**ÇUKUROVA UNIVERSITY**
**INSTITUTE OF NATURAL AND APPLIED SCIENCES**

**PhD THESIS**

**Erdem ÇARŞANBA**

# PRODUCTION OF CITRIC ACID AND SINGLE CELL OIL BY USING THE YEAST *YARROWIA LIPOLYTICA*

**DEPARTMENT OF FOOD ENGINEERING**

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We certify that the thesis titled above was reviewed and approved for the award of degree of the Doctor of Philosophy by the board of jury on 11/01/2018.

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## ABSTRACT

## PHD THESIS

# PRODUCTION OF CITRIC ACID AND SINGLE CELL OIL BY USING THE YEAST *YARROWIA LIPOLYTICA*

**Erdem ÇARŞANBA**

**ÇUKUROVA UNIVERSITY**
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**DEPARTMENT OF FOOD ENGINEERING**

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In this study, 12 different *Yarrowia lipolytica* strains, collected from different laboratories, were used to investigate microbial lipid and citric acid production using a glucose-based media under nitrogen-limited conditions in shake-flask fermentations. As a result, a domestic strain, *Y. lipolytica* K57, was selected. The effect of the initial glucose concentration, agitation speed and aeration rate of the bioreactor on the production of lipids and citric acid by *Y. lipolytica* strain K57 were investigated. The enzymatic hydrolysis of waste bread was optimized and used for the production of citric acid and lipids in a batch-type bioreactor. Fermentation kinetics, fatty acid composition and organic acids were assessed. The results show that a high citric acid yield of 0.77 (g CA/g glucose) but a low lipid yield of 0.14 (g lipid/g dry cell weight) were obtained from glucose, whereas a low citric acid yield of 0.13 g/g, a high lipid yield of 0.32 g/g and biomass of more than 60 g/L were produced from a waste bread hydrolysate by *Y. lipolytica* strain K57 in a bioreactor fermentation. Initial concentrations of glucose of approximately 100 g/L and dissolved oxygen of 90% generally stimulated high citric acid production, whereas initial concentrations of glucose of around 150 g/L and low levels of dissolved oxygen induced the accumulation of lipids. A high nitrogen level in the medium inhibited citric acid synthesis but enhanced biomass production.

**Key Words:** Citric acid, Microbial lipid, *Yarrowia lipolytica*, Waste bread, Bioreactor fermentation

# ÖZ

## DOKTORA TEZİ

## *YARROWIA LIPOLYTICA* KULLANILARAK SİTRİK ASİT VE TEK HÜCRE YAĞI ÜRETİMİ

**Erdem ÇARŞANBA**

**ÇUKUROVA ÜNİVERSİTESİ**
**FEN BİLİMLERİ ENSTİTÜSÜ**
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Bu çalışmada, farklı laboratuvarlardan temin edilen 12 farklı *Yarrowia lipolytica* suşunun azotça sınırlandırılmış sentetik ortamda mikrobiyel yağ ve sitrik asit üretim kabiliyetleri araştırılmıştır. Bu araştırmaların sonucunda, yerli suş *Y. lipolytica* K57 sitrik asit ve yağ üretimi için seçilmiştir. Başlangıçta glikoz konsantrasyonu, biyoreaktör karıştırma hızı ve havalandırma oranının yağ ve sitrik asit üretimine etkisi *Y. lipolytica* K57 suşu kullanılarak araştırılmıştır. Atık ekmeklerin enzimatik hidrolizi optimize edilmiş ve kesikli biyoreaktör de sitrik asit ve yağ üretimi için kullanılmıştır. Fermantasyon sonucu elde edilen kinetik değerler, yağ asidi kompozisyonu ve organik asitler değerlendirilmiştir. Sonuç olarak, 0.77 (g sitrik asit/g glikoz) yüksek sitrik asit verimi, fakat 0.14 (g yağ/g kuru hücre ağırlığı) düşük yağ verimi sentetik ortam içeren kesikli biyoreaktör de elde edilmiştir. Atık ekmek hidrolizatı içeren kesikli biyoreaktör de ise düşük sitrik asit verimi 0.13 g /g, yüksek yağ verimi 0.32 g/g ve 60 g / L'den fazla biyokütle *Y. lipolytica* K57 suşu kullanılarak üretilmiştir. Genel olarak, 100 g/L başlangıç glikoz konsantrasyonu ve % 90 çözünmüş oksijen kullanıldığında yüksek sitrik asit üretimi sağlanırken, başlangıç glikoz konsantrasyonu 150 g /L ve çözünmüş oksijen seviyesi düşük tutulduğunda daha yüksek yağ birikimi elde edilmiştir. Ortamda bulunan yüksek azot içeriği, sitrik asit sentezini engellerken, biyokütle üretimini arttırmıştır.

**Anahtar Kelimeler:** Sitrik asit, Mikrobiyel yağ, *Yarrowia lipolytica*, Atık ekmek, Biyoreaktör fermantasyonu

## EXTENDED SUMMARY

The lipids produced by microorganisms are called microbial lipids or single cell oils (SCOs), which include triacylglycerols (TAGs) (lipids of energy reserve), glycolipids (lipids of membrane structure), phospholipids and sterylesters. Among these lipids, TAGs are the glycerol triesters with non-polar fatty acids and the main synthesized component in microbial cells. In most cases, microbial lipids exhibit compositional similarities to common plant oils (e.g., rapeseed oil). Economic and technological interest increases continuously for lipid-producing microorganisms, from bacteria (the simplest class) to fungi (the most complex). In particular, the production of microbial lipids (in most cases fungal or algal oils) containing γ-linolenic acid (GLA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) is currently of significant interest as these poly-unsaturated fatty acids are valuable as functional foods and have benefits for human health. Moreover, cocoa butter substitutes (CBSs) can be produced with the aid of oleaginous microorganisms, as the microbial lipid composition is similar to exotic fats under several culture conditions. It is known that cocoa butter is an expensive raw material in the chocolate and biscuit industries. As their composition is similar to one of several vegetable oils, microbial lipids could be considered as perfect feedstocks for biodiesel production. Traditionally, biodiesel, i.e., fatty acid methyl esters (FAMEs), is obtained by transesterification of triacylglycerol (TAG) and alcohol using an acid or alkali catalyst. Over recent decades, finding alternative energy sources has been a priority, given that the quantity of frequently used fuels is diminishing; hence, energy producers use vegetable oils to generate biodiesel and as a result the price increases. As production depends on seasons, temperature and the availability of arable lands, researchers started to investigate microbial oil which exhibits desirable properties including, customizable fatty acid profiles, alternative feedstock for biodiesel, high productivity, abundant, reduced labour

required, easier to scale up, less affected by location and no climatic or geopolitics limitations during production.

Citric acid (2-hydroxy-1.2.3-propanetricarboxylic acid, $C_6H_8O_7.H_2O$), symbolized as food additive E330, is an organic acid and naturally found in many plants, animal tissues and physiological fluids. Recently, CA has gained increasing interest due to its important role in food, pharmaceutical and detergent industries, and its global production rate has reached more than 1.6 million tons annually with a growth of 5% per year. Approximately 70% of this quantity is in food, 20% is in the production of washing powders and 10% is in the pharmaceutical and chemical industry in the form of anhydrous, monohydrate and salts, such as calcium citrate, potassium citrate and sodium citrate. CA has a wide range of utilization in various industries, for instance, as a food additive it is employed as an acidifier, antioxidant, emulsifier, flavouring agent, stabilizer and preservative. Due to its GRAS, a biodegradable and environmentally friendly property, CA is also used in manufacturing pharmaceutical, cosmetic and detergent products. In our country, CA is mostly used by juice companies as a food additive. Approximately 950 million litres of fruit juice and nectar are consumed annually in Turkey and the average annual citric acid requirement of the fruit juice industry is 2.850 tons, considering that an average of 3 grams of citric acid is used per 1 litre of juice. According to 2016 TUIK data, 45,341,283 kg of citric acid was imported into the country costing approximately $35,959,020, as the price is approximately $0.8-$1/kg. Due to the wide ranging utilization of CA and its functional properties in food, pharmaceutical and chemistry industries, a high rate production is important. In addition, because of the high price of CA, methods to reduce the production cost and new production techniques should be investigated. From this point of view, in this study, CA was produced from a low-cost substrate (waste bread) using *Yarrowia lipolytica*, and fermentation conditions, productivity and yield were evaluated.

*Y.lipolytica* has been most often studied yeast for production of citric acid because of its numerous advantages than other microorganisms such as GRAS property, high productivity, wide range of substrate usage (hydrophilic and hydrophobic), high tolerance to metal ions, contaminations and salt solutions, capability to grown at low and high pH (pH=4 to 8) and wide range of temperatures (T= 18 to 32 ºC). *Y. lipolytica* which is a model for oleaginous microorganism, is one of the most studied and engineered oleaginous yeast because its genetics information had been discovered and bioinformatics tools are becoming increasingly available and allow to apply metabolic engineering strategies for this microorganism. Recently, *Y. lipolytica* strains which are constructed by recombinant technology have been revealed capable to produce high quantity of lipids as high as 90 % of dry cell weight, with titers up to 85 g/L and lipid productivity of approximately 1 g/L $h^{-1}$.

In this study, the molecular characterization of 12 *Y. lipolytica* strains was done and their citric acid (CA) and single cell oil (SCO) production on glucose-based media using the shake-flask fermentation method were primarily investigated. Fermentation kinetics, optimum fermentation conditions, fatty acid composition and organic acids generated by these strains were examined. An optimum strain was selected for a high level of CA and lipid production. Secondarily, CA and SCO productions by the selected strain on glucose-based media in a batch-type bioreactor fermentation were studied. Aeration and agitation parameters were optimized to achieve high yields of CA and SCO. The effects of initial glucose and dissolved oxygen concentrations on CA and lipid accumulation by the selected strain on glucose-based media were observed. Thirdly, waste bread was selected for CA and SCO production by *Y. lipolytica* and for this reason enzymatic hydrolysis was applied and optimized for large-scale production in a bioreactor. Finally, CA and lipid production and fatty acid composition by *Y. lipolytica* on waste bread hydrolysate media in a batch-type bioreactor fermentation was investigated.

All *Y. lipolytica* strains tested exhibited a high degree of homology (99% to 100%) with various *Y. lipolytica* strains present in the National Center for Biotechnology Information, as a result of BLAST analysis of a partial sequence of the 26S rRNA gene. All strains used for this study belonged to the *Y. lipolytica* species.

Among all the studied strains, CBS 6303, K57, W29 and Zu110 were selected as high CA-producing strains due to the high values of CA concentrations and yields. However, strain K57 was selected for further bioreactor fermentations due to its comparable values of CA yield and concentration, and also being a strain domestically isolated in Turkey. Maximum lipid yields and lipid content varied from 0.40 to 0.61 g/g and 0.40 to 1.28 g/L, respectively. Lipid accumulation was very low, especially for strains K57 and CBS 6303, whereas higher quantities were exhibited by strains Peggy, W29 and DBVPG 5858. In all cases, oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2) are the most abundant FAs produced inside the cell of all examined *Y. lipolytica* strains. The composition of FAs in yeast cells was strain dependent.

An agitation speed of 800 rpm and aeration rate of 1.0 vvm were suitable for a high level of CA production, whereas an agitation speed of 350 rpm and aeration rate of 0.5 vvm were suitable for lipid accumulation. An agitation speed of 800 rpm and aeration rate of 1.0 vvm were selected for use in further bioreactor experiments due to the high maximum production rate of 0.18 g/L/h and growth rate of 0.32 g/L/h.

Comparing yields of CA according to different initial glucose concentrations (Si), the highest value of 0.77 g/g was found at an initial glucose concentration of 107 g/L, the second highest value of 0.70 g/g was obtained at an initial glucose concentration of 50 g/L and the lowest yield of 0.59 g/g was determined at an initial glucose concentration of 166 g/L. Maximum values of CA content of 72.12 g/L, 63.87 g/L and 35.41 g/L were found at initial glucose concentrations of 107 g/L, 166 g/L and 50 g/L, respectively. Lipid yields at initial

glucose concentrations of 107 and 50 g/L were lower at values of 0.03 g/g and 0.04 g/g and the highest lipid yield of 0.14 g/g was determined at an initial glucose concentration of 166 g/L. Moreover, the highest lipid content of 0.32 g/L was also obtained at Si=166 g/L. In waste bread hydrolysis, a glucoamylase loading of 3 U/g was used for 2 kg of waste bread slurry, and the glucose content of WBH after hydrolysis was determined to be 125.75 g/L, corresponding to a conversion yield of approximately 97.10% from starch, which is considered to be quite a high ratio. The FAN content of WBH was found to be 127.88 mg/L with a conversion yield from total Kjeldahl nitrogen (TKN) to FAN of 3.97%.

Generally, CA production on WBH was lower than bioreactor trials in glucose-based media. A higher CA production of 15.03 g/L was obtained at the initial glucose concentration of 142.52 g/L in a batch reactor fermentation at the $47^{th}$ hour. The highest lipid yield of 0.32 g/g was generated from WBH containing an initial glucose concentration of 142 g/L. Moreover, the highest lipid content of 5.12 g/L at the $24^{th}$ hour of fermentation was produced from WBH containing an initial glucose concentration of 142 g/L.

Consequently, it can be concluded that there is no need for any nitrogen source supplement to a waste bread hydrolysate for CA production, as nitrogen-limited conditions are required for high CA productivity. However, a high dissolved oxygen concentration in the medium is a necessity. Moreover, in order to achieve a high level of lipid content and lipid yield from a waste bread hydrolysate by *Y. lipolytica*, nitrogen limitation and low air saturation should be applied. In addition, inhibitory effects of WBH components such as hydroxymethyl furfural on lipid production and growth of *Y. lipolytica* should be investigated. For a high lipid yield, the dissolved oxygen concentration in the exponential growth phase must be kept higher than 20%, while in the stationary phase it should be lower than 20% and these conditions can be achieved in a bioreactor equipped with a “cascade mode”. Lastly, Fed-batch and continuous fermentation methods and other strains

rather than strain K57 can also be employed for high citric acid and lipid production from a waste bread hydrolysate by *Y. lipolytica* in further studies.

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**LİST OF ABBREVIATIONS**

| | |
|---|---|
| CA | : Citric acid |
| SCO | : Single cell oil |
| TAG | : Triacylglycerol |
| MFSP | : A minimum fuel selling price |
| GGE | : Gasoline gallon-equivalent |
| GLA | : γ-linolenic acid |
| EPA | : Eicosapentaenoic acid |
| ARA | : Arachidonic acid |
| DHA | : Docosahexaenoic acid |
| w/w | : weight/weight |
| EPO | : Evening Primrose Oil |
| US | : United States |
| FAMEs | : Fatty acid methyl esters |
| GRAS | : Generally Regarded As Safe |
| TUIK | : Turkish Statistical Institute |
| TL | : Turkish Lira |
| Kg | : Kilogram |
| g | : Gram |
| $ | : Dollar |
| FDA | : American Food and Drug Administration |
| *Y. lipolytica* | : *Yarrowia lipolytica* |
| ºC | : Centigrade degree |
| L | : Litre |
| h | : Hour |
| BLAST | : Basic local alignment search tool |
| rpm | : Rotation per minute |
| vvm | : Vessels volume per minute |

| | |
|---|---|
| U/g | : Units per gram |
| FAN | : Free amino nitrogen |
| WBH | : Waste bread hydrolysate |
| TKN | : Total kjeldahl nitrogen |
| PUFAs | : Poly unsaturated fatty acids |
| FDA | : Food and drug administration |
| EP | : Extracellular protease |
| RAPD-PCR | : Random amplification of poliformic DNA- Polymerization chain reaction |
| G/C | : Guanine/ Cytosine |
| C/N | : Carbon/ Nitrogen |
| DCW | : Dry cell weight |
| wt | : Weight |
| TCA | : (Krebs cycle) Tricarboxylic acid cycle |
| ICA | : Iso- Citric acid |
| mg | : Milligram |
| WBP | : Waste bread powder |
| HPLC | : High performance lipid chromatography |
| DNS | : Dinitro- salicylic acid |
| PDA | : Potato dextrose agar |
| µL | : Micro litre |
| µmol | : Micro mol |
| mM | : Micro molar |
| BP | : Base pairs |
| UV | : Ultra violet |
| mL | : Millilitre |
| GC-MS | : Gas chromatography- Mass spectrophotometry |
| OD | : Optical density |
| $dO_2$ | : Dissolved oxygen |

| | |
|---|---|
| RID | : Refractive Index Detector |
| ANOVA | : Analysis of Variance |
| PCA | : Principle component analysis |
| S | : Substrate concentration |
| X | : Biomass concentration |
| C | : Citric acid concentration |
| L | : Lipid concentration |
| $Y_{X/S}$ | : Biomass yield |
| $Y_{C/S}$ | : Citric acid yield |
| $Y_{L/X}$ | : Lipids yield |
| $S_0$ | : Initial glucose concentration |

# 1. INTRODUCTION

The lipids produced by microorganisms are called microbial lipids or single cell oils (SCOs) (Ochsenreitheri et al, 2016; Qin et al, 2017), which include triacylglycerols (TAGs) (lipids of energy reserve), glycolipids (lipids of membrane structure), phospholipids and sterylesters (Bouchedja et al, 2017). Among these lipids, TAGs are the glycerol triesters with non-polar fatty acids and the main synthesized component in microbial cells. In most cases, microbial lipids exhibit compositional similarities to common plant oils (e.g., rapeseed oil) (Kumar et al, 2017). Storage lipids that are not integrated into the phospholipid bilayers accumulate to constitute the hydrophobic core of oil or lipid bodies (Capus et al, 2016). The aim of TAG biosynthesis by microorganisms is to generate energy reserves which can prolong vital functions during environmental deficiency or stress, such as a limited nitrogen medium (Ochsenreitheri et al, 2016). The accumulation of TAGs by microorganisms, specifically when glucose, other hexoses or polysaccharides like glycerol, starch, etc. are employed as the sole carbon source starts at the stationary phase of growth with the formation of oil droplets in the cytoplasm of the cell (Dourou et al, 2017). As a reserve component, TAGs can be produced in large amounts in the cytosol and the composition and percentage of TAGs vary according to the genetic predisposition of the microorganism, as well as the culture conditions employed (e.g., type of carbon and nitrogen source, medium pH, fermentation configuration, etc.) for the synthesis of microbial lipids (Fakas, 2017).

Over the last few years there have been a large number of publications regarding microbial oil production in the international literature (Bellou et al, 2016a; Finco et al, 2017; Ledesma-Amaro and Nicaud, 2016b; Maina et al, 2017; Ochsenreitheri et al, 2016; Souza et al, 2017). Economic and technological interest increases continuously for lipid-producing microorganisms, from bacteria (the simplest class) to fungi (the most complex). Although the production price of

SCOs is higher than the traditional utilization of common oils and fats, due to the obligatory maintenance of aseptic conditions and the cost of the fermentation per se (Biddy et al, 2016; Koutinas et al, 2014), several alternative options for the large-scale production of SCOs exist, as the price of various naturally occurring lipids and fats of the plant and animal kingdom can vary tremendously (a minimum fuel selling price (MFSP) of USD $9.55/gasoline gallon-equivalent (GGE); see:(Biddy et al, 2016)). Therefore, the determination of microorganisms able to produce high ratio of lipids with a similar composition and structure of high-value fats, and the production of these speciality lipids in large-scale, is of tremendous financial attention (Papanikolaou and Aggelis, 2011a). As a result of this objective, the prospect of the large-scale production of microbial lipids constituting sources of valuable fatty acids, which are essential in the medical-dietary field, is currently a high priority. In particular, the production of microbial lipids (in most cases fungal or algal oils) containing γ-linolenic acid (GLA), eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA) is currently of significant interest as these poly-unsaturated fatty acids are valuable as functional foods and have benefits for human health (Béligon et al, 2016; Bellou et al, 2016a; Ledesma-Amaro and Nicaud, 2016b; Xie et al, 2017). GLA or the lithium salts of microbial lipids containing this fatty acid present important anticancer characteristics (Alakhras et al, 2015), ARA, EPA and DHA aid the foetal brain development, the sight function of the eye, hormonal balance and the cardiovascular system (Ledesma-Amaro and Nicaud, 2016b). The added value of these lipids is of great importance in the food and healthcare industry (Sun et al, 2017). To put into perspective the cost of lipids containing the above-mentioned poly-unsaturated fatty acids, it must be pointed out that there are a limited number of plant lipids that contain the medically important GLA (C18:3, $\omega$-6) that are currently commercialized (generally, the oil derived from the plant ***Oenothera biennis*** contains γ-linolenic acid in concentrations of 8–10%, w/w, also called Evening Primrose Oil (EPO)); the price of these oils is approximately ***c.*** 45–50 US

$ per kg (Koutinas et al, 2014). Moreover, cocoa butter substitutes (CBSs) can be produced with the aid of oleaginous microorganisms, as the microbial lipid composition is similar to exotic fats under several culture conditions. We know that cocoa butter is an expensive raw material in the chocolate and biscuit industry and its availability and price change depending on seasons, climate and cultivation conditions of the cocoa tree (Wei et al, 2017); an "extreme" scenario that has recently been presented in both the scientific literature and the electronic international press indicates that there is a risk of cocoa butter disappearing over the coming years due to the general failure of the cultivation techniques of the cocoa plant; thus, the utilization and application of the various CBSs will be important for the food industry (Koutinas et al, 2014). Due to these reasons, alternative ways to supply CBSs and reduce production costs were sought. Investigations performed over the last few years have indicated that using the yeast *Yarrowia lipolytica* (*Y. lipolytica*) during its cultivation in agro-industrial residues (such as animal fat derivatives blended with biodiesel-derived waste glycerol or hydrolysed rapeseed oil, mutton tallow, etc.) can contribute to the accumulation of lipids resembling the composition of cocoa butter (Wei et al, 2017) and this lipid profile could be used as its substitute. As a result, using low-cost substrates for yeast fermentation, the production of equivalent valuable fats (cocoa butter, sal fat, shea fat etc.) could be achieved.

As their composition is similar to one of several vegetable oils, microbial lipids could be considered as perfect feedstocks for biodiesel production. This is the so-called "2$^{nd}$ generation" biodiesel (synthesized with the aid of heterotrophic oleaginous microorganisms cultivated on low- or negative-cost sugars or glycerol) or the "3$^{rd}$ generation" biodiesel derived via $CO_2$ sequestration by oleaginous algae (Darvishi et al, 2017; Papanikolaou et al, 2017). Traditionally, biodiesel, i.e., fatty acid methyl esters (FAMEs), is obtained by transesterification of triacylglycerol (TAG) and alcohol using an acid or alkali catalyst (Li et al, 2008). Over recent decades, finding alternative energy sources has been a priority, given that the

quantity of frequently used fuels is diminishing; hence, energy producers use vegetable oils to generate biodiesel and as a result the price increases. However, the production of vegetable oils such as palm, soy and rape seed oil is not enough to fulfil electric, heat and fuel energy demands. Additionally, when oil from vegetable seeds is supplied, there are some climatic and geopolitical limitations. As production depends on seasons, temperature and the availability of arable lands (Donot et al, 2014), researchers started to investigate microbial oil which exhibits desirable properties including, customizable fatty acid profiles, alternative feedstock for biodiesel, high productivity, abundant, reduced labour required, easier to scale up, less affected by location and no climatic or geopolitics limitations during production (Soccol et al, 2017). It is evident that concerning the production of liquid biofuels (i.e. biodiesel) realized with the aid of oleaginous microorganisms, only the low-cost hydrophilic carbon sources (i.e. biodiesel-derived “waste” glycerol, low molecular weight organic acids, wastewaters or low-quality waters containing increased concentrations of sugars, glucose syrups, etc.) should be considered as substrates for this purpose; it would certainly not make sense to proceed to fermentation of a fatty substrate in order to create cellular lipid that would subsequently be converted into biodiesel, while the initial fat substrate could have already been directly converted into biofuel without the step of fermentation being implicated.

Citric acid (2-hydroxy-1.2.3-propanetricarboxylic acid, $C_6H_8O_7.H_2O$) symbolised as food additive E330 is an organic acid and naturally found in many plants, animal tissues and physiological fluids (Apelblat, 2014; Kamzolova et al, 2008). Higher amount of citric acid (CA) around 4-8 % in lemon and lime and small amount of it around 1-3 % in other variety of fruits and vegetables such as oranges, tangerines, raspberries and strawberries are contained. Moreover, human body tissues such as blood, bones, semen, urine, human milk have CA quantity ranged from 10-25 ppm (mg/L) to 2000-4000 ppm (mg/L) (Apelblat, 2014). In terms of biochemical way, human and animal body can produce CA in the middle

of tricarboxylic acid cycle called as also Krebs cycle during oxidation of fats, proteins and carbohydrates in order to gain energy by series of enzymatic reactions (Kristiansen et al, 2002). Some physiological properties of this organic acid are colourless, soluble in water and have molecular weight of 210.14 g/mol and the density of 1.665 g/cm$^3$ at 18 ºC. In addition, it has biodegradable, eco-friendly, economical and Generally Recognised as Safe (GRAS) properties.

Although, small amount of CA can be produced by isolation from citrus fruits such as in Greece, Mexica and South America, mostly fermentation process with using cheap substrates like carbohydrates is applied due to low production cost. Generally, CA has utilisation in preparation of medicinal citrates, candy and beverage industry. Its small amount is also used in silvering and engraving and in dying and calico printing (Angumeenal and Venkappayya, 2013).

Recently, CA has increasing interest due to its important roles in food, pharmaceutical and detergent industry and its global production rate have reached more than 1.6 million tons annually with a growth of 5 % per year (Kamzolova et al, 2008; Morgunov et al, 2013). Approximately, 70 % of this quantity in food, 20 % in the production of washing powders and 10 % in pharmaceutical and chemical industries with in the form of anhydrous, monohydrate and salts; calcium citrate, potassium citrate and sodium citrate are used (Kamzolova et al, 2010).

CA has wide range of utilization in various industries. For instance, as a food additive it is employed as acidifier, antioxidant, emulsifier, flavouring agent, stabilizer and preservative. It is preferred to add in beverages in order to enhance their taste (sourness) and obtain natural fruit flavour. It prevents wine and other cider drinks from oxidation and browning reactions and provides clarity. In dairy industry especially in the production of cheese and ice creams it is applied as emulsifier, antioxidant and acidifier. In jellies, gelatine deserts and jams it is used to adjust their pH. As a stabilizer, it is employed in manufacturing of juice, confectionery and canned foods. Due to its GRAS, a biodegradable and environmental friendly property, CA has also usage in manufacturing

pharmaceutical, cosmetic and detergent products. Its high buffer capacity promotes the usage in application of soaps, detergents and cleaning products. In analytical chemistry, it is used as eluting agent in ion exchange chromatography. In industry it has usage in textile, printing, paper, leather, construction industries, in production of reagents and inks and as cleaning agent for hot water systems with removing calcium layers and also some metal plating and cleaning technologies (Apelblat, 2014).In our country, CA is mostly used in fruit juice companies as a food additive. Only about 950 million litres of fruit juice and nectar are consumed annually in our country and the average annual citric acid requirement of the fruit juice industry is reached to 2.850 tons when it is considered that the average of 3 grams of citric acid is used for 1 litre of juice. According to 2012 data of TUIK (Turkish Statistical Institute), about 6 million TL annually was paid for importation of citric acid in order to use in fruit juice sector. In addition, according to 2016 TUIK data, 45.341.283 kg of citric acid was imported to country and it was paid about 35.959.020 dollars. Price of it is approximately 0.8$-1$/kg.

Due to wide range utilization of CA and its functional properties in food, pharmaceutical and chemistry industry, high rate production of it is important. In addition, because of high price of CA, production cost should be decreased and new production techniques should be investigated. From this point of view, in this study, CA was produced from low cost substrate (waste bread) by using *Y.lipolytica* and fermentation conditions, productivity and yield were evaluated.

For this aim; primarily, the most promising *Y. lipolytica* strain was selected for high SCOs and CA productions from glucose in shake-flask fermentation. Secondarily, SCOs and CA were produced from glucose by the selected strain in bioreactor fermentation. Meanwhile, bioreactor conditions were optimized. Effect of initial glucose and $dO_2$ concentrations on CA and SCOs productions were observed by the selected strain on glucose based media in bioreactor fermentation. Lastly, waste breads were hydrolysed and SCOs and CA were produced from waste bread hydrolysate by selected strain in bioreactor fermentation.

# 2. LITERATURE OVERVIEW

## 2.1. History of Microbial Lipids

The initial research involving microbial lipids was performed in Germany during the First World War using *Endomyces* and *Fusarium* sp. to produce lipids in order to solve the cooking oil shortage problem (Sitepu et al, 2014). Before the 1980s, researchers tried to elucidate the metabolism and biochemistry of lipid synthesis by oleaginous microorganisms. The commercial production of microbial lipids did not start until 1985, because SCO was not a cost-effective product compared with vegetable oils (Ratledge, 2004). Over the following years, interest in microbial oil (SCO) production has increased, as SCO can replace high-value lipids such as cocoa butter (Beopoulos et al, 2009b) and help to maintain human health (Sijtsma and De Swaaf, 2004) by means of PUFAs (polyunsaturated fatty acids). SCO was also referred to as an important renewable energy feedstock that could be used in the biodiesel industry because of high energy prices. Studies continued in order to investigate the biochemical pathway involved in the phenomenon of lipid accumulation by oleaginous microorganisms (Tehlivets et al, 2007). Research focusing on microbial lipid accumulation progressed using key enzymes of this metabolic mechanism and regulating their activities (Zhang et al, 2007). In addition, scientists screened maximum lipid-accumulating oleaginous microorganisms (Papanikolaou et al, 2007a; Papanikolaou et al, 2007b). To increase the yield of the SCO content, genetic methods (mutation, gene replacement) of oleaginous yeasts, moulds and algae were studied (Friedlander et al, 2016; Ledesma-Amaro and Nicaud, 2016b). In addition, numerous substrates (hydrophobic and hydrophilic sources), especially low-cost agro-industrial wastes, were examined for lipid accumulation. Furthermore, to achieve the desired fatty acid profile of SCO, different microorganisms and culture conditions (pH, temperature and culture time) were investigated (Papanikolaou et al, 2004) and SCO detecting methods were also studied (Huang et al, 2013).

## 2.2. Oleaginous Microorganisms

Microorganisms that are able to produce lipids at least 20% of their biomass, called as oleaginous microorganisms, were firstly investigated at the end of World War II. Less than 100 species of moulds, algae and yeasts are considered to be oleaginous and oleaginicity is a comparatively unusual physiological speciality in the microbial environment (Liang and Jiang, 2013).

Non-oleaginous organisms are not capable of storing lipids in significant quantities (e.g., >20% w/w) inside their cells, pellets or mycelia under any conditions (Ratledge and Wynn, 2002). They stop cell division and propagation and can store polysaccharides (Diamantopoulou et al, 2014) or secrete secondary metabolites (e.g., acetic acid, citric acid, mannitol, etc.) into the medium under nitrogen-limiting conditions (Papanikolaou et al, 2013). On the other hand, it has recently been demonstrated that in several instances, even when insignificant lipid production existed, microbial lipids were consisted of principally of neutral fractions (e.g., *Y. lipolytica* cultivated on glycerol and mainly producing citric acid without any significant production of SCO) (Papanikolaou et al, 2017).

About 70 of 1600 yeast species can generate lipids to more than 20 % of their dry biomass (Garay et al, 2016). The average accumulated lipid content of these yeasts is around 40% of their biomass and can reach 70% depending upon nutrient limitation (Goncalves et al, 2014). The most well-known genera are *Candida, Cryptococcus, Rhodotorula, Trichosporon,* and *Yarrowia* (Rywinska et al, 2013), which can accumulate 90% w/w of the total storage lipid as TAGs and sometimes higher than 80% w/w of this total lipid includes unsaturated fatty acids (such as linoleic and oleic acids). The principal fatty acids present in microbially produced SCOs are myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) (Uemura, 2012). Oleaginous microorganisms (mostly fungi and algae) are also able to produce PUFAs that in many instances are not frequently present within the Plant or Animal Kingdom, such as dihomo-γ-linolenic acid, eicosapentaenoic acid, docosahexaenoic acid and

arachidonic acid which can be used for medical and dietary purposes (Bellou et al, 2016a). Moreover, over the last few years, a significant amount of work has been devoted to the utilization of genetic engineering tools in order to construct genetically modified yeast strains (mainly the species *Y. lipolytica*), that are able to (super)-express various desaturases (e.g., $\Delta_5$-, $\Delta_6$-, $\Delta_8$-desaturate), elongates and acyl-transferases; as a result, mutant yeast strains capable of synthesizing several of the above-mentioned "rare" PUFAs have been constructed (Béligon et al, 2016; Bellou et al, 2016a; Friedlander et al, 2016; Imatoukene et al, 2017; Ledesma-Amaro and Nicaud, 2016b; Tezaki et al, 2017; Xie et al, 2017).

### 2.3. *Yarrowia lipolytica*

*Yarrowia* was previously known as *Saccharomycopsis lipolytica, Candida* or *Endomycopsis* that is a yeast genus and approved in the class *Hemiascomycetes*. David Yarrow of the Delft Microbiology Laboratory identified a new genus and in acknowledgement; Van der Walt and Von Arx (1980) recommended the name as *Yarrowia*. The name "*lipolytica*" derived from the lipid hydrolysing capability of this yeast (Greek origin "Λιπολυτικός" – "Lipolytikós" which means "fat splitter"). Above 32°C, most of *Yarrowia* strains cannot grow and aerobic condition is required for the species. It has been classified as Generally Regarded As Safe (GRAS) for citric acid production by the American Food and Drug Administration (FDA) and assumed as non-pathogenic yeast (Fickers et al, 2005b; Madzak, 2015).
*Y. lipolytica* strains can be isolated from meat, poultry, dairy products (cheese, yoghurt and kefir), shrimp salads, soy sauce and oil-polluted environments, or marine and hypersaline environments (Liu et al, 2015a). They are obligate aerobic non-conventional yeasts, which tolerate salt, low temperatures and pH. Due to the existence of extracellular lipolytic and proteolytic activities of this organism, proteins and lipids can be degraded. The extracellular protease (EP) can exist up to 1–2 g/L in protein or peptone-rich medium. *Y. lipolytica* accumulates extracellular lipase in lipid-rich media; in addition, it synthesizes different extracellular enzymes

such as esterases, phosphatases and RNases which could help its growth under different conditions (Zinjarde, 2014). Besides SCO, this yeast can also produce organic acids (most commonly citric–acid and to a lesser extent pyruvate, α-ketoglutarate and acetate), polyols (mostly erythritol and to lesser extent mannitol), lipases, emulsifiers, surfactants, $\gamma$-decalactone, intracellular lipids and other related end products (for reviews see: (Madzak, 2015; Nicaud, 2012; Rywinska et al, 2013)).

*Yarrowia* generally grows at the temperature range of 24-33°C. Ethanol can be used as a carbon source by *Y. lipolytica* but this yeast species is not capable to produce it under any circumstances, being Crabtree- and Pasteur-negative species. Hexoses, such as glucose, fructose and mannose can be degraded by *Y. lipolytica* but it is not possible for some carbohydrates to pass freely through the membrane of the yeast; hence, di- and tri-saccharides are hydrolysed outside the cell. For instance, wild-type strains of *Y. lipolytica* are not capable of using sucrose due to the lack of the enzyme invertase (Coelho et al, 2010). Moreover, among the organic acids, acetic, lactic, propionic, malic, citric and succinic acid can be utilized by *Y. lipolytica* as the carbon source (Rodrigues and Pais, 2000); however, propionic, butyric and sorbic acids can inhibit the growth of this yeast. Furthermore, a sodium acetate concentration of more than 1.0% exerts an inhibitory effect on growth. As *Y. lipolytica* is an aerobic yeast, the amount of oxygen in the medium is an important parameter for this microorganism. Oxygen usage by this yeast also depends on components such as iron and perfluorocarbons in the culture. Sufficient concentration of those components can lead to high oxygen uptake due to the higher solubility of oxygen in perfluorocarbons than in water (Amaral et al, 2006).

*Y. lipolytica* which is a model for oleaginous microorganism, is one of the most studied and engineered oleaginous yeast because its genetics information had been discovered and bioinformatics tools are becoming increasingly available and allow to apply metabolic engineering strategies for this microorganism. Recently,

*Y. lipolytica* strains which are constructed by recombinant technology have been revealed capable to produce high quantity of lipids as high as 90 % of dry cell weight, with titers up to 85 g/L and lipid productivity of approximately 1 g/L $h^{-1}$(Ledesma-Amaro and Nicaud, 2016a). Different essential fatty acids such as omega 3 fatty acid, conjugated linoleic acid, gamma linoleic acid and arachidonic acid could be produced significant amount by engineered strains of *Y. lipolytica*. Although wild strains of *Y. lipolytica* cannot metabolize substrates rather than glucose, fructose and glycerol, inexpensive di and polysaccharides such as sucrose (from molasses), xylose, cellobiose, galactose, starch, inulin, xylan and cellulose can be used by engineering modified strains of this microorganism. There are many advantages to choose the yeast *Y. lipolytica* as host microorganism for metabolic engineering because it is safe and robust microorganism with genetic and physiological advantages for the target products such as single cell oil or essential fatty acids (Fakas, 2017; Hussain et al, 2016; Ledesma-Amaro and Nicaud, 2016a).

### 2.4. Taxonomy of *Y. lipolytica*

*Y. lipolytica* is yeast with different physiology to *Saccharomyces cerevisiae*. Taxonomically, the yeast belongs to the class *Hemiascomycetes* and *Dipodascacea* family (Kurtzman Cp, 2000) and was previously called different names such as *Candida, Endomycopsis* and *Saccharomycopsis lipolytica*. Nowadays, a few species of *Candida* such as *Candida deformans, Candida galli, Candida yakushimensisl, Candida oslonensis, Candida alimentaria* and *Candida hollandica,* have been classed as new members of the genus *Yarrowia* (Knutsen et al, 2007). The genome of *Y. lipolytica* strains was analysed via molecular identification methods (such as RAPD-PCR and karyotyping analysis) (Naumova et al, 2010) in order to gain knowledge regarding different properties, such as the genome size is twice that of *S. cerevisiae* (20,5Mbp), the G/C content and tRNA genes are high (49%, 510 respectively), the number of protein coding genes is 6449 (one ORF per 3 kbp) and the number of introns is 1083 (Liu et al, 2015a).

## 2.5. Biochemistry of Microbial Lipids Synthesis

Microbial lipid accumulation takes place in the yeast cell due to unbalanced (unstable) metabolism, which under nutrient-limited conditions can lead to lipogenesis, the lipids production and storage in the yeast cell, whereas in the case of a high level of nutrients in the medium the yeast begins to propagate new cells and as a result microbial growth is increased (Goncalves et al, 2014).

Lipid synthesis in the yeast cell can be divided into two mechanisms; (1) *de novo* synthesis, which comprises the use of hydrophilic substrates by the yeast in nutrient (nitrogen)-limited conditions and (2) *ex novo* synthesis, which comprises the hydrophobic substrates usage (Bellou et al, 2016a).

### 2.5.1. *De Novo* Lipid Accumulation

The intermediate key product of *de novo* lipid accumulation is acetyl-CoA which is a sub-product of fatty acid synthesis. After glycolysis, pyruvic acid passes through the mitochondria where acetyl-CoA is produced from pyruvic acid by the catalysing enzyme pyruvate dehydrogenase; subsequently, acetyl-CoA can be used two ways. It either joins the Krebs cycle or it passes again into the cytoplasm where fatty acid biosynthesis occurs (Ratledge and Wynn, 2002). Since acetyl-CoA is not able to pass through the mitochondrial membrane it needs to be transformed into acetyl-carnitine, which is catalysed by the carnitine-acyl-transferase enzyme, so that it forms acetyl-carnitine and can be transported into the cytosol. Generally, a little amount of acetyl-carnitine can pass through the cytosol from the mitochondrial matrix in non-oleaginous microorganisms (Papanikolaou and Aggelis, 2011a); however, in the case of oleaginous microorganisms citric acid is broken down to acetyl-CoA under some conditions and acetyl-CoA can be accumulated in the mitochondria (Fakas et al, 2009).

There are different ways to generate acetyl-CoA in oleaginous microorganisms; for instance, exhaustion of nutrients (usually nitrogen) from the medium is a key factor. In this situation, the AMP-deaminase enzyme is activated

and catalyses the formation of IMP (inosine monophosphate) and $NH_4^+$ from AMP, as a result the AMP concentration decreases in the mitochondria but the cellular ammonium concentration increases. The reduction of AMP concentration inhibits the isocitrate dehydrogenase enzyme which blocks the Krebs cycle at the level of iso-citrate, simultaneously, the aconitase enzyme catalyses the accumulation of citrate in the mitochondria. When the citrate concentration in the mitochondria reaches a critical value, citric acid passes into the cytosol and existing citrate in the cytoplasm is cleaved into acetyl-CoA and oxaloacetate by ATP-citrate lyase, which is the key enzyme for lipid accumulation in oleaginous microorganisms. At the end of these pathways, accumulated acetyl-CoA is used for fatty acid synthesis. If the ATP-citrate lyase enzyme is not present in the microorganism, there is an accumulation of citric acid in the cytoplasm under nitrogen-limited conditions (Papanikolaou and Aggelis, 2011a) (see Figure 2.1).

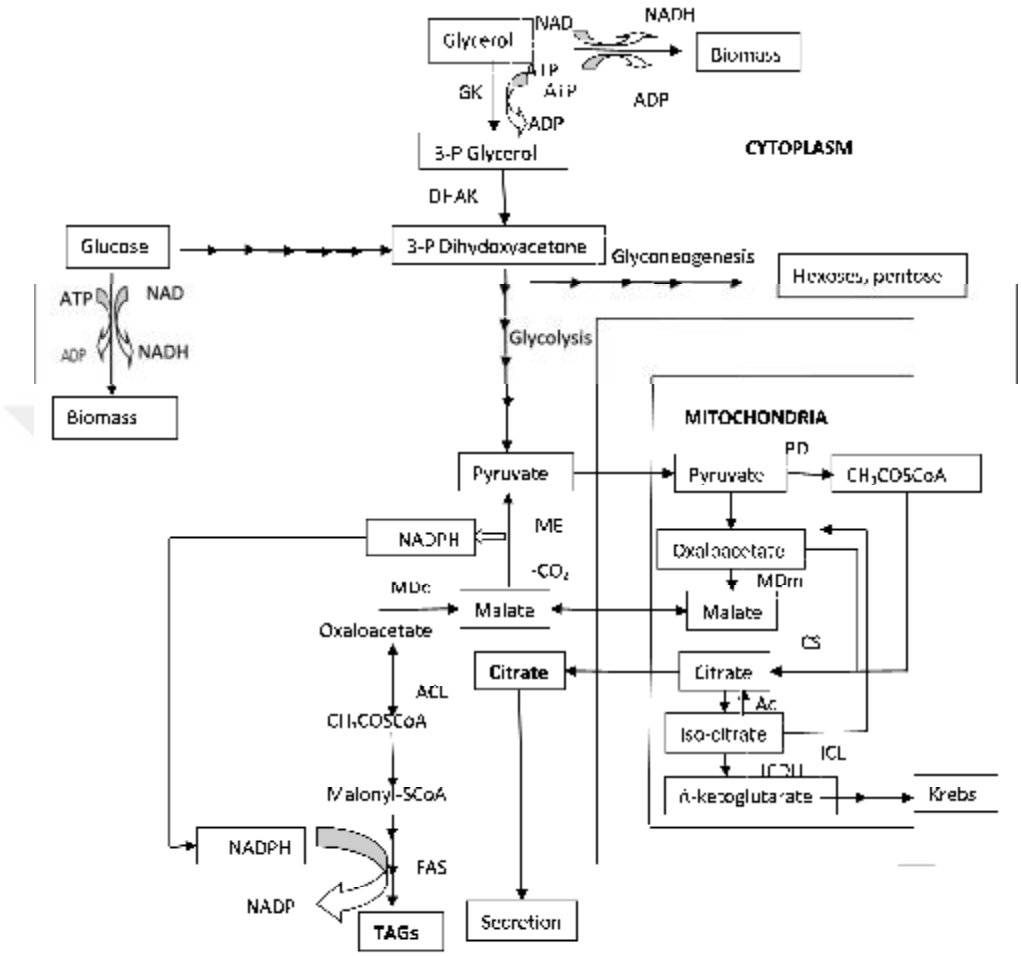



Figure 2.1. Metabolism of oleaginous microorganisms (Enzymes: GK, glycerol kinase; DHAK, dihydoxyacetone kinase; ME, malic enzyme; Ac, acotinase; ACC, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; FAS, fatty acid synthesis; CS, citrate synthese; ICL, iso-citrate lyase; TAGs, triacylglycerols) (Papanikolaou and Aggelis, 2011a).

