

**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE**  
**ENGINEERING AND TECHNOLOGY**

**ENERGY AND BIOMASS PRODUCTION USING *YARROWIA LIPOLYTICA***  
**FROM SANITARY LANDFILL LEACHATE**

**M.Sc. THESIS**

**Ayça BAŞAR**

**Department of Environmental Engineering**  
**Environmental Science and Engineering Programme**

**AUGUST 2016**



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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

***YARROWIA LIPOLYTICA* İLE DEPONİ SIZINTI SUYUNDAN ENERJİ VE  
BİYOKÜTLE ÜRETİMİ**

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*For my dear family, beloved friends and a more peaceful world,*



## **FOREWORD**

The efficient environmental engineer has been completely conscious about understanding of how to satisfy the urgent needs of world. In my opinion, optimization of the production process of biofuels is current need. I have been in close contact with the “waste to energy” concepts. Every aspects of the engineering design has been commercially feasible. Therefore, I has started to study about the optimizayion of production of bioesel from microorganisms.

I would like to thank my advisor Asst. Prof. Dr. Mahmut Altinbas for giving me valuable advice and support always when needed. Other important persons considering my thesis are Biorafinery Working Team members as Dođukan Tunay, Ece Polat, Gzde Yaman, Hande Ermiř, Mehmet Sadık Akça, Neslihan Say, Remziye Ođuz, Umut Metin whom I like to give my special thanks for giving me an opportunity to share their knowledge and support.

I hope that the experience I have gained during the thesis, develop my approach to design “waste to energy” process.

August 2016

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## **ABBREVIATIONS**

<b>C</b>	: Carbon
<b>COD</b>	: Chemical Oxygen Demand
<b>C:N</b>	: Carbon to Nitrogen ration
<b>FAME</b>	: Fatty Acid Methyl Ester
<b>LB</b>	: Lipid Body
<b>LL</b>	: Landfill Lechate
<b>N</b>	: Nitrogen
<b>OD</b>	: Optical Density
<b>P</b>	: Phosphate
<b>SCO</b>	: Single Cell Oil
<b>SS</b>	: Suspended Solid
<b>TKN</b>	: Total Kjeldahl Nitrogen
<b>TP</b>	: Total Phosphate
<b>YPD</b>	: Yeast Peptone D-glucose



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## ENERGY AND BIOMASS PRODUCTION USING YARROWIA LIPOLYTICA FROM SANITARY LANDFILL LEACHATE

### SUMMARY

Approximately 80% of world's energy needs is supplied by fossil fuels. Since the depletion of fossil fuels, climate change, rapid industrialization, increase in waste management requirement, it is urgent to develop alternative sustainable energy systems. For this reason, *Yarrowia lipolytica*, a kind of oleaginous yeast, have received attention to become an alternative. *Y. lipolytica* has high lipid accumulation capacity intracellularly.

To provide cheap way for nutrients provision for *Y. lipolytica*, a nutritionally rich wastestream should be used as raw material. Sanitary landfill leachate can be an alternative for the cultivation of *Y. lipolytica*. It is not only waste valorization but also alternative energy generation.

Within the scope of this approach, growth of *Y. lipolytica* in different dilution rates of sanitary landfill leachate was carried out. All dilutions were made with tap water.

In this study, in YPD medium with minerals resulted in 14,83 g/L biomass concentration. Biomass concentrations at the end of cultivations with leachate were lower than cultures grown in YPD medium. The optimum biomass growth was observed with the cultivation of 60% LL as 4,34 g/L. 60% concentration of leachate was the most available cultivation for *Y. lipolytica*. After addition of external P to the leachate cultivation, the growth of biomass increased. In 60%+P LL cultivation, biomass concentration reached to nearly 8 g/L.

To improve lipid accumulation intracellularly, nitrogen limitation condition was provided by the addition of carbon source. Applied C:N ratio was 20-30.

The removal efficiencies of leachate treatment was determined. The maximum TP removal was observed in 60%+P leachate nearly as 98%. The maximum PO<sub>4</sub>P removal was observed in 100%+P leachate nearly as 96%. Secondly, PO<sub>4</sub>P removal was observed in 60%+P leachate nearly as 95%. The maximum COD removal efficiency was observed in 60%+P leachate nearly as 85%. The maximum TKN removal efficiency was observed in 60%+P leachate nearly as 75%. The maximum ammonium removal efficiency was observed in 60%+C leachate nearly as 76%. Secondly, ammonium removal efficiency was observed in 60% leachate nearly as 61%.

Particle size distribution from the effluent of cultivation medium was done. It was shown that the particle size became bigger at the end of the cultivation. It should be due to colony forming ability of *Y. lipolytica*.

In stress conditions to maximize lipid accumulation, biomass growth should be at stationary phase. Thus, growth should not be observed anymore. The growth of *Y. lipolytica* under stress conditions with 60%+C and 100%+C LL were still observed.

The lipid content of *Y. lipolytica* that was grown in YPDxM was around  $26\pm 0.2\%$ . The lipid content of 60% LL was similar to the lipid content of YPDwM. In 60%+C LL and 100%+C LL cultivations, lipid percentage was not higher than 60% LL and 100% LL cultivations. Since there were not successful stress conditions proving the addition of C source with cultivation of *Y. lipolytica*, lipid accumulation intracellularly was not increased. Adjustment of C:N ratio of leachate from 1-2 to 20-30 was not enough to prove stress conditions for *Y. lipolytica*.

The FAME composition obtained from biomass of the different mediums were calculated. It was observed that most of the FAME were Palmitic acid (C16:0), Oleic acid (C18:1) and Linoleic acid (C18:2) as around 45%. FAME composition with cultivations was suitable for biodiesel production.

## YARROWIA LIPOLYTICA İLE SIZINTI SUYUNDAN ENERJİ VE BİYOKÜTLE ÜRETİMİ

### ÖZET

Artan enerji ihtiyaçlarını karşılayabilecek için petrole alternatif enerji kaynakları aranmaktadır. Dünyadaki birincil enerji rezervlerinin yaklaşık olarak %80'ini fosil yakıtlar oluşturmaktadır ve çeşitli kurumlar tarafından bu değerin 2035 yılında % 63'e kadar düşebileceği belirtilmektedir. Aynı zamanda, petrol, kömür, doğalgaz gibi fosil yakıtların global tüketimi, endüstrileşmiş dünyanın kirlilik problemlerini arttırmaktadır. Gazolin ve dizel gibi petrol türevi yakıtların egzoz gazları, asit yağmurları, iklim değişikliği, sera gazı emisyonlarında artış gibi olumsuz çevresel sonuçlara neden olmaktadır. Diğer bir taraftan, atıklar dünya genelinde çevresel problem olarak düşünölmeye başlanmıştır. İçinde bulunduğumuz yüzyılda gerçekleşen hızlı sanayileşme ve beraberinde artan kirlilik yüklerinin ekosistemde birikmesi nedeni ile atık arıtımı giderek önem kazanması gereken bir konu haline gelmektedir. Ekonomik etkenler göz önünde bulundurulacak olursa, atık geri kazanımı daha yaygın bir şekilde uygulamaya geçilmesi gereken bir konu olmalıdır. Aynı zamanda, atıktan enerji üretimi sağlayacak entegre sistemler üzerinde çalışılmasına ağırlık verilmelidir.

Günümüzde, gelişmiş ve gelişmekte olan ölkelerde araştırma ve geliştirme çalışmaları için önemli bütçelerin ayrıldığı güneş, rüzgar ve biyokütle gibi yenilenebilir enerji kaynakları temelli yakıtlar için akademisyenler; fosil yakıtlara alternatif olabilecek ve global düzeyde artan enerji taleplerini sürdürülebilir olarak karşılayabilecek alternatif kaynaklar olarak ciddiyle ele alınması ve geliştirilmesi gerektiği yönünde görüş bildirmektedirler.

Biyokütle, biyolojik uygulamalar sonucunda etanol, metanol, metan, hidrojen ve dizel gibi geleceğin yakıtı olarak nitelendirilen yakıtlara dönüştürülebilmektedir. . Biyokütle kaynaklarının kullanılarak elde edilen 3 tip (katı, sıvı, gaz) biyoyakıt vardır. Bugün yapılan Ar-Ge çalışmaları biyoetanol ve biyodizel üretimi üzerine odaklanmıştır. Biyoetanol şeker fermentasyonu sonucu elde edilirken diğer biyoyakıt türleri lipitlerden elde edilmektedir.

Biyodizel, petrol türevi yakıt olan dizel ile benzer özelliklere sahip olması sayesinde çeşitli mevcut dizel motorlarında ve ısıtma sistemlerinde kullanılma potansiyeli yüksek bir biyoyakıttır. Aynı zamanda biyodizel, atmosferik karbon dioksit seviyesini artırıcı etkisi olmayan bir yakıt türüdür ve küresel iklim değişikliğine katkıda bulunmadığı için çevre dostu bir yakıt olarak kabul edilebilir. Temiz teknoloji ürünü olmasından dolayı ayrışabilen, toksik olmayan sürdürülebilir bir enerji alternatifi olarak düşünölebilir. Bunlardan dolayı, biyodizel üretimi araştırmacıların dikkatini çekmektedir. 1. nesil biyodizel, kanola, ayçiçeği ve soya fasülyesi gibi yenilenebilir bitki yağları kullanılarak üretilmiştir. Son yıllardaki gıda fiyatlarındaki artış ve bazı ölkelerdeki büyük tarımsal alanların biyodizel üretimi için

hammadde endüstrisine dönüşmesi nedenleri ile hammadde olarak yenilebilir bitkilerin kullanımına karşı tepkiler oluşmuştur. Bu tepkiler nedeni ile jojoba ve jatrofa gibi yenilemeyen bitki yağlardan 2. nesil biyodizel üretilmiştir. Yenilemeyen bitkilerin yetiştirilmesi sürdürülebilirlik açısından sorun oluşturmaktadır ve global enerji ihtiyaçlarını karşılamaya yetmeyeceği belirtilmiştir.

Günümüzde, mikrobiyal lipofil -yağ sever- bileşimler kullanılarak üretilen 3. nesil biyodizel üzerine yapılan çalışmalar gelişim aşamasındadır. Bu mikrobiyal lipofil bileşimler, Tek Hücre Yağları (THY) olarak adlandırılmaktadır; bazı yağlı alg, bakteri, mantar ve maya türleri THY olarak kabul edilir. THY, intraselüler olarak yüksek miktarda yağ depolayabilen, en azından kuru ağırlıklarının %20'si yağdan oluşan yağlı mikroorganizmalar olarak düşünülebilirler.

*Y. lipolytica* karbon fazlalığı ve azot, fosfor veya demir gibi nütrientlerin kısıtlandığı stres koşulları altında, çok miktarda mikrobiyal hücre içinde Yağ Damlacıkları (YD) oluşturmaktadır. Pratikte, medyada kolaylıkla kontrol edilebilir olması sayesinde yaygın olarak kullanılan kısıtlayıcı nütrient azottur. YD, ağırlıklı olarak trigliseridlerden ve steril esterlerden meydana gelen hücre içi nötral yağ depolarıdır. Biyodizel, mikroorganizmalardaki depolama lipitleri olan YD'in yağ asitlerinde bulunan trigliseridlerin, etanol veya metanol gibi alkoller yardımı ile transesterifikasyonu sonucu üretilirler. Biyodizel, Yağ asidi Metil Ester (FAME) olarak da adlandırılmaktadır.

Üretilen lipitin yağ asiti miktarı ve özellikleri biyodizelin kalitesini etkilemektedir. Özellikle karbon zinciri uzunluğu ve yapısındaki çift bağlar daha iyi kalite sağlanmaktadır. 12 ve üzeri karbon içeriği biyodizelin kristal oluşturma sıcaklığını arttırmaktadır. Yağlı bir maya türü olan *Yarrowia lipolytica*'dan izole edilen YD'in yağ asidi kompozisyonu; oleik asit (C18:1), palmitik asit (C16:0), palmitoleik asit (C16:1), stearik asit (C18:0) ve linoleik asit (C18:2)den oluşmaktadır.

Biyodizel üretim proseslerindeki maliyetin büyük kısmını hammadde oluşturmaktadır. Ülkemizde bu sektör duraklamış durumdadır. Üreticiler, ÖTV uygulamasının getirilmesi ile, biyodizel üretimin proseslerinin maliyeti kurtarmadığını belirtmişlerdir.

Ülkemizde ticari amaçlı biyokütle eldesi için işletilen maya üretim tesisi mevcut değildir. Laboratuvar ölçekli biyoreaktör reaktör denemeleri sonucunda göre büyük ölçekli bir tesisin kurulumu için taban teşkil edecek tasarım verileri belirlenmeye çalışılmıştır. Bu çalışma ile, biyokütle olan *Y. lipolytica* maya türünün hammadde ihtiyacı atık akımından karşılanarak üretim maliyetleri azaltılmaya çalışılmıştır ve ticari amaçlı maya üretimi optimize edilmeye çalışılmış ve özel sektör yatırıma katkı sağlamak amaçlanmıştır.

Mevcut teknolojiler ile arıtımında güçlük yaşanan sızıntı suyu beslemesi ile heterotrofik ortamda büyüme gösteren yağlı maya *Y. lipolytica*'nın MUCL 28849 suşunun çoğalma verimi ve lipit üretim kapasitesi optimize edilmesi amaçlanmıştır. Biyodizel eldesi için mikrobiyal lipit üretiminin ekonomik değerlerini ticarileştirmeye uygun hale getirmek adına gerekli optimizasyon çalışmaları yürütülmüştür.

Biyokütle çoğalması 680 nm'de spektrofotometrik optik yoğunluk ölçülerek gözlenmiştir. Ayrıca, biyokütlenin ikiye katlanma süresi hesaplanmıştır. Biyokütlenin optik yoğunluğu ile askıda katı madde konsantrasyonu lineer olarak kalibre edilmiştir. Sızıntı suyunun farklı derişim oranlarındaki *Y. lipolytica* çoğalması

sonucu nütrient giderim verimleri standart metotlara göre yapılan toplam kjeldahl azotu, amonyok azotu, ortofosfat, toplam fosfat ve kimyasal oksijen ihtiyacı deneyleri sonucunda belirlenmiştir. Elde edilen biyokütlenin lipit, protein, karbonhidrat ve yağ asidi metil ester kompozisyon analizleri yapılmıştır. Biyokütle eldesinde artış sağlamak için kültür ortamına dışarıdan fosfor kaynağı eklenmesi yapılmıştır. Hücre içi yağ miktarını arttırmak için ise dışarıdan karbon kaynağı eklenmesi yapılarak biyokütleyle azot limitasyonu ile stres koşulu sağlanmaya çalışılmıştır. Bu kapsamda atıktan biyoenerji üretimi yaklaşımı ile entegre bir sistem oluşturularak enerji üretimi hedeflenmiştir.

Yapılan çalışmaların sonuçlarına göre artan sızıntı suyu derişimi ile birlikte kültürlerdeki biyokütle çoğalmasının azaldığı gözlemlenmiştir. Optimum biyokütle çoğalması sızıntı suyunun %60 derişiminde yaklaşık olarak 4,35 g/L konsantrasyonunda gözlemlenmiştir. Optimum derişimdeki sızıntı suyuna dışarıdan fosfor kaynağı eklenmesi yapılarak biyokütle çoğaltımının artırılması hedeflenmiştir. %60+P derişiminde sızıntı suyunda biyokütle konsantrasyonunun yaklaşık olarak 8 g/L'ye ulaştığı ölçülmüştür. Kültür ortamının karbon-azot oranı 1 ile karbon kaynağı eklenmesi ile bu değer yaklaşık olarak 30'a çıkartılmıştır. Stres koşullarında biyokütle büyümesinin stasyonel fazda olması beklenmektedir. Ancak stres koşulları sağlanan %60+C ve %100+C sızıntı suyu kültür ortamlarında biyokütlerde büyüme fazı görülmüştür. Bu durum, uygulanan karbon-azot oranının yeterli olmadığını göstermektedir.

