이용자는 아래의 조건을 따르는 경우에 한하여 자유롭게

- 이 저작물을 복제, 배포, 전송, 전시, 공연 및 방송할 수 있습니다.
- 이차적 저작물을 작성할 수 있습니다.

다음과 같은 조건을 따라야 합니다:

저작자표시. 귀하는 원저작자를 표시하여야 합니다.

비영리. 귀하는 이 저작물을 영리 목적으로 이용할 수 없습니다.

동일조건변경허락. 귀하가 이 저작물을 개작, 변형 또는 가공했을 경우 에는, 이 저작물과 동일한 이용허락조건하에서만 배포할 수 있습니다.

- 귀하는, 이 저작물의 재이용이나 배포의 경우, 이 저작물에 적용된 이용허락조건 을 명확하게 나타내야합니다.
- 저작권자로부터 별도의 허가를 받으면 이러한 조건들은 적용되지 않습니다.

저작권법에 따른 이용자의 권리는 위의 내용에 의하여 영향을 받지 않습니다.

이것은 이용허락규약(Legal Code)을 이해하기 쉽게 요약한 것입니다.

Disclaimer
Study on Factors Enhancing Biomass Productivity in Green Algae

2012 年 2 月

仁荷大學校 大學院
生物工學科
任나례
Study on Factors Enhancing Biomass Productivity in Green Algae

By

YIM, NA-RAE

A THESIS

Submitted to the faculty of

INHA UNIVERSITY

in partial fulfillment of the requirements

For the degree of

MASTER OF SCIENCE

Department of Biological Engineering

2012
이 논문을 임재혁의 석사학위논문으로 인정함.

2012 년 2 월

주심

부심

위원
# TABLE OF CONTENTS

TABLE OF CONTENTS ................................................................. 1
LIST OF TABLES ........................................................................... 4
ABSTRACT ...................................................................................... 5
1. INTRODUCTION ......................................................................... 7
2. LITERATURE REVIEW ............................................................... 10
   2.1 Microalgae ........................................................................... 10
   2.2 Biodiesel .............................................................................. 11
   2.3 Factors Affecting Biomass Production .................................. 13
      2.3.1 Nutrients ....................................................................... 13
      2.3.2 Light ............................................................................. 15
      2.3.3 Temperature ................................................................... 15
      2.3.4 Carbon Dioxide ............................................................. 16
      2.3.5 pH ................................................................................ 16
   2.4 Factorial Design ................................................................... 17
3. MATERIALS AND METHODS ..................................................... 18
   3.1 Strains and Culture Conditions ............................................ 18
      3.1.1 *Chlorella vulgaris* NIES-2170 ................................... 18
      3.1.2 *Chlorella vulgaris* NIES-2166 ................................... 18
      3.1.3 Culture Condition .......................................................... 19
   3.2 Cell Concentration and Cell Size Distribution ...................... 20
   3.3 Light Sources and Light Intensity Measurement ................... 20
   3.4 Nitrogen Sources .................................................................. 21
   3.5 Nitrate Concentration ........................................................... 21
   3.6 Factorial Design ................................................................... 21
      3.6.1 Fractional Factorial Design ............................................ 22
      3.6.2 Full Factorial Design ....................................................... 22
   3.7 Dimensionless number, B ..................................................... 23
4. RESULTS AND DISCUSSION .................................................... 24
   4.1 Effects of Nutrients and Environmental Factors on Biomass .... 24
4.1.1 Effect of Nutrients on Biomass ............................................... 24
4.1.2 Effect of Environmental Factors on Biomass ....................... 29

4.2 Effects of Nitrogen Sources, Light Intensity and Light Quality which affecting to Photosystem in Chloroplast on Biomass Production .... 33
   4.2.1 Effect of Nitrogen Sources on Biomass .............................. 34
   4.2.2 Effect of Light Intensity on Biomass ................................. 36
   4.2.3 Effect of Light Quality on Biomass .................................. 38

5. CONCLUSION ............................................................................. 41

6. REFERENCES ............................................................................. 43
LIST OF FIGURES

Figure 1. Major events and world oil prices, 1990-2010......................... 7
Figure 2. Transesterification of oil to biodiesel. ................................. 11
Figure 3. Pareto chart of the standardized effects in fractional factorial
design........................................................................................................... 26
Figure 4. Cell concentration of *Chlorella vulgaris* in fractional factorial
design............................................................................................................ 28
Figure 5. Pareto chart of the standardized effects in full factorial design . 30
Figure 6. Cell concentration of *Chlorella vulgaris* in full factorial design. 32
Figure 7. Metabolic pathway of microalgae and factors affecting biomass
production....................................................................................................... 33
Figure 8. Cell growth of *Chlorella vulgaris* cultivated in media consisting
of sodium nitrate, ammonium chloride and urea............................. 34
Figure 9. Cell concentration of *Chlorella vulgaris* with different light
intensity. Cultures were illuminated by fluorescence lamp. ....... 36
Figure 10. Cell concentration of *Chlorella vulgaris* with fluorescent lamp,
red light-emitting diodes (LEDs), green LEDs and blue LEDs as
light source .................................................................................................. 38
LIST OF TABLES

