REPUBLIC OF TURKEY
YILDIZ TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CULTIVATION OF *NANNOCHLOROPSIS OCULATA* AND *TETRASELMIS SUECICA* IN TREATED MUNICIPAL WASTEWATER TOWARD MICROALGAL BIOETHANOL PRODUCTION

ZUBAIDAI REYIMU

MSc. THESIS
DEPARTMENT OF BIOENGINEERING
PROGRAM OF BIOENGINEERING

ADVISER
ASSOC. PROF. DR. DİDEM ÖZÇİMEN

İSTANBUL, 2016

REPUBLIC OF TURKEY

YILDIZ TECHNICAL UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

CULTIVATION OF *NANNOCHLOROPSIS OCULATA* AND *TETRASELMIS SUECICA* IN TREATED MUNICIPAL WASTEWATER TOWARD MICROALGAL BIOETHANOL PRODUCTION

A thesis submitted by Zubaidai REYIMU in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** is approved by the committee on 18.11.2016 in Department of Bioengineering, Bioengineering Program.

Thesis Adviser

Assoc. Prof. Dr. Didem Öz çimen

Yıldız Technical University

Approved By the Examining Committee

Assoc. Prof. Dr. Didem Öz çimen

Yıldız Technical University _____

Prof. Dr. İbrahim Işıldak

Yıldız Technical University _____

Prof. Dr. Fatma Gülen İskender

İstanbul Technical University _____

ACKNOWLEDGEMENTS

This study aims to investigate the influence of ISKI municipal wastewater as a cultural media to two different microalgae samples. Wastewater medium was designed with different ratio in order to find out optimum concentration of wastewater. Moreover, experiments on bioethanol production using microalgae samples which is grown in specific concentration of wastewater medium were implemented and analyzed according to results.

Firstly, I would like to express my sincere gratitude to my advisor Assoc. Prof. Dr. Didem ÖZÇİMEN for the continuous support to my MSc study and research, for her patience, motivation, enthusiasm and immense knowledge. She helped me not only on my research, but also helped me to be a motivated, passionate and cooperative researcher during whole this time. She is one of the most outstanding professors that I ever met.

Besides, I would like to thank Assoc. Prof. Dr. M. Sinan Bilgili for supplying ISKI municipal wastewater for my experiments.

I also would like to thank Bioengineer Benan INAN, Bioengineer Anıl Tevfik KOÇER and Bioengineer Dilek ÖZTAY who provided valuable guidance and help on my research and help me to adjust to school life of TURKEY. They already became my outstanding partner in Laboratory, best friends in life.

Moreover, I would like to thank Yurdışı Türkler ve Akraba Topluluklar Başkanlığı who gave me this amazing opportunity to finish my graduate study in TURKEY with Turkiye burslari scholarship program.

Last but not least, I would like to thank my parents who supported my study abroad and always willing to be by my side. I also would like to thank my husband for his wise counsel and sympathetic ear.

October, 2016

Zubaidai REYIMU

TABLE OF CONTENTS

Page

LIST OF SYMBOLS viii

LIST OF ABBREVIATIONS ix

LIST OF FIGURES x

LIST OF TABLES xi

ABSTRACT xii

ÖZET xiv

CHAPTER 1

INTRODUCTION 1

1.1 Literature review 1
1.2 Algae Classification 2
1.2.1 Macroalgae 2
1.2.2 Microalgae 3
1.3 Objectives of the thesis 4
1.4 Original contribution 5

CHAPTER 2

CULTIVATION OF MICROALGAE 6

2.1 Microalgae cultivation techniques 6
2.1.1 Open pond production system 6
2.2 Closed system 7
2.3 Culture medium and wastewater medium 8
2.3.1 Microalgae growth medium 8
2.3.2 Wastewater medium 9
2.4 Microalgae growth factors 9

2.4.1 Light intensity 10
2.4.2 Photoperiod 10
2.4.3 Temperature 10
2.4.4 Salinity, pH and Mixing/turbulence 10
2.5 Microalgae harvesting and dewatering 11
2.5.1 Gravity sedimentation 12
2.5.2 Flocculation 12
2.5.3 Centrifugation 12
2.5.4 Filtration 12
2.5.5 Flotation 12
2.5.6 Electrophoresis techniques 13
2.6 Measurement of Microalgae Growth 13
2.7 Total carbohydrate content 13
2.8 Microalgae growth kinetics 14
CHAPTER 3
BIOETHANOL PRODUCTION FROM MICROALGAE 17
3.1 Preparation of microalgae biomass 17
3.2 Pretreatment of microalgae biomass 17
3.2.1 Chemical pretreatment 18
3.2.2 Enzymatic pretreatment 18
3.2.3 Mechanical pretreatment 18
3.3 Fermentation 19
3.3.1 SHF 19
3.3.2 SSF 19
3.4 Ethanol recovery 20
CHAPTER 4
EXPERIMENTAL STUDY 21
4.1 Materials and culture conditions 21
4.1.1 Microalgae strain 21
4.1.2 Materials 22
4.1.3 Cultural condition 22
4.2 Analytical method 25
4.2.1 Optical density (OD) 25
4.2.2 Cell counting 26

4.2.3 Microscopic observation 28
4.2.4 Total carbohydrate analysis by phenol-sulfuric method 29
4.3 Evaluation of growth kinetics 30
4.4 Bioethanol production 30
4.4.1 Preparation of microalgae biomass 30
4.4.2 Pretreatment of microalgae biomass 31
4.4.3 Preparation of yeast 31
4.4.4 Fermentation 31
4.4.5 Determination of bioethanol concentration 31
CHAPTER 5
RESULTS AND DISCUSSION 33
5.1 Growth measurements 33
5.2 Total carbohydrate content 38
5.3 Dry weight 39
5.4 Growth kinetics-specific growth rate and cell doubling time 42
5.5 Bioethanol production 43
CONCLUSION 46
REFERENCES 47
CURRICULUM VITAE 54

LIST OF SYMBOLS

ΔN	The rate of increase of cells
Δt	Per unit of time
N	Cell concentration at the given time
μ	Specific growth rate
$N_{\circ}$	Cell density at the beginning of a time interval (t) in the exponential phase
Nt	Cell density at the end of a time interval (t) in the exponential phase
t_d	Cell doubling time

LIST OF ABBREVIATIONS

Daf	Dry and ash free
DT	Doubling time
FASW	Filtered autoclaved seawater
FID	Flame ionization detector
ISKI	Istanbul Water and Sewerage Administration
LB	Luria broth
OD	Optical density
SGR	Specific growth rate
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TOC	Total organic carbon
UV	Ultraviolet
WW	Wastewater

LIST OF FIGURES

Page

Figure 1.1 Seaweed (Macroalgae) [12] 3
Figure 1.2 Marine microalgae [19] 4
Figure 2.1 Open pound system for microalgae cultivation [25] 7
Figure 2.2 Tubular photobioreactor [27] 8
Figure 4.1 Initial culture medium of *N. oculata* (a) and *T. suecica* (b) 23
Figure 4.2 Shaking incubator that used in this study 25
Figure 4.3 T60 UV-VIS Spectrophotometer 26
Figure 4.4 (a) Hemocytometer [70] and (b) counting grid [71] 27
Figure 4.5 Morphological figures of *N.oculata* (A) and *T.suecica* (B) under microscope 28
Figure 4.6 OLYMPUS CX22 Microscope 28
Figure 4.7 Standard samples for total carbohydrate content measurement 30
Figure 4.8 YL 6100 GC gas chromatography 32
Figure 5.1 Optical density versus culture period (day) for *N.oculata* in control and different wastewater concentration (25%, 50%, 75%, and 100%) 34
Figure 5.2 Optical densities versus culture period (day) for *T.suecica* in control and different wastewater concentration (25%, 50%, 75%, and 100%) 34
Figure 5.3 Cell densities versus culture period (day) for *N. oculata* in control and different wastewater concentration (25%, 50%,75% and 100%) 36
Figure 5.4 Cell densities versus culture period (day) for *T. suecica* in control and different wastewater concentration (25%, 50%, 75% and 100%) 36
Figure 5.5 Comparison of cell densities of *N.oculata* (red) and *T.suecica* (blue) in control, and in different wastewater concentration (25%, 50%, 75% and 100%) 37
Figure 5.6 Milled fine powder of *N.oculata* 40
Figure 5.7 Milled fine powder of *T.suecica* 41
Figure 5.8 Bioethanol concentrations of *N.oculata* and *T.suecica* during 24 hours and 48 hours of fermentation 44

LIST OF TABLES

Page

Table 2.1 Growth rate of different microalgae strain in different medium 16
Table 4.1 List of chemicals used in this study 22
Table 4.2 TOC analysis of ISKI wastewater 23
Table 4.3 Cultivation condition of *N.oculata* and *T.suecica* 24
Table 4.4 Standard glucose concentration values 29
Table 5.1 Total carbohydrate content of *N.oculata* and *T.suecica* 38
Table 5.2 Dry weight of *N.oculata* and *T.suecica* in different concentration of wastewater medium 42
Table 5.3 Specific growth rate (μ) and doubling time (td) of *N.oculata* and *T.suecica* 43

ABSTRACT

CULTIVATION OF *NANNOCHLOROPSIS OCULATA* AND *TETRASELMIS SUECICA* IN TREATED MUNICIPAL WASTEWATER TOWARD MICROALGAL BIOETHANOL PRODUCTION

Zubaidai REYIMU

Department of Bioengineering

MSc. Thesis

Adviser: Assoc. Prof. Dr. Didem ÖZÇİMEN

Due to the fast growth of world population and rapid development of economy, energy demands increased all around the world. Today, most of the energy comes from fossil fuels that is the main reason for several problems such as global warming and environmental pollution. To solve these problems and built better future for the next generations, environment friendly sustainable fuels must be developed.

In this case, first generation biofuel feedstocks such as wheat and corn are criticized for low-land use efficiency and huge demand for arable land. As for second generation biofuels feedstocks, they are considered unsustainable and its cost on pretreatment process made them uneasy to develop further. Therefore, attentions on third generation feedstocks are becoming much higher recently. Comparing with other feedtocks, as third generation biofuel feedstocks, algae need less cultivation area and they can be cultivated in different types of water including wastewater. Most importantly, its efficiency of biofuel production is much higher due to their high lipid, protein, as well as carbohydrate content.

In this study, wastewater supplied from Istanbul Water and Sewerage Administration (ISKI) were utilized as a growth medium for *Nannochloropsis oculata* and *Tetraselmis suecica* microalgae strain under the same growth conditions, and its effect on cell proliferation and growth kinetics were investigated. It was found that, both *N.oculata* and *T.suecica* can tolerate and utilize the wastewater and the results showed that, the specific growth rate of *N.oculata* can reach up to 0.5430 d^{-1} (75% of wastewater), and

0.4778 d^{-1} (25% of wastewater) in *T.suecica*. To evaluate the bioethanol production performance of these two strains, samples, which included maximum carbohydrate content as well as control groups, were chosen for further study. The results showed that *T.suecica* is much available for ethanol production using municipal wastewater as a cultural medium due to its higher carbohydrate concentration than those of *N.oculata* .

Key words: Bioethanol, *Nannochloropsis oculata*, *Tetraselmis suecica*, wastewater.

ÖZET

MİKROALGAL BİYOETANOL ÜRETİMİ İÇİN *NANNOCHLOROPSIS OCULATA* VE *TETRASELMIS SUECICA'NIN* BELEDİYE ATIKSUYUNDA YETİŞTİRİLMESİ

Zubaidai REYIMU

Biyomühendislik Anabilim Dalı

Yüksek Lisans Tezi

Tez Danışmanı: Doç. Dr. Didem ÖZÇİMEN

Dünya nüfusunun ve ekonomisinin hızlı gelişimi tüm dünyada enerji taleplerinin artmasına yol açmıştır. Günümüzde kullanılan enerjinin büyük bir kısmının fosil yakıtlardan elde edilmesi, bu tür yakıtların küresel ısınma gibi birçok çevre sorunlarını da beraberinde getirmesine yol açmaktadır. Gelecek nesillerin ihtiyaçlarını karşılamak ve çevre problemlerini en aza indirgemek amacıyla yeni, çevre dostu ve sürdürülebilir yakıtların geliştirilmesi gerekmektedir.

Buğday ve mısır gibi birinci nesil biyoyakıt hammaddeleri, gıda maddesi olarak kullanımları, yüksek üretim maliyeti ve düşük arazi kullanım verimliliği gibi sorunları nedeniyle tartışmalara yol açmaktadır. İkinci nesil biyoyakıt hammaddelerinin ise, ön muamele aşamalarının zor olması ve bu maliyetlerinin yüksek olması nedeniyle biyoyakıtlar üzerine yapılan çalışmalar üçüncü nesil biyoyakıt hammadesi olan mikroalgler üzerine yoğunlaşmıştır. Diğer hammaddelerle karşılaştırıldığında, alglerin büyümeleri için geniş arazilere ihtiyaç yoktur, atıksu da dahil olmak üzere farklı ortamlarda yetiştirilebilir ve yüksek yağ, protein ve karbonhidrat içeriği ile biyoyakıt üretiminde umut vaat etmektedir.

Bu çalışmada, İstanbul Su ve Kanalizasyon İdaresinden (İSKİ) temin edilmiş olan atıksu kullanılarak, *Nannochloropsis oculata* ve *Tetraselmis suecica* türü deniz alglerinin aynı büyüme ortamı ve koşullar altında hücre çoğalması ve büyüme kinetikleri araştırılmıştır. Deneysel çalışmalar sonucunda, *N.oculata* mikroalginin en yüksek hücre büyüme hızının 0.5430 d^{-1} (%75 atıksuda) olduğu görülmüş, *T.suecica* türünde ise maksimum büyüme hızının 0.4778 d^{-1} (%25 atıksuda) olduğu belirlenmiştir. Ayrıca, bu iki tür deniz

mikroalglerinin biyoetanol üretim performansının değerlendirilmesi için, kontrol grubu ve maksimum karbonhidrat içeriğine sahip olanları biyoetanol üretiminde kulanılmış, belediye atıksuyu medium olarak kullanıldığında *T.suecica* türünün yüksek karbonhidrat içeriği nedeniyle etanol üretim verimliliğinin *N.oculata* türüne kıyasla biyoetanol üretimi için daha uygun olduğu tespit edilmiştir.

Anahtar Kelimeler: Atıksu, Biyoetanol, *Nannochloropsis oculata, Tetraselmis suecica*

CHAPTER 1

INTRODUCTION

1.1 Literature review

As the world energy consumption increased due to the expansion of the world population and increase of industrial prosperity, the major energy demand is still supplied from conventional fossil fuels such as oil, coal, and natural gas [1,2].

