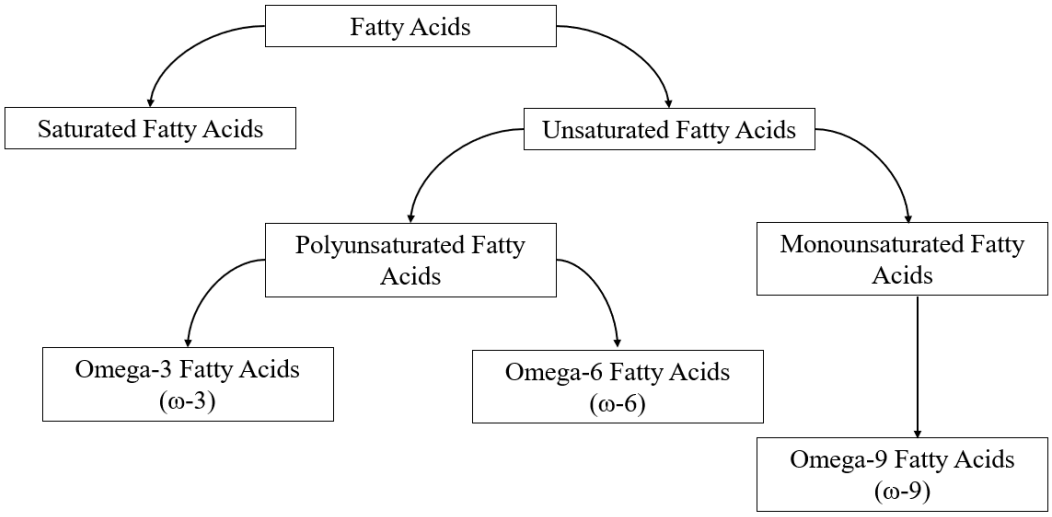

Figure 2.1. Fatty acid classification.

2.2 Microbial Oil Production Mechanism

Microbial oil production from oleaginous microorganisms is focused on two different pathways; ex novo and de novo processes. While in the ex novo fermentation process from hydrophobic substrates such as oils, alkanes are used, the fermentation process from hydrophilic substrates such as sugars and amino acids is called de novo. (Huang et al., 2017). Lipid accumulation in de novo process begins when there is an abundance of carbon source and a scarcity of nitrogen source present in the substrate. The excess carbon source is no longer used for cellular expansion due to limited nitrogen availability, which inhibits both rapid cell growth and protein production. This results in the accumulation of lipids. On the other hand, ex novo process does not depend on nitrogen concentration (Sutanto et al., 2018).

In lipid synthesis pathway, the first step is to metabolize carbon source to for generation of acetyl-CoA. This is achieved through the process of glycolysis, where the carbon source is converted to pyruvate. When pyruvate is transported to mitochondria, it is converted to acetyl-CoA with the help of pyruvate decarboxylative dehydrogenase enzyme (Gurel, 2019). Because of limited concentration of nitrogen source, the adenosine mono phosphate (AMP) deaminase activity increases in the cell. This enzyme causes breaking down AMP and generation of ammonia which is used as nitrogen source. When AMP level reduces, isocitrate dehydrogenase enzyme activity stops since its activity depends on AMP concentration. Consequently, isocitrate accumulation increases and it converts into citrate by the enzyme aconitase. The accumulated citrate is then transferred to cytoplasm through citrate / malate export system to exchange citrate with malate. ATP citrate lyase (ACL), cleaves the citrate to generate acetyl-CoA and oxaloacetate. The acetyl-CoA carboxylate 1 (ACC1) carboxylate converts the formed acetyl-CoA into malonyl-CoA. These two compounds, acetyl-CoA and malonyl-CoA are the starter of fatty acid synthesis. Then, the formed fatty acids are connected to glycerol molecules which is generated by glycerol-3 phosphate dehydrogenase, to form triglycerides (TAGs) (Ratledge, 2004; Sutanto et al., 2018; Tuhanioglu, 2021). A simplified illustration of this process is shown in Figure 2.2.

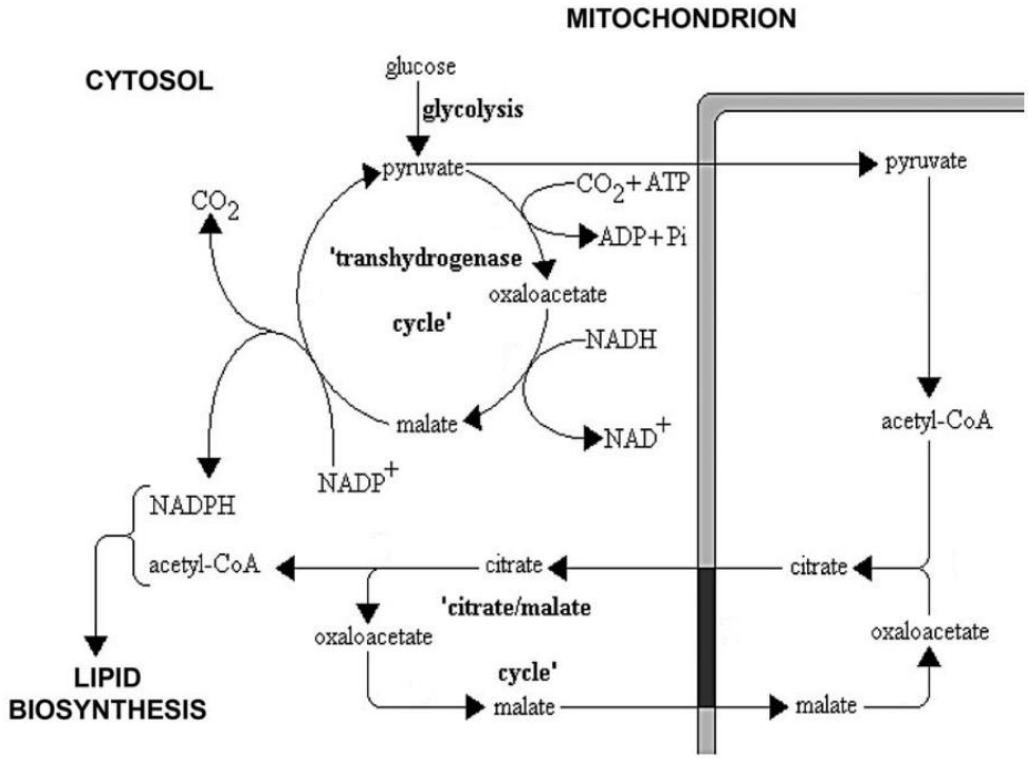

Figure 2.2. A simplified diagram illustrating the process of lipid synthesis in oleaginous microorganisms (Ratledge, 2004)

2.3 Factors Affecting Microbial Oil Synthesis

2.3.1 Carbon and Nitrogen Sources

Carbon is the most essential element for the synthesis of microbial lipids. Microorganisms need an enough supply of carbon sources to increase their growth and accumulation of lipid. Sugars, carbohydrates, and organic acids are mostly used in microbial oil production. Carbon source quality can affect the yield of microbial oil. The most used carbon sources are glucose and xylose. Other than these carbon

sources, fructose, arabinose, mannose, galactose and sometimes glycerol are used (Caporusso et al., 2021).

Another important nutrient for microbial oil production is nitrogen. It is needed for protein synthesis and different metabolic processes in microorganisms. Availability of nitrogen source affects growth of cells, production of biomass and accumulation of microbial oils. Organic and inorganic nitrogen sources are commonly used compounds for microbial oil production. Organic nitrogen sources include yeast extract, tryptone, peptone, and urea, while inorganic nitrogen sources include ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4^+)_2SO_4$), and sodium nitrate ($NaNO_3$). These compounds provide a nitrogen source necessary for the growth of microorganisms and lipid accumulation. In this way, microbial oil production can be optimized by increasing the lipid production potential of microorganisms (Caporusso et al., 2021).

Carbon to nitrogen (C/N) ratio is thus critical for microbial oil production. Generally, high C/N ratio promotes lipid accumulation while minimizing the biomass production. Since cells cannot use nitrogen for growth in medium that contains limited amount of nitrogen, they start to accumulate lipids. To obtain maximum lipid yield the ratio of carbon to nitrogen must be kept under control (Gurel, 2019). However, it is important to consider that the optimal carbon-to-nitrogen ratio can vary depending on the specific microorganism and cultivation conditions. For example, Wang et al. (2014) conducted a study on *Lipomyces starkeyi* and reported that the maximum lipid yield was achieved at a C/N of 200/1, resulting in a lipid yield of 48.6%. In addition, maximum lipid yield for *Rhodosporidium toruloides* was found as 60 % at C/N=120/1 when xylose used as a carbon source (Caporusso et al., 2021), for *Rhodotorula glutinis* was found as 50% at C/N=70/1 when using glucose as a carbon source (Braunwald et al., 2013).

Moreover, C/N ratio affects the biomass production. According to Kraisintu, et al. (2010), when C/N ratio decreases the biomass production increases.

2.3.2 Metal Ions

Some ions such as Mg^{+2}, Ca^{+2}, Mn^{+2}, Fe^{+3}, Cu^{+2} and Zn^{+2} that are used in microbial oil production can affect the biomass amount and lipid content (Caporusso et al., 2021). By adjusting the concentration of manganese sulfate ($MnSO_4$), zinc sulfate ($ZnSO_4$), magnesium sulfate ($MgSO_4$), cobalt chloride ($CoCl_2$), copper (II) sulfate ($CuSO_4$), and iron (II) sulfate ($FeSO_4$), it is possible to enhance cell growth and lipid accumulation (Zhao et al., 2008). According to a study conducted by Kraisintu et al. (2010) it was reported that lipid yield and biomass accumulation vary when different salts and their different concentrations were used. In that study *Rhodosporidium toruloides* was used and the highest lipid yield of 69 % was obtained when 2 g/L $MgSO_4.H_2O$ was used and highest biomass of 13.82 g/L was obtained when 0.5 g/L $MgSO_4.H_2O$ was used. (Kraisintu et al., 2010)

2.3.3 Temperature

Temperature plays a critical role on cell growth and microbial oil production. It shows an important effect on several aspects of microbial metabolism, including enzyme activity, cell growth rate, and pathways included in lipid biosynthesis (Tuhanioğlu, 2021). Usually, microorganisms that has ability to grow at high temperatures are preferred because working at high temperatures reduces the cooling

cost. Most oleaginous yeasts' optimum temperature for growth is around 30 °C (Gong et al., 2013).

Lamers et al. (2016) conducted a study for several oleaginous yeast strains to understand temperature effect on microbial oil and biomass production. In this study there were two different types of yeast, which have a small temperature range and broad temperature range, were used. According to results, for *Saccharomyces cerevisiae* optimum temperature was 29 °C and its optimum temperature range was 5 °C. Any deviation from this temperature can have a negative effect on cell growth and microbial oil production. On the other hand, *Pichia anomala, Waltomyces lipofer,* and *Torulaspora delbrueckii* had an optimum temperature range of 9°C and within these temperatures they do not have a negative effect on microbial growth and lipid production. In another study, it was found that increase in temperature decreases the biomass and lipid yields (Abeln & Chuck, 2020).

2.3.4 pH

In order to achieve optimal cell growth and microbial oil production, it is important to determine the optimum pH value of the growth and fermentation media. This is because pH directly influences the surface properties of the cell membrane, which in turn affects the process of carbon assimilation (Caporusso, 2021).

Different microorganisms have different optimum pH values for their best growth and microbial oil production. Oleaginous yeast can grow and produce microbial oil in a wide range of pH levels, including low pH values, which can inhibit the growth of other microorganisms (Mohammed et al., 2018). This feature presents the

advantage of minimizing the contamination risk by pathogenic microorganisms (Gurel, 2019).

According to Jach and Malm (2022), the pH range of *Yarrowia lipolytica* in which it can grow 2-8 but its optimum pH range for lipid production is 6-6.5 (Papanikolaou et al., 2002). For another oleaginous yeasts *Rhodosporidium toruloides* and *Lipomyces starkeyi*, it was found that although they are capable of surviving in challenging environments, they exhibit optimal growth in pH range of 5 to 7 (Gürel, 2019). On the other hand, it was found that *Rhodosporidium toruloides* lipid production did not change in different pH values of 3, 4.5 and 6 (Sitepu et al., 2014).

2.3.5 Other Factors

Oxygenation rate, aeration rate, agitation rate, incubation period, and fermentation type also affect the microbial oil and biomass production.

The presence of oxygen in the culture medium is often positively correlated with yeast biomass concentration and lipid productivity. Also, oxygen availability affects composition of fatty acid (Bento et al., 2019). For each specific microorganism the aeration rate must be optimized and to achieve the desired lipid yield and compositions oxygen concentration, aeration rate and agitation speed should be controlled carefully. Oxygen could be toxic for some microorganisms at excessive oxygen concentrations (Zhou et al., 2018). Saenge et al. (2011) found aeration rate between 0-2 vvm, which increased the lipid production in *Rhodotorula* glutinis. In another study, it was observed that decreasing aeration rate increased the saturated fatty acids for *Lipomyces starkeyi* and *C. curvatus* (Calvey et al., 2016).

Also, agitation rate affects the oxygen level in the medium when it increases, oxygenation increases, and it enhances the biomass and lipid productions (Suryawati & Ilmi, 2023). Optimum agitation rate depends on experimental conditions and microorganism used. For example, the optimum agitation rate for *Rhodotorula glutinis* was reported as 180 rpm (Elfeky et al., 2020), for *Rhodotorula dibovata* 200 rpm was found and for *Rhodotorula kratochvilovae* it was found as 225 rpm. (Osman et al., 2022). Moreover, in the study that conducted for *Rhodosporidium toruloides* it was found that oxygen demand increased after decreasing nitrogen source in the medium then it was declined (Sitepu et al., 2014).

Other than the factors mentioned, incubation period also plays a critical role for the biomass production and microbial oil accumulation. Generally, at the stationary phase of microbial growth microbial oil content reaches to its highest value. Thus, it is recommended that harvesting should be done at an early stage of stationary phase for preventing lipid deterioration (Jiru et al., 2017).

Fermentation type is another factor that plays an important role in microbial oil production. Different fermenter types influence oleaginous microorganisms' productibility of oil and biomass by providing various levels of oxygen transfer, efficiency of mixing, and nutrient distribution. Moreover, changing in fermentation medium amount can change the lipid and biomass yields. According to Tuhanioğlu (2021), for *Rhodosporidium toruloides* the maximum lipid yield was obtained as 45.7, 40.1 and 50. 9% when 500 mL Erlenmeyer flasks, 1 L batch bioreactor and 10 L batch bioreactor were used, respectively. On the other hand, different studies were conducted by using fed-batch bioreactor for obtaining microbial oil from *Rhodosporidium toruloides*. Some of the obtained results of lipid yields are 40.0 % Afonso et al., 2018), 61.3 % (Diaz er.al., 2018) and 43.3 % (Chen et al., 2018).

2.4 Oleaginous Microorganisms

Oleaginous microorganisms have been defined as oily species that have the ability to accumulate lipid content exceeding 20 % of their total dry biomass. Some of the yeast, algae, bacteria, and fungi are considered as oleaginous microorganisms (Kumar et al., 2019).

Oleaginous bacteria can store specific type of lipids compared to other microbial oils under some specific conditions and compositions (Li et al., 2008). However, their lipid compositions are different from other oleaginous microorganisms. The accumulated lipid by bacteria is not suitable for human consumption (Abeln & Chuck, 2021), it is a good source of TAGs which serve as the primary components necessary for biodiesel production (Patel et al., 2020). The most significant genera of oleaginous bacteria include *Mycobacterium* sp., *Nocardia* sp., *Streptomyces* sp., *Bacillus* sp., *Rhodococcus* sp., and *Gordonia* sp. Among these microorganisms for *Rhodococcus* sp., and *Gordonia* sp., it was found that they can accumulate lipid almost 80 % with a very low biomass concentration under some specific conditions (Liang & Jiang, 2013).

Oleaginous microalgae can produce polyunsaturated fatty acids (PUFAs) such as EPA, DHA, and ARA which are necessary for human health. (Ochsenreither et al, 2016). Most species can accumulate between 20-50 % lipid in their cells. Species such as *Botryococcus, Chlorella*, *Scenedesmus*, and *Monoraphidium* have been extensively studied for their ability to accumulate significant quantities of lipids (Caporusso et al, 2021). They can use sunlight as an energy source and CO_2 as a carbon source when growing (Li et al., 2008). Despite oleaginous bacteria and yeast species, oleaginous microalgae need large surface area and longer growth time to produce desired level of lipid (Kumar et al., 2019).

Some oleaginous fungal species can accumulate lipids, reaching 70% of their dry biomass, under suitable conditions (Ratledge, 2002). *Mortierella alpina*, *Umbelopsis isabellina*, *Mucor circinelloide*, and *Cunninghamella echinulate* are some examples of oleaginous fungal species. These oleaginous microorganisms are of great interest due to their ability to produce significant quantities of long-chain polyunsaturated fatty acids (LC-PUFAs) like DHA, GLA, EPA and ARA (Zhu et al., 2021). For instance, *Umbelopsis isabellina* produces lipid containing around 50 % oleic acid (Economou et al., 2010), *Aspergillus tubingensis* can accumulate almost 50% unsaturated fatty acids which contain 36 % oleic acid (Cheirsilp & Kitcha, 2014).

Oleaginous yeasts are comprising approximately 5 % of the total population of yeast species (Kamineni & Shaw, 2020). Using yeasts in microbial oil production is easier than other oleaginous microorganisms since their cultivation is easier, they have high cell density and their lipid yield is more than the others (Salvador López et al., 2022). According to Abeln & Chuck (2021) there are more than 160 oleaginous yeast species, and this number will increase in the future. Oleaginous yeast can accumulate lipid from 20 to 80% under suitable conditions. Their fatty acid content is similar to the compositions found in several plant oil seeds oils, with up to 80% TAGs composition. (Poontawee et al., 2017). Some of the oleaginous yeast species are *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Trichosporon*, *Myxozyma*, *Sporidiobolus*, *Sporobolomyces*, *Apiotrichum* and *Lipomyces* (Sitepu et al., 2014).

Some oleaginous bacteria, microalgae, fungi and yeasts examples, and their obtained lipid yields are shown in Table 2.1.

Table 2.1. Some examples of oleaginous microorganisms and their lipid yields (w/w %).

Oleaginous Microorganisms	Lipid Yield (w/w%)	Reference
Bacteria		
Arthrobacter sp.	>40	(Subramaniam et al., 2010)
Nocardia globerula 432	>49.7	(Alvarez & Steinbüchel, 2002)
Rhodococcus opacus PD630	33	(Saisriyoot et al., 2019)
Bacillus alcalophilus	18-24	(Subramaniam et al., 2010)
Rhodococcus jostii 602	60-70	(Alvarez et al., 2021)
Gordonia sp. DG	72	(Gouda et al., 2008)
Microalgae		
Botryococcus braunii	25-75	(Subramaniam et al., 2010)
Chlorella protothecoides	49	(Li et al., 2007)
Scenedesmus sp	34	(Srinuanpan et al., 2018)
Monorahidium sp.	19-35	(Díaz et al., 2015)
Auxenochlorella protothecoides	63	(Patel et al., 2018)
Nitzshia sp.	45-47	(Subramaniam et al., 2010)
Phaeodactylum tricornutum	23	(Ova Ozcan & Ovez, 2022)
Chlorella zofingienesis	52	(Liu et al., 2010)
Fungi		
Umbelopsis isabellina	41.8	(Hussain et al., 2014)
Cunninghamella echinulata	46	(Fakas et al., 2009)
Colletotrichum sp.	36-49.1	(Dey et al., 2011)
Alternaria sp.	40.1-58.1	(Dey et al., 2011)
Fusarium oxysporum	42.6	(Matsakas et al., 2017)
Mortierella alpina	25.Eyl	(Chen et al., 2022)

Table 2.1 (continued)

Yeast		
Lipomyces starkeyi	61	(Zhao et al., 2008)
Rhodosporidium toruloides Y4	67.5	(Li et al., 2007)
Yarrowia lipolytica	67	(Niehus et al., 2018)
Trichosporon fermentans	40.1	(Huang et al., 2009)
Candida sp.	56	(Duarte et al., 2013)
Rhodotorula glutinis	53.23	(Verma et al., 2019)
Cryptococcus curvatus	56.7	(Gong et al., 2015)
Sporidiobolus pararoseus	47.1	(Wang et al., 2020)
Sporobolomyces carnicolor	50	(Matsui et al., 2011)
Apiotrichum porosum	36.2	(Qian et al., 2021)

2.4.1 Oleaginous Yeast: *Rhodosporidium toruloides*

Rhodosporidium toruloides is the most researched oleaginous yeast apart from *Yarrowia lipolytica* (Chattopadhyay & Maiti, 2021). This eukaryotic, unicellular, red basidiomycete, and oleaginous yeast is one of the members of four genera, *Rhodotorula, Sporobolomyces* and *Sporidiobolus* (Tuhanioglu, 2021). Its cell wall has a higher amount of glucose, galactose, mannose, and glucosamine (Bonturi et al. 2015). *R. toruloides* can be found in different environments such as water, air, animals, and plants. It also has the capability of surviving in extreme conditions like arctic ice sheets. But *R. toruloides* are mostly found in soil (Dinh et al., 2019). *Rhodosporidium toruloides* has the remarkable ability to accumulate lipids (specifically, single-cell oils or SCOs), which can account for over 70% of its dry

biomass. Additionally, this yeast species has the capacity to produce various beneficial compounds, including carotenoids, essential enzymes like cephalosporin esterase and epoxide hydrolase, as well as sugar alcohols such as arabitol and galactitol. (Park & Ledesma-Amaro, 2018; Jagtap et al., 2019). All these produced compounds can be used by various industries; carotenoids can be used as colorants, antioxidants and precursors of vitamin A, lipids can be used as nutritional supplements and produced sugar alcohols can be used as low-calorie sweeteners (Park & Ledesma-Amaro, 2018; Chattopadhyay & Maiti, 2021).

R. toruloides can use different carbon sources such as glucose, xylose, fructose, and glycerol to accumulate lipids (Bommareddy et al., 2015). Different carbon sources give different lipid yields. The other factors that affect lipid yield of *R. toruloides* are C/N ratio, nitrogen source, temperature, extraction method, and process type. There are many studies on *R. toruloides* yeast in literature. In Table 2.2, the effect of some parameters on lipid yield was shown.

Moreover, produced microbial lipid by *R. toruloides* mostly composed of long chain fatty acids (C14 to C18), the percentage of these are more than 90 %. (Zhang et al., 2022). The most produced fatty acids by *R. toruloides* are myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic acids (18:3) (Fei et al., 2016).

Table 2.2. Factors affecting lipid yield in *Rhodosporidium toruloides* and corresponding lipid yields.

	Parameters	Lipid Yield (w/w %)	References
Different C/N ratio			
R. toruloides 1588	70	43	(Saini et al., 2021)
R. toruloides CCT 0783	120	60	(Lopes et al., 2020)
R. toruloides AS 2.1389	200	48.2	(Huang et al., 2016)
Different carbon source			
R. toruloides CBS14	Glucose	75	(Wiebe et al., 2012)
R. toruloides NRRL Y-27012	Xylose	57	(Michou et al., 2022)
R. toruloides CCT 0783	Glycerol	67.4	(Lopes et al., 2020)

Table 2.2 (continued)

Different nitrogen source			
R. toruloides R-ZL2 strain	Ammonium Sulfate	59	(Ye et al., 2021)
R. toruloides R-ZL2 strain	Ammonium Nitrate	65	(Ye et al., 2021)
R. toruloides DEBB 5533	Urea	41	(Soccol et al., 2017)

2.5 Microbial Oil Extraction Methods

Yeast cells are an important source of single cell oils, proteins, vitamins, enzymes, antioxidants, and several other bioactive compounds (Ganeva et al., 2020). Most of the cell disruption and microbial oil extraction methods include breakage of these cell walls and extract the oil with organic solvents like hexane, chloroform and methanol mixture, toluene, water etc. (Tuhanioglu, 2021; Bonturi et al., 2015). Similar to other microorganisms, yeast cells possess a sturdy cell wall that needs to be disrupted in order to access the cellular contents (Liu et al., 2016). Obtaining lipids from yeast cells can be challenging due to certain features, such as the high density of the yeast cell wall. In general, oleaginous yeast cell wall is composed of β (1 → 3)-D-glucan, β (1 → 6)-D-glucan, mannoproteins, and chitin (Figure 2.3). Glucans and chitin complex provide the shape and cell elasticity while mannoproteins play an important role in cell permeability. Having high amount of mannan and chitin in oleaginous yeast cell walls, make disruption of cell wall more difficult (Khot et al., 2020).

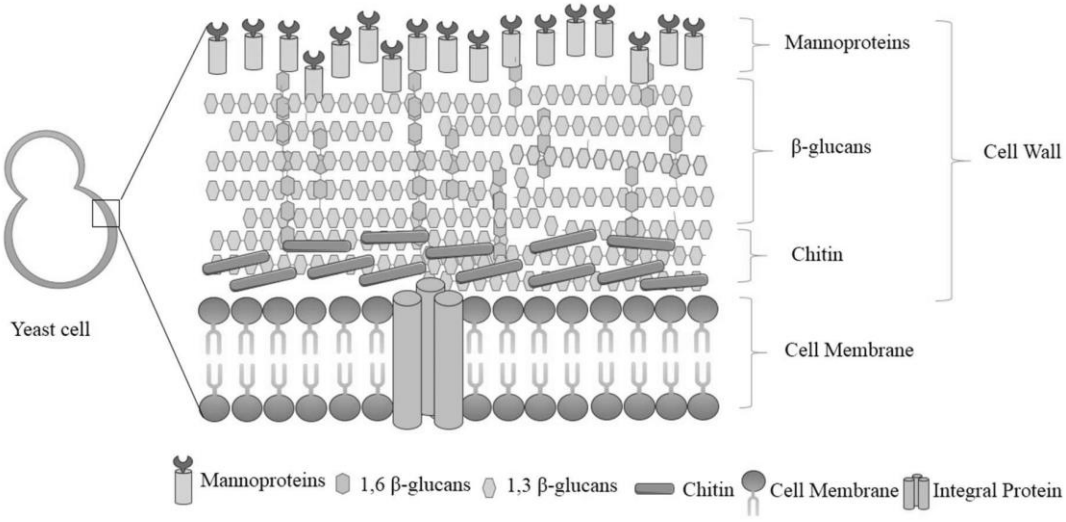

Figure 2.3. Cell wall structure of oleaginous yeast (Anwar et al., 2017)

There are conventional and novel methods used to disrupt cell wall and extract microbial lipid.

2.5.1 Conventional Methods to Extract Microbial Oil

Conventional methods mostly rely on organic solvents for extraction of lipid from microorganisms. Mostly used methods are solvent extraction methods are Folch method, Bligh & Dyer method, and Soxhlet method.

In Folch method, chloroform-methanol mixture with a volume ratio of 2:1 is used with the disrupted cells to obtain homogenous mixture. The Bligh & Dyer method is similar to the Folch method, but it has some differences. The variations between the protocols of Folch and Bligh and Dyer methods include differences not only in the solvent system volume relative to the sample but also in the solvent ratios and the presence or absence of salt in the added water phase, affecting the selectivity and efficiency of lipid extraction. In the Soxhlet method, another commonly used technique, hexane or petroleum ether is typically used. The Soxhlet method employs a repetitive process of extraction and solvent evaporation in order to achieve effective and efficient extraction (Meullemiestre et al., 2015).

2.5.2 Novel Methods to Extract Microbial Oil

Extraction of microbial oils by using novel methods primarily concentrate on achieving cell wall degradation. There are some obstacles to their commercialization due to high cost despite their proven effectiveness (Tuhanioglu, 2021). Some of the

novel extraction methods for cell disruption are ultrasound assisted extraction, pulsed electric field extraction, microwave-assisted extraction, high speed homogenizer, super critical fluid extraction, enzyme-assisted extraction and high-pressure homogenizer, which have been widely studied over the years (Meullemiestre et al., 2015; Patel et al., 2018)

Ultrasound-assisted extraction is conducted by using high frequency sonic waves (ultrasound). High frequency ultrasound cause cell disruption, cell size reduction by creating and collapsing of microscopic bubbles, known as cavitation (Sánchez-Camargo et al., 2020). Collapsing cavitations generate high temperature and pressure, thus cell wall of microorganisms destroyed, and lipids are released to the medium (Liu et al., 2016). This method is cost friendly, simple and time efficient. Also, it can be performed without any chemicals. However, a significant weakness of this method is the emergence of harmful free radicals that can have a detrimental impact on the quality of extracted lipids after a long process time (Patel et al., 2018b). Also, Zhao et al. (2006) found that applying ultrasound method can cause pigment degradation of the compounds (Zhao et al., 2006).

Pulsed electric field extraction is a non-thermal, environmentally friendly, and minimally invasive method (Bocker & Silva, 2022), and its principles relies on applying a short pulses of high intensity electric fields for a short duration (Meullemiestre et al., 2015). Cell walls include dielectric compounds and these materials within the cells undergo a conversion into charged entities, which then migrate towards the electrode with opposite charge when the electric field is applied (Arshad et al., 2020). Movement of ions creates an electro-mechanical stress within the cell walls. This causes an increase in permeability of cell and cellular compounds (Bocker & Silva, 2022). Using this method, it is possible to achieve high extraction efficiency in a short time and with low energy consumption. However, the

application of this method relies on the concentration of the medium and is sensitive to the conductivity of the medium (Karim et al., 2019).

Microwave assisted extraction principle causes the dielectric heating, which is primarily by absorption of energy in the solvent to be available in wet biomass or a given sample. As a result, microwave increases the temperature of the cell intercellular fluids leading to evaporation of water, exerting pressure on cell walls and eventually causing cell disruption (Kapoore et al., 2018). Advantages of this method is reduced time of extraction and consumption of solvent (Llompart et al., 2018). Moreover, this method is safe and relatively cheap, and it can be easily scaled up (Wang et al., 2012; Karim et al, 2019). Conversely, the high energy that occurs may cause lipid oxidation (Danlami et al., 2014).