Biyokütlenin mineral eklenmesi yapılmış olan YPD medyasındaki lipit içeriği %26±0.2 olarak hesaplanmıştır. %60 derişimindeki sızıntı suyu kültür ortamının lipit içeriği YPD medyasındaki lipit içeriği ile yaklaşık olarak aynıdır. Ancak, stres koşulları sağlanan ve daha yüksek lipit içeriğine sahip olması beklenen %60+C ve %100+C sızıntı suyu kültür ortamlarındaki lipit içeriği de YPD medyasındaki lipit içeriği ile yaklaşık olarak aynıdır. Bu durum, stres koşullarının biyokütlenin lipit depolama kapasitesini arttırmayı başaramadığını göstermektedir. Transesterifikasyon prosesi uygulanmış ve ardından gaz kromatografisi yöntemi ile biyokütlenin yağ asidi metil esterleri kompozisyonu hesaplanmıştır. Buna göre, biyokütlerde çoğunlukla gözlenen yağ asidi metil esterleri yaklaşık olarak %45 oranında palmitik asit (C16:0), oleik asit (C18:1) ve linoleik asittir. Gözlenen bu yağ asidi metil esterleri biyodizel üretimi için uygunluk teşkil eden yağ asidi metil esterleridir.

Standart metotlara göre, sızıntı suyunun nütrient arıtım verimleri hesaplanmıştır. Maksimum TP giderimi %98 ile %60+P derişimindeki sızıntı suyunda, maksimum PO<sub>4</sub>-P giderimi %96 ile %100+P derişimindeki sızıntı suyunda, maksimum KOİ giderimi %85 ile %60+P derişimindeki sızıntı suyunda, maksimum TKN giderim verimi %75 ile %60+P, maksimum NH<sub>4</sub>-N giderim verimi %76 ile %60+C derişimindeki sızıntı suyunda gözlemlenmiştir. Giderim verimleri sızıntı suyu arıtımını istenilen şekilde sağlandığını göstermektedir.

Kültür ortamlarının partikül boyut dağılımı ölçülmüştür. Kültürlenme sonrası sızıntı suyu çıkış akımındaki partikül boyutu, sızıntı suyu giriş akımındaki partikül boyutundan daha büyüktür. Bu durum biyokütlenin kolonileşme eğiliminde olduğunun göstergesi olarak düşünülebilir.

Bu çalışma sızıntı suyunda ilk defa *Y. lipolytica* çoğaltılması denemesidir. Bu açıdan, gelecek için umut vaat eden sonuçları sayesinde temel teşkil edeceği düşünülen bir çalışma olmuştur. Stres koşulları optimizasyonu başarıya ulaşamamıştır. Stres koşulları optimize çalışmaları ile gelecekte biyokütlenin lipit içeriği artırılır ise *Y. lipolytica* ile biyodizel üretimi için büyük bir adım atılmış olacaktır.



## 1. INTRODUCTION

Due to global increase in energy consumption, predicted increase in energy needs in the future, the depletion of fossil fuel reserves, climate change and increase in environmental pollution, the creation of alternative energy source is an urgent need to provide sustainable energy systems. Biodiesel as a renewable, environmentally friendly and alternative energy source to petroleum could be produced from Single Cell Oils (SCO) present in oleaginous yeast to satisfy the considerable amount of energy demands of the world. However, biodiesel should be qualitatively and economically comparable to the conventional diesel to be sustainable. To satisfy these conditions, SCO production processes need to be optimized. Decreasing the cost of biodiesel production from SCO results in easy commercialization.

Moreover, waste/wastestream have been considered to be a serious worldwide environmental problem. Due to the fast industrialization and increasing pollution loads to the ecosystem, waste treatment should be a more crucial topic in this century. Waste recovery should be done to provide also economical consideration.

Operational expenses are the main obstacle for the production process. One of the main operational expenses of the SCO production is the substrate supply. The waste/wastewater streams can be a viable and attractive solution to be fed through oleaginous yeasts.

The waste/wastestream feeding to the biomass leads to provide not only economical advantage but also ecological benefit due to being waste valorization.

As a result, high value product obtained from *Y. lipolytica* within waste valorization concept, valorization of sanitary landfill leachate, is provided and it is suggested as a commercially optimized green energy alternative is provided.

## 1.1 Purpose of Thesis

Purpose of this thesis is to determine the suitability of sanitary landfill leachate to sustain appropriate medium for *Yarrowia Lipolytica*, to define the economically biodiesel production conditions by the usage of the intracellular lipid content in *Y. lipolytica*. Moreover, leachate is a strong wastewater and treatment is essential before discharging through either farmlands or water bodies. Thus, the treatment systems are modified in order to reduce carbon amount and save nutrients. In this study, it is also purposed to produce new treatment approach for leachate. For these purposes, *Y. lipolytica* strain was grown on mediums as leachate, of which dilution rate differ from each other. These dilutions were 5%, 10%, 20%, 40% 60%, 80% and 100%. After these steps, *Y. lipolytica* strain was also grown on optimize mediums since necessary nutrient conditions needed to be optimized. For this reason, addition of internal nutrient sources was done to improve the growth. At the end of all cultivations, nutrient removal efficiencies and protein, lipid, carbohydrate content of the *Y. lipolytica* were determined. In this thesis, it was focused on the optimization of growth and lipid accumulation of *Y. lipolytica* grown on sanitary landfill leachate.

## 1.2 Scope of Thesis

The scope of the thesis is given below:

- In the Second Chapter, the current literature survey related to biofuels, biodiesel, leachate, oleaginous yeasts, and *Yarrowia lipolytica*, and comparison of algae and *Y. lipolytica* was given in detail.
- In the Third Chapter, materials and methods used in the experimental set-up and in the theoretical approaches were described in detail.
- In the Forth Chapter, results and discussions were divided into three parts. In the first part, the experimental results to find out the highest dilution rate of leachate where growth of *Y. lipolytica* observed were given. In the second part, the experimental study was extended to observe the effect of cultivations on nutrient removal efficiencies of leachate. In the third part, lipid, carbohydrates, protein, FAME composition of *Y. lipolytica* was explained.
- In the fifth chapter, the conclusions and concluding remarks were explained.

## 2. LITERATURE REVIEW

### 2.1 Biofuel

In recent years, due to the depletion of petroleum reserves, alternative energy sources to petroleum as liquid fuel are being much sought to satisfy the increasing energy demands (Dube et al, 2007).

Also, the global consumption of fossil fuels such as petroleum, coal and natural gas increases the pollution problems in this industrialized world. There are unfavorable environmental consequences of exhaust gases from petroleum derived fuels like gasoline and diesel. It is crucial to develop renewable energy sources such as biodiesel to renewably satisfy increasing energy needs.

On the other hand, wastes have been considered to be a serious worldwide environmental problem. Due to the fast industrialization and increasing pollution loads to the ecosystem, waste treatment should be more crucial topic in this century. Waste recovery should be done to provide also economical consideration. Biofuel is as an alternative energy source to petroleum to satisfy the energy demands of the world.

Biofuel, as a potential alternative for fossil fuel, is a kind of fuel that is produces from biological material (Lee & Lavoie, 2013). It is renewable energy source as an alternative energy. Basically biofuels are generated from biomass or renewable energy resources. There are several feedstocks and methods to produce biofuels. Commonly known biofuel types are as bio-hydrogen, biomethanol, biodimethyl-ether, biomass-to-liquid fuels, bioethanol, biobutanol, biogas and biodiesel (Festel, 2008). Variable process to produce biofuels from biomass are shown in Table 1.

**Table 1:** Variable process to produce biofuels from biomass. Taken from Festel, 2008.

Biofuel name	Biomass feedstock	Production process
--------------	-------------------	--------------------

Bioethanol	Sugar beets, grains	Hydrolysis& fermentation
Pure vegetable oil	Oil crops	Cold pressing/extraction
Biodiesel from energy crops	Oil crops	Cold pressing/extraction& transesterification
Biodiesel from waste	Waste/cooking/ trying oil	Transesterification
Biogas	(Wet) biomass	Digestion
Biohydrogen	Lignocellulosic material	Gasification& synthesis or Biological process

## 2.2 Biodiesel

Due to global increase in energy consumption, predicted increase in energy needs in the future, the depletion of fossil fuel reserves, climate change, emissions from transportation and increase in environmental pollution, the creation of alternative energy source is an urgent need to provide sustainable energy systems.

Biodiesel is the most promising biofuel. Since it has higher a positive net energy balance than bioethanol, easier storage property than biohydrogen and used in existing engine system. Biodiesel, a kind of biofuel, is an renewable, environmentally friendly and alternative energy source to petroleum. Biodiesel is also called as fatty acid methyl ester (FAME). It is degradable, non-toxic. It does not contribute to the atmospheric carbon dioxide levels (Abreu et al, 2005). Emissions comparison of petroleum diesel and biodiesel are shown in Table 2.

**Table 2:** Emissions comparison of petroleum diesel and biodiesel

Emissions	Diesel	Biodiesel
Carbon dioxide	100	-78%
Carbon monoxide	100	-41%
NO <sub>x</sub>	100	5%
Particles	100	-55%
PAH	100	-80%
NPAH	100	-90%
Sulfats	100	-100%

Some properties of petroleum diesel and biodiesel are shown in Table 3.

**Table 3:** Comparison of biodiesel and petroleum diesel

Property	Biodiesel	Petroleum diesel
Explosiveness	Not explosive	Explosive
Flash point, min	130 °C	64 °C
Biodegradability	Biodegradable	Not biodegradable
Toxicity	Nontoxic	Toxic

These properties proof that biodiesel is more sustainable than petroleum diesel.

Considering the similar characteristics to petroleum biodiesel, it has a potential fuel for various diesel engines and heating systems (Sharma et al, 2009). The combustion characteristics of biodiesel is respectably similar to petroleum diesel (Sitepu et al, 2014). Therefore, researchers are focussing on biodiesel production.

1<sup>st</sup>Generation biodiesel has been derived from edible plant oils such as rapeseed, sunflower, palm oil, soybean, etc. As a result of public debate about the necessity of food and fuel, 2<sup>nd</sup>generation biodiesel has been started to be argued from nonedible plant oils such as jojoba, waste oils, animal fats, etc. However, it has been decided that these oils might not be sufficient to satisfy global energy needs. Therefore, 3<sup>rd</sup>generation biodiesel from microbial lipophilic compounds is under development. These are called as SCOs including some oleaginous types of Algae, Bacteria, Fungi and Yeast. (Sitepu et al, 2014).

Potential raw materials that are used to produce biodiesel in the countries are shown in Table 4.

**Table 4:** Potential feedstocks for biodiesel production in some countries.

Taken from Mofijur et al, 2016.

<b>Country</b>	<b>Potential feedstock</b>
Argentina	Soybeans
Australia	Beauty leaf, <i>Jatropha curcas</i> , Pongamia
Bangladesh	Rubber seed, Pongamia Pinnata
Brazil	Soybeans/Palm oil/Castor/Cotton oil
Canada	Rapeseed/Animal fat/Soybeans/Yellow grease and Tallow/Mustard/Flax
China	<i>Jatropha</i> /Waste cooking oil/Rapeseed
Cuba	<i>Jatropha curcas</i> , Moringa, Neem
France	Rapeseed/Sunflower
Germany	Rapeseed
Ghana	Palm
Greece	Cottonseed
India	<i>Jatropha</i> /Pongamia Pinnata (karanja)/Soybean/Rapeseed/Sunflower
Indonesia	Palm oil/ <i>Jatropha</i> /Coconut
Iran	Palm, <i>Jatropha</i> , castor, Algae
Ireland	frying oil/animal fats
Italy	Rapeseed/Sunflower
Japan	Waste cooking oil
Kenya	Castor
Malaysia	Palm oil
Mali	<i>Jatropha curcas</i>
Mexico	Animal fat/ Waste oil
New Zealand	Waste cooking oil/Tallow
Norway	Animal fats
Pakistan	<i>Jatropha curcas</i>
Peru	Palm, <i>Jatropha</i>
Philippines	Coconut/ <i>Jatropha</i>
Singapore	Palm oil
Spain	Linseed oil/Sunflower
Sweden	Rapeseed
Thailand	Palm/ <i>Jatropha</i> /Coconut
Turkey	Sunflower, Rapeseed
UK	Rapeseed/Waste cooking oil
USA	Soybeans/waste oil/Peanut
Zimbabwe	<i>Jatropha</i>

It is seen that 1<sup>st</sup>Generation biodiesel potential is higher than 3<sup>rd</sup>generation biodiesel that is generated from microbial lipophilic compounds. The aim of this study is to contribute 3<sup>rd</sup>generation biodiesel by using *Yarrowia lipolytica*.

Biodiesel has to be satisfy specifications of ASTM D 6751 standart. In this standart, biodiesel, without mixing with other kind of oil, is expressed as B100. ASTM D 6751 standart values for B100 as biodiesel are shown in the Table 5.

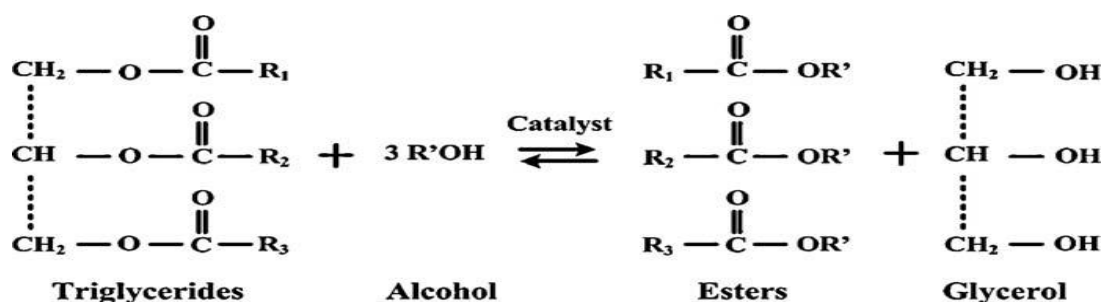
**Table 5:** ASTM D 6751 Specifications for biodiesel. Taken from ASTM D 6751 Standarts.

Property	Limit value
Flash point (closed cup), °C, min	130
Water and sediment, % volume, max	0.050
Kinematic viscosity, mm <sup>2</sup> /s, 40°C	1.9-6.0
Sulfated ash, % mass, max	0.020
Cetane number, min	47
Carbon residuea, % mass, max	0.050
Acid number, mg KOH/g, max	0.80
Free glycerin, % mass, max	0.020
Total glycerin, % mass, max	0.240
Phosphate content, % mass, max	0.001
Distillation temperature, 90% recovered (T90) <sup>b</sup> , °C, max	360

Crude oils of potential feedstocks cannot be directly used in engines because of the properties such as higher viscosity, lower volatility and higher polyunsaturated characteristics(Srivastava& Prasad, 2000). To use crude oils as a fuel in engines, these properties should be optimised. Currently, applied process are mainly as blending of crude oils; thermal cracking, micro-emulsions, and transesterification. In comparison, the most successful process to optimize crued oils is transesterification (Koh&Ghazi, 2011). Crude oil of *Y. lipolytica* is viscous. Therefore, transesterification process should be applied that oleagenous lipid body.

Biodiesel is made of fatty acid/alkyl esters which can be produced by oil species mainly triglycerides, diglycerides, monoglycerides, free fatty acids and phospholipids (Chisti, 2007).

Biodiesel has been commonly produced by transesterification, using an alcohol as ethanol or methanol (Cheirsilp, 2013).Transesterification is a multiple step reaction including three simple steps. Firstly, triglycerides converted to diglycerides. Then, diglycerides converted to monoglycerides. Finally, monoglycerides converted to esters (biodiesel) and glycerol (Mata and Martins, 2010).



**Figure 1:** Transesterification of triglycerides (Mata and Martins, 2010).

Alcohols that are used as a reactant in the transesterification process are methanol, ethanol, propanol and butanol. Because of cost-efficiency and high physical benefits, mainly methanol is used as an alcohols in the transesterification process. Also, acids, bases and enzymes are used as catalysts for transesterification. Alkali catalysts have 4000 times higher conversion rate than acid catalysts and in addition to this, alkali catalysts have higher reaction efficiency than acid catalysts (Hama et al, 2007).