First generation biofuels such as bioethanol from wheat, biogas from corn, and biodiesel from rapeseed oil have been criticized for low land-use efficiency, increasing demand on arable land, and poor carbon balance [3] and the production of first generation biomass also leads to the rising of food price. This increasing concern about the first generation feedstock has raised attention to the potential of second-generation biofuels. Second generation biofuel production has the advantages such as consuming waste residues and making use of abandoned land and promote rural development. However, according to Singh et al., 2011, second-generation biofuel crops and production technologies are not so efficient, their production could become unsustainable as they also compete with food crops for available land. The limitations of first-generation biofuels have put greater emphasis on the use of second-generation biofuels produced from lignocellulosic feedstocks, they still facing more challenges to handle when it comes to the sustainability and renewability [2]. This has led to a renewed interest in renewable energy resources such as solar energy, wind, biomass, geothermal, wave and hydrogen energy. As for sustainable biofuels production, it is recommended to use third generation feedstocks such as algae, as they contain a high level of lipids and carbohydrates that they are considered to be the most promising and environment friendly raw materials for biofuel production. Moreover, algae can grow 20-30 times quicker than food crops holding up to 30 times more fuel than other biofuel sources. It

also can produce up to 10000 gallons per acre of biofuel per year while other sources such as corn would only produce 60 gallons per acre [4].

The main benefits of using algae for biofuel production are: (i) they need less water when growing than terrestrial crops and also can be cultivated in saline water, brackish water or wastewater; (ii) they can be cultivated in non-arable lands and without competing with food crops; (iii) they can contribute to carbon dioxide mitigation, since they may grow in high CO_2 concentrations from coal-fired power stations; and (iv) they have high photosynthetic efficiency compared to terrestrial crops, which may lead to high productivity [5].

However, there are also some drawbacks about using algae for biofuel production. The most crucial problem is its low sufficiency to produce algae on large commercial scale because of high production costs [6]. Huge investments are required in growing algae as flat lands and abundant water are essential [7]. Algae cultivation should be near to water resources and places which are far from seashores are not able to be access to water sources.

1.2 Algae Classification

Algae are simple organisms that are mainly aquatic and microscopic. There are two main populations of algae according to [8], filamentous and phytoplankton algae. They are categorized into four main classes: diatoms, green algae, blue-green algae and golden algae [8]. They are often unicellular (microalgae) or multicellular (macroalgae) [9].

1.2.1 Macroalgae

Macroalgae, also known as seaweeds, are multicellular macroscopic marine organisms (Figure 1.1). According to Peng et al., 2015 [10], marine macroalgae can be divided into three main phyla with their pigmentation differences. Phaeophyta (brown seaweeds); Chlorophyta (green seaweeds); and Rhodophyta (red seaweeds). They are the species that have been used all over the world for thousands of years for different purpose including food and nonfood use. In one hand, marine macroalgae have been used as a food in Asian countries for long time ago, on the other hand, it can also be used by many countries as a nonfood resources such as to feed animals, medicinal purpose, agricultural applications and even for production of fuels in recent years [11].

With the development of technology, recent studies are focusing on the much efficient application processes of marine macroalgae such as biofuel production. According to Marquez et al., 2014 [13], most of the seaweed species are ideal for biofuel production as they met the important criteria for efficient biofuel production such as (i) the amount of seaweeds are large in many costal area; (ii) they can be harvested all year round without effected by season; (iii) the cultivation of marine macroalgae can be sustainable when compared with other resources and do not require land for growth. These criteria made marine macroalgae promising for biofuel production such as biogas, bioethanol and biodiesel.



Figure 1.1 Seaweed (Macroalgae) [12]

1.2.2 Microalgae

As for microalgae, they are known as unicellular microorganisms that can be found in marine and freshwater with different size ranging from a few micrometers to a few hundreds of micrometers (Figure 1.2) [14]. They include prokaryotic microalgae such as cyanobacteria, and eukaryotic microalgae such as green algae, red algae and diatoms [15]. They are submerged in an aqueous environment, where they can get efficiently get access to water, CO_2 and other nutrients, so that they are much more efficient in converting solar energy into biomass [16]. According to Lu ś a, 2011[17], microalgal biomass has a high caloric value, low viscosity and low density that it can be more

suitable for biofuel production than lignocellulosic materials. They are promising and ideal feedstock for different types of renewable biofuels including biodiesel, bioethanol, biohydrogen and methane [18].

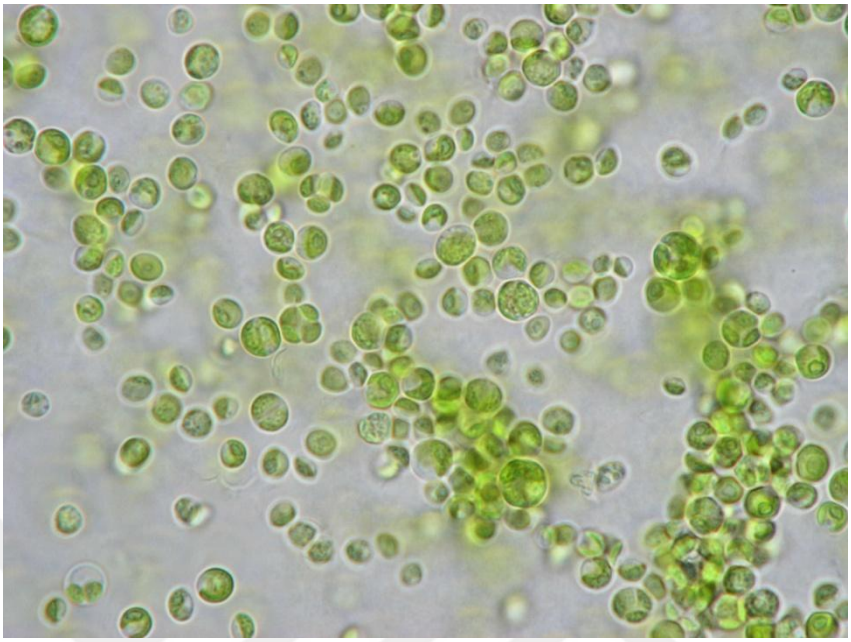


Figure 1.2 Marine microalgae [19]

When comparing macroalgae and microalgae for biofuel production, [14], microalgae have several advantages. Firstly, microalgae have much higher carbohydrate productivity than macroalgae, because of their fast cell growth rate. Secondly, most of the monosaccharide that derived from the carbohydrates produced from some microalgae species is glucose. On the contrary, the major building blocks of polysaccharides that found in macroalgae are alginate and mannitol, which are much more difficult to ferment with yeasts. So that microalgae is much more suitable for converting its biomass to biofuels such as bioethanol [20, 21].

1.3 Objectives of the thesis

In this study, municipal wastewater obtained from ISKI was utilized as a medium with different ratio to cultivate two different types of marine microalgae *Nannochloropsis oculata* and *Tetraselmis suecica*. In addition to evaluating the cultivation of these microalgae species in wastewater of ISKI, the most productive species which have the highest carbohydrate content were chosen for bioethanol production and bioethanol yields of these samples were investigated.

1.4 Original contribution

Recently, more studies are focusing on the cultivation of different types of microalgae in various wastewater medium in order to reduce the production costs. In literature, the sources are limited by only a few numbers of microalgae strains and researches are continued to optimize the wastewater ratios to utilize them as growth medium. In order to suggest a solution to decrease the cost of the algal cultivation, as an alternative growth medium, wastewater supplied from Istanbul Water and Sewerage Administration (ISKI) was utilized for cultivation of *Nannochloropsis oculata* and *Tetraselmis suecica,* in this study. There is not any study which was investigated algal cultivation in municipal wastewater of Istanbul and evaluation of bioethanol production potential of these microalgae cultivated in this wastewater. Obtained data from this study will provide original contributions to the literature.

CHAPTER 2

CULTIVATION OF MICROALGAE

2.1 Microalgae cultivation techniques

As for the mass production of microalgal biomass, different types of culturing systems are used including open ponds and photobioreactor. Many parameters must be taken into account when choosing cultivation type. These can be summarized as biology of the microalgae; the cost of land, energy, water, and nutrients; local climatic conditions; and final product [22]. In this part, these main culturing systems will be specifically introduced.

2.1.1 Open pond production system

Open pond system is an old traditional system which is used since the 1950s (Figure 2.1). According to Suali and Sarbatly, 2012 [23], open ponds can be divided into raceway ponds, circular, inclined and unmixed ponds. Among all, raceway ponds are widely applied due to the fact that race way ponds are easy to set up and the cheapest for large-scale microalgae production [15]. The size of raceway ponds are generally between 0.2 and 0.5 m deep, and often stirred with a paddlewheel to provide circulation of the algae and nutrients [22]. Raceway ponds are easier to build and operate, however, its productivity is not so promising due to the fact that raceway ponds are easily contaminated by other microorganisms, mixing of microalgae and nutrients are poor, uneven illumination, and some growth parameters such as light intensity, temperature, pH and dissolved oxygen concentration are difficult to control [22, 24].

Therefore, more systematic and modern cultivation techniques such as photobioreactors are required to reach maximum productivity of microalgae.



Figure 2.1 Open pound system for microalgae cultivation [25]

2.2 Closed system

As open ponds are more risky when the contamination and other negative influences are considered, researchers are suggested to use closed system to eliminate such disadvantages. Closed photobioreactors are ideal for such requirements (Figure 2.2) [23]. However they are very expensive systems for algal cultivation. Photobioreactor includes different subcategories such as tubular, vertical, flat-plate, annular, fermenter-type and internally illuminated photobioreactors.

Although photobioreactors are often extremely expensive, they are recommended to use for production of highly pure microalgae strains and sensitive strains. Besides, in photobioreactors, essential nutrients and other growth elements such as light intensity, temperature, pH and CO_2 should be set up to its optimum level in advance so that microalgae can reach its ideal growth conditions [16].

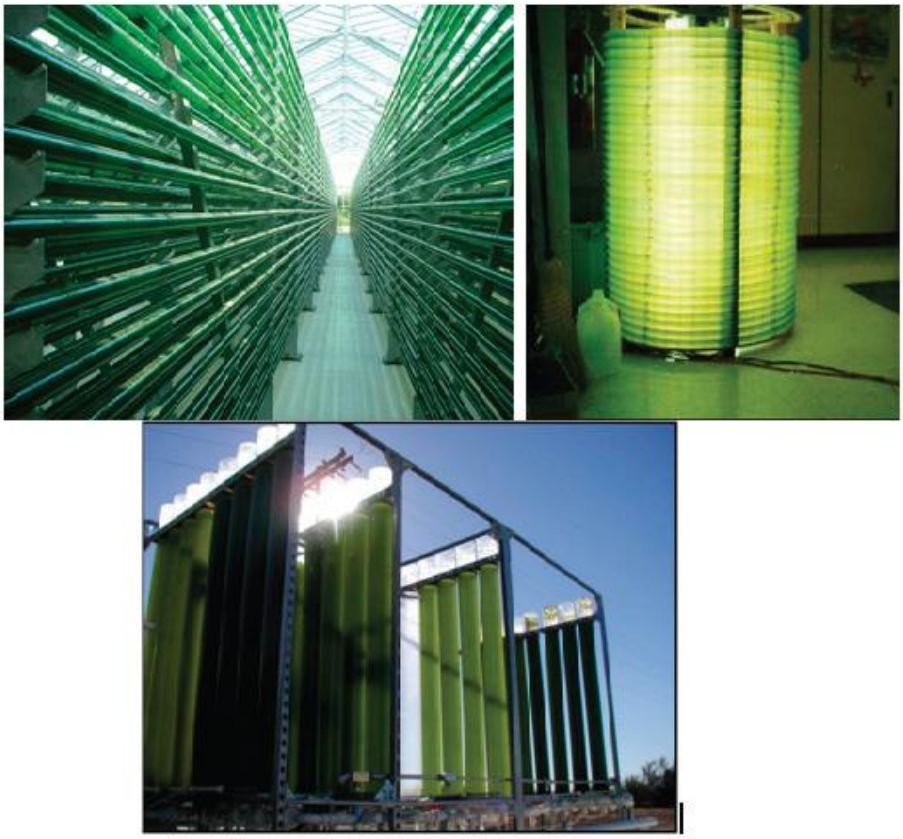


Figure 2.2 Tubular photobioreactor [27]

2.3 Culture medium and wastewater medium

Microalgal growth is widely influenced by nutrient types and amounts. According to P. Singh, Gupta, Guldhe, Rawat, and Bux, 2015 [28], microalgae require plentiful inorganic nutrients such as nitrogen, phosphorus, and vitamins and trace elements such as iron, manganese, selenium, zinc, cobalt and nickel. These nutrients are vital factors when determining the productivity of microalgae. To produce efficient microalgal biomass, should be highly imitated like its natural growth habits including nutrients, lights and other factors. Subsequently, growth medium is essential to provide such optimal growth condition for microalgae.

2.3.1 Microalgae growth medium

Microalgae growth medium can be divided into two groups such as freshwater media and marine algal media. Cultural media that used for freshwater algae are BG11, COMBO medium, DY-V medium, DY-III medium, and MES volvox medium; as for marine algae, Black sea medium, ES medium, ASP medium, Aquil medium, Allen's

Cyanidium medium, CCAP artificial seawater, Chry medium, ESAW medium, ESM medium, f/2 medium, K medium, L1 medium, MNK medium, Pro99 medium, and SN medium, etc. [28].

2.3.2 Wastewater medium

There are still limitation in industrial cultivation of algae such as high cost of culture medium may influence the productivity and sustainability of microalgae. Apart from growth medium, wastewater medium can be an alternative for cultivating microalgae. In some studies related to wastewater cultivation, urban wastewater, domestic wastewater, industrial wastewater and even slaughterhouse wastewater are been used. The advantage of using wastewater as an culture medium are, as wastewater includes plenty of nutrients, they provide microalgae different nutrients that microalgae needed in their growth. Secondly, wastewater treatment process can be performed while they are used as cultural medium. Because the excess of nutrients and other toxic compounds in wastewater is the main cause of eutrophication and ecological damage of natural water basin [29], microalgae can astonishingly remove the major pollutants such as nitrogen and phosphorus up to 41% and 30%, respectively [30]. Therefore, microalgae cultivation can be combined with wastewater treatment to achieve economically productive microalgae biomass yield, as well as removal of pollutants in wastewater.

However, there are some limitations when using wastewater as culture medium. The efficiency of different microalgae in removing nutrients from different types of wastewater as well as achieving higher productivities is different. One of the criteria is to choose microalgae strain that have good wastewater tolerance, so that maximum benefits can be achieved from microalgae [31].

2.4 Microalgae growth factors

Productivity of microalgal biomass is highly influenced by outer factors such as light intensity, photoperiod, temperature, salinity, pH, and mixing/turbulence, etc. [28]. It is essential to adjust these parameters to its optimum level so that ideal amount of microalgal biomass can be produced.

2.4.1 Light intensity

Among all the environmental factors affecting the growth rates of microalgae, light is considered to be the most important factors in photosynthesis [32]. According to Lazaro, Varesche, and Silva, 2015 [33], light intensity vary according to the microalgae species. Besides, microalgae can be better grown and more productive with the increase of light intensity and red light was the optimal light wavelength for microalgae growth. Low light intensities may negatively affect the logarithmic growth of microalgae [32, 34].

