EVALUATION OF MOLASSES AND VINASSE PERFORMANCE FOR LIPID
PRODUCTION FROM GREEN MICROALGAE

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ABSTRACT

EVALUATION OF MOLASSES AND VINASSE PERFORMANCE FOR LIPID PRODUCTION FROM GREEN MICROALGAE

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Microalgae are considered a valuable source of energy that could be an alternative to fossil fuels. Due to their high photosynthetic efficiency and high biomass production, microalgae have drawn attention for biodiesel production. However, cost of carbon source is a major challenge in biodiesel production from microalgae. For this reason, alternative carbon sources are being considered.

In this study, heterotrophic growth conditions for improvement of biomass production from thermo-resistant *Micractinium sp.* METUNERGY1405 (ME05) cells were optimized. Plackett-Burman (PB) design was used to screen glucose and yeast extract concentrations, pH, temperature, and inoculum ratio. Variables that were selected by the PB design (pH, temperature and glucose concentration) were further optimized using Box-Behnken design to increase biomass concentration. Molasses was used as

an alternative carbon source for a cost-efficient biomass production. According to the results of statistical analysis, 0.41 ± 0.02 g.L^{-1}.day^{-1} of maximal biomass productivity was achieved by using 19g/L molasses, which revealed an increase in biomass productivity under optimized conditions and this biomass productivity was further improved to 0.53 ± 0.038 g.L^{-1}.day^{-1} by scaling up to 2L bioreactors.

Also, *Micractinium sp.* ME05 was scaled up successfully in vinasse based media and a two-fold increase in biomass productivity was achieved in 5 L bioreactors (0.32 ± 0.2 g.L^{-1}.day^{-1}) with respect to 500 mL flasks (0.16 ± 0.01 g.L^{-1}.day^{-1}).

High lipid content and rich FAME profile of *Micractinium sp.*ME05 grown in molasses and vinasse based media, could make these by-products desirable carbon sources for biodiesel production from *Micractinium sp.*ME05.

Finally, with industrial by-products, molasses and vinasse, *Micractinium sp.* ME05 demonstrated remarkable biomass productivities, that were higher than those obtained with *Micractinium sp.* in previous studies.

This is the first study that highlights the usage of industrial by-products as nutrients for a cost-efficient biomass and lipid production from native *Micractinium sp.* ME05 cells.

Keywords: *Micractinium sp*., Plackett-Burman, Optimization, Molasses, Vinasse, Heterotrophic, Biodiesel.

ÖZ

YEŞİL MİKROALGLERDEN LİPİT ÜRETİMİNDE MELAS VE ŞİLEMPENİN PERFORMANSININ DEĞERLENDİRİLMESİ

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Mikroalgler fosil yakıtlara alternatif olabilecek önemli enerji kaynakları olarak görülmektedir. Mikroalgler yüksek fotosentez verimleri ve yüksek biyokütle üretimlerinden dolayı biyodizel üretimi için ilgi görmektedirler. Buna karşın, mikroalglerden biyodizel üretiminde, karbon kaynağının maliyeti aşılması gereken bir engeldir. Bu nedenle alternatif karbon kaynakları göz önünde bulundurulmaktadır.

Bu çalışmada özgün ve ısıl dirençli *Micractinium sp.* METUNERGY1405 (ME05) suşunun biyokütle miktarının arttırılması için heterotrofik büyüme koşulları optimize edilmiştir. Plackett-Burman (BP) tasarımı glikoz, maya özütü derişimi, pH, sıcaklık ve inokulum oranı değişkenlerini taramak için kullanılmıştır. PB tasarımı ile seçilen değişkenler (pH, sıcaklık ve glikoz derişimi) Box-Behnken yöntemiyle optimize edilmiştir. Düşük maliyetli biyokütle eldesi için, melas alternatif karbon kaynağı

olarak kullanılmıştır. İstatistik analiz sonuçlarına göre, 19 g/L melas kullanılarak elde edilen biyokütle verimi 0.41 ± 0.02 g.L^{-1}.gün^{-1} olmuştur ve bu verim optimize edilen kültür koşullarında biyokütle veriminin arttığını göstermiştir. Ayrıca bu verim 2 L biyoreaktörlerin kullanılmasıyla 0.53 ± 0.038 g.L^{-1}.gün^{-1} e çıkarılmıştır.

Ayrıca *Micractinium sp.* ME05, şilempe bazlı büyüme ortamında başarılı bir şekilde kültive edilmiştir ve 5 L biyoreaktörde elde edilen biyokütle veriminde (0.32 ± 0.2 g.L^{-1}.gün^{-1}) 500 mL erlenmayerde elde edilen biyokütle verimine (0.16 ± 0.01 g.L^{-1}.gün^{-1}) kıyasla 2 kat artış gözlenmiştir.

Melas ve şilempe büyüme ortamında kültüre alınan edilen *Micractinium sp.*ME05 hücrelerinin yüksek lipid içeriği ve zengin FAME profili, bu endüstriyel yan ürünlerin *Micractinium sp.*ME05 hücrelerinden biyodizel üretiminde kullanılabileceğini göstermektedir.

Sonuç olarak, endüstriyel yan ürünlerin (melas ve şilempe) kullanımı ile *Micractinium sp.* ME05 hücrelerinin biyokütle verimlerinde, daha önce *Micractinium sp.* ile yapılmış çalışmalara kıyasla dikkate değer bir artış elde edilmiştir.

Bu çalışma, yerel *Micractinium sp.* ME05 hücrelerinden düşük maliyet ile biyokütle ve lipid eldesine dikkat çeken ilk çalışmadır.

Anahtar kelimeler: *Micractinium sp.*, Plackett-Burman, Optimizasyon, Melas, Şilempe, Heterotrofik, Biyodizel.

To my family…

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Acetyl-coA: Acetyl CoenzymeA

ANOVA: Analysis of Variance

ASTM: American Society for Testing Materials

ATP: Adenosine Triphosphate

BB: Box Behnken

BBM: Bold Basal Medium

BCE: Bicompost Derived of Chicken Excrement

COD: Chemical Oxygen Demand

DNS: Dinitrosalicylic acid Solution

DO: Dissolved Oxygen

EMP: Embden Meyerhoff Pathway

EU: European Union

HB: Fruit/horticultural biocompost

HUP1: Hexose Uptake Protein Gene

LMP: Liter Per Minute

MAE: Mean Absolute Error

MDS: Municipal Domestic Sewage

MUFA: Monounsaturated Fatty Acid

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

OD: Optical Density

PB: Plackett-Burman

PPP: Pentose Phosphate Pathway

PUFA: Polyunsaturated Fatty Acid

RCM: Raw Chicken Manure

RSM: Response Surface Method

RMSE: Root Mean Square Error

RMSWE: Raw Municipal Wastewater Secondary Effluent

SFA: Saturated Fatty Acid

SS: Stock Solution

TAG: Triacyl Glycerol

TAP: Tris Acetate Phosphate

TCA: Tricarboxylic Acid

VB: Biocompost Derived from Sugarcane and Vinasse

CHAPTER 1

INTRODUCTION

Energy crisis has been a major concern that the world has dealt with in recent years. The use of traditional fossil fuels for energy is not sustainable and gives rise to greenhouse gases emissions, which contribute to global warming (Amaro, Guedes, & Malcata, 2011).

In addition to global warming, greenhouse gases also affect human life and environment. Approximately one third of the CO_2, that comes from anthropogenic emissions such as applying fossil fuels for transport and energy production, is absorbed by the oceans. The increase in the emission of CO_2 to the atmosphere, cause an increase CO_2 levels in the oceans, and this gives rise to a decrease in water pH. As a result, aquatic life diversity and thus earth life is affected negatively from this acidity (Mata, et al. 2010a).

For these reasons, finding alternative energy sources is one of the main considerations that mankind faced in last decades. Recently, liquid fuels obtained from plant materials, which is also named as biofuels, has drawn attention as alternative to petroleum fuels due to being renewable and environmentally beneficial (Amaro et al., 2011; Mata et al., 2010a).

Biofuels diminish greenhouse gases emissions and can be applied in engines and transportation vehicles by mixing with petroleum diesel (Amaro et al., 2011). Biodiesel and bio-ethanol are the most common biofuels. Biodiesel is composed of monoalkyl esters of long chain fatty acids that arise from vegetable and animal oils (Amaro et al., 2011; Mata et al., 2010a). However, these sources cannot fulfil the requirements for transport fuels because of the need for huge area for cultivation of oil

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crops. On the other hand, microalgae have received attention as alternative feedstocks for biodiesel production due to their high growth rate, less land requirement, and high biomass and lipid yields per hectare (Chisti, 2007).

Microalgae are procaryotic or eucaryotic photosynthetic microorganisms that can grow rapidly and able to live in severe conditions due to their unicellular or simple multicellular structure. (Brennan & Owende, 2010).

The widely used cultivation method for microalgae is autotrophic growth since they are photosynthetic organisms that use sunlight or artificial light as the energy source. On the other hand, insufficient light at high cell density cultures generally hinders autotrophic growth (Perez-Garcia et al., 2011a). As another cultivation method, heterotrophic cultivation offers several advantages over autotrophic cultivation such as no need of light requirement, enabling process control, and low harvesting cost due to high cell density obtained (Huang et al., 2010). In heterotrophic cultivation of microalgae, the most important source is the carbon source and it constitutes approximately 50% of the total cost of the cultivation (Cheng et al., 2009). Thus, the cost of fermentation substrate in heterotrophic cultivation is a major problem that prevent in commercial applications. For that reason, there is a need to use alternative organic carbon sources for heterotrophic cultivation of microalgae.

Molasses, which is a by-product of sugar industry, can be considered as an alternative carbon source. It contains approximately 45-50% (w/w) total sugars (mainly sucrose, glucose and fructose), 17-25% water, and 2-5% polysaccharides (Najafpour et al., 2003). Another by-product of sugar industry is vinasse, which is highly acidic and has high organic content with oxygen demand (COD:50-150 g/L) (Espana-Gamboa et al., 2011). Direct disposal of vinasse to the environment is a major problem since its high organic content and dissolved solids can corrupt soil and groundwater and can cause an increase in nutrient supply and eutrophication (nutrient enrichment) (Espana-Gamboa et al., 2011). For that reason, there is a need to find out solutions to reduce the organic content of vinasse before releasing to the environment.

Microalgae, classification of their growth conditions, their large-scale growth in bioreactors, and alternative carbon sources that are applied in cultivation of microalgae such as molasses and vinasse are reviewed in Chapter 2.

To the best of our knowledge this is the first report that evaluated the usage of molasses and vinasse in pilot scale biomass and lipid production from a native thermo-resistant strain *Micractinium sp.* METUNERGY1405 (ME05), which adds extra novelty to our work. The strain used in this study, was previously isolated from hot springs of Haymana and described as native thermo-resistant strain of Central Anatolia by Onay et al. (2014).

The hypothesis is if *Micractinium sp.* ME05 utilizes molasses as a carbon source, then its heterotrophic growth conditions will be optimized and these culture conditions can be applied in bioreactors for a cost efficient large-scale biomass production with molasses. The goal of this research was to optimize culture conditions of *Micractinium sp.* ME05 using molasses as carbon source, to increase biomass concentration of *Micractinium sp.*ME05 by using industrial by-products and thus reduce the cost of cultivation. Beside *Micractinium sp.*ME05, growth of *Chlamydomonas reinhardtii* 137C$^-$ was also evaluated in the molasses based medium. Growth conditions of microalgal cells used in this study were given in Chapter 3.

In preliminary tests that were done with *C. reinhardtii* 137C$^-$ cells, different carbon sources such as acetate, glucose, sucrose, and molasses hyrdolysate were evaluated. With *Micractinium sp.*ME05 cells one factor at a time approach was employed, where different medium compositions were evaluated with different glucose concentrations and different inoculum ratios of microalgal cells. Then, variables that were selected based on literature survey, were screened and optimized with statistical analysis. Under optimized culture conditions 2-L bioreactor experiments were performed and their lipid extractions were done (Chapter 3).

In addition to molasses, another alternative carbon source, vinasse was also evaluated for the cultivation of *Micractinium sp.*ME05 cells. Different vinasse concentrations were studied under heterotrophic and mixotrophic conditions. With the vinasse concentration that gave the highest biomass concentration, 5-L bioreactor experiments were conducted and their lipid extractions were conducted (Chapter 3).

Heterotrophic biomass production conditions with molasses, were optimized using selected factors such as pH, temperature and molasses hydrolysate concentration. Additionally, the constructed model was verified with statistical analysis tools. The

screening and optimization results of heterotrophic biomass production are presented in Chapter 4. Furthermore, vinasse performance on the growth of *Micractinium sp.* ME05 cells were also investigated and presented in Chapter 4. Lipid contents and FAME profiles of *Micractinium sp.*ME05 cells grown with molasses and vinasse were also examined for biodiesel suitability (Chapter 4).

CHAPTER 2

LITERATURE REVIEW

2.1. Microalgae

Algae are plant-like organisms that are capable of performing photosynthesis. According to their morphology and size, they are divided into two groups, which are macroalgae and microalgae. Macroalgae have multiple cellular structures and contain structures like roots, stems, and leaves, while microalgae do not have multiple cellular structures, and instead consist of unicellular structures. Microalgae are subdivided into procaryotic cyanobacteria and eucaryotic microalgae (P. Chen et al., 2009).

Procaryotic cyanobacteria do not have membrane bound organelles such as plastids, mitochondria, nuclei, golgi bodies, and flagella but they contain chlorophyll a in cells. Eucaryotic microalgae include organelles and consist of different types of algae. They are divided into different classes according to their light harvesting pigment, life cycle, and cellular structure. Green algae (Chlorophyta), red algae (Rhodophyta), and diatoms (Bacillarophyta) are few of these classes (Figure 2.1) (Brennan & Owende, 2010).
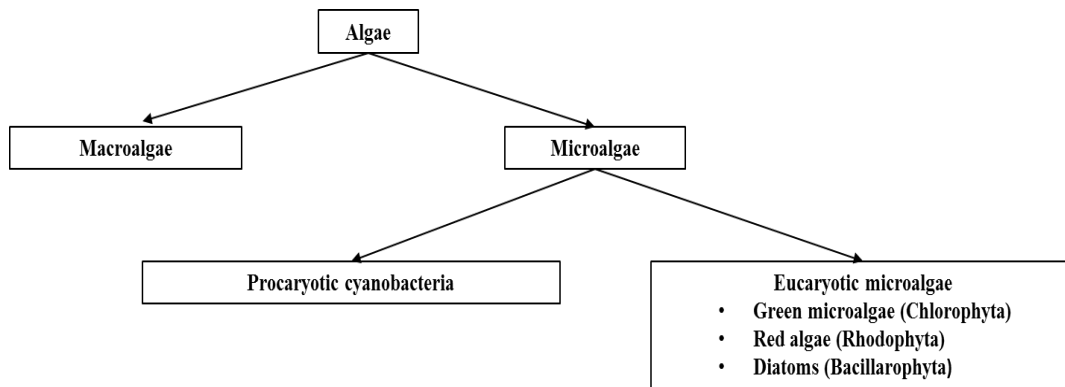
Figure 2.1. Classification of algae (Brennan & Owende, 2010).

2.1.1. Growth metabolisms of Microalgae

In order to obtain high biomass concentrations and high biomass productivities from microalgae, various conditions should be taken into account. Generally, microalgae need inorganic carbon and light energy to perform photosynthesis. However, there are some microalgae that can metabolize organic carbon as an energy and carbon source. There are three different major metabolisms that are involved in different microalgae species. These are autotrophic, heterotrophic and mixotrophic metabolism (Chojnacka et al. 2004).

2.1.1.1. Autotrophic Metabolism

In autotrophic metabolism, light is used as an energy source and inorganic carbon, especially CO_2, is used as a carbon source to produce chemical energy via photosynthesis. Photoautotrophic cultivation is the most frequently used cultivation type for the growth of microalgae. Depending on the microalgae species, there is a large diversity in the amount of lipid which may vary from 5 to 68 % of oil/ per dry weight (C.-Y. Chen et al., 2011). In order to increase the lipid content in microalgae a nitrogen limiting or nutrient limiting condition was examined (Mata et al., 2010). However, lipid content is not the only factor that affects oil producing ability of microalgae. Lipid content and biomass quantity should be taken into account together. Therefore, lipid productivity, which shows the effects of oil content and biomass

production is more appropriate in order to show the ability of microalgae to produce oil. The advantage of phototrophic cultivation is consuming CO_2 for cell growth and oil production and thus providing an effective carbon capture. However, when the only carbon source is CO_2 , the cultivation should be done near factories or power plants that can provide large amounts of CO_2 (Chen et al., 2011).

2.1.1.2. Heterotrophic metabolism

Heterotrophic metabolism is defined as the usage of organic carbon as energy and carbon sources under dark conditions (Chojnacka et al. 2004). The limited light that prevent high cell density in autotrophic cultures, does not cause a problem in heterotrophic cultivation since there is no light requirement (Huang et al ., 2010). Moreover, enabling process control and low harvesting cost due to high cell density are other advantages of heterotrophic metabolism.

Heterotrophic microalgae should have some useful properties which are; the ability to divide and metabolize in the absence of light, to acclimate themselves to a new environment and endure the hydrodynamic stresses in the fermenters (Perez-Garcia et al., 2011)

Different organic carbon sources are being used in heterotrophic metabolism such as glucose, acetate, glycerol, fructose, sucrose and lactose. However the cost of organic carbon source is a major problem in terms of commercial view (C.-Y. Chen et al., 2011). It was reported that 50% of the cost of the medium in algal cultivation comes from the cost of organic carbon source (Cheng et al., 2009). To overcome this problem alternative carbon sources are considered. Xu et al., 2006 cultivated *Chlorella prothecoides* heterotrophically using glucose and corn powder hydrolysate. Higher biomass concentrations were obtained with corn powder hydrolysate (3.92 g/L) than glucose (3.74 g/L). Their result suggested that cell density significantly increased under heterotrophic condition with the usage of corn powder hydrolysate. Gaurav et al., (2015a) applied molasses to increase the biomass and lipid production of *Chlorella pyrenoidosa*. *C. pyrenoidosa* cells were cultivated in 100 mL Fogg's medium that contains different sugars (10 g/L), which included glucose, sugar mixture (8 g/L

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sucrose, 1 g/L glucose and 1 g/L fructose), pretreated and untreated molasses. The biomass obtained was 0.89 g/L, 0.81 g/L, 0.52 g/L and 1.22 g/L with glucose, sugar mixture, untreated molasses and pretreated molasses, respectively. Due to the increase in biomass from 0.89 g/L to 1.22 g/L, and increase in lipid content from 0.27 g/g to 0.66 g/g by using molasses instead of glucose, they reported the feasibility of using molasses as a carbon source for large scale low-cost lipid production by *C. pyrenoidosa*. Sweet sorghum hydrolysate (Gao et al., 2010), waste cane molasses (Jin Liu et al., 2013), and vinasse (Ramirez et al., 2014) are other organic carbon sources that are used for heterotrophic cultivation of microalgae.

2.1.1.2.1. Glucose and Acetate Metabolism in Heterotrophic Microalgae

In heterotrophic cultures of microalgae, glucose is the most common carbon source. With glucose as a carbon source, high growth rates are obtained in comparison to other substrates such as sugar alcohols, sugar phosphates, organic acids, and monohydric alcohols due to carrying more energy content per mole (Perez-Garcia et al., 2011a). For instance with glucose 2.8 kj/mole energy is produced as ATP while with acetate 0.8 kj/mole energy is produced as ATP (Boyle & Morgan, 2009).

Oxidative assimilation of glucose starts with phosphorylation of hexose that yields glucose-6-phosphate. Glucose-6-phopsphate is used for storage, cell synthesis, and respiration. There are two main pathways used by microorganisms for the aerobic breakdown of glucose (glycolysis); Embden-Meyerhoff pathway (EMP) and Pentose Phosphate Pathway (PPP). Under anaerobic dark conditions, the energy released by dissimilation of glucose is low and the quantity of the enzyme lactate dehydrogenase that is required to complete anaerobic fermentation process is also low and as a result glucose cannot be metabolized by algae under anaerobic dark conditions. The main difference in glucose metabolism of autotrophic and heterotrophic microalgae is that under darkness, PPP pathway is used for assimilation of glucose while under light conditions EMP pathway is used (Yang et al.,2000) (Figure 2.2).
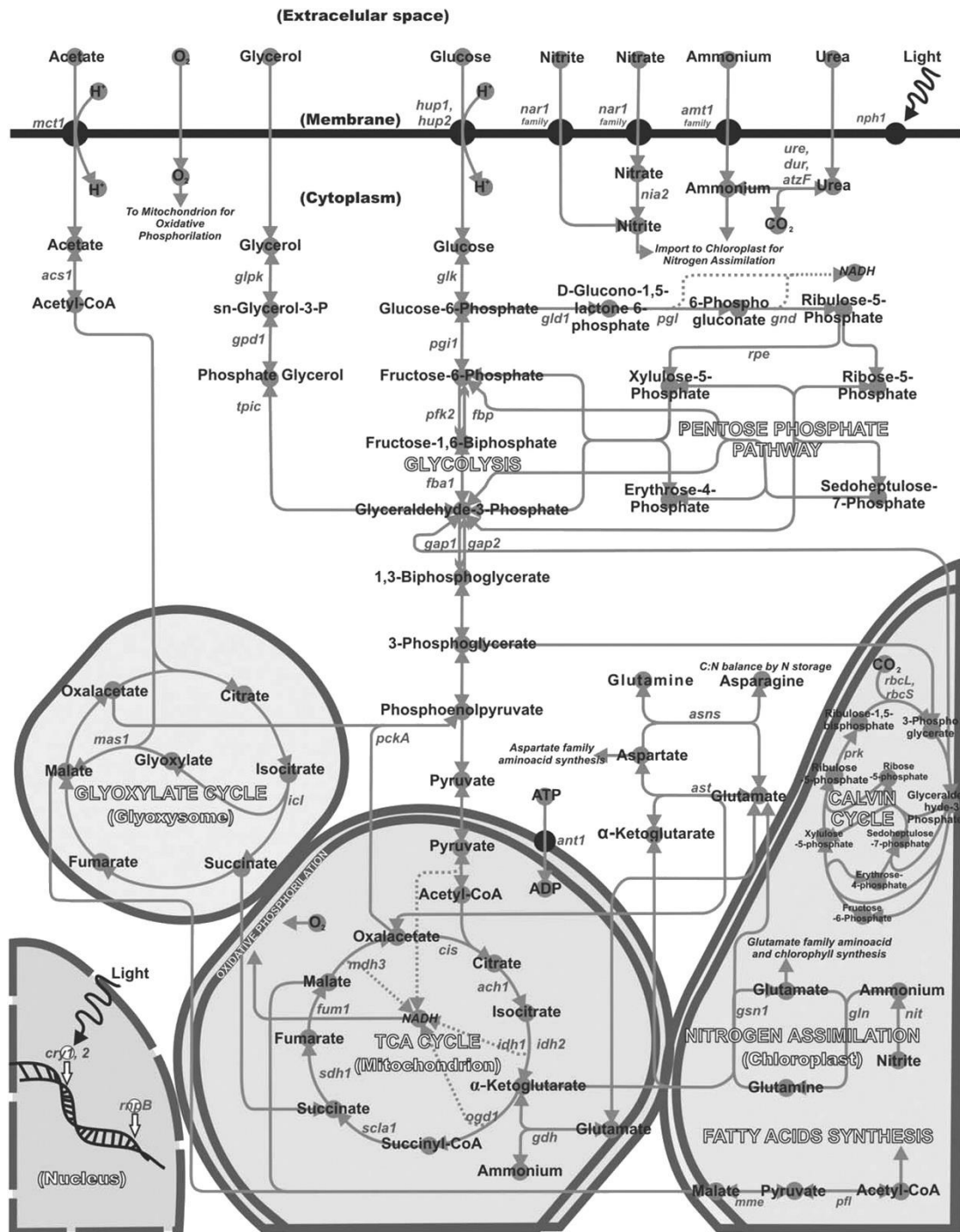
Figure 2.2. Heterotrophic metabolism in microalgae (Adapted from Perez-Garcia et al., 2011b).

Both of the pathways are carried out in cytosol but Pentose Phosphate Pathway (PPP) has a higher flux rate than Embden Meyerhoff Pathway (EMP). It was reported by Yang et al. (2000) that under complete darkness about 90% of glucose metabolized via glucose-6-phosphate dehydrogenase, which is the key component for the

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regulation of PPP. On the other hand, the activity of PP pathway under autotrophic conditions is very small. Under light conditions the only role of PP pathway is to provide synthesis of nucleic acid and amino acid precursors and the activity of glucose-6-phosphate dehydrogenase is restricted in the presence of NADPH. The differences in the percentages of NADPH/NADP$^+$ is responsible for the light mediated regulation of glucose-6- phosphate dehydrogenase. This light mediated control of glucose-6- phosphate dehydrogenase is the reason for the low activity of PP pathway in autotrophic cultures (Yang et al., 2000).

The effect of light on glucose uptake of microalgal cells is also observed in *Chlorella vulgaris* cells. *C. vulgaris* cells include hexose/H$^+$ symport system that is in charge for the uptake of glucose. Hexose/ H$^+$ symport system carries sugars and protons together and with every sugar molecule one molecule of ATP is contributed by cells. The expression of this mechanism is suppressed in the presence of light (Perez-Garcia et al., 2011b).

Beside glucose, acetate is also a common carbon source in microalgal cultures. Acetate assimilation in microalgal cells begins with acetylation of coenzyme A, which is catalyzed by acetyl-CoA synthetase. This reaction is mediated using single ATP molecule and ends up with the production of acetyl-CoA (Figure 1.1).

Acetate is oxidized with glyoxylate cycle and with tricarboxylic acid (TCA) cycle. In glyoxylate cycle acetate is oxidized to produce malate in specialized plastids which are called glyoxysomes. In TCA cycle, acetate is oxidized to form citrate in mithocondria, which is the key component for carbon skeletons, ATP and NADPH. The key enzymes that participate in glyoxylate cycle are isocitrate lyase and malate synthetase. When microalgal cells are grown in media that contain acetate, these enzymes are induced (Boyle & Morgan, 2009; Perez-Garcia et al., 2011a).

2.1.1.2.2. Advantages of Heterotrophic Metabolism

Heterotrophic cultivation of microalgae has some advantages over autotrophic cultivation. These advantages can be listed as;

- No need of light,

- Control of the cultivation process
- Use of any type of fermenter as a bioreactor,
- Low harvesting cost due to high cell density,
- Ability of microalgal strains to use different low cost waste products as carbon source,

Especially the cost effectiveness and easiness of operation are the main advantages that draw attention to heterotrophic cultivation (Perez-Garcia et al., 2011b).

2.1.1.3. Mixotrophic Metabolism

In mixotrophic metabolism, microalgae use both organic compounds and inorganic carbon (CO_2) for growth. In this type of metabolism microalgal cells can live either in autotrophic or heterotrophic conditions. Organic carbon sources and CO_2 are used as carbon source and the CO_2 that is liberated by respiration is captured and used again for autotrophic cultivation (C.-Y. Chen et al., 2011).

2.1.2. Factors Affecting Growth of Microalgae

Protein, lipid and carbohydrate synthesis of microalgae are strongly affected by nutrient conditions and environmental factors. Temperature, availability of light, pH, and availability of nutrients have major impacts on biochemical composition of microalgae. Carbon, oxygen and hydrogen are important for algal growth. Macronutrients such as nitrogen, phosphorus, sulfur, potassium and magnesium; and micronutrients such as iron and manganese are also required for algal growth. Beside macronutrients and micronutrients, some trace elements such as zinc, copper, cobalt and molybdenum are also essential. Altogether the presence of nutrients, macronutrients, micronutrients, and environmental conditions have remarkable effect on the growth and biochemical composition of microalgae (Juneja, Ceballos, & Murthy, 2013)

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2.1.2.1. Effect of Temperature on Microalgae

One of the most important environmental factors that affect algal growth rate, cell size, and biochemical composition is temperature. Under sub-optimal temperatures, alterations occur in the viscosity of cytoplasmic cells and this give rise to a decrease in carbon and nitrogen utilization. Low temperatures reduce the fluidity of cell membrane. To overcome this problem cells start to produce unsaturated fatty acids (Juneja et al., 2013).

In contrast, at high temperatures cells start to increase saturated fatty acids content. This change is affected by alterations in the fluidity of cell membrane phospholipid layers regulated by the degree of fatty acid unsaturation. Moreover increase in temperatures can disturb chain elongation and desaturation of fatty acids (Renaud et al., 2002). Cells manipulate such changes in order to maintain their normal functions. Fatty acids that contain carbon-carbon double bonds that cannot be packed properly like saturated fatty acids and therefore their fluidity starts to increase. At low temperatures membrane fluidity starts to decrease and as an adaptation microalgal cells start to increase their unsaturated fatty acid content. In a study of Thompson (1996); it was shown that a temperature change from 30°C to 12°C caused an increase at the level of unsaturated fatty acids in *Dunaliella salina* (Sharma et al., 2012).

2.1.2.2. Effect of Light on Microalgae

Intensity of light affects microalgae through photosynthesis. Although this mechanism is a result of the strain and temperature of the culture, at saturation intensity the growth rate of the cells is maximum. Light saturation is related with the saturation constant. Saturation constant demonstrates the intensity of light that specific growth rate of microalgal biomass is the half of its maximum value, μ_{max} (Yusuf, 2007).

Adaptation to light intensity in microalgal cells may occur with different mechanisms such as alterations in growth rate, dark respiration rate, alterations in pigment types and quantities. Moreover, some morphological adaptations may occur such as alterations in cell volume and density of thylakoid membranes. Light intensity more than saturation levels can lead to photoinhibition by damaging chloroplast lamellae

and by damaging enzymes used in carbon dioxide fixation. As a result biomass growth rate starts to decrease (Chisti, 2007; Juneja et al., 2013).

For instance in a study of Gordillo et al.,(1998) with an increase in light intensity from 700 μmol.m^2.s^{-1} to 1500 μmol.m^2.s^{-1} the growth rate of *Dunaliella viridis* cells decreased (Juneja et al., 2013).

2.1.2.3. Effect of pH on Microalgae

The solubility of essential nutrients and CO_2 is regulated by pH. With the assimilation of inorganic carbon by cells, pH can increase significantly (Hansen, 2002). The optimum pH of microalgal cells is the pH, in which microalgal cells accustomed to grow, however algal growth is maximum at neutral pH. Alterations in medium pH can inhibit microalgal growth (Goldman et al., 1982; Juneja et al., 2013).

In the presence of alkaline pH conditions, the elasticity of cell wall mother cells increases. The increase in elasticity of the cells walls hinders its rupture and prevents the release of autospores, as a result the time needed for accomplishment of cell cycle becomes longer. Acidic pH conditions can affect microalgal growth by changing nutrient uptake, stimulating metal toxicity (Juneja et al., 2013). For instance in the study of Visviki et al., 2001, *Chlamydomonas aciophila* cells were cultivated in a pH range of 1.4 to 8.4. Between pH 1.4 and 3.4 cells were unable to grow. Between pH 5.4 and 8.4 an exponential growth was observed but the optimum growth was observed around neutral pH.

2.1.3. Growth Dynamics of Microalgae

Under appropriate environmental conditions and in the presence of sufficient nutrients, microalgal cells can grow extensively. Generally, it takes 24 hours for microalgae to double their biomass.

A microalgal growth curve in a batch culture is given in Figure 2.3. In a batch culture of microalgal cells, there are five growth phases which are; lag or induction phase (1), exponential (logarithmic) growth phase (2), linear growth phase (3), stationary growth phase (4), decline (death) phase (5).

In lag phase, microalgal cells adapt themselves to new cultural environment and a slight growth is observed. Exponential growth phase is the phase, where there is maximum cell growth rate and cell concentration increases as a logarithmic function. In stationary phase cell production rate is equal to cell death rate and thus cell concentration remains constant. Environmental conditions start to become unfavourable due to nutrient depletion, accumulation of toxic compounds, etc. At death phase, cell growth decreases rapidly and the cell concentration decreases due to nutrient depletion (Mata et al., 2010b).
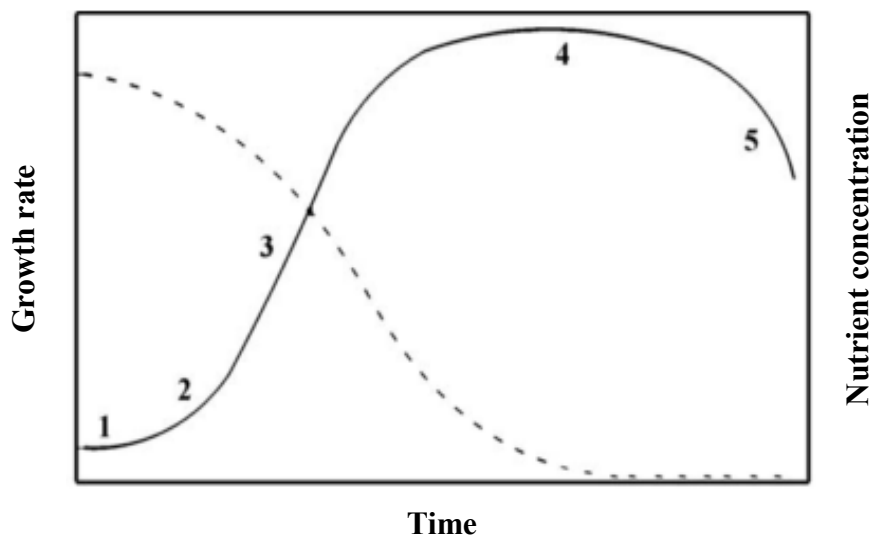
Figure 2.3. Microalgal growth curve in a batch culture. Solid line represents microalgal growth, dashed line represents nutrient concentration (Adapted from Mata et al. ,2010).

2.1.4. Applications of Microalgae

Microalgae are being used in various biotechnological applications. Microalgae are being used for production of different high-value products such as polyunsaturated fatty acids (PUFA), pigments like carotenoids and phycobiliproteins, pharmaceuticals, and food additives (Bux, 2013). *Chlorella* was the first microalgae to be used as a food additive in Japan in the early 1960s (Brennan et al., 2010). Moreover, microalgae are also widely used for wastewater bioremediation. More importantly, microalgae have remarkable applications in biodiesel production (Bux, 2013).

2.2. Biofuels

The area of energy and transportation are the major causes of greenhouse gas emissions in European Union (EU). Agriculture sector is also responsible for greenhouse gas emissions especially for nitrous oxide (N_2O) and methane (CH_4). With developing economies such as China and India, the energy utilization will increase and this will cause more damage to the environment.

Discovering clean and renewable energy sources have been a major concern for mankind for a long time. Different strategies have emerged such as; solar energy, wind energy and biofuels. Among these sources, biofuels seem to be the most promising one in terms of reducing transportation emission. It is believed that biofuel production will provide a chance to increase fuel reserve sources, provide occupation in rural areas and more importantly will decrease greenhouse gas emissions. Biodiesel and bioethanol are the most common biofuels (Mata et al., 2010b).

2.2.1. Biodiesel

Generally biodiesel is produced from soybean oil, vegetable oil, palm oil, sunflower oil, and rapeseed oil (Huang et al., 2010). However, using vegetable oils for biodiesel production can cause an increase in the price of food based oils and biodiesel, since they are also used for human consumption. In order to be an alternative fuel to replace diesel, biodiesel production must be economic. Generally the cost of feedstocks constitutes 65-70 % of total cost of biodiesel (Canakci et al., 2008). For that reason, low cost feedstocks like frying oils and animal fats are considered. But the quantities of waste oils and animal fats are not enough to satisfy the need for biodiesel. Thus, microalgae has drawn attention because of their high energy yields per hectare (Mata et al., 2010b).

Microalgae are favored as potential candidates for biodiesel production due their advantages such as; having similar fatty acid composition to vegetable oils, high growth rate, high photosynthetic efficiency, and producing high quantities of biomass. In addition, according to American Society for Testing Materials (ASTM), the

characteristics of microalgal biodiesel is similar to the characteristics of standard biodiesel (Huang et al., 2010).

2.2.1.1. Biodiesel Production from Microalgae

In microalgal cells, triacylglycerides (TAGs) generally provide energy storage and after being extracted from cells they are converted to biodiesel with transesterification reactions (ester exchange reaction) (Figure 2.4). These neutral lipids (TAGs) contain three long chain fatty acids (Sharma et al., 2012).
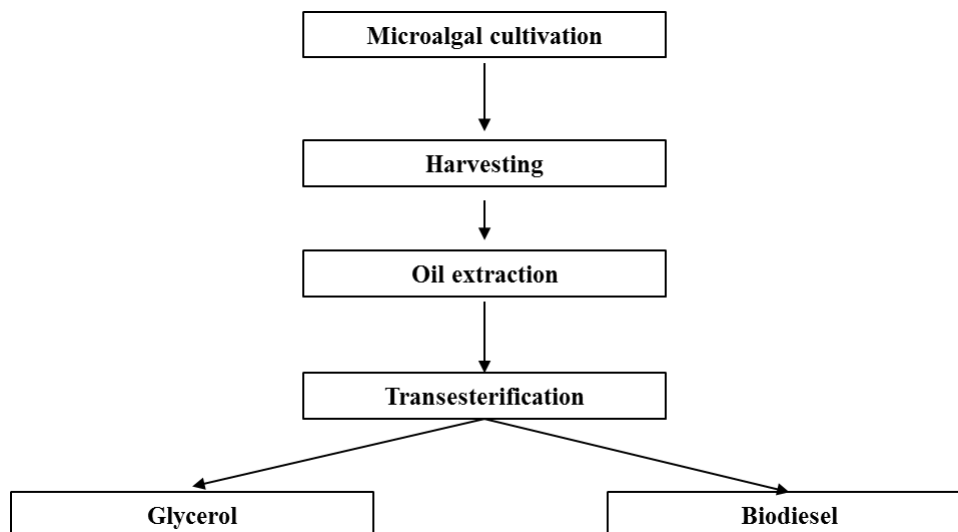
Figure 2.4. Microalgal biodiesel production process (Mata et al., 2010a).

Prior to lipid extraction, microalgae should be harvested. Microalgae can be harvested via filtration, centrifugation, gravity sedimentation and flocculation. The aim of filtration is to dewater of microalgae and small scale microalgal samples can be filtered with this method. However, the cost of membranes constitutes a problem. Centrifugation is a rapid method but its cost is high due to high energy input. Centrifugation can be used for all types of microalgae but during the centrifugation shear stress should also be considered. Gravity sedimentation depends on microalgal density. Flocculation is a preparatory step that can be applied before sedimentation, centrifugation and filtration. It is applied to increase particle size of microalgal cells

by concentrating them. Chemical agents such as ferric sulphate and aluminium sulphate are used for flocculation (Amaro et al., 2011; Barros et al., 2015).

In order to extract lipids from microalgal cells, different methods can be applied, namely; Folch method, Bligh-Dyer method, and Soxhlet extraction method. Folch and Bligh-Dyer extraction methods use methanol and chloroform as solvents. Soxhlet method uses Soxhlet apparatus for lipid extraction (Mercer and Armenta, 2011). Moreover, in large scale microalgal lipid extraction, supercritical CO_2 extraction is reported to a be an efficient method. Supercritical carbon dioxide is a green technology that has low toxicity and generates organic solvent free extract in a short time. On the other hand the high cost of the equipment and high pressure requirement are the main drawback of this process (Amaro et al., 2011).

In this study neutral lipids of *Micractinium sp.* ME05 cells were extracted with ultrasonication and lyophilization assisted Bligh-Dyer Method since this method was reported as an efficient method for lipid extraction (Onay et al., 2016).

