

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**LIPID PRODUCTION BY YARROWIA LIPOLYTICA GROWING ON FOOD
WASTE**



Ph.D. THESIS

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Department of Environmental Engineering

Environmental Biotechnology Programme

MAY 2023

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MAY 2023

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**YEMEK ATIKLARINDA ÇOĞALTILAN YARROWIA LIPOLYTICA İLE
LİPİT ÜRETİMİ**

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To my family,



FOREWORD

The endless path of learning and the advancement in the field of science is an experience of endless wonder. This journey of discovery; this passage of enlightenment and this path to truth, brings me immense excitement and joy. In the words of Socrates: *“The only true wisdom is in knowing you know nothing. I am the wisest man alive, for I know one thing, and that is that I know nothing”*.

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ABBREVIATIONS

C/N	: Carbon/Nitrogen
COD	: Chemical Oxygen Demand
FAME	: Fatty Acid Methyl Ester
FFW	: Fermented Food Waste
FW	: Food Waste
GHGs	: Greenhouse Gases
IS	: Iron Sulphate
MUFA	: Monounsaturated Fatty Acid
PUFA	: Polyunsaturated Fatty Acid
SCOD	: Soluble Chemical Oxygen Demand
SFA	: Saturated Fatty Acid
TAG	: Triacylglycerol
TCOD	: Total Chemical Oxygen Demand
TKN	: Total Kjeldahl Nitrogen
TP	: Total Phosphorus
TS	: Trace Elements Solution
TSS	: Total Suspended Solid
VFA	: Volatile Fatty Acid
VSS	: Volatile Suspended Solid
YE	: Yeast Extract



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LIPID PRODUCTION BY YARROWIA LIPOLYTICA GROWING ON FOOD WASTE

SUMMARY

Biodiesel production from plants and vegetable oils or different organic wastes as feedstock for microorganisms, can help to decrease the consumption of fossil fuels and generation of greenhouse gases along with improve the economy. Applying organic wastes as feedstock for oleaginous yeasts is an economic technique in order to replace fossil-derived diesel with biodiesel as a clean and green fuel. Using food waste (FW) as a rich organic carbon source for cultivation of oleaginous yeast is considered as a promising environmentally friendly approach to achieve microbial lipid as the source of biodiesel.

In this thesis, FW was collected from refectory of Istanbul Technical University. It contained cooked and uncooked food which dried and filtered after collection. Dark fermentation process was carried out with the collected FW and rumen microorganisms as inoculum. The rumen was taken from sheep stomach. The output of fermentation process was collected and applied as substrate for cultivation of oleaginous yeast *Yarrowia lipolytica*. The Soluble COD (SCOD) and TKN concentrations of fermented food waste (FFW) were 48.400 ± 0.49 and 0.907 ± 0.01 g/L, respectively and pH of the medium was 5.44 ± 0.05 .

Different concentrations of FFW (diluted with distilled water) were applied as growth medium of *Y. lipolytica*. The medium which was applied with no dilution was identified as the optimum one. In all applied mediums the growth was monitored as optical density (OD₆₀₀) and the highest OD of 36.11 was observed in medium with FFW with no dilution.

Nitrogen is considered as an essential nutrient for synthesis of cell materials and metabolites by microorganism. In terms of nitrogen depletion along with excess amount of carbon in the culture, carbon uptake rate is limited that causes metabolic activities shift towards lipid storage instead of cell proliferation. This approach was used in this thesis to increase lipid content of *Y. lipolytica* cultivated on FFW. Different carbon sources of glucose, glycerol, and potassium acetate along with five different COD/TKN ratios of 75, 100, 125, 150 and 175 were selected. Carbon sources were added to the medium in early stationary phase to increase carbon concentration of FFW and obtain favorable COD/TKN ratios. In order to identify the appropriate fermentation time to collect the biomass to evaluate its lipid content, biomass samples were collected at both early and late stationary phase in YPD (the optimum growth medium for yeast growth) and FFW medium. The results indicated lipid content of 21.54 ± 1.4 and $14.97 \pm 0.51\%$ lipid along with biomass concentration of 9.63 ± 0.24 and 8.34 ± 0.82 g/L in early and late stationary phase respectively, for YPD medium. These values were 19.5 ± 0.5 and $15.52 \pm 0.31\%$ lipid content along with 8.31 ± 0.51 and 7.10 ± 0.34 g/L biomass generation for *Y. lipolytica* cultivated on FFW medium. Results illustrated the early stationary phase as the optimum fermentation point to obtain highest lipid content and microbial cell because the biodegradation of yeast cell and intracellular lipid is occurred by time during stationary phase. This biodegradation caused drop of intracellular lipid content and yeast cell quantity at the end of stationary phase.

In COD/TKN 75, lipid content of biomass was 26.7 ± 0.5 , 34.2 ± 1.12 and $33.2 \pm 1.59\%$ with biomass concentration of 9.50 ± 0.37 , 8.40 ± 0.72 and 9.32 ± 0.12 g/L for mediums contain glucose, glycerol, and potassium acetate, respectively. By increasing the ratio to 100, lipid content of medium with glucose increased to 29.2 ± 0.28 , in medium contains glycerol lipid content was 34.3 ± 2.16 and in medium with potassium acetate the lipid content was slightly decreased to $31.2 \pm 1.60\%$. In COD/TKN ratio of

125, the significant lipid content of $42.2 \pm 1.72\%$ in the medium supplemented with glycerol was measured. The other mediums contain glucose and potassium acetate have intracellular lipid content of 38.7 ± 0.35 and $34.7 \pm 3.1\%$, respectively. Biomass concentration was measured as 18.52 ± 1.97 , 12.95 ± 1.95 and 17.60 ± 0.75 g/L in culture sets supplemented with glucose, glycerol, and potassium acetate, respectively. In COD/TKN ratios 150 and 175, the amount of accumulated lipid and generated biomass were decreased that demonstrated the adverse effect of high concentration of carbon source on intracellular lipid accumulation of the cell. In these two ratios the amount of lipid content and cell concentration was 36.7 ± 1.3 and 9.77 ± 0.97 ; and $38.1 \pm 3.0\%$ and 11.57 ± 0.77 g/L, respectively.

Highest lipid concentration of 7.61 ± 0.17 g/L was observed in medium with potassium acetate in COD/TKN 100 that is related to high biomass concentration in this experimental set. Lipid concentration started to decrease in higher COD/TKN ratios and dropped to 3.59 ± 0.13 g/L in COD/TKN ratio 150 with glycerol. Although the final pH value of the mediums with COD/TKN 125 and lower was always over 8, in ratios over 125 the pH was dropped to 4.3. This pH indicated formation of secondary metabolites such as organic acids in higher ratios that was due to high glycerol concentration in the mediums. This metabolic shift from lipid accumulation to organic acid generation led to the pH drop of the batch cultures.

In next step of the thesis, Yeast Extract (YE), Iron Sulphate (IS) and Trace elements Solution (TS) was supplied to FFW along with glycerol as second carbon source to boost the lipid content further. Firstly, various amount of mentioned components were added to FFW to investigate the optimum concentration of each one to enhance lipid content of the cell. The concentrations of 1000 mg/L YE, 150 mg/L IS, and 5 ml/L TS were identified as the optimum. Different dual and triple combinations of these components along with glycerol were added to the mediums. In COD/TKN 150, accumulated lipid was reached to $44.72 \pm 0.31\%$ in the medium contains YE+IS+TS. Accumulated lipid reached to its maximum in ratio 175 i.e., $45.94 \pm 0.21\%$ in experimental set supplemented with YE+IS+TS. This value was the highest lipid content obtained in this thesis and by increasing the COD/TKN ratio over 175, the lipid accumulation dropped significantly. The highest concentration of lipid was 7.12 ± 0.12 g/L obtained in ratio 175 in medium supplied with YE+TS. The highest cell production was measured in the same culture with biomass concentration of 16.67 ± 0.27 g/L.

Investigation of metabolic behavior of *Y. lipolytica* in this thesis revealed that in general, by increasing concentration of organic carbon in the medium, the carbon consumption is enhanced as well. In ratio 75, the highest COD consumption was measured in culture contains glycerol as 30.11 g/L COD. In ratio 100, the COD consumption of medium contain glycerol was increased slightly to 32.54 mg/L COD. In ratio 125, the COD consumption of 38.84 g/L was observed that caused the lipid storage of $42.2 \pm 1.72\%$ w/w in the cell. By increasing the COD/TKN ratios to 150 and 175, the microbial lipid content decreased although COD consumption was increased. This demonstrated the metabolic shift of *Y. lipolytica* from lipid generation to secondary metabolite production which caused drop of lipid biosynthesis. By adding YE, IS and TS to the medium, in ratio 150 the lipid content enhanced to $44.72 \pm 0.31\%$ with COD consumption of 48.37 g/L and in ratio 175, highest lipid content of $45.94 \pm 0.21\%$ was obtained by COD consumption of 49.63 g/L. However, decreasing pH value in experimental sets with COD/TKN over 125 indicated the generation of

secondary metabolites in the medium. Additionally, in ratio 200 and 225 despite increase in COD consumption in the culture, the amount of stored lipid was reduced.

Gas chromatography analyses of accumulated lipid revealed the fatty acid (FA) composition of stearic acid (C18:0), linoleic acid (C18:2), pentadecanoic acid (C15:1), palmitic acid (C16:0) and heptadecenoic acid (C17:1), similar to the FA profile of plant oil and appropriate for biodiesel generation.

This thesis presented the efficient conversion of FFW and glycerol to considerable amount of microbial lipid. It indicated that FW and glycerol as two available and economic carbon sources can be evaluated as feedstock for intracellular lipid accumulation of *Y. lipolytica*. Additionally, this study demonstrated that the two-stage batch cultivation method by using FFW as initial carbon source and glycerol as the second one, can improve the lipid content of the microbial cell in significant amount. This thesis provides not only an economic waste treatment strategy, but also a sustainable and profitable method of microbial oil production by using carbon rich wastes as substrate for oleaginous yeast.



YEMEK ATIKLARINDA ÇOĞALTILAN YARROWIA LIPOLYTICA İLE LİPİT ÜRETİMİ

ÖZET

Günümüzde çevreye olan olumsuz etkilerinin açıkça bilindiği ve rezervlerinin de her geçen gün azaldığı fosil yakıtların kullanımı, artan popülasyon ve taleple birlikte ham petrolün fiyatlarının sürekli yükselmesine neden olurken, biyodizel gibi biyoyakıtların üretimi her geçen gün daha da önem kazanmakta ve biyoyakıtlar geleceğe dair ümit veren bir enerji kaynağı olarak düşünülmektedir. Bitkilerden, bitkisel yağlardan veya mikroorganizmaların hammadde olarak kullandığı farklı organik atıklardan biyodizel üretimi, bir yandan fosil yakıtların tüketimini azaltıp enerji üretim verimliliğini iyileştirirken; diğer yandan küresel iklim değişikliğini engellemekte ve ekonomiye de pozitif katkıda bulunmaktadır. Yağlı mayaların organik atıkları kullanarak oluşturduğu hücre içi lipitlerden üretilen ve temiz ve yeşil bir yakıt olan biyoyakıtların, fosil kaynaklı dizel yakıt ile değiştirilmesi ekonomik bir alternatiftir. Dünyada her yıl oluşan milyonlarca ton yemek atığının yağlı maya kültürü için zengin bir organik karbon kaynağı olarak kullanılması ve biyodizel kaynağı olarak mikrobiyal lipit elde etmek için kullanılması, son derece umut verici ve çevre dostu bir yaklaşım olarak kabul edilmektedir.

Bu tezde, İstanbul Teknik Üniversitesi yemekhanesinden pişmiş ve pişmemiş yiyecekler içeren yemek atıkları düzenli olarak toplanmıştır. Her gün toplanan bu yemek atıkları, öğütülmüş ve 2-3 gün boyunca 70°C'de kurutulmuştur. Kurutulan atıklar 3 mm filtreden geçirilmiş ve 3 mm'den küçük parçalar toplanmıştır. Filtrelenmiş kuru yemek atıkları 4°C'de saklanmıştır. Karanlık fermentasyon, direkt olarak koyun midesinden elde edilen Rumen mikroorganizmaları kullanılarak yemek atıkları üzerinde uygulanmıştır. Karanlık fermentasyon işleminin çıkışı da her gün toplanıp, çözünmüş KOİ açısından analiz edilmiştir. *Yarrowia lipolytica*'nın ekimi için substrat olarak uygulanan fermente yemek atığının çözünmüş KOİ konsantrasyonu $48,40 \pm 0,49$ g/L olup, TKN'si $0,907 \pm 0,01$ g/L ve pH değeri $5,44 \pm 0,05$ olarak ölçülmüştür. Fermente yemek atıkları, *Y. lipolytica*'nın büyümesini ve biyokütle üretimine etkisini değerlendirmek için 10 farklı konsantrasyonda (%10 ile %100 arasında, %10 aralıklarla) kullanılmıştır. Büyüme, optik yoğunluk (OD₆₀₀) olarak izlenmiştir. Konsantrasyon arttıkça biyokütle üretiminde artış gözlemlenmiş, çoğalma ve biyokütle üretimi üzerinde herhangi bir inhibisyon gözlemlenmemiştir. En yüksek OD değeri, hiçbir seyreltme yapılmayan %100 fermente yemek atığı içeren medyada gözlemlenmiş ve 36,11 olarak ölçülmüştür. Elde edilen bu sonuçlar, fermente yemek atığının *Y. lipolytica*'nın büyümesi ve hücre çoğalması için uygun bir besin kaynağı olduğunu göstermiştir.

Azot, hücrede bulunan organellerin sentezi ve mikroorganizmaların metabolitleri için temel bir besin kaynağıdır. Kültür ortamındaki azot tükenmesi ile birlikte aşırı karbon miktarının varlığı, mikroorganizmaların karbon alım hızını sınırlı hale getirip, hücre çoğalması yerine lipit depolamaya yönelik metabolik aktivitelere neden olmaktadır. Bu yaklaşım, *Y. lipolytica*'nın fermente yemek atığı üzerinde yetiştirilmiş yağ içeriğini artırmak için kullanılmıştır. Bu çalışmada, glikoz, gliserol ve potasyum asetat olmak üzere üç farklı karbon kaynağı ile 75, 100, 125, 150 ve 175 KOİ/TKN oranından oluşan beş farklı oran seçilmiştir. Fermente yemek atığının karbon konsantrasyonunu artırmak ve uygun KOİ/TKN oranlarını elde etmek için karbon kaynakları medyalarla eklenmiştir. İki aşamalı batch kültür, tüm deney setlerinde uygulanmış olup, fermente yemek atığı, başlangıç karbon kaynağı olarak kullanılmıştır. Diğer karbon kaynakları, durağan fazın başında her bir sette kültür ortamına eklenmiş ve bu da hücrelerde lipit biyosentezini önemli ölçüde artırmıştır.

Fermentasyon işleminin çıktısı, mayanın büyümesi ve lipit birikimini sağlamak için substrat olarak kullanılmıştır. Durağan fazın başında medyaların çözünmüş KOİ ve TKN'si ölçülmüş

ve KOİ miktarını artırmak için medyalara seçilen karbon kaynakları eklenmiştir. 48 saat sonra, biyokütle konsantrasyonu, biyokütlenin lipit içeriği ve yağ asitlerinin bileşimi değerlendirilmiştir. Tüm deney setleri için başlangıç pH'ı, lipit üretimi için uygun olan fermente yemek atığının doğal pH'ı olan 5,44 olup, deneyler sırasında pH ayarlaması yapılmamıştır. Sadece fermente yemek atığı içeren kontrol setinde, maya hücreleri gecikme fazı olmadan çoğalmaya başlamış ve 48 saat sonra maksimum OD 36,35 ile platoya ulaşmıştır. Karbon kaynakları eklendikten sonra, biyokütle üretimi devam etmiş ve OD, potasyum asetat varlığında KOİ/TKN oranı 100 olan medyada maksimuma ulaşmıştır. Gliserol içeren medya, her zaman en düşük OD'yi gösterirken, diğer kültürlerle kıyasla en yüksek hücre içi lipit birikimini göstermiştir.

Biokütleyi toplamak ve lipit içeriğini değerlendirmek için uygun fermantasyon zamanını belirlemek üzere, numuneler YPD (mayanın çoğalması için optimum medya) ve yemek atığı medyasında erken durağan faz ve geç durağan faz olarak toplanmıştır. Sonuçlar, YPD medyası için erken ve geç durağan fazda sırasıyla $9,63 \pm 0,24$ ve $8,34 \pm 0,82$ g/L biyokütle konsantrasyonu ile birlikte $\%21,54 \pm 1,4$ ve $\%14,97 \pm 0,51$ lipit içeriği olarak ölçülmüştür. Bu değerlerle, fermente yemek atığı medyasında yetiştirilen *Y. lipolytica*'nın $8,31 \pm 0,51$ ve $7,10 \pm 0,34$ g/L biyokütlesi ile birlikte $\%19,5 \pm 0,5$ ve $\%15,52 \pm 0,31$ lipit içeriği elde edilmiştir. Sonuçlar, en yüksek mikrobiyal hücre konsantrasyonu ve lipit içeriğini elde etmede optimum fermantasyon noktasının, erken durağan faz olduğunu göstermiştir. Durağan faz sırasında maya hücrelerinin ve hücre içi lipitlerin zamanla gerçekleşen biyolojik bozunması, bu fazın sonunda, maya hücresi ve hücre içi lipit miktarlarının düşmesine neden olmuştur.

KOİ/TKN oranı 75 olan medyada, glikoz, gliserol ve potasyum asetat içeren medyalar için biyokütlenin lipit içeriği sırasıyla $26,7 \pm 0,5$, $34,2 \pm 1,12$ ve $\%33,2 \pm 1,59$ olup, biyokütle konsantrasyonu $9,50 \pm 0,37$, $8,40 \pm 0,72$ ve $9,32 \pm 0,12$ g/L olarak elde edilmiştir. Oran 100'e çıkarıldığında glikozlu besiyerinin lipit içeriği $29,2 \pm 0,28$ 'e yükselirken, gliserol içeren besiyerinde lipit içeriği $34,3 \pm 2,16$ 'e yükselmiştir. Potasyum asetatlı medyadaki lipit içeriğinde ise, $\%31,2 \pm 1,60$ 'a hafif bir düşüş gözlemlenmiştir. Hücrenin yüksek lipit içeriği, gliserol katkılı medyada $\%42,2 \pm 1,72$ olarak KOİ/TKN oranı 125 olan medyada gözlenmiştir. Glikoz ve potasyum asetat içeren diğer medyalar ise sırasıyla $\%38,7 \pm 0,35$ ve $\%34,7 \pm 3,1$ hücre içi lipit içeriğine sahip olmuştur. Biokütle konsantrasyonu glikoz, gliserol ve potasyum asetat eklenmiş kültür setlerinde sırasıyla $18,52 \pm 1,97$, $12,95 \pm 1,95$ ve $17,60 \pm 0,75$ g/L olarak ölçülmüştür. KOİ/TKN oranları 150 ve 175'te, lipit içeriği ve biyokütle konsantrasyonu azalmış olup, bu durum, yüksek karbon konsantrasyonunun, hücrenin lipit birikimini olumsuz yönde etkilediğini göstermiştir. Bu deney setlerinde yüksek biyokütle konsantrasyonuna bağlı olarak en yüksek lipit konsantrasyonu $7,61 \pm 0,17$ g/L ile KOİ/TKN 100'de potasyum asetatlı medyada gözlenmiştir. Lipit konsantrasyonu yüksek KOİ/TKN oranlarında azalmaya başlamış ve gliserol ile KOİ/TKN oranı 150'de $3,59 \pm 0,13$ g/L'ye düştüğü görülmüştür. KOİ/TKN oranı 125 ve altı olan medyaların nihai pH değeri her zaman 8'in üzerinde olup, 125'in üzerindeki oranlarda pH'ın 4,3'e düştüğü görülmüştür. Bu pH, organik asitler gibi ikincil metabolitlerin daha yüksek KOİ/TKN oranlarında üretildiğini göstermiştir. Medyadaki yüksek gliserol konsantrasyonu, mikroorganizmanın lipit üretiminden organik asit üretimine geçişine ve batch kültürde pH düşüşüne neden olmuştur.

Çalışmanın bir sonraki adımında, lipit içeriğini daha da artırmak için gliserol ile birlikte maya özü, demir sülfat ve iz elementler solüsyonu, fermente yemek atığına eklenmiştir. İlk olarak, hücrenin lipit içeriğini arttırmak üzere, her birinin optimum konsantrasyonunu araştırmak için fermente yemek atığına çeşitli miktarlarda bahsedilen bileşenler eklenmiştir. Yapılan yağ ölçüm analizlerine göre, 1000 mg/L maya özü, 150 mg/L demir sülfat ve 5 ml/L iz element solüsyonunun optimum konsantrasyonlar olduğu bulunmuştur. Gliserol ile birlikte bu bileşenlerin ikili ve üçlü kombinasyonları medyalara eklenmiştir. KOİ/TKN oranı 150 olan medyada uygulanan kombinasyonlar arasında en iyi sonuç, $\%44,72 \pm 0,31$ lipit içeriğine sahip biyokütle ile, maya özü + demir sülfat + iz element içeren kültür setlerinde elde edilmiştir. Maya özü + demir sülfat + iz element ile desteklenmiş deney setinde hücrenin lipit birikimi

KOİ/TKN oranı 175 olan medyada, $45,94 \pm 0,21$ değeriyle maksimuma ulaşmıştır. Bu değer, bu tezde elde edilen en yüksek lipit içeriği olmuştur. Lipit birikiminin, KOİ/TKN oranının 175'in üzerine çıkarılmasıyla önemli ölçüde azaldığı görülmüştür. Elde edilen maksimum lipit ve biyokütle konsantrasyonu sırasıyla $7,12 \pm 0,12$ g/L ve $16,67 \pm 0,27$ g/L olup, bu değerler KOİ/TKN oranı 175 olan ve maya özü + iz element eklenen medyada elde edilmiştir.

Bu tezde, *Y. lipolytica*'nın metabolik davranışı incelenerek, genel olarak kültürdeki karbon konsantrasyonunun artmasıyla, karbon tüketiminin de arttığı ortaya koyulmuştur. KOİ/TKN 75 oranında en yüksek KOİ tüketimi 30,11 g/L ile gliserol içeren kültürde ölçülmüştür. 100 oranında, gliserol içeren besiyerindeki KOİ tüketimi 32,54 g/L olarak ölçülmüştür. 125 oranında, hücrede $42,2 \pm 1,72$ 'lik bir lipit birikimi ölçülmüş ve 38,84 g/L KOİ tüketimi gözlenmiştir. KOİ/TKN oranları 150 ve 175 olan medyalarda, KOİ tüketimi artmasına rağmen mikrobiyal lipit içeriğinde düşüş gözlenmiştir. Bu durum medya pH'ının düşmesiyle birlikte, *Y. lipolytica*'nın lipit oluşumundan ikincil metabolit üretimine geçtiğini göstermiş ve bu da lipit biyosentezinde düşüşe neden olmuştur. KOİ/TKN oranı 150 olan medyada, besiyerine maya özü, demir sülfat ve iz element ilave edildiğinde, lipit içeriği $44,72 \pm 0,31$ olup, KOİ tüketimi 48,37 g/L olurken; oranın 175 olduğu medyada en yüksek lipit içeriği $45,94 \pm 0,21$ olup KOİ tüketimi 49,63 g/L olarak ölçülmüştür. Ancak, KOİ/TKN oranının 125'in üzerinde olduğu medyalardaki düşük pH değerleri, ikincil metabolitlerin oluştuğunu göstermiştir. KOİ/TKN 200 ve 225 oranlarındaki medyalarda, KOİ tüketimindeki artışa rağmen lipit içeriğinin azaldığı görülmüştür.

Yapılan GC analizleri sonucunda, biriken lipitin yağ asiti bileşenlerinin, stearik asit (C18:0), linoleik asit (C18:2), pentadekanoik asit (C15:1), palmitik asit (C16:0) ve heptadekanoik asit (C17:1) olduğu saptanmıştır. Elde edilen bu profil, bitkisel yağların, yağ asitleri profiline benzer ve biyodizel üretimi için uygun nitelikte olduğunu göstermiştir.

Bu tez, fermente yemek atığı ve gliserolün önemli miktarda ve etkin bir şekilde mikrobiyal lipide dönüştüğünü göstermiştir. *Y. lipolytica*'nın hücre içi lipit birikimini sağlamada, iki yaygın ve ekonomik karbon kaynağı olarak, yemek atığı ve gliserolün, hammadde olmak üzere kullanılabilirliğini göstermiştir. Ek olarak, bu çalışma, ilk karbon kaynağı olarak fermente yemek atığı ve ikinci olarak gliserol kullanan iki aşamalı batch sistemde, mikrobiyal hücrenin lipit içeriğini önemli miktarda artırabileceğini göstermiştir. Bu yaklaşım, yalnızca ekonomik bir atık işleme stratejisi değil, aynı zamanda karbon açısından zengin atıkları, yağlı maya için substrat olarak kullanarak, sürdürülebilir ve karlı bir mikrobiyal yağ üretim yöntemi sunmuştur.



1. INTRODUCTION

1.1 Background

1.1.1 Fossil fuels and their impact on environment and climate

Environmental pollution and climate change have complex interaction with each other. The main factor which affects the climate change is greenhouse gasses (GHGs). Greenhouse gasses are any compounds in the form of gas which are able to absorb infrared radiation that leads to keep heat in the atmosphere. Increasing human population, energy demand and climate change crisis enhance the general concerns about finding an alternative energy source to traditional fossil fuels. Application of fossil fuels has resulted severe environmental problems like global warming because of GHGs generation through combustion of fossil fuels (Leong et al., 2018). Several human activities impact climate changes by heating and changing composition of the atmosphere via emission of gasses. Fossil fuel combustion releases the heat into the atmosphere and even water body that changes the climate subsequently. The source of atmospheric particles are anthropogenic activities, as well. There is interaction between these particles and atmospheric infrared radiation. Various factors affect the balance of radiation such as dispensation, shape, and size of the particles as well as their absorbance and scattering characteristics. Quantity of CO₂ in the atmosphere is increased also through fossil fuel combustion and can't be absorbed by the oceans rapidly. Greenhouse gasses absorb and keep energy in the lower layers of atmosphere and caused change of Earth's climate. The energy enters and leaves the atmosphere, keeps the balance of the earth's temperature. The sunlight can be either absorbed or reflected by the earth and the absorbed part, heats up the planet. Greenhouse gasses prevent the loss of energy to the space and make the planet warmer. This process which named "greenhouse effect" is naturally essential for different Earth's ecosystems but rising anthropogenic GHG emissions lead to climate change, serious health problems for human and drastic effects on ecosystems. Greenhouse gasses, carbon dioxide and methane can be generated through burning of fossil fuels.

Environmental biotechnology is concentrating on decrease and treatment of environmental pollutions by promoting sustainable processes to generate fewer toxic materials with lower impact on environment.