### 2.5.2. *Ex Novo* Lipid Accumulation

When oleaginous microorganisms use hydrophobic substrates, such as TAGs, free fatty acids, oils and fats, are firstly transformed to free fatty acids outside the cell by a lipase-catalysed hydrolysis process so that they can be

transferred inside the microbial cell by means of active transport. Inside the microbial cell, fatty acids are then modified either for cell propagation or used for the biotransformation of new fatty acid profiles which did not previously exist in the medium ; these newly modified fatty acids are reduced to short chain acyl-CoAs and acetyl-CoAs by different acyl-CoA oxidases via β-oxidation and are used to provide the required energy for growth and cell repair or bioformation of some metabolites which enable the synthesis of new cellular materials (Beopoulos et al, 2009b).

*Y. lipolytica* is one of the known yeast (other yeasts genera such as *Rhodosporidium, Geortichum* and *Trichosporon* can also grow on fats) that can also utilize hydrophobic substrates through existing lipolytic and proteolytic activities using its own metabolism (Barth et al, 2003). In order to uptake these hydrophobic substrates from the medium; it can generate surfactants which help decrease the size of hydrophobic substrate, which increases the contact surface between the yeast and substrates (Mlickova et al, 2004). In addition to producing surfactants, the yeast can also produce an extracellular emulsifier (liposan) and lipase which stimulate TAGs hydrolysis (Barth G, 1996) and increase the number of droplets of small-sized hydrophobic substrates in order to help the transport of substrate inside the yeast cell.

There are several pathways for substrate degradation inside the cell such as alkane oxidation, β-oxidation and mix between citrate and glycosylate cycles which occur in the endoplasmic reticulum, peroxisomes and mitochondria, respectively. The final substrate degradation step takes place in the peroxisome via β-oxidation, but if there is an excess amount of hydrophobic substrate, they are reserved as TAGs and sterol esters in the yeast cell (Beopoulos et al, 2009b).

## 2.6. Factors Affecting the Production of Microbial Lipids by *Y. lipolytica*

The carbon/nitrogen (C/N) ratio, carbon and nitrogen sources, dissolved oxygen, pH and incubation temperature are the main factors affecting SCO

production (Ochoa-Estopier and Guillouet, 2014). Nevertheless, there are also other factors such as minerals (sulphur, zinc, phosphorus etc.) and vitamins (like thiamine) which play a vital role in microbial activity (Donot et al, 2014); in addition, secondary metabolites like citrate can also affect SCO production (Beopoulos et al, 2009b).

The general factors affecting lipid accumulation are detailed below.

### 2.6.1. Factors Affecting Lipid Production

#### 2.6.1.1. Types of Carbon Source Used in *De Novo* Lipid Production

Many researchers have studied different kinds of carbon sources for the optimization of de novo lipid production by *Y. lipolytica* using various fermentation modes, such as batch, fed-batch, continuous and shake-flask. Glucose and other carbon sources such as, raw starch, xylose, fructose, sucrose, hydrolysates of lignocellulose, acetate, wheat straw hydrolysate, rice bran hydrolysate, glucose enriched with municipal waste waters, glucose enriched with volatile fatty acids, glucose enriched with organic acids, sugarcane bagasse hydrolysate, and glycerol have mainly been used for SCO production by wild type and metabolically engineered strains of *Y. lipolytica* (Donot et al, 2014; Leiva-Candia et al, 2014) (Table 1).

*Y. lipolytica* can naturally use glucose, glycerol, fructose and lesser amount of organic acids and alcohols as hydrophilic substrates. In glucose based media it can grow fast and produce high amount of biomass. Although growth of *Y. lipolytica* generally decreased at the higher glucose concentration more than 150 g/L, it was reported that engineering strain of *Y. lipolytica* enabled to achieve high glucose to lipid conversion yield of 0.234 g/g (84.7% of the theoretical maximum) and showed good tolerance to high glucose concentration of 300 g/L (Qiao et al, 2015).

Fructose is another substrate for *Y. lipolytica* and amount of lipid production from fructose by *Y. lipolytica* is strain dependent. Growth rate of *Y.*

*lipolytica* is lower in fructose than in glucose media. Furthermore, glucose uptake was found to be delayed with the presence of fructose at the beginning of fermentation (Lazar et al, 2014; Lazar et al, 2011). Recent studies demonstrated that level of biomass and lipid from fructose can be improved by mutant strains of *Y. lipolytica* by changing hexokinase activity. Lazar et al. (2014) found that mutant strain of *Y. lipolytica* W29 could produce biomass of 21.6 g/L (17.4 g/L produced by wild type) and lipid of 3.02 g/L (1.56 g/L produced by wild type) from 100 g/L fructose as sole carbon source during fermentation time of 120 hours.

Glycerol is another low-cost substrate which is a by-product of biodiesel production. The biodiesel industry has flourished over the last few years, which has increased the availability of glycerol. Research has been focused on glycerol as an important carbon source for SCO production and has been discussed by many scientists (Dobrowolski et al, 2016); for example, Papanikolaou et al. (2002; 2003) studied glycerol with other food industry wastes to produce SCO via *Y. lipolytica*. Applied metabolic engineering methods to strains of *Y. lipolytica* have been improved amount of biomass and single cell oil production during cultivation in glycerol media (Celinska and Grajek, 2013). For instance, Celinska and Grajek (2013) reported that genetically engineered strain of *Y. lipolytica* NCYC3825 could accumulate biomass of 42 g/L and lipid content of 13 g/L (see Table 1).

Sucrose is the main component of molasses from sugar industry which is the low cost by product and is thought as substrate for many oleaginous microorganisms in the production of single cell oil. However, enzyme invertase which cleaves sucrose into glucose and fructose is lack in natural strains of *Y. lipolytica* that cause unable to utilize sucrose as a carbon source. Moreover, high nitrogen concentration of molasses is not preferable for microbial lipid production since research on SCO production has shown that nitrogen limitation is a key parameter for *de novo* lipid synthesis. Therefore, molasses utilized for lipid accumulation should not contain high nitrogen content. In addition, the process of removing it adds to the cost. As a result, molasses has a high proportion of sugar

but is not cost-effective due to the high nitrogen content. However, recent studies showed that metabolic engineered strains of *Y. lipolytica* which had high invertase activity could able to degrade sucrose as carbon source (Lazar et al, 2013). It was obtained by Lazar et al (2014) that maximum lipid content of 9.15 g/L and lipid yield of 0.262 g/g were produced from sucrose in bioreactor trials by strain *Y. lipolytica* Y4086.

Although other mono and disaccharides such as galactose, cellobiose and xylose are also not utilized as sole carbon source by wild type of *Y. lipolytica* strains, recent studies indicated that genetically modified strains able to metabolize these types of carbon sources (Guo et al, 2015; Lazar et al, 2015; Stephanopoulos and Tai, 2013; Wang et al, 2014).

In addition, *Y. lipolytica* can naturally use ethanol as a substrate due to presence of enzymes; alcohol dehydrogenase and aldehyde dehydrogenase and can able to grow in the ethanol concentration up to 3 %. It was indicated that higher concentration more than this value inhibited the growth of *Y. lipolytica.* Moreover, acetate which is another hydrophilic substrate can be utilized as sole carbon source by *Y. lipolytica.* In a study with sodium acetate, it was reported that sodium acetate concentration of 0.4 % (v/v) was tolerable for assimilation and metabolism in *Y. lipolytica* and concentration more than 1.0 % inhibited the growth (Barth G, 1996). Furthermore, volatile fatty acids such as acetic acid and propionic acid can be used as carbon source to increase lipid accumulation and biomass production (Fontanille et al, 2012). For instance, Fontanielle et al (2012) reported that *Y. lipolytica* MUCL 28849 could produce lipids of 16.5 g/L with the yield of 0.4 g/g (lipid/DCW) from volatile fatty acids added subsequently after glucose exhaustion during 60 hours fermentation time in 7 L bioreactor.

Table 2.1. Total dry weight and lipid production by *Y. lipolytica* strains.

| Yeast Strain | Lipid Content (%w/w) | Total Dry Weight (g/L) | Carbon Source | Nitrogen Source | Culture Mode | Type of Synthesis | References |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* NC1 | 37.7 | 9 | Waste oil from chicken products fat | | Flask | *Ex-novo* | |
| *Y. lipolytica* NC1 | 45.49 | 8.4 | Waste oil from frying fish | | Flask | *Ex-novo* | |
| *Y. lipolytica* NC1 | 34.02 | 8.04 | Meat products fat | | Flask | *Ex-novo* | |
| *Y. lipolytica* NC1 | 57.89 | 7.56 | Waste oil from frying vegetables | | Flask | *Ex-novo* | |
| *Y. lipolytica* | n.m | 15.3 | Municipal wastewater + glucose | Peptone + yeast extract | Flask | *De novo* | (Leiva-Candia et al, 2014) |
| *Y. lipolytica* MUCL 28849 | 40.22 | 41.02 | Glucose + volatile fatty acids | Ammonium sulphate | Fed-batch | *De novo* | |
| *Y. lipolytica* MUCL 28849 | 34.59 | 41.02 | Glycerol + volatile fatty acids | Ammonium sulphate | Fed-batch | *De novo* | |
| *Y. lipolytica* ATCC 20460 | 4.4 | 7.2 | Detoxified liquid wheat straw hydrolysate | Yeast extract | Flask | *De novo* | |
| *Yarrowia lipolytica* ATCC 20460 | 4.6 | 7.8 | Non-detoxified liquid wheat straw hydrolysate | Yeast extract | Flask | *De novo* | |
| *Y. lipolytica* Po1g | 58.5 | 11.42 | Detoxified sugarcane bagasse hydrolysate | Peptone | Flask | *De novo* | |

Table 2.1. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* Po1g | 56.01 | 6.8 | Raw glycerol | Yeast extract+ ammonium sulphate | Batch | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 5.5 | 14.5 | Glucose | Yeast extract | Batch | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 19.56 | 4.6 | Raw glycerol | Yeast extract+ ammonium sulphate | Batch | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 50 | 15 | Animal fats | Ammonium sulphate | Fed-batch | *Ex-novo* | |
| *Y. lipolytica* LGAM S(7)1 | 40.1 | 30.83 | Glucose + acetic acid | Ammonium sulphate | Fed-batch | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 38 | 39 | Glucose + propionic acid | | | *De novo* | (Donot et al, 2014) |
| *Y. lipolytica* LGAM S(7)1 | 24 | 31.76 | Glucose + butyric acid | | | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 38.4 | 40.93 | Glycerol+ acetic acid | | | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 38.8 | 36.82 | Glycerol + propionic acid | | | *De novo* | |
| *Y. lipolytica* LGAM S(7)1 | 37.6 | 30.83 | Glycerol + butyric acid | | | *De novo* | |
| *Y. lipolytica* ACA-DC 50109 | 34.2 | 41 | Glycerol + fatty acids | Ammonium sulphate | Fed-batch | *De novo* | |
| *Y. lipolytica* ACA-DC 50109 | 2.74 | 7.3 | Glucose + essential oils from Greek citrus | Yeast extract | Batch | *De novo* | |

Table 2.1. Continued

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* YL-ad9* | 67 | 82 | Glucose | Yeast extract + ammonium sulphate + yeast nitrogen base | Fed-batch | *De novo* | (Qiao et al, 2015) |
| *Y. lipolytica* W29* *(transformat)* | 14 | 21,6 | Fructose | Yeast nitrogen base medium | Flask | *De novo* | (Lazar et al, 2014) |
| *Y. lipolytica* A101 | 25 | 6.76 | Glycerol | Yeast nitrogen base + Ammonium sulphate | Flask | *De novo* | (Dobrowolski et al, 2016) |
| *Y. lipolytica* NCYC3825* | 30 | 42 | Glycerol | Yeast extract+ ammonium sulphate | Fed-batch | *De novo* | (Celinska and Grajek, 2013) |
| *Y. lipolytica* Y4086* | 26.2 | 34.9 | Sucrose | Ammonium chloride | Batch | *De novo* | (Lazar et al, 2014) |
| *Y. lipolytica* Y4588* | 16.6 | 19.4 | Galactose + glucose | Ammonium chloride | Batch | *De novo* | (Lazar et al, 2015) |
| *Y. lipolytica* MTYL085* | 42 | 18 | Glucose + xylose | Yeast extract+ ammonium sulphate | Batch | *De novo* | (Stephanopoulos and Tai, 2013) |
| *Y. lipolytica* Po1g | 48.02 | 10.75 | Detoxified defatted rice bran hydrolysate | Urea, Peptone | Flask | *De novo* | (Tsigie et al, 2012) |
| *Y. lipolytica* XYL+Obese* | 35.5 | 56.6 | Xylose | Ammonium chloride + yeast extract | Fed-batch | *De novo* | (Ledesma-Amaro et al, 2016) |

**Microbial lipid production from lignocellulosic biomass:** Lignocellulosic biomass is considered to be a lower cost substrate than those mentioned above for microbial oil production and usually contains cellulose, lignin and hemicellulose (Huang et al, 2013). There are two steps for the utilization of lignocellulosic biomass for SCO production. First, the biomass is degraded to its corresponding monosaccharides, and then the fermentation of biodegradable sugars takes place. Using this method, the complete hydrolysis of lignocellulosic material is achieved and fermentable sugars like pentoses (arabinose and xylose) and hexoses (glucose and mannose) can be obtained. The fermentable sugar, glucose, is present in the highest concentration, then mannose (from softwoods) or xylose (from hardwoods) and other sugars are found in decreasing concentrations in the lignocellulosic hydrolysate. It is important to consider that the microorganisms used should be able to hydrolyse hexoses and pentoses to enable biodegradation; however, it is particularly difficult to find microorganisms which can utilize pentoses such as xylose. Therefore, the most important factor in the utilization of lignocellulosic biomass is to find pentose-fermenting strains. For instance, Yu et al, (2011) reported that *Y. lipolytica* ATCC 20460 can use hydrolysate from the dilute sulfuric acid pre-treatment of wheat straw for the production of microbial lipid and 0.4 g/L of lipid and 7.8 g/L of dry cell weight were obtained. Also, in another study by using detoxified sugar cane bagasse hydrolysate as a carbon source, *Y. lipolytica* Po1g which is a derivative of the wild-type strain W29 (ATCC 20460) by a series of genetic modifications, can accumulate 6.68 g/L of lipids, 11.42 g/L of biomass and 1.76 g/L-day of lipid productivity under optimum fermentation conditions (Tsigie et al, 2011). Moreover, same strain of *Y. lipolytica* was used for production of single cell oil from detoxified defatted rice bran hydrolysate and 10.75 g/L of dry cell weight, 48.02 % of lipid content were obtained (Tsigie et al, 2012).

Although *Y. lipolytica* cannot fully use lignocellulosic biomass genetically engineered strains enabled to assimilate and produce significant level of lipids (Ledesma-Amaro and Nicaud, 2016a). For instance, Stephanopoulos and Tai

(2013) engineered genetically modified *Y. lipolytica* Po1g to convert xylose mixed with glycerol as carbon source to SCO and a biomass of 18 g/L (13 g/L generated from glycerol), 7.56 g/L of lipids were produced from 20 g/L glycerol and 80 g/L xylose. In addition, Ryu et al. (2015) reported that mutant strain of *Y. lipolytica* enable to use xylose as sole carbon source but obtained biomass and growth rate from xylose was 16 times lower and 10 times slower respectively than in glucose based media that cause unfavourable conditions for large-scale industrial production. Therefore, recent years, Ledesma-Amaro et al. (2016) studied well for lipid production from xylose as the sole carbon source by engineered strain *Y. lipolytica* XYL+Obese. Lipids of 20.1 g/L, lipid yield of 0.08 g/g and productivity of 0.19 g/L/h were obtained from 250 g/L of xylose as the sole carbon source by *Y. lipolytica* XYL+Obese strain.

Research on *Y. lipolytica* shows that this microorganism generally produces more microbial lipid when hydrophobic substrates (the *ex-novo* lipid accumulation process), such as oil materials, are used as the carbon source.

#### 2.6.1.2. Types of Carbon Source Used in *Ex Novo* Lipid Production

As previously mentioned, hydrophobic substrates are also one of the main substrates for SCO production by *Y. lipolytica* and this process does not require nitrogen-limited conditions (Papanikolaou and Aggelis, 2011b). Various types of hydrophobic substrates are produced via industrial processes and, until now, their quantity has been increasing day by day; however, the possibility of low-cost SCO production using these substrates helps to protect the environment from their hazardous effects. Most of hydrophobic carbon sources which are pure fatty acids, animal or vegetable oils fatty acids (Papanikolaou et al, 2008c), fatty acid by-products or wastes (Papanikolaou et al, 2002a), n-alkanes (Fontanille et al, 2012) have been used as carbon sources for *ex novo* lipid accumulation. In addition to these, a combination of hydrophilic substrates (such as glucose) and different kinds of fatty materials are commonly used for *ex novo* lipid synthesis.

There are several hydrophobic substrates that have already been used for SCO production; for example, vegetable oils have been used for the growth of *Y. lipolytica* and SCO production. Moreover, research on *Y. lipolytica* used a small amount of essential oils of fruit and glucose as the culture medium and this essential oil addition raised the growth lag phase and slightly decreased the biomass content (Papanikolaou et al, 2008a). The saturated fatty acid content of the produced lipids increased from 24 to 35–43 wt % of cellular lipids by the essential oils addition. However, in other research, the addition of fat to the culture of *Y. lipolytica* did not change the fatty acid profile. Studies on *Y. lipolytica* cultivation using additional volatile fatty acids with fructose or glucose improved the lipid production and biomass up to 40% with a conversion yield of up to 20% using a two-stage fed-batch fermentation mode (Fontanille et al, 2012).

In addition, industrial fats which were used to increase the C:18 fatty acid content in lipids produced by oleaginous microorganisms for the cocoa butter industry, have also been used as media for the production and accumulation of lipids by *Y. lipolytica* (Papanikolaou and Aggelis, 2003). From this work, Papanikolaou et al. (2003) were able to accumulate cocoa butter-like lipids using *Y. lipolytica* when they employed in the technical glycerol, glucose and industrial derivative of animal fat (stearin) as the cultivation medium. In the case of the utilization of blended hydrophilic and hydrophobic substrates, both the *de novo* and the *ex novo* mechanisms can be implicated, in order SCO to be synthesized; specifically, the pathways implicated could contain but are not limited to the following reactions: a) Incorporation of the extra-cellular aliphatic chains inside the cells; b) Reactions of biomodification of the fatty chains that had previously been incorporated (elongation and desaturation); c) Direct and not random incorporation of several of the previously consumed fatty chains in the triacylglycerols of the microorganisms that are implicated in the process; d) Assimilation of the acyl-CoA units, *via* β-oxidation pathway; e) Potential anabolic reactions of portion of the acetyl-CoAs generated after β-oxidation through the glyoxylic acid by-pass; f) *De*

*novo* biosynthesis of fatty acids from acetyl-CoA deriving from glucose or glycerol catabolism (Papanikolaou and Aggelis, 2011a).

It should be noticed that cocoa butter-like lipids can be also produced by a *de novo* lipid accumulation process as the lipid profile of oleaginous microorganisms does alter throughout lipid fermentation. The quantity of hydrophobic substrates from food industry wastes is less than hydrophilic substrates; however, because of the above mentioned specific properties, such as the synthesis of CBSs, they will be the focus of further investigation in future (Huang et al, 2013).

### 2.6.2. Nitrogen Source

*De novo* lipid accumulation can be affected by the source of nitrogen and its limitation in the medium is important. The general nitrogen sources used for the production of SCO are $(NH_4)_2SO_4$, peptone, yeast extract, urea, corn steep, L-arginine, corn gluten, tomato waste hydrolysate and whey concentrate (Donot et al, 2014); combinations of these have also sometimes been used as the nitrogen source.

Recently, Bellou et al (2016) was studied effect of nitrogen source on lipid production by *Y. lipolytica*. $(NH_4)_2SO_4$ (ammonium sulphate), $NH_4C_2H_3O_2$ (ammonium acetate), yeast extract, tomato extract, meat peptone and their combinations were used as nitrogen sources. Among all nitrogen sources, higher lipid yield of 0.18 g/g (DCW) and biomass of 8.6 g/L were produced from glycerol by using yeast extract as nitrogen source in bioreactor fermentation with the carbon/ nitrogen ratio of 95. Lower biomass and lipid production were observed when ammonium nitrogen was used. Especially when $NH_4C_2H_3O_2$ was used as nitrogen source in glycerol (50 g/L) media, minimum lipid and biomass values were obtained. Yeast extract concentration of 3 g/L (0.32 g/L total nitrogen) was found to be highest lipid yield of 10.9 % (w/w) and higher nitrogen concentrations led to increase biomass production and lower the lipid accumulation. Furthermore,

in another study, it was reported that the critical nitrogen concentration to induce lipid production by *Y. lipolytica* was stated as $10^{-3}$ mol/L (Cescut, 2009) and explained that nitrogen concentration in the medium should be more than this value to prevent the citric acid production, therefore lipid production will not be affected (Beopoulos et al, 2009a).

### 2.6.3. C/N Ratio

In the case of the *de novo* lipid accumulation process, limiting the nitrogen concentration usually determines the biomass content, whilst the concentration of the carbon source generally determines the quantity of lipids (Papanikolaou and Aggelis, 2011b), as a result the C/N ratio is important in determining the lipid content and quantity of oleaginous microbial biomass. According to several research performed in this field, lipid accumulation is stimulated at a molar ratio of C/N>20; when the C/N ratio is higher than 70, lipid production generally decreases (Papanikolaou and Aggelis, 2011b); therefore, to achieve high lipid accumulation, the initial C/N molar ratio should be optimized. On the other hand, in several other cases implicating oleaginous moulds, the production of microbial lipid (in terms of both absolute; g/L and relative; % of lipid in DCW) constantly increases with the rise of initial C/N molar ratio imposed (Zikou et al, 2013).

Moreover, research on *Y. lipolytica*, using glucose and glycerol as the carbon source, employed a C/N ratio of 62 (80 g/L of C and 3 g/L of N) and after 48 hours of fermentation, 16 g/L lipid content was obtained with a 20% conversion yield of carbon into lipids (Fontanille et al, 2012). Ochoa-Estopier et al (2014) studied the effect of N/C ratio on lipid accumulation and stated that N/C range of 0.021-0.084 mol N C mol $^{-1}$ was found to be critical range for lipid accumulation without citric acid production by *Y. lipolytica* W29. Significant specific lipid accumulation rate was observed at the higher N/C molar ratio more than 0.021 with the increased production of citric acid. It was also reported that high initial C/N molar ratio of 80-120 caused cell growth with significant citric acid and low lipid

production by *Y. lipolytica* (Beopoulos et al, 2009a). However, recent studies shows that the optimal molar C/N ratio for high lipid accumulation ranged 75 to 150 when *Y. lipolytica* A101 grown on glycerol (Dobrowolski et al, 2016). Also, Canonico et al (2016) found the maximum lipid production of 2.60 g/L at C/N ratio of 118 when 90 g/L glycerol was used as carbon source by *Y. lipolytica* 347 during fermentation time of 144 hours. In another study with *Y. lipolytica* SKY7, highest lipid production from 82.5 g/L glycerol was found at the C/N ratio of 75 with inoculum volume of 6.25 %, 29.5 g/L biomass and 50 % (w/w) lipid content were obtained (Kuttiraja et al, 2016). Recently genetically engineered strain of *Y. lipolytica* JMY3580 produced 40 % of lipids in biomass with C/N ratio of 90 which did not lead lipid accumulation in wild type strain *Y. lipolytica* more than 20 % in glycerol based media. These results demonstrated that C/N ratio affects the lipid accumulation and it is strain dependent factor for the yeast *Y. lipolytica* (Gajdos et al, 2017).

### 2.6.4. pH

Like the carbon composition, pH is another important factor that influences lipid accumulation. *Y. lipolytica* has unique property than other microorganisms that it has ability to grown on extremely high (up to 10) and low (pH=3) pH values (Barth G, 1996); this advantage can lead to grow in unfavourable media conditions with a minimum minerals, toxic and waste materials (Sekova et al, 2015).

pH of medium was considered an important factor for lipid production by *Y. lipolytica*, with most pH values used for cultivation ranging between 5.0 and 7.0 (Fontanille et al, 2012). A study using *Y. lipolytica* grown on stearin at pH 6.0–6.5 showed that lipid production was only observed at pH 6.0 (Goncalves et al, 2014). Recently, Timoumi et al (2017) demonstrated that kinetic parameters, yield and viability of *Y. lipolytica* W29 were slightly affected at pH values of 4.5, 5.6 and 7.0 and filamentous growth was observed at pH of 4.5 and 7.0 rather than 5.6 in batch experiments.

### 2.6.5. Temperature

The incubation temperature significantly influences lipid production in the yeasts *Y. lipolytica,* Although *Y. lipolytica* does not show high growth at temperatures of 19 and 38°C on low-cost saturated free fatty acids, temperatures from 24 to 33°C favour significant growth with maximum biomass of 7.5–8.7 g/L; however, lipid accumulation is observed at 28°C (Papanikolaou et al, 2002a).

In addition to the quantity of fat accumulation, the lipid profile can be modified via the incubation temperature (Papanikolaou and Aggelis, 2011a); fatty acid composition is dependent on the incubation temperature and usually the degree of saturation decreases at low temperatures (Ageitos et al, 2011). For example, when *Y. lipolytica* grew on glucose, the activity of the enzyme catalysing the transformation of oleoyl-CoA to linoleoyl-CoA (Δ12-desaturase) at T=10°C was doubled compared with that at T=25°C and the $^{\Delta 9,12}$ C18:2 fatty acid concentration increased in the latter case (Papanikolaou and Aggelis, 2011b). Moreover last study done by Tezaki et al (2017) showed that Δ12-fatty acid desaturase enzyme was involved in the growth of *Y. lipolytica* at the low temperature of 12 ºC. Furthermore, the desaturase enzyme activity is reduced with increasing temperature. Although low incubation temperature increases unsaturation, it does not favour lipid accumulation; generally, low temperatures cause cellular activities and metabolism to decrease (Beopoulos et al, 2009b).

### 2.6.6. Dissolved Oxygen

The dissolved oxygen content in the medium affects lipid accumulation and composition. It is known that at the higher dissolved oxygen concentration citric acid production increases with the decrease in lipid accumulation. Therefore, for the optimum lipid production dissolved oxygen value should be kept at lower values. For instance, recently Sabra et al (2017) reported that lipid accumulation by *Y. lipolytica* was decreased in the oxygen controlled cultivation so limited oxygen and nitrogen conditions were the key factor for significant lipid

accumulation. Same results were also obtained by Racicka et al (2015) who explained that unregulated oxygen condition was favourable for lipid accumulation than regulated oxygenation. Moreover, Unrean et al (2017) demonstrated that lower than 0.1 mmol/mmol (oxygen/ glucose) oxygen uptake rate increase the lipid accumulation and reduced the growth of *Y. lipolytica.* At the same study, the decrease of agitation from 300 to 100 rpm resulted in 14 % increase in lipid yield and 48 % increase in lipid content within 5 days of batch shake flask experiment.

### 2.6.7. Fermentation Modes

Several fermentation modes have been investigated with a view to increasing lipid production by oleaginous microorganisms. Generally, four kinds of fermentation modes are used; batch, repeated batch, fed-batch and continuous (Subramaniam et al, 2010). Of these fermentation modes, fed-batch mode has been demonstrated to be effective in increasing the cell density and cellular lipids of oleaginous microorganisms (Beopoulos et al, 2009b; Papanikolaou and Aggelis, 2011b; Zhang et al, 2011). For instance, a fed-batch mode increased biomass (104.1 g/L) and lipid production (86.1 g/L) compared with batch mode (biomass: 51.8 g/L and lipids: 33 g/L), when glucose was used as the sole carbon source (Zhang et al, 2011).

*Y. lipolytica* is able to produce large amounts of intermediates, accumulate large quantities of lipids and break down stored lipids via β-oxidation. Based on this knowledge, the fermentation process for lipid production by *Y. lipolytica* can be directed. According to differences in the C/N flux ratio for a constant nitrogen flux, several metabolic states can be specified (Beopoulos et al, 2009b). There are three different fermentation modes that are generally used, i.e., batch, fed-batch and continuous.

At the beginning of batch mode fermentation, carbon, nitrogen and minerals are firstly mixed with the aim of obtaining a high ratio of rC/rN (residual carbon to residual nitrogen). Nitrogen exhaustion starts at the beginning of the

culture and the rC/rN ratio steadily increases. At this stage, growth is exponential whereas nitrogen is not limiting; then in the second stage, there is an excess concentration of carbon in the culture and a high rC/rN ratio is obtained. As a result of these conditions, biomass production decreases while lipid production remains high. During the third stage, citric acid production is induced due to the rC/rN ratio; the rC/rN ratio increases continuously and results in repression of lipid accumulation in support of citric acid production, which lowers the conversion yield of lipid production. To overcome this situation, controlling the rC/rN ratio is a critical factor in this type of fermentation (Beopoulos et al, 2009b).

Ykema et al (1986) designed a continuous culture mode for lipid accumulation where the C/N ratio in the medium and rC/rN were kept constant for a given dilution rate. The low dilution rates led to high lipid and biomass concentrations compared with higher dilution rates. For effective lipid production, the optimal C/N ratio and dilution rate should be adjusted for a continuous mode.

Changes in nitrogen and carbon concentration are observed continuously in the fed-batch fermentation mode; it is therefore highly advantageous to regularly monitor biomass production and the rC/rN ratio. For instance, in one of the studies involving the fed-batch fermentation of *Y. lipolytica* at 28°C, three phases were obtained when glucose was used as the sole carbon source. At the beginning of fermentation, the growth phase resulted in catalytic biomass production; the second stage was the transition phase where excess carbon and limiting nitrogen resulted in the production of lipids. The last phase was the expanded lipid accumulation phase, consisting of SCO production under nitrogen-limited conditions. During the lipid accumulation phase, the prevention of citric acid production is crucial and can be achieved with a C/N ratio of approximately 20.

## 2.7. History of Citric Acid

CA was first discovered by Swedish chemist Carl Wilhelm Scheele who isolated and crystallized it from lemon juice in 1784. Later, German chemist Justus Liebig defined it as hydroxytricarboxylic acid (Apelblat, 2014; Mattey, 1992).

Beside isolation from lemon and lime, it was also first time synthesized by chemical way by Grimaux and Adam in 1880, with a series of reactions starting from hydrochloric acid reacting on glycerine to obtain propenyl dichlorohydrin. Then, synthesis of CA was discovered by Haller and Held in 1890, Dunschmann and Pechmann in 1891 and Lawrence in 1897, respectively by using different chemical reactions such as ethyl chloroacetoacetate-potassium cyanide, acetonedicarboxylic acid and ethylic oxaloacetate-ethylic bromoacetate (Apelblat, 2014).

First, commercial production of CA started in the middle of 1800s with isolation from lemon and lime fruits of Italy in England. After the discovery of Carl Wilhelm Scheele in 1893 that CA could be produced from sugar by using mould *Penicillium,* it was produced industrial scale by fermentation in Belgium in 1919. Later, most of CA was also produced with high rate by using strains of *Aspergillus niger* in low pH medium containing sucrose and small amounts of inorganic salts that found by the food chemist James Currie and microbiologist Charles Thom in USA. Especially, North America, Europe and Russia were used this fermentation method until World War 2 then other countries such as China, India, Brazil, Japan etc. were used. Molasses, sugar cane and sugar beet were firstly preferred as substrate for *Aspergillus niger* fermentations due to high sugar content and ready to use properties. Later, inexpensive substrates; straw, hydrolysed starch, cellulose and waste products were used as substrate. Surface method, submerged, continues, immobilized, yeast based and koji processes were applied for production of CA (Apelblat, 2014).

After 1965, yeast based production of CA was developed and n-alkanes were fermented especially by *Candida* species such as *C. lipolytica, C. tropicalis,*

*C. guillermondii, C. oleophila and C. intermedia.* In 1970, commercial production of CA by yeast based fermentation was made in Italy at Saline, Reggio, Calabria (Liquid-Chimica), however yield and productivity of CA was not economical as fermentation made by *Aspergillus niger* due to high conversion of alkanes to biomass and carbon dioxide rather than CA.

Recently, new fermentation techniques, microorganisms and substrates for production of CA have been examined. Yeast *Y. lipolyica* is considered to have high potential to produce CA by using low price substrates since it has advantage to use wide range of carbon sources in difficult conditions like low pH, temperature and high salt concentrations. There were numerous researches regarding *Y. lipolytica* to produce CA from low cost substrates such as ethanol (Arzumanov et al, 2000), oils (Kamzolova et al, 2008; Venter et al, 2004), sucrose (Forster et al, 2007a), glucose (Antonucci et al, 2001; Kamzolova et al, 2008), n-paraffin (Crolla and Kennedy, 2004a), glycerol (Imandi et al, 2007; Papanikolaou and Aggelis, 2009; Rymowicz et al, 2010), olive mill wastewater (Gonçalves et al, 2009) and crude glycerol (Ferreira et al, 2015).

## 2.8. Biochemistry of Citric Acid

The formation of citric acid in the cell is a very complex process and it is defined as an organic acid obtained at the end of many metabolic and morphological changes (Anastassiadis et al, 2002). The first studies on citric acid biochemistry were examined on mould *Aspergillus niger* and results showed that citric acid reported as an intermediate product in the Krebs cycle (TCA). The TCA cycle is defined as the last phase of chemical processes that enables living cells to gain energy by oxidizing nutrients (carbohydrates, fat and protein) and plays an important role in all living organisms. The Krebs cycle is the second stage of cellular respiration after glycolysis and its reactions take place in mitochondria. The formation of citric acid depends on the regulation of the enzyme synthesis involved in each step of the TCA cycle and the cofactors involved in the activation

of these enzymes. Metal ions provide cofactor formation and regulate enzyme activation (Angumeenal and Venkappayya, 2013).

Biosynthesis of citric acid consists of three stages. At the first stage, every substrate that can be used by microorganisms is converted to Acetil-CoA and at the second stage, Acetil-CoA joins to TCA cycle and at the final stage, citric acid is generated as by-product of TCA cycle (Figure 2.2).

The reason of high level of citric acid synthesis especially with ***Aspergillus niger*** is originated due to the extraordinary reactions in the TCA cycle. Based on this information, citric acid fermentation was studied in detail on two key enzymes, acotinase and isocitrate dehydrogenase and it was found that these two enzymes were reported to show very low activity during citric acid formation and to increase the activity of enzyme like citrate synthase which is responsible to citric acid synthesis. Therefore, inhibition of these two enzymes is required and change of environmental conditions such as removing iron from medium helps to inhibit especially acotinase enzyme (Grewal and Kalra, 1995). Also in another study it was indicated that citric acid and ferrocyanide in the medium inhibited isocitrate dehydrogenase enzyme (Ramakrishnan et al, 1955).

CA is generated by the combination of Acetyl-CoA and oxaloacetic acid and it has been reported that the rate of citric acid production is regulated by citrate synthase (Papagianni, 2007). It has been also stated that the citrate synthase enzyme has a low allosteric regulation and oxaloacetic acid which plays a role in citric acid production, is generated in an anaplotic reaction in the TCA cycle and provides a high level of citric acid production (Angumeenal and Venkappayya, 2013). The biochemistry of citric acid production in yeasts has been reported to be similar to ***A. niger*** and formation of it takes place in the stationary phase, not in the logarithmic phase of the yeast growth. The increase of citric acid quantity is dependent on the enough energy produced by circle and blocking the metabolism in the citrate compound. The growth of yeast slows down with a decrease of nitrogen and stops with the ending up carbon source available in the medium. As a

result of this, it has been observed that ATP concentration increases and AMP and ADP concentration decrease in the medium. Within the decrease in AMP, the activity of the isocitrate dehydrogenase enzyme is stopped (Cartledge, 1987). In a study about citric acid production by *Candida oleophila*, it has been indicated that citric acid is produced instead of cell growth by the depletion of nitrogen source in the fermentation medium (Anastassiadis and Rehm, 2005). Besides that, in an another study on *Y. lipolytica*, it has been reported that the conditions established for citric acid production are due to restriction of cell growth as a result of nitrogen and carbon deficiency in the culture medium (Fickers et al, 2005a). By the completion of the nitrogen in the stationary phase of growth, the activity of yeast metabolism is kept at the same level, the carbon source continues to be consumed and citric acid production takes place (Yalcin et al, 2009a)

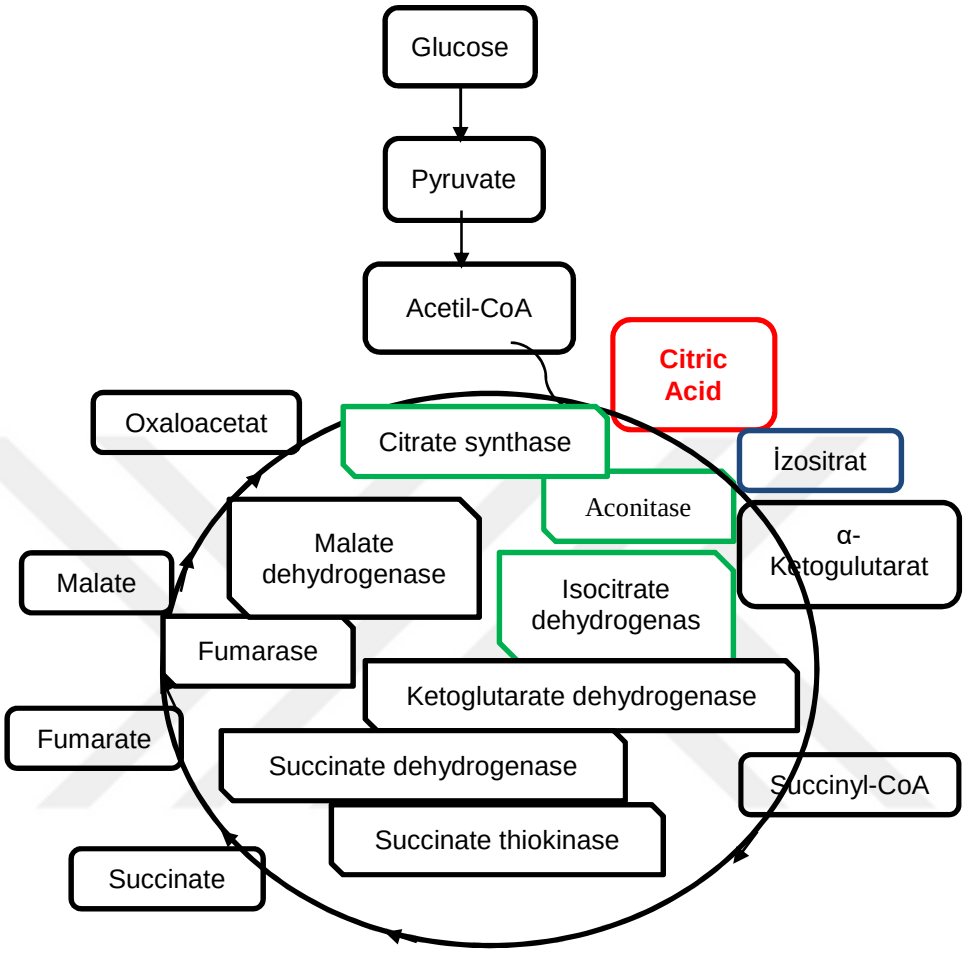



Figure 2.2.The tricarboxylic acid cycle (TCA cycle) (Bubber et al, 2011).

## 2.9. Production of Citric Acid by Yeasts

Mould *Aspergillus niger* has been indicated that it is mainly used microorganism which can produce industrial scale of citric acid. However, interest on citric acid production by yeast in the last 35 years has been increased due to the numerous advantages than *A. niger*. For instance, they have a good tolerance to high substrate concentrations and ability to grow in metal ions that enable them to use wide range of unrefined substrates and waste products. Conversion rate of substrates to citric acid by yeasts is also comparable to production by *A. niger*.

Process control of fermentation is easier than fungi due to their unicellular nature and biomass produced by them can be also used as nutritive animal food. In addition, recombinant technology is more convenient for the yeasts than fungus and with the genetically modified strains the productivity can be increased. However, there is also disadvantage that iso-citric acid is produced high amount during citric acid production sometimes more than 50 %. It has been stated that depend on the yeast strain, carbon source and micronutrient concentrations, citric/ iso-citric acid rate can be varied between 1/1 to 20/1. Therefore, it is important that selected yeast strain should have the high ratio of citric acid/ iso-citric acid (CA/ICA). For this purpose, especially wild *Y. lipolytica* strains were screened and their mutant and recombinant strains were used in order to increase the ratio of CA/ICA (Forster et al, 2007b; Holz et al, 2009; Yalcin et al, 2009a).

Citric acid produced yeast species has been reported as, *Candida (Yarrowia) lipolytica, Candida guilliermondii, Candida oleophila, Candida intermedia, Candida paratropicalis, Candida zeylanoides, Candida catenulata, Candida parapsilosis, Pichia anomala,* and some *Rhodotorula species.* Among them, *Y. lipolytica* has been indicated as the most potential citric acid producer.

It has been reported that more than 90 % of citric acid is produced by fermentation methods such as submerged, surface and solid state (Koji) which are used for industrial scale production. Among them, 80 % of world citric acid production is mostly carried out by submerged process in stirred tanks of 40-200 $m^3$ or airlift fermenters of 200-900 $m^3$ and batch, fed-batch and continues systems can be applied. However, most frequently used one is the batch system. Production of citric acid by yeast is generally carried out by submerged fermentation. There have been also studies that Koji fermentation is also applied (Soccol et al, 2006).

The first studies on production of citric acid by yeast were made by *Candida* species with using n-alkanes as carbon source in 1970s. However, n-alkanes were no longer used due to their high price after 1973/74 with the world oil crisis and low productivity of citric acid since iso-citric acid production was high.

Then, it was discovered that some yeasts strains could also degrade carbohydrates. Especially, *Y. lipolytica* was examined for citric acid production from glucose and found that iso-citric acid production value was lower than when n-alkanes were used as substrate. After that, other carbon sources such as ethanol, plant oils, starch hydrolysate, raw or pure glycerol and some industrial wastes like olive mill waste water, whey and pine apple waste were used for citric acid production especially by *Y. lipolytica* (Table 2.3).