High-speed homogenization is the method that disrupts the cells by creating high shear force with stirring at high rpm between 10,000 to 20,000 (Hua et al., 2017). According to a study conducted by Chen et al., (2014), in long term use high-speed homogenizer can cause disintegration of fiber structure and degradation of polysaccharide chains. The main advantage of this method is that it can be used directly for samples with high humidity (Patel et al, 2018). Generating high heat and consumption of high energy during process are the disadvantages of this method (Karim et al, 2019).

Supercritical fluid extraction method is widely used in lipid extraction from oleaginous microorganisms. This method has more advantages and is ecofriendly compared to other extraction methods, especially the solvent and enzyme extraction methods (Sahena et al., 2009). This method relies on employing solvents at pressures and temperatures that exceed their critical points (Sánchez-Camargo et al., 2020). The most used supercritical fluid is CO_2. The underlying principle of this method is to apply pressure to supercritical fluid to convert it into liquid form, which can be

easily separated from the polar constituents. Subsequently, by reducing the pressure and temperature of the scavenger, the CO2 returns to the gaseous state, leaving the lipid fraction without the scavenger (Hewavitharana et al., 2020). CO2 has several advantages such as it is inert, not expensive, environmentally friendly, not flammable and its availability is high (Meullemiestre et al., 2015).

Enzyme assisted extraction method depends on the characteristic of cell wall of the microorganisms (Nadar et al., 2018). To disrupt cell wall different enzymes such as cellulase, amylase, pectinase and xylanase are used (Patel et al, 2019). Enzymes are employed to selectively bind to specific molecules within the cell wall components, such as the mannoprotein complex and glucan backbone, and hydrolyze the chemical bonds present (Karim et al, 2019; Liu et al., 2016). While the use of enzymes in extraction offers advantages such as specificity, low energy requirements, high extraction efficiency, and non-toxicity, there are also several disadvantages to consider. These include the time-consuming nature of the process, the need for sterile conditions, the cost of enzymes, and the selection of appropriate enzymes for the desired extraction target (Karim et al, 2019).

2.6 High Pressure Homogenizer (HPH)

High-pressure homogenization (HPH) is a non-thermal technique that involves reduction of particle size and mechanical disruption of microorganisms through the application of high pressure, ranging from 50 to 400 MPa. This technology utilizes a narrow gap in the disruption valve, where the product is forced under high pressure, leading to the occurrence of various physical phenomena such as cavitation, turbulence, friction, temperature, and shear forces (Figure 2.4) (Kruszewski et al., 2021). The control parameters of HPH are pass numbers, operating temperature, and

pressure (Karim et al, 2019; Che et al., 2007). According to Samarasinghe (2012), increasing the number of passes and the applied pressure during high-pressure homogenization results in a higher level of cell disruption.

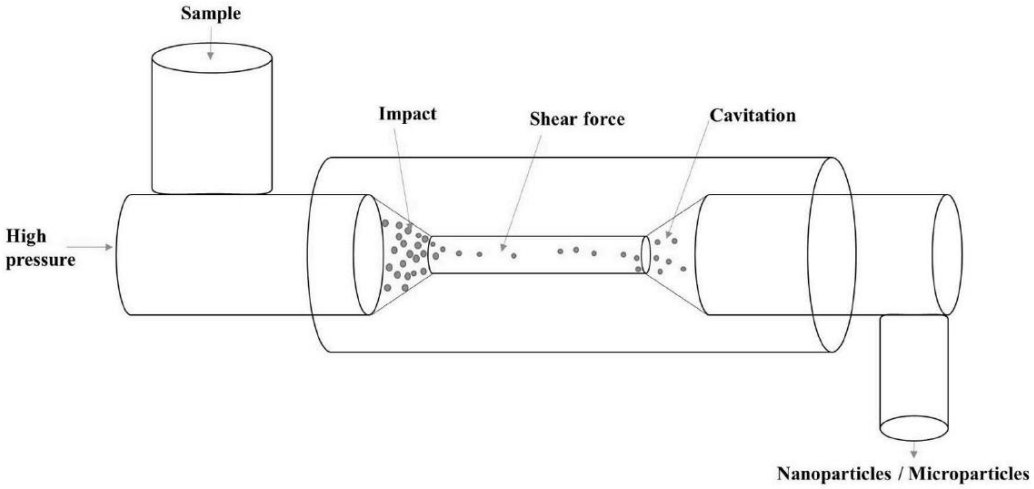

Figure 2.4. Schematic illustration of a HPH equipment (Rajendran et al., 2016).

There are different industries, in which HPH is widely used such as chemical, pharmaceutical, biotechnology and food for emulsions and suspensions stabilization (Comuzzo & Calligaris, 2019; Diels & Michiels, 2006). Also, this method is used comprehensively for yeast, bacteria as well as microalgae cell degradation (Patel et al, 2018). To enhance the extraction of several compounds like proteins and lipids, HPH is used as a pretreatment method for extraction of cellular components (Plazzotta & Manzocco, 2018). HPH can easily be adjustable and since this method is solvent free, it is accepted as one of the most environmentally safe methods (Dias et al., 2022; Karim et al, 2019). After harvesting the biomass, HPH can be carried out instantaneously and this method does not need any chemical use. By using

different solvents that can easily access into the cell, lipids, carotenoids, and other cellular components can be extracted after performing HPH (Dias et al., 2022). On the other hand, temperature of the product increases by about 2-2.5 ℃ with every 10 MPa (Diels & Michiels, 2006). This temperature increase should be taken into account for heat sensitive products (Rendueles et al., 2011).

High pressure homogenizer is mostly used as pre-treatment method to extract lipid from microorganisms. In the literature, there are several studies about cellular component extraction from microorganisms but from oleaginous microorganisms there are limited studies. Some of the obtained lipid yields from conducted studies and their pressure and pass number conditions are shown in Table 2.3.

Table 2.3. Some oleaginous microorganisms are treated with HPH.

Microorganism	**Extraction Method**	**Pressure (MPa)**	**Pass Number**	**Lipid Yield (w/w %)**	**Ref.**
Saitozyma podzolica DSM 27192	HPH with Folch method	200	15	37.8	(Gorte et al., 2020)
Yarrowia lipolytica JMY5578	HPH	150	20	83.9	(Drévillon et al., 2019)
Yarrowia lipolytica JMY5289	HPH with Solvent Extraction	150	5	76	(Drévillon et al., 2018)

Table 2.3 (continued)

Cryptococcus curvatus	HPH with Solvent Extraction	80	5	48	(Thiru et al., 2011)
Pichia kudriavzevii MTCC 5493	HPH with Solvent Extraction	80	5	23	(Sankh et al., 2013)
Rhodosporidium toruloides NCYC 921	HPH with Solvent Extraction	60	3	55.9	(Dias et al., 2022)
Nannochloropsis sp.	HPH with Solvent Extraction	120	1	70	(Olmstead et al., 2013)

2.7 High Hydrostatic Pressure (HHP)

High hydrostatic pressure (HHP) is a non-thermal technique, like HPH, that is used for increasing shelf life and food safety improvement by microbial disruption. In this treatment the food product is exposed to high levels of pressure, generally in a container filled with water (Salazar et al., 2021). The widespread pressure range changes between 100 to 800 MPa, depending on the targeted aim (Rendueles et al., 2011). The HHP process is isostatic since the transmission of pressure is uniform and instant, and it is adiabatic, meaning that there is a little change in temperature (about 3 ℃ increase for 100 MPa) as pressure increases, independent of the shape or size of the food (Rendueles et al., 2011).

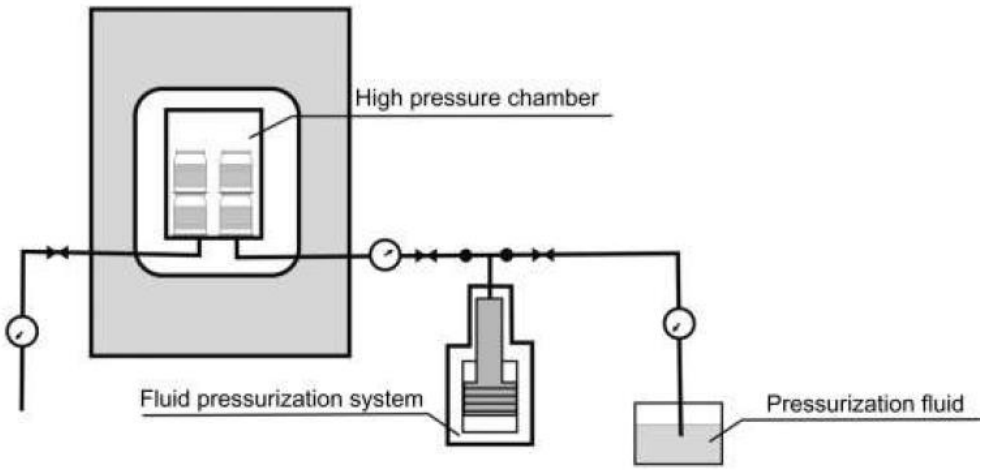

Figure 2.5. Schematic illustration of a HHP equipment (Zhang et al., 2022).

It is assumed that HHP kills cells by affecting permeability of cell membrane, altering cell morphology, changing reactions and biochemical processes in the cells, and disrupting the genetic mechanisms of cells. The simultaneous occurrence of multiple effects, involving both less critical components as well as critical parameters, is of critical importance for cell death (Sehrawat et al., 2020). Although the exact mechanism responsible for triggering cell death is not fully understood, it is hypothesized that the simultaneous action of proteins, ribosomes, and DNA may play a role in this process (Mañas & Pagán, 2005).

HHP has several advantages such as simple handling, short operation time and optimal preservation of the nutritional value of food (Zhang et al., 2022). Besides, when it is used for food products, they must have at least 40 % free water to obtain desired results. Also, when used for food products, they must have at least 40% free water to achieve the desired results and some spores have a high resistance to pressure, so very high pressures are needed to inactivate these microorganisms. Thus,

these factors can be considered as drawbacks of HHP treatment (Naveena & Nagaraju, 2020).

Since high hydrostatic pressure treatment is mostly used for food safety and shelf-life enhancement, there are not enough studies using oleaginous microorganisms, although it has been used on a variety of pathogenic microorganisms. On the other hand, there are studies using it for other microorganisms for the extraction of different bioactive compounds.

Recently, total lipid yield for *Lipomyces starkeyi* DSM 70295 by using high hydrostatic pressure (HHP) was studied, where apple pomace was used as carbon source and the maximum lipid yield was obtained as 45.8 % (w/w) at 200 MPa, 40°C operation temperature and 15 minutes of duration (Tuhanioglu et al, 2022).

2.8 Cheese Whey

Cheese whey, which has a yellow-green color is the by-product of cheese production obtained after the coagulation and removal of the curds. Around 85% of the milk utilized in the process of cheese production is extracted as whey during the process (Yüksel et al., 2019). It contains water, lactose as a milk sugar, proteins, minerals, and low amount of fat (Gutiérrez-Hernández et al., 2022). Also, it has leucine, isoleucine and valine, which cannot be produced in human body, thus consumption of whey is very important for human health (Soltani et al, 2017).

Whey can cause significant environmental problems if not evaluated. Approximately 180-190 million tons of whey waste are generated annually, and this amount is increasing due to the rising demand for coagulated milk products (Chandrapala, 2015). When whey, an organic waste product, is disposed of without undergoing any

treatment, it undergoes fermentation and poses a substantial risk of environmental pollution. The dumping of such waste into water bodies poses a grave threat to the organisms inhabiting those ecosystems (Yüksel et al., 2019).

The content of whey varies depending on how it is obtained (Table 2.4). Whey is divided into two categories depending on how it is produced. One of them is sweet whey which has a higher pH, and it is coagulated by using enzyme without any acidifications. The other one is acid (sour) whey which is formed by increasing acidity and it has a lower pH. (Bleoussi et al., 2020).

Table 2.4. The composition of sweet and acid whey. (Soltani et al, 2017).

Component	Sweet Whey (w/w %)	Acid Whey (w/w %)
Water	93-94	94-95
Dry matter	5.0-6.0	5.0-6.0
Lactose	4.5-5.0	3.8-4.3
Lactic Acid	Traces	Up to 0.8
Total protein	0.8-1.0	0.8-1.0
Whey protein	0.60-0.65	0.6-0.65
Citric acid	0.1	0.1
Minerals	0.5-0.7	0.5-0.7
pH	6.2-6.4	4.6-5.0

Whey can be used in various industries such as agriculture, food, dairy, animal feed, cosmetics, and pharmaceutical industries. In the food industry, whey serves multiple purposes. It can be used to enhance nutritional value of products and as an ingredient in functional beverages like sports drinks. Additionally, whey proteins find extensive use in the food industry due to their physicochemical and nutritional properties, which include acting as emulsifiers, water binders, and foaming agents. (Pires et al., 2021). In pharmaceutical industry, infant milk formulation is enhanced by using whey proteins (Gutiérrez-Hernández et al., 2022). On the other hand, whey finds applications in the dairy industry, such as enhancing the texture in cheese production and providing stability during freeze-thaw cycles in ice cream production. Whey components are used to modify the characteristics of these dairy products, ensuring desirable texture and quality (de Castro et al., 2017). Since whey has a high content of lactose and essential minerals, it is a suitable choice for animal feed (Schingoethe, 1976). Besides these industries, it has an industrial application. For instance, whey not only provides an optimal growth environment for various microorganisms but also serves as an ideal substrate in biotechnological processes. Its utilization as a low-cost substrate in fermentations supports microbial growth and enables the synthesis of value-added compounds. (Buchanan et al., 2023).

2.9 Aim of the Study

This study aims to be the first research in the scientific literature to investigate the application of high-pressure homogenizer (HPH) and high hydrostatic pressure (HHP) techniques for extraction of microbial oil produced by *Rhodosporidium toruloides* using whey hydrolysate, processed by the beta galactosidase enzyme, as a carbon source for the growth and lipid accumulation.

To achieve this goal following objectives were targeted:

- Determining the optimal fermentation conditions for *Rhodosporidium toruloides* in synthetic medium.
- Utilizing whey enzymatically hydrolysed for microbial oil production by *Rhodosporidium toruloides*.
- Disrupting the cells by using HPH and HHP methods.
- Employing response surface method (RSM) for optimization.
- Analyzing the results by conducting fatty acid composition analysis, measuring the peroxide value, and performing living cell counting.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

All the analytic grade chemicals and other materials utilized in this study were acquired from Sigma Aldrich and Merck, Turkey.

3.2 Microorganism activation and inoculum medium preparation

In this study, *Rhodosporidium toruloides* DMS 4444 was used, and this yeast were obtained in freeze dried form from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. To activate *R. toruloides*, a liquid medium containing 30.0 g/L of malt extract and 3.0 g/L of peptone was employed ("German Collection of Microorganisms and Cell Cultures GmbH: Details"). To preserve the cells at -80 °C, the activated cells were mixed with a solution consisting of 25 % glycerol and 25 % distilled water in 1.5 mL Eppendorf tubes. The preserved cells were periodically renewed.

After activation, cells were grown in yeast peptone dextrose (YPD) medium that contained 20.0 g/L glucose, 10.0 g/L peptone and 10.0 g/L yeast extract (Lyu et al., 2021).

Once the initial pH was adjusted to 5, the growth of *R. toruloides* was initiated by placing the culture on an orbital shaker (INFORS AG CH-4103, Bottmingen, Switzerland) set at 130 rpm and 30°C (Tuhanioğlu, 2021) for a duration of 24 hours

in 50 mL of YPD medium in 250 mL Erlenmeyer flasks. For measuring cell growth, a spectrophotometric method at a wavelength of 600 nm (Shimadzu UV-1280 spectrophotometer, manufactured by Shimadzu Corp. in Kyoto, Japan). Once the cells reached the desired optical density (OD) of 0.7 for *R. toruloides*, 3 mL of cell suspensions for 100 mL were added to the fermentation medium.

All the prepared media were sterilized using an autoclave (Autoclave, 56 MaXterile 60, Wisd Laboratory Instruments) at a temperature of 121 °C for a duration of 15 minutes.

3.3 Fermentation Medium

In this study, synthetic and hydrolyzed whey media were used. For starting fermentation process, 3 mL of culture from YPD medium was transferred to both 100 mL synthetic and hydrolyzed whey media in 500 mL Erlenmeyer flasks. The fermentation process was conducted for a duration of 8 days on an orbital shaker at agitation of 130 rpm and a temperature of 30 °C.

3.3.1 Synthetic Medium

The fermentation medium compositions with a C/N ratio of 55/1 included the following components: 1 g/L of di-sodium phosphate (Na_2HPO_4), 0.5 g/L of ammonium sulfate ($(NH_4)_2SO_4$), 2.5 g/L of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 12.5 g/L of potassium dihydrogen phosphate (KH_2PO_4), 0.25 g/L of calcium chloride dihydrate ($CaCl_2.2H_2O$), 1.9 g/L of yeast extract, and 40 g/L of carbon source (Gurel, 2019; Tuhanioglu, 2021)

As a carbon source, glucose, galactose, lactose, and glucose galactose mixture were used. Also, media that had different C/N ratios were prepared and they were obtained by adjusting the amount of nitrogen sources. Fermentation was done with 100 mL fermentation volume in 500 mL in Erlenmeyer flasks. In addition to using Erlenmeyer flasks for the fermentation process, a 2 L fermenter was also used.

3.3.2 Hydrolyzed Whey Medium

In this experiment, hydrolyzed whey was used as the fermentation medium. For the small-scale experiments, 100 mL of fermentation medium was prepared in 500 mL Erlenmeyer flasks, while for the larger-scale experiments, 2 L of fermentation medium was used in a fermenter.

3.3.2.1 Hydrolyzed Whey Medium Preparation

For this study, whey was obtained from a local producer named as Ataturk Orman Ciftligi (AOC), Ankara. Obtained whey was sterilized in an autoclave and stored at a cold room to prevent microbial activity.

The β-galactosidase enzyme used in the experiment was sourced from *Kluyveromyces lactis* (Lactozyme®, liquid form, > 3600 U.ml-1) and was purchased from Sigma-Aldrich (Darmstadt, Germany). This enzyme was employed to hydrolyze the lactose present in the whey. The pH was set to 7, and the 100 mL samples in 500 mL Erlenmeyer flasks were subjected to a 3-hour incubation at 50°C and 150 rpm on an orbital shaker, resulting in an efficiency of 98%. After hydrolysis,

hydrolyzed whey was autoclaved at 121 °C for 15 minutes to stop the enzyme activity.

3.3.2.2 Content of Whey

Elemental analysis was performed at the METU Central Laboratory to determine the concentrations of Potassium (K), Carbon (C), Magnesium (Mg), Nitrogen (N), Calcium (Ca), Phosphate (P), and Sulphur (S) in the samples, both before and after autoclaving.

The lactose concentration in the whey was determined using High Performance Liquid Chromatography (HPLC) at the METU-GAL laboratory. For calibration of HPLC, lactose standard solutions with concentrations of 10, 20, 30, 40, 50, and 60 g/L were used.

3.3.2.3 Culturing and Conditioning of Hydrolyzed Whey

Before autoclaving, pH of the medium was adjusted to 5.0 by using 10 molar NaOH and 10 molar H_2SO_4. Then, 3 % (v/v) of inoculum was added to 100 mL hydrolyzed whey medium in 500 mL Erlenmeyer flasks from YPD medium. Orbital shaker was used at 130 rpm and 30 °C with the initial pH 5 and this process took 8 days.

3.3.3 Fermenter Conditions and Preparation

Two liters of synthetic medium and hydrolyzed whey medium were put into 2-liter fermenter (Sartorius Stedim Systems GmbH, Schwarzenberger, Weg 73-79, DE-34212 Melsungen, Germany) and the samples were sterilized at a temperature of 121 °C for a duration of 30 minutes using an autoclave. For initiating the fermentation process, a 3 % (v/v) inoculum was transferred from YPD medium to the fermenter and this process was carried out at 130 rpm, 25 °C and 1 vvm aeration for 8 days. Besides, by using 10 M H_2SO_4 and 10 M NaOH, pH was kept at 5. To prevent excessive foaming and minimize the loss of the medium, a diluted solution of antifoam was employed.

3.4 Harvesting of Biomass

After an 8-day fermentation period, the biomass was separated from the fermentation medium by centrifugation. The remaining liquid medium from the fermenter and Erlenmeyer flasks were transferred to Falcon tubes, which have a volume of 50 mL. The tubes were then centrifuged at 8000 rpm for 10 minutes and rinsed two times with distilled water to eliminate the supernatant (Zhao et.al, 2008). Obtained biomass at bottom of the Falcon tubes was put into glass petri dishes and dried in an oven at 40 °C for one day.

3.5 Extraction of Lipid

The *R. toruloides* cells were disrupted using methods High-Pressure Homogenizer (HPH) and High Hydrostatic Pressure (HHP). Subsequently, the extraction of lipids was carried out using the HSH-assisted Bligh & Dyer method.

3.5.1 High Pressure Homogenizer (HPH)

The yeast cell disruption process was carried out using the domestically sourced GEA PandaPlus Lab Homogenizer 2000, which has a minimum sample volume of 100 mL and a minimum pressure value of 7 MPa, and a maximum pressure value of 200 MPa. Also, at its highest pressure, its flow rate is 9 L/s. Due to the homogenizer's unsuitability for flammable, volatile, or organic solvent usage, a synthetic fermentation medium and hydrolyzed whey fermentation medium were used without any additional solutions.

In one experimental series, cell disruption was performed using slurry of dry yeast, while in the other series, it was performed directly using the spent fermentation medium. For both series, different pass numbers (3, 5, 7, 9, 11, and 13) and pressure values (50, 100, 150, and 200) were applied to disrupt the cells. A 25 mL sample volume was used for each experiment, and the pressure values were manually adjusted. After each pass, the obtained samples were returned to the machine funnel. The samples that reached the desired number of passes were transferred to 50 mL Falcon tubes for subsequent steps.

3.5.2 High Hydrostatic Pressure (HHP)

A pressure equipment of type 760.0118 provided by SITEC-Sieber Engineering AG, Zurich, Switzerland, was used for the high hydrostatic pressure process. The pressure vessel had a capacity of 50 mL, an inner diameter of 24 mm, and a length of 153 mm. The pressure vessel was equipped with a built-in heating and cooling system, specifically the Huber Circulation Thermostat from Offenburg, Germany. A K-type thermocouple was employed to accurately measure and regulate the initial and processing temperatures during the experiment. The pressure vessel was filled with distilled water to transmit the pressure (Tuhanioglu, 2021).

For cell disruption, two different experimental designs were used by applying high hydrostatic pressure. In one of them, the cells to be disrupted along with the fermentation medium were transferred to 25 mL polyethylene cryotubes. The other one involving dry yeast biomass, dried *R. toruloides* cells were mixed with a chloroform-methanol solution (2:1 v/v) in 25 mL polyethylene cryotubes. After the HHP treatment, the samples were transferred to Falcon tubes for lipid extraction.

Three different parameters were used which were pressure, time, and amount of biomass when the dry biomass was used. On the other hand, when the fermentation medium was directly used, the parameters were pressure, temperature, and time. The experimental design for this study was developed using response surface methodology (RSM) with the assistance of Minitab 13 software (Minitab Inc., State College, PA, USA). The specific details of the RSM design can be found under the Experimental Design section (Chapter 3-3.9).

3.5.3 High Speed Homogenizer (HSH)

By utilizing the HSH method along with the Bligh and Dyer method, a comparative analysis was conducted to assess the differences between the HPH and HHP methods. The dry biomass was placed into 50 mL Falcon tubes with the chloroform-methanol (2:1 v/v) mixture in a ratio of 1:10 (w/v). This means that for every unit weight of the dry biomass, 10 units of chloroform-methanol solution were added. The mixture was processed for 6 minutes at a rotational speed of 12000 rpm using the IKA T18 digital ULTRA TURRAX high-speed homogenizer (HSH). This step was performed to disrupt the cell wall and enhance the extraction of lipids. To prevent excessive heating, homogenization was carried out in intervals of 25 seconds with 5-second breaks. Following the HSH process, 2.5 mL of 1 M NaCl was added to the mixture according to the instructions provided by the Bligh and Dyer method and the mixture was centrifuged at 8000 rpm for 10 minutes at temperature 4 °C in order to observe separation of the phases.

3.5.4 Bligh & Dyer Method

Following the HPH procedure, the samples collected in 50 mL Falcon tubes were combined with a mixture of chloroform-methanol (2:1 v/v) and 2.5 mL of 1 M NaCl. On the other hand, the samples obtained after HHP were only supplemented with 2.5 mL of 1 M NaCl solution (Bligh & Dyer, 1959). Subsequently, these mixtures underwent centrifugation at 8000 rpm for 15 minutes at 4°C using a centrifuge machine to facilitate phase separation which can be seen in Appendix A. The lower layer (red-orange color), which contained the chloroform and lipid solution, was carefully collected in glass petri dishes, and then these petri dishes were put into the drying oven to evaporate the chloroform from the chloroform-oil phase at 40°C and for a duration of 4 hours.

3.6 Measurement of Total Reducing Sugar

In order to measure the total reducing sugar, after every fermentation process residual sugar analysis was performed. To achieve this, a traditional method called the dinitro salicylic acid (DNS) residual sugar assay was conducted through a colorimetric approach. This technique relies on the presence of free carbonyl groups as indicators of reducing sugars (Miller, 1959).

DNS solution was prepared just before measurement. To prepare this solution, 10 g/L 3-5 dinitro salicylic acid, 10 g/L sodium hydroxide (NaOH), 2 g/L phenol, 0.5 g/L sodium sulfite (Na_2SO_3) and 256 g/L Rochelle salt ($KNaC_4H_4O_6.4H_2O$) was mixed. For analysis, 3 mL DNS solution, 2.8 mL distilled water and 0.2 mL cell free media that was centrifuged and diluted with the ratio of 1:100 (v/v) mixed in the glass tubes and for blank sample 3 mL DNS and 3 mL distilled water was used. These tubes were put into the water bath (Şimşek Laborteknik Ltd. Şti. 5BD-313) at 95 °C for 15 minutes. Then, tubes were cooled to room temperature and after cooling at a wavelength of 575 nm optical densities were measured. The obtained absorbance data from the samples were subsequently compared to a standard curve (Appendix B) that had been previously generated using known concentrations of a glucose stock solution.

3.7 Visual Analysis

3.7.1 Viable Yeast Cell Count

First of all, 9 mL of peptone water was prepared by mixing 1 g of peptone with 1 liter of distilled water. The sample was diluted by adding 1 mL of the sample to 9 mL of the prepared peptone water, and this dilution process was repeated until a

dilution rate of up to 10^{-6} was achieved. On the other hand, potato dextrose agar (PDA) was prepared. For preparation of it, 39 g PDA was suspended in 1 liter of distilled water. Then, this mixture was boiled until the all solute was dissolved. Both PDA and prepared peptone water were sterilized by autoclave at 121 °C for the duration of 15 minutes. After sterilization, PDA (Potato Dextrose Agar) was supplemented with ten percent sterilized tartaric acid to inhibit bacterial growth by reducing the pH (*Potato dextrose agar* 2014). This mixture was poured into sterile petri dishes. From every diluted sample 0.1 mL was taken and spread on PDA by using spread plate technique. The Petri dishes were incubated at 30°C for 5 days, and after incubation, the colonies were counted. The results were reported as colony forming units (CFU) per mL.

3.7.2 Scanning Electron Microscope (SEM)

Samples collected from the optimal conditions based on the experimental design for both HHP and HPH methods were subjected to scanning electron microscope (SEM) analysis. The SEM analysis was conducted by the Central Laboratory of Middle East Technical University (METU).

Prior to the SEM analysis, samples were prepared by retrieving the biomass from the layer separated from their lipids using the Bligh & Dyer method. Distilled water was used to dissolve the biomass. Before being subjected to freeze drying, the diluted biomass was kept at -20 °C for one day. The cells were subsequently subjected to freeze-drying (Christ, Alpha 2-4 LD Plus) at a temperature of -85°C and a vacuum pressure of 0.15 mbar for a duration of 24 hours. Freeze dried cells were covered with thin gold layer at METU Central Lab and SEM images were taken at 10000 times magnification.

3.8 Lipid Analysis

3.8.1 Yield of Lipid

The lipid yield (Y) obtained after performing all these processes was calculated as the ratio of grams of lipid to grams of dry biomass.

$$Y = \frac{\text{gram lipid}}{\text{gram dry biomass}} * 100 \quad (1)$$

3.8.2 Peroxide Value

Peroxide value (POV) was determined based on the findings of Low and Ng (1987) with some minor changes. 25 mL chloroform-acetic acid ($CHCl_3$-CH_3COOH) with the ratio of 2:3 (v/v) was used for dissolving 0.5 grams of microbial oil. Ten grams of potassium iodide crystals (KI) were dissolved in 100 mL of distilled water, and then 5 grams of iodine were added to the solution. The solution was vigorously stirred. By using a Pasteur pipette one single drop of iodine solution was added to oil and chloroform-acetic acid solution and this mixture was kept at dark for 10 minutes to release iodine present in the solution. After 10 minutes, 1.5 % starch solution and 75 mL distilled water was added and titrated with 0.01 M sodium thiosulfate ($Na_2S_2O_3$) until colorless solution. The peroxide value was calculated by using the equation given below.

$$POV = \frac{(V_S - V_B) * N * 100}{W} \quad (2)$$

Where V_S; Volume of consumed $Na_2S_2O_3$ for sample (mL),

V_B; Volume of consumed $Na_2S_2O_3$ for blank (mL)

N; Normality of $Na_2S_2O_3$

W; Weight of oil in the volume of extract.

3.8.3 Fatty Acid Composition

The yeast oil samples were prepared for the purpose of determining the fatty acid composition and this fatty acid methyl ester analysis was performed at Middle East Technical University Food Engineering GAL laboratory by using gas chromatography (GC).