1.1.2 Biofuel: A sustainable alternative for fossil fuel

New technologies are required to supply a clean and cost-effective energy in a sustainable manner to decrease GHGs. In recent years, the sustainable generation of fuel in order to transition to renewable energy with low carbon footprint and a sustainable economy is considered to overcome the energy crisis and climate changes. In recent years, transportation sector is recognized as the major source of GHG emissions in the US and it is expected to be the major one in the future (Bleviss D.L., 2021). In this transition, liquid biofuel specially biodiesel is considered as a perfect replacement of traditional diesel. Application of biofuel as a replacement to traditional fuel has been begun at 1900 by Dr. Rudolph Diesel who used peanut oil in compression engine (Kawabe K., 2010). Biodiesel is an environmentally friendly energy source and a high-performance fuel with very low carbon dioxide emission while burning. Generally, it hasn't toxicity effect on nature and shows lower flammability compared to fossil fuels. Biomass is an alternative renewable source for generation of biofuel in forms of liquid, solid or gas. Liquid form of biofuel has some advantages such as high energy density, easier transport, and storage. Additionally, liquid biofuel can be applied in existing engines and turbines (Vamvuka D., 2011).

Biofuels are categorized into various generations according to the exploited feedstock. In 1st generation biofuel, edible material is used such as sugarcane, corn, sorghum, etc. Biodiesel as the end product is generated by fermentation of carbohydrates of food crops. Disadvantage of 1st generation biodiesel is consumption of food crops, competition with food chain, deforestation and change in biodiversity by agricultural land use for substrate cultivation (Kamineni et al., 2021). In 2nd generation of biofuel, non-edible component is used and in 3rd generation, microorganisms are applied for microbial oil production. Generated oil is converted to biodiesel through transesterification.

Biodiesel is generated from the chemical reaction between one triacylglycerol molecule with three alcohol molecules. Applied alcohol is generally ethanol or

methanol. The triacylglycerol contains three long-chain fatty acids and one molecule of glycerol which are attached together. By this reaction, three molecules of methyl esters or ethyl esters (biodiesel) and one molecule of glycerol are generated (Chilakamarthy et al., 2021). The reaction is depicted in Figure 1.1. Microbial lipid is a renewable alternative for vegetable oils or animal fats because its composition is same as vegetable oils (Caporusso et al., 2021).

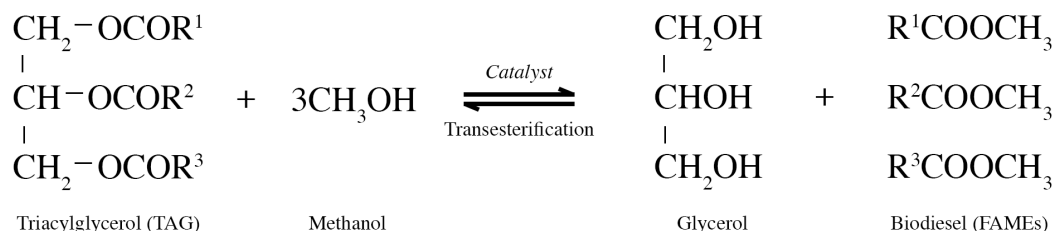


Figure 1.1: General reaction of biodiesel generation (transesterification).

There are different biomass resources that can be applied for generation of biodiesel. Plant resources consist of lignocellulosic and woody residues, agricultural wastes, and vegetables such as sugarcane, wheat, sunflower, soya, jatropha, etc. that used as feedstock for biofuel production. Microbial resources named oleaginous microorganisms can store lipid inside the cell. Stored lipid can be used for biodiesel production further, after conversion to FAMES. These microorganisms are yeast, fungi, microalgae, and bacteria.

1.1.3 Biodiesel production from oleaginous microorganisms

Biodiesel as an energy source is a great demand specially for transportation sector and different industries because of its various characterizations such as: (i) being a clean energy source, it isn't toxic and harmful for the environment as there is no GHGs emission, (ii) higher flash point (2660 °F) compared to traditional diesel that provides safer storage, (iii) high lubricity, (iv) high cetane number which provides desirable combustion even if mixed with petrodiesel; and finally it is regarded as cost effective because can be generated from economic feedstocks (Gujjala et al., 2019).

Applying edible material such as soybean, sunflower, corn, etc., is not preferred for biodiesel production because of interference with food chain as well as high cost of these feedstocks. It is reported that the cost of edible oil is more than 70% of the production cost of microbial lipid which makes it an uneconomic process (Meng et

al., 2009). This issue can be overcome by exploiting non-edible components in the process. Using microorganisms for biodiesel production has different benefits such as avoiding consumption of food crops, short growth rate of microorganisms compared to vegetable resources, independency from seasonal changes and similar lipid composition to vegetable oils.

Microbial lipids which are produced by different oleaginous microorganisms are known as 3rd generation biofuel with focusing on biomass and lipid production with no land use and no competition with food chain, with exploiting low- or negative-cost carbon-based mediums (Yang et al., 2018). This cost-effective biodiesel generation depends on lipid yield, lipid productivity, and lipid content of the microbial cell. Some different types of microorganisms are capable to generate and store lipid more than 20% of cell dry mass which called “oleaginous microorganisms”. The lipid content of these microorganisms can reach up to 70% of dry weight. There are more than 160 species known as oleaginous, but only some of them are well known such as *Yarrowia lipolytica*, *Rhodospiridium/Rhodotorula toruloides*, *Lipomyces starkeyi*, *Rhodotorula glutinis*, *Cutaneotrichosporon oleaginosus* and *Cutaneotrichosporon cutaneum* (Abeln et al., 2021).

Oleaginous microorganisms include various strains of microalgae, bacteria, molds and yeasts. Lipid production and biomass generation is varied depends on the strain. For instance, bacteria show faster growth rate than microalgae but few of them are capable of accumulating lipid. Additionally, generated lipid attaches firmly to the cell membrane in bacteria that makes the extraction process difficult (Qadeer et al., 2017). Microalgae are capable of generating biomass in salty water with no competition with food lands. They can convert carbon dioxide as greenhouse gas to intracellular lipid through photosynthesis. Amount of generated biomass is low and some limiting factors like environment temperature and light intensity affect lipid yield (Qadeer et al., 2017; Park et al., 2019). Oleaginous yeasts are less sensitive to environmental condition such as acidic and alkaline pH, salt, and low temperature. They are capable of consume diverse carbon sources with high growth rate, and there is no need of land use for their cultivation (Abghari et al., 2014). Yeasts are more tolerant to metal ions compared with molds. They show bigger cell size, and their lipid extraction is easier than bacteria.

Oleaginous yeasts are categorized under the kingdom of Fungi and two different phyla i.e., Ascomycota and Basidiomycota. The genera of *Lipomyces*, *Candida* and *Yarrowia* were mostly studied in the phylum Ascomycota, because of their ability to lipid accumulation (Valdés et al., 2020). There are numerous published studies related to *L. starkeyi* as one of species of *Lipomyces* genus, because of its considerable lipid productivity. The dimorphic oleaginous yeast *Y. lipolytica* generally exists in hydrophobic substrates in the environment as well as environments rich in oil, fats and alkanes. This yeast is isolated from oily products like yoghurt, cheese, soy sauce, meat, etc. Among its different strains, *Y. lipolytica* Polf is generally used for metabolic engineering. In the phylum Basidiomycota, *Rhodospiridium toruloides*, *Trichosporon* species and *Cryptococcus curvatus* have lipid accumulation potential. Different species of *Rhodotorula* are exist in vegetables, plants, fresh water, marine environment, and soil and known as red yeasts. The red color is because of carotenoids production by the yeast. The capability of oleaginous yeasts to utilize low-cost carbon source may be due to the ecosystem they isolated from. The soil as their habitat improves the yeast ability to utilize a different type of substrates (Yurkov A.M., 2018). For instance, glycerol as the by-product of biodiesel industry is a favorable substrate which can be consumed by different oleaginous yeasts such as *Y. lipolytica*, *C. curvatus*, *R. toruloides*, *C. boidinii* and *L. starkeyi*. This cheap carbon source can be used for microbial lipid production especially for oleaginous yeasts that are able to tolerate methanol and catabolize it which is the main pollutant in crude glycerol (Klein et al., 2016; Kumar et al., 2019).

Some of best-known strains among oleaginous yeasts are *Yarrowia*, *Lipomyces*, *Rhodospiridium* and *Cryptococcus*. *Y. lipolytica* has the mechanism to utilize hydrophobic substrates include oily wastes or substrates with high amount of protein (dairy and poultry) as carbon source, with capacity of accumulate lipid that can reaches higher than 50% of dry cell weight (Ratledge, 2005). Protrusions on the surface of the cell play important role in lipid accumulation by boosting hydrophobic substrate uptake such as oils, triglycerides, fatty acids, and short chain alkanes from environment. These substrates are used later for growth or accumulate in the cell as single cell oil. Microbial oils are recognized as valuable source for generation of biodiesel as composition of their fatty acids is similar to plant oils. Biodiesel or fatty acid methyl ester (FAME) is generated through transesterification of TAG by using

alcohol and catalysts. Traditionally, vegetable oils such as palm, rapeseed oil or soy is used to generate biodiesel that increase the production cost considerably. Moreover, it cannot fulfil the high energy demand because the production relies on access to arable lands, season and temperature. Single cell oils are regarded as significant alternative feedstock for biodiesel generation with remarkable properties such as high productivity, low labor requirement and no climatic limitation. It should be noted that to enhance feasibility of biodiesel production, low-cost carbon sources like glycerol, food waste (FW) or organic carbon rich wastes/wastewaters should be applied as substrate. The safety of *Y. lipolytica* is identified recently. This safety status along with specific characteristics of this yeast such as ability of utilize hydrophobic substrates and generate valuable products like lipid, make it a significant biotechnological yeast.

1.1.4 Carbon sources for microbial lipid production

Adequate amount of carbon source and nutrients are required to fulfil oleaginous microorganism requirement in order to lipid accumulation. Different low-cost carbon sources like lignocellulosic compounds, different wastes, industrial wastewaters, and by-products have been used as feedstock for microorganisms to store lipid. However, it should be considered that even these economic feedstocks contain carbon and nutrient for microorganism, they consist of inhibitory material as well that affect the metabolism and growth of the cell negatively. Accordingly, the composition and characterization of target feedstock should be known in detail before application.

Lignocellulose is a polysaccharide rich in cellulose, hemicellulose, lignin and low quantities of protein and pectin. More than 60% of total dry weight of lignocellulosic biomass is carbohydrates which can be applied as carbon substrate for microorganisms. Pre-treatment is needed to convert polymers to related monomers for microbial use. Different industrial effluents, agricultural, food industry and livestock wastes can be used as feedstock for cell growth and intracellular lipid production of microorganism. This approach provides bioremediation and organic carbon removal of waste/wastewater along with microbial lipid production that results in saving the cost of commercial carbon sources by adopting low-cost organic carbon substrate. The inorganic portion of wastes also contain considerable amount of heavy metals, magnesium, sodium, sulphur, calcium, potassium, iron, phosphate and ammonium salts (Lim et al., 2010). Heavy metals have toxic effect on cell metabolism and

proliferation which inhibit the enzymatic activities inside the cell. Therefore, quantity of these compounds should be investigated before applying waste/wastewater as substrate for microorganism.

Crude glycerol as a promising feedstock for oleaginous microorganisms contain high amount of carbohydrate such as sucrose, glucose, and starch. It is generated as by-product in production of biodiesel during fats transesterification, as fermentation product of alcoholic beverage production or as by product of saponification process. Discharge of glycerol to the environment will cause sever environmental problems. Crude glycerol contains some macro elements such as magnesium, potassium, calcium, sodium, and sulphur that is beneficial for microbial growth and makes it a promising substrate for lipid accumulation by microorganism (Qin et al., 2017). Composition of crude glycerol may changes depending on the biodiesel generation process. Glycerol can be used as a mixture with other wastes that are rich in carbon, as growth medium of microorganism to obtain microbial lipid (Feng et al., 2014). Crude glycerol contains 70-80% glycerol, alcohols, salts, and catalysts which can be consumed for TAG generation by oleaginous yeasts. Oleaginous yeasts utilize glycerol as substrate via the phosphorylation and oxidative pathways. In oxidative pathway, firstly glycerol is oxidized to dihydroxyacetone by FAD/NAD dehydrogenase. Then dihydroxyacetone is phosphorylated, and dihydroxyacetone phosphate is generated. Apart from these reactions, another reaction can be occurred in which glycerol-3-phosphate is converted to dihydroxyacetone phosphate by 3-phosphoglycerate dehydrogenase in the mitochondria. In the phosphorylation pathway, phosphorylation of glycerol is occurred by glycerol kinase. The glycerol-3-phosphate is generated by this reaction which enter to Kennedy pathway and leads to single cell oil generation in the cell (Klein et al., 2016; Chilakamarry et al., 202). Generally crude glycerol contains considerable amount of alcohol that is due to high amount of methanol used by biodiesel generating industries. This alcohol is toxic and has inhibitory effect on microorganism. Some part of this methanol can be eliminated by thermal treatment, but remained part still has toxic effect on cell bioprocesses. On the other hand, methanol can prevent the bacterial growth in non-sterile medium contains crude glycerol consuming by oleaginous yeasts in order to lipid production (Chen et al., 2018; Fabarius et al., 2021).

Volatile fatty acids (VFAs) are other compounds which can be consumed by oleaginous microorganisms. These compounds are produced through anaerobic biodegradation of organic wastes such as food wastes, after hydrolyses and acidogenic steps in an anaerobic process. The composition of generated VFAs is totally depends on applied anaerobic biodegradation condition, type of substrate and applied microorganisms. VFAs are considered an appropriate and economic feedstock for microbial lipid generation. High amount of VFAs as “acetic acid:propionic acid:butyric acid” with 8:1:1 ratio caused more lipid storage along with higher lipid productivity in comparison with medium with lower amount of acetic acid (Fei et al., 2011).

Oleaginous yeasts are able to use combination of various carbon sources as substrate such as glycerol and VFA, glycerol and lignocellulosic biomasses, sugar and VFA, etc. (Poontawee et al., 2020).

1.2 Hypothesis

The sustainable and economic production of appropriate microbial lipid for biodiesel generation from oleaginous yeast *Y. lipolytica* has received increasing attention these days. The fossil fuel consumption for diesel production has two major problems: (i) narrow global reservoirs; and (ii) climate change due to greenhouse gas generation (Masri et al., 2019). To meet the energy requirement of the world along with generation of an environmentally friendly form of energy for transportation, biodiesel is an attractive replacement of traditional fuels. Plant oil-based or cooking oil-based biodiesel can't compete with traditional diesel as their feedstocks are limited. The current methods to decrease carbon dioxide, hydrocarbons and particulate matter emissions greatly relies on the sustainable biofuel application (Leong et al., 2018). Microbial lipid is regarded as a clean, renewable, and sustainable feedstock which obtained from oleaginous microorganisms to produce biodiesel with the same characteristics of plant oils. In the recent years, the mostly used oleaginous microorganisms for lipid production were *Y. lipolytica* (Gao et al., 2020), *L. starkeyi* (Xavier et al., 2017), *R. fluvialis* (Polburee et al., 2020), *R. toruloides* (Castañeda et al., 2018; Osorio-González et al., 2019), *C. curvatus* (Annamalai et al., 2018), *R. glutinis* (Maza et al., 2021), *Cryptococcus albidus* (Sathiyamoorthi et al., 2019) and *Chlorella vulgaris* (Nguyen et al., 2019).

Among these microorganisms, *Y. lipolytica* is frequently studied and applied one which can utilize a wide range of different organic carbons as substrate for lipid accumulation. By means of optimization of different parameters, high lipid synthesis and accumulation of *Y. lipolytica* is possible. Food waste as a rich organic carbon source can contaminate air, water and soil and causes problems to the environment and public health in terms of discharge with no treatment. This waste can be applied as substrate to generate valuable compounds such as microbial oil through biorefinery approaches (Chen et al., 2020).

Production of biodiesel from microbial lipid is a new research area which is developed in the last decades. The number of studies in this field start to grow from 2008 and increase rapidly in the last few years. Raw materials are playing a key role in operating a feasible and cost-effective process for microbial lipid production from oleaginous microorganisms. Although several researches have been conducted on the topic of biodiesel production from lipids generated by oleaginous yeasts, there is no specific study about optimizing lipid production using FFW supplemented with different carbon sources in two-stage batch strategy by *Y. lipolytica*.

Very few studies are conducted about applying food waste derived VFAs and novel approaches are required to evaluate the worldwide FW as a problematic waste but rich in carbon and nutrient substrate. On the other hand, applying two or more carbon sources in the same cultivation system can supply more feed composition and treat more wastes as well. Many researches have been conducted with the aim of decrease the production cost of biofuels. The most important phase in cost management of biofuel production is preparation of growth medium. Carbon source, nitrogen source and consumed water are the most important costs of the medium. Glucose is one of the most effective carbon sources, but its high price makes the process uneconomic. This is why most of researches have focused on identify and develop a substitute of glucose. Food waste can be considered as an appropriate abundant and unexpensive alternative which allows great performance of lipid synthesis by *Y. lipolytica*. Glycerol is also one of the most applied low-cost substrates in production of microbial lipid. Glycerol availability is increased nowadays by growing biodiesel production industries. This carbon source is generated in biodiesel industry as by-product which

makes it a promising feedstock for microbial oil production because of its low price and high organic carbon content.

Applying organic waste derived VFAs for synthesis of microbial oil by yeast *Y. lipolytica* is becoming a research hotspot these days. To the best of the author's knowledge, this is the first study in which FFW is applied for lipid production by *Y. lipolytica* in two stage batch culture through optimization of COD/TKN ratio, carbon source and nutrients. It is known that lipid biosynthesis by oleaginous yeasts is performed in a medium with high amount of carbon source and limited amount of nitrogen (Beopoulos et al., 2011). High carbon amount leads to lipid generation, but it has negative effect on biomass growth. In applied two-stage batch strategy in this thesis, FFW was applied as the initial carbon source. Three different carbon sources of glucose, potassium acetate and glycerol were added to the batch culture at second stage of cultivation to increase COD/TKN ratio in the medium and improve lipid storage of *Y. lipolytica* growing on FFW.

1.3 Purpose of Thesis

The sustainability of lipid production by oleaginous yeasts from conventional carbon sources like glucose or synthetic VFAs is not profitable due to high price of applied feedstocks. New approaches are needed to reduce process costs and investigate alternative unexpensive carbon sources to make the lipid production process economically viable. This study provided a precious inspiration to apply FW and glycerol as cost-effective feedstocks to generate microbial lipid in two-stage batch culture. Increasing amount of carbon source in the culture medium promotes the microbial lipid generation, although high amount of substrate may cause substrate inhibition on growth of microorganism. In two-stage batch culture, this problem was solved because in first stage by using fermented food waste (FFW) as initial carbon source cell proliferation is increased and biomass concentration in the medium is enhanced. In second stage, lipid accumulation was occurred in the medium that is supplemented with excess carbon. Fermentation was applied on FW in order to hydrolyze the organic matters, increase concentration of SCOD and produce VFAs as valuable substrate for growth and biomass generation. Different supplements i.e., trace elements, iron and yeast extract were supplied to the culture medium to promote the lipid content of the cell because nitrogen source, minerals and trace elements play key

role in microbial activities such as growth and lipid synthesis. The purpose of this thesis was to provide a new strategy to exploit FW as available and economic substrate to produce microbial lipid by oleaginous yeast *Y. lipolytica* and promote this accumulated lipid by supplying selected components. In this approach, glycerol, potassium acetate and glucose were applied at the second stage of cultivation to enhance the lipid generation in batch system. Utilizing of waste resources to produce microbial oil can effectively reduce the cost of biodiesel generation. By applying waste biorefinery strategy in this thesis, a promising approach is provided as well towards solving the environmental impacts of FW.

1.4 Thesis Outline and Scope

Microbial lipid generated by *Y. lipolytica* has been recognized as potential feedstock for biodiesel production. In this thesis the possibility to utilize FW as carbon source by *Y. lipolytica* MUCL 28849 in batch culture and production of single cell oil in nitrogen limited media was evaluated. The soluble organic content of the waste was boosted by means of dark fermentation process and fermented food waste (FFW) was exploited as substrate for growth and lipid storage of *Y. lipolytica*.

Three different carbon sources as glucose, potassium acetate and glycerol were selected and supplied to the medium at early stationary phase to boost the nitrogen deficiency and increase the carbon source concentration in the medium. Various COD/TKN ratios were applied and optimization of type of carbon source and its concentration was performed to obtain high lipid accumulation by the cell. Two-stage batch culture was applied in all experimental sets. In initial stage, growth of *Y. lipolytica* was completed on FFW as initial carbon source. In 2nd stage, different carbon sources were supplied to the culture medium in different individual sets with the aim of increase lipid biosynthesis and accumulation in the cell. The identified optimal carbon source and COD/TKN ratio was selected and used for further investigations.

Moreover, effect of various concentrations of yeast extract, iron and trace elements on biomass growth and lipid accumulation of *Y. lipolytica* was evaluated. The optimum amounts of mentioned components were identified and added to the growth medium

in different combinations, to enhance the lipid content further. The carbon concentration was increased in the culture sets simultaneously.

Accumulated lipid inside the biomass is converted to FAME by transesterification and fatty acid profile of generated lipid is identified to compare with fatty acid composition of traditional diesel and evaluate the properties of generated lipid.



2. LITERATURE REVIEW

2.1 Lipid Accumulation in Oleaginous Microorganisms

All microorganisms have the ability to synthesize lipid to be able to survive, but only the microorganisms capable of storing lipid more than 20% of their dry cell mass are known as oleaginous microorganisms. This accumulated lipid can reach up to 70% based on the species that is performed under nutrient deficiency condition. These specific microorganisms include microalgae, yeast, mold, and bacteria which can generate biomass and intracellular lipid. Application of oleaginous microorganisms as a tool for microbial lipid generation takes a great attention of different industries. The composition of accumulated microbial lipid is mainly triacylglycerol (TAGs) along with small quantity of steryl esters, free fatty acids, sterols, phospholipids, and glycolipids. The amount of accumulated TAGs when growth is occurred on hydrophobic substrates (ex novo lipid biosynthesis), is lower than growth on sugar-based medium (de novo lipid biosynthesis). The microorganism species and different environmental factors have significant effect on the lipid amount and its composition. High level of lipid accumulation can be achieved with suitable culture medium and environmental condition. Table 1.1 shows a list of oleaginous yeasts that accumulate considerable amount of intracellular lipid and can be applied for biodiesel production.

In order to decrease the cost of substrate for microbial lipid production, oleaginous yeasts/ microalgae/ bacteria can be cultivated on different organic wastes. Biodiesel generation using oleaginous microorganisms produce higher “lipid yield per cultivation area” compared with oil crops like corn (Chiu et al., 2015; Fei et al., 2015). These microorganisms can consume different types of wastes such as agricultural, industrial, or municipal (Cho et al., 2017). Food waste can be used as a substrate with high organic carbon content for oleaginous microorganisms for the purpose of lipid generation. Pleissner et al. (2013) applied hydrolyses process on FW by *Aspergillus* spp. and used the hydrolysate as a nutrient source for growth of *Schizochytrium* sp. and *Chlorella* sp. They achieved lipid content of 16 and 20% for *Schizochytrium* sp. and *Chlorella* sp., respectively. The fatty acid composition of both strains was oleic, palmitic, and stearic acid and were appropriate for biodiesel generation. The mixture of crude glycerol and brewer fermentation waste as the by-product of beer production

process can be used also as substrate for microalgae. In an investigation conducted by Feng et al. 2014, growth of *Chlorella protothecoides* on this mixture resulted in 12.7 g/L biomass generation and 52% lipid content.

Table 1.1 : Lipid content in different oleaginous yeasts using different carbon sources.

Yeast Strain	Carbon source	Lipid content (%)	Lipid productivity (mg/L/d)	Lipid concentration (g/L)	Lipid yield (g/g)	Reference
<i>Cryptococcus curvatus</i>	Hydrolysate of sorghum bagasse	52.0	797.3	-	-	Liang et al. (2012)
<i>Cryptococcus curvatus</i>	Pretreated waste activated sludge	23.0	2260.0	-	-	Seo et al. (2013)
<i>Cryptococcus curvatus</i>	Corn stover hydrolysates and glycerol	27.3-49.7	130	-	-	Gong et al. (2016)
<i>Cryptococcus curvatus</i>	Leachate from pretreated food waste	20.0	355.6	-	-	Johnravindar et al. (2017)
<i>Cryptococcus curvatus</i>	Pretreated waste office paper	37.8	-	5.75	-	Annamalai et al. (2018)
<i>Lipomyces starkeyi</i>	Hydrolysate of flour-rich waste	40.4	1540.2	-	-	Tsakona et al. (2014)
<i>Lipomyces starkeyi</i>	Hydrolysate of wheat bran	37.3	-	-	-	Probst and Vadlani (2015)
<i>Lipomyces starkeyi</i>	Corn stover hydrolysate	55	60	-	-	Calvey et al. (2016)
<i>Rhodospiridiobolus fluvialis</i>	Sugarcane top hydrolysate and glycerol	75	-	-	-	Poontawee et al. (2018)
<i>Rhodospiridium toruloides</i>	Acetic acid	38.6-48.2	570	-	-	Huang et al. (2016)
<i>Rhodospiridium toruloides</i>	Food waste saccharified liquid	54.5-63.8	-	-	-	Ma et al. (2018)
<i>Rhodospiridium toruloides</i>	Laminaria residues	37.6	-	-	0.16	Zhang et al. (2016)
<i>Rhodospiridium toruloides</i>	Glucose+Rapeseed meal	44.2	-	15.4	0.15	Uckun Kiran et al. (2012)
<i>Rhodotorula glutinis</i>	Hydrolysate of wheat straw	25	575.0	-	-	Yu et al. (2011)
<i>Rhodotorula glutinis</i>	Brewery effluents	11.5	-	-	-	Schneider et al. (2013)
<i>Trichosporon porosum</i>	Glucose	34.1	100	-	-	Schulze et al. (2014)
<i>Yarrowia lipolytica</i>	Organic waste derived acetic acid/acetate	-	800	115	0.16	Xu et al. (2017)
<i>Yarrowia lipolytica</i>	Synthetic and food waste- derived VFAs	18.1-31.7	110-230	-	-	Gao et al. (2017)
<i>Yarrowia lipolytica</i>	Leachate from pretreated food waste	49	1540.2	-	-	Johnravindar et al. (2017)

Table 1.1 (continued) : Lipid content in different oleaginous yeasts using different carbon sources.

Yeast Strain	Carbon source	Lipid content (%)	Lipid productivity (mg/L/d)	Lipid concentration (g/L)	Lipid yield (g/g)	Reference
<i>Yarrowia lipolytica</i>	Hydrolysis of apple pomace	64.2	-	-	-	Liu et al. (2019)
<i>Yarrowia lipolytica</i>	Glucose, followed by VFAs	25.4	-	-	-	Pereira et al. (2021)
<i>Yarrowia lipolytica</i>	Glycerol	25	-	1.69	-	Dobrowolski et al. (2016)
<i>Yarrowia lipolytica</i>	Glucose + volatile fatty acids	38.43	-	15.73	0.17	Fontanille et al. (2012)
<i>Yarrowia lipolytica</i>	Glycerol + volatile fatty acids	34.59	-	14.19	0.16	Fontanille et al. (2012)
<i>Yarrowia lipolytica</i>	Waste oil of chicken products	37.7	-	-	-	El Bialy et al. (2011)
<i>Yarrowia lipolytica</i>	Waste oil from fried vegetables	57.89	-	-	-	Leiva-Candia et al. (2014)
<i>Yarrowia lipolytica</i>	Detoxified defatted rice bran hydrolysate	48.02	-	5.16	-	Tsigie et al. (2012)
<i>Yarrowia lipolytica</i>	Detoxified sugarcane bagasse hydrolysate + peptone	58.5	1760	6.68	-	Tsigie et al. (2011)

Although oleaginous bacteria have the potential to store TAGs inside the cell, their application for biodiesel generation using organic wastes is not studied as much as yeasts or algae. The most studies oleaginous bacteria are *Bacillus* sp., *Acinetobacter* sp., *Gordonia* sp. and *Rhodococcus* sp. Different organic wastes are applied as substrate for cultivation of *Rhodococcus* sp. (Wei et al., 2015). These oleaginous bacteria can convert lignin to microbial lipid (Kosa and Ragauskas, 2013). In a study, the effluent obtained from pre-treatment of loblolly pine was applied as carbon source for growth of *Rhodococcus opacus* by Wells et al. (2015). The aromatic compounds were degraded as well as the carbohydrates (glucose, mannose, and xylose). The lipid content of the cell was 26.8% and the accumulated fatty acid composition by the cell was mainly C18:1, C16:0 and C18:0 that was suitable for biodiesel generation. In another study conducted by (Zhang et al., 2014), the lipid content of *Bacillus subtilis* cultivated on cotton stalk hydrolysate was 39.8% with cell growth of 5.7 g/L.