Table 2.2. Citric acid production from different carbon sources by *Y. lipolytica* strains.

| Yeast Strain | Citric Acid (g/L) | Citric Acid Yield (g/g) | Initial Glucose Concentration (g/L) | Carbon Source | Nitrogen Source | Culture Mode | References |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* SWJ-1b | 31.7 | 0,4 | 80 | Cooking waste oil | Ammonium sulphate | Batch | (Liu et al, 2015c) |
| *Y. lipolytica* N1 | 14.4-19.2 | n | 0.01-1.0 | Ethanol | Ammonium sulphate | Continues | (Finogenova et al, 2002b) |
| *Y. lipolytica* 57 | 65.1 | 0.38 | 200 | Fructose | Ammonium chloride, yeast extract | Batch | (Yalcin et al, 2009b) |
| *Y. lipolytica* 57 | 49.23 | 0.33 | 150 | Fructose in whey based medium | Ammonium chloride, yeast extract | Batch | (Yalcin et al, 2009c) |
| *Candida lipolytica* Y-1095 | 13.6-78.5 | 0.50-0.79 | 50-150 | Glucose | Ammonium chloride, yeast extract | Batch | (Rane and Sims, 1993) |
| *Candida oleophila* ATCC 20177 | 57.8 | n | 250 | Glucose | Ammonium chloride | Batch | (Anastassiadis and Rehm, 2005) |
| *Candida oleophila* ATCC 20177 | 50-80 | n | 120-209 | Glucose | Ammonium chloride | Batch and Fed-Batch | (Anastassiadis et al, 2002) |
| *Candida oleophila* ATCC 20177 | 167 | n | 336 | Glucose | Ammonium chloride | Continues | (Anastassiadis and Rehm, 2006) |
| *Y. lipolytica* A-101 | 34.3 | 0.84 | 92 | Glucose | Ammonium chloride, yeast extract | Repeated Batch | (Rymowicz et al, 1993) |
| *Y. lipolytica* W29 | 49 | 0.85 | 60 | Glucose | Ammonium sulphate, yeast extract | Batch | (Papanikolaou et al, 2009) |
| *Y. lipolytica* A-101-1.14 | >80 | 0,93 | 400 (mL/L) | Glucose hydrol | Ammonium chloride, yeast extract | Batch | (Wojtatowicz et al, 1991) |
| *Y. lipolytica* ACA-DC 50109 | 28.9 | 0.82 | 65 | Glucose in Olive mill waste water | Ammonium sulphate, yeast extract | Batch | (Papanikolaou et al, 2008b) |
| *Y. lipolytica* NBRC 1658 | 38.88 | 0.38 | 100 | Glucose in whey based medium | Ammonium chloride, yeast extract | Batch | (Yalcin et al, 2009c) |

Table 2.2 Continue

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* 57 | 32.09 | 0.48 | 78.30-85.16 | Grape must | Ammonium chloride, yeast extract | Batch | (Yalcin et al, 2009c) |
| *Y. lipolytica* SWJ-1b | 42.4 | 0.43 | n | Hydrolysate of pre-treated straw cellulose | Ammonium sulphate | Fed-Batch | (Liu et al, 2015d) |
| *Y. lipolytica* Transformat 87 | 77.9 | n | 100 | Inulin | Ammonium sulphate, yeast extract | Batch | (Liu et al, 2010) |
| *Y. lipolytica* 30 | 68.3 | 0,91 | 84.3 | Jerusalem artichoke tubers extract | Ammonium sulphate | Batch | (Wang et al, 2013) |
| *Candida lipolytica* Y-1095 | 9.8 | n | 100-150 | n-paraffin | Urea | Batch | (Crolla and Kennedy, 2001) |
| *Candida lipolytica* Y-1095 | 42 | 0.8-1.0 | 150 | n-paraffin | Urea | Fed-Batch | (Crolla and Kennedy, 2004a) |
| *Y. lipolytica* ACA-YC 5033 | 18.1 | 0.51 | 35.7 | Olive mill waste water | Ammonium sulphate, yeast extract | Batch | (Sarris et al, 2011) |
| *Y. lipolytica* NCIM 3589 | 202.35 (g/kg) | n | n | Pineapple waste | Yeast extract | Solid State | (Imandi et al, 2008) |
| *Y. lipolytica* Wratislavia AWG7 | 157.5 | 0.6 | 300 | Pure and raw glycerol | Ammonium chloride, yeast extract | Fed-Batch | (Rywinska et al, 2010a) |
| *Y. lipolytica* 57 | 32.8 | 0.21 | 160 | Pure glycerol | Ammonium chloride, yeast extract | Batch | (Karasu-Yalcin et al, 2009) |
| *Y. lipolytica* NRRL YB-423 | 21.6 | 0.54 | 40 | Pure glycerol | Ammonium sulphate, yeast extract | Batch | (Levinson et al, 2007) |
| *Y. lipolytica* 187/1 | 135 | 1.55 | 5 | Rapeseed oil | Ammonium sulphate | Batch | (Kamzolova et al, 2005) |
| *Y. lipolytica* NG40/UV7 | 175 | 1.5 | 20 | Rapeseed oil | Ammonium sulphate, yeast extract | Batch | (Kamzolova et al, 2011) |
| *Y. lipolytica* 1.31 | 124.5 | 0.62 | 200 | Raw glycerol | n | Batch | (Rymowicz et al, 2006) |
| *Y. lipolytica* LGAM S(7)1 | 33-35 | 0.42-0.44 | 80-120 | Raw glycerol | Ammonium sulphate, yeast extract | Batch | (Papanikolaou et al, 2002b) |

Table 2.2 Continue

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* NCIM 3589 | 77.39 | n | 54.4 | Raw glycerol | Yeast extract | Batch | (Imandi et al, 2007) |
| *Y. lipolytica* H222-S4(p67ICL1)T5 | 80 | 0,57 | 150 | Sucrose | Ammonium sulphate | Repeated Fed-Batch | (Moeller et al, 2013) |
| *Y. lipolytica* UOFS Y-1701 | 18.7 | n | 30 | Sunflower oil | Ammonium chloride, yeast extract | Batch | (Venter et al, 2004) |
| *Y. lipolytica* H222 | 53-97 | 0.39-0.69 | 150 | Glucose | Ammonium chloride | Fed-Batch | (Moeller et al, 2011) |
| *Y. lipolytica* VKM Y-2373 | 80-85 | 0.70-0.75 | Pulsed addition 30 | Glucose | Ammonium sulphate | Fed-Batch | (Kamzolova and Morgunov, 2017) |

## 2.10. Citric Acid Production by *Y. lipolytica*

*Y.lipolytica* has been most often studied yeast for production of citric acid because of its numerous advantages than other microorganisms such as GRAS property, high productivity, wide range of substrate usage (hydrophilic and hydrophobic), high tolerance to metal ions, contaminations and salt solutions, capability to grown on at the low and high pH (pH=4 to 8) and wide range of temperatures (T= 18 to 32 ºC). Furthermore, it is also convenient yeast for recombinant strain engineering that yield and productivity of citric acid can be increased by regulating genes which are responsible for enzyme activities. The key situation for citric acid accumulation by *Y. lipolytica* is availability of excess carbon source and growth limited nitrogen, salt or thiamine in the fermentation medium (Liu et al, 2015a). The negative side of using *Y. lipolytica* for citric acid production is the high iso-citric acid accumulation during fermentation. Yield and productivity of citric acid synthesised by *Y. lipolytica* are mainly dependent on carbon source and its concentration, limitation of nitrogen and phosphate, fermentation conditions such as aeration, agitation, pH, and temperature. Factors affecting citric acid production by *Y. lipolytica* are reviewed in the next section.

## 2.11. Effect of Carbon Sources for Citric Acid Production by *Y. lipolytica*

Concentration and type of carbon source used for citric acid production by *Y. lipolytica* are important and affect mainly yield and productivity rates of citric acid accumulation. It is known that wild and recombinant strains of *Y. lipolytica* can use wide variety of carbon sources such as n-alkanes, edible oils, animal fats, alcohols (ethanol and methanol), molasses, starch hydrolysates, glucose, fructose, different agricultural residues- wastes (pineapple waste, cooking oil, straw cellulose, corn steep liquor, grape pomace, kiwi fruit peel, kumara, okara, carob pod, apple pomace, whey, olive mill waste water, Jerusalem artichoke tuber extract, inulin) and raw or pure glycerol (Abghari and Chen, 2017; Crolla and Kennedy, 2001; Crolla and Kennedy, 2004a; Darvishi et al, 2009; Imandi et al,

2007; Imandi et al, 2008; Kamzolova et al, 2011; Levinson et al, 2007; Liu et al, 2010; Liu et al, 2015b; Liu et al, 2016; Liu et al, 2015d; Papanikolaou et al, 2006; Papanikolaou et al, 2008b; Papanikolaou et al, 2002b; Sabra et al, 2017; Venter et al, 2004; Wang et al, 2013).

Various carbon sources used for citric acid production by *Y. lipolytica* strains are showed in Table 2.2. Although, most favourable substrate for citric acid production by *Aspergillus niger* is sucrose and molasses are mostly used raw material, due to high citric acid yield and productivity, mainly glucose and glycerol are used for citric acid production by *Y. lipolytica*. However, invert sugar and molasses can also be used as carbon sources for citric acid production by *Y. lipolytica* (Wojtatowicz et al, 1991). While it is indicated that assimilation of fructose by some yeast are low, Yalcin et al (2009a) showed that highest citric acid production by *Y. lipolytica* 57 was obtained by using fructose with the value of 65.1 g/L from 200 g/L initial fructose content and maximum specific citric acid production rate of 0.0179 (g /g $h^{-1}$) was found with 100 g/L initial fructose content.

However, highest specific growth rate was obtained with fructose concentration of 50 g/L. According to these results, it can be said that excess concentration of substrate has to be used in order to achieve high citric acid production. Generally, 150-250 g/L initial substrate usages promotes the citric acid production and lower than 50 g/L substrate usage decreases the accumulation of citric acid (Antonucci et al, 2001; Kubicek, 2001). In addition, it was stated that higher glucose concentration stimulates the glucose transport system and inhibits the hexokinase by trehalose 6-phosphate (Kubicek, 2001). In another study on sucrose as carbon source, recombinant *Y. lipolytica* strain could produce 80 g/L citric acid with the yield and productivity of 0.57 g/g and 1.1 g/L $h^{-1}$ respectively (Moeller et al, 2013).

Besides glucose, fructose and sucrose, ethanol was also used as carbon source for citric acid production by *Y. lipolytica* in submerged continuous fermentations (Arzumanov et al, 2000; Finogenova et al, 2002b). 105 g/L of

highest citric acid production and 88.3 % yield from ethanol were obtained with 50 % feed in each three day. But ethanol concentration should not be more than 1.2 g/L and fermentation conditions should be at pH 4.5 and 28 ºC.

Edible oils such as rapeseed and sunflower were used as substrate for citric acid production by *Y. lipolytica* strains. Kamzolova et al (2011) used rapeseed oil as carbon source and 175 g/L highest citric acid production and 1.5 g/g yield were obtained at suitable fermentation conditions. Moreover, in another study, sunflower oil was used as carbon source and citric acid concentration of 18.7 g /L was obtained with addition of 10 g/L acetate to 30 g/L sunflower oil (Venter et al, 2004).

Glycerol which is by-product of biodiesel industry has been used as carbon source for citric acid production by *Y. lipolytica*. It has been estimated that in order to produce 10 kg of biodiesel, 1 kg of raw glycerol is produced (Rymowicz et al, 2010). Due to being inexpensive industrial raw material, having higher conversion yield to citric acid and best carbon source for *Y. lipolytica*, glycerol was used for citric acid production in many researches (Imandi et al, 2007; Levinson et al, 2007; Morgunov et al, 2013; Papanikolaou et al, 2002b; Rymowicz et al, 2010; Rymowicz et al, 2006; Rywinska et al, 2013; Rywinska et al, 2012; Rywinska and Rymowicz, 2010; Rywinska et al, 2010a; Rywinska et al, 2009). Rymowicz et al (2006) obtained highest citric acid production of 124.5 g/L from initial raw glycerol concentration of 200g/L by acetate mutants of *Y. lipolytica* in batch bioreactor culture. Later, fed-batch culture fermentations were applied and maximum citric acid production of 157.5 g/L from 300 g/L initial glycerol concentration was obtained (Rywinska et al, 2010a).

Recently, researches have been looking for inexpensive carbon sources for citric acid production by *Y. lipolytica* and agricultural residues and wastes were employed as substrate for this purpose. For instance, Jerusalem artichoke tubers extract was used for citric acid production by Wang et al (2013) and 68.3 g/L citric acid with 0.91 g/g yield was obtained. In another study with olive mill waste water

enriched with glucose (contained initial sugar of 65 g/L), 28.9 g/L citric acid was produced (Papanikolaou et al, 2008b). In addition, Imandi et al (2008) was employed solid state fermentation for citric acid production from pineapple waste by using *Y. lipolytica* and obtained citric acid of 202.35 g/kg ds (g citric acid produced/kg of dried pineapple waste as substrate). In the last researches, hydrolysate of pre-treated straw cellulose and cooking waste oil were also used by Liu et al (2015d) and Liu et al (2015c) as carbon sources for citric acid production by *Y. lipolytica* and, citric acid of 42.4 g/L from hydrolysate of pre-treated straw cellulose and 31.7 g/L from waste cooking oil were produced.

Although, agricultural residues and wastes are inexpensive carbon sources for citric acid production by *Y. lipolytica*, it should be noted that these substrates could contain trace metals which can inhibit growth of microorganism and production of citric acid. Therefore, deionization or some chemical pre-treatment methods should be applied before usage of these substrates (Roukas and Kotzekidou, 1997; Yalcin et al, 2009a).

Lately, molecular methods and recombinant technology were often used for *Y. lipolytica* strains in order to improve yield and productivity of citric acid. With the usage of recombinant strains of *Y. lipolytica*, most of the carbon sources such as sucrose, inulin etc. can be used for citric acid production.

## 2.12. Effect of Nitrogen and Trace Elements for Citric Acid Production by *Y. lipolytica*

Type of nitrogen source and its concentration in fermentation medium are critical factor effecting the citric acid production by *Y. lipolytica.* Nitrogen source is mainly required for growth of microorganism and its vital activity. High concentration of nitrogen in the medium can be negative effect for productivity of citric acid (Soccol et al, 2006). The key factor of citric acid accumulation by *Y. lipolytica* is explained that nitrogen source in the fermentation medium should be exhausted and cell growth should be limited by the excess carbon and nitrogen

deficiency (Fickers et al, 2005b). After nitrogen exhaustion in the stationary phase of growth, metabolic activity of yeast still continues with the carbon source assimilation and citric acid production (Morgunov et al, 2004). Generally, ammonium salts (ammonium chloride, ammonium sulphate, ammonium nitrate), potassium nitrate, sodium nitrate, urea, peptone, malt extract, yeast extract and corn steep liquor were used as nitrogen source for citric acid production. Among these nitrogen sources, the most convenient ones were reported as yeast extract and ammonium chloride for citric acid production by yeasts, *Y. lipolytica, C. paratropicalis and C. guilliermondii* (Rane and Sims, 1996). In a research on citric acid production by *C. lipolytica,* 1.5 g/L of ammonium chloride as nitrogen source was found to be optimum concentration for highest citric acid production (Hamissa et al, 1981). In an another study on citric acid production from glycerol by *Y. lipolytica,* yeast extract of 0.2682 g/L was estimated as optimum concentration as nitrogen source (Imandi et al, 2007). Moreover, Yalcin et al (2009a) stated that citric acid production by *Y. lipolytica* was increased with rising ammonium chloride concentration from 0 to 2 g/L. It was also reported that required nitrogen concentration for citric acid production was 0.1 to 0.4 g/L. Higher nitrogen content can lead the pH decrease which is critical factor for citric acid production, while this can also cause increase in fungal growth and sugar consumption but decrease the citric acid accumulation (Soccol et al, 2006). Therefore, at the growth phase of yeast, nitrogen content is required to keep in definite quantity in order to maintain required biomass production without pH decrease (Vandenberghe et al, 1999). It should be noticed that this requirement of limiting nitrogen content medium can be problem for citric acid production from high nitrogen content waste usage by *Y. lipolytica.* For instance, in a one study performed with whey as carbon source, addition of extra nitrogen source to medium leads to decrease citric acid production (Yalcin et al, 2009a).

Phosphate which is another crucial source for citric acid production, should be also in limiting quantity in the fermentation medium like nitrogen source.

Higher concentration of phosphate can promote the cell growth and less citric acid production. Potassium dihydrogen phosphate with the concentration of 0.1 % was reported as most suitable phosphate source for citric acid production (Grewal and Kalra, 1995). Phosphates acts at the level of enzyme activity and not at the level of gene expression. Higher concentration of phosphate in the medium causes the decrease in the fixation of carbon dioxide, which in turn increases the production of certain sugar acids, and the stimulation of growth (Grewal and Kalra, 1995; Max et al, 2010; Vandenberghe et al, 1999).

It was reported that trace elements of zinc, iron, manganese, copper and magnesium have effect on citric acid production (Soccol et al, 2006). These elements are required with trace quantity for inducing cell growth to obtain high citric acid yields (Angumeenal and Venkappayya, 2013). For instance, in a study with manganese concentration of more than 2 mg/L, decrease of 20% in citric acid production was observed. In case of iron on citric acid accumulation, it was stated that limitation of iron inactivates the aconitase enzyme which catalyse the citric acid degradation within the tricarboxylic acid cycle (Kubicek, 2001). Anastassiadis and Rehm (2005) reported that biomass formation was increased with addition of iron to glucose medium in fermentation of *C. oleophila* whereas Finogenova et al (2002a) indicated that iron and zinc limitation cause cell growth without citric acid production and addition of zinc increased the citric acid production. For the same study, it was also stated that optimum iron concentration in the medium for the high citric acid production from ethanol should be in the range of 0.2-2.5 mg/g and not more than 7.0 mg/g which inhibited the citric acid production. In another study, Yalcin et al (2009a) also found that citric acid production by *Y. lipolytica* strains inhibited and cell growth increased with the addition of iron, copper and zinc sulphate to the fermentation medium and optimum zinc sulphate concentration was found as 0.008 g/L for highest citric acid production by *Y. lipolytica* 57.

## 2.13. Effect of Fermentation Conditions for Citric Acid Production by *Y. lipolytica*

Citric acid production by *Y. lipolytica* in submerged fermentations is mainly affected by pH, temperature, aeration and agitation parameters of bioreactors.

pH of the medium is one of the important parameter for citric acid production by yeasts and it should be well studied before citric acid production since each yeast strain shows different citric acid yield at the definite pH. Generally, if yeast is used for citric acid production, initial pH of the medium should be more than 5 since pH values below 5 shows negative effect on citric acid production. It was indicated that the production of some poly alcohols like erythritol, arabitol and mannitol increased instead of citric acid accumulation below pH 5 (Mattey, 1992). In addition, it was explained that citric acid production and its transportation from cells were inhibited at lower pH values. For instance, in a study on citric acid production by *C. oleophile* in continues fermentation, active citric acid transport system was found to be affected by pH of medium. It was also indicated that growth, biomass composition and citric acid synthesis depended on pH and highest citric acid production was found at pH of 5. In another study performed with *Y. lipolytica*, initial fermentation medium contained glucose as carbon source, pH range of 5.2 to 7 was favour for highest citric acid production by *Y. lipolytica* 57 and *Y. lipolytica* NBRC 1658 (Yalcin et al, 2009a). But, Kamzolova et al (2008) examined the citric acid to iso-citric acid ratio in different pH mediums and reported that production of these two acids at pH 4.5 was almost equal and iso-citric acid production increased usually at pH 6. Therefore, it is important to examine effect of pH on citric acid production before starting fermentation. Depend on strain of yeast, citric acid accumulation, growth, biomass composition and ratio of citrate to iso-citrate can change.

Another important parameter on citric acid production by yeast is temperature. Growth of cells, biomass and product formation depends on

temperature of fermentation medium. Various strains can behave in different way at the same temperature and effect of temperature on yeast should be examined before scale up the fermentation. Effect of temperature on citric acid production and biomass formation by *C. lipolytica* was studied and 26-30 ºC was obtained as optimum range (Crolla and Kennedy, 2001). In another study performed by *C. lipolytica* Y 1095, optimum temperature for citric acid production was determined as 27 ºC (Rane and Sims, 1993). Moreover, optimum temperature for citric acid and biomass production by *C. oleophila and Y. lipolyica* 57 was found as 35 and 30 ºC respectively (Anastassiadis and Rehm, 2006; Yalcin et al, 2009a).

Since citric acid production is aerobic process, it is obviously affected by aeration (Kubicek, 2001). Biochemically, this type of fermentation requires oxygen to generate citric acid by *A. niger* as well as the yeast strains (Kamzolova et al, 2003). It was reported that higher citric acid production was obtained at high oxygen concentrations in medium (Rywinska et al, 2012). Availability of oxygen in the medium was a crucial parameter in growth of *Y. lipolytica*, substrate uptake and citric acid synthesis (Workman et al, 2013). Different types of aeration devices were constructed to obtain high oxygen transfer rate (Soccol et al, 2006). Dissolved oxygen ratio in the fermentation medium generally depends on agitation, aeration and at the same time viscosity of medium. Agitation process increases the area of dispersed air bubbles in the fermentation medium that provide high oxygen transfer rate. However, high agitation can lead high shear stress on cell walls and cell-insoluble substrate interface. Therefore, optimum agitation speed should be examined before scale-up of fermentation (Crolla and Kennedy, 2004a; Crolla and Kennedy, 2004b). Some authors reported that increasing agitation speed from 400 to 800 rpm and to 1000 rpm had positive effect on citric acid production (Crolla and Kennedy, 2004a; Rywinska et al, 2012). It was also stated that increasing aeration rate from 0.18 vvm (volume or air/ volume of medium/minute) to 0.6 vvm increased concentration of citric acid (Rywinska et al, 2012). Dissolved oxygen concentration affected also the citrate/iso-citrate concentration ratio and it was

reported that ratio of the citrate/iso-citrate concentration produced by *C. tropicalis* increased with high dissolved oxygen concentration in the medium. In another study performed with *Y. lipolytica* 704, decreasing dissolved oxygen concentration from 60-95 % to 28-30 % inhibited the citric acid production (Kamzolova et al, 2003) and dissolved oxygen concentration of 60 % was found to be optimum for high citric acid production.

# 3. MATERIAL AND METHODS

## 3.1. Materials

### 3.1.1. Microorganisms

12 different strains of *Y. lipolytica* (W29, CBS 6303 (Prof. Dr. Patrick FICKERS, Universite Libre de Bruxelles, Belgium), Peggy (Brewery Industry, Austria), K57 (Prof. Filiz ÖZÇELİK, Ankara University, Turkey), Ain 16, Ain 19, Zu 110, H 917, N 155, Po1 dL (Prof. Dr. Andriy SIBIRNY, Instute of Cell Biology, NAS of Ukraine, Ukraine), DBVPG 5858, DBVPG 4558 (Perugia University, Italia)) were examined for microbial lipid and citric acid production.

### 3.1.2. Raw Materials

Waste breads were collected from local bakery with mould-free status and stale conditions (Figure 3.1). Before each hydrolysis, stale breads were manually cut into cubes of ca. 2–4 cm size. Obtained cubes were dried in a drying oven at 50 ºC for 24 h and grounded in a kitchen type blender. Waste bread powder (WBP) was stored in an air tight jar at room temperature until usage (Pietrzak and Kawa-Rygielska, 2014).

Figure 3.1. Waste breads.

### 3.1.3. Enzymes

Termamyl SC DS alpha-amylase (thermostable α-amylase from *Bacillus licheniformis* with declared activity of 300U/mL), SAN Extra L (glucoamylase from *Aspergillus niger* with declared activity of 300 U/mL) and Neutrase 0.8 L (bacterial protease form *Bacillus amyloliquefaciensis* with declared activity was 0.8 U/g) protease were used for waste bread hydrolysis (Pietrzak and Kawa-Rygielska, 2014).

### 3.1.4. Antifoam Agent

Antifoam agent of Struktol J 673 (Schill+Seilacher "Struktol" GmbH, Germany) was used for bioreactor trials in order to decrease foam formation during fermentation.

### 3.1.5. Chemicals and Salts

Glucose (monohydrate), yeast extract, $(NH_4)_2SO_4$, $KH_2PO_4$, $Na_2HPO_4$, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $FeCl_3.6H_2O$, $ZnSO_4 .7H_2O$, $MnSO_4.H_2O$ were used for fermentation mediums. Methanol and chloroform were used for lipid extraction. $H_2SO_4$ was used to prepare mobile phase solution of HPLC. Perchloric acid was used to precipitate protein contents of HPLC samples. NaOH, KOH, HCl and $H_2SO_4$ were used to regulate pH of fermentation and hydrolysis mediums. 2 N KOH-Methanol and n-heptane were used to esterify fatty acids. GeneMatrix, $MgCl_2$, dNTP, ITS1, ITS4, Tag polymerase, agarose, TBE, SYBR dye and 100 BP markers were used for PCR and electrophorese analysis. 3, 5-di-nitro salicylic acid, phenol, sodium sulphite and sodium hydroxide were used for DNS analysis. $Na_2HPO_4$, $KH_2PO_4$, ninhydrin, fructose, $KIO_3$, ethanol and glycine were used for free-amino nitrogen content analysis. Diethyl ether was used for total fat analysis of waste bread. Kjeldahl tablets and boric acid were used for crude protein analysis of waste bread.

### 3.1.6. Standard Chemicals

Standards of glucose, fructose, arabinose, sucrose, maltose, lactose, glycerol, ethanol, citric, tartaric, malic, lactic, formic, acetic and propionic acids were used to obtain calibration curves of HPLC.

### 3.1.7. Indicators

Methylene blue was used to dye yeast cells in order to count them under the microscope with Toma lam. Methyl orange was used for Kjeldahl experiment.

### 3.1.8. Medium

Potato dextrose agar (PDA) and potato dextrose broth were used for *Yarrowia lipolytica* cultivation and inoculum preparations.

### 3.1.9. Equipment

Autoclave (JSAC-40, JS Research Inc. Korea), incubator (BPH-7091CR, Chincan Instruments, PRC), drying oven (FN 500, Nüve Instruments, Turkey) orbital shaker (KS 501, IKA Laboratechnik, Germany), microscope (BM-2000, BOECO, Germany), pH meter (HI 2211, HANNA Instruments, USA) spectrophotometer (T80, PG Instruments Ltd. United Kingdom), centrifuges (KA-1000, PRC and Hettich, Germany), Bioreactor (Bioflo 110, New Brunswick, USA), High Performance Liquid Chromatography (Schimadzu, Japan), Gas Chromatography-Mass Spectrophotometry (Hewlett-Packard, USA), PCR (Eppendorf, USA), Freeze-dryer (Christ, Germany) were used for required analysis.

## 3.2. Methods

### 3.2.1. Experimental Set Up

Before starting bioreactor trials, strain which produced high quantity of citric acid and microbial lipid, was selected as optimal strain by using shake-flask

fermentation method. After selection of optimal strain, reactor trials were applied to produce microbial lipid and citric acid from waste bread hydrolysate (Figure 3.2).

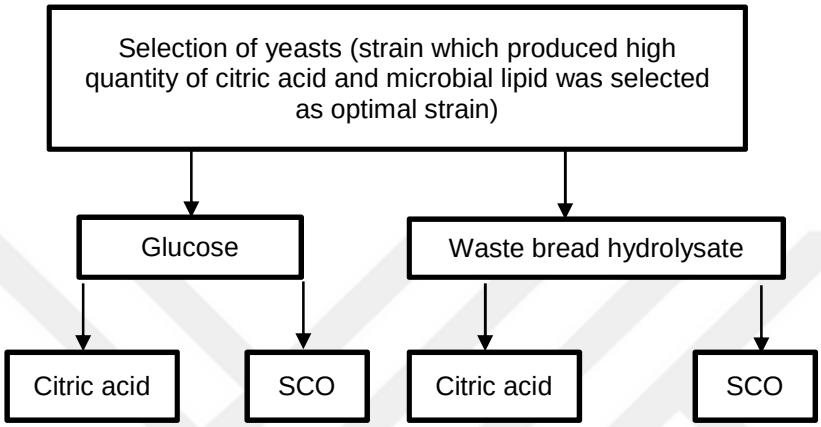



Figure 3.2. Microbial lipid and citric acid production from waste bread hydrolysate.

### 3.2.2. Molecular Characterization of Yeast Strains

Before starting the experiments, collected strains were examined for identification of molecular characterization.

**DNA Isolation of Yeasts:** DNA isolation of yeast strains was carried out according to procedure of InstaGene Matrix Kit (Bio-Rad, Hercules, California, USA).

Firstly, 2 % of yeast strains kept at -40 ºC were inoculated to PDA broth medium and incubated at 30 ºC in Eppendorf tubes for 2-3 days. Incubated strains were centrifuged at 4 ºC and 14000 rpm for 3 minutes. Aliquot of centrifuge was removed and remained part was washed with 1 mL ultra-distilled water. Eppendorf tubes were again centrifuged and aliquot was removed. This step was repeated 3 times. 100 µL solution of genomic DNA isolation was added to fresh cultures and mixed by vortex for 10 seconds. After that, tubes were incubated in water bath at 56 ºC for a half hour. Then, tubes were again mixed by vortex for 10 seconds and

kept at 100 ºC in water bath for 10 minutes. Lastly, tubes were again mixed for 10 seconds by vortex and centrifuged at 4 ºC and 14000 rpm for 3 minutes. Upper parts of centrifuges were removed to another sterile Eppendorf tubes and kept at -20 ºC until subsequent tests were made.

**26S rRNA Sequence Analysis:** Polymerisation of D1/ D2 region of 26S rRNA was proceeded by using NL 1 and NL 4 primers (Table 3.1). 50 µL total solution (Table 3.2) included sterile distilled water, DNA (50-100 mg/ µL), buffer (10x), dNTP (25 µmol), $MgCl_2$ (25 mM), Primer NL 1 (100 µM), Primer NL 4 (100 µM) and Tag polymerase (50 U/µL) were added to tubes.

Table 3.1. Primers for 26S rRNA sequence analysis.

| **Name of Primer** | **DNA sequence (5' —>3')** |
|---|---|
| NL 1 Forward | 5' GCATATCAATAAGCGGAGGAAAAG 3' |
| NL 4 Backward | 5' GGTCCGTGTTTCAAGACGG 3' |

Table 3.2. PCR mixture of 26S rRNA Sequence Analysis.

| **PCR Mix** | **Quantity(µL)** |
|---|---|
| Buffer (10X, 20 mM $MgCl_2$ included) | 5 |
| $MgCl_2$ (25 mM) | 2.5 |
| dNTP (2.5 mM) | 5 |
| Primer NL 1 (100 µM) | 0.126 |
| Primer NL 4 (100 µM) | 0.126 |
| Tag Polymerase (50 U/µL) | 0.15 |
| $dH_2O$ | 34.598 |
| DNA | 2.5 |
| Total Volume | 50 |

PCR started with first cycle of denaturation process at 95 ºC for 5 minutes, then 30 cycles was proceeded by using temperature-time parameters of 95 ºC for 1 minute, 55 ºC for 45 seconds, 72 ºC for 1 minutes, respectively and ended with last cycle at 72 C for 7 minutes.

After PCR process, electrophoresis was carried out. Agarose gel was prepared by using 2 % agarose and 200 mL of 1xTBE buffer (tris/borate/EDTA

solution). SYBER dye was added to cold gel solution which was then poured to electrophoresis rock and left for gelation. Wells were opened in gel by using plastic comb and first and last wells were filled with 100 BP (base pairs) DNA markers (Cat No: 10488058). Other wells were filled with PCR products and electrophoresis was performed at 120 volt for 90 minutes. Then, gel of electrophoresis was screened under UV light.

Positive results of 26S rRNA PCR were analysed for DNA sequence in MACROGEN (Nederland).

### 3.2.3. Selection of Optimal *Y. lipolytica* Strain for Citric Acid and Microbial Lipid Production by Shake Flask Fermentation

12 strains of *Y. lipolytica* were examined for maximum citric acid and microbial lipid production from glucose medium. For each examined strain, 15 flasks (250 mL) including 50 mL medium with salt solution used by Papanikolaou et al, (2002) were prepared (Figure 3.3). Table 3.3 shows fermentation medium consisted of 60 g/L glucose, 0.5 g/L ammonium sulphate, 0.5 g/L yeast extract, $KH_2PO_4$, 7 g/L; $Na_2HPO_4$, 2.5 g/L; $MgSO_4.7H_2O$, 1.5 g/L; $CaCl_2$, 0.15 g/L; $FeCl_3.6H_2O$, 0.15 g/L; $ZnSO_4.7H_2O$, 0.02 g/L; $MnSO_4.H_2O$, 0.06 g/L was used for shake-flask fermentations and autoclaved at 121 ºC for 15 minutes. Before inoculation, inoculum which was same as main fermentation medium composition was prepared in orbital shaker at 185 rpm at 28 ºC for 48 hours. Approximately $10^7$ cell/mL of yeast were inoculated to each flask. pH of fermentation medium was regulated between 5 to 6 with addition of 5 M KOH periodically and aseptically. Fermentations were carried out in orbital shaker at 185 rpm and temperature of 28 ºC for 192 hours. Except fifth and seventh days of fermentations, every day samples were collected from medium and dry cell weight, OD 600, pH, cell count in microscope, titration acidity, total lipid, organic acids and sugars were determined. Fatty acids profile of samples was carried out by using GC-MS.

Table 3.3. Composition of Fermentation Medium.

| **Fermentation Medium** | **Quantity (g/L)** |
|---|---|
| Glucose | 60 |
| Ammonium Sulphate | 0.5 |
| Yeast Extract | 0.5 |
| $KH_2PO_4$ | 7 |
| $Na_2HPO_4$ | 2.5 |
| $MgSO_4.7H_2O$ | 1.5 |
| $CaCl_2$ | 0.15 |
| $FeCl_3.6H_2O$ | 0.15 |
| $ZnSO_4.7H_2O$ | 0.02 |
| $MnSO_4.H_2O$ | 0.06 |
| $dH_2O$ | (added for required volume) |

Figure 3.3. Selection of optimal *Y. lipolytica* strain for citric acid and microbial lipid production by Shake-flask fermentation.

### 3.2.4. Microbial Lipid and Citric Acid Production from Glucose by Bioreactor Fermentation

Bioreactor trials were examined by using glucose as a substrate with the inoculation of selected strain. Concentration of 50, 100 and 150 g/L glucose were

used for three different fermentations (Figure 3.4). Other ingredients of medium were same as in shake flask fermentation (Table 3.3). Fermentation medium (1 L working volume) was autoclaved at 121 ºC for 15 minutes. Inoculum was prepared in orbital shaker at 185 rpm and temperature of 28 ºC for 48 hours. 10 % inoculum (100 mL) was inoculated aseptically to both reactors (twin reactors). Two different bioreactor conditions of 350 rpm (agitation) -1.0 vvm (aeration) and 800 rpm-0.5 vvm were primarily applied for the medium composition of 50 g/L glucose in order to optimise fermentation parameters (Figure 3.5). Fermentations were carried out until whole glucose consumed by *Y. lipolytica* at 28 ºC and pH of 5-6 with addition of 5 M KOH periodically. Temperature, pH and $dO_2$ (dissolved oxygen) were monitored during fermentation. Dry cell weight, OD 600, pH, cell count in microscope, titration acidity, total lipid, organic acids and sugars were determined. Fatty acids profile of samples was carried out by using GC-MS.

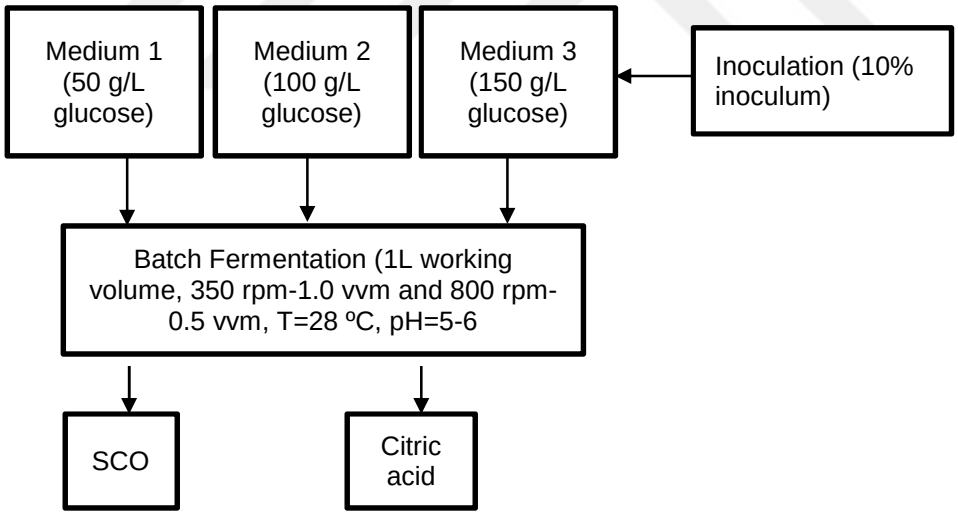



Figure 3.4. Method of microbial lipid and citric acid production from glucose by *Y. lipolytica* bioreactor fermentation.

Figure 3.5. Microbial lipid and citric acid production from glucose by *Y. lipolytica* in bioreactor.

### 3.2.5. Hydrolysis of Waste Bread

First experiments for optimisation of waste bread hydrolyse were performed by using 250 mL beaker in water bath with laboratory stirrer (Figure 3.6-A). 150 mL total volume of mash was worked for trials. Later, bioreactor was used in order to obtain 2 kg of hydrolysate. Figure 3.8 shows procedure of waste bread hydrolyses in bioreactor. 300 gram of WBP was weighed and 1440 mL of distilled water was added. pH of mixture was adjusted to 6.0 by adding 1 M NaOH or $H_2SO_4$. Reactor temperature was fixed to 45 °C and agitation was adjusted to 500 rpm. At the temperature of 60 °C, 0.6 ml of alpha-amylase (1U/gr WBP) was added and mixture was heated until 85 ºC and kept at this temperature for 60 minutes. Then, temperature of mixture was decreased to 55 ºC and pH was adjusted

to 5.8 by adding 7.11 M $H_2SO_4$. 3 mL of gluco-amylase (3 U/ WBP) and 0.263 mL of protease ($8.4x10^{-4}$ U/g WBP) was added and kept at this temperature for 90 minutes. After that, temperature decreased to 20 ºC again and pH was adjusted to 4.5 with addition of 7.11 M $H_2SO_4$. Finally, hydrolysate was weighed and distilled water was added for 2 kg mixture (Figure 3.7). In order to remove precipitant of hydrolysate, at 12000 rpm for 30 minutes centrifugation was carried out (Figure 3.7). Final weigh of obtained hydrolysate was 1.6 kg. FAN, DNS and HPLC analyses were performed in order to determine sugar and free amino nitrogen contents of hydrolysate.

**Lyophilisation:** In order to obtain high glucose concentration of hydrolysate, lyophilisation was carried out for 4 days at -80 ºC by using freeze-dryer (Figure 3.9). 80 % of water was removed by this process. Later, required concentration was adjusted with dilution of hydrolysate.

(A) (B)

Figure 3.6. Hydrolysis of waste bread in small scale in water bath (A) and big scale in bioreactor (B).

(A) (B)

Figure 3.7. Hydrolysate of waste bread (A) and filtration (B).

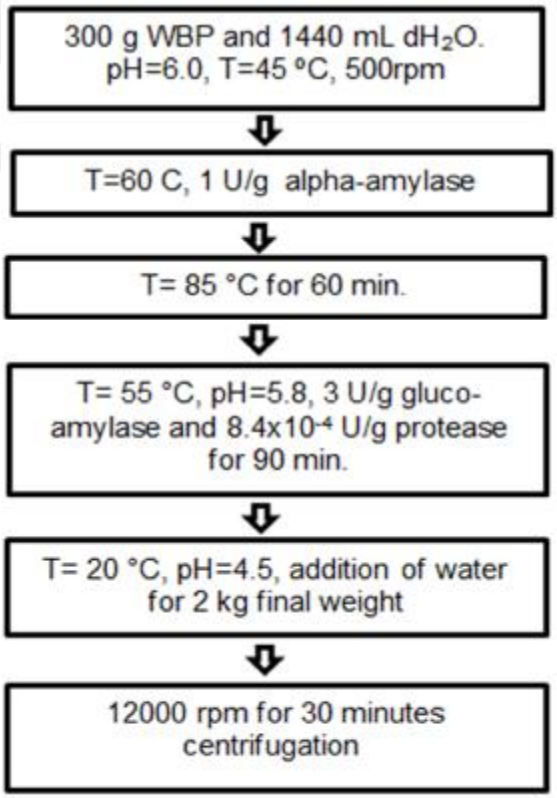



Figure 3.8. Hydrolysis of waste bread.

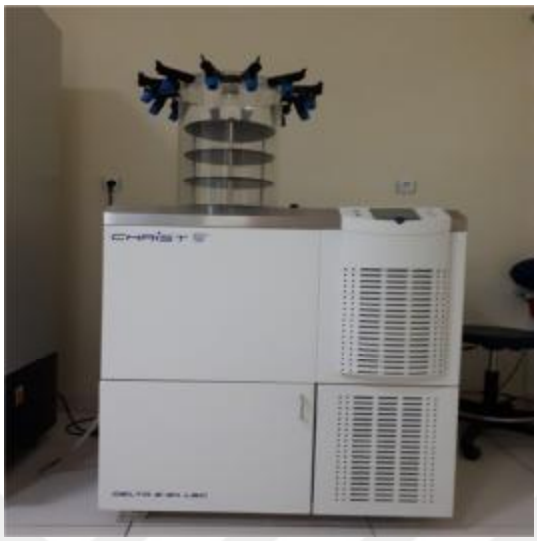


Figure 3.9. Lyophilisation of waste bread hydrolysate.

### 3.2.6. Microbial lipid and Citric Acid Production from Waste Bread Hydrolysate

After hydrolyse, waste bread hydrolysate (WBH) was mixed with salt solution (Papanikolaou et al, 2002b) and autoclaved at 121 ºC for 15 minutes. WBH and salt solution were autoclaved separately and joined together with peristaltic pump after sterilisation. Inoculum was prepared in an orbital shaker at 185 rpm and temperature of 28 ºC for 48 hours. 10 % inoculum (100 mL) was inoculated aseptically to both reactors (twin reactors). Medium composition of 50, 100 and 150 g/L glucose in hydrolysate were used for bioreactor trials (Figure 3.10). Bioreactor conditions of 800 rpm (agitation) -1.0 vvm (aeration) were applied for the all medium compositions (Figure 3.11). Fermentations were carried out until whole glucose consumed by yeast at 28 ºC and pH of 5-6 with addition of 5 M KOH periodically. Temperature, pH and $dO_2$ were monitored during fermentation. Dry cell weight, OD 600, pH, cell count in microscope, titration acidity, total lipid, organic acids and sugars were determined. Fatty acids profile of samples was carried out by using GC-MS.

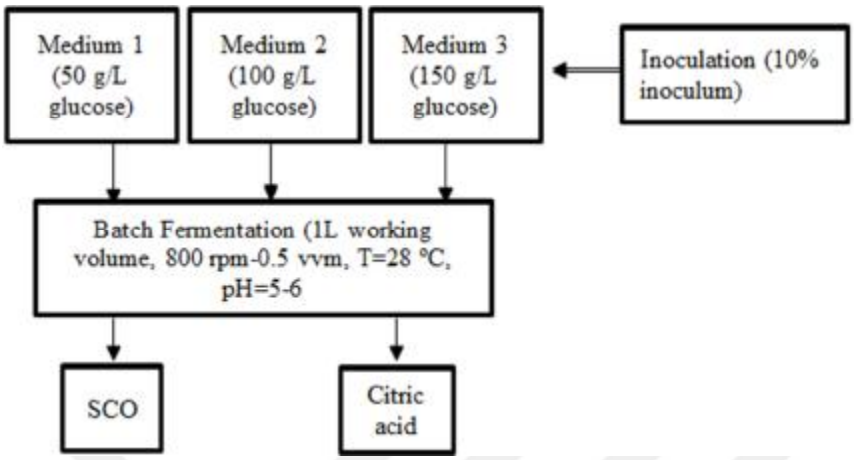



Figure 3.10. Microbial lipid and citric acid production from WBH.