2.4.2 Photoperiod

In the experimental research of [32], the author indicated that with the increase of light intensity from 50 to 200 μmol m^{-2} s^{-1}, photoperiod (L: D cycle) of *Nannochloropsis sp.* reduced from 24:0 at 50 μmol m^{-2} s^{-1} to 12:12 h at 200 μmol m^{-2} s^{-1}. In another similar experiment, author claimed that the specific growth rate of *Chlorella vulgaris* is highest at 24:00 h light and dark cycle at 100 μmol m^{-2} s^{-1}, when comparing to and 12:12 h light and dark cycle at 200 μmol m^{-2} s^{-1} and 12:12 h light and dark cycle at 300 μmol m^{-2} s^{-1} [35]. These results showed that the photoperiod of microalgae is highly related to the light intensity and increase of light and dark cycle corresponds to the increase in microalgae growth. Therefore, optimal photoperiod must be chosen according to the previous researches and analysis.

2.4.3 Temperature

As we all know, temperature is the most important factor for most of the microorganisms growth including microalgae as temperature always comes together with the metabolic activities and enzyme kinetics of microalgae. The average temperature of microalgae is from 16 to 27 ℃, and optimal temperature range is from 18 to 24 ℃ [28]. Although microalgae can be also cultivated well at lower temperature, it is hard to reach our ideal productivity. So the optimal temperature of microalgae differ from each other due to the differences on chemical structure and natural culture habits.

2.4.4 Salinity, pH and Mixing/turbulence

Salinity is another important factor for microalgal growth. According to [36], the experimental result showed that as for microalgae species *Tetraselmis chuii*, the maximum growth rate happens at 40 ppt when comparing to 20, 30 and 50 ppt. another

experiment showed that, the specific growth rate of both *Nannochloropsis sp.* and *Tetraselmis sp.* was maximum when cultured at 30 ppt under control condition than 20 and 40 ppt [37].

The pH of the culture media is also important for microalgae growth. But the specific pH value is different according to the sensitiveness of microalgae species. When adjusting the pH, hydrochloric acid and sodium bicarbonate acid can be used [37].

In addition to these, one of the important factors for microalgae growth is mixing/turbulence. The aim of mixing is to make all the nutrients in medium become available to every single microalgae cell so that we can prevent gravitational sedimentation and uneven dispersing of light [38]. At the same time, microalgae are also sensitive to stress and harsh mechanical stirring, rapid mixing may cause cell damage and this kind of damage is usually related to the cell size and type of cell wall. There are several kinds of mixing including mechanical stirring, aeration with air pumps, paddle wheels and jet pumps, etc [28].

2.5 Microalgae harvesting and dewatering

After production of microalgae biomass, it is essential to remove water in the culture for further biofuel production process. Among all the processes', harvesting is the most costly that accounts for 20%-30% of the total production cost [39]. Therefore, it is important to choose efficient harvesting methods for different types of microalgae.

There are several types of harvesting methods such as gravity sedimentation, flocculation, centrifugation, filtration and microscreening, flotation and electrophoresis techniques [40]. The selection of harvesting method highly depends on the cell properties such as density, size and the value of desired products [15].

Microalgae harvesting can be divided into a two-step process. First step is bulk harvesting during which microalgae biomass can be separated from the bulk culture such as flocculation, flotation, or gravity sedimentation. In this step, the biomass concentration can reach to 2%-7% dry weight. Second step, however, is thickening, where biomass can be concentrate through technologies such as centrifugation and filtration [41].

2.5.1 Gravity sedimentation

Gravity sedimentation is usually used for separating microalgae in water. It is the most common method for algal biomass in wastewater treatment as it can treat the large volumes and generate the low value of biomass. But the method is only suitable for large microalgae (ca.>70 μm) such as *spirulina* [15]

2.5.2 Flocculation

Flocculation is generally used to increase the efficiency of gravity sedimentation. It belongs to the bulk harvesting step that solute particles in a solution join together to form aggregates called floc [39]. As microalgae cells carry negative charge to prevent natural aggregation of cells in suspension, flocculation process can use to reduce or neutralizes the negative charge with the addition of flocculants such as multivalent cations and cationic polymers [15]

2.5.3 Centrifugation

Among all the harvesting, centrifugation is the most useful one for laboratory scale, and it is preferred for harvesting of microalgae biomass which produce extended shelf-life concentrates for aquaculture. But centrifugation is not so productive for large scale biomass harvesting as it is time consuming and also costly [41].

2.5.4 Filtration

In filtration, algae is obtained and waters passes through specific filter to reach harvesting aim. Filtration usually used to harvest larger microalgae and as for smaller microalgae, microfiltration can be implemented. But the process can be costly as it is vital to renew the membrane frequently [39]. There are other problems for using filtration such as membrane clogging, the formation of compressible filter cake and high maintenance costs [40].

2.5.5 Flotation

Flotation is regarded to be more effective for small, unicellular algal biomass harvesting. In the process, air or gas bubbles are attached to solid particles and then carry them to the liquid surface. This method has been indicated to be more effective and beneficial than sedimentation. On the basis of bubble size, flotation can be divided into dissolved

air flotation, dispersed flotation and electrolytic flotation [39, 41].

2.5.6 Electrophoresis techniques

Electrophoresis technique is potential method for separating algae without using any chemicals. In this method, an electric field derives charged algae to move out of the solution. The advantage of using electrochemical methods includes its environmental compatibility, versatility, energy efficiency, safety, selectivity, and cost effectively [41, 42]

2.6 Measurement of Microalgae Growth

There are lots of methods for analyzing the growth productivity. It can be summarized as spectrophotometer, gravimetric biomass determination, cell enumeration using counting chambers, and flow cytometry [43]. Spectrophotometer is for measuring the cell density and chlorophyll content. The wavelength from 400 to 700 μm used to determine the growth.

Cell enumeration is implemented by counting chambers. The most widely used type of chamber is called a hemocytometer [44]. However, when counting cells, other contaminants resembling microalgae might cause inaccurate results. Thus other methods such as flow cytometry is introduced for minimizing the counting errors of counting chambers as flow cytometry measures fluorescence and scattering of light caused by microalgae cell. Because this is a costly and sophisticate method so that for laboratory scale, counting chambers are met with great favor [28].

To evaluate the biomass growth productivity of microalgae, different methods are using in different studies. For example, according to Şirin and Sillanpaa, 2015, to see the growth performance of microalgae and its adaptation to medium, pH was measured by pH meter, cell density was measured by means of daily cell counts with flow cytometer, and even ash-free dry weight (AFDW) was evaluated by filtering the biomass through preweighed, precombsted glass fiber filters [45, 46]. In other studies, biomass productivity was evaluated by counting the cell numbers with hemocytometer [47, 48].

2.7 Total carbohydrate content

Generally, total carbohydrate content is determined by using phenol-sulfuric acid method via glucose solution as standard [49]. Moreover, there is one study used

modified quantitive saccharification (QS) method for carbohydrate content and composition in microalgae reported by the National Renewable Energy Laboratory (NREL), USA [20]. Another two studies, however, used the anthrone spectrophotometric method for estimating the available carbohydrates [50, 51]. Therefore, selection of the proper method depends on the study itself and feasibility. Carbohydrate can be hydrolyzed to fermentable sugar through hydrolysis for different biofuel production such as bioethanol [52].

2.8 Microalgae growth kinetics

The growth of microalgae has five different phases: (1) Lag-phase; (2) Exponential phase; (3) Linear growth phase; (4) Stationary growth phase; (5) Death phase.

In lag-phase, microalgae are started to adapt the environment, the growth of microalgae is not observed or, little increase on cell density occurs. To shorten the lag-phase and increase productivity, cells from exponential phase can be inoculated. As in the next phase, exponential phase, cells starts to divide as an exponential function of time. This phase usually used to measure the specific growth rate of microalgae biomass. The linear growth phase, however, cells growth starts to slow down due to the nutrient depletion and other limitation (light, pH, carbon dioxide and others). As for stationary phase, the growth rate of cells reaches zero due to the fully used up of the nutrient so that the numbers of growing cells and death cells are even. In the last phase, decline or death phase, the numbers of death cells are much more than the growing cells so the whole cell density declines sharply. This is the main result of nutrient depletion, overheating, pH disturbance or contamination [53 54].

To increase productivity of microalgae, the cultures should be maintained in exponential phase to boost growth by adding fresh medium, pH adjustment and providing proper light intensity and heat.

In batch culture, during the exponential phase, the increase of cells (ΔN) per unit of time (Δt) is proportional to the cell concentration at the given time (N).

$$\frac{\Delta N}{\Delta t} \sim N \tag{2.1}$$

As the newly produced cells always add to the population, N increases steadily, so both the time intervals and numbers of newly formed cells can be regarded as small as possible. Then applying this method mathematically as (2.2)

$$\frac{d\mathrm{N}}{d\mathrm{t}} \sim \mathrm{N} \tag{2.2}$$

To get from proportionality to an equation, specific growth rate μ was introduced. Therefore, μ can be calculated with the equation (2.3):

$$\frac{d\mathrm{N}}{d\mathrm{t}} = \mu\mathrm{N} \tag{2.3}$$

After changing the equation (2.3) into differential equation, it will be as (2.4)

$$\int \frac{dN}{N} = \mu \int dt \tag{2.4}$$

$$\ln \frac{Nt}{N0} = \mu t \tag{2.5}$$

$$\mu = \frac{\ln \frac{Nt}{N0}}{\mathrm{t}} \tag{2.6}$$

In this equation, N_0 and N_t are cell density (N) at the beginning and the end of a time interval (t) in the exponential phase [55].

This equation is used for growth kinetics measurement of microalgae in many studies such as [56, 57, 58 and 47]. There are some studies that measured the specific growth rate of different types of microalgae, to better understand the growth of microalgae (Table 2.1).

Table 2.1 Growth rate of different microalgae strain in different medium

Microalgae	Culture medium	Growth rate (μ)/d^{-1}	References
Chlorella vulgaris	Urban wastewater	0.38	[59]
C.kessleri		0.44	
C.sorokiniana		0.37	
B.braunii		0.42	
S.obliquus		0.28	
A.falcatus		0.10	
Tetraselmis F.Stein sp	f/2 medium	0.226	[56]
Nannochloropsis D.J.Hibberd sp		0.250	
Nannochloropsis sp	f/2 medium	0.33	[57]
Tetraselmis sp		0.14	
Isocrysis sp		0.18	
Rhodomonas sp		0.28	
chlamydomonas	BG11	0.131	[30]
C.pyrenoidosa	Fog's medium	0.203	[61]
Nannochloropsis oculata	Walne's medium	0.39	[45]
Nannochloropsis sp	Seawater medium	0.27	[47]
Tetraselmis suecica	AMCONA(Artificial Multipurpose Complement fot nutrient of Algae) medium	0.19	[62]
Dunaliella salina		0.23	
Isocrysis galbana	f/2	0.47	[63]
S.abundansPKUAC 12	BG11	0.11	[64]
M.afer PKUAC 9	BG11	0.09	[64]

Another important factor, cell doubling time (t_d) is regarded as species-specific and unchangeable, so that we can also evaluate the growth of microalgae through calculating the cell doubling time of microalgae using equation (2.7) that recommended by [55] after we obtain the specific growth rate of each sample:

$$t_d = \frac{\ln 2}{\mu} \tag{2.7}$$

CHAPTER 3

BIOETHANOL PRODUCTION FROM MICROALGAE

Bioethanol, is considered to be an environment friendly, sustainable renewable fuel. Apart from the first and second generation biofuel feedstocks such as sugar or starch crops and lignocellulosic biomass, the third generation biofuel feedstock such as algae are considered to be an alternative, promising feedstock for bioethanol production [65]. Especially in some microalgae, they can synthesize and accumulate large quantities of carbohydrate so that microalgae are considered to be an excellent substrate for bioethanol production [20, 65]. Bioethanol production includes different processes such as preparation of biomass, pretreatment of biomass, hydrolysis, fermentation and product recovery [66]. In this chapter, bioethanol production from microalgae will be introduced.

3.1 Preparation of microalgae biomass

After cultivating the microalgae species, harvested biomass should be prepared for next pretreatment process. In this step, the microalgae stock or cultivated microalgae is centrifuged at 4500×g for 10 min. Centrifugation process can be performed repeatedly with fresh de-ionized water to remove residual medium salts from strain. Then the biomass should be dried in oven at 60 ℃ overnight. Lastly, the dried biomass will be milled into fine powder for 1 min [66].

3.2 Pretreatment of microalgae biomass

Before fermentation, the microalgal biomass should be pretreated in order to release fermentable sugars. It is the process for releasing the carbohydrate content of microalgae biomass from its cell wall [52]. There are three different pretreatment

method according to Lam and Lee, 2015, chemical pretreatment, enzymatic pretreatment and mechanical and other alternative pretreatments [52].

3.2.1 Chemical pretreatment

Chemical pretreatment includes acid or alkali pretreatment. In acid pretreatment, sulfuric (H_2SO_4) and phosphoric (H_3PO_4) acids are widely used because of their low cost and efficient for hydrolyzing biomass [31]. Acid pretreatment can be used either as a pretreatment before hydrolysis or as the hydrolyzing method for biomass to be converted to sugars [1]. However, concentrated acid pretreatment may cause inhibition in the fermentation process because of the composition of toxic compounds [20]. Therefore, dilute acid hydrolysis has been recommended to use. Besides, when using acid hydrolysis method, the process time, temperature, amount of substrate loading and acid concentration must be considered carefully [67].

As for alkaline pretreatment, however, it is recommended to use bases such as sodium, potassium, calcium, and ammonium hydroxide [1]. According to Harun, Jason, Cherrington, and Danquah, 2011a, alkaline pretreatment is regarded to be promising because the process can be implemented in low temperature and pressure, and it can reduce the inhibition during fermentation process. Therefore, alkaline pretreatment is recommended for producing bioethanol production as they are more efficient in terms of sugar yields and biomass utilization rate [66].

3.2.2 Enzymatic pretreatment

The advantages of enzymatic hydrolysis are being environmentally friendly and efficient method, they don’t produce many by-products, and have no corrosion issues [51]. However, its disadvantage is that, the process may take for a long time, several factors such as temperature, pH, substrate concentration and enzyme loading must be considered. Besides, it is also a costly method when comparing to other pretreatment processes. Generally, a-amylase is widely use for enzymatic hydrolysis.

3.2.3 Mechanical pretreatment

Mechanical pretreatment methods include milling, grinding, and extrusion [52]. The ideal particle size after mechanical pretreatment is preferred at a range of 3-6 mm, the energy input can be kept below 30 kWh per ton of biomass [1]. But this method is not

recommended and did not reported so often in other studies because when in large-scale bioethanol production from microalgae, mechanical pretreatment method is not so sufficient for pretreatment of large amount of biomass [52]. However, it can be used for pretreatment of macroalgae biomass.

3.3 Fermentation

Fermentation is the conversion of biomass materials that contain glucose, starch or cellulose into ethanol [15]. In fermentation, *Saccharomyces cerevisiae* yeast or bacterium *Zymomonas mobilis* is used to break down the sugars in biomass and convert it to ethanol [20]. To process fermentation, pretreatment is essential because starch or cellulose in biomass cannot be directly fermented by yeast or bacterium. So pretreatment and hydrolyzing method of biomass is required to release and convert the complex carbohydrate into fermentable sugars. During fermentation process, the pH should be maintained at a range of 6-9. There are two different strategies when deciding the accomplishment of hydrolysis and fermentation process. These are separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) [1].