Table 1. Levels of the variables tested in the fractional factorial design (Unit: g/L) ................................................................. 24
Table 2. Fractional factorial design for four variables ....................... 25
Table 3. Estimated regression coefficients of fractional factorial design... 27
Table 4. Levels of the variables tested in the full factorial design......... 29
Table 5. Full factorial design for three variables ................................ 29
Table 6. Estimated regression coefficients of full factorial design ....... 31
Table 7. Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* with different nitrogen sources.......................... 34
Table 8. B values with different nitrogen sources............................. 35
Table 9 Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* with different light intensities .............................. 37
Table 10. B values of between light intensities.................................. 37
Table 11. Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* under different light sources ....................... 39
Table 12. B values between blue LEDs and the other light sources ....... 39
ABSTRACT

Biofuels have become a renewable energy because the oil price fluctuates depending on global major events such as Iran-Iraq War, financial crisis. World carbon dioxide emissions from burning fossil fuels have surged to record levels.

Microalgae, one of the feedstock of biodiesel, have a relatively faster growth rate than land plants and a higher lipid contents under proper condition. Moreover, microalgae are non-edible feedstock and do not require as large space for culture as other oil crops.

However, the production costs of biodiesel from microalgae are higher than those of conventional petroleum diesel. It is necessary that biomass production has to be enhanced and optimized in order to reduce the production costs. Furthermore, to increase biomass production, it is more efficient to use microalgae which have faster growth rates than to use microalgae which are contained a lot of lipid contents.

These studies were carried out in order to investigate significant factors for enhancement of biomass production. In fractional factorial design, magnesium concentration was the significant factor for growth among each component in medium.

B value was used in order to predict how biomass will increase or optimize. B value of magnesium concentration approached to 0.5 and it means that optimization of magnesium concentration can’t bring dramatic increase of biomass.
In full factorial design, experiments were performed to identify environmentally effective factors such as light intensity, CO$_2$ concentration and flow rate. As a result, CO$_2$ concentration was screened as the main factor for growth of *Chlorella vulgaris*. Its B value also approached to 0.5 similar to B value of magnesium concentration.

Optimization of nutrients and environmental factors could not affect the cell growth. It means a medium was already optimized biomass production in some degree.

Nitrogen sources, light intensity and light quality were selected as directly affecting factors to photosystem in chloroplast in order to screen how B value will be decrease. As the results, B values under light quality and light intensity were much lower (0.13 and 0.08, respectively) than nutrients and environmental factors.

Therefore, studies will have to focus on light quality and light intensity because these are more sensitivity than the others.

**Keywords:** microalgae, factorial design, dimensionless number (B)
1. INTRODUCTION

Nowadays, biofuels are an attractive source of energy in order to replace fossil fuels, to increase the security of energy supply and to reduce transportation emissions because fossil fuels are limited and the oil price increase depending on global major events such as invasion and economic crisis [1].

![Figure 1. Major events and world oil prices, 1990-2010](Canadian Petroleum Products Institute, CPPI)
One of the most common biofuels as alternative transportation fuel is biodiesel. Using biodiesel as alternative transportation fuel contains several advantages. First of all, biodiesel can be used with our current fueling infrastructure such as storage and distribution system and biodiesel works in any diesel engine with little or no modifications to the engine [2]. Biodiesel provides safety benefits. Biodiesel is non-toxic, so it causes far less damage than petroleum diesel. It is also safer because of its higher flash point, so it is less combustible [3]. Moreover, use of biodiesel results in substantial reduction of carbon monoxide, hydrocarbons and diesel particulates. Also, biodiesel contains virtually no sulfur or aromatics, so it does not contribute to sulfur dioxide emissions [2].

Increasing biodiesel production on cultured land could have severe consequences for global food supplies. Biodiesel from microalgae is widely regarded as one of the most efficient ways of generating biodiesel and also appears to represent the only current renewable source of oil that could meet the global demand for transport fuels.

The use of microalgae can be more suitable alternative source for biodiesel for several reasons. At first, microalgae have high growth rate and photosynthetic yields. Microalgae have short biomass doubling time than oil crops and show very high lipid accumulation capability [4]. Oil content of microalgae can exceed 80% by weight of dry biomass where as less than 5% for agricultural oil crops [5]. Therefore, microalgae would be the most compatible and realistic biodiesel feedstock.
However, production cost of biodiesel from microalgae is more expensive than that of gasoline and diesel. The microalgal biomass market produces about 5 kt of dry matter/year at production costs of 25,000 $/t [6]. To replace 5% of US demand of fuel for transport it is necessary to produce 66,250 kt of oil rich biomass at production costs lower than 400 $/t [5].

Thus, to make maximal production of algal biomass, it is necessary that biomass production has to be enhanced and optimized. Furthermore, it is more efficient to use microalgae which have fast growth rate than to use microalgae which are contained a lot of lipid content.

The cell growth of microalgae depends on many factors which nutrients, light intensity, light quality, carbon dioxide and so on [7] [8].