In transesterification reactions; a reaction takes place between triglycerides and methanol. As a result of transesterification reaction, methyl esters of fatty acids (biodiesel) and glycerol is produced (Chisti, 2007) (Figure 2.5).
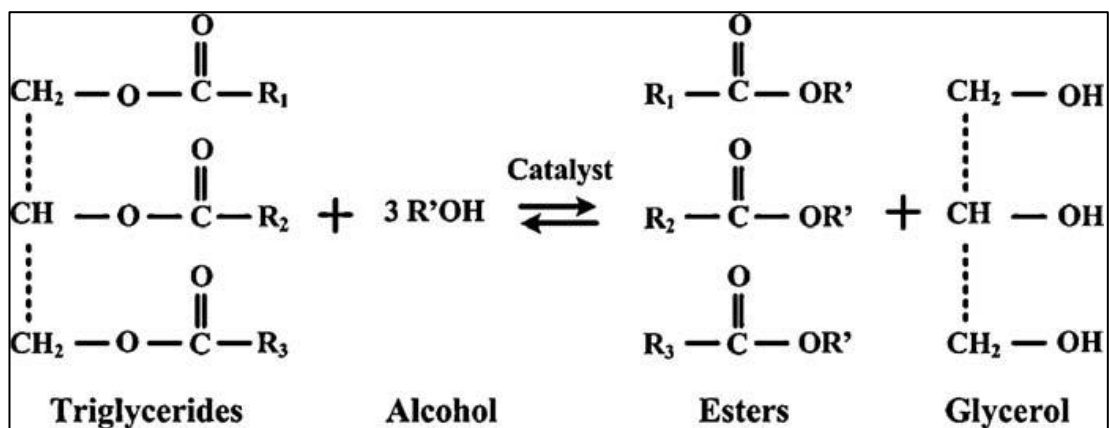
Figure 2.5. Transesterification reaction of triglycerides (Adapted from Mata et al.,2010).

The objective of transesterification process is to reduce the viscosity of the oil. With transesterification process large branched molecules of oils are converted to straight chain molecules , which is a desired property for diesel engines (Demirbas, 2008).

The key component in transesterification reaction is alcohol. The widely used alcohols are methanol, ethanol, propanol, and butanol. But due to its low cost and physical advantages generally methanol is preferred. The reactions are catalyzed by acids, alkalis or lipase enzymes. Due to their high reaction rate, such as catalyzing the reaction 4000 times faster than acid catalysts, alkali catalysts are commonly used. Sodium and potassium hydroxide are widely used alkali catalysts (Huang et al., 2010).

2.2.1.2. Lipids in Microalgae

Generally, lipids produced from microalgae are divided into two groups namely storage lipids (non-polar lipids) and structural lipids (polar lipids). In storage lipids, fatty acids generally consisted of saturated fatty acids and sometimes unsaturated fatty acids that can be converted to biodiesel via transesterification. On the other hand, polar lipids contain high amounts of polyunsaturated fatty acids. Polar lipids are structural lipids that sustain specific membrane properties and also maintain many metabolic processes by attending membrane fusion activities (Sharma et al., 2012)

Microalgae can alter their lipid metabolism according to environmental conditions. Under normal growth conditions microalgae generate large amounts of biomass with low lipid contents (approximately 5-20% of their cell dry weight) that composed of membrane based lipids. Under stress conditions microalgae start to produce neutral lipids (approximately 20-50% of their cell dry weight) generally in the form of triacylglycerol which provide microalgae a resistant to unfavorable conditions (Sharma et al., 2012)

For instance, under nutrient limited conditions, rate of cell division decreases and cells start to produce fatty acids in the form of triacyclglycerols. Under nutrient limited conditions, generation of triacyclglycerols can contribute a protective mechanism. Generally, ATP and NADPH that are generated by photosynthesis, are used up to produce biomass, with ADP and NADP$^+$, which are then act as electron acceptors in photosynthesis. Under nutrient limiting conditions, cell growth slows down, cell

division decreases and the NADP$^+$ repository start to diminish. Under these conditions, NADPH that is produced with photosynthesis is used up in fatty acid biosynthesis. So generation of fatty acids, that are stored in triacyclglycerols, restore the NADP$^+$ repository (Qiang Hu et al., 2008; Sharma et al., 2012).

2.2.1.3. Properties of Microalgal Biodiesel

Feasibility of biodiesel fuel is affected by different factors such as cetane number, viscosity, cold flow, and oxidative stability. The fatty acid composition of biodiesel has a great impact on these properties.

Microalgal fatty acids, generally consist of myrtistic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). However the fatty acid composition of microalgae can differ according to species ,environmental conditions, and culture conditions (Piloto et al., 2017).

Unsaturation levels of fatty acids have a great impact on the oxidative stability, ignition quality and, cold flow properties of biodiesel. On the other hand, the cetane number raises with the increase of saturated fatty acids (Piloto et al., 2017). Shorter chain lengths, high levels of branching, and unsaturation levels reduces cetane number. When the cetane number is high, the ignition quality of biodiesel fuel is better. On the other side, although saturated fatty acids are advantageous in terms of their high cetane numbers, their poor cold flow properties is a disadvantage. Moreover, polyunsaturated fatty acids possess low melting points, which is a good feature especially under low temperatures but their cetane numbers and oxidative stabilities are low, which are undesirable properties for biodiesel fuel (Knothe, 2008).

Accordingly, microalgal species, its culture and environmental conditions and thus fatty acid composition have a great impact on the properties of biodiesel and its engine performance (Piloto-Rodriguez et al., 2017).

2.3. Agro-Industrial By-Products for Microalgal Cultivation

Microalgae use CO_2 and sunlight to grow photoautotrophically. However restricted supply of desired light intensity and lower energy conversion are obstacles that prevent high biomass concentration and thus high lipid content. On the other hand, with heterotrophic cultivation higher biomass concentrations and lipid content are obtained in a shorter time and provides an easier scale up. By this means, heterotrophic cultivation provides a way for large scale biodiesel production.

In order to increase the algal oil and reduce the cost of fermentation, it is crucial to optimize medium composition since it can affect the yield and cost of products to a large extent. The nitrogen and carbon sources are the key factors that affect the oil yield content. The cost of the algal cultivation medium mainly comes from the cost of carbon , which represents approximately 50% of the cost of medium (Cheng et al., 2009). For that reason cheap carbon sources like sweet sorghum, agricultural waste medium, molasses, vinasse, and industrial dairy waste are considered appropriate carbon sources to reduce the cost for microalgal cultivation (Mohammad et al., 2016).

2.3.1. Molasses

Molasses is the final effluent obtained in sugar production by repeated crystallization of raw sugar in sugar factories. Molasses are the final products of sugar factories and contains approximately 45-50% (w/w) total sugars (mainly sucrose, glucose and fructose), 17-25% water and 2-5% polysaccharides (dextrins, pentosanes, polyuronic acid) (Najafpour et al., 2003).The main component of molasses is sucrose rather than glucose, fructose, and raffinose (Olbrich H, 2006).

Molasses is widely used in the cultivation of microalgae as a carbon source to reduce the cost of cultivation. For instance Gaurav et al. (2015b) evaluated molasses to replace glucose for a low-cost lipid production of *Chlorella pyrenoidosa*. *C. pyrenoidosa* cells were cultivated in 100 mL Fogg's medium that contains different sugars (10 g/L), which included glucose, sugar mixture (8 g/L sucrose, 1 g/L glucose and 1 g/L fructose), pretreated and untreated molasses. The biomass obtained was 0.89

g/L, 0.81 g/L, 0.52 g/L and 1.22 g/L with glucose, sugar mixture, untreated molasses and pretreated molasses, respectively. Due to the increase in biomass from 0.89 g/L to 1.22 g/L, and increase in lipid content from 0.27 g/g to 0.66 g/g by using molasses instead of glucose, they reported the feasibility of using molasses as a carbon source for large scale low-cost lipid production by *C. pyrenoidosa*.

Karpagam et al. (2015b) evaluated the feasibility of different carbon sources such as sugarcane industry effluent (0.625, 1.2 and 2.5 mL/L), citric acid (10, 20, 30 mg/L), glucose (0.05, 0.1, 0.15 and 0.2%), and vitamin B12 (0.001, 0.002 and 0.003%) with BG-11 medium to enhance biomass and lipid production of *Micractinium sp*. M-13 cells. Highest biomass productivity of *Micractinium sp.* M-13 cells (0.0615 g/L/day) was obtained in BG-11 medium that were supplemented with 25mg/L citric acid and 1.25ml/L sugarcane industry effluent. Their results suggested that cheap carbon sources such as sugarcane industry effluent was efficient carbon source for cost efficient biomass and lipid production from microalgae.

2.3.2. Vinasse

Vinasse is a byproduct of sugar-ethanol industry. Generally, it is acidic (pH:3.5-5), have dark brown color, high organic content with oxygen demand(COD:50-150 g/L) and undesirable odor (Espana-Gamboa et al., 2011). It was reported that large quantities of vinasse is generated during the production of ethanol from sugar crops, starch crops, and from cellulosic materials. Approximately 10-15L of vinasse is produced from the preparation of 1L ethanol. Vinasse production process is given in Figure 2.6 (Christofoletti et al., 2013).
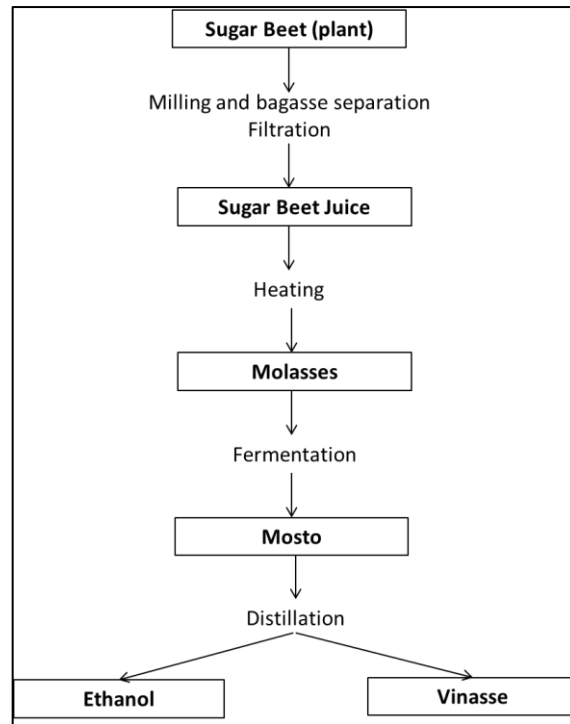
Figure 2.6. Ethanol production process and underproduction of sugarcane vinasse (Adapted from Christofoletti et al., 2013).

Vinasse can be useful as a fertilizer since its organic content especially calcium and potassium content is high. On the other hand, due to its corrosive and toxic structure, disposal of vinasse to the environment is a problem. Its high organic content and dissolved solids can corrupt soil and groundwater. The high organic content of vinasse can cause an increase in nutrient supply and cause eutrophication (nutrient enrichment). Moreover, its direct disposal to the environment can damage microorganisms and plants due to high content of phenols, polyphenols and heavy metals. Dark color of vinasse prevents sunlight penetration in lakes and rivers and thus cause a decrease in photosynthetic activity in aquatic life (Espana-Gamboa et al., 2011). Direct disposal of vinasse to the environment is one of the major problem for molasses fermentation factories. Industries are forced with strict environmental regulations to find out more efficient solutions to reduce vinasse damage to the environment. In order to decrease the organic content of vinasse, some biological treatments such as aerobic or anaerobic treatments are being applied. These treatments

are fertirrigation, concentration by evaporation, use in energy production, and yeast production (Figure 1.5). (Candido & Lombardi, 2016; Christofoletti et al., 2013).

2.3.2.1. Alternatives for Use of Vinasse

The corrosive and fetid structure of vinasse makes its direct disposal to the environment difficult. For that reason, different alternatives such as fertirrigation, concentration by evaporation, use in energy production, and yeast production are being considered for the discharge of vinasse to the environment (Figure 2.7). (Candido & Lombardi, 2016; Christofoletti et al., 2013).
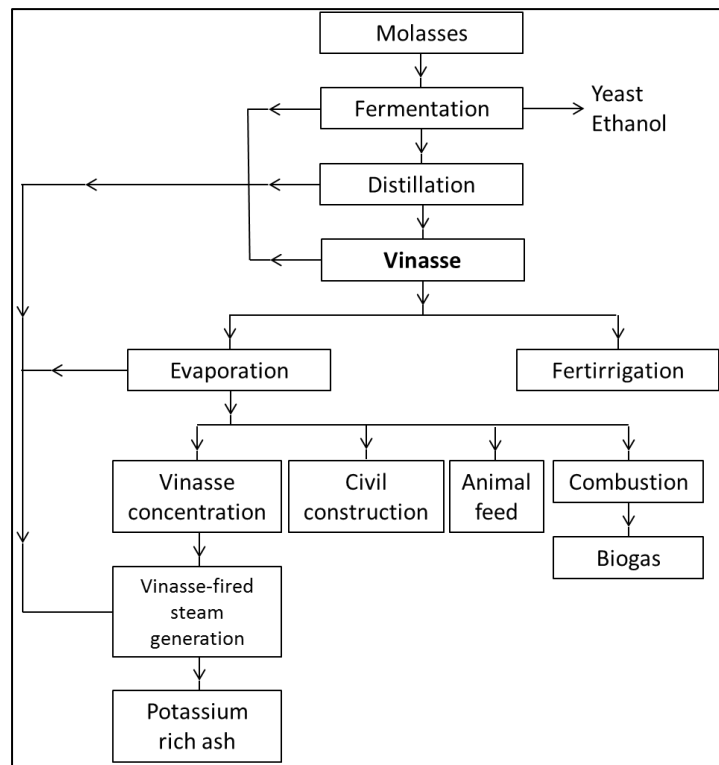
Figure 2.7. Flowchart of the alternatives for the discharge of vinasse (Adapted from (Christofoletti et al., 2013).

2.3.2.1.1. Fertirrigation

Fertirrigation, is the process in which raw vinasse in the soil is infiltrated during the irrigation of sugarcane crops. Applying fertirrigation process to the natura not only irrigates sugar cane crops but also fertilizes the crops and reduce the cost with chemical fertilizers. By fertirrigation process, the use of natural resources comes up and hinder the release of vinasse to the rivers directly (Christofoletti et al., 2013)

Among different processes that can be applied for discharge of vinasse, fertirrigation process is the one that demands low initial payment for tubes, pumps, trucks, and tanks. Moreover its maintenance cost is low and does not need complex technologies (Christofoletti et al., 2013; V. S. Santana et al., 2008).

Direct disposal of vinasse to the soil can lead to salinization, draining of metals present in the soil to groundwater, reduction in alkalinity, and alter the soil quality because of unbalance of the nutrients. V. S. Santana et al. (2008) reported that fertirrigation is a temporary process that provides wrong impression to deal with vinasse disposal problem. Moreover some environmental criteria should be considered in fertirrigation process; such as type of the soil, water holding capacity of salts in the soil, and distance from water (Christofoletti et al., 2013).

2.3.2.1.2. Concentration of Vinasse by Evaporation

Fertirrigation process cannot always deal with the total volume of vinasse, for that reason another alternative is the concentration of vinasse by evaporation. The final product gathered in this process is used to obtain livestock feed and to promote vinasse fertilizer quality. Vinasse can also be fired in boilers to produce energy and the condensate that is discharged by evaporation can be processed and used again by the factory (Christofoletti et al., 2013).

In case of not being used as a fertilizer, concentrated vinasse can be used to obtain livestock feed since its nutrient levels are high. Concentrated vinasse should have low levels of potassium and can be applied to cattle, pigs, and poultry as a feed. Moreover,

Waliszewski et al.,1997); reported that concentrated vinasse is used instead of molasses for feeding ruminants.

Concentration process is the process, in which water is taken out from vinasse without loss of solids and finally volume of vinasse decreases. This process has the advantage of being an alternative when fertirrigation process is not applicable. However, the crystallization that occurs as the concentration of solids raises and rapid incrustation of evaporators are the bottlenecks of concentration process. Requirement of high energy is also another limitation for this process (Christofoletti et al., 2013)

2.3.2.1.3. Anaerobic Biodigestion of Vinasse

Vinasse can be used for alcohol production in plants by anaerobic biodigestion process. In vinasse biodigestion process; organic content of vinasse is biodegraded to obtain biogas and bio digested vinasse (Christofoletti et al., 2013; Espana-Gamboa et al., 2011).

Anaerobic digestion of vinasse is carried out in two phases; acidogenic and methanogenic phases. In acidogenic phase; proteins, carbohydrates, and lipids are decomposed to obtain small carbon chain compounds. These compounds are oxidized biologically and facultative and obligate anaerobic bacteria generate organic acids such as acetic acid (CH_3COOH) and propionic acid (CH_3CH_2COOH). At this phase, the organic content of the effluent decreases. The second phase is methanogenic phase, in which methane, carbon dioxide, and organic acids are produced from acids. This phase regulates the conversion rates and is the slowest phase of the process (Christofoletti et al., 2013) .

Although the final bio digested vinasse has a low organic content, it still preserves its fertilizer ability. In addition, the biogas produced can be used to function in gas turbines, or boilers to produce vapor and to grind sugarcane or can be used in agroindustry fuels (Christofoletti et al., 2013).

Production of biogas that can be used for energy production, low electric energy consumption and the decrease in vinasse organic load since biological oxygen demand is transformed to biogas are advantages of this process. But the high detention time of

the process and development of corrosive gases are the disadvantages of the process (Christofoletti et al., 2013).

2.3.2.1.4. Production of Yeasts from Vinasse

Another alternative for the use of vinasse is yeast production from vinasse. But there are two factors that increase the cost of this process; the requirement of ammonium salts and magnesium and high energy requirement during the evaporation of water from vinasse.

2.3.2.1.5. Use of Vinasse for Microalgal Cultivation

Apart from these applications listed above, the high nutrient content of vinasse make it applicable as a substrate for cultivation of microalgae and therefore can be useful for decreasing vinasse's eutrophic potential and reduce cost of microalgal cultivation (Candido & Lombardi, 2016).

Coca et al. (2015) reported beet vinasse supplementation on the growth medium of *Spirulina platensis* and analyzed the effect of beet vinasse on biomass concentration and protein productivity of *S.planetsis* (Coca et al., 2015). Calixto et al (2016) reported the usage of alternative media compositions such as municipal domestic sewage (MDS), fruit/horticultural bio compost (HB), bio-compost derived from sugarcane and vinasse (VB), raw chicken manure (RCM) and bio-compost of chicken excrement (BCE). *Chlorella* sp., *Chlamydomonas* sp., *Lagerheimia longiseta* and *Pediastrum tetras* were cultivated in these media compositions at 25°C. At the end of the incubation period, *Chlorella sp*. produced the highest biomass (1805 mg/L) in municipal domestic sewage on the other hand in bio-compost derived from sugarcane and vinasse (VB) the biomass obtained was 112mg/L. *Chlamydomonas sp*., produced the highest biomass,483 mg/L, in fruit/horticultural biocompost (HB). *Lagerheimia longiseta* and *Pediastrum tetras* produced 430mg/L and 508mg/L biomass in Zarrouk control medium. They reported that large biomass quantities can be obtained with low cost by using media that contain some domestic and agroindustrial residues (Calixto et al., 2016).

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2.4. Microalgal Bioreactors

Microalgae are generally cultivated in open ponds, raceway ponds or in enclosed photobioreactors. While open ponds are generally placed outdoors and need sunlight, closed photobioreactors can be placed either indoor or outdoor (Bahadar et al., 2013). Advantages and disadvantages of open pond systems and closed photobioreactors are given in Table 1.

Table 2.1. Comparison of open pond system and closed system (Adapted from Zhu et al., 2013).

Parameter	Open System	Closed System
Biomass concentration	Low	High
Harvesting costs	High	Low
Space required	High	Low
Construction costs	Low	High
Contamination risk	High	Low
Water losses	High	Almost none
CO2-losses	High	Low
Biomass quality	Difficult to control	Easy to control
Weather dependence	High	Low
Repeatability	Low	High
Period of culture	long	Short

Large scale production of microalgal biomass is cheaper in open pond systems rather than closed photobioreactors. Open pond systems can be applied in every area, even in areas that have low crop production potential. So there is no competition with current agricultural crops (Chisti, 2008). Moreover their energy input requirement is low and easy to sanitize and therefore their net energy production is high (Rodolfi et al., 2009).

On the other hand, light is a major limiting factor in open pond bioreactors. Different methods have been applied to provide microalgal cells more light. At this point flow

velocity becomes a crucial point since it increases the exposure of microalgal cells to light and CO_2. Flow velocity is selected according to the medium depth and rate of microalgal cells going down and generally a velocity of 10 to 30 cm/s is found efficient. Loss of water through evaporation is another problem in open pond systems. While temperature control in open photobioreactors is easy to handle because of evaporation, it is difficult in closed photobioreactors. Moreover natural environmental conditions should be considered while selecting cultivation area (Zhu et al., 2013).

2.4.1. Open Pond Systems

Microalgal cultivation in open pond systems has been used since the 1950s. Open systems can be divided into neutral waters such as lakes, lagoons, and ponds and artificial ponds. The widely accepted artificial system is raceway ponds (Brennan & Owende, 2010).

Raceway ponds consist of oval shaped recirculation channels that function in mixing and recirculation to maintain algal growth and productivity (Figure 2.8). Generally, algae are put in front of the paddlewheel and dispersed with the loop to the spot, where extraction is harvested. Paddlewheels provide continuous mixing and thus prevents precipitation of microalgae. Generally CO_2 requirement of microalgae is provided from air, but some aerators can also be applied to increase CO_2 assimilation (Brennan & Owende, 2010).
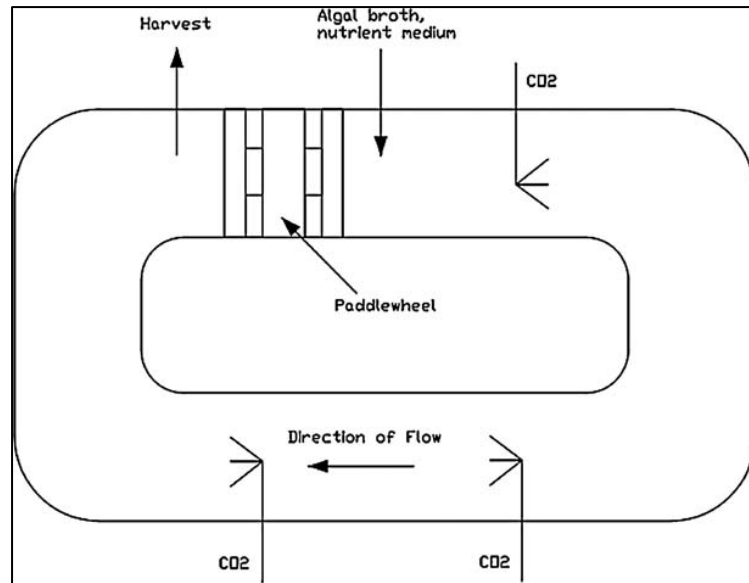
Figure 2. 8. Schematic view of a raceway pond (Adapted from Brennan et al., 2010).

Although there are some limitations such as evaporation losses, temperature variations in culture media and limited light; there are several studies that achieved high biomass concentrations in open pond systems. Jiménez et al., (2003) evaluated mass production of *Spirulina max*ima using raceway ponds with an area of 450 m^2 and depth of 0.30m. A biomass productivity of 8.2 g /m^2 .day was obtained and it was estimated that this would produce 30 tons per hectare of biomass in a year. Weissman et al. (1989) conducted an outdoor open pond photobioreactor and reported a 37 tons per hectare of biomass production rate with four different species (Brennan & Owende, 2010).

2.4.2. Closed photobioreactors

A photobioreactor is described as a closed system, in which energy is supplied with electric lights or natural sunlight. In the design of photobioreactors; uniform distribution of light and an efficient mass transfer of CO_2 and O_2 should be considered (Gupta et al., 2015).

Closed photobioreactors generally contain plates that are located horizontally or vertically or thin panel of transparent tubes. The main types of closed photobioreactors are tubular, flat plate, airlift and bubble column, and stirred tank bioreactors (Bahadar et al., 2013).

2.4.2.1. Tubular photobioreactors

Closed tubular photobioreactors are the most favorable closed photobioreactor type for large scale cultures due to high biomass yields (Bahadar et al., 2013). They consist of parallel tubes that are placed horizontally or vertically to improve sun exposure (Figure 2.9).
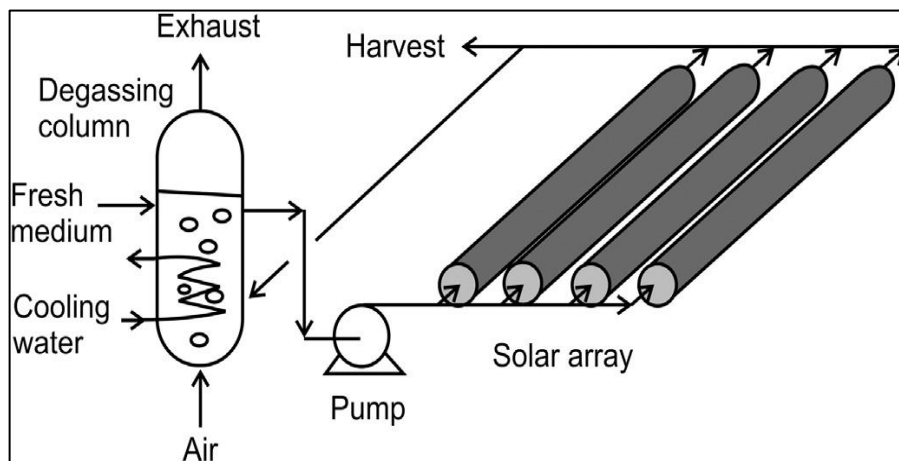
Figure 2.9. Horizontal tubular photobioreactor (Adapted from Bahadar et al., 2013).

Algae are dispersed by the tubes with the aid of a pump or airlift technology (Hall et al., 2003). Airlift apparatus have some advantages; provide CO_2 and O_2 transfer between aeration gas and liquid medium; reduce possible cell damage that may result from mechanical pumping; provide circulation (L. Xu et al., 2009).

The circulation of the culture in the medium is mainly affected by the diameter and length of the tube. The higher tube diameter reduces surface/volume ratio and this greatly affects microalgal culture. When microalgal cells grow and their density becomes larger self-shading occurs and as a result a volumetric decrease in the biomass per unit of incident light is observed. Moreover, the tube length mainly affects the circulation of the medium within the bioreactor. Long tubes cause an increase in in the O_2 concentration that is produced by photosynthesis. In the case of surpassing air saturation, high O_2 concentration can restrict photosynthesis by inhibiting key enzyme of photosynthesis, Rubisco (L. Xu et al., 2009). Because of these problems

tubular photobioreactors cannot applied to indefinite scale up processes (Eriksen, 2008; L. Xu et al., 2009).

2.4.2.2. Flat plate photobioreactors

Flat plate photobioreactors consist of transparent materials to provide highest solar energy capture and a thin layer absorbs radiation (Hu et al., 1998). In comparison to tubular photobioreactors, flat plate photobioreactors are convenient for mass cultivation of microalgae because of the low levels of dissolved oxygen and high levels of photosynthetic activity obtained (Brennan & Owende, 2010; Richmond, 2000)

Flat plate photobioreactors are planned in order to provide an efficient sunlight usage for that reason thin panels generally provide high area /volume ratios. High productivity and equal dispersion of light are the advantages of this system. Moreover, possessing an open gas transfer unit, is the advantage of this system in terms of reducing oxygen concentration that is accumulated. On the other hand, this open gas transfer unit makes the system more susceptible to contamination. These type of bioreactors are also expensive to manufacture and mechanically weaker in many cases.These type of reactors can be placed against the sun to provide more energy absorption (Carvalho et al., 2006) (Figure 2.10).
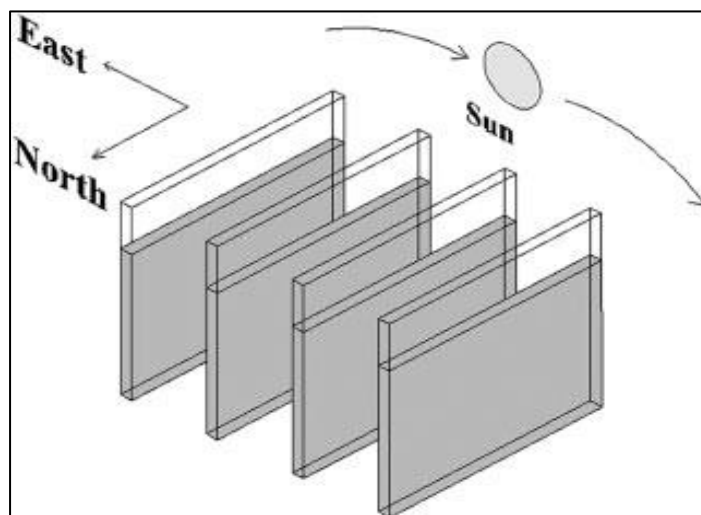
Figure 2.10. Solar orientation of a flat-plate photobioreactor system (Adapted from (L. Xu et al., 2009).

2.4.2.3. Airlift and Bubble Column Photobioreactors

Airlift and bubble column bioreactors are equipment that have been applied in bioprocessing, chemical processes, and in wastewater treatment (L. Xu et al., 2009). They consist of transparent polyethylene or glass tubes to enable light penetration and CO_2 is provided by bubbling. These tubes are generally produced with common materials to be cost effective. In these type of photobioreactors aeration via mixing are supplied by sparging, which consumes less energy than mechanical stirring (Carvalho et al., 2006; Gupta et al., 2015). From the bottom of these reactors air is bubbled to the system to provide adequate mixing and CO_2 supply. Due to being inexpensive and highly transparent, polyethylene bags are widely used (Carvalho et al., 2006).
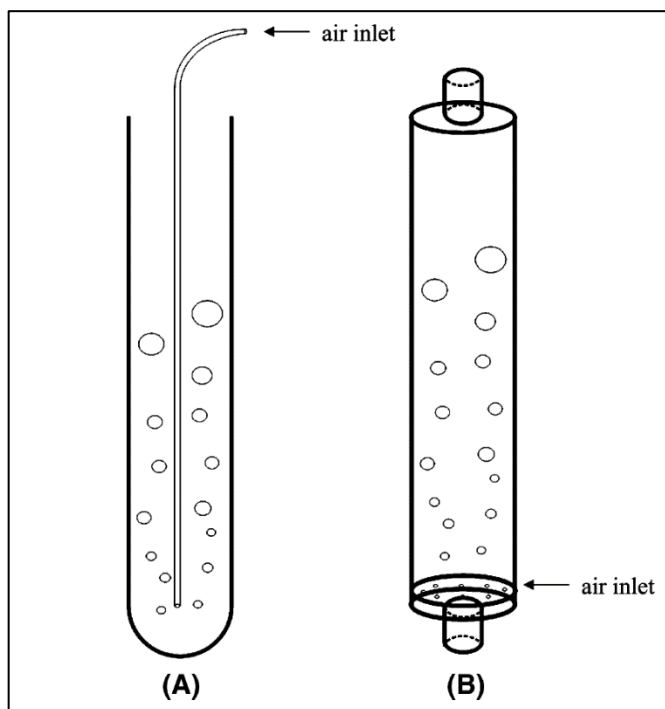
Figure 2.11. Schematic representation of airlift (A) and bubble column (B) bioreactor (Adapted from (Carvalho et al., 2006).

In bubble column photobioreactors, flow profile that carries cells is less uniform and cells are devoid of circulation in low or high light intensities, however in airlift photobioreactors flow profile is more consistent and carries cells from dark side (riser)

to light (down comer) sides (Kaewpintong et al., 2007). Hence, bubble columns enable cells to locate in low or high light intensities for a long time. (L. Xu et al., 2009).

The major cost in airlift and bubble column photobioreactors comes from the cost of added gases. In a study of Merchuk et al., (2000) Porphyridium cultures were evaluated in bubble column and airlift photobioreactor. Airlift photobioreactor was supplemented with a flow device and the cost of the gases required for the generation of per kg biomass was about half of that in the bubble column (L. Xu et al., 2009).

In a study of Silva-Aciares et al. (2008), a bubble column that is made of PVC bristles were used to dislocate the diatom. The resulted biomass yield was about 20% more than the cells that were cultivated in bubble column that do not contain bristles. The movement that is provided by bristles is an efficient method to increase biomass production of adhesive algae.

In order to enhance CO_2 supply and O_2 removal, dual spargers are applied to bubble column photobioreactors. In bubble columns that have dual spargers, CO_2 transfer rate is higher in comparison to bubble columns without dual spargers (Eriksen et al., 1998). Moreover, size of bubbles is important to reduce the harm to cells that result from shear. In a study conducted with *Nannochloropsis gaditana* it was observed that with larger bubbles microalgal cells grew better. Cells with small bubbles were highly affected with high growth rates than the cells with large bubbles (Rocha et al., 2003) (L. Xu et al., 2009).

Low power suspension, not possessing moving parts, providing a homogenous shear and fast mixing, no need of large area, efficient mass transfer rate are advantages of airlift and bubble columns bioreactors. However, when scaled up, light penetration becomes a problem since it is inversely proportional to the length from the light source (L. Xu et al., 2009).

2.4.2.4. Stirred Tank Bioreactors

Stirred tank bioreactors can be used for the heterotrophic cultivation of microalgae (Figure 1.9). These systems have several advantages such as; process parameters such as light, temperature and pH can be fully controlled, with long time of cultivation period of axenic cultures, certain high value metabolites can be produced.

In stirred tank bioreactors mixing and bubble dispersion are provided by mechanical agitation. Baffles are present to decrease vortexing. In order to provide enough space for separation of droplets from exhaust gas, generally 70-80% of the vessel is filled with medium. In the case of a foaming problem, foam breakers or chemical antifoam agents can be applied. Temperature control and heat transfer can be provided by internal cooling coils (Figure 2.12).

On the other hand, low volume to ratio of bioreactors decreases light harvesting capacity of cells. Internal illumination systems are used to distribute light homogenously. For instance Ogbonna et al. (1999) designed a bioreactor that uses both sunlight and artificial light, which can reduce, operational costs.

If high productivities can be achieved, stirred tank bioreactors would compete with other type of reactors for industrial production of various biochemical products by microalgae. Otherwise, bubble column bioreactors can be used (Doran, 1995).
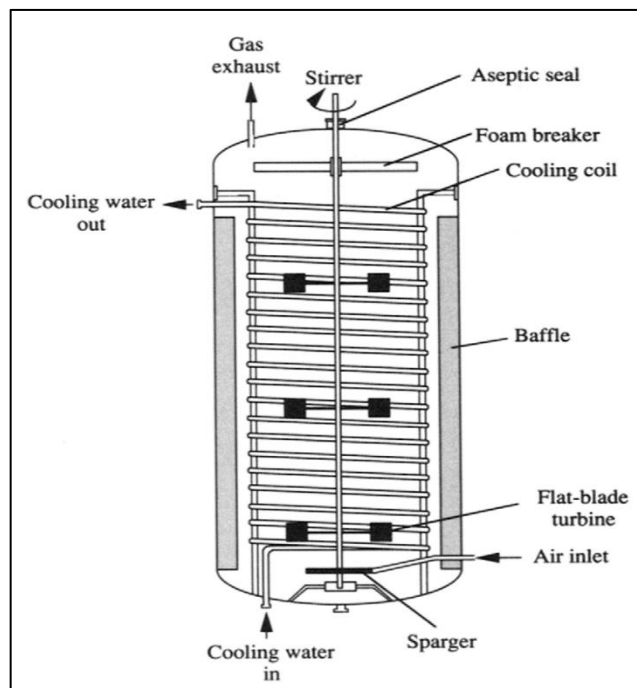
Figure 2.12. Stirred tank bioreactor (Adapted from Doran, 1995).

2.4.3. Operation Modes of Bioreactors

There are three modes of operation for bioreactors: batch, fed-batch, and continuous. Selecting the appropriate operation mode is important for obtaining efficient substrate conversion and product formation.

2.4.3.1. Batch Operation

In batch operation, substrate is placed into reactor at the beginning of the operation and final products are discharged at the end of the process. As long as there is no evaporation loss from the vessel, medium volume in the tank remains constant.

Generally commercial bioreactors are stirred tank bioreactors; however airlift or bubble column bioreactor can also be used in batch mode as long as there is a uniform dispersion of the substrate and product (Doran, 1995).

2.4.3.2. Fed-Batch Operation

Fed batch operation involves periodic or intermittent feeding of nutrients to the reactor. This type of operation mode enables control of substrate concentration. Initiating the process with a diluted substrate solution and adding more nutrients during the process, prevent high growth rates. Starting with a diluted solution may prevent inhibitory effect of high substrate concentration (Doran, 1995).

2.4.3.3. Continuous Operation

In continuous operation, substrate is supplied into the vessel and the product removed from the vessel continuously. To obtain a homogenous product, process is considered to be at steady state and the conditions inside the reactor should not be related with time. To obtain an efficient steady state condition, continuous bioreactors are

processed with constant volume and this provides homogenous substrate and product flow rates (Kadic & Heindel, 2014).

2.4.4. Design Principles of Bioreactors

There are some properties that should be taken into account when designing a bioreactor. These features are light supply, circulation, O_2 supply, pH control, and temperature control.

2.4.4.1. Light

In the design of photobioreactors, the most important feature is light supply. Light can be applied in periodic light-dark cycles or continuously. The change in cell concentration alters their light demand. Too little light can restrict algal growth and too much light can also be harmful to cells. An increase in light causes an increase in growth rate up to saturation point of the culture, but further increase in light beyond the compensation point can cause photoinhibition (Andersen Robert A., 2005).

2.4.4.2. Circulation

Circulation is required in order to obtain a homogenous nutrient dispersion, sufficient gas exchange, and to prevent cells from collapse. Circulation can be provided by aeration or mechanical agitation. Aeration is generally applied to supply oxygen requirement of heterotrophic cells since in heterotrophic cell culture oxygen is a limiting factor. On the other hand, mechanical agitation is applied with baffles and impellers. Too much circulation can produce shear that can harm cell growth, cause cell damage, and finally cell death may occur. The resistance to stress caused by shear is species dependent (Pahl et al., 2010).

2.4.4.3. Oxygen

Excessive O_2 concentration more than air saturation inhibits photosynthesis in microalgae. Moreover, oxygen concentrations more than 35 mg/L is generally toxic to most of the microalgae. In closed photobioreactors, buildup of photosynthetically produced O_2 is a main problem in photobioreactors and to solve this problem degasser systems are applied (Carvalho et al., 2006).

On the other hand, in stirred tank bioreactors providing sufficient oxygen is crucial to obtain high cell yields and high growth rates. Generally, oxygen is provided as compressed air. Dissolved oxygen level in air-saturated water is agreed to be 100% dissolved oxygen (D.O.), and its level in bioreactor should be lower than 100% due to the metabolism of microalgal cells. It is reported that oxygen concentration between 20 and 75% air saturation does not inhibit growth of microalgal cells, while oxygen concentrations from 75 to 250% air saturation have negative effect on microalgal growth (Raso, van Genugten, Vermuë, & Wijffels, 2011).

Besides, as microalgal cells grow and their concentration change, the level of dissolved oxygen changes accordingly (Andersen Robert A., 2005).

2.4.4.4. pH and Temperature Control

pH and temperature are also important parameters to be controlled in a bioreactor. pH and temperature control is achieved by commercially available controllers. On the other hand, cooling is also required. Cooling is provided by a system in which cool water outside the system is circulated via a heat conducting apparatus. Heat is then extracted from the bioreactor with this heat conducting apparatus (Andersen Robert A., 2005).

2.5. Modelling and Optimization

Optimization can be defined as to enhance performance of a process to acquire maximum benefit from it (Bezerra et al., 2008). It is crucial to enhance process yields and enhance the performance of the systems without changing the cost too much.

In order to find out optimum process conditions, generally one parameter is altered while other parameters are kept constant. This process is known as one-factor-at a time technique. This technique is disadvantageous since it does not provide information about the interaction effect of variables and also does estimate the effect of variables on the process (Baş & Boyacı, 2007). Moreover, it causes an increase in number of experiments to be conducted and as a result it takes too much time.