3.3.1 SHF

SHF is the process which hydrolysis and fermentation are carried out separately using two different reactors. As they can be processed independently, their optimum conditions such as temperature, pH and time can be different. This means, except for enzyme hydrolysis, acid or alkaline hydrolysis method is also applicable for SHF. The advantage of this fermentation is that, the costs of chemicals are low, the residence time is short, and the requirement for equipment system is simple. However, SHF also has a lot of disadvantages. When acid or alkaline is used for hydrolysis method, additional neutralizing and purification steps are must be undertaken to neutralize the acid or alkaline. Besides, according to the study [66], during bioethanol production process, yeast metabolism may change such that produced bioethanol may be consumed by yeast as substrate which cause a decrease in bioethanol yield [1, 52].

3.3.2 SSF

In SSF, hydrolysis and fermentation are operated simultaneously in the same reactor.

The advantage of SSF is the process can minimize the inhibition which results from glucose and cellobiose and both compounds are directly converted into bioethanol. Moreover, the operation time is shorter than SHF and it also can reduce the contamination risk of external microflora. However, the drawback of SSF is that, the hydrolysis and fermentation conditions of the process must be compromise so that the conditions can be suitable for both two processes. Another disadvantage of this fermentation is that, after bioethanol production, recycling of enzyme and yeast are difficult, therefore, they are not recommended for scale-up production of bioethanol [1,52].

3.4 Ethanol recovery

After fermentation process, produced ethanol should be drained from the tank and pumped into a holding tank, to get into a distillation unit. In the final step of bioethanol production process, purification is crucial and required to remove the water and other unwanted impurities in the diluted ethanol. Diluted ethanol can be condensed to become concentrated ethanol to be utilized for different purposes [9].

CHAPTER 4

EXPERIMENTAL STUDY

4.1 Materials and culture conditions

4.1.1 Microalgae strain

Two different marine microalgae *Nannochloropsis oculata* and *Tetraselmis suecica* obtained from (Yildiz Technical University) were used in this study. Although *N. oculata* and *T. suecica* are both marine microalgae and both of them are proposed as an excellent candidate for biofuel production, their growth performances during cultivation are quite different. According to other studies, in usual cultural condition, the carbohydrate content of *N. oculata* was only 8% (daf), its lipid content, however, was 32% (daf) [68]. It indicates that the lipid content of *N. oculata* was much higher than its carbohydrate content. Contrarily, carbohydrate content of *T. suecica* was around 15-50% (dry biomass), and its lipid content was around 20-30% (dry weight) [69]. To evaluate the performance of microalgae towards growth in wastewater medium, microalgae which has higher carbohydrate content and lower carbohydrate were chosen, so that the influence of wastewater to the growth and carbohydrate accumulation of these two marine microalgae can be identified.

4.1.2 Materials

F/2 medium which was used for the cultivation of control group was supplied from Sigma-Aldrich. Sodium hydroxide was used for pre-treatments and 96% grade of ethanol was used to determine the concentration of bioethanol. Sulfuric acid (98% concentrated), phenol and D-glucose were used for determination of carbohydrate content. Luria Broth (LB) medium was used for the yeast growth. All these chemicals were supplied from Merck. Chemicals that used in this study given in Table 4.

Table 4.1 List of chemicals used in this study

Chemicals	Company
f/2 medium	Sigma-Aldrich
Glucose solution	Merck
Phenol	Merck
H_2SO_4	Merck
NaOH	Merck
Luria Broth (LB)	Merck

4.1.3 Cultural condition

N. oculata and *T. suecica* were cultivated indoor with f/2 medium (control) as well as different concentration of wastewater medium. Wastewater was collected from Istanbul Water and Sewerage Administration (ISKI) and sterilized before utilization. The result of the total carbon analysis of wastewater was given in Table 4.2. In wastewater medium, the ratio of wastewater and filtered autoclaved seawater (FASW) was adjusted to obtain concentration of 25%, 50%, 75% and 100% wastewater in seawater solution. The study was carried out in 250 ml erlenmeyer flasks containing 20 ml of fresh *N.oculata* and *T. suecica* microalgae strain and 180 ml of f/2 medium as well as different concentration of wastewater medium for each sample. The initial culture medium of *N.oculata* and *T. suecica* were shown in Figure 4.1.

Table 4.2 TOC analysis of ISKI wastewater

	Total carbon concentration	*Total nitrogen concentration*
ISKI WW	7,67 mg/l±655,4 µg/l	3,77 mg/l±117,4 µg/l

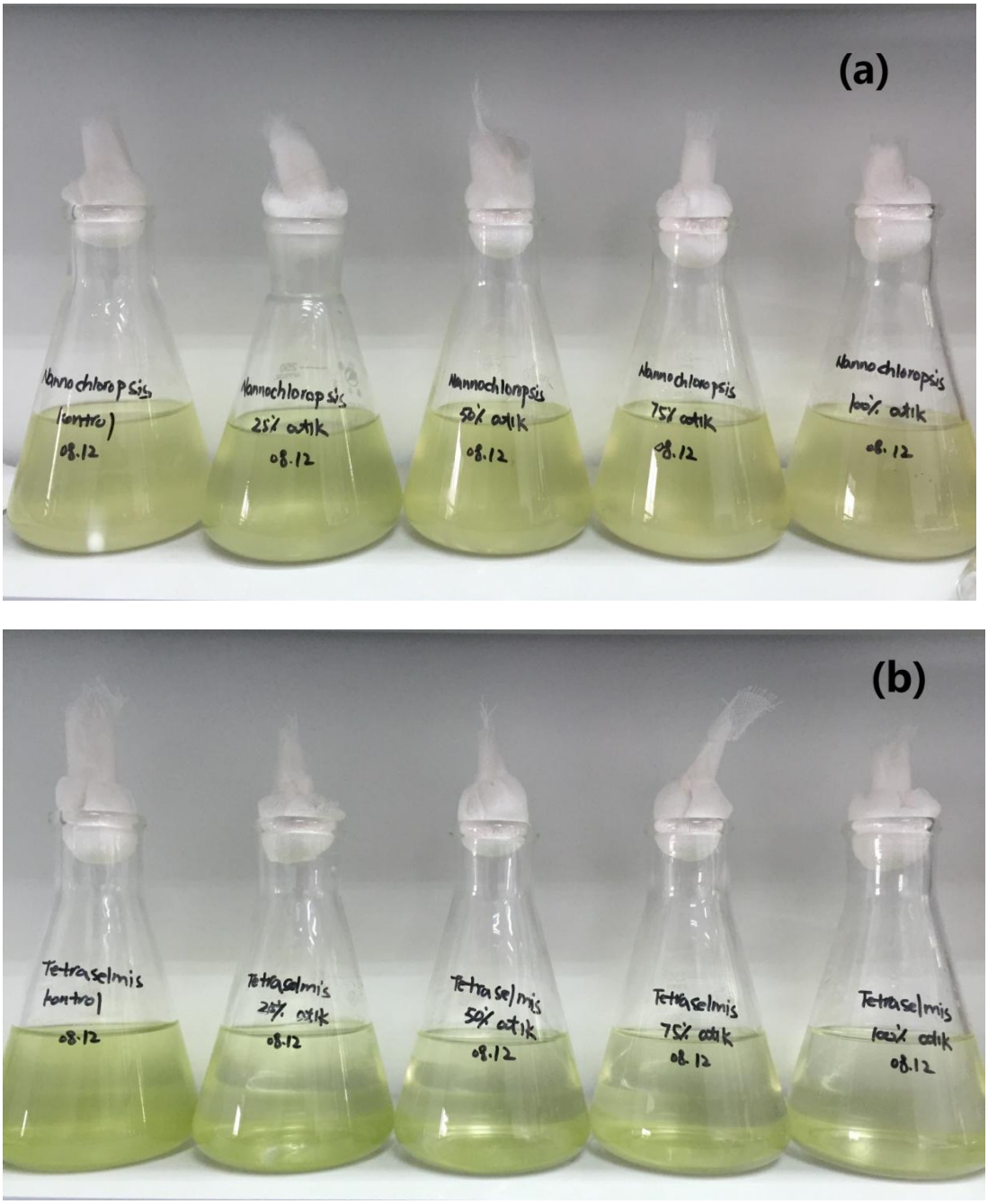


Figure 4.1 Initial culture medium of *N. oculata* (a) and *T. suecica* (b)

Cultures were incubated in a shaking incubator (Figure 4.2) at 24±2 ℃ with 24 h illumination by pink fluorescent lights. pH of the culture medium was maintained during 14 days of cultivation between 7.5-8.5, because this range is regarded to be optimal for growth of *Nannochloropsis sp*. and *Tetraselmis sp*. [37]. The cultivation condition of *N.oculata* and *T.suecica* were shown in Table 4.3

Table 4.3 Cultivation condition of *N.oculata* and *T.suecica*

Temperature	24±2 ℃
pH	7.5-8.5
Photoperiod	24 hours
Mixing rate	150 rpm
Duration	14 days

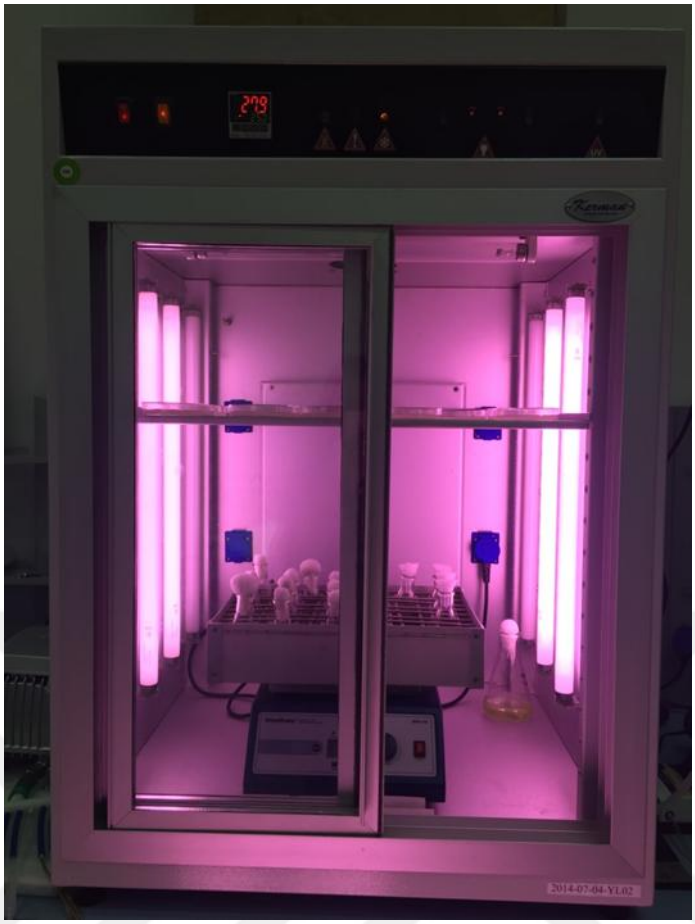


Figure 4.2 Shaking incubator that used in this study

4.2 Analytical method

During cultivation, different types of analytical methods were used such as optical density measurement, cell counting, microscopic observation and total carbohydrate content measurement.

4.2.1 Optical density (OD)

In this study, optical density was measured by spectrophotometer (T60 UV-VIS Spectrophotometer) which shown in Figure 4.3. A spectrophotometer measures the absorbance of light which passes through before and after the sample at specific wavelength [60]. The optical density (OD) of *N. oculata* and *T. suecica* were measured every day at wavelength of 680 nm and 620 nm, respectively.



Figure 4.3 T60 UV-VIS Spectrophotometer

4.2.2 Cell counting

For counting the cell numbers of *N. oculata* and *T. suecica*, hemocytometer was used. This is the most widely used type of chamber for determining the number of cells per unit of a suspension [44]. The volume of hemocytometer is 0.1 m^3 with either 16*25 chambers or 25*16 chambers. But the total chamber remains 400. The counting is implemented under microscope with 10X contrast objective, which allows for 1000X maximum magnification. The figure of the hemocytometer and its counting grid were shown in Figure 4.4.



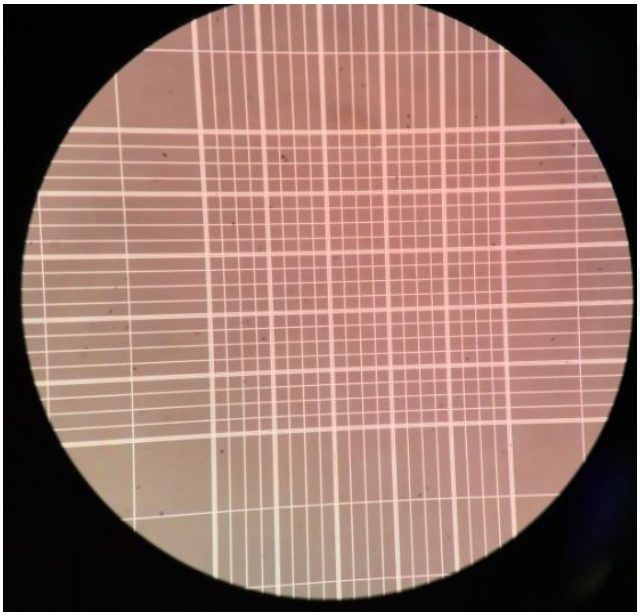


Figure 4.4 (a) Hemocytometer [70] and (b) counting grid [71]

4.2.3 Microscopic observation

Along with hemocytometer, micrographs of *N.oculata* and *T.suecica* was also observed (Figure 4.5) under microscope (OLYMPUS CX22) which is illustrated by Figure 4.6. The microscope was equipped with 4X, 10X, 40X and 100X phase contrast objectives.