The purpose of this work is to screen significant factors for cell growth and to predict how biomass will increase or optimize by introducing B value.
2. LITERATURE REVIEW

2.1 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure [1].

Microalgae present in all existing earth ecosystems, not just aquatic but also terrestrial, representing a big variety of species living in a wide range of environmental conditions. It is estimated that more than 50,000 species exist, but only a limited number, of around 30,000, have been studied and analyzed [9].

Depending on the microalgae species other compounds may also be extracted, with variable applications in different industrial sectors, including a large range of fine chemicals and bulk products, such as fats, polyunsaturated fatty acids, oil, natural dyes, pigments, antioxidants and other fine chemicals and biomass [1] [10] [11].

Most of the commercially produced algal biomass is being marketed as health foods, in the forms of tablets and capsules. Several microalgae, such as species of Chlorella, Spirulina and Dunaliella, are grown commercially and algal products such as β-carotene and phycocyanin are available [12] [13].
2.2 Biodiesel

The major components of vegetable oils and animal fats are triacylglycerols (TAG; often also called triglycerides). Chemically, TAG are esters of fatty acids (FA) with glycerol (1, 2, 3-propanetriol; glycerol is often also called glycerine). To obtain biodiesel, the vegetable oil or animal fat is subjected to a chemical reaction termed **transesterification**. In that reaction, the vegetable oil or animal fat is reacted in the presence of a catalyst (usually a base) with an alcohol (usually methanol) to give the corresponding alkyl esters (or for methanol, the methyl esters) of the FA mixture that is found in the parent vegetable oil or animal fat [14].

![Diagram of transesterification reaction]

**Figure 2. Transesterification of oil to biodiesel.**
Transesterification is a multiple step reaction, including three reversible steps in series, where triglycerides are converted to diglycerides, then diglycerides are converted to monoglycerides, and monoglycerides are then converted to esters (biodiesel) and glycerol (by-product). The overall transesterification reaction is described in Figure 1 where the radicals $R_1$, $R_2$, $R_3$ represent long chain hydrocarbons, known as fatty acids [1].
2.3 Factors Affecting Biomass Production

There are several factors influencing algal growth: nutrients, light intensity and light quality, temperature, carbon dioxide and growth-medium pH. Therefore, factors affecting biomass production have to be regulated in order to enhance algal biomass.

2.3.1 Nutrients

Nitrogen

Nitrogen is quantitatively the most important element in algal nutrition. These are present in form of proteins, vitamins, chlorophylls, nucleic acid in microalgae.

There are different sources of nitrogen form which microalgae can directly or indirectly take or assimilate nitrogen. Generally, algae are able to utilize nitrate, ammonia and other organic sources of nitrogen such as urea. Atmospheric gases contain nitrogen in form of nitrogen gas and oxide of nitrogen majority of microalgae cannot assimilate directly. Certain cyanobacteria are also capable of assimilating nitrogen in its elemental form from the atmosphere [15]. *Neochloris oleoabundans* was cultivated in media consisting sodium nitrate, ammonium bicarbonate and urea. In this experiment, ammonium bicarbonate support rather poor growth of *N. oleoabundans* under the investigated conditions. On the other hand, sodium nitrate was the best nitrogen source among three tested compounds [16].
Phosphorous

Phosphorous is a major nutrients for normal growth of all algae. It is essential for almost all cellular processes (i.e. biosynthesis of nucleic acids). The phosphorus concentration is often growth limiting in the natural aqueous habitat. The major form in which algae acquire phosphorous is as inorganic phosphate, either as $H_2PO_4^-$ or $HPO_4^{2-}$ [15] [17].

Chen et al. (2011) carried out experiments that effect of nutrients on growth and lipid accumulation in Dunaliella tertiolecta. In their studies, phosphate deprivation had little effect growth because of intracellular phosphate stores [18].

Sulfur

Sulfur is vital to all cells because it is a constituent of some essential amino acids (methionine, cysteine) and vitamins, etc. It is generally provided as inorganic sulfate in the culture medium [15].

Iron

The importance of iron for the growth of algae is well substantiated. It is a key element in metabolism, being a constituent of cytochromes. It plays an important role in nitrogen assimilation as a functional part of ferredoxin and affects the synthesis of phycocyanin and chlorophyll [15].
2.3.2 Light

Microalgae grown at various light intensities show remarkable changes in their whole chemical composition, photosynthetic activity and pigment content [19]. Two properties of light energy are important for algal growth and metabolism: spectral quality and intensity. Spectral quality is defined by the absorption spectrum for the chlorophyll and other photosynthetically active pigments, and the photosynthetic efficiency is a function of spectra quality. The spectrum of emitted light from the light source, the delivery and the distribution of the light into the culture, and the light scattering and attenuation in the culture must be considered [20].

2.3.3 Temperature

A difference in the effects of temperature on cell mass increase and cell division has been reported for *Chlorella* [15].