To overcome this, optimization studies should be carried out with Response Surface Methodology (RSM). RSM is a combination of statistical and mathematical techniques used for establishing, expanding, and optimizing the process. It can be used when a response or responses are affected by different variables. The aim is to optimize the levels of these various variables to find out best process performance.

RSM determines the effects of variables on the process and also provides a mathematical model that defines the biochemical or chemical processes (Baş & Boyacı, 2007; Farooq Anjum, Tasadduq, & Al-Sultan, 1997).

Before starting RSM an experimental design that will describe the experiments to be conducted in the field of experiment have to be considered. For that purpose, first order model (factorial designs) experimental designs can be applied when data sets are not curved (Bezerra et al., 2008). For instance, Plackett-Burman design is a factorial design that considers no interactions between process variables and enables us to screen process parameters (Francis et al., 2003).

Besides, when the response cannot be explained by linear functions, experimental designs for quadratic response surfaces such as Box-Behnken, Central Composite, and Doehlert designs should be considered.

Box-Behnken designs need only three levels which are coded as -1, 0 and +1. They are generated by coupling two level factorial design and incomplete block designs.

This method develops a design that have good statistical features and also with experiments that are needed for three level factorial (Francis et al., 2003).

There are several researchers that have studied the optimization of cultivation conditions and medium compositions of microalgae using RSM (Cheng et al., 2009; Kirrolia et al., 2014a). Cheng et al. (2009) used the Central Composite Design of RSM to investigate the effects of C/N (carbon source /nitrogen source) and yeast extract concentration on the biomass and lipid production of *Chlorella prothecodies*. Kirrolia et al. (2014b) used the Box-Behnken Design of RSM for medium optimization of *Chlorella spp*. during biodiesel production (Kirrolia et al., 2014b).

2.6. Microalgae Species Used in This Study

In this study, two different microalgal species were used. These species were *Micractinium sp. ME05* and *Chlamydomonas reinhardtii* 137C$^-$.

2.6.1. *Micractinium sp.* METUNERGY1405

Micractinium was firstly identified by Fresenius (1858). It is defined by ovoid or spherical cells that are generally organized in colonies. Their cell wall contain bristles that include protein and these bristles are produced after cell wall development (Luo et al., 2006).

The primary microalgal strain used in this study was *Micractinium sp.*ME05 (Figure 2.13). This strain was previously isolated and characterized by Onay et al. (2014). *Micractinium sp*. belongs to Chlorophyta division. It was isolated from thermal regions of Haymana, Ankara and described as native thermo-resistant strain of Central Anatolia. It was reported that the strain was able to grow at temperatures up to 50°C (Onay et al., 2014).

This strain was used in optimization studies and in experiments conducted with molasses and vinasse.
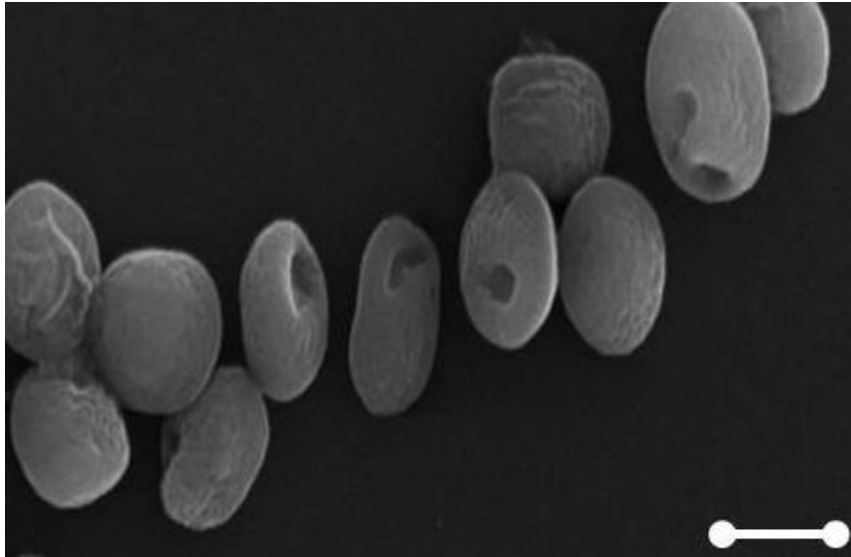
Figure 2.13. Scanning electron microscope image of *Micractinium sp*. ME05. The scale bar is 2µm (Onay et al., 2014).

2.6.2. *Chlamydomonas reinhardtii* **137C-**

Chlamydomonas reinhardtii is a unicellular green microalga with two flagella. It is about 10µm in length and contains multiple mithocondria (Merchant et al., 2010). *Chlamydomonas reinhardtii* belongs to Chlorophyta division and is a model organism that was investigated for photosynthesis, motility, and reproduction. It contains a haploidic life cycle and its sexual life cycle is described well (Aoyama et al., 2014).

Chlamydomonas reinhardtii was used in preliminary experiments of this study. It was evaluated in different medium compositions with different carbon sources.

2.7. Aim of The Study

The aim of this study was to optimize culture conditions of *Micractinium sp*. ME05 using inexpensive substrate molasses under heterotrophic conditions.

Beside *Micractinium sp.* ME05 cells, *Chlamydomonas reinhardtii* 137C^{-} cells were evaluated with different carbon sources (glucose, sucrose, and molasses).

Different concentrations of carbon source, inoculum ratios of cells, and media (basal medium and bold basal medium) were firstly tested by one factor at a time approach. Plackett-Burman design was then applied in order to screen process variables that

significantly affect biomass production of *Micractinium sp.* ME05 cells. The interaction effect between process variables were evaluated by using Box-Behnken Design of Response Surface Method and optimum culture conditions were determined.

Furthermore, effects of different aeration rates and mixing conditions on biomass production of *Micractinium sp.*ME05 cells were evaluated in flasks and 2L bioreactors, respectively. Next, lipid extractions of microalgal cells cultivated in bioreactors were performed according to Bligh-Dyer method and their FAME profile were evaluated for their biodiesel properties.

As another inexpensive by-product of sugar industry, vinasse was adopted for the cultivation of *Micractinium sp.*ME05 cells. Different vinasse concentrations (2%-5%-10% and 20%) were used under mixotrophic and heterotrophic conditions in 500 mL and 2 L flasks. 5 L bioreactors were then used with the vinasse concentration that has given the highest biomass production in flasks. Lipid extractions of microalgal cells cultivated with vinasse were also performed and their FAME profile were evaluated for their suitability for biodiesel production.

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1. Microorganisms

In this study, *Micractinium sp. ME05* and *Chlamydomonas reinhardtii 137C*⁻ were used. *Micractinium sp.ME05* was isolated from Haymana, Ankara (latitude 39.44° N, longitude 32.48° E) geothermal flora and characterized as previously described (Onay et al., 2014). *Chlamydomonas reinhardtii 137C*⁻ was kindly donated by Dr. Clarie Remacle, Genetics of Microorganisms Laboratory, Department of Life Sciences, Université de Liège, B-4000 Liège.

3.1.2. By-products of Sugar Industry

Molasses and Vinasse were kindly provided by Konya Sugar Factory / Konya. The composition of molasses and vinasse which was reported by the supplier is given in Appendix A.

3.1.3. Chemicals

The chemicals used in this study were purchased from Sigma Aldrich, Merck Chemical Company and AppliChem Chemical Company. All of the solutions were prepared by using distilled water.

3.2. Methods

3.2.1. Culture Maintenance

The microalgae were maintained onTris-Acetic Acid- Phosphate (TAP) (Appendix B) agar plates or liquid media at pH:6.8, with constant shaking at 24±1 °C, 16-hour light-8-hour dark photoperiod. *Chlamydomonas reinhardtii* 137C$^-$ and *Micractinium sp.* ME05 cells on agar plates are shown in Figure 3.1. These cells were used as seed cells in the following cultures.
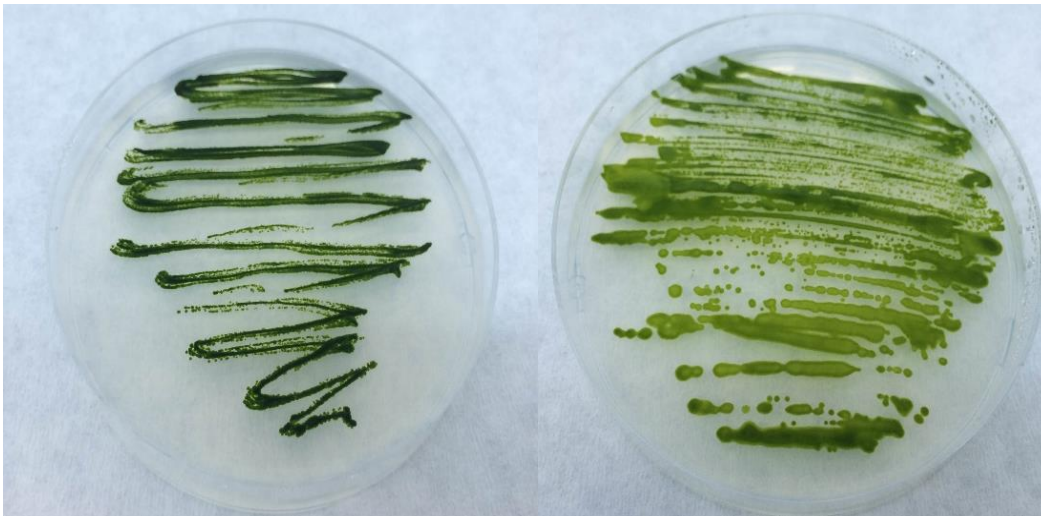
Figure 3.1 *Micractinium sp. ME05* (left) and *Chlamydomonas reinthardii* 137C$^-$ (right) on TAP agar plates.

3.2.2. Shake Flask Culture

Basal medium (Xiong, Li, Xiang, & Wu, 2008) and Bold Basal Medium (Abou-shanab et al., 2012) were used for heterotrophic cultivation of microalgae. Medium compositions are given in Appendix B. For heterotrophic cultivation of microalgae, glucose, sucrose, molasses, and vinasse were used as carbon source. Heterotrophic cultivation was carried out with constant shaking under complete darkness in a growth chamber (Nuve GC 400 Growth Chamber) at 25 ±1°C, 30.7 ±1°C and 37±1°C. Dark condition was achieved by wrapping the cultivation system with aluminum foil.

3.2.3. Aquarium Pump Culture

Basal medium (Xiong et al., 2008) and Bold Basal Medium (Abou-shanab et al., 2012) were used for the heterotrophic cultivation of microalgae. Medium compositions are given in Appendix B. During aquarium pump culture of microalgae, glucose and molasses were used as carbon source. Cultures were aerated with filtered air pumped by aquarium compressors with 0.25 L/min and 0.50 L/min airflow which was controlled by a rotameter. Flask set up with air flow is given in Figure 3.2. Cultivation was carried out in a controlled growth chamber (Nuve GC 400 Growth Chamber) at 25 ±1°C, 30.7 ±1°C and 37±1°C.
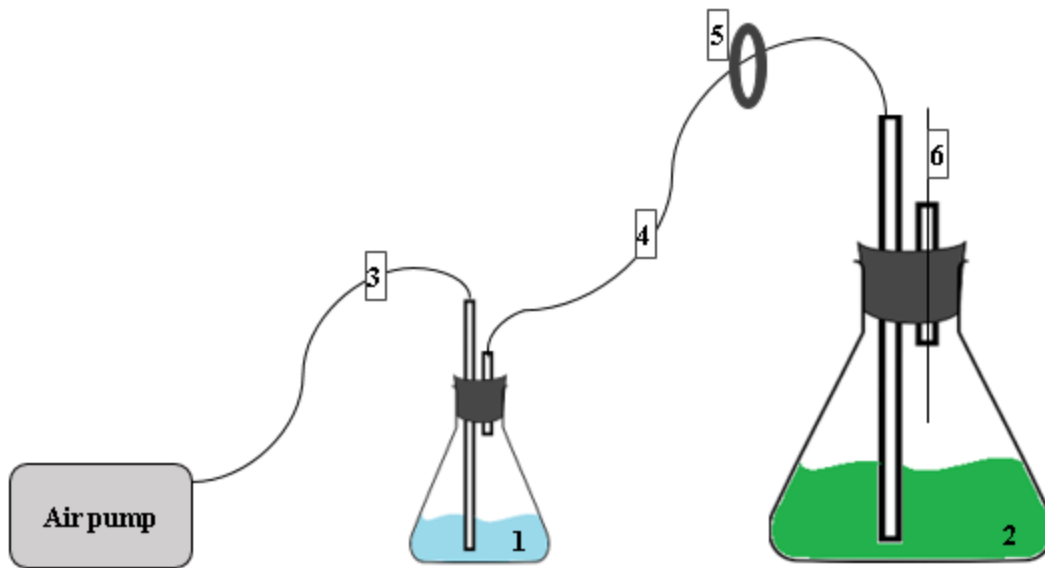
Figure 3.2. Flask set up with air flow. 1) water that humidifies air, 2) microalgal culture, 3) air inlet, 4) humified air, 5) air filter, 6) air exhaust.

3.2.4. Cell Growth, Cell Dry Weight and Cell Density Measurement

Cell growth was monitored by optical density measurements at 680 nm using Thermo Scientific Multiskan GO spectrophotometer. For cell dry weight calculation, 5mL of microalgal cells were harvested in stationary phase by centrifugation at 3000×g for 10 min at 4°C (Onay et al., 2014). The pellet was washed three times with dH_2O and then dried at 70°C in a hot air oven (Nuve ES 500). The biomass was determined gravimetrically (Karpagam, Raj, Ashokkumar, & Varalakshmi, 2015a). The biomass concentration (g/L), biomass productivity (g.L^{-1}.d^{-1}) and biomass yield of glucose ($Y_{x/s}$) were calculated using the following formulas:

$$\text{Biomass concentration (g.L}^{-1}) = \frac{\text{dry weight (g)}}{\text{1L of culture}} \qquad (1)$$

$$\text{Biomass productivity (g.L}^{-1}.\text{day}^{-1}) = \frac{B_2 - B_1}{t_2 - t_1} \qquad (2)$$

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Where, B_1 = Biomass concentration (g/L) on t_1 time, and

B_2 = Biomass concentration (g/L) on t_2 time.

$$\text{Biomass yield on glucose } (Y_{x/s}) = \frac{\text{g biomass produced}}{\text{g susbtrate consumed}} \qquad (3)$$

The cell density (cells/ml) of microalgal culture was determined by using a cell counting hemocytometer (Heinz Herenz Counting Chamber). 5µl of formaldehyde was added to 1 ml of microalgal cells in order to prevent movement of cells. Then 10µl of cells were put between the counting chamber and glass cover and cells were counted with a light microscope (Leica CM E) under 40× objective. The central square that consists of 25 small squares was counted (Figure 3.3.). Following counting the cells, concentration was calculated according to Equation 4:

$$\text{Concentration (cell/mL)} = \text{number of cells} \times 10^4 \qquad (4)$$
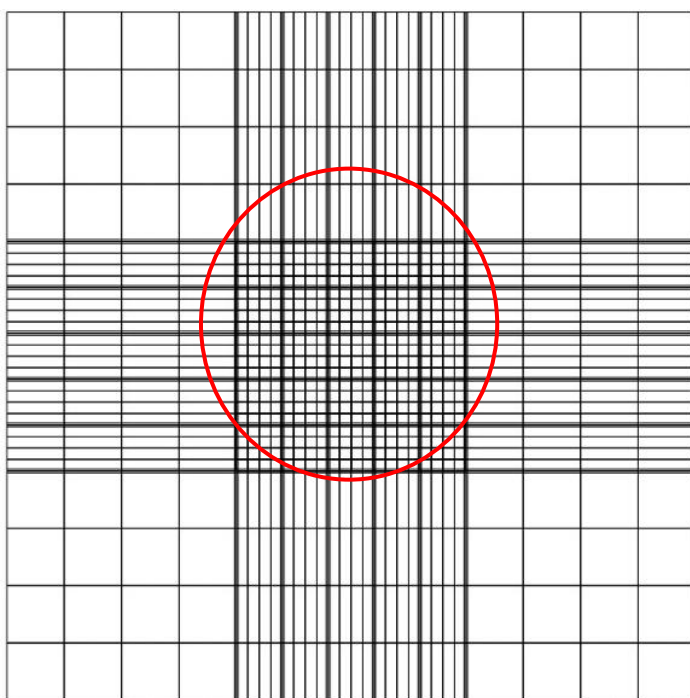
Figure 3.3. Cell counting chamber. Squares in the red circle were counted.

3.2.5. Pretreatment of Molasses and Vinasse

Acidic pretreatment method was applied to molasses. For this purpose, 10 mL of molasses (1.5g/mL) were mixed with 90 mL of 1% (w/v) of H_2SO_4. The solutions were autoclaved at 121°C for 40 minutes. The pretreated samples were filtered and the reducing sugar content was determined using 3,5-dinitrosalicylic acid (DNS) method (G.L.Miller, 1959).

In order to clarify vinasse, it was subjected to pretreatment. For this purpose, one liter of distilled water was mixed with 1 liter of vinasse and heated for 30 min in an autoclave and subsequently centrifuged at 3920× g for 10 min, 10°C (Calixto et al., 2016; Candido & Lombardi, 2016).

3.2.6. Determination of Reducing Sugar Concentration and Glucose Concentration

Reducing sugar concentration was determined with 3,5- dinitrosalicyclic acid DNS method (G.L.Miller, 1959). The detailed description of the preparation of 3,5-

dinitrosalicyclic acid (DNS) solution is given in Appendix B. In DNSA method, 3 mL of DNS reagent was mixed with 3 mL of diluted sample in a tube. The resulting mixture was incubated in a water bath at 90°C for 15 min. After 15 minutes, 1mL of 40% Rochelle salt was added when the tubes were still hot. The tubes were cooled and their absorbance values were measured at 575nm. During absorbance readings, a blank that contain 3mL of dH_2O, DNS and Rochelle salt was used. Following absorbance readings calculations were performed according to the glucose standard curve (Appendix C).

In order to measure remaining glucose in the heterotrophic media, whose carbon source was glucose, glucose oxidase kit was used. Calculations were performed according to the glucose standard curve obtained through serial dilutions from 100mg/dl glucose standard. Glucose standard curve is given in Appendix C. Glucose oxidase kit procedure was applied as follows; after 120 hours of incubation of the cells, 20µl of cells were mixed with 3ml of glucose reagent and their absorbance values were measured at 500nm. By using the glucose standard curve (Appendix C) calculations were performed and remaining glucose in the growth media were determined.

3.2.7. Lipid Content Determination

Lipid extractions of microalgal cells that were cultivated in bioreactors were carried out via lyophilization-assisted Bligh and Dyer method. Initially microalgal cells that reached stationary phase were centrifuged at 3600×g for 20 min at 4°C. Following centrifugation, supernatant was discarded and the pellet was collected in a new falcon tube and centrifuged again at 3600×g for 10 minutes. Supernatant was discarded and the pellet was incubated at -80°C overnight. The pellet was lyophilized in a Heto Maxi Dry Lyo Freeze-Dryer (METU Central Laboratory Molecular Biology and Biotechnology R&D Center, Ankara). Following lyophilization, Bligh Dyer method was applied as given in Figure 3.4.

Weigh 0.1 g of lyohphilized tissue and place into glass test tubes

↓

Add 1.4 mL methanol and 0.7 mL chloroform

↓

Mix 1 minute by vortexing every 10 minutes (3 times)

↓

Sonicate for 15 minutes

↓

Add 0.7 mL chloroform and mix by vortexing for 1 minute

↓

Incubate for 3 hours at room temperature

↓

Add 1.2 mL dH$_2$O and centrifuge at 3600×g for 10 minutes

↓

Collect the lipid phase into a new test tube and discard upper phase

↓

Dissolve remaininig cell debris and pellet in 1.4 mL methanol and 0.7 mL chloroform.

↓

Incubate for 15 minutes and mix by vortexing

↓

Add 0.7mL chloroform and incubate overnight

↓

Add 1.2 mL dH$_2$O and centrifuge at 3600×g for 10 minutes

↓

Transfer dissolved lipids into a new tube

↓

Continue centrifuge until all cell debris was cleaned

↓

After obtaining clean lipid solution, leave lipid collection tube for air drying

↓

Weigh the obtained lipids

Figure 3.4. Lipid extraction with Bligh and Dyer Method.

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3.3. Evaluation of Growth Conditions for Chlamydomonas reinhardtii 137C-

C.reinhardtii 137C⁻ cells, which were maintained in TAP agar slants were inoculated into 25 mL of TAP liquid media. From these starter liquid media, cells were inoculated into 500 mL flasks that contain 200 mL TAP medium. Cultivation was carried out at 25°C under 16 hours light-8 hours dark photoperiod and under complete darkness with constant shaking. On a daily basis 1mL sample was withdrawn from the flasks under aseptic conditions and their optical densities at 680 nm were recorded (Thermo Scientific Multiskan GO spectrophotometer). Growth curves were plotted as absorbance (680nm) versus hour. Measurements given were the averages of three biological replicates.

Further studies performed with *C. reinhardtii* 137C⁻ were growth evaluation studies that were performed with different carbon sources in TAP medium and basal medium to determine the carbon sources that would be metabolized by *C. reinhardtii* 137C⁻ cells.

3.3.1. Growth Evaluation of *C. reinhardtii* 137 C- Cells with Different Carbon Sources

C. reinhardtii 137C⁻ cells were grown up to exponential phase in TAP medium. Grown cells were inoculated into; TAP medium with different glucose concentrations (10g/L-30g/L- 50g/L- 80g/L glucose), Basal medium with different glucose concentrations (10g/L- 30g/L- 50g/L- 80g/L glucose), Basal medium with different sucrose concentrations (10g/L- 30g/L- 50g/L- 80g/L sucrose), and basal medium with different molasses concentrations separately (10g/L- 30g/L- 50g/L- 80g/L molasses). 3 g/L yeast extract was added to these media by autoclaving separately. *C. reinhardtii* 137C⁻ cells that came to exponential growth phase were inoculated into 250 mL erlenmayer flasks containing 100 ml of these media and cultivation was carried out at 25± 1 °C with shaking at 150 rpm. Incubation was carried out for six days under 16 hours light-8 hours dark photoperiod.

3.4. Evaluation of Growth Conditions for *Micractinium sp*. ME05

Micractinium sp. ME05 cells, which were maintained in TAP agar slants were inoculated into 25 mL of TAP liquid media. From these starter liquid media, cells were inoculated into 500 mL flasks that contain 200 mL TAP medium. Cultivation was carried out at 25°C, 30°C and 37°C under 16 hours light-8 hours dark photoperiod with constant shaking. On a daily basis, 1mL sample was withdrawn from the flasks under aseptic conditions and their optical densities at 680 nm were recorded. Growth curves were plotted as absorbance (680nm) versus hour. Measurements given were the averages of three biological replicates.

Following obtaining the growth curve of *Micractinium sp. ME05*, growth conditions were evaluated with experimental design studies to optimize biomass production.

3.4.1. Experimental Design for Biomass Production using *Micractinium sp*. ME05

Optimization of biomass production using *Micractinium sp. ME05* cells were performed in several steps. The first step was one factor at a time approach. One factor at a time approach is the conventional optimization method, in which one factor was varied and all other factors were kept constant. In this study the variables were pH, temperature, inoculum volume (v/v %), yeast extract concentration and glucose concentration. The second step was the selection of variables that significantly affect biomass production by Plackett-Burman Design (PBD). The third step was to use Box-Behnken Design to develop mathematical model for the estimation of optimum conditions, which yield high biomass concentration with *Micractinium sp*. ME05 cells.

3.4.2. Growth Evaluation of *Micractinium sp.* ME05 Cells with Different Concentrations of Carbon Sources Within Different Media

In order to determine the appropriate cultivation condition to obtain high biomass concentration using *Micractinium sp.ME05* cells, one factor at a time approach was used. For this purpose, one factor was varied and other factors were kept constant. For

this purpose, Basal medium and Bold Basal medium were evaluated with 10g/L glucose and 30g/L glucose as carbon sources.

Micractinium sp. ME05 cells were grown up to exponential phase in TAP medium. Grown cells were inoculated (0.2% and 5%, v/v) into 100 mL TAP medium and 100 mL Basal medium that contain 30g/L glucose and 2g/L yeast extract. Cultivation were carried out in a controlled growth chamber at 25±1 °C.

As an alternative medium, grown cells were also inoculated (10 % (v/v)) into 100 mL Bold Basal Medium that contains 10g/L and 30g/L glucose, separately. Cultivation were carried out in a controlled growth chamber at 25± 1°C, either on a constant shaker or with aeration (0.5L/min) provided by an aquarium pump.

3.4.3. Screening of Process Variables by Plackett-Burman Method

A Plackett-Burman Design matrix was set up to screen factors that significantly affect biomass production. The pH, temperature, glucose, yeast extract, and inoculum size were selected as the variables. The range of the variables selected were based on various studies in the literature. Variables were represented at two levels, high (+1) and low (-1), as shown in Table 3.1.

Table 3.1. Coded and uncoded variables at different levels used in Plackett-Burman Design.

Variable	Symbol	Value	
		Low (-1)	High (+1)
pH	X_1	6	8
Temperature (°C)	X_2	25	37
Inoculum (v/v %)	X_3	5	10
Yeast Extract (g/L)	X_4	0	4
Glucose (g/L)	X_5	10	30

Plackett-Burman technique depends on first-order polynomial model (Equation 5).

$$Y= \beta_0 + \sum \beta_i \, X_i \qquad\qquad\qquad (5)$$

Where Y is the response, β_0 is the model intercept, β_i is the linear coefficient and X_i is the level of independent variable (Guo et al., 2009). In this study, the independent variables; pH, temperature, glucose, yeast extract, and inoculum size were coded as X_1, X_2, X_3, X_4 and X_5 respectively. In this respect, the predicted response ($\hat{Y}$) was written with Equation 6.

$$\hat{Y}=\beta_0 + \beta_1 \, X_1 + \beta_2 \, X_2 + \beta_3 \, X_3 + \beta_4 X_4 + \beta_5 X_5 \qquad\qquad (6)$$

where $\hat{Y}$ is the predicted response (biomass concentration), β_0, β_1, β_2, β_3, β_4 and β_5 are regression coefficients (Uncu & Cekmecelioglu, 2011). In order to screen five variables, a 12-run Plackett-Burman Design matrix was built by the statistical software MINITAB 16.0 (Minitab Inc., State College, PA, USA). Table 2.2. illustrates the design matrix of different variables with coded and uncoded values. A set of 12 experiments were carried out in two replicates and the response was the average biomass concentration after 120 h of incubation. A pareto chart was plotted to show the factors that significantly affect biomass concentration (Figure 4.19).

Table 3.2. Plackett-Burman Design Matrix of Different Variables with Coded and Uncoded Values.

Exp.No	pH		Temperature (°C)		Inoculum (v/v %)		Yeast Extract (g/L)		Glucose (g/L)	
	coded	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**	**coded**	**uncoded**
1	+1	8	-1	25	-1	5	-1	0	+1	30
2	-1	6	+1	37	+1	10	+1	4	-1	10
3	-1	6	+1	37	+1	10	-1	0	+1	30
4	-1	6	-1	25	+1	10	+1	4	+1	30
5	-1	6	+1	37	-1	5	-1	0	-1	10
6	+1	8	-1	25	+1	10	+1	4	-1	10
7	+1	8	+1	37	-1	5	+1	4	+1	30
8	-1	6	-1	25	-1	5	-1	0	-1	10
9	-1	6	-1	25	-1	5	+1	4	+1	30
10	+1	8	-1	25	+1	10	-1	0	-1	10
11	+1	8	+1	37	+1	10	-1	0	+1	30
12	+1	8	+1	37	+1	5	+1	4	-1	10

3.4.4. Box-Behnken Design

Based on the Pareto chart results as given in Figure 3.15 and regression analysis; pH, temperature (°C), and glucose were selected for further optimization studies. The individual and combined effects of process variables were studied using the Box-Behnken Design (BBD) with a quadratic model using MINITAB 16.0. During Box-Behnken Design experiment studies and further studies, molasses hydrolysate was used instead of glucose as a carbon source. The BBD matrix was constructed for three significant factors (pH, temperature and molasses hydrolysate). The ranges of process variables were 6-8 for pH, 25-37 °C for temperature and 10-30g/L for molasses hydrolysate concentration (g/L) (Table 3.3.).

Table 3.3. Process variables in Box-Behnken Design

Variable	Low level (-1)	Center point (0)	High Level (+1)
pH	6	7	8
Temperature (°C)	25	31	37
Molasses hydr. (g/L)	10	20	30

A second order polynomial equation (Equation 7.) was developed using regression analysis:

$$\hat{Y}=\beta_0 + \beta_1\,X_1 + \beta_2\,X_2 + \beta_3\,X_5 + \beta_4\,X_1{}^2 + \beta_5 X_2{}^2 + \beta_6 X_5{}^2 + \beta_7\,X_1X_2 + \beta_8\,X_1X_5 + \beta_9 X_2 X_5 \quad (7)$$

Where $\hat{Y}$ is the predicted response (biomass concentration) and β_0 the intercept, β_1, β_2 and β_3 are linear coefficients, β_4, β_5 and β_6 squared coefficients, β_7, β_8 and β_9 interaction coefficients and X_1, X_2 and X_5 independent variables used in this study. Analysis of variance (ANOVA) and regression analysis were conducted with 95 % confidence interval to determine the significant terms and coefficients of predictive model. Box-Behnken Design matrix with coded and uncoded variables is given in Table 3.4. The

optimum conditions for maximizing biomass production was determined by using Response Optimizer Tool in MINITAB16.0.

Table 3.4. Experimental design for optimization of biomass production using Box-Behnken response surface method (RSM).

Exp.No	pH		Temperature (T°C)		Molasses hydr. conc. (g/L)	
	coded	uncoded	coded	uncoded	coded	uncoded
1	0	7	-1	25	-1	10
2	-1	6	0	31	1	30
3	-1	7	1	37	-1	10
4	1	8	1	37	0	20
5	0	7	0	31	0	20
6	1	8	0	31	1	30
7	1	8	-1	25	0	20
8	0	7	-1	25	1	30
9	-1	6	1	37	0	20
10	-1	6	0	31	-1	10
11	0	7	0	31	0	20
12	0	7	0	31	0	20
13	-1	6	-1	25	0	20
14	0	7	1	37	1	30
15	1	8	0	31	-1	10

3.4.5. Model Verification

The constructed model was verified by comparing the experimental biomass results with results of predicted biomass calculations. Besides, a statistical difference measure test was applied to determine the performance of the model. Root mean square error (RMSE) was calculated with Equation 8 and mean absolute error (MAE) was calculated with Equation 9 as follows:

$$RMSE = \left(\frac{1}{N} \sum_{i=1}^{N} \left(X_{pred,i} - X_{exp,i} \right)^2 \right)^{0.5} \qquad (8)$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} \left| X_{pred,i} - X_{exp,i} \right| \qquad (9)$$

where $X_{pred,i}$ and $X_{exp,i}$ are predicted and experimental biomass concentrations, respectively; N is the number of data points (Uncu & Cekmecelioglu, 2011).

3.5. Evaluation of Different Inoculum Ratios, Aeration Rates and Working Volumes in Shake Flasks with *Micractinium sp.* ME05

In shake flasks with *Micractinium sp.*ME05, different inoculum ratios, aeration rates and working volumes were evaluated with molasses hydrolysate. On the other hand, as another by product of sugar industry, vinasse was also evaluated with different concentrations under different cultivation conditions.

For that purpose, firstly optimum growth conditions of *Micractinium sp.*ME05 by using molasses under heterotrophic conditions were evaluated in 500 mL flasks with 250 mL working volume with different inoculum ratios (%5-%7-%10-%15). Then aeration rate was evaluated by comparing the effects of two different aeration rates (0.25L/min and 0.5L/min) on biomass production. After achieving highest biomass concentration, the conditions were evaluated by adapting microalgal cells to molasses.

As another by-product of sugar industry, vinasse was evaluated in terms of supporting the growth of *Micractinium sp*. ME05 cells. For that purpose, 4 different vinasse concentrations; %2-%5-%10-%20 (v/v) vinasse were evaluated in 500 mL flasks with 250 mL working volume under heterotrophic and under mixotrophic conditions. Heterotrophic cultivation was carried out under complete darkness while mixotrophic cultivation was carried out under 16h light-8h dark photoperiod. Following obtaining

higher biomass concentrations under mixotrophic conditions, same experimental conditions were applied to 2-L flasks with 1-L of working volume.

3.5.1. Growth Evaluation of *Micractinium sp.* ME05 with Molasses Hydrolysate in Small Scale Flasks with Different Inoculum Ratios

In order to evaluate the effect of different inoculum ratios of *Micractinium sp.* ME05 under optimized culture conditions (19 g/L molasses hydrolysate, 2 g/L yeast extract, 30.7±1 °C, pH: 6.9) four different inoculum ratios were tested (5%-7%-10% and 15% (v/v)). Cultivation medium was 250 mL bold basal medium (pH: 6.9), that was supplemented with 19 g/L molasses hydrolysate and 2 g/L yeast extract. *Micractinium sp.* ME05 cells in exponential growth phase were inoculated into the media at ratios of 5% (v/v), 7% (v/v), 10% (v/v) and 15% (v/v). Cultures were aerated with filtered air pumped by aquarium compressors with 0.5 L/min airflow. Cultivation was carried out at 30.7±1 °C, under dark conditions for 5 days. Dark conditions were achieved by wrapping the flasks with aluminum foil. Optical density measurements were performed on a daily basis for 5 days. Microalgal cells were grown up to stationary phase and then cell dry weight were calculated. For cell dry weight calculation, microalgal cells were collected with centrifugation (3000×g, 10 minutes) and pellets were washed three times with dH_2O and dried in oven (70°C).

3.5.2. Growth Evaluation of *Micractinium sp.* ME05 with Molasses Hydrolysate in Small and Large-Scale Flasks with Different Aeration Rates

After obtaining highest biomass concentration with 10% (v/v) inoculum under optimized culture conditions the same experimental conditions (pH: 6.9, temperature: 30.7±1 °C, molasses hydrolysate: 19g/L) were applied to 500 mL flasks with 250 mL working volume by reducing the air given to the system from 0.5 L/min to 0.25L/min. Cultivation was carried out under complete darkness with three biological replicates. On the other hand; 2-L flasks with 1-L working volume were also evaluated under optimized culture conditions. For that purpose, 1-L of bold basal medium were supplemented with 19g/L molasses hydrolysate and 2g/L yeast extract. *Micractinium*

sp. ME05 cells in exponential growth phase were inoculated into the media (10% (v/v)) under aseptic conditions. Cultivation was carried out at 30.7±1 °C, under dark conditions for 5 days. 0.25 L/min and 0.50 L/min air were given to the system, separately. Daily measurements of optical density were performed. Experiments were conducted with three biological replicates. Results were given as the average of three measurements. Microalgal cells were harvested in stationary phase at 5th day by centrifugation.

In order to compare biomass concentrations obtained under complete darkness and under 16 hours light-8 hours dark photoperiod, optimum growth conditions of *Micractinium sp.* ME05 were evaluated in 16 hours light-8 hours dark photoperiod. For this purpose, 1-L Bold basal medium was prepared in 2-L flasks and supplemented with 19g/L molasses hydrolysate as previously described. *Micractinium sp.*ME05 cells in exponential growth period were inoculated into the media under aseptic conditions. Incubation was carried out at 30.7±1 °C by giving 0.5L/min air through an aquarium pump under 16h light-8h dark photoperiod. After 120 hours of incubation period, biomass was harvested via centrifugation as described in Section 3.2.4. Experiments were performed with three biological replicates.

Additionally, in order to compare obtained biomass concentrations under complete darkness within TAP medium and bold basal medium that contain molasses hydrolysate and yeast extract, *Micractinium sp.*ME05 cells in exponential growth phase were also inoculated (10% (v/v)) into TAP medium. Cultivation was carried out at 30.7±1 °C, under dark conditions for 5 days. 0.50 L/min air were given to the system. After 120 hours of incubation period biomass was harvested via centrifugation as described in Section 2.2.4. Experiments were performed with three biological replicates.

3.5.3. Growth Evaluation of *Micractinium sp.*ME05 in Molasses Adapted Medium

After obtaining optimum conditions with molasses and optimum aeration rate (0.5 L/min) that gave the highest biomass concentration, these conditions (pH:6.9,

temperature:30.7±1°C, molasses hydrolysate:19 g/L) were applied to *Microactinium sp. ME05* cells by adapting them to molasses gradually.

The steps that was applied during adaptation are given in Figure 3.5. During these experiments, 0.5 L/min air was provided through an aquarium pump and incubation was carried out at 30.7±1 °C under complete darkness. All experiments were conducted with three biological replicates.

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┌─────────────────────────────────────────┐
│  Starter culture (TAP medium, 25±1°C)    │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│         BBM+ 17mM acetate                │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  BBM+ 10mM acetate+ 5g/L molasses hydr.  │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│  BBM+ 5mM acetate+ 10g/L molasses hydr.  │
└─────────────────────────────────────────┘
                    │
                    ▼
┌─────────────────────────────────────────┐
│      BBM+ 19 g/L molasses hydr.          │
└─────────────────────────────────────────┘
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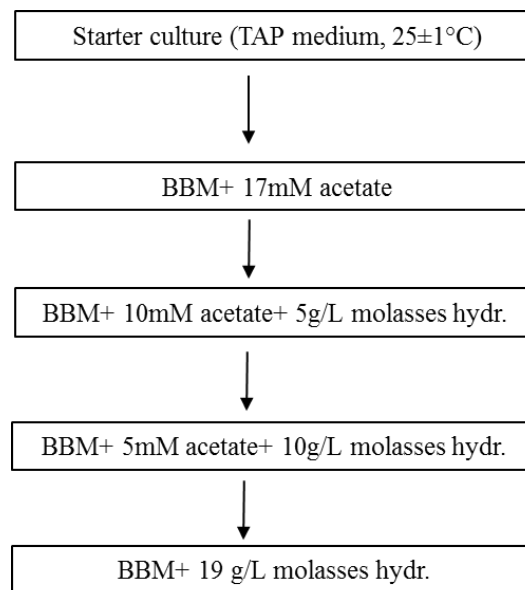
Figure 3.5. Steps applied in adaptation of *Microactinium sp.* ME05 to molasses.

3.5.4. Growth Evaluation of *Microactinium sp.*ME05 in Vinasse in 500 mL Flasks and in 2L Flasks

Vinasse was evaluated in terms of supporting the growth of *Microactinium sp.ME05* cells. For this purpose Bold Basal Medium was supplemented with different ratios of vinasse.

Microactinium sp. ME05 strain, which was normally maintained on slant agars of tap medium was inoculated into 100ml of liquid TAP medium. Cells were incubated at

25± 1 °C with shaking at 150 rpm for 4 days. *Micractinium sp.* ME05 in exponential phase were used as seed cells for following cultures.

In 500mL flasks, in triplicate, 250 mL of Bold Basal medium containing 2%, 5%, 10% and 20% (v/v) vinasse were prepared. Before using vinasse it was clarified as described in Section 2.2.6. Medium was sterilized in autoclave at 121°C for 15 min. Filter sterilized ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) were added to the sterile medium before inoculation (H. Santana et al., 2017).

Microalgal cells in exponential period were inoculated into these media at a ratio of 10% (v/v). Cultivation was carried out in a controlled growth chamber for 7 days at 25±1°C, under heterotrophic and under mixotrophic conditions, separately. Aeration was provided by an aquarium pump (0.5L/min air).

On a daily basis, 1 ml samples were withdrawn from each flask and to monitor cell growth optical density measurement was performed at 680nm. After 7 days of incubation cells were collected with centrifugation at 3000×g for 10 min at 4°C. The pellets were washed with water and incubated in a 70 °C oven for 24 hours and then the biomass was measured gravimetrically.

