PRODUCTION AND HIGH HYDROSTATIC PRESSURE ASSISTED EXTRACTION OF MICROBIAL OIL FROM *LIPOMYCES STARKEYI* AND *RHODOSPORIDIUM TORULOIDES*

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ABSTRACT

PRODUCTION AND HIGH HYDROSTATIC PRESSURE ASSISTED EXTRACTION OF MICROBIAL OIL FROM *LIPOMYCES STARKEYI* AND *RHODOSPORIDIUM TORULOIDES*

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Lipomyces starkeyi and *Rhodosporidium toruloides* are able to accumulate high lipid fractions, so called microbial oil, in their cells. Microbial oils are of high potential as dietary supplements and future energy sources.

In this study, use of HHP in cell disruption of *Lipomyces starkeyi* DSM 70295 was investigated. The HHP process variables (pressure, time, and temp) were optimized for maximal oil yield using response surface method (RSM). The pressurized *L. starkeyi* yeasts were imaged by scanning electron microscopy (SEM). Apple pomace was tested as sole raw material in different conditioned forms, in which only *Rhodosporidium toruloides* DSM 4444 was grown. The best form of apple pomace hydrolysate medium was scaled-up to 1 L and 10 L fermentor. The extracted oil samples were analyzed for fatty acid composition. A maximal lipid yield of 45.8±2.1% was obtained at 200MPa and moderate temperature of 40 ^{o}C, within 15 min, while the lowest yield of 15.2±0.9% was observed at 300 MPa, 40 ^{o}C, and 10 min. Scanning electron microscopy (SEM) analysis revealed that lower pressure (200

MPa) caused cell distortions, whereas higher pressure (400MPa) resulted in surface scars and swellings. It was observed that detoxified apple pomace media gave a maximum lipid yield of 47.5±2.47% at flask scale experiments. In 1 L and 10 L fermentors, the lipid yields were measured as 40.1±5.51% and 50.9%, respectively, for detoxified apple pomace medium. The apple pomace conversion was calculated as 0.08 (g oil/ g dry apple pomace) for the detoxified medium. High palmitic (C16:0) and palmitoleic acid (C16:1) contents imply that *L. starkeyi*-origined oils are favorable to substitute palm oil or cocoa butter. *R. toruloides*-origined oils were rich in oleic (C18:1) and linoleic acids (C18:2). The results proved that HHP can be effective for microbial oil extraction. Also, apple pomace was demonstrated as suitable source for lipid production.

Keywords: *Lipomyces starkeyi, Rhodosporidium toruloides*, high hydrostatic pressure, apple pomace, scale-up.

ÖZ

LIPOMYCES STARKEYI VE *RHODOSPORIDIUM TORULOIDES* MAYALARINDAN MİKROBİYAL YAĞLARIN ÜRETİMİ VE YÜKSEK HİDROSTATİK BASINÇ DESTEKLİ ÖZÜTLENMESİ

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Lipomyces starkeyi ve *Rhodosporidium toruloides,* en yüksek verimle çalışan yağ mayalarından ikisidir. Mikrobiyal yağlar, geleceğin enerji kaynağıve gıda takviyeleri olarak görülmekte ve bu amaç doğrultusunda çalışılmaktadırlar.

Bu çalışmada, yüksek hidrotatik basıncın *Lipomyces starkeyi* DSM 70295 mayasından yağ özütleme potansiyeli üzerinde çalışılmıştır. HHP yardımı ile yağ özütleme işlemi yanıt yüzey yöntemiyle optimize edilmiştir. HHP uygulanan hücreler taramalı elektron miskroskobunda incelenmiştir. Elma posası, farklı koşullarda tek kabon kaynağı olarak test edilmiştir. Elma posası deneylerinde *Rhodosporidium toruloides* DSM 4444 mayası kullanılmıştır. En iyi sonucu veren elma posası ortamı 1 L ve 10 L fermentör deneylerinde kullanılmıştır. Özütlenen yağların yağ asidi kompozisyonları belirlenmiştir. En yüksek verim 200 MPa, 40 ^{o}C, 15 dakikada %45.8 ± 2.1 olarak ölçülürken, en düşük yağ verimi %15.2 ± 0.9 olarak 300 MPa, 40 ^{o}C ve 10 dakikada ölçülmüştür. Elektron mikroskobuyla yapılan gözlemler, kısmen düşük basıncın (200 MPa) hücrelerde şekil bozukluğu yarattığını, kısmen yüksek basıncın (400 MPa) ise yüzeysel yaralara ve şişmelere sebep olduğunu ortaya koymuştur. Maximum yağ verimi %47.5±2.47 olarak, detoks

edilmiş elma posası hidrolizatından elde edilmiştir. Fermentör deneylerinde ise, 1 litrelik ve 10 litrelik hacimlerden sırasıyla %40.1±5.51 ve %50.9 verimleri alınmıtır. Detoks edilmiş ortamda posası-mikrobiyal yağ dönüşümü katsayısı 0.08 olarak hesaplanmıştır. Yüksek palmitik (C16:0) ve palmitoleik asit (16:1) içerikleri, *L. starkeyi* kaynaklı yağların palm yağı veya kakao yağı ikamesi olarak kullanılabileceğini göstermiştir. *R. toruloides* kaynaklı yağların ise oleic (C18:1) ve linoleic asitçe (C18:2) zengin olduğu belirlenmiştir. Sonuçlar, HHP'nin diğer modern yağ özütleme yöntemleri kadar verimli olabileceğini ortaya koymuştur. Ayrıca, elma posasnın büyük ölçekte yağ üretimi için uygun olduğu gösterilmiştir.

Anahtar Kelimeler: *Lipomyces starkeyi, Rhodosporidium toruloides*, yüksek hidrostatik basınç, elma posası, büyük ölçek.

To my mother,

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CHAPTER 1

INTRODUCTION

Growing human population, depletion of agricultural lands, increasing demands for fatty acid supplements and low cost products necessitated to search for alternative lipid and fatty acid sources (Bellou et al., 2016). As microbial oils emerged as potential nutritional supplements, they have been studied heavily for decades to implement into commercial scale (Probst et al., 2016).

Microbial oils are defined as oils that are accumulated within the cell of microorganisms, so-called oleaginous microorganisms, above 20% of their dry cell basis (Meesters et al., 1996). Even though a number of oleaginous molds, algae and bacteria have been identified, yeasts are considered more advantageous due to the convenience of handling and fatty acid compositions that are similar to commercial oils (C. Ratledge, 1994). Among all oleaginous yeasts, *Lipomyces starkeyi* and *Rhodosporidium toruloides* are two of the most studied species. Studies revealed that these two organisms are able to store lipids over 40% of their cell dry basis (Lin et al., 2014) (Chapter 2).

Microbial oil production is a process that requires a carbon rich and nitrogen deprived medium with some trace elements. When the proper conditions are given (temperature, pH, etc.), the yeast depletes the nitrogen in the medium and undergoes a metabolic stress, which eventually results in lipid accumulation (Ratledge, 1994). Since single cell oils are intracellular metabolites, it is imperative to damage the cell wall to create a cytoplasmic leakage or rupture the cells completely to recover the accumulated lipids (Dai et al.,2014).

Although this challenge can be overcome easily at laboratory scale by traditional procedures, continuous and environmentally friendly methods for large scale productions are still under research (Kruger et al., 2018).

High hydrostatic pressure (HHP) is a highly recognized method in food processing, especially as a novel shelf life improvement method (Tokuşoğlu et al., 2010). It is also studied extensively for extraction purposes. Several authors suggested that HHP is a suitable process to extract functional substances with minimum quality losses (Ugur et al., 2020). Nonetheless, the feasibility of HHP for lipid extraction from oleaginous yeasts has not been studied so far. Thus, the first part of this study mainly focused on evaluating the potential of HHP as a novel approach for lipid recovery from the yeast *Lipomyces starkeyi* DSM 70295. Moreover, the process was statistically analyzed and modelled by response surface methodology (Box-Behnken Design). Later, HHP treated cells were imaged under scanning electron microscope to further detect any physical effects of the HHP on the cell wall and add the study a visual feature (Chapter 4).

Increasing supply of agricultural products results in increasing agro-industrial wastes, which are mostly complex lignocellosic materials (Yu et al., 2011). Wheat straw, sugarcane bagasse, rice bran and corn stover are some of the commonly generated wastes (Sutanto et al., 2017; Xavier et al., 2017). These materials were suggested as economical carbon sources for lipid production by several studies (Chapter 2).

According to Food and Agriculture Organization (FAO), apple is the second most cultivated fruit in the world by 87.2 million tons in the year 2019 (FAO, 2021). Furthermore, Turkey is the third biggest apple producer worldwide. Although most of the apple are consumed as fresh fruit, approximately 30% are processed by the industry (FAO, 2021). However, the industry only utilizes 60-70% of the apple, and the rest are wasted. The apple waste is called apple pomace (Appendix D).

In this study, apple pomace was studied as a sole material for lipid synthesis by the

oleaginous yeast *Rhodosporidium toruloides* DSM 4444. Prior to fermentation, apple pomace was instrumentally analyzed to demonstrate the mineral composition and acid hydrolyzed in several conditions to observe the sugar release potential. Based on the efficiency of the flask scale experiments (100 mL fermentation volume), the lipid fermentation process was scaled up gradually in both lab-scale (1 L) and industrial-type fermentors (10 L) (Chapter 5).

One of the two goals of this study was to show use of high hydrostatic pressure as a lipid extraction method. Furthermore, this study aimed to become the first in literature for HHP disruption of yeast *L. starkeyi* over a pre-determined interval of pressure, temperature, and time for microbial oil extraction.

As second goal, apple pomace hydrolysate was investigated as a sole medium for *R. toruloides* to accumulate lipid. Even though lignocellulosic compounds were demonstrated as suitable carbon sources for lipid accumulation by *R. toruloides*, apple pomace was tested for the first time in several conditioned forms for comprehensive evaluation.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Single Cell Oil

Lipid synthesis by microorganisms has been studied for more than a century, starting in Germany (Nägeli & Loew, 1878; Colin Ratledge & Wynn, 2002a). The first attempt to identify the fractions of the oil that originated from biomass was claimed to be a mixture of myristic acid (C14:0), stearic acid (C18:0), oleic acid (C18:1) and lauric acid (C12:0) (C. Ratledge, 1984). Ratledge (1984) stated that it was Lindner who was the prominent botanist in late 19th century made his observations on the "oleaginous" yeast, namely "Torula", and then during the World War I, he made his trials to build up an alternative system for fat supply by using the strain which is knowns as *Trichosporon pullulans* today (*Endomyces vernalis* back in old days) (C. Ratledge, 1984). During the World War II, some German companies accelerated the work of discovering and exploiting alternative food sources for both soldiers and horses. They began to feed their horses by the oleaginous biomass mixed with hay and straw, whereas the soldiers consumed also some of biomass (20 g/day) for the purpose of supplementing their diet with protein (C. Ratledge, 1984; Colin Ratledge, 2010). It must be realized that back in days of World War II (1939-1945), the technological opportunities and accessibilities were nowhere compared to today. Characterization of the ideal strain, maximization of the biomass and extraction of oil/protein from the cell efficiently were out of question in the mid - 20th century such that single cell oil cannot be utilized efficiently even in the 21th century. Considering fermentors and bioreactors were not that much available in 1950's and

chromatographic methods were not invented yet, commercialization of biolipid had to wait for quite a long time since the existing bioreactors were mainly used and studied for penicillin production (Colin Ratledge, 2010). There are a number of studies in literature claiming that new strains and new conditions can be developed in order to maximize the yield of the single cell oil production.

2.2 Single Cell Oil

2.2.1 Introduction to Single Cell Oil

Single cell oils can be defined as the sympathetic expression of all forms of lipids that are produced or accumulated by microorganisms (e.g. yeasts, molds, bacteria, and microalgae) (Colin Ratledge, 2010). Single cell oils are expected to become a serious lipid supplies due to the ability of oleaginous organisms to accumulate lipids from several eco-sources such as food wastes, agricultural residues and industrial by-products (Probst et al., 2016). They possess a great potential to be an alternative to commercial edible oils (exotic oils like cocoa butter, palm oil, etc.) due to their similar fatty acid compositions (Papanikolaou & Aggelis, 2011b).

Among all the microorganisms that are able to accumulate lipids, some are defined as oleaginous, meaning that they can store lipids up to 70% on a dry cellular basis (E. Tsouko et al., 2016). Even the oleaginous microorganisms themselves are classified according to some advantage/disadvantage balance, oleaginous yeasts are consideredmore attractive because of several advantages over other microorganisms. Yeasts arenon-toxic to humans, relatively faster in cell growth, efficient in lipid accumulation,climate independent, easy to scale up, and able to utilize wide range of sugars (Patelet al., 2016). Oleaginous bacteria can also accumulate considerable amount of oil (up to 40% of dry cell weight). Nevertheless, in comparison to oleaginous yeasts and molds, lipid production by bacteria attracts relatively less

attention than yeasts, which can accumulate lipid up to 70% of dry cell weight. Moreover, triacylglycerolfraction of bacterial oil is not considered sufficient (Y. Li et al., 2007; Papanikolaou& Aggelis, 2011b; C. Ratledge, 1994). Furthermore, single cell oil produced by microalgae is in particular interest since they are important industrial products with long chain polyunsaturated fatty acids (Colin Ratledge & Lippmeier, 2017).

Considering there are 1600 known species of yeasts, only about 70 of them are recognized as oleaginous (Garay et al., 2016). Yeast originated single cell oils mostly consist of triacylglycerols and lesser fractions of free fatty acids, phospholipids, and sterols (Probst et al., 2016). The fatty acid fraction of oils produced by wide range of yeasts on various carbon sources is oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), linolenic acid (C18:3) and stearic acid (C16:0) (Colin Ratledge & Wynn, 2002b). Some representative oleaginous yeasts are *Lipomyces starkeyi, Rhodosporidum toruloides, Rhodotorula glutinis, Yarrowia lipolytica, and Cryptococcus curvatus* (Colin Ratledge & Lippmeier, 2017).

2.2.2 Mechanism of Biosynthesis

There are two ways to obtain microbial lipids depending on the substrate, namely *de novo* and *ex novo*. De novo synthesis of single cell oils is defined as formation of complex molecules, which is lipid in our case, from simple molecules such as sugars and amino acids (Sutanto et al., 2018). Lipid synthesis by utilizing hydrophilic carbon sources is called ex novo (Papanikolaou & Aggelis, 2011b). De novo biosynthesis mechanism of single cell oil in yeasts cells has been elucidated by Ratledge (1994).

As nitrogen is exhausted in the medium, the organism experiences a metabolic stress, which is a desired condition for lipid accumulation, unlike cell growth conditions. In nitrogen deficiency, the enzyme AMP deaminase is activated and starts the process

by yielding inosine 5-monophosphate along with ammonium (1).

Adenosine monophosphate $\longrightarrow$ inosine 5-monophosphate + NH_3 (1)

The reaction keeps occurring until AMP depletes. The released ammonium is consumed as the final N reserve. Meanwhile in mitochondria, another enzyme called isocitrate dehydrogenase (ICDH) is activated by AMP actions right after the conversion of pyruvate to citrate (2). Isocitrate dehydroxigenase (ICDH) is responsible for the metabolism of isocitrate in such a way that it turns isocitrate into α-ketoglutarate (3). Because of the fact that ICDH is activated as a result of AMP activity, as AMP stops functioning, ICDH stops functioning, too, causing isocitrate to be converted back to citrate by the enzyme aconitase and citrate accumulates (4).

Pyruvate $\longrightarrow$ Citrate $\longrightarrow$ Isocitrate (2)

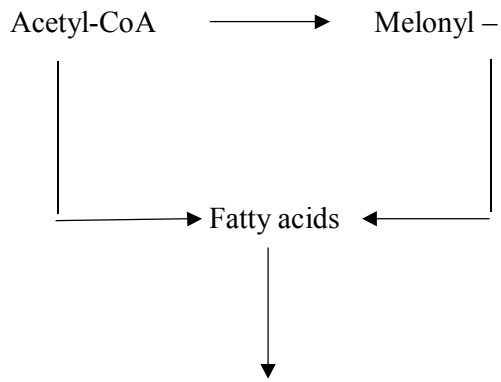
Isocitrate + NAD^+ $\longrightarrow$ α-ketoglutarate + CO_2 + NADH (3)

Isocitrate $\longrightarrow$ Citrate (4)

Further, the accumulated citrate is transferred to cytosol in return for malate, through citrate – malate cycle (6). Citrate in the cytosol is then converted into acetyl-CoA and oxaloacetate by the enzyme ATP Citrate lyase (5). In fact, ATP Citrate lyase is the characteristic enzyme of oleaginous yeast. In other words, oleaginous yeasts can accumulate immense amount of lipid through ATP Citrate lyase (Boulton & Ratledge, 1983a). Acetyl-CoA is further converted into malonyl-CoA by acetyl-CoA carboxylase (7). Acetyl-CoA and Melonyl-CoA are the primers of fatty acid biosynthesis (8). The fatty acids are then linked to glycerol to form triacylglycerol (9). Figure 2.1 also demonstrates how de novo lipid accumulation occurs in oleaginous cells.

Citrate $\longrightarrow$ Acetyl-CoA + oxaloacetate (5)

Oxaloacetate + NADH $\longrightarrow$ Malate + NAD^+ (6)



(7)

(8)

Triacylglycerol (9)

As a result of the metabolic process, the conversion of sugar to microbial oil occurs in 5:1 ratio (w sugar/w oil) (Colin Ratledge, 2010).

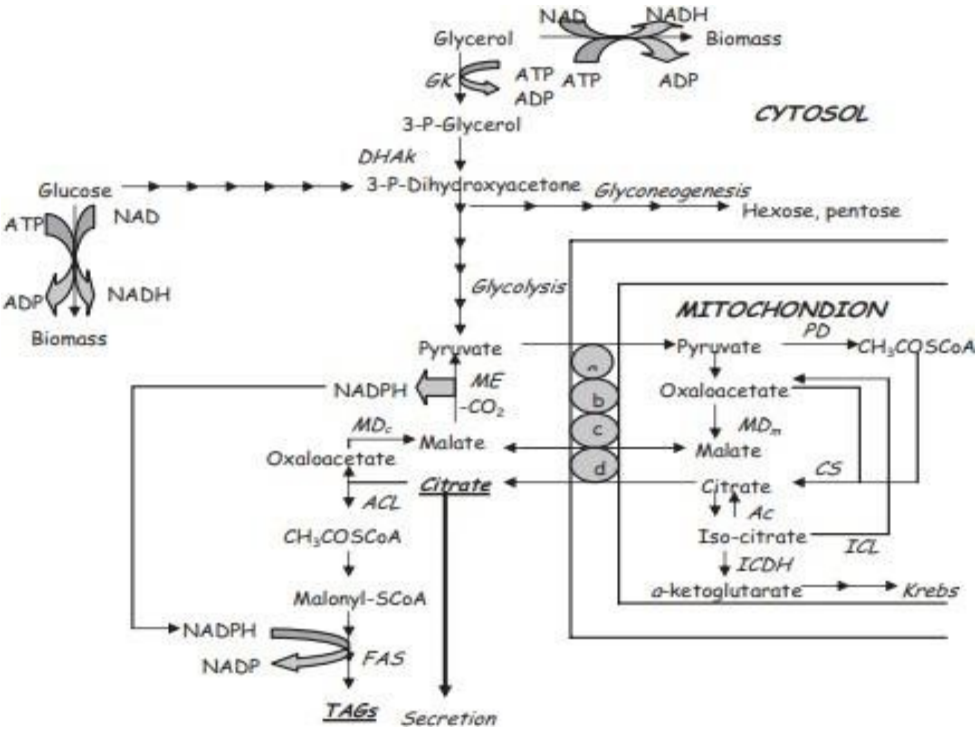


Figure 2.1. Simplified schematic of lipid synthesis in oleaginous yeasts. Ac: Acotniase, ACL: Acetyl CoA carboxylase, ACL: ATP-citrate lyase, FAS: Fatty acid synthetase, ICDH: Iso-citrate dehydrogenase, MD_c: Malate dehydrogenase in cytoplasm, MD_c: Malate dehydrogenase in mitochondria, PD: Pyruvate dehydrogenase, PFK: Phospho-fructo-kinase, PK: Pyruvate kinase (Papanikolaou & Aggelis, 2011a).

2.2.3 Factors Affecting Single Cell Oil Production

Microbial oil accumulation is mainly affected by carbon and nitrogen amount in the medium, processing temperature and initial pH (Amza et al., 2019; Suutari etal., 1993).

2.2.3.1 C/N Ratio

It is well known that lipid accumulation (>20% dry cell mass) occurs only when nitrogen is limited in the fermentation medium (Yang et al., 2014). Abundance of carbon and limited nitrogen cause microorganism to suffer from a metabolic stress, in which the organism starts to accumulate carbon sources in the form of lipids to serve as a long term energy stock. Boulton and Ratledge (1983) stated that in the first 24 hours, yeasts accumulate carbon source as sugar, only after 28 hours begin to store lipids (Boulton & Ratledge, 1983b).

It is demonstrated in Table 2.1 that high C/N ratio yielded higher lipid accumulation in both *L. starkeyi* and *R. toruloides*. However, excessive sugar creates osmotic stress, therefore the microorganisms will not able to utilize the minerals in the medium (Singh et al., 2016). As a result, a proper carbon to nitrogen ratio must be determined prior to the process based on the strain that is desired to use (Calvey et al., 2016).
Types of carbon and nitrogen sources are also as important as the C/N ratio. For instance, Liu et al. (2013) stated that the most effective nitrogen source for cell growth was determined as NH_4Cl, whereas the most suitable nitrogen source for lipid accumulation was $(NH_4)_2SO_4$ (Liu et al., 2013). From synthetic glucose to agro-industrial wastes (corn stover, sugarcane bagasse, wheat straw, rice bran, etc.), several carbon sources have been studied to maximize lipid yield (Dourou et al., 2016; Lin et al., 2014). Different carbon/nitrogen combinations have also been demonstrated by numerous authors (Liu et al., 2017; Matsakas et al., 2014).

Table 2.1. Effect of C/N ratio on lipid yield for *L. starkeyi* and *R. toruloides.*

Yeast	Carbon source	Nitrogen source	C/N ratio	Processing type	Processing conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
L. starkeyi NS	Glucose	$(NH_4)_2SO_4$	58	Flask	30 ^{0}C, 160rpm, 10 days	Ultrasonic homogenizer + solvent extraction	NS	12.1	NS	(Yousuf et al., 2010)
L.starkeyi ATCC 58680	Glucose	$(NH_4)_2SO_4$	114	Flask	30 ^{0}C, 80hours, pH 5.5	Solvent extraction	9.7	53.6	0.17	(Rahman et al., 2017)
R.toruloides DSM 4444	Glucose	$(NH_4)_2SO_4$ + yeast extract	77	Flask	28 ^{0}C, 185rpm, 120hours, pH 5.0 - 6.0	Folch	9.7	39.6	NS	(Papanikolaou et al., 2017)
R.toruloides RT559-AD	Glucose	$(NH_4)_2SO_4$ + yeast extract	339	Flask	30 ^{0}C, 250rpm, 289hours, pH 5.6	Folch	15.6	58.4	0.13	(S. Zhang, Skerker, et al., 2016)

2.2.3.2 Temperature

Due to the direct effect on enzyme activity, temperature is one of the most important variables in cell growth and lipid accumulation. As mentioned in the Mechanism of Biosynthesis section, lipogenesis is a highly enzyme dependent process (Colin Ratledge & Wynn, 2002a). Optimum temperature for *L. starkeyi* varies from 25 to 30 ^{o}C for lipid accumulation (Suutari et al., 1996). In addition, Table 2.2 presents temperatures of *L. starkeyi* and *R. toruloides* in glucose medium. According to Suutari (1993), the optimum temperature for cellular lipid storage, glucose conversion efficiency and the specific lipid production rate was determined as 28 ^{o}C for

L.starkeyi. The study showed that when the cell growth and lipid accumulation temperatures were set to 16-18 ºC, biomass yield and lipid production per dry weight were found as 29.8% and 40% respectively, whereas if both temperatures were set to 28 ºC, biomass yield and lipid production increases to 30.4% and 55%, respectively. (Suutari et al., 1993).

It was also shown that lipid accumulation temperature also affects the lipid composition. A study on *L. starkeyi* demonstrated that as the temperature increases from 10 ºC to 30 ºC, rate of phospholipids (mg/g dry weight) also increases from 19.7% to 42.3%. However, natural lipid rates followed a decreasing trend by ascending temperatures, from 80.3% to 42.9%. Hence, excessive temperature shows a negative effect on triacylglycerol synthesis (Suutari et al., 1996).

Table 2.2. Temperature effects on lipid yield for *L. starkeyi* and *R. toruloides*.

Yeast	Carbon source	Nitrogen source	C/N ratio	Processing type	Processing conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
L.starkeyi AS 2.1560	Glucose	NS	NS	Flask	30 ^{0}C, 200rpm, 36 hours	Solvent extraction	11.2	61.0	0.24	(Lin et al., 2011)
L.starkeyi NRRL Y-11557	Glucose syrup	NS	NS	Flask	25 ^{0}C, 200rpm, 72hours, pH 6.0	Solvent extraction	8.5	16.4	0.04	(Ali El-Naggar et al., 2011)
R.toruloides np11	Glucose	$(NH_4)_2SO_4$ + yeast extract + amino acid supplements	NS	Flask	30 ^{0}C, 220rpm, 168 hours, pH 6.0	Solvent extraction	8.6	43.0	NS	(C. Zhang, Shen, et al., 2016)
R.toruloides DSM 4444	Glucose	Yeats extract + peptone	NS	Flask	26 ^{0}C, 185rpm, 192 hours, pH 6.0	Solvent extraction	9.4	71.3	0.14	(Tchakouteu et al., 2017)

2.2.3.3 Initial pH

Initial pH of a medium is considered a chief factor for cell growth and lipid accumulation. Liu et al. (2017) investigated the effect of initial pH, varying from 4 to 8, on lipid accumulation and concluded that the biomass and lipid content of *L. starkeyi* maximize at pH 6.0. The study further states that pH value of medium decreases to below 3.0 the as fermentation proceeds (Liu et al., 2017). It is also possible to find numerous studies that suggest the optimum pH for cell and lipid accumulation as 5.0 (Amza et al., 2019). Consequently, most of the recent studies on *L. starkeyi* set their initial pH ranging from 5.0 – 6.5 (Table 2.3).

Table 2.3. . Effect of initial pH on lipid yield for *L. starkeyi* and *R. toruloides.*

Yeast	Carbon source	Nitrogen source	C/N ratio	Processing type	Processing conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant(g/g sugar)	Ref.
L.starkeyi HL	Glucose	Yeast extract	53	Flask	30 ^{0}C, 150rpm, 6 days, pH 6.5	Bligh & Dyer	8.6	21.5	0.04	(L. Huang etal., 2011)
L.starkeyi DSM 70296	Glucose	Flour based industrial waste	NS	Flask	30 ^{0}C, 180rpm 200 hours, pH5.2	Folch	30.5	40.4	NS	(Tsakona etal., 2014)
R.toruloides DSM 4444	Glucose	$(NH_4)_2SO_4$ + yeast extract	77	Flask	28 ^{0}C, 185rpm, 120 hours, pH 5.0 - 6.0	Folch	9.7	39.6	NS	(Papanikolaou et al., 2017)
R.toruloides L1-1	Glucose	$(NH_4)_2SO_4$ + yeast extract	140	Flask	37 ^{0}C, 150rpm, 120h, pH5.5	Bligh & Dyer	NS	13.4	NS	(Wu et al., 2018)

Table 2.3 (continued)

AS 2.1560	Crude glycerol	Yeast extract + peptone	NS	Flask	30 ^{0}C, 200rpm, 5 days, pH 6.0	Bligh & Dyer	16.6	29.0	NS	(Liu et al.,2017)
NBRC10381	Glucose	$(NH_4)_2SO_4$ + yeastextract	17	Bioreactor (fed- batch)	30 ^{0}C, 150rpm 120 hours, pH 5.0	Folch	66.6	49.0	0.11	(Amza et al.,2019)

2.3 Oleaginous Yeasts

Oleaginous yeasts are more preferable over molds and bacteria due to multiple reasons. Higher biomass and oil storage, less sensitivity to environmental conditions and high triacylglycerol content are some of advantageous features. *Lipomyces starkeyi, Rhodosporidium toruloides, Yarrowia lipolytica, Rhodotorula glutinis* and *Cryptococcus curvatus* are popular examples of oleaginous yeasts (Colin Ratledge & Lippmeier, 2017). Mutant species of *Yarrowia lipolytica* store 90% lipid yield on a dry cell basis (Blazeck et al., 2014). Nevertheless, *Lipomyces starkeyi* and *Rhodosporidium toruloides* are capable of storing lipid over 70% on a dry cell basis without gene manipulation (Ario Betha Juanssilfero et al., 2018; Singh et al., 2018).

2.3.1 *Lipomyces starkeyi*

2.3.1.1 Taxonomy of *Lipomyces starkeyi*

Lipomyces starkeyi is an eukaryotic, unicellular organism that was first isolated by R.L. Starkey (named as Starkey74) from soil and described by Lodder and Kreger-van Rij in 1952 (Starkey, 1946) (Kreger-van Rij, 1984; Records, 1964) (C. P. Kurtzman & Lodder, 1972). It has a sexual reproduction mechanism such that it develops 4-20 ellipsoidal or round acrospores per ascus then divide by budding (see Figure 2.2 and Figure 2.3) (Sutanto et al., 2018). The taxonomy is kingdom *Fungi*, phylum *Ascomycota*, class *Saccharomycetes*, order *Saccharomycetales*, family *Lipomycetaceae*, genus *Lipomyces* Lodder & Kreger-van Rij, and species *Lipomyces starkeyi* Lodder & Kreger-van Rij. Although, it was isolated from soil by Starkey, he was unable to classify the yeast properly, thereby the taxonomic classification was concluded by Lodder & Kreger-van Rij (1984). They found out that the new "fat" yeast must belong to a new family (at least something different from *Endomycetaceae,* which was suggested before Lodder & Kreger-van Rij) on account of the differences in ascosporal morphology. Thus, the family was named as *Lipomycetaceae.* For detailed taxonomic story of genus *Lipomyces*, an elaborative study was published by Cottrell and Kock (1989).

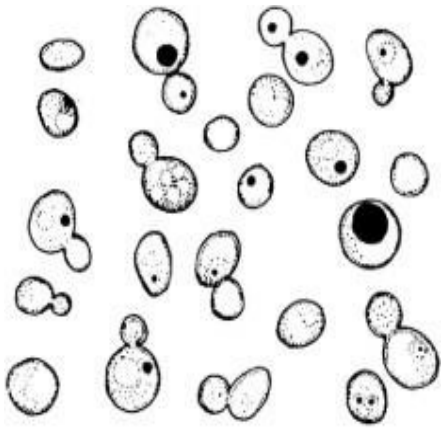


Figure 2.2. An early microscopic image (Kreger-van Rij, 1984).