Discovering of microorganisms with the potential of lipid production or single cell oil (SCO) generation from wastes or by-product in large quantity with similar composition to vegetable oil, provides huge financial interest.

2.2 Lipid Biosynthesis in *Y. lipolytica* as an Oleaginous Yeast

Amongst oleaginous microorganisms, oleaginous yeasts are the most promising and the best studied one because of their potential to store considerable fatty acids content as triglycerides, fast growth rate and ability to generate high biomass amount that are important points in microbial lipid production. Most of the species of oleaginous yeasts are appropriate for large-scale fermentation systems and genetic engineering as well. The feasibility of lipid production bioprocess from microorganism can be promoted by optimizing the environmental and operational condition and applying low-cost carbon sources as substrate i.e., volatile fatty acids, FW, lignocelluloses, glycerol, etc. Accumulated triglycerides are valuable material for conversion to biodiesel and used as jet fuel. *Y. lipolytica* is one of the largely investigated yeasts after *Saccharomyces cerevisiae* in terms of microbial lipid accumulation. It is a Generally Regarded as Safe (GRAS) microorganism for industrial application by the American Food and Drug Administration (FDA) and identified as safe microorganism that can be used in food industry by the European Food and Safety Authority (EFSA BIOHAZ Panel, 2018; Zieniuk & Fabiszewska, 2019). Yeast *Y. lipolytica* firstly was classified as *Candida lipolytica* at 1960s by Wickerham in Peoria. It was re-classified in 1970 as *Endomycopsis lipolytica* and two years later as *Saccharomycopsis lipolytica* in Delft Microbiology Laboratory by David Yarrow and finally in 1980 named as *Yarrowia lipolytica* by van der Walt and von Arx (Barth, 1996). The name “*Yarrowia*” reveals the new discovered genus by David Yarrow and “*lipolytica*” as the species name identifies the origin of yeast i.e., lipid rich mediums like dairy or sausage (Liu et al., 2015). Isolation of this yeast from dairy products such as yoghurt and kefir, soy sauce, rancid margarin, sourdough, oil contaminated soil and marine environment is possible. It can utilize extensive range of hydrophilic and hydrophobic substrate and tolerate severe environmental condition such as salt, different pH ranges from 2.5 to 8 and temperatures from 2 °C to 32 °C. It has also the ability to tolerate metal ions i.e., nickel, cadmium, zinc and cobalt and salty environment. The pathways of microbial oil biosynthesis in *Y. lipolytica* are also a promising topic of metabolic engineering to produce engineered strains which can generate biodiesel with promoted properties. Lipid production and storage in oleaginous yeasts takes place in nitrogen limited condition. In terms of high nitrogen concentration in the culture, microbial growth and proliferation is enhanced (Goncalves et al., 2014). The single cell oils of oleaginous

yeast generally consist of C16-C18 fatty acids that are appropriate for generation of biodiesel. Applying organic wastes as feedstock for oleaginous yeast is an economic technique in order to replace fossil-derived diesel with biodiesel as a clean and green fuel. *Y. lipolytica* can utilize different organic substrates and convert them to intracellular lipid in a short time.

2.3 Lipid Accumulation Pathways

Two different lipid biosynthesis mechanisms are performed by an oleaginous yeast: (1) *de novo* synthesis, in which hydrophilic substrates are utilized by yeast in a medium with limited nutrient content; and (2) *ex novo* synthesis, that occurs in the medium contain hydrophobic substrate.

2.3.1 De novo synthesis

Oleaginous yeast *Y. lipolytica* accumulates lipid in the form of particles inside the cell named lipid body. These lipid bodies are generally neutral lipids consist of TAGs as 85% and steryl esters (SE) in smaller amount as 8% (Athenstaedt et al., 2006). In *de novo* system, fatty acid precursors i.e., acetyl and malonyl-CoA are produced and associated into the Kennedy pathway (lipid biosynthesis pathways). Acetyl-CoA is an important factor in fatty acid synthesis and is generated from breaking down of carbohydrates to pyruvate. Generated pyruvate oxidizes and converted to Acetyl-CoA and enters in Krebs cycle in mitochondria. In nitrogen limited medium, AMP-deaminase enzyme is activated and IMP (inosine monophosphate) and NH_4^+ is formed from APM. Consequently, concentration of AMP drops in the mitochondria and ammonium concentration increased in the cell. By decreasing AMP concentration, isocitrate dehydrogenase enzyme (IDH) is inhibited that blocks the Krebs cycle. Meanwhile, citrate accumulated in the mitochondria is catalyzed by the aconitase enzyme. Citrate passes into the cytoplasm and breaks down into acetyl-CoA and oxaloacetate by ATP-citrate lyase, which is one of important enzymes in lipid accumulation system. At the end, accumulated acetyl-CoA is utilized in fatty acid production process. In terms of absence of ATP-citrate lyase enzyme, citric acid is accumulated in the cytoplasm in nitrogen limited medium. TAGs are produced in Kennedy pathway in which DAG is transformed to TAG from phospholipid by *LROI* gene or from acyl-CoA by *DGAI* and *DGA2* genes. Steryl esters are produced from

acyl-CoA by means of *ARE1* gene (Beopoulos et al., 2012). In metabolic engineered strains, *DGA1* and *DGA2* genes are overexpressed to enhance lipid production in the cell.

2.3.2 Ex novo synthesis

In this lipid synthesis mechanism, hydrophobic carbon sources such as methyl-esters, free fatty acids, TAGs, oil, and fats enter the cell from culture medium which can be accumulated inside the cell or used for growth needs. In this pathway, hydrophobic substrates should be hydrolyzed to fatty acids and generated fatty acids can enter the cell. These free fatty acids are used in lipid accumulation by forming TAGs or oxidized through β -oxidation system in order to ATP production (Papanikolaou et al., 2011). Yeast *Y. lipolytica* has developed sophisticated mechanism to produce surfactants and extracellular emulsifiers to improve transport of hydrophobic substrates from the culture medium inside the cell. The main difference between mentioned two different lipid biosynthesis is in ex novo process lipid storage is not dependent to nitrogen depletion in the cell and lipid storage and cell growth are performed simultaneously. It means by applying hydrophobic substrates as carbon source to *Y. lipolytica*, accumulation of SCO and generation of lipid free materials for cell growth in nitrogen available medium are simultaneous processes. Papanikolaou et al. (2007) cultivated *Y. lipolytica* ACA-DC 50109 on stearin with two different concentrations of 10 and 20 g/L along with various concentrations of nitrogen as 0-1 g/L ammonium sulphate. In all conducted sets significant amount of biomass and intracellular lipid was generated. Although the amount of produced SCO was high regardless of nitrogen existence in the culture medium, its quantity was higher in presence of lower nitrogen amount in the medium.

In growth medium contains fats as sole substrate, de novo lipid biosynthesis form acetyl-CoA is not occurred because FAS (fatty acid synthase) and ACL (ATP-citrate lyase) are inhibited in the medium contain aliphatic chains. Hence, existence of hydrophobic substances in the medium are contradictory with de novo lipid biosynthesis pathway even if hydrophilic carbon sources such as glucose existed in the medium (Papanikolaou and Aggelis, 2011).

2.4 Factors Affecting Lipid Accumulation of *Y. lipolytica*.

There are different physiological and environmental parameters affecting lipid storage and lipid composition of *Y. lipolytica*. These factors are type of yeast (species and strains), growth medium composition such as source of carbon and nitrogen, ratio of carbon to nitrogen, macro/micronutrients, pH, temperature, cultivation time and dissolved oxygen level. High microbial lipid yield can be achieved by optimization of these parameters.

2.4.1 Carbon source

Different studies were conducted for the optimization of microbial lipid accumulation using different carbon sources such as glucose, fructose, sucrose, raw starch, hydrolysates of lignocellulose, acetate, hydrolysate of agricultural waste, etc. Combination of different carbon sources such as glucose along with municipal waste waters, volatile fatty acids or organic acids can be applied as well. Waste oil, food waste, glycerol and glycerol can be combined with glycerol as well for lipid production by *Y. lipolytica* (Tsigie et al., 2012; Donat et al., 2014; Leiva-Candia et al., 2014; Dobrowolski et al., 2016). Some of carbon sources needs to be treated before applying to microorganisms. For instance, lignocellulosic biomass was used in some studies as carbon source for oleaginous yeasts that contains cellulose, carbohydrates, lignin, and hemicellulose. If this carbon source is going to be used as substrate, the carbohydrates should be hydrolyzed. This is difficult because the carbohydrates are attached to lignin. So, pre-treatments are required to transform this complex carbon source to available carbon for microorganism. Glucose is the mostly studied substrate among mentioned carbon sources which can generate considerable amount of microbial lipid by oleaginous yeast (Ledesma-Amaro et al., 2016; Zhang et al., 2019).

Y. lipolytica can grows fast and generates high biomass in terms of using glucose as carbon source, although glucose concentration of higher than 150 g/L has adverse effect on growth in wild type strains. Sucrose is also an economic carbon source and the main component of molasses that can be used for growth and lipid production by engineered types of oleaginous microorganism. The reason is the related enzyme that can breaks down sucrose to glucose and fructose is not exist in wild type strains. High amount of nitrogen in molasses is not suitable for lipid synthesis as nitrogen limited

medium is required for de novo lipid production. However, removing nitrogen from molasses is an additional cost. Ethanol also can be applied as substrate to *Y. lipolytica* as alcohol dehydrogenase and aldehyde dehydrogenase enzymes exist in the microbial cell. Although ethanol concentration of more than 3% is growth limited. Volatile fatty acids can be applied as substrate to improve lipid content and cell growth of the yeast cell. It is shown that *Y. lipolytica* MUCL 28849 can generate lipid concentration of 16.5 g/L and lipid content of 40% in the medium contains glucose supplemented with volatile fatty acids after glucose exhaustion in the medium (Fontanille et al., 2012).

Glycerol, which is the main by-product of biodiesel industry, is a low-cost substrate that is suitable carbon source for microorganism for lipid generation. Generally, during biodiesel production, 10% glycerol is generated. Different studies investigate the ability of wide range of yeasts specially *Y. lipolytica* to utilize glycerol for growth and lipid generation via de novo process (Kuttiraja et al., 2016; Dobrowolski et al., 2019). Glycerol derived from biodiesel contains macro elements like potassium, calcium, magnesium, and sulfur that are useful for growth of microorganisms. It is proved that despite some impurities in raw glycerol like salts, alcohol, metals, and non-glycerol organic substances, it is considered as a suitable substrate for biomass growth and microbial lipid generation by *Y. lipolytica* (Papanikolaou et al., 2017). Applying glycerol as a substrate for oleaginous yeasts leads to decreasing the cultivation costs as well as providing an approach for evaluating this carbon rich by-product. Some advantages of crude glycerol over other carbon sources are its lower cost, availability, higher degree of reduction and lower interference with food production. It contains high amount of soap that works as surfactant which enhances the biomass and lipid amount (Xu et al., 2016). Impurities exist in pure glycerol such as sodium hydroxide has positive effect on biomass and lipid production. Inorganic acid or base catalysts along with high amount of methanol is used in biodiesel industry. This methanol inhibits the biomass growth and lipid accumulation of cell to some degree and can be removed from the culture by evaporation. Increase glycerol concentration in the medium will enhance lipid production by yeast (Lei et al., 2017). Some studies showed that *Y. lipolytica* prefers glycerol over glucose (Abghari et al., 2014; Sestric et al., 2014). Low-cost carbon sources are required in single cell oil production to be able to make biodiesel generation economically viable and

competitive in the fuel market. Glucose shows significant performance in growth and lipid generation by oleaginous yeasts, but it is a high-cost carbon source (\$500/ton) that represents about 60-80% of biodiesel production cost. As the most important barrier for commercialization of biodiesel is its cost, applying cheap substrate is always preferred. Volatile fatty acids are considered as low-cost carbon sources as well with price of about \$30–100/ton. Hydrophobic substrates are consumed by yeast as well and converted into microbial lipid through ex novo lipid synthesis system. This process does not require nitrogen deficiency condition. Some examples of hydrophobic carbon sources are fatty acids originated from animal or plant oils, pure fatty acids, n-alkanes, fatty acid by-products or wastes.

Different investigations are performed by using high-cost materials as substrate for biomass generation and intracellular lipid storage of oleaginous microorganisms. Applying expensive carbon sources makes the lipid-based biodiesel production an uneconomic process and decreases its feasibility. In few studies, FFW was applied with *Y. lipolytica* to lipid generation. Conducting high-cost carbon sources such as synthetic VFA or glucose in these studies to enhance carbon concentration in the culture is not practicable as the cost of process increases significantly. It is vital to find an economic carbon source to enhance carbon concentration in the growth medium. Applying FW as a widely available and an economic carbon source is a sustainable approach for lipid production.

2.4.2 Nitrogen source

Nitrogen source and its limitation has considerable impact on lipid accumulation of oleaginous yeasts. Two types of nitrogen sources are used in a yeast growth medium: organic and inorganic. Organic nitrogen sources like yeast extract, peptone, urea, and fish meal are suitable for lipid production as contain some nutrients like vitamins or amino acid. In some studies, yeast extract is replaced with other cheap organic nitrogen sources such as soybean powder, corn steep liquor, etc. Inorganic nitrogen sources such as potassium nitrate, ammonium nitrate, ammonium sulfate and ammonium chloride are appropriate for biomass growth (Huang et al., 1998). Impact of urine on lipid storage of *Y. lipolytica* was evaluated by Brabender et al. (2018). Obtained lipid was 4.45 g/L which assigned 26% of dry cell weight, higher than cultures contain ammonium sulfate with 2.59 g/L and 22% lipid. For the first time the

human urine was applied directly as nitrogen source without any pre-treatment for intracellular lipid production. In a study conducted by Poontawee et al. (2018), half of the yeast extract was replaced with urine and the lipid concentration decreased from 6.6 g/L to 4.9 g/L. This is due to ammonium ion which resulted from hydrolysis of urea that increased the alkalinity of culture medium. Inorganic carbon sources are generally preferred by industries due to their lower price. Extreme nitrogen deficiency causes sharp decrease in cell viability before the cell starts lipid storage. Therefore, always low nitrogen content is required in the medium for the cell to be able to enter lipid biosynthesis and accumulation stage (Beopoulos et al., 2009). Ammonia in growth mediums has two forms of ammonium ion (NH_4^+) and free ammonia (NH_3). Two factors of temperature and pH affect the free ammonia concentration of medium. Free ammonia can enter cell freely, it has lethal inhibition effect by absorb protons and interrupt proton balance in the cell. It can cause potassium deficiency as well and disturb cytoplasmic pH (Dennis and Jarrel, 1984).

2.4.3 Amount of organic carbon and nitrogen

Bioprocesses for microbial lipid storage should be designed concerning the knowledge of yeast metabolism because oleaginous yeasts can produce and accumulate high quantity of lipid, break and degrade accumulated lipid and generate some secondary metabolites as well. There is four different metabolic condition for oleaginous yeasts, based on carbon to nitrogen ratio in the culture medium. First one is occurred when carbon concentration is less than required amount for microbial growth. In this situation, cell would break down existed lipids to fulfill the substrate deficiency. In second condition, optimum carbon source concentration is existed in the culture, so cell growth is maximum along with consuming required nitrogen amount. In this situation, maximum biomass generation is performed. Third condition is related to the culture medium with high quantity of substrate and low nitrogen content that causes low cell growth but high quantity of SCOs. In the fourth condition, suppression of lipid accumulation is occurred by further enhancement of carbon concentration and metabolic pathway shifts towards secondary metabolite generation. Hereby, in order to obtain microbial lipid as an end product, the bioprocess must be optimized and designed properly in a way to maximize the lipid biosynthesis.

Lipid accumulation of oleaginous microorganism mainly occurs when nutrient is limiting and excess carbon is available in the medium. Medium with nitrogen deficiency is the most efficient one for increase lipid production by the cell. In the growth phase of microorganism, the carbon flux is assigned to protein, carbohydrate, lipid, and nucleic acid. Nitrogen is a crucial element for cell proliferation during biosynthesis of protein and nucleic acid. In terms of nitrogen limitation, cell growth drops quickly, and the carbon flux goes toward microbial lipid biosynthesis instead of cell proliferation that leads to lipid accumulation inside the cell. If non-oleaginous microorganisms were applied in nitrogen exhausted condition, cell growth is stopped but polysaccharides such as glycogen is accumulated in the cell instead of microbial lipid. Various metabolic processes such as lipid generation, cell growth and secondary metabolite production like organic acids can take place in the cell based on the ratio of available organic carbon to nitrogen. In medium with nitrogen deficiency, lipid accumulation is enhanced instead of cell proliferation (Leong et al., 2018). The reason is nitrogen exhaustion activates the adenosine monophosphate (AMP) deaminase. Afterwards, inosine 5'-monophosphate is generated from adenosine monophosphate and ammonium and TAG synthesis process is started (Papanikolaou and Aggelis, 2011). Generally, the amount of accumulated lipid is determined with carbon source concentration. Therefore, carbon concentration to nitrogen ratio is an important factor to specify the accumulated lipid quantity in oleaginous microorganisms. It is important to select this ratio in a way to don't affect the cell proliferation adversely. According to the literature, in $C/N > 20$ the lipid accumulation is enhanced, and it can be decreased in ratio over 70. The ratio of organic carbon to nitrogen should be optimized for the purpose of achieving high lipid generation in the cell. In a study conducted by Canonico et al. (2016), lipid of 2.6 g/L was obtained at C/N ratio of 118 by *Y. lipolytica* applying 90 g/L glycerol as carbon source. Gajdos et al., (2017) obtained 40% microbial lipid by *Y. lipolytica* JMY3580 under C/N ratio of 90. Results indicated the effect of carbon and nitrogen concentration on microbial lipid accumulation varies based on the strain. Kuttiraja et al. (2016) investigated different C/N ratios of 25 to 150 applying *Y. lipolytica* SKY7 and biomass decreasing from 22.62 to 13.57 g/L was observed by increasing C/N ratio. Additionally, the amount of nitrogen in the culture medium is important as well. It means enhancing the carbon concentration would not always improve the lipid biosynthesis. To obtain high lipid content without limiting cell growth, two-stage batch strategy is recommended. In this strategy, biomass

growth and nitrogen depletion are performed in first stage and lipid accumulation is occurred in nitrogen limited medium in second stage (Llamas et al., 2019). Zhang et al. (2021), improved the lipid content of *L. starkeyi* NRRL Y-1388 by using this strategy to 78%. In another study investigated by Slininger et al. (2016), three different oleaginous yeasts of *L. tetrasporus* Y-11562, *L. kononenkoae* Y-7042 and *R. toruloides* Y-1091 were used at C/N ratio of 60 at initial stage. This ratio increased to about 500 in second stage and the amount of lipid content was 3 to 7-fold higher than obtained lipid in first stage of experiment.

Nitrogen is a crucial nutrient that is needed for synthesis of cell materials and metabolites by microorganism. In terms of nitrogen depletion along with excess amount of carbon in the growth media, de novo lipid synthesis mechanism will be activated. This mechanism leads to citrate accumulation in the mitochondria and then its transmission to the cytoplasm. Acetyl CoA will be formed later which is a precursor of fatty acids as building blocks of intercellular lipid. Enhancing carbon to nitrogen ratio of culture is considered as significant approach to promote the lipid accumulation of the oleaginous microorganisms. In nitrogen deficiency condition, carbon uptake rate is limited that causes metabolic activities towards lipid storage instead of cell proliferation. Different studies investigate the impact of various C/N ratios on growth and lipid accumulation of microorganisms. According to Gao et al. (2020), most oleaginous microorganisms prefer C/N ratio of 40-80 for biosynthesis and accumulate microbial lipid. Kuttiraja et al. (2016) investigated different C/N ratios of 25-150 on *Y. lipolytica* SKY7 cultivated on glycerol and assessed the optimum ratio of 100 with lipid content of 50%. Additionally, they obtained lower biomass concentration of 13.57 g/L in high C/N ratio of 150 whereas in lower ratio of 25 biomass concentration of 22.62 g/L was obtained. Optimizing the type of carbon source and its concentration in culture medium is important to increase lipid content of the cell (Papanikolaou et al., 2011).

2.4.4 Temperature, pH, dissolved oxygen, and agitation

The incubation temperature has direct effect on lipid synthesis of *Y. lipolytica*. Studies demonstrated the temperature of 24-33 °C as favorable temperature for growth, although temperature of 28 °C was observed as the optimum one for lipid accumulation by *Y. lipolytica* (Papanikolaou et al., 2002). Additionally, fatty acids

composition and degree of saturation is affected by incubation temperature in a way that saturation degree decreases by applying low temperature during incubation (Ageitos et al., 2011). Amount of dissolved oxygen in the culture also has important effect on lipid accumulation by *Y. lipolytica* and lipid composition as well. Magdouli et al., (2018) investigated the effect of dissolved oxygen on biomass growth and fatty acid composition. Results demonstrated direct impact of dissolved oxygen concentration on accumulated lipid. The highest lipid content of $44.8\% \pm 1.2\%$ was achieved by dissolved oxygen concentration of 30%. Nambou et al. (2014) reported that dissolved oxygen concentration affected the composition of stored lipid by *Y. lipolytica* DSM3286. By applying 20% dissolved oxygen, the amount of C16:1, C16:0, C18:2, C18:1, and C18:0 was 14.32%, 16.04%, 1.25%, 60%, and 4.31%. When dissolved oxygen concentration increased to 50%, the fatty acids proportions were 11.21%, 15.96%, 2.58%, 65.3%, and 4.95%, respectively. Unrean et al. (2017) showed that 0.1 mmol/mmol (oxygen/glucose) oxygen uptake rate leads to enhance lipid storage and decrease cell growth of *Y. lipolytica*. High aeration causes drop of lipid content, but its optimum level varies based on yeast strain (Calvey et al., 2016). It should be noted that oxygen is used by fatty acid desaturases in unsaturation reaction in which saturated fatty acids transformed into unsaturated.

Another important factor in lipid accumulation by oleaginous yeast is pH of the medium. *Y. lipolytica* can grows in medium with unfavorable environmental conditions such as very high pH of up to 10 or very low pH of 3, low mineral concentration or medium contains toxic matter. According to Timouti et al. (2018), the optimum pH for lipid production by microorganism is slightly acidic one like pH of 5-6.5. Papanikolaou et al. (2002) obtained the biomass concentration of 9.5 g/L along with lipid concentration of 2.7 g/L at pH 6 with *Y. lipolytica* ACA-DC 50109 growing on industrial fat. Kuttiraja et al. (2017) achieved high biomass generation of 18 g/L and 20.6 g/L in mediums with controlled and uncontrolled pH with initial pH of 6.5. But accumulated lipid content in medium with controlled pH was higher (45.44%) than uncontrolled pH (33.8%). When VFAs are using as carbon source for oleaginous yeasts, in some studies slightly acidic pH of 5.6-7 was identified as the optimum although in some other investigations higher pH of about 8 was the optimum one specially in presence of high VFA concentration in the medium. This is due to toxicity effect of high VFA content of the culture medium which was alleviated when

higher pH was applied. Results demonstrated the optimum pH varies based on yeast strain and type of substrate in the growth culture. In a culture medium contains glucose, the acidic pH of 5-6 is the optimum for lipid accumulation (Gao et al., 2020).

Agitation was evaluated in the same study which demonstrated 14% increase in lipid yield and also 48% improve in lipid content by decrease agitation from 300 to 100 rpm in 5 days batch experiments. Studies revealed the enhance of microbial lipid production by decreasing the dissolved oxygen concentration. High dissolved oxygen concentration resulted in metabolic shift and decrease lipid generation (Papanikolaou et al., 2007).

2.4.5 Nutrients

Micronutrients play essential role in metabolic actions of oleaginous yeast. Trace amount of some metal salts, sulfur, phosphorus, magnesium, zinc, potassium, sodium, molybdate, cobalt, etc. is sufficient to enhance growth or lipid synthesis of the cell. Wu et al., (2010) investigated the impact of phosphate and sulfur deficiency on lipid production of *R. toruloides*. Lipid content of 62.2% was achieved in C/P molar ratio of 9552. The reason belongs to phosphate interference with chemical oxidative stress that is induced with some minerals. It is shown that sulphate limitation is efficient in lipid synthesis even if high nitrogen was existed in the growth medium (Wu et al., 2011). Hence, some nitrogen rich substrates have potential to be used as feedstock with the aim of lipid generation by oleaginous microorganism. Biomass generation and lipid accumulation can be increased by high magnesium concentration in the medium due to magnesium contribution in different cell activities and enzymatic reactions i.e., association with proteins, phospholipids, and nucleic acids. Nambou et al. (2014) demonstrated the increase of biomass from 1.69 g/L to 6.23 g/L in presence of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ with different concentrations of 0 g/L to 15 g/L. Maximum lipid content of 38% was obtained with 1.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in the medium.

2.4.6 Cultivation mode

Cultivation mode is one of important factors to enhance transformation of carbon source to lipid by the cell. The common cultivation modes applied on *Y. lipolytica* are batch culture, fed batch culture and continuous culture.

The most common mode applied in different studies is batch culture. It is a closed system in which all substrate material and microorganism cells added initially to the system and no addition will be occurred during the fermentation time. Generally, most of the medium optimizations are investigated as batch culture. In a study conducted by Sitepu et al. (2013), a batch culture was used to evaluate a new oleaginous yeast species and identify the effect of nitrogen limitation and fermentation time on biomass growth, lipid storage and fatty acid composition. To optimize the type and quantity of carbon and nitrogen in the medium, environmental conditions such as temperature, pH, oxygen amount, agitation speed, etc., batch culture is conducted as well. In this mode, high initial C/N ratio is needed to improve lipid storage of the cell. If the nitrogen was not rate limiting, the growth continues exponentially but nitrogen limitation reduces the substrate consumption rate. Followingly, the growth decreases and cell begins to lipid production.

In fed-batch culture, substrate is added during fermentation. This is an efficient cultivation mode to achieve high biomass concentration and lipid synthesis. Control of substrate flow rate is essential to optimize the lipid production rate. Feeding strategy can be varied in fed-batch mode. Only one substrate may be added in different fermentation time to maintain the appropriate substrate concentration in the medium. Two or more substrates can be added as well in this cultivation mode. Chen et al. (2017) applied carbon source in an 8 h time interval to increase C/N ratio of the culture to 10, 20 and 45 and obtained high cell concentration of 43.82 g/L with 49.91% lipid content. In another research investigated by Unrean et al. (2017), the biomass concentration generated in fed-batch culture was 23.48 g/L and four times more than the one achieved in batch culture. The lipid content was also increased from 50.6% to 65.9% in that study. Generally, the optimized conditions of a bioprocess identified in batch culture and then applied in fed-batch mode.