Following obtaining highest biomass concentration in bold basal medium that was cultivated under 16h light:8h dark conditions, %2-%5-%10 and %20 vinasse concentration were evaluated in 2-L flasks with 1-L working volume under 16h light:8h dark photoperiod.

*Micractinium sp.*ME05 cells in exponential growth phase were inoculated into bold basal medium that was supplemented with %2, %5, %10 and %20 (v/v) vinasse, separately. Cultivation was carried out in a controlled growth chamber for 7 days at 25±1°C, under 16h light:8h dark conditions with 0.5L/min air provided by aquarium pump. Daily measurements of optical density were conducted at 680nm using a spectrophotometer. At the end of the incubation period, 5 mL of cells were used for dry weight calculation (described in Section 2.2.4) and the remaining cell suspension were used for lipid content determination with Bligh-Dyer method as described in Section. 2.2.8. After lipid content determination, fatty acid methyl ester profile of microalgal oils were determined via Gas Chromatography (GC) (Agilent HP GC 6890) by Sargem /KONYA.

3.6. Bioreactor Studies with *Micractinium sp.*ME05

Optimum growth conditions of *Micractinium sp*.ME05 by using molasses were evaluated in 2L bioreactors with different agitation rates and different inoculum ratios. On the other hand, the conditions that gave the highest biomass concentration from *Micractinium sp*.ME05 cells with vinasse were evaluated in 5-L bioreactors.

3.6.1. Growth Evaluation of *Micractinium sp*.ME05 in 2L Bioreactors with Molasses

Optimum growth conditions that gave the highest biomass concentration with molasses as carbon source (pH:6.9, temperature:30.7±1°C, molasses hydrolysate:19 g/L) were evaluated in 2L stirred tank bioreactors due to lab facilities. 2L bioreactor experiments were conducted in Bioprocess Laboratory in Food Engineering Department, METU. For that purpose, batch cultivations of *Micractinium sp*.ME05 were performed in 2L stirred tank bioreactor (STR) (Biostat® A-plus, Sartorius, Germany) with 1 L working volume. Figure 3.6. shows the diagram of the bioreactor.
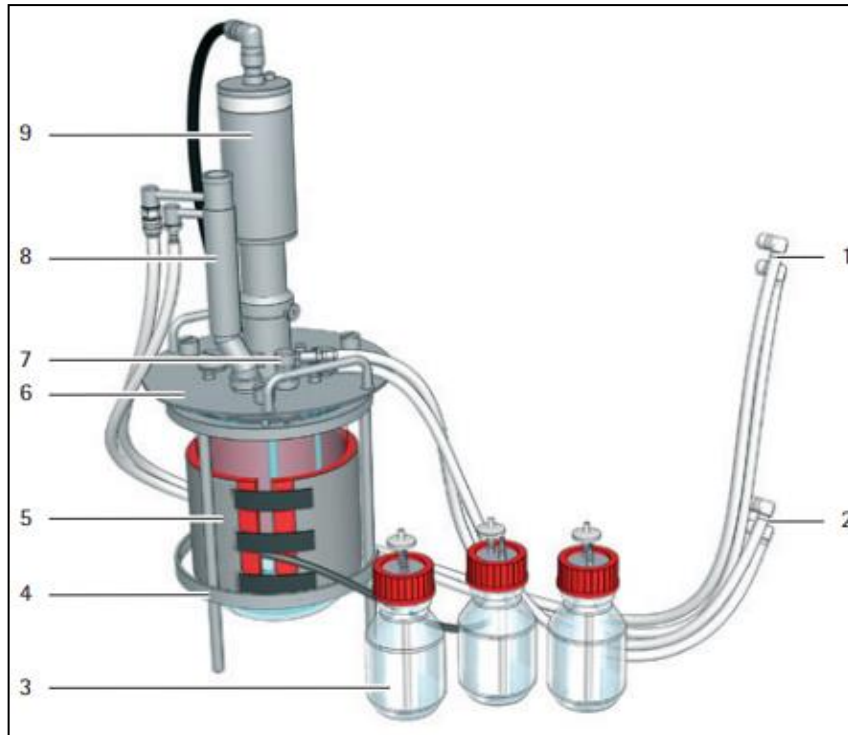
Figure 3.6. Biostat A-Plus Bioreactor. 1) Cooling finger tubing on chiller, 2) Exhaust cooler tubing on chiller, 3) Supply bottles, 4) Support Column and culture vessel, 5) Heating blanket on culture vessel, 6) Cover plate with ports, 7) Cooling finger, 8) Exhaust cooler, 9) Stirrer driver (Adapted from Biostat® A operating manual).

Bioreactor was filled with 1-L of bold basal medium (Appendix A) that was supplemented with 19 g/L molasses hydrolysate. Following probes were set up on the top of the bioreactor: a pH sensor (Hamilton Easyferm K8 200, Bonaduz, Switzerland), a dissolved oxygen sensor (Hamilton Oxyferm) and a temperature sensor. Bioreactor was sterilized in situ at 121 °C for 15 min. Yeast extract (2 g/L) was sterilized separately and added to the medium under aseptic conditions.

After sterilization completed and vessel had cooled, *Micractinium sp. ME05* cells in exponential period were inoculated into the bioreactor with inoculation ratios of 5% and 10 % (v/v), separately. Agitation speed were set at 50, 75, 100 and 200 rpm separately. Microalgal cultivation was carried out for 5 days at 30.7±1 °C. Aeration rate was set initially at 0.5 L/min. A constant pH of 6.9 was maintained in the bioreactor with 1M KOH solution and 1M H_2SO_4 solution dosing. No light was provided. Experiments were conducted with two biological replicates.

On a daily basis 1 mL of the culture was withdrawn from the bioreactor from the sampling port and optical density was measured. During incubation period pH, dissolved oxygen and temperature were monitored.

When microalgal culture reached stationary phase, cells were harvested by centrifugation. 5 mL of microalgal suspension were dried in an oven for dry weight calculation. Remaining microalgal suspension were lyophilized. The lyophilized microalgal biomass samples were stored at -80 °C for lipid content determination.

3.6.2. Growth Evaluation of *Micractinium Sp*.ME05 in 5L Bioreactors with Vinasse

Following obtaining highest biomass concentration with 10% vinasse under 16h light-8h dark photoperiod in 2-L flasks with 1-L working volume, the same experimental conditions were evaluated in 5-L bioreactors (BioFlo-New Brunswick Scientific). 5-L bioreactor experiments were conducted in METU Central Laboratory Molecular Biology and Biotechnology R&D Center.

For that purpose, bioreactor vessel was filled with 4L of bold basal medium and following probes were set up on the top of the bioreactor: a pH sensor (Mettler Toledo) a dissolved oxygen sensor (Mettler Toledo) and a temperature sensor. Bioreactor was sterilized in situ at 121 °C for 15 min.

After the vessel cooled down, filter sterilized ampicillin (100 µg/mL) and chloramphenicol (25 µg/mL) were added to the sterile medium before inoculation (H. Santana et al., 2017). *Micractinium sp*.ME05 cells in exponential growth phase were inoculated (10%v/v) to the bioreactor.

Daily measurements of optical densities were performed during incubation period. Cultivation was carried at 25±1°C, under 16h light:8h dark conditions. Agitation was set at 150 rpm and 0.5L/min air was provided.

3.7. Statistical Analysis

The statistical analyses were performed using statistical software MINITAB 16.0 to test the significance of different glucose concentrations, yeast extract concentrations, pH, temperature and inoculum ratio on biomass production. All statistical analysis was performed at a confidence level of 95% and P values equal and smaller than 0.05 were considered statistically significant.

3.8. Cost Evaluation of Different Medium Compositions

Cost of using bold basal medium that was supplemented with glucose, molasses hydrolysate, and vinasse were calculated according to the current prices of the medium components.

3.9. Content of The Study

The whole strategy applied to *Micractinium sp.* ME05 cells in this study are summarized in Figure 3.7.
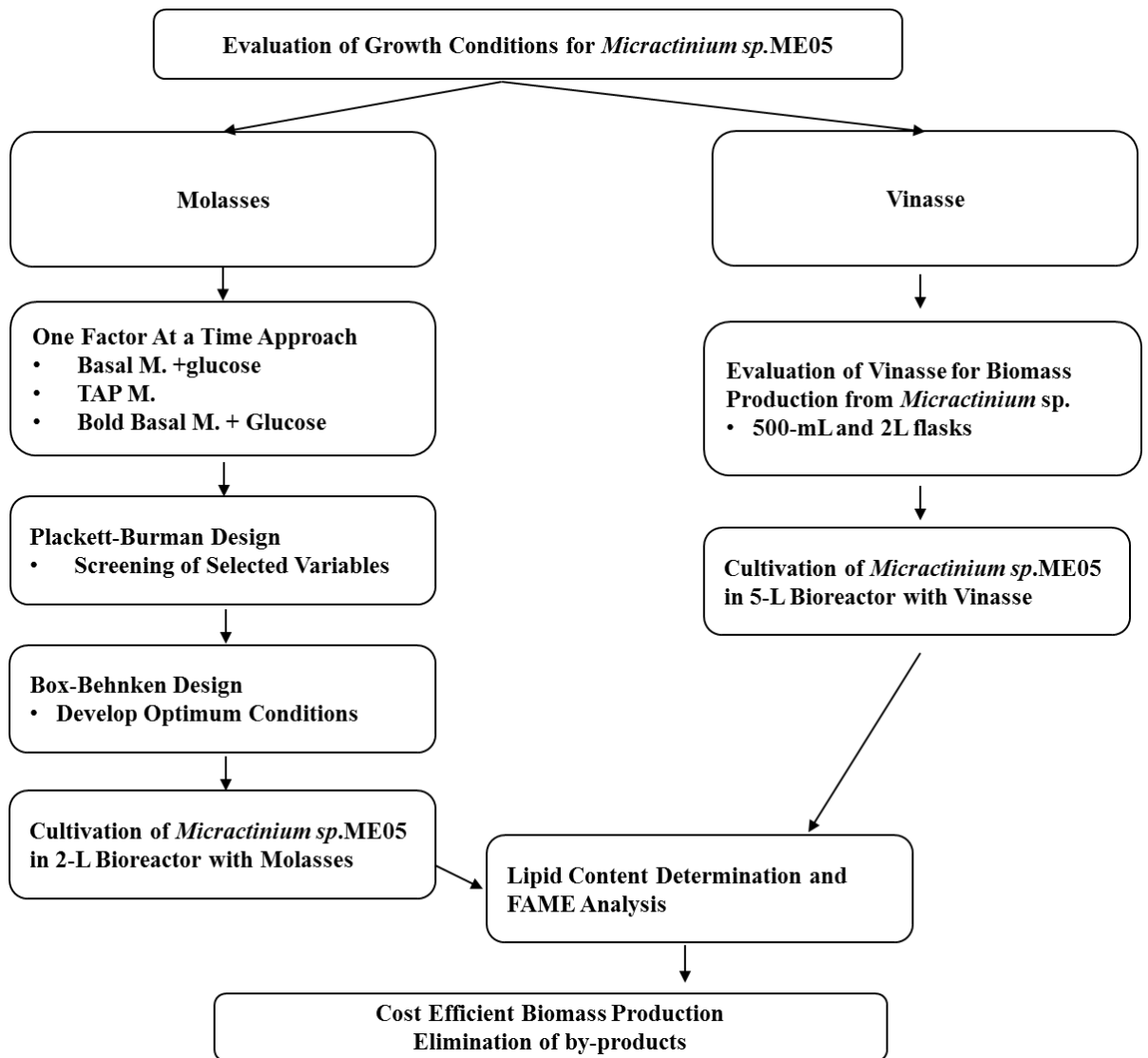
Figure 3.7. The strategy applied to *Microactinium sp.* ME05 cells in this study.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Evaluation of Growth Conditions for *Chlamydomonas reinhardtii* 137C⁻

Growth curves of *C. reinhardtii* 137C⁻ were evaluated in TAP medium at 25°C under 16-hour light-8-hour dark photoperiod with a light intensity of 54 μmol/m²/sec (17.65 W/m²) and under complete dark conditions (Figure 4.1). Light intensity was measured with a luminometer. *C. reinhardtii* 137C⁻ showed a better growth under 16h light-8 h dark conditions and reached stationary phase after 3 days. On the other hand, under dark conditions the cells showed a lower growth during the 7 days on incubation.
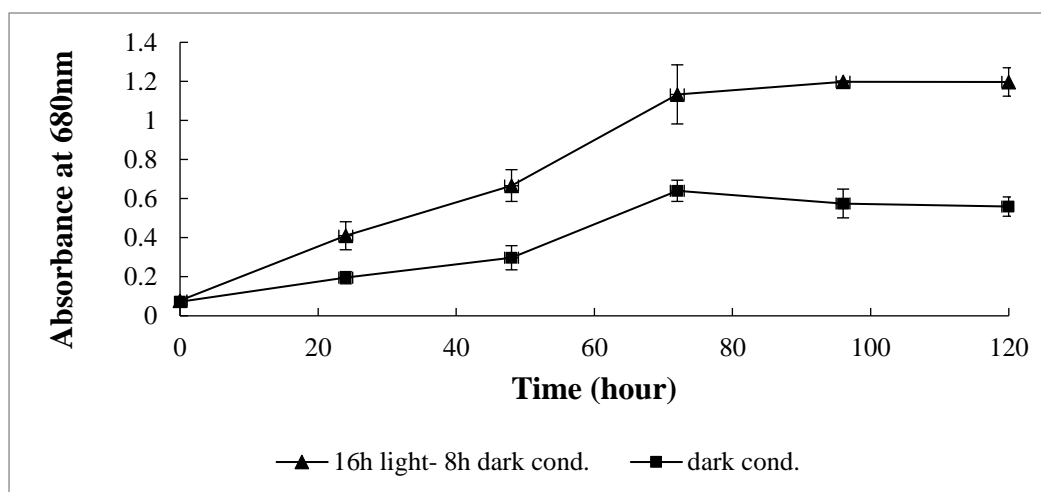
Figure 4.1. Growth curves of *C. reinhardtii* 137C⁻ under 16 h light-8h dark photoperiod and dark period at 25°C. Data are mean ± standard error from three replicates.

4.1.1. Growth Evaluation of *C. reinhardtii* 137C⁻ with TAP medium + Different Glucose Concentrations

In order to see the growth pattern of *C. reinhardtii* 137C⁻ with glucose, TAP medium with different glucose concentration were prepared. Cells were incubated at 25 ±1°C under 16h light-8h dark photoperiod.

It was observed that *C. reinhardtii* 137C⁻ cells showed the highest growth within TAP medium on the other hand it had lower growth with TAP medium that contain different glucose concentrations (Figure 4.2).
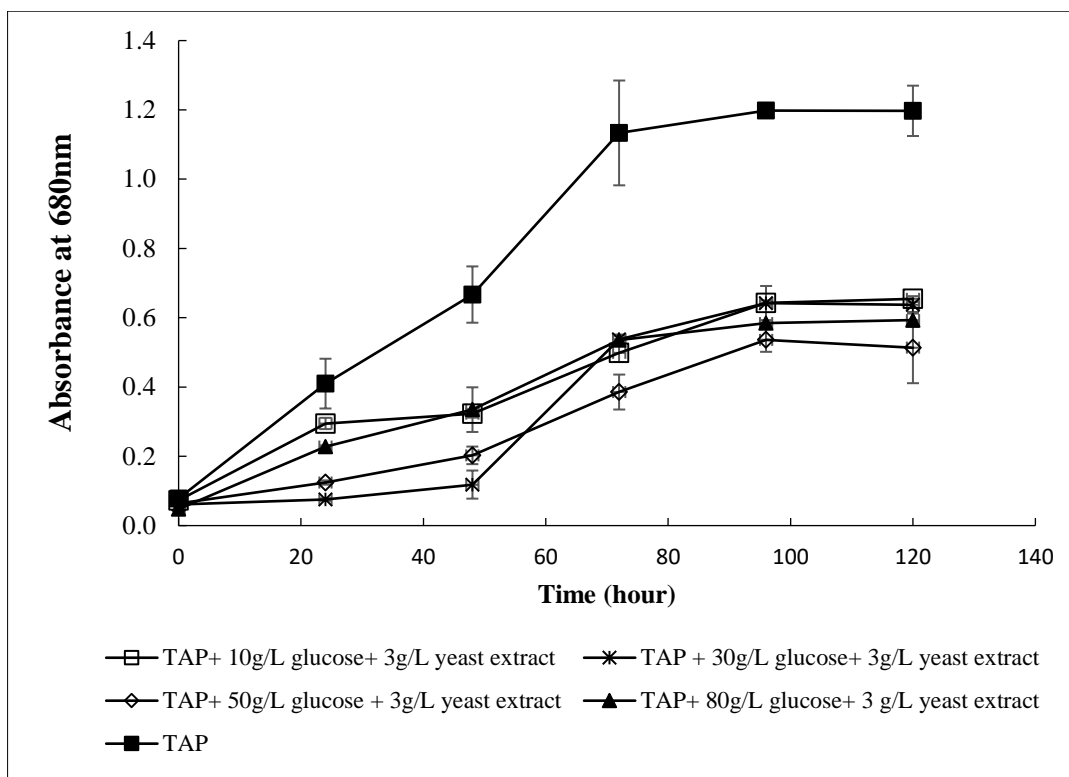
Figure 4.2. Absorbance versus time curve of *C. reinhardtii* 137C ⁻ within TAP medium with different glucose concentrations under 16h light-8 h dark photoperiod. Data are mean ± standard error from three replicates.

In order to see the growth pattern of *C. reinhardtii 137C⁻* with glucose under dark conditions, TAP medium with different glucose concentration were prepared. Cells were incubated at 25 ±1°C under dark conditions. As seen in Figure 4.3. *C. reinhardtii*

137C⁻ cells showed the highest growth within TAP medium and reached stationary phase at 3 days. On the other hand, cells did not show a remarkable growth in TAP medium with glucose under dark conditions at 5 days.
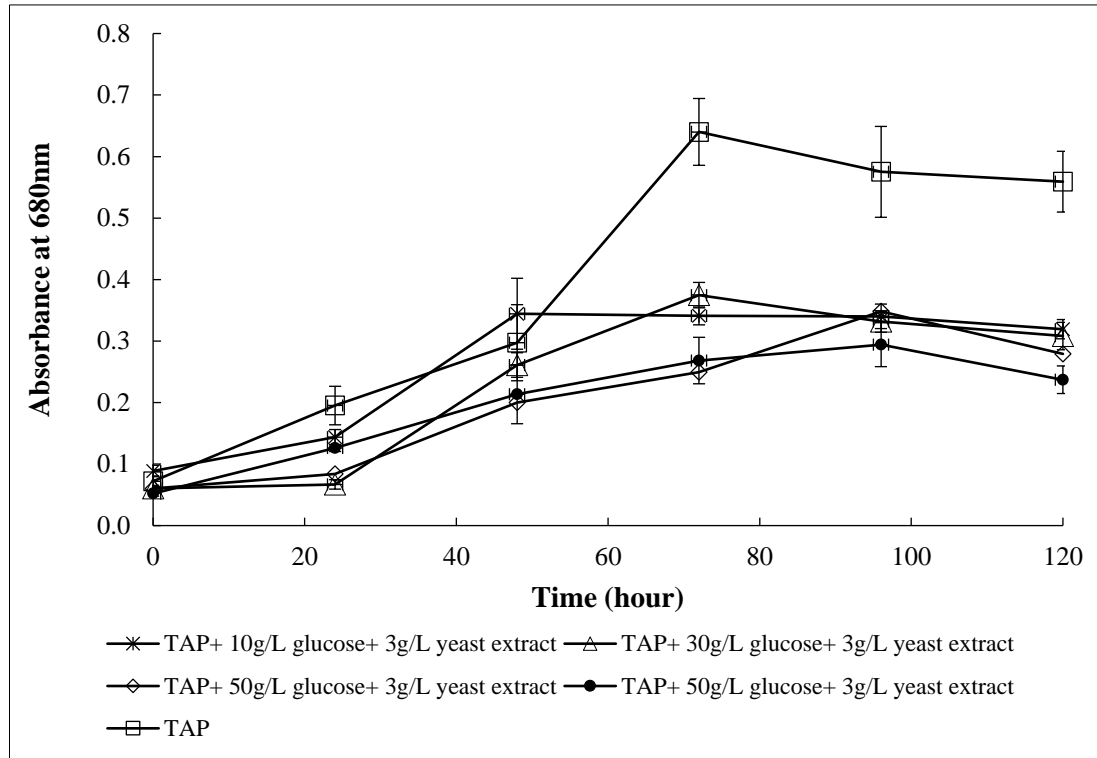
Figure 4.3. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within TAP medium with different glucose concentrations under dark conditions. Data are mean ± standard error from three replicates.

4.1.2. Growth Evaluation of *C. reinhardtii* 137C⁻ with Basal Medium + Different Glucose Concentrations

Basal medium with different glucose concentrations (10 g/L-30 g/L-50 g/L and 80 g/L) were prepared in order to see the growth pattern of *C. reinhardtii 137C⁻*. Microalgal cells that came to exponential phase were inoculated into these media under aseptic conditions. Incubation was carried out at 25±1 °C under 16 h light-8h dark photoperiod and under dark conditions separately. Absorbance versus time

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curves of *C. reinhardtii* 137C⁻ under 16h light-8h dark photoperiod and under dark conditions is given in Figure 4.4 and Figure 4.5, respectively.

According to absorbance versus time curves it was observed that *C. reinhardtii* 137C⁻ cells could not reach an optical density value higher than 0.25, which depicted that microalgal cells could not grow well in basal medium supplemented with glucose for all of the glucose concentrations under 16h light-8h dark and dark conditions separately (Figure 4.4 and Figure 4.5).
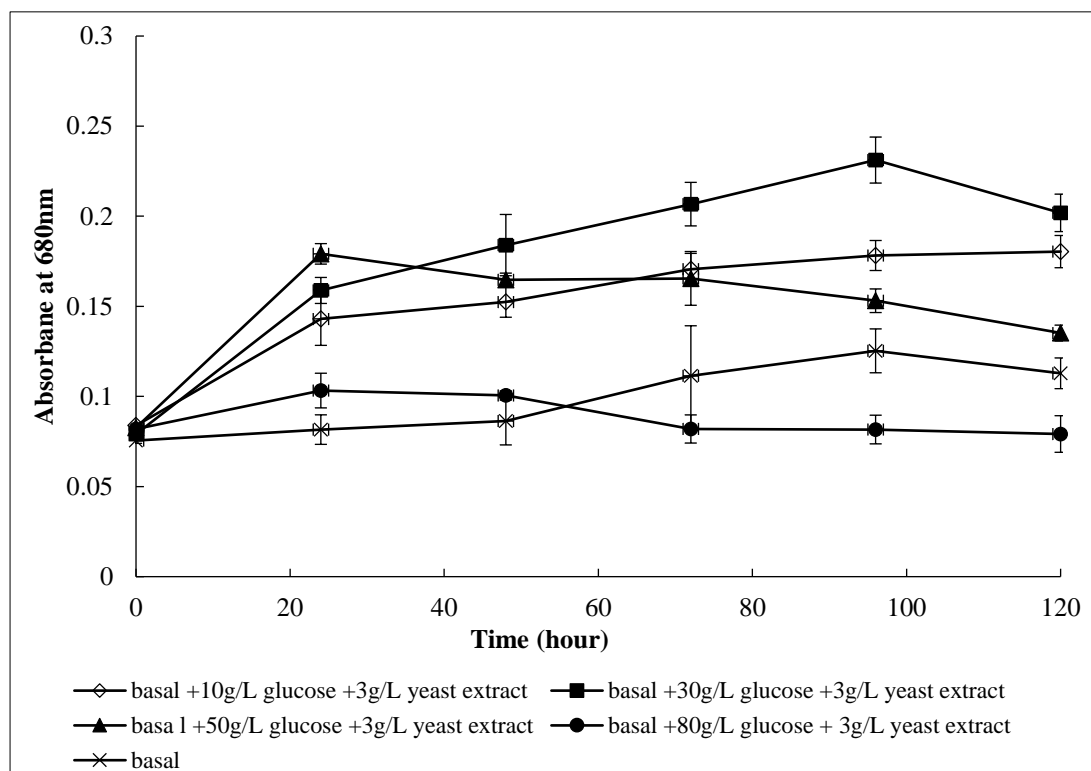
Figure 4.4. Absorbance versus time curve of *C. reinhardtii* 137C ⁻ within Basal medium with different glucose concentrations under 16h light-8 h dark photoperiod. Data are mean ± standard error from three replicates.
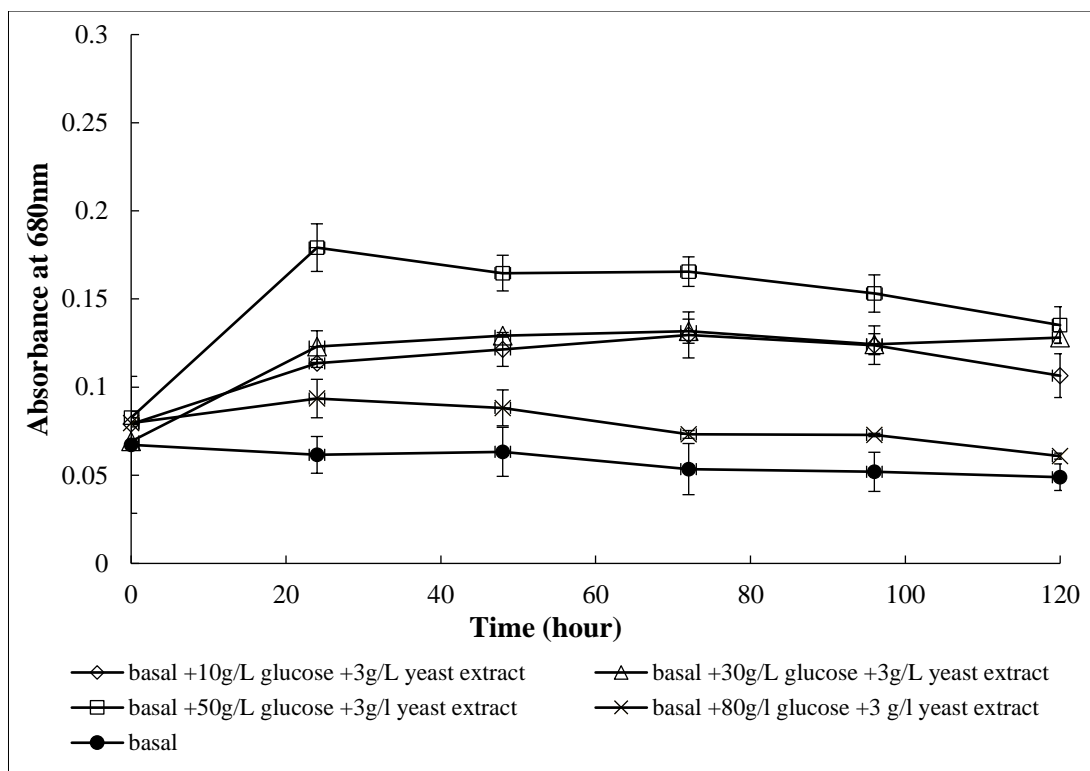
Figure 4.5. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within Basal medium with different glucose concentrations under dark conditions. Data are mean ± standard error from three replicates.

4.1.3. Growth Evaluation of *C. reinhardtii* 137C⁻ With Basal Medium + Different Sucrose Concentrations

Basal medium with different sucrose concentrations (10 g/L-30 g/L-50 g/L and 80 g/L) were prepared in order to see the growth pattern of *C. reinhardtii 137C⁻*. Incubation was carried out at 25±1 °C under 16 h light-8h dark photoperiod and under dark conditions separately.

According to absorbance versus time curve of *C. reinhardtii 137C⁻* within TAP medium supplemented with different sucrose concentrations, it was observed that *C. reinhardtii* 137C⁻ cells did not show a remarkable growth with sucrose either under 16h light-8 h dark photoperiod and under dark conditions, as given in Figure 4.6 and Figure 4.7., respectively.
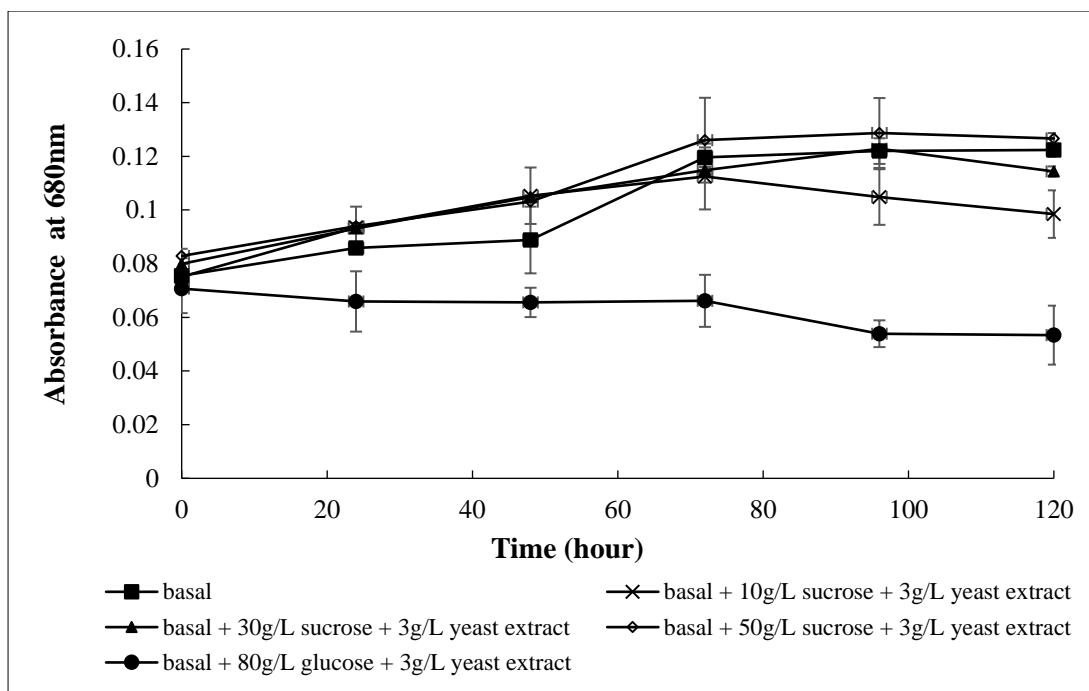
Figure 4.6. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within Basal medium with different sucrose concentrations under 16h light-8h dark photoperiod. Data are mean ± standard error from three replicates.
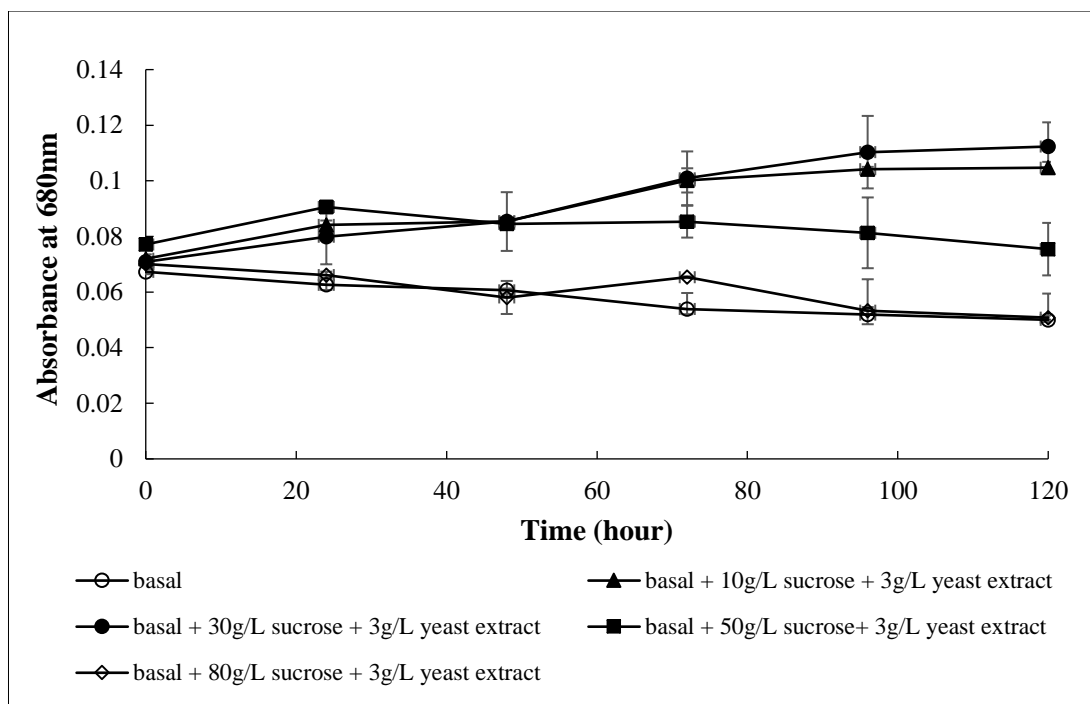
Figure 4.7. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within Basal medium with different sucrose concentrations under dark conditions. Data are mean ± standard error from three replicates.

4.1.4. Growth Evaluation of *C. reinhardtii* 137C⁻ with Basal medium + different Molasses Hydrolysate Concentrations

According to the absorbance versus time curves of *C. reinhardtii 137C⁻* within basal medium supplemented with different molasses hydrolysate concentrations, as given in Figure 4.8 and Figure 4.9, cells did not show a remarkable growth with molasses hydrolysate under 16h light-8h dark photoperiod and under dark conditions.
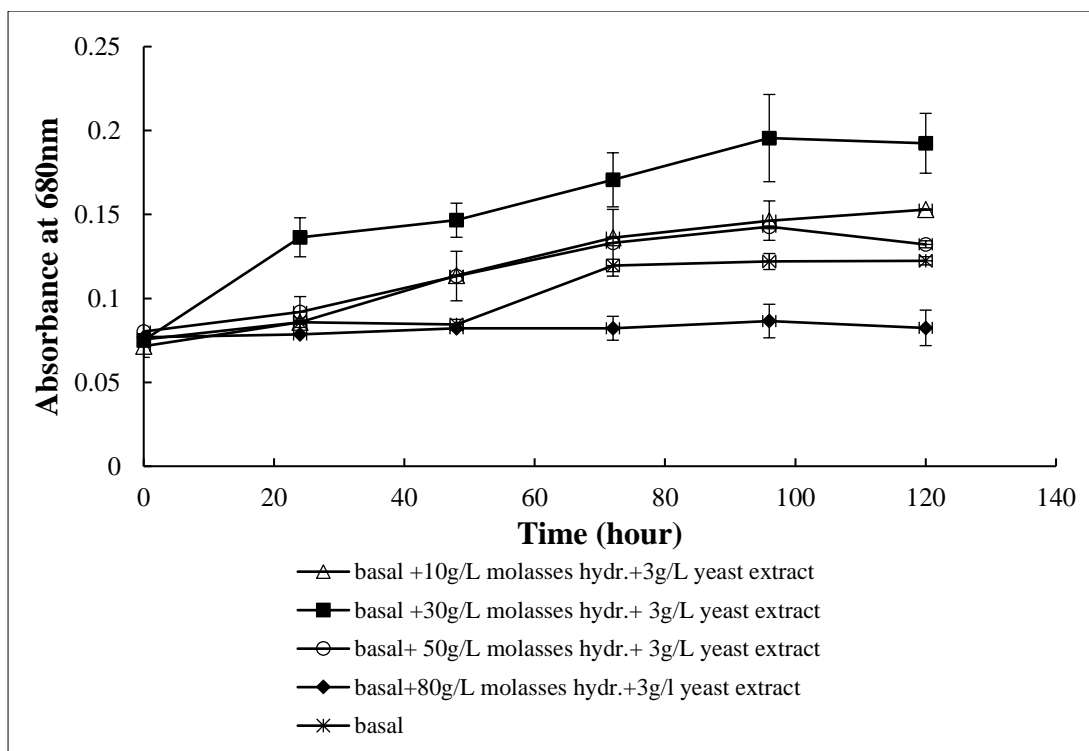
Figure 4.8. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within Basal medium with different molasses hydrolysate concentrations under 16h light-8h dark photoperiod. Data are mean ± standard error from three replicates.
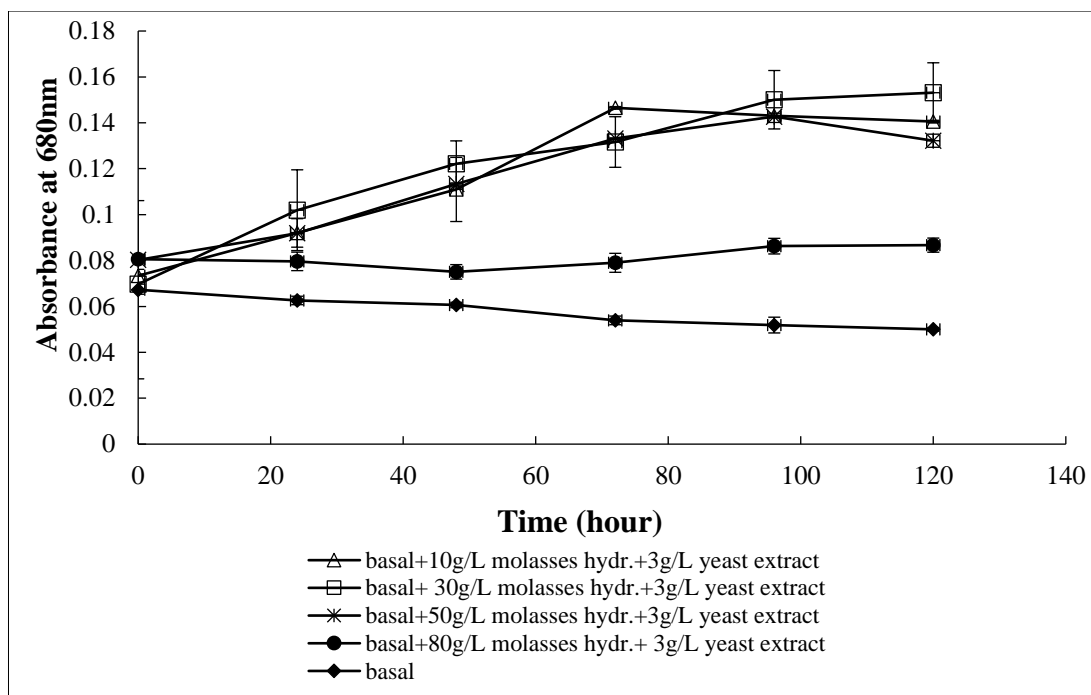
Figure 4.9. Absorbance versus time curve of *C. reinhardtii* 137C⁻ within Basal medium with different molasses hydrolysate concentrations under dark conditions. Data are mean ± standard error from three replicates.

Growth evaluation experiments within TAP medium supplemented with different glucose concentrations and basal medium supplemented with different glucose, sucrose, molasses hydrolysate concentrations demonstrated that, *C. reinhardtii137C⁻* grew better within TAP medium by using acetate in it, rather than other carbon sources. This may result from *C. reinhardtii* being a facultative 'acetate flagellate' that can only grow on acetate (Heifetz, Förster, Osmond, Giles, & Boynton, 2000). On the other hand, since TAP medium contain acetate as a carbon source, within TAP medium cells showed a high growth and reached stationary phase at 3 days, under 16h light-8h dark conditions and under dark conditions (Figure 4.2 and Figure 4.3).

Since *C. reinhardtii* cells could not be able to metabolize glucose, sucrose, and molasses hydrolysate as carbon source, further optimization studies were performed with *Micractinium sp*.ME05.