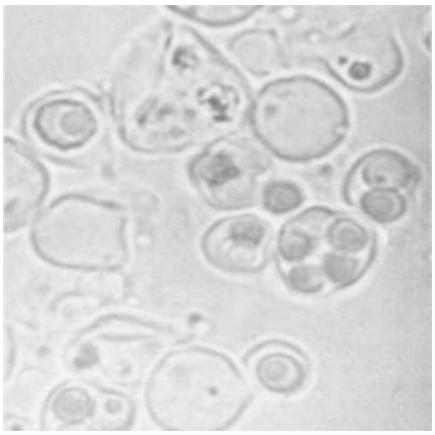


Figure 2.3. Acrospores *L.starkeyi* (Smith & Batenburg-Van derVegte, 1984)

2.3.1.2 Chronological Progress on *Lipomyces starkeyi* and Single Cell Oil

According to British Records (1964), *Lipomyces* was isolated as mucoid colonies from moist soils in such conditions that the cultivation medium was nothing but a 4% (w/w) of glucose solution at 20 ^{o}C. In glucose-peptone medium, a 72h incubation resulted in oval colonies (both single or in pairs) that contains "conspicuous" oil globule (Records, 1964). One of the earliest evidence, which mentions that *Lipomyces* is capable of accumulating oil or so to say oil-like molecules (i.e., sterols) is encountered in Tomkins' study (Tomkins, 1959). However, Tomkins (1959) claimed *L. starkeyi* and *L. lipoferus* as "may be synonymous of each other". In fact, Cottrell and Kock (1989) stated that the differentiation between these two strains was made based on the ability to destroy lactose, which turned out inaccurate as shown in Table 2.4. In the 1960's, it was achieved to distinguish the two strains of *Lipomyces* from each other simply by their intracellular and exocellular sugar polysaccharides compositions (Gorin & Spencer, 1968; Heidelberger & Slodki,

1972). Later studies on cell wall structure of *L. starkeyi* showed that it is a capsule shaped polysaccharide with 1700 cm^3/g intrinsic viscosity, which is mostly made up of D-mannose, D-galactose and uronic acid (D-glucuronic acid) by the proportions of 53.3%, 29.1%, and 17.4%, respectively (Chapman & Lynch, 1985).

Table 2.4. Properties of cellular polysaccharides of *Lipomyces* species (Gorin & Spencer, 1968).

				Sugars Present		
Species	**Source**	**NRRL Number**	**Lactose assimilation**	**Mannose**	**Galactose**	**Glucuronic acid**
Lipomyces starkeyi	Starkey 74	Y-1388	+	+	+	+
Lipomyces starkeyi	Starkey 74b	Y-2543	-	+	+	+
Lipomyces starkeyi	David Jones	Y-6333	-	+	-	+
Lipomyces lipoferus	A.C. Thaysen	Y-1351	+	+	-	+
Lipomyces lipoferus	Type strain CBS	Y-2542	+	+	-	+

During 1970's, for *Lipomyces starkeyi* specifically, numerous aspects were reported besides oil accumulation. *Lipomyces starkeyi* was shown to be capable of oxidizing ethanol (Heick & Barrette, 1970), and decomposing herbicides like paraquat or diquat. (Wright, 1971). However, the main focus of many studies concerning *L. starkeyi* was to understand the composition of the fungal lipids in terms of their polar-nonpolar lipid ratios, amount and types of diols, cholesterols and triacylglycerol, free fatty acids, ergosterols, and fatty acid methyl ester compositions

(Brennan & Lösel, 1978; Schaffner & Matile, 1981; Uzuka et al., 1975). For instance, Suzuki and Hasegawa (1976) performed a study to describe the biomass growth and lipid accumulation behaviour of *L. starkeyi* in glucose (2.5%) and l, 2-propanediol medium (2.5%). They also investigated the lipid composition in detail with respect to cultivation time and cellular mass (Table 2.5) (Suzuki & Hasegawa, 1976).

Table 2.5. Lipid composition of *L. starkeyi* grown in the "glucose-l,2-propanediol medium (Suzuki & Hasegawa, 1976).

Lipid	72h		120h		180h	
	mg/g cell	wt%	mg/g cell	wt%	mg/g cell	wt%
Sterol ester	trace	trace	2.2	2.8	1.6	1.2
Triglyceride	133.0	88.7	66.6	83.2	83.3	64.1
Free fatty acid	6.0	4.0	5.6	7.0	13.0	10.0
Diglyceride	2.7	1.8	1.7	2.1	11.6	8.9
Ergosterol	2.1	1.4	1.1	1.4	2.3	1.8
Monoglyceride	trace	trace	0.4	0.5	trace	trace
Phospholipid	5.9	3.9	2.3	2.9	14.3	11.0

Another striking effort was performed to compile the lipid yield and composition of 30 oil accumulating yeasts by chromatographic methods, in which *L. starkeyi* outstands among all the other ascosporogenous yeasts with the highest total lipid to dry weight ratio (32.3%) of which 93.4% is composed of triglycerides (Kaneko et al., 1976). Even though too much effort devoted to optimize the lipid accumulation process for oleaginous species during the course of 1970's, the main objective was to figure out the biological path of lipid accumulation in the cell. Furhermore, it was discovered that oil accumulation occurs in the nitrogen limited and carbon abundant medium (C. Ratledge, 1979). In the early 1980's, Boulton and Ratladge (1981)

published a number of studies in order to shed a light upon de novo lipogenesis in *L. starkeyi.* By series of inhibition tests, they concluded that ATP citrate lyase was "indeed" one of the most important, maybe the key enzyme for bio-lipogenesis just as it is important as in the animal and plant tissues (Boulton & Ratledge, 1981). As a matter of fact, the idea of ATP citrate lyase being the limiting factor for lipid accumulation might have evoked due to the fact that the enzyme is not present in so called non-oleaginous yeasts (Boulton & Ratledge, 1983a). They further stated that, even in the nitrogen limited medium, *L. starkeyi* stores glucose as an intermediate or short term energy stock and only after a certain time around 24 to 28 hours, transfers glucose to lipids as the long term carbon (energy) source (Boulton & Ratledge, 1983b). Meanwhile, Naganuma et al. (1985) made an extensive study on *L. starkeyi* to test how lipid accumulation process is affected by the presence/absence of each minerals. The most important factors were found to be the six minerals, namely NH^{4+}, K^{+}, Ca^{2+}, Zn^{2+}, Fe^{3+}, and Mn^{2+} which affected the lipid yield and total yeast count critically among which only the presence of Zn^{2+} reduced the lipid yield significantly although Zn^{2+} presence affects yeast growth positively (Naganuma et al., 1985). A brief summary of the study can be seen in Table 2.6. The table was compiled according to the ion concentrations that gave the best lipid yield according to study of Naganuma (1985).

Table 2.6. Mineral effects on cell growth and lipid yield for *L. starkeyi* (Naganuma et al., 1985).

Ion (Compound)	Optimum ion concentration (g/l)	Total cell number ($x10^8$/ml)	Lipid content (mg/10^8 cells)	Lipid yield (g of lipid/100g of glucose consumed)
NH_4^+ [$(NH_4)_2SO_4$)]	0.13	1.5	1.9	13
K^+ [KCl]	0.11	1.3	2.1	10
Mg^{2+} [$MgSO_4.7H_2O$]	0.01	1.9	1.6	10
Ca^{+2} [$CaCl_2.2H_2O$]	1.35	1.3	2.2	11
Na^+ [NaCl]	0.39	2.0	1.2	8
PO_4^{3-} [$NaH_2PO_4.2H_2O$]	3.75	1.7	2.1	11
Cl^- [NaCl]	0.009	2.1	1.2	10
SO_4^{2-} [Na_2SO_4]	0.12	1.8	1.5	10
Zn^{2+} [$ZnSO_4.7H_2O$]	0	0.4	4.5	15
Fe^{3+} [$FeCl_3.6H_2O$]	0.04	1.6	1.6	11
Mn^{2+} [$MnCl_2.4H_2O$]	0.022	1.5	1.7	8

Table 2.6 (continued)

Cu^{2+} [$CuCl_2.2H_2O$]	0.0007	1.6	1.9	10
BO_3^{3-} [H_3BO_3]	0.041	1.6	1.6	9
I^- [KI]	0.009	1.6	1.6	9
MoO_4^{2-} [$Na_2MoO_4.2H_2O$]	0.0022	1.6	1.6	9

2.3.1.3 Current Status of *Lipomyces starkeyi*

One of the most outstanding characteristics of recent studies on microbial oil production by *L. starkeyi*, particularly those were performed in the last 10 years, are the studies that focused on optimizing the process variables (carbon/nitrogen ratio, temperature, pH, inoculum size, etc.) in order to maximize the sugar to lipid conversion and utilization of agro industrial wastes as main carbon and mineral sources (Calvey et al., 2016; Ario B. Juanssilfero et al., 2018; Suutari et al., 1993; Ali El-Naggar et al., 2011; Chaturvedi et al., 2019; C. Huang et al., 2014). A compilation of studies that were conducted in the last 10 years on *L. starkeyi* as a single cell oil producer is presented in Table 2.7. Notice that the C/N ratios and processing conditions shown in Table 2.7 are only the initial conditions. In addition for the initial C/N ratio calculations, yeast extract was considered as a nitrogen source only (approximately 10% of the yeast extract is nitrogen). The data depicted in each row were selected as the most noticeable part of the cited study to give an overview of the recent studies.

Table 2.7. Compilation of microbial oil studies by *L. starkeyi* for the last 10 years.

L.starkeyi strain	Carbon source	Nitrogen source	C/N ratio	Process type	Processing conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
NS*	Olive oil mill wastewaters	NS	NS	Flask	30 ^{0}C, 160rpm, 10 days	Ultrasonic homogenizer + solvent extraction	10.4	22.4	NS	(Yousuf et al., 2010)
NS	Glucose	$(NH_4)_2SO_4$	58	Flask	30 ^{0}C, 160rpm, 10 days	Ultrasonic homogenizer + solvent extraction	NS	12.1	NS	(Yousuf et al., 2010)
HL	Fishmeal wastewater	NS	NS	Flask	30 ^{0}C, 150rpm	Bligh & Dyer	17.6	15.3	NS	(L. Huang et al., 2011)
HL	Glucose	Yeast extract	53	Flask	30 ^{0}C, 150rpm 6 days, pH 6.5	Bligh & Dyer	8.6	21.5	0.04	(L. Huang et al., 2011)
AS 2.1560	Glucose	NS	NS	Flask	30 ^{0}C, 200rpm 36hours	Solvent extraction	11.2	61.0	0.24	(Lin et al., 2011)
AS 2.1560	Glucose	NS	NS	Bioreactor	30 ^{0}C, 200rpm 40hours	Solvent extraction	104.6	64.9	NS	(Lin et al., 2011)
ATCC 12659	Wheat straw hydrolysate	Yeast extract	NS	Flask	28 ^{0}C, 200rpm 6 days, pH 5.5	NS	14.7	31.2	NS	(Yu et al., 2011)
NRRL Y-11557	Glucose syrup	NS	NS	Flask	25 ^{0}C, 200rpm 72 hours, pH 6.0	Solvent extraction	8.5	16.4	0.04	(Ali El-Naggar et al., 2011)
NRRL Y-11557	Tomato peels	NS	NS	Flask	25 ^{0}C, 200rpm 72 hours, pH 6.0	Solvent extraction	5.4	11.1	0.02	(Ali El-Naggar et al., 2011)

Table 2.7 (continued)

NRRL Y-11557	NS	Corn gluten meal	NS	Flask	25 ^{0}C, 200rpm 72 hours, pH 6.0	Solvent extraction	7.4	16.2	0.01	(Ali El-Naggar et al., 2011)
GIM2.142	Monosodium glutamate wastewater	NS	NS	Flask	30 ^{0}C, 150rpm 5 days, pH 5.0	Solvent extraction	5.6	12.5	NS	(J. X. Liu et al., 2012)
AS 2.1560	Glucose + cellobiose + xylose	$(NH_4)_2SO_4$ + yeast extract	107	Flask	30 ^{0}C, 200rpm 108 hours, pH 5.8	Solvent extraction	25.5	52.0	0.20	(Gong et al., 2012)
AS 2.1560	Cellobiose	$(NH_4)_2SO_4$ + yeast extract	113	Flask	30 ^{0}C, 200rpm 103 hours, pH 5.8	Solvent extraction	27.9	50.0	0.20	(Gong et al., 2012)
ATCC 12659	Acetate + formate	$(NH_4)_2SO_4$	NS	Flask	30 ^{0}C, 200rpm 5 days, pH 7.0	Solvent extraction	1.5–2.0	20-25	NS	(Lian et al., 2012)
ATCC 12659	Acetol	$(NH_4)_2SO_4$	NS	Flask	30 ^{0}C, 200rpm 5 days, pH 7.0	Solvent extraction	1.0-1.5	25-30	NS	(Lian et al., 2012)
ATCC 12659	Acetate + acetol	$(NH_4)_2SO_4$	NS	Flask	30 ^{0}C, 200rpm 5 days, pH 7.0	Solvent extraction	1.0-1.5	30-35	NS	(Lian et al., 2012)
GIM2.142	Potato starch wastewater + glucose	$(NH_4)_2SO_4$	76	Flask	30 ^{0}C, 150rpm 4 days, pH 5.5	Solvent extraction	2.5	8.8	NS	(J. X. Liu et al., 2013)
CH010	Corncob hydrolysate	Yeast extract	NS	Flask	38 ^{0}C, 150rpm 8 days, pH 6.5	Folch	17.2	47.0	NS	(C. Huang et al., 2014)

Table 2.7 (continued)

AS 2.1560	Xylose	$(NH_4)_2SO_4$ + yeast extract	59	Flask	30 ^{0}C, 200rpm 8 days, pH 6.0	Solvent extraction	13.2	56.2	NS	(Lin et al., 2014)
AS 2.1560	Xylose	NS	NS	Flask (two-staged)	30 ^{0}C, 200rpm 4 days, pH 6.0	Solvent extraction	11.4	60.4	NS	(Lin et al., 2014)
AS 2.1560	Xylose	NS	NS	Bioreactor (two-staged)	30 ^{0}C, 600-800rpm 80 hours, pH 6.0	Solvent extraction	97.4	65.5	NS	(Lin et al., 2014)
DSM 70296	Sugarcane bagasse hydrolysate	NS	50	Bioreactor (Batch)	28 ^{0}C, 400rpm, pH 5.5	Bligh & Dyer	13.3	17.2	0.13	(Michelle C.A. Xavier & Franco, 2014)
DSM 70296	Glucose + xylose	$(NH_4)_2SO_4$ + yeast extract	50	Bioreactor (fed-batch)	28 ^{0}C, 400rpm 6 days pH 5.5	Bligh & Dyer	42.1	36.7	0.08	(Anschau et al., 2014)
DSM 70296	Xylose + glucose	$(NH_4)_2SO_4$ + yeast extract	50	Bioreactor (fed-batch)	28 ^{0}C, 400rpm 115 hours, pH 5.5	Bligh & Dyer	66.6	36.0	0.31	(Anschau et al., 2014)
DSM 70296	Hemicellulosic hydrolysate	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (continuous)	28 ^{0}C, 400rpm pH 5.5	Bligh & Dyer	13.9	26.7	NS	(Anschau et al., 2014)
AS 2.1560	Spent yeast cell	$(NH_4)_2SO_4$ + yeast extract	NS	Flask	28 ^{0}C, 200rpm 5 days, pH 6.0	Solvent extraction	19.7	30.8	0.12	(Yang et al., 2014)
AS 2.1560	Mannose	$(NH_4)_2SO_4$ + yeast extract	291	Flask	28 ^{0}C, 200rpm 5 days, pH 6.0	Solvent extraction	21.6	58.3	0.18	(Yang et al., 2014)

Table 2.7 (continued)

DSM 70296	Glucose	Flour based industrial waste	NS	Flask	30 ^{0}C, 180rpm 200 hours, pH 5.2	Folch	30.5	40.4	NS	(Tsakona et al., 2014)
DSM 70296	Brazilian molasses	$(NH_4)_2SO_4$ + yeast extract	40	Bioreactor (fed-batch)	28 ^{0}C, 250rpm, 60 hours, pH 5.8	Bligh & Dyer	21.2	32.0	0.06	(Vieira et al., 2014)
CBS 1807	Sweet sorghum juice	NS	NS	Flask	30 ^{0}C, 200rpm 200 hours, pH 6.0	Folch	21.6	29.5	0.07	(Matsakas et al., 2014)
CBS 1807	Synthetic sugar	Urea	100	Flask	30 ^{0}C, 200rpm pH 6.0	Folch	NS	10-20	NS	(Matsakas et al., 2014)
NS	Arundo donax hydrolysate	NS	NS	Flask	30 ^{0}C, 160rpm pH 6.5-6.6	Bligh & Dyer	7.6	20.1	NS	(Pirozzi et al., 2015)
AS 2.1560	Beet pulp hydrolysates	$(NH_4)_2SO_4$ + yeast extract	NS	Flask	30 ^{0}C, 200rpm pH 6.0	Solvent extraction	No growth observed	NS	NS	(Wang et al., 2015)
DSM 70296	Xylose	Urea	50	Bioreactor (fed-batch)	28 ^{0}C, 400rpm 144 hours, pH 5.5	Bligh & Dyer	94.6	37.4	0.17	(Anschau & Franco, 2015)
DSM 70296	Sunflower meal hydrolysate	NS	195	Flask	30 ^{0}C, 180rpm pH 6.0	Solvent extraction	17.4	29.4	NS	(Leiva-Candia et al., 2015)
ATCC 56304	Wheat bran hydrolysate	NS	NS	Flask	26 ^{0}C, 200rpm 120 hours	Bligh & Dyer	17.1	37.3	0.12	(Probst & Vadlani, 2015)
ATCC 56304	Corn bran hydrolysate	NS	NS	Flask	26 ^{0}C, 200rpm 120 hours	Bligh & Dyer	23.5	33.3	0.13	(Probst & Vadlani, 2015)

Table 2.7 (continued)

NRRL Y-11557	Raw glycerol	Yeast extract	NS	Flask	30 ^{0}C, 180rpm 120 hours, pH 5.5	Bligh & Dyer	5.7	50.4	0.13	(Spier et al., 2015)
NRRL Y-11557	Glucose	Urea + NH_4Cl	72	Flask	30 ^{0}C, 200rpm 192 hours	Bligh & Dyer	18.2	54.8	0.17	(Calvey et al., 2016)
NRRL Y-11557	Corn stover hydrolysate	NS	20	Flask	30 ^{0}C, 200rpm 193 hours	Bligh & Dyer	24.6	38.0	0.14	(Calvey et al., 2016)
DSM 70295	Crude glycerol	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (batch)	25 ^{0}C, 220rpm 200 hours, pH 5.5	Solvent extraction	32.7	55.9	NS	(Signori et al., 2016)
DSM 70295	Pure glycerol	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (batch)	25 ^{0}C, 220rpm 200 hours, pH 5.5	Solvent extraction	31.4	48.2	NS	(Signori et al., 2016)
CBS1807	Xylose + acetate	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (fed-batch)	25 ^{0}C, 500rpm 6 days, pH 6.0	Folch	22.1	35.0	0.11	(Brandenburg et al., 2016)
NRRL Y-11557	Olive oil wastewater	$(NH_4)_2SO_4$ + yeast extract	NS	Flask	28 ^{0}C, 180rpm	Folch	2.7	17.8	0.07	(Dourou et al., 2016)
NRRL Y-11557	Olive oil wastewater + glucose	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (fed-batch)	28 ^{0}C, 280rpm pH 6.0	Folch	9.5	25.4	NS	(Dourou et al., 2016)
DSM 70296	Sugarcane bagasse hydrolysate	$(NH_4)_2SO_4$ + yeast extract	50	Flask	28 ^{0}C, 200rpm pH 5.5	Bligh & Dyer	9.3	26.9	0.14	(Xavier et al., 2017)

Table 2.7 (continued)

DSM 70296	Sugarcane bagasse hydrolysate	$(NH_4)_2SO_4$ + yeast extract + $(NH_4)_2Mo_2O_7$	50	Bioreactor (batch)	28 ^{0}C, 400rpm pH 5.5	Bligh & Dyer	9.6	26.1	0.14	(Xavier et al., 2017)
DSM 70296	Sugarcane bagasse hydrolysate	$(NH_4)_2SO_4$ + yeast extract + $(NH_4)_2Mo_2O_7$	50	Bioreactor (continuous)	28 ^{0}C, 400rpm pH 5.5	Bligh & Dyer	11.5	27.3	0.18	(Xavier et al., 2017)
DSM 70296	Palm kernel cake hydrolysate + glucose	Yeast extract + peptone	85	Flask	30 ^{0}C, 180rpm 120-192 hours	Solvent extraction	22.2	36.8	NS	(Erminda Tsouko et al., 2017)
ATCC 56304	Glucose + xylose	Urea + yeast extract	NS	Bioreactor (fed-batch)	26.5 ^{0}C, 300rpm 156 hours	Solvent extraction	188.2	57.4	0.17	(Probst & Vadlani, 2017)
AS 2.1560	Crude glycerol	Yeast extract + peptone	NS	Flask	30 ^{0}C, 200rpm 5 days, pH 6.0	Bligh & Dyer	16.6	29.0	NS	(Liu et al., 2017)
AS 2.1560	Crude glycerol	NS	NS	Bioreactor (batch)	10 days	Bligh & Dyer	25-30	40-45	NS	(Liu et al., 2017)
ATCC 58680	Glucose	$(NH_4)_2SO_4$	114	Flask	30 ^{0}C, 80 hours, pH 5.5	Solvent extraction	9.7	53.6	0.17	(Rahman et al., 2017)
ATCC 58680	Xylose	$(NH_4)_2SO_4$	114	Flask	30 ^{0}C, 80-100 hours, pH 5.5	Solvent extraction	9.6	48.4	0.15	(Rahman et al., 2017)
ATCC 58680	Xylose + glucose	$(NH_4)_2SO_4$	114	Flask	30 ^{0}C, 80-100 hours, pH 5.5	Solvent extraction	9.4	56.0	0.18	(Rahman et al., 2017)

Table 2.7 (continued)

DSM 70296	Glucose	NS	NS	Flask (immobilized cells)	30 ^{0}C, 180rpm 120 hours, pH 5.0	Folch	22.5	33.0	0.12	(Ganatsios et al., 2017)
DSM 70296	Agro-industrial waste + glucose	NS	NS	Flask (immobilized cells)	30 ^{0}C, 180rpm 168 hours, pH 5.0	Folch	26.0	38.0	0.25	(Ganatsios et al., 2017)
BCRC 23408	Rice bran hydrolysate	NS	NS	Flask	28 ^{0}C, 200rpm 84 hours, pH 6.0	Solvent extraction	13.5	52.6	0.24	(Sutanto et al., 2017)
ATCC 56304	Palm oil mill effluent	Not added	NS	Flask	25 ^{0}C, 150rpm 6 days	Solvent extraction	7.6	21.3	NS	(Islam et al., 2018)
NRRL Y-11557	Glucose	$(NH_4)_2SO_4$	NS	Bioreactor (repeated fed-batch)	30 ^{0}C, pH 5.5	Bligh & Dyer	22.7	51.4	NS	(Thirumal et al., 2018)
NRRL Y-11557	Glucose	$(NH_4)_2SO_4$	113	Bioreactor (continuous)	30 ^{0}C, pH 5.5	Bligh & Dyer	NS	NS	0.17	(Thirumal et al., 2018)
DBVPG 6193 + microalgal polyculture	Urban wastewater	NS	NS	Raceway pond	14 days, pH 7.6	NS	NS	14-16	NS	(Iasimone et al., 2018)
NBRC10381	Glucose + xylose	$(NH_4)_2SO_4$ + yeast extract	197	Flask	30 ^{0}C, 190rpm 4 days, pH 5.5	Folch	38.2	52.6	NS	(Ario B. Juanssilfero et al., 2018)
CBS 1807	Wheat straw hydrolysate	Not added	NS	Flask	25 ^{0}C, 125rpm 48 hours, pH 5.0	Folch	11.7	31.8	0.09	(Brandenburg et al., 2018)

Table 2.7 (continued)

NBRC10381	Glucose + xylose	$(NH_4)_2SO_4$ + yeast extract	197	Flask	30 ^{0}C, 190rpm 3 days, pH 5.5	Folch	25.9	77.1	0.19	(Ario Betha Juanssilfero et al., 2018)
CBS 1807	Wheat straw hydrolysate	Not added	NS	Bioreactor (fed-batch)	30 ^{0}C, 7.5 days, pH 5.0	Solvent extraction	47.9	43.0	0.09	(Blomqvist et al., 2018)
NRRL Y-11558	Corn stover hydrolysate	NH_4Cl	NS	Bioreactor (batch)	30 ^{0}C, 600rpm pH 5.5	Solvent extraction	18.6	36.0	0.22	(Collett et al., 2019)
DBVPG 6193 + microalgal culture	Glucose	NH_4Cl + yeast extract	NS	Flask	24 ^{0}C, 120rpm 4 days, pH 6.25	Bligh & Dyer	NS	38.0	NS	(Zuccaro et al., 2019)
NBRC10381	Oil palm trunks	Not added	NS	Flask	30 ^{0}C, 190rpm 4 days, pH 3.2	Folch	27.7	55.2	0.15	(Ario Betha Juanssilfero et al., 2019)
NBRC10381	Oil palm trunks	$(NH_4)_2SO_4$	NS	Flask	30 ^{0}C, 190rpm 4 days, pH 3.2	Folch	30.1	64.4	0.19	(Ario Betha Juanssilfero et al., 2019)
NBRC10381	Oil palm trunks	$(NH_4)_2SO_4$	NS	Flask	30 ^{0}C, 190rpm 4 days, pH 5.0	Folch	28.1	63.1	0.17	(Ario Betha Juanssilfero et al., 2019)
CBC 1807	Starchy waste (rice residue)	Not added	15	Flask	30 ^{0}C, 120rpm 8 days	Ultrasonic homogenizer + solvent extraction	26.7	52.0	NS	(Chaturvedi et al., 2019)
CBC 1807	Starchy waste (cassava peel)	Not added	28	Flask	30 ^{0}C, 120rpm 8 days	Ultrasonic homogenizer + solvent extraction	20.7	38.9	NS	(Chaturvedi et al., 2019)

Table 2.7 (continued)

CBC 1807	Starchy waste (barley residue)	Not added	20	Flask	30 ^{0}C, 120rpm 8 days	Ultrasonic homogenizer + solvent extraction	15.5	31.6	NS	(Chaturvedi et al., 2019)
CBC 1807	Starchy waste (corn residue)	Not added	16	Flask	30 ^{0}C, 120rpm 8 days	Ultrasonic homogenizer + solvent extraction	28.1	15.2	NS	(Chaturvedi et al., 2019)
CBC 1807	Starchy waste (banana peel)	Not added	16	Flask	30 ^{0}C, 120rpm 8 days	Ultrasonic homogenizer + solvent extraction	48.8	11.6	NS	(Chaturvedi et al., 2019)
CBC 1807	Sweet sorghum juice	NS	42	Flask	30 ^{0}C, 250rpm, 7 days, pH 5.0	Folch	24.9	56.3	NS	(Rolz et al., 2019)
DSM 70295	Oil palm empty fruit bunch hydrolysate	$(NH_4)_2SO_4$ + yeast extract	71	Flask	30 ^{0}C, 140rpm 72 hours, pH 5.5	Bligh & Dyer	9.4	41.4	0.15	(Thanapimmetha et al., 2019)
DSM 70295	Oil palm empty fruit bunch hydrolysate + glucose	$(NH_4)_2SO_4$ + yeast extract	71	Flask (repeated batch)	30 ^{0}C, 140rpm 96 hours, pH 5.5	Bligh & Dyer	9.4	45.3	0.16	(Thanapimmetha et al., 2019)
NBRC10381	Glucose	$(NH_4)_2SO_4$ + yeast extract	17	Bioreactor (fed-batch)	30 ^{0}C, 150rpm 120 hours, pH 5.0	Folch	66.6	49.0	0.11	(Amza et al., 2019)
NBRC10381	Xylose	$(NH_4)_2SO_4$ + yeast extract	17	Bioreactor (fed-batch)	30 ^{0}C, 150rpm 120 hours, pH 5.0	Folch	63.4	46.2	0.13	(Amza et al., 2019)

Table 2.7 (continued)

NBRC10381	Glucose + xylose	$(NH_4)_2SO_4$ + yeast extract	17	Bioreactor (fed-batch)	30 ^{0}C, 150rpm 96 hours, pH 5.0	Folch	57.9	42.0	0.11	(Amza et al., 2019)
NBRC10381	Glucose + xylose	$(NH_4)_2SO_4$ + yeast extract	17	Bioreactor (biphasic)	30 ^{0}C, 150rpm 96 hours, pH 5.0	Folch	58.2	43.7	0.10	(Amza et al., 2019)
NRRL Y-1389	Decolorized cellulosic hydrolysate (wheat straw)	Not added	NS	Flask	30 ^{0}C, 250rpm 120 hours	Solvent extraction	12.5	25.6	NS	(Z. Liu et al., 2020)
NRRL Y-1389	Decolorized hemi cellulose hydrolysate (wheat straw)	Not added	NS	Flask	30 ^{0}C, 250rpm 120 hours	Solvent extraction	14.1	10.6	NS	(Z. Liu et al., 2020)

*NS: not specified

2.3.1.4 Different Aspects of *Lipomyces starkeyi*

As the name implies, *Lipomyces starkeyi* is famous for its capability to get "obese" after which we are able to extract the stored lipids. Nonetheless, just before the 2000's, there have been some studies mentioning several other aspects of *Lipomyces starkeyi* beside lipid accumulation. Although some cell-bound amylolytic enzymes such as α-amylase and α-glucosidase were discovered in *L. starkeyi* in previous studies (Kelly et al., 1985; Moulin & Galzy, 1979), enzyme activities, optimum pH and molecular weights of these suspicious enzymes were further clarified by Bignell et al., (2000). Furthermore, it was stated that it is possible to observe α-amylase and glucoamylase activities only after 2 days of cultivation in the culture broth without

protease activity (Punpeng et al., 1992). Both authors agreed that amylolytic activities reached maximum after 6 days and molecular weight of *L. starkeyi* based α-amylase is around 50kDa. Unlike the optimum cell cultivation and lipid accumulation temperature, which is at least 25 ^{o}C, Punpeng et al. (1992) further stated that the relatively higher enzyme activity was observed at 15 ^{o}C, which was attributed to temperature dependence of permeability of cell membrane. On the other hand, the attempts of enzymatic utilization of *L. starkeyi* is not limited with α-amylase. It was discovered that *L. starkeyi* is capable of producing endo-dextranase, which is an enzyme that cleaves α-(1,6)-D-glucopyranosyl linkages in dextrans (D. Kim & Day, 1995). Briefly, dextran is a general name for the group of bacterial polysaccharides that consist of D-glucose chains linked by α-1,6 bonds (Doman Kim & Day, 1994). Low molecular weight dextran is used as blood plasma extender and blood flow improver (Doman Kim, Jhon, et al., 1996). Thus, dextran has an important value for pharmaceutical industry and it is also found in the literature as "clinical dextran", whereas the enzyme dextranase is useful for food industry on account of its ability to remove dextran in sugar processing (D. Kim & Day, 1995). According to Kim (1996), the challenge in commercial small size dextran production is that significant amount of dextran is being lost during acid hydrolysis step, which is the most critical step to obtain the desired dextran size. He suggested that the mutant strain of *L. starkeyi* (*Lipomyces starkeyi* ATCC 74054) should be utilized as a constitutive dextranase producer from fructose, while bacteria *Leuconostoc mesenteroides* is being used as dextransucrase producing strain (Doman Kim, Seo, et al., 1996). He also concluded that this mixed culture processing is more practical and efficient than traditional dextran production. Lastly, novel glucanhydrolase enzymes retrieved from *L. starkeyi* was investigated as a dental plaque cleaning agents. It turned out that *L. starkeyi* originated dextranases can be useful for dental health applications as well (Su-Jin et al., 2000).

