EVALUATION AND OPTIMIZATION OF MICROBIAL OIL PRODUCTION USING SYNTHETIC SUGARS

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ABSTRACT

EVALUATION AND OPTIMIZATION OF MICROBIAL OIL PRODUCTION USING SYNTHETIC SUGARS

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Oleaginous microorganisms produce microbial oil that can be a solution to sustainable oil production. *Lipomyces starkeyi* DSM70295 and *Rhodosporidium toruloides* DSM4444 yeasts were chosen to produce microbial oil in this study.

Synthetic sugars, glucose, galactose,xylose, fructose and 1:1(w/w) combinations of sugars with glucose were used in fermentation medium. Nitrogen is an important criterion for yeast growth and lipid accumulation. To test effects of nitrogen on yeast growth and lipid production, C/N ratio of fermentation medium was at 50 and 110.

It was observed that both yeasts were grown more efficiently at 50 C/N ratio whereas lipid contents of yeasts were higher at C/N=110. Biomass, reducing sugar and lipid content analysis proved *Rhodosporidium toruloides* to be more efficient than *Lipomyces starkeyi* in oil production.

To determine optimum conditions, *Rhodosporidium toruloides* was grown in media with different C/N ratio and pH. The results of response surface method(RSM) gave the highest microbial lipid yield at C/N ratio of 125 and pH 5.0. Lipid content of *R.toruloides* was measured as 60.6%. The coefficient of determination (R^2=

97.0), insignificant lack of fit ($p=0.23>0.05$) showed that model fitted well to experimental data.

The results confirmed that *R.toruloides* was more efficient microbial oil producer than *L.starkeyi* under tested conditions with the highest lipid yield in glucose:galactose containing medium. In future study, food wastes such as wheycan be used as fermentation medium to reduce costs.

Keywords: *Lipomyces starkeyi*, *Rhodosporidium toruloides*, single cell oil, microbial oil

ÖZ

SENTETİK ŞEKER KULLANARAK ÜRETİLEN MİKROBİYAL YAĞIN DEĞERLENDİRİLMESİ VE OPTİMİZASYONU

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Yağ üreten mikroorganizmalar sürdürülebilir yağ elde etme çözümlerinden biri olabilen mikrobiyal yağı üretirler. Bu çalışmada *Lipomyces starkeyi* DSM70295 ve *Rhodosporidium Toruloides* DSM4444 mayaları mikrobiyal yağ üretimi için seçilmiştir.

Glikoz, galaktoz, fruktoz, ksiloz ve 1:1 oranında glikoz ve şeker kombinasyonları içeren fermantasyon ortamı hazırlanır. Azot, maya gelişimi ve lipit birikimi için önemli bir kriterdir. Azotun maya gelişimi ve yağ üretimine olan etkilerini test etmek için C/N oranı 50 ve 110'a ayarlandı. Her iki maya çeşidi C/N oranı 50 ve 110 olan fermantasyon ortamlarında geliştirildi. *Rhodosporidium toruloides* ve *Lipomyces starkeyi* mayalarının C/N oranı 50 olan ortamda daha verimli geliştiği ancak lipit kapasitesinin C/N oranı 110 olan ortamda daha yüksek olduğu anlaşıldı. Ayrıca biyokütle, indirgen şeker analizi ve lipit kapasitesi analizleri mikrobiyal yağ üretiminde *Rhodosporidium toruloides*'in *Lipomyces starkeyi'*den daha verimli olduğunu gösterdi.

Mikrobiyal yağ üretimine uygun koşulların belirlenmesi için *Rhodosporidium toruloides* farklı C/N oranı ve pH değeri olan fermantasyon ortamlarında geliştirildi. Mikrobiyal yağ üretiminde uygun koşulları görmek için yanıt yüzey yöntemi optimizasyonu yapıldı. Optimum koşullar C/N oranı 125 ve pH değeri 5.0 olarak bulundu. *Rhodosporidium toruloides* mayasının lipit kapasitesi uygun koşullarda 60.6±0.4 olarak bulundu. Hesaplanan varyasyon katsayısı (R^2= 97.0) ve önemsiz uyum eksikliği ($p=0.23>0.05$) modelin deneysel verilere uyumunu doğruladı.

Sonuç olarak bu çalışma *Rhodosporidium toruloides'*in mikrobiyal yağ üretim prosesinde kullanılan verimli bir maya çeşidi olduğunu göstermiştir ve *Rhodosporidium toruloides* en yüksek % lipid verimine glucose:galactose (1:1) içeren fermantasyon ortamında ulaşmıştır. İleriki çalışmalarda, gıda atıklarından peynir altı suyu fermantasyon ortamında kullanılabilir. Örneğin, peynir altı suyu glikoz ve galaktoz içerdiğinden fermantasyon ortamı olarak ilerleyen çalışmalarda kullanılabilir.

Anahtar Kelimeler: *Lipomyces starkeyi, Rhodosporidium toruloides, tek hücre yağları, mikrobiyal yağ*

To my family…

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CHAPTER 1

INTRODUCTION

Most of agricultural land is devoted to oil crop agriculture and this threatens biological diversity (Darcan & Sarıgül, 2016). Many types of plant and animal are exhausted due to production of unsustainable oils and these restricted resources cannot meet the increasing requirements in the long term. In recent years, there has been an increasing awareness in search of new oil sources. Among them, microbial oil also called single cell oil (SCO) has been considered as strong substitute of vegetable oils due to their similar fatty acid compositions (Huang, Zong, Wu, & Liu, 2009). Microbial oils are advantageous over plant oils as there is no need of any agricultural lands and long period for the production stage.

SCO is produced by the oleaginous microorganisms involving bacteria, fungi, and microalgae, which can produce and store lipids between 20-80 % of their biomasses under optimum conditions (Hong vd. 2006). Among all microorganisms, yeasts have been used for food commodities since ancient times and yeast oil would have high acceptability of consumers.

In several studies, biomass and lipid content of different microorganisms were compared. It has been understood from those studies that Rhodosporidium toruloides and Lipomyces starkeyi yeasts produce the highest lipid content (Li, Du ve Liu, 2008). Thus, Rhodosporidium toruloides and Lipomyces starkeyi were chosen for production of microbial oil in different fermentation media in this study.

Yeasts use nitrogen in their cells to grow up and increase their biomass. In nitrogen limited conditions, yeast cells stop growth and they start to produce lipids. Therefore, carbon to nitrogen ratio is of critical for SCO production and effects of C/N ratio on lipid production were also investigated in this study.

First of all, Lipomyces starkeyi and Rhodosporidium toruloides yeasts were used for Single Cell Oil production for 8 days. Biomass, sugar utilization and lipid content analysis were done to define the behavior of yeasts in synthetic fermentation media. Since Rhodosporidium toruloides was defined as more efficient than Lipomyces starkeyi, optimization experiments were conducted for microbial oil production using Rhodosporidium toruloides.

The optimization study was carried out by Central-Composite (CC) response surface methodology to determine the highest lipid yield using various values of pH and C/N ratio.

The objectives that support our aim are to analyse glucose, galactose, xylose, fructose and 1:1 combinations of them as substrate of yeasts, to optimize conditions of fermentation medium based on pH and C/N ratio, to indicate correlation between biomass, reducing sugar content, and lipid content at optimum conditions and to improve kinetic model on microbial oil production.

In this context, firstly a literature review was given about microbial oil. It is included the history of microbial oil, the factors affecting the microbial oil production and microbial oil pathway and the aim of this study (Chapter 2). The materials and methods were explained clearly and detailed information about all the experiments carried out (Chapter 3). Then, the results were analysed and interpreted (Chapter 4). Finally, the contribution of this study to microbial oil industry and outcomes were written (Chapter5).

CHAPTER 2

LITERATURE REVIEW

2.1 Microbial Oil History

Microbial oil is currently investigated as substitute of vegetable oils for biodiesel industry. Since it has very similar fatty acid compositions to plant oils, it was considered to be produced for food industry in our study. Furthermore, microbial oil contains important fatty acids such as omega-3 (alfa linolenic acid, ALA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) and omega 6 (linoleic acid, LA; gamma linoleic acid, GLA; arachidonic acid, ARA) which are necessary for human body. Omega 3 and omega 6 fatty acids are found in several plant and fish oils. However, rapid population growth, urbanization and decline of agricultural lands cause source of sustainable plant oil reduction. Separately, due to polluters like dioxin, heavy metals and polychlorin biphenyl (PCBs), safety of fishes is an argument between of scientists (Ochsenreither vd. 2016). For these reasons, microbial oil can be a good alternative for food products.

The first commercial microbial oil, which was called Javanicus Oil was produced by using *Mucor circinelloides* (*Mucor* javanicus) yeast in 1985. However, following years, Javanicus oil which is rich in gamma linoleic acid (GLA) did not compete with “Starflower Oil” because of having higher GLA content (Barre, 2009). In 1988, a Japanese company has got the patent of process that is higher in ARA and this process was used in infant food formula. However, microbial oil industry has reached its real commercial success in 21 century. For instance, microbial oil was used for enrichment of infant foods that are wanted to be rich in essential fatty acids (Ratledge, 2013).

Microbial oil industry has reached the success in infant formula in Europa, Australia and Far East countries. Before then, microbial oil has been used as cocoa oil substitute; however it couldn't compete with cocoa oil due to price reduction of it.

Microbial oil was also used as emulsion of milk and meat products to enrich with polyunsaturated fatty acids (Ochsenreither et al. 2016).

Compared to plant oils, microbial oil is advantages as a sustainable oil source due to its fast production, less labor needs, being independent of climate and no need of agricultural lands (Liu et al. 2017).

2.2 Microbial Oil Compositions

Microorganisms, which can accumulate lipids more than 20% of their dry biomass, are called oleaginous organisms (Ratledge and Wynn, 2002). The percentage can increase up to 70% under nitrogen limited medium. Oleaginous microorganisms store lipids by using carbon sources in cell vacuole in the form of triacylglycerols (Certik et al.1999).

Microbial oils are produced by several bacteria, microalgae, fungi, and yeasts. Among all, yeasts are safer for biotechnological and food processes. Oleaginous yeasts have many advantages over others such as being less affected by climate, consuming many sugars in their metabolism, being less affected by metal ions, and fast growth (Qin, Liu, Zeng, & Wei, 2017). Among these advantages, oleaginous yeasts increase biomass and lipid yield in small time. Table 2.1 shows the comparison of biomass and lipid yield% of some of the oleaginous yeasts.

Table 2.1. Comparison of microbial oil production on crude glycerol (Liu et al., 2017)

Strain	Biomass (g/L)	Lipid Yield% (w/w%)
Rhodotorula glutinis TISTR 5159	8.2	53.0
Rhodosporidium toruloides	19.2	47.7
Rhodosporidium diobovatum	14.1	50.3
Yarrowia lipolytica ACA-YC 5030	5.7	33.5
Trichosporanoides spathulata JU4-57	17.0	43.0
Candida sp. LEB-M3	16.1	56.6
L. starkeyi NRRL Y-11557	5.7	50.5
L. starkeyi DSM 70296	34.4	35.9
L. starkeyi AS 2.1560	21.1	35.7

Lipomyces starkeyi and *Rhodosporidium toruloides* species, which belong to yeast family, are generally known as good microbial oil producers. They exist in soil, atmosphere and wood pulp from conifers ("CBS Database"). They grow fast and produce high lipid yield. Because of their genome and transcriptome are easily sequenced and metabolic system are easily approachable, they are widely studied (Qin, Liu, Zeng, & Wei, 2017).

In recent years, microbial oil which is produced by oleaginous yeasts is used as potential alternatives for biodiesel sector due to their similar fatty acid composition to the plant oils. Microbial oil consists of C16 and C18 fatty acids esterified in the form of triacylglycerols (Christophe et al., 2012). Table 2.1 shows fatty acid compositions of *Rhodosporidium toruloides* and *Lipomyces* starkeyi yeasts grown in pure glycerol. It is obvious that both of the yeasts have high amount of polyunsaturated fatty acid ratio like plant oils.

Table 2.2. Microbial oil compositions of *R. toruloides* and *L.* starkeyi were grown in pure glycerol media (Signori et.al. 2016)

Yeast	Fatty Acids Composition (weight %)						
	C 14:0	**C 16:0**	**C 16:1**	**C 18:0**	**C 18:1**	**C 18:2**	**C 18:3**
R. toruloides	1.5	27.9	2.1	12.3	35.3	17.4	2.8
L. starkeyi	3.1	31.0	4.3	12.9	39.4	7.6	0.5

Microbial oil is produced in fermentation media which contain carbon sources, limited amount of nitrogen sources and various minerals. Yeasts grow and start to produce lipids inside of their cells. At the end of fermentation, yeasts were harvested and dried before extraction (Figure 2.1).

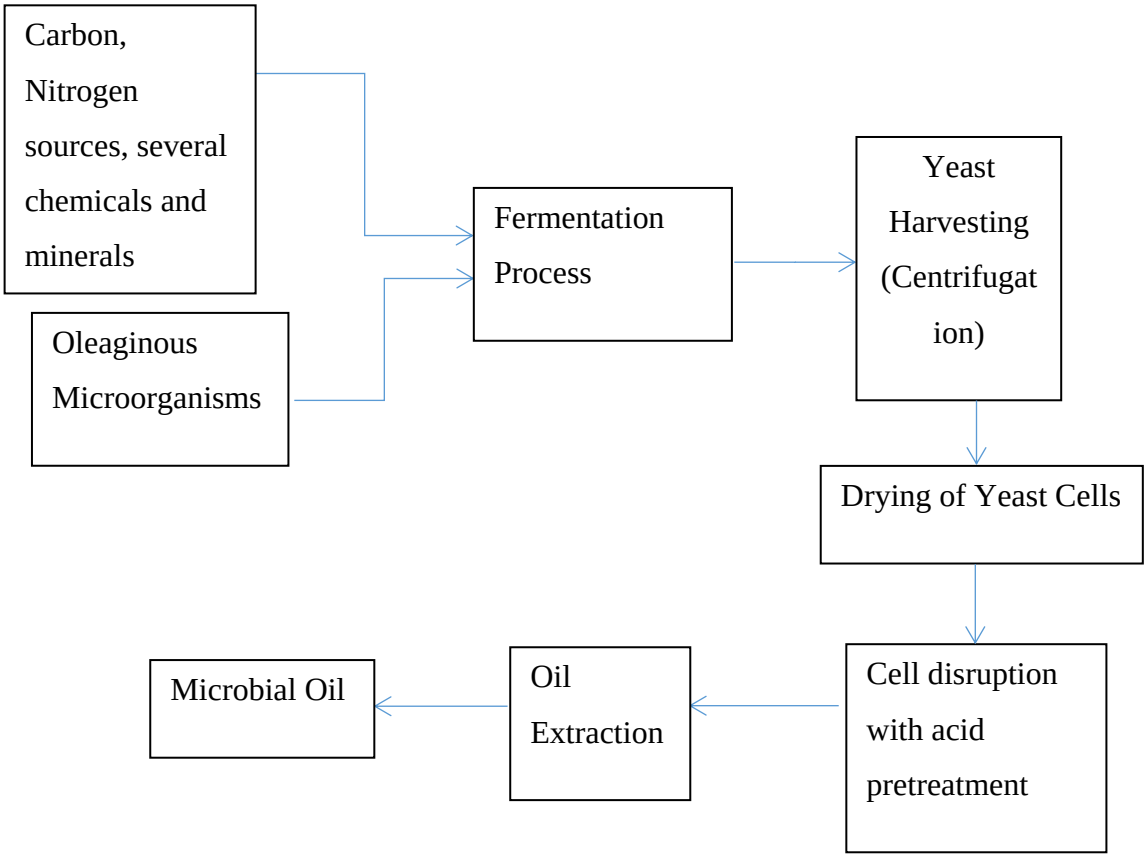

Figure 2.1 Schematic of Microbial Oil Production

As it is obvious from flow chart, microbial oil production process contains fermentation, centrifugation, drying, cell disruption and extraction steps. Microbial oil yields are affected by several factors such as C/N ratio, pH, temperature and aeration.