Continuous culture mode can be considered as a more effective one due to its constant supplying of substrate to the system. It is possible to provide a constant C/N ratio in the system as an important parameter to enhance lipid biosynthesis by the microorganism. Rakicka et al. (2015) applied a continuous fed-batch culture to identify the lipid generation by *Y. lipolytica* cultivated on glycerol. The fed of 100-450 g/L glycerol with 100 h time interval was applied with C/N ratio of 60. By

increasing glycerol concentration in the medium, the biomass concentration was increased from 32.2 g/L to 67.4 g/L. Moreover, the lipid content was improved from 20% to 37.2% but in glycerol concentration more than 350 g/L the amount of accumulated lipid started to reduce.

Rather generally applied cultivation modes, nowadays some other effective modes are applied as well. One of these specific modes which investigated to enhance cell growth and lipid biosynthesis is two-stage batch cultivation method. In this mode, carbon sources are added to the culture medium in two various stages with the aim of enhance cell proliferation in the growth phase and lipid biosynthesis on lipogenic phase. The growth of cell is completed on an initial carbon source in first stage. In second stage, the carbon source is added to the system after nutrient depletion which leads to improve lipid biosynthesis in the cell significantly. In some studies, this method exploited to obtain higher lipid production by the cell. A two-stage fed batch fermentation system was conducted by Fontanille et al. (2012), in which *Y. lipolytica* grown on glucose following with VFAs. They obtained cell concentration of 31 g/L and lipid content of 40% w/w by applying two different carbon sources. In another study investigated by Pereira et al. (2022), 37% w/w lipid content was achieved by applying two-stage batch culture on *Y. lipolytica* cultivated on volatile fatty acids in a stirred tank bioreactor.

2.4.7 Genetic engineering

In recent years *Y. lipolytica* become an attractive genetic engineering host for researchers to generation of single cell oil. Genetically engineered strains of this oleaginous yeast are capable of producing higher amount of intracellular lipid in comparison with wild type strains. In various studies, different genetic tools are conducted to change and modify the genes of *Y. lipolytica* to improve the lipid production (Gajdos et al., 2017; Lazar et al., 2018; Wang et al., 2020). The enzymes involve the pathways of single cell oil generation are the target components in genetic engineering methods to enhance lipid production. Moreover, lipid degradation as a pathway opposite to lipid biosynthesis, reduces the lipid accumulation. Therefore, deletion of the genes responsible for lipid degradation is another target of genetic engineers (Beopoulos et al., 2008). For example, deleting *MFE2* gene leads to over accumulation of lipid in *Y. lipolytica* (Dulermo and Nicaud, 2011).

Modification of genes are also a great tool to expand the range of utilizable carbon source as substrate by *Y. lipolytica* for TAGs production from renewable substrates. For instance, genes modification improves fructose metabolism by the microbial cell and leads to produce high amount of lipid from molasses (Rakicka et al., 2015). Genetically modified *Y. lipolytica* strains are able to grow greatly on lignocellulosic biomasses and accumulate high amount of lipid (Niehus et al., 2018).

Although genetic engineering techniques enhance lipid production of the cell to a great extent, the high cost and required complex techniques are matter of concern. So, the feasibility of conducting this strategy should be considered carefully.

2.5 Microbial Lipid as a Potential Biofuel Source

Biofuel is recognized as a promising replacement for traditional fuel for different reasons. Using biofuel leads to zero carbon emission, decrease the toxicity of greenhouse gasses and mitigate the climate change. Exploiting the most efficient techniques for promoting the biodiesel generation and its quality will assist to solve the climate issues. In this regard, decrease the usage of fossil fuels is important to fix carbon dioxide level in the atmosphere. Using fossil fuels generate 70% of carbon monoxide and 20% carbon dioxide of atmosphere (Dechambre et al., 2017). Oceans absorb about 26% of atmospheric CO₂ (SCOPE, 2015). Followingly, water temperature increases, and ocean acidification happens. According to Feldman et al. (2015), the reason of greenhouse effect and climate change is anthropogenic emissions. To hinder the global climate change and decrease the emissions, alternatives such as sustainable, green and clear energy is required. Biofuel is considered as a carbon-neutralizing agent through decrease the carbon emission to the atmosphere and is regarded as an alternative for traditional diesel fuel which obtained from sustainable sources like microbial lipids or vegetable oils. Biodiesel from soybean as the most common fuel in the US, can decrease the air pollution and GHGs around 41% in comparison with traditional diesel (Hill et al., 2006). Biodiesel generation from edible plant oils causes increase of food price. On the other hand, close to 85% of biodiesel production cost is related to feedstock price. Hence, an alternative feedstock should be investigated for biodiesel production.

Different technologies and processes are being established for production of biodiesel. Conventional biodiesel or first-generation biodiesel is generated by using edible materials like sugar beet, sugarcane, sorghum, wheat, corn, barely and plants oil (sunflower, canola, and rapeseed). Among these, sugarcane is the major one for biofuel generation. One of the most common biodiesels in the US is soybean biodiesel which decrease emissions by 41% compared to traditional diesel. Although, biodiesel is not considered as an economic energy source as 85% of its cost belongs to feedstock. Biofuels such as ethanol, propanol and butanol are generated from seeds or through valorization of sucrose from sugarcane or sugar beets, or starch-derived sugar by yeast (Wu et al., 2014). Ethanol is the product of C₆ sugars fermentation (mainly glucose) by using yeasts like *S. cerevisiae*. Despite the simplicity of bioethanol generation from sugarcane, increasing the sugar price is an important problem in this process. Corn is another substrate that is used for ethanol production although pre-treatment is required to hydrolyze starch to sugar. From each ton corn, 400 to 450 L ethanol can be produced based on the efficiency of applied technique (Lee and Lavoie, 2013). The process of biodiesel generation differs from ethanol production because it is a chemical process that is based on oil extraction and transesterification. Canola, soybean, and palm oils are common feedstocks for microbial lipid generation. The success of first generation of biofuel is limited by its unsustainability. It has some disadvantages such as its interference with food chain, consumption of edible materials along with water and land use, greenhouse gas emissions and water fouling because of high consumption of fertilizers for crops growth (Phwan et al., 2018, Fairley, 2011). Non-edible substrate such as waste frying oils, animal fats and fats from microorganisms can be applied as alternative for food-based feedstock, but high amount of free fatty acid causes biodiesel with high viscosity (Pinzi et al., 2013).

Second generation or advanced biofuel is generated from wide range of various feedstock especially non-edible biomass in a more sustainable way with the aim of decrease the food and biofuel interference. Applied biomass in production of second-generation biofuel can be divided into three main categories: homogenous, like white wood chips; semi- homogenous, like agricultural waste and non- homogenous such as low-price carbon sources like municipal organic waste (Lavoie et al., 2011). These biomasses are remarkably more economic than sugarcane, corn, vegetable oil, etc., but more complex as well. Thus, the cost of convert the biomass to biodiesel needs to be

low enough and profitability of the process should be considered. For instance, lignocellulosic biomass is gained from various sources like agricultural residues and municipal waste. Complex organic wastes need to be hydrolyzed to simpler organic matters and became consumable for microorganism as feedstock. Investment of an efficient treatment methods to release highest sugar amount from lignocellulosic biomass is a matter of concern.

In third generation biofuel, biofuel is generated from oleaginous microorganisms such as algal biomass or yeasts in which lipid content and lipid productivity of microorganism play an important role here. Microorganisms are cultivated on low-cost substrates which are used as feedstock for the cell. In this approach, firstly biomass is cultivated and then harvested to extract accumulated lipid. It has significant advantages like high growth rate of microorganisms, high productivity, non-edible feedstock, no land requirement, ability to utilize different water resources i.e., fresh, saline and wastewaters and potential of recycling carbon dioxide (Singh et al., 2013).

Biodiesel is considered as a renewable alternative for diesel fuel that is generated from triacylglycerols (TAGs). TAGs are generally found in animal fats or plant oils. A common method to produce biodiesel is transesterification in which fatty acid methyl or ethyl esters and glycerol are formed in a reaction between fat or oil with an alcohol. Usually, ethanol or methanol are used as they are cost effective with rapid reactions that can be accelerate more by a catalyst (Ma and Hanna, 1999). Characteristics of generated biodiesel and its performance such as cetane number, oxidative stability, and viscosity can be changed depend on applied feedstock. Oleaginous yeasts are capable of accumulating 20-70% intracellular lipid in form of TAG. Triacylglycerols are esters of glycerol contain three molecule of fatty acids that are insoluble in water and recognized as high energy reservoirs which can be transformed to fatty acid methyl esters (FAME) or biodiesel. The composition of obtained fatty acid such as their chain length defines the quality of the generated biodiesel. Accumulated lipid by oleaginous yeasts is non-polar like TAGs or triacylglycerols and SEs or sterile esters. This lipid mainly consists of C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (alpha-linolenic acid) (Llamas et al., 2019). The profile of accumulated fatty acids by oleaginous yeast is changed based on the culture medium and applied strain. In general, microbial based

fatty acid composition have the same characteristics with vegetable oils such as sunflower or rapeseed oil (Vasconcelos et al., 2019) which indicates them as an excellent sustainable resource for renewable biodiesel generation.

Figure 2.1 depicts synthetize of single cell oil by oleaginous yeast, which is converted to FAME by transesterification further, to biodiesel production. As it is illustrated, by nitrogen depletion in the culture, activity of AMP deaminase increases, and concentration of adenosine monophosphate (AMP) drops immediately in the cell. Followingly, citrate accumulation was occurred in mitochondria, the citrate is transported into the cytoplasm and oxaloacetate and acetyl-CoA is generated by ATP citrate lyase (ACL).

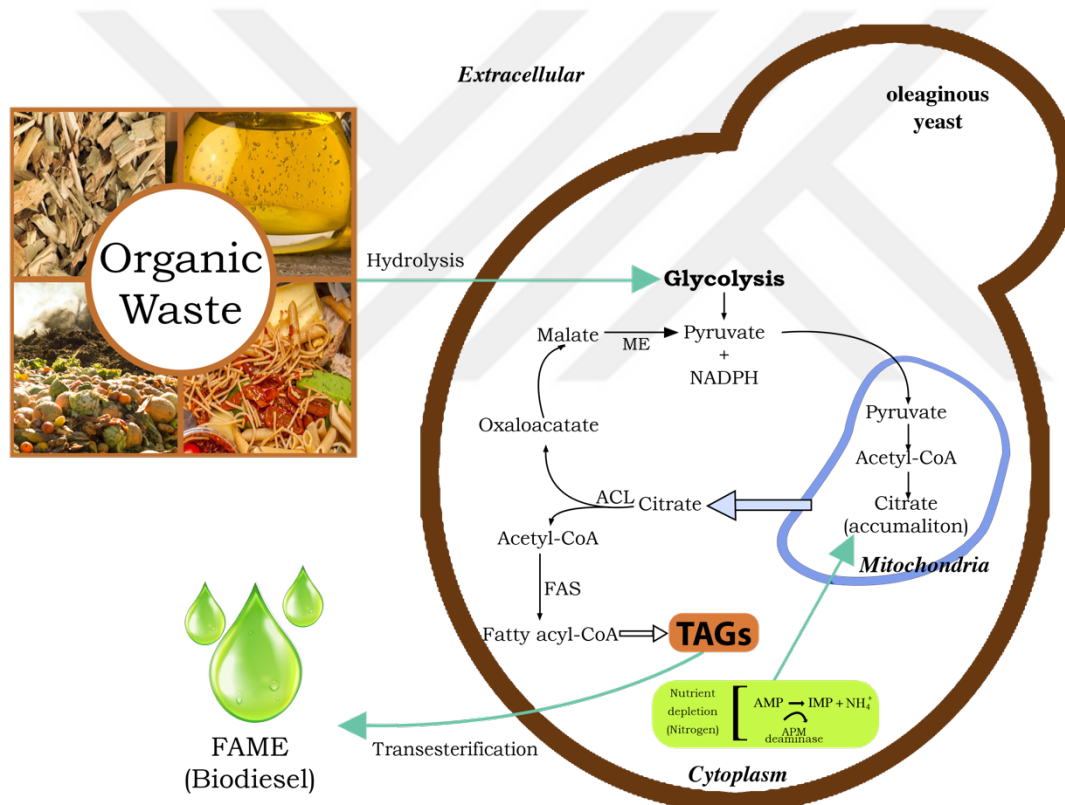


Figure 2.1 : Schematic of triacylglycerides biosynthesis by oleaginous yeast.

The ACL enzyme is the first recognized enzyme that is responsible for different metabolic pathway in oleaginous yeasts. This Acetyl-CoA is used later in microbial oil synthesis and lipid accumulation. In nutrient limitation condition, cell proliferation is ceased, and lipid accumulation is occurred. Lipid storage occurs mainly in stationary phase of oleaginous yeasts (Alvarez et al., 2000) since phospholipids biosynthesis from fatty acids are shifted to TAG biosynthesis (Alvarez and Steinbuchel, 2002).

Additional nutrient substances can promote the substrate composition in a way to enhance biomass and accumulated lipid yield, as well. For instance, in an investigation performed by Kumar et al. (2015), dairy wastewater was used for cultivation of *Rhodococcus opacus*. This wastewater contains carbohydrate, fats, and ammonium. Addition of mineral salts to the medium enhanced the lipid content to 33.3% and the biomass amount increased to 6.6 g/L indicated the effect of mineral salts on improve the efficient utilization of organic matter by the strain. Yeast extract as an organic nitrogen source contains 10% nitrogen, different macro/micronutrients and phosphorus as well. It is known as a suitable supplement for cell growth and lipid generation (Dzurendova et al., 2020). Sriwongchaet et al. (2013) improved biomass concentration from 4.01 to 4.75 g/L, using *Y. lipolytica* JDC 335 and obtained the lipid content of $54.44 \pm 1.01\%$. In another study supplying yeast extract caused generation of 51.8 g/L biomass and 65.1% w/w lipid content using *C. curvatus* O3 (Zhang et al., 2011).

The yeast *Y. lipolytica* can consume different hydrophilic and hydrophobic substrates to generate and store intracellular lipid such as glucose, glycerol, alcohols, fatty acids, alkanes, etc. Glycerol is one of most economic carbon sources because it is generated as a by-product in some industries specially in biodiesel production. By noticeable increase of fuel demand for transportation and decrease of natural reservoirs, biodiesel production is increased as a renewable fuel in recent years, although the high production cost caused the slow rate of its generation. Using glycerol as a by-product is an important option to drop the production cost. During biodiesel production, 10% glycerol is generated. It means that a biodiesel production plant with the ability to produce 30 million gallon/year is going to generate 11,500 tones of pure glycerin (Anand and Saxena, 2011). Therefore, applying a sustainable process to use this valuable raw material is required.

2.6 Food Waste, a Valuable Feed Stock for Biodiesel Production

Microbial lipids have received attention as a promising feedstock for generation of biodiesel in last decades as an eco-friendly alternative for traditional fuel. Biodiesel is the most prevalent type of biofuel in Europe. Applying crops for biodiesel production leads to increase of edible oil price due to utilization of food sources. The most common feedstock for biodiesel generation is plant oil which is different based on

geographical region. In Europe canola and rapeseed oil are used while in North America and Brazil soybean oil is common. Recently by increasing demand of biodiesel production, demand for agricultural land areas is increased as well which leads to competition with crops farms. Microbial lipids as sustainable energy sources can be applied for biodiesel generation with no food competition regarding the fatty acid composition same as plant oils. The major problem regarding large scale application of microbial oil is high cost of processing comparison with plant oil that was estimated around \$2300-\$3400/ton (Braunwald et al., 2014). Nowadays most of research are focusing on developing cost effective methods to achieve microbial lipids. There are different types of microorganisms who can consume various types of low-cost carbon sources and generate microbial lipid. Carbon sources and nutrients available in wastes should be accessible to microorganisms. Therefore, suitable pre-treatment techniques such as hydrolysis needs to be applied.

Food waste is specified as cooked or uncooked food which is wasted in any steps of food manufacturing, transportation, storage, and final consumer. Food waste which generated in pre-consumer stage contains contaminated food, non-edible parts of food or the food that is not according to standard which is discarded during manufacturing and doesn't reach final consumer. Food waste generated by final consumer contains household, hospitals and restaurants waste and composed of rice, beef, peels, cooking oil, etc. EU waste management strategies has changed in recent years. In 2005, over 60% of municipal solid waste (MSW) were discharged in landfills. After 10 years, this amount was decreased to 24%. Additionally, in 2005, the amount of recycled MSW was 10% which was increased to 25% after 10 years. It is planned to enhance the amount of recycled MSW to 60% by 2025 (Eurostat, 2016). A sustainable recovery approach is provided by biorefinery concept in which high value-added chemical is produced from FW as raw material. In biomass-based approach, different products can be obtained i.e., electricity, heat, plastics, biofuels, etc. This technique decreases the greenhouse gasses and the energy costs in comparison with traditional energy production systems.

About 74% of nutrients exist in FW are soluble and don't need a specific refining process. Different FW samples were characterized by Kim et al. (2015) to evaluate characterization of both solid and soluble part. According to this study, 30% of

nitrogen was solubilized and 40% of COD was in liquid phase. Generally, the main carbon source of FW are carbohydrates that have important function on metabolism of applied microorganisms in production of biofuel. Mono and disaccharides are the most favorable substrates for microorganisms because of their biodegradability. Food waste contains mostly high quantity of polysaccharides such as starch and fiber that needs to be hydrolyzed and made consumable for metabolic pathways.

Protein content of FW shows a considerable seasonal change. The amount of nitrogen plays a key role to provide favorable carbon to nitrogen ratio to achieve highest lipid storage by microorganism. On the other hand, excess amount of nitrogen in the medium cause inhibition effect on microorganism. Nitrogen degradation of FW may cause acidification of growth medium as ammonium or ammonia will be generated.

Food waste is one of a main global concern due to its huge generation amount and inappropriate disposal techniques. Additionally, by increasing the world population and energy crisis, dependency to fossil fuel and related environmental problems are increased as well. The biorefinery approach for FW can solve the disposal obstacle and decreases the carbon footprint of it. Compared to other biomass wastes, FW has more significant sustainability in microbial lipid production. Although FW is considered as an advantageous substrate including high number of organic matters, the hydrolysis of its solid part is rate-limiting issue in growth of microorganism. Dark fermentation is a promising biological process which can hydrolyses the organic substrates of FW, converts them to volatile fatty acids and increase soluble COD of the substrate. Food waste with high total COD content is a valuable feedstock for VFA production. The microbial community of the medium affect the performance of dark fermentation process i.e., amount and composition of generated fatty acids. Environmental factors (pH, temperature, etc.) and operational condition like solid retention time (SRT) and hydraulic retention time (HRT) also influence the performance of the system. Different factors affecting VFA production from an organic substrate are:

- Type of bioreactor

Different types of bioreactors are used for VFA production. The FBR (fluidized-bed reactor) is an efficient bioreactor compared to PBR (packed-bed reactor) as there is no clogging problem due to biomass growth in the medium. The CSTR is another appropriate type of reactor which provides complete and constant mixing of the waste and biomass (Conti et al., 2019). There are other types of reactors applied for anaerobic fermentation i.e., up-flow anaerobic sludge blanket (UASB) reactor, two-stage thermophilic anaerobic membrane bioreactor, continuous flow fermentation reactors, anaerobic leach bed reactor, packed-bed biofilm column reactor, etc. Mode of operation is one of important factors in operation of bioreactor. For instance, packed and fluidized-bed reactors and UASB reactor need to be conducted as batch or semi-continuous mode to obtain high VFA yield (Lee et al., 2014).

- Temperature

Temperature has critical role in VFA production during hydrolysis and acidogenesis steps. It not only affects the growth of microorganisms, but also has influence on performance of fermentative bacteria during hydrolysis process. The bacteria responsible for hydrolysis can adopt the different temperatures within the range of 10–60 °C. It should be concerned that higher temperature promotes the solubilization of organic substances, hydrolyses and fermentation process. Research about the impact of temperature on VFA production identified contradictory results. In some studies, temperature of 20–40 °C (mesophilic condition) is reported as the optimum one (Zhou et al., 2018). Although Hao and Wang (2015) assessed thermophilic conditions as the favorable temperature which increases the VFA production about tenfold of mesophilic conditions with no pH change. The reason is in high temperature solubility of hydrocarbons and proteins are increased that leads to enhance hydrolysis process (Zhang et al., 2009). VFA composition also change in different temperatures. Temperatures of 35 °C and 45 °C leads to generation of acetate and propionate, respectively. Whereas at 55 °C the most prevalent one was butyrate (Jiang et al., 2013). He et al. (2012) reported temperature of 55 °C (thermophilic) as the optimum temperature to generate higher amount of VFA.

- Hydraulic retention time (HRT)

HRT is time which soluble compounds is kept inside the reactor. Hydraulic retention time specifies the flow rate which is treated every day. The duration of HRT is important because it should last enough to solubilize the organic matter properly and substrate fermentation. A very high hydraulic retention time has adverse effect on the bioreactor because the VFAs production rate will increase, and the system will be collapsed due to VFA accumulation. On the other hand, in a system with a short HRT, microorganisms with slow growth rate may be washed out. Lim et al. (2008) showed that by improving HRT from 4 to 8 days VFAs production increased, and no further increase was observed by increasing HRT to 12 days. HRT is considered as one of the most important factors to provide a balance between produced VFAs in the bioreactor. No considerable changes were observed in VFA composition through co-fermentation of organic wastes (fruit and vegetable) and waste activated sludge by increasing HRT from 1 to 4 days (Zhou et al., 2018).

- Solid Retention time (SRT)

Solid Retention time is the period in which the biomass is kept in the bioreactor. In a CSTR bioreactor with no biomass recycle, SRT and HRT are the same because biomass and organic substrates remain in the system for the same time. In VFA production from sludge, short SRT is required. Because methanogens have short growth rate and short SRT does not allows them to consume the generated VFAs to produce methane and carbon dioxide in the system. Selected SRT should be long enough to provide efficient hydrolyses in the system. It has also influence on VFAs composition. In a study conducted by Luo et al. (2014), by increasing SRT from 4 to 20 d, acetic acid amount enhanced from 40.6% to 59.5%, while propionic acid amount dropped from 49.4% to 23% that might be due to conversion of propionic acid to acetic acid by acidogens in long SRT. In a study investigated by Yuan et al. (2009), quantity of propionic acid was not changed (16-18%) by increasing SRT from 5 to 10 d. However, acetic acid proportion decrease from 66 to 49%.

- pH

One of the main factors to control VFA generation in fermentation process is pH because most enzymes of the microorganism cell are so sensitive to extreme pH

condition. Hydrolysis and acidogenesis steps are affected by pH during fermentation process. Therefore, selected pH for VFA generation needs to be suitable for these processes as well. Optimum pH for acidogenic microorganisms is 5.5-6.5 while methanogens are active at pH 7 and acidic pH of environment has negative effect on methanogens that leads to VFA accumulation (Liu et al., 2018). Generally, composition of generated VFAs is directly related to the type of applied carbon source. The pH can determine the composition of VFAs as well during acidogenic step. According to Wang et al. (2014), butyric acid was the primary VFA (80%) in anaerobic digestion of FW in pH below 5. When pH was kept unchanged at 5, the predominant one was acetic acid, followed by butyric acid, propionic acid and valeric acid. In another study performed by Begum et al. (2018), acetic acid was reported as predominant VFA at pH 5.5, followed by formic acid, butyric acid and propionic acid. By increasing the pH to 11, butyric acid was the main produced VFA, followed by acetic acid, formic acid and propionic acid. It is demonstrated that by increasing step by step of the pH, the acidogenic fermentation will be improved which leads to enhance the VFA generation in bioreactor.

About 3.7 billion tons of agricultural residues and 1.3 billion tons of food is wasted annually in the world (Pleissner et al., 2016). This amount of FW is equal to one third of global food production. This waste carbon footprint is around 3.3 billion tons of CO₂ emission and annually loss of 936 billion Dollars, exclude seafood (Jouhara et al., 2018). Traditionally, FW is incinerated along with burnable part of municipal wastes for heat generation. Food Waste combustion has negative effects on environment and leads to severe air pollutions along with losing the valuable chemicals. Therefore, a proper FW management is strongly required. Food Waste generally contains carbohydrates such as cellulose, starch, hemicelluloses, proteins, organic acids, lipids, lignin and some inorganic matters. High sugar and protein content of FW make it a promising feedstock for production of different valuable bioproducts i.e., microbial lipid. Market demand for biodiesel production is bigger comparing other biochemicals i.e., enzymes, biodegradable plastics, organic acids, etc. Food waste production is mainly attributed to three main reasons: (i) weak food management system because most of the sectors such as food manufacturers, wholesalers, tourisms, and caterings focus on only customer satisfaction by providing high quality food; (ii) insufficient infrastructure and techniques for food processing

and packaging; (iii) inadequate knowledge of effect of FW on human health and environment. Additionally, food waste can be generated due to break of food supply chain in natural disasters such as earthquake, floods or even bad weather. Buffets in restaurant or hotels are some of main correspondents in FW generation as generally their extra food is going to waste. Figure 2.2 shows the amount of food wasted in the world indicating at least one country from each continent. According to data, the lowest food wasted per capita belongs to the two most populated Asian countries of China and India. Argentina, Australia and UAE have the highest food waste per capita with maximum amount of 363 kg/year in Argentina. The highest annual FW production belongs to China with 89 million tones/year, whereas Singapore shows the lowest amount of 0.8 million tones/year.

The high cost of single cell oil generation from uneconomic substrates like glucose has increased the need for applying different crude renewable substrates such as cheese whey, glycerol, oil palm biomass, sorghum juice and different organic wastes. Tsakona et al. (2014) applied a flour-rich waste hydrolysate in order to obtain lipid through fed-batch cultures using *L. starkeyi* and biomass of 109.8 g/L with 57.8% (w/w) microbial lipid was obtained. In another study conducted by Johnravindar et al. (2018), FW leachate was used as feedstock applying *Rhodotorula glutinis* and *Y. lipolytica*. They achieved 12 g/L biomass with 20% lipid content and 20.9 g/L biomass contains 49% lipid, respectively. Short chain organic acids generated by fermentation of FW is a suitable feedstock for microbial lipid production (Ma et al., 2019). Depending on its source, FW generation takes place in various steps of food supply network: (i) food manufacturing, (ii) logistic and warehouse, (iii) packing food materials, (iv) food distribution, (v) food final destination such as restaurants, household, etc. The FW of catering and restaurants contains high amount of oil, carbohydrate and protein and is suitable substrate for microbial lipid production. Generally, the FW composition in a Western restaurant is about 40–50% (w/w) carbohydrate with 23–29% starch and 18–24% protein (Carmona-Cabello et al., 2020).

Biodiesel generation using algal-derived lipids may cause atmospheric contamination as considerable amount of NO_x is generated along with lipid. According to Bharathiraja et al. 2017, yeast-derived lipids are not contain nitrogen oxides and are

appropriate for biodiesel production. Intracellular lipid and biodiesel which produced from different yeasts varied based on lipid composition and used feedstock i.e., glucose, VFAs, glycerol, bagasse, etc. (Ananthi et al., 2019).