2.3.2 *Rhodosporidium toruloides*

2.3.2.1 Taxonomy of *Rhodosporidium toruloides*

Rhodosporidium toruloides is an eukaryotic, unicellular and a member of red yeasts of 4 genera with *Rhodotorula, Sporobolomyces* and *Sporidiobolus* (Mannazzu et al., 2015) isolated from conifers (Ageitos et al., 2011). The taxonomy is kingdom *Fungi,* phylum *Basidiomycota*, class *Microbotryomycetes*, order *Sporidiobolales*, family *Sporidiobolaceae*, genus *Rhodotorula* F.C. Harrison, and species *Rhodosporidium toruloides* I.Banno, synonym of *Rhodotorula toruloides* I. Banno. Even though red yeasts have been studied since 1852, *Rhodosporidium toruloides* was first discriminated from *Rhodotorula* by Banno (1967). In this famous study, Banno (1967) defines *Rhodosporidium toruloides* as follows: "It has dikaryotic mycelial stage, oval, round or elongated shape, reproducing by multilateral budding; conjugate developing to true mycelium, reproduces by budding, forming monokaryotic yeast stage." (Banno, 1967). A simplified life cycle schema of *Rhodosporidium* illustrated by Banno is depicted in Figure 2.4. For a detailed taxonomic study of *Rhodosporidium toruloides* Banno, see Johnson-Reid and Moore (1972).

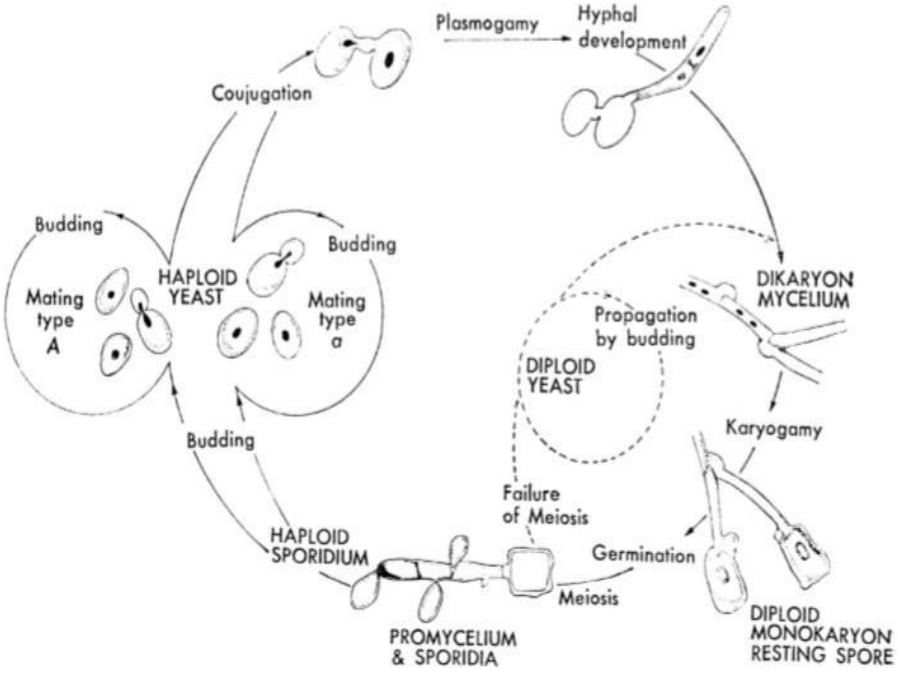


Figure 2.4. Life cycle scheme of *Rhodosporidium toruloides* (Banno, 1967).

It is also possible to discriminate *Rhodosporidium* from *Rhodotorula* simply by biochemical methods. Studies showed that unlike *Rhodotorula glutinis*, *Rhodosporidium toruloides* is able to assimilate lactic acid and grow at 35 - 37 ºC; however *Rhodosporidium toruloides* cannot assimilate sorbose, ethylamine or tartaric acid (Table 2.8) (C. p. Kurtzman et al., 2011).

Table 2.8. Biochemical characterization of *Rhodosporidium toruloides* and *Rhodotorula glutinis* (C. p. Kurtzman et al., 2011).

Genus	L-Sorbose	Ethylamine	L-Tartaric acid	DL-Lactic acid	Growth at 35 ºC	Growth at 37 ºC
Rhodosporidium toruloides	-	-	-	+	+	+
Rhodotorula glutinis	+	+	+	-	-	-

2.3.2.2 Chronological Progress on *Rhodosporidium toruloides* and Single Cell Oil

Earlier, it used to be hard to encounter as many studies as recent years on single cell oil production by *Rhodosporidium toruloides*. The complexity of reproduction mechanism of the "new" species, in other words, understanding the species *Rhodosporidium* that had been newly discriminated from *Rhodotorula,* attracted researchers' attention more than investigating the ways of exploiting the strain. Evans and Ratledge (1985) published a series of reports enlightening the biochemical lipid accumulation mechanism of *Rhodosporidium toruloides.* Five key enzymes were specified as they might be the key biological factors behind lipid accumulation in *Rhodosporidium toruloides* cells, namely pyruvate kinase (Evans & Ratledge, 1985a), isocitrate dehydrogenase (Evans & Ratledge, 1985c), malic enzyme, ADP deaminase, and certainly the famous oleaginous yeast specific enzyme ATP citrate lyase (Evans & Ratledge, 1985b). Justas other oleaginous microorganisms, lipid accumulation mechanism of *Rhodosporidium toruloides* is dramatically affected by carbon and nitrogen sources present in the fermentation medium. Evans and Ratledge

(1984) demonstrated the effects of nitrogen sources on lipid accumulation. Using glucose as the carbon source, as inorganic N sources NH_4CI and $NaNO_3$ gave the lowest lipid yields of 18 and 21%, respectively. However, organic N sources, namely urea, glutamate and arginine showed the highest lipid content of 52, 52 and 54%, respectively (Evans &Ratledge, 1984). As mentioned in the previous sections, lipid storage in oleaginous yeasts is highly dependent on initial C/N ratio, temperature and initial pH (see section1.1.2.). As an oleaginous yeast, *R. toruloides* is significantly stimulated to store lipidwhen nitrogen is limited in the medium (Turcotte & Kosaric, 1989). According to Turcotte and Kosaric (1989), in the synthetic glucose medium, when C/N ratio is 77and temperature at 30 ºC, lipid yield was more or less the same regardless of the nitrogen source, which suggests that C/N ratio is more critical than the nitrogen source in the case of *R. toruloides* utilization (Turcotte & Kosaric, 1989). In addition,an early attempt to examine lipid production capacity of *R. toruloides* in a mixed culture showed that it is possible to obtain lipid up to 36.5% of dry biomass using soluble starch as the only carbon source with some additives (Dostálek, 1986). Furthermore, *R. toruloides* is also capable of assimilating molasses and gives 20.6 g/L of cell mass and 67% of lipids (Kennedy et al., 1992).

2.3.2.3 Different Aspects of *Rhodosporidium toruloides*

Although it has been shown that *R. toruloides* can be used as an alternative enzyme producer such as phenylalanine ammonia lyase (Kawasaki Watanabe et al., 1992), asparaginase (Ramakrishnan & Joseph, 1996) and epoxide hydrolase (Botes et al., 1999), the most important characteristic of *R. toruloides* is to accumulate carotenoids simultaneously with lipids. (Kot et al., 2018). Simply, carotenoids are vitamin A precursors and lipid soluble pigments (orange/red colored) that are used in food and pharmaceutical industries due to their coloring and antioxidant activities (Mata-Gómez et al., 2014). Industrial carotenoids are mostly obtained from fruits and vegetables or by synthetic methods. Nonetheless, recent studies demonstrated that

R.toruloides might be a serious candidate to be a major natural carotenoid producer by 24.5 mg/L carotenoid accumulation (Z. Liu et al., 2020).

2.3.2.4 Current Status of *Rhodosporidium toruloides*

Studies that have been performed in the last 5 years on single cell oil production by *R. torulodies* are shown in Table 2.9. It has been specified by multiple authors that *R. torulodies* might be one of the most serious lipid producer of the future by the lipid yield above 70% of dry cellular mass in relatively short time and higher scales (González-García et al., 2017; Y. Li et al., 2007; J. Ling et al., 2016; Singh et al., 2018; Tchakouteu et al., 2017; S. Zhang, Ito, et al., 2016).

Table 2.9. Compilation of microbial oil studies regarding different strains of *R. toruloides* in the last 5 years.

***R.toruloides* strain**	**Carbon source**	**Nitrogen source**	**C/N ratio**	**Process types**	**Process condition**	**Extraction method**	**Biomass yield (g/L)**	**Lipid yield(% w/w)**	**Lipid constant (g/g sugar)**	**Ref.**
DSM 4444	Pure glycerol	(NH4)2SO 4 + yeast extract	99	Bioreactor (batch)	25 ^{0}C, 300rpm, 200-250 hours,pH 5.5	Bligh &Dyer	40.4	60.9	NS*	(Signori etal., 2016)
DSM 4444	Crude glycerol	(NH4)2SO 4 + yeast extract	99	Bioreactor (batch)	25 ^{0}C, 300rpm, 200-250 hours, pH 5.5	Bligh &Dyer	41.0	60.0	NS	(Signori etal., 2016)
Y4	P-limited Laminaria residues hydrolysate	Yeastextract	NS	Flask	30 ^{0}C, 200rpm,96 hours, pH 5.2	Solvent extraction	12.7	37.6	0.16	(X. Zhang, Shen, etal., 2016)

Table 2.9 (continued)

Y4	Laminaria residues hydrolysate	Yeastextract	NS	Flask	30 ^{0}C, 200rpm 96 hours, pH 5.2	Solvent extraction	11.9	22.2	0.04	(X. Zhang, Shen, etal., 2016)
AS 2.1389	Beer brewery wastewater	NS	NS	Flask	35 ^{0}C, 200rpm, 5 days	Bligh &Dyer	0.6	16.7	NS	(J. Ling etal., 2016)
AS 2.1389	Milk candy wastewater	NS	NS	Flask	25 ^{0}C, 200rpm, 7 days	Bligh &Dyer	1.6	71.3	NS	(J. Ling etal., 2016)
AS 2.1389	Rice wine distillery wastewater	NS	NS	Flask	30 ^{0}C, 200rpm, 4 days, pH 3.5	Bligh &Dyer	1.8	32.6	NS	(J. Ling etal., 2016)
AS 2.1389	Nonsterile distillery wastewater	NS	NS	Flask	35 ^{0}C, 200rpm, 4 days	Bligh &Dyer	5.7	47.0	NS	(J. Ling etal., 2016)
AS 2.1389	Acetic acid	(NH4)2SO4 + yeast extract	200	Flask	30^{0}C, 200rpm, 200-230 hours, pH 6.0	Solvent extraction	4.3	48.2	0.18	(X. F. Huang etal., 2016)
AS 2.1389	Acetic acid	((NH4)2SO4 + yeast extract	100	Flask (sequencing batch)	30^{0}C, 200rpm, pH 6.0	Solvent extraction	4.2	38.6	NS	(X. F. Huang etal., 2016)
DSM 4444	Glucose	Flour rich waste hydrolysate	80	Bioreactor (fed- batch)	27 ^{0}C, 150-500rpm, 150 hours, pH 6.0	Folch	23.3	61.8	0.17	(Tsakonaet al., 2016)

Table 2.9 (continued)

DSM 4444	Glucose	Flour rich waste hydrolysate	100	Bioreactor (fed-batch)	27 ^{0}C, 150-500rpm, 150 hours, pH 6.0	Folch	20.9	57.5	0.14	(Tsakonaet al., 2016)
RT880-AD	Glucose	(NH4)2SO4 + yeast extract	339	Flask	30 ^{0}C, 250rpm, 217 hours, pH 5.6	Folch	26.8	61.1	0.23	(S. Zhang, Skerker, et al., 2016)
RT880-AD	Xylose	(NH4)2SO4 + yeast extract	339	Flask	30 ^{0}C, 250rpm, 385 hours, pH 5.6	Folch	21.9	43.4	0.14	(S. Zhang, Skerker, et al., 2016)
RT559-AD	Glucose	(NH4)2SO4 + yeast extract	339	Flask	30 ^{0}C, 250rpm, 289 hours, pH 5.6	Folch	15.6	58.4	0.13	(S. Zhang, Skerker, et al., 2016)
DSMZ4444	Ligno- cellulosic sugar mixture	NS	NS	Bioreactor (pulsed-fed- batch)	30 ^{0}C, 300 - 700rpm, 96 hours pH 5.2	Solvent extraction	43.0	61.4	0.24	(Fei et al., 2016)
DSMZ4444	Ligno- cellulosic sugar mixture	NS	NS	Bioreactor (Onlinefed-batch)	30 ^{0}C, 300 - 700rpm, 96 hours pH 5.2	Solvent extraction	54.0	58.7	0.29	(Fei et al., 2016)
AS 2. 1389	Glycerol	(NH4)2SO4 + yeast extract	75	Flask	30 ^{0}C, 200rpm, 136 hours, pH 6.0	Solvent extraction	8.5	51.0	0.16	(Xu et al., 2016)
AS 2. 1389	Glycerol + sodium oleate	(NH4)2SO4 + yeast extract	NS	Flask	30 ^{0}C, 200rpm, 136 hours, pH 6.0	Solvent extraction	8.4	67.0	0.20	(Xu et al., 2016)

Table 2.9 (continued)

NCYC 921	Glucose	(NH4)2SO4 + yeast extract	54	Flask	30 ^{0}C, 150rpm, 72 hours, pH4.0	Flow cytometry	5.9	21.8	NS	(Dias etal., 2016)
32489	Crude glycerol	(NH4)2SO4 + peptone	60	Flask	30 ^{0}C, 200rpm, 7 days, pH 7.0	Solvent extraction	14.8	41.7	NS	(Gao et al., 2016)
32489	Pure glycerol	(NH4)2SO4 + peptone	60	Flask	30 ^{0}C, 200rpm, 7 days, pH 7.0	Solvent extraction	15.1	34.2	NS	(Gao et al., 2016)
np11	Glucose	(NH4)2SO4 + yeast extract + amino acid supplements	NS	Flask	30 ^{0}C, 220rpm, 168 hours , pH6.0	Solvent extraction	8.6	43.0	NS	(C. Zhang, Shen, etal., 2016)
RT880-ADS	Glucose	Yeats extract + peptone	NS	Bioreactor (fed- batch)	30 ^{0}C, 144 hours, pH5.6	Folch	118.4	75.6	0.22	(S. Zhang,Ito, et al., 2016)
RT880-AD	Glucose	Yeats extract + peptone	NS	Bioreactor (fed- batch)	30 ^{0}C, 144 hours, pH5.6	Folch	86.2	72.9	0.14	(S. Zhang,Ito, et al., 2016)
np11	Galactose + raffinose	(NH4)2SO4	136	Flask	30 ^{0}C, 120 hours, pH6.0	Solvent extraction	8.8	55.0	NS	(Yanan Wang etal.,2016)
np11-FAD1	Galactose + raffinose	(NH4)2SO4	136	Flask	30 ^{0}C, 120hours, pH6.0	Solvent extraction	9.9	42.0	NS	(Yanan Wang etal.,2016)

Table 2.9 (continued)

DEBB5533	Sugarcanejuice	Urea	NS	Bioreactor (batch)	30 ^{0}C, 200rpm , 24 hours pH 5.0	Three staged extraction	24.5	41.0	0.36	(Soccol etal., 2017)
DEBB5533	Sugarcanejuice	Urea	NS	Bioreactor (fed- batch)	30 ^{0}C, 200rpm, 45 hours pH 5.0	Three staged extraction	40.3	45.0	NS	(Soccol etal., 2017)
A29	Glucose +glucose syrup	Yeats extract	42	Bioreactor (fed- batch)	30 ^{0}C, 250rpm, pH 5.5	Folch	23.3	53.5	NS	(Saran etal., 2017)
DSM 4444	Glucose	Yeats extract + peptone	120	Flask	26 ^{0}C, 185rpm, 192 hours, pH 6.0	Solvent extraction	9.4	71.3	0.14	(Tchakouteu et al., 2017)
DSM 4444	Glucose	Yeats extract + peptone	NS	Bioreactor (fed- batch)	26 ^{0}C, 200-500rpm, 312 hours, pH 6.0	Solvent extraction	36.2	61.5	0.21	(Tchakouteu et al., 2017)
ATCC10788	Crude glycerol	(NH4)2SO4	100	Flask	30 ^{0}C, 200rpm, 168 hours, pH 6.0	Bligh &Dyer	21.1	53.2	0.43	(Uprety,Dalli, et al., 2017)
ATCC10788	Pure glycerol	(NH4)2SO4	100	Flask	30 ^{0}C, 200rpm, 168 hours, pH 6.0	Bligh &Dyer	10.1	34.8	0.09	(Uprety,Dalli, et al., 2017)
DSM 4444	Glucose	(NH4)2SO4 + yeast extract	77	Flask	28 ^{0}C, 185rpm, 120hours, pH 5.0-6.0	Folch	9.7	39.6	NS	(Papanikolaou et al.,2017)
CCT 0783	Sugarcane bagasse hemicellulosic hydrolysate + glycerol	NS	47	Bioreactor (batch)	28 ^{0}C, 350rpm, 72 hours, pH 6.0	Folch	6.6	55	NS	(Bonturi et al., 2017)

Table 2.9 (continued)

ATCC10788	Crude glycerol	NH4Cl	100	Bioreactor (batch)	30 ^{0}C, 300rpm, 7 days, pH 6.0	Bligh &Dyer	27.4	68.0	NS	(Uprety, Reddy, et. al., 2017)
ATCC204091	Glucose	NaNO3	NS	Flask	30 ^{0}C, 150rpm, 240-264 hours, pH 5.5	Solvent extraction	60.0 -70.0	77.0	0.51	(González - García etal., 2017)
NCYC1576	Birch chips hydrolysate	NS	NS	Bioreactor	30 ^{0}C, 800rpm, 8 days, pH 5.5	Folch	7.1	39.0	0.07	(Matsakaset al., 2017)
CECT13085	Corn steepliquor + sucrose	NH4NO3	NS	Bioreactor (fed-batch)	30 ^{0}C, 500-1000rpm 96 hours pH 5.0	Soxhlet	88.1	8.2	NS	(Fillet etal., 2017)
AS 2.1389	Spent yeast culture	Yeats extract +peptone	NS	Flask	30 ^{0}C, 200rpm, 2 days	Bligh &Dyer	5.7	37.8	NS	(Jiayin Ling etal., 2017)
AS 2.1389	Spent yeast culture + glucose	Yeats extract + peptone	NS	Flask	30 ^{0}C, 200rpm, 2 days	Bligh &Dyer	5.4	41.8	NS	(Jiayin Ling etal., 2017)
CECT1499	Carob extract	(NH4)2SO4 + yeast extract	330	Flask	30 ^{0}C, 200rpm, 200-216 hours	Nile Red	5.7	9.7	NS	(Afonso etal., 2018)
CECT1499	Carob extract + acetic acid	(NH4)2SO4 + yeast extract	NS	Bioreactor (fed- batch)	30 ^{0}C, 200rpm	Nile Red	NS	40.0	NS	(Afonso etal., 2018)
0047-17	Wheat straw hydrolisate +corn steep liquor	NH4NO3 + NH4Cl	NS	Bioreactor (fed- batch)	30 ^{0}C, 500-1000rpm, pH 6.0	Nile Red	64.5	61.3	0.17	(Díaz etal., 2018)

Table 2.9 (continued)

NRRL Y-1091	Switchgrass	(NH4)2SO4	NS	Bioreactor (fed- batch)	30 ^{0}C, 200rpm, 120 hours, pH 5.5	Phosphoric acid- vanillin assay	19.4	43.8	NS	(Chen etal., 2018)
2.1389	Food waste saccharified liquid	(NH4)2SO4	73	Flask	30 ^{0}C, 200rpm, 6 days, pH 4.0-5.0	Solvent extraction	12.0	52.6	NS	(Ma et al., 2018)
Y2 + microalgae	Food waste hydrolysate	NS	14	Flask	28 ^{0}C, 180rpm, 5 days, pH 6.5	Bligh &Dyer	12.3	29.3	0.12	(Zeng etal., 2018)
ATCC20409	Mixed foodwaste extracts	NS	30	Flask	30 ^{0}C, 120rpm	Bligh &Dyer	38.7	76.0	NS	(Singh etal., 2018)
L1-1	Glucose	(NH4)2SO4 + yeast extract	140	Flask	37 ^{0}C, 150rpm , 120 hours, pH 5.5	Bligh &Dyer	NS	13.4	NS	(Wu et al., 2018)
NCYC1576	Brewers' spent grain hydrolysate	(NH4)2SO4+ yeast extract + amino acids	500	Flask	25 ^{0}C, 180rpm, 168hours, pH 5.5	Solvent extraction + sonication + microwave	18.4	56.4	NS	(Patel et al., 2018)
RP 15	Sugarcane bagasse hydrolysate + glycerol	(NH4)2SO4	140	Flask	28 ^{0}C, 180rpm, 14 days, pH 5.5	Bligh &Dyer	12.0 -14.0	59.8	0.15	(Hassanpour et al., 2019)
RP 15	Glycerol	(NH4)2SO4 + yeast extract	160	Flask	28 ^{0}C, 180rpm, 6 days, pH 5.5	Bligh &Dyer	7.1	57.4	0.18	(Hassanpour et al., 2019)
AS 2.1389	Corn stover	NS	NS	Flask	37 ^{0}C, 200rpm, 72 hours pH 5.2	Solvent extraction	NS	NS	0.06	(Dai et al., 2019)

Table 2.9 (continued)

AS 2.1389	Corn stover	NS	NS	Flask	30^{0}C, 200rpm, pH 5.2	Solvent extraction	NS	NS	0.08	(Dai et al., 2019)
2.1389	Food waste	NS	64	Flask	27 ^{0}C, 180rpm, 7 days	Solvent extraction	NS	50.1	NS	(Gao et al., 2019)
2.1389	Food waste saccharified liquid	(NH4)2SO4	30 - 73	Flask (two-staged)	27 ^{0}C, 180rpm, pH 4.0	Solvent extraction	15.1	60.6	0.20	(Ma et al., 2019)
1588	Wood hydrolysate(C6)	(NH4)2SO4	95	Flask	25 ^{0}C, 180rpm, 112 hours, pH 6.0	Solvent extraction	100 - 110	35.0	NS	(Osorio-Gonzálezet al., 2019)
7191	Wood hydrolysate(C5)	(NH4)2SO4	95	Flask	25 ^{0}C, 180rpm ,112 hours, pH 6.0	Solvent extraction	90 - 100	32.0	NS	(Osorio-Gonzálezet al., 2019)
CCT 0783	Glycerol	(NH4)2SO4 + yeast extract	100	Flask	120 hours, pH 6.0	Folch	17.7	42.1	NS	(Lopes et al., 2020)

2.4 Extraction Methods for Single Cell Oil

Lipid extraction from oleaginous yeasts is a unit operation that must be investigated in two categories; cell wall disruption and lipid recovery. The complex cell wall must be unfolded in some way possible prior to lipid recovery for an utmost effective lipid extraction. Although direct application of appropriate solvents (alcohols, chloroform, hexane, toluene, n-heptane, water, etc.) to intact cells might result in lipid recovery to some extent this would be far from being an efficient extraction (Dong et al., 2016). Oleaginous yeasts are surrounded by a rigid cell wall, which is made of complex covalent bonded structural networks of celluloses such as glucans with β(1, 3) and β(1, 6) glucose linkages, chitin, mannan, mannoprotein, phosphates and lipids (Jacob, 1992). Cell wall structures of oleaginous and non-oleaginous yeasts differ from each as shown in Figure 2.5.

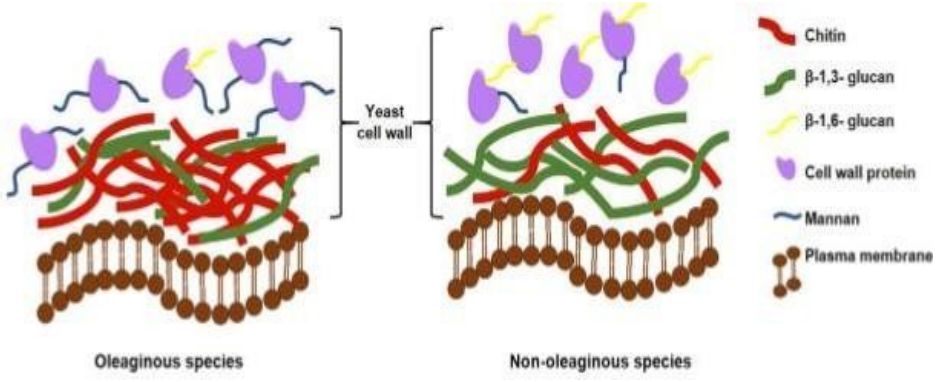


Figure 2.5. Comparative cell wall structures for oleaginous and non-oleaginous cells (Khot et al., 2020).

Briefly, oleaginous cell walls comprise a higher ratio of mannan and chitin in comparison to non-oleaginous yeasts. Thus, oleaginous cell walls are harder to destroy, which makes lipid recovery harder (Khot et al., 2020). As a result, the rigid

cell wall of oleaginous yeasts might be considered as the most critical hurdle to overcome in order to achieve a satisfactory lipid extraction.

2.4.1 Conventional Methods

Lipids are hydrophobic substances, which means to be insoluble in water. As a result of this fact, lipid extractions from microorganisms are performed with the help of organic solvents such as hexane, methanol, chloroform, etc. Conventional solvent extraction methods are highly preferred since they do not require expensive high technological equipment. As it can be understood from Table 2.7 and Table 2.9, most of the extraction steps are done by solvent extraction methods; namely, soxhlet apparatus extraction (Fillet et al., 2017), Folch method (Folch et al., 1957), Bligh & Dyer method (Bligh & Dyer, 1959), or some modified versions of them (Ario B. Juanssilfero et al., 2018). Briefly, after cells are harvested from fermentation medium; they are treated with dilute acids under high temperatures to unfold/weaken rigid cell wall, then organic solvents are applied to extract the accumulated lipids from the cells. Lastly, the solvent is evaporated for maximum purification and reuse. Detailed explanation of Bligh & Dyer methods can be found in the Material and Methods section.

2.4.2 Conventional Methods

Novel extraction methods mostly focus on advanced cell wall destruction (Patel, Mikes, & Matsakas, 2018). Although they have been proved to be effective, there are still some challenges to commercialize these techniques since they are costly (Patel, Mikes, & Matsakas, 2018). They are mostly applied with the combination of solvent extraction methods to achieve maximum lipid recovery (Yousuf et al., 2010). Some widely studied examples for novel cell disruption methods are: High pressure homogenizer, ultrasonication, pulsed electric field, microwave irradiation, and

enzymatic lysis (Kapoore et al., 2018; Karim et al., 2018; Kruger et al., 2018; Patel, Mikes, & Matsakas, 2018).

A high pressure homogenizer is a machine used for homogenizing by forcing heterogeneous mixtures (such as biomass-solvent) to flow through a narrow path at high speed, which generates high pressures between 40 to 120 MPa (Sankh et al., 2013). Although this method was suggested as an alternative lipid extraction method, it was stated ineffective in cell wall disruption by one flow cycle; therefore, the method is energy-intensive (Shang et al., 2015). Ultrasonication is a widely utilized cell disruption method in laboratory scale experiments. The principle of ultrasound assisted extraction relies on disrupting cell wall by shear stress generated by sound wave pulses, then recovering lipids by organic solvents (Chemat et al., 2011). Even though ultrasonication is found to be effective in cell wall disruption of oleaginous microorganisms, it is not suitable to scale-up (Patel, Mikes, & Matsakas, 2018). Pulsed electric field (PEF), also known as electroporation, is an efficient method to damage cell wall to recover intracellular components such as microbial oil (Weaver et al., 1988). The cells are exposed to electric pulses in an applied electric field to cause membrane porosity, through which lipid is recovered by solvent (Sheng et al., 2011). The disadvantages of PEF are stated as dependency of medium composition, and disintegration of sensitive compounds (Patel, Mikes, & Matsakas, 2018). Another widely used method for lipid extraction is microwave irradiation. In this method, biomass-organic solvent suspension is exposed to electromagnetic waves, which finally generates heat as the polar compounds move at high speed to align themselves to the direction of electric field (Budarin et al., 2015). Microwave irradiation is proven to be environmentally friendly method due to low energy and solvent requirement; however, since free radicals form in high temperatures, it is a poor method in preserving the quality of lipid (Zheng et al., 2011). Lastly, cell walls are treated with cellulose-degrading enzymes. Nevertheless, complex structure of cell walls (see Figure 2.5) necessitates high purity enzyme cocktails, which makes the enzymatic methods costly at large scale processes (Khot et al., 2020).

Consequently, the main requirenments of a novel extraction method are applicability at large scales and high maintenance costs. High hydrostatic pressure (HHP) was studied as a lipid extraction method from oleaginous yeasts for the first time in this study. The efficiency of HHP at cell wall disruption of yeasts (Marx et al., 2011), and availability of commercialization make HHP a promising method for lipid extraction from oleaginous yeasts.