For this analysis 80 mg dry oil sample was put into Eppendorf tubes and 800 µL isooctane was added to tubes to dissolve the samples. Then, 500 µL 2 molar methanolic potassium hydroxide was added to the tubes and tubes were centrifuged for 6 minutes. After the phase separation occurred and for the GC analysis sample was collected from the upper phase and injected to the GC.

The GC instrument used in the analysis was equipped with a CP-Sil 88 capillary column (50m x 0.25mm ID, 0.20 µm film), an FID detector, and an automatic injection system. The analysis was performed using a flow rate of 1 mL/min of helium gas as the carrier gas. The injector temperature was adjusted to 225 °C, and the detector temperature was set to 285 °C. The total analysis time was 15 minutes.

3.9 Experimental Design

The experiments conducted in the study were designed using the MINITAB 13 software. MINITAB 13 is a statistical software package that offers various tools and features for experimental design and data analysis. Response Surface Methodology (RSM) is a statistical technique used for optimizing and modeling the relationship between multiple independent variables and a response variable of interest.

To create a model for lipid extraction using the HHP (High Hydrostatic Pressure) technique, the Box-Behnken response surface method was employed. The model incorporated the following parameters: pressure, time, dry biomass amount (when dry biomass was used), and pressure, temperature, and time (when the fermentation medium was directly used). The Box-Behnken design is a widely used response surface methodology that allows for the efficient exploration and optimization of multiple factors and their interactions. Table 3.1 displays the parameters and their maximum and minimum values used for the dry biomass experiment, while Table 3.2 illustrates the parameters used for the experiment where the fermentation medium was directly employed. On the other hand, using these parameters in the MINITAB 13 software, 13 different combinations were generated, and a total of 30 runs were conducted separately for each of the two experiments (Table 3.4 and Table 3.5). The response variable measured in both experiments was the lipid yield on a dry biomass basis. Also, the custom-determined parameters for HPH were illustrated in Table 3.3 and according to these parameters conducted 24 runs were displayed at Table 3.6.

Table 3.1. Parameters used in dry biomass experiment in Box-Behnken design for HHP assisted lipid extraction.

Parameter	Low (-1)	High (+1)
Pressure	100	300
Time	5	15
Dry biomass	1	3

Table 3.2. Parameters for spent fermentation medium in the Box-Behnken design for HHP assisted lipid extraction.

Parameter	Low (-1)	High (+1)
Pressure	100	300
Time	5	15
Temperature	30	50

Table 3.3. Parameters for HPH assisted lipid extraction.

Parameter	Low (-1)	High (+1)
Pressure	50	200
Pass Number	3	13

Table 3.4. The Box-Behnken response design for dry biomass experiment of HHP assisted lipid extraction.

Run	Pressure (MPa)	Time (min)	Dry biomass (g)
1	200	5	3
2	100	5	2
3	300	5	2
4	100	10	1
5	300	15	2
6	200	15	1
7	100	15	2
8	200	10	2
9	300	10	3
10	300	10	1
11	200	10	2
12	100	10	3
13	200	15	3
14	200	5	1
15	200	10	2

Table 3.5. The Box-Behnken response design for spent fermentation medium experiment of HHP assisted lipid extraction.

Run	Pressure (MPa)	Time (min)	Temperature (°C)
1	200	5	30
2	300	10	30
3	300	10	50
4	200	15	50
5	200	10	40
6	300	5	40
7	200	5	50
8	300	15	40
9	200	10	40
10	200	15	30
11	100	15	40
12	100	5	40
13	200	10	40
14	100	10	50
15	100	10	30

Table 3.6. Experimental design for HPH assisted lipid extraction.

Run	Pressure (MPa)	Time (min)
1	50	3
2	50	5
3	50	7
4	50	9
5	50	11
6	50	13
7	100	3
8	100	5
9	100	7
10	100	9
11	100	11
12	100	13
13	150	3
14	150	5
15	150	7
16	150	9

Table 3.6 (continued)

17	150	11
18	150	13
19	200	3
20	200	5
21	200	7
22	200	9
23	200	11
24	200	13

In order to develop a predictive model for the impact of HHP on lipid yield, the experimental data was fitted to a quadratic polynomial equation, which can be expressed as follows:

$$Y = \beta_0 + \sum_{i=1}^{2} \beta_i X_i + \sum_{i=1}^{2} \beta_i X_i^2 + \sum_{i=1}^{2} \sum_{j=1}^{2} \beta_{ij} X_i X_j \quad (3)$$

The equation relates the response variable, denoted as Y, to the independent variables were represented by i and X_j. The coefficients in the equation, β_0, β_i, β_{ij}, and β_{ii}, represent the constant term, linear coefficients, cross-product coefficients, and quadratic coefficients, respectively.

For analyzing error between predicted and experimental data, root mean square error (RMSE) and mean absolute error (MAE) were calculated by following equations.

$$\mathrm{RMSE} = \left[\sum_{i=1}^{n} \frac{(y_i - y_p)^2}{n}\right]^{\frac{1}{2}} \quad (4)$$

$$\mathrm{MAE} = \left(\frac{1}{n}\right) * \sum_{i=1}^{n} |y_i - y_p| \quad (5)$$

In the equation, N represents the number of data points. y_i refers to the predicted values, while y_p represents the experimental values.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth Curve and Sugar Consumption of *Rhodosporidium toruloides* DSM 4444 in Fermentation Medium

4.1.1 Growth in Synthetic Medium

Different synthetic media were prepared to determine the optimum conditions for maximum lipid yields. Therefore, obtained results were used as a guide for fermentation conditions for hydrolyzed whey.

4.1.1.1 Different Carbon Sources

As mentioned before, *Rhodosporidium toruloides* can grow on various carbon sources, including glucose, galactose, fructose, xylose, and glycerol (Bommareddy et al., 2015). *R. toruloides* showed different growing behavior in different synthetic sugars. Results of growth rate and sugar consumption of *R. toruloides* in different fermentation media are shown in the following figures. These media contained glucose, galactose, lactose and the mixture of glucose and galactose (ratio of 1:1).

As seen in the Figure 4.1, the growth rate of *R. toruloides* increased rapidly from 4th day and 6th days, and then continued to rise with a decreasing rate until the 8th day. At the end of the 8th day obtained OD_{600} value was 18.1±0.5. Sugar consumption rate was proportional to increase in growth rate. Between days 2 and 4, sugar content of medium slightly decreased but at day 4 it was decreased dramatically until the end

of fermentation. Glucose concentration was initially set at 40 g/L, and it was measured as 4.9 ± 0.5 g/L at the 8th day by using DNS assay. The reduction in sugar concentration shows *R. toruloides* can utilize almost 88% of glucose within 192 hours fermentation. According to Osorio-González et al. (2022), the maximum consumption of glucose utilization was achieved as 99.7% after 144 hours when 50 g/L glucose was used. In the same study, for 75 g/L and 100 g/L glucose, the consumption was found as 97 and 86.5% at 144 hours, respectively while for 120 g/L glucose, 80% consumption was observed at 96 hours. This study clearly demonstrated that the initial glucose concentration and fermentation time directly affect the consumption rate of glucose. The mentioned study supports the result obtained from this study.

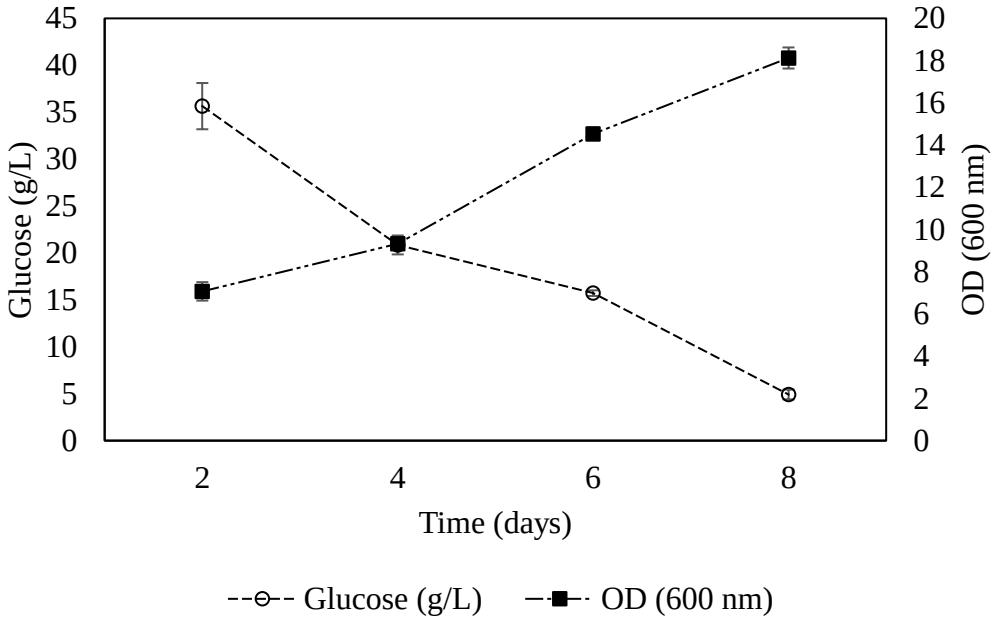

Figure 4.1. Growth and glucose consumption of *R. toruloides* in synthetic medium.

In the galactose medium, cell growth and sugar consumption showed almost the same pattern to in glucose medium. However, different from the other medium in this medium cell growth rate experienced a notable increase from 2nd day until 6th day of fermentation. After day 6, the growth rate almost came to stop and showed minimal increase until the day 8. At the end of the process, OD_{600} value and sugar concentration were found to be 17.9±0.03 and 1.9±0.05 g/L, respectively (Figure 4.2). In the study conducted by Jagtap et al., (2019), it was reported that *R. toruloides* has an ability to produce sugar alcohol, galactitol via conversion of galactose. Therefore, the reason for increasing sugar consumption rate after 6th day even not too much change in growth could be attributed to the galactitol production in the medium.

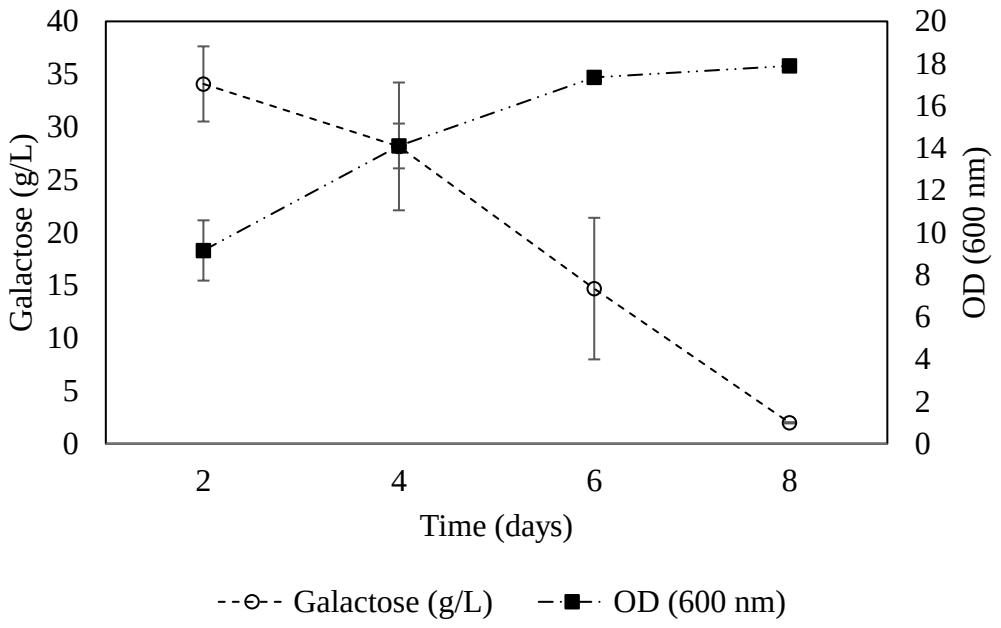

Figure 4.2. Growth and galactose consumption of *R. toruloides* in synthetic medium.

Unlike the noticeable growth observed in media that contained glucose and galactose, there was minimal growth observed in media that contained lactose. As shown in Figure 4.3, growth of *R. toruloides* displayed a gradual and slight increase until day 2. However, after reaching its peak, the growth started to decline steadily. In proportion to this, not much change was observed in sugar consumption. The maximum and final OD_{600} values and final sugar concentration were found as 5.8±1.0, 4.2±0.9 and 31.6±0.7 g/L, respectively (Figure 4.3). There is a scarcity of consumption of lactose by *R. toruloides* in the literature. In one study, it was reported that *R. toruloides* cannot consume lactose (Sampaio, 2011). Nevertheless, the decrease in the growth declines and the increase in sugar consumption after the 6th day could potentially serve as indicators of an ongoing adaptation process. Therefore, it was recommended to investigate this phenomenon in further studies.

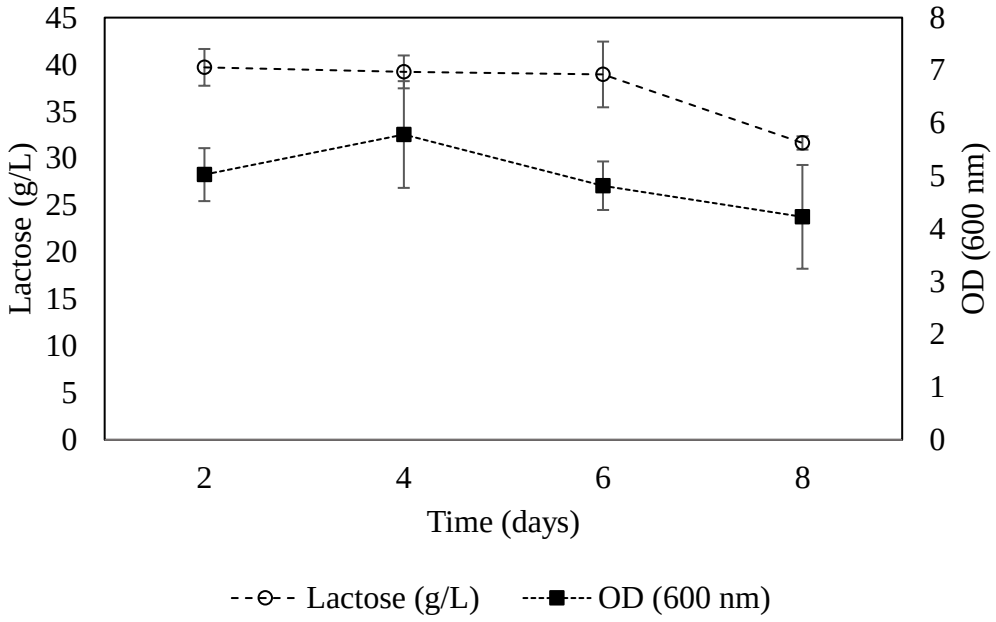

Figure 4.3. Growth and lactose consumption of *R. toruloides* in synthetic medium.

Since lactose was not utilized by *R. toruloides*, the medium contained glucose and galactose mixture (1:1) was prepared to mimic lactose hydrolysate. It was observed that the growth and sugar consumption had similar patterns in the medium containing glucose and galactose. In this experiment, growth increased from the 2nd day to 6th day gradually then its increase accelerated. On the other hand, sugar consumption was dramatically increased from day 2 to 4 and 6 to 8. The final optical density was 18.6±0.3 and sugar concentration was 3.8±0.2 g/L (Figure 4.4).

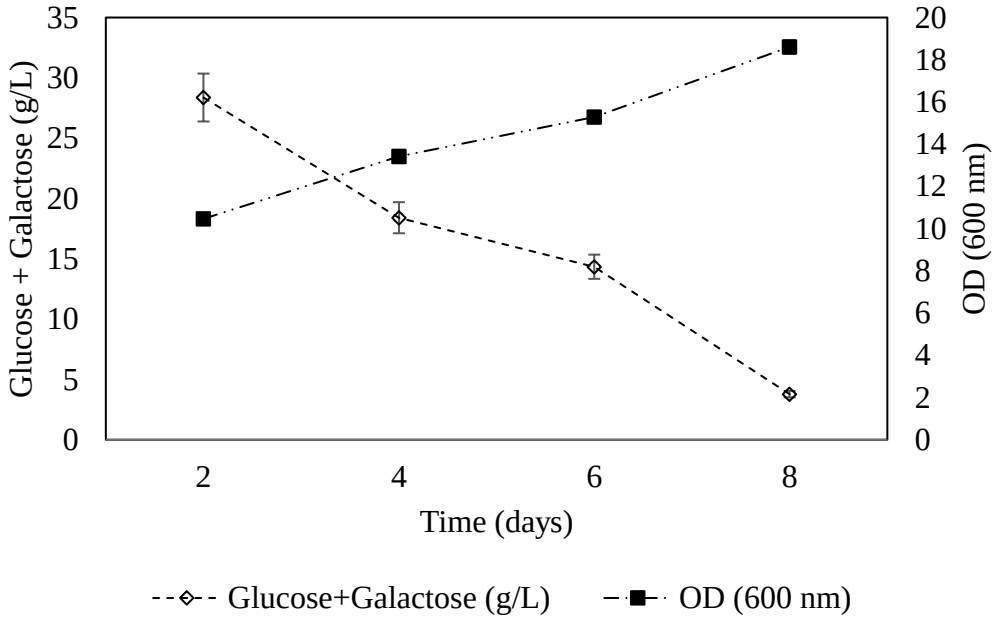

Figure 4.4. Growth and glucose + galactose consumption of *R. toruloides* in synthetic medium.

4.1.1.2 Different Nitrogen Sources

Nitrogen source is one of the important factors for growth of oleaginous microorganisms. Therefore, different nitrogen sources were tested. According to results, the maximum optical density was obtained when only yeast extract was used in the medium as a N source. The values of OD at the end of the 8 days were found as 16.3±2.6, 12.7±0.9, and 17.8±1.1 when ammonium sulfate + yeast extract, ammonium sulfate and yeast extract was used, respectively (Figure 4.5).

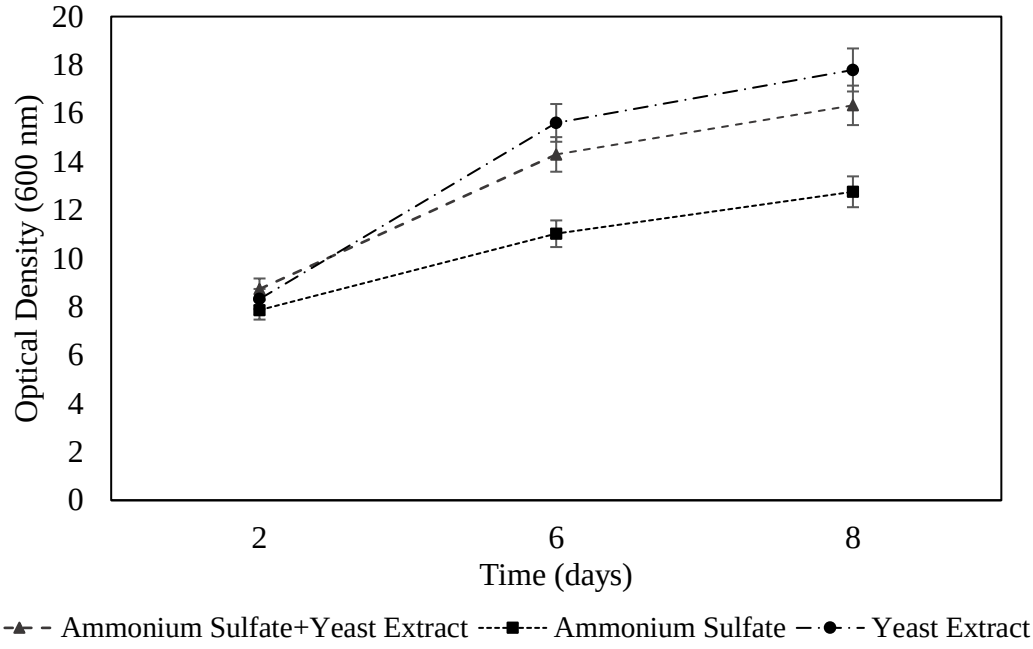

Figure 4.5. Growth of *R. toruloides* by using different nitrogen sources in synthetic medium.

4.1.1.3 Different C/N Ratios

C/N ratio is an important and critical factor for cell growth. To understand the effect of C/N ratio on growth of *R. toruloides,* different experiments were conducted at C/N ratio of 30/1, 55/1, 100/1, and 170/1 with glucose, galactose, and glucose + galactose mixture (1:1 w/w) as carbon sources.

The results revealed that the best growth was consistently observed at C/N=55/1 for all carbon sources tested. Furthermore, the final optical density values were measured as 18.1±0.5 for the glucose medium, 17.9±0.03 for the galactose medium, and 18.6±0.03 for the glucose + galactose medium. These results indicate that C/N 55 provides an optimal environment for growth across various carbon sources, with glucose + galactose medium showing the highest final optical density value among the tested conditions. In contrast, the lowest growth was consistently observed at C/N=170/1 for all carbon sources investigated. The final optical density values were measured as 14.8±0.7 for the glucose medium, 11.5±0.4 for the galactose medium, and 15.2±0.2 for the glucose + galactose medium (Figure 4.6). These findings highlight the inhibitory effect of a high C/N ratio on microbial growth. Gurel (2019) studied the effects of C/N ratios of 50/1, and 110/1 for *R. toruloides* and reported the optical density values at C/N=50/1 to be significantly higher than those obtained at a C/N ratio of 110/1.

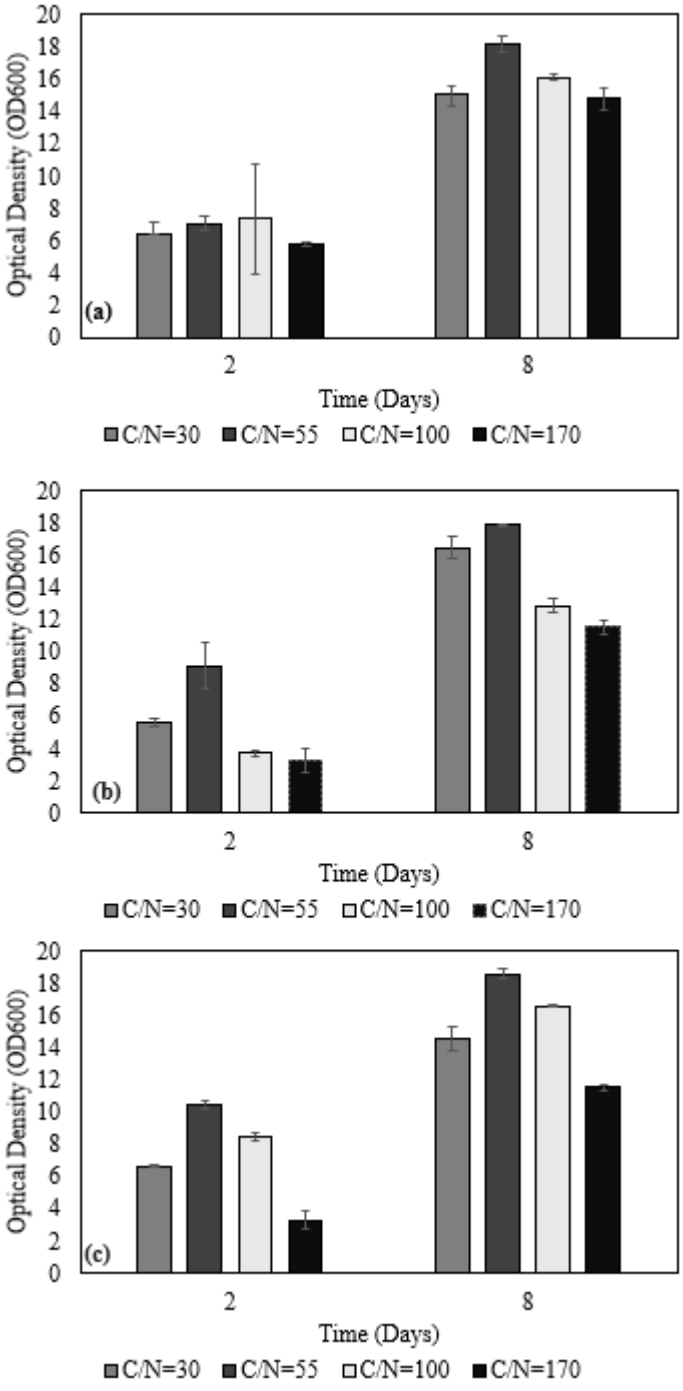

Figure 4.6. Growth of *R. toruloides* at different C/N ratios in (a) glucose, (b) galactose, and (c) glucose + galactose media.

4.1.1.4 Different Inoculum Amount and Fermentation Duration

Besides the factors mentioned above, different inoculum amount and fermentation duration also affect growth of *R. toruloides*. The obtained results are illustrated in Figures 4.7-4.8. When fermentation was initiated with different amounts of inoculum almost the same tendency in growth and final optical density values were observed. According to results the final optical density values were 14.6±0.5, 15.2±0.1, 15.5 and 16.5±0.6 for inoculation amount of 3 mL, 4 mL, 5 mL, and 6 mL, respectively.

On the other hand, when fermentation time was considered, the same trend was observed. Between days 2 and 6, there were significant rises and after 6 days a decrease was observed. The determined optical densities were 13.5±0.9, 14.7±0.5 and 15.1±0.7 for fermentation durations of 6,8, and 10-days, respectively.

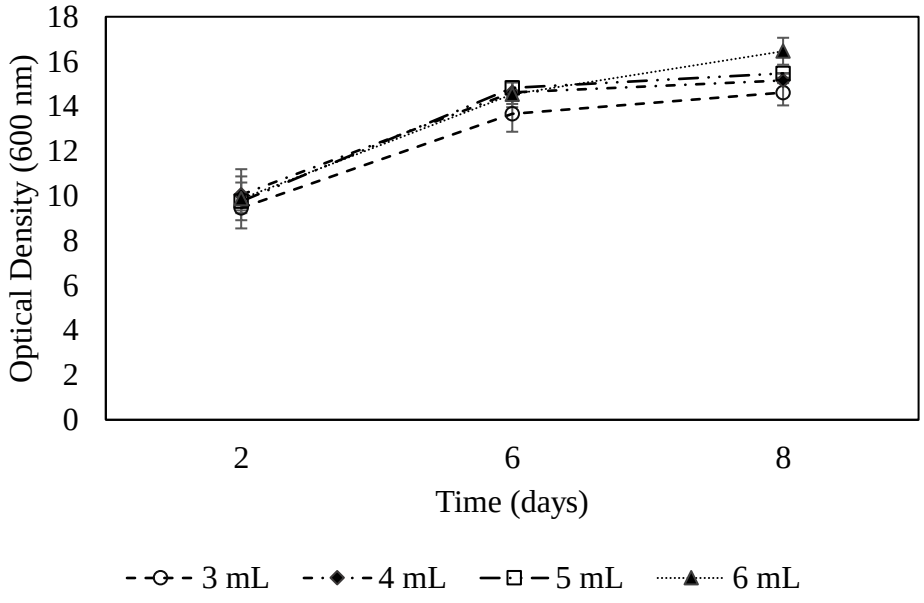

Figure 4.7. Growth of *R. toruloides* with different initial inoculum in synthetic medium

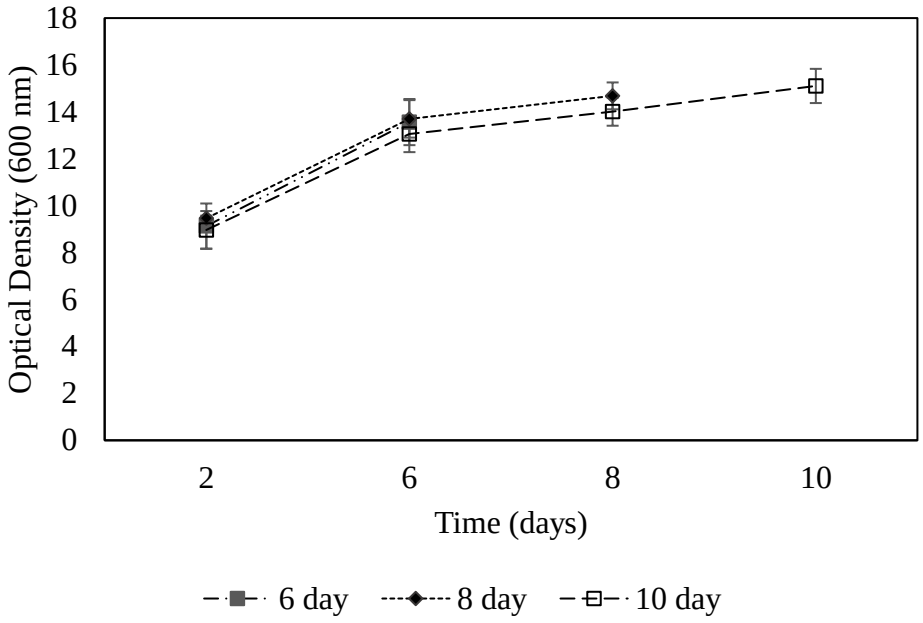

Figure 4.8. Growth of *R. toruloides* with different fermentation times in synthetic medium.

4.1.2 Growth in Hydrolyzed Whey Media

With the light of previous experiments for growth of *R. toruloides* under different medium and environmental conditions, the yeast was grown in hydrolyzed whey media. For that experiment, other than mentioned factors also temperature and initial pH effects on growth, biomass, and lipid production were evaluated. According to results, it is obvious that growth is much lower at 35 ℃ than the other investigated factors. While at 30 ℃ and at the initial pH 5.0 growth dramatically increased between days 2 and 4, this increase was observed at days 2 to 6 and 6 to 8 for the experiments of 35 ℃ and initial pH of 5.5, respectively. For all conditions, the maximum optical density was observed as 16.58±0.8 for 30 ℃, 12.85±1.6 for 35 ℃, 16.35±0.8 for initial pH 5.0 and 15.55±1.2 for initial pH 5.5 (Figure 4.9).