Temperature is the most important limiting factor. Many microalgae can easily tolerate temperatures up to 15°C lower than their optimal, but exceeding the optimum temperature by only 2-4°C may result in the total culture loss [1].
2.3.4 Carbon Dioxide

Since about 50% of the algal biomass consists of carbon, a sufficient supply of carbon is of vital importance for successful cultivation. Carbon can be supplied as an inorganic substrate in the form of gases carbon dioxide. Algae require an inorganic carbon source to perform photosynthesis [15]. *Dunaliella tertiolecta* was grown using ten different carbon dioxide concentrations (0%, 0.03%, 0.1%, 2%, 4%, 6%, 10%, 20% and 100%) in air under fluorescent light of 100 μE·m⁻²·s⁻¹, a photoperiod of 15 h light: 9 h dark and temperature of 23°C in culture flasks. The cultures with 2%, 4% and 6% carbon dioxide concentrations had comparable cell growth while 1% and 10% carbon dioxide concentrations had lower growth rates and algae did not grow at all without carbon dioxide [21].

2.3.5 pH

The pH of algal cultures can be influenced by various factors such as composition and buffering capacity of the medium, amount of carbon dioxide dissolved, temperature and metabolic activity of the algal cells [15].
2.4 Factorial Design

Factorial design was one of the statistical methods and powerful to screen several factors at the same time. It is widely accepted that the most commonly used experimental designs in many companies are full and fractional factorial designs at two-levels and three-levels. Factorial designs use studying to joint effect of the factors (or process/design parameters) on a response. A factorial design can be either full or fractional factorial.

A full factorial designed experiment consists of all possible combinations of levels for all factors. The total number of experiments for studying $N$ factors at 2-levels is $2^N$. The $2^N$ full factorial design is particularly useful in the early stages of experimental work, especially when the number of process parameters or design parameters (or factors) is less than or equal to 4.

If the experimenters can reasonably assume that certain higher-order interactions (third-order and higher) are not important, then information on the main effects and tow-order interactions can be obtained by running a fraction of full factorial experiment. This design is generally represented in the form $2^{(k-p)}$, where $k$ is the number of factors and $1/2^p$ represents the fraction of the full factorial $2^k$ [22].
3. MATERIALS AND METHODS

3.1 Strains and Culture Conditions

3.1.1 *Chlorella vulgaris* NIES-2170

The unicellular green algae *C. vulgaris*, NIES-2170 was purchased from the National Institute for Environmental Studies (NIES), and was cultivated photoautotrophically in N-8 medium consisting of 1000 mg/L of KNO$_3$, 740 mg/L of KH$_2$PO$_4$, 212 mg/L of Na$_2$HPO$_4$, 50 mg/L of MgSO$_4$·7H$_2$O, 13 mg/L of CaCl$_2$·2H$_2$O, 10 mg/L of C$_{10}$H$_{16}$N$_2$O$_8$ (EDTA), 1mL of micronutrients in distilled water. The micronutrients stock contains 3,580 mg/L of Al$_2$(SO$_4$)$_3$·18H$_2$O, 12,980 mg/L of MnCl$_2$·4H$_2$O, 1,830 mg/L of CuSO$_4$·5H$_2$O, 3,200 mg/L of ZnSO$_4$·7H$_2$O in distilled water. The medium was autoclaved after adjusting the initial pH to 6.5±0.5 for sterilization.

3.1.2 *Chlorella vulgaris* NIES-2166

The green algae *C. vulgaris*, NIES-2166 was purchased from the NIES, and was cultivated photoautotrophically. The name of *C. vulgaris* NIES-2166 was changed into *Coccomyxa subellipsoidea* C-169 after whole genome sequencing. Algae of the genus *Coccomyxa* occur as free-living form and as photobiont of symbiotic system in lichen [23]. This strain was cultivated in Bold’s basal medium (BBM) consisting of 246.5 mg/L of NaNO$_3$, 24.99 mg/L of CaCl$_2$·2H$_2$O, 73.95 mg/L of MgSO$_4$·7H$_2$O, 4.98
mg/L of FeSO₄·7H₂O, 74.9 mg/L of K₂HPO₄, 175.57 mg/L of KH₂PO₄, 25.13 mg/L of NaCl, 49.68 mg/L of C₁₀H₁₆N₂O₈ (EDTA), 1.57 mg/L of CuSO₄·5H₂O, 11.42 mg/L of H₃BO₃, 1.44 mg/L of MnCl₂·4H₂O, 8.82 mg/L of ZnSO₄·7H₂O, 0.49 mg/L of Co(NO₃)₂·6H₂O, 0.71 mg/L of MoO₃, 30.86 mg/L of KOH, and 0.98 mg/L of H₂SO₄ in distilled water. The medium was autoclaved after adjusting the initial pH to 6.8±0.5 for sterilization.