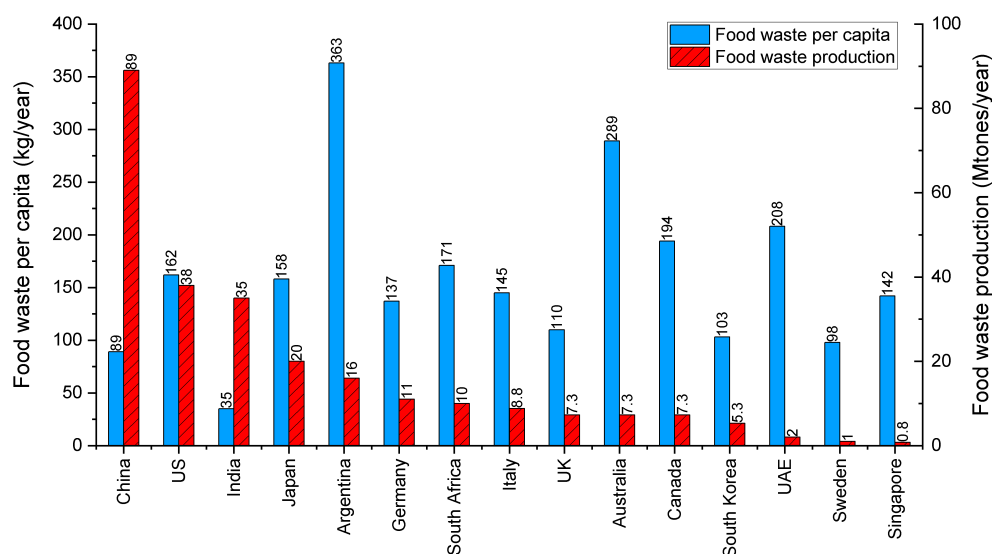


Figure 2.2 : Amount of food waste in selected countries (Pour & Makkawi, 2021)

European biodiesel EN 14214 and American ASTM D6751 standards define appropriate biodiesel characterization which describes its behavior while combustion in engines such as yield, or its physical properties i.e., cetane number (CN), oxidation stability, kinematic viscosity (ν) and cold-weather behavior properties. According to study conducted with Chebbi et al. (2019), produced biodiesel by *Meyerozyma caribbica* MH26779 applying crude glycerol was according to the EN 14214 standard excluding FAME composition and oxidation stability. Soccol et al. (2017) applied the biodiesel generated from lipid produced by *Rhodosporidium toruloides* in diesel engines and great performance was observed when mixing it with diesel fuel. Two-time lower carbon dioxide, 7 times lower carbon monoxide, half of NO_x and less than 0.1 ppm hydrocarbons was observed compared with mix of diesel and soybean oil biofuel. It is expected to biofuels make up more than 30% of the transportation fuel in the world till 2060 (Oh et al., 2018). The important matter in biodiesel commercialization is defining a cost-effective feedstock with a view to sustainable generation of biodiesel. Up to now, in investigations related to microbial lipid accumulation by oleaginous yeast, glucose was the main carbon source. Efficient transformation of glucose to microbial lipid was observed in different

studies (40-70%) under nutrient deficiency condition. However, price of glucose as a substrate is about 60% of biodiesel generation cost (Fei et al., 2011).

The results of different studies demonstrated that amount of FW-derived microbial lipids was under the average level that was because of high amount of protein in the food waste that led to high ammonia nitrogen concentration in the medium after hydrolyses process. This high nitrogen concentration has an adverse effect on lipid accumulation process as the carbon nitrogen ratio decreases in the medium. FW as an unexpensive feedstock for microbial growth is demonstrating a complex chemical matrix. About 74% of nutrients available in food waste are water soluble and there is no need to complicated treatment process. Generally, the main carbonous composition of FW are carbohydrates which is important factor in biomass production and lipid storage of the cell. Source of nitrogen in FW as a necessary parameter for cell growth in bioprocesses is proteins that generate amino acids and peptides through hydrolysis. Lipids also exist in FW which can be metabolized by microorganisms through β -oxidation to generate energy for the cell. Yeasts can utilize the lipid available in growth medium and accumulate it as microbial lipid (Papanikolaou et al., 2011). Rice bran oil, coffee oils, orange peels and brown seaweed oil are some examples of valuable lipophilic materials which are conducted in microbial lipid production. Organic food wastes mostly contain considerable quantity of organic carbon and can be assumed as a great and cheap substrate for microbial lipid generation. On the other hand, the yeast-derived microbial lipid mainly includes C16 and C18 fatty acids that are appropriate for biodiesel application. High concentration of some components such as hydrocarbon, lipid and salt make this waste harmful for the environment. Exploiting FW as a rich and valuable economic organic substrate for growth of oleaginous microorganism is considered as a promising environmentally friendly approach for the purpose of achieving single cell oil as a source of biodiesel. In this concept, new methods should be developed to obtain cost-effective microbial oil by oleaginous microorganisms.

Volatile fatty acids that are known as short chain fatty acids with carbon quantity of C1 to C6, can be generated during fermentation of different organic substrate and are attractive raw materials for microbial growth and lipid generation. According to Fei et al. (2011), 1 ton of food waste derived VFAs cost \$30, less than 10% of price of one

tone of glucose. Different factors can alter the quantity and composition of VFAs generated in fermentation of FW i.e., substrate composition, pH, temperature, and design of reactor. In comparison with other carbon sources, theoretical conversion efficiency of VFAs is higher along with shorter metabolic pathway for intracellular lipid production (Gong et al., 2016). Thus, VFAs generated from FW are considered as a favorable alternative substrate for production of SCO. According to Chang et al. (2010), some oleaginous yeasts and molds have the potential to utilize VFAs as substrate to generate lipid, such as *Y. lipolytica*, *C. curvatus* and *C. albidus*. Fermentation of FW as carbon source to VFAs is significantly more effective (10–40 g/L) compared with fermentation of sewage excess sludge as 2–8 g/L (Yin et al., 2014; Me et al., 2015). In terms of composition of generated VFAs, highest quantity belongs to acetic acid (10–54%), following by propionic acid as 10–54% and butyric acid with 9–46.9%. Acetic acid is considered as an excellent substrate for lipid production due to its short conversion pathway to acetyl-CoA during lipid biosynthesis. High amount of VFA in the medium has adverse effect on biomass growth. This negative effect can be observed in VFA concentration of more than 10 g/L and can be considered as a fatal parameter for microorganism in 20 g/L concentration (Gao et al., 2017).

Y. lipolytica can grow efficiently in medium contain VFAs. However, lipid biosynthesis is increased by adding suitable co-substrates i.e., glucose or glycerol to the medium. The two-stage batch cultivation method can improve the lipid production of oleaginous yeast. In the initial stage, yeast is cultivated in a growth medium contains carbon and nutrient for the purpose of improving cell propagation and increasing biomass density. In second stage, the second carbon source is added to the medium after nutrients completion to enhance lipid synthesis in nutrient limited medium. Nutrient limitation (specially nitrogen) ends the biosynthesis of cell material and inhibit cell proliferation. So, the substrate is converted into lipid through lipid biosynthesis mechanism inside the cell. Two stage cultivation method has been investigated in various research with the aim of enhance lipid accumulation using different oleaginous yeasts (Kitcha et al., 2013; Anschau et al., 2014; Sathiyamoorthi et al., 2019; Pereira et al., 2021).

Different oleaginous microorganisms like *Yarrowia* sp., *Lipomyces* sp., *Cryptococcus* sp., *Rhodotorula* sp. and *Rhodospiridium* sp. have been applied for SCO generation

due to their magnificent capacity to utilize different types of organic carbons and produce lipid inside the cell.

2.7 Economic Feasibility

Basically, oleaginous yeasts have significant privileges over microalgae such as production of high amount of biomass and less sensitivity to environmental condition and contamination (Koutinas et al., 2014). Additionally, high growth rate and easy handling are some of important characteristics of oleaginous yeasts compared molds (Papanikolaou et al., 2011).

The cost of microbial oil generation is estimated between US\$ 1300-9900/tonne oil. This cost is mainly depending on the used feedstock in the system, type of fermentation process, value of generated co-product during bio-oil production i.e., the biomass or generated metabolites, and the production scale.

The main process cost in lipid generation from oleaginous yeast is feedstock or organic carbon cost. To generate 1 tonne of microbial lipid, 4-5 tons of sugar is needed even if the yeast with highest lipid production yield was used. If the source of sugar was sugarcane or corn, it would cost approximately US\$ 430/tonne oil (Caspeta and Nielsen, 2013). Lignocellulosic biomass has lower cost of around US\$ 50-60/tonne oil. This cost doesn't include the pre-treatment cost of lignocellulosic substrate (Humbird et al., 2011). Agro-industrial and industrial wastes such as glycerol, whey permeate, different wastewaters, food waste and sewage sludge are another possible feedstock which are suitable for lipid accumulation by microorganisms. If wastes required further treatment before disposal, it considered as negative cost in terms of applying as feedstock for biodiesel generation.

Karamerou et al. (2021) identified a model to design a biological reactor for microbial lipid generation with cheap carbon source that theoretically has the lowest price of US\$ 1200/tonne oil. The most important factor affects the capital cost is type of bioreactor. Bioconversion of substrate to microbial lipid is occurred in bioreactor that accounts about 90% of equipment cost including the air compressor (Koutinas et al., 2014). Additionally, to improve the economic feasibility of SCO production from yeast, high biomass concentration is needed that followingly needs aeration and

mixing and consequently the operating cost. According to Koutinas et al. (2014), to have a cell productivity of 0.8 g/L/h, the electric consumption of a system consisting of compressor is near to 63% of utility cost of SCO generation. The cost of oil recovery from microbial cell with solvent extraction should be regraded as well, which is close to 13% of production cost (Braunwald et al., 2016).

Generation of different metabolites can reduce the cost of biodiesel generation from microbial oils such as carotenoids, polyols, lipases, emulsifiers, etc. Koutinas et al. (2014), identified that if a biodiesel producer sells the produced metabolites and spent biomass, its benefit could decrease the production cost of SCO to US\$ 1000/tonne. In a hypothetical bioreactor designed by Karamerou et al. (2021) with lowest process cost, if the price of spent biomass was US\$ 1.75/kg it could reduce the cost of SCO generation to about zero. The spent biomass can be applied as animal feed because it contains considerable amount of protein and carbohydrates. Developing an economically viable industrial process for biodiesel generation from SCOs is a matter of concern to meet future demand of energy.

2.8 Challenge and Future Opportunities

To replace the conventional oil with microbial oil, estimated microbial lipid generation cost is very high. Various lipid yields or productivity along with different fatty acid profiles are observed when different wastes are exploited that is due nutrient composition, different inhibitory compounds, type of carbon source, etc. (Abeln et al., 2019). The challenge is to find a proper yeast strain (wild or engineered type) and optimize the process to fill the gaps and improve microbial lipid production. Additionally, economic alternatives of equipment and reactors should be investigated to make the technology of SCO generation as a cost effective one. Brevenue et al. (2016), evaluated the break-even price (the point in which cost, and revenue are equal) as 27% lower when STR system is replaced with open bond for yeast-derived microbial lipid. Regarding high electric consumption of system for agitation and aeration, a balance between yeast productivity and operating cost should be investigated as well.

However, the major obstacle towards the commercialization of biodiesel derived from microbial lipid is its production cost and more investigations of process design,

product and marketing would improve the vital role of oleaginous yeast in biodiesel production processes.





3. MATERIALS AND METHODS

3.1 Food Waste Processing and Dark Fermentation

3.1.1 Collection and preparation of food waste

The FW was obtained from refectory of Istanbul Technical University. It contained cooked and uncooked food with the major components of beans, chicken, beef, bread, rice, and vegetables. Wastes were collected daily, grinded at the same day by blender and dried at 70 °C for 2-3 days. Dried wastes were passed through 3 mm filter and the parts less than 3 mm were collected. Filtered dry waste was stored at 4 °C for using in fermentation process. Figure 3.1 shows different steps of food waste preparation.



Figure 3.1 : Food waste preparation.

3.1.2 Dark fermentation experimental setup

Bottles with total volume of 1 L were used in fermentation process. Rubber stopper and aluminum cap was used to close the bottles in anaerobic batch experiment. The active volume of each bottle was 750 ml, contains 10% dry FW and remaining as tap water and anaerobic inoculum. Two types of different microorganisms were applied in two different individual sets with the aim of evaluate the impact on solubilization

of dry FW: (a) cow manure; (b) rumen microorganisms which obtain directly from sheep stomach. SRT of 5 days was selected and pH was controlled and adjusted at 7. The bottles were incubated in temperature of 55 °C. Feeding solution with the same composition of bottle contents was prepared and 150 ml of each bottle was replaced every day with feeding solution. Figure 3.2 shows the experimental setup of dark fermentation process.



Figure 3.2 : Setup of dark fermentation process.

The bottles were controlled in terms of methane generation to be sure that there is no conversion of organic matter to methane gas. This test was applying by using 20% KOH solution. Samples were collected from input and output of fermentation step to measure different parameters such as total COD, SCOD, ammonia, TKN, total phosphorus, ortho phosphate and pH. The soluble COD of bottles was controlled every day to identify the impact of fermentation on degradation and solubilization of organic matter. Output of fermentation step was collected, filtered, and applied as growth medium for cultivation of *Y. lipolytica* later. The output was autoclaved at 121 °C and 15 psi for 15 min before cultivation to get rid of any other microorganism that may exist.

3.2 Inoculum Preparation

Y. lipolytica strain MUCL 28849 was gained from BCCM/MUCL (Agro) Industrial Fungi & Yeasts Collection, Belgium. The strain was kept at 4 °C on potato dextrose agar and was sub-cultured on YPD medium weekly. YPD medium consists of 10 g/L yeast extract, 20 g/L d-glucose and 20 g/L peptone. The medium was autoclaved at 121 °C and 15 psi for 15 min. Analytical grade chemicals was obtained from Sigma-Aldrich or Merck. The inoculum was cultivated on YPD medium in 1 L Erlenmeyer flask with active volume of 400 ml. The cultivated medium was incubated at 28 ± 1

°C for 1 day. Aeration and a rotary shaker at 100 rpm were applied. The culture was used for inoculation in all experimental sets.

3.2.1 Experimental set-up

To evaluate the suitability of FFW and optimizing its concentration as substrate for biomass generation and lipid production of *Y. lipolytica*, its ten different concentration was prepared and applied as substrate for growth of the yeast in different individual sets. Distilled water was used for dilution. The 250 ml Erlenmeyer flasks were applied with active volume of 100 ml for batch cultures. The yeast *Y. lipolytica* which was cultivated previously on YPD medium, was used as inoculum. Fermented food waste was used as growth medium with initial pH of 5.44 that was the natural pH of FFW. pH adjustment was not applied during cultivation. Cultures were aerated and a shaking incubator (NB-205, N-BIOTEK) at 100 rpm was applied to provide completely mixed condition during incubation. Samples were collected every day to determine cell growth. The yeast growth was monitored by measuring optical density at 600 nm (OD_{600}) with a spectrophotometer device (SmartSpec Plus, Bio-Rad). All glassware and equipment were sterilized in autoclave at 121 °C for 20 minutes. One step acclimation was applied and thereafter batch experiments were conducted as duplicate. Experimental set is depicted in Figure 3.3.



Figure 3.3 : Fermented food waste cultivated with *Y. lipolytica*.

3.2.2 Analytical Methods

Nitrogen as Total Kjeldahl Nitrogen (TKN), Ammonia (NH₃-N), Phosphorus as Total Phosphorus (TP) and Orthophosphate (PO₄³⁻) were measured according to Standard Methods (APHA, 2012). In SCOD analyses, sample was passed through 0.45 µm Millipore PVDF filter and Standard method was used for analyses. The pH measurement was carried out with a pH meter (Thermo Orion 720A). To determine cell dry weight of yeast culture, 5 ml of broth was passed through 0.45 µm filter that was washed, dried and weighed previously. Vacuum pump was used during filtration. The sample was dried at 550 °C and the constant weight of the cell was obtained.

The total organic carbon (TOC) was evaluated with a Shimadzu TOC-VWP analyzer with an ASI-V autosampler. Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Perkin Elmer, Optima 7000) was used to investigate the concentrations of Ca, Mg, K, Si, Fe, Mn and Al in FFW medium.

3.3 Growth and Lipid Accumulation of *Y. lipolytica* on Fermented Food Waste with Different COD/TKN Ratios and Carbon Sources

3.3.1 Experimental setup

250 ml Erlenmeyer flask with active volume of 100 ml was applied in all experimental sets. Fermented food waste was applied as carbon source and *Y. lipolytica* cultivated on YPD medium was used as inoculum. Aeration was applied and shaking incubator (NB-205, N-BIOTEK) was used at 100 rpm. According to literature, agitation of 100 rpm improves intracellular lipid accumulation compared with other agitation speed (Unrean et al. 2017). The flasks contain inoculated medium were incubated at 28 ± 1 °C for 96 h. The pH for all culture flasks was 5.44 and was not adjusted during cultivation. Growth of the strain was monitored every day as optical density by spectrophotometer device (SmartSpec Plus, Bio-Rad). Fermented food waste along with all glassware were sterilized at 121 °C for 15 and 20 min, respectively. The cultivated flasks are illustrated in Figure 3.4.



Figure 3.4 : Incubation of *Y. lipolytica* cultivated on fermented food waste.

Three different carbon sources as second substrate and five different COD/TKN ratio was selected. Samples were collected at early stationary phase to evaluate SCOD and TKN concentration after passing through 0.45 μm filter. The COD/TKN ratio of the medium was calculated, and second carbon source was added to the system at early stationary phase based on calculated COD/TKN ratio. The purpose of adding second carbon source was increase COD concentration in the medium and obtain favorable COD/TKN ratio. Then, 48 h after addition of second carbon source the biomass was collected from each experimental set and accumulated lipid and generated biomass was measured. Each carbon source was applied in an individual set.

3.3.2 Analytical methods

In order to measure SCOD and TKN, collected samples were filtered (0.45 μm Millipore PVDF filter) and experiment were performed according to Standard method (APHA, 2012).

To assess biomass concentration of the yeast culture, 5 ml of broth was passed through a pre-washed, dried and weighed nitrocellulose filter (0.45 μm). Vacuum pump was

used during filtration. The sample was dried at 550 °C and the constant weight of the cell was obtained. The pH was measured using pH meter (Thermo Orion 720A).

3.3.3 Microbial lipid extraction and quantification

Quantification of lipid content of the cell was according to Phospho-vanillin method in lyophilized cell (Izard et al., 2003; Wang et al., 2009). The yeast cells were separated from culture medium by centrifuge. The PBS buffer solution was applied for washing the biomass. The cells were lyophilized by freeze dryer device and lyophilized cells were disrupted by bead beating technique in high-speed cell disruptor (Disruptor Genie, Scientific Industries, SI-D236).

2 ml concentrated sulfuric acid (98%) was added to disrupted cells in glass tubes and tubes were heated for 10 min on 100 °C. Thereafter, the glass tubes were cooled down in room temperature for 10 minutes. After adding 4 ml of the freshly prepared vanillin-phosphoric acid reagent to the samples, the tubes were incubated for 15 min in 37 °C. Then samples were cooled down for 10 min. A pink color was generated by reaction between phosphor-vanillin reagent and lipid and the intensity was measured at 530 nm using spectrophotometer (SmartSpec Plus, BioRad).

The Phospho-vanillin method was conducted in various studies to quantify intracellular lipid (Frings et al., 1970; Turki et al., 2010; Chen et al., 2018; Díaz et al., 2018; Ananthi et al., 2019; Hashem et al., 2020).

3.3.4 Transesterification and fatty acid analyses

In situ transesterification was conducted to transform the accumulated microbial lipid into fatty acid methyl ester (FAME). Chloroform, methanol, and methanolic hydrochloric acid was used. Methyl tridecanoate (C13:0 ME) was applied as internal standard and hexane was used for phase separation. Lyophilized yeast biomass was used for transesterification. 20 µL internal standard, 200 µL chloroform-methanol (2:1, v/v) and 300 µL methanolic hydrochloric acid were added to yeast biomass in a glass tube. Tubes were incubated at 85 °C for 1 h. After incubation, 1 ml of hexane was added to the tubes to provide phase separation and the phase contains methyl ester was used to evaluate fatty acid profile by gas chromatography (GC) device (GC- 2010, Shimadzu, Japan). The device was equipped with a flame-ionization detector with a

100 m × 0.25 mm inner diameter and film thickness of 0.2 µm TR-CN100 capillary column. The carrier gas was helium at 30 ml/min. The hydrogen gas and air had flow rates of 40 ml/min and 400 ml/min, respectively. The temperature of both detector and injector were 260 °C.

3.4 Effect of Yeast Extract, Iron and Trace elements on Biomass Growth and Lipid Accumulation of *Y. lipolytica*

3.4.1 Experimental design

Different concentrations of iron sulphate (10, 50, 100 and 150 mg/L), yeast extract (10, 100, 1000 and 10000 mg/L) and trace elements solution (0.5, 1, 2.5 and 5 ml/L) were supplemented to batch cultures in individual sets contain FFW as initial carbon source. The 250 ml Erlenmeyer flasks with active volume of 100 ml were used and *Y. lipolytica* cultivated on YPD medium was used as inoculum. Aeration was applied and shaking incubator (NB-205, N-BIOTEK) was used at 100 rpm. The flasks contain inoculated medium were incubated at 28 ± 1 °C for 48 h. The cell growth was measured every day and at the end of growth, the samples were collected to measure the biomass concentration and lipid content of the microbial cell.

Stock solutions of yeast extract, iron sulphate and trace elements were prepared by using distilled water. The stock solutions were filtered (0.45 µm) for sterilization. The trace elements solution included: (g/L) CaCl_2 3.6 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.75 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.13 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.5 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.13 g and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ 0.17 g.

3.4.2 Analytical methods

To assess biomass concentration of the yeast culture, 5 ml of broth was passed through a pre-washed, dried and weighed filter (nitrocellulose, 0.45 µm). Vacuum pump was used during filtration. Cells were dried at 550 °C and the cell constant weight was measured. Lipid quantification was performed by using Phospho-vanillin method. Lyophilized yeast cells were applied. The collected biomass was washed with PBS buffer solution previously. The bead beating technique was used to disrupt lyophilized cell walls.

For lipid quantification, 2 ml concentrated sulfuric acid (98%) was added to disrupted cells in glass tubes and tubes were incubated for 10 min in 100 °C. Thereafter, the glass tubes were cooled down in room temperature for 10 minutes. After adding 4 ml of the freshly prepared vanillin-phosphoric acid reagent to the samples, the tubes were incubated for 15 min in 37 °C. Then samples were cooled down for 10 min. The intensity of generated pink color was measured by spectrophotometer at 530 nm.

3.5 Growth and Lipid Accumulation of *Y. lipolytica* on Fermented Food Waste Along with Glycerol, Yeast Extract, Iron and Trace elements

3.5.1 Experimental design

Different concentrations of iron sulphate, yeast extract and trace elements solution were supplemented to growth medium contains FFW as initial substrate and glycerol as the optimal second carbon source identified in previous section. Different COD/TKN ratios were applied. Stock solutions of yeast extract, iron sulphate and trace elements were prepared, and 250 ml Erlenmeyer flask was used in all batch cultures with working volume of 100 ml.

Fermented FW was applied as carbon source and *Y. lipolytica* cultivated on YPD medium was applied as inoculum. Aeration was applied and shaking incubator (NB-205, N-BIOTEK) was used at 100 rpm. The flasks contain inoculated medium were incubated at 28 ± 1 °C for 96 h. The initial pH for all culture flasks was 5.44. The pH was not adjusted during cultivation. The yeast extract, iron sulphate and trace elements solution were supplied to the batch cultures at $t = 0$ and the glycerol was added at early stationary phase. The growth was monitored as OD by spectrophotometer device and the samples were collected at early stationary phase. TKN and SCOD of filtered samples (0.45 μm) were measured. Glycerol as second carbon source was added to each experimental set to increase COD concentration and obtain favorable COD/TKN ratio. The biomass was collected 48 h after glycerol addition. Collected cells were lyophilized by freeze dryer device and lipid content of the lyophilized cells were identified.

Two-stage batch culture was conducted in all experimental sets. In the first stage, growth of *Y. lipolytica* was completed on FFW along with iron sulphate, yeast extract

and trace elements solution. In 2nd stage, glycerol was supplemented to the culture mediums with the aim of increase COD/TKN ratio of the culture and boost lipid storage in the microbial cell.

3.5.2 Analytical methods

To measure SCOD and TKN, collected samples were filtered (0.45 µm Millipore PVDF) and the parameters were measured according to Standard method (APHA, 2012).

To assess biomass concentration of yeast culture, 5 ml of broth was passed through a pre-washed, dried and weighed nitrocellulose filter (0.45 µm) using vacuum pump for filtration. The cell constant weight was obtained after drying the sample at 550 °C. The pH was measured using pH meter device (Thermo Orion 720A).

3.5.3 Microbial lipid extraction and quantification

Quantification of accumulated lipid was according to phospho-vanillin method in lyophilized cell (Izard et al., 2003; Wang et al., 2009). The yeast cells were separated from culture medium by centrifuge. The PBS buffer solution was applied for washing the biomass. The cells were lyophilized by freeze dryer device and lyophilized cells were disrupted by bead beating technique in high-speed cell disruptor (Disruptor Genie, Scientific Industries, SI-D236).

2 ml concentrated sulfuric acid (98%) was added to disrupted cells in glass tubes and tubes were heated for 10 min in 100 °C. Thereafter, the glass tubes were cooled down in room temperature for 10 minutes. After adding 4 ml of the freshly prepared vanillin-phosphoric acid reagent to the samples, the tubes were incubated for 15 min in 37 °C. Then samples were cooled down for 10 min. A pink color was generated by reaction between phosphor-vanillin reagent and lipid and the intensity was measured at 530 nm by spectrophotometer (SmartSpec Plus, BioRad).

3.5.4 Transesterification and fatty acid analyses

The fatty acid composition of accumulated SCO was identified by gas chromatography (GC) device. To transform the microbial lipid into fatty acid methyl ester (FAME), in situ transesterification was applied. Chloroform, methanol, and

methanolic hydrochloric acid was used. For phase separation, hexane was used and methyl tridecanoate (C13:0 ME) was applied as internal standard. Obtained methyl esters was analyzed in GC device (2010, Shimadzu, Japan) with a flame-ionization detector. TR-CN100 capillary column as 100 m $\times$ 0.25 mm inner diameter and film thickness of 0.2 μ m was applied (Teknokroma, Barcelona, Spain). The carrier gas was helium with flow rate of 30 ml/min. Flow rate of hydrogen gas and air were 40 ml/min and 400 ml/min, respectively. Temperature of the detector and injector was 260 °C for both.



4. RESULTS AND DISSCUSION

4.1 Fermented Food Waste Characterization

The output of fermentation process was analyzed in terms of different parameters. As it is illustrated in Table 4.1, the fermented food waste (FFW) contained total COD concentration of 51.255 ± 0.50 g/L and SCOD concentration of the medium was 48.400 ± 0.49 g/L. TKN content of the medium was 0.907 ± 0.01 g/L and low amount of ammonia as 0.028 ± 0.003 g/L was available in FFW. Total phosphorus and orthophosphate concentration of FFW was 0.242 ± 0.008 and 0.143 ± 0.023 g/L, respectively.