2.3 Factors that affect the microbial oil production

2.3.1 C/N Ratio

Carbon is essential for energy requirement of microorganisms and nitrogen is required for reproduction of cells. Oleaginous yeasts including *Lipomyces starkeyi* and *Rhodosporidium toruloides* produce and accumulate microbial oil under nutrient deficient conditions. As limiting nutrient, nitrogen is commonly used. Sugars (glucose, xylose, fructose, galactose, etc.) are transformed to fatty acids via tricarboxylic acid cycle in microbial oil production process. In order to maximize oil accumulation in yeast cells, carbon to nitrogen ratio (C:N ratio, $molmol^{-1}$) has to be under control.

It is a critical factor to increase oil production, cellular growth and prevent losses to secondary metabolites formation (Beopoulos et al., 2009). In limitation of nitrogen, oleaginous yeasts cannot use nitrogen for excess growth and, they start to store lipid in their cells. High lipid yields are best reached in nitrogen limited media (Lin et al., 2011).

Effects of C/N ratio on microbial oil production have been investigated in many studies. For example, in the study of Angerbauer et. al.(2008), the highest lipid content was calculated at a C/N-ratios of 150 as 68%, and reported only 40% of lipid content with C/N ratios of 60. Therefore, C/N-ratio is very important factor affecting microbial oil yield.

2.3.2 pH Value

Ph value of fermentation media is very important since microorganism cannot tolerate harsh conditions of pH value. However, yeasts can even grow in acidic

conditions and they are chosen to produce microbial oil in many studies. This is also a good advantage to reduce contamination risks by pathogenic bacteria.

Holdsworth and Ratledge (1988) worked with glucose at pH 5.5, and compared *L. starkeyi* with other organisms. It was reported that *Lipomyces starkeyi* and *Rhodosporidium toruloides* can even live in harsh conditions; however, they grow best in the range of pH 5.0 to 7.0. As it is obvious from Table 2.2 from the study of Angerbauer et. al.(2008), fermentation is carried out at different pH values.

Table 2.3. Lipid Production by *Lipomyces starkeyi* at different pH-values(Angerbauer et. al.,2008)

pH	5.0	5.5	6.0	6.5	7.0
Accumulated lipids (%)	56 ± 1	51 ± 1	50 ± 1	51 ± 1	7 ± 1
Lipid Yields% (w/w)%	7.5 ± 0.2	7.1 ± 0.2	5.6 ± 0.2	**7.7 ± 0.2**	1.1 ± 0.2

The highest lipid content was measured at pH 5.0 as 56± 1. The difference was not high in the range of 5.0 to 6.5. However, at pH 7.0 lipid accumulation has decreased. In the literature, different pH-values are studied for ideal lipid accumulation. An optimum pH value of fermentation medium for lipid accumulation by using *Lipomyces starkeyi* was found as 5.0 (Naganuma et al., 1985a).

2.3.3 Temperature

Like pH and C/N ratio, temperature is a very important criterion for growth and lipid production of yeasts. In one study, to observe the effect of temperature on yeast cell growth and microbial lipid production, several temperature values such as 25 °C, 28 °C, 30 °C and 32 °C were applied in fermentation (Liu et al., 2017). Although the biomass of yeast cells slightly increased as temperature increased from 25 ^{0}C to 30 ^{0}C, it was clear that temperature impact on cell growth and microbial lipid production was small on *Lipomyces starkeyi* and *Rhodosporidium toruloides.* The highest lipid yield was 5.7 g/L at 30°C. As the temperature increased to 32 °C, biomass and lipid production showed slight decrease. Therefore, the most effective temperature on *Lipomyces starkeyi* and *Rhodosporidium toruloides* is between 25 ^{0}C and 30 ^{0}C (Liu et al., 2017).

2.3.4 Aeration

Lipomyces starkeyi and *Rhodosporidium toruloides* grow under aerobic conditions (Anschau et al., 2014). Aeration is an important factor for yeast growth and lipid accumulation. To supply aerobic conditions for growth and lipid production, system is designed to give enough oxygen. Microorganisms get oxygen by diffusion. Therefore, by changing speed of shaking incubator or forced aeration, oxygen is supplied to fermentation process (Bari & Koenig, 2012).

Higher oxygen transfer rates were related with faster growth rates in fermenting yeast species (Su et al., 2014). There are several studies about effects of aeration on lipid production in yeast cells. For example, in the study of Anschau et al.,2014, *Lipomyces starkeyi* was cultivated in completely aired (1000 rpm) bioreactors. This culture stored only 25% lipid yield. In the same study, *L. starkeyi* accumulated 30% lipid content in (400 rpm). Lipid content and lipid yield at 300 rpm were

about 50% lower than the values at 200 rpm. The high aerated conditions cause less lipid production and the low lipid yields.

2.4 Microbial Oil Production Pathway and Lipogenesis

Microbial lipid production and accumulation can be expressed in two parts. First part is acetyl CoA production and second part is transformation of acetyl CoA into lipids. Lipid production scheme in yeast cell is shown in Figure 2.2. Glucose, which is obtained from glycolysis cycle causes proton-linked pyruvate to transport into the mitochondrion. Pyruvate decarboxylative dehydrogenase enzyme changes pyruvate to acetyl-CoA in mitochondria. Acetyl-CoA reacts with oxaloacetate to form citrate, which participate in Krebs cycle under oxygenated conditions. Reaction1 leads to ATP generation by means of electron transport chain in the membrane of mitochondria. Citrate are replaced with intracellular malate by the help of a citrate/malate translocase enzyme and disrupted by the ATP.

Reaction 1: Citrate + ATP + CoA →AcetylCoA + Oxaloacétate +ADP + Pi (Christophe et al., 2012)

In the second part of lipid production, Acetyl-CoA is transformed into palmitic acid that is the primer of long chain fatty acids in Reaction 2. Furthermore, Acetyl-CoA becomes primer of malonyl-Acyl carrier protein and acetyl-ACP. Acetyl-CoA Carboxylation by acetyl-CoA transacetylase or carboxylase resulted in Malonyl-CoA and by the help of malonyl transacetylase enzyme, Malonyl-CoA is relocated to an acyl carrier protein (ACP) with discharge of CoA. Malonyl-ACP adds C2 units to acetyl-ACP primer by acetyl group of acetyl-CoA transferation on an ACP with CoA releasing in Reaction 2. A β-ketoacyl-ACP synthase enzyme put in

malonyl-ACP C2-unit on an acetyl ACP and it is décarboxylated the malonyl group. Then, it results in an ACP releasing to make acetoacetyl-ACP. Acetoacyl-ACP reductase enzyme causes β- hydroxybutyryl-ACP by redevelopment of $NADP^+$. β-hydroxybutyryl-ACP dehydration by a β-hydroxyacyl-ACP dehydratase enzyme generates a crotonyl-ACP with regeneration of a NAD^+. Fatty acid chain extension is made by C2 unit addition of acyl group form from malonyl-ACP (Ratledge 2002; Ratledge and Wynn 2002).

The overall reaction 2: AcetylCoA + 7malonylCoA + 14NADPH + $14H^+$ → palmitic acid + $7CO_2$ + 8CoA + $14NADP^+$ + $6H_2O$

After all, palmitic acid is transformed into polyunsaturated fatty acids by the help of desaturase and elongase enzymes. These enzymes catalyze double bonds to fatty acid chain and add structure to C2-unit cycling.

In overall reactions, oleaginous yeasts have three critical enzymes that are not found in non-oleaginous yeasts. These are isocitrate dehydrogenase (IDH), ATP: citrate lyase (ACL) and malic enzyme.

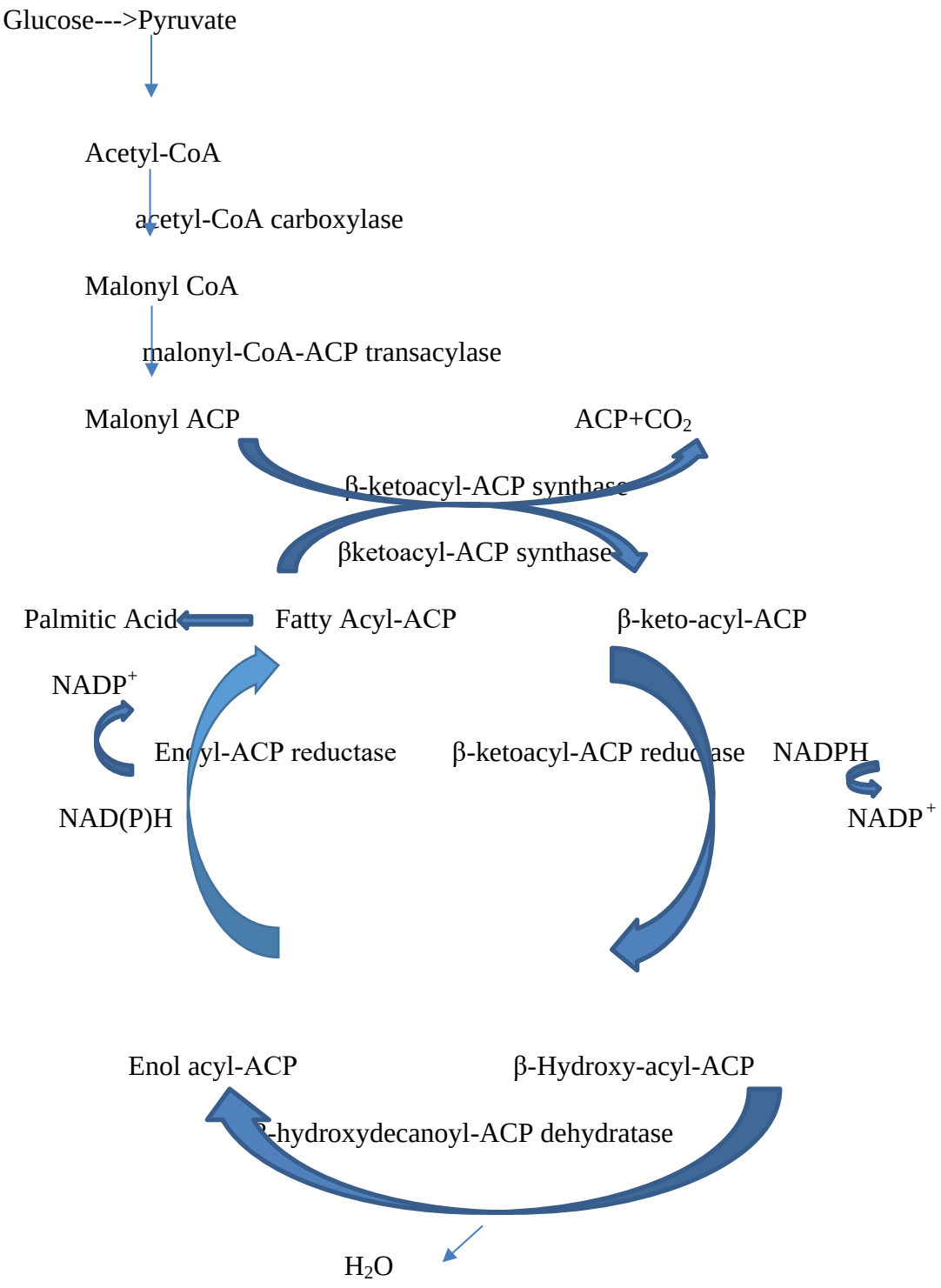

Fig 2.2 Schematic of Lipogenesis in yeast cell

2.5 The characteristic properties of microbial oil

Microbial oil is solid at room temperature and colour of microbial oil from *Lipomyces starkeyi* is yellowish. *Rhodosporidium toruloides* has microbial oil which has pigments like beta caroten and it has orange colour. The fatty acid composition of microbial oil is similar to plant oil. In the study of Liu et al.(2017), microbial oil contains mainly palmitic acid (27 %), stearic acid (15%), oleic acid (44%) and linoleic acid (11%) that are similar to those of plant oils. Unsaturated fatty acid amounts are found as above 55% of total fatty acids. Therefore, it can be used as alternative oil to plant oils.

2.6 Mass Balance for Microbial Oil Production

Yeast cells use carbon source for their cell growth and microbial oil production. Yeasts use 3 tons of sugar to produce 1 ton of microbial oil and they use about 2 tons of sugar for cell growth (Li, Liu, Zhao, & Bai, 2006). As a result, yeasts use 5 tons of sugars to produce 1 tons of microbial oil.

In this study, 4 g/100 ml of sugars were used in fermentation medium and around 800 mg of microbial oil were obtained in 100 ml of fermentation medium. It means that the microbial oil to sugar ratio is around 1:5. Therefore, the result of this study is compatible with literature data.

2.7 Aim of the study

Microbial oil is renewable and sustainable oil that similar to plant oils in terms of fatty acid compositions. Because of similarity to plant oils, it can be used in food industry. In order to obtain microbial oil, advantageous oleaginous yeasts such as

Lipomyces starkeyi DSM 70295 and *Rhodosporidium Toruloides* DSM 4444 are used to produce microbial oil.

The aim of this study is to obtain high microbial lipid yield% by using synthetic sugars such as glucose, xylose, fructose, galactose and 1:1 (w/w) combinations of them.

To achive this goal, the following objectives were applied;

1- Monitoring growth of *Lipomyces starkeyi* and *Rhodosporidium toruloides*

2- Growth of *Lipomyces starkeyi* and *Rhodosporidium toruloides* in several synthetic sugars containing fermentation medium

3- Comparison of microbial lipid yield% of both yeasts

4- Optimization of lipid yield% of ideal yeast

Biomass, sugar utilization and lipid content of yeasts give information about the growth and microbial lipid production capacity of yeasts. The purpose is to define the conditions which high amount of lipid content has been obtained.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Raw Materials and Chemicals

The chemicals and synthetic sugars for growth media and fermentation media were obtained from Sigma-Aldrich and Merck, Turkey.

All of the chemicals that are used in this study are listed in Appendix A.

3.1.2. Microorganisms, activation and growth media

Lipomyces starkeyi DSM70295 and *Rhodosporidium toruloides* DSM4444 yeasts were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany as freeze dried powdered form. Freeze dried microorganisms were firstly activated in liquid medium. The medium for *Lipomyces starkeyi* DSM70295 contained 3.0 g/L yeast extract, 3.0 g/L malt extract, 5.0 g/L peptone, 10.0 g/L glucose and distilled water ("German Collection of Microorganisms and Cell Cultures GmbH: Details”). On the other hand, medium for *Rhodosporidium toruloides* DSM4444 consists of 30.0 g/L malt extract, 3.0 g/L peptone and distilled water ("German Collection of Microorganisms and Cell Cultures GmbH: Details"). Both yeasts were activated in defined liquid media.

Activated microorganisms were taken into YEPD (yeast extract peptone dextrose) growth media to prepare seed cultures of each fermentation experiment. YEPD media contain 20 g/L glucose, 10 g/L bacto-peptone, 10 g/L yeast extract and 1000 ml distilled water (Angerbauer et al., 2008). They were prepared as 50 ml inside of 250 ml Erlenmeyer flask. As in the study of Angerbauer et al., growth condition was fixed at 5.0 pH, 25 °C, 130 rpm and 48 h. The pH of YEPD media were adjusted to 5.0 by using 10 M sulfuric acid and 10 M sodium hydroxide with distilled water (Wang, Wang, Xu, Fang, & Liu, 2014).