### 2.3 Sanitary Landfill Leachate

Wastewater management at a landfill is critical to operations as well as the protection of human health and the environment. Landfill leachate is liquid that collects from the landfill system either produced by the waste within the system or occurring from infiltration, e.g., groundwater or rainfall (Ganesh, K. and Jambeck, J.R., 2013). Leachate is a strong wastewater and treatment is essential. Due to the mixture of solid waste deposited in sanitary landfill areas, leachate includes a variety of harmful micropollutants. These pollutants can be listed as dissolved organic matter (DOM) , inorganic macro-components, heavy metals (e.g., Cr<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Pb<sup>2+</sup>) , xenobiotic organic compounds and microorganisms (Hu et al, 2016). The synergistic, additive or antagonistic effects of the contaminants present in leachate may lead to toxicity, carcinogenicity or estrogenicity. Hence, if leachates are not collected, treated and disposal seriously, public and environmental safety should be threaten that it could contaminate near bysoil, ground water and surface water. Therefore, it is crucial to find a sustainable option to treat leachate effectively before discharging it in to the environment. (Kumari et al, 2016).

Treatment strategies include treating for discharge or pre-treatment prior to transport to a municipal wastewater treatment facility. Some wastewater treatment facilities

have the capability and capacity to accept landfill leachate with no pre-treatment; however, the large organic load of leachate can be expensive to manage. A wide variety of wastewaters have been treated in microbial fuel cells (Ganesh, K. and Jambeck, J.R., 2013). In the recent years, the bio-based production of unusual fatty acids has attracted significant attention in both plants and microbial sources such as oleaginous microorganisms. Among oleaginous microorganisms, *Y. lipolytica* is the most studied due to both its interesting biotechnological characteristics and its suitability to be manipulated in the laboratory (Ledesma-Amaro, R. and Nicaud, J.M., 2016). Landfill leachate, especially the mature leachate, contains high strength ammonium. The average concentration was about 1000-3000 mg l<sup>-1</sup>, the highest can reach to 13,000 mg l<sup>-1</sup>. Ammonium concentration between 1500 mg l<sup>-1</sup> and 3000 mg l<sup>-1</sup> has an inhibitory effect on microorganisms particularly at higher pH. It shows to be toxic to some microbes when it is above 5800 mg l<sup>-1</sup> (Zheng et al, 2005). The general characteristics of landfill leachate is shown in the Table 6:

**Table 6:** Composition of Sanitary Landfill Leachate (Akkaya et. Al., 2011)

Parameter	Analysis number	Max. value	Min. value	Gap	Average
COD	151	40600	4158	36442	12062,6
TKN	114	4368	1478	2890	2867,7
NH4-N	118	4088	1271	2817	2558
Org-N	110	902,4	0	902,4	249,3
TP	140	45,36	8,51	36,85	25,3
pH	151	8,97	6,3	2,67	8,1
Electrical conductivity	151	35	16,04	18,96	30,5
Alkalinity	147	23600	9000	14600	17234,3
Chlorur	151	6517,98	3099,04	3418,94	4962
Colour	88	9060	1700	7360	3963,9
Turbidity	87	4380	780	3600	2221,9
Total VFA	98	26188,7	41,3	26147,4	3269

To provide cheap way for nutrients provision for microorganisms, current agricultural and industrial applications should be used as raw materials due to being nutritionally rich waste/wastestreams generation. It also provides to valorize wastes by biotechnological practices. *Y. lipolytica* is used to treat wastewater in this study as biotechnological practice. The selected wastewater to cultivate *Y. lipolytica* was leachate since the treatment of leachate was harder than other types of wastewater.

To develop a new alternative approach for the treatment of leachate was aimed. Recovery rather than treatment is the hot topic in the world for the waste management. In this new approach, wastewater recovery for the energy generation as biodiesel was tried. Therefore, decrease the cost of biodiesel generated from *Y. lipolytica* as microbial oil is to provide easily commercialization. It should be highlighted that the presence of trace elements in wastes are also valorized, therefore; it provides economical advantage. For that reason, it is aimed to develop cheaper raw wastewater as leachate. Then, optimization of the growth condition of *Y. lipolytica* in landfill leachate for the high lipid accumulation is done in this study. Different concentration of leachate is treated with *Y. lipolytica* and the effect of growth and treatment efficiency is observed. Thus, treatment techniques are modified in order to reduce carbon amount and save nutrients.

## 2.4 Oleaginous Yeasts

Biodiesel could be produced from oleaginous microorganisms. Oleaginous microorganisms present in oleaginous yeast to satisfy the considerable amount of energy demands of the world. 3<sup>rd</sup> generation biodiesel from these oleaginous microorganism, also called as microbial lipophilic compounds, is under development. These oleaginous microorganisms called as SCOs include some oleaginous types of Algae, Bacteria, Fungi and Yeast (Sitepu et al, 2014). SCOs should be considered as oleaginous microorganism, accumulating large amount of lipids, at least 20% of dry weight (Ratledge, 1979).

Under the nutrients limitations (nitrogen, phosphate or iron) in the presence of excess carbon, Lipid Body (LB) could be formed in SCOs due to survive in stress conditions. LB is considered as an intracellular depot for neutral lipids mainly TAG and/or steryl esters (SE). In practice, the commonly used limiting-nutrient is nitrogen due to being easily controllable in medium (Beopoulos et al, 2009). Fatty acid composition of LB isolated from *Y. lipolytica* is oleic acid (C18:1), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and linoleic acid (C18:2). Oleic acid is the major fatty acid esterified to lipids of LP as up to 77% (Athenstaeds et al, 2006).

LB can result from two methods: de novo lipid synthesis that includes the production step of fatty acid precursors, such as acetyl and malonyl-CoA and the following step called as the Kennedy pathway or ex novo lipid synthesis that includes the directly uptake of hydrophobic substrates as alkanes, fatty acids, and triacylglycerols (TAG) from the environment and their accumulation within the cell. Ex novo lipid synthesis needs the hydrolysis of the hydrophobic substrates, transportation of the released fatty acids and their storage within the LB (Beopolous et al, 2009).

Yeasts are unicellular eukaryotic microorganisms. There are a variety of oleaginous yeast species. High-lipid accumulating yeasts have been *Lipomyces tetrasporus* (syn. *Zygotlipomyces lactosus*) with 66.5% of lipid content (LC) (Eroshin et al, 1983), *Lipomyces lipofera* with 43% of LC (Nilsson et al, 1943), *Lipomyces starkeyi* with 62 % of LC (Starkey, 1946), *Lipomyces kockii* with 77.8% of LC ( Whiffin et al, 2016), *Rhodotorula glutinis* with 57.2% of LC (Yoon et al, 1983), *Cryptococcus curvatus* (syn. *Candida curvata*, *Apiotrichum curvatum*) having 57% of LC (Moon et al, 1978), *Cryptococcus albidus* with 34% of LC (Sitepu et al, 2013), *Rhodospiridium toruloides* (syn. *Rhodotorula gracilis*) with 59.80% of LC (Tatsumi et al,1977), *Metschnikowia gruessii* (syn. *Nectaromyces reukaufii*) with 34% of LC (Stanier, 1946), *Guehomyces pullulans* (syn. *Trichosporon pullulans*, *Endomycopsis vernalis*, *Endomyces vernalis*) with 29.3% of LC( Nilsson et al, 1943), *Cryptococcus aerius* (syn. *Cr. albidus* var. *aerius*) with 63.3% of LC (Eroshin et al, 1983). Lipid content of *Y. lipolytica* is between 37.6 to 54.8% (Bati et al, 1984). *Y. lipolytica* concentration can be between 7.2 and 41.42 g/L in the bioreactor (Candia et al, 2014; Fontanille et al, 2012).

## **2.5 *Yarrowia lipolytica***

*Y. lipolytica* is a dimorphic fungus. It forms yeast cells. It is a kind of oleagenous yeast called as SCOs. It occurs naturally in food – especially in fermented dairy and meat products (Groenewald, 2014) such as cheese, yoghurts, kefir, shoyu, or from salads containing meat or shrimps (Barth et al, 1999). However, it should be also isolated from soil and petroleum products. They are observed in nature with colonies that are white in color. It is a strickly aerobic.

It has been developed in multiple biotechnological applications due to safe-to-use organism (Groenewald et al, 2013) , for instance (i) extracellular enzymes

production as lipases and esterases, proteases, phosphatases, rnaase, asparaginase, laccase, mannosidase and inulinase, (ii) organic acids production as citric and isocitric acids, a-ketoglutaric acid, pyruvic acid, succinic acid, (iii) fatty acid and alkane bioconversions, (iv) importance in food and feed industries as traditional food making, single cell protein and carotenoids, (v) fine chemistry and pharmaceutical applications, (vi) environmental applications as waste treatment, bioremediation and biodegradation of environmental pollutants, bioaccumulation of heavy metals, trinitrotoluene biotransformation (Harzevili, 2014).

Biodiesel should be qualitatively and economically comparable to the conventional diesel to be sustainable. Decreasing the cost of biodiesel production from SCO as *Y. lipolytica* will result in easily commercialization. On the contrary, operational expenses are the main obstacle for the production process. One of the main operational expenses of the SCO production is the substrate supply. The waste/wastewater streams can be viable and attractive solution to be fed through oleaginous yeasts. Although waste/wastewater contains variety of valuable compounds, the heterogenous structure will be undesirable due to its instability. To satisfy these conditions, SCO production processes need to be optimized within the optimized treatment method to provide process stability. *Y. lipolytica* consume not only hydrophilic carbon sources (e.g., glucose, glycerol, fructose, organic acids and alcohols) but also hydrophobic carbon sources (e.g., lipids, alkanes). Generally, oleaginous yeasts have not a mechanism to ferment carbohydrates. *Y. lipolytica* is an oleaginous yeast species taking an attention of scientists throughout the world since it is known as “nonconventional” yeast having the unique mechanisms for breaking down and using a variety of hydrophobic substrates as the sole carbon source.

*Y. lipolytica* has a skill to ferment carbohydrates. The range of substrates used by *Y. lipolytica* include hydrophobic substrates such as *n*-alkanes, fatty acids, fats and oils. *Y. lipolytica* should ferment some waste streams as valorization such as food waste hydrolysate (Chi et al, 2011), olive mill waste water (Gomes et al, 2008), detoxified hydrochloric acid hydrolysate of sugarcane bagasse (Tsigie et al, 2011), detoxified sulfuric acid hydrolysate of rice bran (Tsigie, 2012), detoxified liquid wheat straw hydrolysate (Yu et al, 2011), meat products fat (El Bialy et al, 2011), waste oil from chicken products fat (El Bialy et al, 2011), waste oil from frying fish (El Bialy et al, 2011), waste oil from frying vegetables (El Bialy et al, 2011), municipal wastewater

(Chi,2011), casein hydrolysate (Fickers et al, 2012), molasses (Karatay *et. al.*, 2010), hydrolyzed starch (Coelho et al, 2010), glycerol as by product of biodiesel production process(Fickers et al, 2005), brewery waste (Poli et al, 2014), barley bran and trituated nut wastes (Dominguez et al, 2003), sweet sorghum bagasse as molasses cane (Liang, 2012).

In this study, leachate wastewater stream feeding to *Y. lipolytica* leads to provide not only economical advantage but also ecological benefit due to being waste valorization. While optimization of lipid accumulation intracellularly, stress conditions is applied to increase the size of lipid body considering the biodiesel production.

## **2.6 Comparison of single cell oils: Algae vs *Yarrowia lipolytica***

It is stated that microalgae seem to be the only biodiesel feedstock that can completely displace petroleum diesel when oil yields and land areas of microalgae and other biodiesel sources are compared (Chisti, 2007). There are some advantages of microalgal biodiesel production. However, comparing with *Y. lipolytica*, microalgal biodiesel production is not the best way as an alternative for petroleum diesel.

*Y. lipolytica* has higher growth rates and lipid content than microalgae. Algal biomass productivity is set up to 4 g/L for photobioreactors (Sitepu et al, 2014). However, *Y. lipolytica* can grow quickly to high density as 41 g/L (Fontanille et al, 2012) and they can accumulate up to 54.8% oil by dry weight (Bati et al, 1984). The doubling time of *Y. lipolytica* is also high and ranging between 3 h and 4 h (Beopolous et al, 2009; Papanikolaou et al, 2001). However, commonly used microalgae as *Chlorella* and *Spirulina* has 7h and 24 h as doubling time.

In contrast, the disadvantage of *Y. lipolytica* is the need of organic carbon. Since photosynthetic microalgae has the ability to produce oxygen by the mechanism of photosynthesis. However, oleaginous yeasts utilize organic carbon and the cost of substrates should be accounted for oleaginous yeasts. Within the concept of waste valorization, as usage landfill leachate to feed *Y. lipolytica*, advantages of yeasts over algae include potentially faster growth, higher density growth, less susceptibility to viral infection, and ability to control bacterial contamination using low pH growth

conditions. (Sitepu et al, 2014). Considering waste treatment ability of *Y. lipolytica*, it was reported that 80% decrease in the COD of oil mill wastewater after treatment for 24h by using of *Y. lipolytica* has been observed (Scioli et al, 1997), and it also found that *Y. lipolytica* had been a capable of decreasing COD by 90% in palm oil mill wastewater (Oswal et. al., 2002). Therefore, *Y. lipolytica* could be used to treat wastewater.

According to Ratledge and Cohen, in 2008, 1 liter of biodiesel generated from yeast has the production cost approximately as 3 USD. According to Christi, in 2007, tax-freely 1 liter of algal biodiesel has the production cost as 2,8 USD while the 1 liter of biodiesel generated from plant oil has the production cost as 0,52 USD. 1 liter of commonly used petro-biodiesel has the production cost as 0,49 USD. Therefore, biodiesel production from SCOs should be optimized.

### 3. METATERIALS AND METHODS

#### 3.1 Microorganism, culture conditions and inoculum preparation

*Y. lipolytica* strain MUCL 28849 was obtained from BCCM/MUCL (Agro) Industrial Fungi & Yeasts Collection, Belgium. The yeast culture was maintained on YPD medium at 28 °C and sub-cultured every week. The cultivation medium recipe was given in Table 7.

**Table 7:** YPD medium (Barth&Gaillardin, 1997)

Component	Amount, g/L
D-glucose	20
Bacto Peptone	20
Yeast extract	10

The minerals addition was done to the YPD medium. The additional minerals were given in Table 8.

**Table 8:** The mineral composition for yeasts (Fontanille, 2012)

Component	Amount, g/L
KH <sub>2</sub> PO <sub>4</sub>	3,0
Na <sub>2</sub> HPO <sub>4</sub> xH <sub>2</sub> O	3,0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3,0
MgSO <sub>4</sub> x7H <sub>2</sub> O	1,0
ZnSO <sub>4</sub> x7H <sub>2</sub> O	0,040
FeSO <sub>4</sub> x7H <sub>2</sub> O	0,016
MnSO <sub>4</sub> xH <sub>2</sub> O	0,0038
CaCl <sub>2</sub> x2H <sub>2</sub> O	0,023
CoCl <sub>2</sub> x6H <sub>2</sub> O	0,0005

CuSO <sub>4</sub> ·5H <sub>2</sub> O	0,0009
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0,00006
H <sub>3</sub> BO <sub>3</sub>	0,003
Vitamin solution	1,0 *

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\* indicates mL/L

Vitamin stock solution was preserved at 4 °C and added at the time of inoculation while the rest of the medium was steam autoclaved at 121 °C for a batch time of 20 min. The inoculum was grown in a 1 M Erlenmeyer flask containing 400 mL of culture medium (as mentioned above). Based on the literature survey, pH was 6 (Papanikolaou, 2001), 5.6 (Fontanille, 2012), 4.5-6 (Dominguez, 2003), 6.1 (Chatzifragkou,2010); aeration rate was between 0,5 L/min – 5 L/min (Fontanille, 2012); temperature was around 30°C (Fontanille, 2012). Optimum operational conditions were selected. Bioreactors were operated at 28 ±2 °C with an initial pH of 5.8. The pH was adjusted to 5.8 by adding a solution of sulfuric acid. To avoid the pH change during autoclave, the pH was adjusted to 5.8 after sterilization. Aeration rate was provided as 0,5 L/min by air diffusers located at the bottom of bioreactors. Each bioreactor was equipped with flowmeter to control aeration rate.