4.2. Evaluation of Growth Conditions for *Micractinium sp.* ME05, at 25°C, 30°C and 37°C

Growth performance of *Micractinium sp.*ME05 were assessed in TAP medium at 25°C, 30°C and 37°C under 16h light-8h dark photoperiod.
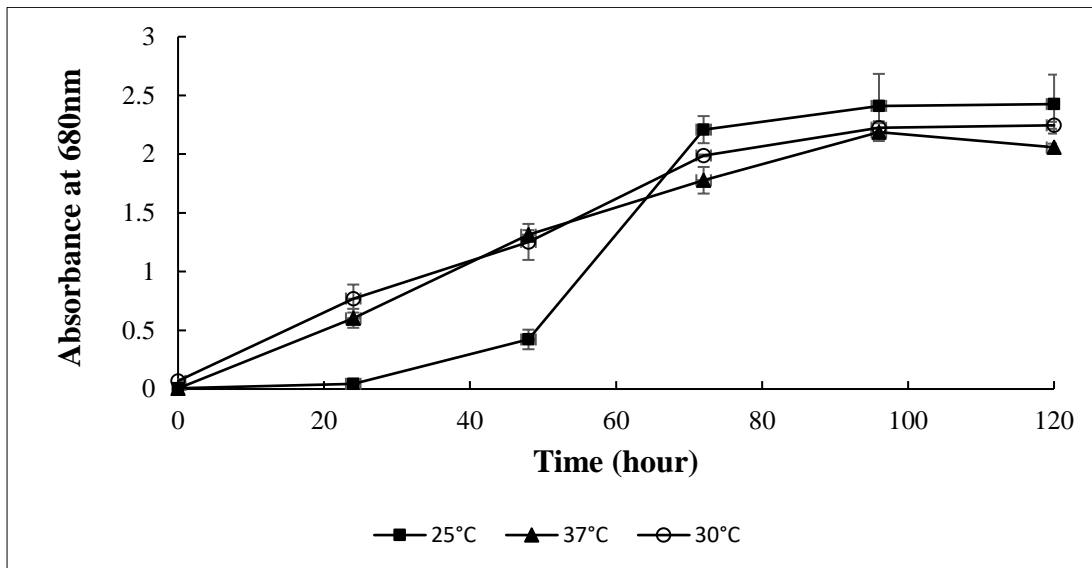
Figure 4.10. Growth curves of *Micractinium sp.* ME05 under 16h light-8h dark photoperiod at 25°C, 30°C and 37C°. Data are mean ± standard error from three replicates.

In a previous study of Onay et al. (2014), *Micractinium sp. ME05* was shown to grow in a temperature range of 25-50°C (Onay et al., 2014). It is clear from Figure 4.10 that *Micractinium sp.*ME05 cells were able to grow well at 25°C, 30°C and 37°C. It was observed that the cells reached stationary phase with 3 days for three of the incubation temperatures. These results agreed well with the study of Sonmez et al. (2016), who observed that *Micractinium sp.ME05* could grow at 16°C, 25°C and 30°C and reached stationary phase in 3.5 days at 25 and 30°C (Sonmez, Elcin, Akın, Avni, & Yucel, 2016).

4.2.1. Experimental Design

Experimental design studies were performed with *Micractinium sp.*ME05 cells. For a quick review of biomass production, one factor at a time approach was employed. To obtain optimum conditions that would give highest biomass concentration the selected parameters were initially screened using Plackett-Burman Design and consequently Box-Behnken Design was applied to determine the optimal conditions.

4.2.1.1. Growth Evaluation of *Micractinium sp*. ME05 Within Basal Medium and TAP Medium

One factor experiments were conducted with randomly selected variables. Firstly, basal medium supplemented with 30 g/L glucose and 2 g/L yeast extract were evaluated for biomass production of *Micractinium sp.*ME05. During these experiments 0.5 L/min air were provided to the flasks through an aquarium pump (Figure 4.11).

Figure 4.11. *Micractinium sp.* ME05 cells in TAP medium (dark green) and in basal medium supplemented with 30 g/L glucose and 2 g/L yeast extract (light green).

Biomass concentrations obtained after 120 hours of incubation period are given in Table 4.1. When basal medium that contain, 30 g/L glucose were inoculated with 0.2 % (v/v) microalgal cells, the biomass obtained was 1.33±0.02 g/L. On the other hand, with 5% (v/v) inoculum, the biomass increased to 2.63±0.10 g/L. When TAP medium were inoculated with 0.2 % (v/v) cells, the biomass obtained was 1.01±0.06 g/L. With 5%(v/v) inoculum the biomass increased to 1.23±0.003 g/L. From these data, as also given in Table 3.1., it was clear that the highest biomass concentration (2.63±0.10 g/L) and thus highest biomass productivity (0.52±0.02 g.L^{-1}day^{-1}) was obtained with basal medium that was inoculated with 5% (v/v) of microalgal cells.

In terms of growth rates, within TAP medium increasing inoculum ratio from 0.2% (v/v) to 5% (v/v) caused a decline in growth rate from $1.38\pm0.01d^{-1}$ to $1.34\pm0.06d^{-1}$. Similarly, in basal medium, increasing inoculum ratio from 0.2% to 5% caused a decline in growth rate from 1.44 ± 0.003 to 1.38 ± 0.06. A decline in growth rate with an increase in inoculum ratio, may result from limiting key nutrients especially carbon sources with increasing cell concentration. Similar results were reported by Zhang et al. (2003), who studied the effects on inoculum size and inoculum age on biomass and taxol production from *Taxus yunnannensis* cells. In their study, it was observed that with an increase in inoculum size, biomass productivity increased while growth rate decreased.

Table 4.1. Biomass concentration, biomass productivity and specific growth rates of *Microctinium sp.ME05* within different media with different inoculum ratios. Data are mean ± standard error from three replicates.

	0.2 % (v/v) inoculum		5% (v/v) inoculum	
	Basal*	**TAP**	**Basal***	**TAP**
Biomass concentration (g/L)	1.33±0.02	1.01±0.06	2.63±0.10	1.23±0.003
Biomass productivity (g.L^{-1}.day^{-1})	0.27±0.05	0.20±0.01	0.52±0.02	0.24±0.03
Growth rate (μ) (d^{-1})	1.44±0.003	1.38±0.01	1.40±0.09	1.34±0.06

*: Basal medium that contain 30g/L glucose +2g/L yeast extract.

Growth curves of *Microctinium sp.ME05* in basal medium containing 30g/L glucose and in TAP medium are given in Figure 4.12.and Figure 4.13, respectively. In basal medium, with 5% (v/v) inoculum, microalgal cells showed high growth and reached stationary phase at 4 days. With 0.2 % (v/v) inoculum, cells showed slower growth than 5% (v/v) inoculum and reached stationary phase at 3 days. In TAP medium,

similar to basal medium, cells showed high growth with 5 % (v/v) inoculum and reached stationary phase within 4 days. So, within basal medium and TAP medium it was clear that the lag phase of the cells was shorter with higher inoculum ratios.

Similar results were obtained in the study of Ma et al. (2013) in which, a proteomic analysis was conducted to figure out the effect of inoculum concentration on *Chlorella sorokiniana* cellular mechanism. BG11 medium was inoculated with *C. sorokiniana* cells at ratios of 1×10^4, 1×10^5, 1×10^6, 1×10^7 and an increase in biomass productivity and shortened lag phase was observed with an increase in inoculum size.
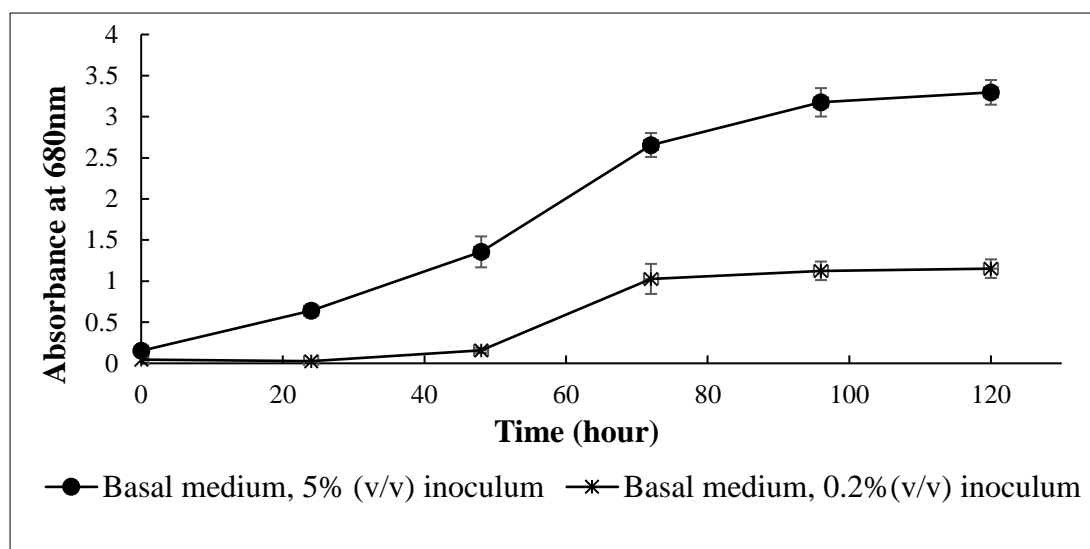
Figure 4.12. Growth curves of *Micractinium sp.* ME05 in basal medium with different inoculum ratios. Data are mean ± standard error from three replicates.
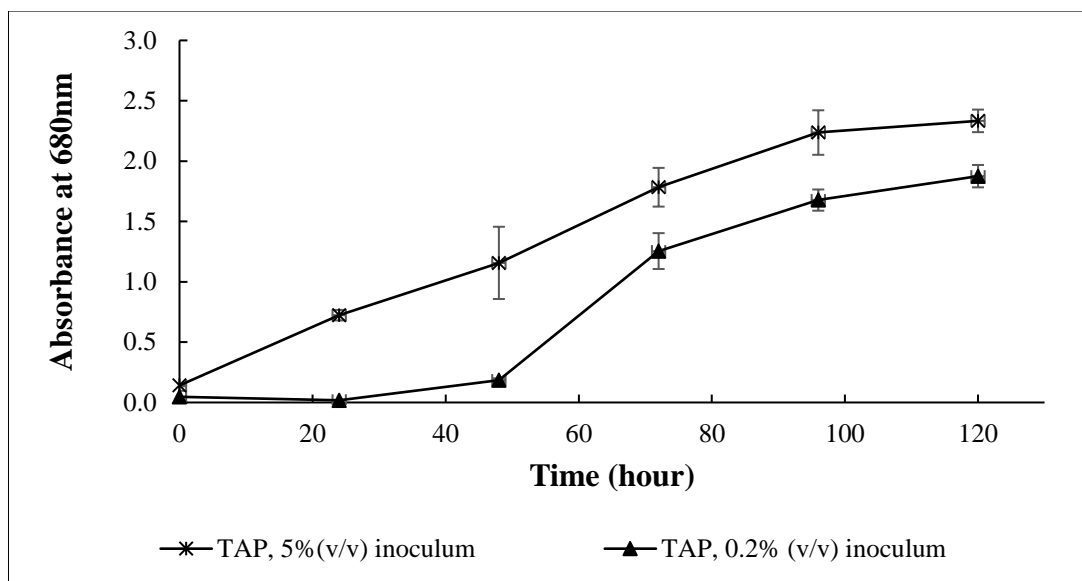
Figure 4.13. Growth curves of *Micractinium sp.* ME05 in TAP medium with different inoculum ratios. Data are mean ± standard error from three replicates.

4.2.1.2. Growth of Evaluation *Micractinium sp.* ME05 in Bold Basal Medium with Different Glucose Concentrations

Growth and biomass production of *Micractinium sp.* ME05 were evaluated in bold basal medium that contains 30g/L glucose as a carbon source. Cultivation was carried out either on a constant shaker with 200 rpm agitation or with aeration (0.5 L/min) provided by aquarium pump.

Obtained biomass concentrations and biomass productivities are given in Table 4.2. At the end of the incubation period it was observed that the biomass concentration and biomass productivity obtained from the cells that were aerated through an aquarium pump was 2.23 ± 0.42 g/L and 0.44 ± 0.08 g.L^{-1}.day^{-1}, respectively. On the other hand, the biomass concentration and biomass productivity obtained from the cells that were cultivated on a shaker were 1.43 ± 0.21 g/L and 0.28 ± 0.01 g.L^{-1}.day^{-1}, respectively.

These results demonstrated that microalgal cells produced more biomass when cultivated with an aquarium pump than on a shaker. Following obtaining high biomass concentration and biomass productivity in bold basal medium by giving air through aquarium pump, another experiment was set up under the same conditions except for

glucose concentration; glucose concentration was reduced from 30 g/L to 10 g/L. At the end of the incubation period the biomass concentration and biomass productivities were 0.97 ± 0.07 g/L and 0.18 ± 0.01 g.L^{-1}.day^{-1}, respectively. These results showed that within the cultures cultivated with an aquarium pump, increasing glucose concentration from 10 g/L to 30 g/L caused an increase in biomass concentration from 0.97 ± 0.07 g/L to 2.23 ± 0.42 g/L. Similarly, an increase in biomass productivity from 0.18 ± 0.01 g.L^{-1}.day^{-1} to 0.44 ± 0.08 g.L^{-1}.day^{-1} was also observed.

These results were consistent with the studies performed by other researchers. Zheng et al. (2012) studied the effects of two stage heterotrophic and phototrophic culture method for biomass and lipid production from *C. sorokinana* cells. In their study increasing glucose concentration from 5 g/L to 20 g/L in heterotrophic cultures of *C. sorokiana* cells caused an increase in productivity from 31 ± 8 cells.mL^{-1}.d^{-1} to 182 ± 3 cells.mL^{-1}.d^{-1} (Y. Zheng, Chi, Lucker, & Chen, 2012).

Shi et al. (1999) also reported similar results. In their study, the effect of glucose concentration on biomass and lutein production by *Chlorella prothecodies* cells were investigated. The cultivation medium was basal medium and supplemented with 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 g/L glucose separately. It was reported that an increase in glucose concentration from 10 g/L up to 60g/L caused an increase in biomass productivity from 0.8 g.L^{-1}.day^{-1} to 2.5 g.L^{-1}.day^{-1}.
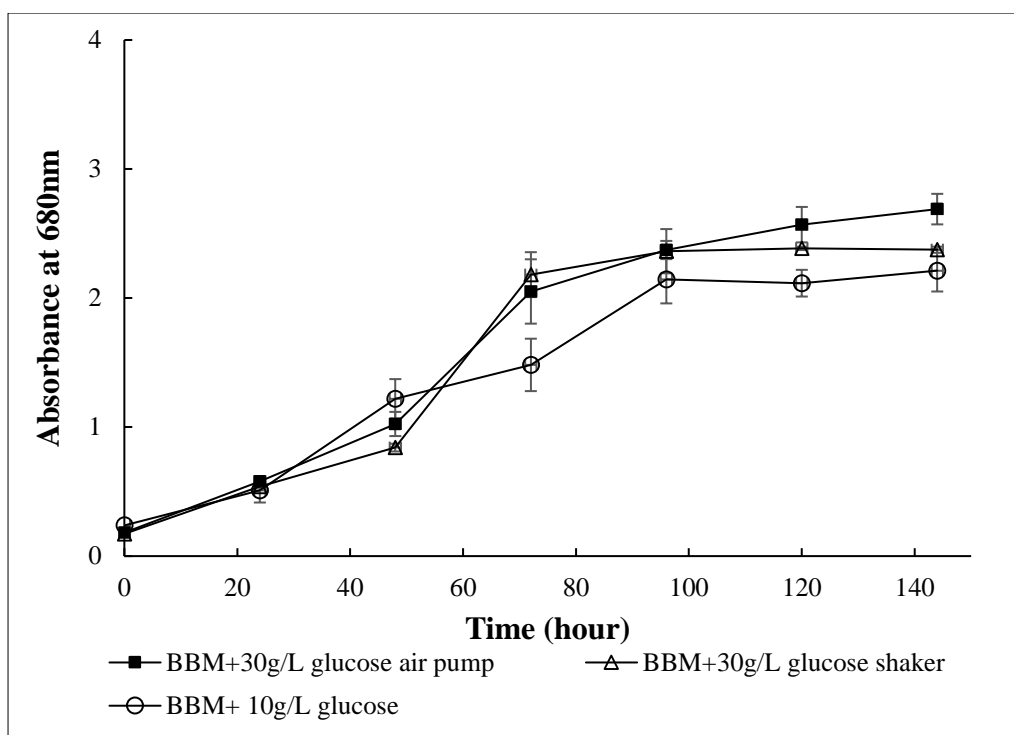
Figure 4.14. Growth curves of *Micractinium sp.* ME05 in bold basal medium (BBM) + 30g/L glucose cultivated on a shaker and with an aquarium pump, in BBM +10 g/L cultivated with an aquarium pump. Data are mean ± standard error from three replicates.

Table 4.2. Biomass concentrations and biomass productivities of *Micractinium sp. ME 05* in basal medium with different glucose concentrations. Data are mean ± standard error from three replicates.

	BBM +30g/L glucose		BBM+10g/L glucose
	Shaker	**Pump***	**Pump***
Biomass concentration (g/L)	1.43±0.21	2.23±0.42	0.97±0.07
Biomass productivity (g.L⁻¹.day⁻¹)	0.28±0.04	0.44±0.08	0.18±0.01

* 0.5 L/min air was given to the system through an aquarium pump.

Up here, one factor at a time approach was used to explore the potential of biomass generation with *Micractinium sp.* ME05 cells. The selected variables were changed randomly and the effects of variables were evaluated.

Although one factor at a time approach is a simple applicable method, it was not used in further optimization studies due to its inability to determine the interaction effects between process variables and due to being inefficient to determine optimal experimental conditions. On the other hand, response surface methodology have the advantages of combining different statistical and mathematical techniques to improve and optimize a process (Mandenius & Brundin, 2008). Therefore, Plackett-Burman method was applied in this study to screen process variables. After selecting variables that significantly affect biomass concentration, Box-Behnken Design of Response Surface Methodology (RSM) was applied to determine the interaction effect between process variables and to determine optimum conditions.

4.2.1.3. Screening of Process Variables by Plackett Burman Method

Plackett-Burman Design is as an efficient tool for medium-component optimization (Guo et al., 2009) and was used in this study to select variables that significantly affect biomass concentration of *Micractinium sp.* ME05.

Selection of appropriate cultivation conditions is important for the development of an economic and efficient process. In this regard, 5 variables were selected; pH, temperature (°C), inoculum ratio (v/v %), yeast extract concentration (g/L), and glucose concentration (g/L). The concentration of variables was selected according to literature. Actual levels of variables are given in Table 4.3. Plackett-Burman Design matrix, obtained biomass concentrations and biomass yields on glucose ($Y_{x/s}$) are given in Table 4.4.

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Table 4.3. Variables with coded and uncoded values used in Plackett-Burman Design

Variable	Symbol	Low (-1)	Medium (0)	High (+1)
pH	X_1	6	7	8
Temperature	X_2	25	30	35
Inoculum (v/v %)	X_3	5	7.5	10
Yeast Extract (g/L)	X_4	0	2	4
Glucose (g/L)	X_5	10	20	30

The first order equation expressed in coded units for biomass production is given in Equation 10 as follows:

$$\hat{Y} = 1.29 + 0.34X_1 + 0.35X_2 - 0.039X_3 - 0.18X_4 - 0.021X_5 \qquad (10)$$

Where X_1, X_2, X_3, X_4 and X_5 are coded variables for pH, temperature, inoculum ratio (v/v %), yeast extract concentration (g/L) and glucose concentration (g/L), respectively.

According to Plackett-Burman experimental results, a maximum biomass concentration of 2.38 g/L was obtained when *Micractinium sp.ME05* cells were inoculated with 5% (v/v) to the medium (pH 6.0) that contain 4 g/L yeast extract and 30 g/L glucose. Under these conditions the biomass yield of glucose was calculated as 0.09 g.g^{-1}. On the other hand the highest biomass yield on glucose ($Y_{x/s}$) was calculated as 0.22 g.g^{-1} at pH of 8, temperature of 25°C, inoculum ratio of 10 % (v/v), and glucose concentration of 10 g/L (Table 3.4.).

Similar observations were reported by Leesing et al. (2011), who evaluated the effects of different glucose concentrations and carbon sources on the growth kinetic and lipid production of Chlorella *sp.*KKU-S2, *Chlorella sp.*KKU-W7, and *Chlorella* KKU-W9.

In their study, with 20 g/L glucose; the biomass concentrations obtained were 4.82 g/L, 1.24 g/L and 2.81 g/L and biomass yields on glucose were 0.25 g cell/ g glucose, 0.14 g cell/ g glucose and 0.20 g cell/ g glucose for *Chlorella sp*.KKU-S2, *Chlorella sp*.KKU-W7 and *Chlorella* KKU-W9, respectively (Leesing & Kookkhunthod, 2011). Moreover, the highest biomass concentration obtained with 30 g/L glucose (2.38 g/L) was comparable with the study of Singhasuwan et al. (2015), who studied the effects of different carbon nitrogen ratios (C/N), agitation speed on the biomass concentration and fatty acid content of *Chlorella sp.* TSIR 8990. In their study, three different carbon to nitrogen ratios (29:1, 63:1 and 95:1 g C/g N) were evaluated with 100, 150 and 200 rpm agitation speed. Highest biomass concentration obtained was 2.71 g/L at a C/N ratio of 29:1 and with 200 rpm agitation (Singhasuwan, Choorit, Sirisansaneeyakul, Kokkaew, & Chisti, 2015).

Similar observations were also reported by Xu et al. (2006), who evaluated the heterotrophic cultivation of *Chlorella prothecodies*. Heterotrophic cultivation of *C. prothecodies* cells were conducted with 10 g/L glucose at 28±1°C with air flowing in the dark. *C. prothecoides* cells in exponential growth phase were inoculated 10% (v/v) into basal medium that contain 10 g/L glucose. After 144 h of incubation, maximum biomass obtained was 3.74 g/L (H. Xu et al., 2006). Higher quantity of biomass (3.74 g/L) than that obtained in our study (2.38 g/L) may resulted from the higher inoculum ratio (10% v/v) of microalgal cells than in our study (5% v/v).

Table 4.4. Plackett Burman experimental design and obtained biomass results

Run No.	pH	Temperature (°C)	Inoculum (v/v %)	Yeast Ext. (g/L)	Glucose (g/L)	Biomass * (g/L)	$Y_{x/s}$ ** (g g^{-1})
1	+1	-1	-1	-1	+1	1.12±0.04	0.06±0.003
2	-1	+1	+1	+1	-1	1.26±0.10	0.13±0.009
3	-1	+1	+1	-1	+1	1.25±0.05	0.05±0.002
4	-1	-1	+1	+1	+1	1.75±0.03	0.07±0.001
5	-1	+1	-1	-1	-1	1.70±0.02	0.17±0.002
6	+1	-1	+1	+1	-1	1.26±0.07	0.15±0.001
7	+1	+1	-1	+1	+1	1.68±0.04	0.06±0.001
8	-1	-1	-1	-1	-1	1.08±0.04	0.15±0.004
9	-1	-1	-1	+1	+1	2.38±0.04	0.09±0.002
10	+1	-1	+1	-1	-1	1.66±0.14	0.22±0.026
11	+1	+1	+1	-1	+1	1.93±0.05	0.07±0.002
12	+1	+1	-1	+1	-1	2.06±0.05	0.21±0.005

* Results are mean± standard error from three biological replicates.

** Biomass yield on glucose (g.g^{-1}).

Pareto chart of variables is given in Figure 3.15. The standardized pareto chart includes a bar for each variable and these variables are aligned from the most significant to the less significant. The variable bars that passes to the right of the vertical line states that the variable is statistically significant ($P<0.05$) (Barrocal, García-Cubero, González-Benito, & Coca, 2010). According to Figure 4.15. pH, temperature, yeast extract concentration, and glucose concentration had significant effect ($P<0.05$) on biomass concentration.
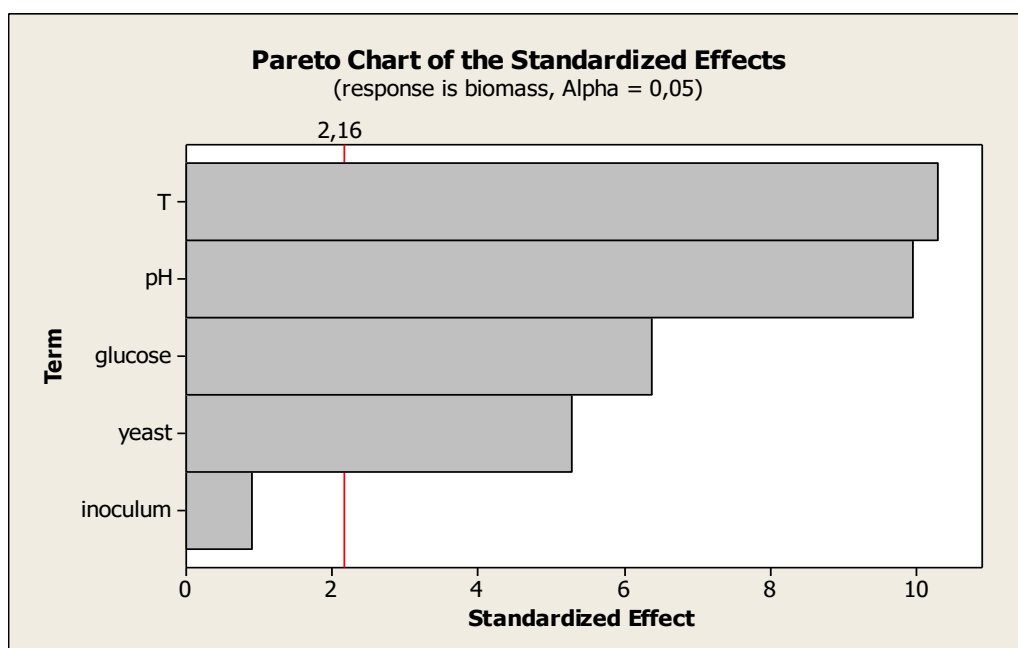
Figure 4.15. Pareto chart of standardized effects for the Plackett-Burman Design.

Further statistical analysis as given in Table 4.5., revealed that pH and temperature displayed positive effects on biomass production; whereas inoculum ratio, yeast extract concentration, and glucose concentration displayed negative effects on biomass concentration.

Moreover, the test model was statistically significant at the 95% level of significance. The coefficient of determination (R^2) was 0.9443, which demonstrated that the model could explain 94.43% of the variability in the response (biomass concentration). Adjusted coefficient of determination was R^2 (Adj): 0.8751, which indicated high significance of the model. Moreover, pH, temperature, yeast extract concentration, and glucose concentration significantly ($P<0.05$) affected biomass production. On the other hand, inoculum ratio was found to have no significant ($P>0.05$) effect on biomass production.

These results were in consistent with the findings of Li et al. (2011). In their study; carbon, nitrogen, and phosphorus concentrations in the cultivation medium of *Chlorella minutissima* UTEX2341 were optimized via Box-Behnken Design. According to Design-Expert 8.0.4. maximum biomass productivity of 1.78 g.L^{-1}·day^{-1}

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was obtained with 26.37 g/L carbon, 2.61 g/L nitrogen, and 0.03 g/L phosphorus. On the other hand, carbon and nitrogen concentration were reported to significantly affect ($P<0.05$) biomass concentration, and phosphorus concentration had no significant ($P>0.05$) effect on biomass concentration (ZhaoSheng Li et al., 2011). Isleten et al. (2012) applied Box Behnken and Central Composite Design of Response Surface Methodology to evaluate the effects of glucose, glycerol and peptone concentration on cell dry weight of *Chlorella saccharophila.* It was observed that glucose and peptone concentrations had significant effect ($P<0.05$) on biomass concentration.

Table 4.5. Regression analysis of Plackett-Burman design for biomass concentration.

Term	Effect	Coefficient	SE coefficient	t-value	P value*
Constant		1.2883	0.02894	44.51	0.000
pH	0.6883	0.3442	0.02736	12.58	0.000
T°C	0.7068	0.3534	0.02894	12.21	0.000
inoculum (v/v %)	-0.0769	-0.0385	0.02194	-1.75	0.103
yeast extract (g/L)	-0.3650	-0.1825	0.02736	-6.67	0.000
glucose (g/L)	-0.4218	-0.2109	0.02894	-7.29	0.000

* Result is significant when $P<0.05$. $R^2 = 0.9442$, R^2 (adj) = 0.8751% and R^2(pred) = 0.9227.

4.2.1.4. Box-Behnken Design

Box-Behnken design is a statistical tool that is used for estimation and interpretation of interactions and relations between process variables. It enables us to find out linear quadratic responses to create a first or second order polynomial model with few runs (Palukurty, Telgana, Sundar, Bora, & Mulampaka, 2008).

According to Plackett-Burman results, Box-Behnken design matrix was created with 3 significant variables; pH (X_1), temperature (X_2), and molasses hydrolysate concentration (g/L) (X_3). During Box-Behnken design experiments, molasses

hydrolysate was used as carbon source instead of glucose. The ranges of process variables were 6-8 for pH, 25-37 °C for temperature and 10-30 g/L for molasses hydrolysate concentration. A set of 45 experiments were carried out. The experimental plan, experimental biomass concentrations and fitted biomass concentrations are given in Table 4.6. Each response was expressed as the average of two replicates. The highest biomass concentration (2.08 g/L) was obtained with pH 7, temperature 31°C and 20g/L molasses hydrolysate. On the other hand, lowest biomass concentration was obtained with pH 7, temperature 37°C and 10 g/L molasses hydrolysate (Table 4.6.).

In order to optimize biomass concentration, a second order polynomial model was developed by RSM. This model predicted the response as a function of three variables and as a function of the interaction of variables. Second order polynomial equation obtained with coded values is given in Equation 11 as follows:

$$\hat{Y}= 1.95- 0.027X_1 -0.034X_2 - 0.03375X_3 -0.48X_1^2-0.708X_2^2-0.25X_3^2-0.0045X_1X_2-0.0124X_1X_3 + 0.298X_2 X_3 \tag{11}$$

where $\hat{Y}$ is the predicted biomass concentration (g/L), X_1, X_2 and X_3 are coded variables for pH, temperature and molasses hydrolysate, respectively.

Table 4.6. Box-Behnken Design, experimental and predicted values of biomass concentration

Run	pH	T°C	Molasses hydrolysate (g/L)	Y (g/L) [1]	Ŷ (g/L) [2]
1	7	25	10	1.32±0.11	1.41
2	6	31	30	1.31±0.14	1.32
3	7	37	10	0.62±0.04	0.68
4	8	37	20	0.70±0.04	0.64
5	7	31	20	1.88±0.03	1.95
6	8	31	30	0.95±0.02	1.01
7	8	25	20	0.82±0.02	0.82
8	7	25	30	0.76±0.02	0.69
9	6	37	20	0.79±0.01	0.78
10	6	31	10	1.22±0.07	1.15
11	7	31	20	1.92±0.07	1.95
12	7	31	20	2.08±0.08	1.95
13	6	25	20	0.73±0.03	0.78
14	7	37	30	1.25±0.03	1.24
15	8	31	10	1.36±0.09	1.35

Y(g/L)[1] is the experimental biomass concentration

Ŷ (g/L)[2] is the fitted biomass concentration.

By comparing the magnitude of the coefficients of the second order model, it is possible to determine the measure of the efficiency of different variables (Uzuner & Cekmecelioglu, 2015). Molasses hydrolysate (g/L) was the most important variable with the highest coefficient (0.03375) in finding the biomass concentration. Temperature and pH followed molasses hydrolysate with their coefficient values, which were 0.03333 and 0.020708, respectively. These results demonstrated that molasses hydrolysate concentration was the most important variable affecting obtained biomass concentration (Table 4.7).

The significance of each variable was determined by *P*-values, which are given in Table 4.7. The *P*-value is possibility that the difference between conditions may have generated by chance. The smaller *P*-value indicates that the corresponding coefficient

is more significant (Palukurty et al., 2008). According to the regression analysis, the linear effects of molasses hydrolysate, pH and, temperature were found insignificant ($P>0.05$). This was an expected result due to inability of microalgal cells to increase their growth with continuous increase of pH, temperature and molasses hydrolysate concentration. On the other hand, the quadratic effect of each variable was found significant ($P<0.05$). The interaction effect between pH and molasses hydrolysate and the interaction effect between temperature and molasses hydrolysate were significant ($P<0.05$). The interaction effect between pH and molasses hydrolysate were insignificant (P>0.05) (Table 4.7).

Table 4.7. ANOVA results of Box-Behnken Design in coded values.

Term	Coefficient	P-value
Constant	1.95000	0.000*
pH (X_1)	-0.02708	0.284
Temperature (°C) (X_2)	-0.03333	0.189
Molasses hydrolysate (g/L) (X_3)	-0.03375	0.184
X_1*X_1	-0.48542	0.000*
X_2*X_2	-0.70792	0.000*
X_3*X_3	-0.25375	0.000*
X_1*X_2	-0.04500	0.209
X_1*X_3	-0.12417	0.001*
X_2*X_3	0.29833	0.000*

* Result is significant when $P<0.05$.

The lack of fit value measures the difference between the deviation caused by the divergence of the model to data and the deviation result from random error. A significant lack of fit values indicates that the model does not fit the data (Androga et al., 2014). In this study, the insignificant lack of fit ($P=0.131>0.05$) demonstrated that the model fitted well to the experimental data.

The coefficient of determination (R^2) was 0.9470 for biomass concentration, demonstrating that 94.70% of the variability in biomass concentration could be described by the model. Since the R^2 value that is closer to 1.0 indicates that the model

is stronger (Gurkok, Cekmecelioglu, & Ogel, 2011), the R^2 value (R^2=0.9470) demonstrated that the quadratic model fitted well to the data. Moreover, the estimated R^2 (adj) and predicted R^2 (pred.) were found as 0.93 and 0.91, respectively, which also gives certain results about the goodness of fit (Ohtani, 2000). R^2 (adj) value of 0.93 demonstrated that only 7% variation in response (biomass concentration) cannot be clarified by the model.

The three-dimensional (3D) response surface plots were used to determine the relationship between different variables and to determine their optimum values to obtain highest biomass concentration. Figure 4.16. represents the response surface plots for biomass concentration at varying concentration of a) pH vs temperature (°C), b) pH vs molasses hydrolysate concentration (g/L) and c) Temperature (°C) vs molasses hydrolysate concentration (g/L), respectively. The third factor, in all cases, was held constant at the center point (i.e. pH:7, temperature: 30.7±1 °C and molasses hydrolysate: 20 g/L).

In all cases it was observed that biomass concentration increased up to a certain level as the level of variables increased. From the response surface plot Fig.4.16.a. biomass concentration demonstrated a non-linear increase pattern, with the increase of temperature from 25 to 37°C and with the increase of pH from 6 to 8. Highest biomass concentration was observed beyond 30°C and pH 7. Converti et al. (2009) evaluated the effects of temperature and nitrogen concentration on the growth and lipid content of *Chlorella vulgaris*. In the study *C. vulgaris* cells showed a decrease in cell growth at temperatures above 30°C. When temperature was altered to 38°C cell death was observed.

From the response surface plot in Figure 4.16.b biomass concentration can be observed to increase as the pH and molasses hydrolysate concentration increase, up to their optimal values, then started to decrease even when pH and molasses hydrolysate continue to increase. Since maximum algal growth occurs nearly at neutral pH, a decrease in biomass concentration beyond neutral pH was an expected result. On the other hand, alkaline pH changes the flexibility of the cell wall of mother cells that limits the release of autospores and thus extend the time need for cell cycle completion. Similarly, acidic pH affects microalgal growth by changing nutrient uptake or by promoting metal toxicity (Juneja et al., 2013).

Figure 4.16.c depicted that high and low temperatures affected biomass concentration negatively. High and low concentrations of molasses hydrolysate also affected biomass concentration negatively. At constant pH, increase in temperature approximately up to 30°C and increase in molasses hydrolysate concentration approximately up to 20 g/L gave rise to an increase in biomass production, which was also given as the significant term (P=0.000<0.05) in Table 3.7.

Considering the fact that carbon source is important for biomass production, an increase in biomass concentration with a simultaneous increase in molasses hydrolysate concentration was a fair result. Besides low temperature conditions may affect biomass production by altering cytoplasmic viscosity, which would give rise to a decrease in carbon and nitrogen utilization. On the other hand, and increase in temperature above the optimal values, causes a decline in protein synthesis and eventually cell death occurs (Juneja et al., 2013). Lakshmikandan and Murugesan (2016) used Response Surface Methodology to evaluate the effect of substrate concentration, temperature, pH, and carbon dioxide on the growth and biohydrogen production of *Chlorella vulgaris* MSU-AGM 14. In their study, 3D response surface plots depicted that an increase in substrate concentration and temperature caused an increase in biohydrogen production up to a certain level and then started to decrease.
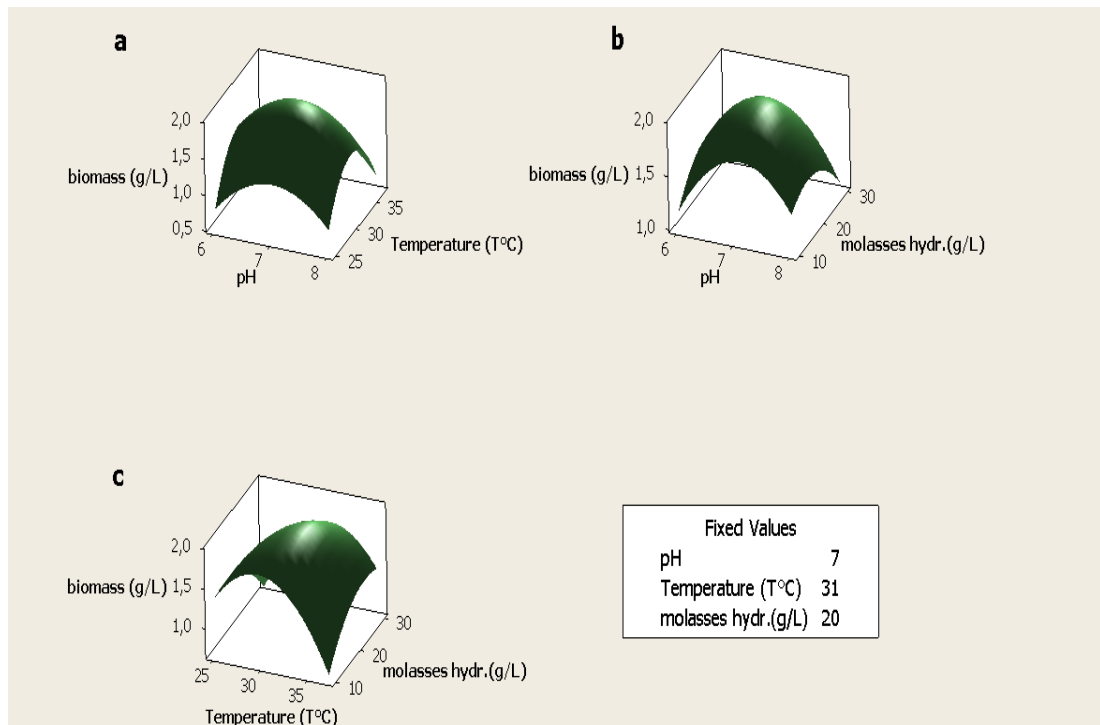
Figure 4.16. Response surface plots showing the effects of a) pH and temperature (°C), b) pH and molasses hydrolysate concentration (g/L), c) temperature and molasse hydrolysate concentration (g/L).

Response surface plots symbolize the regression equation and used to determine the relationship between different process variables. Contour plots also enables to find out the type of the interactions between test variables. A circular contour of response demonstrates negligible interaction between process variables. On the other hand, an elliptical or saddle contour plots shows significant interaction between process variables (Murthy, Swaminathan, Rakshit, & Kosugi, 2000).