At the end of the 48 h, the yeasts have reached the expected optical density. All of the experiments were made at constant optical density(OD_{600}) after 48 h inoculum (Angerbauer et al., 2008).

3.1.3 Fermentation Medium

In order to start the fermentation, 3 mL of inoculum culture was mixed with 100 mL of fermentation medium in 500 mL Erlenmeyer flasks. Fermentation was carried out under 130 rpm at 25 ^{o}C for 8 days in a shaker incubator (Huang et al., 2013).

Fermentation medium compositions (C/N=50) were di-sodium phosphate (Na_2HPO_4) 1 g/L, ammonium sulphate $(NH_4)_2SO_4$ 0.5 g/L, magnesium sulphate heptahydrate($MgSO_4.7H_2O$) 2.5 g/L, potassium di-hydrogen phosphate (KH2PO4) 12.5 g/L, calcium chloride di-hydrate ($CaCl_2.2H_2O$) 0.25 g/L, yeast extract 1.9 g/L and carbon source 40 g/L (Angerbauer et. al., 2008).

Fermentation medium has different C/N ratios and they were achieved by changing the nitrogen source, ammonium sulphate and yeast extract amount. The pH of fermentation media was arranged by using 10 M NaOH and/or 10 M Sulfiric acid.

Monosaccharides and 1:1 (w/w) combinations of them were used in fermentation media as shown in Table 3.1.

Table 3.1. Monosaccharides that are used in fermentation media

Flask Number	Monosaccharide Content (40 g/L)
1	Glucose
2	Fructose
3	Xylose
4	Galactose
5	Glucose+Fructose
6	Glucose+Xylose
7	Glucose+Galactose

Carbon and nitrogen are significant nutrients for growth and lipid production of yeast cells. Carbon is required for energy (ATP synthesis) and nitrogen is essential for cell production. Therefore, C/N ration of fermentation medium was calculated and some of the experiments were done to investigate the effect of C/N ration on growth and lipid production of yeast cells.

3.1.4. Solutions

Solutions that are used in the study are written in Appendix B.

3.2 Methods

3.2.1 Culture Activation and Seed Culture Preparation

Media were prepared as 5 ml in several glass tubes and were sterilized in autoclave at 121 ^{o}C for 15 minutes. When it is cooled down to 25 ^{o}C, powdered form freeze-dried yeasts were added into them and they were shaked to make homogenous disperse for 30 minutes. End of this duration, they were divided into several tubes which consist of 5 ml sterile liquid media and they were incubated at 25 ^{o}C and 130 rpm for 24 hours in shaking incubator to form stock cultures.

Microorganisms were grown in YEPD medium for 48 hours to prepare.Their optical densities were measured at 600 nm by spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan) and they were subcultured twice a month for storage at 4 ^{0}C, -20 ^{0}C and -80 ^{0}C.

3.2.2 Cell density

Cell density was obtained by measuring optical density at 600 nm (OD_{600}) absorbance by using spectrophotometer (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan). All the experiments were conducted at constant OD for good incubation in fermentation process (Vyas et al., 2016).

3.2.3 Inoculum preparation

Growth culture was autoclaved at 121°C for 15 min for sterilization. After cooling to room temperature 1 ml of yeast culture from either microorganism was added to growth culture in Erlenmeyer flasks and, incubated at 25°C, pH: 5.0, 130 rpm in the incubator (INFORS AG CH-4103, Bottmingen, Switzerland) for 48 hours.

3.2.4 C/N Ratio Determination of Fermentation Medium

To investigate C/N ratio of fermentation medium, carbon and nitrogen determinations of medium are done separately. Since carbon amount of yeast extract is negligible, carbon determination of media is a simple calculation. The only carbon source of medium is assumed as synthetic sugars (Kim et al., 2019). The molecular weights of synthetic sugars are known and the carbon amount is calculated easily. Nitrogen sources of fermentation medium are ammonium sulphate $(NH_4)_2SO_4$ and yeast extract. Although the molecular weight of inorganic ammonium sulphate is known for simple nitrogen determination, molecular weight of organic yeast extract is not known. Therefore, nitrogen content of yeast extract was determined by Kjeldahl method (Labconco Corporation, 2005).

The Kjeldahl method contains three main steps such as digestion, distillation and titration. Digestion is the step of breakdown of nitrogen in yeast extract by the help of heat and sulfuric acid solution. At the end of digestion, an ammonium sulphate solution has been occurred according to reaction;

Organic N+ H_2 $SO_4 \rightarrow (NH_4)_2$ SO_4 + H_2O+CO_2 + by products (Labconco Corporation, 2005)

In digestion process, 1 gram of yeast extract was put into Kjeldahl flask, 15 ml of H_2SO_4, 5 gram of K_2SO_4 and $CuSO_4$ were added into tubes. After all, the flasks were brought to digestion unit for 2 hours at 380 - 400°C until to get green colored solution (Labconco Corporation, 2005).

After digestion, distillation step starts. This is for convertion of NH_4 to NH_3 and it can be written as;

$$(NH_4)_2\ SO_4 + 2NaOH\ heat \rightarrow 2NH_3 + 2Na_2SO_4 + 2H_2O$$

The flask was taken into the Kjeldahl apparatus and a 50 ml of 0.1 N HCl solution with methyl red was added into receiving unit. The condenser tip is immersed in acidic delivery solution. Around 75 ml of % 40 NaOH was added to obtain the color change (Solutions were listed in Appendix D). The below reaction helps to understand the procedure;

$$2NH_3 + 2HCl \rightarrow NH_4Cl + \text{excess acid}$$

The final step is titration which consists of quantifying the ammonia in the receiving solution. The nitrogen amount in a sample can be calculated from the ammonia ions amount in the receiving solution.

The final mixture (ammonium chloride + excess hydrochloric acid) was titrated with 0.1 N NaOH solution to the pink colour turned to yellow:

$$NH_4Cl + HCl + NaOH \rightarrow NaCl + NH_4Cl + H_2O$$

NaOH consumption amount was calculated and by using the value of that nitrogen amount was calculated as follows ;

$$N\ \% = (\text{moles of HCl} + \text{moles of NaOH}) \times 1.4\ W\ \text{dry sample}$$

3.2.5 Microbial oil production

Since carbon and nitrogen ratio of fermentation medium is important for growth of yeasts, the C/N ratio was predetermined and adjusted as required. The C/N ratio of typical fermentation medium was calculated as 50 by using Kjeldhal method (Labconco Corporation, 2005) and this was considered as control. According to several studies, experiments were done at C/N ratio equals to 110 (Angerbauer et. al.(2008)). Therefore, fermentation trials were carried out in the range of 50 to 110.

Fermentation media were autoclaved at 121 C^{o} for 15 minutes in autoclave (Autoclave, MaXterile 60, Wisd laboratory Instruments) and inoculated with 3.1 ml of culture (3% amount of fermentation media). *Lipomyces starkeyi* and *Rhodosporidium toruloides* were grown in synthetic sugars combinations of media at 25^{o}C, pH 5.0, and 130 rpm for 192 hours (8 days) and cell density was measured periodically (Shimadzu UV-1700, Shimadzu Corp., Kyoto, Japan) (Table 13 & 14).

3.2.6 Downstream Processes

3.2.6.1 Yeast Harvesting (Centrifugation)

At the end of the fermentation, all media were centrifuged at 8720xg (10000 rpm) (Sigma 2-16PK, Germany) for 10 minutes to collect yeasts, which were consequently washed twice with distilled water (Zhao et al., 2008). The yeast cells were accumulated at the bottom of centrifugation tubes and supernatant were removed by washing with distilled water (Huang et al., 2013).

3.2.6.2 Drying of yeasts

After centrifugation, yeast cells were put into petri glasses and dried at 70 ^{o}C for 24 h in drying oven (Oven ST-120, Şimşek Laborteknik Ltd. Şti). Dry weight of cells were calculated using the formula;

Dry weight of yeast cells= Dry yeast cells in petri glasses – Empty petri glasses

3.2.6.3 Acid hydrolysis, cell disruption and oil extraction

Since microbial oil is produced and stored inside the yeasts cells, dry yeast cells were disrupted with 5 ml of hydrochloric acid solution (1M) and boiled at 100 ^{o}C in hot water bath for 1 hour to take out lipids from cells (Tapia V, Anschau, Coradini, T Franco, & Deckmann, 2012).

Hydrochloric acid pretreatment helps to obtain microbial oil from cells and then they are mixed with hexane and extracted at 70 ^{o}C for 2 hours in the Soxhlet-apparatus (PI-400, Şimşek Laborteknik Ltd. Şti).

Soxhlet extraction is a conventional method to extract lipid in the system. An organic solvent such as hexane in this study was used to solubilize and extract lipids from yeast cells (Nafiu, Hamid, Muritala, & Adeyemi, 2017).

Microbial oil extraction does not completely finished at Soxhlet apparatus because of presence of n-hexane in microbial oil. The mixture of hexane and microbial oil was evaporated at 70 ^{o}C in drying oven for 24 hours to be sure there is no hexane in it("Soxhlet Extraction Method- Estimation Of Fat," 2017).

The lipid yield was calculated using the formula;

Lipid yield (%, w/w) = [Microbial oil amount (g/L) / Dry weight of yeast cells (g/L)]*100

3.2.7 Total reducing sugar

Total reducing sugar analysis is a method to measure initial and final fermentable sugar concentration of fermentation media and reducing sugar content is related with yeast growth rate and microbial oil production rate. Thus, total reducing sugar amount was analysed in this study.

The Dinitrosalicylic (DNS) Colorimetric method which qualify the existence of free carbonyl group (C=O) as reducing sugar source was used to calculate total reducing sugar content (Miller, 1959).

DNS reagent was used as fresh and is described in Appendix B.

In order to obtain standard curve for reducing sugar amount; glucose was used as reducing sugar standard at six different values (0.10, 0.20, 0.40, 0.60, 0.80 and 1.0 g/L); in distilled water.

Standard curve of DNS method is shown in Appendix D.

In DNS reagent method, to measure the initial and final fermentable sugar concentration, fermentation media samples were centrifuged to get cell free fermentation media (MPW-15 Mini Centrifuge, MPW Med. Instruments Co., Warsaw, Poland). The DNS solution for reducing sugar analysis is given in Appendix B.

In DNS method; 3 ml of fermentation medium was mixed with 3 ml of 3,5-dinitrosalicylic acid (DNS) and placed into 100 ^{o}C water bath for 15 minutes. The

colour turns to the brownish colour in this duration. To hot tubes 1 ml of 40% Rochelle salt was added and were cooled to room temperature. The absorbance of samples was measured with spectrophotometer at 575 nm wavelength.

3.2.8 Statistical Analysis

Statistical analyses were done by using MINITAB 16.0 to test the importance of pH and C/N ratio of fermentation media . Response surface methodology is a good way to study the effects of the factors and to reach maximum levels of variables (Deka et al., 2013). Central-Composite design of response surface methodology was employed for microbial oil production in different fermentation media. The pairwise comparisons were made by Tukey's test with a significance level of 0.05.

3.3. Optimization of microbial oil production by response surface methodology (RSM)

To increase the efficiency of the microbial oil production process by increasing the lipid yield% while changing C/N ratio and pH of fermentation medium RSM optimization was carried out using *Rhodosporidium toruloides*. Microbial oil accumulation is known to be highly affected by C/N ratio of media. Several studies were conducted on the microbial oil production process of oleaginous yeasts, whereas some of them were published about oil production capacity of yeasts in different synthetic sugars and different C/N ratio containing fermentation media. One of the critical stages in microbial oil production is to find suitable carbon and nitrogen sources. Carbon and nitrogen sources of fermentation media should be

arranged according to utilization by oleaginous yeasts to grow and produce microbial lipids.

Response surface methodology (RSM) is used to obtain the relations between parameters by offering a few numbers of experiments (Haaland, 1989).

Response surface methodology has a collection of statistical techniques. Interactions between several variables can be identified with fewer experimental trials by the help of RSM (Tepe and Dursun, 2014). The series of experiments were designed using Minitab® 16.1.1 software (Minitab Inc., State Collage, PA, USA). The RSM with Central-Composite design was created by 2 parameters (pH and C/N ratio of fermentation medium) with 3 levels (Table 3.15). The response was the lipid yield as measured by ratio of microbial oil and dry yeast cell amounts.

A set of 13 experiments was constructed in two replicates (Table 3.16). Therefore, twenty-six experimental run were carried out with 10 centre runs. The effects of two variables were analysed.

Table 3.2. Range of variables for Central Composite design for microbial oil production

Variable	**Low Level (-1)**	**Centre (0)**	**High Level(+1)**
pH	3	5	7
C/N	50	110	170

Table 3.3. Experimental Design Matrix of CC design for microbial oil production

RUN ORDER	**pH**	**C/N**
1	3	50
2	5	110
3	5	170
4	3	170
5	7	110
6	5	110
7	5	110
8	5	110
9	3	110
10	5	50
11	7	50
12	7	170
13	5	110

Minitab® 16.1.1 with 95 % confidence level optimized experimental designs with two replications. ANOVA and regression were done.

Experimental data were fit to the quadratic equation.

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{12} X_1 X_2 + b_{11} X_1^{\ 2} + b_{22} X_2^{\ 2}$$

where Y is response (lipid yield%), b's are regression coefficients, and

X_1, X_2 are C/N ratio and pH respectively.

ANOVA and regression were performed at 95% confidence interval to describe the predictive model and significant factors. The ideal conditions for increasing lipid yield% was found by using Response Optimizer tool in MINITAB 16.0. The differences between predicted and experimental data was evaluated by root mean square error (RMSE) and mean absolute error (MAE) values as written in the following equations;

$$\mathbf{RMSE}= \frac{\sqrt{1}}{N} * \left(\sum_{i=1}^{N}[(P)i - (O)i]^2\right)$$

$$\mathbf{MAE}= \frac{1}{N} * \sum_{i=1}^{N} |(P)i - (O)i|$$

where *Pi* and *Oi* are predicted and experimental lipid yield values respectively, and *N* represents the number of data points.

The reproducibility of the verification experiments (CV) were calculated as follows;

$$\mathbf{CV}= \frac{\sigma}{\bar{X}} * 100$$

where σ is standard deviation and $\bar{X}$ is sample mean.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Growth curve of *Lipomyces Starkeyi* DSM70295 and *Rhodosporidium Toruloides* DSM4444

Two yeast strains, *Lipomyces starkeyi DSM70295* and *Rhodosporidium toruloides DSM4444* were grown on YEPD (yeast extract peptone dextrose) for two days. One mililiter of seed culture were inoculated to fermentation media which contain various sugars such as glucose, xylose, fructose, galactose and 1:1 (w:w) combinations of them at C/N 50 and C/N 110 and incubated at 25 °C and 130 rpm for 8 days (Akhtar, Gray, & Asghar, 1998).

Optical densities of fermentation media were measured every day and growth behaviour of two yeasts were obtained. Fig 4.1 and Fig 4.2 show growth of *Lipomyces starkeyi* and *Rhodosporidium toruloides* respectively.

4.1.1. C/N ratio of 50

Lipomyces starkeyi shows different growing behaviour in various synthetic sugars. Optical density of *Lipomyces starkeyi* increases between day 1 and day 6; however, it reaches constant level between day 6 and 8. Optical density of *Lipomyces starkeyi* reaches to the highest value as 29.2 ± 0.3 in fructose containing fermentation media at C/N ratio of 50. It has the least optical density as 19.1 ± 0.2 in xylose containing media under same conditions at 25 C^o and 130 rpm.