2.5 High Hydrostatic Pressure

2.5.1 A Brief Overview of HHP

High hydrostatic pressure (HHP) is an emerging non-thermal technique that is highly used and studied for microbial inactivation, food preservation and food quality enhancement (Tokuşoğlu et al., 2010). In high hydrostatic pressure processing, the food product is exposed to extreme pressures in a vessel filled with fluid (mostly water) to spread the pressure homogeneously (up to 600 MPa) at low-mild temperatures (up to 50 ^{o}C) leading to inactivation of microorganisms and enzymes without harming the sensory and nutritional quality of food (Ascencio et al., 2020; Tokuşoğlu & Swanson, 2014). There are several theories about the microbial inactivation mechanism of high hydrostatic pressure. One of them is that HHP alters or damages the membrane structure, which leads to changes in membrane permeability (Ross et al., 2003). On the other hand, it was reported that high pressure causes physical imbalances and internal structural damage in the cell, which hinders them from growing (Marquis, 1976). Inactivation effects of high hydrostatic pressure on bacteria have been studied extensively (Alpas & Bozoglu, 2002; Bayindirli et al., 2006; Bozoglu et al., 2004). However, extraction aspects of this method have been scarcely studied. Some studies on the extraction capacity of HHP on food-related compounds are shown in Table 2.10. Oleaginous yeasts aside, in comparison to bacteria, there is lack of information about HHP effects on any types of yeasts cells.

Saccharomyces cerevisiae has been studied extensively being the most common yeast in food industry (Shimada et al., 1993).

Table 2.10. Applications of HHP as an extraction method.

Source	Extracted Material	Yield (%)	Reference
Mealworm	Protein	74.5	(Bolat et al., 2021)
Cricket	Protein	81.5	(Bolat et al., 2021)
Mealworm	Lipid	24.0	(Ugur et al., 2020)
Cricket	Lipid	18.0	(Ugur et al., 2020)
Palmaria palmata	Polysaccharides	27.3	(Suwal et al., 2019)
Betula pendula	Protein	38.3	(Altuner et al., 2014)
Propolis	Flavonoids	1.9	(Shouqin et al., 2005)

2.5.2 HHP Effects on Yeasts

As mentioned above, the novel extraction methods are mainly new, and information on cell wall destruction is scarce. Thus, it is imperative to know how HHP changes the yeast cell wall structure and morphology in order to predict the HHP extraction efficacy. Since *Saccharomyces cerevisiae* and *Lipomyces starkeyi* share the same taxonomic order as *saccharomycetales*, it may be possible to have an idea about how HHP affects *Lipomyces starkeyi* in terms of cell wall and membrane damage by

checking the studies on *S. cerevisiae*. It was stated that when *S. cerevisiae* is exposed to 100 MPa at room temperature for 10 minutes, nuclear cell membrane and internal structure begin to change, whereas the cell wall does not change significantly (Shimada et al., 1993). Nevertheless, when the pressure reaches up to 600 MPa, leakage of cytoplasmic substances was observed.

According to another promising study on *S. cerevisiae*, HHP damages the cell wall severely (Marx et al., 2011). Under 600 MPa at 21 ^{o}C for 7 minutes, lethal damages on *S. cerevisiae* occurs such that the cell wall is broken and cytoplasm exposes (Figure 2.6). According to Brul (2000), typical conditions to change the cell wall protein structure of *S. cerevisiae* are 300 MPa, 25 ^{o}C and 15 min exposure; nonetheless, if the cell membrane protecting agent (namely trehalose) is removed, the minimal effective pressure drops to 200 MPa with the same time/temperature settings.

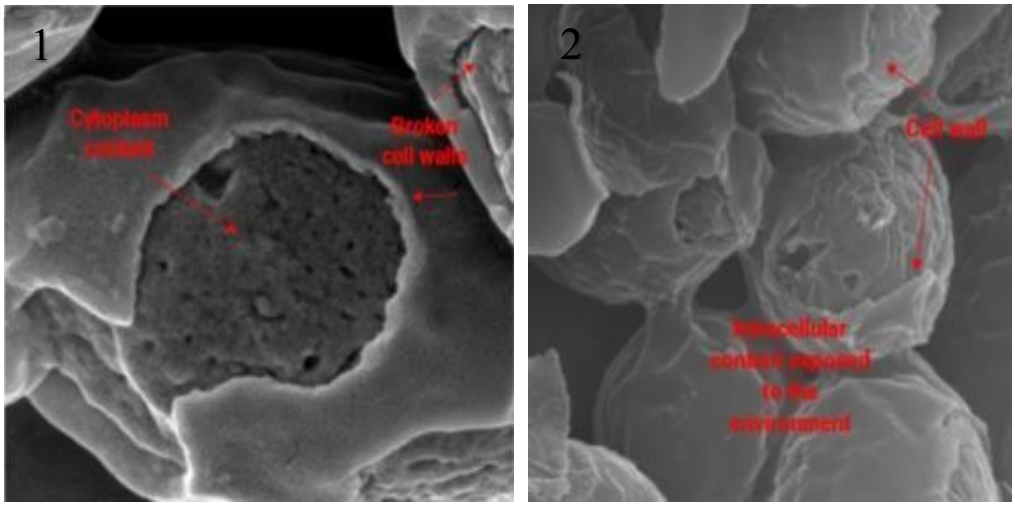


Figure 2.6. Pictures of the cytoplasm exposure and extracellular damages of *S. cerevisiae* ATCC 4113 under electron microscope after 600 MPa pressure at 21 ^{0}C for 7 mins (Marx et al., 2011).

To sum up, *S. cerevisiae* is not classified as barotolerant, meaning that HHP has lethal impact on the famous baker's yeast (Brul et al., 2000). Pressing up to 200 MPa damages the intracellular structure of cells (bud scars and cell nucleus membrane damages) whilst above 400 MPa, cell wall begin to unfold and cytoplasmic substances is transferred into the environment (Shimada et al., 1993). Considering lipid is accumulated in the cytoplasm, it might be a promising statement for those, who plan to utilize HHP for an efficient lipid extraction process.

2.6 Apple Pomace

Utilization of organic agricultural and industrial organic wastes in single cell oil studies has drawn too much attention such that many lignocellulosic structured wastes such as cassava, wood and sugarcane bagasse and much more has been investigated as a potential carbon and nutrient source for microbial growth and lipid accumulation (see Table 2.7 and Table 2.9).

Apple (*Malus domestica*) has been one of the most consumed fruit worldwide due to its essential nutrient content and high production rate, which makes it available nearly every part of the world (Khanali et al., 2020). During the last 15 years, apple production and harvested area has been followed an increasing trend. Apple comprised 10% of the worldwide fruit production (Figure 2.7) in 2019 by the rate of 87 million tone approximately, of which China outstands as the predominant producer by 40%, whereas the USA, Turkey and Poland follow by 4.9%, 3.6% and 3.0% respectively (FAO, 2019). Although most of them are consumed as fresh apple, 25 to 30% are processed to industrial products such as apple juice, in which only 65% is converted into yield (Cruz et al., 2018). Apple juice production comes along with a serious amount of agro-industrial wastes known as apple pomace, which contains 20-30% of dry matter (Yates et al., 2017). Roughly, 7.7 million tones apple pomace was generated in 2018 worldwide (FAO, 2018).

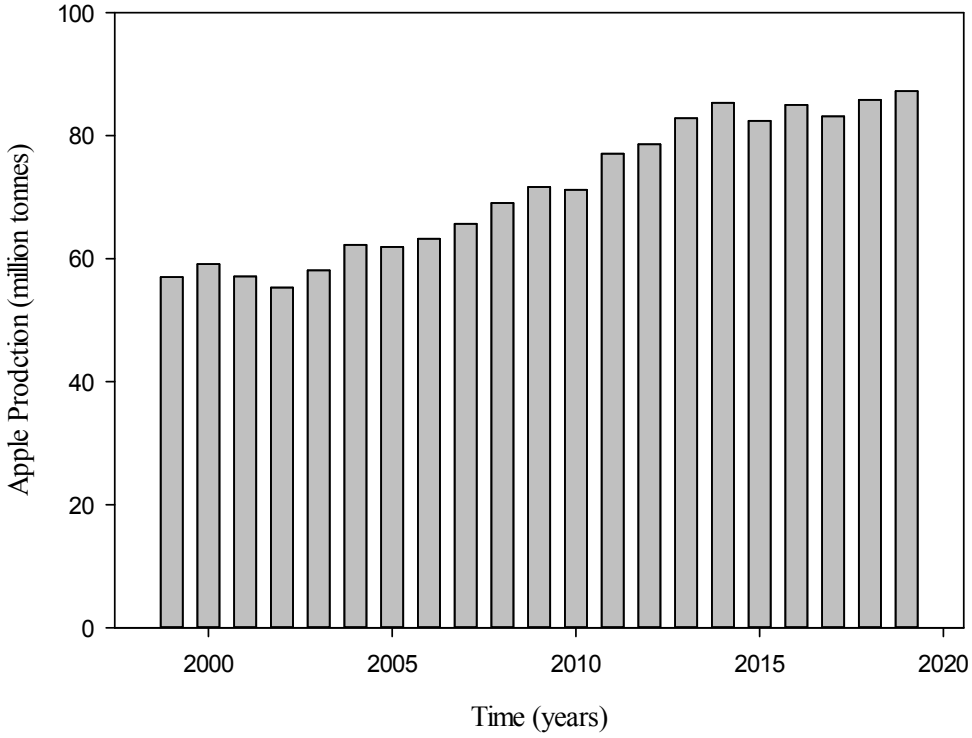


Figure 2.7. Apple production between the years 1999 – 2019 (FAO, 2019).

Macro molecules and nutrients such as protein (up to 5.8%), reducing sugars (up to 10.8%), lipids (up to 4.2%), fibers (up to 40.3%), pectin (up to 7%) and ashes (3.5%) may be present in the apple pomace in different compositions as compiled from various studies by Vendruscolo et al., (2008) (Table 2.11). Due to its high carbohydrate content (up to 22% in wet basis), apple pomace has been reported as suitable to utilize as fuel for steam generation, edible products (jams, sauce, powders etc.), source of pectin, source of dietary and soluble fibers, and carbon supply for biogas and microbial enzymes (Shalini & Gupta, 2010). However, utilization of apple pomace as a nutrient source for microbial lipid accumulation is yet to be studied.

Table 2.11. Example of wet apple pomace composition (Vendruscolo et al., 2008).

Composition (%)	Hang & Woodams, 1987
Moisture	75.6
Protein	5.1
Lipid	4.2
Fiber	4.3 – 10.5
Ash	2.8
Carbohydrates	9.5 – 22.0
Reducing sugars	5.7
Pectin	1.5 – 2.5

2.7 Aim of the Study

This study aims to become the first in the literature for HHP-assisted oil extraction from *L. starkeyi*. Further, apple pomace hydrolysate was investigated as a sole medium for *R. toruloides* to accumulate lipid. Although lignocellulosic compounds were demonstrated as potential mineral sources for lipid production by *R. toruloides,* apple pomace was studied for the first time for such a purpose in various conditioned forms for comprehensive understanding. To meet these goals, the following objectives were achieved:

- *L. starkeyi* and *R. toruloides* biomass were grown in synthetic medium.
- *L. starkeyi* was subjected to HHP for oil extraction. HHP-assisted extraction was optimized by RSM.
- Apple pomace hydrolysate was used as sole carbon source in different conditions. Also, apple pomace hydrolisate was tested in larger scales.
- Fatty acid compositions of oils were determined.

CHAPTER 3

MATERIAL AND METHODS

3.1 Materials and Chemicals

All materials used in this study were purchased from Sigma-Aldrich and Merck, Turkey, and the chemicals are of analytical grade (Appendix A).

3.2 Yeast activation and seed culture preparation

The yeasts *Lipomyces starkeyi* DSM 70295 and *Rhodosporidium toruloides* DSM4444 were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany in freeze dried form. *Lipomyces starkeyi* cells were activated in liquid medium that contains 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, and 10.0 g/L glucose, whereas *Rhodosporidium toruloides* cells were activated in liquid medium that contains 30.0 g/L malt extract and 3.0 g/L peptone ("German Collection of Microorganisms and Cell Cultures GmbH: Details"). Activated cells were grown inYPD medium (10 g/L peptone, 10 g/L yeast extract and 20 g/L glucose), and preserved under cryogenic conditions (25% glycerol solution in 1.5 mL Eppendorf tubes) at -80 ^{o}C as stock cultures.

Frozen cells were activated in an orbital shaker (INFORS AG CH-4103, Bottmingen, Switzerland) in 50 mL YPD medium (10 g/L peptone, 10 g/L yeast extract and 20 g/L glucose) in 250 mL Erlenmeyer flask at 130 rpm and 25 ^{o}C with the initial pH 5.0 (Angerbauer et al., 2008). pH was adusted with 10M H_2SO_4 and 10M NaOH (R. Wang et al., 2014). Growth media were sterilized by an autoclave (Autoclave,

MaXterile 60, Wisd laboratory Instruments) at 121 ºC for 15 minutes. Cell growth was measured by spectrophotometric methods in 600nm wavelength (OD_{600nm}) (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). Cultures were kept at 25 ºC and 130 rpm for 48 hours for *Rhodosporidium toruloides* and 72 hours for *Lipomyces starkeyi* to prepare the seed culture.

3.3 Lipid Production Media

Lipid production were evaluated for both synthetic media and apple pomace hydrolysate media. Both *L. starkeyi* and *R. toruloides* were grown in synthetic glucose media, whereas only *R. toruloides* was grown in apple pomace hydrolysate.

3.3.1 Synthetic Media

For *L. starkeyi,* 40 g/L glucose was used in the synthetic medium as a sole carbon source. The nitrogen sources were 1.9 g/L yeast extract (10% of nitrogen) and 0.5 g/L $(NH_4)_2SO_4$. Other minerals were 12.5 g/L KH_2PO_4; 1.0 g/L Na_2HPO_4; 2.5 g/L $MgSO_4 \cdot 7H_2O$ and 0.25 g/L $CaCl_2.2H_2O$ (Angerbauer et al., 2008). The initial C/N ratio was calculated as 55/1. Three percent (v/v) of inoculum was transferred from YPD medium into 100 mL lipid accumulation medium in a 500 mL Erlenmeyer flasks, which were placed in the orbital shaker at 130 rpm and 25 ºC with the initial pH of 5.0 (pH was adjusted by 10M H_2SO_4 and 10M NaOH). The yeast growth was traced daily by measuring optical density at 600nm wavelength using spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan) for 192 hours (Huang et al., 2014).

For *R. toruloides,* fermentation was carried out with various volumes of media with the same mineral composition and processing parameters as used for *L. starkeyi*.Three percent (v/v) of inoculum was transferred from YPD medium into 50 mL, 100mL, 150 mL, 250 mL media in 500 mL Erlenmeyer flasks, and 500 mL medium in 1 L

Erlenmeyer flask. Flasks were placed in the orbital shaker at 130 rpm and 25 ºCwith the initial pH of 5.0 for 192 hours (Huang et al., 2014).

3.3.2 Apple Pomace Hydrolysate

Experiments using conditioned apple pomace hydrolysate were performed with 100 mL fermentation volume in 500 ml Erlenmeyer flask. The medium that gave the best result in terms of lipid yield was used for scale up trials in 1 L and 10 L fermentors.

3.3.2.1 Preparation of Apple Pomace

Apple pomace was kindly provided by Elite Naturel Organik Gıda San. ve Tic. A.Ş. Ankara, Turkey. Apple pomace was dried in tray dryer (EKSİS Industrial Dryer Systems, Isparta, Turkey) at 80 ºC, 5% RH, with the air velocity of 1 m/s, for 5 hours. Dried apple pomace was ground (FRITSCH Industries. 8 55743 Idar-Oberstein, Germany) into 1 mm particle size. Ground apple pomace was treated with H_2SO_4 in several concentrations (from 0.5 to 5% w/v). The acidic suspension was autoclaved at 121 ºC for 30 minutes to perform acidic hydrolysis. The hydrolysate was vacuum filtered to separate solid particles. In order to meet the same reducing sugar content of the synthetic media, several solid/liquid ratios (from 5 to 10% w/v) for different acid concentrations were tested.

3.3.2.2 Content of Apple Pomace

Elemental analysis was performed for dried apple pomace powders. Potassium (K), Sodium (Na), Phosphate (P), Magnesium (Mg), Calcium (Ca), and Sulphur (S) contents were analyzed with inductively coupled plasma optical emission spectral analysis, whereas Carbon (C) and Nitrogen (N) contents were determined with elemental analysis device by METU Central Laboratory

3.3.2.3 Detoxification

Apple pomace hydrolysates were detoxified by activated charcoal method according to study of Guo et al., (2013). Two grams of activated charcoal was added into 100 mL hydrolysate, and the mixture was shaken vigorously for 5 minutes. The suspension was centrifuged (NUVE, NF 1200R, Bench-Top Cooled Centrifuge) at 8000*g*, 4 ºC for 10 minutes. The supernatant was separated from precipitate by vacuum filter, and centrifuged at the same conditions for further clarification. Finally, the transparent medium was adjusted to pH 5.0 using 10M NaOH.

3.3.2.4 Conditioning and Culturing of Apple Pomace Hydrolysate

Apple pomace hydrolysate was used as a sole raw material in 6 different conditioning methods as shown in Table 3.1. All media were adjusted to pH 5.0 using 10M NaOH. Three percent (v/v) of inoculum was transferred from YPD medium into 100 mL fermentation medium in 500 mL Erlenmeyer flasks. Flasks were placed in the orbital shaker at a rotary rate of 130 rpm and 25 ºC with the initial pH of 5.0. The processes were terminated after 192 hours.

Table 3.1. Conditioning of apple pomace based media.

Name of Medium	Detoxification	Minerals Added
Standard Apple Pomace Hydrolysate (S medium)	-	-
Apple Pomace Hydrolysate with Mineral Additionwithout Nitrogen (SM medium)	-	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H2O$
Apple Pomace Hydrolysate with Mineral Addition Including Nitrogen (SMN medium)	-	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$, $(NH)_2SO_4$, yeast extract
Detoxed Apple PomaceHydrolysate (D medium)	+	-
Detoxed Apple PomaceHydrolysate with Mineral Addition without Nitrogen (DM medium)	+	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$
Detoxed Apple PomaceHydrolysate with Mineral Addition including Nitrogen (DMN medium)	+	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$, $(NH_4)_2SO_4$, yeast extract

3.3.2.5 Fermentor Experiments

One liter of detoxed apple pomace hydrolysate (D medium) was put into 2L Sartorius A Plus fermentor (Sartorius Stedim Systems GmbH, Schwarzenberger, Weg 73-79, DE-34212 Melsungen, Germany) and autoclaved at 121 ^{o}C for 30 minutes. Three percent (v/v) of inoculum was injected from YPD medium into fermentor to start the fermentation process. Fermentation was performed at 25 ^{o}C, 130 rpm agitation and 0.5 vvm aeration for 192 hours. Also, the pH of the media kept constant at 5.0 by 2M H_2SO_4 and 2M NaOH. Diluted antifoam solution (3:11 v/v) was used to avoid overfoaming and prevent medium loss.

3.4 Yield Assessments

3.4.1 Biomass Harvesting

After 8 days of biomass generation, cells were harvested by centrifugation at 8000*g* for 10 minutes at 4 ^{o}C. The supernatant was stored for the residual sugar determination and precipitated biomass was washed with distilled water and centrifuged again under the same conditions. Collected biomass was placed in glass petri dishes and dried in an oven (Oven ST-120, Şimşek Laborteknik Ltd. Şti) at 70 ^{o}C for 24 hours to determine dry biomass content.

3.4.2 Lipid Extraction

Lipid extraction was performed by two methods. High hydrostatic pressurewas used for cell disruption to improve lipid recovery from *L. starkeyi*, whilst high speed homogenizer (HSH) assisted Bligh & Dyer method was applied to *R. toruloides* (Singh et al., 2018).

3.4.2.1 High Hydrostatic Pressure-Assisted Extraction

In this study, HHP was utilized as a cell disruption method that aimed to unfold the rigid cell wall of *L. starkeyi* and to cause cytoplasmic leakage that mostly consists of intracellular oil. The high hydrostatic pressure (HHP) equipment utilized in this study was a 760.0118 type supplied by SITEC-Sieber Engineering, Zurich, Switzerland. The 100 mL pressure vessel had 24 mm of internal diameter and 153 mm of length. The vessel was also connected to a built-in heating cooling system (Huber Circulation Thermostat, Offenburg, Germany) with a thermocouple type K, which was used to measure and control the initial and processing temperatures. As the pressure-transmitting medium, distilled water was filled into the pressure vessel. Dried *L. starkeyi* cells were suspended in the organic solvent mixture, which consisted of chloroform-methanol (2:1 v/v) (Singh et al., 2018). The suspensions were placed in 25 mL polyethylene cryotubes (LP Italiana SPA) and the tubes were heated in the water bath (Şimşek Laborteknik Ltd. Şti. 5BD-313) until the required temperatures were achieved uniformly. Finally, the tubes were fit into the pressure vessel and the processes were performed adiabatically.

HHP is a process that depends on three main process variables, namely pressure, time and temperature. The experimental design was created by response surface methodology using Minitab 13 software (Minitab Inc., State Collage, PA, USA). Detailed RSM design is given in the Experimental Design section.

3.4.2.2 High Speed Homogenizer-Assisted Extraction

Collected biomass was placed into falcon tubes and mixed with chloroform-methanol (2:1 v/v) mixture and 2.5 ml of 1M NaCl solution as suggested by Bligh & Dyer (1959). Sample to solvent ratio was set to 1:20 (w/v). The mixture was treated with high speed homogenizer (IKA T18 digital ULTRA TURRAX) at 12000 rpm for 6 minutes to damage cell wall and facilitate the lipid recovery. In order to avoid

overheating, homogenization was performed intermittently (25 seconds on, 5 seconds off). After homogenization, the tubes were centrifuged at 8000*g* for 10 minutes at 4 ºC to observe the phase separation. The bottom phase (pink-red colored for *R. toruloides*, transparent for *L. starkeyi* as shown in Appendix H) was collected and poured into glass petri dishes and further purification was achieved by a vacuum oven (UNITERM UVT-20) at 40 ºC, 160 mmHg for 2 hours.

3.4.3 Lipid Yield

Lipid yield ($Y_{L/B}$) is expressed as ratio of gram lipid to gram dry biomass (Eqn. 3.1). Lipid efficiency constant ($Y_{L/S}$) was calculated as gram of lipid per gram of reducing sugar consumed (Eqn. 3.2). Lipid efficiency constant based on apple pomace ($Y_{L/A}$) was estimated as gram lipid per gram of dry apple pomace (Eqn. 3.3).

$$Y_{L/B} = \frac{\text{g lipid}}{\text{g biomass}} * 100 \qquad \text{(Eqn. 3.1)}$$

$$Y_{L/S} = \frac{\text{g lipid}}{\text{g reducing sugar consumed}} \qquad \text{(Eqn. 3.2)}$$

$$Y_{L/A} = \frac{\text{g lipid}}{\text{g dry apple pomace}} \qquad \text{(Eqn. 3.3)}$$

Specific growth rate (μ) and sugar consumption rate (k) were calculated according to first order kinetics (Eqn. 3.4, 3.5). Slope of the natural logarithm of optical density vs time curve was taken as specific growth rate.

$$\ln\left(\frac{N_t}{N_0}\right) = \mu * t \qquad \text{(Eqn. 3.4)}$$

where μ is specific growth rate, t is time, N_t is optical density at t, and N_o is initial optical density

and,

$$\ln\left(\frac{S_t}{S_0}\right) = -k * t \qquad \text{(Eqn. 3.5)}$$

where k is sugar consumption rate, t is time, S_t is sugar amount at t, and S_o is initial sugar amount (g/L).

3.4.4 Total Reducing Sugar

In order to calculate the lipid efficiency ($Y_{L/S}$), residual sugar analysis was conducted after each fermentation period. Total reducing sugar was determined colorimetrically by using the dinitrosalicylic acid (DNS) residual sugar assay (Miller, 1959). It is a traditional method that depends on the existence of free carbonyl group as the reducing sugar indicator.

DNS solution consisted of 10 g/L 3-5 dinitrosalicylic, 10 g/L NaOH, 2 g/L phenol, and 0.5 g/L Na_2SO_3,. and 256 g/L Rochelle salt ($KNaC_4H_4O_6 \cdot 4H_2O$). Three milliliter of DNS solution and 3 mL 1:100 (v/v) diluted fermentation mediumwere mixed in glass tubes. The tubes were placed into 95 ^oC water bath (Şimşek Laborteknik Ltd. Şti. 5BD-313) for 15 minutes. Then, the tubes were removed fromthe water bath and cooled down to room temperature. Optical densities were measured at 575nm. The absorbance data were then compared to the standard curve that was created previously by the known concentrations of glucose stock solution. Standard curve is given in Appendix B.

3.4.5 Fatty Acid Methyl Ester Analysis

The FAME analysis were performed by METU Food Analysis Laboratory at Food Engineering Department.

3.4.6 Visual Analysis

The *L. starkeyi* cells that were disrupted by HHP were observed under light microscope and scanning electron microscope in different magnification rates.

3.4.6.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy analysis was performed by METU Central Laboratory. Based on the experimental design, the cells that were collected from the experiments, which gave the maximum and minimum yields were investigated under SEM. In addition, intact cells were also investigated under SEM to observe the HHP effects on the cells.

As soon as HHP was done, the cells were collected by centrifuging at 3000*g*, and 4 ^{o}C for 10 minutes. The cells were then spread onto glass petri dishes and freeze dried (CHRIST, ALPHA 2-4 LD plus) at -85 ^{o}C, under 0.15 mbar vacuum for 24 hours. After freeze drying, the cells were sent to METU Central Laboratory, where the SEM images were captured.

3.5 Experimental Design

HHP experiments were designed by using software MINITAB 13. Response surface methodology offers a set of experiments to build a model for a process without requiring to test all the interactions between the parameters one by one. In order to create a model for HHP on biolipid extraction, Box-Behnken response surface methodology was used with three continuous factors as pressure, temperature and time (Table 3.2). The program created 13 different pressure-time-temperature combinations with 3 central point runs in duplicates which made 30 runs in total (Table 3.3). The response was lipid yield on a dry biomass. In addition,ANOVA was employed to evaluate the significance of the variables.

Table 3.2. Variables used in Box-Behnken design for HHP assisted extraction.

Factor	Low (-1)	High (+1)
Pressure	200	400
Temperature	30	50
Time	5	15

Table 3.3. The Box-Behnken response design for HHP treatment.

Run Order	Pressure (MPa)	Temperature (^{o}C)	Time (min)
1	300	30	15
2	300	40	10
3	200	40	15
4	300	50	5
5	400	50	10
6	200	40	5
7	200	30	10
8	300	50	15
9	400	30	10
10	400	40	15
11	200	50	10
12	300	40	10
13	400	40	5
14	300	40	10
15	300	30	5

To develop a predictive model for effect of HHP on oil yield, the experimented data was fitted to a quadratic polynomial equation expressed by:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \text{(Eqn. 3.6)}$$

where Y stands for the response (oil yield), β_0 is constant , β_i is the linear coefficient, β_{ij} is the cross-product coefficient, β_{ii} is the quadratic coefficient, $_i$ and X_j are the independent variables (pressure and temperature).

To analyze error between experimental and predicted data, root mean square error (RMSE) (Eqn. 3.7) and mean absolute error (MAE) (Eqn. 3.8) were calculated.

$$RMSE = [\sum_{i=1}^{N} (P_i - O_i)^2 / N]^{\frac{1}{2}} \quad \text{(Eqn. 3.7)}$$

$$MAE = \left(\frac{1}{N}\right) \sum_{i=1}^{N} |P_i - O_i| \quad \text{(Eqn. 3.8)}$$

where N is number of data, P_i and O_i are predicted and observed values,respectively.

3.6 Determination of Peroxide Value

Peroxide value was calculated according to Ugur et al., (2020) with some modifications. Half gram of microbial oil was dissolved in 25 mL of chloroform-acetic acid mixture (2:3 v/v). Ten grams of potassium iodide crystals were dissolved in 100 mL distilled water and 5 grams of iodine then added to the solution, and stirred vigorously. One drop of iodine solution was added to the solvent-oil solution with the help of a Pasteur pipette, and kept in a dark place for 10 min as closed. Then, 1 mL of starch solution and 75 mL of distilled water added to the solution. The solution then titrated with 0.01 M of sodium thiosulfate. Peroxide value (PV) was calculated according to following equation:

$$PV = \frac{(V_2 - V_1) * N * 1000}{m} \text{ mEq } O_2 \text{/kg oil} \quad \text{(Eqn. 3.9)}$$

where V2 is consumption of sodium thiosulfate for sample (mL), V1 is consumption of sodium thiosulfate for blank (mL), N is the normality of sodium thiosulfate, m is gram of oil taken for the test.

3.7 Color Analysis

Color analysis was performed photometricaly. To begin the analysis, 0.05 gram of oil was dissolved in 1 mL hexan, and centrifuged in Eppendorf tubes to clarify the solution. Photometric analysis was carried out in 1 mL plastic cuvettes. In order to obtain maximum absorption value, samples were scanned by spectrophotometer in different wavelengths, from 200 to 700 nm with the increments of 20 nm.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Growth of *Lipomyces starkeyi* and *Rhodosporidium toruloides* in YPD Medium

4.1.1 *Lipomyces starkeyi*

Growth of *Lipomyces starkeyi* in YPD medium was traced daily by measuring optical density. Figure 4.1 shows that the yeast was in the lag phase for 3 days. It is worthy of notice that OD_{600} value rose sharply after day 3, which indicated that log phase started between day 3 and 4. Therefore, seed culture was used shortly after 72th hour for inoculating the fermentation medium (Dias et al., 2016).

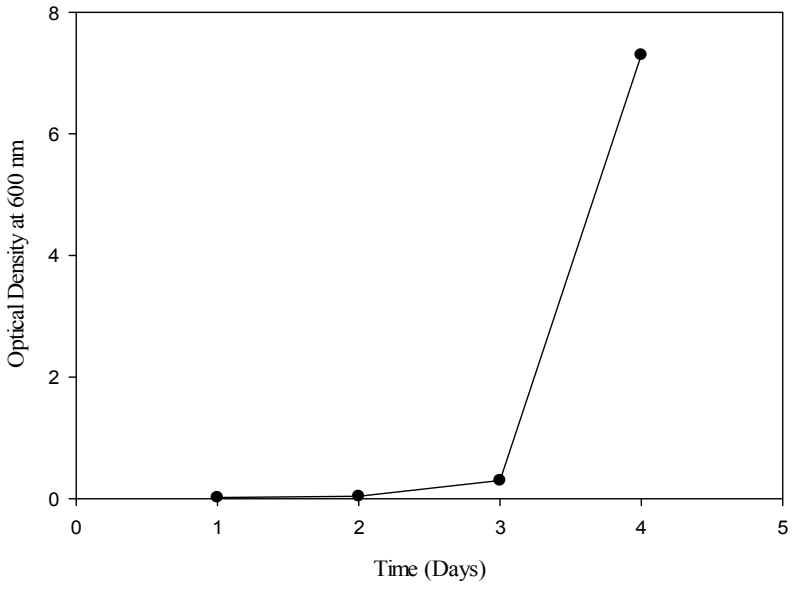


Figure 4.1. Growth of *Lipomyces starkeyi* in YPD medium.