Singh (2021) studied the effect of different temperatures and different pH values for *R. toruloides.* In that study, similar results were obtained and the best temperature for growth found as 27 ℃ over the temperatures 33 ℃ and 37 ℃. Also, in that study the highest optical density was obtained at pH 6.5 and the lower one was found at pH 4.0. Although the temperature results are similar to this study, pH experiment results show differences. Using different strains of *R. toruloides* could be one reason for obtaining different results.

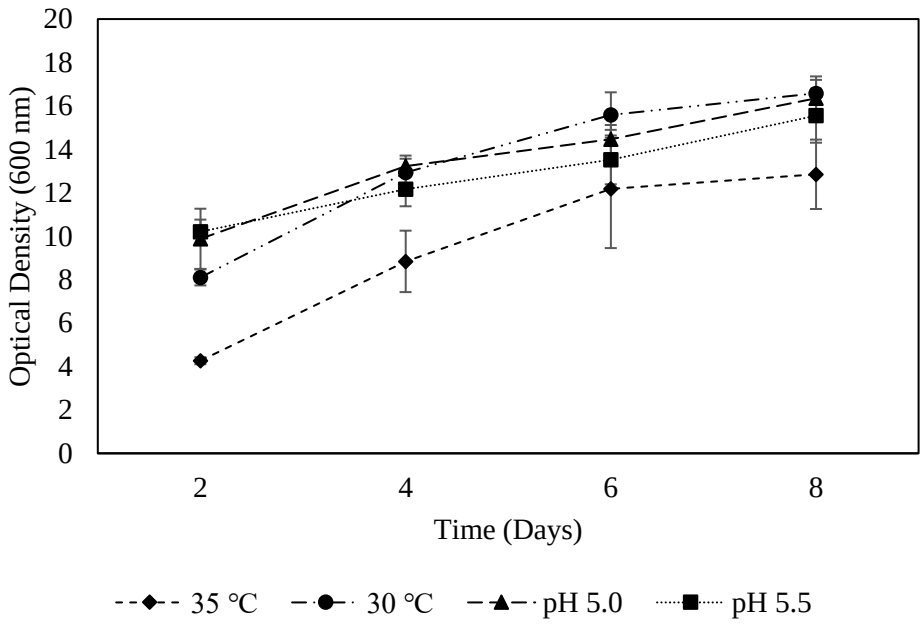

Figure 4.9. Growth of *R. toruloides* with different fermentation temperature and initial pH values in hydrolyzed whey medium.

4.2 Biomass Production and Lipid Yield

4.2.1 Synthetic Medium

After fermentation process, dry biomass of *R. toruloides* was obtained by using centrifuge and dry oven. Lipid yields for all conditions were calculated and these results were used for determining the conditions of fermentation process in whey hydrolyzed medium. In this step, to extract lipid from yeast high speed homogenizer (HSH) was used.

R. toruloides demonstrates enhanced growth in a medium containing galactose when the C/N ratio is 30. Conversely, there is a decline in biomass accumulation when the C/N ratio is 55/1, 100/1, and 170/1. The obtained value of maximum biomass is 13.7±0.25 g/L. On the other hand, the biomass accumulation in lactose medium is remarkably low compared to others, (3.4±0.12 g/L) at C/N=55. The maximum and minimum biomass contents in glucose medium were found as 11.44±0.23 g/L and 9.02±1.87, respectively at C/N=30/1 and C/N=170/1.

In the study of Kraisintu et al. (2010), different C/N ratios (65/1, 90/1, 115/1, 140/1) were examined to optimize lipid and biomass accumulation. The study reported a direct impact of C/N ratio on biomass production, as evidenced by varying biomass production at different C/N ratios. Biomass production decreased from 10.52 to 6.79 g/L at C/N 65/1 and 140/1, respectively. Consequently, increasing C/N ratio resulted in a decrease in biomass production. This mentioned study confirms that biomass production is reduced in media containing glucose and galactose at high C/N ratio, as depicted in Table 4.1.

Table 4.1. Biomass and lipid content of *R. toruloides* by utilizing different carbon sources at different C/N ratios in synthetic medium.

Carbon Source	C/N Ratio	Biomass (g/L)	Lipid Yield (w/w %)
Glucose	30	11.44	17.37
Glucose	55	10.83	41.38
Glucose	100	10.06	41.63
Glucose	170	9.02	43.50
Galactose	30	13.68	11.62
Galactose	55	12.07	27.25
Galactose	100	6.14	13.98
Galactose	170	4.43	17.12
Glucose+Galactose	30	9.52	10.75
Glucose+Galactose	55	11.24	35.62
Glucose+Galactose	100	9.70	26.38
Glucose+Galactose	170	8.13	33.25
Lactose	55	3.36	9.20

The maximum lipid yield (w/w %) was found as 43.5±5.3 % for glucose containing medium at the C/N ratio of 170/1. In the other media containing galactose, and

glucose-galactose mixture lipid yields were determined as 17.12±3.85, and 35.62±6.72% at C/N=170/1 and C/N=55/1, respectively. On the other hand, for the lactose-containing medium, only the C/N ratio of 55/1 was tested, and the oil yield was found to be 9.2±3.12%.

In the literature, the impact of C/N ratio on lipid production was also investigated, revealing that an increase in C/N ratio resulted in an increase in lipid yield. In one study, when the ratios were 65/1 and 140/1, the lipid yields were determined as 25.84 and 62.3 (w/w %), respectively (Kraisintu et al. 2010). The results of this study are supported by previous findings as they show that maximum lipid yield is observed at the highest C/N ratios for media containing glucose and glucose-galactose media.

The study also examined the influence of nitrogen sources on biomass and lipid accumulation. The highest lipid yield of 38.7±4.3% (w/w) was achieved when both yeast extract and $(NH_4)_2SO_4$ were used. Meanwhile, the lipid yields for other nitrogen sources were determined as 34.6±4.9% for only yeast extract and 32.7±5.6% for only $(NH_4)_2SO_4$ containing media. Regarding biomass production, the values at the end of the fermentation were found to be 10.02±1.1 g/L, 10.64±0.4 g/L, and 6.44±1.0 g/L for $(NH_4)_2SO_4$+yeast extract, yeast extract, and $(NH_4)_2SO_4$ containing media, respectively (Figure 4.10).

There are several studies in the literature about the effect of nitrogen sources on biomass and lipid productions in oleaginous microorganisms. Alakraa et. al (2020) also conducted a similar study by using *R. toruloides* and in this study the highest biomass and lipid yield were obtained as 35.4±1.28 and 44.8±5.42, respectively, when yeast extract was used.

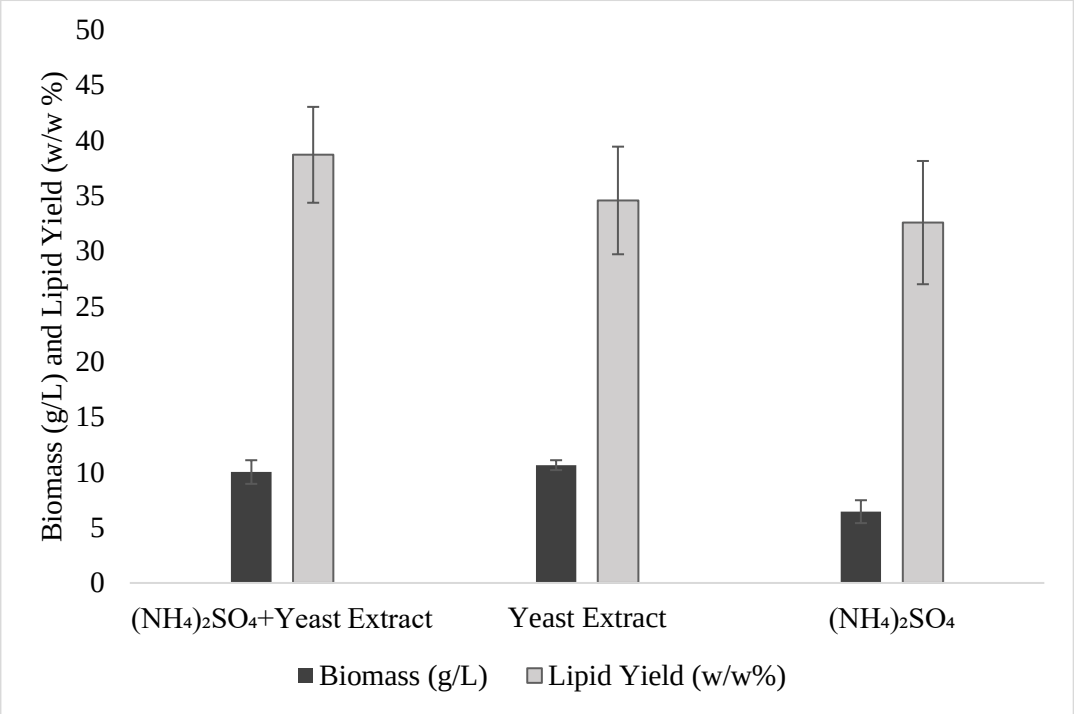

Figure 4.10. Biomass and lipid content for *R. toruloides* by using different nitrogen sources.

Although fermentation duration seems to have an insignificant effect on biomass production, it was observed that increasing time, also increases the lipid accumulation in the cell at some point. Moreover, after that point, lipid accumulation decreases (Figure 4.11). The maximum biomass production of 7.6±1.02 g/L and lipid yield of 44.3±0.35% were obtained on day 8. Ye et al., (2021) also reported similar results by changing process time and stated day 8th to be the optimum duration for lipid yield of *R. toruloides.*

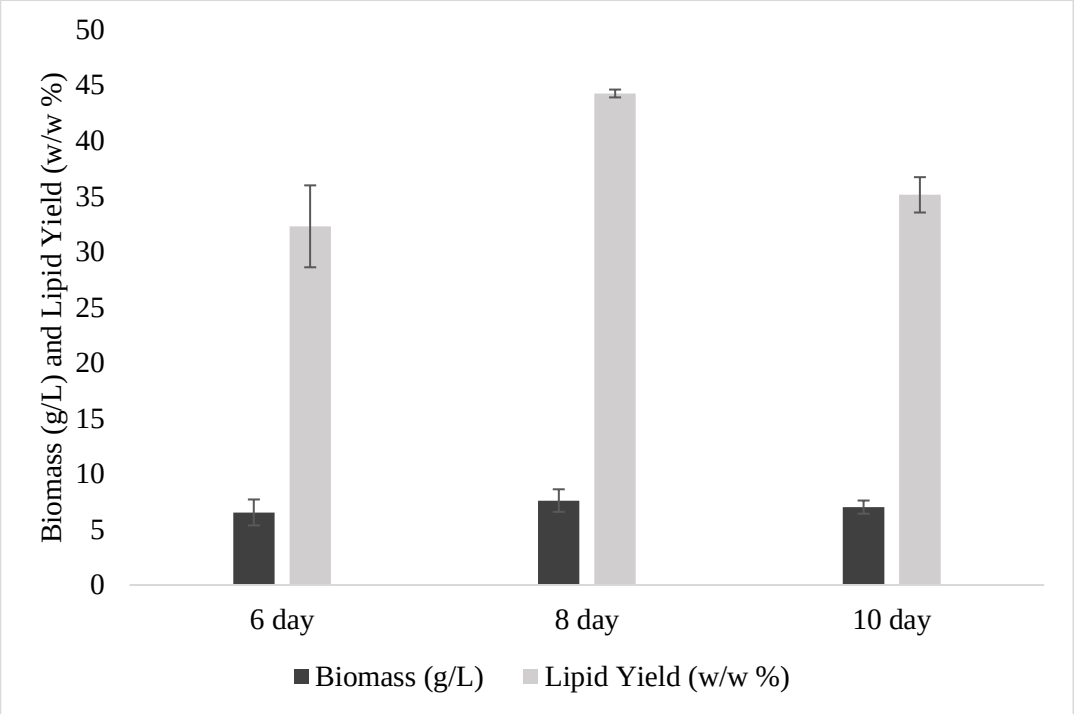

Figure 4.11. Biomass and lipid content of *R. toruloides* at different fermentation durations.

To complete this study on determining the optimal fermentation conditions, the inoculum size was also investigated. According to results, biomass production did not exhibit a significant increase across different inoculum amounts. However, the maximum lipid yield was obtained with 3 mL giving 41.9±2.92% lipid yield. It is noteworthy that the highest biomass value observed was 9.2±1.13 g/L for a 6 mL inoculation amount, but this value did not show a substantial difference compared to the other inoculation amounts tested (Figure 4.12).

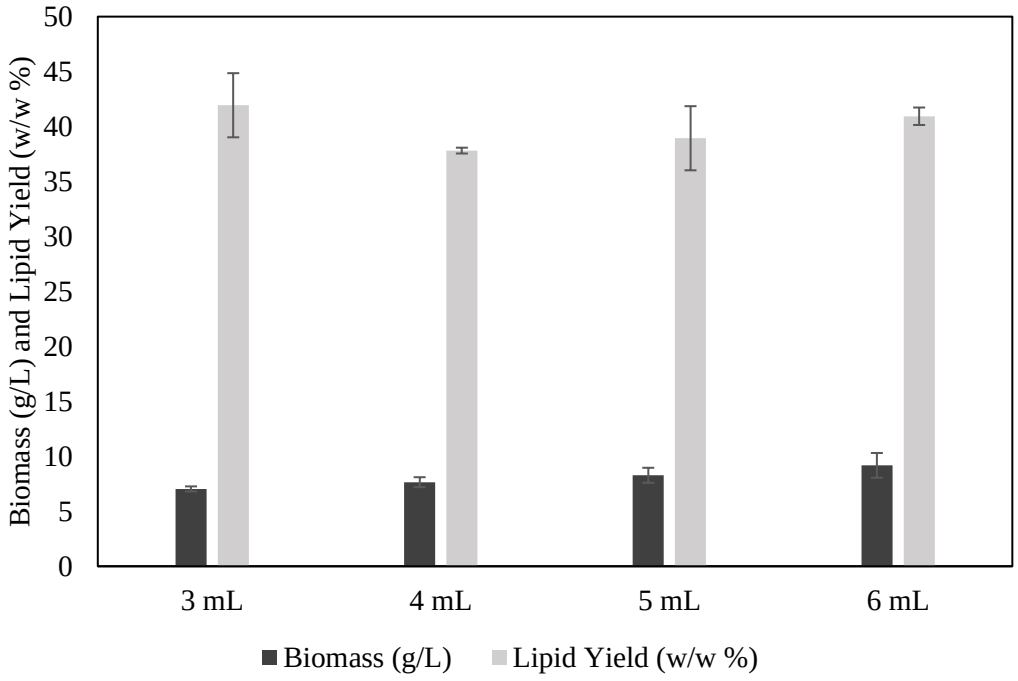

Figure 4.12. Biomass and lipid content of *R. toruloides* with different inoculum amounts.

4.2.2 Hydrolyzed Whey Medium

In this step, temperature and initial pH effects on biomass and lipid yield were investigated. For this purpose, hydrolyzed whey medium at 30℃, 35℃, and at initial pH values 5 and 5.5 media were prepared. For the temperature experiments initial pH was not arrange and pH value experiments temperature was set at 30℃. As an extraction method, high speed homogenizer (HSH) was used. Regarding biomass production and lipid accumulation it was found that at the temperature of 30℃, higher biomass was obtained than that of 35℃ (Figure 4.13). Likewise, when initial pH was set to 5.0 biomass production was higher than when pH was 5.5. Moreover, the results for lipid yield also showed the same trend with biomass production. The

maximum biomass and lipid production was found as 12.6±0.9 g/L and 34.3±0.5 % for 30 ℃, 12.9±1.2 g/L and 43.9±4.4 % for initial pH 5.0.

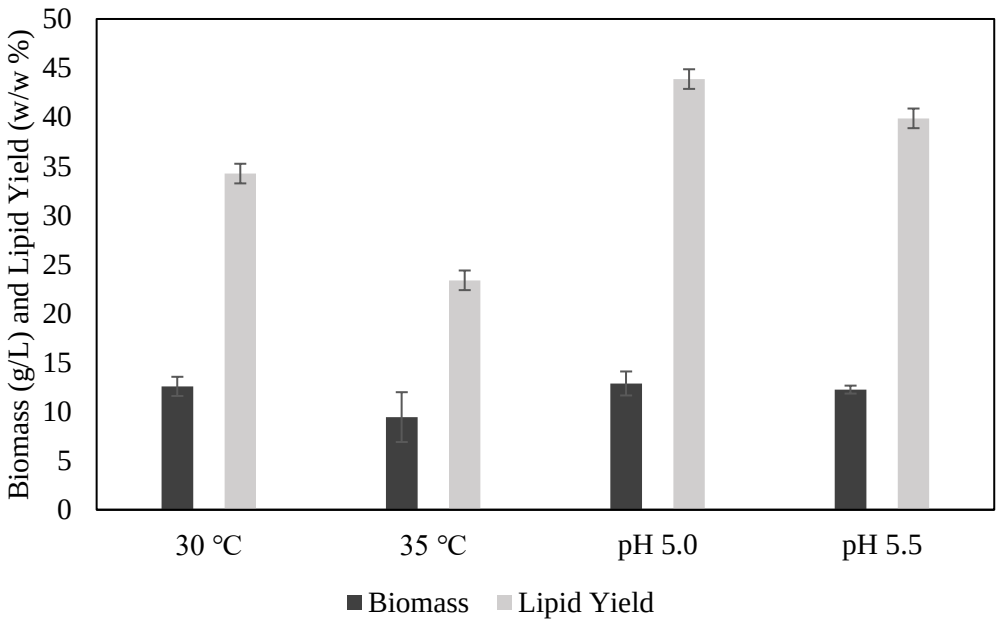

Figure 4.13. Biomass and lipid content of *R. toruloides* at different temperatures and initial pH values.

Moreover, to compare the lipid yield results obtained from HPH and HHP assisted extraction method. The HSH assisted method was also used as a control in hydrolyzed whey as a fermentation medium.

4.3 HPH Assisted Microbial Oil Extraction and Lipid Yields

4.3.1 Lipid Extraction from Synthetic Media

In this experiment, microbial lipid production was achieved by combining two approaches: mixing the dry biomass obtained from yeast cultivated in a synthetic medium with an organic solvent, and direct pressurization of the spent fermentation medium.

Both approaches resulted in the production of microbial lipids, with the extraction and yield depending on the specific experimental conditions, such as the pressure applied and the number of passes. These methods allowed for the efficient extraction of lipids from *R. toruloides* cultivated in synthetic media, providing alternative strategies for microbial lipid production.

According to the results (Figure 4.14) of which fermentation medium was directly pressurized, the highest lipid yield of 52% was observed at a pressure of 200 MPa, with 7 passes. At a lower pressure of 50 MPa, the lipid yield showed an increase from 20 to 32% when 3 to 9 pass numbers were used. At a pressure of 100 MPa, the lipid yield changed between 28 to 40%.

Further experiments were conducted at a pressure of 150 MPa, where all passes resulted in a lipid yield ranging from 40 to 47 %. Finally, at a pressure of 200 MPa, all passes consistently yielded a lipid yield greater than 44%, with the maximum lipid yield of 52% achieved with 7 passes.

These results highlight the influence of pressure and the number of passes on the lipid yield of *R. toruloides*. Higher pressures, particularly at 200 MPa, and a greater number of passes, such as 7, proved to be favorable conditions for obtaining higher lipid yields.

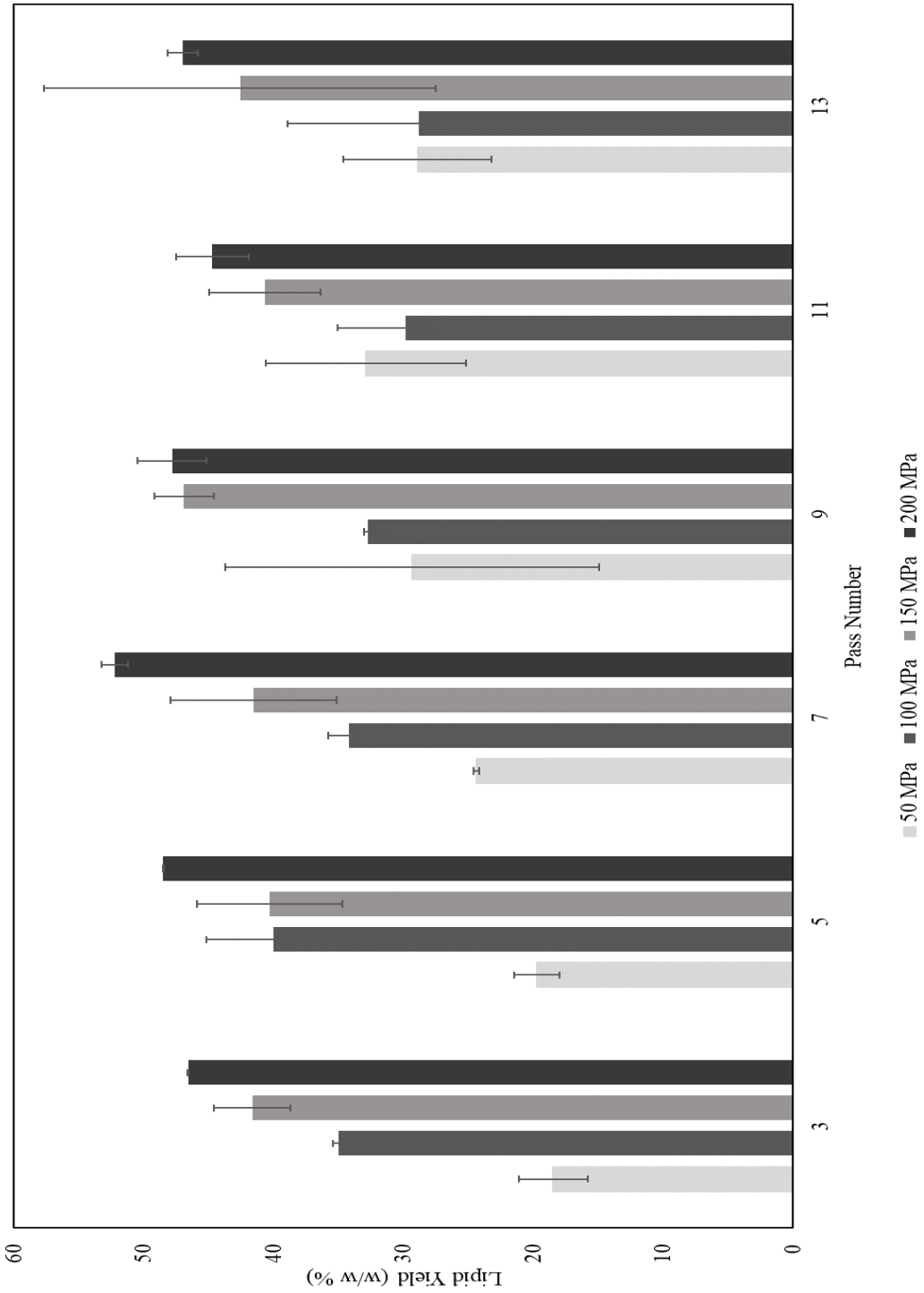

Figure 4.14. Lipid yield (w/w %) of HPH experiment (using spent fermentation medium)

On the other hand, after culturing in a synthetic medium for 8 days, the biomass of *Rhodosporidium toruloides* was dried, and for each experiment, a solution of 200 mL of pure water and 1.6 grams of dried biomass was used in the HPH device. The optical density (OD_{600}) of the yeast represented the solid-liquid ratio, and the OD value for *R. toruloides* was determined as 12.8. In this experimental design, pressures ranging from 50-200 MPa and passage numbers ranging from 3 to 13 were applied and lipid yield was calculated.

The highest lipid yield of 40% for *R. toruloides* was obtained at a pressure of 200 MPa with 9 passes (Figure 4.15). Sequentially, at 50 MPa pressure, a decrease in lipid yield was observed after 3 passes (26%), but it reached 30 % at 13 passes. At 100 MPa pressure, the number of passes did not lead to a significant increase, and the lipid yields varied between 10 and 26 %. At 150 MPa pressure, a lipid yield of 33.3% was obtained at 9 passes, while the lipid yield ranged from 16.7 to 33.3 %. Lastly, at 200 MPa pressure, the lipid yield varied between 25 and 40 %, with a maximum lipid yield of 40% achieved at 9 passes. In the HPH experiment conducted using the fermentation medium, the highest lipid yield was 52.2 % at 200 MPa and 7 passes, whereas in the HPH experiments conducted using the solution of dried biomass with pure water, the highest lipid yield of 40 % was observed at 200 MPa and 9 passes.

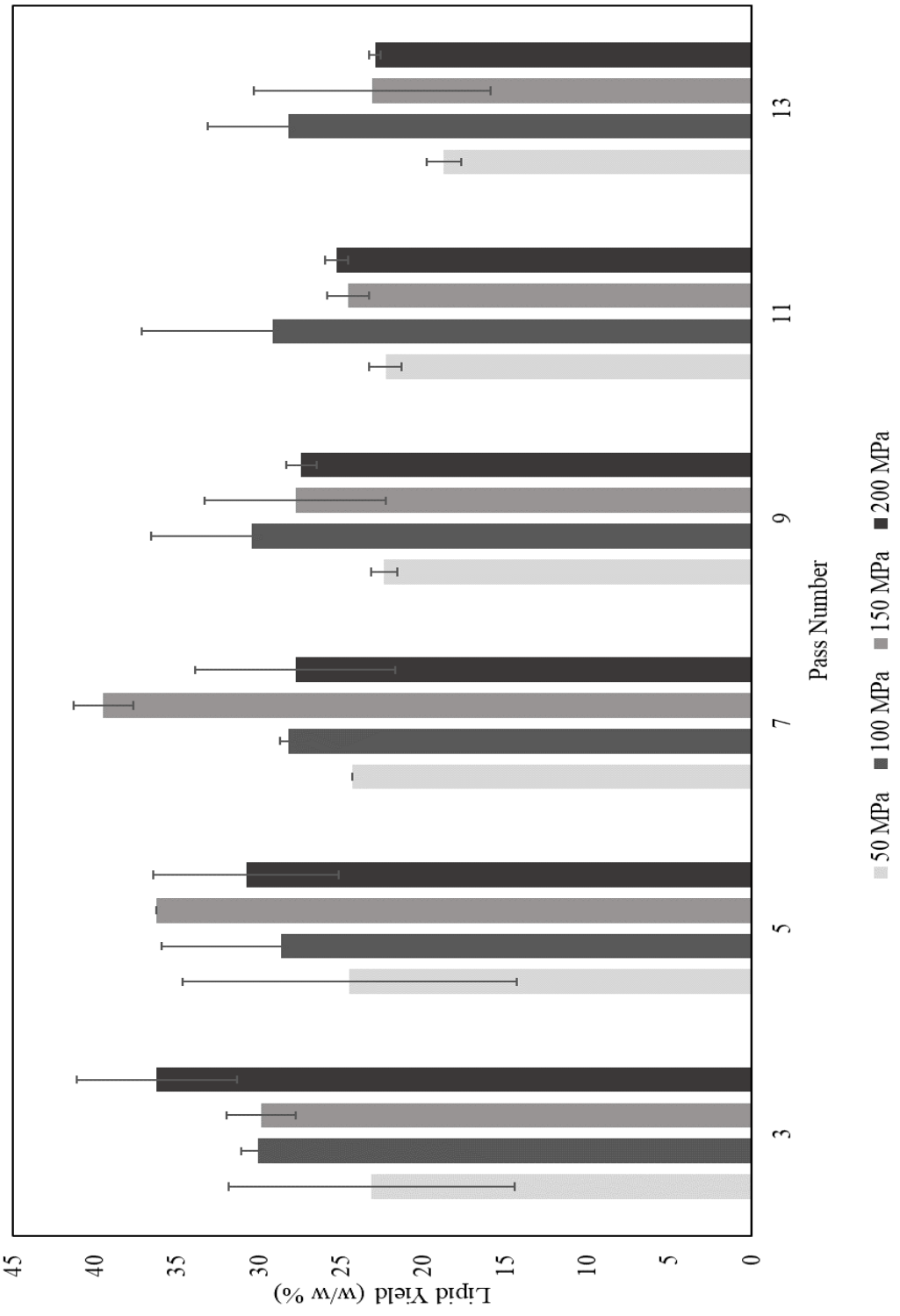

Figure 4.15. Lipid yield (w/w %) of HPH experiment (using dry biomass slurry)

4.3.2 Lipid Extraction from Spent Hydrolyzed Whey Medium

Due to lower results of the lipid extraction from the dry biomass of yeast grown in synthetic medium, the lipid extraction from hydrolyzed whey-based biomass was performed solely using the spent fermentation medium.

For this experiment, the same parameters, pressure (50-200 MPa) and pass number (3-13), were used. Also, the C/N ratio of hydrolyzed whey medium was calculated as 40. The results depicted in Figure 4.16 represent the average values obtained from two repetitions for *R. toruloides*. The figure demonstrates the impact of applying yeast cell disruption using whey hydrolysate combined with HPH on lipid yield under various pressure conditions and pass numbers.

According to the results displayed in Figure 4.16, it is evident that the highest lipid yield of 39.5±1.8% was achieved when the process was conducted at 150 MPa pressure and with 7 passes. At this condition, the lipid yield reached its peak value. In contrast, at the lowest pressure of 50 MPa, the lipid yield exhibited a range between approximately 18.7 and 24.5%. As the pressure increased to 100 MPa, the number of passes did not significantly affect the yield, which remained within the range of 28.2 to 30.0%. When the maximum pressure of 200 MPa was applied, the lipid yield ranged between 23 and 36.2%. Although this range indicates a relatively lower yield compared to the maximum yield condition, it still demonstrates the potential of lipid extraction at higher pressures.