Lipomyces starkeyi grows higher in fructose and glucose-fructose containing fermentation media than xylose containing media.

In the literature, optical density of *L. starkeyi* reaches to the highest value as 12.4 in glucose containing media and the least amount as 10.4 in xylose containing media at day 4 (Wang, Wang, Xu, Fang, & Liu, 2014). In this study, optical density of *L.starkeyi* is 10.0 in xylose containing fermentation media and 17.5 in glucose containing media at day 4. The results of this study and literature match each other.

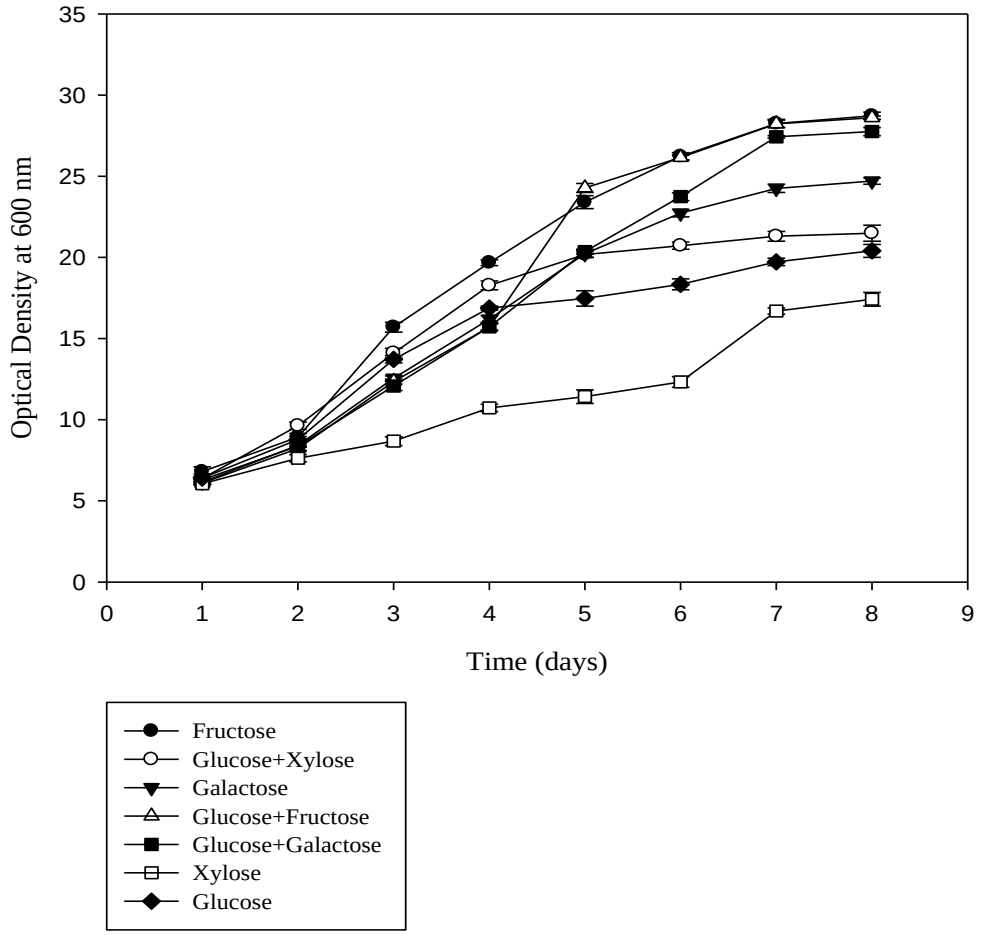

Figure 4.1. Growth of *Lipomyces starkeyi* in fermentation media that contain different sugars at C/N=50

In the study of Zhu et al., excess amount of glucose, 70 g/L, is used in fermentation media. The biomass has reached to 12.2 g/L at day 4. The biomass has reached to 16 g/L in fermentation process in 40 g/L of glucose. The excess amount of substrate affected growing behaviour negatively. In another study, different amounts of glucose have been used as substrate and *Rhodosporidium toruloides* showed the highest optical density in 40 g/L of glucose containing media (Li et al., 2007).

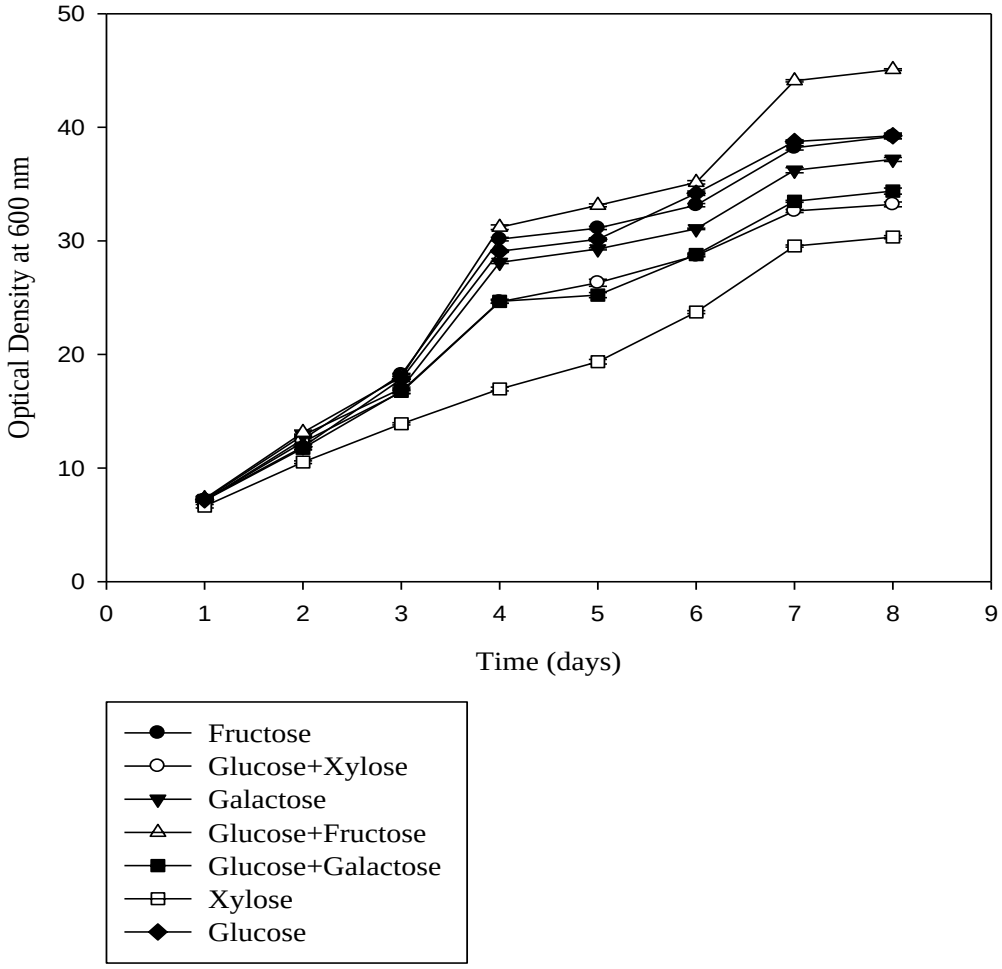

Figure 4.2. Growth of *Rhodosporidium toruloides* in fermentation media that contain different sugars at C/N=50

Rhodosporidium toruloides grows differently in various synthetic sugars. Optical density of *Rhodosporidium toruloides* shows increment between day 1 and day 6; however, it reaches constant values between day 7 and 8. The optical density of *Rhodosporidium toruloides* is the highest as 45.1±0.1 in glucose:fructose (1:1)(w/w) containing media at C/N ratio of 50 and the least as 30.3±0.2 in xylose containing media. *Rhodosporidium toruloides* yeast cells use glucose and fructose (1:1) more than xylose for growth.

According to results, although *Lipomyces starkeyi* grows high in fructose containing media and *Rhodosporidium toruloides* grows well in glucose-fructose (1:1) (w/w) containing fermentation media, they both grow poor in xylose containing fermentation media.

Rhodosporidium toruloides grows faster and to higher OD_{600} values than *Lipomyces starkeyi* in every condition tested in this study. They both reached their stationary phase at 7 to 8th day of their fermentation.

4.1.2. C/N ratio of 110

The carbon to nitrogen ratio of the fermentation medium is critical for microbial lipid production in yeast cells (Akhtar et al., 1998). To understand the effects of nitrogen limitation to biomass and lipid yield in *Lipomyces starkeyi* and *Rhodosporidium toruloides*, sugar of fermentation media was arranged as 40 g/L and C/N ration of 50/1 and 110/1 by changing amount of ammonium sulphate $(NH_4)_2SO_4$ and yeast extract.

The biomasses of *Lipomyces starkeyi* and *Rhodosporidium toruloides* were measured daily for C/N ratio of 110 to understand the effects of higher C/N ratio on yeast growth

and lipid production capacity. Figure 4.3 and Figure 4.4 show optical density of *Lipomyces starkeyi* and *Rhodosporidium toruloides* in various sugars containing fermentation media. It is obvious that both of the yeasts grow faster and higher OD in C/N ratio of 50 with respect to C/N ration of 110. Therefore, it can be said that higher C/N ration affect yeasts growth negatively.

It has been investigated that high nitrogen concentration in the culture medium develops biomass production; however, decreases the microbial oil synthesis (Moreton 1988). In nitrogen limitation conditions, oleaginous yeast cells produce microbial lipids well in their cells.

Optical density of *Lipomyces starkeyi* increases fast until day 6 and reaches the constant between day 6 and 8 at C/N ratio of 110 fermentation media. *Lipomyces starkeyi* has the highest optical density as 28.6 ±0.1 in fructose containing media and the least optical density as 17.4±0.6 in xylose containing media.

In the study of Liu et al., (2017), microbial oil was produced by *Lipomyces starkeyi* in crude glycerol containing fermentation media which have C/N ratio of 40, 60, 80, 110 and 140. The maximum optical density of yeast cell is obtained as 39.0 ±0.1 in C/N ratio of 60 and the minimum optical density of yeast cell is obtained as 13.8 ± 0.0 in C/N ration of 140. The biomass of yeasts decreases with increasing C/N ratio. The literature data and our study are compatible.

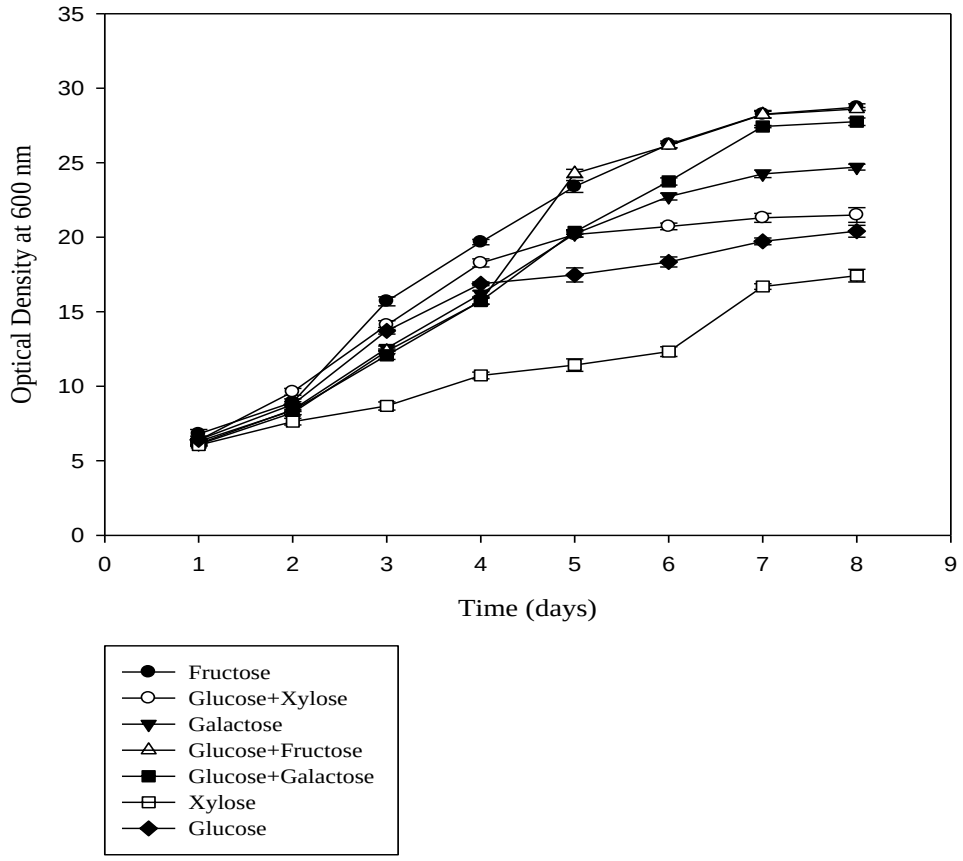

Figure 4.3. Growth of *Lipomyces starkeyi* in fermentation media that contain different sugars at C/N=110

Similar to *Lipomyces starkeyi*, *Rhodosporidium toruloides* grows fast until day 7 and stays constant between day 7 and 8.

Rhodosporidium toruloides has the highest optical density as 43.7±0.2 in glucose:fructose (1:1) (w/w) containing media and the least optical density as 27.3±0.4 in xylose containing media.

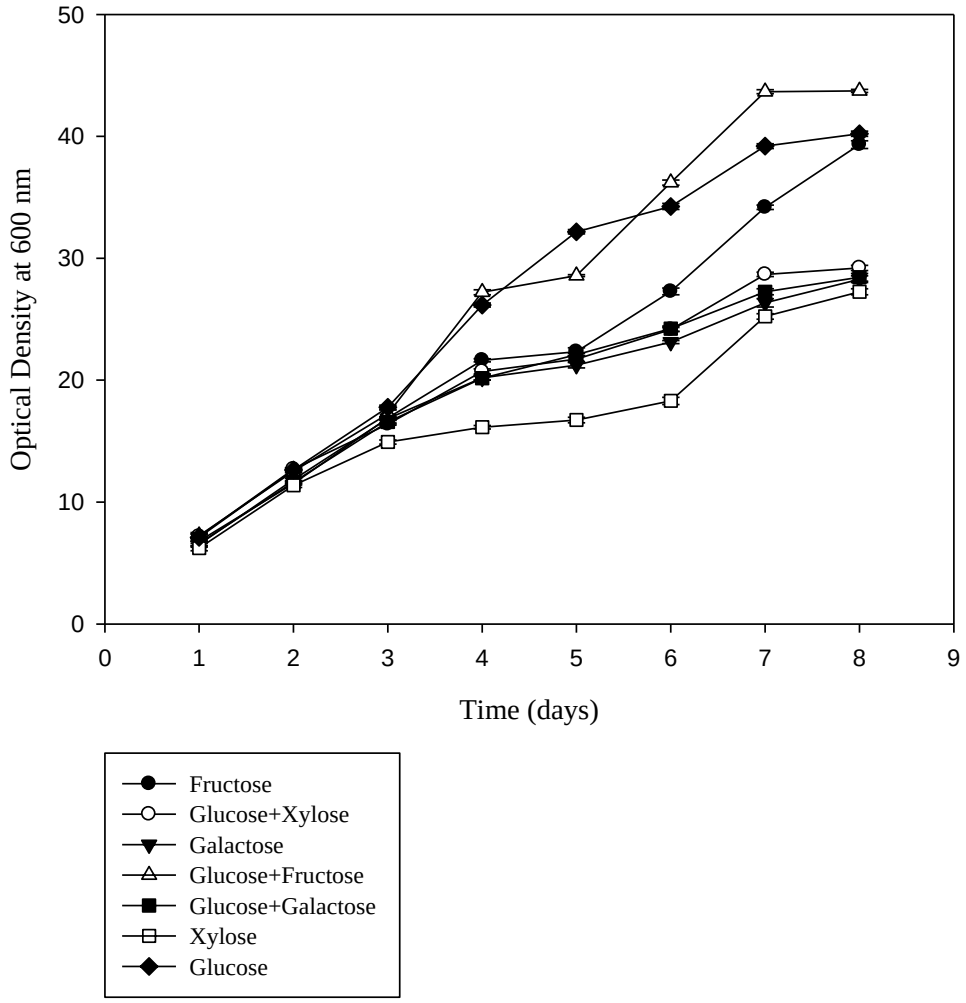

Figure 4.4. Growth of *Rhodosporidium toruloides* in fermentation media that contain different sugars at C/N=110

According to Li et al., (2007), *Rhodosporidium toruloides* Y4 yeast has been grown in glucose containing fermentation media. The optical density of *R. toruloides* Y4 was measured daily. The optical density has reached to 39.0 in 16 hours. At the end of fermentation, the biomass of *R.toruloides* has reached to 83.5 in glucose containing fermentation media. Because the fermentation was preceded in bioreactor, all the data are under control and high biomass results were obtained for the yeast *R. toruloides* Y4 (Li et

al., 2007). Although fermentation process was done in flasks in this study, the biomass of *R.toruloides* is compatible with literature.