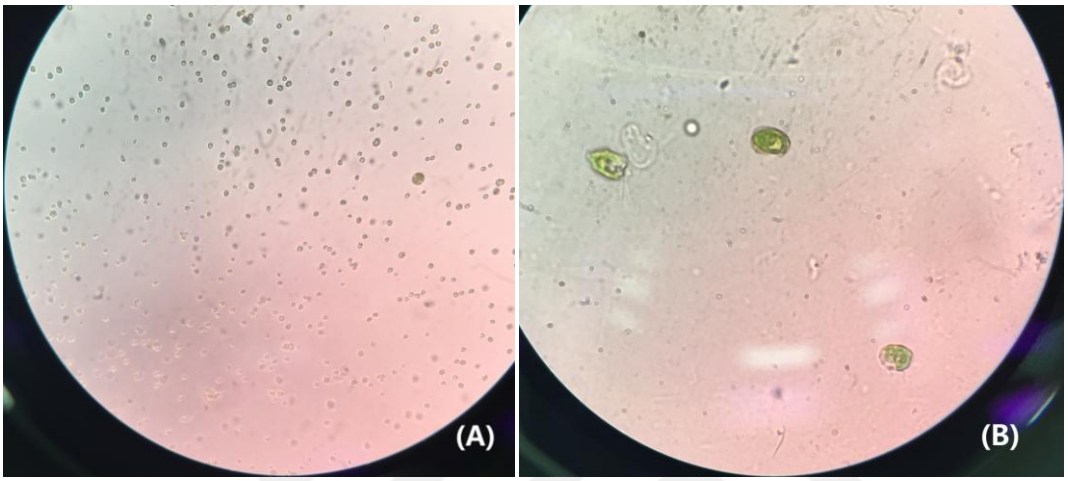


Figure 4.5 Morphological figures of *N.oculata* (A) and *T.suecica* (B) under microscope

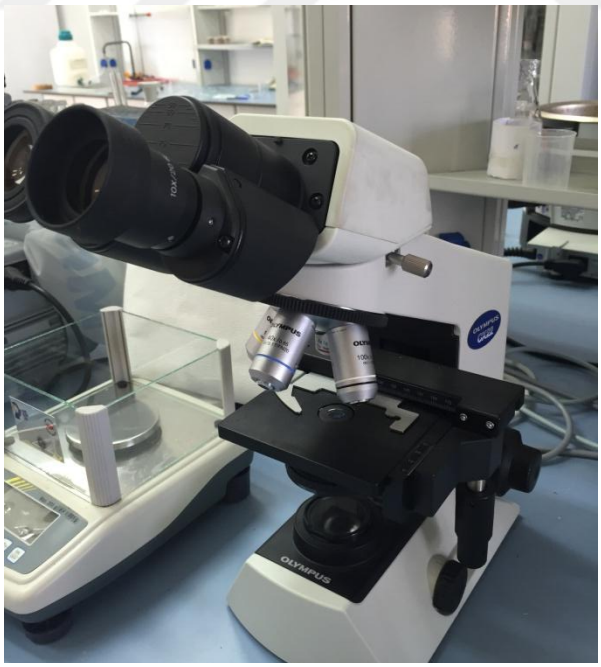


Figure 4.6 OLYMPUS CX22 Microscope

4.2.4 Total carbohydrate analysis by phenol-sulfuric method

Phenol-sulfuric acid method was utilized for determining the total carbohydrate content of *N.oculata* and *T.suecica*. In this method, glucose solution was used as standard [49]. 80% (w/w) phenol reagent was prepared by adding 80 g phenol to the 20 g distilled water. To obtain the standard curve tubes, different concentration of glucose solutions were adding into the 0.05 ml phenol reagent followed by adding 5 ml of H_2SO_4. The tubes contain 0, 20, 40, 60, 80, and 100 µg/ml glucose, with total volume of 2 ml. These tubes are used to create a standard curve. The standard glucose concentration value was given in Table 4.4 [72], and the standard samples for measuring the total carbohydrate content of *N.oculata* and *T.suecica* were given in Figure 4.7. After adding 0.05 ml phenol reagent followed by 5 ml of H_2SO_4, the optical densities (OD) of samples were determined using the spectrophotometer. Afterwards, total carbohydrate contents of *N.oculata* and *T.suecica* were calculated by using the equation which is obtained by standard curve.

Table 4.4 Standard glucose concentration values

µg Glucose/2 ml						
	0	20	40	60	80	100
Glucose stock solution (ml)	0	0.2	0.4	0.6	0.8	1.0
Distilled water (ml)	2.0	1.8	1.6	1.4	1.2	1.0

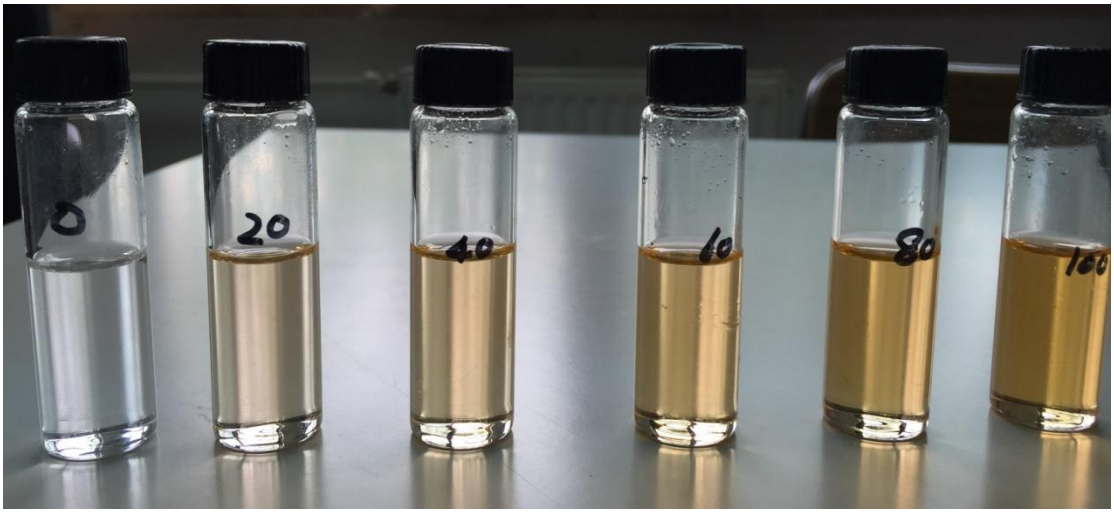


Figure 4.7 Standard samples for total carbohydrate content measurement

4.3 Evaluation of growth kinetics

Growth kinetics of *N.oculata* and *T. suecica* were expressed as the determination of specific growth rate (μ) and doubling time (t_d) of cells. The specific growth rate can be measured using the equation (2.6) that mentioned in chapter 2.

$$\mu = \frac{\ln\frac{Nt}{N0}}{t}$$

As for doubling time of cells, it can be evaluated by another equation (2.7) [55]

$$td = \frac{\ln 2}{\mu}$$

4.4 Bioethanol production

4.4.1 Preparation of microalgae biomass

For preparation of microalgae biomass, the *N.oculata* and *T.suecica* sludge were centrifuged at 4500 rpm for 10 min. The resulting microalgae sedimented on the bottom were gathered and oven dried at the temperature of 60 ℃ for one night. In the next day, the dried biomass were milled for one minute to become a fine powder and weighing for determining the biomass productivity of different concentration of wastewater.

4.4.2 Pretreatment of microalgae biomass

After drying and milling, the highest carbohydrate amount of group and the control group of *N.oculata* and *T.suecica* were chosen (four samples) and dissolved in 15 mL of de-ionized water. 0.75% (w/v) concentration of NaOH were added to the mixture according to the Harun, Jason, Cherrington, and Danquah, 2011b, and stirred at 500 rpm for 10 min. Then the four samples were incubated at the temperature of 120 ℃ for 30 min in oven [66]. After incubation, samples were cooled into room temperature and centrifuged for 10 min at 4500 rpm. The supernatants were collected for bioethanol production.

4.4.3 Preparation of yeast

Yeast and Luria Broth (LB) were also prepared according to Harun et al., 2011b. Prepared LB medium sterilized at the temperature of 121 ℃ for 1 hour and then cooled into room temperature. 5 g yeast (*Saccharomysis cerevisiae*) were dissolved in 50 mL de-ionized water and incubated at the temperature of 43 ℃ for 15 min with mixing. Then 3% of yeast suspension was transferred into prepared LB medium and cultured in incubator at 30 ℃ for 24 h and stirred with 150 rpm [66, 73].

4.4.4 Fermentation

3% (v/v) yeast was transferred in to the pretreated four different microalgae biomass samples. Then the mixture samples were incubated at the temperature of 30 ℃ and 150 rpm for more than 48 hours.

4.4.5 Determination of bioethanol concentration

Bioethanol concentration was measured using YL 6100 GC gas chromatography (Figure 4.8). At first, samples that obtained during the bioethanol fermentation process (24 h and 48 h) were taken and filtered using 0.45 μm filters to avoid blocking in column. The GC contains flame ionization detector (FID) and 30m x 0.32 mm x 0.25 μm ZB-FFAP column oven. The temperature of injector, detector and oven

were maintained at 150, 250 and 100 ℃, respectively. Hydrogen was used as carrier gas. Bioethanol concentration was calculated using calibration curve that prepared by the different concentration of bioethanol standards (0.1% -10% (v/v)) [73].



Figure 4.8 YL 6100 GC gas chromatography

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Growth measurements

As it was mentioned in chapter 4, the growth of *N.oculata* and *T.suecica* were measured using different methods during 14 days of cultivation. Optical density of *N.oculata* and *T.suecica* were measured every day at wavelength of 680 nm and 620 nm, respectively. The results that illustrated by Figure 5.1 and Figure 5.2 showed that the maximum absorbance of *N.oculata* was obtained with the 50% wastewater concentration on the 14th cultivation day (OD=0.542). This result was ideal when comparing with the absorbance of control group. Similarly, the maximum optical density of *T.suecica*, was obtained with the 50% wastewater concentration on the 14^{th} of cultivation day (OD=0.161). It can be concluded from the results that, adding optimal amount of wastewater into growth medium of *N.oculata* and *T.suecica* indeed has positive effect on their growth, especially at 50% of wastewater. According to Kothari, Prasad, Kumar, and Singh, 2013, when *Chlamydomonas polypyrenoideum* was cultivated in different concentration (10%, 25%, 50%, 75% and 100%) of industry daily wastewater, it is showed that the growth of algae was stimulated by about 25% to 75% concentration of daily wastewater. Among all, 75% concentrations of wastewater represent highest optical density during 15 days of cultivation [30]. A similar study about the cultivation of *C. pyrenoidosa* microalgae in different

concentration of untreated and treated wastewater (25%, 50%, 75% and 100%) as well as in tap water (control). Results showed that the algae grow faster in 75% wastewater sample. Moreover, the result showed better biomass yield of *C. pyrenoidosa* in 75% of untreated wastewater than in the treated medium [61].

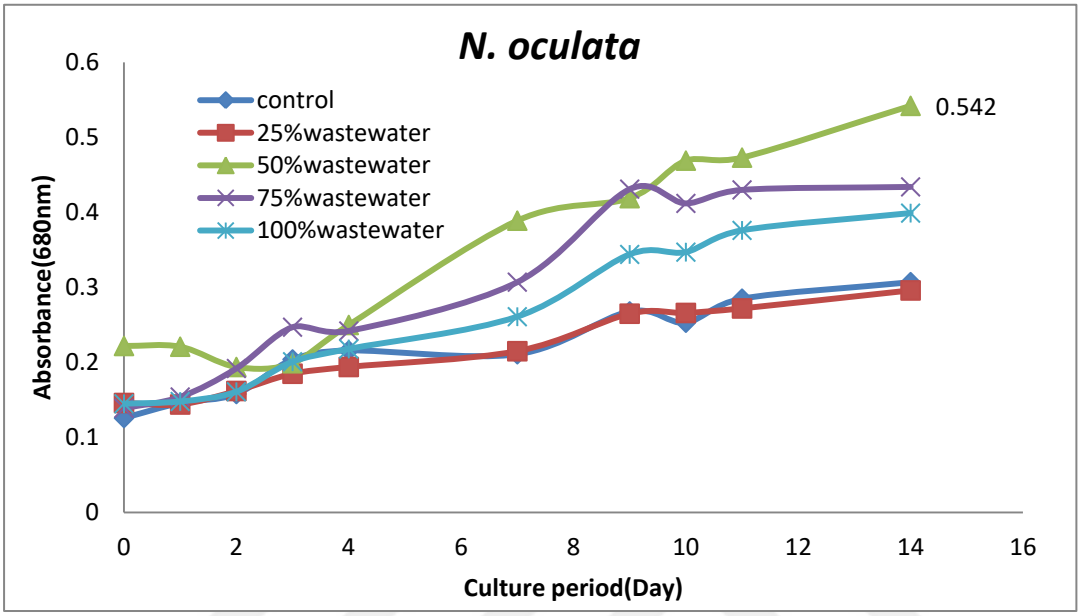


Figure 5.1 Optical density versus culture period (day) for *N.oculata* in control and different wastewater concentration (25%, 50%, 75%, and 100%)

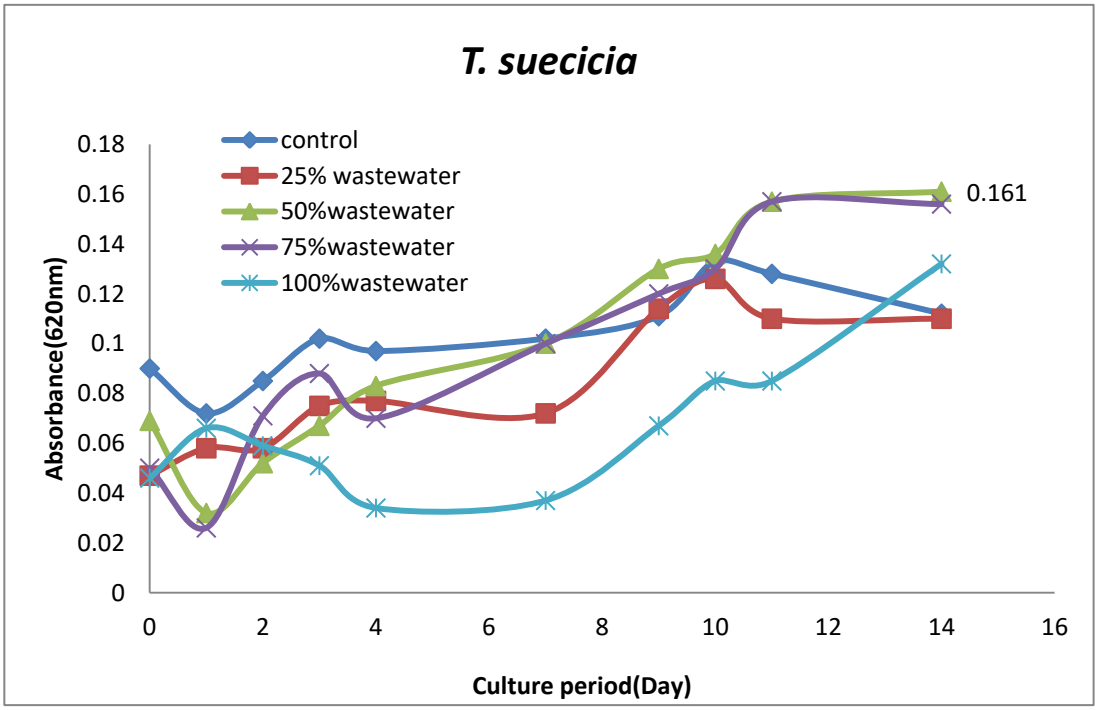


Figure 5.2 Optical densities versus culture period (day) for *T.suecica* in control and different wastewater concentration (25%, 50%, 75%, and 100%)

Cell densities of *N.oculata* and *T.suecica* in control and different concentration of wastewater medium were measured using hemocytometer under microscope during 14 days of cultivation. The results were given in Figure 5.3 and Figure 5.4. The maximum cell density of *N.oculata* was observed in 75% of wastewater on the 7^{th} cultivation day ($12.75*10^6$ cells/ml) which was two times higher than the control group. Furthermore, it was obvious on the growth curve that, in high concentration of wastewater, *N.oculata* entered the exponential phase much slower than the ones with low concentrations. Specifically, 100% and 75% of wastewater entered the exponential phase on the 4th day of cultivation, whereas the other groups entered on 0-2^{nd} cultivation day. This can be explained that *N.oculata* might be suffered from nutrient inhibition at the beginning because of the high concentration of wastewater medium. So it might take for a while to adapt to the cultural environment that high concentration of wastewater group to adjust themselves for better growth. However, when it came to the growth rate, *N.oculata* in 75% and 100% grew ideal than the other group, which was because the adequate amount of nutrient accelerate the growth of *N.oculata* in 75% and 100% of wastewater medium and depleted or inhibited the other concentration group [41,30].