3.1.3 Culture Condition

A single colony of cells grown on an agar plate was inoculated into 10 mL medium in a 50 mL Scott-Duran flask. After a week, 50 mL was scaled up 100 mL and 250 mL flask. The inoculated flasks were incubated at 23°C under continuous shaking (140 rpm) and irradiated at 40 μE·m⁻²·s⁻¹ with fluorescent lamps (Model FL 18D, OSRAM Korea Co., Korea). The seed cultures were grown in 0.5 liter bubble column photobioreactors (BC-PBRs) containing 0.4 L N-8. After 3 days of cultivation, the cells reached the exponential. The cells were transferred into same-scaled 0.5 L BC-PBRs containing 0.4 L of culture broth with 0.2 vvm aeration containing 5% CO₂ gas and 95% air under constant continuous light intensity of 100 μE·m⁻²·s⁻¹ at the column surface. Compact fluorescent lamps (Model DULUX L®, 55 W/865, OSRAM Korea, Korea) were used for all the external illumination of BC-PBRs. The temperature and pH were kept at 24±1°C during cultivation time. Samples were collected every day.
3.2 Cell Concentration and Cell Size Distribution

The cell concentration and the average cell size were measured with a Coulter Counter (Model Multisizer 3, Beckman Coulter, Inc., Brea, CA, USA). Then the data from Coulter counter were exported to an Excel spreadsheet to calculate the cell concentrations and cell size distribution and the average cell size.

3.3 Light Sources and Light Intensity Measurement

Four different light sources were used: fluorescent lamps (Model DULUX L® 55 W/865, OSRAM Korea, Korea), blue LEDs ($\lambda_{\text{max}}$ 470 nm, U-Jin LED Co., Ltd., Koyang, Korea), red LEDs ($\lambda_{\text{max}}$ 660 nm, U-Jin LED Co., Ltd., Koyang, Korea) and green LEDs ($\lambda_{\text{max}}$ 525 nm, U-Jin LED Co., Ltd., Koyang, Korea). The LEDs were powered by DC power supplies (made by BIOTRON, Korea) and the light intensities of each LED were adjusted by varying the supplied voltage. The light intensities were measured using a quantum sensor (Model LI-190SA, LI-COR, Inc., Lincoln, NE, U.S.A.).
3.4 Nitrogen Sources

The nitrogen source was sodium nitrate (SIGMA S-5022, 2.94 mM), ammonium chloride (SIGMA, A-0171, 2.94 mM) and urea (USB, 75826, 1.47 mM). These were dissolved equal molar concentrations of nitrogen.

3.5 Nitrate Concentration

Nitrate and chlorophyll concentrations were determined by a spectrophotometer (Model HP8453B, Hewlett-Packard, Waldbronn, Germany). Nitrate concentration was analyzed after treating the centrifuged sample (3,000 rpm for 10 min) with 1N HCl according to the standard method [24].

3.6 Factorial Design

This study is chosen factorial design at 2-levels only. To investigate the effects of nutrients in medium for the cell growth, fractional factorial design was used. Full factorial design was used to identify the significant environmental factors for the cell growth.

All statistical analysis from data was performed by statistical software, MINITAB (V14, Minitab Inc., State College, PA, USA), in this study.
3.6.1 Fractional Factorial Design

In this study, to identify the effects of nutrients in medium have a significant effect on cell growth in *Chlorella vulgaris*. The components of N-8 medium were grouped into four factors (KNO$_3$, KH$_2$PO$_4$, Na$_2$HPO$_4$, MgSO$_4$·7H$_2$O and CaCl$_2$·2H$_2$O). Therefore four will lead to 16 experiments. We chose to do 1/2 of the 16 experiments, giving 10 experiments including 2 central points in 1 block by Fractional factorial design.

3.6.2 Full Factorial Design

Second experiment was designed to screen the significant environmental factors for the cell growth. The environmental factors were light intensity, carbon dioxide concentration and flow rate. Full factorial design was chosen because the number of factors was only three. Moreover, if we use fractional factorial design, resolution of the experiment would be reduced and the main effect could not find. Therefore three factors will lead to 9 experiments including 1 central point in 1 block by full factorial design.
3.7 Dimensionless number, B

To compare biomass fluctuation between each sample, dimensionless number was used in this study.

\[ B = \frac{X_{min}}{X_{max}} \]

We assume that a factor is an important factor in an experiment of microalgae culture. And then we get the graphs as the result of experiment. In the graphs, \( X_{min} \) means minimum point of biomass (cells/mL) at final day and \( X_{max} \) means maximum point of biomass (cells/mL) at same condition. These values are not points in the same graph (or curve). The points are chosen among graphs (or curves) at the final day in the graphs and a experiment.

When B value approaches to “1”, it has lower possibility to enhance its biomass, on the other hand, when B value approaches to “0”, it has higher possibility to enhance its biomass.
4. RESULTS AND DISCUSSION

4.1 Effects of Nutrients and Environmental Factors on Biomass

4.1.1 Effect of Nutrients on Biomass

The first experiment is the investigation of the effect of four factors on the cell growth in *Chlorella vulgaris*. In this study, the components of N-8 medium were grouped into four factors. Therefore four will lead to 16 experiments. We chose to do 1/2 of the 16 experiments, giving 8 experiments including 2 central points in 1 block by Fractional Factorial Design. Table 1 shows the levels in natural units and the levels in coded units. Table 2 shows the design of this FFD with seven variables.