It is obvious that optical densities of *Lipomyces starkeyi and Rhodosporidium toruloides* are less in higher C/N ratio of fermentation media. The optical density decreases with increasing C/N ratio.

Figure 4.3 and Figure 4.4 are for optical density of *Lipomyces starkeyi* and *Rhodosporidium toruloides* respectively. Since *Rhodosporidium toruloides* grows better and faster than *Lipomyces starkeyi* in the same conditions, it can be interpreted that *Rhodosporidium toruloides* is more efficient and compatible than *Lipomyces starkeyi*.

4.2. Total biomass of yeasts at the end of fermentation

After fermentation process, centrifugation was done and *Lipomyces starkeyi* and *Rhodosporidium toruloides* were dried in drying oven. Yeast cells grow differently in various synthetic sugars. By measuring the biomass of yeasts, growth behaviours of yeasts were obtained. In Figure 4.5 and 4.6, the biomass of *Lipomyces starkeyi* and *Rhodosporidium toruloides* are shown respectively.

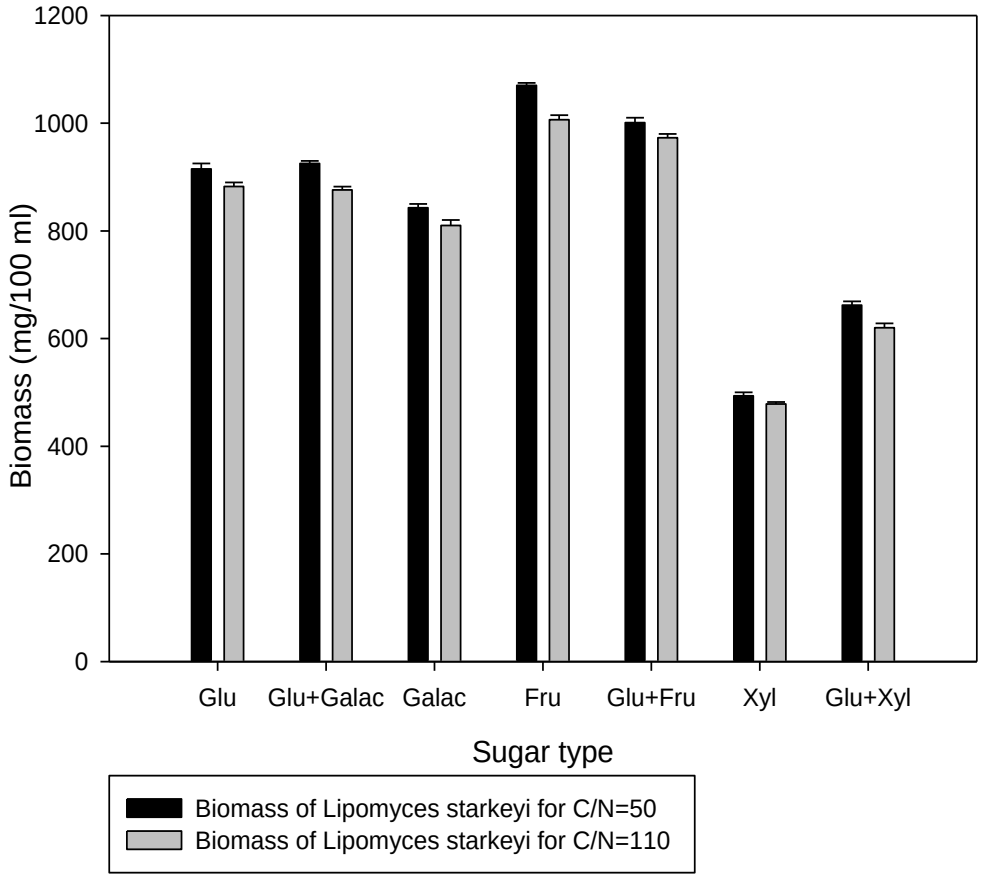

Figure 4.5. Biomass of *Lipomyces starkeyi* at the end of fermentation

Lipomyces starkeyi grows more in fructose containing media and less in xylose containing media. Biomass of *Lipomyces starkeyi* is around 1100 g/L in fructose containing media and 500 g/L in xylose containing fermentation media. Furthermore, *Lipomyces starkeyi* grows more efficiently in C/N=50 than C/N=110 media. In the literature, generally *Lipomyces starkeyi* grows well in glucose containing media and less in xylose containing media. Data are compatible with literature.

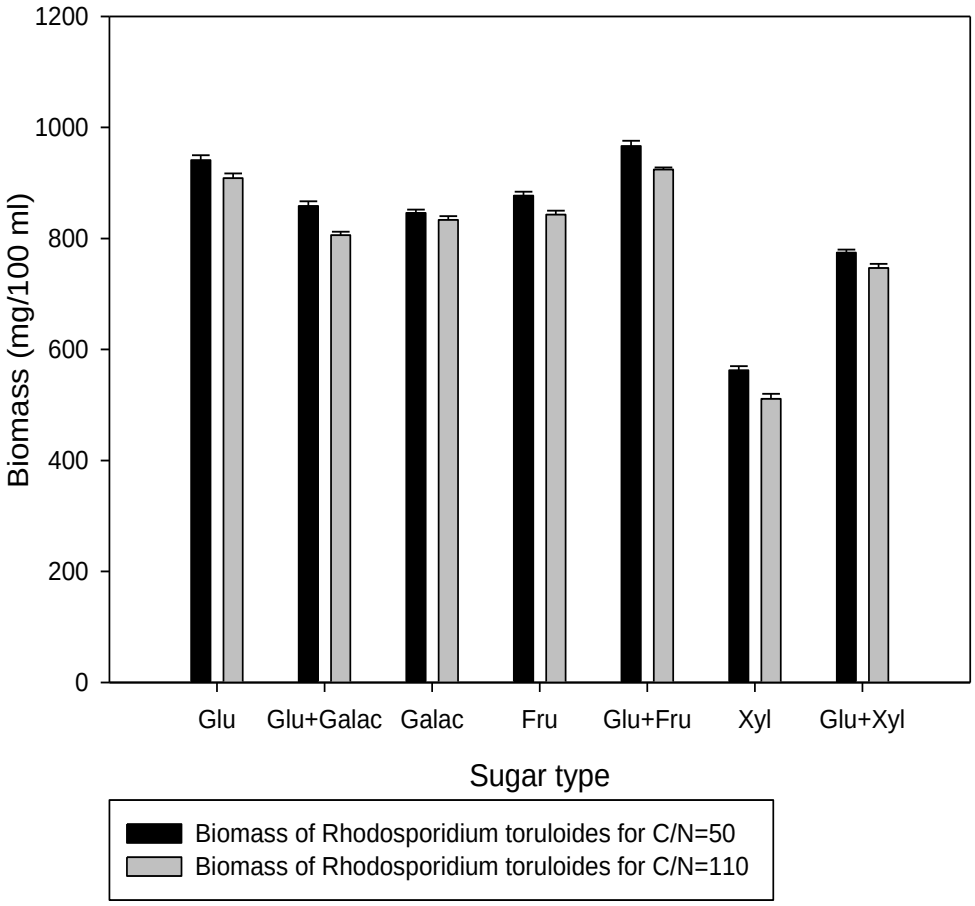

Figure 4.6. Biomass of *Rhodosporidium toruloides* at the end of fermentation

At the end of fermentation process, *Rhodosporidium toruloides* grows the best in glucose:fructose (1:1) containing media and the least in xylose containing media. Also, *Rhodosporidium toruloides* grows better in C/N=50 than C/N=110 media.

In the literature, yeasts are generally grown in glucose containing fermentation media and one type of sugar (Lin et al., 2011). In this study, the effects of different sugars and two sugars on yeast growth and lipid production were obtained. Glucose, fructose, xylose, galactose and combinations of them were used in fermentation media to observe the

biomass and lipid yield of *Lipomyces starkeyi* and *Rhodosporidium toruloides.* All the sugars that used in fermentation media affect yeast growth in positive way; however, yeast cells grow more in glucose and fructose containing media and less in xylose containing media. They also like two sugar combinations.

In the study of J. Lin et al., C/N ratio of fermentation media have been arranged between 40 to 140 and C/N ratio's effects on the cell growth and lipid accumulation of yeasts were investigated. When the C/N ratio of fermentation media raised from 40 to 140, biomass of yeast decreased from 18.3 g/L to 16.8 g/L (J. Lin et al., 2011).

Furthermore, substrates are important factor to increase biomass of yeasts. When glucose was used as substrate, biomasses of 100 g/L were obtained and when glycerol was used as substrate in fermentation medium, the biomass also has reached to 100 g/L. However, biomass was much lower in xylose containing media (Zhao, Kong, Hua, Feng & Zhao, 2008).

4.3 Lipid Yield% of *Lipomyces starkeyi* and *Rhodosporidium toruloides*

Lipomyces starkeyi and *Rhodosporidium toruloides* grow and store microbial lipids in their cells. At the end of fermentation process, lipid extraction has been made, and left hexane was removed by evaporating in drying oven and microbial lipid has been obtained. The lipid yield was calculated by using the ratio of lipid amount to dry cell weight.

Because high lipid yield is the main purpose of this study, lipid yield of *Lipomyces starkeyi* and *Rhodosporidium toruloides* were calculated with respect to various sugars

and C/N ratios. Figure 4.7 and Figure 4.8 are plotted to investigate the effects of sugars and C/N ratio on lipid yield.

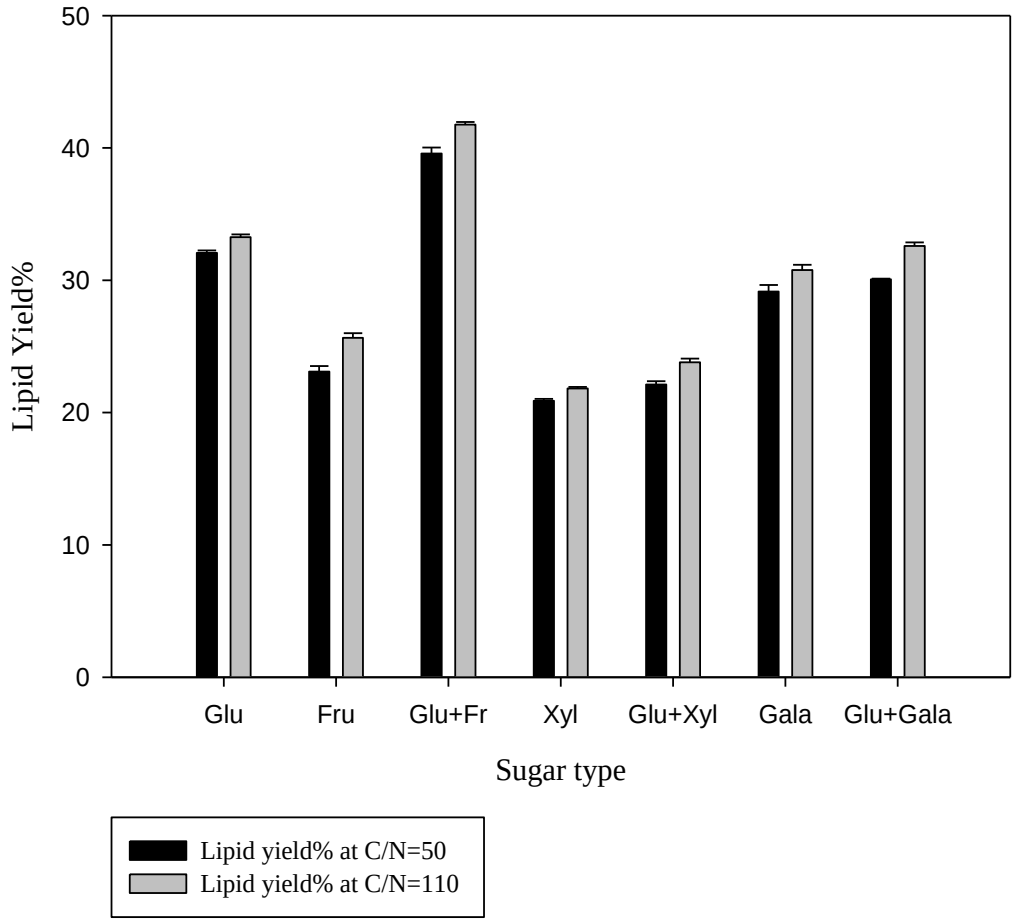

Figure 4.7. Lipid yield% of *Lipomyces starkeyi* in various sugars and C/N ratio

Lipomyces starkeyi produces microbial lipid in various sugars and C/N ratio that are arranged for fermentation media. However, different sugars and C/N ratio affect lipid yield of *Lipomyces starkeyi* in different level. In C/N ratio of 50 fermentation media, *Lipomyces starkeyi* has the highest lipid yield% as 39.6 ± 0.5 in glucose:fructose (1:1)(w/w) containing fermentation media and the least lipid yield% as 20.9 ± 0.1 in xylose containing media. In C/N ratio of 110 fermentation media, *Lipomyces starkeyi*

produce the maximum lipid yield% as 41.8 ± 0.2 in glucose:fructose (1:1) containing fermentation media and the minimum lipid yield% as 21.8 ± 0.1 in xylose containing media. *Lipomyces starkeyi* has maximum lipid yield in glucose:fructose (1:1) containing fermentation media and minimum lipid yield in xylose containing fermentation media for both of the C/N ratios. It is clear that C/N ratio difference affects the lipid yield amount in same sugar.

In this study, maximum lipid yield% of *Lipomyces starkeyi* is 41.8 ± 0.2 in glucose:fructose (1:1) containing fermentation media with C/N ratio of 110 fermentation media and minimum lipid yield% is 20.9 ± 0.1 in xylose containing media with C/N ratio of 50. Lipid yield% increases with increasing C/N ratio.

In the study of Zhao et al.,2008, substrate of fermentation medium affects lipid yield%. The researchers reported that lipid yield% of oleaginous yeast is higher than 50% in glucose containing fermentation media and lipid yield% is 37% in xylose containing fermentation media.