On the cultivation of *T.suecica*, the maximum cell density was occurred in control group also on the 7^{th} day of cultivation ($6.25*10^5$cells/ml). This result was much lower than the maximum cell density of *N.oculata* in 75% of wastewater. Besides, the growth curve showed that, *T.suecica* in higher concentration of wastewater entered into the exponential phase much later than lower concentration groups as well. The lower concentration group, however, started the exponential phase almost without lag phase. This can be explained that *T.suecica* may be highly affected by the nutrient level or culture condition [56], and the higher nutrient level may hindered the strain from entering into the logarithmic phase because intensive amount of nutrient may cause the prompt change in medium conditions.

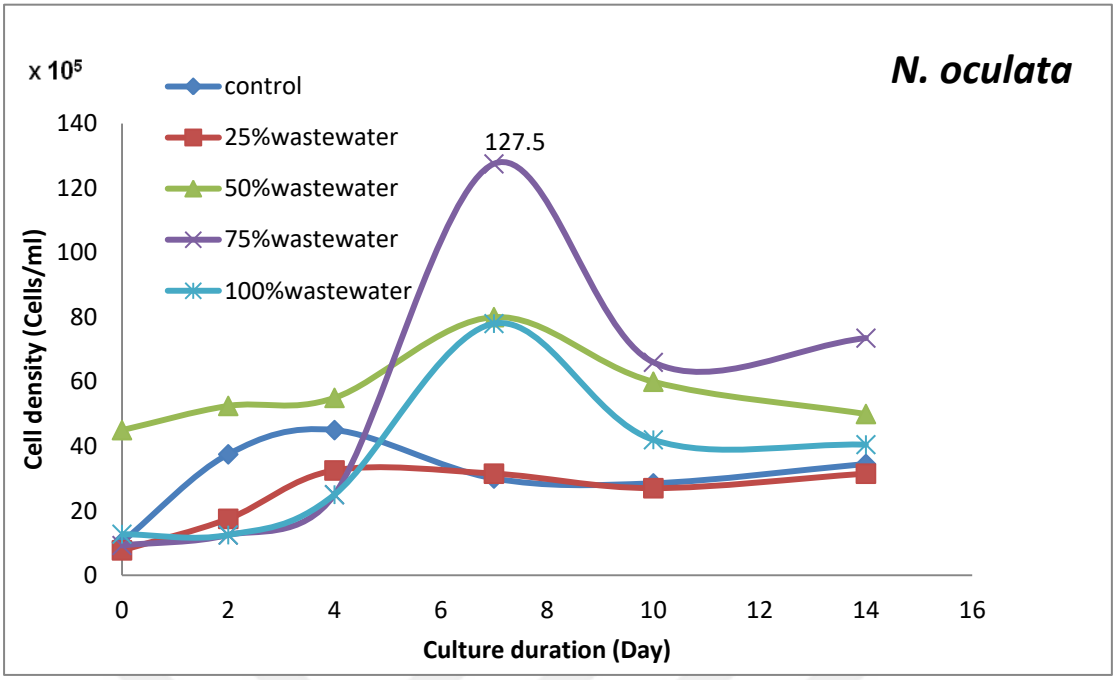


Figure 5.3 Cell densities versus culture period (day) for *N. oculata* in control and different wastewater concentration (25%, 50%,75% and 100%)

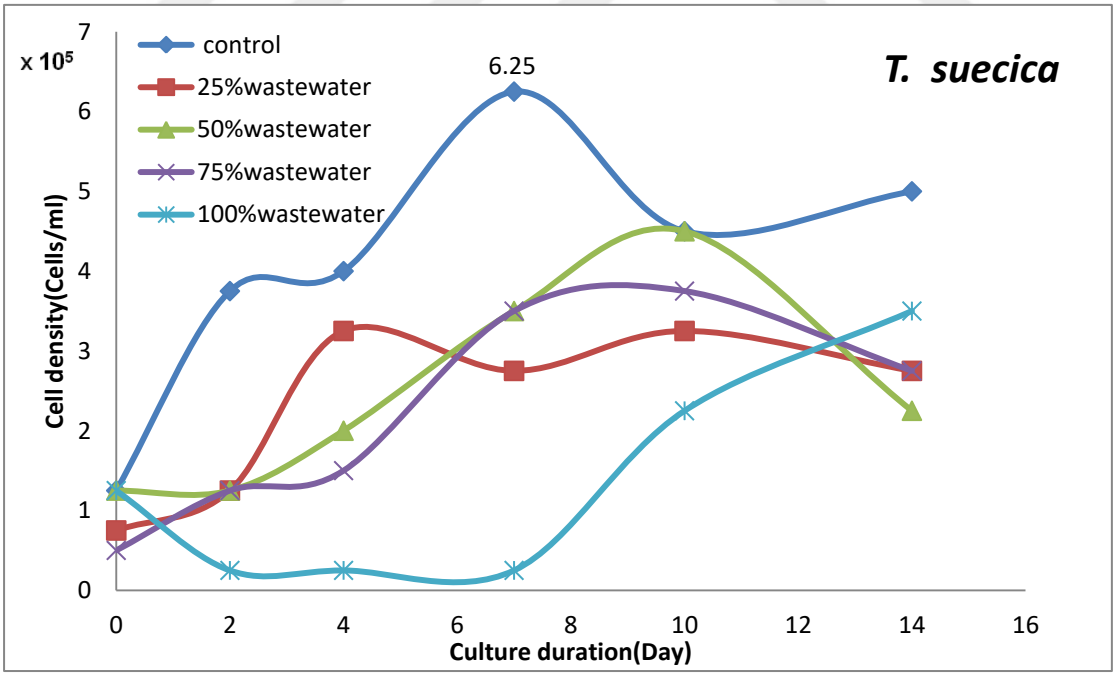


Figure 5.4 Cell densities versus culture period (day) for *T. suecica* in control and different wastewater concentration (25%, 50%, 75% and 100%)

Moreover, when comparing cell densities of *N.oculata* and *T.suecica* in control, and in different wastewater concentration (25%, 50%, 75% and 100%), it can be seen from the Figure 5.5 that, cell densities of *N.oculata* in every group were always higher than cell densities of *T.suecica*. There is not much change in the cell densities of *T.suecica* when adding different concentration of wastewater. This is also true when the optical densities of two microalgae were analyzed that, the optical densities of *T.suecica* showed much lower value than *N.oculata* uring the cultivation. This may be because *T.suecica* cannot well adapt to the wastewater medium because of the change in nutrient level and composition.

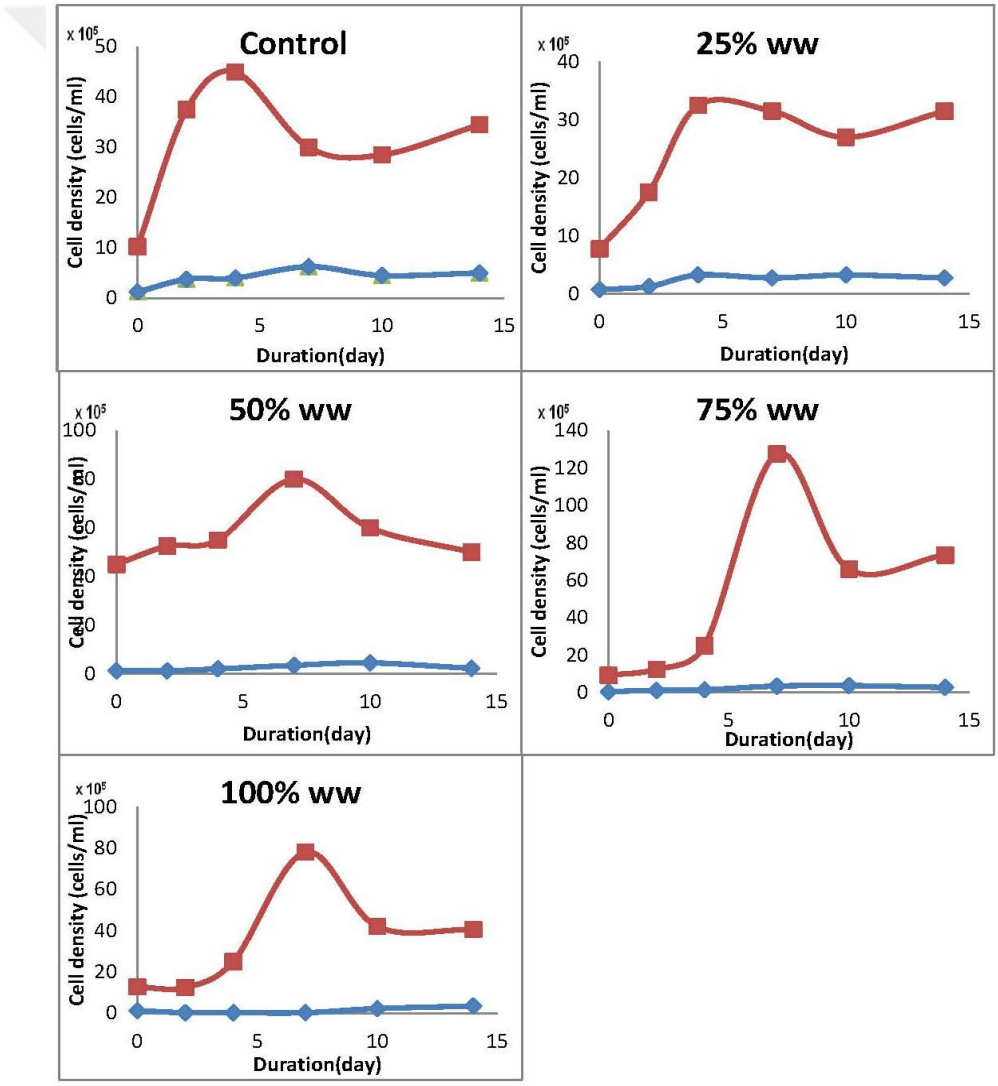


Figure 5.5 Comparison of cell densities of *N.oculata* (red) and *T.suecica* (blue) in control, and in different wastewater concentration (25%, 50%, 75% and 100%)

5.2 Total carbohydrate content

Total carbohydrate content of *N.oculata* and *T.suecica* were determined using phenol-sulfuric method that mentioned in chapter 4. The results in Table 5.1 showed that, after 14 days of cultivation, *T.suecica* accumulate much more carbohydrate than *N.oculata*. This is also mentioned in other studies that, *T.suecica* is among the microalgae species that could accumulate high carbohydrate content and suitable for bioethanol production. Moreover, its carbohydrate content can range from 21% to 64% of their cell dry weight [52]. In this study, the highest carbohydrate content in wastewater group of *T. suecica* occurred in control group (27% dry weight) followed by 100% of wastewater (6.52% dry weight). This is promising when considering *T.suecica* with this concentration of wastewater as an optimum feedstock for bioethanol production.

As for *N. oculata*, however, maximum carbohydrate concentration in wastewater group was found in 75% concentration of wastewater sample (2.39% dry weight). The results were low when comparing with other studies that this may be because the existence of ash content in dry biomass which influence the overall carbohydrate content.

Table 5.1 Total carbohydrate content of *N.oculata* and *T.suecica*

	N.oculata **(% dry weight)**	***T.suecica*** **(% dry weight)**
Control	1	27
25% WW	2.26	4.23
50% WW	2.30	4.54
75% WW	2.39	3.42
100% WW	2.11	6.52

5.3 Dry weight

Milled fine powder figures and the data of dry weight (g) of *N.oculata* and *T.suecica* in different concentration of wastewater medium were shown in Figure 5.6, Figure 5.7 and Table 5.2, respectively. When the dry weights of *N.oculata* together with its milled fine powder were considered, it can be easily observed that there are white unknown ingredients in powder. Especially in control group, there are much more white ingredients than any other groups and its dry weight showed highest result (2.41g/L) than other groups. This cannot be regarded as pure microalgae biomass due to the interruption of this unknown ingredient. In the literature, it is reported that there are ash, and slag deposits were observed. These ingredients were explained as the presence of the inorganic components in the biomass which influence the formation of them [47]. In another study, it is claimed that the ash content in *N.oculata* were measured ranged from 26% to 27% [58]. Therefore, according to literature studies, the ash content of *N.oculata* must be taken into consideration because it may directly influence the carbohydrate concentration in dry weight. Other groups with different concentration of wastewater, however, showed the lowest ash content, especially in 75% and 100% of wastewater. After comparing the figures with dry weight content, it can be concluded that 75% of wastewater medium presents the highest biomass (1.055 g/L) for *T.suecica*, and the milled fine powder can be regarded as pure green and such ash content was not observed when taking its dry weight into consideration.

This results matches with a similar study implemented by Beuckels, Smolders, and Muylaert, 2015 that microalgae can adjust the N and P concentration of their biomass depending on their supply of nutrients in the medium, so that it may result in a low dry weight when the supply of a nutrient is low [74]. In our study, however, as 75% of wastewater contains higher N and P, so that it accumulate much more biomass that other groups.