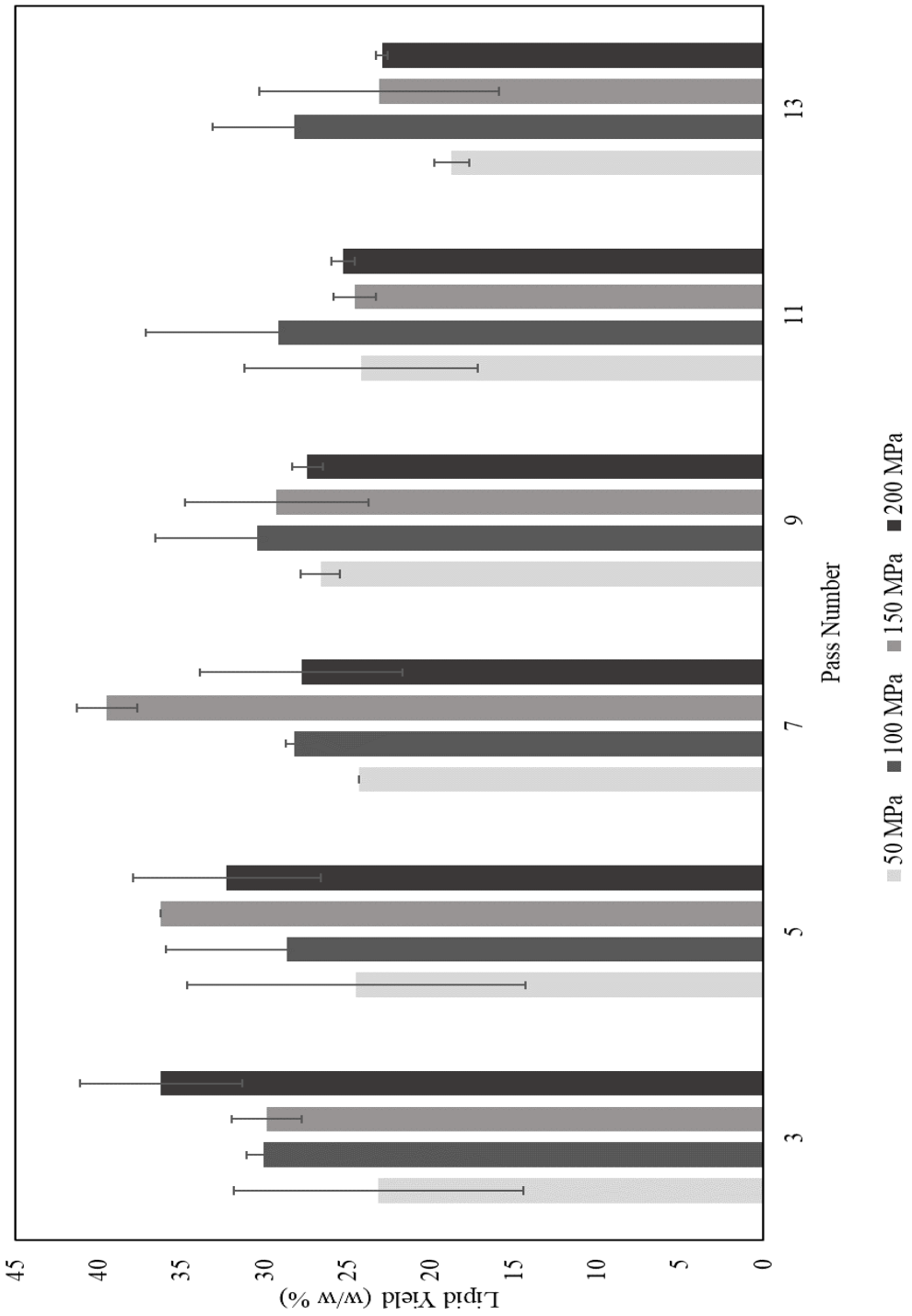

Figure 4.16. Lipid yield (w/w %) of HPH experiment (using hydrolyzed whey medium)

A similar study was carried out by Dias et al, (2022) for the extraction of lipids from *R. toruloides* cultivated in a broth medium. In this study growth cells were disrupted by using high pressure homogenizer, applying 60 MPa pressure for 3 passes. Before and after HPH treatment, Soxhlet method was employed to obtain lipid from the cells. The results indicated that lipid yield was found as 34.7% before performing HPH while lipid yield was increased to 46.4% after HPH. In another study, biomass of *Y. lipolytica* was used to extract microbial oil and by using HPH the cells were disrupted, and it was found that the maximum lipid yield after applying 150 MPa for 5 passes was almost 80% (Drévillon et al., 2018). These results and obtained results in our study show the efficiency of HPH assisted microbial oil extraction. However, it is important to note that lipid yield can change in different studies due to some other factors that can alter the lipid accumulations in the cell.

To investigate the effect of C/N ratio on lipid production of *R. toruloides* when hydrolyzed whey medium was used as fermentation medium, different whey media with different C/N ratios (40/1, 80/1 and 120/1) were prepared. To obtain different C/N ratios, glucose and galactose were added externally to the hydrolyzed whey media. The obtained results from optimal conditions of HPH (125 MPa, 5 passes) were compared with the high speed homogenizer method results. After HPH, the lipid yields obtained for different carbon/nitrogen ratios were 32.4±1.0, 33.7±3.3, and 42.2±0.4% for the respective ratios of 40, 80, and 120/1. In contrast, the lipid yields for the HSH method were 41.9±3.3, 44.5±1.4, and 49±0.4% for the same carbon/nitrogen ratios (Figure 4.17). It is seen that the values obtained from HSH for each C/N ratio are higher than the HPH values and the differences of yield are about the same. This can show the consistency of HPH assisted extraction.

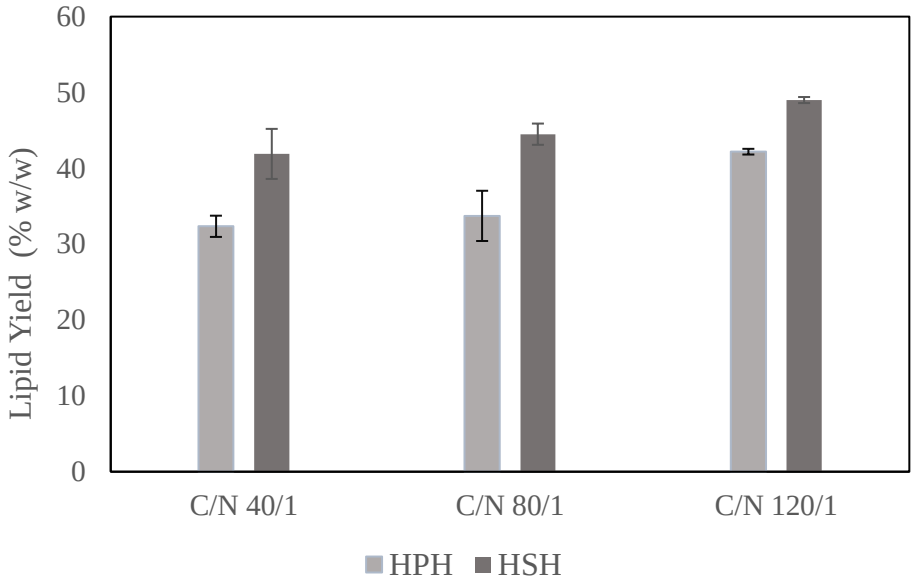

Figure 4.17. Lipid yields of *R. toruloides* at different C/N ratios in hydrolyzed whey medium.

4.3.3 Optimization of HPH Assisted Extraction from Synthetic Medium Based Biomass

4.3.3.1 Optimization Using the Spent Fermentation Medium

Optimization was done by using response surface methodology (RSM) with the results shown in Table 4.2. Some results were removed since they were incompatible.

Table 4.2. Experimental plan for HPH optimization with customized RSM and lipid results for *R. toruloides* on a dry cell basis (% w/w) (using spent fermentation medium)

	Pressure (MPa)	**Pass Number**	**Lipid Yield (w/w%)**
1	50	3	18.5±2.7
2	50	5	19.7±1.7
3	50	7	24.4±0.2
4	50	9	29.3±14.4
5	50	11	32.9±7.7
6	50	13	28.9±5.7
7	100	3	35.0±0.4
8	100	5	40.0±5.2
9	100	7	34.2±1.6
10	100	9	32.7±0.3
11	100	11	29.8±5.2
12	100	13	28.8±10.1
13	150	3	41.6±2.9
14	150	5	40.3±5.6

Table 4.2 (continued)

15	150	7	41.6±6.4
16	150	9	46.9±2.3
17	150	11	40.7±4.3
18	150	13	42.6±15.1
19	200	3	46.6±0
20	200	5	48.5±0
21	200	7	52.2±1.0
22	200	9	47.8±2.7
23	200	11	44.7±2.8
24	200	13	47.0±1.2
HSH	-	-	49.5±1.9

According to conducted statistical analysis, the coefficient of determination (R^2) value was found to be 0.848, while the adjusted value (adj-R^2) was 0.826. The model lack-of-fit value was found to be 0.099 (Table 4.3). Thus, adequacy of the model was confirmed by the insignificant result of the lack-of-fit ($P=0.099 > 0.05$). This implies that there is no significant lack of fit in the model, supporting its suitability for explaining the data (Ekpenyong et al., 2017).

Table 4.3. ANOVA results of *R. toruloides* lipid yields extracted with HPH (using spent fermentation medium)

Source	Coded Coefficient	P
Regression	38.39	0.000
Linear	30.39	0.000
Square	11.31	0.000
Interaction	11.40	0.002
Lack-of-Fit	1.87	0.099
Constant	0.08042	0.000
Pressure	-0.00507	0.000
Pass Number	-0.00316	0.008
Pressure*Pressure	0.000001	0.000
Pass Number*Pass Number	0.000077	0.238
Pressure*Pass Number	0.000011	0.001

P<0.05 values show significant results.

This analysis demonstrated that the linear effect (P=0.000<0.05), square effect (P=0.000<0.05), and interaction effect (P=0.002<0.05) were statistically significant. While the interaction between pressure and pass number was significant, interaction

of pass number with itself (pass number with pass number) was found to be insignificant.

To achieve a better fit. the response values (lipid yield) were transformed using the reciprocal function (1/Y). For *R. toruloides*, the regression model developed as a result of the modeling was found as;

$$\frac{1}{Y} = 8.04 * 10^{-2} - 5.07 * 10^{-4} * P - 3.17 * 10^{-3} * X + 1.0 * 10^{-6} * P^2 + 7.7 * 10^{-5} * P * X + 1.1 * 10^{-5} * X^2 \quad (6)$$

In this equation, Y, P and X represent lipid yield (% w/w), pressure (MPa) and pass number, respectively.

In order to evaluate the interaction effects visually for HPH method, a three-dimensional response surface plot was depicted (Figure 4.18).

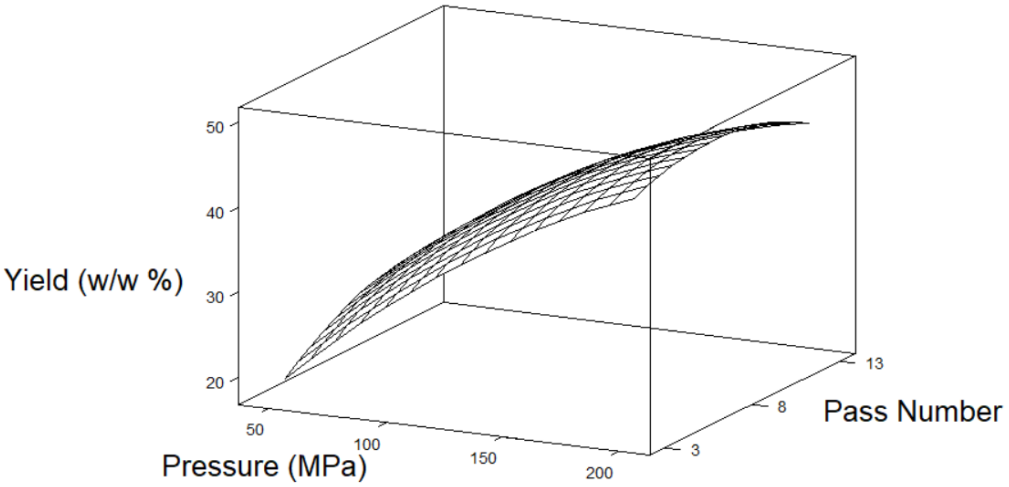

Figure 4.18. Response surface plot showing the effects of pressure and pass number on lipid yield.

According to the figure, it is observed that the optimum conditions for lipid yield are 200 MPa for applied pressure and nearly 8 for the pass number. Also, considering this figure it can be assumed that increasing the pressure would lead to an increase in lipid yield. However, due to limitations of the device used in this study, experiments could not be conducted at pressures higher than 200 MPa.

Optimum conditions were then determined as 200 MPa and 7 pass number. Under these conditions, the oil yield was predicted to be 0.019 (Y= 52.6%) with the desirability of 0.95. After making verification experiment under determined optimum conditions, the lipid yield was found as 45.7±4.4%. Error analysis was also performed, and the root mean square error (RMSE) was calculated as 0.004, while the mean absolute error (MAE) was determined to be 0.05. These small error values indicate that the model is accurate and suitable for the given data (Uzuner & Cekmecelioglu, 2014).

4.3.3.2 Optimization Using Dry Biomass Slurry

Optimization was performed using response surface methodology (RSM), and the used data are presented in Table 4.4.

Table 4.4. Experimental plan for HPH optimization with customized RSM and results of lipid results for *R. toruloides* on a dry cell basis (% w/w) (using dry biomass)

	Pressure (MPa)	**Pass Number**	**Lipid Yield (w/w%)**
1	50	3	26.7
2	50	5	23.3
3	50	7	20.0
4	50	9	13.3
5	50	11	21.7
6	50	13	30.0
7	100	3	13.3
8	100	5	26.7
9	100	7	20.0
10	100	9	23.3
11	100	11	23.3

Table 4.4 (continued)

12	100	13	10.0
13	150	3	16.7
14	150	5	23.3
15	150	7	16.7
16	150	9	33.3
17	150	11	30.0
18	150	13	26.7
19	200	3	36.7
20	200	5	30.0
21	200	7	33.3
22	200	9	40.0
23	200	11	26.7
24	200	13	25.0
HSH	-	-	40.88

According to the results obtained from the statistical analysis, the coefficient of determination (R^2) value is 0.549, and the adjusted value (adj-R^2) is 0.361. The low R^2 value suggests that the model does not effectively fit the data. This analysis

demonstrated that the linear (P=0.167>0.05), square (P=0.088>0.05), and interaction terms (P=0.406>0.05) were statistically insignificant. While the squared of pressure term (P=0.04<0.05) was found significant, the other parameters and their interactions were found to be insignificant (Table 4.5).

Table 4.5. ANOVA results of *R. toruloides* lipid yields extracted with HPH (using dry biomass)

Source	Coded Coefficient	P
Regression	440.160	0.059
Linear	236.677	0.167
Square	181.089	0.088
Interaction	22.394	0.406
Constant	19.1743	0.116
Pressure	-0.1930	0.195
Pass Number	3.0482	0.180
Pressure*Pressure	0.0012	0.040
Pass Number*Pass Number	-0.1336	0.308
Pressure*Pass Number	-0.0055	0.406

Due to insufficient R^2 results (0.549), slurries of dry biomass were not used furthermore.

4.3.4 Optimization of HPH Assisted Extraction from Spent Whey Hydrolysate Based Biomass

RSM was used to optimize the results obtained from HPH assisted extraction and used data are shown in Table 4.6.

Table 4.6. Experimental plan for HPH optimization with customized RSM and lipid results for *R. toruloides* on a dry cell basis (w/w %) (using spent whey hydrolysate medium)

	Pressure (MPa)	Pass Number	Lipid Yield (w/w %)
1	50	3	23.2 ± 2.0
2	50	5	24.5 ± 3.0
3	50	7	24.3 ± 0.0
4	50	9	22.4 ± 0.8
5	50	11	22.3 ± 1.0
6	50	13	18.8 ± 1.1
7	100	3	30.1 ± 1.1
8	100	5	28.7 ± 1.5

Table 4.6 (continued)

9	100	7	28.2 ± 0.6
10	100	9	30.5 ± 6.2
11	100	11	29.2 ± 2.0
12	100	13	28.3 ± 4.9
13	150	3	29.9 ± 2.1
14	150	5	36.3 ± 0.0
15	150	7	39.5 ± 1.8
16	150	9	27.8 ± 5.5
17	150	11	24.6 ± 1.3
18	150	13	23.1 ± 2.0
19	200	3	36.3 ± 4.9
20	200	5	30.8 ± 5.7
21	200	7	27.8 ± 6.1
22	200	9	27.5 ± 0.9
23	200	11	25.3 ± 0.7
24	200	13	23.0 ± 0.4
HSH	-	-	43.5±3.5

According to statistical analysis results, the coefficient of determination (R^2) value and adjusted R^2 value (adj-R^2) were found as 0.783, and 0.746, respectively. The lack of fit value of the model was determined as 0.051, which proved the model adequate.

Table 4.7. ANOVA results of *R. toruloides* lipid yields extracted with HPH (using spent whey hydrolysate medium)

Source	**Coded Coefficient**	**P**
Regression	0.001075	0.000
Linear	0.000850	0.043
Square	0,000211	0.000
Interaction	0.000013	0.265
Lack-of-Fit	0.000242	0.051
Constant	0.044090	0.000
Pressure	-0.000145	0.028
Pass Number	-0.001671	0.089
Pressure*Pressure	0.000001	0.015
Pass Number*Pass Number	0.000208	0.001
Pressure*Pass Number	-0.000003	0.265

The statistically significant effects according to ANOVA results (Table 4.7) were linear, and square effects. Also, pressure, pass number and interaction of pressure with pass number showed significant effect. However, interaction between pressure and pass number and squared pressure and pass number terms showed insignificant effect on lipid yield.

The reciprocal transformation (1/Y) was used to obtain a better fit, and the response values (lipid yield) were modelled. The regression model developed modeling process for *R. toruloides* is as follows:

$$\frac{1}{Y} = 4.41 * 10^{-2} - 1.45 * 10^{-4} * P - 1.67 * 10^{-3} * X + 1.0 * 10^{-6} * P^2 + 2.08 * 10^{-4} * X^2 + 3.4 * 10^{-6} * P * N \quad (7)$$

In this equation, Y, P and X represent lipid yield (% w/w), pressure (MPa) and pass number, respectively.

Three-dimensional response surface plot was generated (Figure 4.19). This plot ensures a visual representation of the multiple variables and to visualize response relationships.

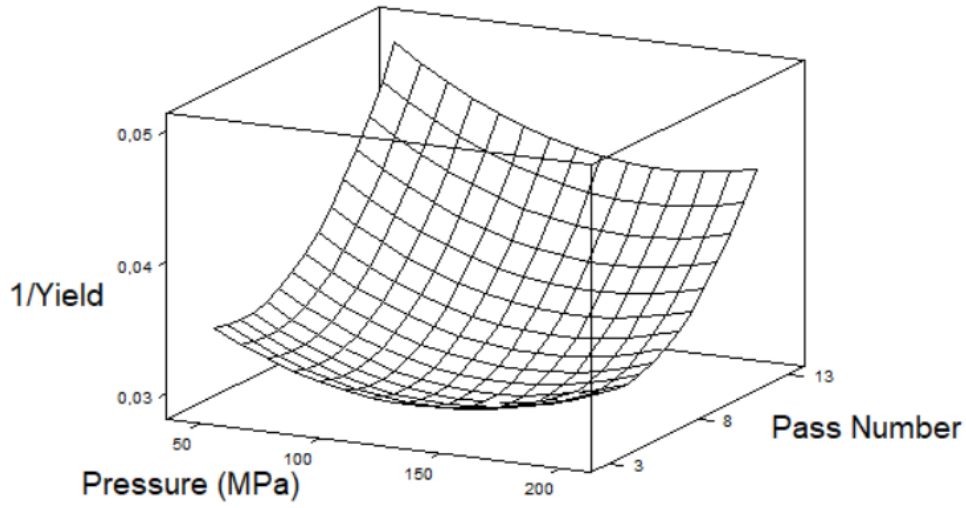

Figure 4.19. Response surface plot showing the effects of pressure and pass number on lipid yield.

According to Figure 4.19, the optimum pressure for lipid extraction seems to be 150 MPa pressure while the optimum pass number is between 3 to 8 passes.

Optimum conditions were then determined as 125 MPa and 5 passes. Under these conditions, lipid yield was predicted to be 0.03 (Y= 33.3%) with the desirability of 1.0. According to determined optimum conditions verification experiments were performed and the lipid yield was found 32.4±3.3%. After performing error analysis and the root mean square error (RMSE) and mean absolute error (MAE) were determined to be 0.01 and 0.05, respectively. The small values obtained show that the model is accurate and fit the well to data.

4.4 HHP Assisted Microbial Oil Extraction and Lipid Yields

4.4.1 Lipid Extraction from Slurry of Dry Biomass of *R. toruloides*

R. toruloides was grown for 8 days in hydrolyzed whey (medium C/N=40/1), then cells were collected to extract lipids by using HHP. In this experiment the temperature was set at 40 °C. The aim was to investigate the effect of pressure, biomass/solvent ratio, and duration on lipid yields. The lipid yields are shown in Figure 4.20.

Lipid yields were between 5.1±0.2 and 10.1±0.9% at the lowest pressure of 100 MPa and between 7.4±0.2 and 17.8±1.3% at 200 MPa. At the maximum pressure of 300 MPa, the lowest and the highest lipid yields were measured as 8.4±0.2 and 14.9±0.9%, respectively. For varying biomass content, lipid yields ranged between 9.0±0.2-17.8±1.3%, 7.1±1.9-14.9±0.9% and, 5.1±0.2-9.3±0.2% for 1,2 and 3 grams of dry biomass used, respectively. Lipid yield also changed from 5.9±0.6 to 11.7±2.6% for 5 minutes pressurization duration, from 5.1±0.2 to 10.1±0.9% for 10 minutes process while lipid yield after HHP applied for 15 minutes varied from 7.1±1.9 to 17.8±1/3%. According to these results, the highest lipid yield was obtained when used 1 g dry biomass/25 mL chloroform: methanol solution (2:1 v/v) at 200 MPa and duration of 15 minutes (Figure 4.20). As a result, longer periods with lower biomass increases the oil yield, but pressure was not clearly distinct.

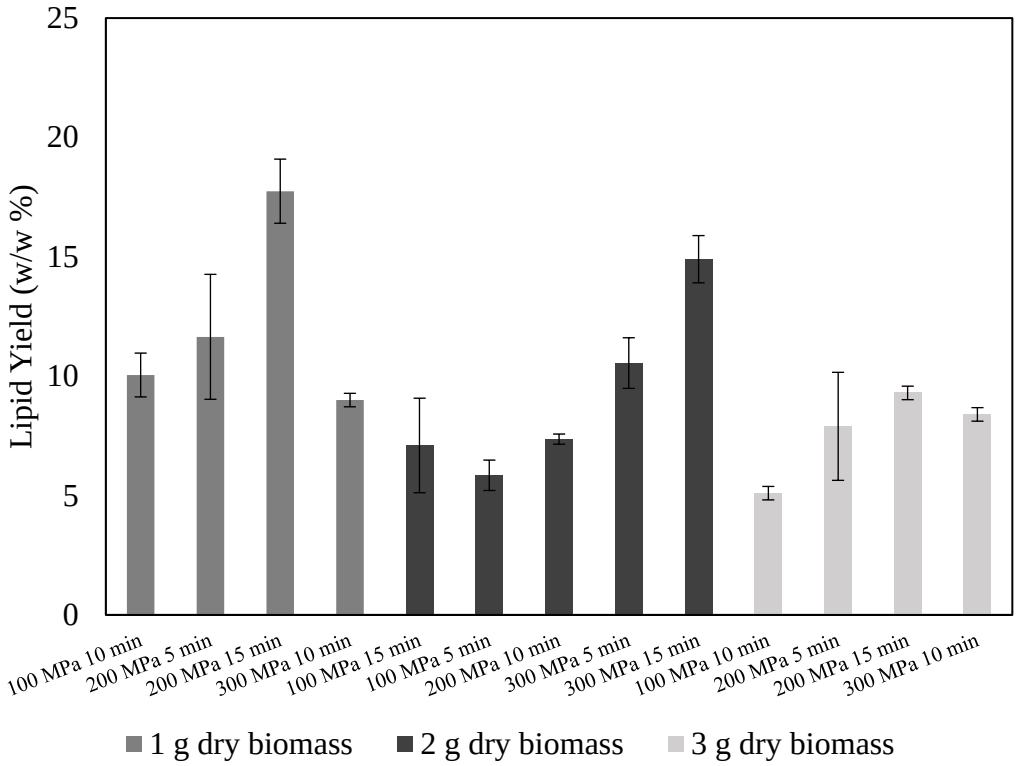

Figure 4.20. Lipid yields from *R. toruloides* dry biomass after HHP assisted extraction.

4.4.2 Lipid Extraction from Spent Hydrolyzed Whey Medium with *R. toruloides.*

The lipid was extracted by using HHP without separating the *R. toruloides* from the spent fermentation medium and the variables for this experiment were temperature (30, 40, and 50°C), duration (5, 10, and 15 min) and pressure (100, 200, and 300 MPa). Obtained results are shown in Figure 4.21.

According to results, the highest oil yield was achieved at 300 MPa, 10 min and 30 °C, while the lowest oil yield was obtained at 100 MPa, 10 min and 50 °C. These results show that the increase in temperature decreases the lipid yield. It was conducted that exposure to sudden high temperature like 50 °C causes yeast cells to exhibit inherent tolerance and this results in the yeast more resistant to HHP (Catarine Tosi-Costa et al., 2019). For 100 MPa, the lowest yield was found to be 11.4% at 50 °C and 10 minutes, and the lowest yield when pressure was doubled was 11.9% at 50 °C and 15 minutes, and for highest pressure (300 MPa), this value was found as 14.7% at 50 °C and 10 minutes duration. The highest lipid yields were measured at 20.4% for 100 MPa, 40 °C and 5 minutes, and 20.5% at 200 MPa, 40 °C and 10 min, while the highest yield for 300 MPa was 22.8±3.7%, at 30 °C and 10 minutes.

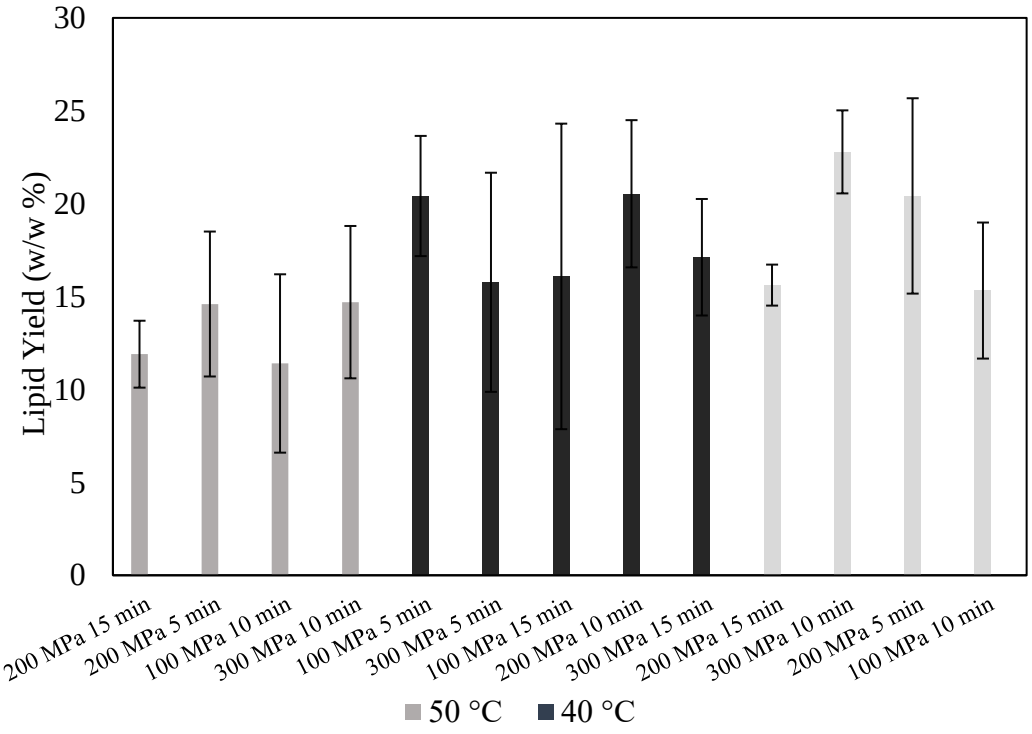

Figure 4.21. Lipid yield of *R. toruloides* by using spent fermentation medium.

4.4.3 Optimization of HHP Assisted Extraction from Whey

4.4.3.1 Optimization of Experiment Using Biomass Slurry

The data used for the optimization of the HHP assisted extraction for the dry biomass experiment are shown in Table 4.8.

Table 4.8. Experimental plan for HHP optimization with customized RSM and lipid results for *R. toruloides* on a dry cell basis (w/w %) (dry biomass)

Pressure (MPa)	Dry Biomass (g)	Time (min)	Lipid Yield (w/w %)
100	2	15	7.1±1.9
100	1	10	10.1±0.9
100	2	5	5.9±0.6
100	3	10	5.1±0.2
200	3	5	7.9±2.2
200	1	5	11.7±2.6
200	3	15	9.3±0.2
200	2	10	7.4±0.2
200	1	15	17.8±1.3
300	2	5	10.6±1.0
300	3	10	8.4±0.2
300	2	15	14.9±0.9
300	1	10	9.0±0.2
HSH	-	-	45.5±3.18

The statistical analysis results showed determination coefficient (R^2) value and adjusted determination coefficient (adj-R^2) value were 0.841 and 0.77, respectively while the lack of fit value was 0.098, which confirms the suitability of the model (P=0.098>0.05).