4.1.2 *Rhodosporidium toruloides*

Figure 4.2 demonstrates the growth of *R. toruloides* in YPD medium based on optical density at 600 nm of wavelength. The figure shows that between day 1 and 3, the optical density increased sharply, almost tripled the initial value, indicating that the microorganism was going through a log phase, which is the ideal time to use for inoculation (Dias et al., 2016). After 3 days, the optical density increased slightly (stationary phase), whereas after the 6th day, the decline in optical density started. In order to reach the optimal fermentation activity, the inoculation from YDP to fermentation media was performed at 48th hour of the growth as suggested by Angerbauer et al., (2008).

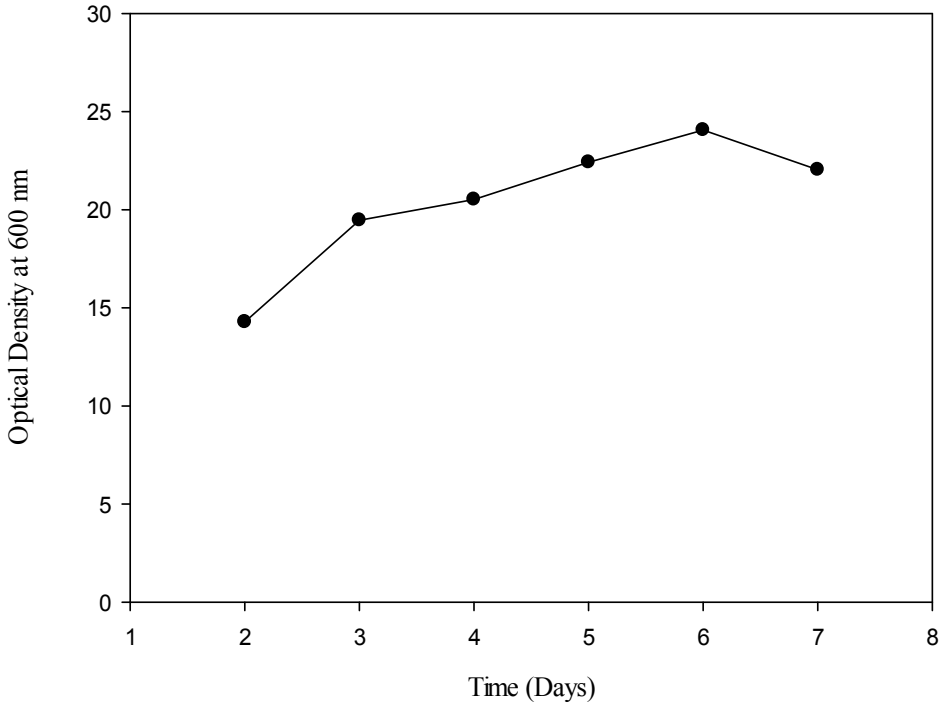


Figure 4.2. Growth of *Rhodosporidium toruloides* in YPD medium.

4.2 Production of Microbial Oil by *Lipomyces starkeyi* and *Rhodosporidium toruloides* in Synthetic Sugar Media

Both *L. starkeyi* and *R. toruloides* were grown in synthetic sugar media containing 40g/L glucose and minerals (C/N = 55). Furthermore, different fermentation volumes were tested for *R. toruloides* in order to determine the optimum volume for lipid accumulation. Thus, the results that were collected from synthetic sugar media served as guidance for the experiments of apple pomace as a sole raw material for lipid production by *R. toruloides*.

4.2.1 *Lipomyces starkeyi*

4.2.1.1 Growth in Fermentation Media

The growth of *L. starkeyi* was measured at OD_{600nm}, which is given in Figure 4.3. From the 1st to 6th day, roughly 45 fold of increase in OD_{600nm} value was observed (from 0.4 to 18.6). Neglecting the slight increase, after 6th day, OD_{600nm} value did not change significantly and remained at 19.8 until the end of fermentation. Also, specific growth rate (μ_L) was estimated as 0.44 day^{-1} (Appendix J).

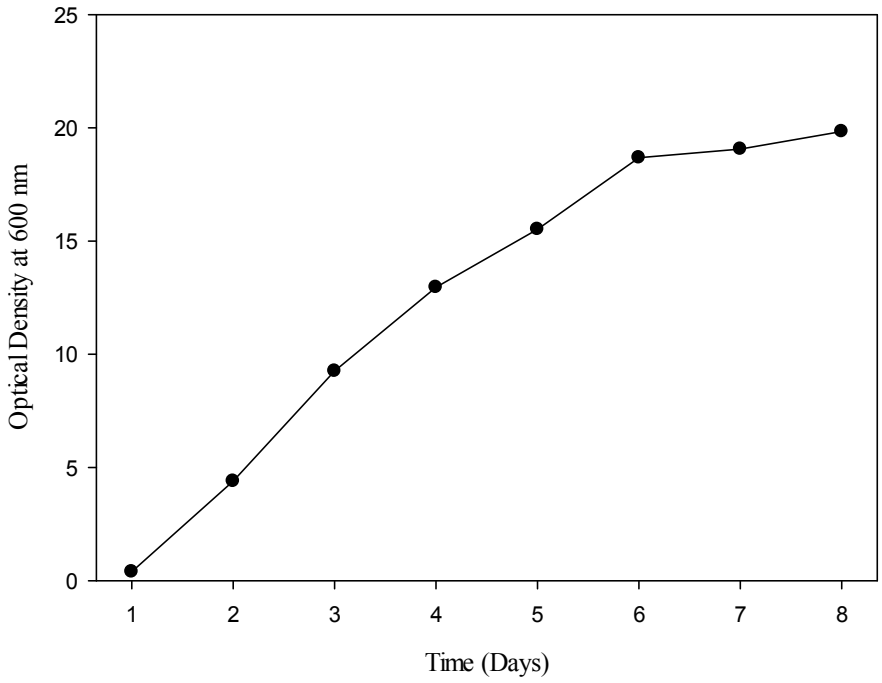


Figure 4.3. Growth of *Lipomyces starkeyi* in fermentation media that contain synthetic sugar as a sole carbon source (C/N = 55).

4.2.1.2 Sugar Consumption

Glucose consumption of *L. starkeyi* was traced on a daily basis (Figure 4.4). After 24 hours, sugar concentration of the medium decreased from 40 g/L to 35.1±0.43 g/L. The coherence between optical density and reducing sugar consumption trends in the fermentation medium draws attention, such that as the optical density increases between 1st to 6th day, sugar concentration of the media reduced remarkably. Glucose concentration dropped to 0.23±0.001 g/L at the end of day 6. Finally, it was day 7 when glucose in the medium almost exhausted by the organism (0.04 g/L). Rate of sugar consumption (k_L) was calculated as 5.29 day^{-1} according to first order kinetics.

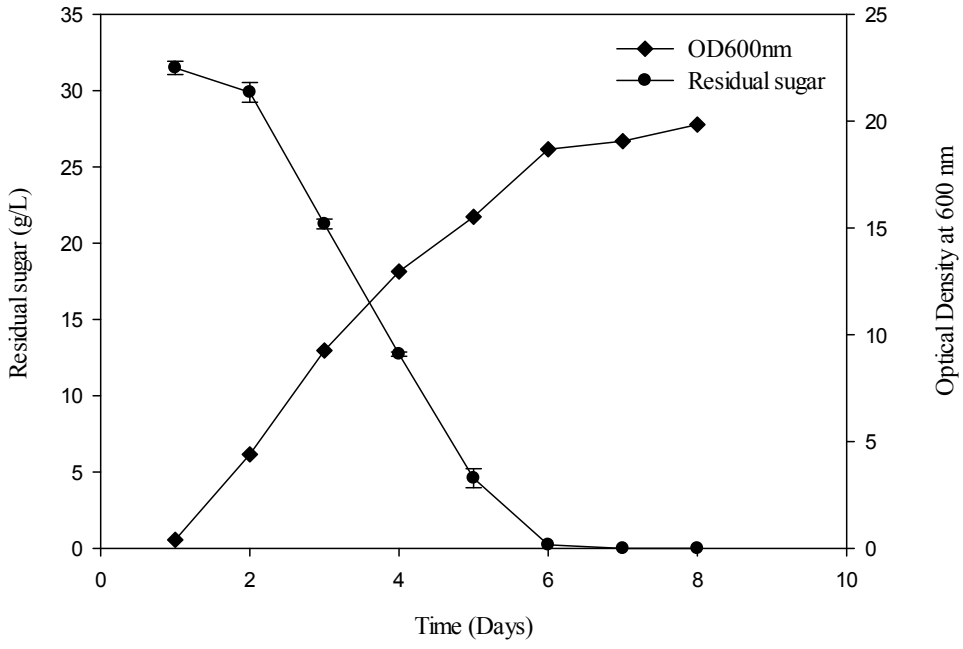


Figure 4.4. Dual graph of sugar consumption and growth of *Lipomyces starkeyi* in fermentation media.

4.2.1.3 HHP-Assisted Extraction and Lipid Yields

Results of the experiments carried out according to RSM are illustrated in Table 4.1. The high speed homogenizer (HSH) assisted extraction was used as a control and it yielded 40.8 ± 1.9% on a dry cell basis. Among all the 13 different pressure, time, temperature combinations, only 3 of them exceeded the control experiment. The best results were observed at 200 MPa, 40 ºC, 15 min (run 3) and 200 MPa, 40 ºC, 5 min (run 6) with oil yield values of 45.8 ± 2.1% and 42.9 ± 1.6%, respectively. Thus, as 200 MPa seems the optimal pressure, yield was higher at 15 than 5 min at constant temperature (40 ºC). In addition, when the fixed variable was time (at 10 min), yield increased from 26.7 ± 2.2% (run 7) to 37.7 ± 0.8% (run 11) with increasing

temperature at 200 MPa. It was also achieved 41.4 ± 1.0% of yield at 300 MPa, 50 °C, and 15 min (run 8), which is the closest value to the maximum yield (run 3). However,when the time was dropped to 5 min at 300 MPa, and 50 °C, lipid yield decreased to 31.9± 0.7% (run 4).

Furthermore, the yield was decreased further to 27.5 ± 0.8% as the temperature dropped from 50 to 30 °C at 300 MPa, and 15 min (run 1). At 400 MPa, and 40 °C, decreasing the time from 15 to 5 min affected the yield positively, by increasingyield from 18.8 ± 1.5% (run 10) to 20.8 ± 1.8% (run 13). Nonetheless, the increase inyield was more outstanding when temperature rose from 30 to 50 °C at 400 MPa, 10min. For instance, at 400 MPa and 10 min, 30 °C, the yield was 16.9 ± 0.2% (run 9), whereas at 50 °C, the yield was 37.9 ± 1.6% (run 5), which is more than double the value of run 9. In fact, it was outstanding that yield dropped considerably with the increasing pressure. The yield difference between run 3 and run 10 demonstrates the effect of pressure clearly. At 40 °C, 15 min, lipid yield was 45.8 ± 2.1% at 200 MPa, which is the maximum value, and 18.8 ± 1.5% at 400 MPa, which was almost the minimum value. The lowest yields were 15.2 ± 0.9% for 300 MPa, 40 °C, and 10 min (run 12) and 16.9 ± 0.2% for 400 MPa, 30 °C, and 10 min (run 9).

Since this is the first study investigating HHP assisted extraction of microbial oil from oleaginous yeasts, it is not possible to make direct comparison with literature. However, HHP was investigated as a lipid extraction method from insects. Ugur et al., (2020) investigated HHP-assisted lipid extraction from crickets and mealworms. Coherent with the current study, it was observed that oil yield was lower at higher pressure. It was stated that lipid yield was 24.2% at 0.1 MPa, and 22.7% at 500 MPa for mealworms. Thus, pressure affected lipid yield negatively at 30 °C, and 15min. Also for crickets, the yield dropped from 18.0 (at 0.1 MPa) to 16.1 (at 500 MPa) at the same conditions. It is noteworthy that yields were higher at 40 °C than 30 °C at 500 MPa, which illustrates another similarity with our study. The decrease in yield was attributed to the destructive effect of HHP on triglycerides and insufficiency of

extraction time (Ugur et al., 2020). Triglyceride determination was not performed in this study; however, extraction time was significant ($p<0.05$). Lipid yield increased significantly from 31.9 ± 0.7% (run 4) to 41.4 ± 1.0% when the time increased from 5 to 15 min at 300 MPa, 50 ^{o}C (run 8).

Table 4.1. Lipid yields (% w/w) of the HHP experiments.

Run Order	Independent variables			Yield (% w/w)
	Pressure (MPa)	Time (min)	Temperature (^{o}C)	
1	300	15	30	27.5 ± 0.8
2	300	10	40	15.2 ± 0.9
3	200	15	40	45.8 ± 2.1
4	300	5	50	31.9 ± 0.7
5	400	10	50	37.9 ± 1.6
6	200	5	40	42.9 ± 1.6
7	200	10	30	26.7 ± 2.2
8	300	15	50	41.4 ± 1.0
9	400	10	30	16.9 ± 0.2
10	400	15	40	18.8 ± 1.5
11	200	10	50	37.7 ± 0.8
13	400	5	40	20.8 ± 1.8
15	300	5	30	25.4 ± 2.0
HSH (Control)	-	-	-	40.8 ± 1.9

HSH: High speed homogenizer extraction yield was taken as the control.

4.2.1.4 RSM Optimization and Statistical Analysis of HHP Extraction

HHP-aided lipid extraction from *L. starkeyi* biomass was optimized by RSM based on the results in Table 4.1. Box-Behnken design with 3 continuous factors was employed. In order to obtain a better fit, natural logarithms of response values (lipid yields) were taken. It was shown by the statistical analysis that coefficient of determination value (R^2) was 0.932 and adjusted value (adj-R^2) was 0.903, and lack-of-fit was 0.08. High R^2 value, close values of R^2 and adj- R^2, and insignificant lack-of-fit showed that the predicted model was adequate ($p<0.05$). The estimated regression coefficients and P values are shown in Table 4.2.

Table 4.2. ANOVA results and estimated regression coefficients for lipid yield.

Term	Coded coefficient	P value
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.003
Lack-of-fit		0.080
Constant	2.7196	0.000
Pressure	-0.2615	0.000**
Temperature	0.1973	0.000**
Time	0.0620	0.078**
Pressure*Pressure	0.3385	0.000**
Temperature*Temperature	0.3383	0.000**
Time*Time	0.3752	0.000**
Pressure*Temperature	0.1666	0.003**

** *P<0.05.*

Table 4.2 shows that linear, square and interactions effects are significant. However, interactions of time with pressure and temperature were insignificant, therefore they

were omitted from the quadratic equation. Thus, the model equation was proposed as:

$$\ln(Y) = 14.5507 - 0.0295877*X_1 - 0.300908*X_2 - 0.287792*X_3 + 0.0000338495*X_1^2 + 0.00338331*X_2^2 + 0.0150100*X_3^2 + 0.000166564*X_1*X_2$$

In the equation, Y represents the response (lipid yield), X_1, X_2, and X_3 represent pressure (MPa), temperature (^oC) and time (min), respectively.

To explore the effect of HHP variables in pairs, surface plots of lipid yields vs all factors are shown in Figure 4.5.

The error analysis were also conducted. Root mean square error (RMSE) was calculated as 0.1, and mean absolute error (MAE) was calculated as 0.08. Therefore, the small value of errors supported that the model is convenient (Uzuner & Cekmecelioglu, 2014).

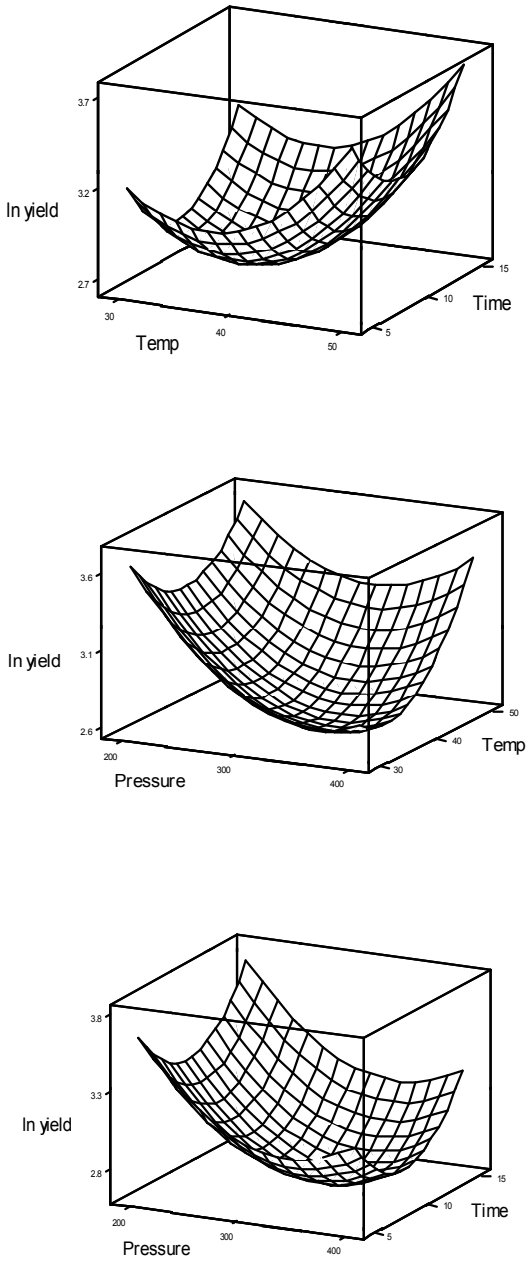


Figure 4.5. Response surface plots showing the effects of pressure, temperature and time. A: Temperature-Time graph at 300 MPa. B: Pressure-Temperature graph at 10 min. C: Pressure-Time graph at 40 ^{o}C.

Response surface plots showing the effects of the HHP process variables are shown in Figure 4.5. According to Figure 4.5 (A) and (B), the optimum pressure for extraction seems to be 200 MPa. Although the yield sharply decreased until 300 MPa, a slight increase was observed from 300 to 400 MPa at 40 ^{o}C. It is noteworthy that maximum yields were obtained in either 5 or 15 minutes, while 10 minutes of treatments minimized the lipid yields (A). When the time was fixed at 10 minutes (B), high yields were achieved at low pressure (200 MPa) - low temperature (30 ^{o}C) and high pressure (400 MPa) - high temperature (50 ^{o}C). Intermediate values of the variables lead to lower lipid yields among all the trials (C) (i.e. 300 MPa, 40 ^{o}C, 10 min).

4.2.1.5 Verification of Optimum HHP Conditions

Figure 4.6 illustrates a linear relation between experimental and predicted values with determination coefficient of 0.932 (R^2). The Box-Behnken model showed 200 MPa, 50 ^{o}C, and 5 min as the optimum conditions, by which lipid yield of 54.6% is predicted with desirability 1.0. To verify the model, lipid extraction was performed at 200 MPa, 50 ^{o}C, and 5 min, which resulted in 43.4±2.7 % lipid yield. Angerbauer et al., (2008) reported that with C/N ratio of 60, maximum lipid yield achieved by *L. starkeyi* was 45%. Given the fact that the C/N ratio in this study was 55, maximum lipid yield was achieved under the suggested conditions by the model.In addition, root mean square error (RMSA) and mean absolute error (MAE) were calculated as 0.1 and 0.08, respectively. Therefore, the model was verified.

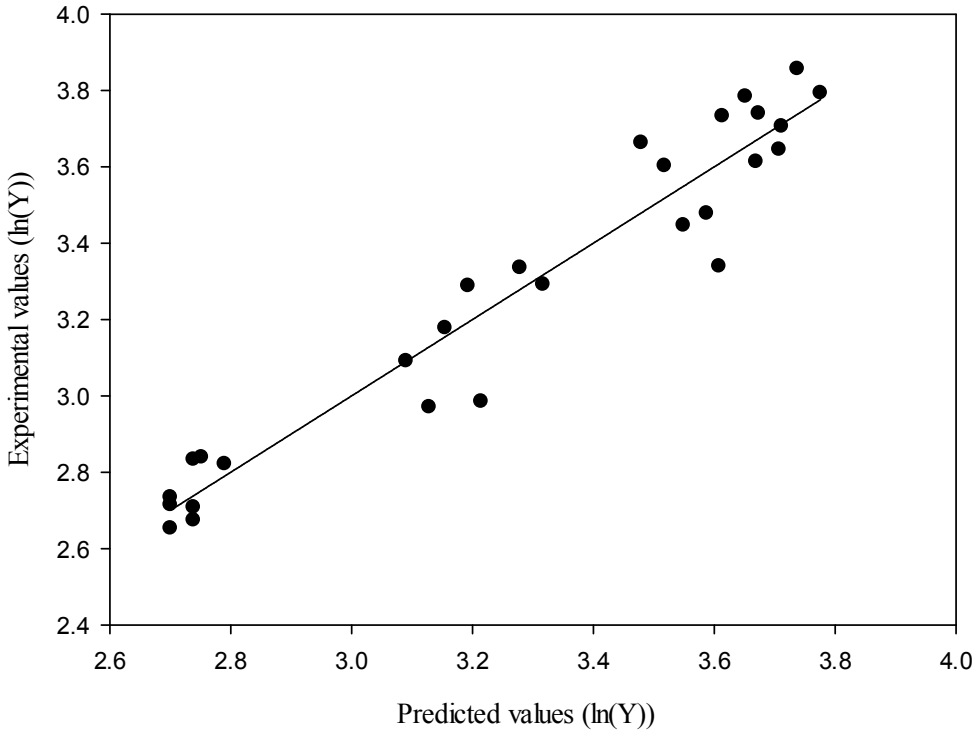


Figure 4.6. Comparison of experimental and predicted values for natural logarithms of lipid yields.

4.2.1.6 Morphological Effects of HHP on *L. starkeyi*

Images that were captured under light microscope at 100x magnification are shown in Figures 4.7 - 4.10. First of all, the cells from growth medium (YPD) were investigated as positive control (Figure 4.7). As shown in the Figure 4.8, cells started to accumulate lipid within 3 days of fermentation. Although lipid accumulation started in the first 3 days as mentioned by Boulton & Ratledge (1983),it looks like cell sizes did not change significantly, though the scale is not certain. Finally, at the end of day 8, cells were significantly bigger and fatty (Figure 4.9).

HHP treated cells at 200 MPa, 50 °C, and 5 min seem distorted and emptied (Figure 4.10(D)). On the other hand, HHP treated cells at 400 MPa, 40 °C, and 15 min were damagedor crushed while oil containing cells were still abundant in the medium (Figure 4.10(E)).

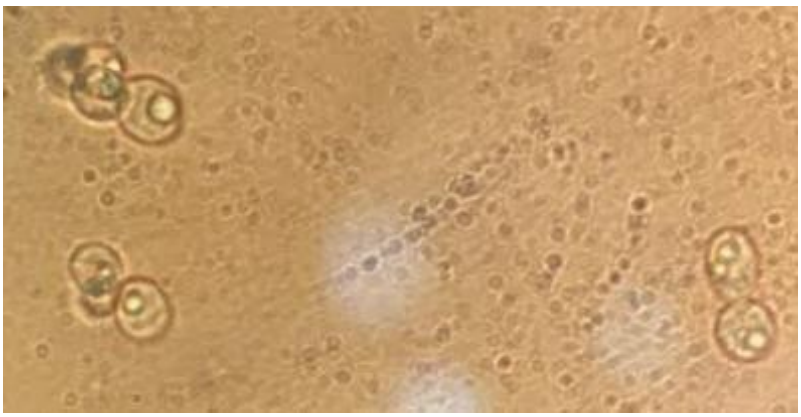


Figure 4.7. Light microscope images of young *L. starkeyi* cells from YDP medium (100x magnification).

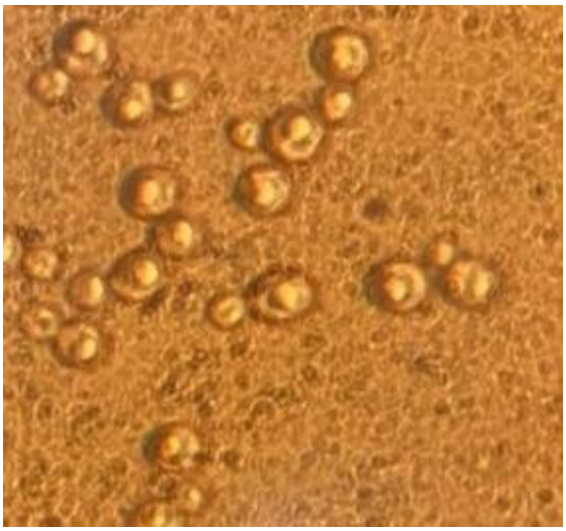


Figure 4.8. Light microscope images of *L. starkeyi* from fermentation medium. The samples were taken in the 3rd day of fermentation (100x magnification).

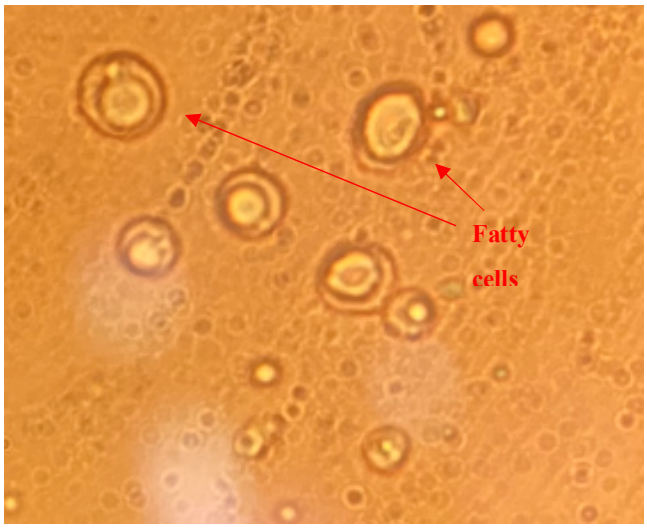


Figure 4.9. Light microscope images of *L. starkeyi* from fermentation medium. Thesamples were taken in the 8th day of fermentation (100x magnification).

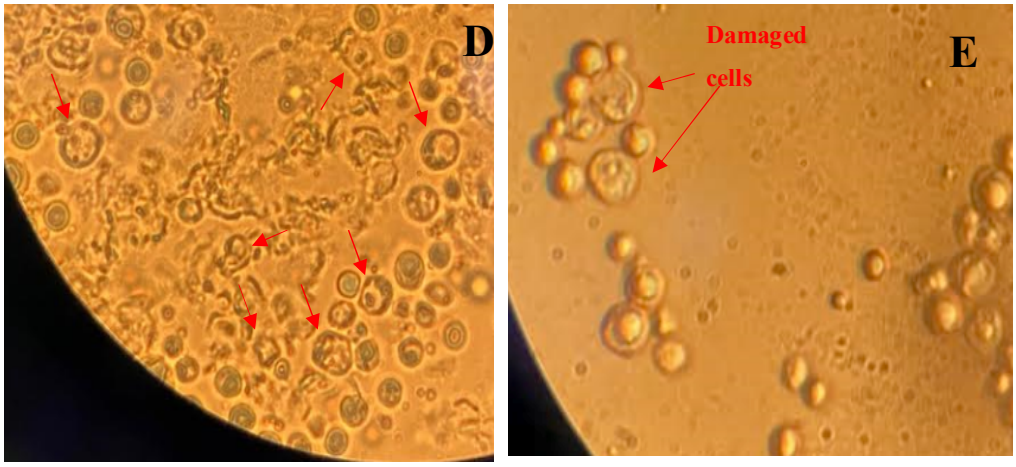


Figure 4.10. Light microscope images of *L. starkeyi* after HHP treatments. D: 200 MPa, 40 ^{o}C, 15 min, E: 400 MPa, 40 ^{o}C, 15 min (100x magnification).

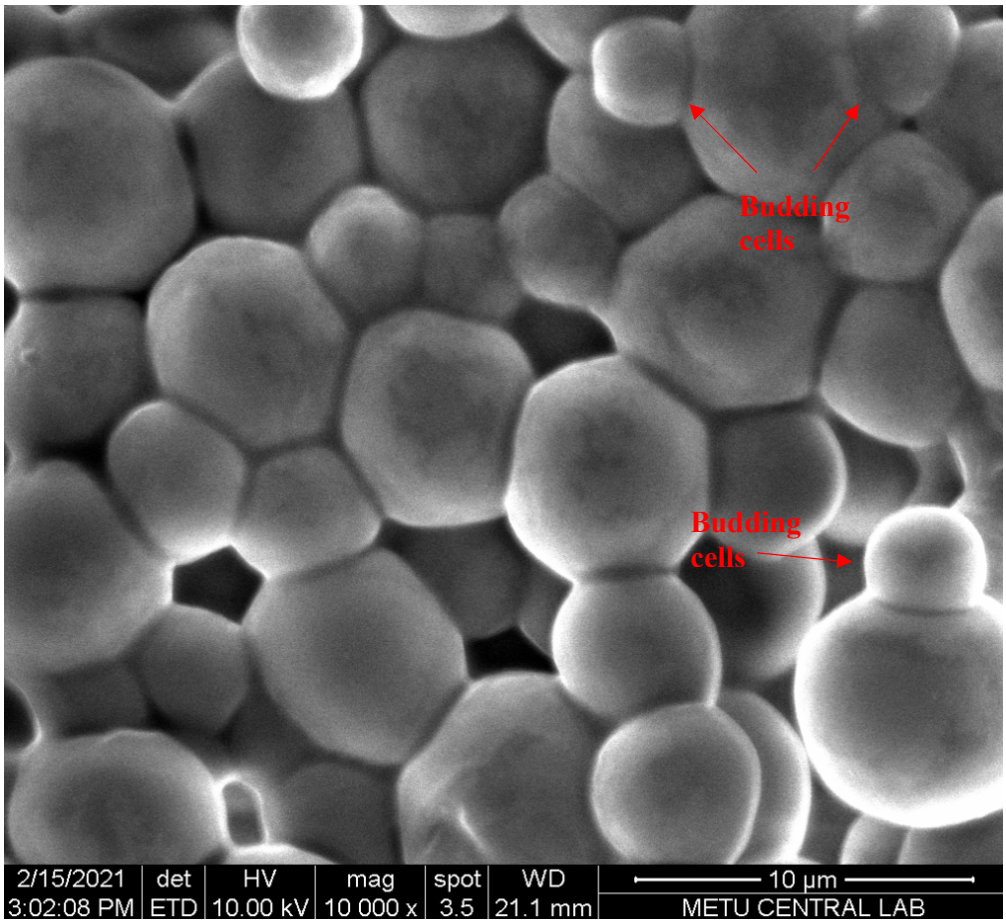


Figure 4.11. Scanning electron microscope (SEM) image of intact *L. starkeyi* cells after fermentation (10000x magnification).