Figure 3.11. Microbial lipid and citric acid production from WBH by *Y. lipolytica* in batch type bioreactor fermentation.

### 3.3. Methods of Analysis

#### 3.3.1. Enumeration of Yeast Cells

Samples were performed for yeast counting by using microscope in order to monitor growth of yeast during fermentations (Figure 3.12).

1 mL of fermentation medium was transferred aseptically to 9 mL of dilution tube which include physiological saline (0.85 % NaCl). As yeast cells were too many to count, 100 factor dilutions were applied. After dilution, 750 µL of diluted sample was mixed with same volume of methylene blue in Eppendorf tube. Later, 50-100 µL coloured sample was transferred to Thoma lam. Microscope was adjusted to screen by objective of 40X/0.65. Uncoloured cells were defines as live cells. Cell density was calculated as below formula;

$$\text{Cell Density (cell/mL)} = \frac{\text{average live cells x dilution factor (100 x 2)}}{\text{Volume of square (mL)}}$$

Cells located in 160 of Thoma lam squares were counted and volume of square was calculated as $4 \times 10^{-6}$ mL. Two repetitions were applied for each test.

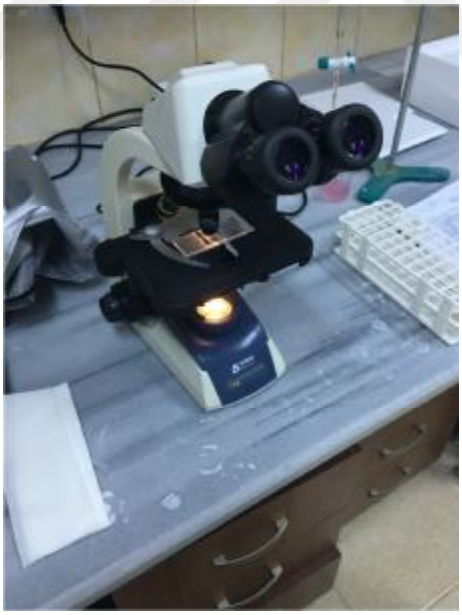


Figure 3.12. Enumeration of yeast cells in microscope.

### 3.3.2. Determination of Dry Cell Weight

Biomass of fermentation medium was harvested by centrifugation (5000 rpm /15 minutes) and washed two times with distilled water. After centrifugation, biomass was dried in drying oven at 105 ºC for 12 hours until reaching constant weight. Desiccator was used in order to avoid moisture loss or gain of tubes before each drying process. Empty and biomass filled test tubes were weighed. Dry cell weight was calculated as below formula;

$$\text{Dry Cell Weight (g/L)} = \frac{\text{Weight of filled tube (g) - weight of empty tube (g)}}{\text{Volume of Sample (mL)} \times 10^{-3}}$$

### 3.3.3. Measurement of Optical Density (OD 600)

1 mL of fermentation medium was transferred to dilution tube contained 9 mL of physiological saline and then mixed by vortex. After that, 1mL from diluted solution was added to other dilution tube and mixed. Spectrophotometer was calibrated with blank solution (distilled water) and adjusted to 600 nm wavelength. Cell of spectrophotometer was filled with diluted sample and optical density (OD 600) was measured at 600 nm (Figure 3.13). Two repetitions were applied for OD 600 test.

Figure 3.13. Measurement of optical density in Spectrophotometer.

### 3.3.4. Measurement of pH

pH of medium at the shake-flask fermentation was measured directly by pH meter on every days and regulated by using 5 M KOH periodically. At the bioreactor trials pH of medium was monitored by pH electrode of fermenter and regulated manually adding 5 M KOH.

### 3.3.5. Measurement of Dissolved Oxygen ($dO_2$)

Dissolved oxygen was monitored by $dO_2$ electrode of fermenter. $dO_2$ level of medium was recorded at selected fermentation time.

### 3.3.6. Analysis of Titration Acidity

5 mL of collected sample was diluted with 30 mL of distilled water and titrated by 0.1 N NaOH after addition of 2-3 drops of phenolphthalein indicator.

Titration acidity was calculated as below formula;

$$\% TA = \frac{(\text{Vol. of titrate}) \times (\text{Normality of titrate}) \times (\text{milliequivalent factor}) \times (100)}{\text{Volume of Sample} \times 1000}$$

Milliequivalent factor of citric acid is 0.064.

### 3.3.7. Determination of Total Lipids of Biomass

Total lipid of harvested biomass was determined according to Folch et al (1957). Lipids were extracted from biomass by keeping 5 days in 2/1 concentration of chloroform/methanol solution. After extraction, organic phase will be filtrated by using Watman filter paper to other fresh tubes. After drying of chloroform/methanol solution in drying oven at 50 ºC, tubes were weighed and quantity of total lipid was calculated as below formula;

$$\text{Total Lipid (g/L)} = \frac{(\text{Weight of filled tube}) - (\text{Weight of empty tube})}{\text{Volume of sample (mL)} \times 10^{-3}}$$

### 3.3.8. Determination of Organic Acids and Sugars

Quantity of organic acids, glycerol and sugars were determined by Shimadzu brand LC-20AD model HPLC (Figure 3.14) with two different detectors, Ultra Violet Detector (UV) for organic acids and Refractive Index Detector (RID) for sugars and glycerol. Bio-Rad HPX-87H (300 x 7.8 mm) colon and 5 mM $H_2SO_4$ solution as mobile phase were used. Flow rate and oven temperature were adjusted as 0.5 mL/ min and 50 ºC respectively. Before starting measurements, calibration curves of organic acids, sugars and glycerol were prepared by using 7 different concentrations of standard solutions, obtaining regression coefficients (r2) above 0.996 in all cases.

Before each injection, 1 mL of fermentation medium was mixed with 50 µL of perchloric acid in order to precipitate protein particles. Centrifugation at 12000 rpm for 30 minutes was carried out and after that upper part of solution was

filtered by 0.45 nm of membrane syringe filter. Generally, 20 to 35 fold dilutions were applied to samples.

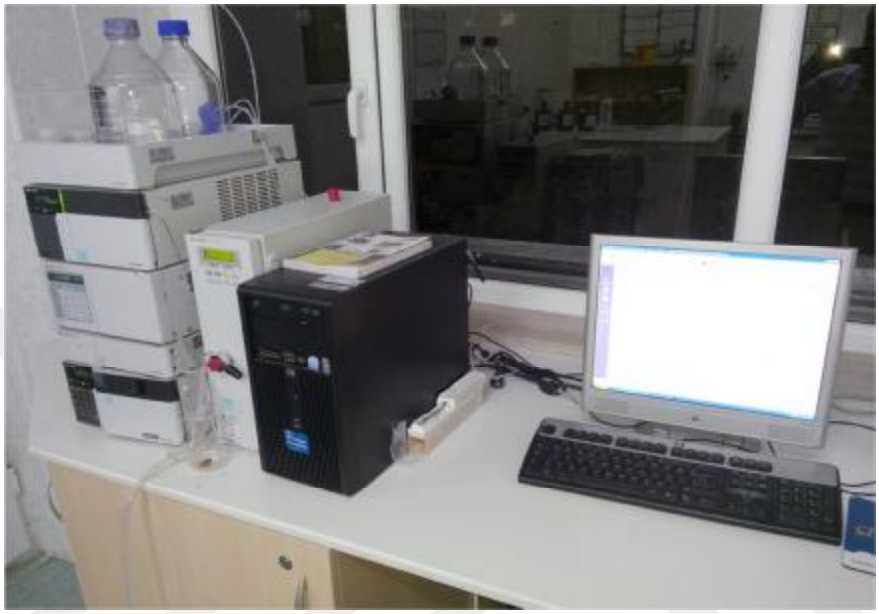


Figure 3.14. HPLC system used for determination of sugars and organic acids in this study.

### 3.3.9. Determination of Fatty Acid Profile

The total lipid of biomass was esterified for fatty acid profile analysis. N-heptane and 2 N KOH in methanol were used for esterification process. In order to prepare fatty acid methyl esters (FAMEs), 3-5 mL of lipid solution in chloroform/methanol (2/1) was mixed with 1.5 mL of 2 N KOH in methanol and then 6 mL of n-heptane was added to solution and mixed. 2 mL of this mixture was then transferred to vials for a further injection to GC-MS (Hewlett Packard, USA).

Conditions of GC-MS are showed in Table 3.4.

Fatty acids were determined from selected library of GC-MS and calculated as percentage in total quantity.

.

Table 3.4. Conditions of GC-MS.

| **Conditions of GC-MS** | |
|---|---|
| Injection-volume | Splitless-1.0µL |
| Detector | MS detector |
| Colon | HP-NOWAX (Code:1909IN-136), 60m length, 0.25mm inner diameter, 0.25mm film thickness, temperature range of 40 to 260 ºC. |
| Flow rate | 1mL/minute |
| Mobile gas | Helium |
| Colon Temperature | 3 min. in 120 ºC,<br>Increase in temperature by 10 ºC/min. from 120 ºC to 180 ºC and wait 10 min. at 180 ºC,<br>Increase in temperature by 10 ºC/min. from 180 ºC to 250 ºC and waiting 19 min. at 180 ºC |

### 3.3.10. Determination of Alpha- Amino Nitrogen (FAN)

FAN analyses were carried out according to EBC analysis method (1972). It is also called as Ninhydrin method. 2 mL of diluted samples were transferred to test tubes and 1 mL of colour reagent (Table 3.5) was added to each. First, the mixtures were heated for 16 minutes in boiling water bath and later cooled for 20 minutes in a water bath at 20 ºC. After that 5 mL of diluted solution was added to each tubes, mixed and read optical densities against solution of colour reagent plus 2 mL of distilled water at 570 nm in Spectrophotometer.

Alpha-amino nitrogen quantities of samples were calculated as below equation;

$$\text{FAN (mg/L)} = \frac{\text{OD of test solution- OD of distilled water}}{\text{Mean OD of standard- OD of distilled water}} \times 2 \times \text{dilution}$$

Mean OD of standard was determined by reading absorption of glycine stock solution (107.2 mg in 100 mL distilled water) at 570 nm in Spectrophotometer. Three replicates were used and stock solution was diluted 100 times (1 mL stock solution diluted by 100 mL distilled water) in order to contain 2 mg/L α-amino nitrogen.

Table 3.5. Reagents for FAN analysis.

| **Reagents of FAN analysis** | |
|---|---|
| Colour reagent | Dissolve in distilled water: 100g $Na_2HPO_4.12H_2O$, 60g $KH_2PO_4$, 5g Ninhydrin, 3g Fructose for 1 litre solution |
| Dilution reagent | Dissolve 2g KIO3 in 600 mL distilled water and add 400mL of 96% ethanol |
| Standard | Dissolve 107.2 mg glycine in 100 mL distilled water |

### 3.3.11. Analysis of Reducing Sugars

Quantity of reducing sugars was determined according to (Miller, 1959) and defined as glucose. 3 mL of di-nitro salicylic acid reagent (Table 3.6) was added to 3 mL of glucose sample in test tube and the mixture was heated at 90 ºC for 5-15 minutes to develop the red-brown colour. Then, 1 mL of 40 % potassium sodium tartrate (Rochelle salt) solution was added to mixture to stabilize the colour. After cooling to room temperature in a cold water bath, absorbance of mixture was read at 575 nm by Spectrophotometer. Calibration curve of absorbance versus different glucose concentrations was prepared before the test. Amount of reducing sugar was determined by using this calibration curve.

Table 3.6. Reagents of DNS analysis.

| **DNS Reagents** | |
|---|---|
| Di-nitro salicylic acid reagent | Di-nitro salicylic acid 10g, phenol 2g, sodium sulphite 0.5g, sodium hydroxide 10g and mix with distilled water for 1 litre solution |
| Potassium sodium tartrate solution | Rochelle salts (Na-K tartarate) solution %40 (w/v)-40g Na-K tartrate and addition of distilled water for 100 mL solution |

### 3.3.12. Analysis of Total Protein of Waste Bread Powder

Total raw protein content of waste breads was determined according to Kjeldahl method. 1 g of WBP was weighed to digestion tube and 15 mL of $H_2SO_4$ and 1 tablet of $K_2SO_4$ + Se catalyst were added. Then, solution was heated at 250 ºC for 30 minutes and at 400 ºC for 1 hour until developing open blue colour. After digestion, tubes were kept to cool and then plugged to distillation instrument. 50 mL of 40 % NaOH and 15 mL of distilled water were used for distillation and distillate was collected in 25 mL of 4 % (w/v) boric acid with 3-4 drops of methyl orange indicator. After that, distillate was titrated with 0.1 N HCl and consumed HCl was recorded. Finally, amount of % protein was determined by below equation;

$$\% \text{ Protein} = \frac{(V) \times (N) \times 0.014 \times 6.25}{W} \times 100$$

V; volume of consumed HCl for titration (mL)

N; Normality of HCl

W; Weight of sample in dry matter (g)

Three replicate were performed for protein analysis.

### 3.3.13. Determination of Total Fat of Waste Bread Powder

Total fat of WBP was determined by classic Soxhlet method. 10 gram of WBP was weighed to Soxhlet extractor and then 200-300 mL of diethyl ether was added. After connection of cooling cycle, diethyl ether was heated. Extraction was carried out for 5-6 hours. Later, solvent was removed by using rotary evaporator and remained solvent and sample was kept in drying oven for a night. After drying step, the sample was cooled in desiccator and weighed. Fat content was calculated as;

$$\% \text{ Fat} = \frac{(F) - (I)}{S} \times 100$$

F; Final weight of sample + Soxhlet balloon

I; Initial weight of Soxhlet balloon

S; Weight of sample in dry matter

Fat content analyses were repeated 3 times.

### 3.3.14. Determination of Moisture Content of Waste Bread Powder

Moisture content of WBP was determined by drying process in oven. 5 gram of WBP was weighed to glass petri and dried in oven at 105 ºC for 12 hours until reaching constant weigh. Dried sample was first cooled in desiccator and then weighed. Moisture content was determined by below equation;

$$\% \text{ Moisture} = \frac{(I) - (F)}{S} \times 100$$

I; Initial weight of sample + glass petri,

F; Final weight of sample + glass petri,

S; Weight of sample,

Moisture content analyses were repeated 3 times.

### 3.3.15. Determination of Ash Content of WBP

Ash content of WBP was examined by burning in furnace at high temperatures. 3 grams of sample were weighed to porcelain crucible. After addition of small amount of alcohol, sample was burned at the door of furnace at 200 ºC. Later, temperature of furnace was increased first to 500 ºC and then to 900 ºC during 2 hours. Door of furnace was opened and temperature was decreased to 200 ºC. Crucibles were cooled in desiccator and weighed after 30 minutes. Ash content was calculated as below;

$$\% \text{ Ash} = \frac{(\text{Final weight of sample + crucible}) - (\text{Weight of empty crucible})}{\text{Weight of Sample}} \times 100$$

### 3.4. Calculation of Fermentation Parameters

- The biomass yield was calculated as follows;

  $Y_{X/S}$= X/S (g/g),

  Where X is the total amount of biomass in the culture liquid (g/L) and S is the amount of carbon source consumed (g/L) (Kamzolova et al, 2011; Moeller et al, 2011; Moeller et al, 2007; Moeller et al, 2013; Morgunov et al, 2013; Rywinska et al, 2012).

- The CA yield was calculated as follows;

  $Y_{C/S}$=C/S (g/g),

  Where C is the total amount of CA in the culture liquid (g/L) and S is the amount of carbon source consumed (g/L) (Kamzolova et al, 2011; Moeller et al, 2011; Moeller et al, 2007; Moeller et al, 2013; Morgunov et al, 2013; Rywinska et al, 2012).

- The lipid yield was calculated as follows;

  $Y_{L/X}$=L/X (g/g),

  Where L is the total amount of lipid in the culture liquid (g/L) and X is the amount of biomass produced (g/L).

- The volumetric CA production rate ($Q_C$), expressed in grams per litre per hour, was calculated from;

  $Q_C$= C/t, ((g/L)/ h))

Where, C is citric acid concentration in the culture liquid at the end of cultivation (g/L) and t, the fermentation duration (h) (Kamzolova et al, 2011; Moeller et al, 2011; Moeller et al, 2007; Moeller et al, 2013; Morgunov et al, 2013; Rywinska et al, 2012).

- The volumetric glucose consumption rate ($R_S$), expressed in grams per litre per hour, was calculated from;

$R_S$= S consumed/t, ((g/L)/h)

Where, S is the consumed glucose concentration in the culture liquid at the end of fermentation (g/L) and t, the fermentation duration (h) (Kamzolova et al, 2011; Moeller et al, 2011; Moeller et al, 2007; Moeller et al, 2013; Morgunov et al, 2013; Rywinska et al, 2012).

- The maximum volumetric production rate ($Q_{Cmax}$), consumption rate ($R_{Smax}$) and growth rate, expressed in grams per litre per hour, were calculated from;

$Q_{Cmax}$= Steepest slope of CA curve, ((g/L)/h)

$q_{max}$ = Steepest slope of biomass curve, ((g/L)/h) and

$R_{Smax}$ = Steepest slope of glucose consumption curve, ((g/L)/h)

Where, slope of these curves were calculated by using the program Microsoft EXCEL (Shuler and Kargi, 2002).

- The specific growth rate (μ), expressed in $h^{-1}$ and was calculated from;

$dX/dt = \mu.X$,

$\ln (X) = \mu.t$,

$\mu=\ln(X)/t$, ($h^{-1}$)

Where, X is the amount of biomass produced (g/L) and t, the fermentation duration (h) (Moeller et al, 2011; Moeller et al, 2007; Moeller et al, 2013; Shuler and Kargi, 2002).

- The doubling time ($t_d$), expressed in h and was calculated from;

  $t_d= \ln (2)/ \mu$, (h)

  Where, μ is the specific growth rate (Shuler and Kargi, 2002).

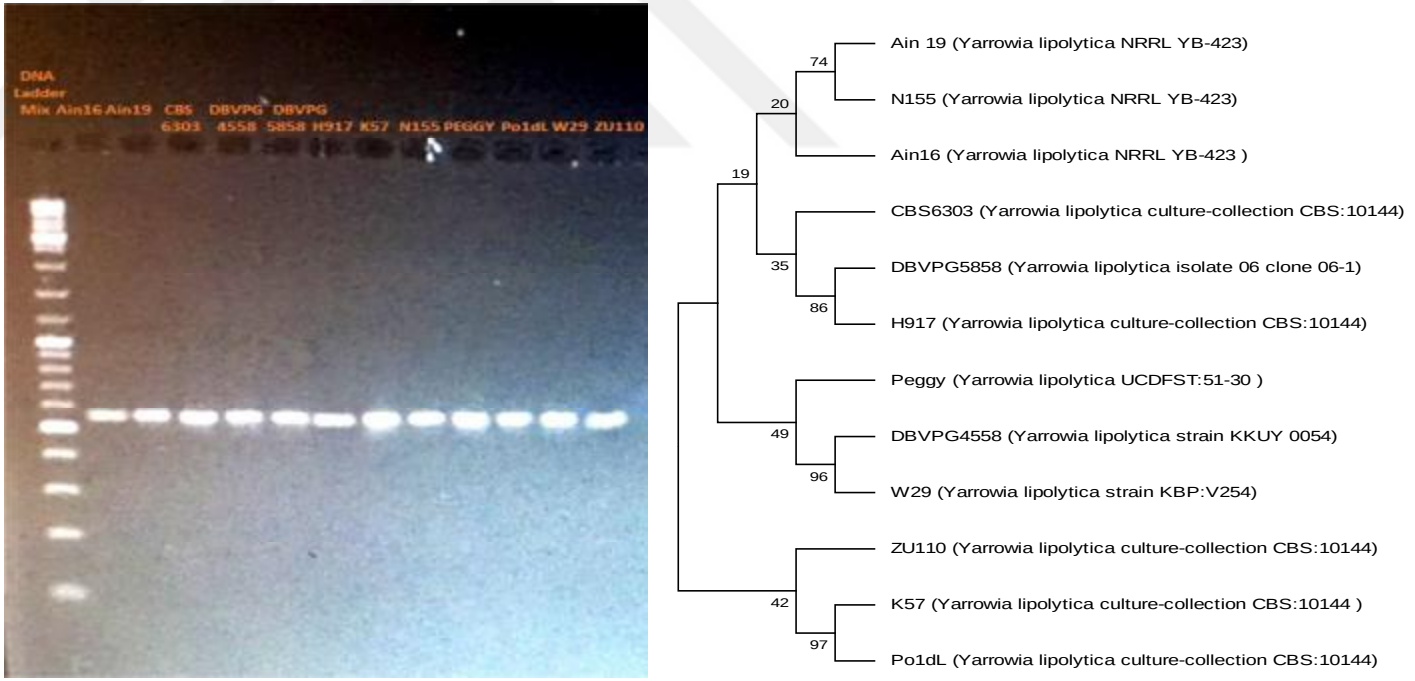
### 3.5. Statistical Analysis

The obtained results were subjected to statistical analysis using SPSS and XLSTAT (version free 2017) programs for Analysis of Variance (ANOVA), multiple range tests, principle component analysis (PCA) and correlation analysis. Sequences analysis results of yeast strains were evaluated by MEGA6 statistic program (Tamura et al, 2013).

# 4. RESULTS AND DISCUSSION

## 4.1. Molecular Characterization of *Y. lipolytica* Strains

It was imperative that all strains used for this study were *Y. lipolytica* strains; therefore, the molecular characterization of *Y. lipolytica* strains was an important step. Prior to starting any experiments producing single cell oil (SCO) and citric acid (CA), 12 different strains of *Y. lipolytica,* collected from different laboratories, were selected for molecular characterization analysis. The 26S rRNA regions were then reproduced using PCR and sent for sequence analysis. Figure 4.1-A illustrates the gel image resulting from reproduction of the 26S rRNA regions of the strains. As can be seen in Figure 4.1-A, all examined strains appear on the same line on the electrophoresis gel and demonstrate similar molecular characterization. According to the sequence results, all examined strains exhibited similarity with other *Y. lipolytica* strains identified in previous studies using the BLAST program. Table 4.1 shows the results of sequence analysis of the 26S rRNA region of examined *Y. lipolytica* strains. It was determined that all *Y. lipolytica* strains tested exhibited a high degree of homology (99% to 100%) with various *Y. lipolytica* strains present in the National Center for Biotechnology Information, as a result of BLAST analysis of a partial sequence of the 26S rRNA gene. The majority were similar to *Y. lipolytica* strains CBS 10144 (5 out of 12) and *Y. lipolytica* NRRL YB-423 (3 out of 12). In conclusion, it can be confirmed that all strains used for this study belonged to the *Y. lipolytica* species, and were expected to produce a significant amount of CA and SCO in further experiments. In addition, Figure 4.1-B shows phylogenetic relationship between 12 *Y. lipolytica* strains and bootstrap values are obtained.




(A) (B)

Figure 4.1. Gel image results (A) from reproduction of the 26S rRNA region and phylogenetic relationship (B) of 12 *Y. lipolytica* strains obtained by BOOTSTRAP method.

Table 4.1. BLAST results of the 26S rRNA region of *Y. lipolytica* strains used in this study.

| *Y. lipolytica* Strains | Species Name | BLAST Identification | Gen Bank Accession Number | Number of Divergent Bases (bp)* |
|---|---|---|---|---|
| Ain 16 | *Yarrowia lipolytica* NRRL YB-423 | 99% | NG_055393.1 | 2 |
| Ain 19 | *Yarrowia lipolytica* NRRL YB-423 | 99% | NG_055393.1 | 2 |
| CBS 63003 | *Yarrowia lipolytica* culture-collection CBS:10144 | 99% | KY110196.1 | 1 |
| DBVPG 4558 | *Yarrowia lipolytica* strain KKUY 0054 | 99% | JQ690248.1 | 1 |
| DBVPG 5858 | *Yarrowia lipolytica* isolate 06 clone 06-1 | 99% | MF148892.1 | 1 |
| H917 | *Yarrowia lipolytica* culture-collection CBS:10144 | 99% | KY110196.1 | 1 |
| K57 | *Yarrowia lipolytica* culture-collection CBS:10144 | 99% | KY110196.1 | 1 |
| N155 | *Yarrowia lipolytica* NRRL YB-423 | 100% | NG_055393.1 | 1 |
| PEGGY | *Yarrowia lipolytica* UCDFST:51-30 | 99% | KY037831.1 | 1 |
| Po1dL | *Yarrowia lipolytica* culture-collection CBS:10144 | 99% | KY110196.1 | 1 |
| W29 | *Yarrowia lipolytica* strain KBP:V254 | 99% | MG367323.1 | 1 |
| ZU110 | *Yarrowia lipolytica* culture-collection CBS:10144 | 99% | KY110196.1 | 0 |

*Number of base changes within the sequence of the identified strain compared with the corresponding type strain.

### 4.2. Selection of the Optimal *Y. lipolytica* Strain for Citric Acid and Microbial Lipid Production using Shake-flask Fermentation

The amount of CA and SCO produced by *Y. lipolytica* depends on the type of strain even under identical fermentation conditions. In the literature, various *Y. lipolytica* strains have been investigated in terms of CA and SCO production capacities (Coelho et al, 2010; Donot et al, 2014; Leiva-Candia et al, 2014; Papanikolaou and Aggelis, 2011a; Papanikolaou and Aggelis, 2011b; Yalcin et al, 2009a; Zinjarde, 2014). It is known that *Y. lipolytica* is an oleaginous microorganism and can accumulate a high quantity of SCO and CA. Different strains of this yeast can accumulate lipid to more than 20% of their dry cell weight. Mutant, wild or recombinant properties of the selected strain affect the accumulation of CA and SCO in the fermentation medium. Therefore, CA and SCO production by different strains should be determined prior to a scale-up process. Based on this knowledge, in this study 12 different strains of *Y. lipolytica* were selected and examined for CA and SCO production using the shake-flask fermentation method. Fermentation conditions, including temperature, pH, medium composition and shaking rate, were kept the same for each strain, and biomass, sugar content, organic acids, total microbial lipid and fatty acid profiles were observed during the fermentation time of 8 days. Moreover, pH, optical cell density (OD 600), cell concentration (cells/mL) and titration acidity were also monitored. The results are shown and discussed in the following sections.

#### 4.2.1. Biomass (Dry Cell Weight) Values of *Y. lipolytica* Strains in Shake-flask Fermentations

Biomass concentration is important for the production of microbial lipid and growth characteristics of *Y. lipolytica*. As it is an oleaginous yeast, it can produce microbial lipid to more than 20% of its biomass, which means that a higher biomass concentration leads to higher a production of microbial lipid. Furthermore, the biomass value is important in calculating the lipid yield of

microorganisms, which is frequently used to compare the microbial lipid production capacity of oleaginous yeasts. The growth characteristics of *Y. lipolytica* can also be identified using the biomass calculation, which is generally low in the beginning and highest at the stationary phase of fermentation. A low level of biomass content indicates that the examined strain cannot grow or is inhibited in the selected fermentation medium.

Beside strain type, the biomass concentration of *Y. lipolytica* depends on the medium used and environmental conditions of fermentation, such as temperature, pH and aeration. Due to these related factors, it was important to keep the fermentation conditions and medium composition constant for each strain trial. Therefore, 12 shake-flask fermentations (one trial per test strain) were performed at 28 ºC, an initial pH of 6 and a shaking speed of 185 rpm. Other factors, such as concentration of nitrogen and carbon sources used in the fermentation medium, which can affect the biomass content, were kept constant for each tested strain. For all experiments the concentration of both ammonium sulphate and yeast extract was 0.5 g/L and the glucose concentration was 60 g/L. The salt concentration was chosen based on the study of Papanikolaou et al (2002b).

Generally, the biomass of *Y. lipolytica*, which contains high amounts of protein and DNA, is used in the production of single cell protein (SCP) (Goncalves et al, 2014; Rywinska et al, 2013). However, in this study, it was important to produce more microbial lipid and citric acid than biomass. For this reason, the biomass production by *Y. lipolytica* should be sufficient to produce a high concentration of citric acid and microbial lipid. It is known that the biomass concentration in a fermentation medium increases due to the usage of a high concentration of a high nitrogen source. As it is thought that both microbial lipid and citric acid production are nitrogen-limited processes, the nitrogen content in the fermentation medium should be minimal but should not inhibit the growth of *Y. lipolytica* cells. In a study performed with *Candida oleophila*, excessive biomass

production with insignificant citric acid production was observed when 3 or 6 g/L $NH_4Cl$ was present in the medium (Anastassiadis et al, 2002).

Figure 4.2 shows example kinetic values of CA, lipid, biomass concentrations and glucose consumption by strain K57 during a fermentation time of 192 hours. Citric acid accumulation by *Y. lipolytica* in the fermentation medium is initiated in the exponential growth phase and increases during the stationary phase. Conversely, the biomass concentration increases during the exponential growth phase and stays constant during the stationary phase. These results were in agreement with work performed by Papanikolaou et al (2009); Papanikolaou et al (2002b) who examined the synthesis of organic acids and lipids by *Y. lipolytica* strains in a fermentation medium containing glucose.

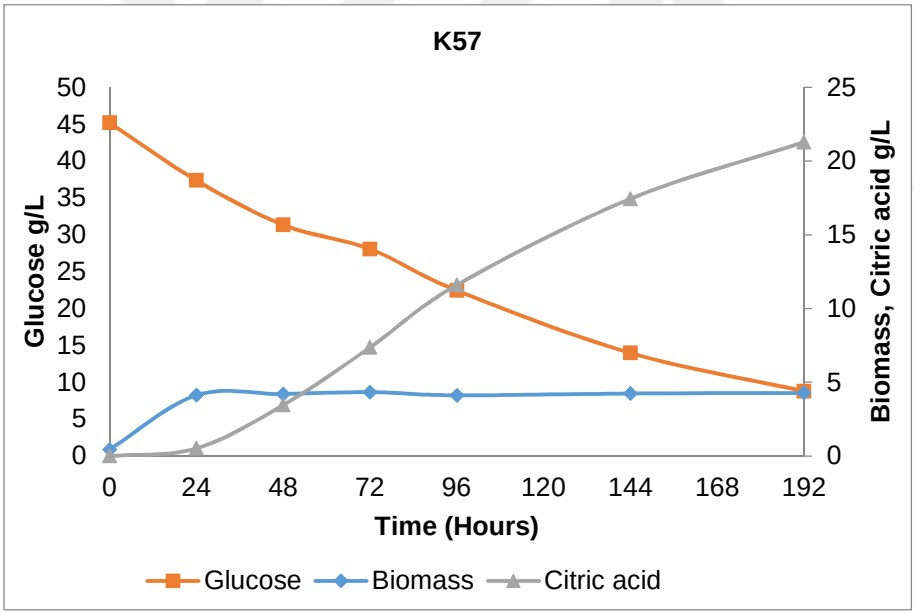



Figure 4. 2. Kinetic values (glucose, biomass and CA) of strain K57 during 192 hours shake-flask fermentation.

Another study examined the effect of biomass concentration on CA production using by *Y. lipolytica* NRRL Y-1095. A decrease in citrate yield from

0.77 g/g to 0.58 g/g was observed along with a high biomass concentration at the stationary phase, which can be explained by the lower yields being related to the higher total maintenance energy requirements of the culture at higher biomass concentrations (Rane and Sims, 1996). As demonstrated by this approach, biomass production should be low in order to obtain high amounts of CA production.

Table 4.2 shows the values of biomass, biomass yield and consumption of glucose for 12 different *Y. lipolytica* strains after 192 hours of fermentation. Results indicate that the biomass values of 12 different strains ranged between 1.29 and 5.09 g/L; with the highest biomass value of 5.09 g/L obtained for strain CBS 6303 and the lowest biomass value of 1.29 g/L observed for strain Po1dL. Furthermore, large differences in the biomass values of the *Y. lipolytica* strains were observed. Among the studied strains, the lowest biomass values ranged between 1.29 and 2.01 g/L and were obtained for strains Ain 16, Ain 19, H917 and Po1dL, whilst the highest biomass values ranged between 3.09 to 5.09 g/L for strains Peggy, CBS 6303, K57, W29, DBVPG 5858, DBVPG 4558, N155 and Zu110. These results were slightly similar to the study performed by Papanikolaou et al (2002b) who used an initial glucose concentration of 30 g/L for citric acid production by *Y. lipolytica* strains in a shake-flask fermentation and obtained biomass values ranging between 5.2 and 6.1 g/L. Moreover, Papanikolaou et al (2009) studied microbial lipid and citric acid production from an initial glucose concentration of 60 g/L using different strains of *Y. lipolytica* (AC-YC 5028, AC-YC 5029, AC-YC 5030 and AC-YC 5033) and observed biomass values ranging between 3.9 and 5.8 g/L.

When the results were examined in terms of biomass yield ($Y_{X/S}$), it was estimated that the highest yield of 0.41 g/g was obtained for strain H917, whereas glucose consumption and biomass production for the same strain were very low due to its low growth rate in the selected fermentation medium. Apart from this strain, two groups were formed in terms of biomass yield. The first group consisted of strains DBVPG 5858, N155, Ain 19 and Peggy ($Y_{X/S}$ ranged from 0.21 to 0.29),

and the second group comprised strains CBS 6303, K57, W59, ZU110, DBVPG 4558, Ain 16 and Po1dL ($Y_{X/S}$ ranged from 0.12 to 0.16). The lower $Y_{X/S}$ was explained by the "over-flow metabolism" phenomenon, in which a high quantity of carbon was efficiently channelled towards the glucose-citric acid pathway, which is generally observed for citric-acid-producing strains rather than non-citric-acid-producing strains (Papanikolaou et al, 2009).

The results of biomass content for different strains indicated that among the 12 strains of *Y. lipolytica*, only 8 produced an acceptable amount of biomass and it was interesting that strain Peggy accumulated one of the highest amounts of biomass, in contrast, glucose consumption for the same strain was lower than other strains with similar biomass values reached for CBS 6303, K57 and W29.

Table 4.2. Values of biomass, biomass yield and glucose consumption for 12 *Y. lipolytica* strains (N=2).

| Strains | Biomass (X g/L) | Biomass Yield ($Y_{X/S}$ g biomass/g glucose) | Consumed Glucose (S g/L) |
|---|---|---|---|
| **CBS 6303** | $5.09\pm0.01^{h}$ | $0.14\pm0.01^{a}$ | $36.62\pm2.69^{g}$ |
| **Peggy** | $5.05\pm0.10^{h}$ | $0.29\pm0.00^{b}$ | $17.41\pm0.42^{c}$ |
| **K57** | $4.28\pm0.06^{g}$ | $0.12\pm0.00^{a}$ | $36.39\pm0.42^{g}$ |
| **W29** | $4.04\pm0.06^{fg}$ | $0.13\pm0.00^{a}$ | $31.44\pm0.38^{f}$ |
| **DBVPG 5858** | $4.00\pm0.01^{f}$ | $0.21\pm0.00^{ab}$ | $18.71\pm0.07^{c}$ |
| **N155** | $3.93\pm0.07^{ef}$ | $0.23\pm0.01^{ab}$ | $17.33\pm0.53^{c}$ |
| **Zu110** | $3.68\pm0.05^{e}$ | $0.14\pm0.01^{a}$ | $27.46\pm0.04^{e}$ |
| **DBVPG 4558** | $3.09\pm0.01^{d}$ | $0.13\pm0.00^{a}$ | $23.62\pm0.42^{d}$ |
| **Ain19** | $2.01\pm0.23^{c}$ | $0.21\pm0.06^{ab}$ | $9.64\pm1.41^{b}$ |
| **Ain16** | $1.69\pm0.01^{b}$ | $0.16\pm0.01^{ab}$ | $10.64\pm0.35^{b}$ |
| **H917** | $1.31\pm0.08^{a}$ | $0.41\pm0.11^{c}$ | $3.31\pm1.09^{a}$ |
| **Po1dL** | $1.29\pm0.05^{a}$ | $0.16\pm0.01^{ab}$ | $8.19\pm0.11^{b}$ |

Results are the mean of two replications ± standard deviations;
$^{a,b,c,d,e,f,g,h}$ Various superscript letters in the same row demonstrate significant differences at the 0.05 level among samples ($p< 0.05$)

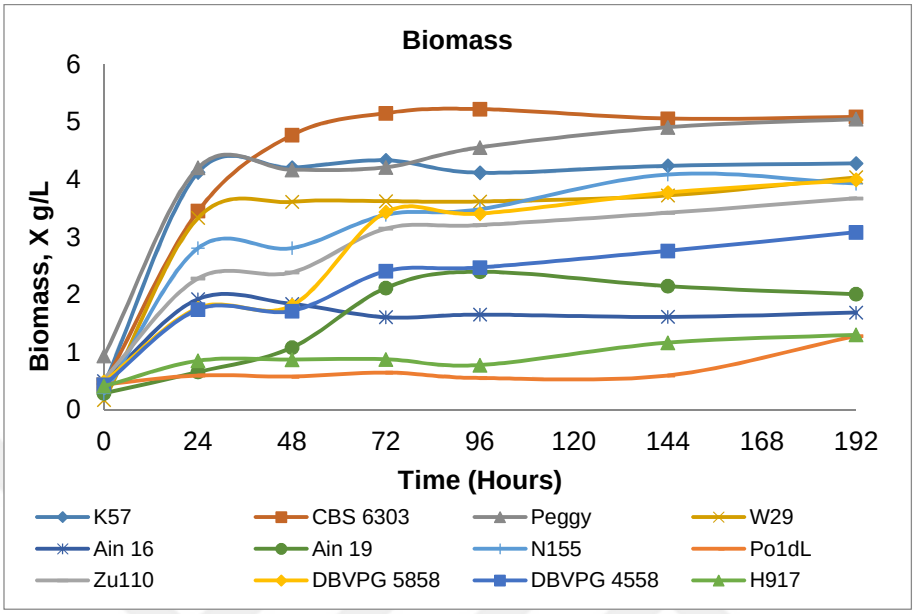



Figure 4.3. Variation of biomass values for the *Y. lipolytica* strains during 192 hours shake-flask fermentations.

Figure 4.3 shows the variation of biomass values of the 12 different strains of *Y. lipolytica* during a fermentation period of 8 days. It is obvious that biomass production generally increases until the first day of fermentation and remains constant for the duration of the fermentation. This was also observed in other similar studies (Papanikolaou et al, 2009; Papanikolaou et al, 2006; Papanikolaou et al, 2002b; Rywinska et al, 2010b). However, in some cases, increased growth was observed until the third day of fermentation for strains DBVPG 5858, DBVPG 4558, N155, Zu110 and Ain 19. This may be explained by the fluctuation of medium pH during fermentation, as sudden drops were observed after 24 hours (see Figure 4.4). In order to regulate the pH of the medium, a 5M KOH solution was used every 12 hours. It can also be seen from Figure 4.4 that the pH of the medium, for most of the strains on the second day of fermentation, was lower than 6 and ranged between 4.5 and 5.5. It is known that medium pH is one of the main inhibition factors for the growth of *Y. lipolytica* strains, which is generally

decreased at a pH lower than 4.5. Therefore, it was important to regulate the pH to be higher than 4.5 during all fermentations.

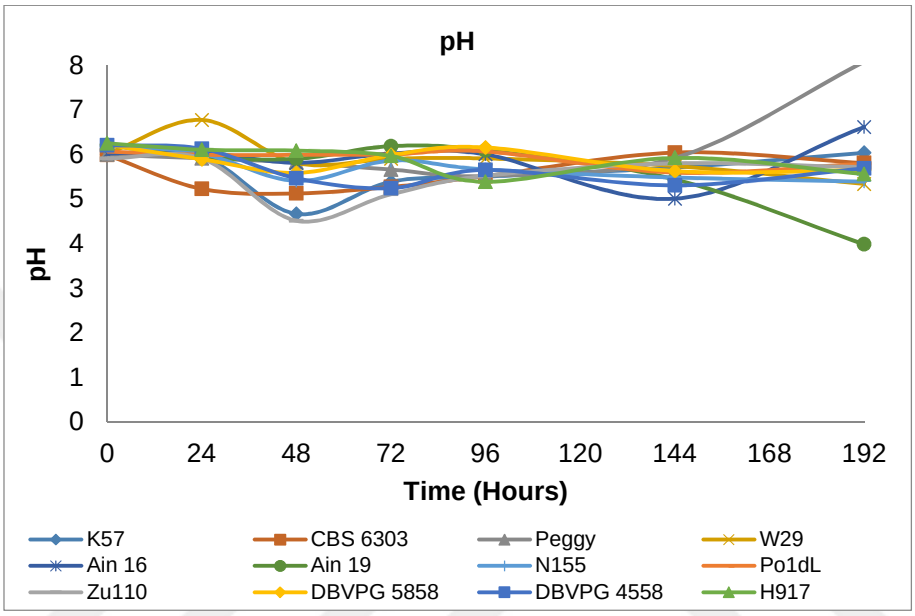



Figure 4.4. Variation of pH values for the *Y. lipolytica* strains during 192 hours shake-flask fermentations.

### 4.2.2. Glucose Consumption of *Y. lipolytica* Strains during Shake-flask Fermentation

A higher glucose consumption by *Y. lipolytica* during fermentation indicates that a high amount of the carbon source is converted to fermentation products such as biomass, organic acids (commonly citrate) and microbial lipids. Glucose consumption is also used for the calculation of the maximum consumption rate by the yeast. Usage of glucose by the *Y. lipolytica* yeast started after inoculation and was mainly used for biomass production during the exponential growth phase and at the later stationary phase for the production of citric acid and microbial lipids. As glucose consumption is also affected by the medium composition and fermentation conditions such as pH, temperature and aeration, it

was important to keep them constant during the shake-flask fermentations. Even though the fermentation conditions were the same for all strains, the glucose consumption varied according to the type of strain used.

Figure 4.5 shows the variation of glucose consumption by the 12 different strains of *Y. lipolytica.* It can be seen that there are differences between strains regarding glucose usage. While some strains, such as H917, Ain 19, Ain 16 and Po1dL, metabolized a lower amount of glucose, others consumed it almost up to the end (Table 4.2); however, there was still unconsumed glucose in the fermentation medium. It may be possible to use all the glucose present in the medium by increasing the fermentation time. In addition, Figure 4.5 shows that initial glucose concentrations of some strains, like K57 and CBS 6303, were higher than others. This may be explained by the interaction of sugar and nitrogen during autoclaving. Although prior to the sterilization process the initial glucose concentration was 60 g/L for all strains, lower initial glucose concentrations were obtained after autoclaving.