The contour plots that show the interactive effects between process variables are given in Fig. 4.17, Fig.4.18 and Fig 4.19. The smallest ellipse in the contour plot demonstrates the maximum response. Moreover, elliptical contours shows the perfect interaction between independent variables (Alam, Muyibi, & Wahid, 2008). An increase in temperature from 25°C to 30°C increased biomass concentration to 1.8 g/L as given in Fig. 4.17. The circular shape of the contour plots given in Fig. 4.17 indicated that the interaction effects between process variables were negligible. The

ANOVA results in Table 3.7., which gave the *P* value of X_1*X_2 as *P*=0.209>0.05 also confirmed that the interaction between pH and temperature was insignificant.
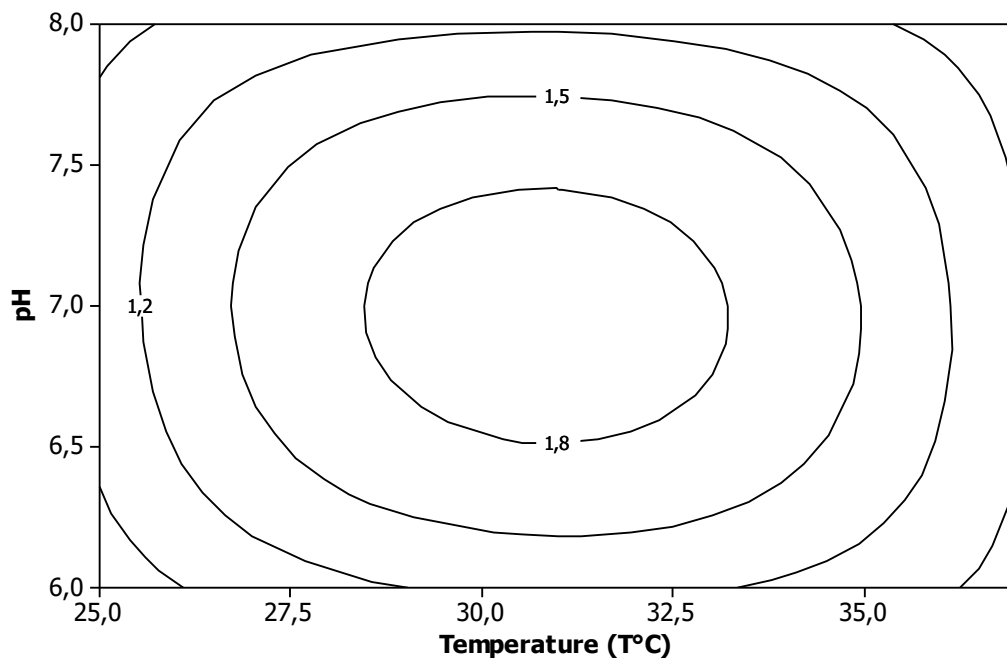
Figure 4.17. Contour plot showing the interactive effect of pH and temperature on biomass concentration.

Contour plots in Fig. 4.18 indicated that with an increase in pH from 6.0 to approximately to 7.0 caused almost a two-fold increase in biomass concentration (from 0.9g/L to 1.8 g/L). The interactive effect of pH with temperature had significant effect.

Contour plots in Fig. 4.19. demonstrated the effect of temperature and molasses hydrolysate concentration on biomass concentration. The interaction effect of temperature and molasses were significant. Maximum biomass concentration was observed when temperature was beyond 30°C and molasses hydrolysate concentration was 19 g/L.
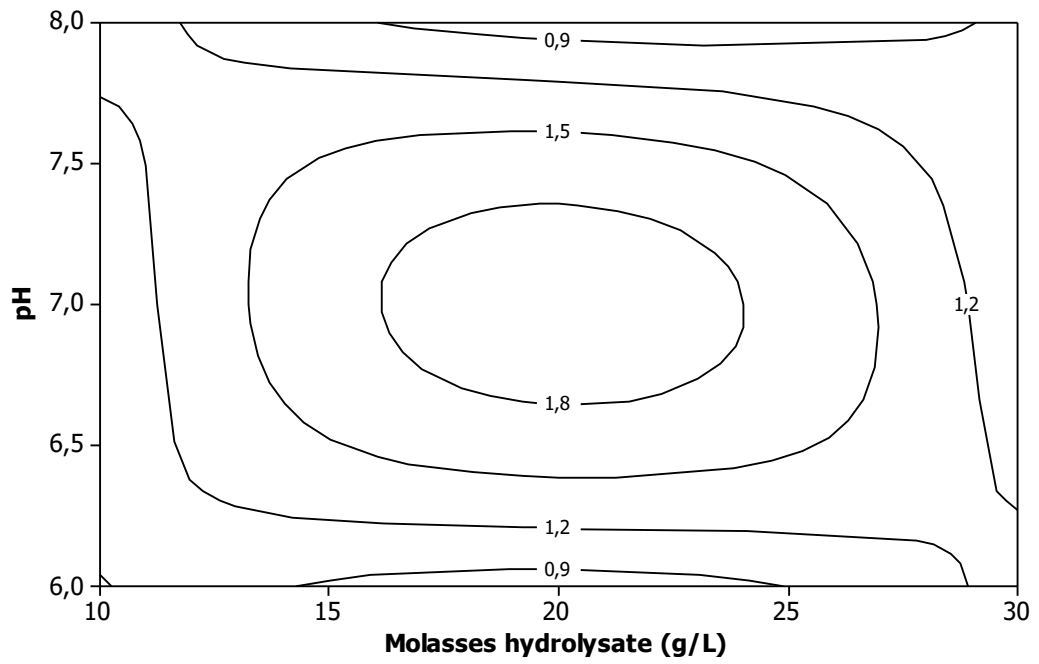
Figure 4.18. Contour plot showing the interactive effect of pH and molasses hydrolysate on biomass concentration.
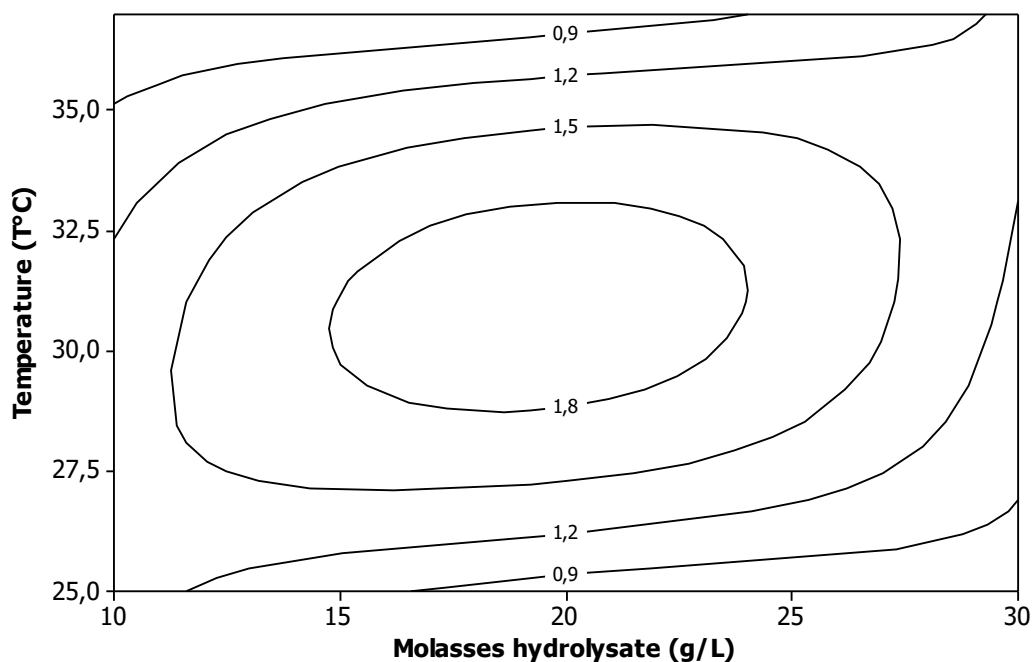
Figure 4.19. Contour plot showing the interactive effect of temperature and molasses hydrolysate on biomass concentration.

4.2.1.5. Model Verification

In order to determine optimum cultivation conditions, Response Optimizer Tool of Minitab 16.0 was used. The optimum conditions for biomass concentration were found as 30.7±1°C of temperature, 19 g/L molasses hydrolysate, and pH of 6.9. Under these conditions the biomass was measured as 2.08g/L. The biomass obtained was very close to corresponding predicted value of 1.95g/L with a very low standard deviation (0.09) and very low coefficient of variation (0.04 or 4%). These results indicated that the model adequately estimates the biomass produced. The constructed model was also checked using error analysis. The RMSE and MAE values were calculated as 0.10 and 0.08, respectively, which both indicate low error and high accuracy of prediction.

4.3. Shake Flask Studies with *Micractinium sp*.ME05

Following obtaining optimum conditions that gave the highest biomass concentration with molasses hydrolysate, the same experimental conditions were evaluated using different inoculum ratios (5%-7%-10%-15%), different aeration rates (0.25L/min and 0.5 L/min) and with small (250 mL of working volume) and large (1-L of working volume) scale flasks.

As another by product of sugar industry, vinasse was also evaluated for growth of *Micractinium sp*.ME05 cells. For that purpose, 4 different vinasse concentrations (%2-%5-%10 and %20 v/v) were evaluated under mixotrophic and heterotrophic conditions.

4.3.1. Growth Evaluation of *Micractinium sp*.ME05 with Molasses Hydrolysate in Small Scale Flasks with Different Inoculum Ratios

After determining optimum conditions that gave highest biomass concentration from *Micractinium sp*.ME05 cells, these conditions (30.7±1°C of temperature, 19 g/L of molasses hydrolysate and pH of 6.9) were applied to 500mL flasks (250-mL of working volume) with different inoculum ratios. 0.5 L/min air were applied during incubation period. Inoculum ratios that were evaluated under these conditions were 5%, 7%, 10% and 15% (v/v). Absorbance versus time curve of *Micractinium sp. ME05* cells are given in Figure 3.20.

According to absorbance versus time curve, it was observed that with 7% (v/v) and 10% (v/v) inoculum of microalgal cells reached stationary phase in 96 hours, while with 15% (v/v) inoculum the cells reached stationary phase in 48 hours. These results demonstrated that higher levels of inoculum shortened the time needed to reach stationary phase. This may result from the immediate decline in nutrient concentration with an increase in inoculum ratio.

Biomass concentrations (g/L), biomass productivities ($g.L^{-1}.day^{-1}$) and growth rates (d^{-1}) of *Micractinium sp*.ME05 with different inoculum ratios are given in Table 3.8. According to these results, with 5% (v/v) inoculum ratio the biomass concentration

was 2.08±0.094 g/L. With 7% (v/v) inoculum the biomass obtained was 2.90±0.121 g/L. The highest biomass concentration that was obtained with 10% (v/v) inoculum was 3.73±0.098 g/L. On the other hand, with 15% (v/v) inoculum of microalgal cells the biomass concentration slightly decreased to 2.14±0.058 g/L.

In terms of biomass productivity, similar results to biomass concentrations were obtained since biomass productivity is defined as biomass concentration per day. Biomass productivity increased from 0.41± 0.02 g.L^{-1}.day^{-1} to 0.74± 0.02 g.L^{-1}.day^{-1} with an increase in inoculum ratio from 5%(v/v) to 10%(v/v), however a further increase in inoculum ratio from 10% (v/v) to 15% (v/v) caused a decline in biomass productivity from 0.74± 0.02 g.L^{-1}.day^{-1}to 0.41± 0.01 g.L^{-1}.day^{-1} (Table 3.8.). The decline in both biomass and biomass productivity with 15% (v/v) inoculum may have resulted from oxygen or nutrient limitation. A similar result was obtained in the study of Zhang et al. (2003), who reported a reduction in biomass production of *Taxus yunnanensis* with an increase in inoculum size.

The lowest growth rate (1.53±0.009) was observed with 5% (v/v) inoculum. With the increase of inoculum ratio from 5% (v/v) up to 10%(v/v), the growth rate was also increased from 1.53±0.009 to 1.64±0.005. However, with 15% (v/v) inoculum the growth rate was also decreased to 1.53±0.005 as also seen in biomass concentration and biomass productivity.

A similar trend was reported in a study conducted with *Chlorella vulgaris.* In that study *C. vulgaris* cells were used at different ratios; 0.017 g/L, 0.060 g/L, 0.103 g/L, 0.135 g/L and 0.160g/L to the cultivation medium. The increase in inoculum concentration from 0.017 g/L to 0.103 g/L, increased biomass productivity from 0.068 g.L^{-1}.day^{-1} to 0.12 g.L^{-1}.day^{-1}. However, a further increase in inoculum concentration, reduced the biomass productivity to 0.11 g.L^{-1}.day^{-1}. On the other hand, with high inoculum ratios of *C. vulgaris* cells, the time needed for the cells to reach stationary phase decreased (Zhang Li et al., 2017).

These results demonstrated that increasing inoculum size caused an increase in biomass concentration, which may result from the increasing concentration of cells that participate in reproduction. On the other hand after a point, cell concentration

started to decrease, which may result from nutrient and oxygen limitation (Zhang Li et al., 2017).
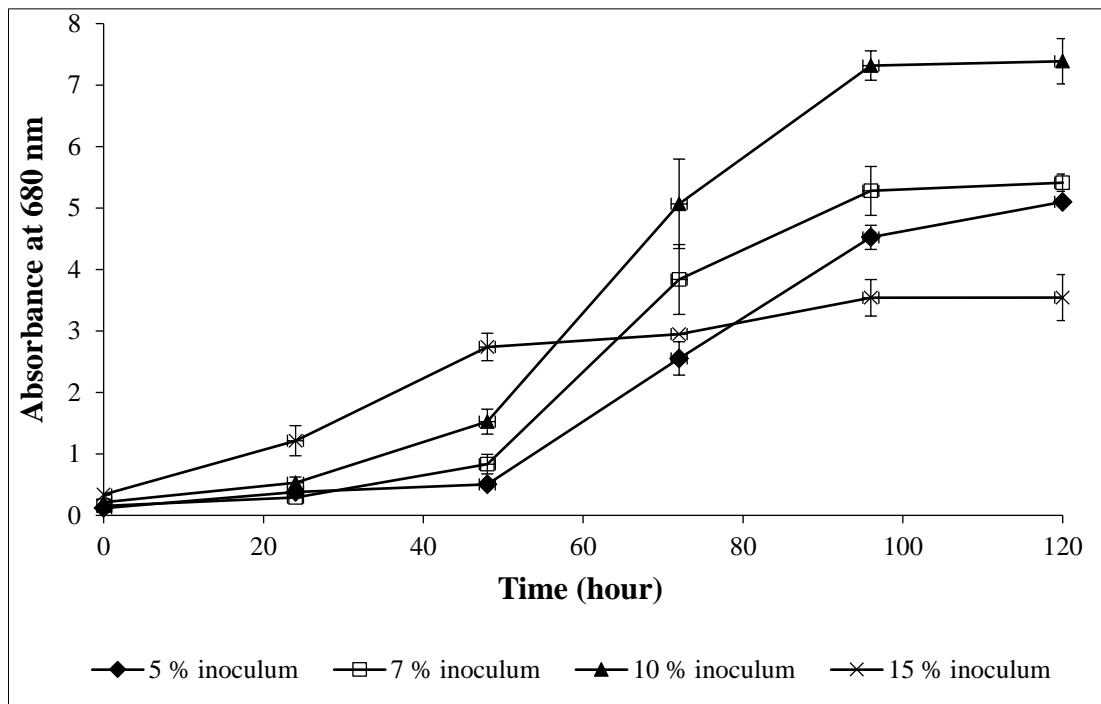
Figure 4.20. Absorbance versus time curve of *Micractinium sp. ME05* with different inoculum ratios in bold basal medium. Data are mean ± standard error of three replicates.

Table 4.8. Biomass concentration, biomass productivity and specific growth rate of *Microactinium sp. ME05* with different inoculum ratios at 30.7±1°C, pH:6.9 with 19g/L molasses hydrolysate in 500-mL flasks with 250-mL working volume. Data are mean ± standard error from three replicates.

	Inoculum ratios (v/v)			
	5% (v/v)	**7 % (v/v)**	**10% (v/v)**	**15 % (v/v)**
Biomass concentration (g.L^{-1})	2.08±0.094	2.90±0.121	3.73±0.098	2.14±0.058
Biomass Productivity (g.L^{-1}.day^{-1})	0.41± 0.02	0.57± 0.02	0.74± 0.02	0.41± 0.01
Growth rate (μ)(d^{-1})	1.53±0.009	1.59±0.008	1.64±0.005	1.53±0.005

4.3.2. Growth Evaluation of *Microactinium sp.*ME05 with Molasses Hydrolysate in Small and Large-Scale Flasks with Different Aeration Rates

The highest biomass concentration of *Microactinium sp.* ME05 cells under optimized culture conditions (pH:6.9, temperature: 30.7±1°C, molasses hydrolysate :19g/L) with 250 mL working volume, were obtained as 3.73 g/L with 10% (v/v) inoculum of microalgal cells as stated in section 4.3.1.

The same conditions were applied to *Microactinium sp.* ME05 cells by reducing the air given to system from 0.5 L/min to 0.25 L/min. 250 mL of bold basal medium was supplemented with 19 g/L molasses hydrolysate and 2 g/L yeast extract. *Microactinium sp.*ME05 cells in exponential growth phase were inoculated into the media (10% v/v) under aseptic conditions.

On the other hand, 2 L flasks were also evaluated with 1-L working volume under optimized culture conditions. 0.25 L/min and 0.50 L/min air were given to the system separately (Figure 4.21). Obtained biomass concentrations are given in Table 4.9.
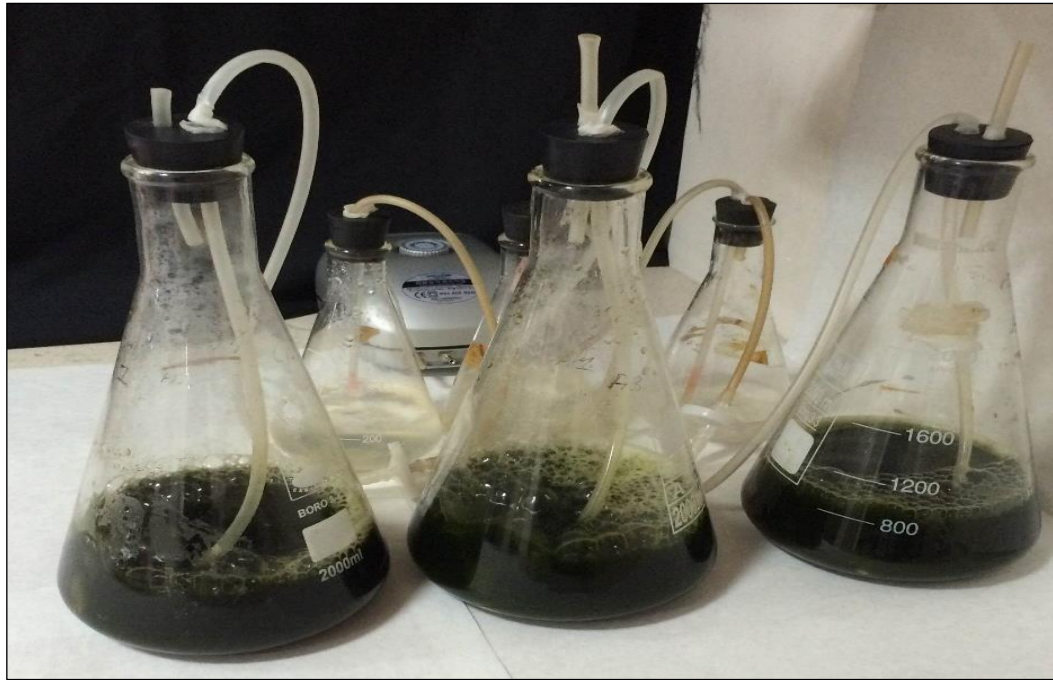
Figure 4.21. *Micractinium sp.*ME05 cells in 1-L bold basal medium supplemented with 19g/L molasses hydrolysate. 0.5L/min aeration was provided.

In 500 mL flasks with 250 mL working volumes, increasing aeration rate from 0.25 L/min to 0.5 L/min caused an increase in biomass concentration from 2.47±0.15 g/L to 3.73±0.14 g/L. Similarly, in 2-L flasks with 1-L working volumes, an increase in aeration rate from 0.25L/min to 0.5L/min caused an increase in biomass concentration from 2.35±0.17 g/L to 3.06±0.06 g/L. These results showed that, an increase in aeration rate caused an increase in biomass concentration, which may result from an increase in dissolved oxygen concentration with high aeration rate.

Similar results were obtained in the study of Pahl et.al. (2010), who studied the effects of agitation and aeration rates on the heterotrophic growth of *Cyclotella cryptica.* In their study 0.28 v/v/min, 0.44 v/v/min and 1.07 v/v/min were evaluated. An increase in aeration rate from 0.28 v/v/min to 0.44 v/v/min, resulted in an increase in cell dry weight from 0.81±0.06 g/L to 1.09±0.126 g/L. It was also stated that aeration rate significantly (*P*<0.05) affected specific growth rate of *C. cryptica* cells (Pahl et al., 2010).

Yen et al. (2014) evaluated the operation conditions of an airlift bioreactor for the cultivation of *Rhodotorula glutinis*. Three different aeration rates;1 vvm, 1.5. vvm, and 2 vvm were evaluated and the biomass concentrations obtained were 16.6 g/L, 18 g/L and 25.4 g/L, respectively. It was reported that increasing aeration rate caused an increase in biomass concentration and specific growth rate of *R. glutinis* cells (Yen & Liu, 2014).

Table 4.9. Biomass concentration of Micractinium sp. ME05 under different working volumes (L) and different air flow rates. Data are mean ± standard error from three replicates.

Volume (L)	Air flow (LPM*)	Biomass concentration (g/L)
0.25	0.25	2.47±0.15
0.25	0.50	3.73±0.14
1	0.25	2.35±0.17
1	0.50	3.06±0.06

*: Liter per minute.

After applying two different aeration rates (0.25 L/min and 0.50 L/min) to two different working volumes (0.25L and 1L), analysis of variance (ANOVA) and regression analysis were performed to determine the coefficients of predictive model and determine significant terms. The *P* values show the pattern of the interactions between process variables. As the *P* values gets smaller, the related coefficient becomes more significant (Jian-zhong Liu, Weng, Zhang, Xu, & Ji, 2003).

According to ANOVA results, it was observed that aeration rate (*P*=0.000<0.05) and working volume of the flask (*P*=0.020<0.05) significantly affected biomass concentration. On the other hand, the interaction effect of aeration rate and working volume on biomass concentration were insignificant (*P*=0.078> 0.05) (Table 4.10).

Table 4.10. Two-way ANOVA of working volume (L) and air flow (LPM) for biomass concentration.

Factors	Degrees of freedom	Sums of squares	Mean squares	F-value	*P*-value[1]
Volume[2]	1	0.46413	0.46413	8.46	0.020
LPM[3]	1	2.94030	2.94030	53.58	0.000
Volume.LPM	1	0.22413	0.22413	4.08	0.078
Error	8	0.43900	0.05488		
Total	11	4.06757			
S=0.2343 R^2=89.21 % R^2(adj)=85.16%					

[1] Result is significant when $P<0.05$

[2] Working volume of the flasks.

[3] LPM: Liter per minute.

From the regression analysis, a linear equation (Equation 11) was developed as follows:

$$\text{Biomass concentration (g/L)} = 1.74 + 3.96 \text{ LPM} - 0.262 \text{ Volume} \qquad (11)$$

Where biomass concentration is the obtained biomass at the end of the incubation period, LPM (liter per minute) is the air given to the system, and volume (liter) is the working volume of the flasks.

The coefficient of determination (R^2) and the adjusted coefficient of determination (R adj^2) are the measures used to estimate goodness of fit (Ohtani, 2000). The closer the R^2 value to 1.0 ,the stronger the model (Gurkok et al., 2011). The coefficient of determination was obtained as 0.89 showed that the model was adequate in representing the experimental data.

4.3.3. Growth Evaluation of *Microctinium sp.*ME05 with Molasses Hydrolysate Under Light Conditions and with TAP Medium Under Complete Darkness

In order to compare biomass concentration of *Microctinium sp.*ME05 cells under complete darkness and under light conditions, microalgal cells were cultivated under optimized culture conditions (19g/L of molasses hydrolysate, temperature of 30.7±1°C, pH:6.9) under 16 h light-8h light conditions. At the end of the incubation period the biomass concentration was 2.67±0.11 g/L.

Moreover, biomass concentration of *Microctinium sp.* ME05 cells were also evaluated in TAP medium under complete darkness and a biomass concentration of 0.47±0.02 g/L was obtained.

In brief, the biomass concentration of *Microctinium sp.* ME05 with molasses hydrolysate under complete darkness and under 16 h light:8h dark photoperiod was 3.73±0.098 g/L (as stated in Section 3.3.1) and 2.67±0.11 g/L, respectively. On the other hand, in TAP medium the biomass concentration of *Microctinium sp.* ME05 under complete darkness and under 16 h light:8h dark photoperiod was 0.47±0.02 g/L and 1.23±0.003 g/L, respectively (Table 4.11).

These results demonstrated that, with molasses hydrolysate as carbon source, *Microctinium sp.*ME05 cells produced more biomass under dark conditions (3.73±0.098) than light (2.67±0.11) conditions. The reason for the production more biomass under heterotrophic conditions may result from more ATP is produced with the energy liberated from glucose rather than the energy supplied by light under autotrophic or mixotrophic conditions (Yang et al., 2000). Moreover, light may have an inhibition effect on hexose/H^+ symport system which is responsible for the uptake of reducing sugars from the medium (Perez-Garcia et al., 2011a)

Under dark conditions glucose is assimilated via Pentose Phosphate Pathway (PPP), while under light conditions glucose is assimilated via Embden Meyerhof Pathway (EMP) (Perez-Garcia et al., 2011b). Yang et al., (2000) reported that PPP might have a higher flux rate than EMP pathway. For instance, in *Chlorella pyrenoidosa* cells, under complete darkness, PPP pathway is responsible for the 90% of metabolic flux distribution with glucose-6-phosphate dehydrogenase enzyme and on the other hand the reaction of EMP pathway that is catalyzed by glucose-6-phosphate isomerase is depressed.

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Moreover, *Micractinium sp.* ME05 cells may possess a symport system similar to *Chlorella* cells that is responsible for more biomass production under dark conditions. *Chlorella* cells include a hexose/H^+ symport system that enables to uptake glucose from the culture medium. This system is activated in the presence of glucose, fructose and galactose. Hexose Uptake Protein Gene (hup1) regulates this system and it was reported that when autotrophically cultivated *Chlorella* cells started to grow heterotrophically with glucose, as this hup1 gene was activated. Moreover light was reported to hinder the expression of hexose/H^+ symport system (Perez-Garcia et al., 2011b).

In terms of TAP medium; under light conditions *Micractinium sp.*ME05 cells generated more biomass (1.23±0.003 g/L) than complete dark conditions (0.47±0.02 g/L). Acetate is the carbon source in TAP medium and acetate is metabolized with glyoxylate cycle to produce malate or with tricarboxylic acid cycle (TCA) to produce citrate that contributes energy in the form of ATP and carbon skeletons. The key enzymes in glyoxylate cycle are isocitrate lyase and malate synthetase and these enzymes are activated in the presence of acetate (Boyle & Morgan, 2009). On the other hand, light has an inhibition effect on the activation of isocitrate lyase enzyme. In this study, light may have suppressed the activation of glyoxylate cycle and cells tended to use TCA cycle for the assimilation of acetate, which gave more energy and more carbon skeletons. Similar observations were reported for *Euglena gracilis* cells; the cells metabolized acetate more efficiently under light rather than dark conditions (Perez-Garcia et al., 2011b).

Higher biomass concentrations in bold basal medium under both light and complete darkness conditions than TAP medium may result from more energy content of glucose than acetate. Glucose generates 2.8kJ/moL energy, while acetate generates 0.8kJ/moL energy (Boyle & Morgan, 2009).

Table 4.11. Biomass concentrations and biomass productivities of *Micractinium sp*. ME05 in different media under different cultivation conditions. Data are mean standard error from three biological replicates.

	TAP medium		Bold Basal Medium	
	16h light:8h dark photoperiod	Complete darkness	16h light:8h dark photoperiod	Complete darkness
Biomass concentration (g.L^{-1})	1.23±0.003	0.47±0.02	2.67±0.11	3.73±0.098
Biomass productivity (g.L^{-1}.day^{-1})	0.22± 0.03	0.09±0.003	0.53± 0.022	0.74± 0.02

4.3.4. Growth Evaluation of *Micractinium* sp. ME05 with Acclimation to Molasses

Micractinium sp.ME05 cells subjected to an acclimation process before inoculation to bold basal medium that was supplemented with 19 g/L molasses. For that purpose, after they came to exponential growth phase in TAP medium, they were transferred to bold basal medium in which acetate concentration was decreased while molasses hydrolysate concentration was increased in each inoculation step. With the final transfer, cells were inoculated at (10% v/v) into bold basal medium that was supplemented with 19g/L molasses hydrolysate.

The obtained growth curve was compared with the growth curve of *Micractinium sp*.ME05 cells that were directly inoculated from TAP medium to bold basal medium that was supplemented with 19g/L molasses hydrolysate (Figure 4.22). According to this growth curve, it was observed that the cells that were adapted to molasses demonstrated a shorter lag period than the cells that were directly transferred to molasses based media. On the other hand, the cells that were adapted to molasses reached stationary phase in 72 hours while the cells that were transferred to molasses based media directly, reached stationary phase in 96 hours.

In terms of biomass concentration and biomass productivity, it was observed that with the cells that were directly transferred to molasses based media, the biomass concentration and biomass productivity was 3.73±0.14 g/L and 0.74±0.02 g/L.day, respectively. On the other hand, with the cells that were adapted to molasses the biomass concentration and biomass productivity was 3.84±0.05 g/L and 0.76±0.01, respectively. The growth rate of *Micractinium sp*. ME05 that were directly inoculated into molasses based media was 1.64±0.005, while the growth rate of *Micractinium* sp.ME05 that were adapted to molasses was 1.65±0.002 (Table 4.12).

These results demonstrated that acclimation process enabled microalgal cells to adapt themselves to the changing environment and thereby reduced their lag phase in which physiological adaptation of cell metabolism takes place. The reduction of lag phase with acclimation process was also reported by Andersen Robert A. (2005).

Similar observations were reported in the study conducted by Osundeko (2014). In this study, different microalgal strains were acclimated to raw municipal wastewater secondary effluent (RMSWE). Biomass productivities and growth rates of acclimated and non-acclimated strains were compared. Acclimated strains demonstrated higher growth rates and thus higher biomass productivities than non-acclimated strains. In terms of growth curves, acclimated strains showed a shorter lag phase in comparison to non-acclimated strains (Osundeko, 2014).

Table 4.12. Biomass concentration, biomass productivity and growth rate of *Micractinium sp*. ME05 without any adaptation and with adaptation to molasses. Data are mean±standard error from three biological replicates.

	Micractinium sp.ME05 without any adaptation	*Micractinium sp*.ME05 adapted to molasses
Biomass concentration (g.L^{-1})	3.73±0.14	3.84±0.05
Biomass Productivity (g.L^{-1}.day^{-1})	0.74±0.02	0.76±0.01
Growth rate (µ)(d^{-1})	1.64±0.005	1.65±0.002
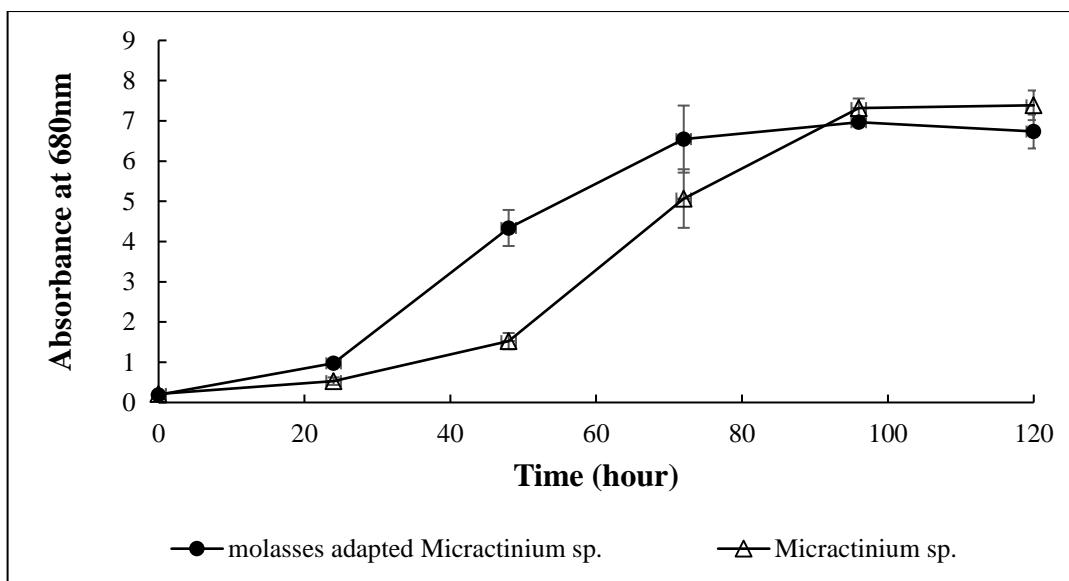
Figure 4.22. Absorbance versus time curve of *Micractinium sp.* ME05, which were adapted to molasses based media and transferred to molasses based media directly. Data are mean ± standard error from three replicates.

4.3.5. Growth Evaluation of *Micractinium sp.* ME05 with Vinasse in 500mL Flasks

Heterotrophic and mixotrophic growth of *Micractinium sp.* ME05 were firstly evaluated in 500 mL flasks with 250 mL bold basal medium that was supplemented with 2%, 5%, 10% and 20% (v/v) vinasse, separately.

Absorbance versus time curve of *Micractinium sp.* ME05 cells in 500 mL flasks with 250 mL working volume, under mixotrophic and heterotrophic conditions are given in Figure 4.23 and Figure 4.24, respectively. Under heterotrophic cultivation, with 10% (v/v) and 20% (v/v) vinasse, cells reached stationary phase approximately in 72 hours of growth, whereas under mixotrophic cultivation, cells demonstrated an increase in the growth phase approximately about 120 hours.

Under mixotrophic growth conditions, the highest cell density in terms of optical density was observed with 10% (v/v) vinasse followed by 5% (v/v) vinasse. The lowest growth was observed within medium that was supplemented with 20% (v/v) vinasse (Figure 4.23).

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Under heterotrophic cultivation, similar results to mixotrophic conditions were obtained. Highest growth was observed within the medium that was supplemented with 10% (v/v) vinasse. The lowest growth was observed within the medium that was supplemented with 20% (v/v) vinasse (Figure 4.24).

To compare mixotrophic and heterotrophic cultivation of *Micractinium sp.*ME05, it was observed that under mixotrophic conditions cells grew better than heterotrophic conditions. These results indicated that algal growth was diminished in the absence of light. In addition, decrease in cell growth after 10% (v/v) vinasse, could be caused by decrease in light penetration with increasing vinasse concentration.

Similar results were obtained in the study performed with *Scenedesmus sp.* in which different incubation temperatures (from 20°C to 35°C), different vinasse concentrations (from 0% to 50% vinasse), and different light intensities (from 2400 to 10000 Lux) were evaluated with Central Composite Design. It was observed that light had a significant effect on cell growth and biomass production of *Scenedesmus sp.* cells and as light intensity increased biomass production increased. On the other hand, increasing vinasse concentration more than 40% had a negative effect on biomass production due to preventing light penetration to the medium (Ramirez et al., 2014).
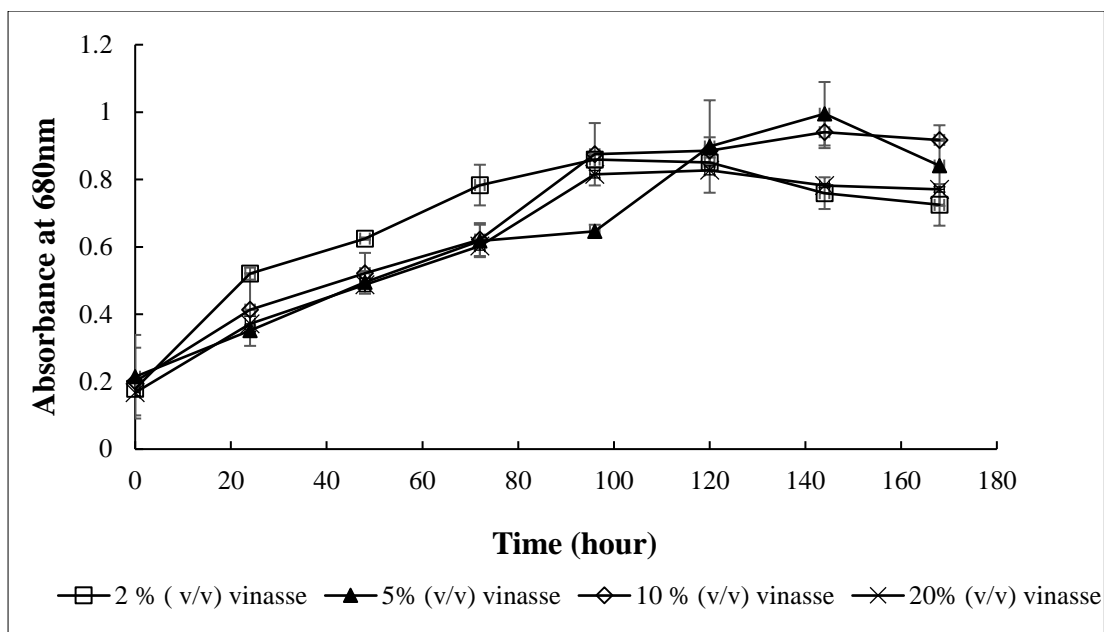
Figure 4.23. Absorbance versus time curve of *Micractinium sp*. ME05 in different vinasse concentrations under mixotrophic conditions. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.

Figure 4.24. Absorbance versus time curve of *Micractinium sp*.ME05 in different vinasse concentrations under heterotrophic conditions. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.

Biomass concentrations of *Micractinium sp*. ME05 during incubation period under mixotrophic and heterotrophic cultivation conditions are given in Figure 4.25 and Figure 4.26, respectively.

Under mixotrophic conditions, biomass concentration was obtained with %5 (v/v) and with 10%(v/v) vinasse were 1.21±0.38 g/L and 1.20±0.108 g/L, respectively. With 20% (v/v) vinasse, obtained biomass concentration was 1.07±0.074 g/L and with 2% (v/v) vinasse, obtained biomass concentration was 0.68± 0.084 g/L (Figure 4.25 and Table 4.13).