Figure 4.11 demonstrates the SEM images of *L. starkeyi* at the end of fermentation. The size of cells in the figure changed between 4.0 – 6.8 µm, which is consistent with the literature (Smith & Batenburg-Van der Vegte, 1984). The cells were attached to each other because of the dense sampling, and the samples were not fixed by aldehydes prior to SEM analysis (Karimy et al., 2020).

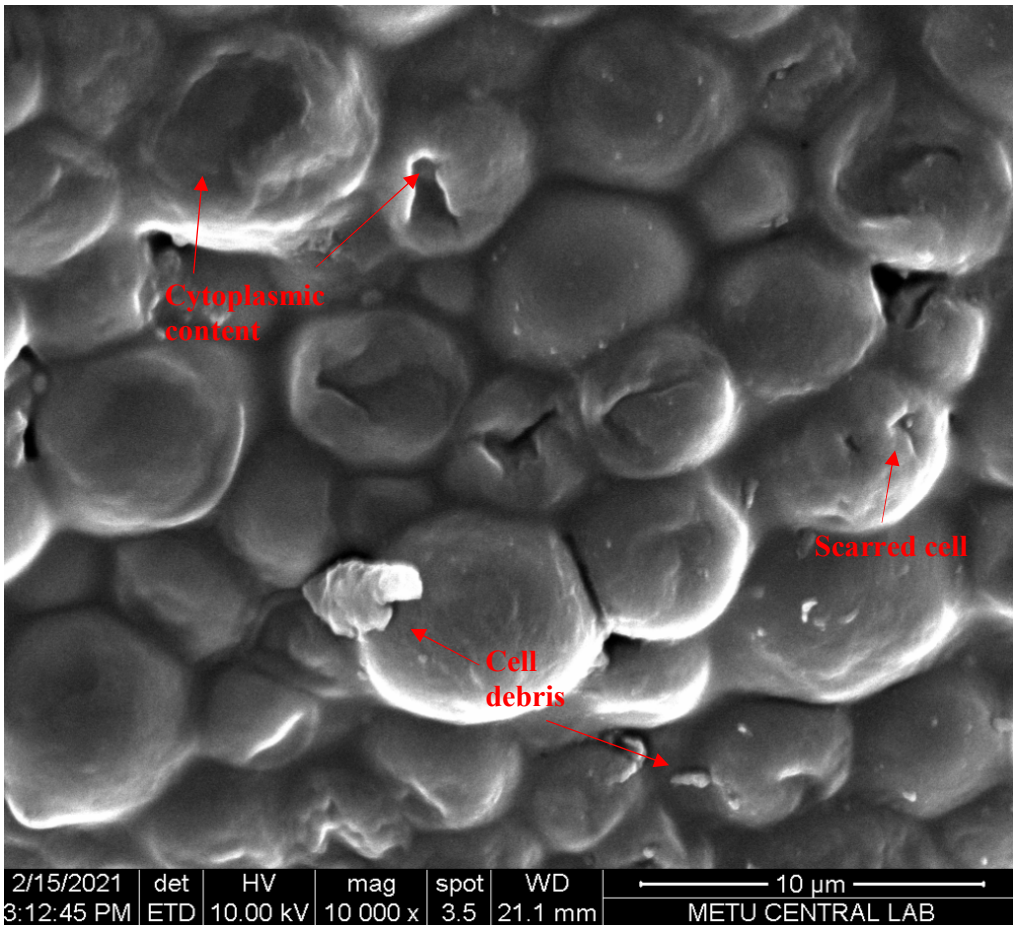


Figure 4.12. Scanning electron microscope (SEM) image of *L. starkeyi* after HHP at400 MPa, 40 ºC, 15 min (10000x magnification).

It was expected to observe high pressure leading to higher mass transfer of the solvent through cell borders and therefore higher lipid extraction yield (Ugur et al., 2020). Nonetheless, the results showed that lipid yield decreased with increasing pressure (Table 4.1). It is evident from SEM analysis that 200 MPa affected the cell morphology more severely than 400 MPa. Marx et al. (2011) reported that HHP(600 MPa) caused cell wall damages in *Saccharomyces cerevisiae,* and some cell wall fragments (cell debris) were present in the environment, which is compatible with the Figure 4.12. Also, cytoplasmic content of some cells were exposed to disruption.

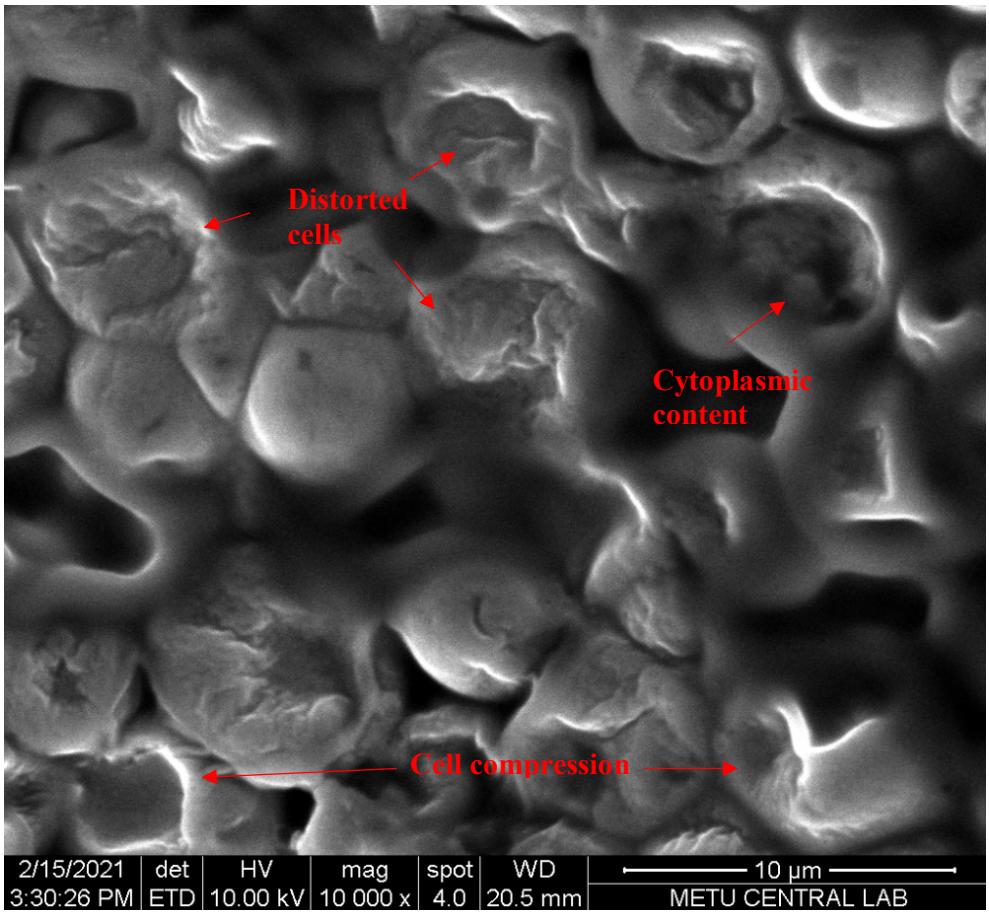


Figure 4.13. Scanning electron microscope (SEM) image of *L. starkeyi* after HHP at200 MPa, 40 ºC, 15 min (10000x magnification).

Figure 4.13 illustrated that cells treated at 200 MPa showed a distorted morphology rather than ruptured or crushed view. It was reported that HHP caused irreversible volume reduction in yeasts up to %35 at 250 MPa, which was attributed to the mass transfer through the cell borders (Pilavtepe-Çelik et al., 2008). Considering the lipid yields varied between 25 – 45% at 200 MPa, the study was compatible with the literature. As a matter of fact, this was the first study which investigated the effects of HHP on *L. starkeyi*, and considering that oleaginous yeasts have distinctive cell wallcharacteristics, further studies are required to explain the morphological effects of high pressure on oleaginous yeasts (Khot et al., 2020).

4.2.1.7 Fatty Acid Methyl Ester (FAME) Analysis of *L. starkeyi* Lipids

Fatty acid methyl ester composition of microbial lipids that were retrieved from *L. starkeyi* by HHP extraction and HSH assisted extraction are listed in Table 4.3. The results are the mean values of the duplicates. Palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) were detected asthe major components of lipids. Other fatty acids such as myristic acid (C14:0), pentadecylic acid (C15:0), linoleic acid (C18:2), linolenic acid (C18:3), eicosanoic acid (C20:0), and tetracosanoic acid (24:0) were also detected in trace amounts (<3% of the whole composition in total).

As can be seen from results, over 40% of the lipid was made up of saturated fatty acids (palmitic and stearic acid), which is one of the main reasons for the lipid to appear solid at the room temperature (Angerbauer et al., 2008; Gong et al., 2012). Oleic acid occupied the most part of unsaturated fatty acid content by over 40% whereas polyunsaturated fatty acids comprised only 2-3% of the total FAME composition, of which 70% are trans fatty acids. One-Way ANOVA showed that HHP had significant effect ($p<0.05$) on palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1) compositions, whereas showed no significant effect on linoleic acid (C18:2) and linolenic acid (C18:3) ($p>0.05$). On the other hand, one-way ANOVA within HHP revealed that pressure shows no significant effect onFAME composition ($p>0.05$).

Table 4.3. GC results of the HHP extracted lipids (%).

Run Order	Pressure (MPa)	Time (min)	Temperature (°C)	C16:0	C16:1	C18:0	C18:1 cis	C18:2 trans	C18:2 cis	C18:3 trans	C18:3 cis
1	300	15	30	38.47	3.97	14.49	39.28	0.77	0.47	0.94	0.12
2	300	10	40	38.35	3.88	12.23	41.67	0.70	0.53	0.85	0.14
3	200	15	40	40.12	4.02	11.22	40.83	0.69	0.47	0.96	0.11
4	300	5	50	36.90	2.27	14.44	42.49	1.09	0.56	0.80	0.19
5	400	10	50	35.64	3.88	11.70	44.90	1.06	0.55	0.82	0.15
6	200	5	40	38.65	3.98	14.45	39.23	0.71	0.30	0.88	0.12
7	200	10	30	46.40	4.68	4.68	39.78	0.86	0.58	1.11	0.14
8	300	15	50	35.20	3.84	13.22	43.79	1.08	0.56	0.83	0.19
9	400	10	30	38.96	3.76	10.25	42.93	0.66	0.53	0.90	0.14
10	400	15	40	38.68	3.79	10.17	43.42	0.66	0.53	0.95	0.14
11	200	10	50	34.36	3.90	9.24	48.69	1.09	0.54	0.85	0.12
13	400	5	40	38.84	3.84	9.51	43.90	0.71	0.28	0.95	0.13
15	300	5	30	38.87	3.81	11.58	41.88	0.69	0.48	0.92	0.11
HSH (Control)[a]	-	-	-	38.41	0.82	8.24	48.57	1.18	0.54	0.91	0.12

[a] HSH: High speed homogenizer extraction yield was taken as the control.

The compositions presented in Table 4.3 are compatible with the literature. Gong et al., (2012) determined that lipids accumulated by *L. starkeyi* has palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0) and oleic acid (C18:1) as major components. For instance, Gong et al., (2012) reported that palmitic acid (C16:0) comprise 34 – 40%, palmitoleic acid (C16:1) 2.9 – 3.7%, stearic acid (C18:0) 4.2 – 5.7%, andoleic acid (C18:1) 49.5 – 55.7% of the total composition, whereas, Yang et al., (2014) determined the limits of stearic acid (C18:0) as 4.4 -11.5 when the rest of the composition was similar to Gong et. al., (2012) (Table 4.4). Slight differences are

attributed to different culturing conditions and variability between strains (Calvey et al., 2016).

Table 4.4. FAME composition of *L. starkeyi* lipids from different studies.

Strain	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	Reference
AS 2.1560	34.1	3.2	4.3	55.7	-	1.3	(Gong et al., 2012)
AS 2.1560	40.0	3.4	5.3	49.5	-	1.1	(Gong et al., 2012)
AS 2.1560	32.4	3.8	4.4	57.3	0.7	0.5	(Yang et al., 2014)
AS 2.1560	36.2	3.7	5.7	52.2	0.7	0.4	(Yang et al., 2014)
AS 2.1560	41.1	4.6	5.1	45.3	3.2	3.2	(Lin et al., 2014)
CBS 1807	42.9	2.15	4.9	49.8	-	-	(Matsakas etal., 2014)

4.2.1.8 Peroxide Value (PV)

Peroxide value was determined as 5.7±0.2 for the crude microbial oil extracted from *L. starkeyi* by HHP-aided method. The extraction conditions were 200 MPa, 50 ^{o}C, and 5min, as determined optimal by RSM. According to Codex Alimentarius, upper limitof PV for virgin oils and cold pressed fats and oils are 15 milliequivalents of active oxygen/kg oil, which means *L. starkeyi*-origined oil is below the international PV limit. Table 4.5 compares PV of microbial oil to commercial vegetable oils.

Table 4.5. Upper limits ofperoxide values (Codex Alimentarius, 2017).

Oil	Sunflower Seed Oil	Extra Virgin Olive Oil	Virgin Olive Oil	Refined Olive Oil	Corn Oil	Soya Oil	Sesame Oil
PV	10	15	15	10	10	10	10

4.2.1.9 Color Analysis

For color analysis, sunflower seed oil was taken as control. The highest absorbance was obtained in 340 nm for both microbial oil and sunflower seed oil by the values of 0.514 and 0.125, respectively (Figure 4.14). Higher absorbance at 340nm showed that microbial oil contains more color pigments than sunflower seed oil.

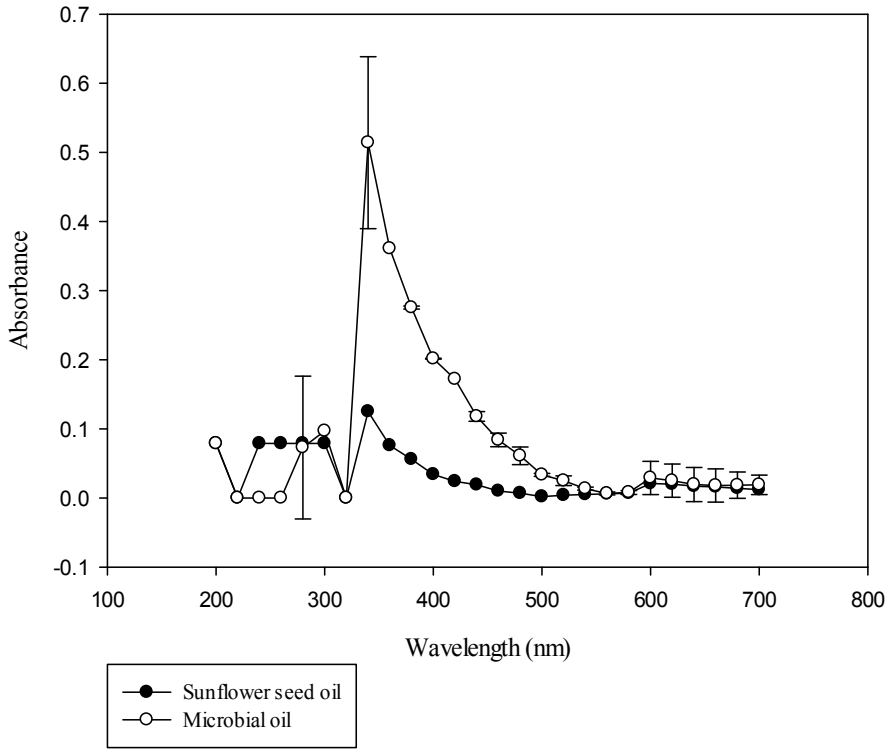


Figure 4.14. Absorbances of microbial and sunflower seed oil in a range of wavelengths.

4.2.2 *Rhodosporidium toruloides*

4.2.2.1 Growth in Different Volumes of Fermentation Media

R. toruloides was studied in five different volumes of 40 g/L synthetic sugar containing media (C/N = 55). The fermentation medium was placed into 500 mL Erlenmeyer flasks except the 500 mL medium, which was placed into 1 L flask. The aim of this experiments was to determine the optimum volume for *R. toruloides* that maximizes the lipid accumulation. Optical densities were also monitored from the first dayto the end of the predetermined fermentation time (Figure 4.15).

In Figure 4.15, optical densities followed an increasing pattern during the whole process in all media except for 50 mL, maximal optical density was reached in 100 mL of fermentation medium using 500 mL Erlenmeyer flasks.

Even though the lowest biomass and lipid yield were obtained in 500 mL, the OD_{600nm} rate was the 2nd biggest value by 0.20 day^{-1} (Table 4.6). n addition, the lowest growth rate of 0.11 day^{-1} was observed for 50 mL medium, which is half the rate of 100 mL medium (0.22 day^{-1}). The growth rates of the othermedia are 0.19 and 0.17 day^{-1} for 150 and 200 mL, respectively. The differences between growth rates might result from the different amounts of air in the Erlenmeyer flasks. Considering the air inside the Erlenmeyer flasks, the highest aerated samples were 50 mL and 100 mL, which lead to the best yields of biomassand lipid (see Table 4.6). Furthermore, Yen and Liu, (2014) reported that high aeration rate causes a rapid growth in oleaginous yeast *Rhodotorula glutinis*, which might explain the sharp increase seen in Figure 4.15 for 50 mL until day 5. The decrease observed after day 5 probably resulted from depletion of sources in the medium due to high metabolic activity. In addition, it was reported for *L. starkeyi,* which is also an oleaginous yeast, that excessive aeration affects lipid accumulationnegatively (Ageitos et al., 2011). This might be the reason that 100 mL was the optimal volume in 500 mL Erlenmeyer

flask for both biomass and lipid yield, even though it was not the highest aerated medium. Thus, aeration must be maintained between certain limits that could be different for each organism.

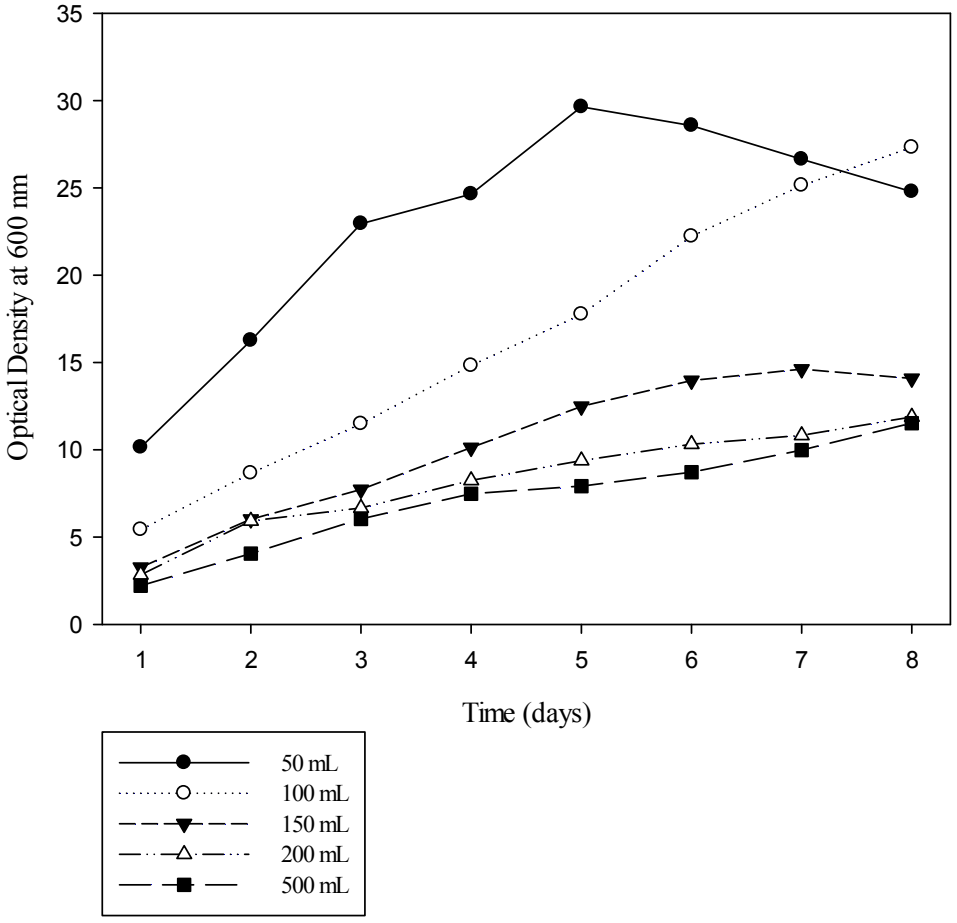


Figure 4.15. Growth of *Rhodosporidium toruloides* in fermentation media with respect to time.

4.2.2.2 Biomass and Lipid Yields from Different Volumes of Fermentation Media

At the end of 8 days of fermentation, the *R. toruloides* cells were collected by centrifugation and dried 24 hours in a conventional oven to estimate the total dry biomass and to express the lipid content on a daily basis. As the optical densities varied with fermentation volume, biomass and lipid contents also changed (Table 4.6).

Table 4.6. Biomass, lipid yield and lipid efficiency of *R. toruloides* for different fermentation volumes.

Volume of Medium/ Volume of Erlenmeyer (ml/ml)	**Biomass (g/L)**	**Lipid yield ($Y_{L/B}$)**	**Efficiency ($Y_{L/S}$)**	**Specific growth rate (μ day^{-1})**
50 / 500	7.6±0.02	27%±0.02	0.05	0.11
100/ 500	11.25±0.02	28%±0.01	0.07	0.22
150 / 500	6.21±0.02	13%±0.01	0.02	0.19
200 / 500	5.07±0.04	8%±0.01	0.01	0.17
500 / 1000	2.41±0.02	5%±0.01	0.003	0.20

For 50, 100, 150, 200, and 500 mL volumes of fermentation media, the dry biomass contents were measured as 7.6±0.02 g/L, 11.25±0.02 g/L, 6.21±0.02 g/L, 5.07±0.04 g/L, and 2.41±0.02 g/L, respectively. These numbers show that 100 mL is the optimum fermentation volume to maximize biomass in glucose containing medium. Similarly, the maximum lipid accumulation of 28%±0.01 was also observed in 100 mL medium (Figure 4.16). Moreover, the least amount of lipid was extracted from 500 mL medium (5%±0.01). Lipid yield of other media were 27%±0.02, 13%±0.01, and

8%±0.01 for 50, 150 and 200 mL, respectively. Pictures of the oils can be seen at Appendix C.

Table 4.7. Comparison of the study to the recent studies on lipid accumulation by *R. toruloides* in synthetic sugar media.

R.toruloides strain	Carbon source	Nitrogen source	C/N ratio	Process type	Process conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
L1-1	Glucose	$(NH_4)_2SO_4$ + yeast extract	140	Flask	37 ^{0}C, 150rpm, 120 hours, pH 5.5	Bligh & Dyer	NS*	13.4	NS	(Wu et al., 2018)
DSM 4444	Glucose	Yeats extract + peptone	120	Flask	26 ^{0}C, 185rpm, 192 hours, pH 6.0	Solvent extraction	9.4	71.3	0.14	(Tchakouteu et al., 2017)
DSM 4444	Glucose	$(NH_4)_2SO_4$ + yeast extract	77	Flask	28 ^{0}C, 185rpm, 120 hours, pH 5.0 - 6.0	Folch	9.7	39.6	NS	(Papanikolaou et al., 2017)
np11	Glucose	$(NH_4)_2SO_4$ + yeast extract + amino acid supplements	NS	Flask	30 ^{0}C, 220rpm, 168 hours, pH 6.0	Solvent extraction	8.6	43.0	NS	(Zhang et al., 2016)
NCYC 921	Glucose	$(NH_4)_2SO_4$ + yeast extract	54	Flask	30 ^{0}C, 150rpm, 72 hours, pH 4.0	Flow cytometry	5.9	21.8	NS	(Dias et al., 2016)
RT880-AD	Glucose	$(NH_4)_2SO_4$ + yeast extract	339	Flask	30 ^{0}C, 250rpm, 217 hours, pH 5.6	Folch	26.8	61.1	0.23	(Zhang et al., 2016)
DSM 4444	Glucose	$(NH_4)_2SO_4$ + yeast extract	54	Flask	25 ^{0}C, 130rpm, 192 hours, pH 5.0	Bligh & Dyer	11.2	28.0	0.07	This Study

*NS: not specified

It is also possible to compare this study with some similar studies in the literature with the help of Table 4.7. All of the studies shown in the table were flask scale, and utilized synthetic glucose as carbon source. Zhang et al., (2016) andZhang, et al., (2016) achieved high lipid yields of 40-60% with recombinant strains of *R. toruloides*. The most outstanding yield was reported by Tchakouteu et al., (2017) by the rate of 71.3% in high C/N ratio with a non-mutant strain in an optimization study. The lipid yield of the study performed by Dias et al., (2016) seems the closest to the current study by 21.8% lipid yield. The differences might come from the unlike process conditions and strain differences.

The results of the synthetic sugar experiments demonstrated that further studies with *R. toruloides* must be conducted using 100 mL of fermentation media in 500 mL Erlenmeyer flask. Therefore, apple pomace experiments were carried out using 100 mL of fermentation media in 500 ml Erlenmeyer flask to analyze the efficiency of apple pomace as a mineral source, while avoiding the potential misleading effects of improper aeration.

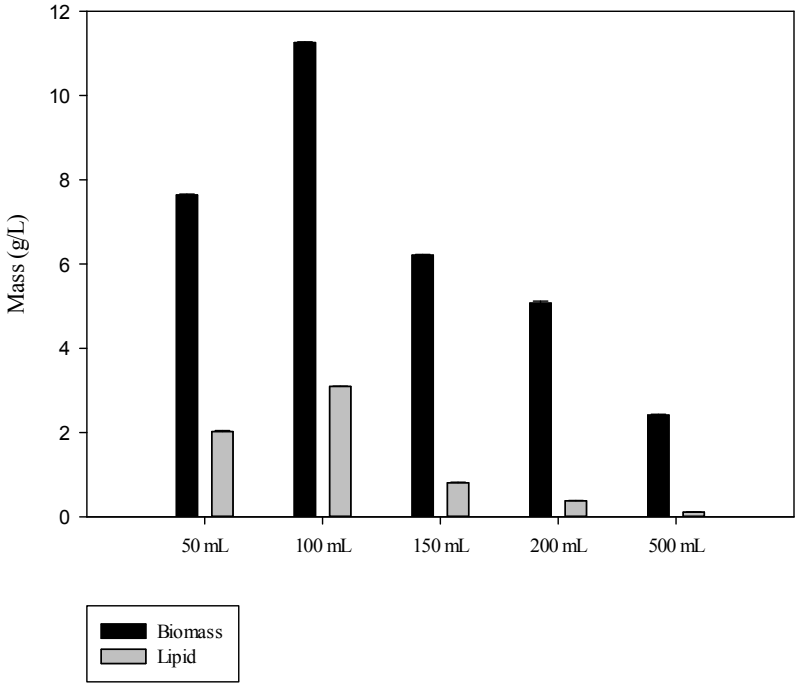


Figure 4.16. Biomass and corresponding lipid yields of *Rhodosporidium toruloides* in different volumes of fermentation media.

4.3 Analysis of Apple Pomace

Since apple pomace is the residue of fruit juice industry, it was received as a moist massof apple waste. Thus, apple pomace was dried to 4% of moisture in a tray dryer andpowdered into 1 mm particle size prior to bioprocessing (Appendix D).

4.3.1 Reducing Sugar Analysis

Apple pomace was acid hydrolyzed in an autoclave to damage the cellulosic structure and to release various types of reducing sugars. In order to assess the effect of apple pomace to lipid accumulation as the only carbon source, approximately 40 g/L of

reducing sugar (the amount in the synthetic medium) was aimed to obtain from apple pomace by acidic hydrolysis. For this purpose, apple pomace was suspended in distilled water and autoclaved in the absence of any additional acids or base to estimate the inherent sugar content. The acidic hydrolysis experiments were performed for 5, 7 and 10 grams of apple pomace in various acidic solutions of 0.5%, 1%, 2%, 3%, 4% and 5% (w/v).

The reducing sugar contents are shown in Table 4.8. Two-way ANOVA revealed that apple pomace mass, acid amount and their interaction affect the reducing sugar yield significantly ($p<0.05$). As a result, hydrolysis of 7 grams of apple pomace per 100 ml of 2% (w/w) H_2SO_4 dilution gave the desired result of 43.4±2.89 g/L of reducing sugar, which is almost equivalent to synthetic medium.

Table 4.8. Reducing sugar yields of apple pomace after acidic hydrolysis in different rates.

	Reducing Sugar Yield (g/L)						
Acid dilution (w/v %) / Apple pomace weight (g/100ml)	**0%**	**0.5%**	**1%**	**2%**	**3%**	**4%**	**5%**
5	24.5±0.43	33.5±1.75	36.4±1.19	35.9±0.92	35.7±1.10	28.6±1.30	35.6±0.76
7	35.6±0.11	50.6±0.94	50.9±2.59	43.4±2.89	43.3±0.32	39.1±0.35	36.3±0.57
10	52.1±2.08	65.3±2.32	65.2±1.78	67.8±2.64	60.7±2.54	62.3±3.13	59.1±0.59

4.3.2 Reducing Sugar Analysis

Effects of trace minerals on single cell oil accumulation has been studied for more than 30 years (Naganuma et al., 1985). Mineral content of dried apple pomace can

be seen in Table 4.9. Potassium, calcium, magnesium phosphor, sulphur, and sodium (the minerals which were used in the synthetic medium) were detected in apple pomace. Furthermore, potassium is the predominant mineral present in apple pomace at 1.29%±0.02. Carbon and nitrogen amounts were also detected by instrumental methods; however, the values that are shown in Table 4.9 are on a dry basis.

Table 4.9. Results of elemental analysis using 1 g of dried apple pomace.

Minerals (%)							
C	**N**	**K**	**Ca**	**Mg**	**P**	**S**	**Na**
45.0	1.12	1.29± 0.02	0.21± 0.01	0.134± 0.004	0.142± 0.006	0.09± 0.004	0.0127

4.4 Lipid Production by *Rhodosporidium toruloides* in Apple Pomace Hydrolysates

It was demonstrated that apple pomace has a potential to break into certain amount of reducing sugars. Thus, the lipid accumulation characteristic of *R. toruloides* was surveyed in several apple pomace media.

4.4.1 Apple Pomace Hydrolysate Medium Design in Erlenmeyer Flasks

Process of lipid accumulation is affected by several factors such as trace mineral content, C/N ratio, and presence of inhibitors (Li et al., 2008). The experiments shown in Table 4.10 were carried out with the intent of investigating the sufficiency of the trace mineral concentration in the apple pomace hydrolysate and the effect of detoxification on the biomass and lipid production.

Table 4.10. Experimental design of lipid fermentation of *Rhodosporidium toruloides* in apple pomace hydrolysate based media.