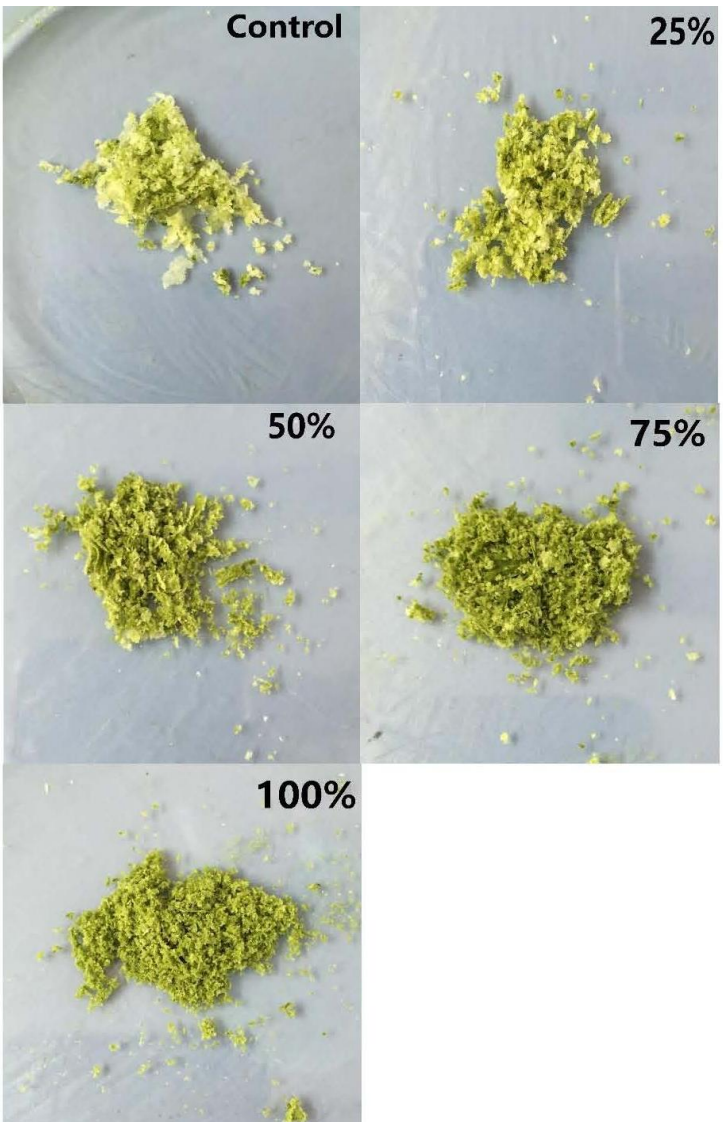


Figure 5.6 Milled fine powder of *N.oculata*

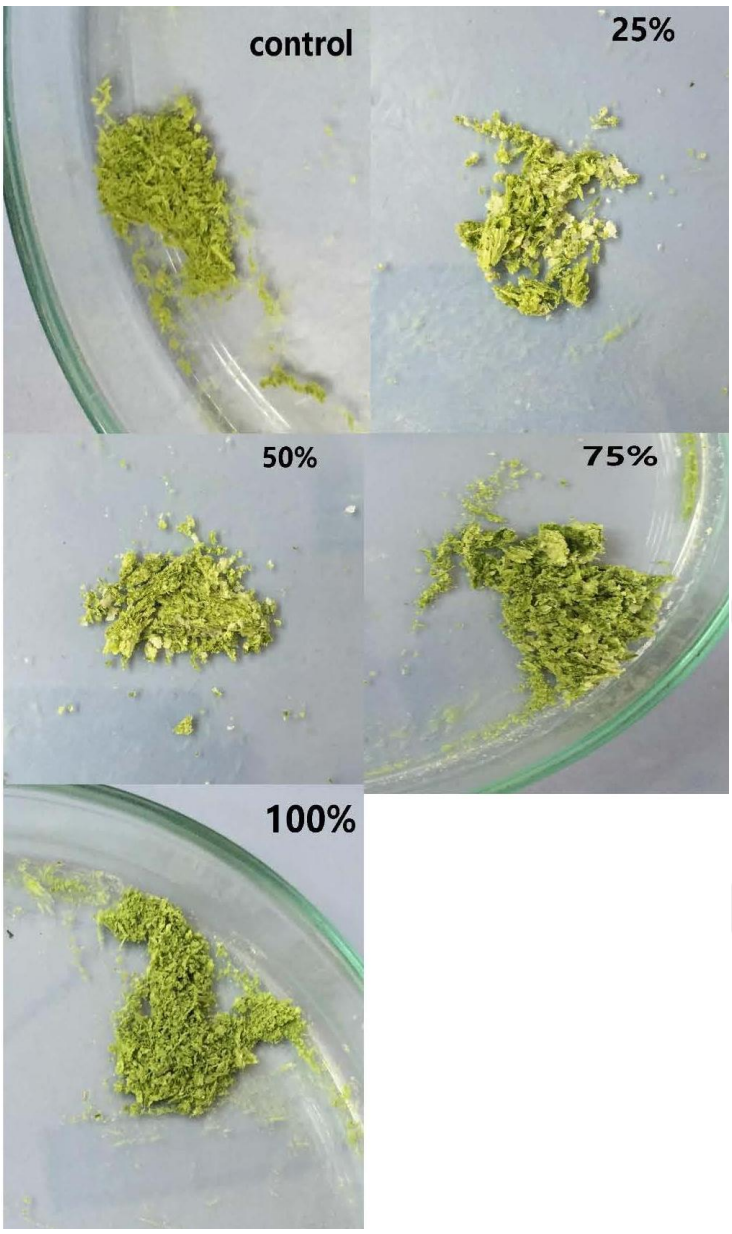


Figure 5.7 Milled fine powder of *T.suecica*

Table 5.2 Dry weight of *N.oculata* and *T.suecica* in different concentration of wastewater medium

	N.oculata **(g/L)**	***T.suecica*** **(g/L)**
Control	2.41	0.14
25% WW	1.19	0.76
50% WW	1.28	0.855
75% WW	1.285	1.055
100% WW	0.975	0.48

5.4 Growth kinetics-specific growth rate and cell doubling time

In this study, the productivity of *N.oculata* and *T.suecica* were also expressed with growth kinetics. Specific growth rate (μ) and doubling time (t_d) of *N.oculata* and *T.suecica* were calculated according to the cell density change during specific time period of exponential phase that expressed as equation (2.6).

According to Table 5.3, the specific growth rate of *N.oculata* in different concentration of wastewater was consistently higher than the specific growth rate of *T.suecica* at same concentration of wastewater. Based on the Table 2.1 that was made in chapter 2, it can be found that the growth rate of *N.oculata* and *T.suecica* were measured as 0.39 d^{-1} and 0.19 d^{-1}, respectively in other studies [45, 62]. In this study, however, the maximum specific growth rate of *N.oculata* and *T.suecica* reached to 0.5430 d^{-1} in 75% of wastewater and 0.4778 d^{-1} in 25% of wastewater, respectively. Therefore, it can be said that the presence of wastewater with 75% was the ideal concentration for cultivating *N.oculata* with high growth rate and doubling time, and the same result can be made for 25% of wastewater in *T.suecica*.

Table 5.3 Specific growth rate (μ) and doubling time (t_d) of *N.oculata* and *T.suecica*

	N.oculata		**T.suecica**	
	SGR(μ) (d^{-1})	**t_d (h)**	**SGR(μ) (d^{-1})**	**t_d (h)**
Control	0.3698	1.874	0.1488	4.657
25% WW	0.3095	2.239	0.4778	1.450
50% WW	0.1248	5.553	0.1352	5.126
75% WW	0.5430	1.276	0.2824	2.454
100% WW	0.3793	1.837	0.1105	6.271

5.5 Bioethanol production

In order to further investigate the bioethanol production from microalgae cultivated in municipal wastewater medium, *N.oculata* and *T.suecica* samples were chosen due to their high carbohydrate content (75% of wastewater in *N.oculata* and 100% of wastewater in *T.suecica*) as well as control groups to compare their bioethanol productivity. Results of bioethanol yield of *N.oculata* and *T.suecica* at 24 hours and 48 hours were given in Table 5.4.

Table 5.4 Bioethanol yield of *N.oculata* and *T.suecica* in control groups and 75%, 100% of wastewater

Microalgae	**Sample**	**Total carbohydrate content (% dry weight)**	**Dry weight (g/L)**	**Sampling time**	**Bioethanol yield (%)**
N.oculata	**Control group**	1	2.41	24 h	0.41
				48 h	2.96
	75% of wastewater	2.39	1.285	24 h	1.21
				48 h	3.68
T.suecica	**Control group**	27	0.14	24 h	2.46
				48 h	7.26
	100% of wastewater	6.52	0.48	24 h	1.38
				48 h	4.12

It can be seen on Table 5.4, bioethanol yield of *N.oculata* and *T.suecica* ranges from 0.41% to 7.26%. In a study carried out by Chng, 2016, bioethanol yield of different microalgae under various type of pretreatment and fermentation were given in a table [26]. It clearly shows that bioethanol yield of different microalgae in over 48 hours ranges from 0.131% (*Chlorella vulgaris*) to 0.26% (*Chlorococcum infusionum*, *Scenedesmus dimorphus*) with SHF fermentation method. In this study however, bioethanol yield were much higher and in agreement with the literature.

When comparing bioethanol concentration of samples which use *N.oculata* and *T.suecica*, it is apparent that *T.suecica* samples showed better ethanol yield both at 24 h and 48 h of fermentation than *N.oculata*. This is maybe due to a reason that samples used *T.suecica* as biomass, accumulated much more carbohydrate than *N.oculata* samples during cultivation.

Specifically, in *N.oculata* results, it was observed that both at 24 h and 48 h of fermentation, the bioethanol yield of 75% of wastewater sample was higher than control group that increased from 1.21% to 3.68%. As 75% of wastewater group also showed highest carbohydrate yield, it can be concluded that 75% of wastewater can be the optimum medium for *N.oculata* growth.

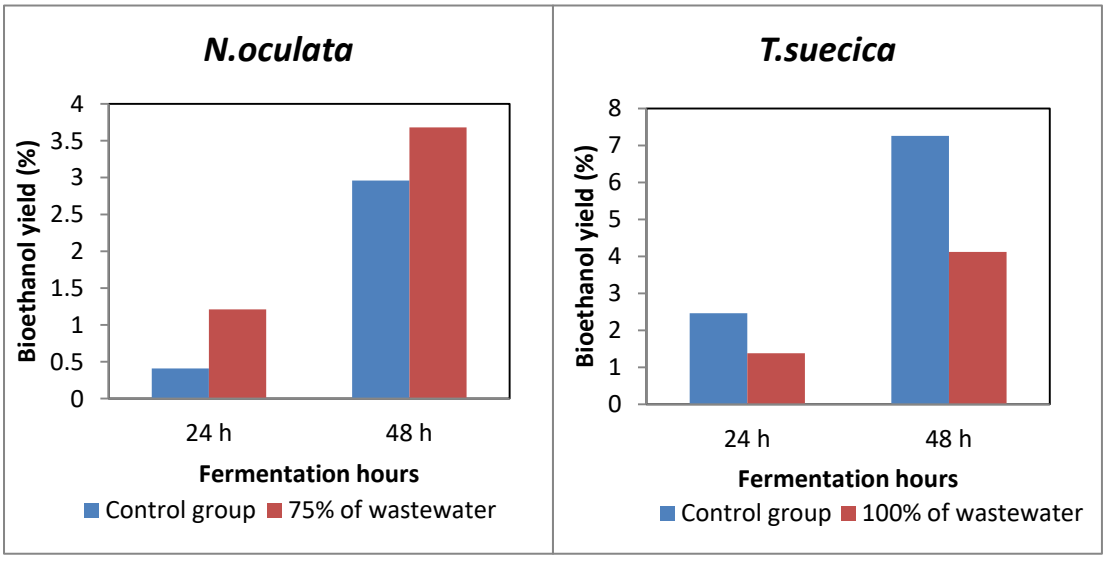


Figure 5.8 Bioethanol concentrations of *N.oculata* and *T.suecica* during 24 hours and 48 hours of fermentation

As for *T.suecica*, ethanol yield showed higher results in control group than 100% of wastewater group that increased from 2.46 % to 7.26 % during fermentation in control. This is mostly because the control group accumulated highest amount of carbohydrate (27% of dry weight) regardless of its lower dry weight (0.14 g/L). In 100% concentration of wastewater group, however, although it did not showed highest ethanol yield, it is still higher when comparing with both control and 75% of wastewater sample of *N.oculata*.

When bioethanol concentration of *N.oculata* and *T.suecica* was compared, it can be easily seen from Figure 5.8 that, *T.suecica* samples showed higher bioethanol productivity than *N. oculata* samples during fermentation. According to Lam and Lee, 2015, *T.suecica* is among the microalgae species that could accumulate high carbohydrate content and suitable for bioethanol production [52], so much more ethanol can be produced than *N.oculata* samples. However, it is possible that produced bioethanol in both samples of *T.suecica* might be consumed by yeast during the fermentation process, because with respect to their carbohydrate content (27% in control group and 6.52% in 100% of wastewater group), their ethanol concentration is not so ideal (only reached to 7.26% in control group and 4.12% in 100% of wastewater group). This may be due to the yeast consumed the fermentable sugar rapidly, so they consumed the produced bioethanol as a carbon source and lead to the decreasing trend in bioethanol concentration [66, 67].

CONCLUSION

This study demonstrates that for effective cultivation of microalgae species such as *N. oculata* and *T. suecica*, municipal wastewater can be utilized at a specific concentration as a medium. According to specific growth rate, after 14 days of cultivation, it was found that 75% concentration of wastewater can be an ideal concentration for *N. oculata* medium, while 25% concentration of wastewater was optimum for *T.suecica*. When *N.oculata* and *T.suecica* were compared, adaptation of *N. oculata* to wastewater medium resulted better than *T. suecica* according to their growth rate profile. After 14 days of cultivation, bioethanol productivities of two species were also investigated. The results showed that *N.oculata* cultivated at 75% of wastewater represents highest carbohydrate content; hence its ethanol concentration was higher than control group. On the other hand, the carbohydrate content of control group of *T.suecica* showed highest result, *T.suecica* cultivated at 100% concentration of wastewater showed the second highest result. When comparing ethanol concentration of *N.oculata* and *T.suecica*, it was obvious that, *T.suecica* samples showed higher ethanol yield than *N.oculata*. These results indicated that *T.suecica* was more suitable for ethanol production using wastewater as a cultural medium, as had higher carbohydrate content than *N.oculata*. In conclusion, these results suggested that municipal wastewater from ISKI can be utilized as growth medium for *N. oculata* and, as well as *T.suecica* and it can be a remarkable solution for waste utilization and bioethanol production can be improved with further studies.

REFERENCES

[1] Li, K., Liu, S. and Liu, X., (2014). "An overview of algae bioethanol production", International journal of Energy Research, 965-977.

[2] A. Singh, P. Singh Nigam and J. D. Murphy, (2011). "Renewable fuels from algae: An answer to debatable land based fuels", Bioresource Technology, 102:10-16.

[3] Andersson, V., Broberg Viklund, S., Hackl, R., Karlsson, M., and Berntsson, T., (2014). "Algae-based biofuel production as part of an industrial cluster", Biomass and Bioenergy, 71:113–124.

[4] Ullah, K., Ahmad, M., Sofia, Sharma, V. K., Lu, P., Harvey, A., Sultana, S., (2015). "Assessing the potential of algal biomass opportunities for bioenergy industry: A review", Fuel, 143:414–423.

[5] F. Passos, E. Uggetti, H. Carrere and Ivet Ferrer, (2014). "Pretreatment of microalgae to improve biogas production: A review", Bioresource Technology, 172:403-412.

[6] Vassilev, S. V., and Vassileva, C. G., (2016). "Composition, properties and challenges of algae biomass for biofuel application: An overview", Fuel, 181:1–33.

[7] Bungay, H. R., (2004). "Confessions of a bioenergy advocate", Trends in Biotechnology, 22(2):67–71.

[8] Demirbas, A., (2010). "Use of algae as biofuel sources", Energy Conversion and Management, 51(12): 2738–2749.

[9] Suganya, T., Varman, M., Masjuki, H. H., and Renganathan, S., (2016). "Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach", Renewable and Sustainable Energy Reviews, 55:909–941.

[10] Peng, Y., Hu, J., Yang, B., Lin, X., Zhou, X., Yang, X., and Liu, Y., (2015). "Chemical composition of seaweeds. Seaweed Sustainability", Elsevier Inc., UK.

[11] Tiwari, B. K., and Troy, D. J., (2015). "Seaweed Sustainability: Food and Non-Food Applications", Elsevier Inc., UK.

[12] Seaweed benefits. Seaweed Pete, http://www.seaweedpete.com/seaweed-benefits-2, 08 October 2012.

[13] Marquez, G. P. B., Santianez, W. J. E., Trono, G. C., Montano, M. N. E., Araki, H., Takeuchi, H., and Hasegawa, T.,(2014). "Seaweed biomass of the Philippines: Sustainable feedstock for biogas production", Renewable and Sustainable Energy Reviews, 38:1056–1068.