Table 1. Levels of the variables tested in the fractional factorial design (Unit: g/L)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Component</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>KNO₃</td>
<td>0.1</td>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td>B</td>
<td>KH₂PO₄</td>
<td>0.074</td>
<td>0.74</td>
<td>1.406</td>
</tr>
<tr>
<td></td>
<td>Na₂HPO₄</td>
<td>0.021</td>
<td>0.21</td>
<td>0.399</td>
</tr>
<tr>
<td>C</td>
<td>MgSO₄·7H₂O</td>
<td>0.005</td>
<td>0.05</td>
<td>0.095</td>
</tr>
<tr>
<td>D</td>
<td>CaCl₂·2H₂O</td>
<td>0.00132</td>
<td>0.0132</td>
<td>0.02508</td>
</tr>
</tbody>
</table>
### Table 2. Fractional factorial design for four variables

<table>
<thead>
<tr>
<th>Run</th>
<th>Blocks</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>CP*</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CP*</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

(CP*: Central point)

Figure 3 illustrates a Pareto plot of effect which indicates that main effect C (concentration of MgSO$_4$·7H$_2$O) is considered to have significant impact on cell growth at 2% significance level.

From the results of FFD, MgSO$_4$·7H$_2$O was selected as significant factors which effect on cell growth in *Chlorella vulgaris* in this experiment ($p \leq 0.02$).
Magnesium is an essential component of chlorophyll and thus an important factor for photosynthesis. When magnesium is deficient, microalgae could not produce energy such as ATP and NADPH for growth [15] [25].
Table 3. Estimated regression coefficients of fractional factorial design

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coefficient</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>0.2378</td>
<td>55.94</td>
<td>0.011</td>
</tr>
<tr>
<td>A</td>
<td>0.1295</td>
<td>0.0648</td>
<td>15.24</td>
<td>0.042</td>
</tr>
<tr>
<td>B</td>
<td>0.2365</td>
<td>0.1183</td>
<td>27.82</td>
<td>0.023</td>
</tr>
<tr>
<td>C</td>
<td>0.3030</td>
<td>0.1515</td>
<td>35.65</td>
<td>0.018</td>
</tr>
<tr>
<td>D</td>
<td>0.2500</td>
<td>0.1250</td>
<td>29.41</td>
<td>0.022</td>
</tr>
<tr>
<td>A*B</td>
<td>0.1345</td>
<td>0.6273</td>
<td>15.82</td>
<td>0.040</td>
</tr>
<tr>
<td>A*C</td>
<td>0.1730</td>
<td>0.8650</td>
<td>20.35</td>
<td>0.031</td>
</tr>
<tr>
<td>A*D</td>
<td>0.1800</td>
<td>0.9000</td>
<td>21.18</td>
<td>0.030</td>
</tr>
</tbody>
</table>

The statistical analysis results from FFD were shown in Table 3. This FFD gave information, which medium components in random range tested had a significant influence on cell growth in *Chlorella vulgaris*. 
In this graph (Figure 4), growth of R7 and R8 was excluded for comparison of B value because their cell concentrations have no difference with initial concentration. B value of magnesium concentration approached to 0.5 and it means that optimization of magnesium concentration can’t bring dramatic increase of biomass.

In case of growth of R3 and R6, it is effect of calcium concentration. Its B value also approached to 0.5 similar to B value of magnesium concentration.
4.1.2 Effect of Environmental Factors on Biomass

We carried out an experiment with three factors at 2-levels. The response of interest for the experiment was fresh cell weight/day. The list of process parameters and their levels are presented in Table 4.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Component</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Light intensity (μE·m⁻²·s⁻¹)</td>
<td>50</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>B</td>
<td>CO₂ conc. (mmol/hr)</td>
<td>0</td>
<td>10.7</td>
<td>21.4</td>
</tr>
<tr>
<td>C</td>
<td>Flow rate (mL/min)</td>
<td>40</td>
<td>80</td>
<td>120</td>
</tr>
</tbody>
</table>

Table 5. Full factorial design for three variables

<table>
<thead>
<tr>
<th>Run</th>
<th>Blocks</th>
<th>Variable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>CP*</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

(CP*: Central point)
In order to screen the significant environmental factors for the cell growth, it was decided to construct a coded design matrix (Table 5).

Figure 5 illustrates the Pareto plot of effects. The graph shows that main effect B (concentration of carbon dioxide) is significant at 5% significance level.

![Pareto Chart of the Standardized Effects](chart)

**Figure 5. Pareto chart of the standardized effects in full factorial design**

From the results of full factorial design, carbon dioxide concentration was found as significant factors which effect on cell growth in *Chlorella vulgaris* in this experiment ($p \leq 0.05$). The statistical analysis results from full factorial design were shown in Table 6.
Table 6. Estimated regression coefficients of full factorial design