Name of Medium	Detoxification	Minerals Added
S medium	-	-
SM medium	-	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$
SMN medium	-	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$, $(NH_4)_2SO_4$, yeast extract
D medium	+	-
DM medium	+	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$
DMN medium	+	KH_2PO_4, Na_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2.2H_2O$, $(NH_4)_2SO_4$, yeast extract

S: Standard medium, **SM**: Mineral added standard medium, **SMN**: Mineral and nitrogen added standard medium, **D**: Detoxified medium, **DM**: Mineral added detoxified medium, **DMN**: Mineral and nitrogen added detoxified medium.

According to Figure 4.17, *R. toruloides* possessed a log phase for the first 4 days regardless of the detoxification or mineral addition to the media. Nevertheless, the transition from log phase to stationary phase varies for each media. It was right after the 4^{th} day when the yeast reached the stationary phase in the D medium, whereas in the DM and S medium, the stationary phase started after 5 days. In SMN medium, log phase ended at some point between day 6 and 7. It is not easy to decide at which point the log phase stopped in the DMN and SM medium since they indicate downward curvature just after they reached their peak $OD_{600nm.}$ The highest OD_{600nm} value of 27.9 was reached by the DMN medium on 7^{th} day. Nonetheless, the optical density of the same medium dropped sharply to 20.8 at the end of 8^{th} day. At the end of fermentation, the highest OD_{600nm} value of 22.5 was observed for S medium.

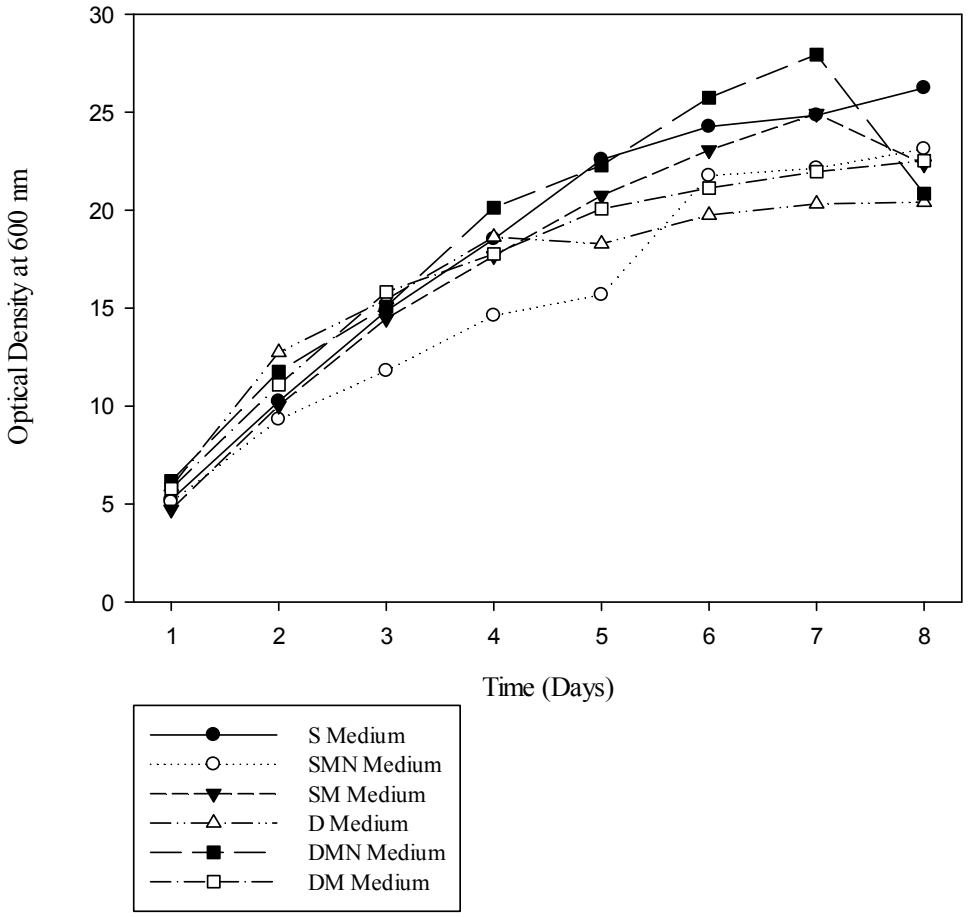


Figure 4.17. Growth of *Rhodosporidium toruloides* in different apple pomace media during fermentation.

Specific growth rates (μ) were also calculated according to kinetic analysis of OD_{600nm} values (Table 4.11) (Appendix I). An equal rate of 0.20 day^{-1} was calculated for the three standard media (S, SMN, SM), which indicates mineral addition did not affect the growth rate significantly in non-detoxified media ($p>0.05$). Further, detoxification decreased the growth rate from 0.20 to 0.13 day^{-1}, which is significant ($p<0.05$). Mineral addition to D medium increased the growth rate from 0.13 to 0.16 day^{-1}, whilst nitrogen addition to DM medium increased growth rate further to 0.19

day^{-1}. Although specific growth rate rose from 0.13 to 0.19 day^{-1} with mineral and nitrogen addition to D medium, one-way ANOVA revealed that the effect is insignificant ($p>0.05$). Thus, none of the media reached the growth rate of synthetic medium (0.22), though three of them surpassed the synthetic medium in lipid yield, which will be discussed in the following sections.

4.4.2 Sugar Consumption

It is noticeable that sugar consumption trends of *R. toruloides* in the S, SM, D, and DMN media are similar. In other words, other than DM and SMN media, the sugar consumption curves almost overlap.

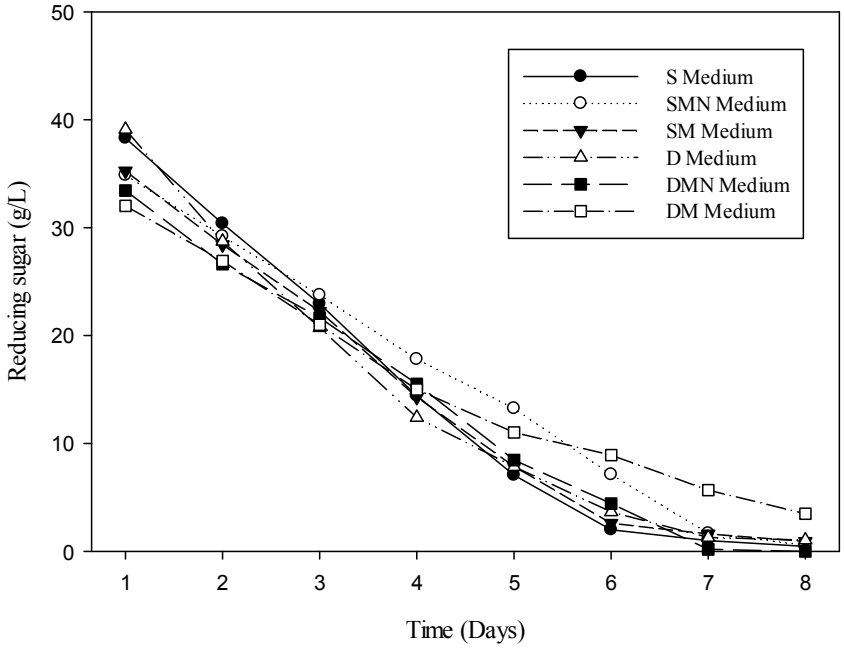


Figure 4.18. Sugar consumption curves of *Rhodosporidium toruloides* in different apple pomace media during fermentation.

As shown in Figure 4.18, all the sugar consumption curves followed an exponential path. The fastest sugar consumption was measured for DM medium in the first 24

hours by approximately spending one-fifth of the total reducing sugar (7.9±1.03 g/L). In fact, sugar was almost depleted in all the media at the end of fermentation except for DM medium with 3.5±0.43 g/L residual sugar. Residual sugar values were determined 0.46±0.11, 0.57±0.16, 0.92±0.16, 1.03±0.11, and 0.04±0.01 g/L for S, SMN, SM, D, and DMN respectively. The fastest (k_{DMN} = 6.96 day^{-1}) sugar consumption was observed in the DMN medium, which is mineral and nitrogen added detoxified apple pomace medium (Table 4.11). The rate was 4.48 day^{-1} for S medium. Also, sugar consumption rates were statistically analyzed by one-way ANOVA. Mineral addition to S medium decreased the k value, such that k values of 3.76 and 4.24 day^{-1} were calculated for both SM and SMN medium respectively, which were similar ($p>0.05$). Detoxification resulted in a significant decline in consumption rate, from 4.48 day^{-1} (for S medium) to 3.60 day^{-1} (for D medium) ($p<0.05$). When D medium was fortified with minerals and nitrogen (DMN), an outstanding rise in consumption rate (k_{DMN}=6.96 day^{-1}) was achieved, whereas mineral addition without nitrogen (DM) displayed a reverse effect in consumption rate (k_{DM}=1.20 day^{-1}).

Cell growth may be limited because of some toxic compounds (acetic acid, HMF, phenoliccompounds etc.) present in lignocellulosic hydrolysates (Palmqvist & Hahn-Hägerdal, 2000). Detoxification was shown to be effective in both biomass and lipid yields by various studies (Matsakas et al., 2017; Yu et al., 2011). Although the yeasts followed different paths of sugar consumption in detoxified media, sugar was almost depleted in D medium and fully depleted in DMN medium in the same way as standard media. The only exception was observed in the DM medium with the residual sugar content of 3.5±0.43 g/L. Moreover, DMN medium showed a linear growth (Figure 4.17) similar to the SMN medium. Thus, presence of nitrogen might regulate sugar consumption whereas nitrogen deprivation accelerates the rate of sugar consumption (Andrade et al., 2012).

4.4.3 Biomass and Lipid Yields from Different Apple Pomace Media

After 8 days of fermentation in various apple pomace media, cells were harvested by centrifugation and dried in an oven for 24 hours in petri dishes. The appearances of biomasses from different media are shown in Appendix E. Since the aim of the study is to analyze the biomass and lipid yields, it was not possible to reach a certain conclusion about the visual differences; however, different colors might result from different solid material density, apple pomace hydrolysate residuals, carotenoid contents, amount of accumulated lipids or other metabolic residues due to different mineral compositions.

The highest biomass was obtained in DMN medium at 11.67±0.33 g/L, which is the closest value to the synthetic sugar counterpart in the same fermentation volume. The second medium, in which biomass exceed 1% of fermentation volume was SMN medium with 10.35±0.33 g/L. Since SMN and DMN are both nitrogen enriched media, and nitrogen addition decreased the C/N value, it is not unexpected that these were the most efficient media for biomass production (Papanikolaou & Aggelis, 2011a). Nonetheless, these two are the least efficient media for lipid yield ($Y_{L/B}$) of 17.1±0.35 % and 21.1±2.40% respectively. Thus, decreasing C/N ratio affected lipid yield negatively, as reported in the literature (Angerbauer et al., 2008).

Table 4.11. Quantitative results of lipid fermentation in different apple pomace media.

Name of Medium	Biomass (g/L)	Lipid Yield (w/w dry cell %) ($Y_{L/B}$)	Sugar Efficiency ($Y_{L/S}$)	Apple Pomace Efficiency ($Y_{L/A}$)	Rate of Consumption (k)	Specific Growth Rate (μ)
S medium	8.56±0.04	26.8±0.85	0.06	0.03	4.48	0.20
SMN medium	10.35±0.34	17.1±0.35	0.04	0.03	4.24	0.20
SM medium	9.11±0.37	37.2±4.10	0.08	0.05	3.76	0.20
D medium	6.32±0.13	47.5±2.47	0.08	0.04	3.60	0.13
DMN medium	11.67±0.33	21.1±2.40	0.07	0.04	6.96	0.19
DM medium	7.69±0.05	44.5±1.27	0.09	0.05	1.20	0.16

S and SM media yielded higher biomasses than detoxified equivalents (Table 4.11). That is, detoxification did not affect biomass production positively if additional nitrogen was not introduced along with other minerals. On the contrary, detoxification increased lipid accumulation significantly. Furthermore, D medium was decided to be the most efficient medium for lipid accumulation with 47.5±2.47%, which is considerably higher than synthetic sugar medium. In other words, by only detoxifying the standard apple pomace hydrolysate, lipid yield ($Y_{L/B}$)

was approximately doubled. On the other hand, detoxification reduced biomass significantly ($p<0.05$). Decrease in biomass, while increasing lipid yield by charcoal-detoxification of lignocellulosic wastes were also reported by other authors (Z. Liu et al., 2020; Matsakas et al., 2017). Mineral addition to D medium slightly increased biomass to 7.69 g/L, whereas nitrogen addition proliferated cell growth (11.67 g/L). However, lipid yield declined to less than half the value of D medium, which is undesired (21.1±2.40). Also, detoxification decreased specific growth rate to the lowest value among all media (0.13 for D medium). One-way ANOVA between DM and DMN medium showed that both biomass and lipid yield were affected by nitrogen presence significantly ($p<0.05$). Thus, impacts of detoxification on C/N ratio must be further studied to explain the dramatic drop of biomass.

Mineral addition to D medium (DM medium) caused a remarkable lipid accumulation of 44.5±1.27%, which is slightly lower than D medium. Even so, as mentioned in the section, there was still some residual sugar left in the DM medium at the end of 8 days. Therefore, it could be worthy to see if DM medium is capable of surpass the D medium in terms of $Y_{L/B}$ value if fermentation period was extended.

Sugar and apple pomace efficiencies were also calculated to decide which medium is the most suitable for the scale up experiments. Figure 4.19 is a bar graph that illustrates the biomass obtained from each media at the end of 8 days and the amount of lipids, which were extracted from the biomass. Based on Table 4.11, SM, D and DM media are the three most efficient media with sugar efficiencies ($Y_{L/S}$) of 0.08, 0.08 and 0.09, respectively. Moreover, apple pomace efficiencies ($Y_{L/A}$) for the same media were estimated as 0.05, 0.04 and 0.05, respectively. In other words, 100 grams of dried apple pomace can give 5 grams of biolipid in SM and DM media, and 4 grams of biolipid in D medium. In spite of the fact that SM and DM seem more efficient, this does not satisfactorily suggest SM and DM media were good for scale up experiments. Note that additional minerals were not added to the efficiency calculations, nor a feasibility study were conducted. Therefore, the constants do not

represent the ultimate economical efficiencies. The $Y_{L/S}$ and $Y_{L/A}$ values of D medium is quite close to the mineral added media (SM and DM). As mentioned before, D medium was the most efficient medium in terms of lipid yield on a dry cell basis (47.5%), almost doubling the standard medium (S). As a result, D medium was decided as the most suitable one to proceed in the fermentor experiments.

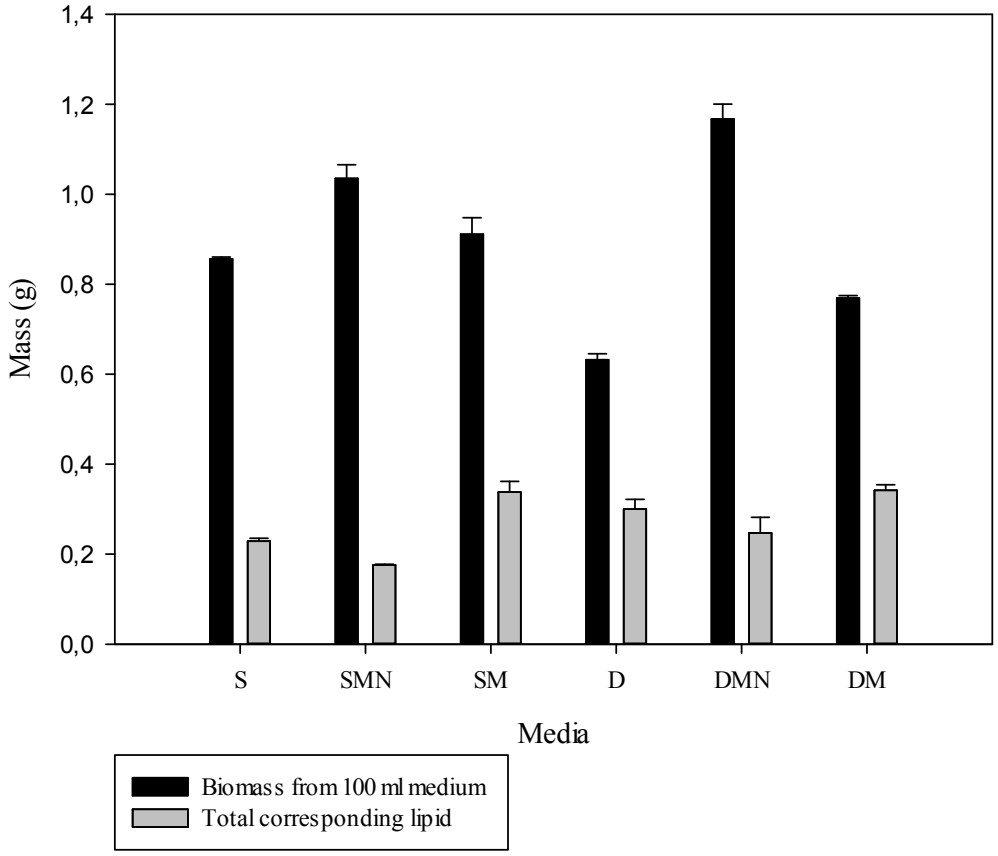


Figure 4.19. Bar chart of biomass and corresponding lipid amount from different apple pomace media.

Some of the microbial oil studies that utilize lignocellulosic materials as a carbon source for the oleaginous yeast *R. toruloides* are compiled in Table 4.12.

Table 4.12. Comparison of the study to the recent studies on lipid accumulation by *R. toruloides* in lignocellulosic hydrolysate contained media.

R.toruloides strain	Carbon source	Nitrogen source	C/N ratio	Process type	Process conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
1588	Wood hydrolysate (C6)	$(NH_4)_2SO_4$	95	Flask	25 ^{0}C, 180rpm, 112 hours, pH 6.0	Solvent extraction	100 - 110	35.0	NS	(Osorio-González et al., 2019)
7191	Wood hydrolysate (C5)	$(NH_4)_2SO_4$	95	Flask	25 ^{0}C, 180rpm, 112 hours, pH 6.0	Solvent extraction	90 - 100	32.0	NS	(Osorio-González et al., 2019)
NCYC 1576	Brewers' spent grain hydrolysate	$(NH_4)_2SO_4$ + yeast extract + amino acids	500	Flask	25 ^{0}C, 180rpm, 168 hours, pH 5.5	Solvent extraction + sonication + microwave	18.4	56.4	NS	(Patel, Mikes, Bühler, et al., 2018)
RP 15	Sugarcane bagasse hydrolysate + glycerol	$(NH_4)_2SO_4$	140	Flask	28 ^{0}C, 180rpm , 14 days pH 5.5	Bligh & Dyer	12.0 - 14.0	59.8	0.15	(Hassanpour et al., 2019)
Y2 + microalgae	Food waste hydrolysate	NS	14	Flask	28 ^{0}C, 180rpm, 5 days, pH 6.5	Bligh & Dyer	12.3	29.3	0.12	(Zeng et al., 2018)
CCT 0783	Sugarcane bagasse hemicellulosic hydrolysate	NS	17	Flask	28 ^{0}C, 200rpm, 72 hours, pH 6.0	Folch	7.6	16.0	NS	(Bonturi et al., 2017)
DSM 4444	Apple pomace hydrolysate	-	40	Flask	25 ^{0}C, 130rpm, 8 days, pH 5.0	Bligh & Dyer	6.3	45.7	0.08	This Study

4.4.4 Fermentor Experiments

Based on flask study, detoxified apple pomace hydrolysate was chosen the best medium for fermentor experiments. First, in order to estimate the efficiency of *R. toruloides* in detoxified apple pomace hydrolysate at large scale biolipid production, fermentation volume was increased to 10 times the flask experiments, which corresponded to 1 L of volume (Appendix F). Finally, the volume was increased to 10 L in an industrial type of fermentor (Appendix G).

4.4.4.1 Biomass and Lipid Yields at Benchtop Fermentor

Batch processing was chosen as the processing method with controlled pH and temperature (PID control) during 8 days of oil fermentation. Biomass was found as 2.8 ±0.33 g/L, which is nearly half the value of expected biomass based on the flask experiments. Also, lipid yield ($Y_{L/B}$) was estimated as 40.1 ±5.51 % (Table 4.13). Lipid yield was approximately 7% less than flask scale experiments; nevertheless, it is still higher than the value that was obtained from synthetic sugar medium. The reason for less biomass than expected was probably the aeration rate or controlled pH which need to be studied in a further work. Ageitos (2011) suggested that the media must be highly aerated (Ageitos et al., 2011). Even though agitation speed was the same, dissolved oxygen levels might not be the same because of uncontrolled aeration in Erlenmeyer flask. In addition, it was observed that pH tends to increase in the course of growth. However, pH was kept at 5.0 throughout the process, which could dictate testing a range of pH.

Sugar efficiency constant ($Y_{L/B}$) and apple pomace efficiency constant ($Y_{L/S}$) were 0.04 and 0.02, respectively. Although the process does not seem as efficient as low scale, residual sugar analysis revealed that 11.0±0.81 g/L sugar was present in the medium at the end of fermentation, which means a quarter of initial sugar remained unused. The value is only 1.0±0.11 g/L for the same medium in flask experiments.

Thus, 8 days was not sufficient for the yeast to metabolize all the available sugar with the given parameters in the fermentor.

4.4.4.2 Biomass and Lipid Yields at 10 L Fermentation Volume

Table 4.13 demonstrates the result of 10 L experiment carried out in 30 L steam heated fermentor (Appendix G). A close final biomass content of 5.1 g/L, and lipid yield of 50.9 % were achieved. Also, sugar and apple pomace efficiency values were estimated as 0.06 and 0.04 respectively, which are both greater than 1 L experiments. In addition, residual sugar analysis showed that 16.2 g/L sugar was present in the medium at the end of fermentation, which means approximately one-third of initial residual sugar remained unused.

Table 4.13. Quantitative results of lipid fermentation in 1 and 10 L of D medium in 2 and 30 L fermentors, respectively.

Name of Medium	Biomass (g/L)	Lipid Yield (w/w dry cell %) ($Y_{L/B}$)	Sugar Efficiency Constant ($Y_{L/S}$)	Apple Pomace Efficiency ($Y_{L/A}$)
D medium (1 L)	2.8 ±0.33	40.1 ±5.51	0.04	0.02
D medium (10 L)	5.1	50.9	0.06	0.04

As explained before, both temperature and pH was controlled during 1 L fermentor experiments; however, neither temperature nor pH was controlled in the 10 L fermentor. Starting from 25 ºC, the temperature fluctuated between 17 ºC and 25 ºC depending on the thermal fluctuation of day-night cycle. Similar to the previous experiments, initial pH was set to 5.0. At the end of the 8 days fermentation, pH was measured as 9.1. Dias (2016) showed that pH tends to decrease for the first 72 hours

in the fermentation medium (Dias et al., 2016). Just as suggested, the pH decreased from 5.0 to 4.2 until the end of 48th hour. Nevertheless, pH started to rise on day 3 (between 48th - 72th hours) until the end of day 8. Thus, this study is compatible with the literature in terms of the pH changing behavior of *R. toruloides* in the first 72 hours. Moreover, this study further demonstrated that pH was peaking after certain point, which proves the need for a pH-control.

In order to make comparison with larger scale studies conducted in the last 5 years, in which agro-industrial wastes was used as the main carbon source, the data from literature was tabulated (Table 4.14). Based on the lipid yields in the table, apple pomace is more efficient than sugarcane juice, birch chips hydrolysate, carob extract, switch grass, and almost as efficient as sugarcane bagasse hemicellulosic hydrolysate. In addition, it is also clear that fed-batch processes are strikingly more effective than batch processes in biomass production.

Table 4.14. Comparison of the study to the recent studies on lipid accumulation by *R. toruloides* in bioreactors with lignocellulosic hydrolysates as fermentation media.

R.toruloides strain	Carbon source	Nitrogen source	C/N ratio	Process type	Process conditions	Extraction method	Biomass yield (g/L)	Lipid yield (% w/w)	Lipid constant (g/g sugar)	Ref.
DSMZ 4444	Lignocellulosic sugar mixture	NS	NS	Bioreactor (pulsed fed-batch)	30 ^{0}C, 300 - 700rpm, 96 hours pH 5.2	Solvent extraction	43.0	61.4	0.24	(Fei et al., 2016)
DEBB 5533	Sugarcane juice	Urea	NS	Bioreactor (fed-batch)	30 ^{0}C, 200rpm, 45 hours pH 5.0	Three staged extraction	40.3	45.0	NS	(Soccol et al., 2017)
CCT 0783	Sugarcane bagasse hemicellulosic hydrolysate + glycerol	NS	47	Bioreactor (batch)	28 ^{0}C, 350rpm, 72 hours, pH 6.0	Folch	6.6	55	NS	(Bonturi et al., 2017)
NCYC 1576	Birch chips hydrolysate	NS	NS	Bioreactor	30 ^{0}C, 800rpm, 8 days, pH 5.5	Folch	7.1	39.0	0.07	(Matsakas et al., 2017)
CECT 1499	Carob extract + acetic acid	$(NH_4)_2SO_4$ + yeast extract	NS	Bioreactor (fed-batch)	30 ^{0}C, 200rpm	Nile Red	NS	40.0	NS	(Afonso et al., 2018)
0047-17	Wheat straw hydrolisate + corn steep liquor	NH_4NO_3 + NH_4Cl	NS	Bioreactor (fed-batch)	30 ^{0}C, 500-1000rpm, pH 6.0	Nile Red	64.5	61.3	0.17	(Díaz et al., 2018)
NRRL Y-1091	Switchgrass	$(NH_4)_2SO_4$	NS	Bioreactor (fed-batch)	30 ^{0}C, 200rpm, 120 hours, pH 5.5	Phosphoric acid-vanillin assay	19.4	43.8	NS	(Chen et al., 2018)
DSM 4444	Apple pomace hydrolysate	-	40	Bioreactor (Batch) (1 L)	25 ^{0}C, 130rpm, 8 days, pH 5.0	Bligh & Dyer	2.8	40.1	0.04	This Study
DSM 4444	Apple pomace hydrolysate	-	40	Bioreactor (Batch) (10L)	25 ^{0}C, 130rpm, 8 days, pH 5.0	Bligh & Dyer	5.1	50.9	0.06	This Study

NS: not specified

4.4.5 Fatty Acid Methyl Ester Analysis

Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2) were detected as the major components of lipids. Linolenic acid also detected in less than <1% of the composition (Table 4.15). Statistical analysis revealed that effect of mineral addition and detoxification were significant factors on the major fatty acid percentages ($p<0.05$). As mentioned before, D medium was determined as the optimum medium for lipid yield. However, D medium also drew attention with the highest saturated fatty acid content, which may not be a desirable feature considering the dietary concerns (Tibbetts et al., 2020).

Table 4.15. FAME composition of microbial lipids from *R. toruloides* grown in synthetic glucose and different apple pomace media.

Name of Media	14:0	15:0	16:0	16:1	18:0	18:1 cis	18:2 trans	18:2 cis	18:3 trans	18:3 cis	20:0
Glucose	1.47	0.31	28.36	0.72	5.48	54.78	6.32	0.83	0.07	0.14	0.33
S	1.23	0.15	25.41	0.57	5.49	55.98	8.22	2.12	0.36	0.07	0.35
SM	1.08	0.13	24.53	0.55	7.47	55.34	7.76	2.20	0.41	0.03	0.40
D	1.09	0.13	25.48	0.42	15.93	43.57	9.52	3.02	0.32	0.02	0.41
DMN	1.36	0.24	26.13	1.73	12.40	50.24	5.38	1.00	0.55	0.13	0.67
DM	0.54	0.13	23.50	0.61	7.17	53.95	10.05	2.47	0.37	0.02	0.51

In the fermentor experiments carried out with medium D, the FAME content varied slightly from the flask experiment. Oleic acid (C18:1) and linoleic acid (C18:2) ranged at 44-55 and 2-3.7, respectively in fermentors (Table 4.16). Given the fact that flask experiments were not pH-controlled, and 1 L fermentor experiment was both temperature and pH controlled, it was suggested that process control has considerable effects on FAME composition of microbial lipids. In the 10 L experiment, palmitic acid (C16:0) and stearic acid (C18:0) values increased from 21.56 and 7.60 to 26.05 and 10.84 respectively. Oleic acid (C18:1) value decreased

to 47.49, and linoleic acid (C18:2) remained almost the same at 12.74 (Table 4.16). It is concerning that majority (80-85%) of the linoleic and linolenic acid of the lipids are in trans-isomerization. According to World Health Organization (WHO), trans-fatty acids are limited to 2 grams per 100 ml of fat (WHO, 2020). However, there are studies claiming that ruminant (natural) trans fatty acids might not be as harmful as those formed by partially hydrogenation (artificial) of vegetable oils (Coyle, 2018). For instance, Mozaffarian and Willet (2009) stated that contribution of ruminant trans fatty acids to coronary heart diseases is not as evident as trans fatty acids from partially hydrogenated oils, though the effects of specific trans fatty acid isomers must be further investigated. Microbial oils are formed naturally by microorganisms, therefore further studies are required to classify microbial oil-originated trans fatty acids.

Table 4.16. FAME composition of microbial lipids from *R. toruloides* grown in D medium using 1 L and 10 L fermentors.

Volume	14:0	15:0	16:0	16:1	18:0	18:1 cis	18:2 trans	18:2 cis	18:3 trans	18:3 cis	20:0
Ferm. in 1L	0.80	0.14	21.56	1.14	7.60	55.16	10.32	2.17	0.12	0.05	0.55
Ferm. in 10L	1.10	0.10	26.05	0.22	10.84	47.49	9.04	3.70	0.10	0.06	0.28

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In this study, HHP assisted lipid extraction and use of apple pomace as sole medium for lipid production were investigated. *Lipomyces starkeyi* and *Rhodosporidium toruloides* were used as the oil producing microorganisms (oleaginous yeasts). Biomass of *Lipomyces starkeyi* was used in the HHP experiments while *Rhodosporidium toruloides* was utilized only in the apple pomace experiments. In addition, the optimal apple pomace based medium was used to maximize lipid accumulation by *Rhodosporidium toruloides*.

Lipomyces starkeyi was cultivated in synthetic glucose containing medium (C/N ratio of 55) to perform lipid fermentation. After 8 days of fermentation, cells were collected, dried and subjected to HHP to extract lipid from the cells. The results revealed optimal HHP conditions of 200 MPa,50 ºC, and 5 min, which achieved 44.72±2.7% of lipid yield, which is higher than high speed homogenizer assisted extraction. Lipid yield decreased with the increasing pressure. HHP showed no impact on fatty acidmethyl ester composition of the microbial lipid. The FAME composition of the lipidfrom *L. starkeyi* mostly consisted of palmitic (C16:0), stearic (18:0) and oleic acid (C18:1).