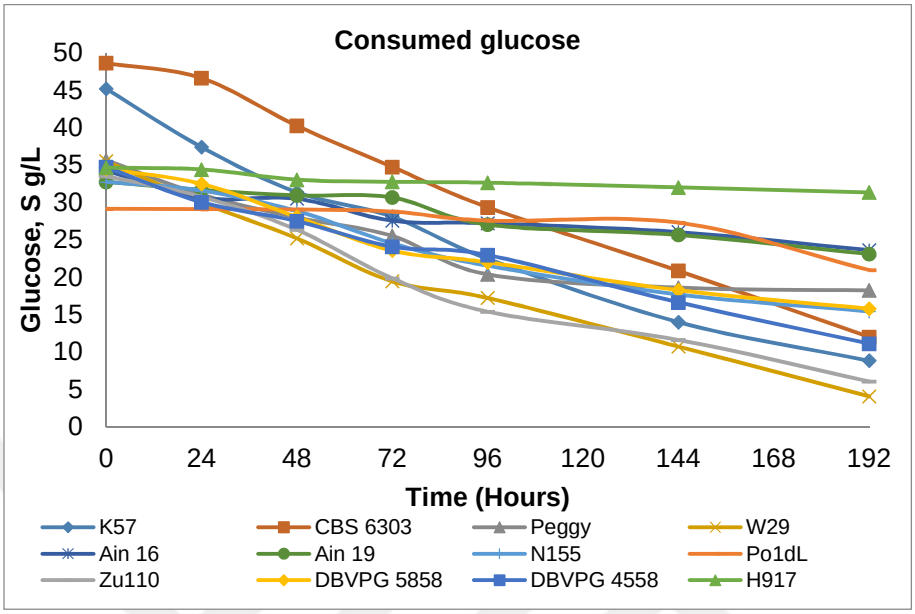



Figure 4.5. Variation of glucose concentration for the *Y. lipolytica* strains during 192 hours shake-flask fermentations.

Table 4.3 shows the glucose consumption values of each strain used in shake-flask fermentations. It can be seen that strains CBS 6303 and K57 had higher initial glucose concentrations (48.62 g/L and 45.19 g/L, respectively) and consumed more glucose (36.62 g/L and 36.39 g/L) than the other strains. Similarly, considering the biomass results, strains Ain 19, Ain 16, H917 and Po1dL didn't exhibit significant values of glucose consumption and among all the strains the lowest glucose consumption value of 3.31 g/L was obtained for strain H917. Papanikolaou et al (2009) also studied the production of citric acid and microbial lipids by strain W29 from an initial glucose concentration of 30 g/L and obtained a glucose consumption of 29.2 g/L during a fermentation time of 142 hours. This value was in slight agreement with our results that estimated values of glucose consumption, initial glucose and fermentation time for strain W29 to be 31.44 g/L, 35.48 g/L and 192 hours, respectively. As can be seen from Table 4.3, the formation of different initial glucose concentrations can be due to the Maillard

reaction between nitrogen and sugar compounds and prevented by autoclaving sugar and nitrogen solutions separately.

Table 4.3. Glucose consumption values of each strain used in shake-flask fermentations (N=2).

| Strains | Consumed Glucose (S, g/L) | Initial Glucose Concentration ($S_0$, g/L) |
|---|---|---|
| **CBS 6303** | 36.62±2.69$^{g}$ | 48.62±0.74$^{d}$ |
| **K57** | 36.39±0.42$^{g}$ | 45.19±1.62$^{c}$ |
| **W29** | 31.44±0.38$^{f}$ | 35.48±1.17$^{b}$ |
| **Zu110** | 27.46±0.04$^{e}$ | 33.50±0.30$^{b}$ |
| **DBVPG 4558** | 23.62±0.42$^{d}$ | 34.71±0.29$^{b}$ |
| **DBVPG 5858** | 18.71±0.07$^{c}$ | 34.47±1.65$^{b}$ |
| **Peggy** | 17.41±0.42$^{c}$ | 35.63±0.10$^{b}$ |
| **N155** | 17.33±0.53$^{c}$ | 32.73±0.23$^{b}$ |
| **Ain16** | 10.64±0.35$^{b}$ | 34.24±0.27$^{b}$ |
| **Ain19** | 9.64±1.41$^{b}$ | 32.73±0.23$^{b}$ |
| **Po1dL** | 8.19±0.11$^{b}$ | 29.13±0.01$^{a}$ |
| **H917** | 3.31±1.09$^{a}$ | 34.64±0.08$^{b}$ |

Results are the mean of two replications ± standard deviations;
$^{a,b,c,d,e,f,g}$ Various superscript letters in the same row demonstrate significant differences at the 0.05 level among samples ($p < 0.05$)

### 4.2.3. Growth Characteristics of *Y. lipolytica* Strains during Shake-flask Fermentation

The growth characteristics of *Y. lipolytica* play a key role in the production of a significant amount of citric acid and microbial lipids. This type of microbial growth is known as nitrogen-limited growth during which the cell population increases until nitrogen is depleted from the medium. The nitrogen concentration recommended for CA and SCO production ranges between 0.5 to 2 g/L (Imandi et al, 2007; Yalcin et al, 2009a) and it is stated that a higher concentration of nitrogen

in the medium leads to an increased cell population as well as biomass production, which is not desirable for CA and SCO production.

An inoculum of 500-1000 µL, which consisted of approximately 1-3 x $10^7$cells/mL, produced during an inoculation of 48 hours was added to the fermentation medium (Papanikolaou et al, 2009). Up to 24 hours after inoculation, the growth of many of the strains was exponential and a later stationary phase was observed (see Figures 4.3, 4.6 and 4.7). When the nitrogen concentration in the medium reached a critical value of 0.02 g/L, the growth of *Y. lipolytica* remained constant and CA biosynthesis occurs (Papanikolaou et al, 2002b), which means CA production begins after 24 hours of fermentation. This growth trend can be seen in Figures 4.6 and 4.7, which show the variation of optical (OD 600) and cell density (cells/mL) for 12 *Y. lipolytica* strains after 8-day shake-flask fermentations, respectively. The obtained values can also be seen in Table 4.4, in which the highest OD 600 value is 0.180 for strain CBS 6303, while the lowest value is 0.025 for strain Po1dL. In addition, when considering cell density, higher cell density values of 3.03 x $10^8$ and 2.90 x $10^8$ cells/mL were observed for strains Peggy and CBS 6303, whereas the lowest amounts were estimated for strains Po1dL, Ain 16, Ain 19 and H917. According to these results, it can be said that although strains CBS6303, N155, K57, W29, Peggy, Zu110, DBVPG 5858 and DBVPG 4558 displayed significant growth, the other four strains didn't exhibit enough growth during the shake-flask fermentations.

Table 4.4. Optical (OD 600) and cell density values of the *Y. lipolytica* strains after 192 hours shake-flask fermentations (N=2).

| Strains | OD 600 (Abs) | Cell Density (cells/mLx$10^8$) |
|---|---|---|
| CBS 6303 | 0.180±0.00$^{d}$ | 2.90±0.35$^{ef}$ |
| N155 | 0.160±0.01$^{cd}$ | 2.60±0.07$^{e}$ |
| K57 | 0.145±0.01$^{bcd}$ | 2.51±0.08$^{e}$ |
| W29 | 0.135±0.01$^{bcd}$ | 2.59±0.08$^{e}$ |
| DBVPG 5858 | 0.125±0.01$^{bc}$ | 0.93±0.04$^{b}$ |
| Peggy | 0.115±0.04$^{bc}$ | 3.03±0.03$^{f}$ |
| Zu110 | 0.110±0.01$^{b}$ | 2.10±0.14$^{d}$ |
| DBVPG 4558 | 0.110±0.01$^{b}$ | 1.51±0.01$^{c}$ |
| Ain 16 | 0.050±0.00$^{a}$ | 0.73±0.04$^{ab}$ |
| Ain 19 | 0.045±0.01$^{a}$ | 0.81±0.01$^{b}$ |
| H917 | 0.035±0.01$^{a}$ | 0.53±0.04$^{ab}$ |
| Po1dL | 0.025±0.01$^{a}$ | 0.33±0.04$^{a}$ |

Results are the mean of two replications ± standard deviations;
$^{a,b,c,d,e,f}$ Various superscript letters in the same row demonstrate significant differences at the 0.05 level among samples ($p< 0.05$)

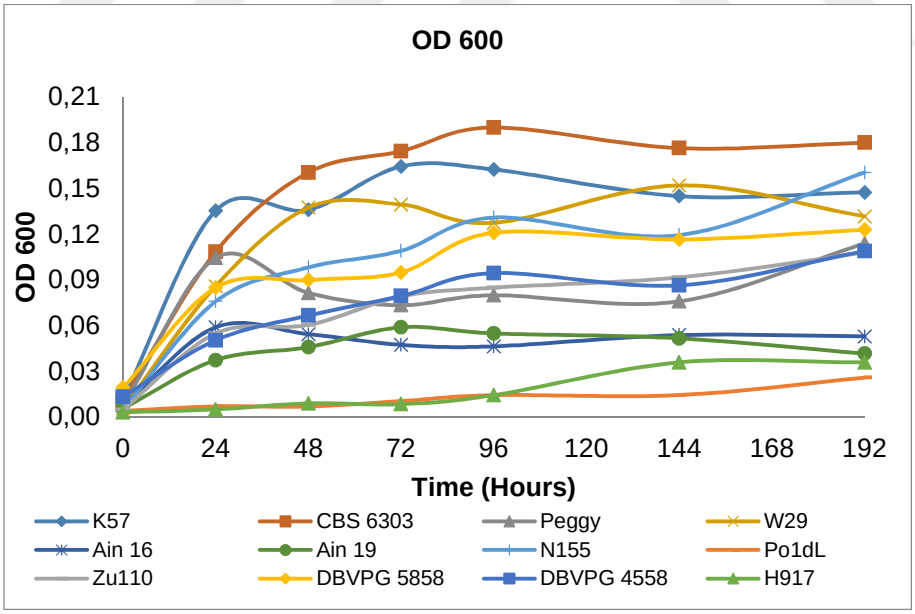



Figure 4.6. Variation of optical density absorbance (OD 600) for the *Y. lipolytica* strains during 192 hours shake-flask fermentations.

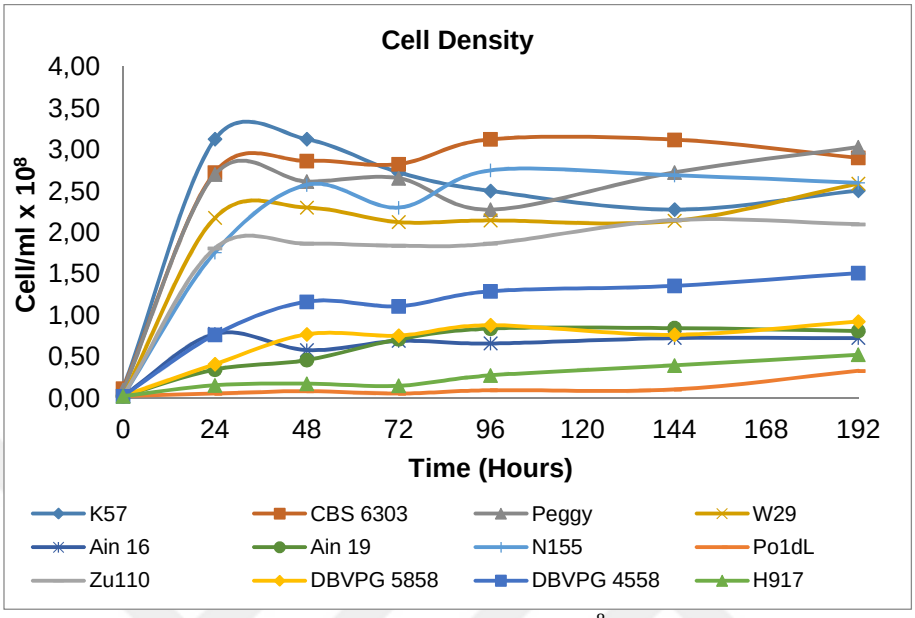



Figure 4.7. Variation of cell density (cells/mL x $10^8$) for the *Y. lipolytica* strains during 192 hours shake-flask fermentations.

### 4.2.4. Organic Acid Production of *Y. lipolytica* Strains during Shake-flask Fermentation

*Y. lipolytica* is a well-known yeast that produces a high amount of organic acids, in particular, citric acid, which is one of the target products of this study. In order to determine CA productivity, 12 different strains of *Y. lipolytica* were cultivated aerobically on an orbital shaker for 8 days with growth limited by low nitrogen and an excess of glucose. Variations of CA values were monitored during the shake-flask fermentations and Figure 4.8 shows that the highest amount of CA was obtained at the end of fermentation. According to Figure 4.8, strains CBS 6303, K57, W29 and Zu110 exhibited higher CA production values of around 20.50 to 24.67 g/L, while strains H917, Ain 16, Ain 19 and Po1dL produced the lowest CA accumulation, ranging between 0.48 and 1.68 g/L during shake-flask fermentations of 8 days.

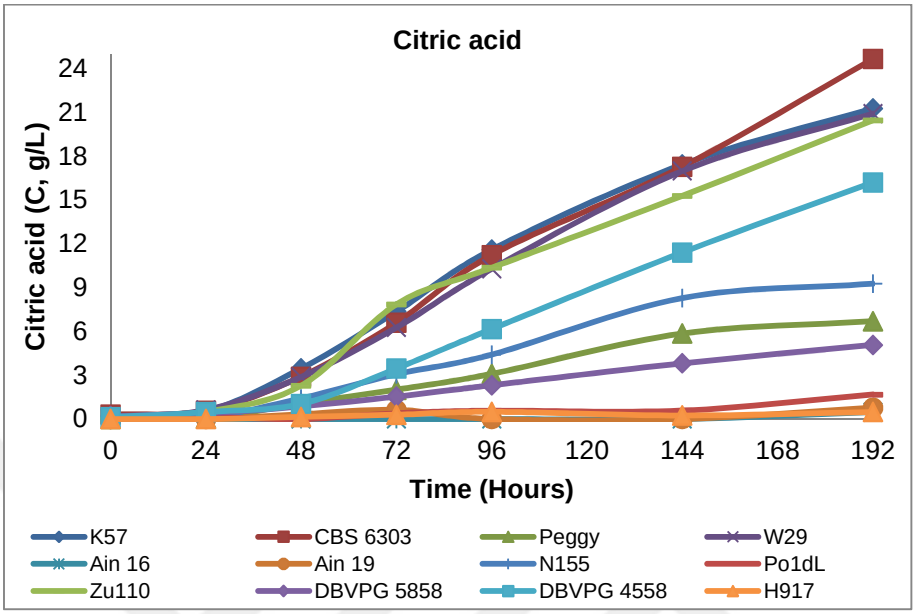



Figure 4.8. Variation of citric acid quantities for the *Y. lipolytica* strains during 8-day shake-flask fermentations.

In addition to Figure 4.8, Table 4.5 shows the concentrations and yield of CA for each studied strain. It can be seen from the biomass and cell growth results that strains Ain 16, Ain 19, H917 and Po1dL produced a lower amount of CA, ranging from 0.48 to 1.69 g/L. While the highest CA concentration of 24.68 g/L was obtained for strain CBS 6303, and the highest CA yield of 0.75 g/g was determined to be for strain Zu110. This can be explained by the low carbon usage accompanying the remarkable amount of citric acid production by this strain. Among all the studied strains, only four of them, i.e., CBS 6303, K57, W29 and Zu110, were high citric acid producers; the other strains didn't accumulate a high amount and were not selectable strains for CA production. In addition, Figure 4.8 illustrates that after 24 hours of fermentation, CA production is exponential. CA accumulation in the fermentation medium was not observed until the first day of fermentation, which is the time range for nitrogen usage by the yeast, and after depletion of the nitrogen source in the medium CA accumulation usually begins (Papanikolaou et al, 2009; Papanikolaou et al, 2006; Papanikolaou et al, 2002b).

When the results from this study are compared with other results published in the literature, it can be said that a remarkably high amount of CA concentration and productivity were obtained by certain strains, especially CBS 6303, K57, W29 and Zu110. For instance, the study of strain W29 by Papanikolaou et al (2009) determined a CA quantity of 18 g/L and yield of 0.62 g/g when 29.2 g/L glucose was consumed during 142 hours of fermentation. Results from that study were close to the present study where 20.94 g/L CA and 0.67 g/g yield were observed for strain W29. In addition, the same study by Papanikolaou et al (2009) obtained the highest CA value of 49 g/L and yield of 0.85 g/g when 57.7 g/L glucose was consumed after 315 hours of fermentation. In another study, using *Candida oleophila* ATCC 20177 performed by Anastassiadis et al (2002), 50.1 g/L of CA was obtained from 120 g/L glucose in the medium after 139.5 hours of a shake-flask fermentation; this can be explained by excess carbon usage promoting high CA production. Moreover, Moeller et al (2007) produced 41 g/L of CA and a yield of 0.55 (g CA/g glucose) from 100 g/L glucose when using strain H222 during a 92-hour fermentation. Later, a fed-batch process for CA production was carried out by Moeller et al (2011), in which the CA production obtained was 53 to 97 g/L with the CA yield of 0.39 to 0.69 g/g. Rywinska et al (2010b) also studied CA production with strain A-101 and a concentration of 69.3 g/L and yield of 0.45 g/g were obtained. Recently, recombinant strains of *Y. lipolytica* were studied in order to achieve high values of CA productivity. From this research, Tan et al (2016) produced 95 g/L of CA and a yield of 0.75 g/g using a recombinant strain of *Y. lipolytica,* PG86. Similarly, Fu et al (2016) obtained a high amount of CA at 111.1 g/L and a yield of 0.93 g/g using the recombinant strain *Y. lipolytica* $PR_{32}$. Recently, Kamzolova and Morgunov (2017) found that, from natural strains of *Y. lipolytica,* strain VKM Y-2373 was a higher CA producer, capable of accumulating 80 g/L of CA with a yield of 0.75 g/g. The CA yield results of this study ranged between 0.59 g/g and 0.74 g/g for the highest CA-producing strains, revealing that

they were comparable to those listed in the literature which were reported to be within the range of 0.40 and 0.85 g/g.

Among all the studied strains CBS 6303, K57, W29 and Zu110 were selected as high CA-producing strains due to the high values of CA concentrations and yields. However, strain K57 was selected for further bioreactor fermentations due to its comparable values of CA yield and concentration, and also being a strain domestically isolated in Turkey. There are not many published results regarding this strain in the literature except for work performed by Yalcin et al (2009c) and Yalcin et al (2009b).

Table 4.5. Citric acid concentrations of *Y. lipolytica* strains after 192 hours shake-flask fermentations (N=2).

| Strains | CA (g/L) | CA Yield, $Y_{C/S}$ (g CA/g consumed glucose) |
|---|---|---|
| CBS 6303 | 24.68±0.78$^{i}$ | 0.67±0.03$^{f}$ |
| K57 | 21.29±0.02$^{h}$ | 0.59±0.01$^{e}$ |
| W29 | 20.94±0.02$^{gh}$ | 0.67±0.01$^{f}$ |
| Zu110 | 20.47±0.01$^{g}$ | 0.75±0.01$^{g}$ |
| DBVPG 4558 | 16,22±0.05$^{f}$ | 0.69±0.01$^{fg}$ |
| N155 | 9,28±0.04$^{e}$ | 0.54±0.02$^{e}$ |
| Peggy | 6.71±0.06$^{d}$ | 0.39±0.01$^{d}$ |
| DBVPG 5858 | 5.09±0.02$^{c}$ | 0.27±0.00$^{c}$ |
| Po1dL | 1.69±0.02$^{b}$ | 0.21±0.01$^{bc}$ |
| Ain19 | 0.79±0.05$^{a}$ | 0.09±0.01$^{a}$ |
| H917 | 0.49±0.05$^{a}$ | 0.16±0.06$^{b}$ |
| Ain16 | 0.48±0.06$^{a}$ | 0.05±0.01$^{a}$ |

Results are the mean of two replications ± standard deviations;
$^{a,b,c,d,e,f,g,h}$ Various superscript letters in the same row demonstrate significant differences at the 0.05 level among samples ($p < 0.05$)

### 4.2.5. Microbial Lipid Production by *Y. lipolytica* Strains

As a yeast, *Y. lipolytica* can accumulate lipid to more than 20% of its dry biomass, consisting of mostly unsaturated fatty acids, as in plant oils, and is of

industrial importance in food biotechnology. Microbial lipids produced via cost-effective fermentation methods, can be used as raw materials for biodiesel, the production of essential fatty acids and cacao butter substitutes. Due to these benefits of microbial lipids, in recent years there has been tremendous effort focused on investigating lipid-producing oleaginous microorganisms. During this study, lipid productivities of selected strains were also investigated. The second main product studied in this research, besides citric acid, was microbial lipid. In order to determine the lipid content of the 12 different strains, biomass was collected every day during fermentation, dried and subjected to an extraction process according to the Folch et al (1957) method.

Variation of the lipid content of the 12 different strains of *Y. lipolytica* cultivated over 8 days in flasks is presented in Figure 4.9 and, as can be seen, there is wide diversity between strains regarding lipid accumulation. The lipid accumulation graph also shows there are, in most cases, big peaks at the early stage of cultivation, between 24 to 48 hours, and at the later stage lipid accumulation remains constant or even decreases (Sabra et al, 2017). In general, higher lipid content varied between 0.4 to 1.30 g/L on the second or third day of fermentation. In some cases, lipid accumulation was very low, especially for strains K57 and CBS 6303, whereas higher quantities were exhibited for strains Peggy, W29 and DBVPG 5858. In the literature, there are three different types of lipid accumulation metabolism; in the first type, called typical oleaginous metabolism, high quantities of lipid accumulate after nitrogen exhaustion in the medium with a simultaneously lower amount of extracellular metabolite production, such as CA and polyols. The second type, referred to as atypical oleaginous metabolism, at the beginning of fermentation lipid is stored in the medium after nitrogen depletion and at a later stage citric acid production occurs and continues uninterrupted accompanied by decreasing lipid concentration. The third type of metabolism is an atypical metabolism, in which lipids in yeast cells accumulate at slow rates without degradation and with the simultaneous secretion of citric acid (Sabra et al, 2017).

According to this assumption, as can be seen in Figure 4.9, while strains Peggy and DVPG 5858 show atypical oleaginous metabolism, strains CBS 6303 and K57 display atypical metabolism. Moreover, the third type of atypical oleaginous metabolism is observed for strains Zu110 and W29, in which lipid accumulation occurred with continuous citric acid production (Tables 4.5 and 4.6).

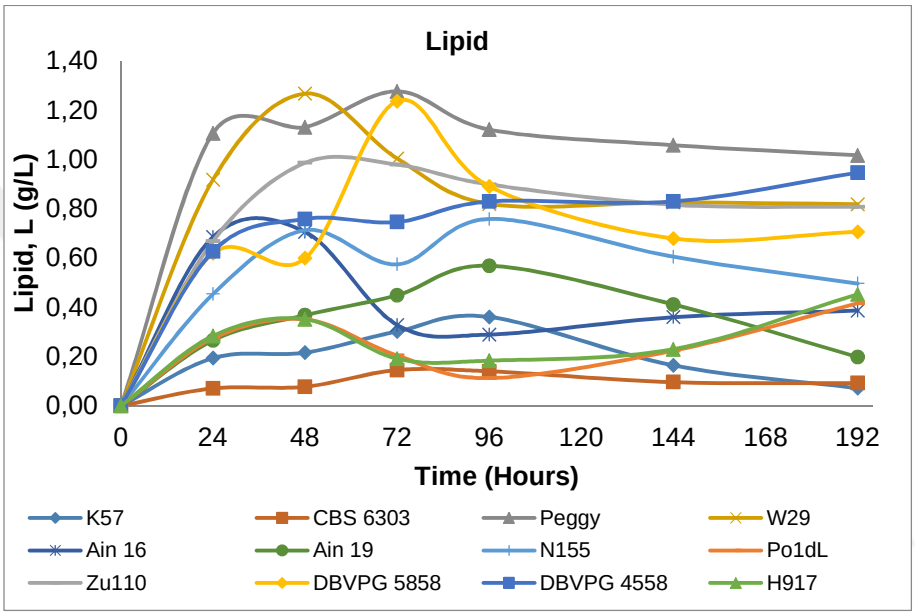



Figure 4.9. Variations of the lipid content of *Y. lipolytica* strains during 192 hours shake-flask fermentations.

Generally, maximum lipid yield values rather than lipid content values are preferred to evaluate the lipid-producing capacity of oleaginous microorganisms. Here, the maximum lipid yields and lipid content of 12 *Y. lipolytica* strains were determined and are illustrated in Table 4.7, showing that strains K57 and CBS 6303 obviously did not display lipid-producing characteristics with yield values of 0.09 and 0.03, which were very low quantities when compared with the other strains. This indicated that cultivation of these two *Y. lipolytica* strains in a glucose-based nitrogen-limited medium in a shake-flask fermentation did not exhibit the *de novo* lipid accumulation of TAG synthesis in the yeast cell, as the

percentage of lipid in the microbial cell biomass in *de novo* accumulation should be more than 20%. This behaviour can be explained by changing the direction of metabolic pathways toward the synthesis of citric acid instead of lipid accumulation (Papanikolaou et al, 2009). However, strains Po1dL, DBVPG 4558, Zu110, Ain 19, Ain 16 and H917 had higher lipid yields ranging between 0.39 and 0.61 g/g, respectively. In addition, the variation of lipid yields of the 12 strains during shake-flask fermentation is illustrated in Figure 4.10, in which higher values are generally observed on the second day of fermentation and later stayed constant or even decreased. This characteristic of strains was also observed in other studies reported in the literature (Papanikolaou et al, 2009; 2006; 2017; Sabra et al, 2017). Dourou et al, (2017) demonstrated the lipid metabolism of *Y. lipolytica* during glucose consumption in shake-flask fermentation occurs in three different phases, i.e., balanced growth, and oleaginous and lipid turnover phases. The balanced growth phase concluded with the exhaustion of nitrogen in the growth medium. The oleaginous phase began after depletion of nitrogen and finished with glucose in the medium. The final phase, the “lipid turnover phase”, occurred after glucose depletion in the fermentation medium. Lipid was stored to a significant degree from glucose during the transition from the early oleaginous to late oleaginous phase. As Figure 4.9 shows, different stages of lipid production by *Y. lipolytica* strains can be seen; the time interval from 0 to 24 hours of fermentation shows a balanced growth phase, 24 to 48 hours (some cases until 72 hours) indicates the oleaginous phase and the sixth day of fermentation can be inferred as the lipid turnover phase of most strains (except strain H917).

Table 4.6. Values of maximum lipid content and lipid yields for *Y. lipolytica* strains in shake-flask fermentations (N=2).

| | Maximum Lipid Content, $L_m$ (g/L) | Maximum Lipid Yield $Y_{mL/X}$ (g/g) |
|---|---|---|
| Po1dL | 0.42±0.01$^{c}$ | 0.61±0.06$^{e}$ |
| DBVPG 4558 | 0.95±0.01$^{h}$ | 0.45±0.08$^{d}$ |
| Zu110 | 0.99±0.01$^{i}$ | 0.42±0.06$^{cd}$ |
| Ain19 | 0.57±0.00$^{e}$ | 0.41±0.01$^{cd}$ |
| H917 | 0.46±0.01$^{d}$ | 0.41±0.01$^{cd}$ |
| Ain16 | 0.71±0.01$^{f}$ | 0.39±0.02$^{cd}$ |
| DBVPG 5858 | 1.24±0.01$^{j}$ | 0.36±0.04$^{bcd}$ |
| W29 | 1.27±0.02$^{k}$ | 0.35±0.00$^{bcd}$ |
| Peggy | 1.28±0.01$^{k}$ | 0.31±0.01$^{bc}$ |
| N155 | 0.76±0.00$^{g}$ | 0.25±0.00$^{b}$ |
| K57 | 0.36±0.00$^{b}$ | 0.09±0.00$^{a}$ |
| CBS 6303 | 0.15±0.01$^{a}$ | 0.03±0.00$^{a}$ |

Results are the mean of two replications ± standard deviations;
$^{a,b,c,d,e,f,g,h,l,j,k}$ Various superscript letters in the same row demonstrate significant differences at the 0.05 level among samples ($p< 0.05$)

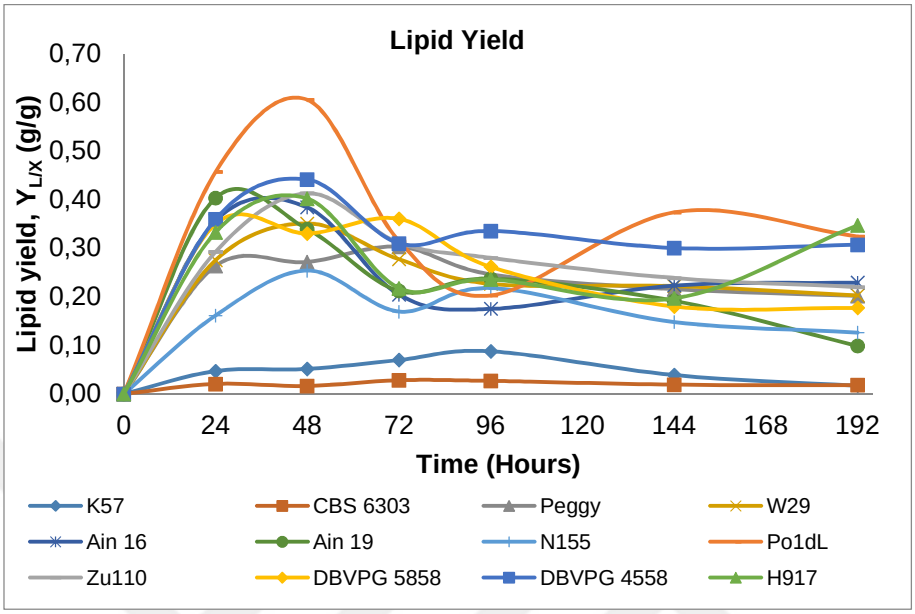



Figure 4.10. Variations of the lipid yield of *Y. lipolytica* strains during 192 hours shake-flask fermentations.

In conclusion, it can be said that maximum lipid yields and lipid content varied from 0.03 to 0.61 g/g and 0.15 to 1.28 g/L, respectively, and were remarkably high values when compared with results obtained by *Y. lipolytica* in shake-flask fermentations using glucose-based media reported in other studies. For instance, maximum lipid yields lower than 0.04 g/g were obtained for strains ACA-YC 5033, ACA-YC 5029, JMY 798, JMY 794 and W29 (Papanikolaou et al, 2009). In the present study, only strains K57 and CBS 6303 presented lipid yield values lower than 0.09 g/g. Moreover, strain W29 generated a higher lipid yield, at 0.35 g/g, than reported by Papanikolaou et al (2009). Similar results were also reported by Papanikolaou et al (2006) with regards to the lipid yield of *Y. lipoytica* ACA-DC 50109, which was found to be between 0.05 and 0.12 g/g in different concentrations of glucose (34 to 150 g/L glucose concentrations) during shake-flask fermentations. Moreover, in another study with the same strain (*Y. lipolytica* ACA DC 50109), Dourou et al (2017) reported a maximum lipid yield of 0.33 g/g

from a glucose concentration of 40 g/L in the late oleaginous phase of a shake-flask fermentation. Recently, Sabra et al (2017) also reported a lipid yield of 0.12 g/g from a glucose concentration of 50 g/L using the same strain in shake-flask fermentation. Despite these low values of lipid yields produced by wild-type *Y. lipolytica* strains, a metabolically engineered strain of *Y. lipolytica,* YL-ad9, was capable of accumulating a lipid content of 67% (g/g) from glucose in a fed-batch fermentation (Qiao et al, 2015). Although this value was found to be close to 0.61 g/g obtained by strain Po1dL in the current study, the generated biomass and lipid content by this strain were very low (1.29 and 0.42 g/L, respectively).

Table 4. 7. Kinetic values for *Y. lipolytica* strains in shake-flask fermentations (N=2)

| | K57 | CBS 6303 | Peggy | W29 | Ain 16 | Ain 19 | N155 | Po1dL | Zu110 | DBVPG 5858 | DBVPG 4558 | H917 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $X_{max}$ | 4,34±0,26 | 5,23±0,29 | 5,05±0,10 | 4,04±0,06 | 1,93±0,09 | 2,40±0,13 | 4,09±0,20 | 1,29±0,05 | 3,68±0,05 | 4,00±0,01 | 3,09±0,01 | 1,31±0,08 |
| $Y_{X/S}$ | 0,25±0,04 | 0,27±0,04 | 0,29±0,00 | 0,13±0,00 | 0,53±0,07 | 0,42±0,05 | 0,27±0,01 | 0,16±0,00 | 0,13±0,00 | 0,21±0,00 | 0,13±0,00 | 0,39±0,11 |
| $Y_{C/S}$ | 0,43±0,05 | 0,58±0,08 | 0,39±0,01 | 0,67±0,01 | 0,00±0,00 | 0,00±0,00 | 0,55±0,00 | 0,21±0,01 | 0,75±0,00 | 0,27±0,00 | 0,69±0,01 | 0,15±0,07 |
| $Y_{L/S}$ | 0,02±0,00 | 0,007±0,00 | 0,06±0,00 | 0,03±0,00 | 0,19±0,02 | 0,10±0,02 | 0,04±0,00 | 0,05±0,00 | 0,03±0,00 | 0,04±0,00 | 0,04±0,00 | 0,14±0,05 |
| $Q_C$ | 0,10±0,00 | 0,12±0,01 | 0,03±0,00 | 0,11±0,00 | 0,00±0,00 | 0,00±0,00 | 0,06±0,00 | 0,01±0,00 | 0,11±0,00 | 0,03±0,00 | 0,08±0,00 | 0,003±0,00 |
| $L_{max}$ | 0,36±0,00 | 0,15±0,00 | 1,28±0,01 | 1,27±0,02 | 0,71±0,01 | 0,57±0,00 | 0,76±0,00 | 0,42±0,01 | 0,99±0,01 | 1,24±0,01 | 0,95±0,01 | 0,45±0,01 |
| $Y_{X/S}$ | 0,18±0,01 | 0,37±0,12 | 0,42±0,03 | 0,35±0,00 | 0,49±0,07 | 0,42±0,05 | 0,31±0,05 | 0,16±0,00 | 0,33±0,06 | 0,31±0,05 | 0,13±0,00 | 0,39±0,11 |
| $Y_{C/S}$ | 0,51±0,05 | 0,48±0,15 | 0,20±0,01 | 0,28±0,00 | 0,00±0,00 | 0,00±0,00 | 0,39±0,06 | 0,21±0,01 | 0,32±0,01 | 0,14±0,01 | 0,69±0,01 | 0,15±0,07 |
| $Y_{L/S}$ | 0,02±0,00 | 0,01±0,00 | 0,13±0,01 | 0,12±0,00 | 0,19±0,02 | 0,10±0,02 | 0,07±0,01 | 0,05±0,00 | 0,14±0,01 | 0,11±0,01 | 0,04±0,00 | 0,14±0,05 |
| $Q_C$ | 0,12±0,01 | 0,09±0,00 | 0,03±0,00 | 0,06±0,00 | 0,00±0,00 | 0,00±0,00 | 0,05±0,00 | 0,01±0,00 | 0,05±0,00 | 0,02±0,00 | 0,08±0,00 | 0,003±0,00 |
| $C_{max}$ | 21,29±0,02 | 24,67±0,78 | 6,71±0,06 | 20,94±0,02 | 0,47±0,06 | 0,79±0,05 | 9,28±0,03 | 1,68±0,02 | 20,47±0,01 | 5,08±0,02 | 16,21±0,05 | 0,48±0,05 |
| $Y_{X/S}$ | 0,12±0,00 | 0,14±0,01 | 0,29±0,00 | 0,13±0,00 | 0,16±0,01 | 0,21±0,05 | 0,23±0,01 | 0,16±0,00 | 0,13±0,00 | 0,21±0,00 | 0,13±0,00 | 0,39±0,11 |
| $Y_{C/S}$ | 0,59±0,01 | 0,67±0,03 | 0,39±0,01 | 0,67±0,01 | 0,04±0,00 | 0,08±0,01 | 0,54±0,02 | 0,21±0,01 | 0,75±0,00 | 0,27±0,00 | 0,69±0,01 | 0,15±0,07 |
| $Y_{L/S}$ | 0,002±0,00 | 0,003±0,00 | 0,06±0,00 | 0,03±0,00 | 0,04±0,00 | 0,02±0,00 | 0,03±0,00 | 0,05±0,00 | 0,03±0,00 | 0,04±0,00 | 0,04±0,00 | 0,14±0,05 |
| $Q_C$ | 0,11±0,00 | 0,13±0,00 | 0,03±0,00 | 0,11±0,00 | 0,002±0,00 | 0,004±0,00 | 0,05±0,00 | 0,01±0,00 | 0,11±0,00 | 0,03±0,00 | 0,08±0,00 | 0,003±0,00 |

($X_{max}$-Maximum biomass (g/L), $C_{max}$-Maximum citric acid (g/L), $L_{max}$-Maximum lipid (g/L), $Y_{X/S}$-biomass yield (g/g), $Y_{C/S}$-Citric acid yield (g/g), $Y_{L/S}$-Lipid yield (g/g), Qc-volumetric productivity of citric acid (g/L/h)).

### 4.2.6. Principle Component Analysis (PCA) Results of Kinetics Parameters for 12 *Y. lipolytica* strains in Shake-flask Fermentations

PCA was applied to investigate the relationship between 12 *Y. lipolytica* strains and their kinetic results after an 8-day shake-flask fermentation. According to Figure 4.11, an overall 80.55% of variance, which includes the first component (F1) of 66.63% and second component (F2) of 13.92%, was observed. A distinct discrimination with regard to kinetics parameters expressed in the *Y. lipolytica* strains was proven along F1. Strains CBS 6303, K57, W29, Zu110, N155, DBVPG 4558 and, to a lesser degree, Peggy were located at the positive side of F1 and characterized by CA, CA yield, maximum lipid, consumed glucose, biomass, cell density and OD600, while strains H917, Po1dL, Ain16, Ain19 and, to a lesser degree, DBVPG 5858 were located at the negative side of F1 and explained by unconsumed glucose, biomass yield and maximum lipid yield. In addition, when considering the second component, strains Zu110, W29, DBVPG 4558, DBVPG 5858, Po1dL and Ain16 were more characterized by maximum lipid and lipid yield. According to these results, overall, it can be said that strains CBS 6303, K57, W29 and Zu110 exhibited CA-producing characteristics, whereas strains Zu110, DBVPG 4558, DBVPG 5858, W29, Po1dL and Ain16 displayed lipid-producing characteristics.

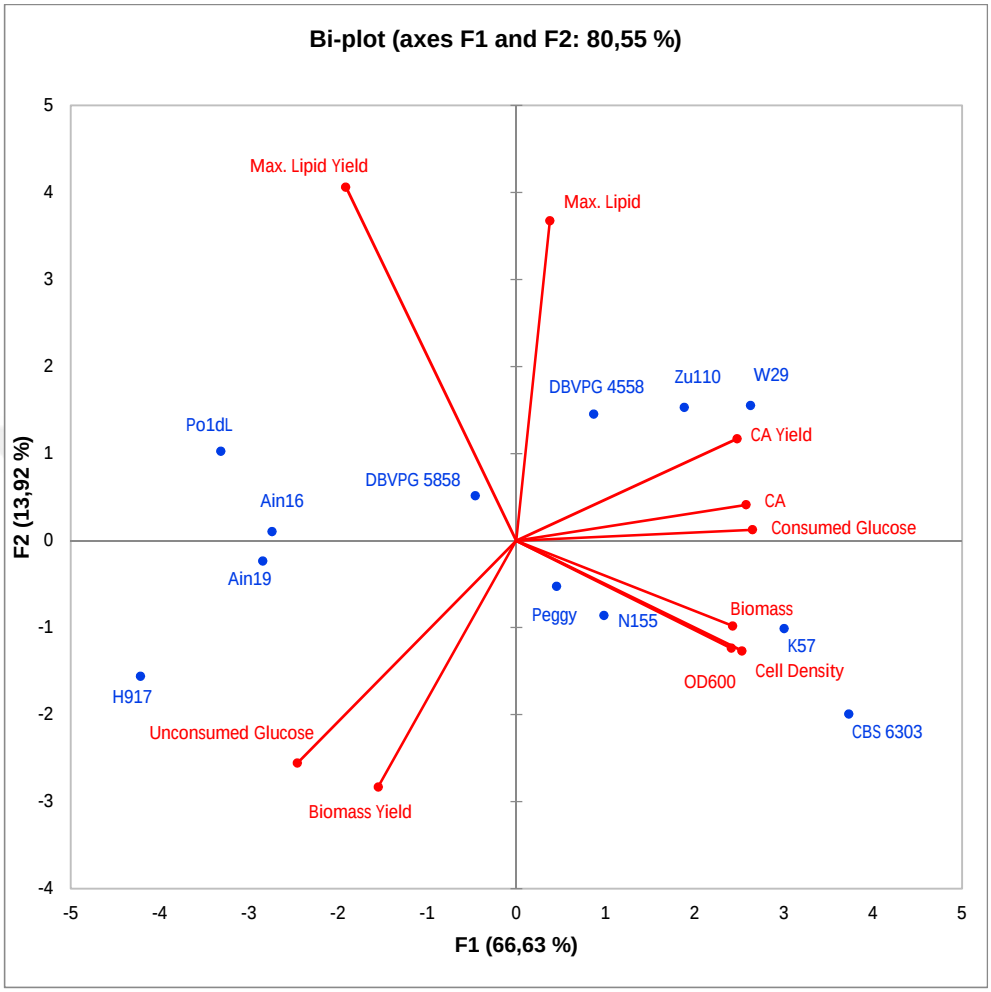



Figure 4.11. Bi-plot of PCA regarding kinetics parameters for *Y. lipolytica* strains in shake-flask fermentations.

### 4.2.7. Composition of Microbial Lipids Produced by *Y. lipolytica* Strains

Lipids produced by oleaginous microorganisms are mainly comprised of triacylglycerols, phospholipids, glycolipids and sterylesters. Among these lipids, TAGs are the main synthesized components in the microbial cells and are composed of glycerol with non-polar fatty acids. However, it was reported that lipids produced by *Y. lipolytica* from glucose were mainly formed from polar fractions such as phospholipids with the fatty acid (FA) concentrations of $^{\Delta 9}$C18:1

43 ± 4% wt/wt, C18:0 7 ± 3% wt/wt, C16:0 16 ± 5% wt/wt, $^{\Delta 9,12}$C18:2 18 ± 4% wt/wt and $^{\Delta 9}$C16:1 7 ± 4% wt/wt (Papanikolaou et al, 2006). As can be observed in the FA composition, the lipid produced by *Y. lipolytica* has an unsaturated characteristic due to high concentrations of unsaturated FAs (C18:1, C18:2 and C16:1 are approximately 68% of the total cellular lipid). The ratio of unsaturated to saturated FAs was found to be 3.5 in the same study. This trend of FA composition can also be seen in this study, in that synthesized FAs mostly range from highest to lowest concentrations in the following order: C18:1, C16:0, C18:2, C16:1, C18:0, C18:3 and others in a small amount (C17:1, C:17:0 and C15:0) (Table 4.8 and Figure 4.12).

On the basis of the lipid yield graph (Figure 4.10), the FA composition of each strain was examined at two different stages of the stationary phase; early (48 hours after inoculation) and late stationary phases (144 hours after inoculation). The early stationary phase is a period in which lipid accumulation increases, on the other hand, during the late stationary phase, referred to as the lipid turnover period, lipid accumulation and stored lipid in the medium decreases (Papanikolaou et al, 2009). The results of FA composition are illustrated in Table 4.8 and show that in all cases, oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2) are the most abundant FAs produced inside the cell of all examined *Y. lipolytica* strains. Moreover, it is interesting that linolenic acid (C18:3) was produced in significant quantities (between 10 and 20%, wt/wt) especially by strains H917 and Po1dL. These values are important when the plethora of health benefits of linolenic acid is considered. Other FAs, such as C15:0, C17:0 and C17:1, were also found in small amounts for all the strains except strain H917 (in which around 20% of C17:0 and 6% of C17:1 were found). In general, it can be revealed that, according to these results, the composition of FAs in yeast cells is strain dependent. Another point of view regarding FA composition involves comparing the FA profile in early and late stationary phases, as a slight change in FA percentage was obtained for strains Ain 16, Ain 19, DBVPG 4558, K57 and N155; whereas large differences were found

for strains CBS 6303, DBVPG 5858, H917, Po1dL, Peggy, W29 and Zu110. For instance, quantities of C16:0, C18:2 and C18:3 in the early stationary phase increased whereas the amount of C16:1, C18:0 and C18:1 decreased in the late stationary phase. Generally, conversion into the group of C18 chains (C18:0, C18:1, C18:2 and C18:3) and C16 chains (C16:0 and C16:1) was observed at different stationary phases with varying concentrations.