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect</th>
<th>Coefficient</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td></td>
<td>0.3916</td>
<td>31.65</td>
<td>0.020</td>
</tr>
<tr>
<td>A</td>
<td>0.1653</td>
<td>0.0826</td>
<td>6.68</td>
<td>0.095</td>
</tr>
<tr>
<td>B</td>
<td>0.3998</td>
<td>0.1999</td>
<td>16.15</td>
<td>0.039</td>
</tr>
<tr>
<td>C</td>
<td>0.1703</td>
<td>0.0851</td>
<td>6.88</td>
<td>0.092</td>
</tr>
<tr>
<td>A*B</td>
<td>0.1158</td>
<td>0.0579</td>
<td>4.68</td>
<td>0.134</td>
</tr>
<tr>
<td>A*C</td>
<td>0.0593</td>
<td>0.0296</td>
<td>2.39</td>
<td>0.252</td>
</tr>
<tr>
<td>B*C</td>
<td>0.0138</td>
<td>0.0069</td>
<td>0.56</td>
<td>0.677</td>
</tr>
</tbody>
</table>

Carbon dioxide has an important role in cell growth of photosynthetic microorganism as a sole carbon source. In addition, dissolved carbon dioxide provides buffering capacity for preventing pH drifts [9] [15].

As the results, B value of carbon dioxide concentration also approached to 0.5. Its B value also approached to 0.5 similar to B values of magnesium concentration and calcium concentration (Figure 6).
Figure 6. Cell concentration of *Chlorella vulgaris* in full factorial design

In two experiments, magnesium and carbon dioxide concentration were the significant factors for growth of *Chlorella vulgaris*. However, these B value approached 0.5 and it means that optimization of these concentration cannot bring dramatic increase of biomass.

Therefore, the next experiment, we chose nitrogen source, light intensity and light quality because there are high sensitivity for cell growth and directly affected factors to photosystem and fatty acid synthesis in chloroplast.
4.2 Effects of Nitrogen Sources, Light Intensity and Light Quality which affecting to Photosystem in Chloroplast on Biomass Production

Nitrogen sources, light quality and light intensity were selected as directly affecting factors to photosystem and fatty acid in chloroplast.

**Figure 7. Metabolic pathway of microalgae and factors affecting biomass production**

Nitrogen sources, light intensity and light quality were selected.
4.2.1 Effect of Nitrogen Sources on Biomass

![Graph showing cell growth of Chlorella vulgaris cultivated in media consisting of sodium nitrate, ammonium chloride, and urea.](image)

**Figure 8.** Cell growth of *Chlorella vulgaris* cultivated in media consisting of sodium nitrate, ammonium chloride, and urea.

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>NO$_3$</th>
<th>NH$_4$</th>
<th>Urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum fresh cell weight (g/L)</td>
<td>3.98</td>
<td>3.09</td>
<td>3.83</td>
</tr>
<tr>
<td>Biomass productivity (g/L/day)</td>
<td>0.66</td>
<td>0.51</td>
<td>0.64</td>
</tr>
</tbody>
</table>

**Table 7.** Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* with different nitrogen sources
Nevertheless three nitrogen sources have each pathway with its own energy level in order to assimilate nitrogen, the cell growth under three conditions showed no difference (Figure 7, Figure 8 and Table 7).

Especially, ammonium, one of nitrogen source and the final form of inorganic nitrogen, needs smaller energy than the others in order to assimilate nitrogen because its pathway is shorter than the others (Figure 7).

In nitrogen metabolism pathway of *Chlamydomonas reinhardtii*, nitrate needs 2-step for conversion of ammonium. Nitrate is reduced to nitrite by nitrate reductase (NADH). And then, nitrite is reduced to ammonium by nitrite reductase.

Urea is one of the organic nitrogen sources to photosynthetic organisms such as algae and plants and converted to ammonium by urease, which catalyses the reaction of urea hydrolysis.

Biomass was not significantly different for any nitrogen sources. Because B value approaches over 0.5, optimization of biomass will be expected to be not easier than the other cases.

Table 8. B values with different nitrogen sources

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>B value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$_3$ and NH$_4$</td>
<td>0.96</td>
</tr>
<tr>
<td>NH$_4$ and Urea</td>
<td>0.56</td>
</tr>
<tr>
<td>NO$_3$ and Urea</td>
<td>0.59</td>
</tr>
</tbody>
</table>
4.2.2 Effect of Light Intensity on Biomass

Figure 9. Cell concentration of *Chlorella vulgaris* with different light intensity. Cultures were illuminated by fluorescence lamp.

Figure 9 shows the results of cell concentration. Although the cell concentration between 50 μE·m⁻²·s⁻¹ and 100 μE·m⁻²·s⁻¹ had no difference, *Chlorella vulgaris* in light intensity of 100 μE·m⁻²·s⁻¹ shows the largest amount of biomass (Table 9) taking into consideration of cell size. The cell concentration and fresh cell weight of 200 μE·m⁻²·s⁻¹ was the smallest.
Table 9 Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* with different light intensities

<table>
<thead>
<tr>
<th>Light intensity (µE·m⁻²·s⁻¹)</th>
<th>50</th>
<th>100</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum fresh cell weight (g/L)</td>
<td>1.92</td>
<td>3.17</td>
<td>0.71</td>
</tr>
<tr>
<td>Biomass productivity (g/L/day)</td>
<td>0.36</td>
<td>0.59</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 10. B values of between light intensities