Under heterotrophic cultivation conditions, maximum biomass concentration of 0.93±0.092 g/L was obtained with 10% (v/v) vinasse. The minimum biomass concentration of 0.62±0.133 g/L was obtained with 2% (v/v) vinasse (Figure 4.26 and Table 4.13).
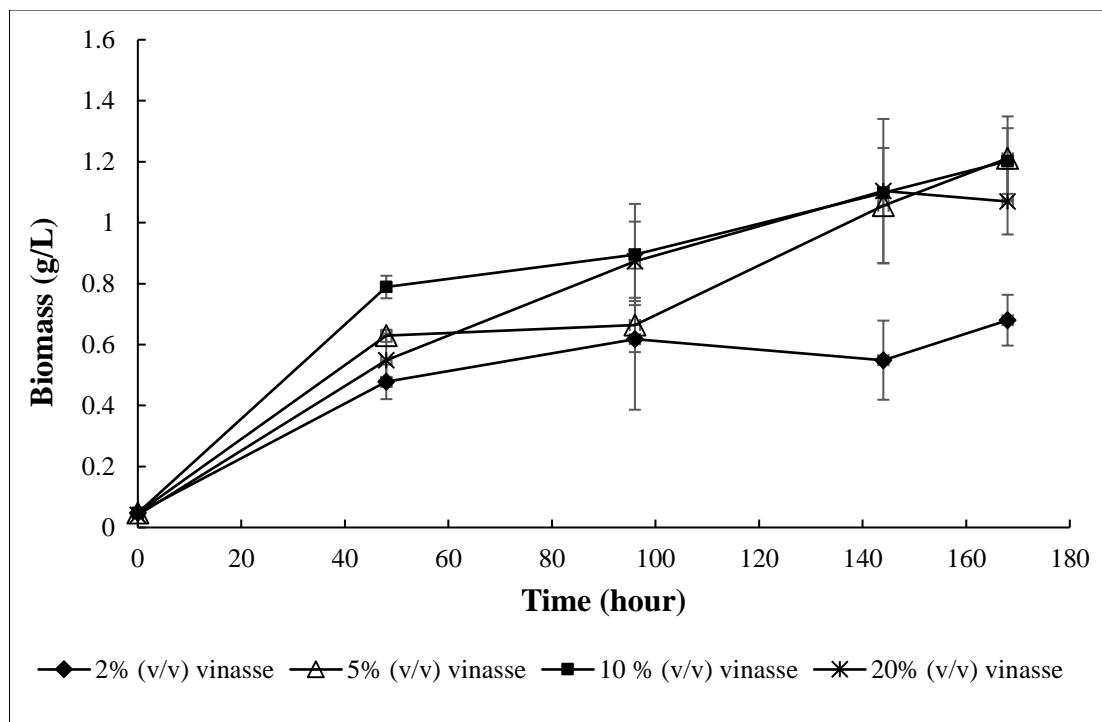
Figure 4.25. Observed biomass quantities *of Micractinium sp.ME05* in different vinasse concentrations under mixotrophic cultivation. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.
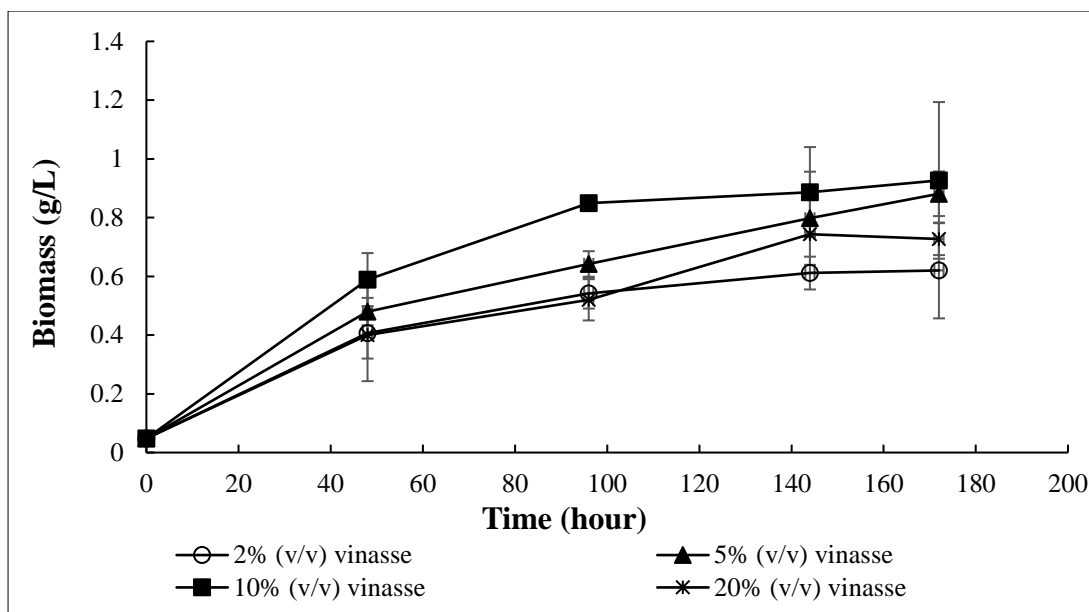
Figure 4.26. Observed biomass quantities *of Micractinium sp.ME05* in different vinasse concentrations under heterotrophic cultivation. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.

Biomass concentrations (g/L), biomass productivities (g/L.day) and growth rates (μ) of *Micractinium sp.*ME05 cells under mixotrophic and heterotrophic conditions at the end of the incubation period are given in Table 4.13.

Under mixotrophic conditions highest biomass productivity (0.17 ± 0.02 g/L.day) was observed with 10% (v/v) vinasse concentration, which was followed (0.16 ± 0.01 g/L.day) by 20% (v/v) vinasse concentration. Under heterotrophic conditions, highest biomass productivity of 0.13 ± 0.01 g/L.day was observed with 10% (v/v) vinasse followed by 0.12 ± 0.01 g/L.day with 5% (v/v) vinasse. For both of the cultivation conditions the lowest biomass productivities were observed with 2% (v/v) vinasse.

Growth rates of *Micractinium sp*.ME05 were also affected by different vinasse concentrations and by different incubation conditions. Under mixotrophic conditions, highest growth rate of 1.01 ± 0.01 d^{-1} was observed with 5% and 10% (v/v) vinasse, which were followed by 20 % (v/v) vinasse with a growth rate of 0.99 ± 0.01 d^{-1}. Under heterotrophic conditions, similar to mixotrophic conditions, highest growth rate of

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0.13 ± 0.01 d^{-1} was observed with 10% (v/v) vinasse, which was followed by 5% (v/v) vinasse with a growth rate of 0.12 ± 0.01 d^{-1}.

These results were comparable with a study conducted with *Spirulina maxima* with different vinasse concentrations under autotrophic, heterotrophic and mixotrophic growth conditions. In the study, 0.1% v/v and 1% v/v of vinasse was used and after 25 days of cultivation under mixotrophic conditions, 0.676 g/L and 0.716 g/L of biomass were produced with 0.1 % (v/v) and 1% (v/v) vinasse, respectively. Moreover under heterotrophic conditions, with 0.1% (v/v) and 1% (v/v) vinasse concentrations the specific growth rates were 0.54d^{-1} and 1.02d^{-1}, respectively (dos Santos, Araújo, de Medeiros, & Chaloub, 2016).

Table 4.13. Biomass concentrations, biomass productivities and growth rates of *Micractinium sp.ME05* cells with different vinasse concentrations, under mixotrophic and heterotrophic growth conditions. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.

		Vinasse Concentration (v/v)			
		2%	**5%**	**10%**	**20%**
Mixotrophic conditions	**Biomass concentration (g.L^{-1})**	0.68±0.08	1.21±0.38	1.20±0.10	1.07±0.07
	Biomass Productivity (g.L^{-1}.day^{-1})	0.09±0.01	0.17±0.02	0.16±0.01	0.15±0.01
	Growth rate (μ)(d^{-1})	0.92±0.02	1.01±0.01	1.01±0.01	0.97±0.01
Heterotrophic conditions	**Biomass concentration (g.L^{-1})**	0.62±0.13	0.88±0.07	0.93±0.09	0.73±0.05
	Biomass Productivity (g.L^{-1}.day^{-1})	0.08±0.01	0.12±0.01	0.13±0.01	0.10±0.01
	Growth rate (μ)(d^{-1})	0.90±0.04	0.96±0.01	0.97±0.01	0.93±0.01

Cell count results of *Micractinium sp*. ME05 with different vinasse concentrations under mixotrophic and heterotrophic growth conditions are given in Figure 4.27 and Figure 4.28, respectively. Cell count results obtained at the end of the incubation period, supported biomass concentrations of microalgal cells.

At the end of the incubation period, under mixotrophic conditions with 5% (v/v) vinasse the highest cell concentration of 610×10^4 cells/mL was obtained. The highest cell dry weight was also obtained with 5% (v/v) vinasse as given in Table 4.13. Similar to biomass concentration results given in Table 3.12, cell count results of 5% (v/v) vinasse and 10% (v/v) vinasse were so close to each other; with 10% (v/v) vinasse the

final cell concentration was 601×10^4 cells/mL. On the other hand, cell count results obtained with 2% (v/v) vinasse and 20% (v/v) vinasse were 499×10^4 cells/mL and 503×10^4 cells/mL, respectively.

Under heterotrophic conditions, the final cell count results obtained with 2% (v/v), 5% (v/v), 10% (v/v) and 20% (v/v) vinasse were 516×10^4 cells/mL, 594×10^4 cells/mL, 572×10^4 cells/mL and 465×10^4 cells/mL, respectively. These cell count results were consistent with the final biomass concentrations since the final biomass concentrations obtained with 2% (v/v), 5% (v/v), 10% (v/v) and 20% (v/v) vinasse were 0.62±0.13 g/L, 0.88±0.07g/L, 0.93±0.09g/L and 0.73±0.05 g/L, respectively (Table 4.13).

Similar cell densities were obtained in the study of Calixto et al. (2016), who evaluated the usage of agro-industrial residues for the cultivation of *Chlorella* sp., *Chlamydomonas* sp., *Lagerheimia longiseta*, and *Pediastrum tetras*. Fruit-vegetable bio compost (HB), bicompost of sugarcane industry residue (VB), raw chicken manure (RCM), bio-compost obtained from chicken excrement (BCE), municipal domestic sewage (MDS) were evaluated. Microalgal cells were cultivated under mixotrophic conditions at 25 °C in 5 L culture medium with aeration. After 16 days of cultivation period, highest cell densities were observed in culture medium supplemented with fruit-vegetable bio compost (HB) and municipal domestic sewage (MDS) which were varied from 5×10^5 cells/mL to 1×10^8 cells/mL. On the other hand, the cell densities of *Chlorella* sp., *Chlamydomonas* sp., *Lagerheimia longiseta* and *Pediastrum tetras* in medium supplemented with bicompost of sugarcane industry residue (VB) were 1×10^5 cells/mL, 3.5×10^6 cells/mL, 3×10^7 cells/mL and 5×10^5 cells/mL, respectively. The higher cell densities in medium supplemented with HB and MDS than medium supplemented with VB were reported to result from the high concentrations of nitrogen in the form of ammonia. Ammonia (NH_4^+) concentrations in MDS, HB and VB were 8.7 mg/L, 1.3mg/L and 0.6 mg/L. Since microalgal cells is known to assimilate nitrogen in the form of ammonia, the low values of ammonia (0.4g/kg) in our study may have caused low cell densities similarly to the study of Calixto et.al (2016).
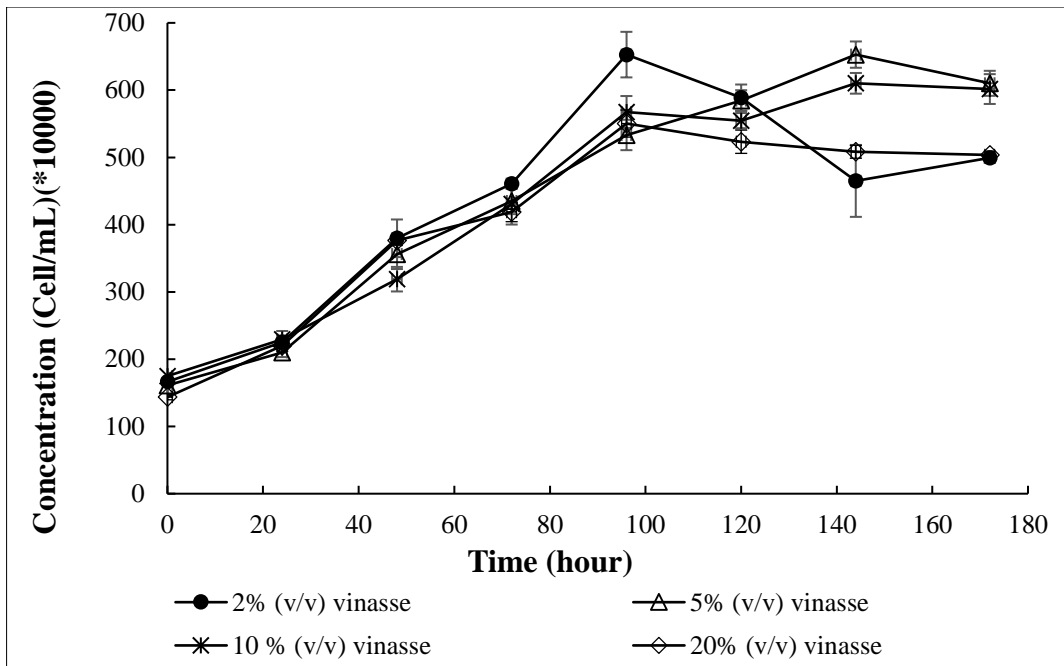
Figure 4.27. Cell enumeration results of *Micractinium sp.* ME05 with different vinasse concentrations under mixotrophic growth conditions. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.
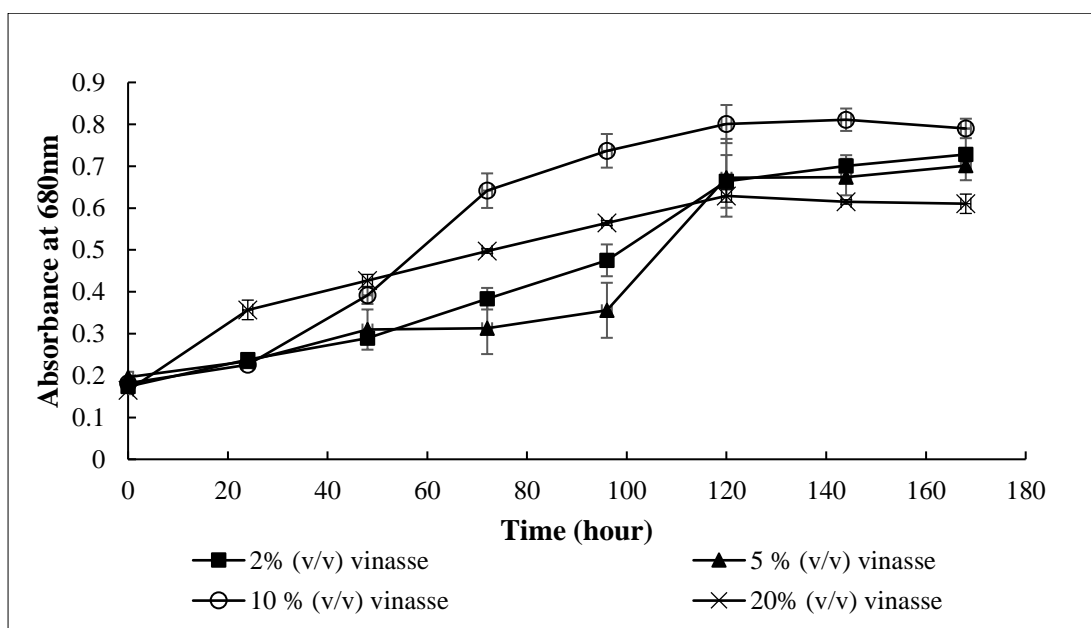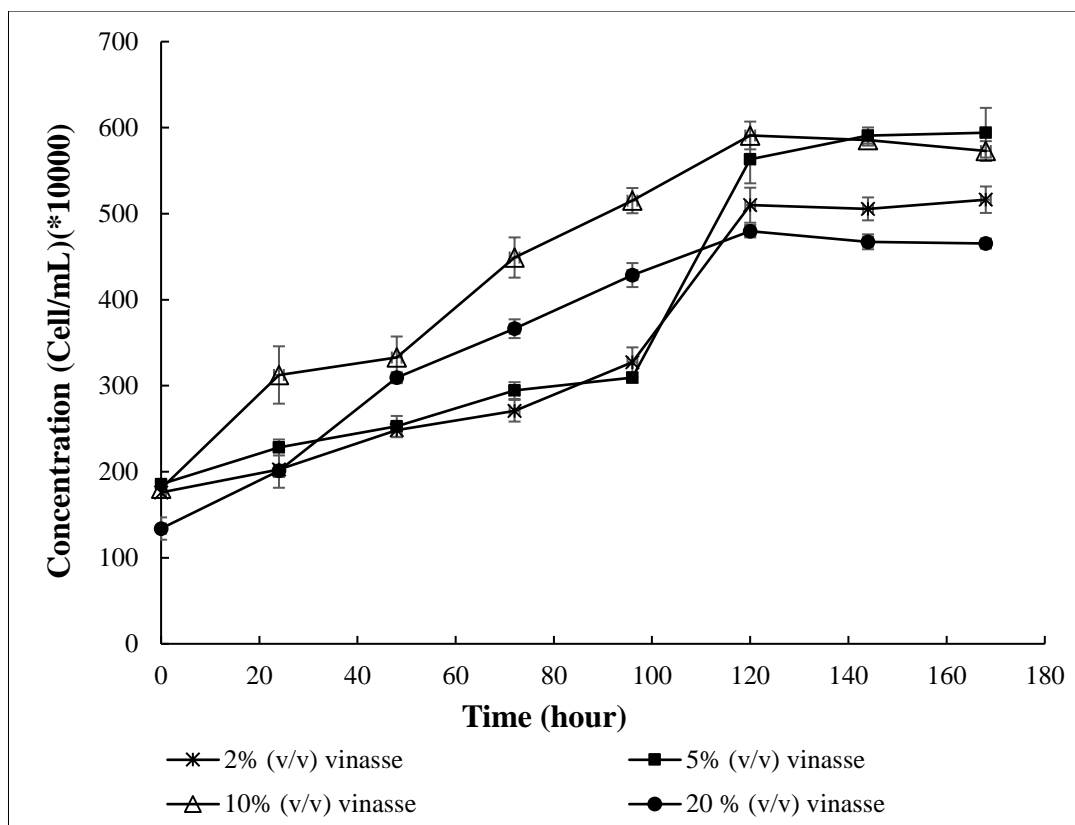
Figure 4.28. Cell enumeration results of *Micractinium sp.* ME05 with different vinasse concentrations under heterotrophic growth conditions. 500-mL flasks with 250-mL working volume. Data are mean± standard error from three replicates.

After all, optical density, biomass concentrations and cell count results demonstrated that microalgal cells grew better under mixotrophic conditions rather than heterotrophic conditions in medium supplemented with vinasse. Higher biomass concentrations, biomass productivities, specific growth rates and cell densities may result from the presence of more energy available under mixotrophic conditions rather than heterotrophic conditions. This high energy may result from the coupling of aerobic respiration with assimilation of carbohydrates present in cultivation medium (Mitra, van Leeuwen, & Lamsal, 2012).

4.3.6. Growth Evaluation of *Micractinium sp.* ME05 with Vinasse in 2L Flasks

Following 500-mL flasks with 250-mL working volume, mixotrophic cultivation conditions of *Micractinium sp.*ME05 cells with 2% (v/v), 5% (v/v), 10% (v/v) and 20% (v/v) vinasse were evaluated in 2-L flasks with 1-L working volume. Absorbance versus time curve of microalgal cells in 2-L flasks with 1-L working volume is given in Figure 4.29.

After 7 days of cultivation, *Micractinium sp.* ME05 reached OD values of 1.20±0.05, 1.57±0.02,1.66±0.02 and 1.18±0.16 with 2% (v/v), 5% (v/v), 10% (v/v) and 20% (v/v) vinasse, respectively.

Final biomass results (g/L), biomass productivities (g.L^{-1}.day^{-1}) and growth rates (d^{-1}) of microalgal cells are given in Table 4.14. Highest biomass concentration (1.38±0.095 g/L), biomass productivity (0.27±0.019 g.L^{-1}.day^{-1}) and growth rate (1.97±0.010 d^{-1}) was obtained with 10% (v/v) vinasse. On the other hand, the lowest biomass concentration (0.60±0.134 g/L), biomass productivity (0.11±0.027 g. L^{-1}.day^{-1}) and growth rate (1.25± 0.046 d^{-1}) was obtained with 2% (v/v) vinasse. A similar trend was reported in a study conducted with *Chlorella vulgaris* with different vinasse concentrations. In the study 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % vinasse concentrations were evaluated under mixotrophic growth conditions. Highest biomass yield was obtained with 10-20% vinasse concentration. Besides, with 10% and 20% vinasse (v/v) the growth rates were approximately 1.2 d^{-1} and 1 d^{-1}, respectively (Candido & Lombardi, 2016).

These results showed that increasing vinasse concentration may increase biomass concentration (g/L), biomass productivity (g. L^{-1}.day^{-1}), and growth rate (d^{-1}) up to a certain point, however after 10% (v/v) vinasse concentration they started to decrease. These results may have arisen from the inhibitory effect of high vinasse concentrations to microalgal cells. For that reason, further vinasse experiments were conducted with 10% (v/v) vinasse concentration.
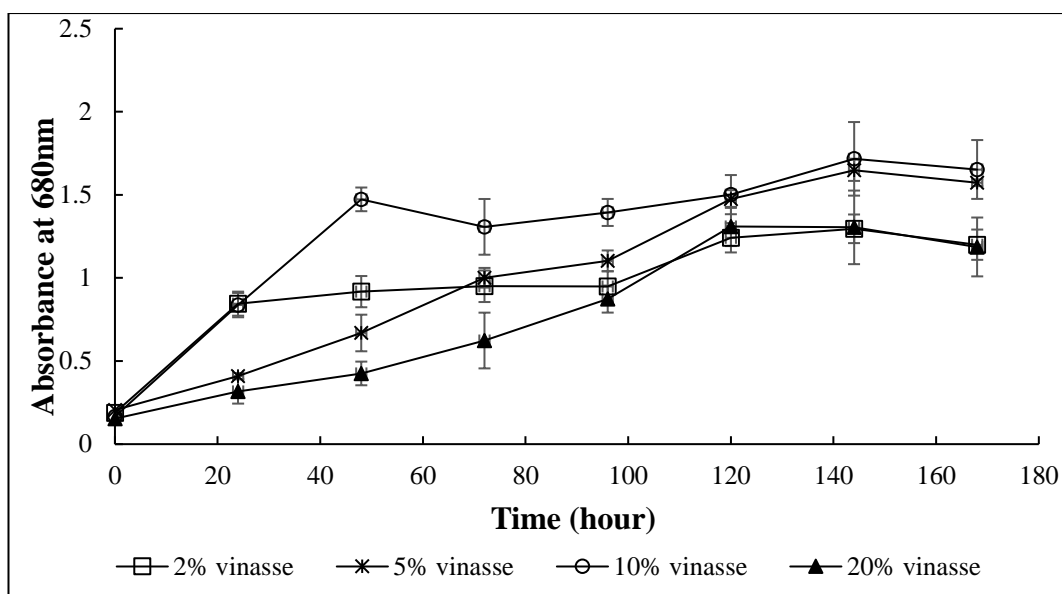
Figure 4.29. Absorbance versus time curve of *Micractinium sp*. ME05 in 1-L medium supplemented with different vinasse concentrations under mixotrophic conditions. Data are mean± standard error from three replicates.

Table 4.14. Biomass concentrations, biomass productivities and growth rates of *Micractinium sp.*ME05 in with different vinasse concentrations under mixotrophic conditions. 2-L flasks with 1-L working volume**.** Data are mean± standard error from three replicates.

	Vinasse concentration (% v/v)			
	2%	**5%**	**10%**	**20%**
Biomass concentration (g/L)	0.60±0.134	1.26±0.106	1.38±0.095	1.28±0.101
Biomass productivity (g.L^{-1}.day^{-1})	0.11±0.027	0.24±0.021	0.27±0.019	0.25±0.020
Growth rate (μ)(d^{-1})	1.25±0.046	1.42±0.017	1.97±0.010	1.42±0.016

4.3.7. Lipid Content and FAME Profile of *Micractinium sp*. ME05 Cultivated with Vinasse in 2L Flasks

Total lipid content and lipid productivities of *Micractinium sp*. ME05 grown in 2L flasks with different vinasse concentrations are given in Table 4.15. With 2% (v/v) vinasse the lipid content and lipid productivities were 5.94±0.62 % and 0.70±0.07 g.L^{-1}.day^{-1}, respectively. The highest lipid content (7.21±0.44 %) and lipid productivity (1.87±0.11 g.L^{-1}.day^{-1}) were obtained with 5% vinasse concentration. Although the highest biomass productivity was observed with 10% vinasse (Table 4.14), higher lipid content of 5% vinasse (7.21±0.44 %) than 10% vinasse (4.90±0.57 %) resulted in higher lipid productivity with 5% vinasse. After 5% vinasse, the lipid content and lipid productivities demonstrated a decreasing pattern. With 10% vinasse the lipid content and lipid productivities were 4.90±0.57 % and 1.30±0.15 g.L^{-1}.day^{-1}, respectively. With 20% vinasse the lipid content and lipid productivities were 4.13±0.12 % and 1.08±0.03 g.L^{-1}.day^{-1}, respectively. A similar trend was observed in the study of Marques et al., (2013), who evaluated the feasibility of anaerobically digested vinasse for cultivation of *Chlorella vulgaris*. Anaerobically digested vinasse were added to the media in ratios of 25%, 50% and 100%. With increasing vinasse concentration, a decreasing pattern was observed in their lipid content and lipid productivities. With 25%, 50% and 100% vinasse, their lipid contents were 26.65%, 24.95% and 23.68%, respectively.

Table 4.15. Lipid content and lipid productivities of *Micractinium sp.* ME05 grown in 2-L flasks with 1-L working volume under different vinasse concentrations. Data are mean± standard error from three biological replicates

Vinasse Concentration (v/v %)			
2%	5%	10%	20%
Lipid Content (% w/w)			
5.94±0.62	7.21±0.44	4.90±0.57	4.13±0.12
Lipid productivity (g.L^{-1}.day^{-1})			
0.70±0.07	1.87±0.11	1.30±0.15	1.08±0.03

Fatty acid methyl ester profile of *Micractinium sp.* ME05 cells cultivated in 2L flasks with different vinasse concentrations (2%-5%-10% and 20%) are given in Table 4.16. It was observed that within all tested vinasse concentrations, the predominant fatty acid was linoleic acid (C18:2), which accounted for 34-45 % total fatty acid present in microalgal lipids. Linoleic acid was followed by palmitic acid (C16:0), which accounted for 16-24% total fatty acid present in microalgal lipids. Oleic acid (C18:1) content was between 5- 23% and linolenic acid (C18:3) content was between 6- 19%.

On the other hand, variations were observed in the fatty acid methyl ester profile of *Micractinium sp.*ME05 cells that were grown with different vinasse concentrations. With 2% vinasse concentration, *Micractinium sp.*ME05 cells were mainly composed of linoleic acid (C18:2), which was 34.1±0.14%. This was followed by palmitic acid (C16:0), whose percentage was 21.3±0.10. These methyl esters were followed by oleic acid (C18:1) and linolenic acid (C18:3), which were close to each other. Oleic acid (C18:1) was 18.6±0.27 % and linolenic acid (C18:3) was 18.1±0.12.

With 5% vinasse concentration, the majority of methyl esters were composed of linoleic acid (C18:2) with a ratio of 44.8±0.15 %. This was followed by palmitic acid (C16:0), oleic acid (C18:1) and linolenic acid (C18:3) with percentages of 24.3±0.20 %, 5.5±0.01 %, and 19.8±0.03 %, respectively.

With 10% vinasse concentration, similarly to 2% and 5%, linoleic acid (C18:2) was the predominant fatty acid (36.3±0.46 %). This was followed by palmitic acid (C16:0) with a content of 22.6±0.25 %. Oleic acid (C18:1) percentage was 14.5±0.24 % and linolenic acid content was 15.0±0.59%.

 With 20% vinasse concentration, linoleic acid (C18:2) was the predominant one with a percentage of 45.4±0.79 %. Linoleic acid was followed by oleic acid (C18:1), palmitic acid (C16:0) and linolenic acid (C18:3), with percentages of 23.1±0.05 %, 16.2±0.24 % and 6.7±0.66 %, respectively

In general, increasing vinasse concentration from 2% (v/v) to 5% (v/v) increased saturated (SFA) and polyunsaturated fatty acid(PUFA) content on the other hand reduced monounsaturated fatty acid (MUFA) content. On the contrary, increasing vinasse concentration from 5% to 10% caused a decline in saturated (SFA) and

polyunsaturated fatty acid(PUFA) content and increase in monounsaturated fatty acid (MUFA) content.

Such variations in methyl ester compositions with different vinasse concentrations were also observed in a study of Zheng et al. (2016). In their study *Chlorella* sp., *Chlorella sorokinana* and *Phaeodactylum tricornutum* cultures were cultivated with different kelp residues concentrations (1%-2%-4%-6% and 8%). They observed an increase in saturated and monounsaturated fatty acids, and a decline in polyunsaturated fatty acids with increasing kelp concentrations from 6% to 8% (S. Zheng et al., 2016).

Moreover, the lipid content results and fatty acid methyl ester profiles obtained in our study were comparable to a study of Calixto et al. (2016). In their study, different bio composts were evaluated for biochemical compositions and fatty acid profile of *Chlorella* sp., *Chlaydomonas* sp., *Lagerheimia longiseta* and *Pediastrum tetras*. Bio-composts that were evaluated were fruit/horticultural wastes (HB), sugarcane waste and vinasse (VB), raw chicken manure (RCM), chicken excrements (BCE) and municipal domestic sewage (MDS). In the study, the lipid contents of *Chlorella* sp., *Chlaydomonas* sp., *Lagerheimia longiseta*, and *Pediastrum tetras* that were grown in sugarcane waste and vinasse (VB) were approximately 8%, 25%, 15% and 14%, respectively. In *Chlorella* sp. and *Chlamydomonas* sp. highest methyl ester was palmitic acid (C16:0), which accounted for 32-38% of total fatty acid methyl ester profile. In *Lagerheimia longiseta* and *Pediastrum tetras* species highest methyl ester was oleic acid (C18:1), which accounted for 37-51% of total fatty acid methyl ester profile (Calixto et al., 2016).

The possibility to use microalgae in biodiesel production depends on the concentrations of fatty acid methyl esters. The chain length and number of double bonds that they contain specifies the physical characteristics of fatty acids. The most common fatty esters that are mainly preferred in biodiesel production are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) methyl esters (Onay et al., 2014). Moreover, the quantity of saturated and unsaturated fatty acids has a great impact on the quality of biodiesel. For instance, saturated fatty acids can resist to deterioration and thus provide endurance to biodiesel;

on the other hand, unsaturated fatty acids increase cold flow properties. Saturated fatty acids also provide protection to oxidation under hot climatic circumstances. Although fatty acid composition has a great impact on the quality of biodiesel, the feasibility of biodiesel is related with the climate condition that the biodiesel is applied. For that reason, to obtain a biodiesel with stable properties, a suitable ratio of unsaturated and saturated fatty acids should be produced (Talebi et al., 2013).

From the results obtained in our study, the high levels of unsaturated fatty acids, especially linoleic acid (C18:2), which accounted for 34-45% of total fatty acids can make biodiesel useful in terms of providing good cold flow properties; and high levels of saturated fatty acid (C16:0), which accounted for 16-24% of total fatty acids can make biodiesel advantageous in terms of providing resistance to oxidative stability and possessing high cetane numbers.

Table 4.16. Percent fatty acid methyl ester amounts (w/w %) of *Micractinium sp.*ME05 grown in 2L flasks with different vinasse concentrations under mixotrophic conditions. Error bars are calculated from mean± standard error from three biological replicates.

	Vinasse Concentration (v/v %)			
FAME	**2%**	**5%**	**10%**	**20%**
Palmitic acid (C16:0)	21.3±0.10	24.3±0.20	22.6±0.25	16.2±0.24
Oleic acid (C18:1)	18.6±0.27	5.5±0.01	14.5±0.24	23.1±0.05
Linoleic acid (C18:2)	34.1±0.14	44.8±0.15	36.3±0.46	45.4±0.79
Linolenic acid (C18:3)	18.1±0.12	19.8±0.03	15.0±0.59	6.7±0.66
Other fatty acids	7.9±0.21	5.6±0.13	11.6±0.15	8.6±0.18

4.4. Bioreactor Studies with *Micractinium sp.*ME05 in Medium Supplemented with Molasses

Optimum cultivation conditions of *Micractinium sp.*ME05 with molasses (19 g/L of molasses hydrolysate, 2 g/L of yeast extract, pH of 6.9, temperature of 30.7 $\pm$1°C) were used for the cultivation of microalgal cells in 2-L bioreactors with 1 L working volume (Figure 4.30). Four different agitation rates (50 rpm- 75 rpm- 100 rpm and 200 rpm) were evaluated with two different inoculum ratios (5% v/v and 10 % v/v).

Figure 4.30. Cultivation of *Micractinium sp.* ME05 cells in 2-L bioreactor with 1-L working volume under heterotrophic conditions.

Obtained biomass concentrations, biomass productivities and growth rates are given in Table 4.17. With 5% (v/v) inoculum, the highest biomass concentration (2.70$\pm$0.12 g/L) and accordingly the highest biomass productivity (0.53$\pm$0.025 g.L^{-1}.day^{-1}) and highest growth rate was obtained with 75 rpm agitation rate. On the other hand, the lowest biomass concentration (2.06$\pm$0.14 g/L) was obtained with 100 rpm agitation

rate. Similarly, with 10% (v/v) inoculum, the highest biomass concentration (2.45±0.015 g/L) was obtained with 75 rpm agitation rate. The biomass productivity and specific growth rate with 75 rpm agitation speed and 10% (v/v) inoculum were 0.48±0.003 g.L^{-1}.day^{-1} and 1.56±0.001, respectively. The lowest biomass concentration (1.95±0.09 g/L) with 10% (v/v) inoculum was obtained with 200 rpm agitation rate.

Table 4.17. Biomass concentrations, biomass productivities and growth rates of *Micractinium sp.* ME05 with different stirring conditions in 2-L bioreactor with 1-L working volume. Data are mean± standard error from two biological replicates.

		Stirring condition			
		50rpm	75rpm	100rpm	200rpm
5% (v/v) inoculum	Biomass concentration (g.L^{-1})	2.69±0.19	2.70±0.12	2.06±0.14	2.18±0.20
	Biomass Productivity (g.L^{-1}.day^{-1})	0.53±0.038	0.53±0.025	0.41±0.029	0.43±0.041
	Growth rate (μ)(d^{-1})	1.58±0.012	1.58±0.008	1.52±0.012	1.53±0.016
10%(v/v) inoculum	Biomass concentration (g.L^{-1})	2.38±0.07	2.45±0.015	2.36±0.28	1.95±0.09
	Biomass Productivity (g.L^{-1}.day^{-1})	0.47±0.014	0.48±0.003	0.46±0.056	0.38±0.019
	Growth rate (μ)(d^{-1})	1.55±0.006	1.56±0.001	1.55±0.024	1.51±0.010

These results demonstrated that agitation rate and inoculum ratio affect biomass concentration and accordingly biomass productivity and growth rate of *Micractinium sp.*ME05 in different ways. To understand their effects analysis of variance (ANOVA) were performed. According to ANOVA results as given in Table 4.18, it was observed that agitation rate had a significant (P=0.035<0.05) effect on biomass concentration, while inoculum ratio (P=0.337>0.05) and the interaction effect of mixing and

inoculum ratio ($P=0.279>0.05$) had non-significant effect on biomass concentration of microalgal cells.

Table 4.18. Two-way ANOVA of agitation speed (rpm) and inoculum ratio (v/v %) for biomass concentration.

Factors	Degrees of freedom	Sums of squares	Mean squares	F-value	p-value
rpm	3	0.73232	0.244106	4.71	0.035
Inoculum	1	0.05406	0.054056	1.04	0.337
Inoculum.rpm	3	0.23847	0.079490	1.53	0.279
Error	8	0.41465	0.051831		
Total	15	1.43949			
S=0.2277 R^2=71.9 % R^2(adj)=45.99%					

Biomass concentration results given in Table 4.17 and ANOVA results given in Table 4.18 showed that biomass concentration increased with an increase in agitation speed. On the other hand, further increase in agitation speed decreased biomass concentration. Agitation changes the intensity of turbulence in a culture medium and turbulence is important in terms of providing homogeneity and oxygen supply while too much turbulence may be harmful to cells and also increases energy consumption. In our study, within 5% (v/v) inoculum the decrease in biomass concentration observed at agitation speed higher than 100 rpm and the decrease in biomass concentration observed at 200 rpm agitation with 10% (v/v) may have resulted from the damage caused by too much agitation speed (Singhasuwan et al., 2015).

A similar trend was reported by Sobczuk et al. (2006), who evaluated the effects of different agitation rates on the growth of *Phaedactylum tricornutum* and *Porphyridium cruentum*. In their study, microalgal cells were cultivated in 5-L bioreactors under various agitation speeds (150-250-350-450-550-650 rpm). The biomass concentration of *P. tricornutum* showed an increase up to 350 rpm, while further increase in agitation speed caused a decline in biomass concentration. On the other hand, the biomass concentration of *P. cruentum* cells tended to increase up to 550 rpm but further

increase in agitation speed caused a decline in biomass concentration. It was reported that although both of the species affected negatively from too much agitation speed, maximum agitation speed that the cell could resist depended on the microalgal species. Lin et al. (2014) evaluated the usage of ketchup for the cultivation of *Ochromonas danica* cells. During the experiments various agitation rates (100 rpm, 200 rpm, 400 rpm and 600 rpm) were evaluated. It was reported that with 600 rpm agitation rate, all of the cells died in 5h. Reducing agitation rate from 600 rpm to 400 rpm also caused damage to *O. danica* cells, but at 100 rpm and 200 rpm agitation rate, no apparent damage occurred (Lin, Raya, & Ju, 2014).

These results demonstrated that too much agitation harmed microalgal cells. Excessive agitation can harm microalgal cells by diminishing the size of the gas bubbles that ruptures at the surface of the culture broth and small bubbles are more harmful to cells (Sobczuk et al., 2006).

4.4.1. Lipid Analysis of *Micractinium sp.*ME05 grown in 2-L Bioreactor with Molasses

Lipid extractions of *Micractinium sp.*ME05 cells grown in 2-L bioreactor with different agitation rates and different inoculum ratios were performed according to Bligh-Dyer method as described in Section 3.2.7. Lipid contents and lipid productivities of *Micractinium sp.*ME05 cells are given in Table 4.19.

With 5% (v/v) inoculum of microalgal cells, the highest lipid content (14.5±1.95 %) and lipid productivity (7.7±1.03 g.L^{-1}.day^{-1}) were observed at 50 rpm agitation rate. Lipid productivity is a result of biomass productivity and lipid content. The biomass productivities of 50 rpm and 75 rpm were the same; 0.53±0.038 g.L^{-1}.day^{-1} for 50 rpm and 0.53±0.025 g.L^{-1}.day^{-1} for 75 rpm (Table 4.17). Although their biomass productivities were the same, the difference in their lipid content (14.5±1.95 % for 50 rpm and 12.2±0.36 % for 75 rpm) led to the difference in their lipid productivity; 7.7±1.03 g.L^{-1}.day^{-1} and 6.5±0.19 g.L^{-1}.day^{-1} for 50 rpm and 75 rpm agitation speed, respectively. On the other hand, the lowest biomass productivity (0.41±0.029 g.L^{-1}.day^{-1}) and lipid content (11.3±1.0 %) that were obtained with 100 rpm agitation

speed caused the lowest lipid productivity (4.6±0.40 g.L^{-1}.day^{-1}) to be observed at 100 rpm agitation speed.

With 10% (v/v) inoculum of microalgal cells, the highest lipid content (16.4±0.92 %) and lipid productivity (6.6±0.37 g.L^{-1}.day^{-1}) were observed at 200 rpm agitation rate. With an increase of agitation speed from 75 rpm to 200 rpm an increase in lipid content from 10.9±0.79 % to 16.4±0.92 % and an increase in lipid productivity from 5.2±0.37 g.L^{-1}.day^{-1} to 6.6±0.37 g.L^{-1}.day^{-1} were observed. The increase in lipid content and accordingly lipid productivity with the increase in agitation speed, may result from supplying sufficient oxygen to microalgal cells. On the other hand, with 50 rpm and 75 rpm agitation speed the lipid productivities were 5.8±0.28 g.L^{-1}.day^{-1} and 5.2±0.37 g.L^{-1}.day^{-1}. The insufficient oxygen at these agitation speed may have resulted in the inadequate growth of microalgal cells.