In the study of Angerbauer et al., *Lipomyces starkeyi* grew up and produced microbial lipid in sewage sludge medium. After that, fermentation medium was adjusted to C/N ratio of 100 and 60 by adding glucose in sewage sludge medium. In C/N ratio of 100 and 60, lipid yield% has been found as 57.2% and 50.8% respectively. Lipid yield% of *Lipomyces starkeyi* increases with increasing C/N ratio. Literature data and data of this study are compatible.

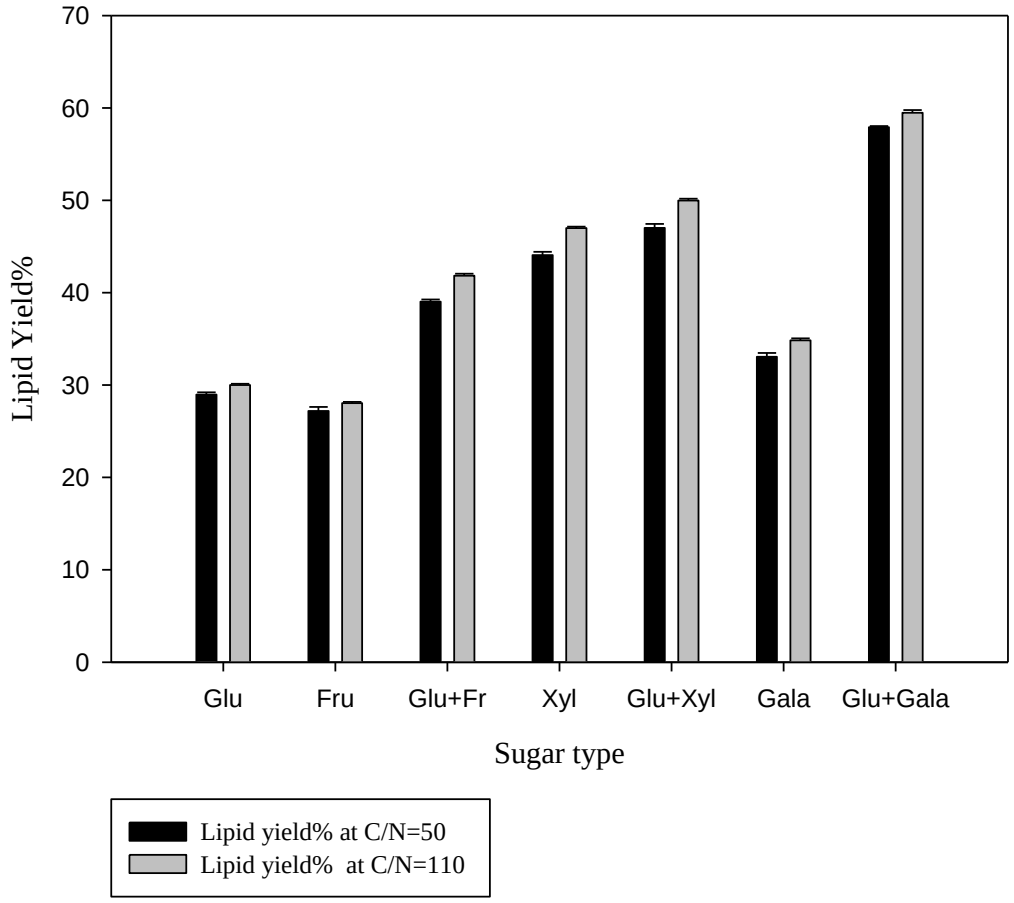

Figure 4.8. Lipid yield% of *Rhodosporidium toruloides* in various sugars and C/N ratio

Rhodosporidium toruloides was used to produce microbial lipid in different synthetic sugars and C/N ratios that are arranged for fermentation. In C/N ratio of 50 fermentation media, *Rhodosporidium toruloides* has the highest lipid yield% as 58 ±0.1 in glucose:galactose (1:1) containing media and the least is 27.2 ±0.4 in fructose containing media. In 110 C/N ration of fermentation media, the highest lipid yield of *Rhodosporidium toruloides* is 59.5 ±0.3 in glucose:galactose (1:1) containing and the least is 28.1 ±0.1 in fructose containing media. *Rhodosporidium toruloides* has maximum lipid yield in glucose:galactose (1:1) containing fermentation media and minimum lipid

yield in fructose containing fermentation media for both of the C/N ratios. It is clear that C/N ratio difference affects the lipid yield.

Maximum lipid yield% of *Rhodosporidium toruloides* is 59.5 g/L ±0.3 in glucose:galactose (1:1) containing fermentation media with C/N ratio of 110 fermentation media and minimum lipid yield% is 27.2 ±0.4 in fructose containing media with C/N ratio of 50. Lipid yield% increases with increasing C/N ratio.

In the study of Zhu et al., researchers reported that when the glucose concentration was 150 g/L, lipid yield% was 55.6% and when glucose concentration amount was 180 g/L, lipid yield% even decreases to 54.8%. Therefore, high amounts of substrate show inhibitory effects on lipid yield. The reason of this phenomenon is that a high concentration of substrate causes high osmotic pressure and this decreases lipid accumulation and biomass (Zhu et al., 2008).

Lipid yield% and biomass do not change as the same way. For example, *Lipomyces starkeyi* use glucose:fructose(1:1)(w:w) more for lipid production than growth and *Rhodosporidium toruloides* use glucose:galactose (1:1)(w:w) more for lipid production than growth. It was investigated that both of the yeasts like to produce microbial lipid in the combinations of two sugars than single sugar in fermentation medium.

4.4. Sugar Utilization of Yeasts

The fermentation process was ended when the carbon source in the medium was almost fully exhausted and the cell growth was in the stationary phase. Thus, by conducting the reducing sugar analysis with DNS reagent method, sugar amount of blank fermentation media and fermentation media at the end of fermentation were investigated and sugar

consumption of yeast species was measured. Consumption of sugars were defined in Table 4.1 and Table 4.2 for both of the yeasts. According to plots, they both consume pentose and hexose that are added in fermentation media.

In C/N ratio of 50 fermentation media, *Lipomyces starkeyi* consumes fructose more than other sugars and utilizes around 94% of fructose during fermentation.

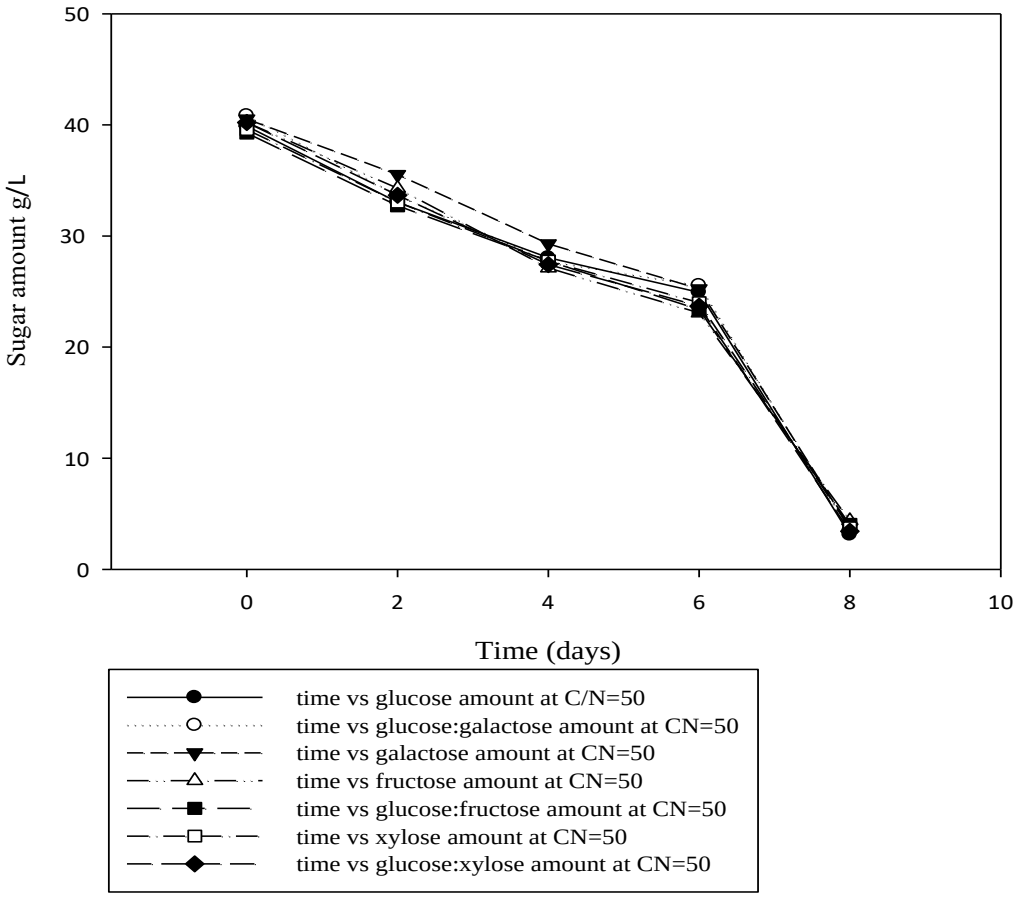

Figure 4.9. Sugar Utilization of *Lipomyces starkeyi* at C/N ratio of 50 with respect to time

It is obvious from Figure 4.9, sugar utilization increases when optical density of yeast increases. *Lipomyces starkeyi* grows higher amount in fructose and less in xylose containing fermentation media.

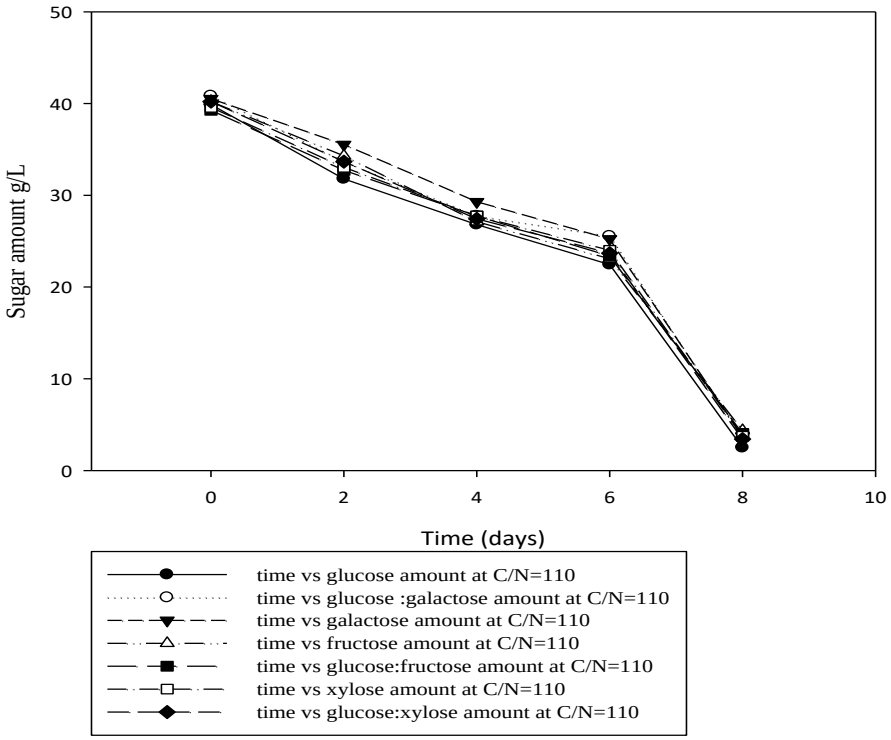

Figure 4.10. Sugar Utilization of *Lipomyces starkeyi* at C/N ratio of 110 with respect to time

Lipomyces starkeyi consumes glucose more and utilizes around 94% of it at C/N ration of 110. The yeast utilizes fructose for growth and glucose for lipid production. Fructose showed the increasing effect on growth of *Lipomyces starkeyi*. Therefore, it can be investigated that C/N ratio of media affects the sugar utilizations of yeast cells.

Table 4.1. Sugar Utilization of *Lipomyces starkeyi* at the end of fermentation

	Lipomyces starkeyi in C/N=50 fermentation media		*Lipomyces starkeyi* in C/N=110 fermentation media	
SUGAR TYPE	Sugar Amount g/L at DAY 0	Sugar Amount g/L at DAY 8	Sugar Amount g/L at DAY 0	Sugar Amount g/L at DAY 8
Glucose	39.89	3.11	39.5	2.5

Glucose-Galactose (1:1) (w/w)	40.83	5.61	40.4	3.4
Galactose	40.89	6.54	40.5	3.7
Fructose	40.52	2.50	40.2	4.4
Glucose-Fructose (1:1) (w/w)	41.14	3.18	39.9	4.05
Xylose	40.83	4.05	39.6	3.7
Glucose-Xylose (1:1) (w/w)	40.21	3.74	40.1	3.4

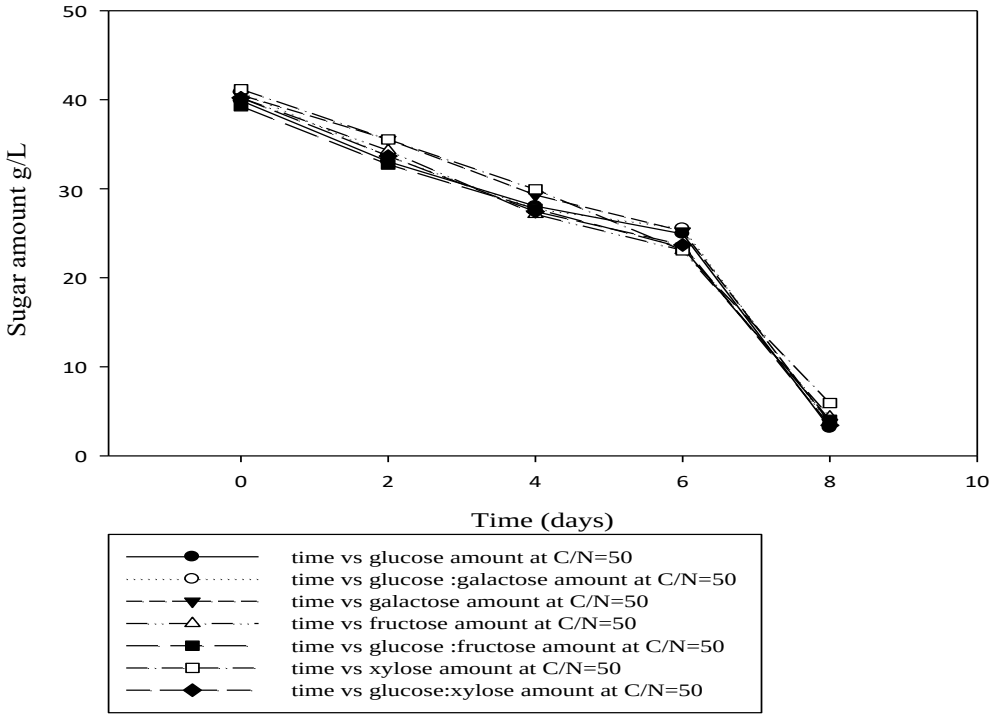

Figure 4.11. Sugar Utilization of *Rhodosporidium toruloides* at C/N ratio of 50 with respect to time

The sugar is used for growth and lipid production of yeast cells. Sugar utilization was measured by DNS reagent method. *Rhodosporidium toruloides* consumes

glucose:galactose (1:1) more than other sugars and utilizes around 96% of glucose:galactose (1:1) during fermentation in C/N ration of 50 and 110 media. It utilizes the highest percentage of glucose:galactose (1:1) media for growth and lipid production.