[14] Venkatesan, J., Manivasagan, P., and Kim, S.-K., (2015). Marine Microalgae Biotechnology, Handbook of Marine Microalgae, First Edition, Elsevier Inc.,UK

[15] Brennan, L., and Owende, P., (2010). "Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products", Renewable and Sustainable Energy Reviews, 14(2):557–577.

[16] Demirbas, M. F., (2011). "Biofuels from algae for sustainable development", Applied Energy, 88(10):3473–3480.

[17] Lu śa, G., (2011). SpringerBriefs in Microbiology, Micoralgae as Feedstock for Biofuels. Springer, Berlin.

[18] Mata, T. M., Martins, A. A., and Caetano, N. S., (2010). "Microalgae for biodiesel production and other applications: A review", Renewable and Sustainable Energy Reviews, 14(1):217–232.

[19] Breakthrough in Microalgae Bio-refinery process, BusinessKorea, http://www.businesskorea.co.kr/english/news/sciencetech/3957-green-fuel-breakthrough-microalgae-bio-refinery-process, 07 April 2014.

[20] Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A., and Chang, J. S., (2013). "Bioethanol production using carbohydrate-rich microalgae biomass as feedstock", Bioresource Technology, 135:191–198.

[21] Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D., Yoshikuni, Y., (2012). "An engineered microbial platform for direct biofuel production from brown macroalgae", Science 335 (6066):308–313.

[22] Costa, J. A. V., and de Morais, M. G., (2013). An Open Pond System for Microalgal Cultivation. Biofuels from Algae, Elsevier B.V., USA

[23] Suali, E., and Sarbatly, R., (2012). "Conversion of microalgae to biofuel", Renewable and Sustainable Energy Reviews, 16(6):4316–4342.

[24] Christenson, L., and Sims, R., (2011). "Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts", Biotechnology Advances, 29(6):686–702.

[25] Amit Garg, Bruce A. Kimball, D.C.Uprety, Dong Hongmin, Jigeesha Upadhyay, Subash Dhar.,(2006). Microalgae for mitigating carbon dioxide.,http://www.climatetechwiki.org/technology/co2-mitigation-micro-algae, 2006.

[26] Chng, L. M., (2016). "Sustainable production of bioethanol using lipid-extracted microalgae from *Scenedesmus dimorphus*", Journal of Cleaner production, 1-10.

[27] National Research Council, (2012). Sustainable Development of Algal Biofuels in the United States, The National Academies Press, USA.

[28] Singh, P., Gupta, S. K., Guldhe, A., Rawat, I., and Bux, F., (2015). Microalgae Isolation and Basic Culturing Techniques, Handbook of Marine Microalgae: Biotechnology Advances, First Edition, Elsevier Inc., UK.

[29] Drira, N., Piras, A., Rosa, A., Porcedda, S., and Dhaouadi, H., (2016). "Microalgae from domestic wastewater facility's high rate algal pond: Lipids extraction, characterization and biodiesel production", Bioresource Technology, 206:239–244.

[30] Kothari, R., Prasad, R., Kumar, V., and Singh, D. P., (2013). "Production of biodiesel from microalgae *Chlamydomonas polypyrenoideum* grown on dairy industry wastewater", Bioresource Technology, 144:499–503.

[31] Jebali, A., Acien, F. G., Gomez, C., Fernandez-Sevilla, J. M., Mhiri, N., Karray, F., Sayadi, S., (2015). "Selection of native Tunisian microalgae for simultaneous wastewater treatment and biofuel production", Bioresource Technology, 198:424–430.

[32] Wahidin, S., Idris, A., and Shaleh, S. R. M., (2013). "The influence of light intensity and photoperiod on the growth and lipid content of microalgae *Nannochloropsis* sp.", Bioresource Technology, 129:7–11.

[33] Lazaro, C. Z., Varesche, M. B. A., and Silva, E. L., (2015). "Effect of inoculum concentration, pH, light intensity and lighting regime on hydrogen production by phototrophic microbial consortium", Renewable Energy, 75:1–7.

[34] Yongjun Zhao, Juan Wang, Hui Zhang, Cheng Yan, Yuejin Zhang,(2013). "Effects of various LED light wavelengths and intensities on microalgae-based simultaneous biogas upgrading and digestate nutrient reduction process", Bioresource Technology,136:461-468.

[35] Atta, M., Idris, A., Bukhari, A., and Wahidin, S., (2013). "Intensity of blue LED light: A potential stimulus for biomass and lipid content in fresh water microalgae *Chlorella vulgaris*", Bioresource Technology, 148:373–378.

[36] F. Ghezelbash, T. Farboodnia, R. Heidari and N. Agh.,(2008). "Effects of Different Salinities and Luminance on Growth Rate of the Green Microalgae *Tetraselmis chuii*", Research Journal of Biological Sciences,3(3): 311-314.

[37] Khatoon, H., Abdu Rahman, N., Banerjee, S., Harun, N., Suleiman, S. S., Zakaria, N. H., Endut, A., (2014). "Effects of different salinities and pH on the growth and proximate composition of *Nannochloropsis* sp. and *Tetraselmis* sp. isolated from South China Sea cultured under control and natural condition", International Biodeterioration and Biodegradation, 95(PA):11–18.

[38] Laing, I., (1991). "Cultivation of marine unicellular algae", MAFF Laboratory Leaflet, (67):32.

[39] Pragya, N., Pandey, K. K., and Sahoo, P. K., (2013). "A review on harvesting, oil extraction and biofuels production technologies from microalgae", Renewable and Sustainable Energy Reviews, 24:159–171.

[40] Prabandono, K., and Amin, S.,(2015). Chapter 10 - Biofuel Production from Microalgae, Handbook of Marine Microalgae, First Edition, Elsevier Inc., UK.

[41] Chen, C. Y., Yeh, K. L., Aisyah, R., Lee, D. J., and Chang, J. S., (2011). "Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review", Bioresource Technology, 102(1):71–81.

[42] Mollah, M. Y. A., Morkovsky, P., Gomes, J. A. G., Kesmez, M., Parga, J., and Cocke, D. L., (2004). "Fundamentals, present and future perspectives of electrocoagulation", Journal of Hazardous Materials, 114(1-3):199–210.

[43] Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., and Bux, F., (2011). "Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production", Bioresource Technology, 102(1):57–70.

[44] Experimental Biosciences, Using a counting champers, http://www.ruf.rice.edu/~bioslabs/methods/microscopy/cellcounting.html, 10 August 2012.

[45] S. Şirin and M. Sillanpaa, (2015). "Cultivating and harvesting of marine alga Nannochloropsis oculata in local municipal wastewater for biodiesel", Bioresource Technology, 191:79-87.

[46] Michels, M. H. A., Vaskoska, M., Vermuë, M. H., and Wijffels, R. H., (2014). "Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm", Water Research, 65(0):290–296.

[47] Sukarni, Sudjito, Hamidi, N., Yanuhar, U., and Wardana, I. N. G., (2014). "Potential and properties of marine microalgae *Nannochloropsis oculata* as biomass fuel feedstock", International Journal of Energy and Environmental Engineering, 5(4):279–290.

[48] Neto, A. M. P., Sotana de Souza, R. A., Leon-Nino, A. D., da Costa, J. D. arc A., Tiburcio, R. S., Nunes, T. A., Gianesella, S. M. F., (2013). "Improvement in microalgae lipid extraction using a sonication-assisted method", Renewable Energy, 55:525–531.

[49] Alsull, M., and Omar, W. M. W., (2012). "Responses of *Tetraselmis* sp. and *Nannochloropsis* sp . Isolated from Penang National Park Coastal Waters , Malaysia , to the Combined Influences of Salinity , Light and Nitrogen Limitation", International Conference on Chemical, Ecology and Environmental Sciences (ICEES'2012), 20 July 2012, Phnom Penh, Cambodia.

[50] M.M.Rebolloso-Fuentes, A.Navarro-perez, F.Garcia-Camacho, J.J.Ramos-Miras, and J.L.Guil-Guerrero., (2011). "Biomass Nutrient Profiles of the Microalgae *Nannochloropsis*", J.Agric.Food Chem, 49:2966-2972

[51] Choi, S. P., Nguyen, M. T., and Sim, S. J., (2010). "Enzymatic pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production", Bioresource Technology, 101(14):5330–5336.

[52] Lam, M. K., and Lee, K. T., (2015). Bioethanol Production from Microalgae, Handbook of Marine Microalgae, First Edition, Elsevier Inc., UK.

[53] Jose C.M. Pires.,(2015). Mass production of Microalgae, Handbook of Marine Microalgae, First Edition, Elsevier Inc.,UK.

[54] Eunyong Lee, Mehregan Jalalizadeh, Qiong Zhang., (2015). "Growth kinetic models for microalgae cultivation: A review", Algal Research, 12:497-512.

[55] Widdel, F., (2007). "Theory and measurement of bacterial growth", Di Dalam Grundpraktikum Mikrobiologie, 1–11.

[56] Imamoglu, E., Demirel, Z., and Conk Dalay, M., (2015). "Process optimization and modeling for the cultivation of *Nannochloropsis* sp. and *Tetraselmis striata* via response surface methodology", Journal of Phycology, 453:442–453.

[57] Huerlimann, R., de Nys, R., and Heimann, K., (2010). "Growth, lipid content, productivity, and fatty acid composition of tropical microalgae for scale-up production", Biotechnology and Bioengineering, 107:245–257.

[58] Campa ña-Torres, a, Mart ínez-C órdova, L., Mart ínez-Porchas, M., L ópez-El ás, J., and Porchas-Cornejo, M., (2012). "Productive response of *Nannochloropsis oculata* , cultured in different media and their efficiency as food for the rotifer *Brachionus rotundiformis*", International Journal of Experimental Botany, 9457(81):45–50.

[59] Mennaa, F. Z., Arbib, Z., and Perales, J. A.,(2015). "Urban wastewater treatment by seven species of microalgae and an algal bloom: Biomass production, N and P removal kinetics and harvestability", Water Research, 83:42–51.

[60] Lowrey, J. B., (2011). Seawater / wastewater production of microalgae- based biofuels in closed loop tubuluar, Msc.thesis, The Faculty of California Polytechnic State University, San Luis Obispo.

[61] Kothari, R., Pathak, V. V, Kumar, V., and Singh, D. P.,(2012). "Bioresource Technology Experimental study for growth potential of unicellular alga *Chlorella pyrenoidosa* on dairy waste water : An integrated approach for treatment and biofuel production", Bioresource Technology, 116:466–470.

[62] Giordano, M., Palmucci, M., and Raven, J. A., (2015). "Growth rate hypothesis and efficiency of protein synthesis under different sulphate concentrations in two green algae", Plant, Cell and Environment, 38(11):2313–2317.

[63] Alkhamis, Y., and Qin, J. G., (2013). "Cultivation of *Isochrysis galbana* in phototrophic, heterotrophic, and mixotrophic conditions", BioMed Research International. Article ID 983465, 9 pages.

[64] Guo, H., Daroch, M., Liu, L., Qiu, G., Geng, S., and Wang, G., (2013). "Biochemical features and bioethanol production of microalgae from coastal waters of Pearl River Delta", Bioresource Technology, 127:422–428.

[65] John, R. P., Anisha, G. S., Nampoothiri, K. M., and Pandey, A., (2011). "Micro and macroalgal biomass: A renewable source for bioethanol", Bioresource Technology, 102(1):186–193.

[66] Harun, R., Jason, W. S. Y., Cherrington, T., and Danquah, M. K., (2010). "Exploring alkaline pre-treatment of microalgal biomass for bioethanol production", Applied Energy, 88(10):3464–3467.

[67] Harun, R., and Danquah, M. K., (2011). "Influence of acid pre-treatment on microalgal biomass for bioethanol production", Process Biochemistry, 46(1):304–309.

[68] Biller, P., and Ross, A. B., (2011). "Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content", Bioresource Technology, 102(1):215–225.

[69] Bondioli, P., Della Bella, L., Rivolta, G., Chini Zittelli, G., Bassi, N., Rodolfi, L., Tredici, M. R., (2012). "Oil production by the marine microalgae *Nannochloropsis* sp. FandM-M24 and *Tetraselmis suecica* FandM-M33", Bioresource Technology, 114:567–572.

[70] MARIENFELD, Counting chambers with V-Slash, http://www.marienfeld-superior.com/index.php/counting-chambers/articles/counting-chambers-with-v-slash.html, 14 April 2016.

[71] Microbehunter Microscopy Magazine, The hemocytometer (counting chamber) http://www.microbehunter.com/the-hemocytometer-counting-chamber, 01 November 2015.

[72] S. Suzanne Nielsen., (2010). Food Analysis Laboratory Manual. 2nd Edition, Springer, USA.

[73] Inan, B., (2014). Alglerin biyoetanol üretiminde değerlendirilmesi, MSc.thesis, Yıldız Technical University, Graduate School of Natural and Applied Sciences, Istanbul.

[74] Beuckels, A., Smolders, E. and Muylaert, K., (2015). "Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment", Water Research, 77:98–106.

CURRICULUM VITAE

PERSONAL INFORMATION

Name Surname	: Zubaidai REYIMU
Date of birth and place	:April 12th, 1990, Korla (China)
Foreign Languages	:Chinese, English and Turkish
E-mail	:zubeyde_rehim@163.com

EDUCATION

Degree	**Department**	**University**	**Date of Graduation**
Undergraduate	Bioengineering	Tianjin University of Science and Technology,CHINA	2013
High School	Applied Science	Shanghai Qibao High school,CHINA	2009

WORK EXPERIENCE

Year	**Corporation/Institute**	**Enrollment**
2013	Food and Drug Administration (FDA), Korla, Xinjiang, China	Intern

PUBLISHMENTS

Papers

1. YANG Jia;ZU Baidai;YANG Qing;DING Xuemei;ZHAO Shuxin;LI Xianshu;YANG Yi, (2014). “Isolation and Aroma-producing Study of Bacteria from Xiaoerbulake High-temperature Daqu”, Liquor-Making Science and Technology, 2014.

Conference Papers

1. Özçimen, D., Vehapi, M., Karakaş, C. Y., Demirer, B. Ö., İnan, B., Akış, S., Koçer, A. T., and Rehim, Z., “Investigation of Growth Kinetics of *Nannochloropsis*, *Isochrysis* and *Tetraselmis* sp.”, BIO ENG’14 Bioengineering Conference. (2014, Kasım)

2. Koçer, A. T., Özçimen, D., İnan, B., Mutlu, B., Balkanlı, N. E., and Reyimu, Z. ,“Utilization of Macroalgae via Thermochemical Process for Biochar Production”. 3rd International Symposium on Innovative Technologies in Engineering and Science. (2015, Haziran)

3. Zubaidai Reyimu, Didem Özçimen, Anıl T.Koçer, Benan Inan,”Marine Microalgae growth in batch culture treating municipal wastewater”, Eurasia 2016 waste management symposium, 2016

Books

1. Özçimen, D., İnan, B., Koçer, A. T., and Reyimu, Z. (2015) Sustainable Design and Synthesis of Algal Biorefinery for Biofuel Production. Biofuels: Production and Future Perspectives: Taylor and Francis (in press).