Table 4.1 : Fermented food waste characterization.

Parameter	Unit	Value
TCOD	g/L	51.255 ± 0.500
SCOD	g/L	48.400 ± 0.490
TOC	g/L	18.700 ± 0.390
TKN	g/L	0.907 ± 0.010
Ammonia	g/L	0.028 ± 0.003
TP	g/L	0.242 ± 0.008
Ortho P	g/L	0.143 ± 0.023
Ca	ppm	125 ± 7
Mg	ppm	77 ± 5
K	ppm	924 ± 42
Si	ppm	14.68 ± 1
Total Fe	ppm	1.45 ± 0.02
Mn	ppm	0.68 ± 0.02
Al	ppm	0.42 ± 0.01

4.2 Growth Optimization of *Y. lipolytica* on FFW

The FFW was applied as substrate for *Y. lipolytica* in 10 different concentrations from 10-100% (diluted with distilled water) to estimate the cell growth and biomass generation of *Y. lipolytica* in this medium. As it is depicted in Figure 4.1, by increasing the concentration of FFW from 10% to 100%, growth and biomass generation of *Y. lipolytica* was increased consequently. The lowest growth belonged to the culture

medium contained 10% FFW with OD of 4.23. By increasing the concentration of FFW to 20%, the OD reached to 4.60. This value was equal to 5.39, 6.18, 8.15, 9.137, 15.44, 17.11 and 30.63 for FFW concentrations of 30, 40, 50, 60, 70, 80 and 90%, respectively. Measured OD value reached to its maximum of 38.11 in medium contained 100% FFW with no dilution.

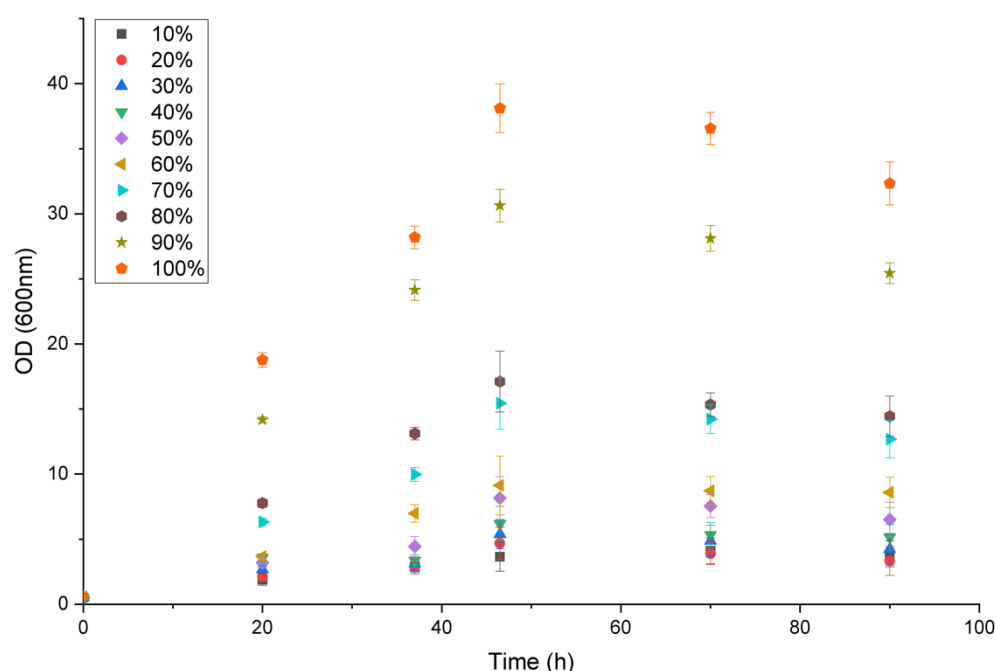


Figure 4.1 : Growth of *Y. lipolytica* on different concentrations of fermented food waste.

4.3 Intracellular Lipid and Microbial Growth in Different Fermentation Times

Microorganisms start to lipid accumulation in stationary phase of growth in presence of suitable metabolized compound in the medium such as monosaccharide, polysaccharide, etc. (Carsanba et al., 2020). Studies conducted by Leiva-Candia et al. (2014) and Qin et al. (2017) revealed that *Y. lipolytica* is able to accumulate different quantity of intracellular lipid with various fatty acid composition based on applied substrate in growth medium. This oleaginous yeast has great potential for biomass growth, biosynthesis and storage of lipid on various carbon sources like glucose, alkanes, alcohols, etc. (Ratledge & Wynn, 2002). Lipid content and biomass generation of *Y. lipolytica* on FFW and YPD as the optimum medium for growth of yeast is presented in Figure 4.2. As it is shown, at the end of log phase of growth, the biomass generation was 9.63 ± 0.24 and 8.31 ± 0.51 g/L which decreased by time to

8.34 $\pm$ 0.82 and 7.1 $\pm$ 0.34 in YPD and FFW, respectively. The accumulated lipid was higher as well at the end of log phase as 21.54 $\pm$ 1.4 and 19.50 $\pm$ 0.50% w/w which decreased to 14.97 $\pm$ 0.51 and 15.52 $\pm$ 0.31% w/w for YPD and FFW medium, respectively. The biomass and lipid reduction are occurred when the substrate uptake rate in the medium decreases by the cell and microorganism faces carbon and nutrient deficiency. Therefore, the cell compensates inadequate materials by biodegradation of accumulated lipid that will end with cell hydrolyses, consequently.

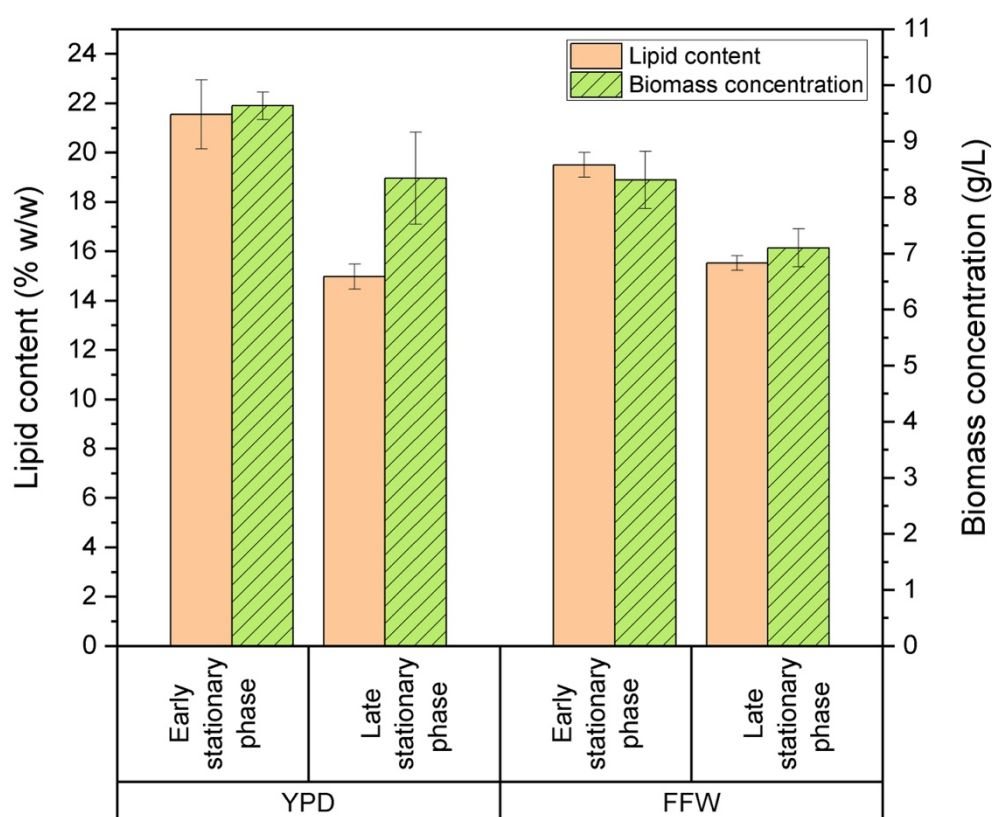


Figure 4.2 : Changes of accumulated lipid and biomass concentration of *Y. lipolytica* cultivated on YPD and FFW by time.

Lipid bodies inside the cell can be visualized by confocal microscopy. Biomass samples were collected from YPD medium and bodipy staining method was applied on the cells. Figure 4.3 shows the microscope image of SCOs in *Y. lipolytica*.

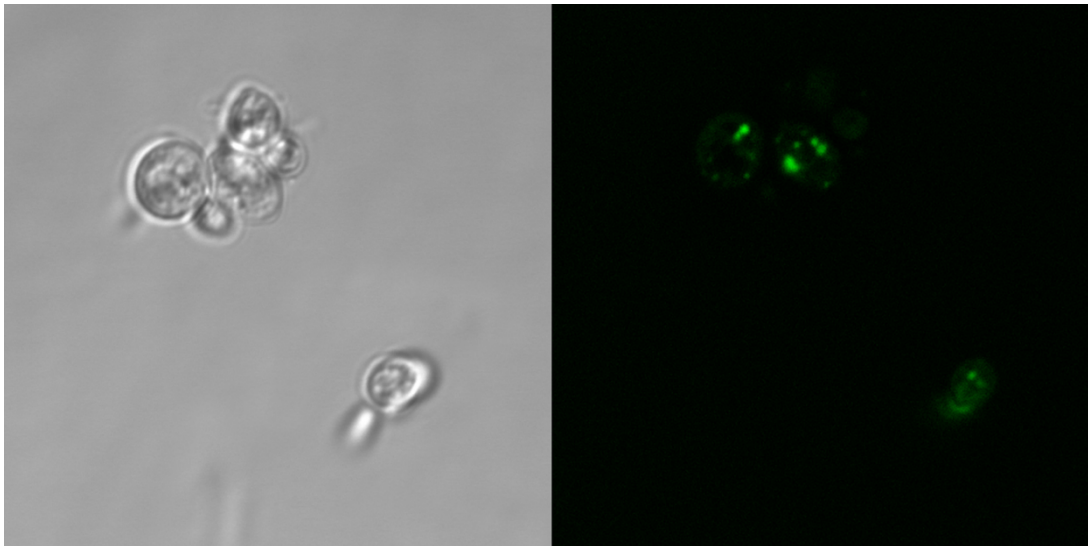


Figure 4.3 : Confocal microscope image of yeast cells and lipid droplets.

4.4 Optimization of Lipid Accumulation of *Y. lipolytica* Growing on Fermented Food Waste with Different COD/TKN Ratios and Carbon Sources

4.4.1 Microbial growth

The output of FFW was applied as carbon substrate for growth and lipid storage of strain. Effect of three different carbon sources i.e., glucose, potassium acetate and glycerol along with different COD/TKN ratios on microbial lipid accumulation by this oleaginous yeast was evaluated. Initial pH of the medium was 5.44, appropriate for lipid biosynthesis (Zhang et al., 2019) and pH adjustment was not applied during experiment. All batch experiments were inoculated with pre-cultured inoculum grown on YPD medium previously. Carbon concentration and nitrogen limitation are two factors that play important role in lipid accumulation of oleaginous yeasts (Ratledge C., 2004; Christophe et al., 2012). After growth of the strain on FFW as initial carbon source, the SCOD and TKN concentration of the medium was measured at early stationary phase. The selected carbon sources were added to the medium in individual sets at early stationary phase to obtain COD/TKN ratios of 75, 100, 125, 150 and 175. Growth of *Y. lipolytica* based on optical density (OD 600nm) in each individual set is shown in Figure 4.4.

According to Figure 4.4 (a), in control medium contain only FFW, growth of yeast cells reached to plateau after 48 h with the maximum OD of 36.35. By increasing the ratio to 75 after supplying different carbon sources, the maximum OD of cultures

contain glucose, glycerol and potassium acetate were 41.2, 35.46 and 39.33, respectively indicated medium contains glucose had the higher growth of yeast. Growth in the medium contains potassium acetate was slightly lower than medium with glucose and the lowest growth belonged to the medium contains glycerol (Figure 4.4 (b)).

In ratio 100, the considerable increase of OD was observed, and biomass growth of the mediums showed OD of 61.12, 41.78 and 81.88 in the mediums contain glucose, glycerol, and potassium acetate, respectively. Increase of OD in medium contain glucose indicated the positive effect of higher concentration of this substrate on growth of the strain. Medium contain potassium acetate showed great increase of OD from 39.33 in ratio 75 to 81.88 in ratio 100 illustrated the high growth of the cell. Among all applied cultivation sets, this was the highest OD that indicated the impact of potassium acetate on biomass growth of *Y. lipolytica* in ratio 100 (Figure 4.4 (c)).

The Figure 4.4 (d) illustrates that by increasing the COD/TKN to 125, the biomass growth in the mediums with glucose and potassium acetate had the same OD of 70 and in the medium with glycerol growth reached to OD of 52.77. Medium contains glucose had higher cell growth compared COD/TKN 100, as the OD was increased from 61.12 to 70. However, increasing the potassium acetate concentration in ratio 125 didn't increase growth of the strain because the OD of 81.88 in COD/TKN 100 was dropped to 70 in COD/TKN 125. In culture medium contains glycerol, the OD of 41.78 in ratio 100 was increased to 52.77, showed the enhance of biomass growth by increasing glycerol concentration.

In higher COD/TKN ratios of 150 and 175, the batch cultures were supplied only with glycerol because as it will be demonstrated in next section, among applied experimental sets the highest lipid content of the cell was always belonged to the mediums contain glycerol. According to Figures 4.4 (e) and 4.4 (f), in the mediums with COD/TKN over 125 the OD started to decrease and dropped to 40.99 and 49.49 in ratio 150 and 175, respectively. This indicated the substrate inhibition in these ratios caused by high glycerol concentration in the culture mediums which had negative effect on microbial growth and cell proliferation due to high carbon source concentration.

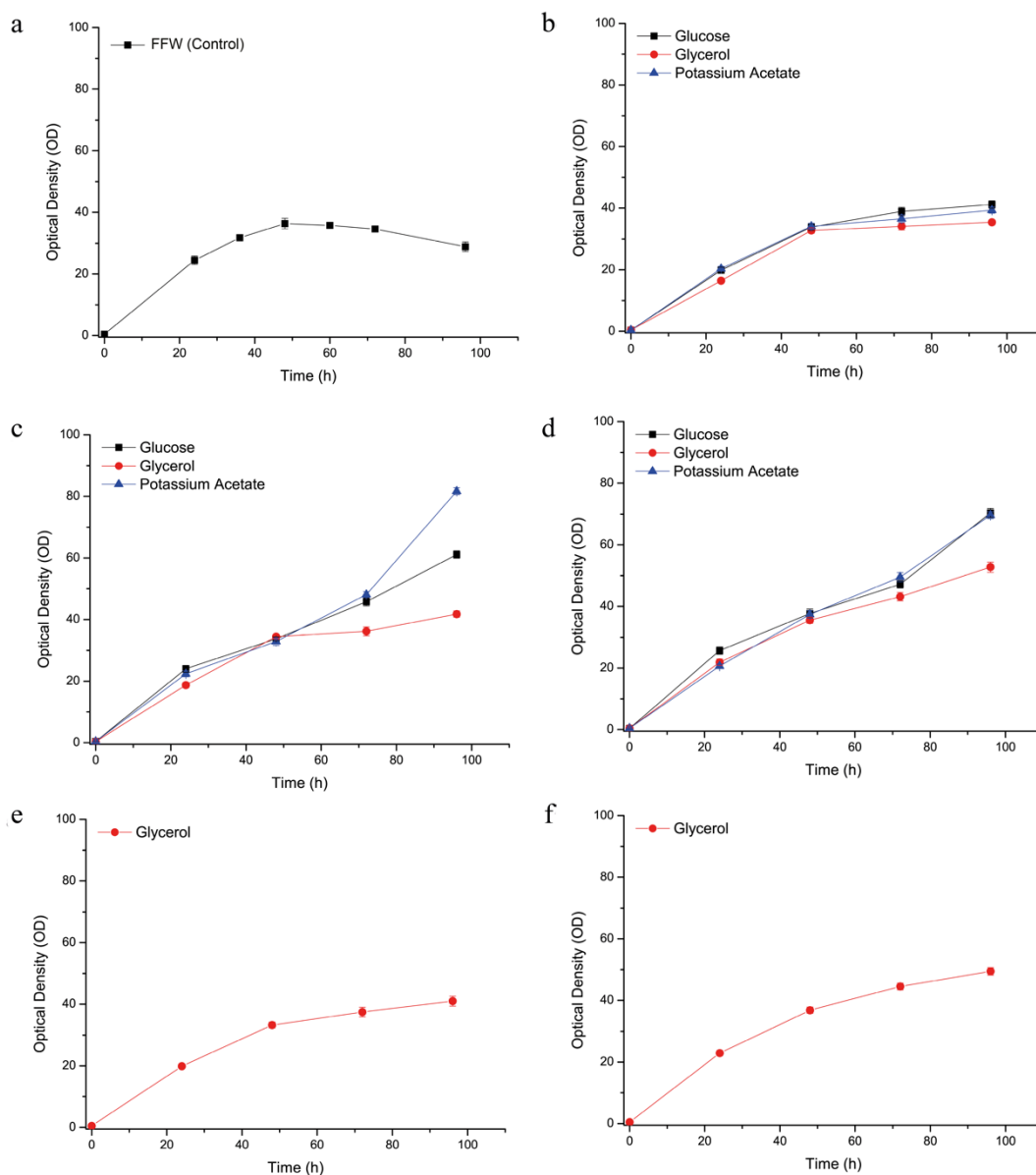


Figure 4.4 : Growth of *Y. lipolytica* on FFW in two stage batch culture: (a) FFW, (b) COD/TKN 75, (c) COD/TKN 100, (d) COD/TKN 125, (e) COD/TKN 150, (f) COD/TKN 175.

4.4.2 Microbial lipid accumulation and biomass generation

Two stage batch cultivation strategy was performed to increase biomass and lipid generation of the strain. In the initial stage, growth of the yeast was completed in the first 48 h of incubation. In this stage, a significant portion of initial carbon source (FFW) was consumed, and nitrogen concentration of the medium was exhausted. In the second stage, lipid accumulation was occurred by adding carbon sources to the FFW. Therefore, growth of *Y. lipolytica* with low amount of lipid accumulation in first stage was completed by higher lipid accumulation of the cell under controlled COD/

TKN in second stage. The amount of carbon added in each experimental set, biomass growth and microbial lipid content of all conducted sets are shown in Table 4.2.

As it is obvious from the results, in control set with only FFW, 8.31 ± 0.51 g/L biomass generated which contain $19.50 \pm 0.5\%$ w/w lipid. The same result was reported by Gao et al. (2017) in which 18.23% lipid was accumulated by *Y. lipolytica* consuming FW-derived VFAs as substrate. By increasing the COD/TKN ratio to 75 by supplying different carbon sources, generated biomass in the first stage (8.31 ± 0.51 g/L) increased to 9.50 ± 0.37 , 8.40 ± 0.72 and 9.32 ± 0.12 g/L in the second stage in the mediums contain glucose, glycerol and potassium acetate, respectively. The lipid content of the mediums with glycerol and potassium acetate were higher as 34.2 ± 1.12 and $33.2 \pm 1.59\%$ compared to medium with glucose with $26.7 \pm 0.5\%$ lipid, illustrated that in ratio 75 glucose participated in cell proliferation rather than lipid synthesis. In an investigation performed by Pereira et al. (2021), the same amount of lipid content of $26.7 \pm 1.6\%$ was achieved that represented lipid concentration of 3.5 ± 0.2 g/L in two-stage batch culture applying *Y. lipolytica*. In that study during the first stage, biomass growth on 20 g/L glucose took place and in the second stage, 18 g/L VFA was supplied to the growth medium. The same amount of accumulated lipid emphasized the merit of FFW as an economic carbon source against glucose as a high-cost one, because the results showed it is possible to obtain the same lipid content by oleaginous yeast using FFW instead of glucose.

In COD/TKN of 100, the same trend was observed in lipid accumulation of the cell. The lowest lipid accumulation in this ratio was belonged to the medium contains glucose with lipid content of $29.2 \pm 0.28\%$ that showed increase comparing to the ratio 75. Lipid content of the medium contains glycerol was measured as $34.3 \pm 2.16\%$ and not changed compared to lower COD/TKN ratio, whereas lipid content of the medium contains potassium acetate slightly decreased to $31.2 \pm 1.60\%$. In ratio 100 supplying potassium acetate had positive effect on biomass growth and 24.40 ± 2.75 g/L biomass was produced, more than 2.5-fold of that generated in COD/TKN 75. According to results, this is the highest biomass concentration obtained in this study, amongst all conducted experimental sets. High biomass generation using acetate is confirmed by different studies. However, *Y. lipolytica* grows well in VFA, but lipid generation is enhanced by adding some supplements or co-substrates such as glucose or glycerol to

the medium containing acetate (Pereira et al., 2021). As it is illustrated in Figure 4.3C, the highest growth belonged to the medium contain potassium acetate whit OD of more than 81 in COD/TKN 100, indicated the highest biomass generation among applied carbon sources.

Table 4.2 : Amount of supplied carbon sources, biomass growth and lipid content of *Y. lipolytica*.

COD/ TKN	Carbon source	Fermentation time (h)	Carbon added at early stationary phase (g/L COD)	Biomass concentration (g/L)	Lipid content (% w/w)
53	FW (Control)	48	—	8.31±0.51	19.50±0.5
75	FFW + Glucose	96	9.32	9.50±0.37	26.7±0.5
	FFW + Glycerol	96	9.32	8.40±0.72	34.2±1.12
	FFW + Potassium Acetate	96	9.32	9.32±0.12	33.2±1.59
100	FFW + Glucose	96	21.49	14.80±0.25	29.2±0.28
	FFW + Glycerol	96	21.49	10.90±0.75	34.3±2.16
	FFW + Potassium Acetate	96	21.49	24.40±2.75	31.2±1.60
125	FFW + Glucose	96	33.6	18.52±1.97	38.7±0.35
	FFW + Glycerol	96	33.6	12.95±1.95	42.2±1.72
	FFW + Potassium Acetate	96	33.6	17.60±0.75	34.7±3.1
150	FFW + Glycerol	96	45.8	9.77±0.97	36.7±1.3
175	FFW + Glycerol	96	57.9	11.57±0.77	38.1±3.0

Despite efficient transformation of substrates such as glucose or acetate to biomass by oleaginous yeasts, these carbon sources are expensive substrates for intracellular lipid production. Cost of carbon source is about 60% of total biodiesel production cost (Fei et al., 2011). In terms of using glucose with price of \$500/ton as substrate, the process cost will enhance drastically.

By increasing the ratio to 125, the cell growth was enhanced as well. In the medium with glucose, the biomass concentration reached to 18.52 ± 1.97 g/L and the medium with glycerol showed higher cell growth of 12.95 ± 1.95 g/L compared to lower ratio of 100. Conversely, increasing concentration of potassium acetate had adverse effect on biomass generation and decreased it to 17.60 ± 0.75 g/L as it is shown in Table 4.2. That is due to substrate inhibition induced by high VFA concentration which varies based on the strain. Though, compared with other VFAs, acetate is a favorable one because of less inhibitory effect on biomass growth (Fontanille et al., 2012).

Lipid content of the cell in the medium contain glucose with COD/TKN 125 was $38.7 \pm 0.35\%$. Most studies have suggested that applying glucose as carbon source can provide high biomass concentration in the medium. Therefore, in different studies glucose was used as substrate during the first stage of cultivation which resulted high cell growth in the culture. In research conducted by Katre et al. (2012), total lipid content of 22–31% was assessed by using various strains of *Y. lipolytica* cultivated on glucose. However, the lipid content of *Y. lipolytica* in current thesis was higher compared with their investigation that was related to the yeast strain, applied two stage cultivation mode, growth on FFW and nutritional factor of medium along with glucose addition. Fontanille et al. (2012) applied two stage cultivation strategy in which the initial carbon source was glucose with concentration of 40 g/L followed by volatile fatty acids addition that led to improve lipid content of the cell from 19.5% to 40.69% with biomass generation of 30.83 g/L. These results indicated considerable increase of lipid content of the cell in the second stage of cultivation during two-stage batch cultivation. According to Table 4.2, lipid content of the cell in the medium contains glycerol increased remarkably and reached to $42.2 \pm 1.72\%$.

According to the results, the highest lipid content among all conducted batch cultures was always achieved with glycerol. This assessment is comparable with the results indicated in the literature by cultivation of *Y. lipolytica* on various carbon sources of

glucose, glycerol and different VFAs. Fontanille et al. (2012) achieved lipid content of 30.76, 36.73 and 38.15% using acetic acid, glucose and glycerol, respectively. High biomass concentration of 42.23 g/L was achieved in glycerol-based medium supplemented with $(\text{NH}_4)_2\text{SO}_4$ indicated the positive effect of ammonium sulphate on cell growth. In current thesis, considerable amount of lipid was generated by applying low-cost carbon sources of FFW and glycerol, compared to the results reported in the literature. Generated lipid content in this thesis was higher than the results of experiment who applied genetically engineered strains that indicate the effect and importance of two-stage batch culture strategy along with rich carbon substrates. A recombinant strain of *Y. lipolytica* was applied by Celińska and Grajek (2013) by using heterologous glycerol dehydratase genes along with related reactivator, alcohol oxidoreductase and glycerol-induced promoter. In medium contains glycerol they obtained microbial lipid content of 40%. Sestric et al. (2014) obtained lower value of $30.9 \pm 1.3\%$ using wild strain and nitrogen limited media contains glycerol. In a study investigated by Dobrowolski et al. (2016), an engineered type of *Y. lipolytica* cultivated on fresh water-based medium with 50 g/L glycerol and 11 g/L biomass and $38.04 \pm 0.79\%$ lipid was obtained. They obtained 25% lipid content with 7.79 g/L biomass by wild type of *Y. lipolytica* in which the strain was grown on glycerol with no addition of supplemental material.

Lipid concentration and lipid content of dry biomass in various COD/TKN ratios is illustrated in Figure 4.5. The lowest lipid concentration was in control medium contain only FFW as 1.62 ± 0.09 g/L. The reason was lower lipid content of biomass and lower biomass concentration in this experimental set compared with other conducted cultures. In all other batch cultures, amount of lipid concentration was between 2.54 and 7.61 g/L. By increasing the COD/TKN ratio to 75 by adding different carbon sources to the system, lipid concentrations of 2.54 ± 0.09 , 3.09 ± 0.04 and 2.87 ± 0.24 were obtained for the mediums contain glucose, potassium acetate and glycerol, respectively. The highest lipid concentration of 7.61 ± 0.85 g/L was achieved in the medium supplied with potassium acetate in ratio 100 that was due to high biomass concentration produced in this culture. In mediums contain glycerol and glucose, 3.74 ± 0.25 and 4.32 ± 0.07 g/L lipid concentration was generated in COD/TKN 100, respectively. In higher COD/TKN 125, lipid concentration of medium contains glucose increased considerably and reached to 7.17 ± 0.76 g/L. Mediums contain

potassium acetate and glycerol had the lipid concentration of 6.11 ± 0.26 and 5.46 ± 0.82 g/L, respectively which showed increase compared with lower COD/TKN ratio. By increasing the ratio to 150 and 175, the lipid concentration in the system was dropped because of high concentration of glycerol in the culture medium that had negative effect on cell proliferation. The lipid concentration in ratios 150 and 175 decreased to 3.59 ± 0.35 and 4.41 ± 0.29 g/L, respectively. The same trend was observed in biomass generation and lipid content of the cell in ratios 150 and 175, because of adverse effect of high substrate content in the medium.