In microalgal cultivation, agitation is an important parameter due to hindering cell sedimentation, enhancing mass and energy transfer rates, providing air bubbles and homogenous distribution of nutrients that can lead to higher biomass concentration. On the other hand, too much mixing can produce shear conditions that can worsen cell growth. (Pahl et al., 2010).

Similar observations to *Micractinium sp*.ME05 cells, were obtained in a study that were conducted with *Chlorella prothecoides* cells. In the study different carbon sources, different pH levels, different agitation speed were evaluated in terms of their effect on cell growth and lipid production of *C. prothecoides* cells. It was observed that lipid content of microalgal cells tended to increase from 15% (w/w) to 23 % (w/w) with the increase in agitation rate from 100 rpm to 200 rpm. It was also reported that agitation provided homogenous distribution of nutrients and increased air bubbles in the culture medium (Heredia-Arroyo, Wei, & Hu, 2010).

Among 5% (v/v) and 10% (v/v) inoculum size of microalgal cells, the highest biomass concentration (2.69±0.19g/L) and lipid productivity (7.7± 1.03 g.L^{-1}.day^{-1}) were observed with 5% inoculum and 50 rpm agitation speed. Similar results were obtained in a study of Stephenson et al. (2010) who studied the effects of nutrient limitation and initial cell density on lipid production of *Chlorella vulgaris* cells. In the study with

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initial cell densities of 1.3×10^7 cells/mL, 2.5×10^7 cells/mL and 1.8×10^8 cells/mL, the lipid contents were 24±3 %, 19±2 % and 14±1 %, respectively.

Table 4.19. Lipid contents and lipid productivities of *Micractinium sp*.ME05 grown in 2-L bioreactor with 1-L working volume. Data are mean±standard error from three biological replicates.

		Agitation speed			
		50rpm	75rpm	100rpm	200rpm
5% (v/v) inoculum	**Lipid content (% w/w)**	14.5±1.95	12.2±0.36	11.3±1.0	11.2±0.51
	Lipid productivity (g.L^{-1}.day^{-1})	7.7±1.03	6.5±0.19	4.6±0.40	5.8±0.27
10%(v/v) inoculum	**Lipid content (% w/w)**	12.3±0.6	10.9±0.79	14.3±0.83	16.4±0.92
	Lipid Productivity (g.L^{-1}.day^{-1})	5.8±0.28	5.2±0.37	6.4±0.16	6.6±0.37

4.4.2. Fatty Acid Methyl Ester (FAME) Analysis of *Micractinium sp.* ME05 grown in 2-L Bioreactor with Molasses

In order to evaluate the suitability of microalgal cells to be used in biodiesel synthesis, their fatty acid content is a crucial factor. The chain length and number of double bonds that they contain specifies the physical characteristics of fatty acids. The most common fatty esters that are mainly preferred in biodiesel production are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3) methyl esters (Onay et al., 2014). Moreover, the raw materials that are intended to be used for biodiesel production should meet some conditions published by some local governments. As reported by European Biodiesel Standards, fatty acid methyl esters that contain four or more double bonds should not exceed 1%, and linolenic acid methyl ester (C 18:3) should not exceed 12 % (Chisti, 2007). Therefore to make a

decision about microalgal cells potential to be used for biodiesel production, it is necessary to evaluate the fatty acid profile of microalgal cells (Huang et al., 2010). Fatty acid methyl ester composition of *Micractinium sp.*ME05 that were cultivated under different agitation rates (50 rpm-75 rpm-100 rpm and 200 rpm) and inoculum ratios are given in Table 4.20.

It was observed that with 5% (v/v) inoculum of *Micractinium sp*. ME05 cells to the bioreactor, the main fatty acid methyl ester was linoleic acid (18:2), which accounted for 40-44 % total fatty acid present in microalgal lipids for all agitation rates. Linoleic acid (18:2) was followed by palmitic acid (16:0) and oleic acid (18:1), which accounted for 19-27% and 16-25% of total fatty acid present in the microalgal lipids, respectively.

Similarly, with 10% (v/v) inoculum of *Micractinium sp.* ME05 cells to the bioreactor, linoleic acid (18:2) was the predominant fatty acid methyl ester constituting 37-45 % of the total amount of fatty acid methyl esters present in microalgal lipids. Palmitic acid (16:0) and linoleic acid (18:1) followed linoleic acid (18:2) within the range 23-30% and 12-17%, respectively.

As a whole, palmitic acid (16:0) and linoleic acid (18:2) generated about 70% of total fatty acids present in *Micractinium sp.* ME05 lipids, which would be a valuable source for biodiesel production. Since fatty acid profile of biodiesel feedstocks generally consists of palmitic acid (16:0), stearic acid (18:0), palmitoleic acid (16:1), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3), the abundance of palmitic acid (16:0) and linoleic acid (18:2) in microalgal lipids in this study, can make *Micractinium sp*. ME05 desirable for biodiesel application (Knothe, 2008). The linolenic acid methyl esters (18:3) contributed 4-9% of total FAME, which is lower than the maximum value that is acceptable by European Biodiesel Standards (Chisti, 2007).

Moreover, saturated fatty acid methyl esters are advantageous in terms of their high cetane numbers, which is related to the ignition quality of diesel fuel. However, saturated fatty acids demonstrate poor cold flow properties. Polyunsaturated fatty acids possess low melting points, which is important for low temperature properties

however their cetane number is generally low, which is undesirable for biodiesel (Knothe, 2008).

The fatty acid methyl ester results of *Micractinium sp*. ME05 cells obtained in this study were comparable with those reported in the literature. In a study conducted with *Chlorella zoefingiensis* cells, the usage of cane molasses for cell growth, lipid production, and astaxanthin production were evaluated. For that purpose, *C. zoefingiensis* cells were cultivated heterotrophically with different molasses concentrations (5 g/L- 10 g/L-15 g/L-20 g/L-30 g/L-40 g/L and 50 g/L). It was reported that palmitic acid (16:0) and linoleic acid (18:2) constituted about 50 % of total fatty acid methyl esters present in *C. zoefinginesis* lipids (Jin Liu, Huang, Jiang, & Chen, 2012). Karpagam et al.(2015) evaluated different nutrients such as sugarcane industry effluent, citric acid, glucose, and vitamin B_{12} for cultivation of *Coelastrella sp*.M-60 and *Micractinium sp*.M-13 species. With 2.5mL/L effluent applied to the cultivation medium, the lipid content of *Micractinium sp*.M-13 cells were about 17% (w/w). Within the cultivation medium that contained 25 mg/L citric acid and 1.25mL/L sugarcane industry effluent, palmitic acid constituted about 25% of total fatty acid methyl esters. Gaurav et al.(2015) evaluated cane molasses for the heterotrophic cultivation of *Chlorella pyrenoidosa* cells. With 10 g/L molasses as carbon source, the fatty acid composition of *C. pyrenoidosa* cells were mainly consisted of palmitic acid (16:0) and linoleic acid (18:2), which constituted 81% of total fatty acid methyl esters.

In addition, an increase in palmitic acid content (16:0) from 21.7±0.3 to 23.0±0.6, linoleic acid content (18:2) from 44.3±1.0 to 45.2±0.8, and linolenic acid content (18:3) from 5.1±0.0 to 8.1±0.5 was observed by changing agitation speed from 50 rpm to 200 rpm and inoculum ratio from 5% (v/v) to 10 (%). On the other hand, a decrease in biomass concentration from 2.69±0.19 g/L to 1.95±0.09 g/L was observed by changing agitation speed from 50 rpm to 200 rpm and inoculum ratio from 5% (v/v) to 10 (%) (Table 4.17). The increase in palmitic acid, linoleic acid and linolenic acid content with an increase in agitation speed and inoculum ratio can be explained by the increase in total lipid content from 14.5±1.95 to 16.4±0.92 under these conditions.

Table 4.20. Percent fatty acid methyl ester amounts of *Micractinium sp.*ME05 with 5% (v/v) and 10% (v/v) inoculum to the bioreactor. Data are mean± standard error from three biological replicates.

FAME	5% (v/v) inoculum			
	50 rpm	75 rpm	100 rpm	200 rpm
Palmitic acid (16:0)	21.7±0.3	19.6±0.2	26.4±0.3	27.5±0.9
Oleic acid (18:1)	23.0±0.1	25.1±0.0	17.4±0.8	16.1±0.6
Linoleic acid (18:2)	44.3±1.0	43.7±1.1	40.4±0.9	42.7±1.0
Linolenic acid (18:3)	5.1±0.0	4.0±0.1	7.8±0.2	8.9±0.1
10% (v/v) inoculum				
	50 rpm	75 rpm	100 rpm	200 rpm
Palmitic acid (16:0)	30.2±0.3	26.1±0.5	28.1±0.6	23.0±0.6
Oleic acid (18:1)	12.3±0.5	14.5±0.3	16.7±0.4	17.2±0.0
Linoleic acid (18:2)	44.1±1.1	39.6±0.9	37.7±0.4	45.2±0.8
Linolenic acid (18:3)	7.9±0.2	8.3±0.4	9.0±0.5	8.1±0.5

4.5. Growth Evaluation of *Micractinium sp.*ME05 Cultivated in 5-L Bioreactor with 10% (v/v) Vinasse

In previous vinasse experiments as depicted in Section 4.3.6., it was observed that with 10% (v/ v) vinasse concentration under mixotrophic cultivation conditions *Micractinium sp.* ME05 cells gave the highest biomass concentration, biomass productivity and growth rate. Therefore 5-L bioreactor experiments were conducted with 10% (v/v) vinasse concentration (Figure 4.31).

Figure 4.31. Growth of *Micractinium sp.* ME05 in 5-L bioreactor with 10% (v/v) vinasse.

Biomass concentrations obtained during incubation period is given in Figure 4.32. It was observed that biomass growth started from the beginning of the inoculation period. A biomass concentration of 0.89 ± 0.16 g/L was obtained within 48 hours. After 72 hours and 96 hours of incubation period the biomass concentration were increased to 1.23 ± 0.22 g/L and 1.76 ± 0.1 g/L, respectively. At the end of the incubation period the biomass obtained was 1.95 ± 0.2 g/L. The biomass productivity at the end of the incubation period was 0.32 ± 0.2 g.L^{-1}.day^{-1}. These results demonstrated that microalgal cells could be able to utilize organic matter present in vinasse. However the decrease in biomass increase rate after 120 hours may resulted from the inability of microalgal cells' utilizing large molecules which was also reported in a study of de Mattos & Bastos, 2015.
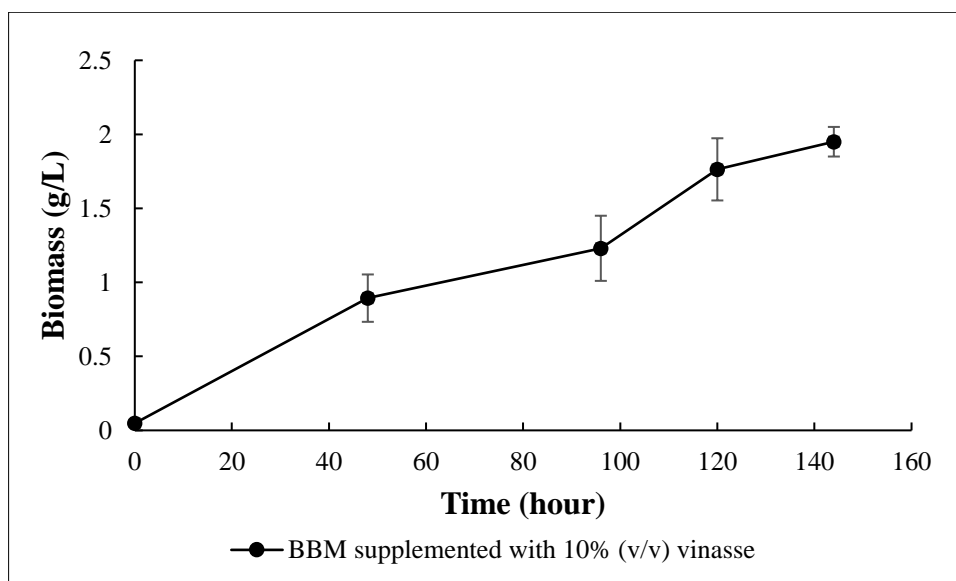
Figure 4.32. Observed biomass quantities of *Micractinium sp.*ME05 in BBM supplemented with 10% (v/v) vinasse in 5-L bioreactor. Error bars are calculated from standard error from two biological replicates.

The biomass concentration (1.95±0.2 g/L) and biomass productivity (0.32±0.2 g.L^{-1}.day^{-1}) obtained at the end of the incubation period were comparable with the studies performed by others. Santana et al. (2017) evaluated the usage of sugarcane vinasse for the growth of *Micractinium sp*. Embrapa LBA32 and *C. biconvexa* Embrapa LBA40. Large scale cultivation was carried out in 15 L flat-plate bioreactors with 13-L working volume. Medium was supplemented with 50% (v/v) vinasse. At the end of the incubation period the biomass productivities of *Micractinium sp*. Embrapa LBA32 and *C. biconvexa* Embrapa LBA40 were 0.17g.L^{-1}.day^{-1} and 0.18 g.L^{-1}.day^{-1}, respectively.

On the other hand, too much vinasse concentration looks like to inhibit microalgal growth. Barrocal et al. (2010) also observed a decrease in biomass productivity of *Spirulina maxima* cells with the increase in vinasse concentration. The biomass productivity of *S. maxima* cells decreased from 240 mg.L^{-1}.day^{-1} to 0 mg. L^{-1}.day^{-1} with the increase of vinasse concentration from 1 g/L to 7 g/L. Similarly, in our study vinasse concentration more than 10% (v/v) vinasse decreased microalgal growth.

These findings highlighted that *Micractinium sp.* ME05 cells were able to grow in medium supplemented with vinasse. However, vinasse concentration >10% (v/v) vinasse reduced cell concentration, which may result from the decrease in light transmittance with increasing vinasse concentration. Besides, addition of antibiotics to the cultivation medium and regular pH control prevented the growth of fungal contaminants, which is a major problem in studies performed with vinasse.

4.6. Lipid content and FAME Profile of *Micractinium sp.* ME05 cultivated in 5L bioreactor with 10% (v/v) vinasse

Micractinium sp. ME05 cells that were cultivated in 5-L bioreactor were harvested by centrifugation and lipid extractions were performed as described in Section 3.2.7. According to lipid extraction results, the total lipid content and lipid productivities were 10.7 ± 0.57 % and 3.4 ± 0.20 $g.L^{-1}.day^{-1}$, respectively. The fatty acid profile demonstrated that the highest fatty acid content was linoleic acid (C18:2), which constituted 49.98 ± 1.07 % of total fatty acid present in microalgal lipids. Linoleic acid was followed by palmitic acid (C16:0), which constituted 21.03 ± 0.62 % of total fatty acid content. The remaining fatty acids were oleic acid (C18:1) and linolenic acid (C18:3) with percentages of 16.43 ± 1.36 % and 7.00 ± 0.18 %, respectively (Table 4.21).

The lipid productivity and FAME profile obtained in 5-L bioreactors were similar to the study of Mitra et al. (2012), who evaluated the application of ethanol thin stillage (vinasse) and soy whey as a nutrient for the cultivation of *Chlorella prothecoides*. The lipid productivity of *C. prothecoides* cells that were cultivated in 6-L bioreactor with soy whey and thin stillage were 0.2 ± 0.02 $g.L^{-1}.day^{-1}$ and 1.1 ± 0.1 $g.L^{-1}.day^{-1}$, respectively. The fatty acid methyl ester profile consisted of high percentages of PUFA's. Oleic acid (C18:1) and linoleic acid (C18:2) content of microalgal cells grown with thin stillage were 21.4 ± 0.2 % and 52.2 ± 0.4 %, respectively.

In comparison to 2-L flask experiments as reported in Section 4.3.7, the lipid content (10.7 ± 0.57 %) and lipid productivities (3.4 ± 0.20 $g.L^{-1}.day^{-1}$) obtained in 5L bioreactors were higher than the lipid content (4.90 ± 0.57 %) and lipid productivities (1.30 ± 0.15 $g.L^{-1}.day^{-1}$) obtained in 2-L flasks with 10% vinasse. Since lipid productivity was a result of lipid content and biomass productivity, higher lipid

productivities in 5-L bioreactors may have resulted from the higher biomass concentration (1.95±0.2 g/L) obtained in 5-L bioreactor. Moreover, the reason for obtaining better results in 5-L reactor than 2-L flasks may have resulted from controlled environment provided by reactor. Agitation may have contributed a positive effect by providing homogenous distribution of nutrients and oxygen and providing sufficient light distribution to microalgal cells.

Palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), and linolenic acid (18:3) methyl esters are most commonly preferred fatty esters for biodiesel production. So, the high content of fatty acids such as palmitic acid, linoleic acid, and oleic acid obtained in our study could make vinasse derived microalgal oils applicable to biodiesel production.

Moreover, since lipid productivity demonstrates the simultaneous effect of biomass productivity and lipid content, lipid productivity is the key criterion in order to assess and determine the success of a microalgal cultivation system (Heidari, Kariminia, & Shayegan, 2016). According to the bioreactor experiment results obtained in our study, it is clear that the high lipid productivity resulted from high biomass productivity of *Micractinium sp.*ME05 in vinasse. High biomass productivity of *Micractinium sp.* ME 05 in a low-cost material like vinasse makes the strain desirable for further studies in optimization of lipid production from vinasse.

Table 4.21. Percent fatty acid methyl ester amounts of *Micractinium sp.*ME05 with 10% (v/v) inoculum to the 5-L bioreactor. Data are mean± standard error from two biological replicates.

FAME	10 %(v/v) vinasse
Palmitic acid (C16:0)	21.03±0.62
Oleic acid (C18:1)	16.43±1.36
Linoleic acid (C18:2)	49.98±1.07
Linolenic acid (C18:3)	7.00±0.18

4.7. Cost Evaluation of Cultivating Microalgal Cells with Glucose, Molasses Hydrolysate and Vinasse

The main barrier for the commercialization of microalgal biomass for biodiesel production is its high capital and operating costs (Zhou et al., 2014). It was reported that the production cost of microalgal biomass that contains 40% oil by weight should not exceed $ 227/m^3, to be able to compete with petroleum whose price is $ 629 /m^3 ($100/barrel). Acien et al. (2012) stated that generating microalgal biomass in 3m^3 tubular bioreactors cost about € 69/ kg and with a more simple and economic technology this cost was reduced to € 12.6/ kg. It was also stated that even if a raceway pond can produce biomass at 10% of this price, the total biomass production cost would still be higher than $ 0.25/kg (Chisti, 2013). Nagarjan et al. (2013) estimated that biodiesel production was between $1.68 to $ 75 per liter. This cost was calculated according to a biomass productivity more than 30 g.m^{-2}.d^{-1} and oil content of 50% of total biomass. For that reason, producing microalgal oil that can compete with petroleum fuels requires further efforts (Chisti, 2013)

Moreover 50% of the cost of microalgal cultivation comes from the cost of carbon source (Cheng et al., 2009). For that reason, the cost of the bold basal medium that was supplemented with glucose and molasses hydrolysate were calculated according to current prices of the components in these media. 1-L of bold basal medium (BBM) cost that was supplemented with 10g/L and 30g/L glucose was 7.85 TL and 15.71 TL, respectively. The cost of cultivation of microalgal cells in 1-L bold basal medium supplemented with molasses and vinasse were 2.74 TL and 0.41 TL, respectively. Price of BBM that was supplemented with glucose was calculated based on the current prices of medium components and glucose. Price of BBM that contain molasses hydrolysate and vinasse were calculated according to current prices of medium components and chemicals used in pretreatment.

By taking into consideration the prices calculated above, it was observed that cultivation in glucose (10g/L) supplemented medium were 2.8 and 19 fold more expensive than that of supplemented with molasses hydrolysate and vinasse, respectively. L. Xiufeng et al.(2007) also stated that the cost of glucose constituted approximately 80% of total medium and suggested the usage of low cost raw materials

that can substitute glucose. For that reason, using raw materials like molasses and vinasse was a good strategy to reduce the cultivation cost. Moreover, by using molasses as carbon source, a biomass productivity of 0.74 ± 0.02 g.L^{-1}.day^{-1} was achieved at a cost of 2.74 TL, whereas by using vinasse as the carbon source, a productivity of 0.27 ± 0.019 g.L^{-1}.day^{-1} was achieved at a lower cost of 0.41 TL. Although biomass productivity of molasses based media was higher than vinasse based media, by taking into consideration the medium costs, using vinasse would be more advantageous for cost efficient biomass production.

The costs calculated above were only the costs of the variables for three different substrates (glucose, molasses and vinasse). To determine the total production cost of microalgal cultivation for all of the culture conditions tested (cultivation with glucose, molasses and vinasse); reactor operating costs, pretreatment costs, harvesting costs, oil extraction costs, power consumption and labor costs should be considered and should be added to the costs calculated above.

CHAPTER 5

CONCLUSION

This study investigated the potential use of sugar industry by-product -molasses- as carbon source for the cultivation of *Chlamydomonas reinhardtii* 137C⁻ and *Micractinium sp.* ME05. For this purpose, different carbon sources were evaluated in the growth media of these species.

C. reinhardtii 137C⁻ cells were tested in basal medium and TAP medium with different glucose, sucrose, and molasses concentrations. *C. reinhardtii* 137C⁻ cells could only grow in TAP medium which indicated that microalgal cells could not metabolize carbon sources other than acetate. For that reason, further studies were performed with *Micractinium sp.* ME05 cells and focused on optimization of culture conditions *Micractinium sp.* ME05 cells with molasses as carbon source.

Variables which were selected based on literature survey (pH, temperature, inoculum ratio, carbon source concentration, and yeast extract concentration), were employed in optimization studies.

Molasses, an inexpensive by-product of sugar industry, was shown to serve as an appropriate carbon source for heterotrophic biomass production from *Micractinium sp.* ME05 cells by optimizing cultivation conditions using Plackett-Burman and Response Surface Method.

It was concluded that biomass production of *Micractinium sp.* ME05 cells with molasses as carbon source, was affected significantly by the concentration of carbon source, pH, and temperature.

A 2-fold increase in biomass productivity was achieved by optimizing culture conditions of *Micractinium sp*.ME05 cells. The optimization results demonstrated that a maximum biomass concentration (2.08g/L) and biomass productivity (0.41±0.02 g/L) was achieved at pH 6.9, 30.7±1°C, and with 19 g/L molasses hydrolysate. This biomass productivity was scaled up to 2-L bioreactors and an improvement in biomass productivity was achieved (0.53± 0.038 g.L^{-1}.day^{-1}).

Vinasse, as another inexpensive by-product of sugar industry, was shown to be an alternative carbon source for the cultivation of *Micractinium sp.* ME05 cells. It was evaluated with different concentrations (2%-5%-10% and 20% v/v) for biomass and lipid production from *Micractinium sp.* ME05 cells. 500-mL and 2-L flask trials demonstrated that microalgal cells produced highest biomass concentration when supplemented with 10 % (v/v) vinasse under mixotrophic conditions. When scaled up to 5-L bioreactors a 2-fold increase in biomass productivity was achieved compared to 500-mL flask trials.

High lipid productivity and rich fatty acid methyl ester contents of *Micractinium sp.* ME05 grown with molasses and vinasse, revealed the feasibility of these inexpensive by-products for biodiesel production.

In terms of biomass productivity (g.L^{-1}.day^{-1}) and lipid productivity (g.L^{-1}.day^{-1}), higher results were obtained with molasses than that of vinasse. Both of the substrates are by-products of sugar industry and their application is advantageous for a cost-efficient biomass production, but direct disposal of vinasse to the environment is a major concern. For that reason, efficient usage of vinasse for microalgal cultivation would be advantageous in terms of eliminating vinasse from the environment.

As a conclusion, present study is the first study that focused on the optimization of heterotrophic culture conditions for native thermo-resistant *Micractinium sp.* ME05 cells by using industrial by-products. This is also the first report that evaluated the usage of vinasse for the cultivation of native strain *Micractinium sp.* ME05. For these reasons, this study has a crucial importance in terms of producing large biomass quantities, while eliminating industrial by-products in an environment-friendly way.

In future studies, optimization of culture conditions with vinasse could be advantageous in terms of eliminating by product-vinasse and producing microalgal biomass in a cost-efficient way.

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COMPOSITION OF MOLASSES AND VINASSE

Table A.1. Composition of undiluted molasses

Parameters	Unit	Result of Chemical Analysis
Total sugar	mg/kg	60
Calcium (Ca)	mg/kg	0.7
Potassium (K)	mg/kg	10.7
Sodium (Na)	mg/kg	0.17
Iron (Fe)	mg/kg	0.01
Magnesium (Mg)	mg/kg	0.01
Copper (Cu)	mg/kg	0.65
Invert sugar	g/kg	0.3
Glucose	g/kg	71
Fructose	g/kg	2.7
Sucrose	g/kg	440
Crude Fat	g/kg	0
Crude Protein	g/kg	112
Ash	g/kg	115

Table A.2. Composition of undiluted vinasse

Parameters	Unit	Result of Chemical Analysis
BOI	mg/L	>35700
COD	mg O_2/L	2346880
Total solids	mg/kg	65
Total Phosphorus	mg/kg	0.1
Ammonia	mg/kg	0.4
Calcium (Ca)	mg/kg	1.3
Potassium	mg/kg	15
Magnesium	mg/kg	0.01
Total nitrogen	g/kg	31
Crude Ash	g/kg	187

BUFFERS AND SOLUTIONS

Tris-Acetate-Phosphate (TAP) Medium

Table B.1. Composition of TAP medium (Adapted from Culture Collection of Cryophilic Algae)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
Tris Base	2.42 g	$H_2NC(CH_2OH)_3$	-	$2.00 \times 10^{-2}M$
B-solution	50 mL	NH_4Cl	15.0 g/L	$7.00 \times 10^{-3}M$
		$CaCl_2.2H_2O$	4.00 g/L	$8.30 \times 10^{-4}M$
		$MgSO_4.2H_2O$	2.00 g/L	$4.50 \times 10^{-4}M$
Phosphate Buffer	1 mL	K_2HPO_4	288 g/L	$1.65 \times 10^{-3}M$
		KH_2PO_4	144 g/L	$1.05 \times 10^{-3}M$
Trace Elements Solution	1 mL	$Na_2EDTA.2H_2O$	50.0 g/L	$1.34 \times 10^{-4}M$
		$ZnSO_4.7H_2O$	22.0 g/L	$1.36 \times 10^{-4}M$
		H_3BO_3	11.4 g/L	$1.84 \times 10^{-4}M$
		$MnCl_2.4H_2O$	5.00 g/L	$4.00 \times 10^{-5}M$
		$FeSO_4.7H_2O$	5.00 g/L	$3.29 \times 10^{-5}M$
		$CoCl_2.6H_2O$	1.60 g/L	$1.23 \times 10^{-5}M$
		$CuSO_4.5H_2O$	1.60 g/L	$1.00 \times 10^{-5}M$
		$(NH_4)_6MoO_3$	1.10 g/L	$4.44 \times 10^{-6}M$
Acetic Acid	1 mL	CH_3COOH	-	-

Indicated quantities of medium components in Table B.1 are dissolved in approximately 850 mL distilled water and then completed to 1 L final volume. Final pH is adjusted to 6.5-7.0 and sterilized by autoclave at 121°C for 20 minutes.

For solid medium 1.5% Agar is added to TAP medium before autoclaving.

Basal Medium

Table B.2. Composition of basal medium (adapted from Xiong et al., 2008)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
Dipotassium phosphate	0.7g	K_2HPO_4	-	$4.011 \times 10^{-3} M$
Potassium diphosphate	0.3g	KH_2PO_4	-	$2.204 \times 10^{-3} M$
Magnesium sulfate heptahydrate	0.3g	$MgSO_4.7H_2O$	-	$1.217 \times 10^{-3} M$
Glycine	0.1g	$C_2H_5NO_2$	-	$1.332 \times 10^{-3} M$
Iron(II) sulfate heptahydrate	0.003g	$FeSO_4.7H_2O$	-	1.079×10^{-5}
Vitamin B_1	0.1 mL	$C_{12}H_{17}N_4OS+$	0.1g/L	$0.004 \times 10^{-4} M$
Trace elements solution	1 mL	H_3BO_3	2.86 g/L	$0.047 \times 10^{-3} M$
		$MnCl_2. 4H_2O$	1.81 g/L	$0.009 \times 10^{-3} M$
		$ZnSO_4. 7H_2O$	0.22 g/L	$0.007 \times 10^{-4} M$
		$Na_2MoO_4. 2H_2O$	0.39 g/L	$0.016 \times 10^{-4} M$
		$CuSO_4. 5H_2O$	0.08 g/L	$0.003 \times 10^{-4} M$

Indicated quantities of medium components in Table B.2 (except vitamin B_1) are dissolved in approximately 850 mL distilled water and then completed to 1 L final volume. Final pH is adjusted to 6.8 and sterilized by autoclave at 121°C for 20

minutes. Vitamin B_1 is filter sterilized and added to the sterile medium under aseptic conditions.

Bold's Basal Medium (BBM)

Table B.3. Composition of bold basal medium (Adapted from Culture Collection of Cryophilic Algae)

Stock Solution (SS)	Volume	Component	Concentration in SS	Concentration in Final Medium
SS 1	10 mL	$NaNO_3$	25.0 g/L	2.98×10^{-3}M
SS 2	10 mL	$MgSO_4.7H_2O$	7.5 g/L	3.04×10^{-4}M
SS 3	10 mL	$NaCl$	2.5 g/L	4.28×10^{-4}M
SS 4	10 mL	K_2HPO_4	7.5 g/L	4.31×10^{-4}M
SS 5	10 mL	KH_2PO_4	17.5 g/L	1.29×10^{-3}M
SS 6	10 mL	$CaCl_2.2H_2O$	2.5 g/L	1.70×10^{-4}M
SS 7	1 mL	H_3BO_3	11.4 g/L	1.85×10^{-4}M
EDTA-KOH Solution	1 mL	$EDTA.Na_2$	50.0 g/L	1.71×10^{-4}M
		KOH	31.0 g/L	5.53×10^{-4}M
Ferric Solution	1 mL	$FeSO_4.7H_2O$	4.9 g/L	1.79×10^{-5}M
		H_2SO_4	1 mL	-
Trace Elements Solution	1 mL	$ZnSO_4.7H_2O$	8.8 g/L	3.07×10^{-5}M
		$MnCl_2.4H_2O$	1.4 g/L	7.28×10^{-6}M
		MoO_3	0.7 g/L	4.93×10^{-6}M
		$CuSO_4.5H_2O$	1.5 g/L	6.29×10^{-6}M
		$Co(NO_3)_2.6H_2O$	0.5 g/L	1.68×10^{-6}M

Indicated quantities of medium components in Table B.3 are dissolved in approximately 850 mL distilled water and then completed to 1 L final volume. Final pH is adjusted to 6.8 and sterilized by autoclave at 121°C for 20 minutes.

DNS Reagent

Table B. 4. Composition of Dinitrosalicylic Acid Solution, 1%

Component	Quantity
Dinitrosalicylic acid	10 g/L
Phenol	2 g/L
Sodium sulfite	0.5 g/L
Sodium hydroxide	10 g/L
Potassium sodium tartrate solution	40%

STANDARD CURVES OF SUGAR DETERMINATION

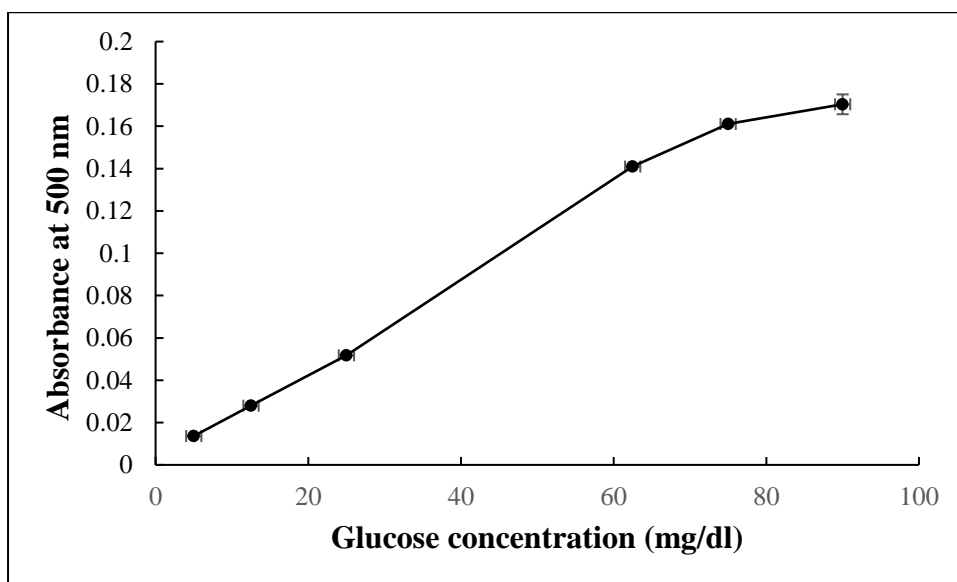**Standard Curve for Glucose in Glucose Oxidase Kit**

Figure C.1. Glucose standard curve for glucose oxidase kit
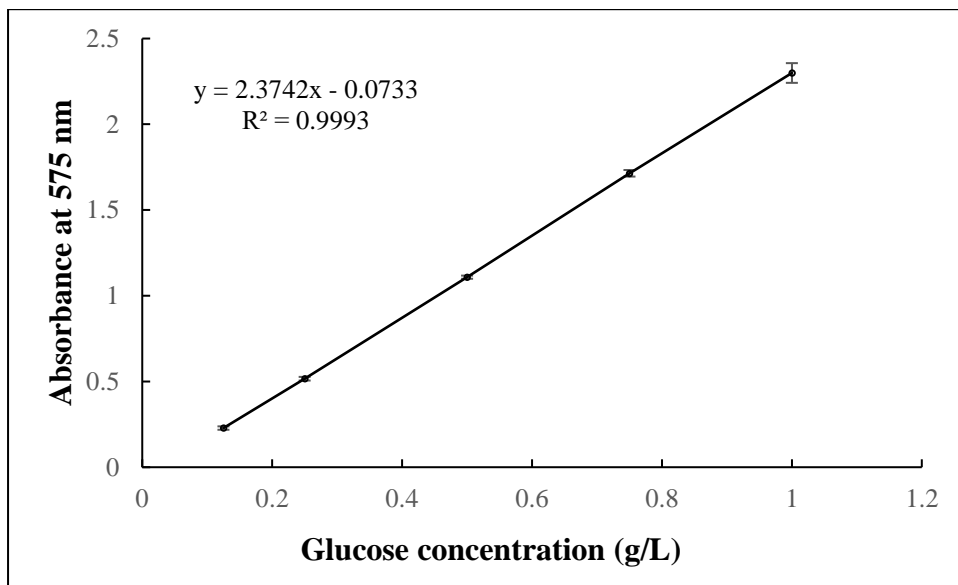
Standard Curve for Total Reducing Sugar

Figure C.2. Standard curve for DNS method

APPENDIX D

BIOMASS AND LIPID FORMULAS

Biomass Formulas

$$\text{Biomass concentration (g.L}^{-1}) = \frac{\text{dry weight (g)}}{\text{volume of culture (L)}}$$

$$\text{Biomass productivity (g.L}^{-1}.\text{day}^{-1}) = \frac{B_2 - B_1}{t_2 - t_1}$$

*B_1: Biomass concentration (g/L) on t_1 time,

*B_2: Biomass concentration (g/L) on t_2 time.

$$\text{Specific growth rate } (\mu) = \frac{ln(X_1\text{-}X_2)}{t_2 - t_1}$$

X_1: Biomass concentration at the end of the time interval,

X_2: Biomass concentration at the beginning of the time interval,

t_2-t_1: time elapsed between selected interval.

$$\text{Biomass yield on glucose } (Y_{x/s}) = \frac{\text{g biomass produced}}{\text{g susbtrate consumed}}$$

Lipid Formulas

$$\text{Lipid content (\%)} = \frac{\text{weight of extracted oil (g)}}{\text{dry weight (g)}} \times 100$$

$$\text{Lipid productivity (g.L}^{-1}.\text{day}^{-1}) = \text{Biomass productivity X lipid content}$$

CURRICULUM VITAE

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EDUCATION

2011-Present	Middle East Technical University, Ankara /Turkey
	PhD in Department of Biotechnology (C.GPA: 3.50/4.00)
2008-2011	Middle East Technical University, Ankara /Turkey
	MSc. in Department of Biotechnology (C.GPA:3.07/4.00)
2004-2008	Ege University, İzmir/Turkey, BSc in Department of Biology
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PROJECT WORK and SCHOLARSHIPS

1. Improvement of microalgal based recombinant protein expression platform. The Scientific and Technological Research Council of Turkey (TUBITAK)-

1001- Scientific and Technological Project (Project No:114Z487) (Postgraduate Project Scholar) (January 2016- July 2017).

2. In-house Validation of DNA Extraction Method from Rye, Maize, Oat, and Wheat Flour Samples. The Scientific and Technological Research Council of Turkey (TUBITAK)-1001- Scientific and Technological Project (Project No:112O779) (Postgraduate Project Scholar) (March 2013- March 2015).

3. Optimization of Heterotrophic Growth Conditions of Microalgae Using Agricultural Wastes. METU Research Fund Project, METU DAP, January 2014-January 2015, Researcher.

4. Identification, Subtyping of Probiotic Microorganisms Isolated from Kefir and Their Usage in Probiotic Yoghurt. METU BAP Research Fund Project, BAP-03-14-2011-006, 2010-2011, Researcher.

WORK EXPERIENCE and INTERNSHIPS

July 2012- October 2017   Metu Central Laboratory Molecular Biology and Biotechnology R&D Center, Ankara

Part-time biologist in Genome Analysis Laboratory

August 2007- October 2007   Mersin Food Control Laboratory Directorate, Mersin

Intern at Physical Analysis Laboratory, Chemical Analysis Laboratory and Microbiology Laboratory.

July 2006-August 2006   Pınar Meat Inc., İzmir

Intern at Microbiology and Chemistry Laboratories. Research about food pathogens and their molecular identification.

PUBLICATIONS AND POSTER PRESENTATIONS

Publications

1. **Engin, K.I.,** Cekmecelioglu, D., Yücel M., Öktem, H.A. (2017). Enhancement of heterotrophic biomass production by *Micractinium sp.* ME05. *Waste and Biomass Valorization.* doi:10.1007/s12649-017-9846-8.

2. **Engin, K.I.,** Cekmecelioglu, D., Yücel M., Öktem, H.A. Heterotrophic growth and oil production using *Micractinium sp.* ME05 with molasses. Manuscript submitted to *Journal of Bioscience and Bioengineering*.

3. **Engin, K.I.,** Cekmecelioglu, D., Yücel M., Öktem, H.A. Evaluation of Heterotrophic and Mixotrophic Cultivation of Novel *Micractinium sp.* on Vinasse and its Scale Up. Manuscript under preparation.

Poster Presentations

1. **Engin, K.I.,** Cekmecelioglu, D., Yücel M., Öktem, H.A. (2015). Biomass production from *Micractinium sp.*ME05 cells under heterotrophic conditions. (Poster No: 17). Poster presentation in 18. Ulusal Biyoteknoloji Kongresi.

2. **Engin, K.I**., Cekmecelioglu, D., Yücel M., Öktem, H.A. (2013). A rapid approach to evaluation of heterotrophic biomass production by *Micractinium sp.* ME05 (Poster No: 11). Poster presentation in International Conference on Microalgae and Biofuels, 30-31 July, Koç University, Istanbul, Turkey.

3. Yılmaz, R., Köksel, H., Bayraç, C., Başman, A., **Köse, I.** (2015). In-house Validation of DNA Extraction Method from Rye, Maize, Oat and Wheat Flour Samples. Poster presentation in 7th international qPCR & NGS Symposium, 23-27 March, Technical University of Munich, Freising, Germany.