From another point of view, the ratio of unsaturated to saturated fatty acids (UFAs/SFAs) indicated that lipids produced in the early stationary phase are, in general, characterized as more unsaturated than ones in the late stationary phase, as the ratio of UFAs/SFAs decreased, i.e., the unsaturated profile decreased through the late stationary phase. In addition, among the FA profiles, only strains Peggy and Po1dL in the stationary phase, strain H917 in the early phase and strain Zu110 in the late stationary phase showed a saturated profile.

When the results from the FA composition in this study are compared with outcomes from similar studies in the literature (Papanikolaou et al, 2009; Papanikolaou et al, 2006; Sabra et al, 2017), it can be revealed that similar FA compositions were determined. The most dominant FAs in cellular lipid produced by all strains were oleic, palmitic, linoleic and stearic acids. The only difference was the linolenic acid content that in some cases was higher than previous results reported in the literature. In addition, the cellular FA composition produced by strain W29 during cultivation on 30 g/L glucose-based media investigated by Papanikolaou et al (2009) was different to this study in that the concentration of C16:0 (around 19% in the work performed by Papanikolaou et al (2009) and 25% in this study) was lower but C18:1 (around 55% in the work performed by Papanikolaou et al (2009) and 39% in this study) was higher.

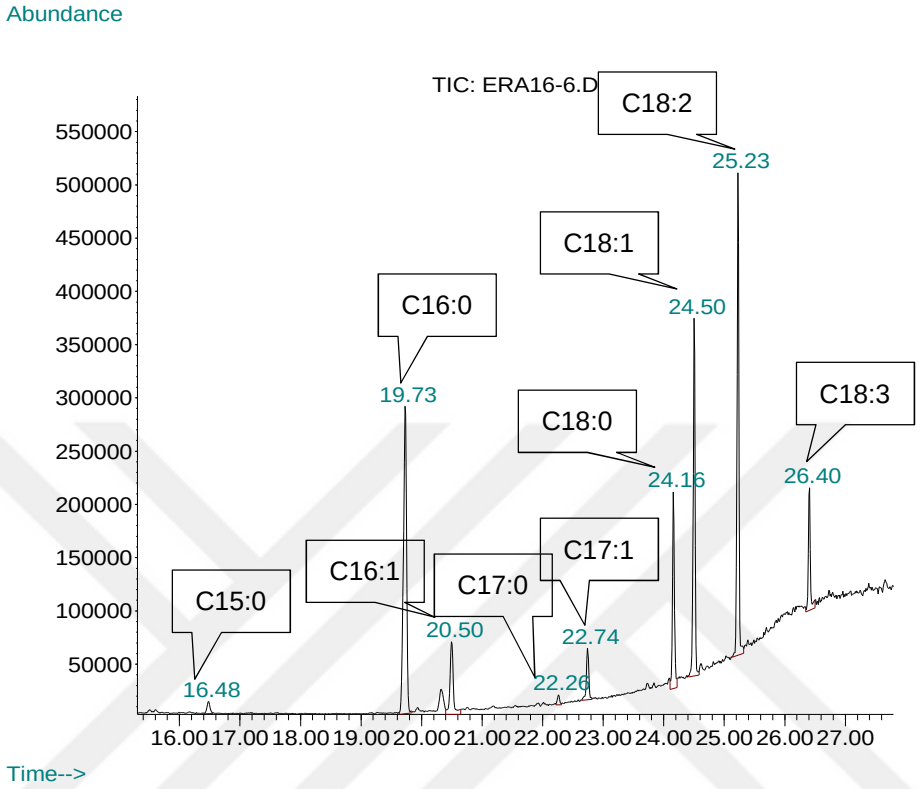



Figure 4.12. Example of a GC-MS (Gas Chromatography-Mass Spectrophotometry) chromatogram of FA of Ain 16 strain in the late stationary phase.

Table 4.8. Fatty acid composition of total lipids produced by *Y. lipolytica* strains during early (2nd day of fermentation) and late stationary (6th day of fermentation) growth phases on glucose-based media.

| | | Fatty Acid Composition % | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Strain** | **Growth Phase** | **15:0** | **16:0** | **16:1** | **17:0** | **17:1** | **18:0** | **18:1** | **18:2** | **18:3** | **UFAs/SFAs** | **Lipid Yield $Y_{L/X}$, g/g** |
| Ain 16 | Early Stationary | 0.95 | 26.95 | 7.18 | 0.72 | 3.35 | 10.51 | 17.80 | 25.15 | 7.39 | 1.56 | 0.38 |
| | Late Stationary | 1.02 | 27.15 | 7.09 | 0.68 | 3.28 | 10.56 | 18.84 | 24.33 | 7.05 | 1.54 | 0.22 |
| Ain 19 | Early Stationary | 0.90 | 23.40 | 9.30 | 1.17 | 4.32 | 7.98 | 24.60 | 24.35 | 3.98 | 1.99 | 0.34 |
| | Late Stationary | 0.96 | 24.22 | 9.08 | 1.01 | 3.17 | 9.54 | 27.64 | 21.09 | 3.29 | 1.80 | 0.19 |
| CBS 6303 | Early Stationary | - | 21.51 | 9.28 | - | - | 8.77 | 46.99 | 13.45 | - | 2.30 | 0.02 |
| | Late Stationary | - | 31.76 | 5.67 | - | - | 2.84 | 21.73 | 28.08 | 9.92 | 1.89 | 0.02 |
| DBVPG 4558 | Early Stationary | 0.65 | 20.64 | 9.43 | 3.50 | 0.54 | 8.60 | 43.56 | 11.48 | 1.60 | 1.99 | 0.44 |
| | Late Stationary | 0.79 | 20.38 | 8.71 | 5.60 | 0.94 | 8.49 | 41.57 | 12.34 | 1.18 | 1.84 | 0.30 |
| DBVPG 5858 | Early Stationary | 0.75 | 23.00 | 12.61 | - | - | 5.89 | 38.57 | 15.55 | 3.63 | 2.37 | 0.33 |
| | Late Stationary | 0.20 | 29.46 | 11.66 | - | - | 19.95 | 24.93 | 7.80 | 6.00 | 1.02 | 0.18 |
| H917 | Early Stationary | 1.14 | 23.38 | 2.09 | 17.16 | 6.15 | 12.23 | 14.64 | 9.01 | 14.20 | 0.85 | 0.40 |
| | Late Stationary | 0.52 | 17.09 | 4.26 | 21.84 | 1.74 | 6.64 | 22.50 | 13.61 | 11.80 | 1.17 | 0.20 |

Table 4.8 Continued

| Strain | Growth Phase | Fatty Acid Composition % | | | | | | | | | UFAs/SFAs | Lipid Yield $Y_{L/X}$, g/g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 15:0 | 16:0 | 16:1 | 17:0 | 17:1 | 18:0 | 18:1 | 18:2 | 18:3 | | |
| K57 | Early Stationary | - | 26.66 | 7.45 | 3.00 | - | 11.79 | 38.54 | 12.56 | - | 1.41 | 0.05 |
| | Late Stationary | 0.24 | 26.51 | 7.56 | - | - | 13.59 | 35.79 | 15.82 | 0.49 | 1.48 | 0.04 |
| N155 | Early Stationary | 0.73 | 26.56 | 8.76 | 0.91 | 1.51 | 12.85 | 34.28 | 13.86 | 0.54 | 1.44 | 0.25 |
| | Late Stationary | 0.63 | 28.64 | 8.66 | 0.60 | 1.22 | 10.06 | 30.70 | 18.80 | 0.69 | 1.50 | 0.15 |
| Po1dL | Early Stationary | 1.55 | 37.21 | 2.26 | - | - | 19.05 | 9.97 | 10.02 | 19.94 | 0.73 | 0.61 |
| | Late Stationary | 1.06 | 34.01 | 2.62 | - | - | 16.40 | 15.43 | 12.22 | 18.26 | 0.94 | 0.37 |
| Peggy | Early Stationary | 0.83 | 37.42 | 6.75 | 2.55 | 0.82 | 15.52 | 22.84 | 10.40 | 2.87 | 0.78 | 0,27 |
| | Late Stationary | 1.00 | 31.64 | 6.43 | 0.44 | 0.48 | 30.44 | 17.27 | 8.83 | 3.47 | 0.57 | 0.22 |
| W29 | Early Stationary | 0.19 | 25.18 | 8.31 | - | 0.79 | 8.33 | 34.48 | 18.60 | 4.12 | 1.97 | 0.35 |
| | Late Stationary | 0.32 | 23.07 | 6.28 | 0.31 | 0.42 | 13.68 | 38.77 | 16.09 | 1.06 | 1.68 | 0.22 |
| Zu110 | Early Stationary | 0.79 | 21.91 | 6.19 | 0.62 | 1.03 | 9.93 | 35.47 | 23.16 | 0.90 | 2.01 | 0.41 |
| | Late Stationary | 0.45 | 28.92 | 5.43 | 0.26 | 1.11 | 25.64 | 19.99 | 18.20 | - | 0.81 | 0.24 |

### 4.2.8. Principle Component Analysis (PCA) Results of Fatty Acid Composition Generated by 12 *Y. lipolytica* Strains in Shake-flask Fermentations

PCA analysis was performed to characterize the fatty acid composition generated by 12 *Y. lipolytica* strains in shake-flask fermentations. For each strain two samples, collected on the second and sixth day of fermentation, were used. In general, on the second day of fermentation, the lipid yield was higher and on the sixth day of fermentation, lipid turnover was observed. Figure 4.13 shows a bi-plot of PCA which explains an overall 63.48% of total variance composed of 41.37% of F1 and 22.10% of F2. Three different distinct groups of strains on the bi-plot are noticeable due to their fatty acid composition. In general, fatty acids of palmitic, stearic, linoleic, linolenic, palmitoleic, oleic, pentadecanoic, margaric and heptadecenoic acids were observed. The first group was generated by strains Ain19, CBS 6303, W29, K57, DBVPG 4558, N155, Zu110-2 and DBVPG 5858-2, characterized by palmitoleic, oleic, linoleic acids and UFAs/SFAs. The second group consists of strains, H917 and Ain16, identified by heptadecenoic, margaric, pentadecanoic and linolenic acids. Finally, the third group was formed by strains Po1dL, Peggy, Zu110-6 and DBVPG 5858-6, differentiated by palmitic and stearic acids. Considering the PCA results, it can be said that most of the strains were characterized by the property of unsaturation, i.e., oleic, palmitoleic and linoleic acids. However, strains Peggy, Po1dL and sixth days samples of Zu110 and DBVPG 5858 showed more saturated characteristics by consisting of a high level of palmitic and stearic acids. In addition, linolenic acid has many health benefits and was generated to a greater degree by strains H917 and Ain16.

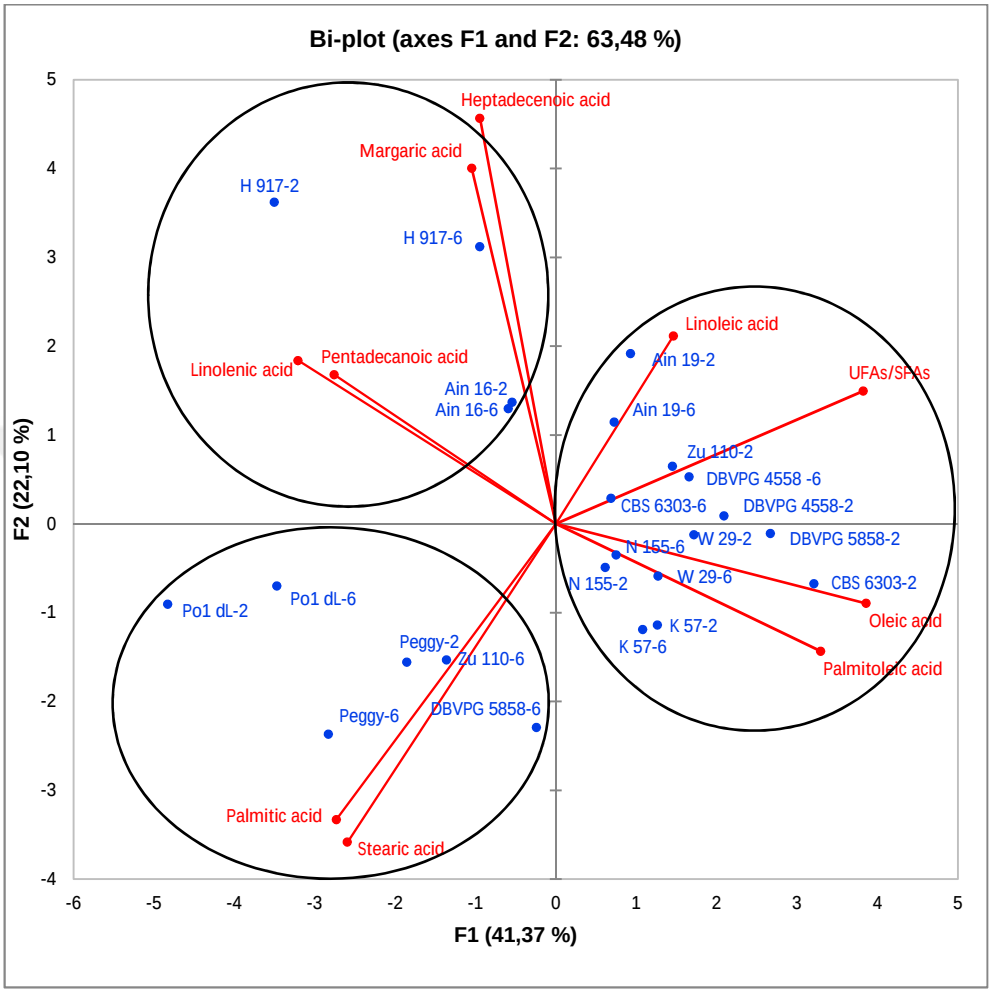



Figure 4.13. Bi-plot of PCA regarding fatty acid composition for *Y. lipolytica* strains in shake-flask fermentations.

### 4.3. Microbial Lipid and Citric Acid Production from Glucose using *Y. lipolytica* Strain K57 in Bioreactor Fermentations

After the selection of an optimum strain for the production of citric acid and microbial lipids via the shake-flask fermentation method, bioreactor trials on glucose-based media at different agitation (350 and 800 rpm) and aeration rates (0.5 and 1.0 vvm) were performed for the large-scale production of key products (CA and SCO) using *Y. lipolytica* strain K57. It is known that dissolved oxygen

and glucose concentrations generally affect the concentration of lipids and citric acid accumulated by *Y. lipolytica*. Therefore, at first, factors such as agitation and aeration were optimized and later three different glucose concentrations of 50, 100 and 150 g/L were used in the fermentation medium in order to examine their effect on lipid and citric acid production. As mentioned in the literature overview section, factors other than glucose and dissolved oxygen concentrations, such as carbon and nitrogen source, pH, temperature and fermentation mode, also have a significant effect on the production of SCO and CA, and were kept constant for all trials in the bioreactor experiments. Glucose was used as a carbon source, with ammonium sulphate and yeast extract as the nitrogen source, the pH ranged between 5 and 6 (by regulating with 5M KOH), the temperature was 28 ºC and a batch fermentation method was used.

It was reported that higher glucose concentrations of 150-200 g/L generally promote CA production, while a concentration lower than 50 g/L decreased CA productivity (Antonucci et al, 2001; Kubicek, 2001). Papanikolaou et al (2006) also studied the influence of the initial glucose concentration on CA and lipid production by *Y. lipolytica* and reported that under nitrogen-limited conditions, a significant amount of CA, at 42.9 g/L, with a yield of 0.56 g/g (g CA per g consumed glucose) and low quantities of cellular lipids (5-12 % of dry cell weight) were produced by *Y. lipolytica* when the initial glucose concentration was 150 g/L. Moreover, in the same study, it was found that increasing the glucose concentration led to a relatively lower unsaturated fatty acid proportion in the FA composition. In another study, performed by Papanikolaou et al (2009), it was reported that an increase in the initial glucose concentration from 30 to 60 g/L caused a significant amount of CA production (49 g/L), with a high conversion yield of 0.85 g/g (g CA per g glucose consumed). Moreover, in the same study, lipid production in a high concentration of glucose was increased compared with a low glucose concentration. Qiao et al (2015) also indicated that a glucose concentration of more than 150 g/L inhibited the growth of *Y. lipolytica*. Therefore, in this study, the

influence of glucose concentrations on CA and SCO production by *Y. lipolytica* were investigated using initial concentrations of 50, 100 and 150 g/L.

In addition to the glucose concentration, dissolved oxygen concentration also affects CA and SCO production. At lower oxygen concentrations, citric acid accumulation and growth by *Y. lipolytica* decreases whereas lipid production increases. There is an inverse relationship between these factors depending on the dissolved oxygen concentration (Rakicka et al, 2015; Sabra et al, 2017; Unrean et al, 2017). Growth of *Y. lipolytica*, substrate uptake and citric acid synthesis are generally high with sufficient oxygen availability in the fermentation medium (Workman et al, 2013). It has also been reported that higher citric acid production is obtained at high concentrations of oxygen in the medium (Rywinska et al, 2012). The dissolved oxygen concentration is regulated by adjusting the agitation and aeration rates of the bioreactor. High agitation and aeration values increase the dissolved oxygen concentration, which can lead to high shear stress on the cell walls and the cell-insoluble substrate interface. Therefore, optimum agitation and aeration rates should be examined prior to fermentation scale-up (Crolla and Kennedy, 2004a; Crolla and Kennedy, 2004b). Considering these approaches, different agitation and aeration rates were also applied to examine their effects on CA and SCO production.

### 4.3.1. Effect of Different Agitation and Aeration Rates on Microbial Lipid and Citric Acid Production by *Y. lipolytica* Strain K57

Before starting to examine the influence of glucose concentration on the production of SCO and CA, it was crucial to optimize certain bioreactor parameters, i.e., agitation speed and aeration rate. As previously mentioned, two different conditions, i.e., 350 rpm - 1 vvm and 800 rpm - 0.5 vvm, agitation-aeration values were adjusted on the bioreactor before SCO and CA production from 50 g/L glucose by *Y. lipolytica* strain K57. A 10% inoculum, a cell density of approximately $10^7$ cells/mL, was inoculated into a total volume of 1 litre of

medium comprising 0.5 g/L yeast extract and 0.5 g/L ammonium sulphate as a nitrogen source, 50 g/L glucose as a carbon source and a salt solution used previously by (Papanikolaou et al, 2002a). Here, it should be noted that the sugar used and nitrogen solutions should be autoclaved separately in order to prevent initiation of the Maillard reaction between proteins and sugar components. Another important point is that the inoculum and nitrogen solution should be transferred to the reactor (including the main sugar solution) aseptically using sterile silicone pipes, air filters and a peristaltic pump in order to avoid contamination. Aeration was supplied by atmospheric air using an air pump, which was regulated via an air rotameter. 0.5 litres per minute of air was pumped to 1 litre of medium for a 0.5 vvm aeration rate and 1.0 litre per minute of air was pumped to 1 litre of medium for a 1 vvm aeration rate. The agitation speed was adjusted via the control unit of the bioreactor and for a long-term fermentation; the power supply would be maintained even if there was an electricity power cut.

Previous studies have shown that agitation speed and aeration rate influence the dissolved oxygen concentration in the medium and since production of CA and SCO by *Y. lipolytica* occurs during aerobic fermentation, the dissolved oxygen level is important for growth, biomass, SCO and CA formation. Different agitation speeds and aeration rates were tested for the highest productivity of CA and SCO by *Y. lipolytica*. Rywinska et al (2012) studied the effect of agitation and aeration on CA production by *Y. lipolytica* grown in glycerol and reported that a dissolved oxygen (DO) concentration higher than 40%, which was generated by agitation speeds of 800-900 rpm and aeration rates within a range of 0.24-0.48, were the optimum conditions for CA production from glycerol. DO was indicated as the main factor, rather than varying the agitation and aeration rates, on biomass and CA production. In another study on the influence of the oxygen mass transfer rate (OTR) on CA accumulation by *Y. lipolytica* from crude glycerol, Ferreira et al (2016a) reported that the volumetric oxygen mass transfer coefficient ($k_La$) was an important parameter when optimizing CA accumulation, rather than DO, and its

value increases with a rise in aeration rate and agitation speed. CA production was found to be higher at high $k_La$ values. It was also indicated in the same study that a DO value of 60% led to optimum CA accumulation as opposed to 20% and 40%. In addition, Ferreira et al (2016b) reported that an increase in OTR resulted in a two- and three-fold improvement of CA accumulation by strains CBS 2073 and W29. Crolla and Kennedy (2004a) also studied the effect of varying agitation speeds on the production of CA by *Y. lipolytica* grown on *n*-paraffins and indicated that an agitation speed range of 800-1000 rpm had the best effect on biomass and CA formation. Moreover, an agitation rate of 800 rpm was found to be the optimum parameter for CA synthesis from raw glycerol by *Y. lipolytica* (Morgunov et al, 2013). In another study, performed by Avila Neto et al (2014), it was reported that the best CA production was obtained with an agitation speed of 800 rpm and aeration rate of 1 vvm. Recently, an improved bioreactor control unit, "Cascade mode", which enables the provision of a constant DO value during fermentation by regulating a predetermined range of agitation speed and aeration rate, has been developed. Papanikolaou et al (2017) subsequently used the "Cascade mode", maintaining the dissolved oxygen tension (DOT) value at more than 20% (by adjusting the agitation to 800 rpm and aeration range to 0.1 to 2.0 vvm) for the production of SCO and CA from glycerol by *Y. lipolytica*.

As mentioned in the Previous Studies section, although a high DO concentration is favourable for CA production by *Y. lipolytica*, it generally leads to decreased lipid accumulation. Rakicka et al (2015); Sabra et al (2017) reported that lipid accumulation by *Y. lipolytica* was decreased during oxygen controlled cultivation, hence limited oxygen and nitrogen conditions were the key factors for significant lipid accumulation. This situation was also proved by Unrean et al (2017), i.e., that decreasing the agitation rate from 300 to 100 rpm resulted in a 14% increase in lipid yield and 48% increase in lipid content within 5 days of a batch shake-flask experiment. Qin et al (2017) used a limited DO concentration of 20% during the growth phase and later 5% at the lipid accumulation phase (after 36

hours of inoculation to the end of fermentation), leading to high lipid productivity, at 1.2 g/L/h, by the best engineered strain of *Y. lipolytica*.

On the basis of this information, regarding the influence of DO concentration on CA and SCO production by *Y. lipolytica*, two different agitation-aeration conditions, i.e., 350 rpm -1.0 vvm and 800 rpm - 0.5 vvm, were used in this study. As the reactor employed in this work lacked the "Cascade mode" property, defined agitation speeds and aeration rates were kept constant during fermentation. Biomass, CA and lipid formation, glucose consumption as well as DO level were determined for these two different conditions (Figures 4.14, 4.15 and 4.16). Kinetic values of fermentation were also calculated (Table 4.9). Variation of DO concentrations for both conditions during fermentation, illustrated in Figure 4.14, demonstrate that an agitation-aeration of 800 rpm - 0.5 vvm generated a better DO concentration than 350 rpm - 1 vvm. Sudden drops at the $12^{th}$ hour and later a rise in DO level were observed; this trend has also been obtained in other studies (Ferreira et al, 2016a; Papanikolaou et al, 2017; Rywinska et al, 2012; Sabra et al, 2017) and Wentworth and Cooper (1996) explained the decrease in growth and respiration rates of *Y. lipolytica* was due to nitrogen exhaustion in the medium resulting in an increased DO concentration. It can also be seen in this study that the DO concentration stayed at the same level or even increased after the growth phase. Moreover, from the DO level (Figure 4.14), it is noticeable that while a high agitation speed of 800 rpm after the growth phase (from 24 hours to the end of fermentation) provided a more than 90% DO level, a low agitation speed of 350 rpm generated unstable DO levels which varied between 85 to 90%.

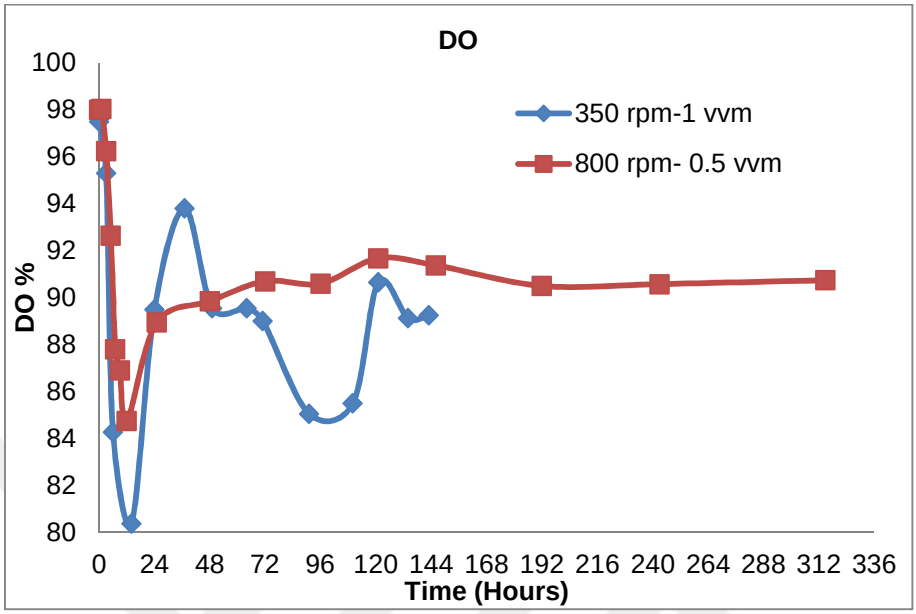



Figure 4.14. Variations of dissolved oxygen concentrations in two different agitation-aeration conditions during fermentation by *Y. lipolytica* strain K57 on glucose-based media.

Figures 4.15 and 4.16 illustrate the kinetics of biomass, CA, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of c. 50 g/L under limited nitrogen conditions at the two different agitation and aeration values. It is obvious that in general, an agitation speed of 800 rpm and aeration rate of 0.5 vvm generated higher kinetic values than 350 rpm and 1.0 vvm. The reason for low CA production under low aeration conditions has been explained by some authors to be due to a decrease in the activity of enzymes involved in the tricarboxylic acid cycle and glyoxylate cycle(Finogenova et al, 2002b; Kamzolova et al, 2003). The activity of citrate synthase, aconitate hydrate and NAD-dependent isocitrate dehydrogenase were decreased, whereas ATP-citrate lyase activity, responsible for the cleavage of citrate, increased under lower aeration conditions. Moreover, the activity of isocitrate lyase and malate synthase, which are involved in the cleavage of isocitrate to succinate and malate, decreased at lower DO concentrations. This

causes more isocitrate production than CA. In addition to the decrease of enzyme activity in the glyoxylate cycle, an increase of ATP-citrate lyase activity occurs as an alternative source for mitochondrial activity, hence, no CA is accumulated (Ferreira et al, 2016a; Finogenova et al, 2002a). Under nitrogen and oxygen limitation, lipid production is enhanced while CA production decreases (Sabra et al, 2017). This trend can be observed in Figure 4.15, where a lower amount of CA, at 6.26 g/L, glucose consumption, at 19.98 g/L and biomass, at 1.14 g/L, but slightly higher lipid yield of 0.11 g/g were observed (see also Tables 4.9 and 4.10). However, in the reverse case, at a high level of DO concentration, a higher CA amount, at 35.41 g/L, glucose consumption, at 50.80 g/L, biomass at 5.79 g/L, and lower lipid yield value of 0.03 g/g were observed (Figure 4.16, Tables 4.9 and 4.10).

Table 4.9. Kinetic parameters of *Y. lipolytica* strain K57 under two different conditions in the bioreactor (A: 350 rpm - 1.0 vvm, B: 800 rpm - 0.5 vvm)

| Parameters | A | B | Units |
|---|---|---|---|
| Glucose consumption (S) | 19.98 | 50.80 | g/L |
| Acetic acid production | 1.13 | 5.81 | g/L |
| Citric acid production (C) | 6.26 | 35.41 | g/L |
| Yield citric acid ($Y_{C/S}$) | 0.31 | 0.70 | g/g |
| Consumption rate ($R_S$) | -0.10 | -0.18 | g/L/h |
| Production rate ($Q_C$) | 0.04 | 0.12 | g/L/h |
| Max. consumption rate ($R_{Smax}$) | -0.48 | -0.44 | g/L/h |
| Max. production rate ($Q_{Cmax}$) | 0.07 | 0.18 | g/L/h |
| Max. growth rate ($q_{max}$) | 0.13 | 0.32 | g/L/h |
| Specific growth rate (μ) | 0.15 | 0.13 | 1/h |
| Doubling time ($t_d$) | 4.67 | 5.30 | h |
| Max. lipid production ($L_{max}$) | 0.11 | 0.18 | g/L |
| Max. lipid yield ($Y_{(L/S)max}$) | 0.11 | 0.03 | g/g |

Table 4.10. Kinetics of *Y. lipolytica* strain K57 on glucose-based media under two different conditions in the bioreactor (1: 350 rpm - 1 vvm, 2: 800 rpm - 0.5 vvm, a: kinetics at the max. lipid yield, b: kinetics at the max. citric acid yield).

| | | Time (h) | Biomass X (g/L) | Biomass Yield $Y_{X/S}$ (g/g) | Consumed Glucose S (g/L) | Citric Acid C (g/L) | Citric Acid Yield $Y_{C/S}$ (g/g) | Lipid L (g/L) | Lipid Yield $Y_{L/X}$ (g/g) | Acetate A (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | a | 121 | 0.87±0.13 | 0.04±0.00 | 20.07±1.80 | 4.38±0.34 | 0.22±0.00 | 0.10±0.01 | 0.11±0.01 | 0.87±0.04 |
| 1 | b | 143 | 1.14±0.22 | 0.06±0.01 | 19.98±1.36 | 6.26±0.46 | 0.31±0.00 | 0.10±0.00 | 0.09±0.02 | 1.13±0.18 |
| 2 | a | 25 | 5.10±0.43 | 0.53±0.10 | 9.82±2.60 | 1.39±0.09 | 0.14±0.03 | 0.16±0.01 | 0.03±0.00 | 0.73±0.10 |
| 2 | b | 315 | 4.86±0.49 | 0.11±0.02 | 50.80±0.01 | 35.41±1.52 | 0.70±0.03 | 0.12±0.01 | 0.02±0.00 | 5.81±0.98 |

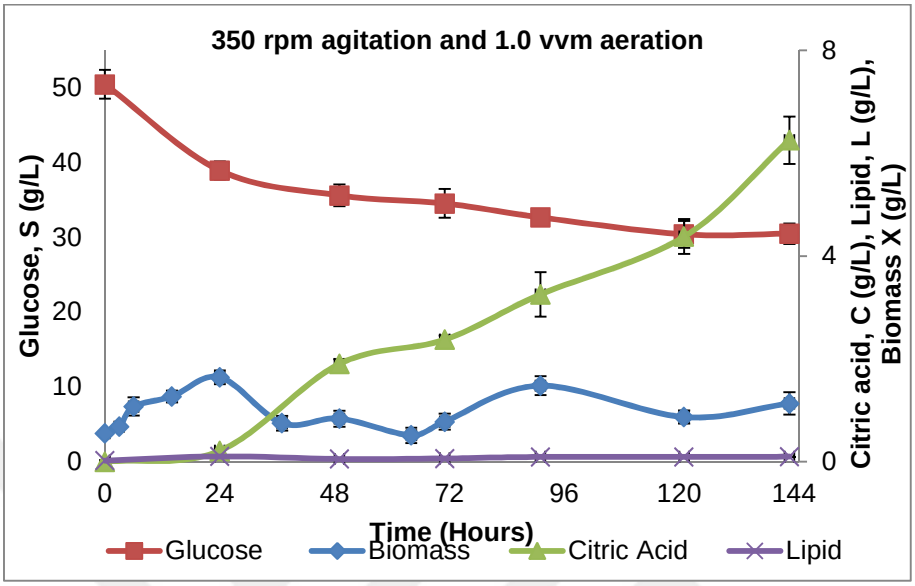



Figure 4.15. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of c. 50 g/L under a limited nitrogen concentration at an agitation of 350 rpm and aeration of 1.0 vvm.

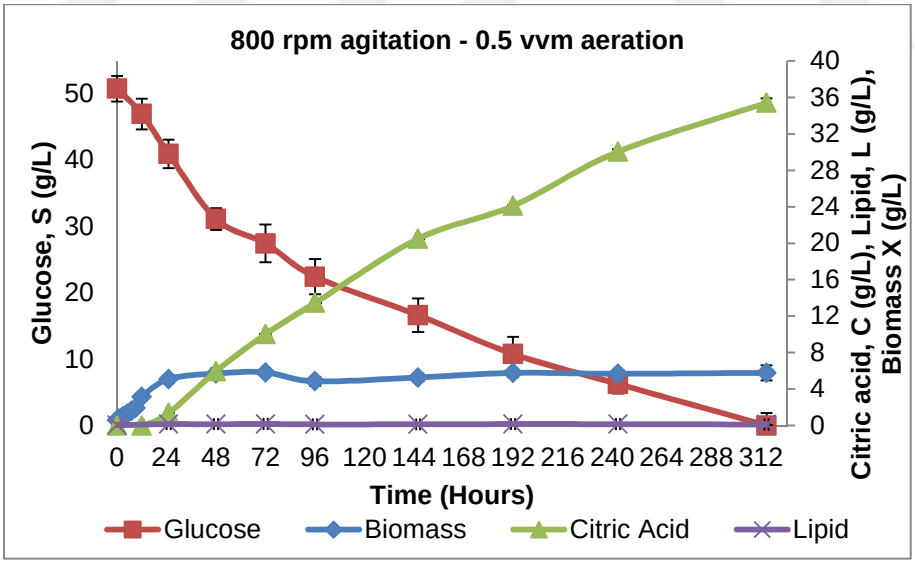



Figure 4.16. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of c. 50 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

Moreover, a very high CA yield of 70% was obtained at a high DO concentration. In contrast to a high level of DO concentration, a higher lipid yield value of 11% was observed at a reduced DO concentration. In conclusion, it can be revealed that an agitation speed of 800 rpm and aeration rate of 1.0 vvm were suitable for a high level of CA production, whereas an agitation speed of 350 rpm and aeration rate of 0.5 vvm were convenient for lipid accumulation. These observations can also be seen in Figures 4.17 and 4.18. On the basis of the obtained results, from these two conditions, an agitation speed of 800 rpm and aeration rate of 1.0 vvm were selected for use in further bioreactor experiments due to the high maximum production rate of 0.18 g/L/h and growth rate of 0.32 g/L/h (Table 4.9). Due to low biomass formation, poor CA formation, minimal glucose consumption and the small difference between the lipid yield value obtained, compared with the value in a high DO concentration, an agitation speed of 350 rpm and aeration rate of 1.0 vvm were found to be unsuitable for CA and SCO production by *Y. lipolytica* strain K57 on glucose-based media. In addition, instead of using a high aeration rate of 1.0 vvm, 0.5 vvm was preferable as high aeration rates lead to a high amount of foam formation during fermentation, which is not economical and requires a considerable antifoam usage.Figures 4.17 and 4.18 also show the variance of CA and lipid yield values during fermentation at the two different agitation and aeration parameters. It is apparent in Figure 4.17 that the lipid yield is higher at an agitation speed of 350 rpm and aeration rate of 1.0 vvm but lower at an agitation speed of 800 rpm and aeration rate of 0.5 vvm; this result was in agreement with studies performed by other researchers (Papanikolaou et al, 2017; Rakicka et al, 2015; Sabra et al, 2017; Unrean et al, 2017). In addition, a higher agitation speed of 800 rpm increased the DO concentration in the medium and thus provided a higher CA yield than produced at a lower agitation speed of 350 rpm (Figure 4.19), which was also observed by Avila Neto et al (2014); Crolla and Kennedy (2004a); Ferreira et al (2016a); Ferreira et al (2016b); Finogenova et al (2002a); Kamzolova et al (2003); Morgunov et al (2013); Rywinska et al (2012).

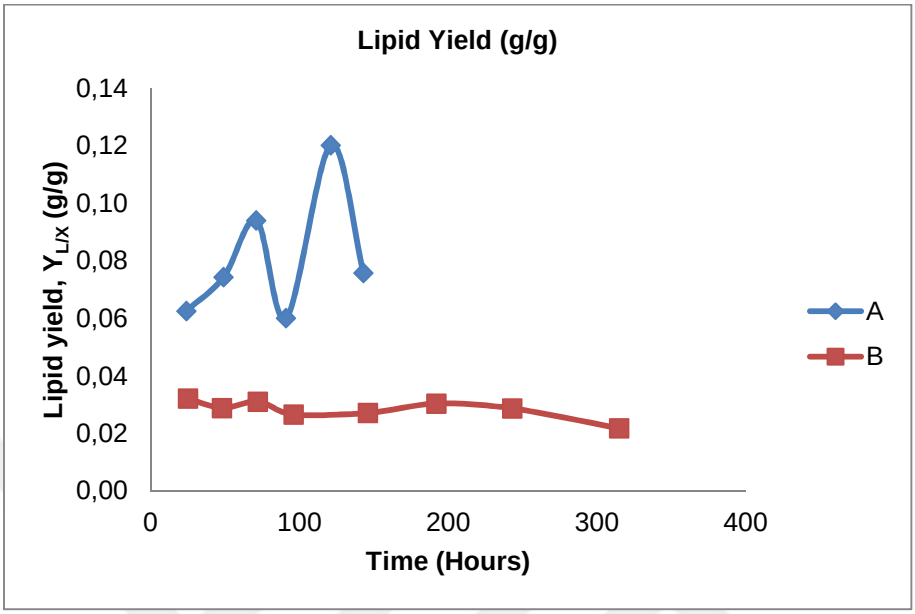



Figure 4.17. Variations of lipid yield of *Y. lipolytica* strain K57 under two different conditions (A: 350 rpm - 1.0 vvm, B: 800 rpm - 0.5 vvm).

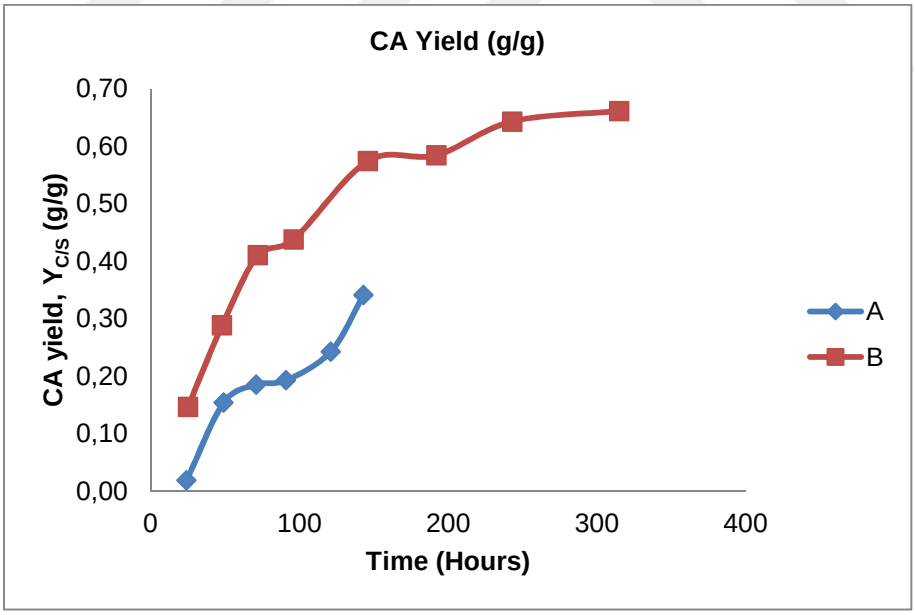



Figure 4.18. Variations of CA yield of *Y. lipolytica* strain K57 under two different conditions (A: 350 rpm - 1.0 vvm, B: 800 rpm - 0.5 vvm).

### 4.3.2. Microbial Lipid and Citric Acid Production from Glucose by *Y. lipolytica* Strain K57 in Bioreactor Fermentations

After optimizing bioreactor parameters, i.e., agitation of 800 rpm and aeration of 0.5 vvm, high initial glucose concentrations of 50 g/L, 107 g/L and 166 g/L were used for SCO and CA production by *Y. lipolytica* strain K57 in bioreactor experiments. It has been indicated that for a high quantity of CA and SCO accumulation, excess glucose concentration in the medium is required under nitrogen-limited conditions (Kamzolova and Morgunov, 2017; Papanikolaou and Aggelis, 2009; Papanikolaou and Aggelis, 2011a; Papanikolaou et al, 2006; Rywinska et al, 2012; Sabra et al, 2017). From this point of view, different concentrations of initial glucose were used in order to observe its influence on CA and SCO accumulation and as well as FA composition in the medium. Concentrations of 0.5 g/L ammonium sulphate and 0.5 g/L yeast extract were used as nitrogen sources, which were kept constant in all bioreactor experiments for each initial glucose concentration.