### 3.2 Bioreactor operation with sanitary landfill leachate

Inoculum cells were grown in YPD medium with minerals for 2 days . After cells pre-grown are harvested from the preculture, they were resuspended in the waste stream-based medium as leachate medium in batch mode.

Waste stream-based medium as leachate medium was the sanitary landfill leachate collected from ISTAC in İstanbul, Turkey. Leachate was stored at +4°C, and been used after leachate was steam autoclaved at 121 °C for a batch time of 20 min for the growth of *Y. lipolytica* (MUCL 028849) in this study. To preserve sterile conditions, bioreactors were siliconized. In Figure 2, cultivation chamber was given.



**Figure 2:** Cultivation chamber of *Y. lipolytica* MUCL 28849

Operation of batch mode were carried out for between 4 and 8 d with different leakage water loading ratios (5%, 20%, 40%, 60%, 80%, and 100%). Dilution of leachate was done with the addition of sterile tap water.

### 3.3 Operational Technique of Bioreactor

Sampling was regularly done with cultivation. Samples were taken as raw and also centrifuged in a Sorvall RC 6 Plus centrifuge (Thermo Scientific, Waltham, MA, USA) following with filtered by microfilters with the pore size of 0.45  $\mu\text{m}$ . Cultures were centrifuged at 9000 rpm for 15 min. In Table 9, sampling period was given.

**Table 9:** Sampling period with cultivation

Parameters	Influent (raw)	Influent (filtered)	Reactor	Effluent (raw)	Effluent (filtered)
COD	+	+	-	+	+
TKN	+	+	-	+	+
NH <sub>4</sub> -N	-	+	-	-	+
TP	+	+	-	+	+
PO <sub>4</sub> -P	-	+	-	-	+
Optical Density analysis	-	-	+	-	-
Biomass concentration analysis	-	-	+	-	-
Protein analysis	+	-	-	+	-
Carbohydrate analysis	+	-	-	+	-
Lipit analysis	+	-	-	+	-
Lipit composition analysis	+	-	-	+	-
Particle size distribution	+	-	-	+	-

- The approximate number of cells in a culture was determined with a spectrophotometer by measuring the optical density (OD) at 680 (Jenway 6300, Staffordshire, UK). For the loss of medium volume due to evaporation, sterile tap water was added to bioreactors daily to keep culture volume constant. The OD of each bioreactor was measured daily. In Figure 3, spectrometric OD analysis was given.



**Figure 3:** Spectrometric OD analysis

- Suspended solid (SS) concentration was defined daily by using of ash-free dry weight. The linear correlation between OD values and SS values were done to define biomass concentration.
- To observe the particle size distribution in sample whether it is *Y. lipolytica* or raw leachate particle and to determine the size of *Y. lipolytica*, master sizer was used. In Figure 4, master sizer was given.



**Figure 4:** Master sizer to determination of particle size distribution

- At the end of cultivations, biomass pellets were separated by the centrifugation. Suspended biomass was kept in freeze drier (Thermo Savant, ModulyoD, USA) for 48 hours for lipid, protein, carbohydrate, fatty acid analysis. In Figure 5, freeze-dryer was given.



**Figure 5:** Freeze-dryer provinig dry biomass

- The doubling time of *Y. lipolytica* with cultivation was determined.
- Nutrient removal efficiencies of each bioreactor was determined.
- To prove biomass growth, external addition of P source was done to the leachate medium to reach P level of YPD medium. Orthophosphoric acid was used as P source.
- To prove stress conditions improving lipid content intracellularly in *Y. lipolytica*, N limitation was done with the addition of external C source. Glucose was used as C source.

### 3.4 Laboraty Equipment

Laboratory Equipment used in this study given in Table 10.

**Table 10:** List of equipment used in this study

Laboratory Equipment	Firma / Model
Micropipettes	Biohit, m10, m100, m1000, m5000
pH-meter	İno-lab pH 720
Magnetic Shaker	Yellow Line MSH Basic
Laminar Flow	Faster BH-EN 2003

Fume	Waldner N-TA 1500x900-900
Deep Freeze (-80 °C)	New Brunswick Scientific U410 Premium
Fridge (-20 °C)	Bosch KIL38A41NE
Fridge (4 °C)	Beko 7125 A+
Vortex	Stuart Vortex mixer SA8
Orbital Mixer Incubator	Certomat S-2
Nanodrop	Thermo Scientific Nanodrop 2000
Sonicator	ELMA Transsonic TP690
Serological Pipettes	Axygen Scientific, 5 ml, 10 ml, 25 ml
Pasteur Pipettes	İsolab Pasteur Pipette Glass-225 mm
Centrifuge (big)	Beckman Coulter Allegra 25 Centrifuge
Centrifuge (small)	ScanSpeed Scanspeed Mini Blue
Centrifuge (small)	CMS Harmony
Shaker	Thermo electron Corporation Forma orbital shaker
Rotary Evaporator	Heirdolp laborata 4000
Microscope	Leica TCS SP5 X
Inverted Microscope	Accu-scope 3032 Inverted BrightField Microscope

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### 3.5 Analytical Methods

Suspended Solids (SS), Chemical Oxygen Demand (COD), Total Kjeldahl Nitrogen (TKN), Ammonium (NH<sub>3</sub>-N), Total Phosphate (TP), Orthophosphate (PO<sub>4</sub>-P) analysis were all analyzed according to Standard Methods (APHA, 2005).

For lipid analysis modified Bligh and Dyer (1959) methods was used (Li et al, 2014). Pre weighted the algal powder was eluted by 5 ml of chloroform and methanol (1:2, v/v; CHCl<sub>3</sub>/MeOH) in a 15 ml falcon tube. Tube placed in an Ultrasonic Cleaner at room temperature (25 derece) for 90 minute. Then, sample kept at this condition one day (approx. 20 hrs). After that, 2 ml CHCl<sub>3</sub> and 3.6 ml water added, vigorously vortexed and centrifuged at 1,000 × g for 5 min. The organic phase was pipetted into new tube for lipid analysis (GC analysis and weighing). The remaining phase was pleaced into the freeze dryer again to determine the pure lipid amount.

The lipid composition of algal biomass was determined by a gas chromatograph (Shimadzu GC-2010) equipped with a flame-ionisation detector and a 100 m × 0.25 mm inner diameter and film thickness 0.2 µm, TR-CN100 capillary column (Teknokroma, Barcelona, Spain). The temperature of the injection port and detector were 260°C and 260°C, respectively. The oven temperature reached 140°C in first 6 min and then 140°C to 240°C (4°C/min) and fixed at 240 °C in 10 min. Helium was

the carrier gas at 30 ml/min. In addition, hydrogen gas was used at 40 ml/min flow rate and air flow was used at 400 ml/min. 1.0  $\mu$ L injection at 100:1 split ratio was used to transfer the sample to gas chromatography. Fatty Acid Composition analysis is done by Gas Chromatography GC-2010.

Carbohydrate analysis was carried out according to Gerdhart et al (1994).

Protein by Folin Reaction (Lowry et al, 1951) was used to estimate the amount of proteins (already in solutions or easily-soluble in dilute alkali) in biological samples.

In Figure 6, suspended solid examination of *Y. lipolytica* (MUCL 28849) on AP 40 filters for the determination of the linearly correlated OD result was given.



**Figure 6:** Suspended Solid examination of *Y. lipolytica* (MUCL 28849) on AP 40 filters

The biomass was subjected to the in-situ transesterification to observe the FAME quality of the generated lipid. Wychen & Laurens (2013) and El-Shimi et.al. (2013) transesterification methods were modified to analyze FAME content of the *Y. lipolytica* biomass that was grown under different mediums.

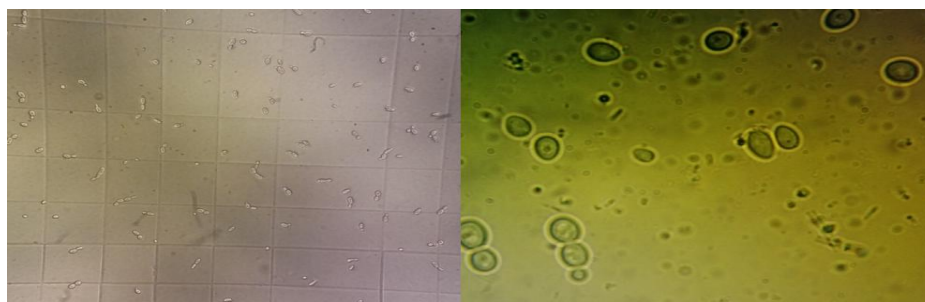
In-situ transesterification method, the yeast biomass was subjected to the direct transesterification process using acid catalyzed named HCl. The transesterification temperature, catalyze concentration are important parameters that effect the transesterification efficiency. In this study, an internal standard named Methyl tridecanoate (C13:0ME), chloroform, methanol and methanolic HCl was put together and incubated on hot plate. After that, hexane was added and obtained upper phase was loaded to the gas chromatography. Firstly, the FAME conversion efficiency under different parameters was investigated and the analysis was continued with the most efficient method. FAME composition was derived with gas chromatography.



## 4. RESULTS AND DISCUSSION

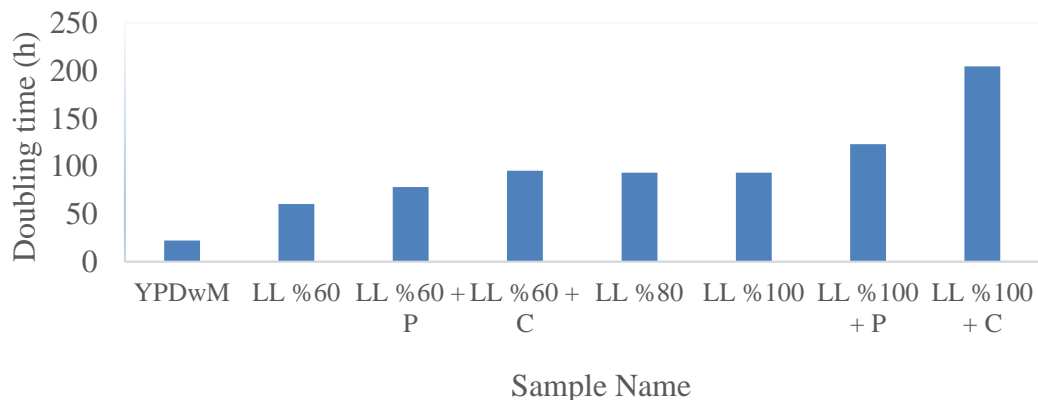
### 4.1 Growth of *Yarrowia lipolytica*

In this study, cultivations of *Y. lipolytica* (MUCL 28849) was carried out with YPD medium. To improve the growth of *Y. lipolytica*, mineral additions were done to the medium. To control the formation of *Y. lipolytica*, microbial examination was done regularly during cultivation. In Figure 7, image of *Y. lipolytica* MUCL 28849 from microscopic examination was given.



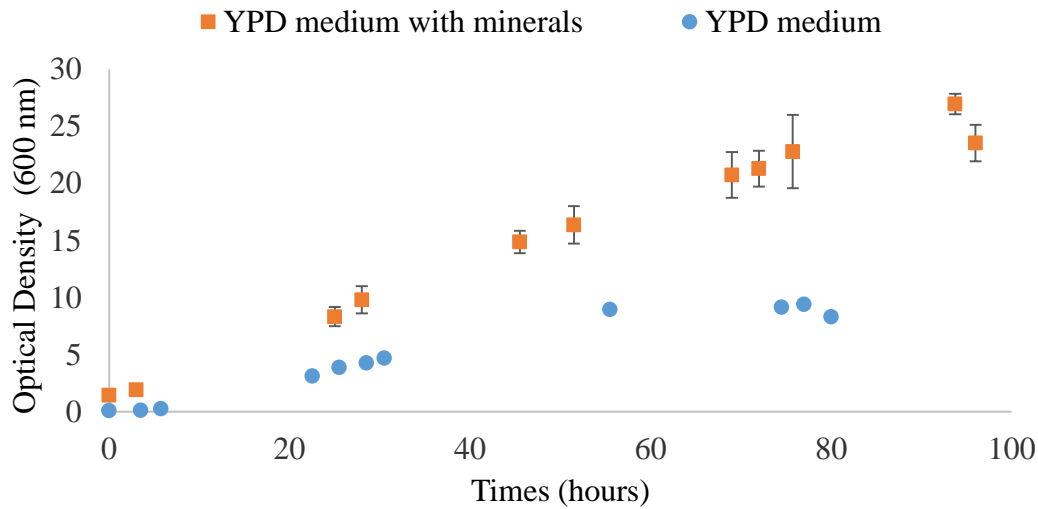
**Figure 7** Image of *Y. lipolytica* MUCL 28849 from microscopic examination

In Figure 8, comparison of doubling time of *Y. lipolytica* was given. It was shown that the doubling time of *Y. lipolytica* increases while the dilution rate also increased. The adaptation of *Y. lipolytica* became hard in higher dilution rate due to high doubling time.



**Figure 8** Doubling time of the growth of *Y. lipolytica*

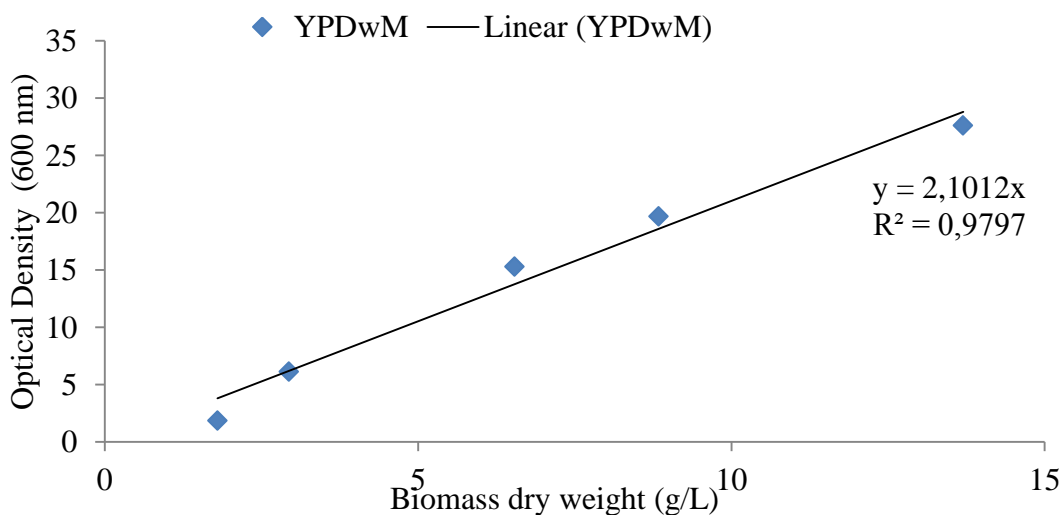
Optical density change in YPD medium with/without minerals was given in Figure 9.



**Figure 9 :** Comparison of average optical density change during cultivation of *Y. lipolytica* MUCL 28849 with YPD medium and YPD medium with minerals

The maximum OD was 26,96 at 93h with the cultivation of YPD with minerals. It was seen that addition of minerals improved the growth of *Y. lipolytica* with the medium.

Within the aim of determination biomass concentration, linear correlation between OD and dry weight was done with YPD medium with minerals. average dry weight change linearly correlated with OD with YPD medium with minerals is in Figure 10.