In general, in all batch cultures lipid concentration of the medium was improved by increasing substrate concentration, although it started to decrease in COD/TKNs of above 125.

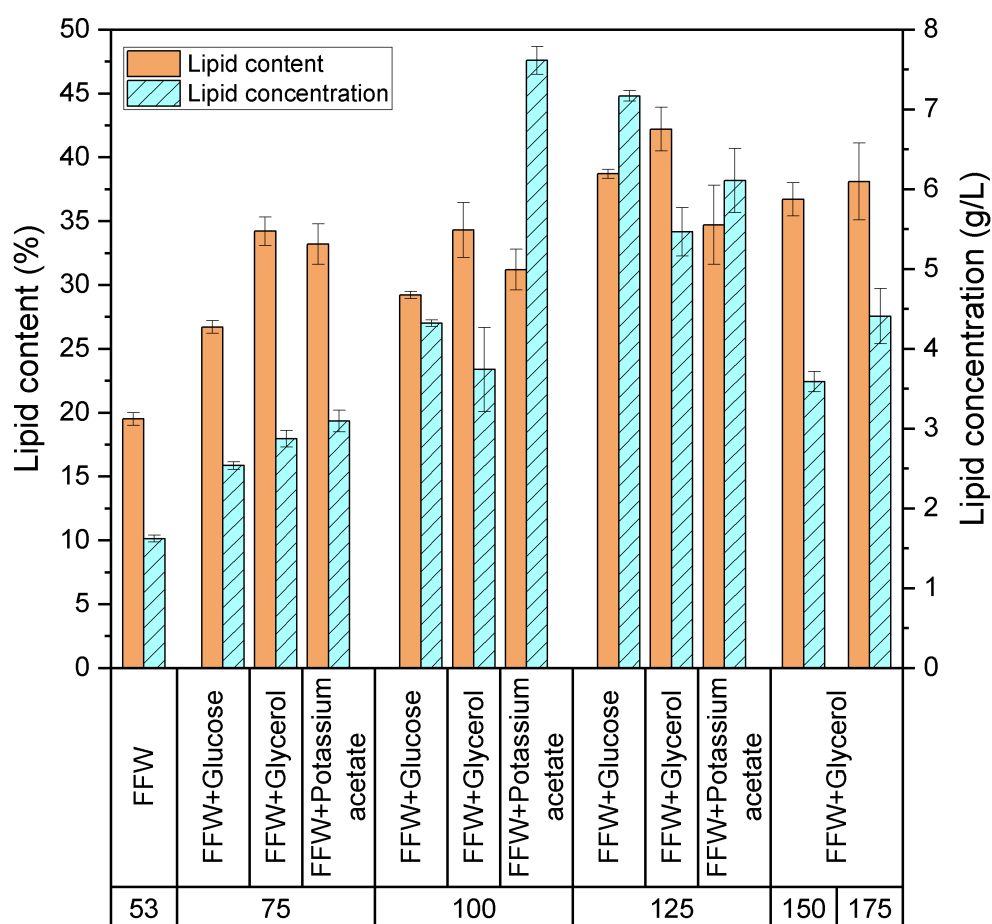


Figure 4.5 : Lipid concentration and lipid content of *Y. lipolytica* growing on different COD/TKN ratios. Error bars represent standard deviation.

The yeast *Y. lipolytica* can consume wide range carbon sources which glycerol is one of the most rich and economic option among them. This carbon source is generated in high quantity these days especially due to worldwide growth of biodiesel production. Different studies in the literature indicated the transformation of glycerol to various secondary metabolites beside SCO₂ during growth of *Y. lipolytica* on glycerol-based medium. Microbial secondary metabolites are low weight molecules which are products of secondary metabolism and generally produced during the late growth phase of the microbial cell. These products are not necessary for the growth of related culture. Generation of different secondary metabolites i.e., polyols (generally erythritol, mannitol and arabitol) or organic acids such as citric acid (CA) are strongly depend on different environmental factors specially pH of the medium. *Y. lipolytica* can survives and grows well under vast pH range but its metabolic pattern is affected by culture pH. Egermeier et al. (2017) investigated the metabolite generation of various strains of *Y. lipolytica* growing on medium contains glycerol under different pHs. According to the results, in the medium with pH of 3.5 glycerol was converted to polyols by *Y. lipolytica*. They assessed pH of 5.5 as the optimum for generation of organic acid by this oleaginous yeast. In another study investigated by Papanikolaou et al. (2017), transformation of glycerol into different secondary metabolites by *Y. lipolytica* and *R. toruloides* in carbon-excess condition was investigated. At the pH between 4.8 and 5.8, polyols were generated by applied strains. Tomaszewska et al. (2012, 2014) indicated production of mannitol and erythritol at pH = 3 by various strains of *Y. lipolytica* in medium contains glycerol and high NaCl concentration while generation of mannitol was decreased by pH increase.

In this thesis, all experimental sets were conducted with no pH adjustment at the beginning or during the yeast fermentation. The initial pH of all cultures was 5.44 which increased by time during yeast growth and lipid biosynthesis and reached to over 8 in the cultures with COD/TKN of lower than 150. This pH value is not suitable for generation of secondary metabolites and excess carbon source is consumed for cell growth and lipid accumulation rather than generation of other metabolites. In experimental sets with COD/ TKN > 125, pH drop was observed during growth and lipid accumulation of the cell and decreased to 4.3. In this case, the carbon source catabolism was changed towards the secondary metabolites production because of high glycerol content in the medium (Beopoulos et al., 2008 and 2009). This metabolic

shift caused production of secondary metabolites such as organic acid in the culture medium and reduction of pH in the batch cultures, consequently.

Growth of various oleaginous strains on glycerol or glucose in nitrogen deficiency medium was investigated in the literature. In a study investigated by Diamantopoulou et al. (2020), different strains of *Y. lipolytica*, *Metschnikowia* sp., *Rhodotorula* sp. and *R. toruloides* were grown on crude glycerol. The strains of *Y. lipolytica* stored maximum lipid of 18.8% inside the cell that decreased to 7.1% after 117 h. The other oleaginous yeasts showed the same behavior as well except *R. toruloides*, even if the substrate was not exhausted and was still available in the medium, but nitrogen was finished. The results demonstrated biosynthesis and accumulation of secondary metabolites like citric acid and mannitol in culture medium. Based on that study, the $\text{pH} \geq 4.5$ was the optimum pH for wild type of *Y. lipolytica* utilizing glycerol as substrate for polyols biosynthesis in nitrogen limited medium, while the citric acid production was performed in pH of 3–3.5. Papanikolaou et al. (2020) conducted an experiment with a new wild type of *Y. lipolytica* LMBF Y-46 growing on nitrogen limited medium contains glycerol and significant amount of polyols was generated under pH of ≥ 4.5 . Additionally, they indicated that generation of polyols begins at $t \approx 50$ h of incubation and reached to its maximum amount after 150 h in the medium contain glycerol in controlled pH.

In some investigations due to biodegradation of lipid and its conversion to secondary metabolites by time, low amount of intracellular lipid was measured (Chatzifragkou et al., 2011; Bellou et al., 2013; Papanikolaou et al., 2013). In a study investigated by Papanikolaou et al. (2020), the amount of accumulated lipid using *Y. lipolytica* LMBF Y-46 in shake flask experiment grown on glycerol was 13–15% w/w. This amount of lipid was decreased to 3–6% w/w by time after more than 170 h and polyols was generated simultaneously with degradation of accumulated lipid.

In this thesis, growth of the yeast was observed at first 48 h of fermentation. After 48 h, the biomass growth reached to its plateau and at that point 19.5% w/w microbial lipid was measured (Table 4.2). Selected carbon sources were added at $t = 48$ h and generated biomass was harvested after 48 h at $t = 96$ h. After this fermentation point, biodegradation of stored lipid was started, and the amount of lipid was dropped by time during stationary phase. This fermentation point was different based on the

applied strain and applied environmental condition. For example, in an investigation conducted by Mathiazhakan et al. (2016), *Y. lipolytica* SKY7 was used as a wild type strain. The substrate was glycerol and experiment was performed in a fermenter. The considerable lipid content of 54.1% w/w was achieved at fermentation time of $t = 72$ h which started to degradation after more than 60 h. Very small quantity of citric acid was measured in that study (2.52 g/L) after 72 h along with lipid biodegradation.

Remarkable quantity of stored microbial lipid was obtained in current thesis at $t = 96$ h before degradation starts in batch cultures which compared well with different published studies (Mathiazhakan et al., 2016; Kumar et al., 2020). It should be noted that amount of accumulated lipid achieved in this thesis was the result of effective application of dual-stage batch culture on the strain and addition of glycerol to the FFW at second stage. According to the results reported in the literature, accumulated lipid of this study was comparable with stored lipid of various species of *Rhodotorula*, *Lipomyces* and *Rhodospiridium* (Yen et al., 2012; Liu et al., 2017; Diamantopoulou et al., 2020).

According to the results, by increasing COD/TKN to 150 and 175, microbial lipid storage decreased to 36.7 ± 1.3 and $38.1 \pm 3.0\%$, respectively. The biomass growth was also lower in COD/TKN ratios higher than 125. This indicated the key role of substrate concentration for efficient cell growth and lipid biosynthesis. It is reported that in medium with very high carbon concentration, the citric acid generated inside mitochondria is excreted out and couldn't be split completely by ATP-citrate lyase. This decreases the lipid generation performance considerably because secretion of excess citrate out of the microbial cell (Beopoulos et al., 2009; Christophe et al., 2012).

According to the results obtained in this thesis, among applied carbon sources glycerol was identified as the most effective one to promote accumulated lipid inside the cell. Therefore, it was applied as the optimal carbon source in the next step of this study.

4.4.3 Metabolic behavior of *Y. lipolytica*

Physiological behavior of *Y. lipolytica* in terms of substrate utilization in FFW medium supplemented with glucose, glycerol and potassium acetate is presented in Table 4.3. Different quantities of selected carbon sources were supplied to the system at $t = 48$ h with the purpose of enhance COD content of the medium. The substrate

consumption increased by increasing lipid accumulation in the cell in experimental sets with COD/TKN of 75, 100 and 125. Moreover, in these ratios the COD consumption were higher than the COD quantity added to the system at $t = 48$. This indicated the FFW was not fully consumed in the initial stage of cultivation and by adding the carbon substrates in second stage along with increasing the incubation time, more FFW was assimilated by *Y. lipolytica*.

Table 4.3 : Amount of carbon source supplied and utilized in different experimental sets.

COD/ TKN	Carbon source	Carbon added at $t = 48$ the beginning of stage 2 (g/L COD)	Carbon consumed at $t = 96$ (g/L COD)
75	Glucose	9.32	23.35
	Potassium acetate	9.32	29.63
	Glycerol	9.32	30.11
100	Glucose	21.49	26.81
	Potassium acetate	21.49	30.45
	Glycerol	21.49	30.54
125	Glucose	33.62	33.57
	Potassium acetate	33.62	31.76
	Glycerol	33.62	38.84
150	Glycerol	45.77	39.83
175	Glycerol	57.92	43.65

By adding carbon sources to the FFW and increase the COD/TKN ratio to 75, the COD consumption in mediums supplemented with glucose, potassium acetate and glycerol was 23.35, 29.63 and 30.11 g/L. The lipid generation was enhanced as well in these mediums by increasing substrate consumption.

By increasing the COD/TKN ratio to 100, the COD consumptions of 26.81, 30.45 and 30.54 g/L was observed in mediums contain glucose, potassium acetate and glycerol, respectively that shows a slight increase compared with COD/TKN 75.

On the other hand, in ratio 125 the carbon consumptions enhanced and COD utilization of 33.57, 31.76 and 38.84 g/L was observed in mediums contain glucose, potassium acetate and glycerol, respectively. Among all conducted sets, the highest carbon

source consumption was belonged to the medium contains glycerol with COD/TKN 125 that caused the highest lipid content of $42.2 \pm 1.72\%$.

By increasing the COD/TKN ratio more than 125 in the medium, the COD consumption was enhanced to 39.83 and 43.65 g/L for ratio 150 and 175, respectively. It should be noticed that the microbial lipid content in ratio 150 and 175 was lower than ratio 125 though the COD consumption was higher. This demonstrated the metabolic shift of *Y. lipolytica* from lipid accumulation to secondary metabolite generation which caused drop of lipid biosynthesis. As it was stated before, pH of the cultures with COD/TKN ratios of lower than 150 was higher than 8 at $t = 96$ h. Whereas, in cultures with COD/TKN 150 and 175 the pH was dropped to under 5. It demonstrated the utilized carbon source was used to synthesize the extra-cellular metabolites by the yeast that decreased pH of the medium. This condition illustrated the impact of high glycerol concentration on generation of secondary metabolites even if the pH was not adjusted during the experiment.

4.4.4 Fatty acid composition of accumulated lipid

Biomass samples were collected 48 h after supplying glucose, glycerol and potassium acetate to the experimental sets. Total lipids were converted into fatty acids by transesterification of lyophilized cell. Fatty acid composition of lipids is presented in Table 4.4. The results indicated the accumulated fatty acids by *Y. lipolytica* grown on FFW was stearic acid (C18:0), linoleic acid (C18:2), pentadecanoic acid (C15:1), palmitic acid (C16:0), and heptadecenoic acid (C17:1) in smaller quantity. This fatty acid composition showed significant similarities with fatty acid profile for different strains of *Y. lipolytica* using glycerol as substrate (André et al., 2009; Chatzifragkou et al., 2011; Sarris et al., 2019).

In control set, *Y. lipolytica* cultivated on FFW as the only carbon source and generated fatty acids consist of 40.65, 31.27, 14.54 and 13.54% of C18:0, C18:2, C16:0 and C15:1, respectively. By supplying different carbon sources, amount of C18:0 was increased in all experimental sets with COD/TKN 75, 100 and 125 except medium contain glucose in ratio 100. It reached to its maximum in ratio 125 with 46.70, 48.17 and 46.03% in cultures contain glycerol, glucose and potassium acetate, respectively. In ratio 150 and 175, quantity of C18:0 decreased to 42.87 and 39.72% of total

generated fatty acid respectively, whereas amount of C18:2 increased to 29.58% in ratio 175 which was the highest amount among experimental sets supplied with different carbon sources. Between all conducted sets, the highest quantity of C18:0 was measured in experimental sets with COD/TKN 125 contain glucose and glycerol with 48.17 and 46.70, respectively. The highest C18:2 was belonged to control set with 31.27% linoleic acid.

Table 4.4 : Fatty acid composition of microbial lipid generated (% w/w) with *Y. lipolytica* cultivated on FFW with different COD/TKN ratios.

COD/ TKN	Carbon source	Pentadecanoic acid (C15:1)	Heptadecenoic acid (C17:1)	Linoleic acid (C18:2)	Palmitic acid (C16:0)	Stearic acid (C18:0)
53	FFW (control)	13.54	-	31.27	14.54	40.65
75	Glycerol	14.90	5.88	23.87	10.49	44.86
	Glucose	17.25	5.93	21.53	14.10	41.19
	Potassium acetate	16.11	4.11	21.77	13.33	44.68
100	Glycerol	15.50	6.45	23.44	10.54	44.06
	Glucose	18.09	6.70	21.05	13.54	40.61
	Potassium acetate	16.51	4.42	20.98	13.19	44.91
125	Glycerol	20.05	12.73	20.53	-	46.70
	Glucose	15.01	5.14	20.11	11.57	48.17
	Potassium acetate	14.61	4.25	22.28	12.83	46.03
150	Glycerol	17.06	10.76	20.81	8.52	42.87
175	Glycerol	19.21	11.49	29.58	-	39.72

Among conducted sets, obtained fatty acids was mostly stearic acid. It could be related to different parameters like phosphate concentration of medium. According to results obtained by Wu et al. (2010), amount of generated C18:0 was 19% in a medium with C/P ratio of 72 mol/moles and by increasing this ratio to 9552, amount of C18:0 reduced to 6% and generated monounsaturated fatty acids were raised. According to the literature, lipid composition of the cell growing in shake-flask, contain more saturated fatty acids in comparison with the one in bioreactor, because of less aeration in shake-flask system (Papanikolaou et al., 2020). This is because of the microorganisms firstly produced saturated fatty acids, then saturated fatty acids are

converted to unsaturated ones by double bonds which formed in desaturation reactions. This reaction is directly related to oxygen and in a bioreactor with high amount of oxygen, conversion of saturated FAME to unsaturated one is enhanced (Davies & Holdsworth, 1992; Rzechonek et al., 2019). The highest content of palmitic acid (C16:0) was observed in control set as 14.54%

Although heptanoic acid (C17:1) was not generated in control set, its amount was increased by adding second carbon sources to the mediums. Its highest amount was measured in medium contains glycerol at COD/TKN 125 as 12.73%.

As it is illustrated in Figure 4.6, in all conducted experimental sets except mediums contain glycerol in COD/TKN 125 and 175 the quantity of saturated fatty acids (SFA) include palmitic acid and stearic acid was more than 50% of all generated fatty acids. In control set, 55.2% of fatty acids consisted of SFA. In COD/TKN 75, adding glucose and glycerol didn't change the amount of SFA and it was measured as 55.4 and 55.3% respectively, whereas in medium contains potassium acetate its increase to 58% was observed. By enhancing the concentration of carbon sources in COD/TKN 100, amount of SFA in the medium contains potassium acetate was measured as 58.1% and mediums contain glycerol and glucose showed around same amount of 54.6 and 54.2%.

Amount of SFA decreased by increasing glycerol concentration in experimental sets contain glycerol. In ratios 125, 150 and 175 SFA amount was 46.7, 51.4 and 39.7%. As it is obvious, its lowest amount was in COD/TKN ratio 175. This indicated the effect of glycerol in higher concentrations on generation of unsaturated fatty acids by *Y. lipolytica*.

In COD/TKN ratios of 75 and 100, amount of monounsaturated fatty acids (MUFA) includes pentadecanoic acid and heptadecanoic acid was higher in experimental sets contain glucose whereas in higher ratios glycerol showed the best performance to enhance the portion of MUFA. It reached to its maximum of 32.8% in ratio 125 with glycerol. The contribution of polyunsaturated fatty acid (PUFA) include linoleic acid in control set was 31.3% which was decreased in other experimental sets with additional carbon sources, although in COD/TKN ratio 175 it reached to 29.6% that

indicated the effect of high glycerol content in the medium on generation of PUFA by *Y. lipolytica*.

Fatty acid composition of generated microbial lipid is crucial parameter in biodiesel generation. Saturated and unsaturated fatty acids have different functions in biodiesel properties. For instance, saturated fatty acids boost cetane number, combustion properties, oxidative stability, and viscosity of produced biodiesel (Ramos et al., 2009). On the other hand, high amount of unsaturated fatty acids mainly PUFAs boost cold flow property of generated fuel but has negative effect on its combustion and oxidative stability (Knothe 2013). The main fatty acid components of biodiesel are C16-C18 (Miao and Wu, 2007), that is according to generated fatty acids in this study.

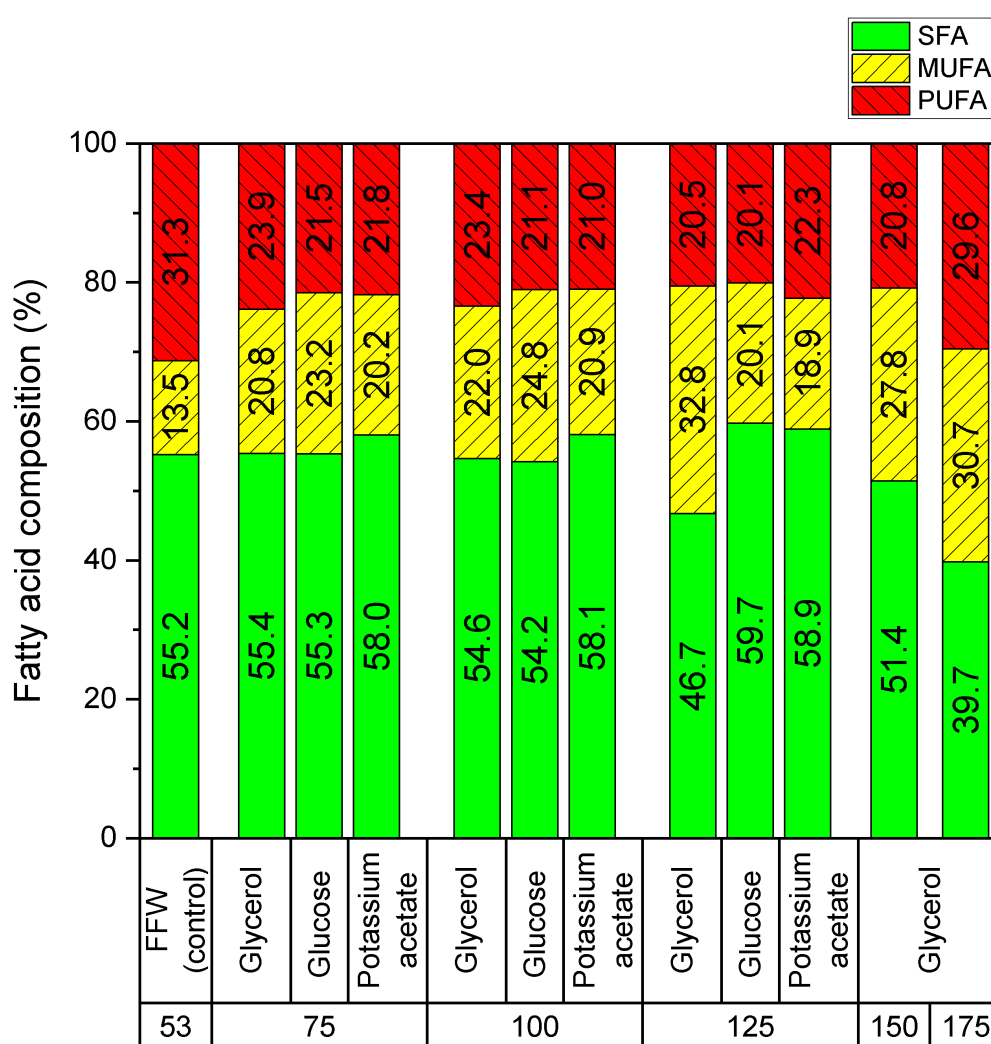


Figure 4.6 : Effect of different carbon sources and COD/TKN ratios on fatty acid composition of cellular lipid.

4.5 Optimization of Lipid Accumulation by *Y. lipolytica* Growing on Fermented Food Waste Supplemented with Yeast Extract, Iron and Trace Elements

4.5.1 Cell growth and microbial lipid production in fermented food waste

The quantity and composition of accumulated microbial lipid and biomass growth of the microorganism is affected by many factors. Different investigations are conducted to optimize these parameters. One of important factors which has important function in biomass growth and lipid generation by microorganism is nitrogen. Both organic and inorganic nitrogen sources can be applied to a growth medium. The organic one like yeast extract, peptone and urea are proper sources for lipid biosynthesis and accumulation (Bao et al., 2021). Inorganic nitrogen sources like potassium nitrate, ammonium chloride and ammonium sulphate are more suitable for biomass generation and growth of microorganism. In some studies, yeast extract was identified as the more effective one in intracellular lipid storage compared with other nitrogen sources. Lipid content of biomass was enhanced two times with yeast extract compared to culture mediums contain $(\text{NH}_4)_2\text{SO}_4$ or $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ in a study conducted by Bellou et al. (2016).

According to the literature, a small amount of nitrogen is needed as cofactor in growth medium for lipid biosynthesis, this cofactor is required for enzymatic activities of the process. Therefore, oleaginous microorganism needs a certain amount of nitrogen to be available for lipid generation (Papanikolaou et al., 2002; Beopoulos et al., 2008). Trace elements are another important factor in lipid biosynthesis by oleaginous microorganism i.e., manganese, cobalt, copper, zinc, magnesium, iron, etc. Some metal ions like Fe^{2+} and Mg^{2+} are cofactors for an enzyme named ACL (ATP citrate lyase) that has important effect on lipid production. Citric acid plays important role in lipid storage mechanism and acts as acetyl donor together with ACL in cytoplasm. When this enzyme is inhibited, citrate is secreted from cytoplasm to out of the cell and decreases lipid storage (Katre et al., 2012).

Iron has important effect on cell metabolism through DNA synthesis and electron transfer (Concas et al., 2014). It acts also as cofactor of ACL enzyme that has important role in lipid accumulation inside the cell. Moreover, during sterilization of culture medium, some components such as minerals are precipitated and can't be

consumed by the cell which caused decrease of cell growth and lipid biosynthesis. In a study performed by Kumar et al. (2020), 51.67 g/L and 19.47 g/L biomass and lipid concentration was achieved respectively by *Y. lipolytica* SKY7 cultivated on purified glycerol along with trace elements. The amount of generated cell and accumulated lipid was 27.67 g/L and 9.35 g/L in same culture medium without trace elements.

In current study, different concentrations of yeast extract (YE), iron sulphate (IS) and trace elements solution (TS) were supplied to FFW culture medium individually and in different combinations as well, at the beginning of cultivation, to identify their impact on biomass production and lipid storage. Fermented food waste was used as initial carbon source. As it is shown in Table 4.5, four different concentrations of yeast extract i.e., 10, 100, 1000 and 10000 mg/L was applied. By increasing yeast extract content in the culture, biomass content and lipid production was enhanced as well. Yeast extract concentrations of 10 and 100 mg/L resulted in approximately the same amount of biomass generation and lipid accumulation. $31.8 \pm 1.4\%$ (w/w) lipid content and 7.79 ± 0.12 g/L biomass was generated in medium contains 10 mg/l yeast extract, these values were $31.4 \pm 0.05\%$ and 7.74 ± 0.87 g/L in the medium with 100 mg/L yeast extract. In medium contains 1000 mg/L yeast extract, higher lipid content of $33.2 \pm 0.5\%$ was obtained with biomass content of 8.87 ± 0.50 g/L in the culture. By increasing yeast extract concentration to 10000 mg/L, lipid synthesis was decreased to $28.8 \pm 0.2\%$ whereas cell proliferation was increased and reached to 9.79 ± 0.87 g/L.

In culture sets provided with iron, different concentrations of iron sulphate as 10, 50, 100 and 150 mg/L was applied. The highest biomass concentration was achieved in the mediums supplied with 10 and 100 mg/L iron sulphate as 12.24 ± 0.49 , 12.30 ± 0.20 g/L, respectively. The lipid content of these two culture mediums was almost the same as 28.2 ± 0.1 and $28.7 \pm 1\%$, respectively. By adding 50 mg/L iron sulphate to the medium, lipid content was enhanced to $30.7 \pm 1.61\%$. The highest lipid content of $33.7 \pm 0.8\%$ and the lower biomass content of 8.49 ± 0.54 g/L was observed in the medium with highest iron concentration of 150 mg/L. In a study performed by Cordova et al. 2022, effect of iron sulphate on both wild and engineered strains of *Y. lipolytica* (Po1f and Po1fpmD, respectively), cultivated on YSC (Yeast Synthetic Complete) medium was assessed. Biomass generation was improved near twice along

with threefold lipid production on wild type strain which was comparable quantity with genetically engineered strain.