<table>
<thead>
<tr>
<th>Light intensity (µE·m⁻²·s⁻¹)</th>
<th>B value</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 and 100</td>
<td>0.72</td>
</tr>
<tr>
<td>100 and 200</td>
<td>0.08</td>
</tr>
<tr>
<td>50 and 200</td>
<td>0.11</td>
</tr>
</tbody>
</table>

As the results, B value is 0.08 and 0.11 except for light intensity between 50 µE·m⁻²·s⁻¹ and 100 µE·m⁻²·s⁻¹ (0.72). This means light intensity will be controlled and changed between 50 µE·m⁻²·s⁻¹ and 200 µE·m⁻²·s⁻¹ (or 100 µE·m⁻²·s⁻¹ and 200 µE·m⁻²·s⁻¹). B value 0.72 means that although light intensity is controlled this range, biomass will not fluctuated dramatically. However, between blue LEDs and green LEDs, B value is 0.73. It means that optimization of blue LEDs cannot bring dramatic increase of biomass, although we will use green LEDs.
4.2.3 Effect of Light Quality on Biomass

Cultures were illuminated with light intensity of 100 μE·m\(^{-2}\)·s\(^{-1}\). Under the fluorescent lamp, the fresh cell weight was the highest and reached 3.17 g/L on the sixth day. Among three LEDs, biomass of red LEDs was higher than that of the others because wavelength of red LEDs was adjacent that of photosystem.
Table 11. Maximum fresh cell weight and biomass productivity of *Chlorella vulgaris* under different light sources

<table>
<thead>
<tr>
<th>Light source</th>
<th>Fluorescent lamp</th>
<th>Red LEDs</th>
<th>Green LEDs</th>
<th>Blue LEDs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum fresh cell weight (g/L)</td>
<td>3.17</td>
<td>1.98</td>
<td>0.56</td>
<td>0.41</td>
</tr>
<tr>
<td>Biomass productivity (g/L/day)</td>
<td>0.59</td>
<td>0.37</td>
<td>0.11</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 11 is the result that *Chlorella vulgaris* in blue LEDs is the smallest biomass. Biomass productivities with red, green and blue LEDs showed 37, 81 and 89 percentages lower than it was from fluorescent lamp.

Table 12. B values between blue LEDs and the other light sources

<table>
<thead>
<tr>
<th>Light source</th>
<th>B value</th>
</tr>
</thead>
<tbody>
<tr>
<td>B and G</td>
<td>0.73</td>
</tr>
<tr>
<td>B and R</td>
<td>0.21</td>
</tr>
<tr>
<td>B and F</td>
<td>0.13</td>
</tr>
</tbody>
</table>

(B: blue LEDs, G: green LEDs, R: red LEDs and F: fluorescent lamp)
Table 12 shows the B values between light sources. B value is 0.13 between growth of fluorescent lamp and that of blue LEDs. However, between blue LEDs and green LEDs, B value is 0.73. It means that optimization of blue LEDs cannot bring dramatic increase of biomass, although we will use green LEDs.
5. CONCLUSION

To screen factors for enhancement of biomass production, this study carried out effects of environmental factors and photosynthetic factors on cell growth of microalgae. Environmental factors mean nutrients and culture condition which is related to cell growth. The experiments were designed by using statistical method.

In fractional factorial design, magnesium concentration was the significant factor for growth of *Chlorella vulgaris*. However its B value approached to 0.5 and it means that optimization of magnesium concentration can’t bring dramatic increase of biomass.

As a result of full factorial design, followed experiment, carbon dioxide was screened as the main factor for growth of *Chlorella vulgaris*. Its B value also approached to 0.5 similar to B value of magnesium concentration.

Magnesium and carbon dioxide were statistically main factor for cell growth while they have low possibility to enhance their biomass. This means biomass production will be not proportional to amount of nutrients and environmental factors.

Optimization of nutrients and environmental factors could not affect the cell growth. It means a medium was already optimized biomass production in some degree.
In the next experiment, nitrogen sources, light intensity and light quality were selected as affecting directly to photosystem in chloroplast.

As the results, although three nitrogen sources have each pathway with its own energy level in order to assimilate nitrogen, the cell growth under three conditions showed no difference. In case of light intensity, *Chlorella vulgaris* in light intensity of 100 μE·m$^{-2}$·s$^{-1}$ shows the largest amount of biomass. Among red LEDs, green LEDs and blue LEDs, biomass of red LEDs was higher than that of the others because wavelength of red LEDs was adjacent that of photosystem.

B values under light quality and light intensity were much lower (0.13 and 0.08, respectively) than nutrients and environmental factors (factors chosen we used for statistical method). However, Cell growth and B value have no differences under three different media containing nitrate, ammonium and urea. Their B values have the number over 0.5.

All factors considered, non-manipulated environmental factors couldn’t affect the cell growth. In comparison, photosynthetic factors could change biomass quantity dramatically.

For enhancing biomass production, studies have to focus on light intensity and light quality because these factors have more sensitive than the others.
6. REFERENCES


intensity, reactor design, and algal physiology. Biotechnology and Bioengineering Symposium.