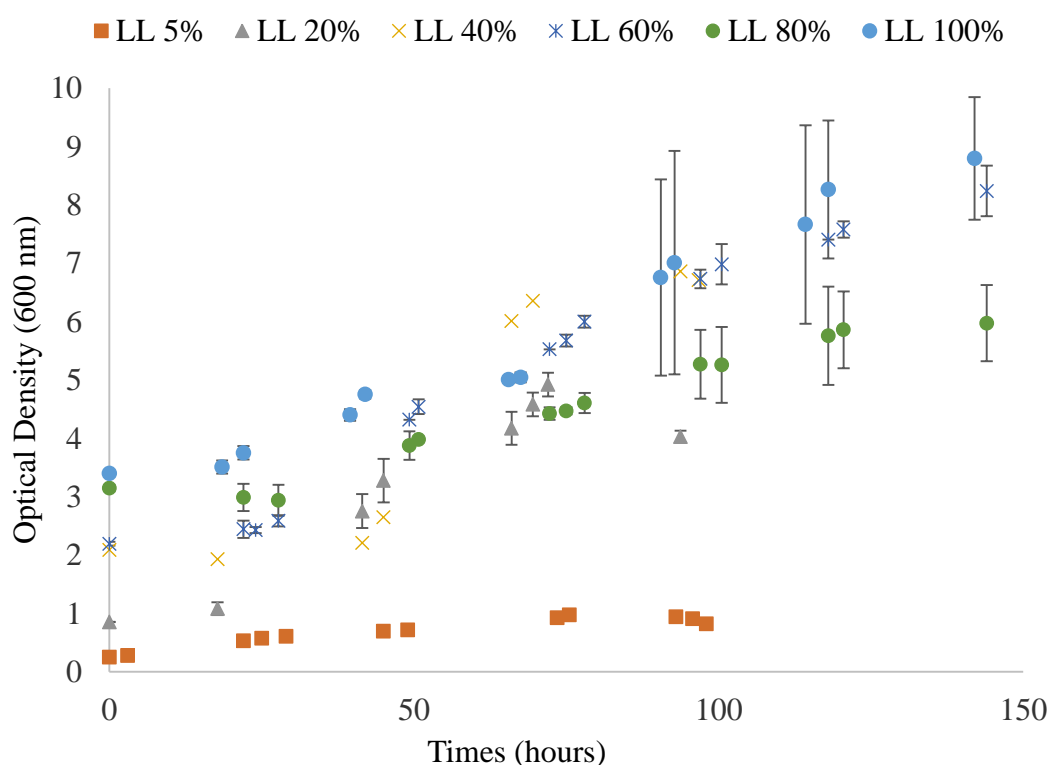


**Figure 10:** Average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* MUCL 28849 with YPD medium with minerals

In the literature, 80 g/L glucose in the medium with minerals results in 43,38 g/L biomass concentration (Fontanille et. Al., 2011). In this study, in YPD medium with

minerals, included 20 g/L glucose, resulted in 14,83 g/L biomass concentration. It showed that similar results were observed with the literature.

Cultivations of *Y. lipolytica* (MUCL 28849) on leachate were carried out up to 100% dilution with water to find out the maximum ratio where the *Y. lipolytica* could grow. The dilution of leachate was managed with tap water to adjust the concentrations of leachate as 5, 20, 40, 60, 80 and 100%. OD and dry weight results of biomass cultivation on leachate demonstrated that how the dilution rate affect the growth efficiency. While the growth of *Y. lipolytica* (MUCL 28849) was achieved at 5, 20 and 40% dilution successfully, at the 60% and less dilutions, not any growth was observed firstly. For this reason, inoculation was done in leachate with the addition of YPD medium. Then, cultivation was done from these acclimated inoculation. The optical density changes during cultivation of *Y. lipolytica* (MUCL 28849) with 5, 10, 20, 40, 60, 80 and 100% LL was given in Figure 11.

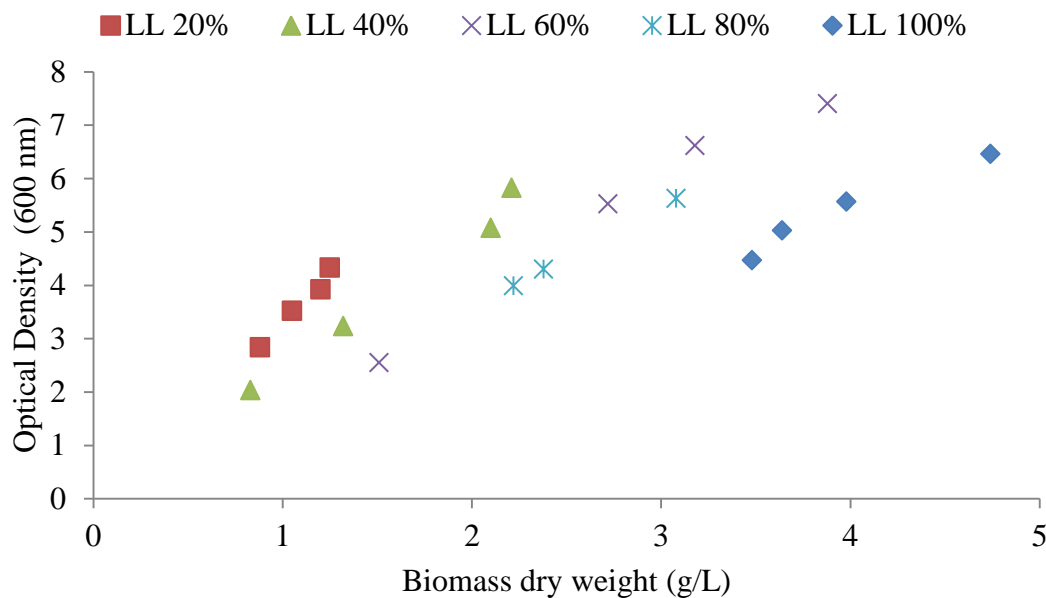


**Figure 11:** Comparison of average optical density change during cultivation of *Y. lipolytica* MUCL 28849 with 5, 10, 20, 40, 60, 80 and 100% LL

The maximum OD was observed in LL 100%. At LL 100% growth study, the OD was the maximum as 8,7 at 142 h. However, the change of OD during cultivation was maximum in LL 60%. Therefore, the optimum medium was LL 60% that was the OD as 8,2 at 144h . The reason why OD was higher with the cultivation of LL

100% than LL 60% was that there was particles in the leachate medium. OD included both biomass and leachate particles. Also, the period of life of *Y. lipolytica* changed with different LL medium. Acclimation problem was observed in LL 60%, LL 80% and LL100%. Therefore, period of life were longer in these medium.

Comparison of the correlation between OD and dry weight of biomass was given in Figure 12.

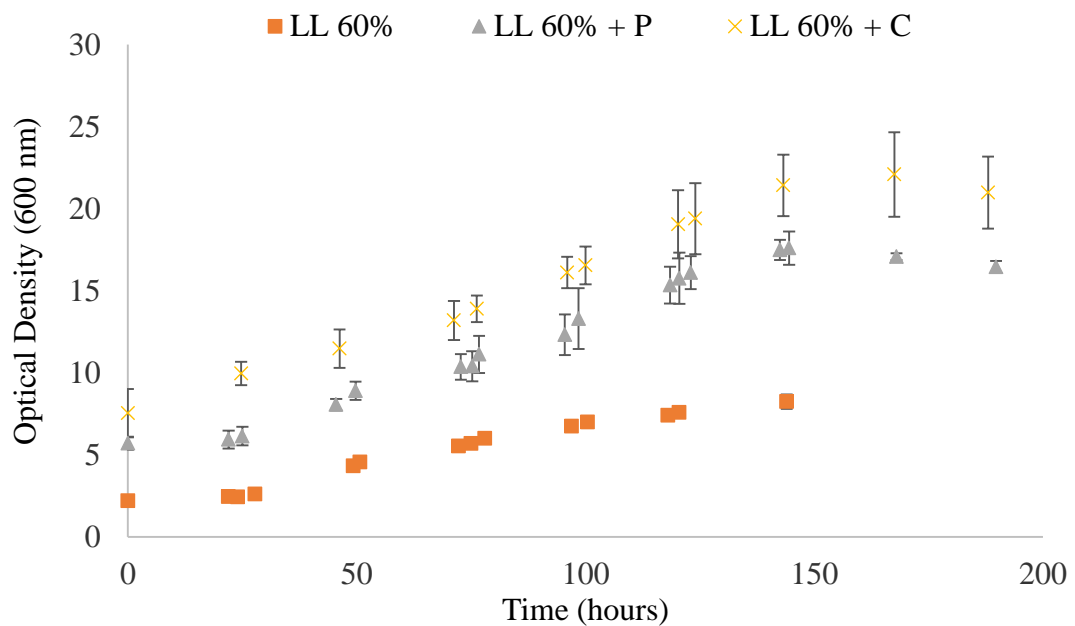


**Figure 12:** Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* MUCL 28849 with 20, 40, 60, 80 and 100% LL

The maximum biomass growth was 100% L as 6, 40 g/L. The optimum biomass growth was calculated by using of linear correlation. It was observed LL 60% as 4,34 g/L. After this concentration, more concentrated leachate had less efficiencies in terms of biomass growth. However, the increase ratio was not proportional as the dilution ratio. This manner can be attained as inhibition of high LL concentrations. Heavy metals (Ca, Mg, K, Na, Pb, Cd, Cr, Cu, Fe, Ni, Zn) are high concentrations in leachate (Akkaya et al, 2011). These could be the reason of acclimation of *Y. lipolytica* through leachate. Therefore, while the leachate concentrations increased, acclimation of *Y. lipolytica* became hard considering typical growth period of yeast cells.

After determination of optimum leachate concentration for *Y. lipolytica*, phosphate was added externally to provide the need in comparison with the YPD medium.

Then, to provide nitrogen stress, carbon addition was done to improve lipid accumulation in biomass. Since *Y. lipolytica* have the property to deposit lipid intracellularly under nitrogen stress (Rakicka, 2015). N limitation carried gradually based on the literature. Gradually, N concentration should be reduced to less than 10% of normal medium concentration (Kavšček, 2015). C:N ratio was reached from 1-2 to 20-30. Stress condition was applied at the biomass that were in the death phase in %60 and %100 +P LL. Comparison of OD of LL 60%, %60+P, %60+C was given in Figure 13.

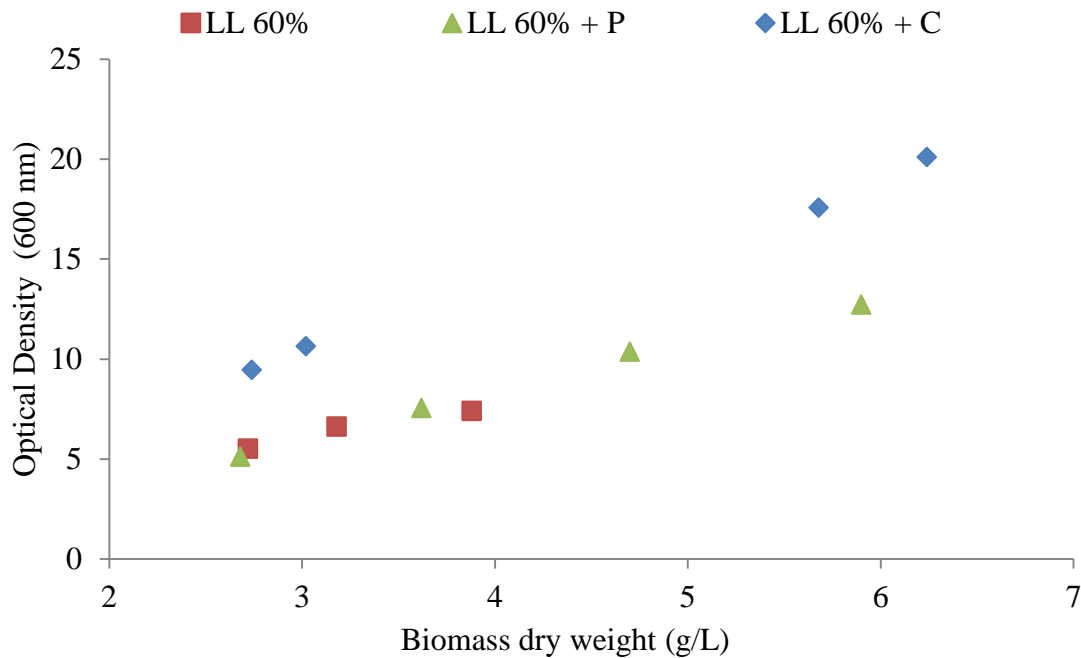


**Figure 13:** Comparison of average optical density change during cultivation of *Y. lipolytica* MUCL 28849 with 60, 60+P and 60+C% LL

External P addition increased OD. LL 60%+P had higher OD than LL 60%. OD was maximum with LL 60%+C as 22,1 at 167 h. However, optimum medium was LL 60% +P. that had OD as 17,6 at 144 h. Addition of P resulted in longer period of life of *Y. lipolytica*.

In stress conditions to maximize lipid accumulation, biomass growth should be at stationary phase (Qiao et al, 2009). Thus, growth should not be observed anymore. In Figure 12, the growth of *Y. lipolytica* with 60% +C LL was still observed. Therefore, stress conditions were not achieved successfully. Adjustment of C:N ratio of leachate from 1-2 to 20-30 was not enough to prove stress for biomass. Much higher C:N ratios were needed to achieve stress conditions to obtain stationary phase.

Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* with 60, 60+P and 60+C% LL was given in Figure 14.

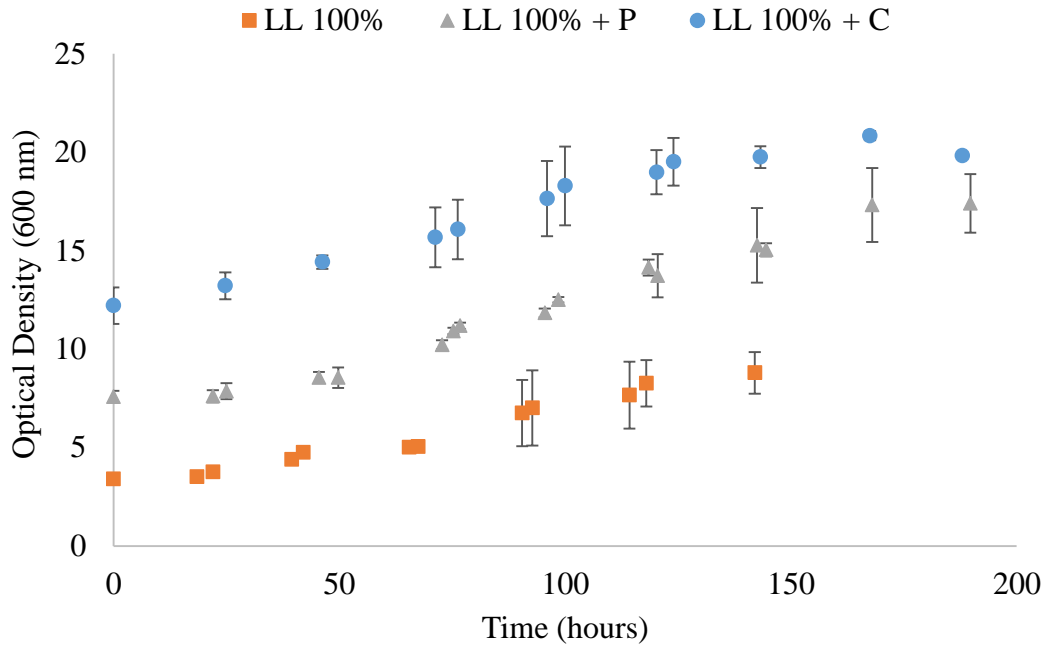


**Figure 14:** Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* MUCL 28849 with 60, 60+P and 60+C% LL

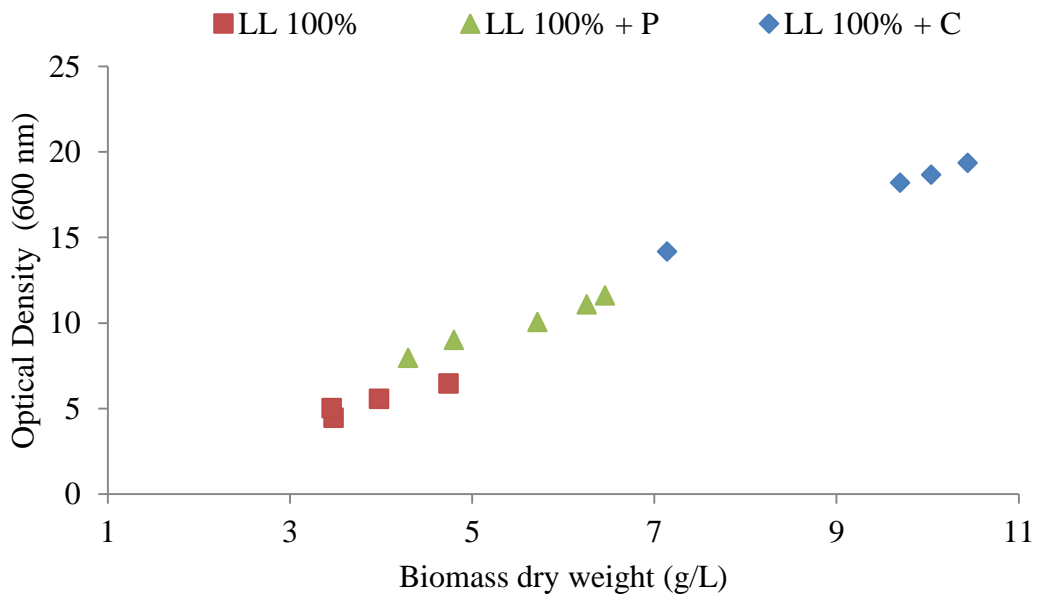
The optimum biomass growth was LL 60%+P as 7,95 g/L.