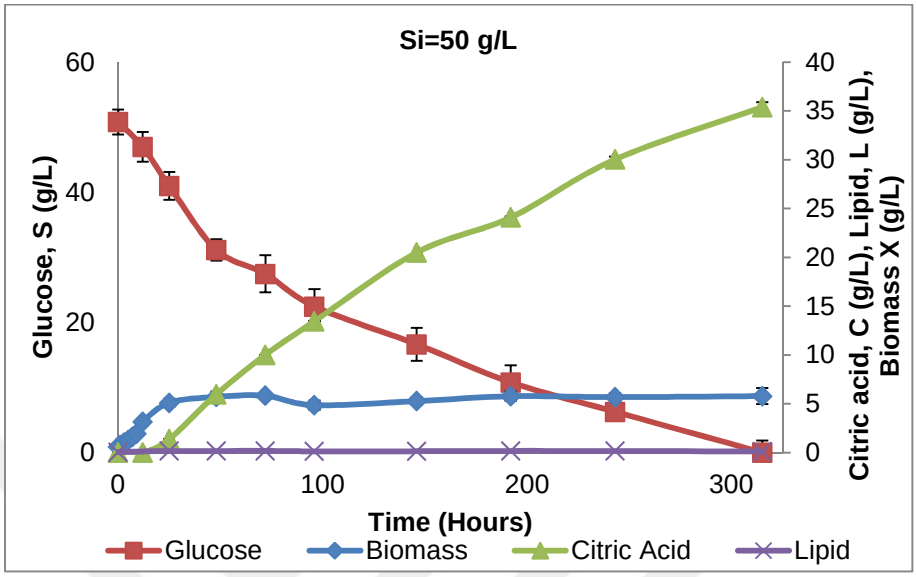



Figure 4. 19. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of ca. 50 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

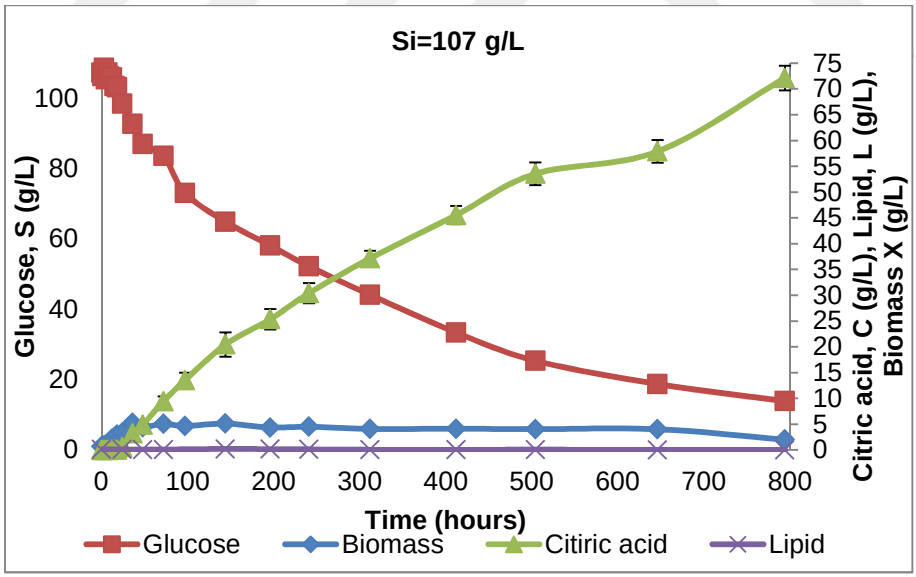



Figure 4.20. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of ca. 107 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

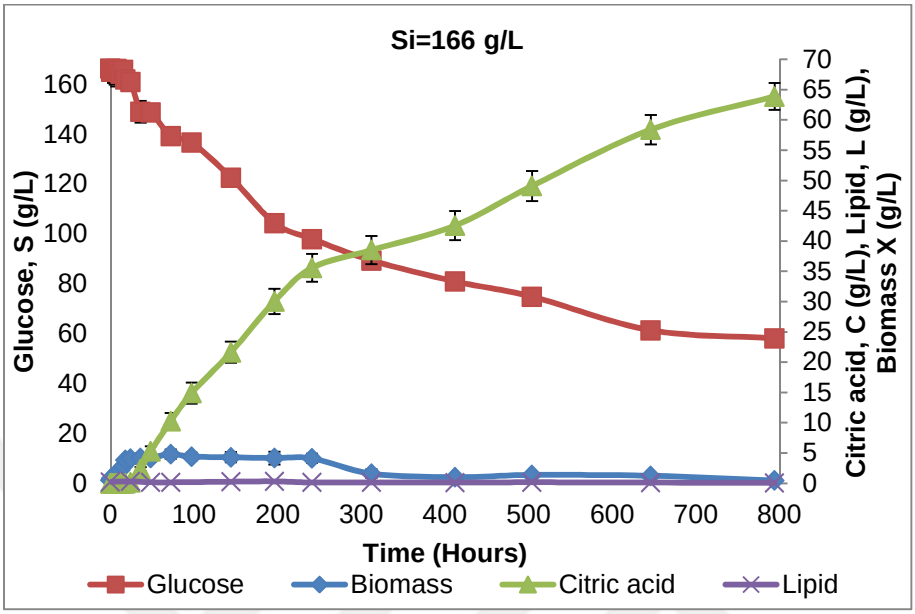



Figure 4.21. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on glucose-based media at an initial glucose concentration of ca. 166 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

Table 4.11. Kinetic parameters of *Y. lipolytica* strain K57 on three different glucose concentrations in a bioreactor (Si: initial glucose concentration).

| Parameters | Si, 50 g/L | Si, 107 g/L | Si, 166 g/L | Unit |
|---|---|---|---|---|
| Glucose consumption (S) | 50.81±0.01 | 93.43±0.63 | 108.18±1.41 | g/L |
| Acetic acid production | 5.81±0.98 | 9.47±1.11 | 6.84±0.73 | g/L |
| Citric acid production (C) | 35.41±1.52 | 72.12±2.40 | 63.87±1.41 | g/L |
| Yield citric acid ($Y_{C/S}$) | 0.70±0.03 | 0.77±0.02 | 0.59±0.01 | g/g |
| Consumption rate ($R_S$) | -0.13 | -0.10 | -0.11 | g/L/h |
| Production rate ($Q_C$) | 0.11 | 0.08 | 0.08 | g/L/h |
| Max. consumption rate ($R_{Smax}$) | -0.44 | -0.54 | -0.45 | g/L/h |
| Max. production rate ($Q_{Cmax}$) | 0.17 | 0.39 | 0.21 | g/L/h |
| Max. growth rate ($q_{max}$) | 0.21 | 0.13 | 0.15 | g/L/h |
| Specific growth rate (μ) | 0.14 | 0.06 | 0.09 | 1/h |
| Doubling time ($t_d$) | 4.95 | 12.37 | 8.04 | h |
| Max. lipid production ($L_{max}$) | 0.18±0.01 | 0.21±0.01 | 0.32±0.01 | g/L |
| Max. lipid yield ($Y_{(L/S)max}$) | 0.03 | 0.04 | 0.14 | g/g |

#### 4.3.2.1. Effect of Glucose Concentration on Growth Characteristics

The content of biomass, CA and SCO, and glucose consumption were monitored periodically during all fermentations, and are illustrated in Figure 4.19 (for Si=50 g/L), Figure 4.20 (for Si=107 g/L) and Figure 4.21 (for Si=166 g/L). Firstly, all growth of *Y. lipolytica* strain K57 on different glucose concentrations was compared, and it can be seen that the biomass content increases until 24 hours of fermentation and then remains constant, with the values ranging between 4.5 and 5.5 g/L. However, decreased biomass values were also observed, especially for 107 g/L initial glucose usage (Figure 4.20) at the end of fermentation and for 166 g/L initial glucose usage after 241 hours (Figure 4.21). As previously mentioned, an increase in biomass was observed until the depletion of nitrogen in the medium. Several similar studies on glucose-based media show similar results, Papanikolaou et al (2006) observed a maximum biomass content of 7.1 g/L and 7.2 g/L from initial glucose concentrations of 52 g/L and 149.5 g/L using the shake-flask fermentation method. Anastassiadis et al (2002) obtained a biomass value of 12 g/L in a glucose medium with 3 g/L $NH_4Cl$ by *Candida oleophila* after 42.5 hours of a fed-batch fermentation. Moreover, Papanikolaou et al (2009) studied *Y. lipolytica* strains and reported biomass values ranging from 4 to 6 g/L using initial glucose concentrations of 30 and 60 g/L in a shake-flask fermentation medium. Recently, Sabra et al (2017) reported a maximum biomass concentration of 7.1 g/L from an initial glucose concentration of 51 g/L at the $185^{th}$ hour of a shake-flask fermentation. Kamzolova and Morgunov (2017) also examined different *Y. lipolytica* strains for CA production using an initial glucose concentration of 30 g/L in a shake-flask fermentation and obtained a biomass content ranging from 1 to 3.5 g/L depending on the strain. In other work, Moeller et al (2007) studied CA production by *Y. lipolytica* H222 using different glucose concentrations and reported that a glucose concentration of more than 75 g/L inhibited the growth rate. In particular, inhibition of the maximum growth rate was observed at an initial glucose concentration of 200 g/L. This result is also exhibited in the present study

in that the maximum growth rate of 0.21 g/L/h at an initial glucose concentration of 50 g/L decreased to 0.13 and 0.15 g/L/h at initial glucose concentrations of 107 and 166 g/L, respectively (Table 4.11). From the results it can be concluded that different initial glucose concentrations did not significantly affect the biomass concentration; however, high glucose concentrations led to a decreased biomass content at a later fermentation stage. Biomass formation generally depends on the strain type and quantity of nitrogen source used in the medium (Kamzolova and Morgunov, 2017). However, high initial glucose concentrations of more than 100 g/L inhibit the growth rate of *Y. lipolytica*.

#### 4.3.2.2. Effect of Glucose Concentration on CA Production

Figures 4.19, 4.20 and 4.21 also show kinetics of the glucose consumption of different initial glucose concentrations, in which glucose was completely consumed, as shown in Figure 4.19, after 315 hours of fermentation, while 13.90 g/L and 58.15 g/L of glucose remained in the fermentation medium, as shown in Figure 4.20 and Figure 4.21, respectively, after long periods of fermentation (794 hours). This result trend was also observed in a study using *Y. lipolytica* performed by Papanikolaou et al (2006), who obtained an unconsumed glucose concentration of 74.1 g/L after 555 hours of a shake-flask fermentation using an initial glucose concentration of 149.5 g/L. Moreover, in the same study, a glucose concentration of 52 g/L was completely consumed after 220 hours of a shake-flask fermentation. In another study performed by Papanikolaou et al (2009), 57.7 g/L glucose was consumed in 315 hours in a shake-flask fermentation by *Y. lipolytica* strain W29. Anastassiadis and Rehm (2006) also studied CA production from glucose by *C. oleophila* in a batch fermentation and 4.4 g/L glucose was obtained after 7.4 days of fermentation.

Estimated kinetic results for CA production by *Y. lipolytica* strain K57 at different initial glucose concentrations are shown in Table 4.11, and Figures 4.19, 4.20 and 4.21. Results show that when comparing yields of CA according to

different initial glucose concentrations (Si), the highest value of 0.77 g/g was found at an initial glucose concentration of 107 g/L, the second highest value of 0.70 g/g was obtained for an initial glucose concentration of 50 g/L and the lowest yield of 0.59 g/g was determined for an initial glucose concentration of 166 g/L. CA production exhibited maximum values of 72.12 g/L, 63.87 g/L and 35.41 g/L at initial glucose concentrations of 107 g/L, 166 g/L and 50 g/L, respectively. According to these results it can be concluded that for high CA production, an initial glucose concentration of 107 g/L was the optimum value, compared with the two other concentrations. Estimated results of CA and yield in this study were comparable to other results obtained in previous similar studies, with CA values varying between 40 g/L and 111.1 g/L, and yield values ranging between 0.39 and 0.93 g/g depending on the type of strain (wild or recombinant) used. For instance, Papanikolaou et al (2006) produced 42.9 g/L of CA with a yield of 0.56 g/g by *Y. lipolytica* ACA-DC 50109 from an initial glucose concentration of 149.5 g/L using the shake-flask fermentation method. Moreover, in another study performed by Papanikolaou et al (2009), 49 g/L of CA with a yield of 0.85 g/g was produced by *Y. lipolytica* W29 from an initial glucose concentration of 60 g/L in a shake-flask fermentation. The same strain of *Y. lipolytica* (K57) used by Yalcin et al (2009b), resulted in a maximum CA concentration of 44.39 g/L at an initial glucose concentration of 150 g/L, and it was reported that an initial glucose concentration of more than 150 g/L caused the CA concentration to decrease. This result was lower than the CA values obtained in this study of 72.12 g/L at an initial glucose concentration of 106 g/L. Moeller et al (2007) also observed 41 g/L CA and 0.55 g/g CA yield produced by *Y. lipolytica* H222 from an initial glucose concentration of 100 g/L in a batch bioreactor fermentation. In addition, with the same strain of *Y. lipolytica* (H222) in another study (Moeller et al, 2011), the CA concentration varied between 53 and 97 g/L and the CA yield ranged between 0.39 and 0.69 for different glucose concentrations in a fed-batch fermentation. Rywinska et al (2010b) also reported a maximum CA concentration of 78.5 g/L with a yield of

0.75 g/g from an initial glucose concentration of 150 g/L using *Y. lipolytica* Wratislavia AWG7. Furthermore, Kamzolova and Morgunov (2017) recently reported a maximum CA concentration of 80-85 g/L and CA yield of 0.70-0.75 g/g by a natural strain, *Y. lipolytica* VKM Y-2373, on glucose-based media in a 10 litre bioreactor fermentation. Moreover, Sabra et al (2017) studied the use of different substrates (sole glucose, sole glycerol and a mixture of the two) and oxygen-dependent citric acid production by *Y. lipolytica*, and produced a maximum CA value of 55 g/L and CA yield of 0.58 g/g from sole glucose usage as a carbon source under controlled dissolved oxygen conditions in bioreactor trials. Besides the wild-type *Y. lipolytica* strains, recombinant ones have also been used in order to achieve a higher quantity of CA and productivity, for instance, Tan et al (2016) obtained 95 g/L of CA with a yield of 0.75 g/g from glucose by using the recombinant strain *Y. lipolytica* PG86. In other work carried out using a recombinant strain, *Y. lipolytica* $PR_{32}$, the highest CA value of 111.1 g/L with a yield of 0.93 g/g were produced from glucose (Fu et al, 2016). In order to achieve high CA productivity, it was also reported that the CA concentration increases with increasing glucose concentration in the medium, which was explained by the "metabolic overflow" mechanism under excess substrate conditions (Papanikolaou et al, 2006). Furthermore, CA productivity depends on the type of carbon source used by *Y. lipolytica*, which present different energy capacities in equal masses, for instance, while CA productivity on hydrophobic substances achieved higher than 1.0 g/g, it reached 0.86 g/g on ethanol-based media (Papanikolaou et al, 2009; Papanikolaou et al, 2006). Moreover, Moeller et al (2010) noted that a bioreactor generated higher CA quantities than shake-flask fermentations due to supplying a high level of oxygen in the fermentation, and when comparing the batch, fed-batch, repeated-batch and repeated fed-batch fermentations, the best CA productivity was achieved using repeated fed-batch fermentation. In conclusion, it can be said that a CA concentration of 72.12 g/L with a CA yield of 0.77 g/g from an initial glucose concentration of 107 g/L were quite high and comparable with the results obtained

in previous studies. It was also found that the maximum CA value and yield were obtained using an initial glucose concentration of 107 g/L rather than 50 g/L and 166 g/L (Figure 4.22). Generally, it has been reported that CA production increases in high glucose concentrations; however, this was not the case in the current study and it was also observed that the generated dissolved oxygen concentration at the same agitation and aeration conditions was higher in the medium with an initial glucose concentration of 106 g/L compared with the others (Figure 4.23). This may be the reason that higher CA values were achieved using this glucose concentration, as oxygen is also an important factor in CA production. Besides CA, acetic acid was also produced in some cases at a significant quantity of 9.47 g/L from an initial glucose concentration of 107 g/L, which was the highest value for acetic acid production compared with the two other glucose concentrations used. Papanikolaou et al (2009) reported that acetic acid production was generally observed when the glucose concentration was increased in the medium. They obtained a higher acetic acid yield, varying from 0.21 to 0.48 g/g, and an even higher acetic acid concentration ranging from 11.5 to 24.1 g/L by some *Y. lipolytica* strains. It was assumed by Papanikolaou et al (2009) that at high glucose concentrations (in which higher intracellular carbon flow occurs), the glucose–citric acid pathway seemed to be saturated, possibly due to a bottleneck in the level of the citrate synthase (CS). Although they did not carry out an assay of the intracellular enzymes, saturation of CS could result in a carbon flow bottleneck in the level of conversion of acetyl-CoA to citric acid with the concomitant intracellular accumulation of acetyl-CoA and subsequent secretion of acetic acid.

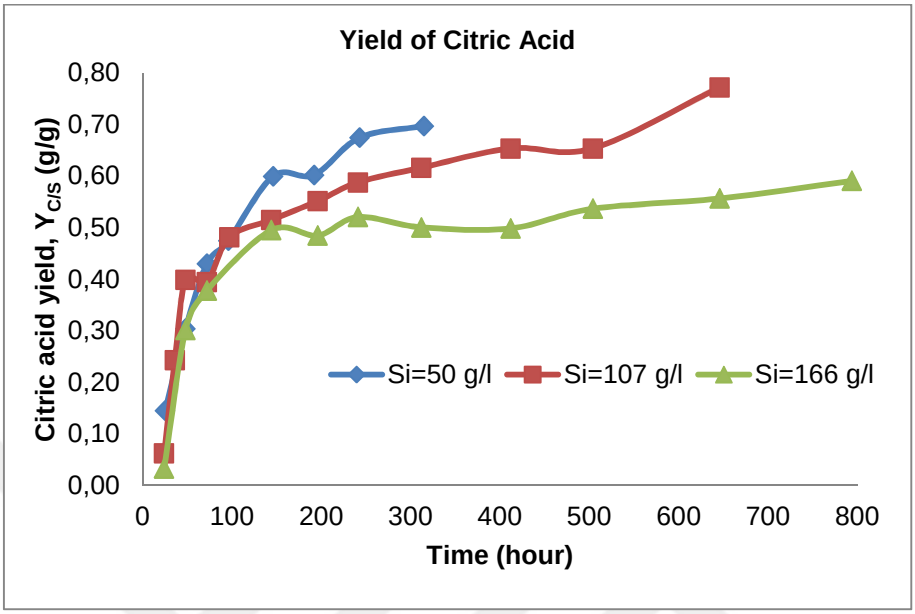



Figure 4.22. Variations of CA yield of *Y. lipolytica* strain K57 on different initial glucose concentrations.

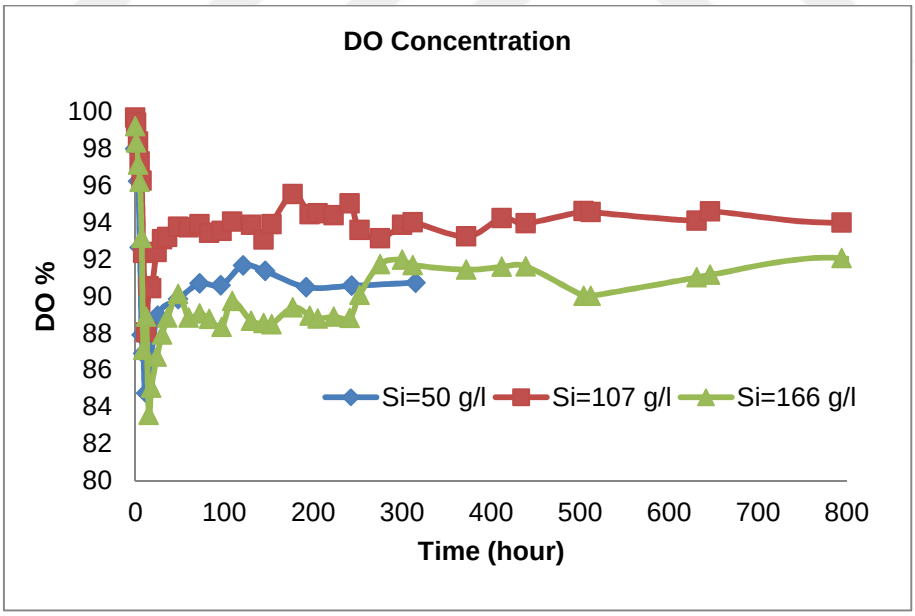



Figure 4.23. Variations of the dissolved oxygen concentrations of *Y. lipolytica* strain K57 on the different initial glucose concentrations.

#### 4.3.2.3. Effect of Glucose Concentration on Lipid Production

While CA yields were higher at initial glucose concentrations of 107 and 50 g/L than at 166 g/L, lipid yields were lower with values of 0.03 g/g and 0.04 g/g, and the highest lipid yield of 0.14 g/g was obtained at an initial glucose concentration of 166 g/L (Figure 4.24, Table 4.12). Moreover, the highest lipid content of 0.32 g/L was also obtained at Si=166 g/L (Table 4.11). According to these results, it can be revealed that lipid production increases at the higher initial glucose concentration of 166 g/L whereas CA yield decreases. This result is in agreement with work carried out by Papanikolaou et al (2009); Papanikolaou et al (2006); Sabra et al (2017) who also observed significant lipid accumulation at high glucose concentrations. Beopoulos et al (2009a) stated that *Y. lipolytica* is able to accumulate lipids to more than 50% of dry cell weight. However, in many studies on lipid production by *Y. lipolytica* using glucose-based media, lower lipid yields were observed. For instance, 5-9% lipid in the dry cell weight by *Y. lipolytica* ACA-DC 50109 was observed in the study undertaken by Papanikolaou et al (2006), and a low concentration of lipid (3-14% lipid in DCW depending on the initial glucose concentration) was also produced in the current study. Although *Y. lipolytica* is known as an oleaginous yeast, it did not demonstrate high lipid production in appropriate culture conditions (excess carbon source and high C/N ratio). Instead of lipid accumulation, in high concentrations CA production was observed. Low lipid accumulation by *Y. lipolytica* has also been obtained in other studies especially when glucose was used as a carbon source, whereas lipid production from hydrophobic substrates such as triglycerides, fatty acids, animal fats, glucose enriched with organic acids was significantly high (Table 2.1). In another study carried out by Papanikolaou et al (2009), the total lipid content was not higher than 0.14 g/g when glucose was used as the sole carbon source and lipid degradation was observed during the stationary phase of fermentation. It can also be seen in Figure 4.24 that lipid degradation occurs, in particular, at the initial glucose concentrations of 107 g/L and 50 g/L. However, in high glucose

concentrations such as 166 g/L, lipid yield and production increased continuously with values of 0.14 g/g and 0.32 g/L, respectively. This result is also in agreement with the study performed by Papanikolaou et al (2009), who reported that increasing the initial glucose concentration in the medium unambiguously increased the lipid yield. The same researchers also stated that lipid turnover occurred particularly in high glucose concentrations; however, in our case the reverse was found, i.e., a high lipid yield was obtained at a high initial glucose concentration. Another point reported by Bellou et al (2016b) was that some strains of *Y. lipolytica* displayed lipid turnover even at a high glucose concentration in the fermentation medium and CA was accumulated instead of lipid during the lipid turnover stage. In another study performed by Sarris et al (2011), a maximum lipid yield of 0.18 g/g with a lipid content of 1.2 g/L were observed from a 35 g/L initial glucose concentration by *Y. lipolytica* ACA-YC 5028. Katre et al (2012) also investigated lipid production on glucose-based media using the marine yeast *Y. lipolytica* NCIM 3589 and observed a maximum 0.29 g/g lipid yield when 30 g/L glucose was used as the sole carbon source. In the same study, they also examined the effect of initial glucose concentrations on lipid yield and stated that a 30 g/L initial glucose concentration was the best concentration for the highest lipid yield (0.29 g/g) and 100 g/L decreased the lipid yield to 0.14 g/g. This is not in agreement with our lipid yield results, in which significant lipid production at a high initial glucose concentration of 166 g/L was observed. Recently, Sabra et al (2017) studied lipid production from glucose using *Y. lipolytica* and produced 3 g/L lipid with 0.23 g/g lipid yield under uncontrolled dissolved oxygen conditions. However, the highest lipid yield value of 0.48 g/g with a biomass quantity of 12.2 g/L was obtained by Bellou et al (2016b) under double nitrogen and magnesium limitation on glucose-based media. According to these results from the literature, it can be concluded that a lipid yield of 0.03-0.14 g/g with a lipid content of 0.18-0.32 g/L produced by *Y. lipolytica* strain K57 at different initial glucose concentrations was not as high as the lipid yield obtained by Bellou et al (2016b);

Katre et al (2012); Sabra et al (2017), who also used wild-type *Y. lipolytica* strains in order to produce SCO. It can be said that lipid productivity by *Y. lipolytica* mainly changes depending on the initial substrate concentration, type of strain, dissolved oxygen concentration in the medium, limitation of nitrogen and magnesium in the culture, and can be increased by using limited nitrogen and magnesium, a high initial glucose concentration of 100 g/L, a low dissolved oxygen concentration and metabolic engineering techniques on *Y. lipolytica* strains. For instance, Qiao et al (2015) recently reported that genetically engineered strain YL-ad9 generated a lipid content of 55 g/L with the yield of 0.67 g/g from glucose as the sole carbon source. In our case, a high dissolved oxygen concentration ranging between 90 and 95% was applied in three different initial glucose concentrations. Maybe applying high oxygenation was the reason for low lipid production. This approach has been explained by some researchers (Rakicka et al, 2015; Sabra et al, 2017; Unrean et al, 2017) who found that in low dissolved oxygen concentrations, lipid productivity was higher. Also in agreement is that lipid production was induced when a high initial glucose concentration was present in the medium.

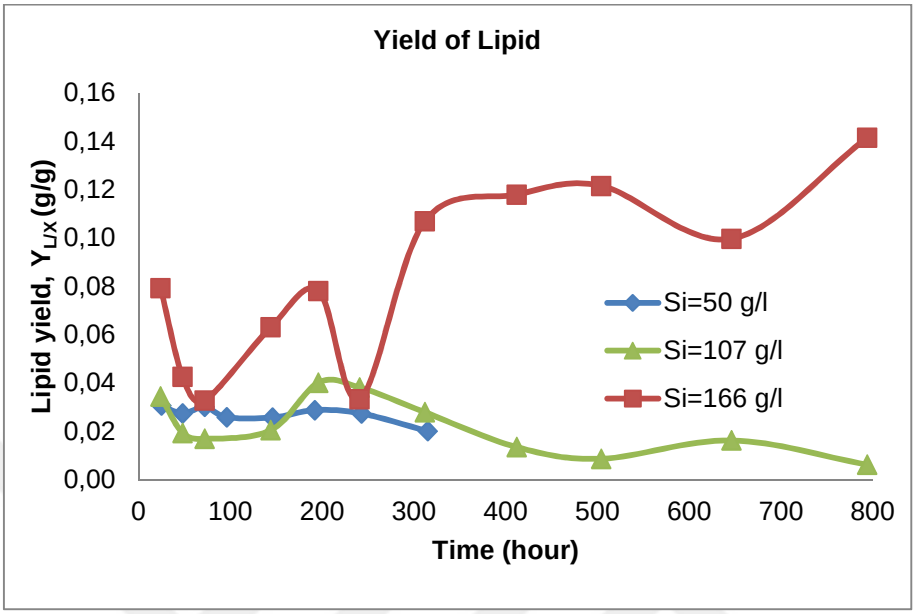



Figure 4.24. Variations of lipid yield of *Y. lipolytica* strain K57 on different initial glucose concentrations.

Table 4.12. Kinetics of *Y. lipolytica* strain K57 on three different glucose-based media in a bioreactor (1: 50 g/L initial glucose, 2: 107 g/L initial glucose, 3: 166 g/L initial glucose, a: kinetics at the max. lipid, b: kinetics at the max. citric acid yield).

| | | Time (h) | Biomass, X (g/L) | Biomass Yield $Y_{X/S}$ (g/g) | Consumed Glucose, S (g/L) | Citric Acid, C (g/L) | Citric Acid Yield, $Y_{C/S}$ (g/g) | Lipid, L (g/L) | Lipid Yield, $Y_{L/X}$ (g/g) | Acetate, A (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | a | 72 | 8.83±0.21 | 0.25±0.01 | 23.34±1.87 | 10.02±0.48 | 0.43±0.01 | 0.18±0.01 | 0.03±0.00 | 2.81±0.22 |
| 1 | b | 315 | 5.79±0.81 | 0.11±0.02 | 50.80±0.01 | 35.41±1.52 | 0.70±0.03 | 0.12±0.01 | 0.02±0.00 | 5.81±0.98 |
| 2 | a | 144 | 5.13±0.31 | 0.12±0.00 | 42.43±2.33 | 20.44±2.36 | 0.48±0.03 | 0.21±0.01 | 0.04±0.00 | 3.23±0.80 |
| 2 | b | 794 | 2.02±0.25 | 0.02±0.00 | 93.43±0.63 | 72.12±2.40 | 0.77±0.02 | 0.03±0.01 | 0.01±0.00 | 9.47±1.11 |
| 3 | a | 24 | 4.07±0.80 | 0.90±0.59 | 5.36±2.63 | 0.17±0.15 | 0.03±0.01 | 0.32±0.01 | 0.08±0.02 | 0.51±0.13 |
| 3 | b | 794 | 0.46±0.06 | 0.00±0.00 | 108.18±1.41 | 63.87±2.23 | 0.59±0.01 | 0.07±0.01 | 0.14±0.00 | 6.84±0.73 |

#### 4.3.2.4. Principle Component Analysis (PCA) Results of Kinetics Parameters for *Y. lipolytica* K57 on Glucose-based Media in a ReactorFermentation

PCA analysis was applied to observe the effect of different initial glucose concentrations on fermentation kinetics generated by *Y. lipolytica* K57 in batch-type reactor fermentations. The bi-plot of PCA analysis in Figure 4.25 shows that an overall 93.34% of variance was explained by the first component (F1 of 71.29%) and second component (F2 of 22.05%). Considering F1, two distinct groups are represented in the bi-plot. The first one on the positive side of F1 corresponds to fermentation samples collected at the highest CA content, explained by consumed glucose, CA, acetate and yield of CA. The second one on the negative side of F1 was formed by samples collected at the highest lipid production, characterized more by lipid, biomass and yield of biomass. However, the lipid yield parameter was placed close to the zero intercept of F1, not characterized by fermentation samples. However, as F2 was considered, sample 3b-794h (Si=166 g/L-max.CA-794$^{th}$ hour) was discriminated more by lipid yield. In conclusion, it can be stated that sample 2b-794h was explained more fully by CA, CA yield, consumed glucose, acetate than others, whereas it was negatively correlated by lipid, biomass and biomass yield. Lastly, sample 3a-24h is also more fully explained by lipids than others.

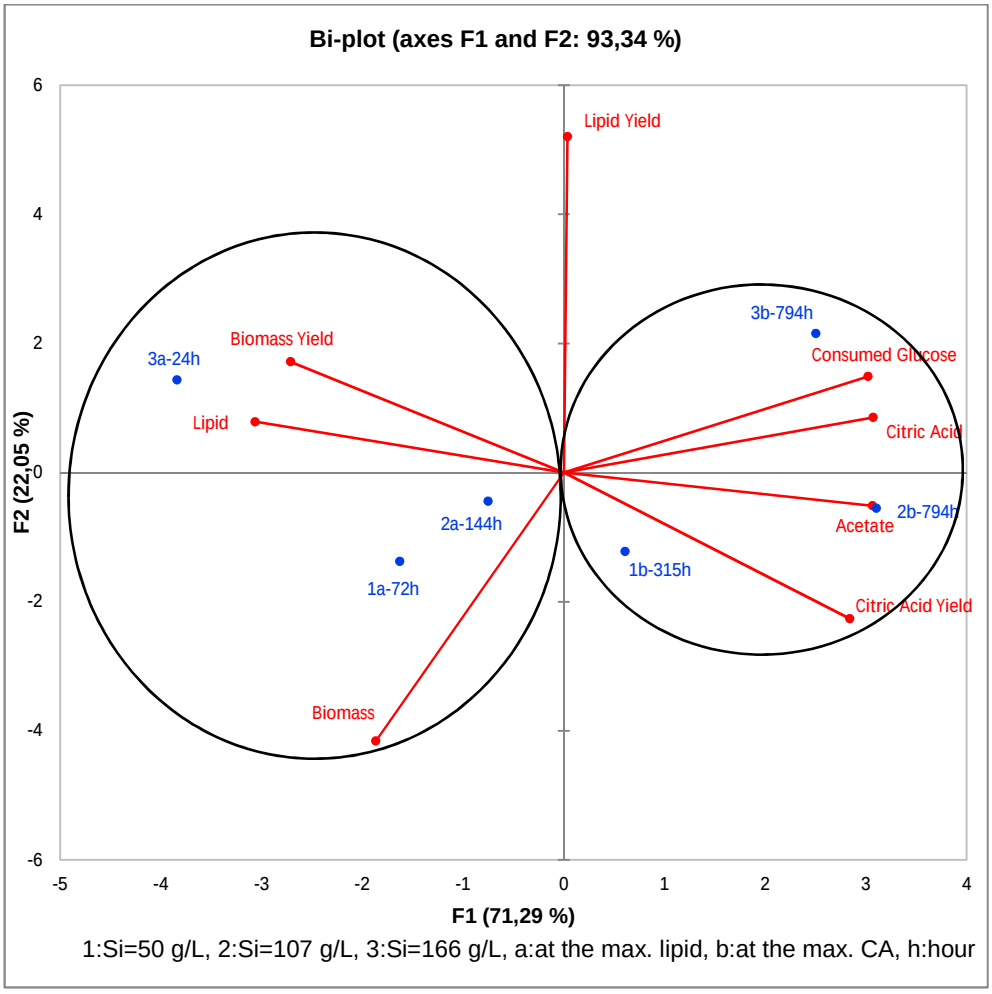



Figure 4.25. Bi-plot of PCA regarding fermentation kinetics for *Y. lipolytica* K57 on glucose-based media in a reactor fermentation.

### 4.4. Chemical Composition of Waste Bread Powder

In order to use waste bread powder (WBP) as a substrate for SCO and CA production, enzyme hydrolysis should first be performed as it is crucial that wild-type *Y. lipolytica* strains cannot metabolize polysaccharides such as starch, which is a main component of bread. Monosaccharides such as glucose, generated by enzyme hydrolysis from waste bread, can be used by *Y. lipolytica* to produce CA and SCO. Besides the conversion of starch to mainly glucose, protein degradation

also occurs using a protease enzyme during hydrolysis. Due to the nitrogen-limited growth fermentation conditions required for CA and SCO production by *Y. lipolytica*, it was also important to consider the nitrogen content of WBP as well as the waste bread hydrolysate. It is well known that waste bread is rich in starch and protein and with this nutritional content it can be considered as a cheap alternative substrate for CA and SCO production by *Y. lipolytica*.

Table 4.13. Chemical composition of waste bread powder (N=3).

| | % Chemical Composition |
|---|---|
| Ash | 1.30±0.03* |
| Crude Protein | 11.99±0.18* |
| Crude Fat | 0.71±0.38* |
| Total Carbohydrate | 82.01±0.19* |
| Moisture | 3.99±0.04 |

(*corresponds to dry matter)

For the enzymatic hydrolysis of WBP, the total sugar and protein content of WBP should be determined prior to hydrolysis, in order to control the conversion yield of starch to glucose and protein to free amino nitrogen, and also the activities of alpha-amylase, glucoamylase and protease. Therefore, the chemical composition of WBP was determined and the results are illustrated in Table 4.13, which corresponds to approximately 12% crude protein, 86% carbohydrate, 0.71% crude fat and 1.30 g/L in dry matter of ash. The moisture content of WBP was determined to be 4%, as the drying process was applied before grinding waste bread suring the sample preparation for WBP, causing a low moisture content. When these results were compared with the composition of other waste bread chemicals, published in the literature, similar values were obtained. For instance, Leung et al (2012) examined the chemical composition of waste bread to use for fermentative succinic acid production and observed a protein content of 8.9% (dry basis) and starch of 59.8% (dry basis). According to Kent and Evers (1994) white

bread consists of 47% starch (94% of total carbohydrate) and 8% protein. In other work performed by Kafle and Kim (2013), the crude protein content and total carbohydrate were found to be 14.6% and 80%, respectively. The results obtained for the chemical composition of waste bread powder in this study are in agreement with reported results in the literature.

When the chemical composition of WBP was examined, it can be seen that it is rich in carbohydrate and protein which can be used as carbon and nitrogen sources for SCO and CA production by *Y. lipolytica* during microbial fermentation. However, crude protein and carbohydrate (rich in starch) should be converted to reduced sugars and free amino nitrogen via enzyme hydrolysis in order for *Y. lipolytica* to use them for growth and metabolite production. Therefore, enzyme hydrolysis should be optimized before starting fermentations. The next section presents results from enzyme hydrolysis.

### 4.5. Hydrolysis of Waste Bread Powder

As previously mentioned, the hydrolysis of bread was required to convert starch into glucose and protein to FAN components. For this purpose, the method applied by Pietrzak and Kawa-Rygielska (2014) was used, in which enzyme hydrolysis was achieved using alpha-amylase, glucoamylase and protease on waste bread. They used a small-scale production of waste bread hydrolysate (WBH) in order to investigate the effect of using granular starch hydrolysing enzymes and raw material pre-treatments. However, in this study, a reactor was used to achieve high quantities of WBH by modifying the method, i.e., the separate hydrolysis and fermentation used by Pietrzak and Kawa-Rygielska (2014). Therefore, the reactor with a 2 L working volume was used for hydrolysis. After hydrolysis, 2 kg of mash (including 150 g WBP/ kg mash equal to 168 g WBP/litre mash) was obtained and used for SCO and CA fermentation by *Y. lipolytica* strain K57. To achieve maximum glucose content, several small-scale hydrolysis trials were performed in 250 mL flasks using various quantities of enzymes. Bioreactor hydrolysis was then

carried out and the enzyme concentrations used were: 0.6 mL alpha-amylase (loading 1 U/g substrate), 3 mL glucoamylase (loading 3 U/g substrate) and 0.263 mL protease (loading $8.4x10^{-4}$ U/g substrate) in order to obtain 2 kg of WBH slurry.

The results for WBH are shown in Table 4.14. Two different trials were undertaken by adding the same quantity of alpha-amylase and protease but varying glucoamylase loadings, which for the first one (A), 1 U/g substrate was used and for the second (B) a 3 U/g substrate of glucoamylase loading was applied to the waste bread slurry during saccharification. The second condition was used for 2 kg of waste bread slurry, and the glucose content of WBH after hydrolysis was determined to be 125.75 g/L, which corresponds to the conversion yield of approximately 97.10% from starch and can be considered a quite high ratio. In addition, the FAN content of WBH was found to be 127.88 mg/L with a conversion yield from total Kjeldahl nitrogen (TKN) to FAN of 3.97%. However, yield values for WBP hydrolysis were lower (63.60% from starch into glucose and 3.39% from TKN into FAN) at the glucoamylase loading of 1 U/g. Due to higher conversion yields, a glucoamylase loading of 3 U/g was used for further hydrolysis.

Table 4.14. Conversion yield values of waste bread hydrolysis (A: glucoamylase loading of 1 U/g substrate, B: glucoamylase loading of 3 U/g substrate and the other 2 enzymes loadings kept constant) (N=3).

| | Starch to glucose conversion yield (%), glucose yield (g G/ g substrate) | TKN to FAN conversion yield (%), FAN yield (mg FAN/g substrate) | Glucose (g/L) | FAN (mg/L) |
|---|---|---|---|---|
| A | 63.60%, 0.49 g/g | 3.39%, 0.64 mg/g | 82.37±3.30 | 108.73±8.39 |
| B | 97.10%, 0.75 g/g | 3.97%, 0.76 mg/g | 125.75±4.35 | 127.88±5.27 |

Comparing these values with results reported in the literature, it can be said that the conversion yield from starch to glucose is very high but the conversion yield from TKN to FAN is lower than values obtained in previous studies. For example, Tsakona et al (2014) studied the hydrolysis of flour-rich waste (FRW) by

enzymes, mainly glucoamylase and protease, produced from the solid state fermentation of *Aspergillus awamori* on wheat milling by-products and achieved higher than 90% (w/w) starch to glucose conversion yields and 40% (w/w) TKN to FAN conversion yields using FRW concentrations up to 205 g/L in batch hydrolysis. In another study on succinic acid production from waste bread performed by Leung et al (2012), the total sugar yield (g total sugar/g substrate) and FAN yield (mg FAN/ g substrate) were determined to be 0.47 and 2.55, respectively. Using 100 g/L glucose, a starch to glucose conversion yield of 90.8% and 490 mg/L FAN were estimated after the hydrolysis of waste bread. Recently, Han et al (2017) worked on the hydrolysis of waste bread by enzymes produced from a solid state fermentation on *Aspergillus awamori* and *Aspergillus oryzae* and reported the highest starch conversion of 96.6% and glucose yield of 0.52 g glucose/g waste bread. In addition, Han et al (2016) stated that 49.78 g/L glucose and 284.12 mg/L FAN could be produced with a waste bread mass ratio of 15% (w/v) by a similar hydrolysis method. Demirci et al (2017) also reported that a maximum predicted glucose yield of 86% was produced by the enzymatic hydrolysis of waste bread during two stages of hydrolysis (liquefaction and saccharification). They used the same hydrolysis method (with the enzyme combinations) as in this study and stated that the highest yield was achieved by liquefying 0.05 (g/g) waste bread with 0.03 (Kilo Novo Units/g substrate) of alpha-amylase and subsequently saccharifying with 3.6 (U/g substrate) of glucoamylase. Despite these results, in this study, the highest yields of 0.75 (g glucose/g substrate) and 0.76 (mg FAN/g substrate) were acquired using 1 U/g of alpha-amylase, 3 U/g of glucoamylase and 8.4 x $10^{-4}$ U/g protease for 0.15 (g/g) waste bread. According to these results, it can be revealed that the glucose conversion yield is comparable but FAN conversion exhibited lower values than previously published for waste bread hydrolysis. It is well known that a high FAN content increases the growth and biomass values of microorganisms. But in our case, nitrogen-limited conditions in a fermentation medium should be provided to produce high CA and SCO by *Y.*

*lipolytica*. For this reason, for a low ratio of FAN conversion yield, nitrogen-limited conditions were required.

Moreover, during waste bread hydrolysis, the solid state fermentation of filamentous fungus such as *Aspergillus awamori* and *Aspergillus oryzae* was used in order to produce fungal glucoamylase and protease, instead of using commercial thermostable alpha-amylase, glucoamylase and protease, in an attempt to decrease the cost of the process (Dorado et al, 2009; Du et al, 2008; Melikoglu et al, 2008). However, in this study, commercial enzymes, thermostable alpha-amylase, glucoamylase and protease, were used as CA and SCO production by *Y. lipolytica* from waste bread hydrolysate was the main focus of this study and not low-cost enzymatic hydrolysis.

### 4.6. Microbial lipid and Citric Acid Production from a Waste Bread Hydrolysate by *Y. lipolytica* Strain K57

After hydrolysis of WBP, glucose concentrations of three different WBHs (50, 100 and 150 g/L) were prepared to use as substrates for SCO and CA production by *Y. lipolytica* strain K57 in batch bioreactor fermentations. Similar concentrations of carbon sources were selected in order to compare results obtained from bioreactor fermentations of glucose-based media. The glucose concentration of WBH after hydrolysis was ca. 125 g/L and was adjusted to 50 g/L and 100 g/L glucose concentrations by diluting the solution or to 150 g/L by removing the water phase via lyophilization. FAN contents were also calculated by multiplying dilution factors. Glucose and FAN contents of WBHs used in bioreactor trials are presented in Table 4.15.

Table 4.15. Glucose and FAN concentrations of WBH used in bioreactor experiments.

| | Glucose (g/L) | FAN (mg/L) |
|---|---|---|
| 1 | 49.42±1.98 | 65.23±5.03 |
| 2 | 96.82±3.35 | 98.47±4.06 |
| 3 | 146.00±5.95 | 134.53±5.47 |

Together with these WBH solutions, a salt solution used in the shake-flask fermentation (with ammonium sulphate of 0.5 g/L and yeast extract of 0.5 g/L), was also prepared, autoclaved in different bottles and mixed together. The final medium was made up to 1 L by the addition of 10% of inoculum (100 mL inoculum) to WBH and salt solutions. At this point it should be noted that the final medium consisted of 0.5 g/L ammonium sulphate, 0.5 g/L yeast extract and FAN of WBH which increased the nitrogen content. Due to the higher nitrogen content, prior to fermentation it was expected that the biomass value and growth of *Y. lipolytica* strain K57 on WBH would be higher than values obtained in bioreactor fermentations of glucose-based media. Moreover, extreme foam formation also occurred during fermentation and was inhibited by using antifoam at a 10% dilution, especially during the growth phase. It was observed that silicon oil used as an antifoam agent lowered the dissolved oxygen concentration and inhibited cell growth. Because of the high biomass concentration, foam formation increased continuously after inoculation until the end of the growth phase. Hence, it was important to choose an appropriate antifoam for this fermentation. Structol J 673 antifoam was found to be suitable due to its properties of good solubility in the water phase and not inhibiting the growth of *Y. lipolytica*. The addition of approximately 0.5% (v/v) to the fermentation volume was enough to prevent foam formation. Initial glucose concentrations were changed after the addition of inoculum to the fermentation medium. Glucose concentrations of 68 g/L, 101 g/L and 142 g/L were obtained at the beginning of three different fermentations.