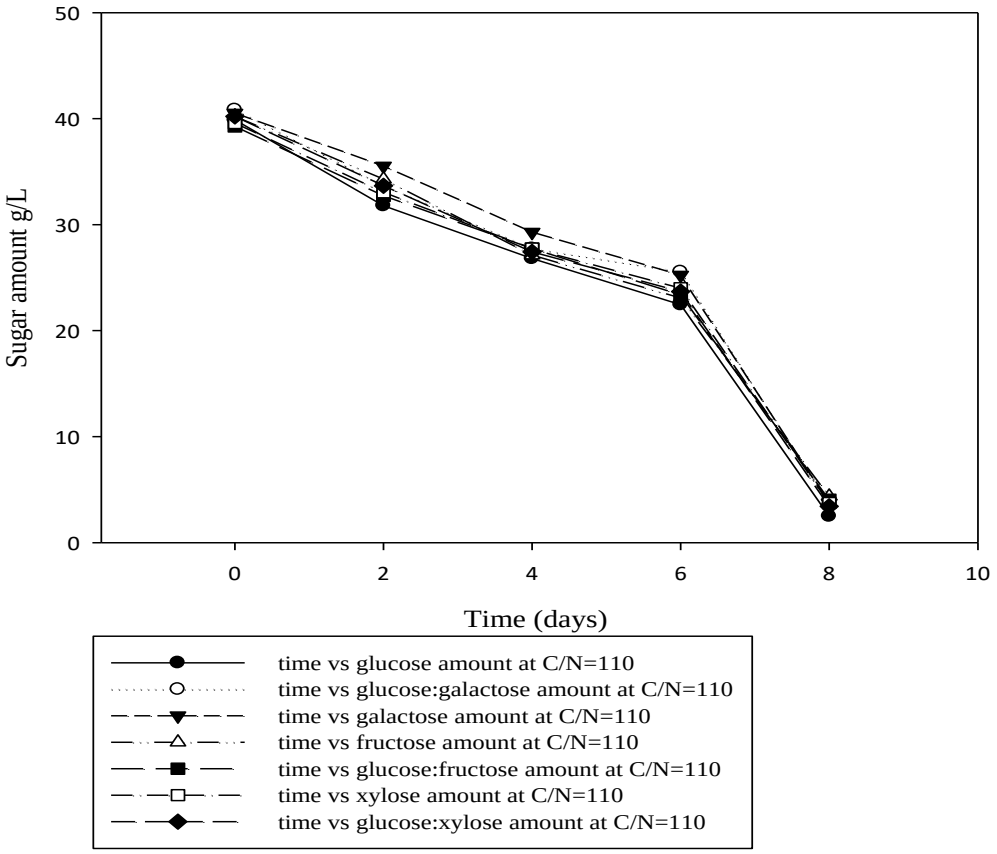

Figure 4.12. Sugar Utilization of *Rhodosporidium toruloides* at C/N ratio of 110 with respect to time

Although, the biomass of *Rhodosporidium toruloides* is more in glucose:fructose (1:1) media, *Rhodosporidium toruloides* utilize glucose:galactose (1:1) more than glucose:fructose(1:1)(w/w) containing fermentation media. It has been investigated that

glucose:galactose (1:1) combination was used in lipid production more than growth and yeast cells use high sugar amount by producing microbial lipid.

Table 4.2. Sugar Utilization of *Rhodosporidium toruloides* in various sugars and C/N ratio

	Rhodosporidium toruloides in C/N=50 fermentation media		*Rhodosporidium toruloides* in C/N=110 fermentation media	
SUGAR TYPE	DAY 0	DAY 8	DAY 0	DAY 8
Glucose	38.3	3.4	39.7	3.3
Glucose-Galactose (1:1) (w/w)	38.9	1.9	40.8	1.7
Galactose	40.2	9.9	41.1	9.04
Fructose	39.6	3.1	40.2	3.1
Glucose-Fructose (1:1) (w/w)	39.9	3.7	40.5	3.7
Xylose	40.5	6.9	41.2	5.9
Glucose-Xylose (1:1) (w/w)	40.8	6.5	40.9	5.6

As a result, *Lipomyces starkeyi* and *Rhodosporidium toruloides* consume almost all of the monosaccharaides in fermentation media at the end of fermentation. They both utilize sugars for growth and lipid production.

4.5 Optimization by using response surface method

Optimization experiments were carried out to define the effects of pH and ratio of carbon to nitrogen on microbial oil production. The optimal results of lipid yield% were determined.

Central Composite response surface optimization method was used since two factors were tested for oil production. The statistical analysis showed that the predicted model was appropriate, possessing no significant lack of fit and satisfactory values of the R^2 for the response.

Table 4.3. Central Composite RSM and lipid content of *Rhodosporidium toruloides*

RUN ORDER	**pH**	**C/N**	**Experimental data of lipid yield%**	**Predicted data of lipid yield%**
1	3	50	53.2±0.4	53.4
2	5	110	59.5±0.2	60.1
3	5	170	59.4±0.4	59.5
4	3	170	60.0±0.2	60.1
5	7	110	57.9±0,5	57.6
6	5	110	60.0±0.2	60.1
7	5	110	60.3±0.2	60.1
8	5	110	60.1±0.2	60.2
9	3	110	58.4±0.6	58.3
10	5	50	55.3±0.2	55.9
11	7	50	51.4±0.2	52.2
12	7	170	57.5±0.4	57.3
13	5	110	60.1±0.2	60.2

The insignificant lack of fit for lipid yield was (P = 0.23 >0.05), that proved that the model fit the experimental data. The effects of C/N ratio and pH values on lipid yield are written (Table 4.2) by the regression coefficients.

Table 4.4 ANOVA results and estimated regression coefficients for lipid yield

Term	**Coefficient**	**P Value**
Regression		0.000
Linear		0.000
Square		0.000
Interaction		0.003
Lack-of-fit		0.23
Constant	60.093	0.000
pH	-0.36	0.002*
C/N	2.39	0.000*
pH*pH	-2.13	0.000*
C/N*C/N	-2.95	0.000*
pH*C/N	0.41	0.003*

R-Sq = 97,62% R-Sq(pred) = 95,21% R-Sq(adj) = 97,02%

*Result is significant when P<0.05

Linear (P=0.000), Square (P=0.000) and interaction effects (P=0.003) were obtained statistically significant. C/N ratio and pH values showed significant effect (P<0.05) on

lipid yield. The results showed significant interaction effects (P<0.05) between pH and C/N ratio on lipid yield (Table 4.2).

From Regression analysis of Central Composite design, optimal conditions for high lipid yield were defined. In order to validate the model, more experiments were carried out.

Estimated Regression Coefficients for Lipid Yield using data in uncoded units

Table 4.5 Estimated regression coefficients for lipid yield

Term	**Coefficient**
Constant	37.8524
pH	4.79
C/N	0.175473
pH*pH	-0.531466
C/N*C/N	-7.67654E-04
pH*C/N	0.00332661

$$Y=37.85 + 0.175\ X_1 + 4.79\ X_2 + 0.00333\ X_1 X_2 - 7.677*10^{-4}\ X_1^{\ 2} - 0.5315\ X_2^{\ 2}$$

Where Y is lipid yield, X_1 and X_2 represent C/N ratio and pH respectively.

The optimum condition for obtaining high lipid yield is defined by drawing a surface Plot of lipid yield with respect to C/N and pH.

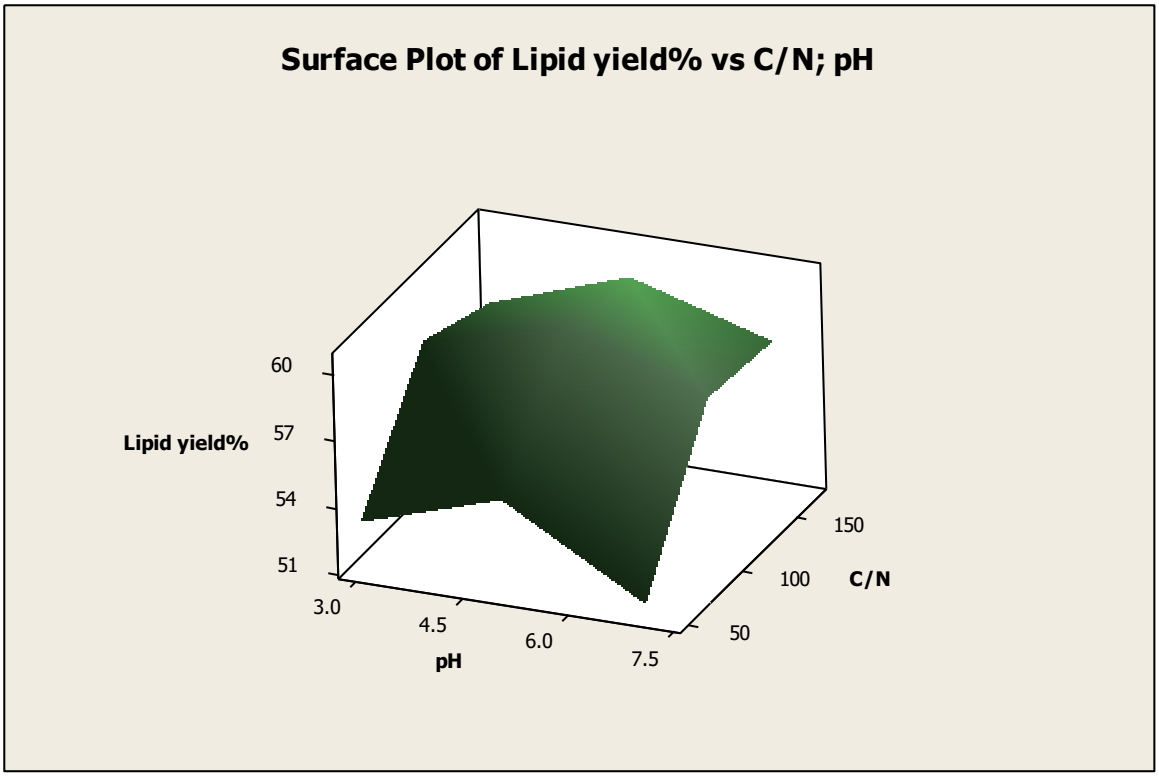

Figure 4.13. Surface Plot of lipid yield% of *Rhodosporidium toruloides*

The optimum conditions were found as pH equals to 5.02 and C/N ratio of 126. Predicted responses of experiments shows that lipid yield% is 60.1 in optimum conditions with 0.92 desirability.

To validate results, experiments were done to find the lipid yield of *Rhodosporidium toruloides* on this conditions. The model was tested by making experiments in shaking

flasks with different C/N ratio and different pH medium compositions. *Rhodosporidium toruloides* has experimental data of lipid yield% as 60.6±0.4. The experimental value was similar to the predicted value of the lipid yield (60.1%). Therefore, the model was successful to increase lipid yield of Rhodosporidium toruloides in glucose:galactose (1:1)(w:w) containing fermentation media. It is obvious that experimental data fits with predicted data and the developed model is accurate and reliable for lipid yield.

The model was also evaluated with error analysis. Root mean square error (RMSE) and mean absolute error (MAE) values were calculated as 1.01 and 0.28, respectively. Coefficient of variation of data were also found as 0.26. Small values of RMSE and MAE show that the model was appropriate for this study (Uncu & Cekmecelioglu, 2011).

Experimental and predicted data were compared in a graph and R^2 of this comparison was found as 0.97 in figure 4.12. As it is clear from graph, experimental data and predicted data were similar and R^2 is high enough to accept this study as appropriate.

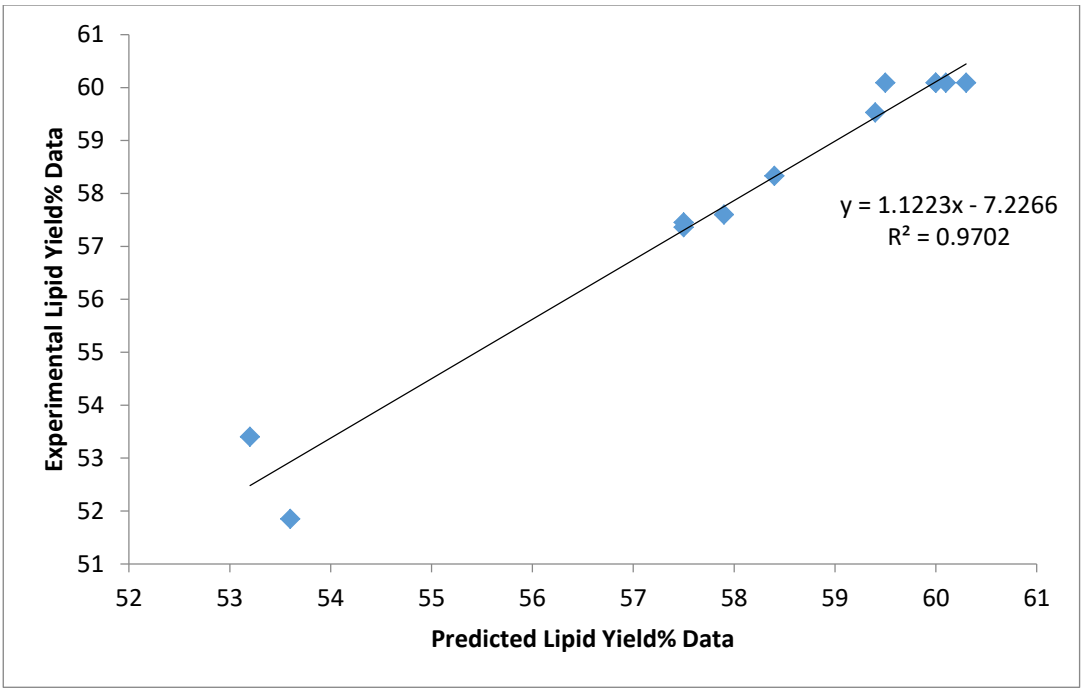

Figure 4.14. Comparison of Experimental and Predicted Data for Lipid Yield% of *Rhodosporidium toruloides* in glucose:galactose (1:1) (w:w) containing medium

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

In this study, oleaginous yeasts, *Lipomyces starkeyi* and *Rhodosporidium toruloides* were used for microbial oil production. Capacities of *Lipomyces starkeyi* and *Rhodosporidium toruloides* yeasts for microbial oil production were evaluated. The optimum conditions for microbial oil production were achieved and the effects of C/N ratio and synthetic sugar types on lipid production of two yeasts were investigated.

First of all, yeasts were grown in fermentation media which contained different synthetic sugars and different C/N ratio. Fermentation process lasted 8 days in Erlenmeyer flasks in a shaking incubator. Experiments were conducted at C/N ratio of 50 and 110. Biomass, optical density, sugar utilization, and reducing sugar analysis were monitored daily.

Secondly, after defining the ideal sugar and efficient yeast, experiments were conducted to investigate the optimum C/N ratio and pH of fermentation medium for lipid production. For this reason, the Central Composite (CC) design of response surface methodology was applied to determine ideal conditions for high lipid yield. The optimum pH and C/N ratio were predicted and compared with experimental data.

The study shows that *Lipomyces starkeyi* grows better in fructose containing fermentation medium with C/N ratio of 50 and less in xylose containing media with C/N ratio of 110. However, *Lipomyces starkeyi* gave the highest lipid yield in fructose containing media and the least lipid yield in xylose containing fermentation media with C/N ration of 110.

Rhodosporidium toruloides grows better in glucose:fructose (1:1) containing media with C/N ration of 50 and the least lipid yield in xylose containing fermentation media with C/N ration of 110. *Rhodosporidium toruloides* achieved the highest lipid yield in glucose:galactose (1:1) containing media with C/N ration of 110 and the least lipid yield in xylose containing media with C/N ration of 50.