Comparison of average optical density change during cultivation of *Y. lipolytica* with 100, 100+P and 100+C% LL was given in Figure 15. Same procedure of addition of P and C with the cultivation of 60% LL, 60%+P LL, 60%+C LL was provided for 100, 100+P and 100+C% LL. The yield of 100, 100+P and 100+C% LL cultivations were less than 60% LL, 60%+P LL, 60%+C LL cultivations.

The OD was maximum with LL 100%+C as 20,8 at 167 h. However, optimum medium was LL 100% +P as 17,3 at 168h. Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* with 100, 100+P and 100+C% LL was given in Figure 16.



**Figure 15:** Comparison of average optical density change during cultivation of *Y. lipolytica* MUCL 28849 with 100, 100+P and 100+C% LL

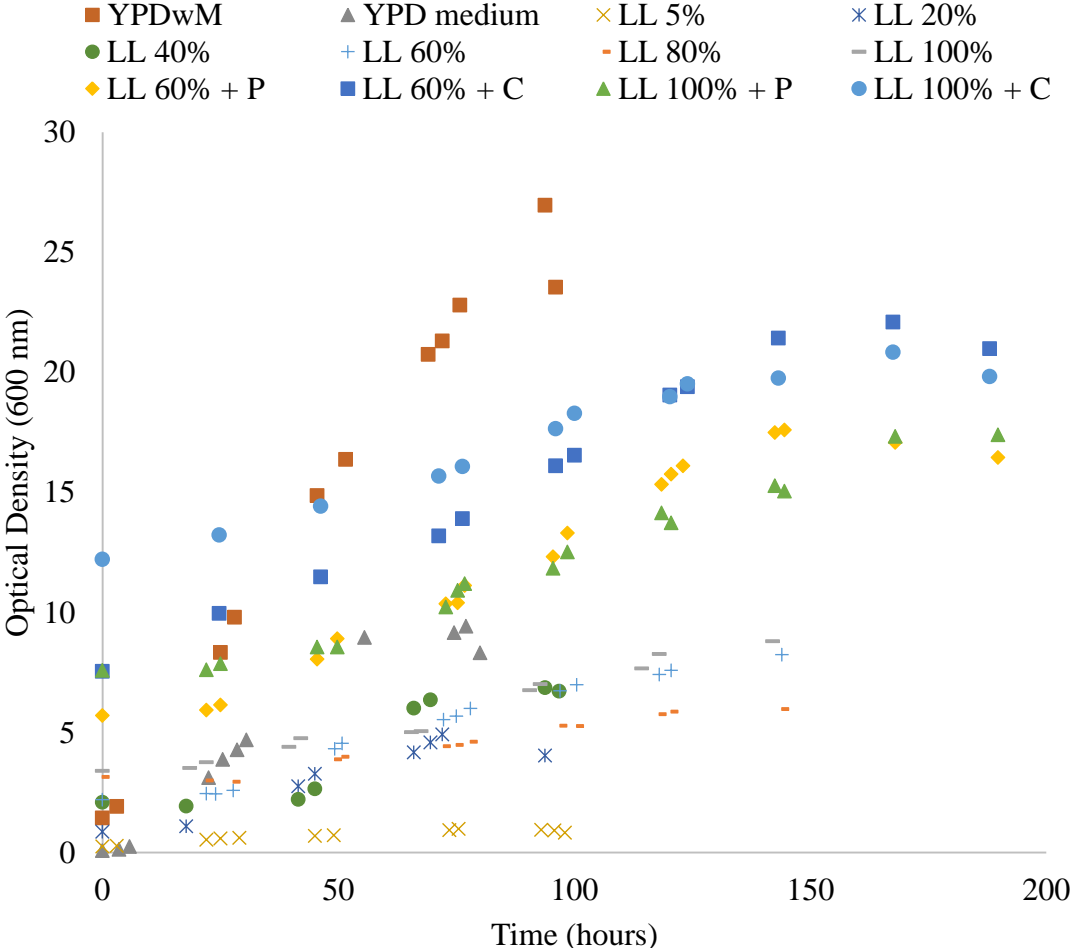


**Figure 16:** Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* MUCL 28849 with 100, 100+P and 100+C% LL

The maximum biomass growth was 100%+C LL as 11,26 g/L; however, the optimum biomass growth was 100%+P LL as 9,62 g/L.

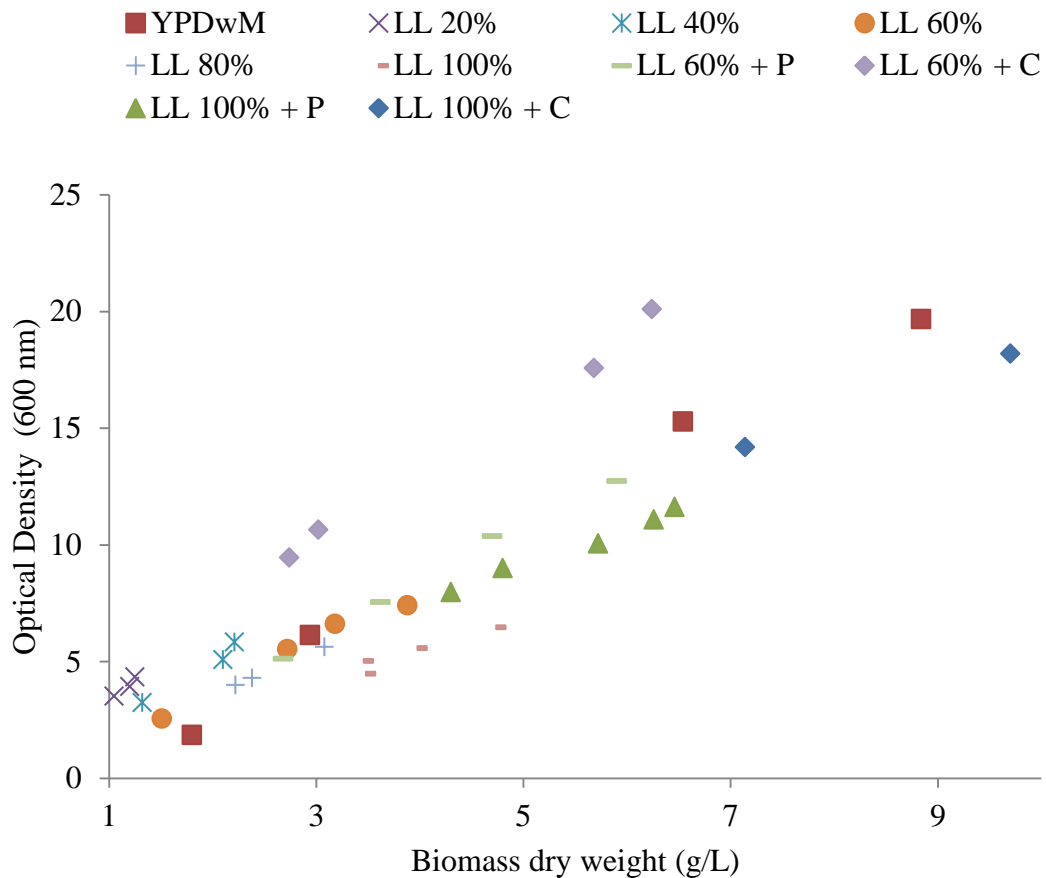
Based on Figure 15 and 16, addition of C was not successful to achieve stress conditions with 100% +C LL, with the similarity of 60, 60+P and 60+C% LL.

In Figure 17, comparison of average optical density change during cultivation of *Y. lipolytica* MUCL 28849 in all medium was summarized.



**Figure 17:** Comparison of all over average optical density change during cultivation of *Y. lipolytica* MUCL 28849 with all medium

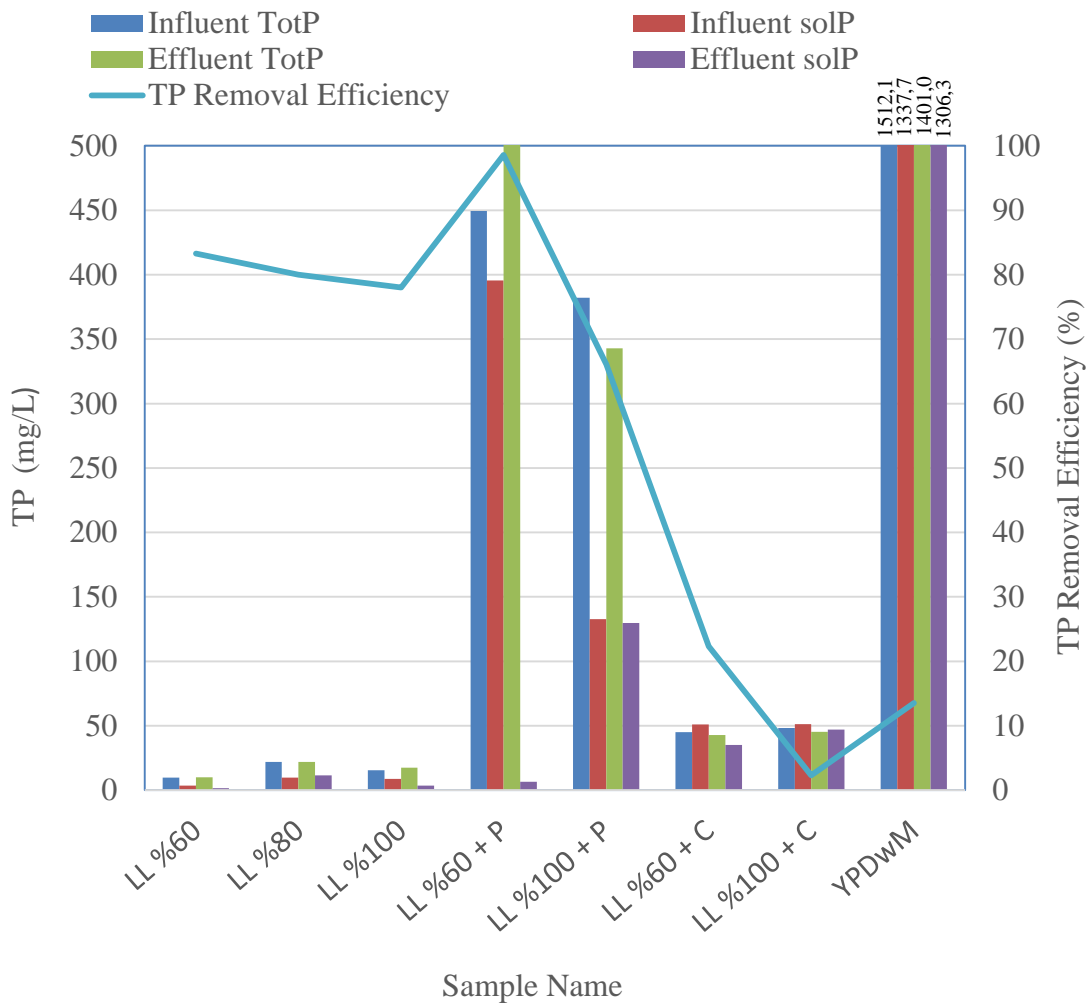
In Figure 18, comparison of average dry weight change linearly correlated with optical density optical density change during cultivation of *Y. lipolytica* MUCL 28849 in all medium was summarized.



**Figure 18:** Comparison of average dry weight change linearly correlated with optical density during cultivation of *Y. lipolytica* MUCL 28849 with YPD medium with all medium

#### 4.2 Characteristic of Medium After Yeast Cultivation

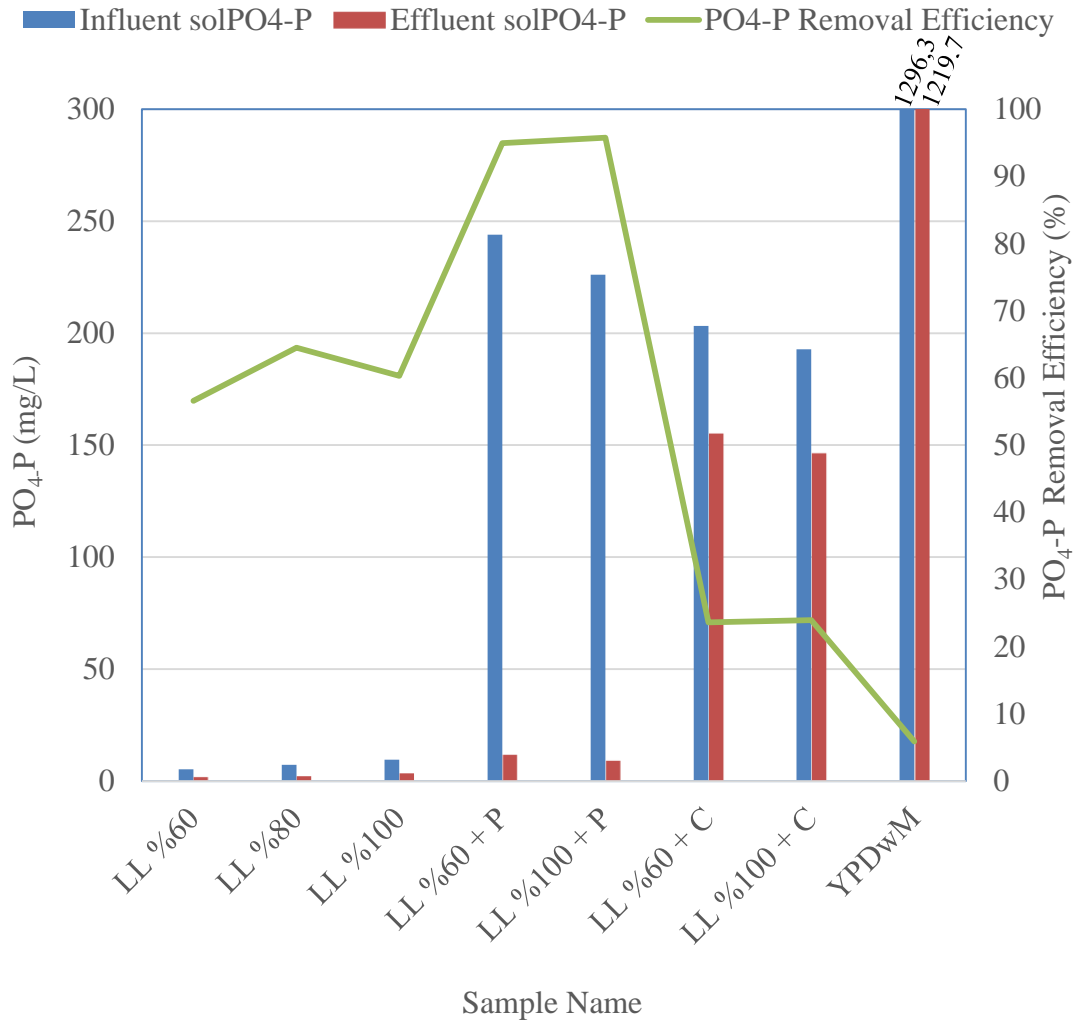
Sampling were done from influent and the effluent during the cultivation. COD, TKN, ammonium, TP, orthophosphate experimental procedures run. Experimental removal efficiencies of nutrients that were present in LL were calculated. Total and soluble form of nutrients were measured. Soluble form was obtain at the end of filtration of raw LL from the filter having 0.45  $\mu\text{m}$ . Average total phosphate change during cultivation of *Y. lipolytica* (MUCL 28849) with all media was given in Figure 19.