Experiments on *Rhodosporidium toruloides* showed that optimum fermentation volume was 100 mL in 500 mL flask. The maximum lipid yield ($Y_{L/B}$) achieved in the synthetic medium was 28.0±0.01%. In contrast, the results of apple pomace experiments confirmed that apple pomace carries a potential to replace synthetic sugar media giving 47.5±2.47% yield in 100 mL detoxified apple pomace hydrolysate in 500 ml flask.

Similarly, when 1 L of detoxified apple pomace hydrolysate was tested in 2 L lab-scale fermentor, a lipid yield of 40.1±5.51 was obtained, while detoxified apple

pomace hydrolisate tested in 30 L industrial-type fermentor with a working volume of 10 L resulted in 50.9% oil yield. Lipids were mostly composed of palmitic (C16:0), oleic (C18:1), and linoleic acid (C18:2).

This study casted light upon HHP as a novel extraction procedure in the area of microbial oil research. It was shown that HHP can be as effective as other novel extraction methods. Considerable yield efficiency, unnecessity of strong acid applications, and relatively low pressure and temperature requirements for the optimal yield make HHP a potential green approach for lipid extraction from oleaginous yeasts. However, further studies are required to explain mechanism of pressure effect on lipid yield.

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APPENDICES

A. CHEMICALS AND SUPPLIER INFORMATION

Table A.1. Chemicals and suppliers.

Chemical	**Supplier**
D-glucose	Merck
Bacto-Pepton	Becton, Dickinson (BD)
Malt Extract	Merck
Di-sodium Phosphate (Na_2HPO_4)	Merck
Ammonium Sulphate $(NH_4)_2SO_4$	Merck
Magnesium Sulphate Heptahydrate ($MgSO_4.7H_2O$)	Merck
Calcium Chloride dehydrate ($CaCl_2.2H_2O$)	Merck
3'-5'-Dinitrosalcylic acid	Sigma-Aldrich
Nutrient Agar	Merck
Potato Dextrose Agar	Merck
Phenol	Merck
Potassium di-hydrogen Phosphate (KH_2PO_4)	Merck
Rochelle salt	Merck
Sulphuric acid	Merck
Sodium hydroxide	Sigma-Aldrich
Sodium sulphite	Merck
Yeast extract	Merck

n-Hexane	Merck
Glycerol	Merck
Methanol	Merck
Chloroform	Merck
Antifoam	Sigma-Aldrich

B. STANDARD CURVE OF DNS ANALYSIS

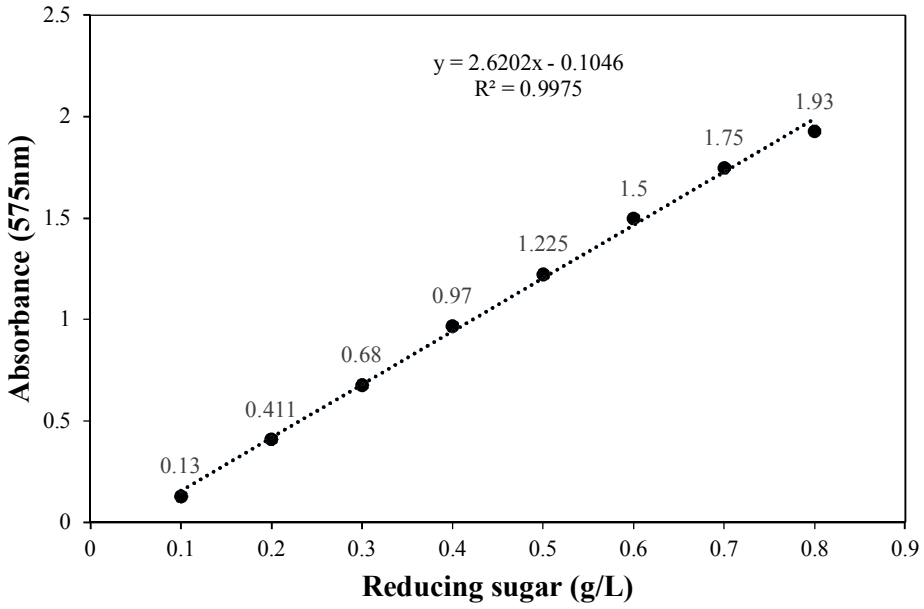

Figure B.1. The standard curve of DNS analysis.

Total reducing sugar content was measured according to the equation below:

Total reducing sugar (g/L) = [(Absorbance)/2.6202]*dilution rate

C. PICTURES OF MICROBIAL OILS

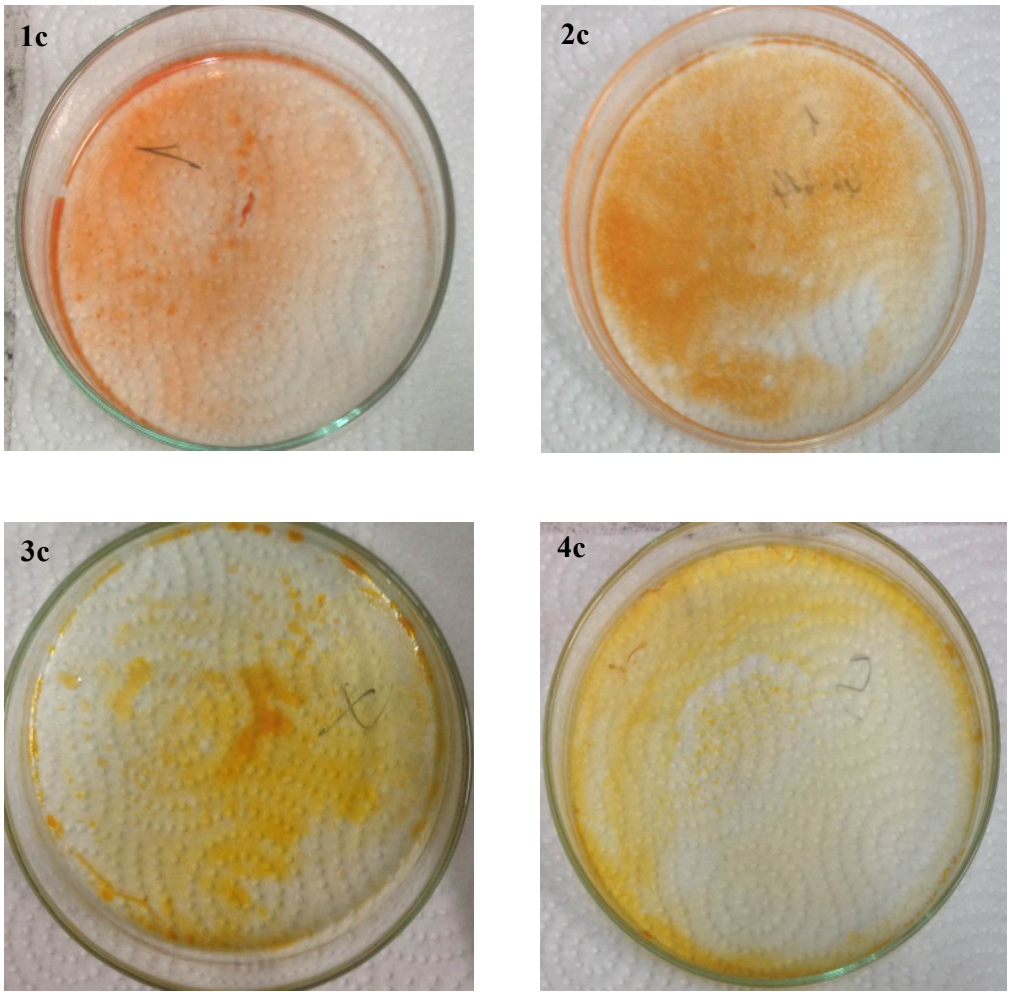

Figure C.1. Pictures of microbial oils which were extracted from *R. toruloides* grown in synthetic sugar media in different volumes. **1c**: Oil from 50 ml medium in 500 ml Erlenmeyer flask, **2c**: Oil from 100 ml medium in 500 ml Erlenmeyer flask, **3c**: Oil from 150 ml medium in 500 ml Erlenmeyer flask, **4c**: Oil from 200 ml medium in 500 ml Erlenmeyer flask.

D. PICTURES OF APPLE POMACE

Figure D.1. Pictures of wet apple pomace (**1d**) and dried apple pomace powders (**2d**).

E. PICTURES OF FLASK SCALE APPLE POMACE EXPERIMENT

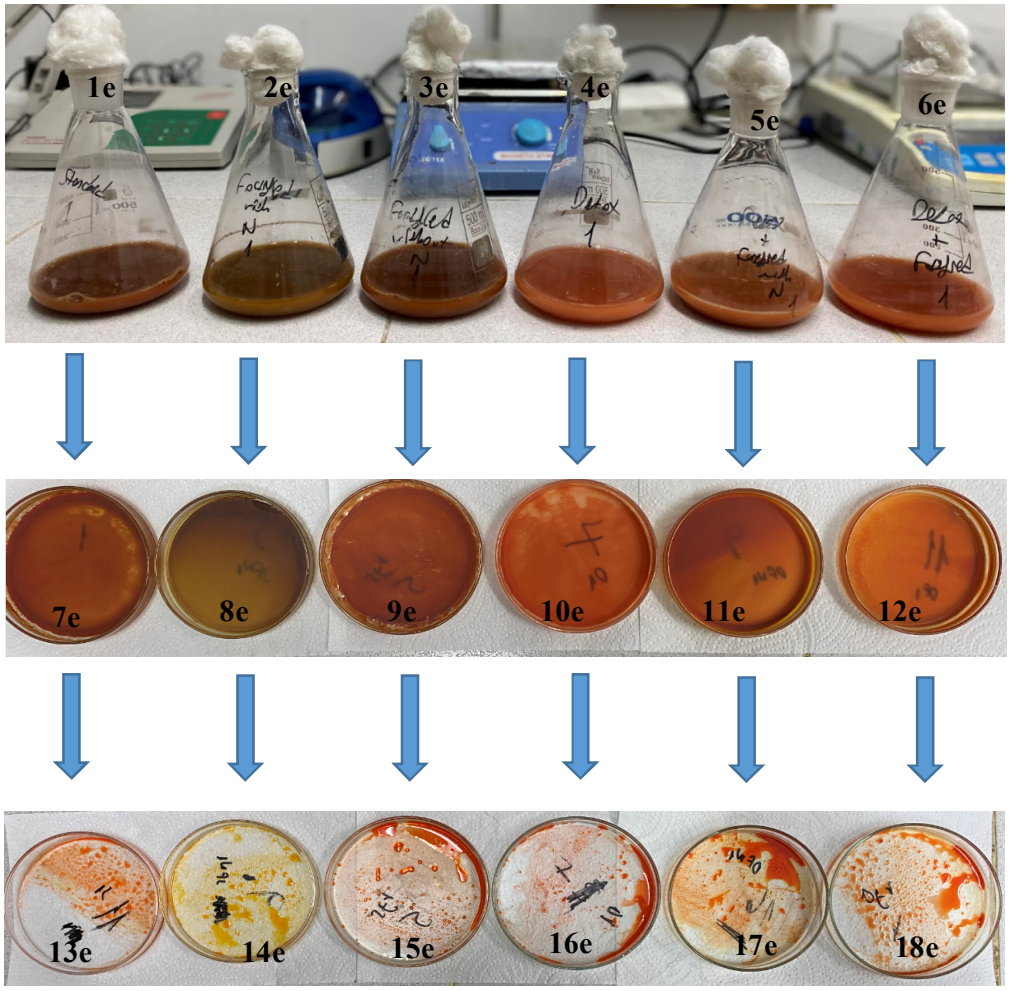

Figure E.1. The flow chart demonstrates the cell suspensions, dried biomasses, and extracted lipids. **1e**: S Medium, **2e**: SMN Medium, **3e**: SM Medium, **4e**: D Medium, **5e**: DMN Medium, **6e**: DM Medium.**7e**: Biomass from S, **8e**: Biomass from SMN, **9e**: Biomass from SM, **10e**: Biomass frım D, **11e**: Biomass from DMN, **12e**: Biomass from DM. **13e**: Lipid from S, **14e**: Lipid from SMN, **15e**: Lipid from SM, **16e**: Lipid from D, **17e**: Lipid from DMN, **18e**: Lipid from DM.

F. PICTURES OF 1 L FERMENTOR EXPERIMENTS

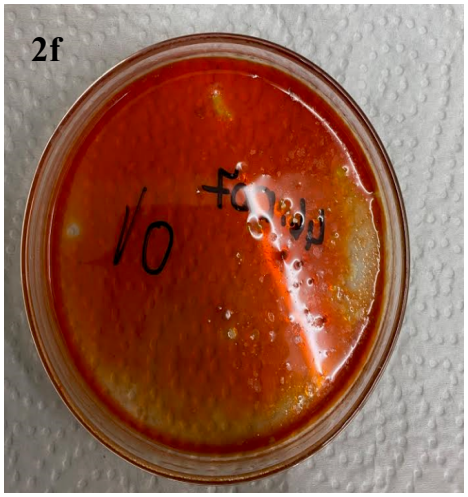

Figure F.1. Post fermentation views of the medium D and microbial lipid. **1f**: Post-fermentation view of D medium in 2 L fermentor prior to cell collecting. **2f**: Total microbial lipid that was extracted from the cells which were grown in 1 L D Medium in a 2 L volume fermentor.

G. PICTURES OF 10 L FERMENTOR EXPERIMENTS

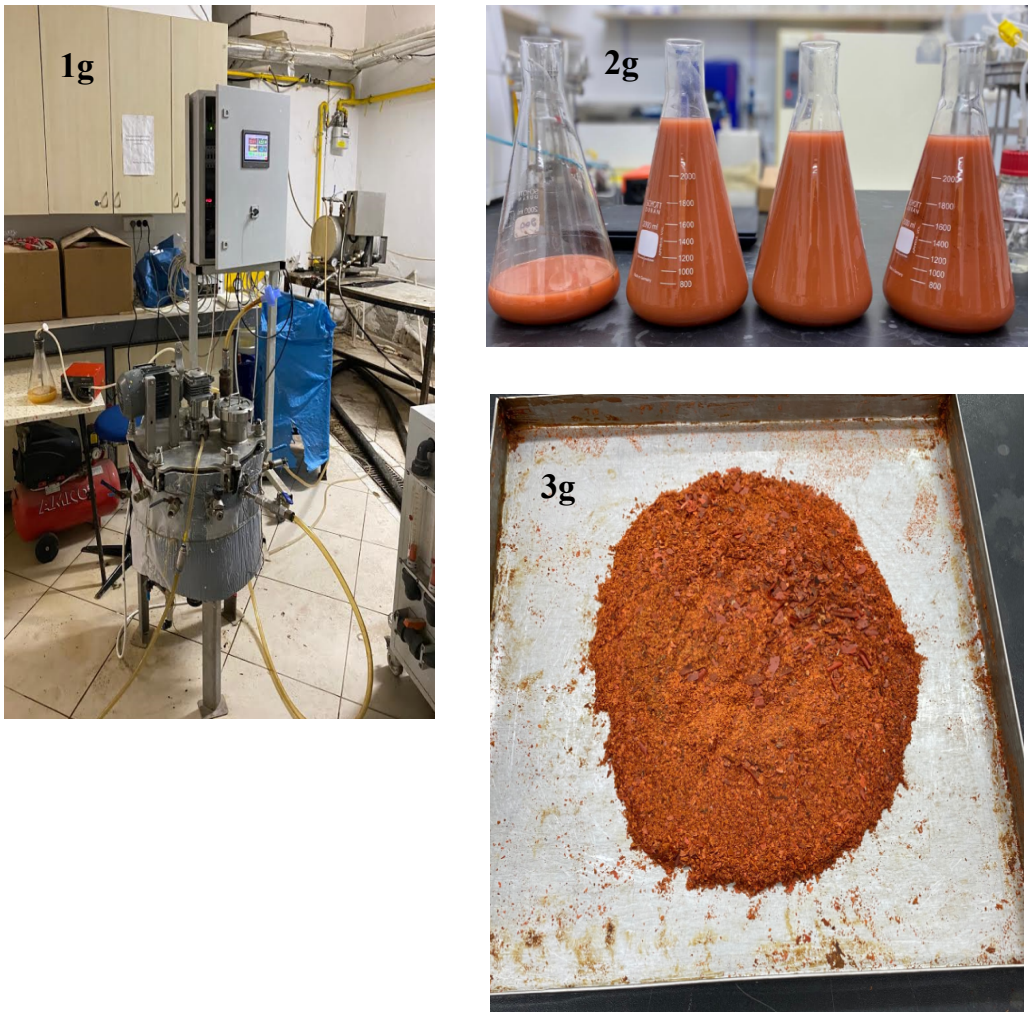

Figure G.1. Pictures of the 10 L scale up experiment. **1g**: 30 L industrial type fermentor with 10 L fermentation medium, **2g**: Post-fermentation view of D medium prior to cell collecting, **3g**: Total biomass collected from 10 L fermentation medium.

H. *L. STARKEYI* AND *R. TORULOIDES* IN SOLVENT MIXTURE

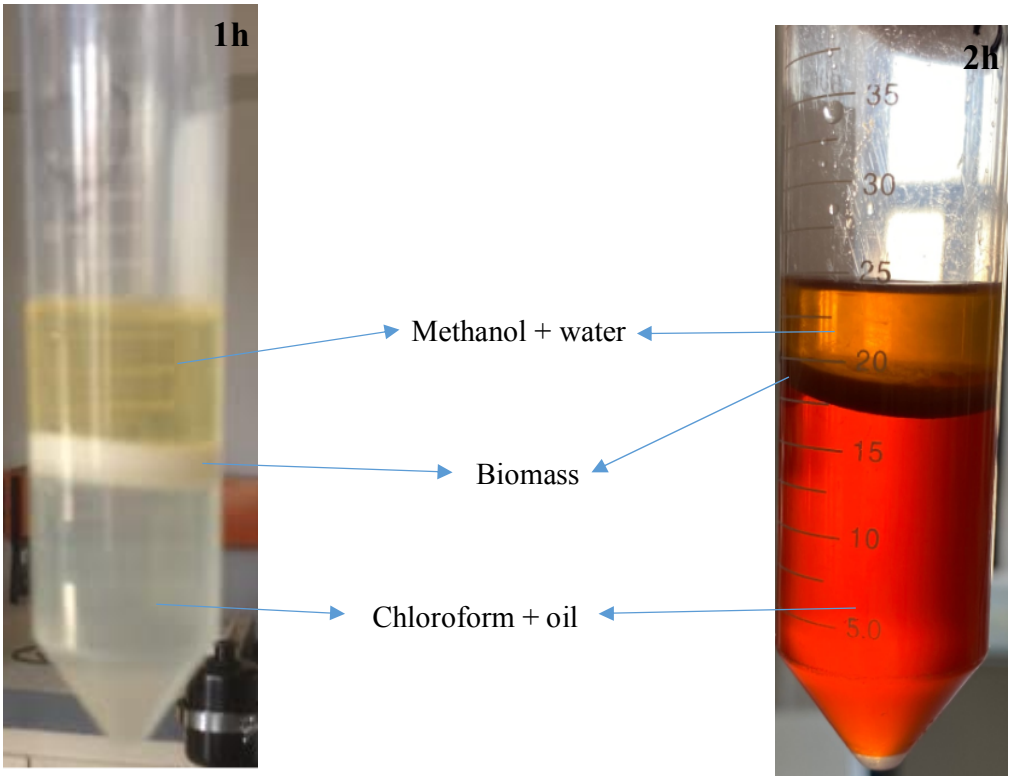

Figure H.1. Pictures of *L. starkeyi* (1h) and *R. toruloides* (2h) biomasses in solvent mixture after centrifugation.

I. GROWTH KINETICS OF *R. TORULOIDES* IN APPLE POMACE MEDIA

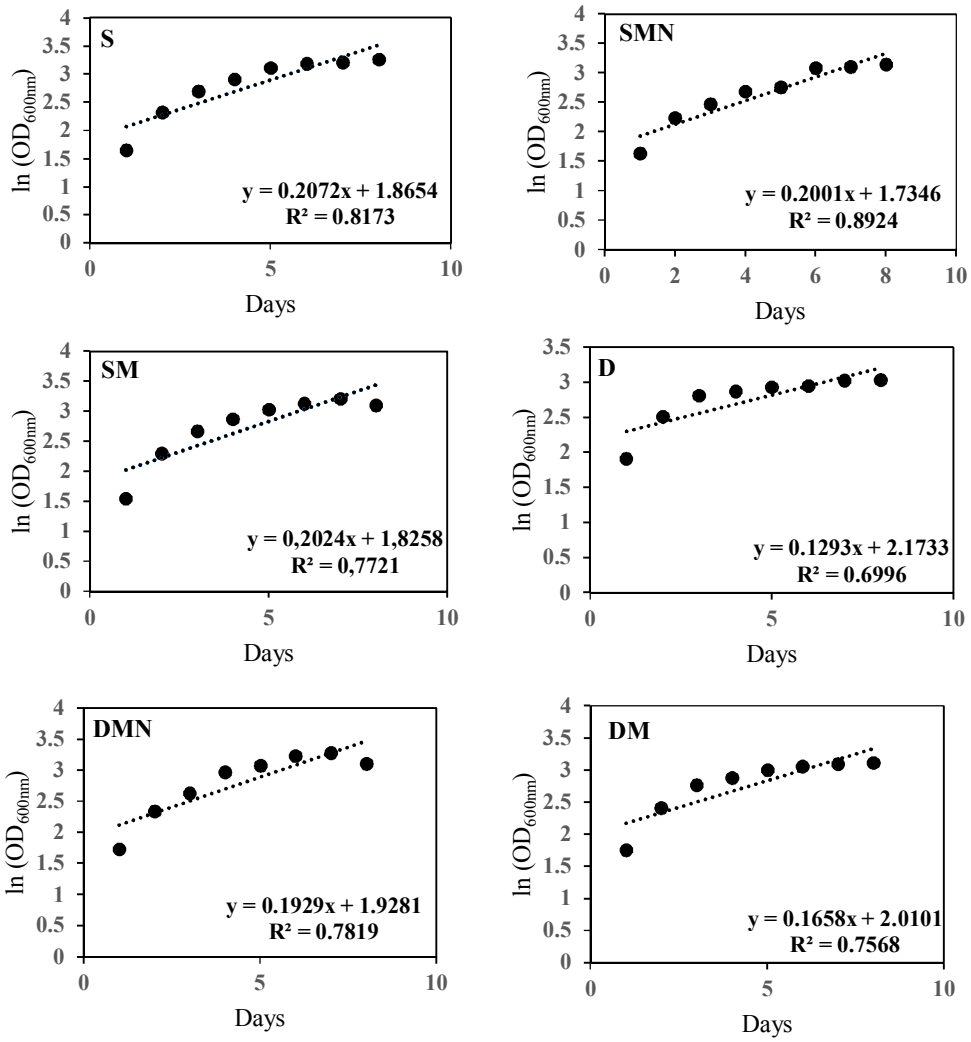

Figure I.1. Graphs used in the determination of specific growth rate of *R. toruloides* in apple pomace media. **S**: Standard medium, **SM**: Mineral added standard medium, **SMN**: Mineral and nitrogen added standard medium, **D**: Detoxified medium, **DM**: Mineral added detoxified medium, **DMN**: Mineral and nitrogen added detoxified medium.

J. GROWTH KINETICS OF *L. STARKEYI* IN SYNTHETIC MEDIUM

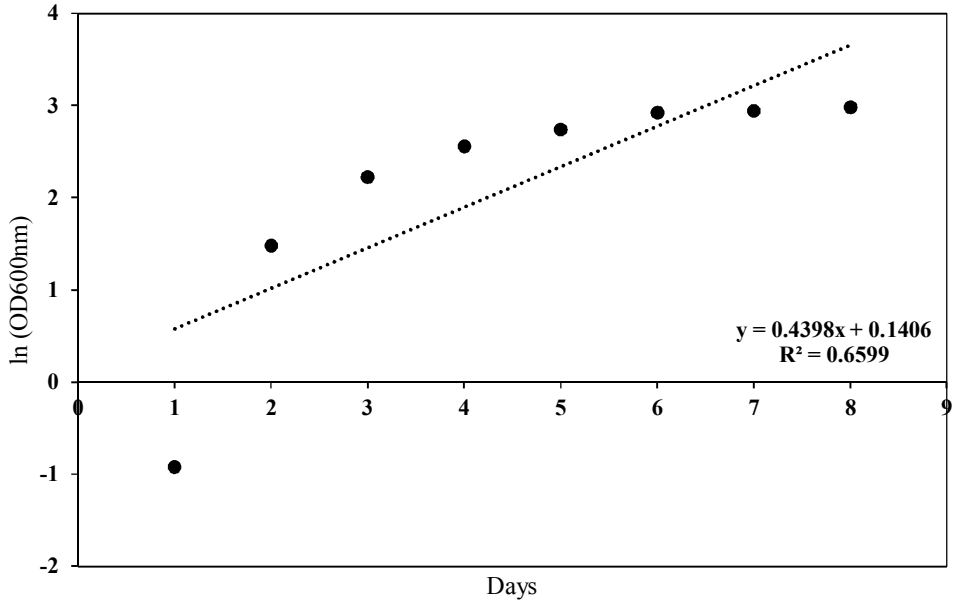

Figure J.1. Graph used in the determination of specific growth rate of *L. starkeyi* in synthetic medium.

K. GROWTH KINETICS OF *R. TORULOIDES* IN SYNTHETIC MEDIA

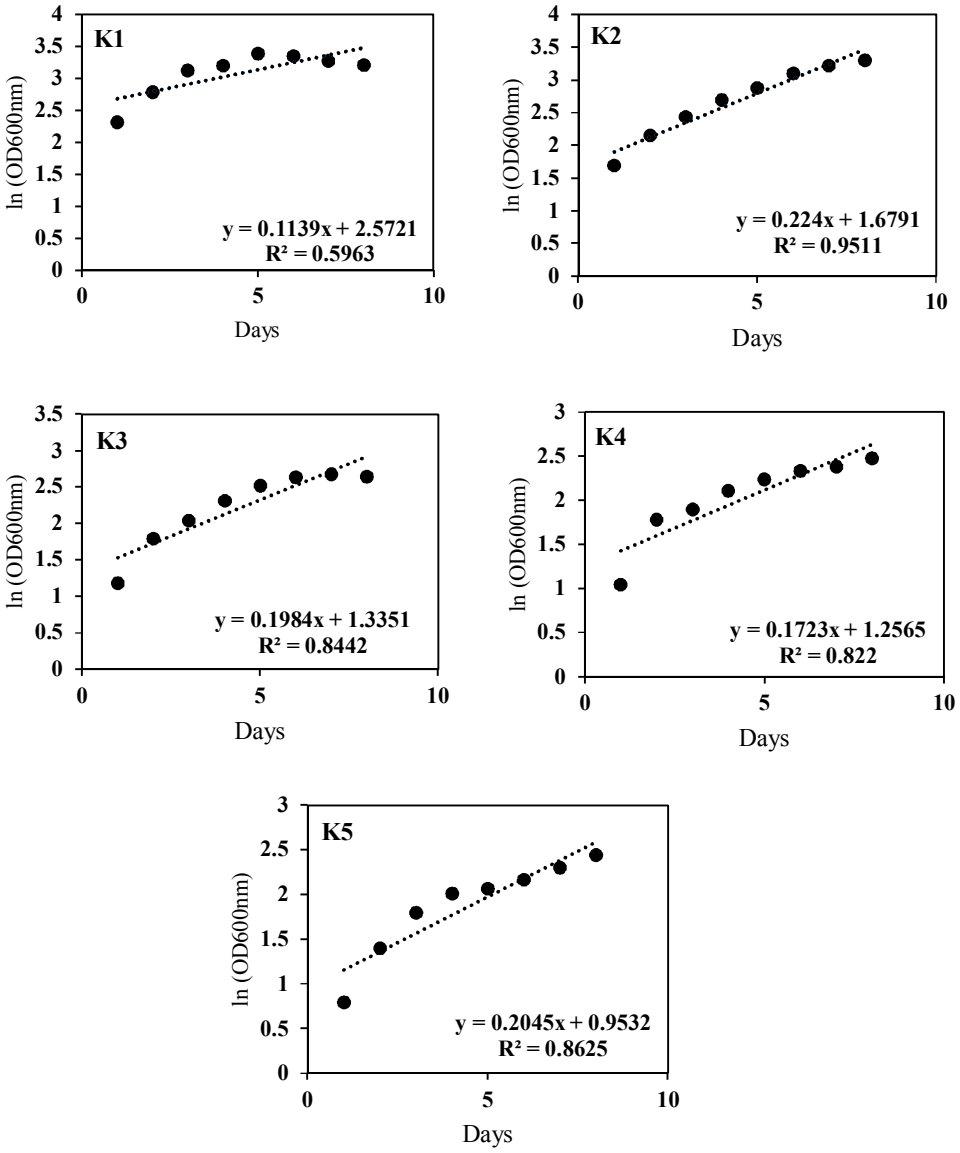

Figure K.1. Graphs used in the determination of specific growth rate of *R. toruloides* in 50 (**K1**), 100 (**K2**), 150 (**K3**), 200 (**K4**), and 500 mL (**K5**) synthetic media.

L. RESPONSE SURFACE REGRESSİON: lnYIELD VERSUS P; TEMP; TIME

Estimated Regression Coefficients for lnYield

Term	Coef	SE Coef	T	P
Constant	2.7196	0.05220	52.101	0.000
Block	0.0191	0.02447	0.780	0.445
P	-0.2615	0.03504	-7.464	0.000
Temp	0.1973	0.03361	5.868	0.000
Time	0.0620	0.03333	1.861	0.078
P*P	0.3385	0.04902	6.906	0.000
Temp*Temp	0.3383	0.04919	6.877	0.000
time*time	0.3752	0.04919	7.628	0.000
P*Temp	0.1666	0.04976	3.347	0.003

S = 0.1279 R-Sq = 93.2% R-Sq(adj) = 90.3%

Analysis of Variance for lnYield

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Blocks	1	0.00159	0.00994	0.009942	0.61	0.445
Regression	7	4.23992	4.23992	0.605703	37.05	0.000
Linear	3	1.77739	1.72691	0.575638	35.21	0.000
Square	3	2.27936	2.31985	0.773285	47.30	0.000
Interaction	1	0.18317	0.18317	0.183172	11.20	0.003
Residual Error	19	0.31062	0.31062	0.016348		
Lack-of-Fit	15	0.29300	0.29300	0.019533	4.43	0.080
Pure Error	4	0.01762	0.01762	0.004405		
Total	27	4.55213				

Estimated Regression Coefficients for lnYield Using Data in Uncoded Units

Term	Coef
Constant	14.5507
Block	0.0190797
P	-0.0295877
Temp	-0.300908
time	-0.287792
P*P	0.0000338495
Temp*Temp	0.00338331
time*time	0.0150100
P*Temp	0.000166564