Results indicated no considerable increase of cell growth in the mediums supplemented with trace elements solution. Different concentrations of trace elements solution were applied as 0.5, 1, 2.5 and 5 ml/L. The biomass generation was changed between 6.90 ± 0.18 and 7.75 ± 0.21 g/L. Comparing amount of biomass generation in mediums supplemented with trace elements with control set contain only FFW, indicated the trace elements available in FFW was adequate for yeast proliferation as biomass content was not improved by trace element addition. Although the cell growth was not improved significantly by adding trace elements to the experimental set, the lipid accumulation was enhanced by increasing the trace element concentration. Supplying 0.5 ml/L trace elements led to lipid content of $24.8 \pm 0.60\%$ and this value increased to 25.9 ± 0.31 , 26.3 ± 0.50 and $30.3 \pm 0.62\%$ by adding 1, 2.5 and 5 ml/L trace elements solution, respectively.

Table 4.5 : Effect of different concentrations of yeast extract, iron and trace elements solution on biomass generation and lipid accumulation of *Y. lipolytica*.

YE (mg/L)	IS (mg/L)	TS (ml/L)	Lipid content (% w/w)	Biomass concentration (g/L)
10	-	-	31.8 ± 1.4	7.79 ± 0.12
100	-	-	31.4 ± 0.05	7.74 ± 0.87
1000	-	-	33.2 ± 0.5	8.87 ± 0.50
10000	-	-	28.8 ± 0.2	9.79 ± 0.87
-	10	-	28.2 ± 0.1	12.24 ± 0.49
-	50	-	30.7 ± 1.6	10.96 ± 0.56
-	100	-	28.7 ± 1.0	12.30 ± 0.20
-	150	-	33.7 ± 0.8	8.49 ± 0.54
-	-	0.5	24.8 ± 0.6	7.75 ± 0.21
-	-	1	25.9 ± 0.3	6.90 ± 0.18
-	-	2.5	26.3 ± 0.5	7.30 ± 0.29
-	-	5	30.3 ± 0.6	6.95 ± 0.25

According to the results, 1000 mg/L YE, 150 mg/L IS and 5 ml/L TS was identified as the optimum concentrations to increase microbial lipid content of *Y. lipolytica* growing on FFW.

4.5.2 Lipid accumulation in fermented food waste with different COD/TKN ratios along with glycerol, yeast extract, iron sulphate and trace elements

The optimum concentrations of YE, IS and TS regarding microbial lipid content, along with glycerol as the optimal substrate was applied to *Y. lipolytica* cultivated on FFW. The optimum COD/TKN ratio was identified as 125 in previous step. So, different COD/TKN ratios over 125 was applied to promote lipid content of the cell even further. As it is illustrated in the Figure 4.7, different combinations of yeast extract+iron sulphate (YE+IS), iron sulphate+trace elements solution (IS+TS), yeast extract+trace elements solution (YE+TS) and yeast extract+iron sulphate+trace elements solution (YE+IS+TS) was applied. In COD/TKN 125 in culture sets contain YE+IS, IS+TS and YE+IS+TS, accumulated lipid was 29.3 ± 0.3 , 37.9 ± 1.51 and $36.2 \pm 0.9\%$, respectively and lower than the lipid content of $42.2 \pm 1.72\%$ that was achieved in COD/TKN 125 in experimental set contains only glycerol as explained in previous section. Supplying the combination of YE+TS resulted almost the same lipid content in the cell i.e., $42.3 \pm 1.3\%$, although the generated biomass was slightly lower (11.65 ± 0.92 g/L). The YE+IS combination was not applied in higher COD/TKN ratios as it was observed that this combination decreased the lipid content in ratio 125. In ratio 150, obtained lipid content in the medium contain YE+TS was $42.53 \pm 0.65\%$ that indicated increase of lipid accumulation compared to experimental set with ratio 150 with only glycerol. By supplying YE+IS+TS to the culture medium, lipid content of the cell increased to $44.72 \pm 0.31\%$.

Lipid accumulation of the cell reached to its plateau in ratio 175 i.e., $45.94 \pm 0.21\%$ in experimental set supplemented with YE+IS+TS. This value was the highest lipid content obtained in this thesis and by increasing the COD/TKN ratio over 175, the lipid accumulation dropped significantly. Two combinations of YE+TS and YE+IS+TS was applied on cultures with COD/TKN 200 and 225. In mediums supplemented with YE+TS, lipid content dropped to 36.9 ± 1.1 and $29.5 \pm 1.23\%$, and in mediums contain YE+IS+TS it decreased to 27.99 ± 0.88 and $27.5 \pm 0.72\%$, respectively.

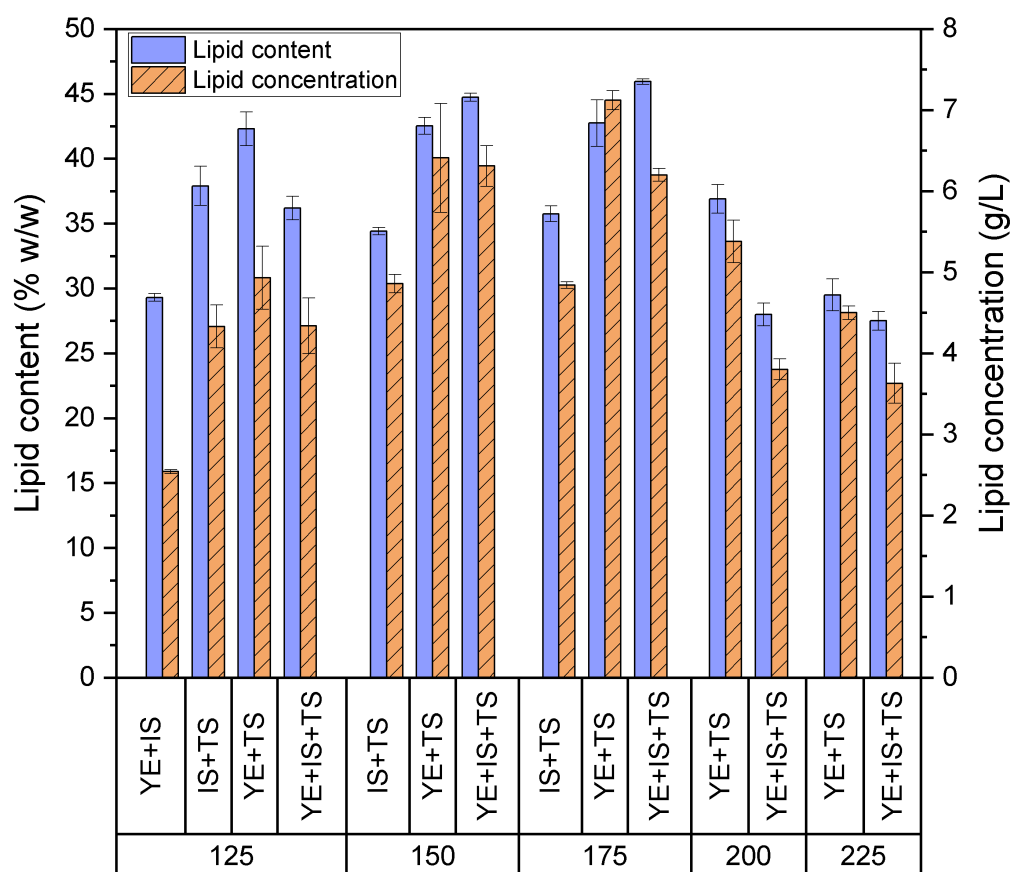


Figure 4.7 : Lipid content and lipid concentration of *Y. lipolytica* growing on FFW supplemented with glycerol, YE, IS and TS. Error bars represent the standard deviations.

Moreover, a significant development was observed in lipid concentration of the mediums with supplemented components compared with the experimental sets with only glycerol (Figure 4.5). In experimental set conducted with only glycerol, the highest lipid concentration of 5.46 g/L was achieved in medium with COD/TKN ratio of 125. According to the results showed in Figure 4.7, the lipid concentrations varied between 2.54 - 7.12 g/L in cultures supplemented with YE, IS and TS. In all conducted batch cultures with supplemented compounds, the most effective approach belonged to dual combination of YE+TS to improve lipid concentration in the medium. In COD/TKN 125, lipid concentration of 2.54 ± 0.02 , 4.33 ± 0.26 , 4.93 ± 0.38 and 4.34 ± 0.43 g/L was obtained in mediums supplemented with YE+IS, IS+TS, YE+TS and YE+IS+TS, respectively. By increasing COD/TKN of the medium to 150, in the mediums contain IS+TS, YE+TS and YE+IS+TS lipid concentration was measured as 4.86 ± 0.114 , 6.41 ± 0.67 and 6.31 ± 0.25 g/L, respectively. The highest lipid

concentration was observed in COD/TKN 175 in medium contain YE+TS with lipid content of 7.12 ± 0.11 g/L. Obtained lipid concentration in mediums with IS+TS and YE+IS+TS was 4.84 ± 0.03 and 6.20 ± 0.07 g/L. In higher ratios of 200 and 225, lipid concentration reduced and dropped to 5.38 and 4.50 g/L respectively in cultures contain YE+TS.

The biomass concentration of each culture is illustrated in Table 4.6. In all conducted COD/TKN ratios except 125, cultures supplemented with YE+TS showed highest cell growth. In COD/TKN 125, the highest biomass concentration of 12 ± 0.95 g/L belonged to the culture set contains YE+IS+TS. By increasing the ratio to 150, mediums contain IS+TS and YE+IS+TS had almost the same biomass concentrations of 14.12 ± 0.33 and 14.1 ± 0.56 g/L, respectively and by supplying YE+TS to the medium, biomass growth reached to 15.08 ± 1.58 g/L.

Table 4.6 : Biomass concentration of *Y. lipolytica* growing on FFW with different COD/TKN ratios, supplemented with YE, IS, TS and glycerol.

COD/TKN	Supplemented components	Biomass concentration (mg/L)
125	YE+IS	8.67 ± 0.85
	IS+TS	11.42 ± 0.70
	YE+TS	11.65 ± 0.92
	YE+IS+TS	12.00 ± 0.95
150	IS+TS	14.12 ± 0.33
	YE+TS	15.08 ± 1.58
	YE+IS+TS	14.10 ± 0.56
175	IS+TS	13.54 ± 0.11
	YE+TS	16.67 ± 0.27
	YE+IS+TS	13.50 ± 0.17
200	YE+TS	14.57 ± 0.71
	YE+IS+TS	13.57 ± 0.46
225	YE+TS	15.25 ± 0.28
	YE+IS+TS	13.20 ± 0.89

Biomass growth was increased by improving the COD/TKN ratio to 175 in all cultures and reached to its maximum amount of 16.67 ± 0.275 g/L in ratio 175 by adding YE+TS to the medium, while culture sets contain IS+TS and YE+IS+TS has almost

the same biomass growth of 13.54 ± 0.11 and 13.5 ± 0.17 g/L, respectively. By increasing the ratio to 200 and 225, the cell growth started to decrease slightly due to adverse effect of high glycerol concentration on cell proliferation. The biomass concentration dropped to 14.57 ± 0.71 and 13.57 ± 0.46 g/L in cultures with YE+TS and YE+IS+TS, respectively in ratio 200 and 15.25 ± 0.28 and 13.20 ± 0.89 g/L in mediums contain YE+TS and YE+IS+TS, respectively in ratio 225.

Results demonstrated the optimum COD/TKN ratio of 175 to achieve both highest cell and lipid content of *Y. lipolytica* growing on FFW as initial substrate which supplemented with glycerol, YE, IS and TS.

4.5.3 Substrate utilization

Table 4.7 illustrates the substrate consumption by *Y. lipolytica* growing on FFW as initial carbon source supplied with yeast extract, iron sulphate, trace elements solution and glycerol as second carbon source, in two stage batch cultivation system. According to the results, in COD/TKN 125 the cultures contain IS+TS, YE+TS and YE+IS+TS the carbon source consumption was 35.4, 39.7 and 32.18 g/L COD, respectively. By increasing the ratio to 150 carbon consumption was increased and mediums supplemented with IS+TS, YE+TS and YE+IS+TS showed the COD consumption of 54.37, 45.92 and 48.82 g/L, respectively.

In higher ratio of 175 which had maximum lipid accumulation in medium contains YE+IS+TS, COD consumption of 50.22 g/L was observed. In COD/TKN 200, organic carbon consumption was 53.80 and 48.97 g/L in mediums contain YE+TS and YE+IS+TS. In higher ratio of 225 these values increased to 57.57 and 51.99 g/L.

As it is obvious from the results, although by increasing COD/TKN ratios the carbon consumption was increased in mediums as well, the lipid content of the cell reached to its maximum in ratio 175 and started to decrease in higher ratios. The reason was the metabolic shift of the yeast cell in which the consumed substrate contributed into the extra-cellular metabolites such as organic acids or polyols such as erythritol, mannitol, etc. The pH is crucial factor to understand metabolic behavior of microorganism. In experimental sets with COD/TKN less than 150, pH of the mediums increased to about 8, but in experimental sets with higher ratios (150, 175, 200 and 225) the lower pH of about 4 was observed in experimental sets. It indicated

that despite uncontrolled pH, some part of consumed COD was used to produce the secondary metabolites rather than lipid accumulation that decreased pH of the medium. The reason was high concentration of glycerol that caused production of extracellular metabolites that is conforming to the previous investigations reported in the literature (Christophe et al., 2012). According to Bao et al. 2021, in the medium with very high or very low nitrogen concentration, citric acid as a secondary metabolite is generated.

Table 4.7 : Amount of organic carbon source supplied and utilized in each experimental set.

COD/TKN	Experimental set	Carbon substrate added at t = 48 at the beginning of stage 2 (g/L COD)	Carbon consumed at t = 96 (g/L COD)
125	Glycerol+IS+TS	33.62	35.4
	Glycerol+YE+TS	33.62	39.7
	Glycerol+YE+IS+TS	33.62	32.18
150	Glycerol+IS+TS	45.77	54.37
	Glycerol+YE+TS	45.77	45.92
	Glycerol+YE+IS+TS	45.77	48.82
175	Glycerol+IS+TS	57.92	51.94
	Glycerol+YE+TS	57.92	49.16
	Glycerol+YE+IS+TS	57.92	50.22
200	Glycerol+YE+TS	70.07	53.80
	Glycerol+YE+IS+TS	70.07	48.97
225	Glycerol+YE+TS	82.22	57.57
	Glycerol+YE+IS+TS	82.22	51.99

4.5.4 Fatty acid composition of accumulated lipid

The fatty acid composition of microbial lipid generated in batch cultivation of *Y. lipolytica* in all experimental sets was determined by gas chromatography device. The results are illustrated in Table 4.8. Total microbial lipid was converted into fatty acid methyl esters by transesterification. The biomass was collected 48 h after supplying different carbon sources at 2nd stage of cultivation and FAME composition was identified. According to the results, the fatty acids were stearic acid (C18:0), linoleic acid (C18:2), pentadecanoic acid (C15:1), palmitic acid (C16:0), and heptadecenoic

acid (C17:1) in smaller quantity. The stored fatty acids contained carbon chain lengths of C16-C18 that were the same as fatty acid profile of vegetable oil (Zlatanov et al., 2014). Obtained fatty acids were the same as the fatty acid composition of other *Y. lipolytica* strains cultivated on glycerol (Papanikolaou et al., 2008; Sarris et al., 2019). Accumulated fatty acid was mainly stearic acid which was above 40% in experimental sets.

Table 4.8 : Fatty acid profile of microbial lipid.

COD/ TKN	Carbon source	Penta- decenoic acid (C15:1)	Hepta- decenoic acid (C17:1)	Linoleic acid (C18:2)	Palmitic acid (C16:0)	Stearic acid (C18:0)
125	Glycerol+YE +IS	16.36	-	31.01	-	52.64
	Glycerol+IS+ TS	16.84	5.29	20.40	12.70	44.77
	Glycerol+YE +TS	19.11	8.43	17.40	11.01	44.05
	Glycerol+IS+ TS+YE	18.01	5.61	18.88	12.35	45.16
150	Glycerol+IS+ TS	16.00	5.00	13.90	11.10	54.00
	Glycerol+YE +TS	17.03	8.47	12.37	17.03	51.15
	Glycerol+YE +IS+TS	19.77	5.18	19.01	13.23	42.81
175	Glycerol+IS+ TS	16.01	3.55	14.36	13.00	53.08
	Glycerol+YE +TS	16.10	7.30	12.65	10.94	53.02
	Glycerol+YE +IS+TS	17.91	11.35	12.24	9.56	48.94
200	Glycerol+TS +YE	16.55	4.78	25.70	11.25	41.73
	Glycerol+YE +IS+TS	18.35	5.37	20.15	13.11	43.02
225	Glycerol+YE +TS	16.95	5.49	23.23	11.50	42.82
	Glycerol+YE +IS+TS	16.11	3.70	25.84	12.82	41.53

The same result was observed in an investigation conducted by Papanikolaou & Aggelis (2011) in which *A. curvatum* was cultivated on whey-permeate. Their results demonstrated the potential of *Y. lipolytica* to generate and store significant quantity of saturated fatty acid.

The highest content of linoleic acid was measured in COD/TKN 125 contains YE+IS as 31.01%. By supplying glycerol and different supplemented components to experimental sets, amount of linoleic acid (C18:2) was started to decrease. In terms of stearic acid content (C18:0), its amount reached to its maximum of 54% in experimental sets contain glycerol+IS+TS in COD/TKN 150.

According to the Figure 4.8, by supplying YE, IS and TS the amount of SFA include stearic acid and palmitic acid in experimental sets was increased. In COD/TKN 125, in culture set with only glycerol the amount of SFA was 46.7% which was increased to 53.3, 57.5, 55.1 and 57.5 in mediums contain YE+IS, IS+TS, YE+TS and IS+TS+YE. Though in ratios 150 and 175 increase of SFA was observed as well, in COD/TKN 200 and 225 it started to decrease, and the lowest value was measured at ratio 200 in culture medium supplemented with TS+YE as 53%.

According to the results, by supplying YE, IS and TS and increasing COD/TKN ratio, amount of PUFA was decreased in ratios < 200. Lowest amount of PUFA (12.2%) and highest total quantity of SFA and MUFA (87.8%) was observed in medium with COD/TKN 175 contains YE+IS+TS. Highest lipid content of cell was observed in this batch culture as well.

Biodiesel properties is defined with fatty acid composition of its microbial oil. Saturated and unsaturated fatty acids have different functions in biodiesel properties. High amount of SFAs increase some properties such as cetane number, combustion, oxidative stability, and viscosity of produced biodiesel (Ramos et al., 2009). High amount of unsaturated fatty acids mainly PUFAs boost cold flow property of generated fuel but has negative effect on its combustion and oxidative stability (Knothe 2013). According to the results, fatty acid composition of accumulated lipid showed the similarity with vegetable oil (Christophe e al., 2012) and was appropriate for biodiesel production.

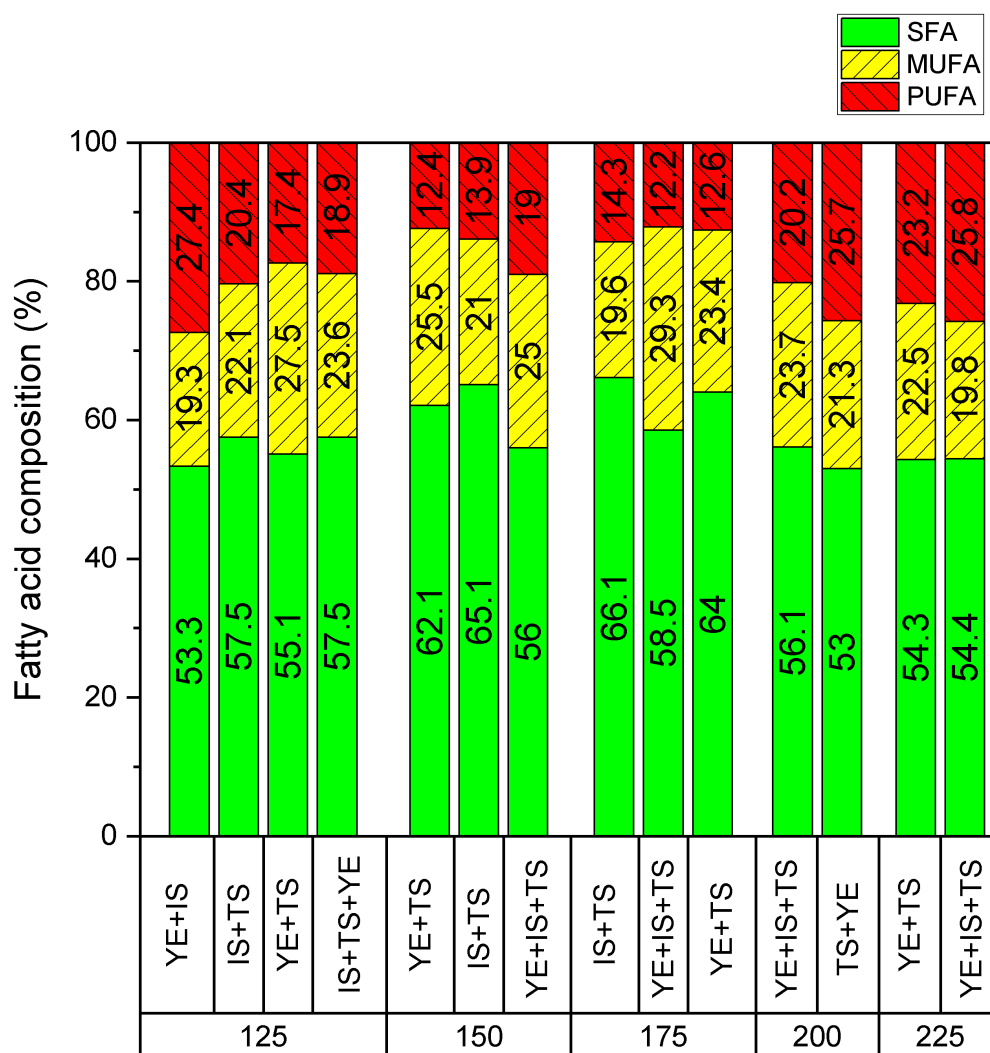


Figure 4.8 : Effect of YE, TS and IS on fatty acid composition of *Y. lipolytica* growing on FFW and glycerol at different COD/TKN ratios.

4.6 Discussion

Results of this thesis indicate FFW as an appropriate substrate for growth, biomass generation and lipid accumulation of *Y. lipolytica*. Lipid content of the cell was promoted by applying glycerol as second carbon source in the medium. Although large scale SCO production as substance for biodiesel generation has been investigated by many researchers, the process feasibility is hampered by the remarkable processing cost. Two important points which make the process economically viable, are the selection of carbon source and cultivation mode. This thesis demonstrates that applying two stage batch cultivation mode and adding extra carbon source in second stage can greatly improve lipid generation of oleaginous yeast. The two-stage

cultivation method is considered as the most appropriate strategy to improve lipid content of the cell, in which biomass production in medium with high carbon concentration is performed prior to lipid biosynthesis. Additionally, this study indicates that FW and glycerol as two economic and suitable carbon sources are appropriate substrates for *Y. lipolytica* which makes the process more profitable. This study provided not only an economic waste treatment strategy, but also a sustainable method of microbial oil production. The fatty acid composition of accumulated lipid was in accordance with those reported in the literature and consists stearic acid (C18:0), palmitic acid (C16:0), linoleic acid (C18:2), pentadecanoic acid (C15:1) and heptadecanoic acid (C17:1) (Wissebach et al., 1969).





5. RECOMMENDATIONS AND CONCLUSION

5.1 Conclusion

This thesis demonstrated the successful usage of FW as a valuable and economic carbon source with oleaginous yeast *Y. lipolytica*. This waste generally contains cooked and uncooked animal and plant waste and food residue which shows very heterogeneous composition. So, the property of this waste is important factor in single cell oil generation. Applied fermentation process was effectively hydrolyzed the FW and by increasing solubility of organic matter content of the waste, made it appropriate for utilization by the strain. *Y. lipolytica* as an oleaginous yeast applied in this thesis was successfully grown on FFW and biomass in considerable amount was generated. Regarding considerable environmental problems caused by FW and the waste management expenses, utilization of FW by *Y. lipolytica*, its biomass generation and lipid accumulation has great advantage to environment protection and waste management. Landfills or incineration technique is using for FW that leads to different environmental problems such as air pollution, ash release, landfill leachates, emission of methane or hydrogen sulfide, etc. Hence, this study with a biorefinery concept to using FW as a rich bio-based carbon source, could be a proper technology for FW management and its valorization along with generation of a valuable product such as microbial lipid.

On the other hand, glycerol as one of the promising substrates for biodiesel generation is a main byproduct of biodiesel industries. The most economical way to renewable energy generation is applying waste as feedstock. This thesis, by considering the concept of waste to wealth, focused on this byproduct as an economic source of organic carbon. Generation of considerable amount of microbial oil indicates the effective combination of FFW and glycerol as valuable carbon sources for lipid production. Efficient utilization of glycerol to produce single cell oil as a value-added product decrease the operation cost of biodiesel generation as the 60% of operation cost belongs to the carbon source. Nutrient limited condition along with excess carbon source in this thesis, directs the carbon flux towards the lipid biosynthesis by *Y. lipolytica*.

Moreover, this thesis proves that two-stage batch cultivation strategy by using FFW as initial carbon source and glycerol as the second one, could be applied as an efficient method for cultivation and intracellular lipid accumulation by *Y. lipolytica*. Fatty acid composition of obtained microbial oil showed that the lipid generated from utilization of FFW and glycerol can be used as a sustainable alternative to conventional fuel. Fermented food waste and glycerol can be used as suitable source of energy and decrease the hazardous emissions and related environmental problems.

5.2 Challenges and Recommendations

By increasing energy demand in the world following with high application of fossil fuels, it is essential to provide an eco-friendly and cost-efficient alternative energy source. Biodiesel is considered as an appropriate source of energy that decrease the greenhouse gas emission and improve the air quality. The most important parameters to evaluate the profitability of biodiesel generation are feedstock cost and energy consumed during the process. Mainly, the cost of feedstock is considered as the principle one. Exploiting low-cost and abundant substrates such as food waste and glycerol provides an economically feasible waste valorization and energy generation. However, the cost of waste collection and transportation needs to be considered and try to minimize it. Additionally, complex organic matters in wastes needs to be pretreated and break down to simple one to be available for microbial use. Hence, the most feasible energy efficient techniques for successful FW pretreatment for recovering high value product such as biodiesel should be employed.

The lipid yield of *Y. lipolytica* needs to be sufficient for an economic production and profitable revenue. The economic viability of SCO generation for biodiesel production greatly relies on applied lipid extraction process. The yield of extracted lipid along with the cost of exploited method needs to be concerned.

Challenges of economic viability of biodiesel generation have been highlighted in different studies by many researchers. Biodiesel generation from oleaginous yeast are economically feasible if the cheap carbon source was used. Therefore, using wastes or by-product is considered as a great option for sustainable production of biofuel.

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PUBLICATIONS/PRESENTATIONS ON THE THESIS

- **Salimi Khaligh, S.,** Polat, E. & Altinbas, M. Optimization of Lipid Accumulation by *Yarrowia lipolytica* Growing on Fermented Food Waste in Two-Stage Batch Strategy. *Waste Biomass Valor.* 14, 2037–2059 (2023).
- **Salimi Khaligh, S. & Altinbas, M.,** (2022) “Lipid accumulation by *Yarrowia lipolytica* growing on food waste under nitrogen deficiency”, 9th International Conference on Engineering for Waste and Biomass Valorisation, Copenhagen, Denmark.
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