**Figure 19:** Average total phosphate change during cultivation of *Y. lipolytica* (MUCL 28849). Experiments run with both raw wastewater (total) and supernatant (soluble) filtered by filter that has 0.45 pore size

Total phosphate concentration was as low as 15,55 mg/L in raw 100% LL. Therefore, external phosphate was supplied to increased the yield of *Y. lipolytica* mass. External addition of orthophosphate was done by phosphoric acid, then the pH was rearranged to set at 5.8. The maximum TP removal was observed in 60% + P LL as 98,57%. TP removal with the cultivation of 60%+P LL was 83,30%. Better P removal was observed 60, 80, 100%, respectively.

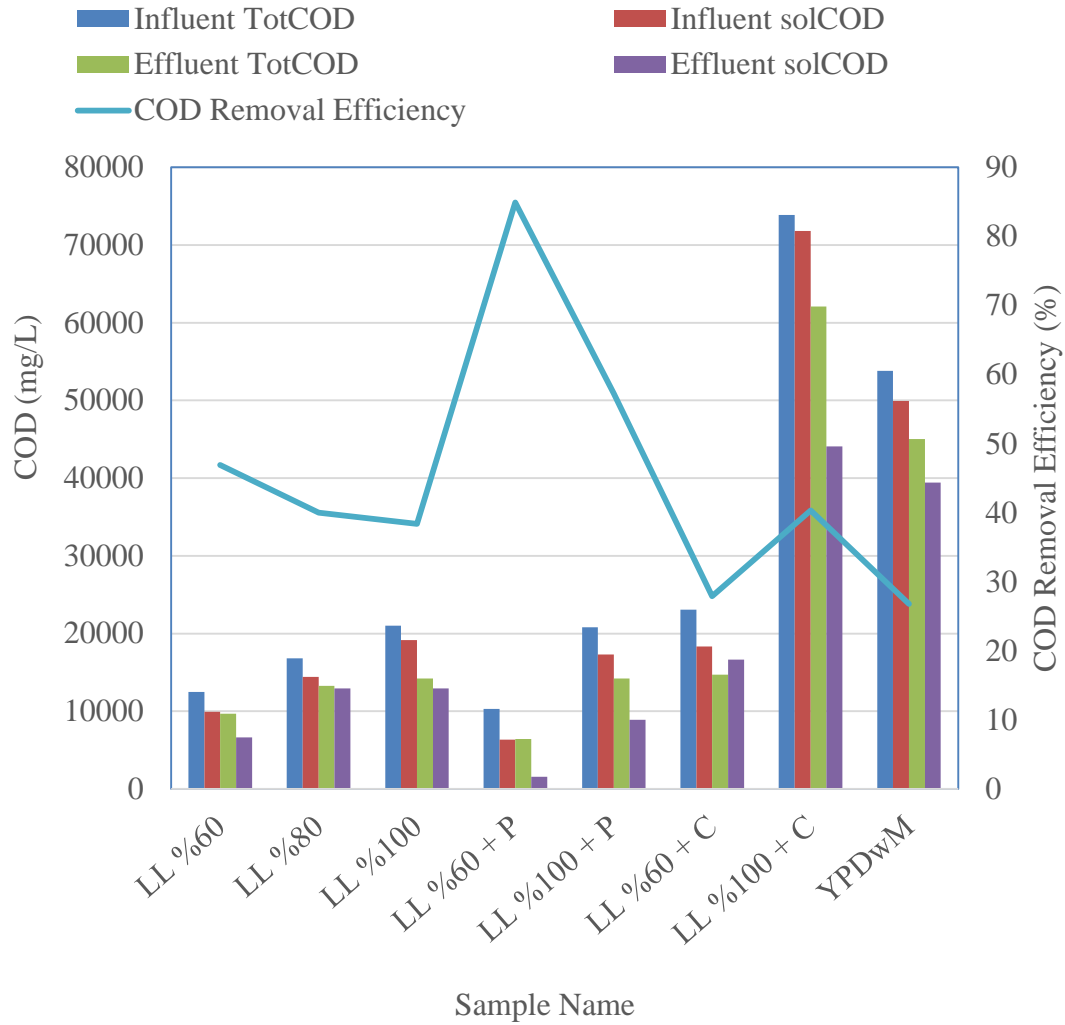
Average orthophosphate change during cultivation of *Y. lipolytica* (MUCL 28849) with all media was given in Figure 20.



**Figure 20:** Average orthophosphate change during cultivation of *Y. lipolytica* (MUCL 28849). Experiments run with only supernatant (soluble) filtered by filter that has 0.45 pore size

The maximum PO<sub>4</sub>-P removal was observed in 100% + P LL as 95,77%. Secondly, PO<sub>4</sub>-P removal was observed in 60% + P LL as 94,98%.

Average COD change during cultivation of *Y. lipolytica* (MUCL 28849) with all media was given in Figure 21.



**Figure 21:** Average chemical oxygen demand change during cultivation of *Y. lipolytica* (MUCL 28849). Experiments run with both raw wastewater (total) and supernatant (soluble) filtered by filter that has 0.45 pore size

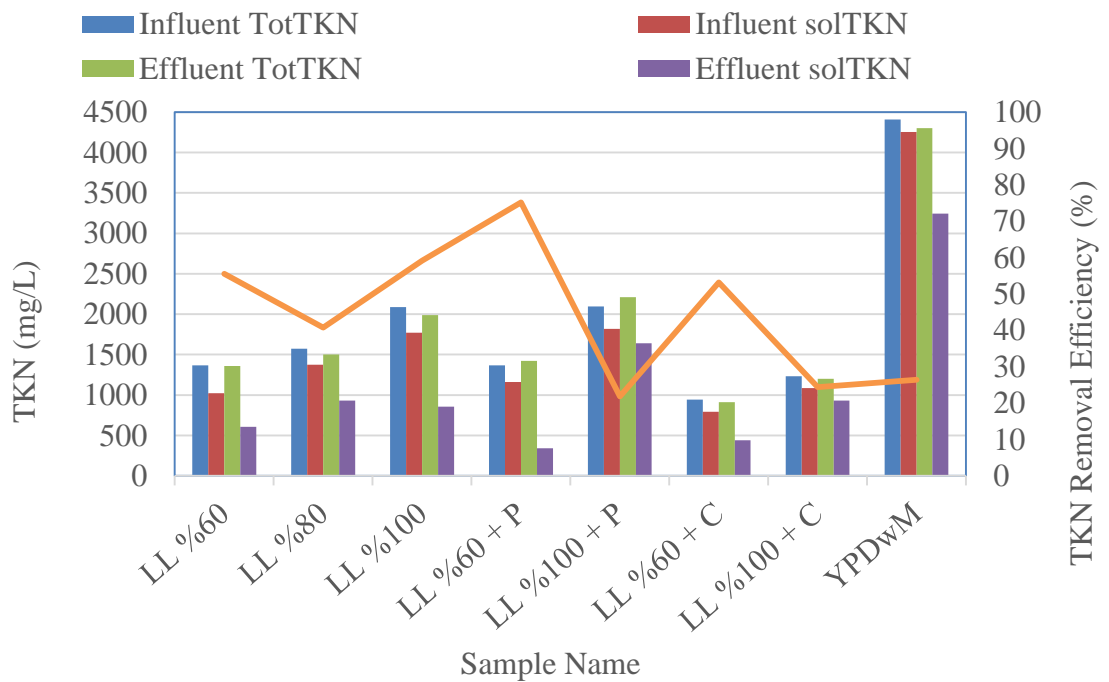
During cultivation on 60, 80, 100% leachate concentrations, without addition of nutrients as N and P, COD removal efficiencies were lower than 50%.

The maximum COD removal efficiency was observed in LL 60% +P as 84,89% .

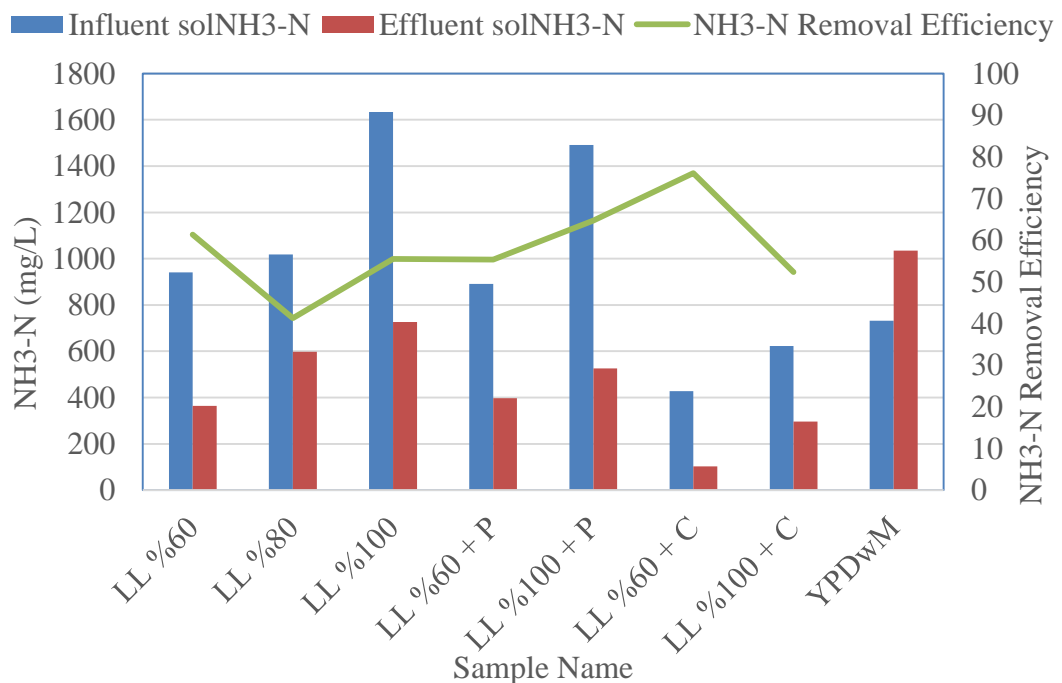
Average TKN change during cultivation of *Y. lipolytica* (MUCL 28849) with all media was given in Figure 22.

The maximum TKN removal efficiency was observed in 60% LL+P as 75,14% .

Average ammonium change during cultivation of *Y. lipolytica* (MUCL 28849) with all media was given in Figure 23.



**Figure 22:** Average total kjeldahl nitrogen change during cultivation of *Y. lipolytica* (MUCL 28849). Experiments run with both raw wastewater (total) and supernatant (soluble) filtered by filter that has 0.45 pore size



**Figure 23:** Average ammonium change during cultivation of *Y. lipolytica* (MUCL 28849). Experiments run with only supernatant (soluble) filtered by filter that has 0.45 pore size

The maximum ammonium removal efficiency was observed in 60% LL+C as 76,10%. Secondly, ammonium removal efficiency was observed in 60% LL as 61,33%.

It was also shown that the removal of ammonium could not be achieved in the YPD medium. Since organic nitrogen in the medium broke down to ammonium. It is called as ammonification. Therefore, at the end of the cultivation in YPD medium, ammonium level was high.

Cultivations were done with raw wastewater as leachate. Since there was particles in raw wastewater, dry weight results included both leachate particles and *Y. lipolytica*. In Table 11, analysis of particle size distribution in the cultivation medium was shown.

**Table 11:** Comparison of particle size distribution change during cultivation of *Y. lipolytica* MUCL 28849

	Influent			Effluent		
	d(0.1), µm	d(0.5), µm	d(0.9), µm	d(0.1), µm	d(0.5), µm	d(0.9), µm
LL % 60	9,2	30,3	89,4	6,5	83,2	364,3
LL % 80	7,6	26,6	77,3	1,4	10,1	35,0
LL % 100	6,0	24,0	83,7	2,1	14,5	81,9
LL % 60 + P	8,4	43,3	134,4	10,3	251,9	715,9
LL % 100 + P	7,1	38,0	169,9	4,7	114,8	538,3
LL % 60 + C	2,7	9,3	48,8	2,9	13,8	72,6
LL % 100 + C	2,9	6,6	175,7	2,8	11,0	411,3
YPDwM	-	-	-	2,9	4,2	6,0

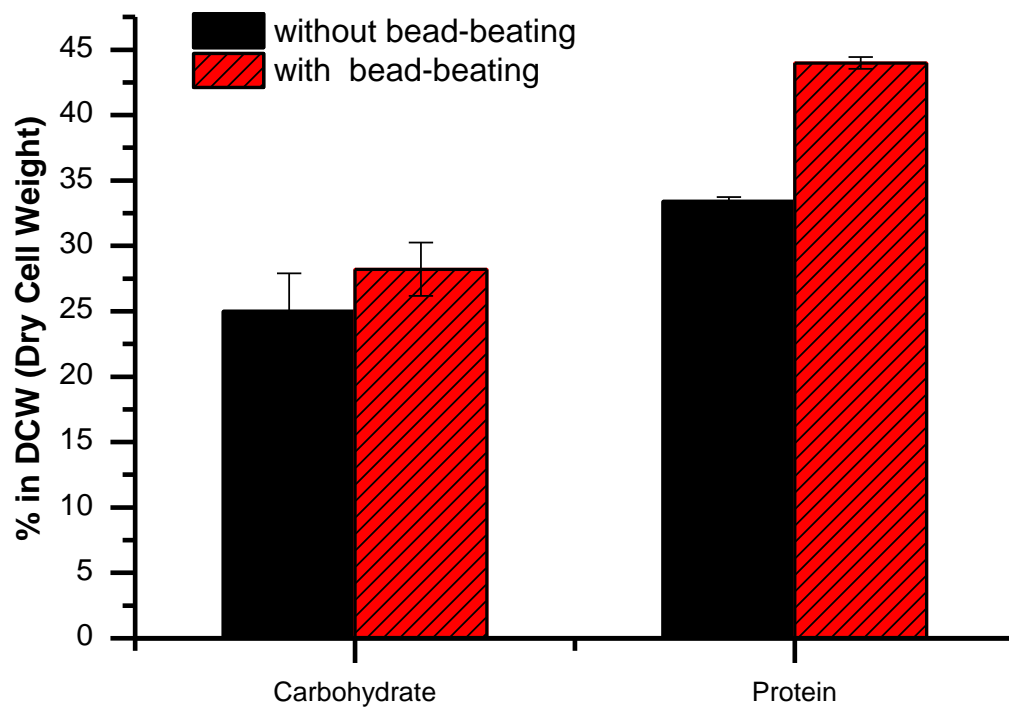
It was expected that the difference between the size of *Y. lipolytica* and leachate particles would be noticeable. However, bigger particle size was observed after cultivations. It should be due to colony forming ability of *Y. lipolytica*. The floc formation ability of *Y. lipolytica* should be convenient.