Table 4.9. ANOVA results of *R. toruloides* lipid yields extracted with HHP (using dry biomass)

Source	Coded Coefficient	P
Regression	0.036624	0.000
Linear	0.025269	0.002
Square	0.007323	0.002
Interaction	0.004033	0.024
Lack-of-Fit	0.002094	0.098
Constant	-0.03900	0.558
Pressure	-0.00020	0.551
Biomass	0.12419	0.001
Time	0.01761	0.016
Pressure*Pressure	0.00000	0.175
Biomass*Biomass	-0.01450	0.047

Table 4.9 (continued)

Time*Time	-0.00104	0.001
Pressure*Biomass	-0.00022	0.003
Pressure*Time	-0.00000	0.925
Biomass*Time	0.00035	0.793

While pressure, pressure-pressure interaction, pressure-time interaction, and biomass-time interaction were insignificant, the others showed significant effects on lipid yield (Table 4.9).

To fit the model, reciprocal (1/Y) transformation was used. The model equation is provided in the following equation.

$$\frac{1}{Y} = -3.39 * 10^{-2} - 2 * 10^{-4} * P - 1.24 * 10^{-1} * B + 1.76 * 10^{-2} * t - 1.45 * 10^{-2} * B^2 - 1.04 * 10^{-3} * t^2 - 2.2 * 10^{-4} * P * B \quad (8)$$

In this equation Y, P, B and t represent lipid yield (w/w%), pressure (MPa), dry biomass (g) and time (min), respectively.

The response surface plots were generated to understand the effect of variables on lipid yield and determine the optimum conditions for HHP assisted extraction experiment for dry biomass with chloroform: methanol mixture (2:1 v/v). These plots are shown in Figure 4.22.

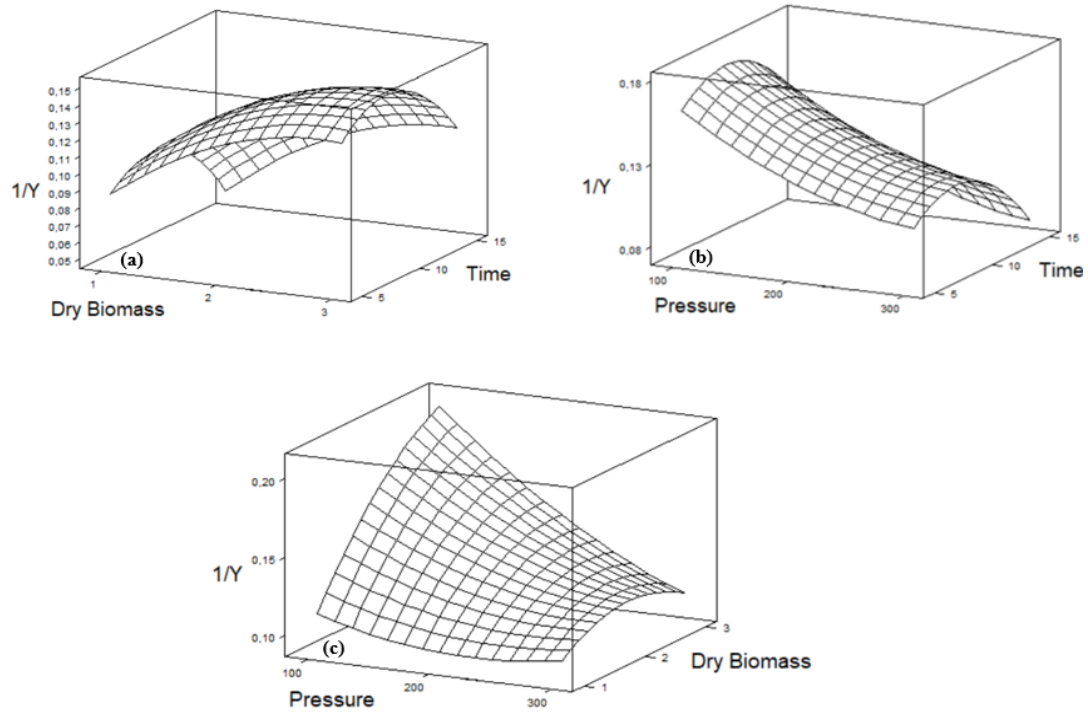

Figure 4.22. For *R. toruloides* effect of **(a)** dry biomass and time, **(b)** pressure and time, **(c)** pressure and dry biomass.

According to Figure 4.22 (a), at same pressure if the used dry biomass amount decreased to 1 gram and process time increased to 15 minutes, the yield increased. On the other hand, when using the same amount of dry biomass, the lipid yield increased sharply up to the highest pressure (300 MPa), while considering the time, the yield decreased from 5 minutes to 10 minutes and increased again from 10 minutes to 15 minutes. (Figure 4.22 (b)). Moreover, in Figure 4.22, (c) it can be seen that at 100 MPa, while the dry biomass amount increases, lipid yield dramatically decreases and also the same thing is acceptable for using 3 g dry biomass at different pressures.

From ANOVA results, the optimum conditions were determined as 230 MPa, 1 g dry biomass/25 mL solvent and 15 minutes, and the lipid yield under these conditions was estimated as 20% (w/w) with a desirability value of 0.98. The lipid yield was determined as 20±0.7% under optimum conditions. Root mean square error (RSME) and mean absolute error (MAE) values of the model were calculated as 0.05 and 0.2, respectively.

4.4.3.2 Optimization of Experiment Spent Hydrolyzed Whey Medium

To optimize the results from HHP assisted extraction for biomass in spent hydrolyzed whey medium are shown in Table 4.10

Table 4.10. Experimental plan for HHP optimization with customized RSM and results of lipid results for *R. toruloides* on a dry cell basis (w/w %) (using hydrolyzed whey medium)

Pressure (MPa)	Temperature (°C)	Time (min)	Lipid Yield (w/w %)
200	50	15	11.9±1.8
200	50	5	14.6±3.9
100	50	10	11.4±4.8
300	50	10	14.7±4.1
100	40	5	20.4±3.2

Table 4.10 (continued)

300	40	5	15.8±5.9
100	40	15	16.1±8.2
200	40	10	20.5±4.0
300	40	15	17.1±3.1
200	30	15	15.6±1.1
300	30	10	22.8±2.2
200	30	5	20.4±5.3
100	30	10	15.3±3.7
HSH	-	-	47.6±2.7

According to results of statistical analysis, the coefficient of determination (R^2) value and adjusted R^2 value (adj-R^2) were found as 0.86, and 0.755, respectively. The lack of fit value of the model was found as 0.533. The lack of fit value confirms the fit of the model ($P=0.533 > 0.05$).

Table 4.11. ANOVA results of *R. toruloides* lipid yields extracted with HHP (using hydrolyzed whey medium)

Source	Coded Coefficient	P
Regression	2.90345	0.001
Linear	0.40586	0.000
Square	0.40586	0.019
Interaction	1.41690	0.001
Lack-of-Fit	0.05574	0.553
Constant	-5.956	0.006
Pressure	0.037	0.000
Time	-0.150	0.146
Temperature	0.321	0.001
Pressure*Pressure	-0.000	0.013
Time*Time	0.005	0.177
Temperature*Temperature	-0.003	0.012
Pressure*Time	-0.000	0.620
Pressure*Temperature	-0.001	0.000
Time*Temperature	0.002	0.315

The time, time-time interaction, pressure-time, and time-temperature showed insignificant effect on lipid yield while the other effects were significant for lipid yield according to ANOVA results (Table 4.11).

In order to improve the fit of the model obtained based in these results, the natural logarithm of lipid yield (lnY) was taken and shown the following equation:

$$\ln(Y) = -5.956 + 0.037 * P - 0.150 * t + 0.321 * T + 0.005 * t^2 - 0.003 * T^2 - 0.001 * P * T + 0.02 * t * T \quad (9)$$

In this equation Y, P, T and t represent lipid yield (w/w%), pressure (MPa), temperature (°C) and time (min), respectively.

In order to investigate the factors in pairs for HHP assisted extraction experiment for biomass in spent fermentation medium, three-dimensional response surface plots were generated (Figure 4.23). This plot provides a visual depiction of the relationships between multiple variables and their corresponding responses in the HHP treatment.

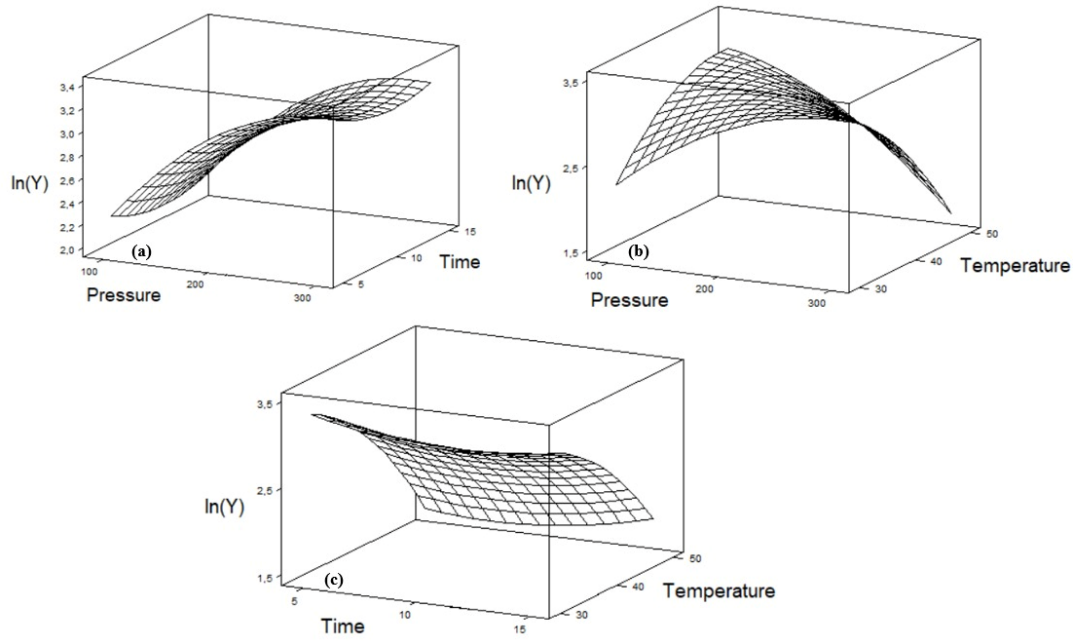

Figure 4.23. Effect of (a) time and pressure, (b) pressure and temperature, (c) time and temperature on lipid yields.

According to Figure 4.23 (a) the maximum lipid yield obtained at 300 MPa for 5 and 15 minutes when temperature at 30℃ and the lipid yield dramatically decreased as pressure decreased. On the other hand, highest lipid yield was observed at 300 MPa, 30℃ and 100 MPa, 50℃ for the duration 5 min (b). Moreover, the maximum lipid yield was at 30℃ for all durations (5, 10, and 15 min) but yield sharply decreased until the temperature 50℃ when the pressure 300 MPa (c).

The optimum conditions from this modelling were determined as 300 MPa, 5 minutes and 30 ℃ with the lipid yield 27.6 % and desirability of 1.0. The experimental result for 300 MPa, 5 minutes and 30℃, the lipid yield was found 23.4±2.7%. Following an error analysis, the root mean square error (RSME) and mean absolute error (MAE) were computed as 1.73 and 1.23, respectively.

4.5 Visual Analysis

4.5.1 Viable Cell Count

After HPH and HHP treatments of *R. toruloides* grown in hydrolyzed whey medium viable cell counts were performed in potato dextrose agar to understand the effect of pressurization methods on cells. After 5 days of incubation in petri dishes, viable cells colonies were counted, and the results are shown in the Tables 4.12-4.13.

As evident in Table 4.12 which shows cell count after HPH, *R. toruloides* was inactivated for 5 to13 passes at 150 MPa and from 3 to 13 passes at 200 MPa with high pressure homogenizer. Although there was a decrease in viable cell numbers at 50 and 100 MPa pressures, inactivation was not observed. According to the results, an increase in applied pressure leads to a decrease in alive yeast cell counts. Additionally, even when the pressure remains constant, an increase in pass numbers also results in a decrease in viable yeast cell counts. Therefore, both pressure and pass numbers are important factors in yeast inactivation through high-pressure homogenization (HPH). In the HPP method, due to an approximate temperature increase of 2-2.5 ℃ for every 10 MPa pressure increment (Diels & Michiels, 2006), temperature can be also considered an important factor in cell disruption along with the two mentioned factors.

Table 4.12. Viable cell count of *R. toruloides* after HPH treatment.

Run	Pressure (MPa)	Pass Number	Viable Cell Count
1	50	3	$18x10^5$
2	50	5	$11x10^5$
3	50	7	$9x10^5$
4	50	9	$155\ x10^4$
5	50	11	$69x10^5$
6	50	13	$17x10^4$
7	100	3	$258x10^4$
8	100	5	$193x10^4$
9	100	7	$84x10^4$
10	100	9	$22x10^4$
11	100	11	$13x10^3$
12	100	13	$8x10^3$
13	150	3	$48x10^2$
14	150	5	X
15	150	7	X

Table 4.12 (continued)

16	150	9	X
17	150	11	X
18	150	13	X
19	200	3	X
20	200	5	X
21	200	7	X
22	200	9	X
23	200	11	X
24	200	13	X

**X refers to the counted number is less than 30.*

To observe the effect of HHP on cells, cell counts were made only for the spent fermentation medium, since a chloroform: methanol mixture was used in the dry biomass experiment. Based on the data in Table 4.13, it was observed that *R. toruloides* was inactivated at 300 MPa, 40 ℃ after 5 and 15 minutes, and 30 ℃ after 10 minutes. While yeast inactivation was not observed at 50 ℃, viable cell counts did not decrease at 100 and 200 MPa pressures at 30 and 40 ℃. In HHP, pressure was the dominant factor affecting cell fragmentation the most, among temperature, pressure, and time. The reason for decreasing viability of cells after HHP treatment is mostly due to delay or inhibition of some cellular activities, such as enzymatic

activities (Catarine Tosi-Costa et al., 2019). On the other hand, although not as significant as pressure, the duration of the process also played an important role in cell disruption, while temperature was considered a critical factor.

Table 4.13. Viable cell count of *R. toruloides* after HHP treatment.

Run	Temperature (°C)	Pressure (MPa)	Time (min)	Viable Cell Count
1	50	100	10	$23x10^6$
2	50	200	5	$293x10^3$
3	50	200	15	298
4	50	300	10	>300
5	40	100	5	$125x10^6$
6	40	100	15	$33x10^4$
7	40	200	10	$2x10^5$
8	40	300	5	X
9	40	300	15	X
10	30	100	10	$13x10^6$
11	30	200	15	$59x10^5$
12	30	200	5	$62x10^4$
13	30	300	10	X

**X refers to the counted number is less than 30.*

4.5.2 SEM Analysis

Figures 4.24-4.25 demonstrate the SEM images of *R. toruloides* cells after the treatments of HPH and HHP. SEM analysis was performed only for samples treated under the optimum experiment conditions (125 MPa, 5 passes for HPH and 230MPa, 40°C, 15 mins for HHP) at METU Central Laboratory, Ankara, Turkey.

There is no specific study about *R. toruloides* cell size, but Shakeri (2021) reported size of *Rhodosporidium* sp. DR37 between 2-8 μm. Since they are the same genus, it was considered as they have similar cell sizes. Thus, the size of the displayed cells can be considered consistent (Shakeri et al., 2021).

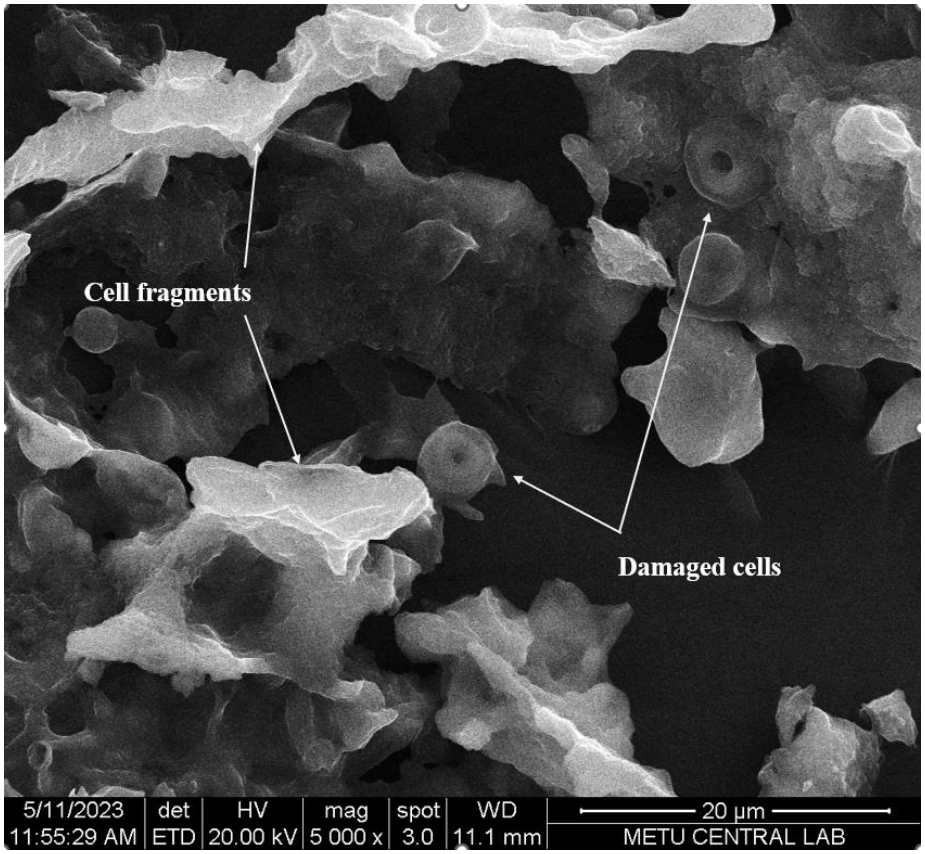

Figure 4.24. SEM image of *R. toruloides* after HPH (125 MPa and 5 passes) treatment (5000x).

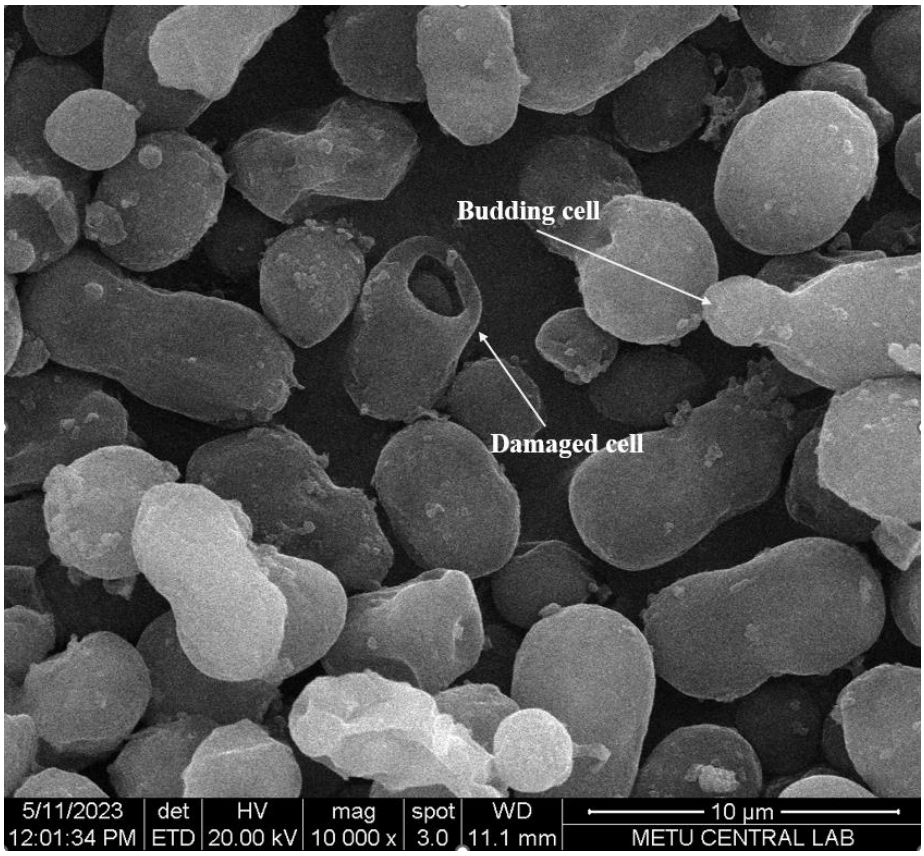

Figure 4.25. SEM image of *R. toruloides* cells after HHP (230 MPa, 40°C, 15 min, 1 g dry biomass) treatment (10000x).

Although applied pressure in HPH (125 MPa) is much lower than the pressure in HHP (230 MPa), it is clear that HPH is more effective than HHP treatment on cells since HHP is more effective to inactivate the cell activities but not destroys cell morphology up to pressure of 200 MPa. However, when applied pressure is increased over the 200 MPa the disruption of cell wall can be observed (Catarine Tosi-Costa et al., 2019). While both methods result in cell damage, the sample that was obtained after HHP method exhibits a higher number of intact cells compared to the HPH method. In addition, obtained microbial oils under optimum conditions for HPH and

HHP assisted extraction were found as 32.4 and 20%. The provided figures (Figure 4.24-4.25) offer supportive evidence for the variations among obtained lipids.

Apart from SEM imaging of the optimum conditions, SEM analysis was also performed with 13 passes at the optimum pressure of 125 MPa and at the highest pressure of 200 MPa with an optimum 5 passes. While Figure 4.26-4.27 show the SEM image of the processes at 125 MPa with 13 passes and 200 MPa with 5 passes, respectively.

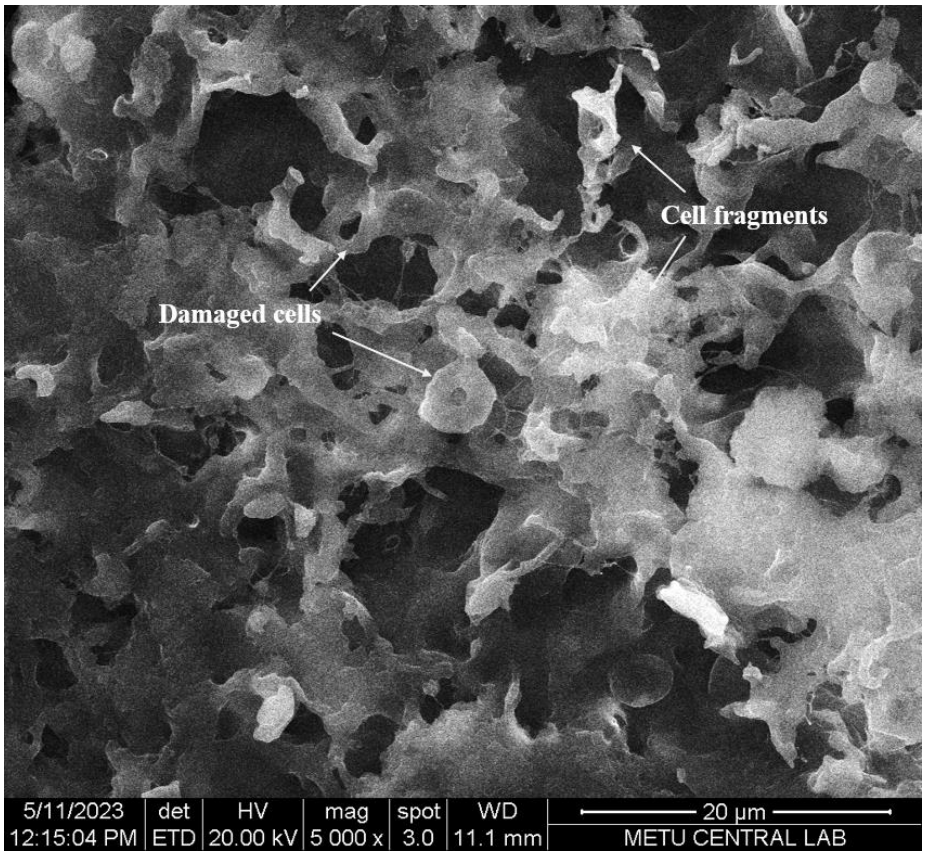

Figure 4.26. SEM image of *R. toruloides* cells after HPH (125 MPa, and 13 passes) treatment (5000x).

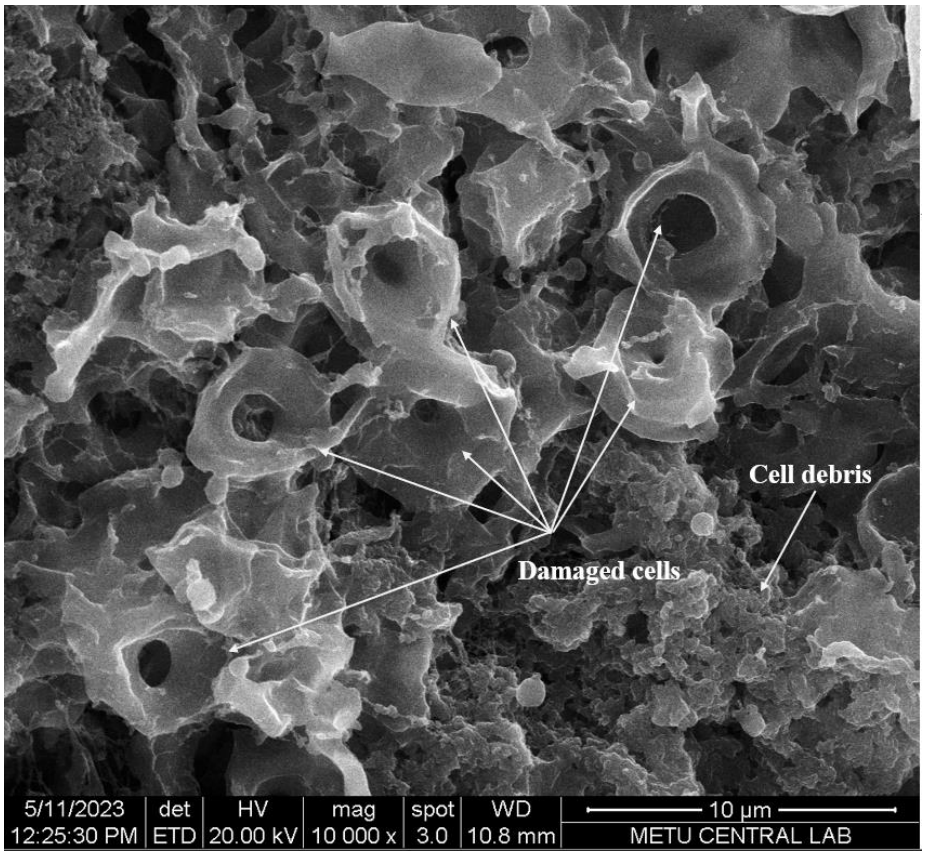

Figure 4.27. SEM image of *R. toruloides* cells after HPH (200 MPa, and 5 passes) treatment (10000x).

It is clear from Figures 4.26-4.27 that applying high pressure and high number of passes almost have same effect on cell morphology. Obtained lipid yields from these conditions and viable cell count after the disruption of cells by high pressure and passes, show consistency with SEM images.

4.6 Lipid Analysis

4.6.1 Peroxide Value (PV) Determination

For the determination of peroxide value, the lipid samples were collected from optimal conditions of HPH and HHP. Peroxide values were determined as 20 milliequivalents of active oxygen/kg oil (meq O_2/kg) for extracted microbial lipid from *R. toruloides* undergone HPH and HHP assisted extractions. In literature there are upper limits. For example, the upper limits for corn oil and sunflower oil are 10 meq O_2/kg, and for extra virgin oil and virgin oil are 15 meq O_2/kg Codex Alimentarius, 2017). On the other hand, according to Gilbraith et al., (2021), the peroxide values of fresh oils are generally below 10 meq O_2/kg and when this number exceeds 30 meq O_2/kg oils become rancid (Gilbraith et al., 2021). Therefore, the obtained peroxide values are on the edge of rancidity.

4.6.2 Fatty Acid Compositions of *R. toruloides* Lipids

The most abundant components are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) for all applications of HPH and HHP. Also, a very low amount (less than 1 %) of several types of fatty acid components were detected. Saturated and unsaturated fatty acid content changed with different applied parameters. For lipids extracted by HPH assisted treatment, while saturated fatty acid amount decreased, unsaturated fatty acid content increased. Also, lipids from HHP assisted treatment have the highest amount of unsaturated fatty acids. This is the main reason for lipid to be liquid at room temperature.

According to Wen et al. (2020), lipid of *R. toruloides* contains 23-30% palmitic acid, 30-37% oleic acid, 32-33.7% stearic acid, and 2-4% linoleic acid. Besides this study,

in another study 22% palmitic acid, 11% linoleic acid and 50% oleic acid were found for lipid of *R. toruloides* (Wiebe et al., 2012). Therefore, reported results in Table 4.14 are consistent with the mentioned findings. There are minor differences that may result from fluctuation in culture medium or fermentation conditions.

Table 4.14. FAME analysis of microbial oils obtained from *R. toruloides*.