As C/N ratio of fermentation medium increases, lipid yields of *Lipomyces starkeyi* and *Rhodosporidium toruloides* increase. However, *Rhodosporidium toruloides* showed higher lipid yield than *Lipomyces starkeyi* in all tested conditions.

Since *Rhodosporidium toruloides* was found more efficient yeast for microbial oil production, experiments were continued with *Rhodosporidium toruloides* in different pH and C/N ratio.

The maximum microbial lipid yield was found with *Rhodosporidium toruloides* as (60.1%) at pH 5.0 and C/N ration of 125 in glucose:galactose (1:1)(w/w) containing fermentation media after 8 days incubation in shaking incubator with agitation speed of 130 rpm and at 25 ^{o}C. According to statistical results, the effects of pH and C/N ratio on lipid yield were significant ($p<0.05$). The increase and decrease of pH from 5.0 had negative impact on lipid yield of *Rhodosporidium toruloides*. The optimum conditions were verified experimentally and lipid yield was obtained as 60.6 ±0.4 %.

For future study, lignocellulosic, agricultural and food wastes can be used as carbon source to reduce final product costs.

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APPENDICES

A. CHEMICALS AND SUPPLIER INFORMATION

Table A.1 Chemicals and supplier information

Chemical	**Supplier**
D-glucose	Merck
Fructose	Merck
Galactose	Merck
Xylose	Merck
Bacto-Pepton	Becton, Dickinson (BD)
MaltExtract	Merck
Di-sodiumphosphate(Na_2HPO_4)	Merck
AmmoniumSulphate$(NH_4)_2SO_4$	Merck
MagnesiumSulphateHeptahydrate($MgSO_4.7H_2O$)	Merck
CalciumChloridedi-hydrate($CaCl_2.2H_2O$)	Merck
3'-5'-Dinitrosalcylicacid	Sigma-Aldrich
NutrientAgar	Merck
Phenol	Merck
Potassiumdi-hydrogenphosphate(KH2PO4)	Merck
Rochelle salt	Merck

Sulphuric acid	Merck
Sodium hydroxide	Sigma Aldrich
Sodium sulphite	Merck
Yeast extract	Merck
Hydrochloric Acid(37%)	Merck
n-Hexane	Merck
Glycerol	Merck

B. COMPOSITIONS OF SOLUTIONS

1-Composition of DNS reagent:

1.00 g- Dinitrosalicylic acid

0.2 g- Phenol

0.05 g- Sodium sulfite

1.00 g- Sodium hydroxide

100 ml Distilled Water

Rochelle salt (40%): Potassium sodium tartrate

C. THE KJELDAHL METHOD SOLUTIONS FOR NITROGEN DETERMINATION

0.1 N NaOH solution: 0.4 gram of NaOH is dissolved in distilled water in volumetric flask.

1 N HCl solution: 4.14 ml of 37 % HCl is mixed with distilled water and completed the flask to 500 ml.

40% NaOH solution: 40 gram of NaOH is dissolved in distilled water and completed to 100 ml in flask.

Methyl red indicator: 100 mg of methyl red (solid) is dissolved in 100 ml alcohol.

D. STANDARD CURVE OF DNS METHOD

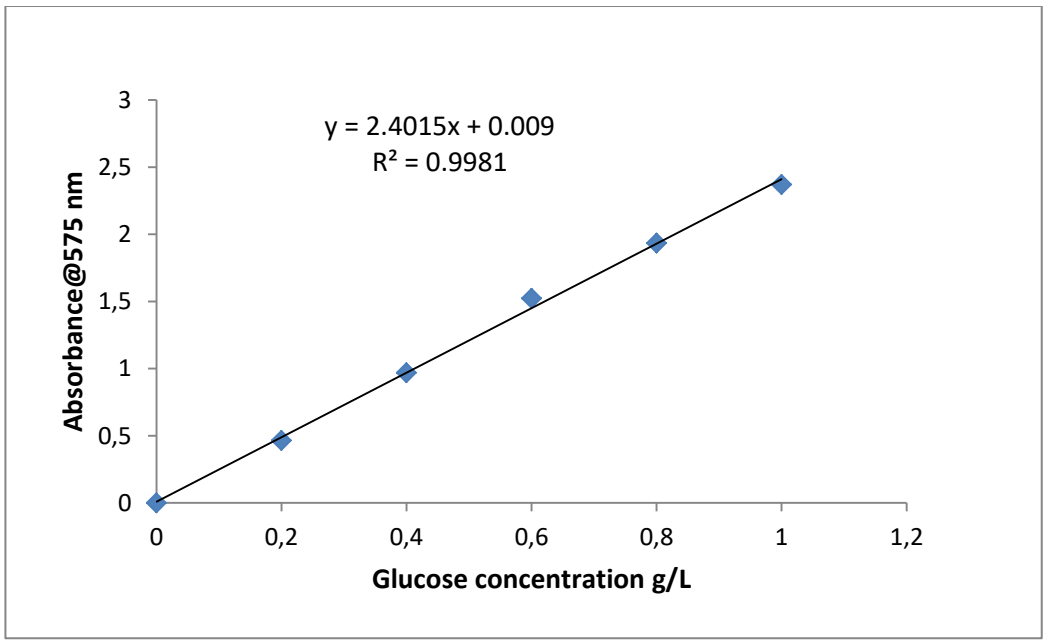

Figure D.1 The standard curve of DNS method

Total reducing sugar content was measured according to standard curve as below:

Total reducing sugar(g/L)= [(Absorbance-0.009)/2.4015]*dilution rate

E. OPTICAL DENSITY AND DRY WEIGHT COMPARISON

To understand the efficiency and suitability of yeasts for microbial oil process, optical density and dry weight of the yeasts were measured daily. It is obvious that yeasts show different growing behaviour. Both of yeast cells grow and increase their biomass linearly in synthetic sugar containing fermentation media. The relations between dry cell weight and biomass are showed in Figure E.1. for both of the yeasts.

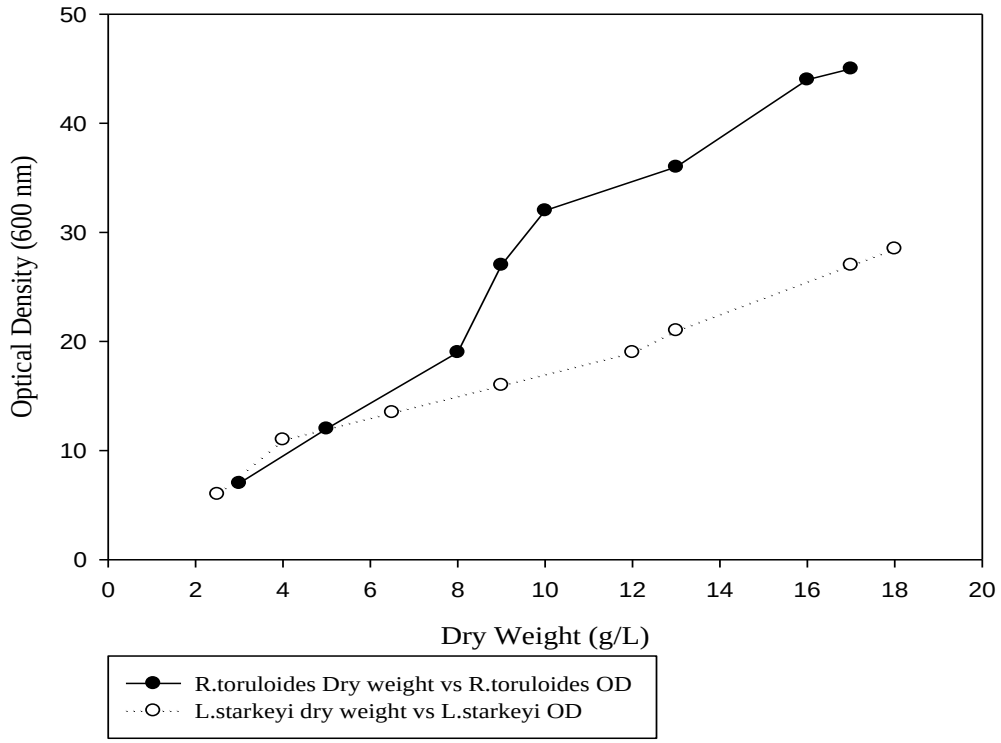

Figure E.1. Dry weight vs optical density plots of *Lipomyces starkeyi* and *Rhodosporidium toruloides*

Optical density of yeast cells were measured by spectrophotometer at 600 nm and dry weight of yeast cells were calculated by using the formula;

Net biomass (g/L) = wet biomass (g/L)-dry biomass (g/L)

It is seen in Figure E.1, both of the yeasts grow linearly; however, *Lipomyces starkeyi* shows less development than *Rhodosporidium toruloides*. *Rhodosporidium toruloides* has higher optical density than *Lipomyces starkeyi*'s at the same value of dry weight. For example, at 12 g/L of dry cell weight, optical density of *Rhodosporidium toruloides* was measured as 35 abs and optical density of *Lipomyces starkeyi* was measured as 19 abs.

In the study of J. Lin et al., the dry cell weight of *Lipomyces starkeyi* are 11.2 g/L, 12.1 g/L and 13.3 g/L at 36, 40 and 48 hours respectively. Dry cell weight and absorbance increase linearly. As similar to literature, dry cell weight and optical density increase linearly in this study.

F. STATISTICAL ANALYSIS

Two-way ANOVA and Tukey's comparison test with 95% confidence level for lipid yield% of Rhodosporidium toruloides

General Linear Model: Lipid Yield% versus C/N; pH

Factor	Type	Levels	Values
C/N	fixed	3	50; 110; 170
pH	fixed	3	3; 5; 7

Analysis of Variance for Yield, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
C/N	2	160.026	116.740	58.370	319.06	0.000
pH	2	26.577	26.577	13.289	72.64	0.000
Error	21	3.842	3.842	0.183		
Total	25	190.445				

S = 0.427717 R-Sq = 97.98% R-Sq(adj) = 97.60%

Unusual Observations for Yield

Obs	Yield	Fit	SE Fit	Residual	St Resid
8	51.4000	52.2580	0.2223	-0.8580	-2.35 R

R denotes an observation with a large standardized residual.

Two-way ANOVA and Tukey's comparison test with 95% confidence level for lipid yield

Grouping Information Using Tukey Method

pH	N	Mean	Grouping
5	14	59.250	A
3	6	56.367	B
7	6	55.633	B

Means that do not share a letter are significantly different.

Grouping Information Using Tukey Method and 95.0% Confidence

Carbon Source	N	Mean	Grouping
4	10	0.3	A
5	10	0.3	A
3	10	0.2	A B
1	10	0.2	A B
2	10	0.2	B

Means that do not share a letter are significantly different.

Response Surface Regression: Lipid yield% versus C/N; pH

The analysis was done using uncoded units.

Estimated Regression Coefficients for Lipid yield%

Term	Coef	SE Coef	T	P
Constant	60.0931	0.1034	581.161	0.000
pH	-0.3667	0.1017	-3.607	0.002
C/N	2.3917	0.1017	23.525	0.000
pH*pH	-2.1259	0.1498	-14.187	0.000
C/N*C/N	-2.9509	0.1498	-19.693	0.000
pH*C/N	0.4125	0.1245	3.313	0.003

S = 0.399802 PRESS = 6.43133

R-Sq = 97.62% R-Sq(pred) = 95.21% R-Sq(adj) = 97.02%

Analysis of Variance for Lipid yield%

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	5	187.964	187.964	37.5929	303.10	0.000
Linear	2	70.254	70.254	35.1271	283.22	0.000
pH	1	1.613	1.613	1.6133	13.01	0.002
C/N	1	68.641	68.641	68.6408	553.44	0.000
Square	2	116.349	116.349	58.1745	469.05	0.000
pH*pH	1	68.250	24.964	24.9637	201.28	0.000
C/N*C/N	1	48.099	48.099	48.0991	387.81	0.000
Interaction	1	1.361	1.361	1.3613	10.98	0.003
pH*C/N	1	1.361	1.361	1.3613	10.98	0.003
Residual Error	20	2.481	2.481	0.1240		
Lack-of-Fit	3	1.040	1.040	0.3465	4.09	0.23
Pure Error	17	1.441	1.441	0.0848		
Total	25	190.445				

Response Optimization

Parameters

	Goal	Lower	Target	Upper	Weight	Import
Lipid yield%	Maximum	50	61	61	1	1

Starting Point

C/N = 50

pH = 3

Global Solution

C/N = 126.364

pH = 5.02020

Predicted Responses

Lipid yield% = 60.0960 , desirability = 0.917816

Composite Desirability = 0.917816

Optimization Plot

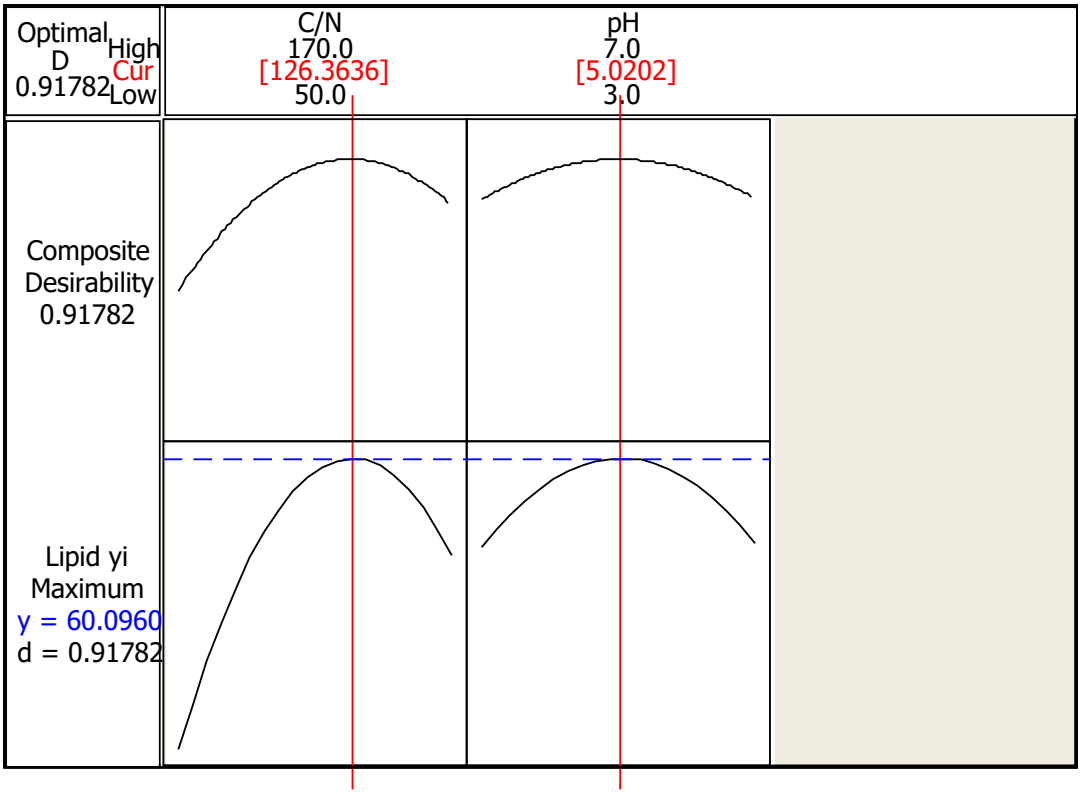

Figure F.1. Optimization plot for lipid yield