Kinetic values of biomass, citric acid, lipid and consumed glucose are presented in Figures 4.26, 4.27 and 4.28. One of the differences between the bioreactor trials on glucose-based and WBHs media was that the fermentation time was shorter in WBHs experiments. At approximately 36 hours, the glucose was completely consumed and fermentation ended. In comparison to bioreactor trials on glucose-based media, CA production was lower while biomass content and lipid production were higher in WBHs fermentations. CA concentration values ranged between around 8 g/L and 15 g/L, the biomass content varied between 36 g/L and 62 g/L and the lipid content ranging from 1.30 to 5.12 g/L during WBHs bioreactor experiments at three different initial glucose concentrations (68, 101 and 142 g/L), which were totally consumed in almost 36 hours (Table 4.16 and Table 4.17).

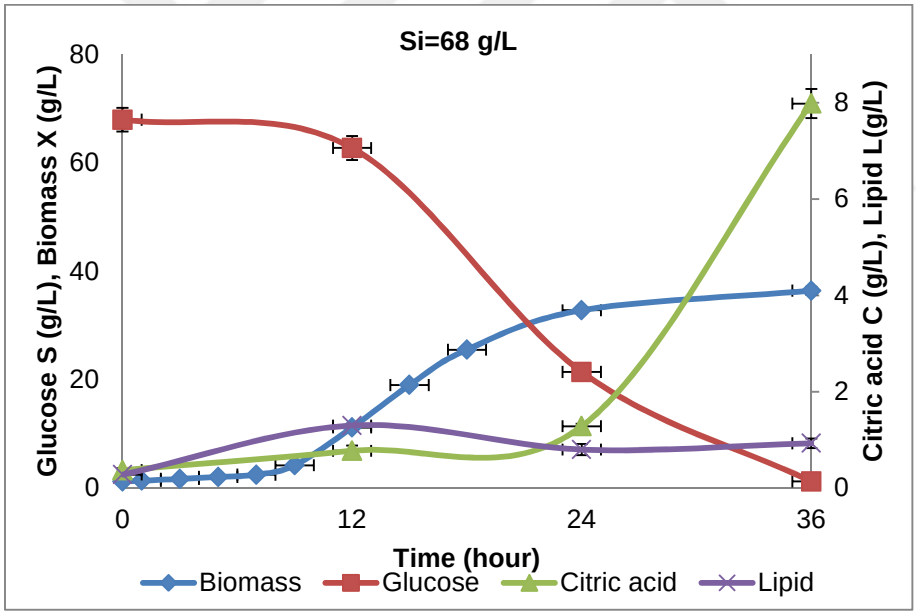



Figure 4.26. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on waste bread hydrolysate media at an initial glucose concentration of ca. 68 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

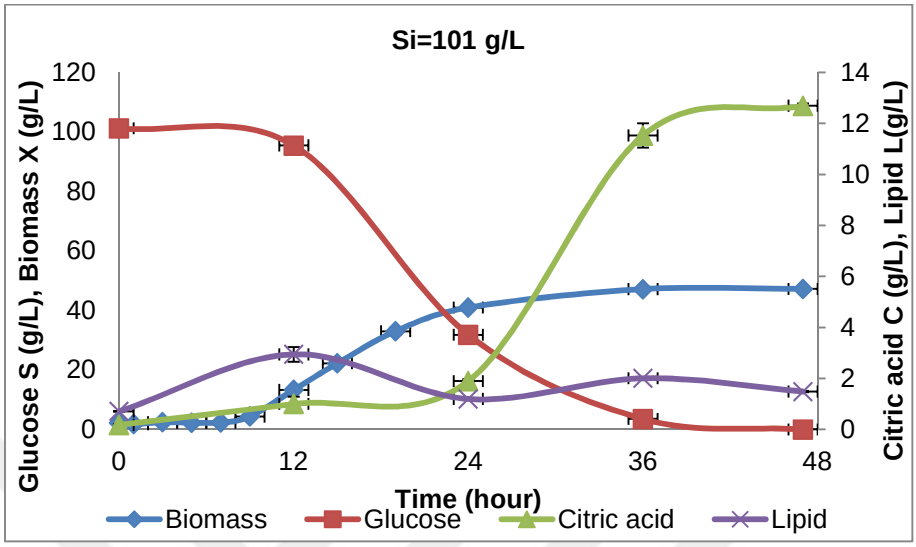



Figure 4.27. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on waste bread hydrolysate media at an initial glucose concentration of ca. 101 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

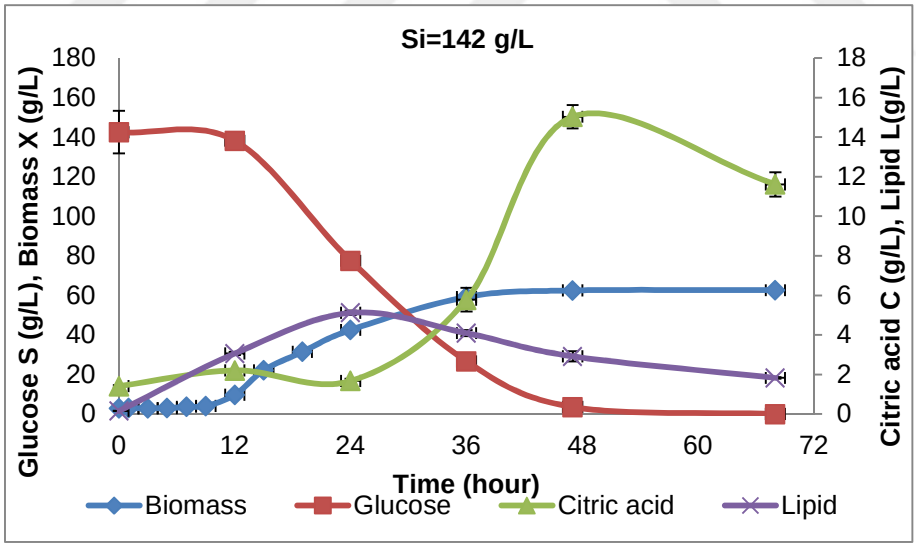



Figure 4.28. Kinetics of biomass, citric acid, lipid and glucose consumption of *Y. lipolytica* strain K57 during growth on waste bread hydrolysate media at an initial glucose concentration of ca. 142 g/L under a limited nitrogen concentration at an agitation of 800 rpm and aeration of 0.5 vvm.

Table 4.16. Kinetic parameters of *Y. lipolytica* strain K57 on three different glucose concentrations of a waste bread hydrolysate in a bioreactor (Si: initial glucose concentration).

| Parameters | Si=68 g/L Value | Si=101 g/L Value | Si=142 g/L Value | Unit |
|---|---|---|---|---|
| Glucose consumption (S) | 66.78±0.03 | 101.23±0.00 | 142.51±0.00 | g/L |
| Acetic acid production | 1.35±0.06 | 1.49±0.09 | 3.30±0.29 | g/L |
| Citric acid production (C) | 7.99±0.30 | 12.69±0.08 | 15.03±0.59 | g/L |
| Yield citric acid ($Y_{C/S}$) | 0.12 | 0.13 | 0.11 | g/g |
| Consumption rate ($R_S$) | -1.29 | -3.00 | -2.46 | g/L/h |
| Production rate ($Q_C$) | 0.19 | 0.34 | 0.30 | g/L/h |
| Max. consumption rate ($R_{Smax}$) | -3.44 | -5.30 | -5.06 | g/L/h |
| Max. production rate ($Q_{Cmax}$) | 0.56 | 0.80 | 0.84 | g/L/h |
| Max. growth rate ($q_{max}$) | 2.47 | 2.20 | 2.49 | g/L/h |
| Specific growth rate (μ) | 0.30 | 0.30 | 0.37 | 1/h |
| Doubling time ($t_d$) | 2.28 | 2.30 | 1.87 | h |
| Max. lipid production ($L_{max}$) | 1.30±0.03 | 2.95±0.29 | 5.12±0.11 | g/L |
| Max. lipid yield ($Y_{(L/S)max}$) | 0.12 | 0.22 | 0.32 | g/g |

### 4.6.1. Effect of Glucose Concentration on Growth Characteristics

The growth of strain K57 on WBH-based media was generally increased after the 9$^{th}$ hour and remained constant for 24 hours until the end of fermentation. Very high biomass concentrations of 36.44 g/L, 47.23 g/L and 62.61 g/L were observed during WBH fermentations at the initial glucose concentrations of 68 g/L, 101.23 g/L and 142.51 g/L, respectively. These values were higher than the biomass content of around 5.00 g/L obtained in bioreactor fermentations of glucose-based media. This difference could be explained by the effect of the nitrogen content on the growth of *Y. lipolytica* strain K57, i.e., cell growth increased with high nitrogen content, in particular, the FAN concentration in the medium (Dorado et al, 2009; Leung et al, 2012). WBH consisted of a high FAN value, in addition to ammonium sulphate and yeast extract concentrations, which led to increased biomass and growth values of *Y. lipolytica* strain K57. Moreover,

WBH was also composed of a high crude protein content at approximately 12% (dry basis). It is well known that nitrogen-limited conditions, with excess carbon in the medium, should be supplied during CA and SCO production by *Y. lipolytica* and an optimum amount of biomass (not very high content) should be obtained. Waste bread is rich in carbon and nitrogen and is a very nitrous substrate for the fermentation process. Tsakona et al (2014) studied lipid production from flour-rich waste by *Lipomyces starkeyi* and they also observed a high biomass content of 30.5 g/L in a shake-flask fermentation and 113 g/L in a fed-batch fermentation when initial concentrations of around 110 g/L-90 g/L glucose and 140 mg/L-180 mg/L FAN were used in a shake-flask fermentation and fed-batch fermentation, respectively. Similarly, when a WBH solution consisting of 96.82 g/L initial glucose with 98.47 mg/L initial FAN, was used as the fermentation medium, a maximum biomass value of 47.23 g/L was observed at the $36^{th}$ hour of fermentation. In another study by the same researchers (Tsakona et al, 2016), a biomass content of around 30 g/L was obtained when flour-rich waste comprising initial concentrations of approximately 100 g/L glucose, as the carbon source, and 200 mg/L FAN, in a batch fermentation for SCO production by *R. toruloides*. According to these results, it can be revealed that biomass values obtained in this study are in agreement with reported values in the literature.

Initial glucose and FAN concentrations of WBH affected the biomass formation by *Y. lipolytica* strain K57. A high FAN content enhanced the biomass content and a higher biomass value of 62.61 g/L was obtained at higher initial concentrations of 142.51 g/L glucose and 134.53 g/L of FAN (Figure 4.28). Moreover, growth inhibition of *Y. lipolytica* strain K57 when using a high initial glucose concentration of 142 g/L in WBH was not observed. A high biomass content is favoured for lipid production and as dry cell weight increases, the produced lipid content also increases. However, this condition is not preferable for CA productivity as the carbon source in the medium can be further used for cell growth and lipid production instead of CA synthesis.

### 4.6.2. Effect of Glucose Concentration on Citric Acid Production

Generally, CA production on WBH was lower than bioreactor trials of glucose-based media. A higher CA production of 15.03 g/L was obtained at the initial glucose concentration of 142.52 g/L in a batch reactor fermentation at the 47$^{th}$ hour. However, the highest CA value of 72.12 g/L was observed from an initial glucose concentration of 107 g/L in a batch fermentation on glucose-based media. In comparison it can be said that the 15.03 g/L CA value was too low. The main reason for this result can be explained by extreme biomass formation and this led to the low concentration of CA synthesis. Various industrial by-products have been used as a substrate for CA production by *Y. lipolytica* strains, of these, the most commonly used substrate was glycerol. Agricultural waste such as olive mill waste water, animal and vegetable fats, and waste cooking oil have been used as a carbon source for CA production by *Y. lipolytica* but these products included a lower amount of nitrogen content. Waste bread consists of a high nitrogen content with a FAN value of more than 100 mg/L and protein content of around 10 % (dry basis). Liu et al (2015d) studied CA production from a hydrolysate of straw cellulose by *Y. lipolytica* and produced 26.7 g/L and 42.4 g/L of CA from the straw cellulose hydrolysate in batch and fed-batch fermentations, respectively. They explained that the yield of CA from the hydrolysate of straw cellulose by *Y. lipolytica* was relatively low, which could be due to the existence of inhibitory components in the hydrolysate; thus, removing the inhibitory components can improve the yield of CA from the straw cellulose hydrolysate. In another study performed by Yalcin et al (2009c), whey and grape must were used as substrates for CA production by the domestic strain *Y. lipolytica* 57 and a maximum CA of 49.23 g/L and 32.09 g/L were obtained from whey fortified with fructose and sole grape must, respectively. When whey was used for CA production by *Y. lipolytica*, very low values were obtained and this was explained by the fact that protein and other nitrogen components, as well as lipids, lactic acid, and minerals in the whey substrate, can exert an inhibitory effect on growth and CA production of *Y.*

*lipolytica*. They also observed a lower CA values with increasing ammonium chloride and yeast extract in the whey medium and concluded that a high amount of nitrogen content may have a negative effect on CA production. In our case, higher nitrogen compounds (FAN, ammonium sulphate and yeast extract) were also present in the fermentation medium and thus could lead to lower CA production. It has previously been reported that CA production increased when nitrogen limitation occurred in the medium, especially during the stationary growth phase. In addition, Imandi et al (2008) studied optimum conditions for CA production from pineapple waste and 202.35 g/kg ds of CA (g citric acid produced/kg of dried pineapple waste as a substrate) was produced in selected conditions. The optimum conditions were determined for this fermentation to be a moisture content of 70.71 (%), yeast extract of 0.34 (%w/w), $Na_2HPO_4$ of 0.69 (%w/w) and $KH_2PO_4$ of 0.64 (%w/w). A 0.34% concentration of yeast extract was found to have a significant effect on CA production. In another study performed by Wang et al (2013), an extract of Jerusalem artichoke tubers was used for CA production by *Y. lipolytica* strain 30 and CA of 68.3 g/L with a yield of 0.91 g/g was obtained in a 10 litre batch reactor fermentation within 336 hours. In the same study, it was observed that the addition of ammonium sulphate to the extract of Jerusalem artichoke tubers led to a decreased production of CA and increased biomass. Therefore, there was no need to add any nitrogen source to the extract of Jerusalem artichoke tubers which is rich in nutrients including a high nitrogen source. In this study, a waste bread hydrolysate was used as a substrate for CA production and it is known that waste bread hydrolysate is also rich in nitrogen and glucose. Therefore, it can be concluded that there is no need to supplement any nitrogen source to waste bread for CA production, as nitrogen-limited conditions should be provided for high CA productivity.

The CA values in three different initial glucose concentrations of WBH were compared and it can be seen that the highest value of 15.03 g/L was obtained from WBH containing an initial glucose concentration of 142 g/L. However, the

highest CA yield of 0.13 g/g (Figure 4.29 and Table 4.16) was observed at the initial glucose concentration of 101.23 g/L. Similar results were also observed in the reactor fermentation experiments on glucose-based media, detailed in Section 4.5, where the highest CA yield of 0.77 g/g was also found at the initial glucose concentration of 107 g/L. Figure 4.29 illustrates the variation of CA yields at the three different glucose concentrations of WBH and it can be observed that at the $12^{th}$ hour of fermentation, the yields of CA were higher for all cases during fermentation and lower CA yield values were observed after 24 hours of fermentation. From these results, it can be concluded that CA production was low during the growth phase and increased at the stationary phase.

DO concentration is one of the main factors affecting CA production and as the DO concentration in the medium increases, CA production also increases. Figure 4.30 shows variations of DO concentrations during fermentation. At the $18^{th}$ hour of fermentation, DO concentrations were lowest with values of 30%, 11% and 1.78% for the initial glucose concentrations of 68, 101 and 142 g/L, respectively, and then remained constant ranging between 80 and 85%. This indicates that the DO concentration was adequate for CA production by *Y. lipolytica* K57 under these conditions. However, it may be better to increase aeration conditions during the time interval between the $12^{th}$ and $18^{th}$ hour of fermentation, as during this time DO values were lower than 20% for the higher initial glucose concentrations (101 g/L and 142 g/L), which has been reported to be a critical DO % for CA production in previous studies. From Figures 4.28, 4.29 and 4.30 it can be seen that this time interval represents the growth phase of *Y. lipolytica* K57 and CA production continuously increased after this period. For this reason, a cascade mode can be used for the regulation of aeration during the whole fermentation. This sudden drop near to 0% DO can be explained by the high cell density of *Y. lipolytica* K57 in the WBH medium. While a maximum of 5.5 g/L biomass was obtained in glucose-based media reactor fermentations, in the WBH medium, more than 60 g/L biomass was produced requiring a high oxygen concentration.

Besides CA, acetic acid concentrations for all scenarios stayed at low concentrations (a maximum of around 7 g/L at the initial glucose concentration of 142 g/L and 12$^{th}$ hour of fermentation). The same content of acetic acid was also observed for a bioreactor fermentation on glucose-based media, as detailed in Section 4.5. However, when acetate values of 7 g/L were compared with a maximum CA content of around 15 g/L, it can be thought of as significant acetate production.

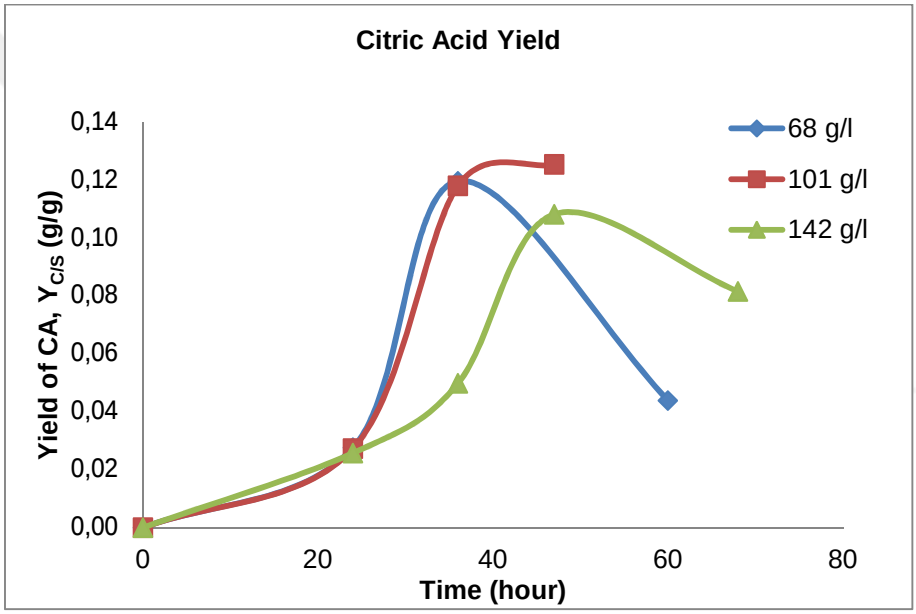



Figure 4.29. Variations of CA yield of *Y. lipolytica* strain K57 on the three different initial glucose concentrations.

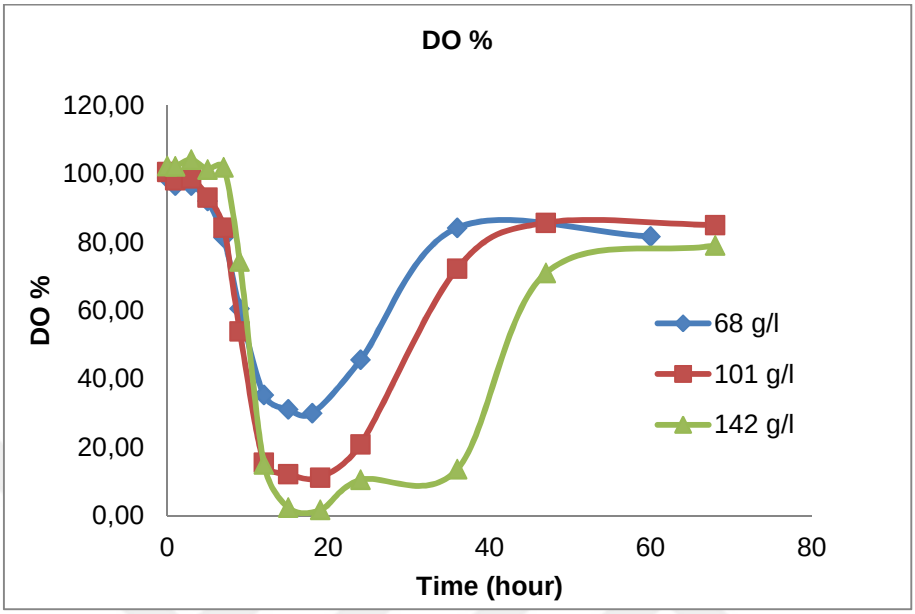



Figure 4.30. Variations of dissolved oxygen concentrations of *Y. lipolytica* strain K57 on the three different initial glucose concentrations.

Table 4.17. Kinetics of *Y. lipolytica* strain K57 on three different waste bread hydrolysate media in a bioreactor (1: 68 g/L initial glucose, 2: 101 g/L initial glucose, 3: 142 g/L initial glucose, a: kinetics at the max. lipid yield, b: kinetics at the max. citric acid yield)

| | | Time (h) | Biomass X (g/L) | Biomass Yield $Y_{X/S}$ (g/g) | Consumed Glucose S (g/L) | Citric Acid C (g/L) | Citric Acid Yield $Y_{C/S}$ (g/g) | Lipid L (g/L) | Lipid Yield $Y_{L/X}$ (g/g) | Acetate A (g/L) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | a | 12 | 11.18±0.23 | 2.36±1.05 | 5.23±2.23 | 0.78±0.11 | 0.16±0.05 | 1.30±0.03 | 0.12±0.00 | 0.81±0.08 |
| 1 | b | 36 | 36.44±0.75 | 0.55±0.01 | 66.78±0.03 | 7.99±0.30 | 0.12±0.00 | 0.93±0.10 | 0.03±0.00 | 1.35±0.06 |
| 2 | a | 12 | 13.30±0.20 | 2.40±0.68 | 5.78±1.72 | 1.00±0.28 | 0.17±0.00 | 2.95±0.29 | 0.22±0.02 | 0.74±0.02 |
| 2 | b | 47 | 47.23±0.83 | 0.47±0.01 | 101.23±0.00 | 12.69±0.08 | 0.13±0.00 | 1.49±0.02 | 0.03±0.01 | 1.49±0.09 |
| 3 | a | 12 | 9.72±1.19 | 3.45±2.75 | 4.32±3.79 | 2.21±0.06 | 0.82±0.70 | 3.06±0.08 | 0.32±0.05 | 6.95±0.07 |
| 3 | b | 47 | 62.48±1.43 | 0.45±0.01 | 138.95±0.08 | 15.03±0.59 | 0.11±0.00 | 2.92±0.26 | 0.05±0.00 | 4.05±0.21 |

### 4.6.3. Effect of Glucose Concentration on Lipid Production

Figures 4.26, 4.27 and 4.28 show lipid values produced by *Y. lipolytica* strain K57 from WBH media. It can be seen that the highest lipid content was observed at 12$^{th}$ hour of fermentation and then lipid degradation was observed. Figure 4.31 illustrates lipid yield values during WBH fermentation, with the highest yield values obtained at the 12$^{th}$ hour of fermentation and later lower values were observed. This means that the carbon source was used for cell growth instead of lipid production, as lipid production decreased while exponential cell growth was observed after the 12$^{th}$ hour of fermentation (Figures 4.26, 4.27 and 4.28). The highest lipid yield of 0.32 g/g was generated from WBH containing an initial glucose concentration of 142 g/L. Moreover, the highest lipid content of 5.12 g/L, after 24 hours of fermentation, was produced from WBH with an initial glucose concentration of 142 g/L. From these results, it can be revealed that the higher initial glucose concentrations of WBH promoted an increased lipid content and lipid yield. It has also been observed in other studies that higher lipid concentrations were generated by increasing the initial glucose concentrations (see section 4.5). In previous studies, a hydrolysate of wheat straw was used for lipid production by *Y. lipolytica* (Yu et al, 2011) and around 4.5% (w/w) of lipid content with biomass formation of 7.2 g/L was produced in a 6-day shake-flask fermentation. The initial glucose concentration of the wheat straw hydrolysate in that study was very low with the value of approximately 3.5 g/L and some other monosaccharides, such as xylose, arabinose, galactose, acetic acid, furfural and HMF, were present in the fermentation medium. Due to the inhibitory effect of HMF and furfural on microorganisms, a detoxification process was applied but there was no significant difference to lipid production in a detoxified or non-detoxified medium. In both detoxified and non-detoxified wheat straw hydrolysate media, lower than 1.0 g/L lipid was produced by *Y. lipolytica*. In another work, Tsigie et al (2011) produced a lipid content of 6.68 g/L and yield of 0.58 g/g from a detoxified sugarcane bagasse hydrolysate with a peptone medium (initial total

sugar available concentration of 20 g/L and peptone of 5 g/L) by *Y. lipolytica* Po1g in a shake-flask fermentation. Moreover, Tsigie et al (2012) also studied lipid production from a detoxified rice bran hydrolysate by *Y. lipolytica* Po1g and a dry cell weight and lipid content of 10.75 g/L and 48.02% were obtained, respectively. In the same study, the effect of nitrogen content and initial glucose concentration of a detoxified rice bran hydrolysate on lipid productivity were also examined and the highest lipid productivity was found to be without the addition of any nitrogen source to a medium containing a 30 g/L initial glucose concentration instead of 20 or 40 g/L. They observed that at a high nitrogen usage in the medium, biomass formation and glucose consumption increased, and more than 30 g/L initial glucose usage inhibited the growth of *Y. lipolytica* Po1g. In this study, using a high nitrogen content also stimulated biomass formation and glucose consumption. Ratledge and Wynn (2002) explained that as the nitrogen source is exhausted in the medium, excess carbon is converted to lipid production by oleaginous microorganisms. Therefore, maybe the key factor for WBH hydrolysis was nitrogen limitation, which can inhibit high biomass formation and fast glucose consumption causing higher lipid production. Recently, Sutanto et al (2017) also studied lipid production from a detoxified rice bran hydrolysate by *Y. lipolytica* Po1g and the highest lipid content of 0.24 g/g (biomass of 14.3 g/L) was determined upon the addition of 10 g/L yeast extract to a 30 g/L initial glucose concentration of detoxified rice bran medium. According to results obtained in previous studies, it can be concluded that a maximum lipid yield value of 0.32 g/g with 5.12 g/L lipid content (Table 4.16 and Table 4.17) was found at the WBH initial glucose concentration of 142 g/L and are comparable to values reported in the literature. However, very high biomass formation and fast glucose consumption were observed. In order to increase lipid content and lipid yield, nitrogen limitation and low air saturation should be applied. In addition, the inhibitory effects of WBH components, such as hydroxyethyl furfural, on lipid production and growth of *Y. lipolytica* should be investigated.

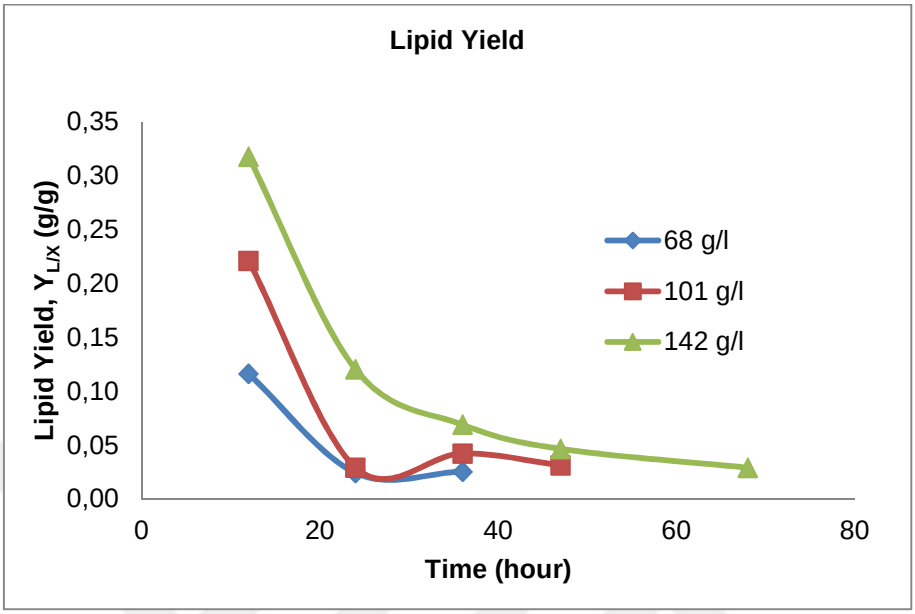



Figure 4.31. Variations of lipid yield of *Y. lipolytica* strain K57 on the three different initial glucose concentrations.

### 4.6.4. Principle Component Analysis (PCA) Results of Kinetics Parameters for *Y. lipolytica* K57 on a Waste Bread Hydrolysate Media in a Reactor Fermentation

To determine the effect of different initial glucose concentrations on fermentation kinetics for *Y. lipolytica* K57 on waste bread hydrolysate media, PCA analysis was performed. The total variance explained by two principle components was 92.37%; with the first component (F1) comprising 65.80% and the second component (F2) 26.57%. Figure 4.32 illustrates the bi-plot of PCA, composed of two distinct groups with regards to F1. The first one was generated by samples 3a-12h, 2a-12h and 1a-12h, characterized by CA yield, lipid, lipid yield, biomass yield and acetate. The second group was formed by samples 3b-47h, 2b-12h and 1b-36h, explained by CA, consumed glucose and biomass. As can be seen from Figure 4.32, sample 3a-12h is obviously described more by lipid yield, CA yield, biomass yield, while sample 3b-47h is determined mostly by CA, biomass and consumed

glucose. Consequently, it can be revealed that the highest initial glucose concentration of 142 g/L had a better effect on lipid and citric acid production. Furthermore, lipids produced in the 12$^{th}$ hour were higher than those generated in the 36$^{th}$ and 47$^{th}$ hours, while CA production was highest at the end of fermentation when glucose in the media was completely consumed. A lower initial glucose concentration usage of 68g/L cannot provide higher CA and lipid values.

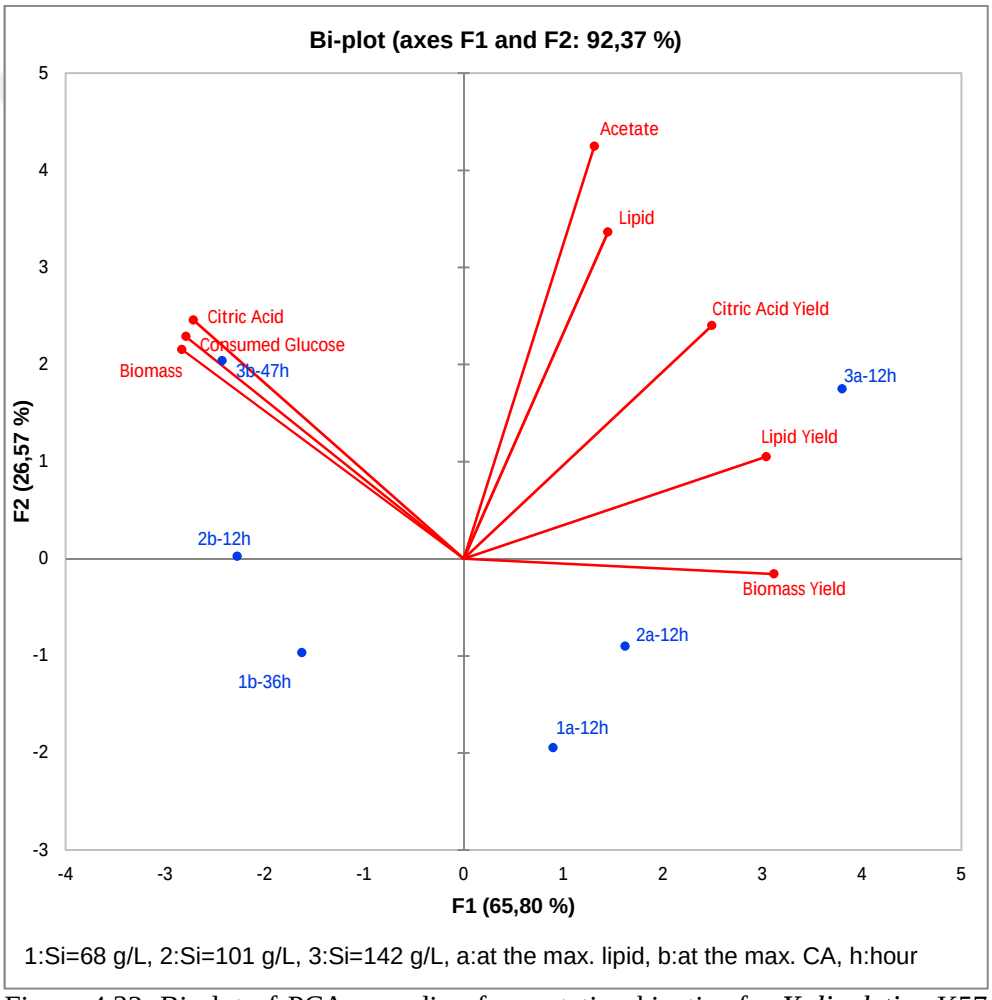



Figure 4.32. Bi-plot of PCA regarding fermentation kinetics for *Y. lipolytica* K57 on WBH media in a reactor fermentation.

# 5.CONCLUSION

In this study, the molecular characterization of 12 *Y. lipolytica* strains and their citric acid (CA) and single cell oil (SCO) production on glucose-based media using the shake-flask fermentation method were primarily investigated. Fermentation kinetics, optimum fermentation conditions, fatty acid composition and organic acids generated by these strains were examined. An optimum strain was selected for a high level of CA and lipid production. Secondarily, CA and SCO production by the selected strain on glucose-based media in a batch-type bioreactor fermentation were studied. Aeration and agitation parameters were optimized to achieve high yields of CA and SCO. The effects of initial glucose and dissolved oxygen concentrations on CA and lipid accumulation by the selected strain on glucose-based media were observed. Thirdly, waste bread was selected for CA and SCO production by *Y. lipolytica* and for this reason enzymatic hydrolysis was applied and optimized for large-scale production in a bioreactor. Finally, CA and lipid production and fatty acid composition by *Y. lipolytica* on waste bread hydrolysate media in a batch-type bioreactor fermentation was investigated. The results can be summarized as follows:

Results of the molecular characterization analysis of 12 different *Y. lipolytica* strains:

- All *Y. lipolytica* strains tested exhibited a high degree of homology (99% to 100%) with various *Y. lipolytica* strains present in the National Center for Biotechnology Information, as a result of BLAST analysis of a partial sequence of the 26S rRNA gene.
- The majority were similar to *Y. lipolytica* strains CBS 10144 (5 out of 12) and *Y. lipolytica* NRRL YB-423 (3 out of 12).
- All strains used for this study belonged to the *Y. lipolytica* species.

Results of the selection of the optimal *Y. lipolytica* strain for citric acid and microbial lipid production using a shake-flask fermentation:

- The results of biomass content for different strains indicated that among the 12 strains of *Y. lipolytica*, only 8 produced an acceptable amount of biomass.
- Strains CBS 6303 and K57 had tolerance to higher initial glucose concentrations (48.62 g/L and 45.19 g/L, respectively) and consumed more glucose (36.62 g/L and 36.39 g/L) than the other strains.
- The highest OD 600 value of 0.180 was obtained for strain CBS 6303, while the lowest value of 0.025 was exhibited by strain Po1dL. Considering cell density, higher cell density values of $3.03 \times 10^8$ and $2.90 \times 10^8$ cells/mL were observed for strains Peggy and CBS 6303, respectively, whereas the lowest amounts were estimated for strains Po1dL, Ain 16, Ain 19 and H917.
- Strains CBS 6303, K57, W29 and Zu110 exhibited higher CA production values of around 20.50 to 24.67 g/L, while strains H917, Ain 16, Ain 19 and Po1dL exhibited the lowest CA accumulation, ranging between 0.48 and 1.68 g/L during 8-day shake-flask fermentations. The highest CA yield of 0.75 g/g was determined to be for strain Zu110. Among all the studied strains, CBS 6303, K57, W29 and Zu110 were selected as high CA-producing strains due to the high values of CA concentrations and yields. However, strain K57 was selected for further bioreactor fermentations due to its comparable values of CA yield and concentration, and also being a strain domestically isolated in Turkey.
- Among the organic acids, the highest acetic acid production, ranging between 13.31 and 19.83 g/L, was observed for strains K57, Ain 19 and Ain 16. Moreover, a significant amount of tartaric acid (13.07 g/L) was produced by strain H917 and malic acid (14.15 g/L) by strain Ain 16.

- Maximum lipid yields and lipid content varied from 0.40 to 0.61 g/g and 0.40 to 1.28 g/L, respectively. Lipid accumulation was very low, especially for strains K57 and CBS 6303, whereas higher quantities were exhibited by strains Peggy, W29 and DBVPG 5858.
- In all cases, oleic acid (C18:1), palmitic acid (C16:0) and linoleic acid (C18:2) are the most abundant FAs produced inside the cell of all examined *Y. lipolytica* strains. Linolenic acid (C18:3) was produced in significant quantities (between 10 and 20%, wt/wt), especially by strains H917 and Po1dL. Other FAs, such as C15:0, C17:0 and C17:1, were also found in small amounts for all strains except strain H917 (in which case around 20% of C17:0 and 6% of C17:1 were found). The composition of FAs in yeast cells was strain dependent.

Results of the effect of different agitation and aeration rates on microbial lipid and citric acid production by *Y. lipolytica* strain K57

- An agitation-aeration of 800 rpm - 0.5 vvm generated a better DO concentration than 350 rpm - 1 vvm.
- At a low level of DO concentration, a lower amount of CA, at 6.26 g/L, glucose consumption, at 19.98 g/L and biomass, at 1.14 g/L, but slightly higher lipid yield of 0.11 g/g were observed. However, in the reverse case, at a high level of DO concentration, a higher CA amount, at 35.41 g/L, glucose consumption, at 50.80 g/L, biomass at 5.79 g/L, and lower lipid yield value of 0.03 g/g were observed. An agitation speed of 800 rpm and aeration rate of 1.0 vvm were suitable for a high level of CA production, whereas an agitation speed of 350 rpm and aeration rate of 0.5 vvm were suitable for lipid accumulation.
- An agitation speed of 800 rpm and aeration rate of 1.0 vvm were selected for use in further bioreactor experiments due to the high maximum

production rate of 0.18 g/L/h and growth rate of 0.32 g/L/h. Due to low biomass formation, poor CA formation, minimal glucose consumption and the small difference between the lipid yield value obtained, compared with the value in a high DO concentration, an agitation speed of 350 rpm and aeration rate of 1.0 vvm were found to be unsuitable for CA and SCO production by *Y. lipolytica* strain K57 on glucose-based media.

Results of the effect of initial glucose concentration on microbial lipid and citric acid production from glucose using *Y. lipolytica* strain K57 in bioreactor fermentations

- Different initial glucose concentrations did not significantly affect the biomass concentration but the only difference was that in high glucose concentrations, the biomass content tended to decrease at the later stage of fermentation. High initial glucose concentrations of more than 100 g/L inhibited the growth rate of *Y. lipolytica*. The maximum growth rate of 0.21 g/L/h at the initial glucose concentration of 50 g/L decreased to 0.13 and 0.15 g/L/h at the initial glucose concentration of 107 and 166 g/L, respectively.
- Comparing yields of CA according to different initial glucose concentrations (Si), the highest value of 0.77 g/g was found at an initial glucose concentration of 107 g/L, the second highest value of 0.70 g/g was obtained at an initial glucose concentration of 50 g/L and the lowest yield of 0.59 g/g was determined at an initial glucose concentration of 166 g/L. Maximum values of CA content of 72.12 g/L, 63.87 g/L and 35.41 g/L were found at initial glucose concentrations of 107 g/L, 166 g/L and 50 g/L, respectively.
- Lipid yields at initial glucose concentrations of 107 and 50 g/L were lower at values of 0.03 g/g and 0.04 g/g and the highest lipid yield of 0.14 g/g

was determined at an initial glucose concentration of 166 g/L. Moreover, the highest lipid content of 0.32 g/L was also obtained at Si=166 g/L. According to these results, it can be revealed that lipid production increases at the higher initial glucose concentration of 166 g/L whereas CA yield decreases.

Results of the chemical composition of waste bread powder

- Waste bread powder was composed of 12% crude protein, 86% carbohydrate, 0.71% crude fat and 1.30 g/L of ash in dry matter. The moisture content of WBP was determined to be 4 %.

Results of waste bread hydrolysis

- A glucoamylase loading of 3 U/g was used for 2 kg of waste bread slurry, and the glucose content of WBH after hydrolysis was determined to be 125.75 g/L, corresponding to a conversion yield of approximately 97.10% from starch, which is considered to be quite a high ratio.
- However, yield values for WBP hydrolysis were lower (63.60% from starch into glucose and 3.39% from TKN into FAN) at the glucoamylase loading of 1 U/g. Due to higher conversion yields, a glucoamylase loading of 3 U/g was used for further hydrolysis.
- The FAN content of WBH was found to be 127.88 mg/L with a conversion yield from total Kjeldahl nitrogen (TKN) to FAN of 3.97%.

Results of the effect of initial glucose concentration on microbial lipid and citric acid production from a waste bread hydrolysate using *Y. lipolytica* strain K57 in bioreactor fermentations

- Very high biomass concentrations of 36.44 g/L, 47.23 g/L and 62.61 g/L were observed during WBH fermentations at initial glucose concentrations of 68 g/L, 101.23 g/L and 142.51 g/L, respectively. Initial glucose and FAN concentrations of WBH affected biomass formation by *Y. lipolytica* strain K57. A high FAN content enhanced the biomass content and a higher biomass value of 62.61 g/L was obtained in higher initial concentrations of 142.51 g/L glucose and 134.53 g/L of FAN.
- Generally, CA production on WBH was lower than bioreactor trials in glucose-based media. A higher CA production of 15.03 g/L was obtained at the initial glucose concentration of 142.52 g/L in a batch reactor fermentation at the $47^{th}$ hour.
- Higher nitrogen compounds (FAN, ammonium sulphate and yeast extract) were present in the fermentation medium and thus led to lower CA production.
- DO concentrations were lowest with the values of 30%, 11% and 1.78% for the initial glucose concentrations of 68, 101 and 142 g/L, respectively, and remained constant between 80 and 85%.
- The highest lipid yield of 0.32 g/g was generated from WBH containing an initial glucose concentration of 142 g/L. Moreover, the highest lipid content of 5.12 g/L at the $24^{th}$ hour of fermentation was produced from WBH containing an initial glucose concentration of 142 g/L.
- Higher initial glucose concentrations of WBH promoted an increase in lipid content and lipid yield.

The following suggestions can be given for further studies based on the results of this thesis:

- There is no need for any nitrogen source supplement to a waste bread hydrolysate for CA production, as nitrogen-limited conditions are required

for high CA productivity. However, a high dissolved oxygen concentration in the medium is a necessity.

- In order to achieve a high level of lipid content and lipid yield from a waste bread hydrolysate by *Y. lipolytica*, nitrogen limitation and low air saturation should be applied. In addition, inhibitory effects of WBH components such as hydroxymethyl furfural on lipid production and growth of *Y. lipolytica* should be investigated.
- For a high lipid yield, the dissolved oxygen concentration in the exponential growth phase must be kept higher than 20%, while in the stationary phase it should be lower than 20% and these conditions can be achieved in a bioreactor equipped with a "cascade mode".
- Fed-batch and continuous fermentation methods and other strains rather than strain K57 can also be employed for high citric acid and lipid production from a waste bread hydrolysate by *Y. lipolytica* in further studies.

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