Fatty Acids	HPH				HHP
	125 MPa 3 Passes	**125 MPa 5 Passes**	**125 MPa 13 Passes**	**200 MPa 5 Passes**	**230 MPa, 1 g dry biomass, 15 min**
10:0	0.15	0.96	0.99	0.94	-
12:0	0.31	1.34	1.31	1.27	0.14
14:0	1.64	5.45	5.35	5.22	1.45
14:1	0.27	0.64	0.64	0.62	-
15:0	0.28	0.69	0.67	0.66	0.13
16:0	36.84	26.97	26.66	27.40	24.38
16:1	3.74	4.00	4.10	3.99	2.30
18:0	15.53	10.87	10.89	11.04	9.25
18:1cis	38.19	41.68	43.12	42.12	56.38
18:2 cis	1.38	5.18	4.89	4.95	4.62

Table 4.14 (continued)

20:0	0.49	0.41	0.43	0.42	0.37
20:1	0.96	0.53	0.55	0.59	0.41
18:3 cis	0.12	-	-	-	0.27
SFA[a]	55.2	47.7	46.7	47.7	36.0
UFA[b]	44.8	52.3	53.3	52.3	64.0
MUFA[c]	43.2	46.9	48.4	47.3	59.1
PUFA[d]	1.6	5.4	4.9	5.0	4.9

[a] SFA: Saturated Fatty Acids

[b] UFA: Unsaturated fatty acids

[c] MUFA: Monounsaturated fatty acids

[d] PUFA: Polyunsaturated fatty acids

CHAPTER 5

CONCLUSION AND RECCOMENDATION

In this study, two extraction methods were used as pretreatment methods to extract microbial oil from oleaginous yeast biomass. These methods were high pressure homogenizer (HPH) and high hydrostatic pressure (HHP). *Rhodosporidium toruloides* DSM 4444 was used for microbial oil production in whey, hydrolyzed by beta galactosidase enzyme. Moreover, synthetic medium with varying composition and fermentation conditions was used to investigate optimal conditions for growth of *R. toruloides*.

Firstly, synthetic medium was used as cultivation medium for *R. toruloides,* and this yeast was cultivated in this medium for 192 hours (8 days). According to results, it was found that lactose contained medium was not suitable for *R. toruloides* thus it was hydrolyzed before using as a carbon source.

HPH assisted extraction was conducted with biomass suspended in spent fermentation medium and the maximum lipid yield was found to be 39.5±1.8% at 150 MPa and 7 passes. On the other hand, for HHP assisted extraction two different experimental designs were conducted. In the first experiment, collected cells were dried and mixed with solvent mixture, then HHP was applied to extract lipid. In the other experiment, a spent fermentation medium containing biomass was subjected to HHP for lipid extraction. The maximum lipid yields were 17.8±1.3% for dry biomass experiment at 200 MPa, 1-gram dry biomass and 15 minutes and for the spent fermentation medium experiment 22.8±3.7% at 300 MPa, 30 °C and with the duration of 10 minutes. Also, HSH method applied as a control method yielded 47.6±2.6%.

After pressurization, fatty acid content of the lipid was examined, and it was observed that fatty acid content changed with pressure and the applied method. Oleic

acid (C18:1) and palmitic acid (C16:0) were the most abundant fatty acids for both HPH and HHP treatments.

According to results, it was concluded that *R. toruloides* can grow in hydrolyzed whey medium and produce high amounts of biomass and lipid. Moreover, during this study HPH assisted extraction was found to be more efficient than HHP method.

In future studies, it would be beneficial to investigate the lactose utilization mechanism of *R. toruloides*. The observed variations in beta-carotene production under different conditions in this study suggest its potential for various industries. While this study focused on a single oleaginous yeast species, exploring the use of different yeast species for lipid production may result in higher lipid yields. Furthermore, the study demonstrated that utilizing hydrolyzed whey resulted in a significant lipid yield for *R. toruloides*, indicating the potential use of this waste material for biomass and lipid production by other microorganisms as well. Finally, both HPH and HHP methods can be employed as pre-treatment methods for lipid extraction from whey-based biomass.

REFERENCES

Abeln, F., & Chuck, C. J. (2020). The role of temperature, pH and nutrition in process development of the unique oleaginous yeast *Metschnikowia pulcherrima*. *Journal of Chemical Technology & Biotechnology*. https://doi.org/10.1002/jctb.6301

Abeln, F., & Chuck, C. J. (2021). The history, state of the art and future prospects for Oleaginous Yeast Research. Microbial Cell Factories, 20(1). https://doi.org/10.1186/s12934-021-01712-1

Afonso, V., Tangerino, L., Oliveira, D., & Raposo, S. (2018). Culture Strategies for Lipid Production Using Low-cost Carbon Sources by *Rhodosporidium toruloides*. INCREaSE, 103–116. https://doi.org/10.1007/978-3-319-70272-8_7

Alakraa, F., Saygün, A., & Şahin Yeşilçubuk, N. (2020). Biotechnological production of lipids and carotenoids from *Rhodosporidium toruloides* Y27012. European Journal of Science and Technology, 156–164. https://doi.org/10.31590/ejosat.708556

Alvarez, H., & Steinbüchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. *Applied Microbiology and Biotechnology*, *60*(4), 367–376. https://doi.org/10.1007/s00253-002-1135-0

Alvarez, H. M., Hernández, M. A., Lanfranconi, M. P., Silva, R. A., & Villalba, M. S. (2021). Rhodococcus as biofactories for Microbial Oil Production. *Molecules*, *26*(16), 4871. https://doi.org/10.3390/molecules26164871

Anwar, M. I., Muhammad, F., Awais, M. M., & Akhtar, M. (2017). A review of β-glucans as a growth promoter and antibiotic alternative against enteric pathogens in Poultry. *World's Poultry Science Journal*, *73*(3), 651–661. https://doi.org/10.1017/s0043933917000241

Arshad, R. N., Abdul-Malek, Z., Munir, A., Buntat, Z., Ahmad, M. H., Jusoh, Y. M. M., Bekhit, A. E.-D., Roobab, U., Manzoor, M. F., & Aadil, R. M. (2020). Electrical systems for pulsed electric field applications in the food industry: An engineering perspective. *Trends in Food Science & Technology*, *104*, 1–13. https://doi.org/10.1016/j.tifs.2020.07.008

Bento, T. F., Viana, V. F., Carneiro, L. M., & Silva, J. P. (2019). Influence of agitation and aeration on single cell oil production by Rhodotorula glutinis from Glycerol. *Journal of Sustainable Bioenergy Systems*, *09*(02), 29–43. https://doi.org/10.4236/jsbs.2019.92003

Bleoussi, R. T. M., Christian, K. T. R., Tchekessi, C. C. K., Sachi, P. A., Banon, J. S. B., Djogbe, A. A., Assogba, K. T., & Innocent, B. Y. (2020). Nutritional Quality and use of whey in Human Food for its valorization. *World Journal of Advanced Research and Reviews*, *8*(1), 284–293. https://doi.org/10.30574/wjarr.2020.8.1.0356

Bligh. E. G. & Dyer. W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*. *37*(1). 911–917. https://doi.org/10.1139/y59-099

Bocker, R., & Silva, E. K. (2022). Pulsed electric field assisted extraction of natural food pigments and colorings from plant matrices. *Food Chemistry: X*, *15*, 100398. https://doi.org/10.1016/j.fochx.2022.100398

Bommareddy. R. R., Sabra. W., Maheshwari. G., & Zeng. A.-P. (2015). Metabolic network analysis and experimental study of lipid production in *Rhodosporidium toruloides* grown on single and mixed substrates. Microbial Cell Factories. 14(1). https://doi.org/10.1186/s12934-015-0217-5

Bonturi, N., Matsakas, L., Nilsson, R., Christakopoulos, P., Miranda, E., Berglund, K., & Rova, U. (2015). Single cell oil producing yeasts Lipomyces starkeyi and *Rhodosporidium toruloides*: Selection of extraction strategies and biodiesel property prediction. Energies, 8(6), 5040–5052. https://doi.org/10.3390/en8065040

Braunwald, T., Schwemmlein, L., Graeff-Hönninger, S., French, W. T., Hernandez, R., Holmes, W. E., & Claupein, W. (2013). Effect of different C/N ratios on carotenoid and lipid production by Rhodotorula Glutinis. *Applied Microbiology and Biotechnology*, *97*(14), 6581–6588. https://doi.org/10.1007/s00253-013-5005-8

Buchanan, D., Martindale, W., Romeih, E., & Hebishy, E. (2023). Recent advances in Whey Processing and valorisation: Technological and environmental perspectives. *International Journal of Dairy Technology*, *76*(2), 291–312. https://doi.org/10.1111/1471-0307.12935

Calvey, C. H., Su, Y.-K., Willis, L. B., McGee, M., & Jeffries, T. W. (2016). Nitrogen limitation, oxygen limitation, and lipid accumulation in Lipomyces

starkeyi. *Bioresource Technology*, *200*, 780–788. https://doi.org/10.1016/j.biortech.2015.10.104

Caporusso, A., Capece, A., & De Bari, I. (2021). Oleaginous yeasts as cell factories for the sustainable production of Microbial Lipids by the valorization of Agri-Food Wastes. *Fermentation*, *7*(2). https://doi.org/10.3390/fermentation7020050

Catarine Tosi-Costa, A., Turbay-Vasconcelos, C., Adami, L., Favarato, L., Bolivar-Telleria, M., Carneiro, T., Santos, A., R. Fernandes, A., & M.B. Fernandes, P. (2019). High hydrostatic pressure process to improve ethanol production. *Fuel Ethanol Production from Sugarcane*. https://doi.org/10.5772/intechopen.78712

Chandrapala, J., Duke, M. C., Gray, S. R., Zisu, B., Weeks, M., Palmer, M., & Vasiljevic, T. (2015). Properties of acid whey as a function of ph and temperature. *Journal of Dairy Science*, *98*(7), 4352–4363. https://doi.org/10.3168/jds.2015-9435

Chattopadhyay, A., & Maiti, M. K. (2021). Lipid production by oleaginous yeasts. *Advances in Applied Microbiology*, 1–98. https://doi.org/10.1016/bs.aambs.2021.03.003

Che, L., Li, D., Wang, L., Özkan, N., Chen, X. D., & Mao, Z. (2007). Effect of high-pressure homogenization on the structure of cassava starch. *International Journal of Food Properties*, *10*(4), 911–922. https://doi.org/10.1080/10942910701223315

Chen, J., Wu, S.-S., Liang, R.-H., Liu, W., Liu, C.-M., Shuai, X.-X., & Wang, Z.-J. (2014). The effect of high speed shearing on disaggregation and degradation of pectin from creeping fig seeds. *Food Chemistry*, *165*, 1–8. https://doi.org/10.1016/j.foodchem.2014.05.096

Chen, Z., Reznicek, W. D., & Wan, C. (2018). Deep eutectic solvent pretreatment enabling full utilization of switchgrass. Bioresource Technology, 263(April), 40–48. https://doi.org/10.1016/j.biortech.2018.04.058

Chen, Y.-H., Ong, C.-C., & Lin, T.-Y. (2022). Effect of sea salt and taro waste on fungal mortierella Alpina cultivation for arachidonic acid-rich lipid production. *Fermentation*, *8*(2), 81. https://doi.org/10.3390/fermentation8020081

Cheirsilp, B., & Kitcha, S. (2014). Solid state fermentation by cellulolytic oleaginous fungi for direct conversion of lignocellulosic biomass into lipids: Fed-batch

and repeated-batch fermentations. Industrial Crops and Products, 66, 73–80. https://doi.org/10.1016/j.indcrop.2014.12.035

Comuzzo, P., & Calligaris, S. (2019). Potential applications of high pressure homogenization in winemaking: A Review. *Beverages*, *5*(3), 56. https://doi.org/10.3390/beverages5030056

Danlami, J. M., Arsad, A., Ahmad Zaini, M. A., & Sulaiman, H. (2014). A comparative study of various oil extraction techniques from plants. *Reviews in Chemical Engineering*, *30*(6). https://doi.org/10.1515/revce-2013-0038

Darcan, S., Sarigül, N. (2016). Single cell oil production from microorganisms. Türk Mikrobiyoloji Cemiyeti Dergisi, 45(2), 55–67. https://doi.org/10.5222/tmcd.2015.055

de Castro, R. J., Domingues, M. A., Ohara, A., Okuro, P. K., dos Santos, J. G., Brexó, R. P., & Sato, H. H. (2017). Whey protein as a key component in food systems: Physicochemical properties, production technologies and applications. *Food Structure*, *14*, 17–29. https://doi.org/10.1016/j.foostr.2017.05.004

Dey, P., Banerjee, J., & Maiti, M. K. (2011). Comparative lipid profiling of two endophytic fungal isolates – colletotrichum sp. and Alternaria sp. having potential utilities as biodiesel feedstock. *Bioresource Technology*, *102*(10), 5815–5823. https://doi.org/10.1016/j.biortech.2011.02.064

Dias, C., Nobre, B. P., Santos, J. A. L., Lopes da Silva, T., & Reis, A. (2022). Direct lipid and carotenoid extraction from *Rhodosporidium toruloides* broth culture after high pressure homogenization cell disruption: Strategies, methodologies, and yields. Biochemical Engineering Journal, 189, 108712. https://doi.org/10.1016/j.bej.2022.108712

Díaz, G. C., Cruz, Y. R., Carliz, R. G., Paula, R. C., Aranda, D. A., Dario, M. A., Marass, G. S., & Furtado, N. C. (2015). Cultivation of microalgae Monoraphidium sp., in the plant pilot the Grand Valle Bio Energy, for biodiesel production. *Natural Science*, *07*(07), 370–378. https://doi.org/10.4236/ns.2015.77040

Díaz, T., Fillet, S., Campoy, S., Vázquez, R., Viña, J., Murillo, J., & Adrio, J. L. (2018). Combining evolutionary and metabolic engineering in *Rhodosporidium toruloides* for lipid production with non-detoxified wheat straw hydrolysates. Applied Microbiology and Biotechnology, 102(7), 3287-3300. https://doi.org/10.1007/s00253-018-8810-2

Diels, A. M., & Michiels, C. W. (2006). High-pressure homogenization as a non-thermal technique for the inactivation of Microorganisms. *Critical Reviews in Microbiology, 32*(4), 201–216. https://doi.org/10.1080/10408410601023516

Dinh, H. V., Suthers, P. F., Chan, S. H., Shen, Y., Xiao, T., Deewan, A., Jagtap, S. S., Zhao, H., Rao, C. V., Rabinowitz, J. D., & Maranas, C. D. (2019). A comprehensive genome-scale model for *Rhodosporidium toruloides* IFO0880 accounting for functional genomics and phenotypic data. *Metabolic Engineering Communications*, *9*. https://doi.org/10.1016/j.mec.2019.e00101

Drévillon, L., Koubaa, M., & Vorobiev, E. (2018). Lipid extraction from Yarrowia lipolytica biomass using high-pressure homogenization. *Biomass and Bioenergy*, *115*, 143–150. https://doi.org/10.1016/j.biombioe.2018.04.014

Drévillon, L., Koubaa, M., Nicaud, J.-M., & Vorobiev, E. (2019). Cell disruption pre-treatments towards an effective recovery of oil from Yarrowia lipolytica oleaginous yeast. *Biomass and Bioenergy*, *128*, 105320. https://doi.org/10.1016/j.biombioe.2019.105320

Duarte, S. H., de Andrade, C. C., Ghiselli, G., & Maugeri, F. (2013). Exploration of Brazilian biodiversity and selection of a new oleaginous yeast strain cultivated in raw glycerol. *Bioresource Technology*, *138*, 377–381. https://doi.org/10.1016/j.biortech.2013.04.004

Economou, Ch. N., Makri, A., Aggelis, G., Pavlou, S., & Vayenas, D. V. (2010). Semi-solid state fermentation of sweet sorghum for the biotechnological production of Single Cell Oil. Bioresource Technology, 101(4), 1385–1388. https://doi.org/10.1016/j.biortech.2009.09.028

Ekpenyong, M., Antai, S., Asitok, A., & Ekpo, B. (2017). Response surface modeling and optimization of major medium variables for glycolipopeptide production. Biocatalysis and Agricultural Biotechnology, 10, 113–121. https://doi.org/10.1016/j.bcab.2017.02.015

Elfeky, N., Elmahmoudy, M., & Bao, Y. (2020). Manipulation of culture conditions: Tool for correlating/improving lipid and carotenoid production by Rhodotorula glutinis. *Processes*, *8*(2), 140. https://doi.org/10.3390/pr8020140

Fakas, S., Papanikolaou, S., Batsos, A., Galiotou-Panayotou, M., Mallouchos, A., & Aggelis, G. (2009). Evaluating renewable carbon sources as substrates for single cell oil production by Cunninghamella echinulata and Mortierella Isabellina. *Biomass and Bioenergy*, *33*(4), 573–580. https://doi.org/10.1016/j.biombioe.2008.09.006

Farag, M. A., & Gad, M. Z. (2022). Omega-9 fatty acids: Potential roles in inflammation and cancer management. *Journal of Genetic Engineering and Biotechnology*, *20*(1). https://doi.org/10.1186/s43141-022-00329-0

Fei, Q., O'Brien, M., Nelson, R., Chen, X., Lowell, A., & Dowe, N. (2016). Enhanced lipid production by *Rhodosporidium toruloides* using different fed-batch feeding strategies with lignocellulosic hydrolysate as the sole carbon source. *Biotechnology for Biofuels*, *9*(1). https://doi.org/10.1186/s13068-016-0542-x

Ganeva, V., Angelova, B., Galutzov, B., Goltsev, V., & Zhiponova, M. (2020). Extraction of proteins and other intracellular bioactive compounds from Baker's yeasts by pulsed electric field treatment. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.552335

Ghazani, S. M., & Marangoni, A. G. (2022). Microbial lipids for foods. *Trends in Food Science & Technology*, *119*, 593–607. https://doi.org/10.1016/j.tifs.2021.10.014

Gilbraith, W. E., Carter, J. C., Adams, K. L., Booksh, K. S., & Ottaway, J. M. (2021). Improving prediction of peroxide value of edible oils using regularized regression models. *Molecules*, *26*(23), 7281. https://doi.org/10.3390/molecules26237281

Gong, Z., Shen, H., Wang, Q., Yang, X., Xie, H., & Zhao, Z. K. (2013). Efficient conversion of biomass into lipids by using the simultaneous saccharification and enhanced lipid production process. Biotechnology for Biofuels, 6(1). https://doi.org/10.1186/1754-6834-6-36

Gong, Z., Shen, H., Zhou, W., Wang, Y., Yang, X., & Zhao, Z. K. (2015). Efficient conversion of acetate into lipids by the oleaginous yeast Cryptococcus curvatus. *Biotechnology for Biofuels*, *8*(1). https://doi.org/10.1186/s13068-015-0371-3

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. *Frontiers in Bioengineering and Biotechnology*, *8*. https://doi.org/10.3389/fbioe.2020.00355

Gouda, M. K., Omar, S. H., & Aouad, L. M. (2008). Single cell oil production by Gordonia sp.. DG using agro-industrial wastes. *World Journal of Microbiology*

and Biotechnology, *24*(9), 1703–1711. https://doi.org/10.1007/s11274-008-9664-z

Gurel, N. (2019). Evaluation And Optimization Of Microbial Oil Production Using Synthetic Sugars [Thesis (M.S.) – Graduate School of Natural and Applied Sciences. Food Engineering.] Middle East Technical University.

Gutiérrez-Hernández, C. A., Hernández-Almanza, A., Hernández-Beltran, J. U., Balagurusamy, N., & Hernández-Teran, F. (2022). Cheese whey valorization to obtain single-cell oils of industrial interest: An overview. *Food Bioscience*, *50*, 102086. https://doi.org/10.1016/j.fbio.2022.102086

Hewavitharana, G. G., Perera, D. N., Navaratne, S. B., & Wickramasinghe, I. (2020). Extraction methods of fat from food samples and preparation of fatty acid methyl esters for gas chromatography: A Review. *Arabian Journal of Chemistry*, *13*(8), 6865–6875. https://doi.org/10.1016/j.arabjc.2020.06.039

Hua, X., Xu, S., Wang, M., Chen, Y., Yang, H., & Yang, R. (2017). Effects of high-speed homogenization and high-pressure homogenization on structure of tomato residue fibers. *Food Chemistry*, *232*, 443–449. https://doi.org/10.1016/j.foodchem.2017.04.003

Huang, C., Zong, M., Wu, H., & Liu, Q. (2009). Microbial oil production from rice straw hydrolysate by Trichosporon Fermentans. *Bioresource Technology*, *100*(19), 4535–4538. https://doi.org/10.1016/j.biortech.2009.04.022

Huang, C., Luo, M.-T., Chen, X.-F., Qi, G.-X., Xiong, L., Lin, X.-Q., Wang, C., Li, H.-L., & Chen, X.-D. (2017). Combined "de novo" and "Ex novo" lipid fermentation in a mix-medium of corncob acid hydrolysate and soybean oil by Trichosporon Dermatis. *Biotechnology for Biofuels*, *10*(1). https://doi.org/10.1186/s13068-017-0835-8

Huang, X.-F., Liu, J.-N., Lu, L.-J., Peng, K.-M., Yang, G.-X., & Liu, J. (2016). Culture strategies for lipid production using acetic acid as sole carbon source by *Rhodosporidium toruloides*. *Bioresource Technology*, *206*, 141–149. https://doi.org/10.1016/j.biortech.2016.01.073

Hussain, J., Ruan, Z., Nascimento, I. A., Liu, Y., & Liao, W. (2014). Lipid profiling and corresponding biodiesel quality of Mortierella Isabellina using different drying and extraction methods. *Bioresource Technology*, *169*, 768–772. https://doi.org/10.1016/j.biortech.2014.06.074

Jach, M. E., & Malm, A. (2022). Yarrowia lipolytica as an alternative and valuable source of nutritional and bioactive compounds for humans. Molecules, 27(7). https://doi.org/10.3390/molecules27072300

Jagtap, S. S., Bedekar, A. A., Liu, J.-J., Jin, Y.-S., & Rao, C. V. (2019). Production of galactitol from galactose by the oleaginous yeast *Rhodosporidium toruloides* IFO0880. *Biotechnology for Biofuels*, *12*(1). https://doi.org/10.1186/s13068-019-1586-5

Jiru, T. M., Groenewald, M., Pohl, C., Steyn, L., Kiggundu, N., & Abate, D. (2017). Optimization of cultivation conditions for biotechnological production of lipid by Rhodotorula kratochvilovae (syn, Rhodosporidium kratochvılovae) SY89 for biodiesel preparation. *3 Biotech*, *7*(2). https://doi.org/10.1007/s13205-017-0769-7

Kamineni, A., & Shaw, J. (2020). Engineering triacylglycerol production from sugars in oleaginous yeasts. *Current Opinion in Biotechnology*, *62*, 239–247. https://doi.org/10.1016/j.copbio.2019.12.022

Kannan, N., Rao, A. S., & Nair, A. (2021). Microbial production of Omega-3 Fatty Acids: An overview. *Journal of Applied Microbiology*, *131*(5), 2114–2130. https://doi.org/10.1111/jam.15034

Kapoore, R., Butler, T., Pandhal, J., & Vaidyanathan, S. (2018). Microwave-assisted extraction for microalgae: From biofuels to Biorefinery. *Biology*, *7*(1), 18. https://doi.org/10.3390/biology7010018

Karim, A., Islam, M. A., Khalid, Z. B., Faizal, C. K., Khan, Md. M., & Yousuf, A. (2019). Microalgal cell disruption and lipid extraction techniques for potential biofuel production. *Microalgae Cultivation for Biofuels Production*, 129–147. https://doi.org/10.1016/b978-0-12-817536-1.00009-6

Khot, M., Raut, G., Ghosh, D., Alarcón-Vivero, M., Contreras, D., & Ravikumar, A. (2020). Lipid recovery from oleaginous yeasts: Perspectives and challenges for industrial applications. *Fuel*, *259*, 116292. https://doi.org/10.1016/j.fuel.2019.116292

Kraisintu, P. & Yongmanitchai, W. & Limtong, Savitree. (2010). Selection and optimization for lipid production of a newly isolated oleaginous yeast, *Rhodosporidium toruloides* DMKU3-TK16. Kasetsart Journal - Natural Science. 44. 436-445.

Kruszewski, B., Zawada, K., & Karpiński, P. (2021). Impact of high-pressure homogenization parameters on physicochemical characteristics, bioactive

compounds content, and antioxidant capacity of blackcurrant juice. Molecules, 26(6), 1802. https://doi.org/10.3390/molecules26061802

Kumar, L. R., Kaur, R., Yellapu, S. K., Zhang, X., & Tyagi, R. D. (2019). Biodiesel production from oleaginous microorganisms with wastes as Raw Materials. Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels, 661–690. https://doi.org/10.1016/b978-0-12-816856-1.00027-0

Lamers, D., van Biezen, N., Martens, D., Peters, L., van de Zilver, E., Jacobs-van Dreumel, N., Wijffels, R. H., & Lokman, C. (2016). Selection of oleaginous yeasts for fatty acid production. BMC Biotechnology, 16(1). https://doi.org/10.1186/s12896-016-0276-7

Lappa. I. K., Kachrimanidou. V., Papadaki. A., Stamatiou. A., Ladakis. D., Eriotou. E. & Kopsahelis. N. (2021). A comprehensive bioprocessing approach to Foster Cheese Whey Valorization: On-site β-galactosidase secretion for lactose hydrolysis and sequential bacterial cellulose production. *Fermentation*. *7*(3). 184. https://doi.org/10.3390/fermentation7030184

Li, Q., Du, W., & Liu, D. (2008). Perspectives of microbial oils for biodiesel production. Applied Microbiology and Biotechnology, 80(5), 749–756. https://doi.org/10.1007/s00253-008-1625-9

Li, X., Xu, H., & Wu, Q. (2007). Large-scale biodiesel production from Microalgachlorella protothecoides through heterotrophic cultivation in bioreactors. *Biotechnology and Bioengineering*, *98*(4), 764–771. https://doi.org/10.1002/bit.21489

Li, Y., Zhao, Z. (Kent), & Bai, F. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture. *Enzyme and Microbial Technology*, *41*(3), 312–317. https://doi.org/10.1016/j.enzmictec.2007.02.008

Liang, M.-H., & Jiang, J.-G. (2013). Advancing oleaginous microorganisms to produce lipid via Metabolic Engineering Technology. Progress in Lipid Research, 52(4), 395–408. https://doi.org/10.1016/j.plipres.2013.05.002

Lin, H., Wang, Q., Shen, Q., Zhan, J., & Zhao, Y. (2013). Genetic engineering of microorganisms for biodiesel production. Bioengineered, 4(5), 292–304. https://doi.org/10.4161/bioe.23114

Liu, D., Ding, L., Sun, J., Boussetta, N., & Vorobiev, E. (2016). Yeast cell disruption strategies for recovery of intracellular bio-active compounds — a review.

Innovative Food Science & Emerging Technologies, *36*, 181–192. https://doi.org/10.1016/j.ifset.2016.06.017

Liu, J., Huang, J., Fan, K. W., Jiang, Y., Zhong, Y., Sun, Z., & Chen, F. (2010). Production potential of Chlorella Zofingienesis as a feedstock for biodiesel. *Bioresource Technology*, *101*(22), 8658–8663. https://doi.org/10.1016/j.biortech.2010.05.082

Llompart, M., Garcia-Jares, C., Celeiro, M., & Dagnac, T. (2018). Microwave-assisted extraction. *Reference Module in Chemistry, Molecular Sciences and Chemical Engineering*. https://doi.org/10.1016/b978-0-12-409547-2.14442-7

Lopes, H. J., Bonturi, N., Kerkhoven, E. J., Miranda, E. A., & Lahtvee, P.-J. (2020). C/N ratio and carbon source-dependent lipid production profiling in Rhodotorula toruloides. *Applied Microbiology and Biotechnology*, *104*(6), 2639–2649. https://doi.org/10.1007/s00253-020-10386-5

Low. L. K., & Ng. C. S. (1987). Analysis of oils: Determination of peroxide value. In Laboratory Manual on Analytical Methods and Procedures for Fish and Fish Products (pp. 71–73). essay. Marine Fisheries Research Department. Southeast Asian Fisheries Development Center.