### **4.3 Protein, Carbohydrate, Lipid, Fatty Acid Composition Analysis of Medium After Yeast Cultivation**

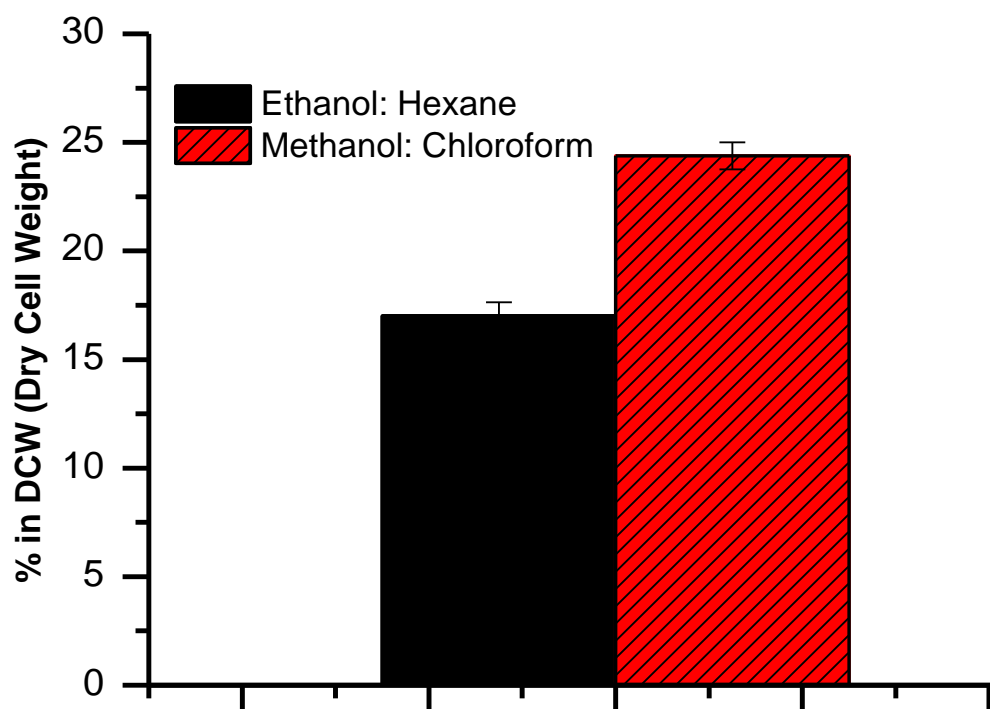
To determine the availability of *Y. lipolytica* as a raw material for biodiesel production, protein, carbohydrate, lipid and FAME composition analysis of medium were done.

For protein, carbohydrate and lipid analysis of *Yarrowia lipolytica* biomass, preliminary studies was done with the efficiency of the extraction method. The yeast cell wall with and without mechanical cell wall distribution method named “bead-beating” was investigated. In Figure 24, mechanical cell wall distribution was given.

It was clearly observed that bead-beating method has positive extraction efficiency. Moreover, co-solvents named hexane-ethanol and chloroform-methanol on lipid extraction from yeast was investigated. In Figure 25, co-solvents on lipid extraction was given. Lipid extraction from yeast results indicated that chloroform-methanol solvent extraction has higher efficiency as compared to ethanol and hexane.

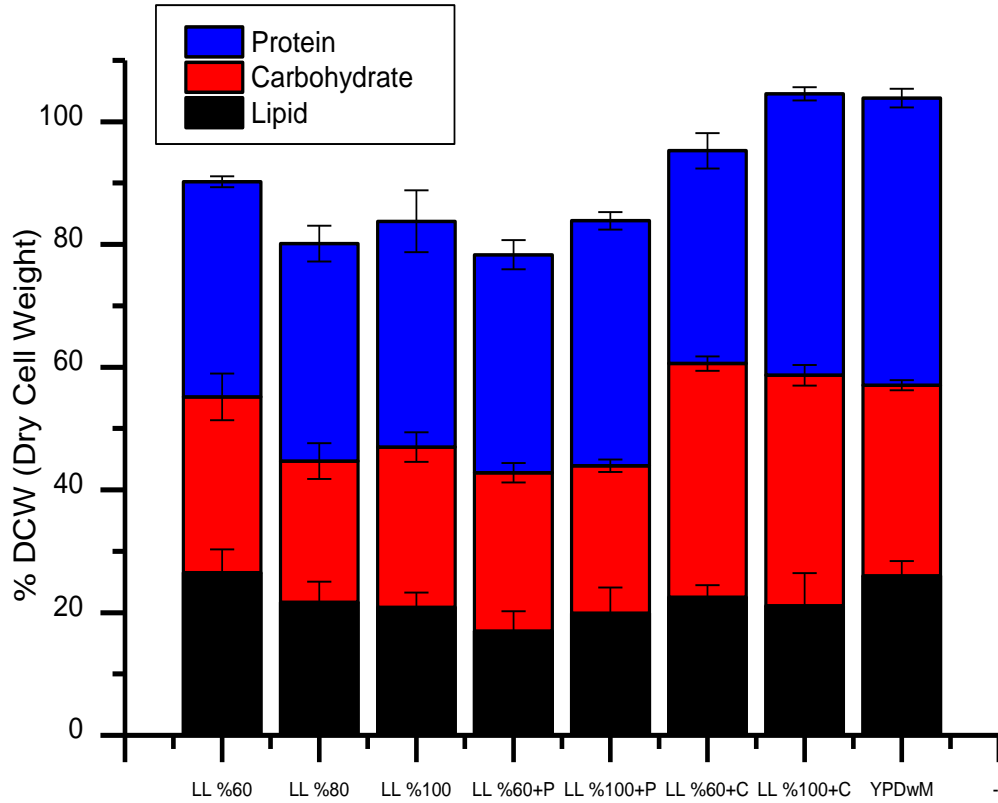


**Figure 24:** Mechanical cell wall distribution



**Figure 25:** Co-solvents on lipid extraction

In Figure 26, protein, carbohydrate, lipid analysis of all medium was given.



**Figure 26:** Protein, carbohydrate, lipid analysis of all cultivations  
In Table 12, percentage of lipid, carbohydrate and protein in DCW was given.

**Table 12:** Percentage of lipid, carbohydrate and protein in DCW

	Lipid	Carbohydrate	Protein
LL % 60	27±4	29±4	35±1
LL % 80	22±0	23±3	35±3
LL % 100	21±2	26±2	37±5
LL % 60+P	17±3	26±2	36±2
LL % 100+P	20±4	24±1	40±1
LL % 60+C	23±2	38±1	35±3
LL % 100+C	21±5	38±1	46±1
YPDwM	26±2	28±1	47±2

It was measured that the total percentages remains the 85-95% of the total biomass.

The residue was defined as ash content of the biomass.

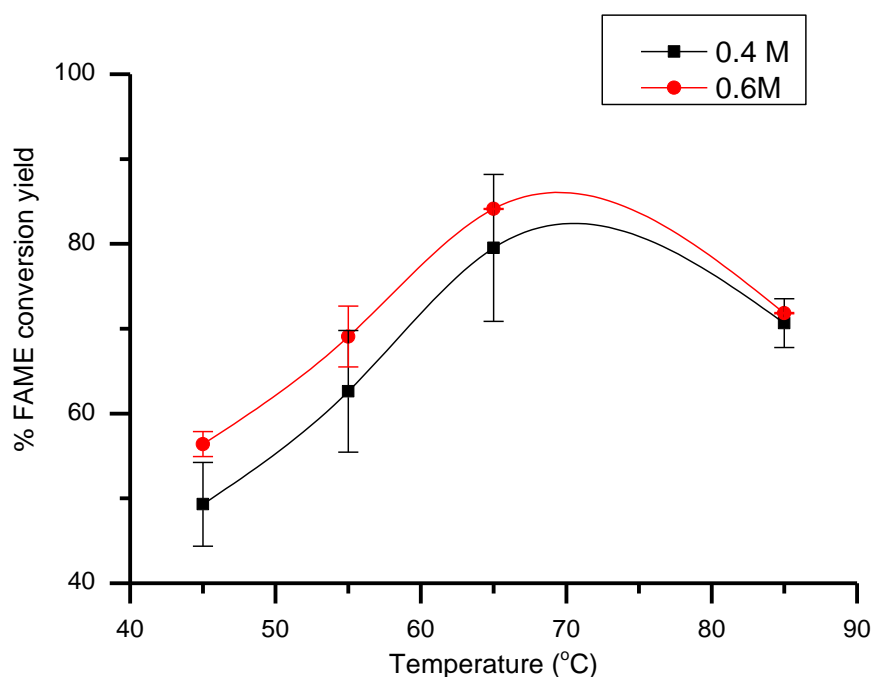
The lipid content of *Y. lipolytica* that was grown in YPDxM was around 26±0.2 %.

The data is in good agreement with previous studies like Friendlander et al, 2016.

The lipid content of 60% LL was similar to the lipid content of YPDwM. In 60%+C LL and 100%+C LL cultivations, lipid percentage was not higher than 60% LL and

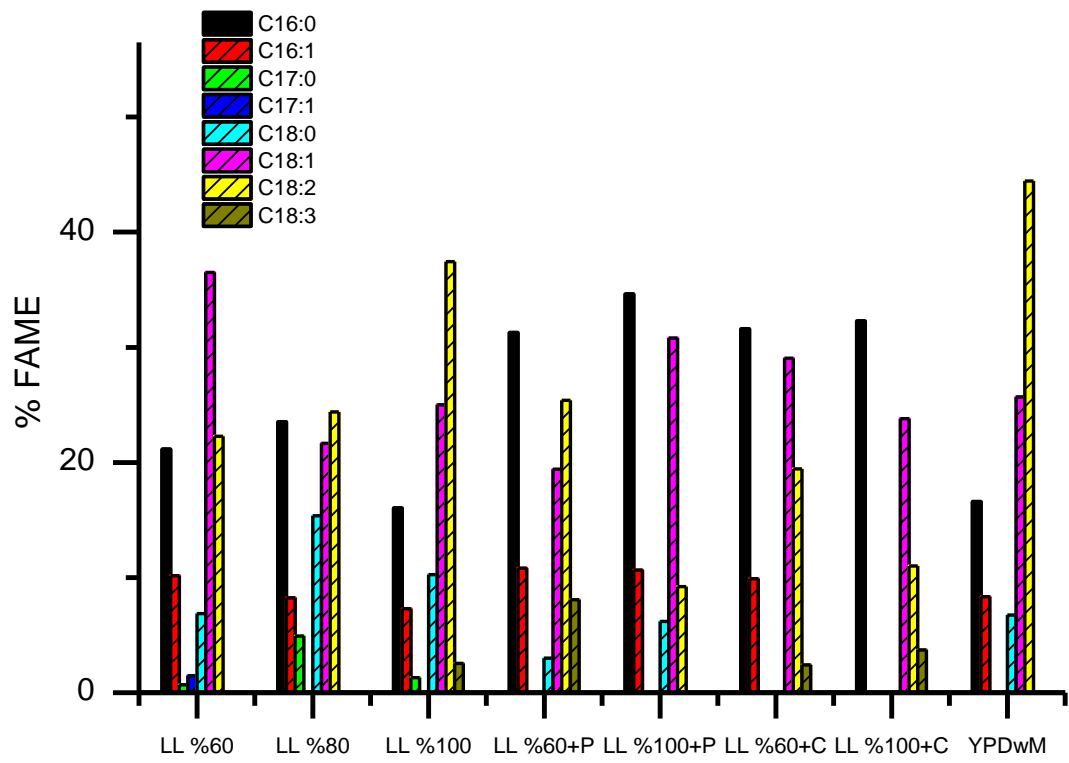
100% LL cultivations. Since there was not succesful stress conditions proving the addition of C source with cultivation of *Y. lipolytica*, lipid accumulation intracellularly was not be increased.

During in-situ transesterificcation process, the most efficient transesterification parameters were chosen as 0.6 M HCl and 65°C incubation temperature as shown in Figure 27.



**Figure 27:** Most efficient transesterification parameters analysis

The FAME composition obtained from biomass of the different mediums were calculated. It was observed that most of the FAME were Palmitic acid (C16:0), Oleic acid (C18:1) and Linoleic acid (C18:2) as around 45%. The observed FAME data was in good agreement with previous studies like Fontainelle et al, 2012. FAME composition were given in Figure 28. FAME composition with cultivations was suitable for biodiesel protuction (National Centre for Agricultural Utilization Research, 2012).



**Figure 28:** FAME Composition with all cultivations



## 5. CONCLUSIONS AND RECOMMENDATIONS

To the *best* of our *knowledge*, this is the first study about the growth of *Y. lipolytica* on Sanitary Landfill Leachate. Results of this thesis showed that *Y. lipolytica* can grow efficiently in sanitary landfill leachate. The nutrient removal efficiencies of leachate was significant. This study is as a first step of this new approach. It is promising for future.

In general, biomass concentrations at the end of experiments were lower than cultures grown in synthetic medium. The optimum biomass growth was observed with the cultivation of 60% LL as 4,34 g/L. 60% concentration of leachate was the most available cultivation *for Y. lipolytica*. To develop the growth, additional P supply was done to the culture. After addition of external P to the leachate cultivation, the growth of biomass increased. In 60%+P LL cultivation, biomass concentration reached to 7,95 g/L.

All dilutions were applied with the addition of tap water. As an commercial aspect, water usage is not preferred. Dilution is not applicable technique due to the usage of water. Therefore, the improvement of the cultivation with 100% LL should be studied. Also, the working temperature was 28 °C. As an commercial aspect, higher temperature than the local climate is not also preferred due to the higher energy cost. The improvement of the adaptation of *Y. lipolytica* with the lower working temperature should be studied.

Based on the particle size distribution results, it was observed that the particle size was bigger after cultivation. Colony forming ability of *Y. lipolytica* should be monitored for the floc formation characteristic of the cultivation.

To improve lipid accumulation intracellularly, nitrogen limitation condition was provided by the addition of C source. Applied C:N ratio was 20-30. In stress conditions to maximize lipid accumulation, biomass growth should be at stationary phase. Thus, growth should not be observed anymore. The growth of *Y. lipolytica*

with 60%+C and 100% leachates were still observed. Therefore, stress conditions were not achieved successfully. Adjustment of C:N ratio of leachate from 1-2 to 20-30 was not enough to prove stress for biomass.

The removal efficiencies of leachate treatment was determined. The maximum TP removal was observed in 60%+P leachate as 98,57%. The maximum PO<sub>4</sub>P removal was observed in 100%+P leachate as 95,77%. Secondly, PO<sub>4</sub>P removal was observed in 60%+P leachate as 94,98%. The maximum COD removal efficiency was observed in 60%+P leachate as 84,89%. The maximum TKN removal efficiency was observed in 60%+P leachate as 75,14%. The maximum ammonium removal efficiency was observed in 60%+C leachate as 76,10%. Secondly, ammonium removal efficiency was observed in 60% leachate as 61,33%.

Particle size distribution from the effluent of cultivation medium was done. It was shown that the flocculation occurred in the cultivation. Since the particle size after cultivation became bigger.

Acclimation of yeast cells to wastewater was thought to affect the growth. More adapting yeast generations should be worked on.

Applied C:N ratio with cultivation did not result in the stress conditions to increase lipid accumulation noticeably. Higher C:N ratios should be applied in the future.

It was observed that most of the FAME has Palmitic acid (C16:0), Oleic acid (C18:1) and Linoleic acid (C18:2). FAME composition with cultivations was suitable for biodiesel production.

It was not possible to determine accurate biomass concentration due to raw wastewater cultivations. The method that differentiate wastewater particles and yeast cells should be developed.

For sustainable and commercial growth of *Y. lipolytica*, optimization studies of lipid accumulation capacity of *Y. lipolytica* should be completed.

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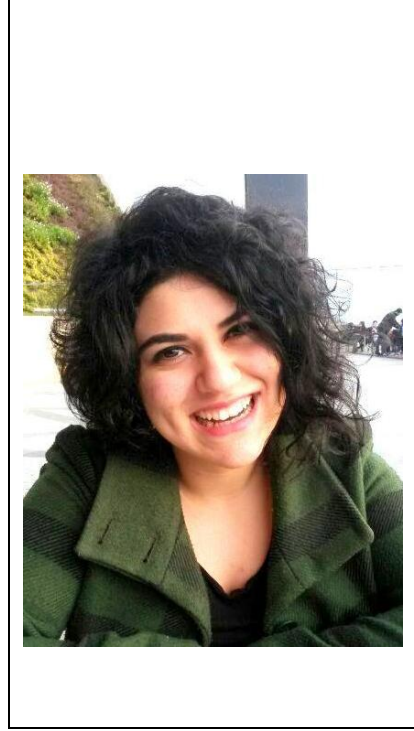
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