Lyu. L., Chu. Y., Zhang. S., Zhang. Y., Huang. Q., Wang. S., & Zhao. Z. K. (2021). Engineering the oleaginous yeast *Rhodosporidium toruloides* for improved resistance against inhibitors in biomass hydrolysates. *Frontiers in Bioengineering and Biotechnology*. *9*. https://doi.org/10.3389/fbioe.2021.768934

Mañas, P., & Pagán, R. (2005). Microbial inactivation by new technologies of Food Preservation. *Journal of Applied Microbiology*, *98*(6), 1387–1399. https://doi.org/10.1111/j.1365-2672.2005.02561.x

Matsui, T., Otsuka, K., & Sato, S. (2011). Microbial oil production from carbohydrates using Sporobolomyces carnicolor strain O33. *Annals of Microbiology*, *62*(2), 861–864. https://doi.org/10.1007/s13213-011-0316-4

Matsakas, L., Giannakou, M., & Vörös, D. (2017). Effect of synthetic and natural media on lipid production from fusarium oxysporum. *Electronic Journal of Biotechnology*, *30*, 95–102. https://doi.org/10.1016/j.ejbt.2017.10.003

Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., & Xian, M. (2009). Biodiesel production from Oleaginous microorganisms. Renewable Energy, 34(1), 1–5. https://doi.org/10.1016/j.renene.2008.04.014

Meullemiestre, A., Breil, C., Abert-Vian, M., & Chemat, F. (2015). Modern techniques and solvents for the extraction of microbial oils. *SpringerBriefs in Molecular Science*. https://doi.org/10.1007/978-3-319-22717-7

Mhlongo, S. I., Ezeokoli, O. T., Roopnarain, A., Ndaba, B., Sekoai, P. T., Habimana, O., & Pohl, C. H. (2021). The potential of single-cell oils derived from filamentous fungi as alternative feedstock sources for biodiesel production. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.637381

Michou, S., Tsouko, E., Vastaroucha, E.-S., Diamantopoulou, P., & Papanikolaou, S. (2022). Growth potential of selected yeast strains cultivated on xylose-based media mimicking lignocellulosic wastewater streams: High production of microbial lipids by *Rhodosporidium toruloides*. *Fermentation*, *8*(12), 713. https://doi.org/10.3390/fermentation8120713

Miller. G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. *31*(3). 426–428. https://doi.org/10.1021/ac60147a030

Mohammed, D., Zaher, F., Hassan, E., Maksoud, H., & Ramadan, E. (2018). Factors affecting microbial oil accumulation by oleaginous yeast. Annual Research & Review in Biology, 23(2), 1–12. https://doi.org/10.9734/arrb/2018/38425

Nadar, S. S., Rao, P., & Rathod, V. K. (2018). Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A Review. *Food Research International*, *108*, 309–330. https://doi.org/10.1016/j.foodres.2018.03.006

Naveena, B., & Nagaraju, M. (2020). Review on principles, effects, advantages and disadvantages of high pressure processing of food. *International Journal of Chemical Studies*, *8*(2), 2964–2967. https://doi.org/10.22271/chemi.2020.v8.i2at.9202

Niehus, X., Crutz-Le Coq, A.-M., Sandoval, G., Nicaud, J.-M., & Ledesma-Amaro, R. (2018). Engineering yarrowia lipolytica to enhance lipid production from lignocellulosic materials. *Biotechnology for Biofuels*, *11*(1). https://doi.org/10.1186/s13068-018-1010-6

Ochsenreither, K., Glück, C., Stressler, T., Fischer, L., & Syldatk, C. (2016). Production strategies and applications of Microbial Single Cell Oils. Frontiers in Microbiology, 7. https://doi.org/10.3389/fmicb.2016.01539

Olmstead, I. L. D., Kentish, S. E., Scales, P. J., & Martin, G. J. O. (2013). Low solvent, low temperature method for extracting biodiesel lipids from

concentrated microalgal biomass. *Bioresource Technology, 148*, 615–619. https://doi.org/10.1016/j.biortech.2013.09.022

Osman, M. E., Abdel-Razik, A. B., Zaki, K. I., Mamdouh, N., & El-Sayed, H. (2022). Isolation, molecular identification of lipid-producing Rhodotorula diobovata: Optimization of lipid accumulation for biodiesel production. *Journal of Genetic Engineering and Biotechnology*, *20*(1). https://doi.org/10.1186/s43141-022-00304-9

Osorio-González, C. S., Saini, R., Hegde, K., Brar, S. K., Lefebvre, A., & Avalos Ramírez, A. (2022). Crabtree effect on *Rhodosporidium toruloides* using wood hydrolysate as a culture media. *Fermentation*, *9*(1), 11. https://doi.org/10.3390/fermentation9010011

Ova Ozcan, D., & Ovez, B. (2022). Phaeodactylum tricornutum as a potential feedstock for an integrated biorefinery process under varying cultivation conditions. *Biocatalysis and Agricultural Biotechnology*, *45*, 102508. https://doi.org/10.1016/j.bcab.2022.102508

Papanikolaou, S., Aggelis , G., Marc, I., Komaitis, M., & Chevalot, I. (2002). Single cell oil production by Yarrowia lipolytica growing on an industrial derivative of animal fat in batch cultures. Applied Microbiology and Biotechnology, 58(3), 308–312. https://doi.org/10.1007/s00253-001-0897-0

Park, Y. K., & Ledesma-Amaro, R. (2018). The engineering potential of *Rhodosporidium toruloides* as a workhorse for biotechnological applications. *Trends in Biotechnology*, *36*(3), 304–317. https://doi.org/10.1016/j.tibtech.2017.10.013

Patel, A., Matsakas, L., Rova, U., & Christakopoulos, P. (2018). Heterotrophic cultivation of auxenochlorella protothecoides using forest biomass as a feedstock for sustainable biodiesel production. *Biotechnology for Biofuels*, *11*(1). https://doi.org/10.1186/s13068-018-1173-1

Patel, A., Mikes, F., & Matsakas, L. (2018). An overview of current pretreatment methods used to improve lipid extraction from oleaginous Micro-Organisms. *Molecules*, *23*(7), 1562. https://doi.org/10.3390/molecules23071562

Patel, A., Karageorgou, D., Rova, E., Katapodis, P., Rova, U., Christakopoulos, P., & Matsakas, L. (2020). An overview of potential oleaginous microorganisms and their role in biodiesel and omega-3 fatty acid-based industries. Microorganisms, 8(3), 434. https://doi.org/10.3390/microorganisms8030434

Plazzotta, S., & Manzocco, L. (2018). Effect of ultrasounds and high pressure homogenization on the extraction of antioxidant polyphenols from lettuce waste. *Innovative Food Science & Emerging Technologies*, *50*, 11–19. https://doi.org/10.1016/j.ifset.2018.10.004

Poontawee, R., Yongmanitchai, W., & Limtong, S. (2017). Efficient oleaginous yeasts for lipid production from lignocellulosic sugars and effects of lignocellulose degradation compounds on growth and lipid production. *Process Biochemistry*, *53*, 44–60. https://doi.org/10.1016/j.procbio.2016.11.013

Potato dextrose agar. (October) https://www.dalynn.com/dyn/ck_assets/files/tech/PP85.pdf

Pires, A. F., Marnotes, N. G., Rubio, O. D., Garcia, A. C., & Pereira, C. D. (2021). Dairy by-products: A review on the valorization of whey and second cheese whey. *Foods*, *10*(5), 1067. https://doi.org/10.3390/foods10051067

Qian, X., Zhou, X., Chen, L., Zhang, X., Xin, F., Dong, W., Zhang, W., Ochsenreither, K., & Jiang, M. (2021). Bioconversion of volatile fatty acids into lipids by the oleaginous yeast apiotrichum porosum DSM27194. *Fuel*, *290*, 119811. https://doi.org/10.1016/j.fuel.2020.119811

Rajendran, S. R., Udenigwe, C. C., & Yada, R. Y. (2016). Nanochemistry of protein-based delivery agents. *Frontiers in Chemistry*, *4*. https://doi.org/10.3389/fchem.2016.00031

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for Single Cell Oil Production. *Biochimie*, *86*(11), 807–815. https://doi.org/10.1016/j.biochi.2004.09.017

Ratledge, C. (2010). Single Cell Oils for the 21st century. Single Cell Oils, 3–26. https://doi.org/10.1016/b978-1-893997-73-8.50005-0

Ratledge, C. (2013). Microbial production of polyunsaturated fatty acids as nutraceuticals. Microbial Production of Food Ingredients, Enzymes and Nutraceuticals, 531-558. doi:10.1533/9780857093547.2.531

Ratledge, C., & Wynn, J. P. (2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Advances in Applied Microbiology*, 1–52. https://doi.org/10.1016/s0065-2164(02)51000-5

Rendueles, E., Omer, M. K., Alvseike, O., Alonso-Calleja, C., Capita, R., & Prieto, M. (2011). Microbiological food safety assessment of high hydrostatic

pressure processing: A review. *LWT - Food Science and Technology*, *44*(5), 1251–1260. https://doi.org/10.1016/j.lwt.2010.11.001

Saenge, C., Cheirsilp, B., Suksaroge, T. T., & Bourtoom, T. (2011). Potential use of oleaginous red yeast Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochemistry*, *46*(1), 210–218. https://doi.org/10.1016/j.procbio.2010.08.009

Sahena, F., Zaidul, I. S. M., Jinap, S., Karim, A. A., Abbas, K. A., Norulaini, N. A. N., & Omar, A. K. M. (2009). Application of supercritical CO2 in Lipid Extraction – A Review. *Journal of Food Engineering*, *95*(2), 240–253. https://doi.org/10.1016/j.jfoodeng.2009.06.026

Saini, R., Osorio-Gonzalez, C. S., Hegde, K., Brar, S. K., & Vezina, P. (2021). Effect of creating a fed-batch like condition using carbon to nitrogen ratios on lipid accumulation in *Rhodosporidium toruloides*-1588. *Bioresource Technology*, *337*, 125354. https://doi.org/10.1016/j.biortech.2021.125354

Saisriyoot, M., Thanapimmetha, A., Suwaleerat, T., Chisti, Y., & Srinophakun, P. (2019). Biomass and lipid production by Rhodococcus Opacus PD630 in molasses-based media with and without osmotic-stress. *Journal of Biotechnology*, *297*, 1–8. https://doi.org/10.1016/j.jbiotec.2019.02.012

Salazar, F. A., Yildiz, S., Leyva, D., Soto-Caballero, M., Welti-Chanes, J., Anubhav, P. S., Lavilla, M., & Escobedo-Avellaneda, Z. (2021). HHP influence on food quality and bioactive compounds: A review of the last decade. *Innovative Food Processing Technologies*, 87–111. https://doi.org/10.1016/b978-0-08-100596-5.22984-3

Salvador López, J. M., Vandeputte, M., & Van Bogaert, I. N. (2022). Oleaginous yeasts: Time to rethink the definition? Yeast, 39(11–12), 553–606. https://doi.org/10.1002/yea.3827

Samarasinghe, N. (2012). Effect of high pressure homogenization on aqueous phase solvent extraction of lipids from Nannochloris oculata microalgae. *Journal of Energy and Natural Resources*, *1*(1), 1. https://doi.org/10.11648/j.jenr.20120101.11

Sampaio, J. P. (2011). Rhodosporidium. *The Yeasts*, 1523–1539. https://doi.org/10.1016/b978-0-444-52149-1.00127-0

Sánchez-Camargo, A. P., Montero, L., Mendiola, J. A., Herrero, M., & Ibáñez, E. (2020). Novel extraction techniques for bioactive compounds from herbs and

spices. *Herbs, Spices and Medicinal Plants*, 95–128. https://doi.org/10.1002/9781119036685.ch5

Sankh, S., Thiru, M., Saran, S., & Rangaswamy, V. (2013). Biodiesel production from a newly isolated pichia KUDRIAVZEVII strain. *Fuel*, *106*, 690–696. https://doi.org/10.1016/j.fuel.2012.12.014

Schingoethe, D. J. (1976). Whey utilization in animal feeding: A summary and evaluation. *Journal of Dairy Science*, *59*(3), 556–570. https://doi.org/10.3168/jds.s0022-0302(76)84240-3

Sehrawat, R., Kaur, B. P., Nema, P. K., Tewari, S., & Kumar, L. (2020). Microbial inactivation by high pressure processing: Principle, mechanism and factors responsible. *Food Science and Biotechnology*, *30*(1), 19–35. https://doi.org/10.1007/s10068-020-00831-6

Shakeri, S., Khoshbasirat, F., & Maleki, M. (2021). Rhodosporidium sp.. Dr37: A novel strain for production of squalene in optimized cultivation conditions. *Biotechnology for Biofuels*, *14*(1). https://doi.org/10.1186/s13068-021-01947-5

Singh, A. (2021). *Characterization and optimization of Rhodosporidium toruloides for production of lipids* [Thesis (M.S.)- Graduate School of Natural and Applied Sciences. Chemical Engineering.] Lund university.

Sitepu, I. R., Garay, L. A., Sestric, R., Levin, D., Block, D. E., German, J. B., & Boundy-Mills, K. L. (2014). Oleaginous yeasts for biodiesel: Current and future trends in biology and production. Biotechnology Advances, 32(7), 1336–1360. https://doi.org/10.1016/j.biotechadv.2014.08.003

Soccol, C. R., Dalmas Neto, C. J., Soccol, V. T., Sydney, E. B., da Costa, E. S., Medeiros, A. B., & Vandenberghe, L. P. (2017). Pilot scale biodiesel production from microbial oil of *Rhodosporidium toruloides* debb 5533 using sugarcane juice: Performance in diesel engine and preliminary economic study. *Bioresource Technology*, *223*, 259–268. https://doi.org/10.1016/j.biortech.2016.10.055

Soltani, M., Say, D. & Guzeler, N. (2017). Functional Properties and Nutritional Quality of Whey Proteins. Journal of International Environmental Application and Science, 12 (4), 334-338. Retrieved from https://dergipark.org.tr/en/pub/jieas/issue/40226/479139

Srinuanpan, S., Cheirsilp, B., & Prasertsan, P. (2018). Effective biogas upgrading and production of biodiesel feedstocks by strategic cultivation of oleaginous microalgae. *Energy*, *148*, 766–774. https://doi.org/10.1016/j.energy.2018.02.010

Subramaniam, R., Dufreche, S., Zappi, M., & Bajpai, R. (2010). Microbial lipids from renewable resources: Production and characterization. *Journal of Industrial Microbiology & Biotechnology*, *37*(12), 1271–1287. https://doi.org/10.1007/s10295-010-0884-5

Sutanto, S., Zullaikah, S., Tran-Nguyen, P. L., Ismadji, S., & Ju, Y.-H. (2018). Lipomyces starkeyi: Its current status as a potential oil producer. *Fuel Processing Technology*, *177*, 39–55. https://doi.org/10.1016/j.fuproc.2018.04.012

Thiru, M., Sankh, S., & Rangaswamy, V. (2011). Process for biodiesel production from Cryptococcus curvatus. *Bioresource Technology*, *102*(22), 10436–10440. https://doi.org/10.1016/j.biortech.2011.08.102

Tuhanioğlu, A. (2021). Production and high hydrostatic pressure assisted extraction of microbial oil from Lipomyces starkeyi and *Rhodosporidium toruloides* [Thesis (M.S.) – Graduate School of Natural and Applied Sciences. Food Engineering.] Middle East Technical University.

Tuhanioglu, A., Alpas, H., & Cekmecelioglu, D. (2022). High hydrostatic pressure-assisted extraction of lipids from *lipomyces starkeyi* biomass. *Journal of Food Science*, *87*(11), 5029–5041. https://doi.org/10.1111/1750-3841.16347

Uzuner, S., & Cekmecelioglu, D. (2014). Hydrolysis of hazelnut shells as a carbon source for bioprocessing applications and fermentation. International Journal of Food Engineering, 10(4), 799–808. https://doi.org/10.1515/ijfe-2014-0158

Verma, G., Anand, P., Pandey, S., & Nagar, S. (2019). Optimization of cultivation conditions for microbial lipid production by Rhodotorula Glutinis, an oleaginous yeast. *Bioscience Biotechnology Research Communications*, *12*(3), 790–797. https://doi.org/10.21786/bbrc/12.3/36

Wen, Z., Zhang, S., Odoh, C. K., Jin, M., & Zhao, Z. K. (2020). *Rhodosporidium toruloides* - a potential red yeast chassis for lipids and beyond. *FEMS Yeast Research*, *20*(5). https://doi.org/10.1093/femsyr/foaa038

Wang, C., Chen, L., Rakesh, B., Qin, Y., & Lv, R. (2012). Technologies for extracting lipids from oleaginous microorganisms for biodiesel production.

Frontiers in Energy, *6*(3), 266–274. https://doi.org/10.1007/s11708-012-0193-y

Wang, H., Hu, B., Liu, J., Qian, H., Xu, J., & Zhang, W. (2020). Co-production of lipid, exopolysaccharide and single-cell protein by sporidiobolus pararoseus under ammonia nitrogen-limited conditions. *Bioprocess and Biosystems Engineering*, *43*(8), 1403–1414. https://doi.org/10.1007/s00449-020-02335-3

Wiebe, M. G., Koivuranta, K., Penttilä, M., & Ruohonen, L. (2012). Lipid production in batch and fed-batch cultures of *Rhodosporidium toruloides*from 5 and 6 carbon carbohydrates. *BMC Biotechnology*, *12*(1). https://doi.org/10.1186/1472-6750-12-26

Ye, Z., Sun, T., Hao, H., He, Y., Liu, X., Guo, M., & Chen, G. (2021). Optimising nutrients in the culture medium of *Rhodosporidium toruloides* enhances lipids production. AMB Express, 11(1). https://doi.org/10.1186/s13568-021-01313-6

Yüksel, M., Kavaz Yüksel, A., & Ürüşan, H. (2019). Various properties of whey and possibilities of its utilisation. *Kahramanmaraş Sütçü İmam Üniversitesi Mühendislik Bilimleri Dergisi*, *22*(3), 114–125. https://doi.org/10.17780/ksujes.565372

Zhang, Y., Kamal, R., Li, Q., Yu, X., Wang, Q., & Zhao, Z. K. (2022). Comparative fatty acid compositional profiles of Rhodotorula toruloides haploid and diploid strains under various storage conditions. *Fermentation*, *8*(9), 467. https://doi.org/10.3390/fermentation8090467

Zhang, Y., Zhang, X., Zhang, Z., Chen, Z., Jing, X., & Wang, X. (2022). Effect of high hydrostatic pressure treatment on the structure and physicochemical properties of millet gliadin. *LWT*, *154*, 112755. https://doi.org/10.1016/j.lwt.2021.112755

Zhao, L., Zhao, G., Chen, F., Wang, Z., Wu, J., & Hu, X. (2006). Different effects of microwave and ultrasound on the stability of Astaxanthin. *Journal of Agricultural and Food Chemistry*, *54*(21), 8346–8351. https://doi.org/10.1021/jf061876d

Zhao, X., Kong, X., Hua, Y., Feng, B., & Zhao, Z. K. (2008). Medium optimization for lipid production through co-fermentation of glucose and xylose by the oleaginous yeast *lipomyces starkeyi*. *European Journal of Lipid Science and Technology*, *110*(5), 405–412. https://doi.org/10.1002/ejlt.200700224

Zhou, Y., Han, L.-R., He, H.-W., Sang, B., Yu, D.-L., Feng, J.-T., & Zhang, X. (2018). Effects of agitation, aeration and temperature on production of a novel glycoprotein GP-1 by streptomyces Kanasenisi ZX01 and scale-up based on volumetric oxygen transfer coefficient. *Molecules*, *23*(1), 125. https://doi.org/10.3390/molecules23010125

Zhu, S., Bonito, G., Chen, Y., & Du, Z.-Y. (2021). Oleaginous fungi in biorefineries. *Encyclopedia of Mycology*, 577–589. https://doi.org/10.1016/b978-0-12-819990-9.00004-4

APPENDICES

A. PHASE SEPERATION OF *R. TORULOIDES*

Figure A.1. Phase separation of *R. toruloides* after centrifuge. Upper phase: Methanol + medium, Interphase: Biomass, Lower Phase: Chloroform + microbial oil

B. STANDARD CURVE FOR SUGAR ANALYSIS

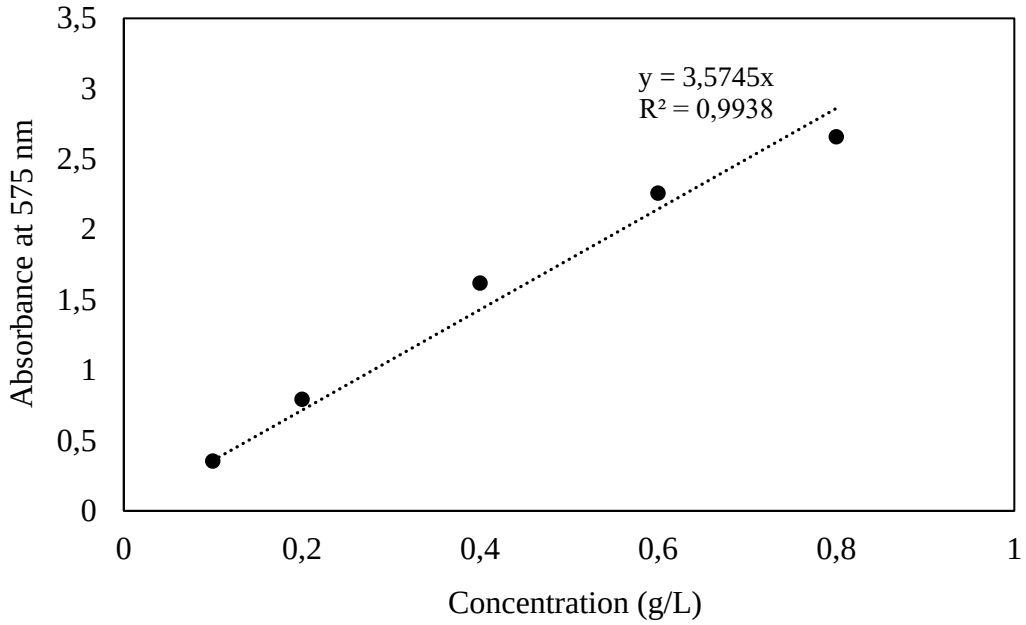

Figure B. 1. Standard curve for sugar analysis

The equation derived from this curve is used to convert absorbance values into corresponding concentrations.

$$y = 3.5745x \quad (10)$$

Total reducing sugar was calculated by the following equation.

$$\text{Total reducing sugar} = \left[\frac{y}{3.5745}\right] * \text{Dilution rate} \quad (11)$$

Where the y and x represent absorbance and concentration, respectively.

C. VERIFICATION OF OPTIMUM HPH AND HHP CONDITIONS

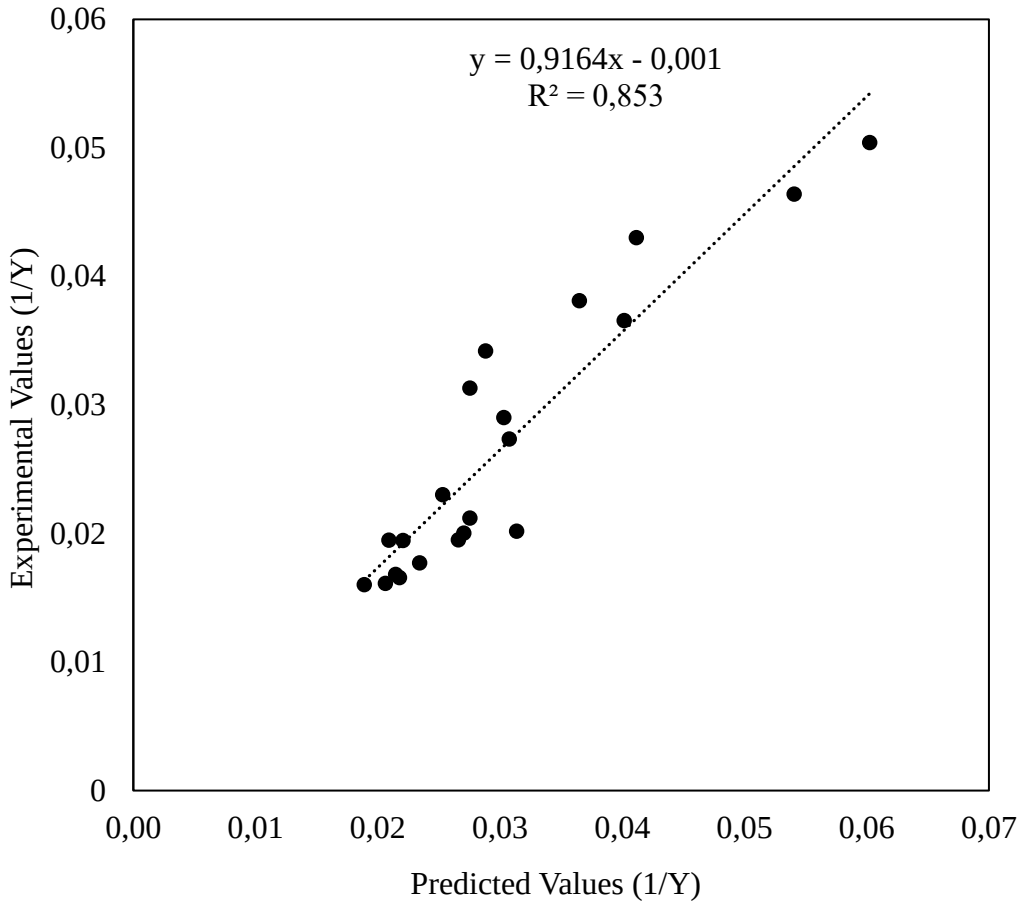

Figure C. 1. Comparison of predicted and experimental values from HPH assisted extraction for reciprocal of lipid yield from synthetic medium.

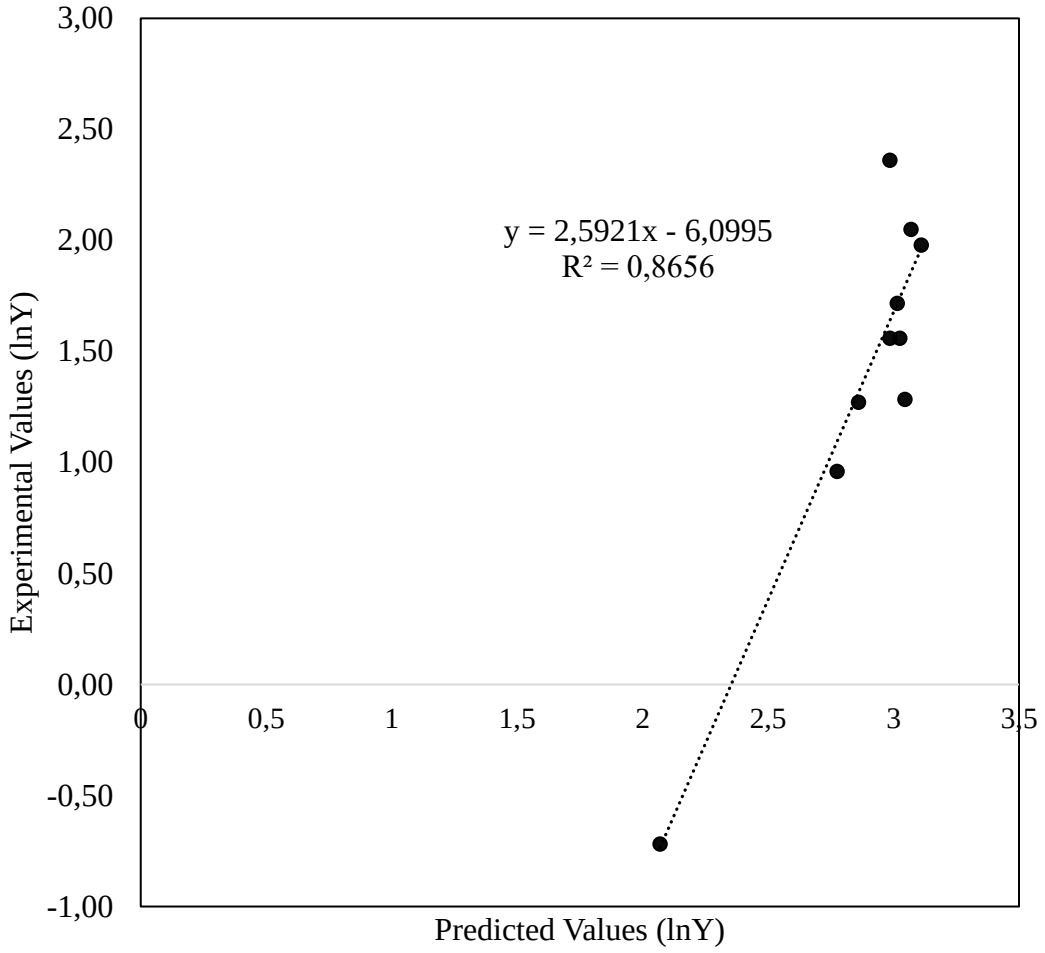

Figure C. 2. Comparison of predicted and experimental values from HHP assisted extraction for natural logarithms of lipid yield from spent hydrolyzed whey medium.

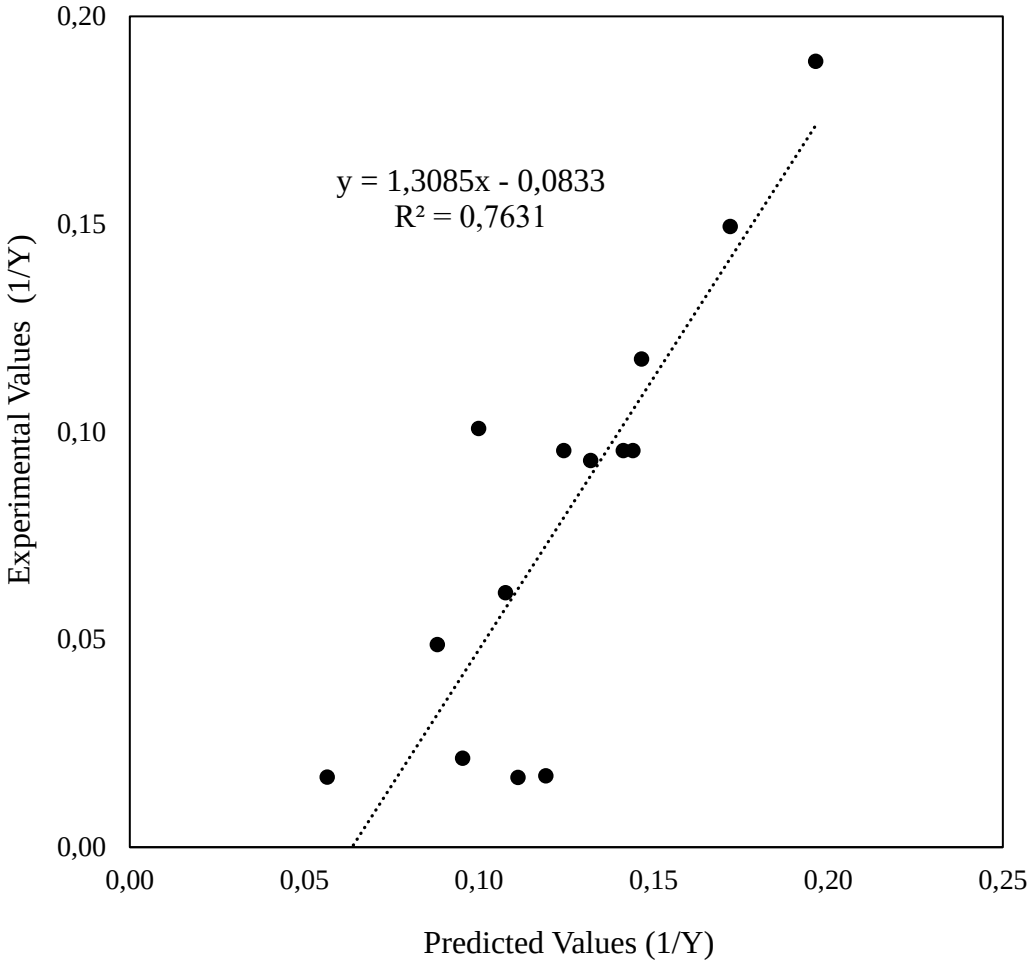

Figure C. 3. Comparison of predicted and experimental values from HHP assisted extraction for reciprocal of lipid yield from dry biomass slurry.