

REPORT DOCUMENTATION PAGE			Form Approved OMB NO. 0704-0188		
<p>The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA, 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</p>					
1. REPORT DATE (DD-MM-YYYY) 24-11-2015		2. REPORT TYPE Ph.D. Dissertation		3. DATES COVERED (From - To) -	
4. TITLE AND SUBTITLE MICROALGAE BIOPHOTONIC OPTIMIZATION OF PHOTOSYNTHESIS BY WEAKLY ABSORBED WAVELENGTHS			5a. CONTRACT NUMBER W911NF-11-1-0218		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER 206022		
6. AUTHORS ERICO ROLIM DE MATTOS			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES AND ADDRESSES University of Puerto Rico at Rio Piedras P. O. Box 21790  San Juan, PR 00931 -1790			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES) U.S. Army Research Office P.O. Box 12211 Research Triangle Park, NC 27709-2211			10. SPONSOR/MONITOR'S ACRONYM(S) ARO		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S) 59089-LS-REP.113		
12. DISTRIBUTION AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.					
13. SUPPLEMENTARY NOTES The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.					
14. ABSTRACT Adjusting the light supply to microalgae cultures at high cell density can enhance photosynthetic efficiency at latter stages of cultivation providing extra biomass growth and production. First we investigate inoculum cultivation based on physical and physiological characteristics. Chlorella sorokiniana cultures inoculated with inoculum at three different physiological stages (lag, exponential and stationary) diluted to the same optical density were cultivated for 12 days under three different CO2 concentrations (0.038%, 5% or 10% CO2					
15. SUBJECT TERMS Algae, growth, photosynthesis, bio-fuel					
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT UU	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON Kai Griebenow	
a. REPORT UU	b. ABSTRACT UU			c. THIS PAGE UU	19b. TELEPHONE NUMBER 787-764-0000

## Report Title

# MICROALGAE BIOPHOTONIC OPTIMIZATION OF PHOTOSYNTHESIS BY WEAKLY ABSORBED WAVELENGTHS

## ABSTRACT

Adjusting the light supply to microalgae cultures at high cell density can enhance photosynthetic efficiency at latter stages of cultivation providing extra biomass growth and production. First we investigate inoculum cultivation based on physical and physiological characteristics. *Chlorella sorokiniana* cultures inoculated with inoculum at three different physiological stages (lag, exponential and stationary) diluted to the same optical density were cultivated for 12 days under three different CO<sub>2</sub> concentrations (0.038%, 5% or 10% CO<sub>2</sub> v/v) and growth pattern and biomass production was observed. Samples inoculated with lag phase inoculum supplied with 5% CO<sub>2</sub> achieved the maximum biomass production whereas samples supplied with 0.038% CO<sub>2</sub> never reached exponential growth. The better growth of samples inoculated with lag phase inoculum was attributed to its increased number of cells compared to the other two inocula.

In another set of experiments, we investigate changes in the light supply to optimize biomass growth at high culture cell densities. First, using chlorophyll fluorescence measurements we evaluated the effects of 6 monochromatic wavelengths (627nm, 617nm, 590nm, 530nm, 505nm, 470nm) and a full spectrum neutral white LED at three different light intensities (100, 250 and 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the photosynthetic parameters quantum yield of photosystem II ( $\Phi\text{PSII}$ ) and non-photochemical quenching (NPQ) in the microalgae *Chlorella sorokiniana* at three different cell densities (OD 0.5, 1.0 and 1.5). An inverted correlation between  $\Phi\text{PSII}$  and light intensity was found across the whole experiment.

ii

Continuously decrease in  $\Phi\text{PSII}$  measured under the green light spectrum was observed as *C. sorokiniana* cell density increased whereas NPQ had a noticeable decrease under all light sources as the culture density increased from OD 0.5 to 1.0.

To confirm the indications found on the previously experiment, in a second experiment photosynthetic activity and biomass production induced by 4 different LEDs (blue-470nm, green-530nm, red-655nm, and white-3000K) were analyzed on high-density cultures of *Scenedesmus bijuga*. Light penetration and oxygen evolution were measured on cultures at different cell densities (0.7, 0.8, 1.0 and 1.45 g/L) and final dry biomass production was measured on samples with initial high biomass concentration (2.19 g/L). As culture density increased the weakly absorbed green light became more photosynthetically efficient than the red light, thereby inducing significantly higher oxygen evolution at culture concentration of 1.45 g/L. High-density culture (2.19 g/L) cultivated under the green light showed higher biomass production rate (30 mg/L/d) with a 8.43% dry biomass growth in a 6-day period compared to the red light that induced 4.35% dry biomass growth on the same period.

MICROALGAE BIOPHOTONIC OPTIMIZATION OF PHOTOSYNTHESIS BY  
WEAKLY ABSORBED WAVELENGTHS

by

ERICO ROLIM DE MATTOS

(Under the Direction of Keshav Das and Miguel Cabrera)

ABSTRACT

Adjusting the light supply to microalgae cultures at high cell density can enhance photosynthetic efficiency at latter stages of cultivation providing extra biomass growth and production. First we investigate inoculum cultivation based on physical and physiological characteristics. *Chlorella sorokiniana* cultures inoculated with inoculum at three different physiological stages (lag, exponential and stationary) diluted to the same optical density were cultivated for 12 days under three different CO<sub>2</sub> concentrations (0.038%, 5% or 10% CO<sub>2</sub> v/v) and growth pattern and biomass production was observed. Samples inoculated with lag phase inoculum supplied with 5% CO<sub>2</sub> achieved the maximum biomass production whereas samples supplied with 0.038% CO<sub>2</sub> never reached exponential growth. The better growth of samples inoculated with lag phase inoculum was attributed to its increased number of cells compared to the other two inocula.

In another set of experiments, we investigate changes in the light supply to optimize biomass growth at high culture cell densities. First, using chlorophyll fluorescence measurements we evaluated the effects of 6 monochromatic wavelengths ( $\lambda_{627\text{nm}}$ ,  $\lambda_{617\text{nm}}$ ,  $\lambda_{590\text{nm}}$ ,  $\lambda_{530\text{nm}}$ ,  $\lambda_{505\text{nm}}$ ,  $\lambda_{470\text{nm}}$ ) and a full spectrum neutral white LED at three different light intensities (100, 250 and 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the photosynthetic parameters quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ) and non-photochemical quenching (NPQ) in the microalgae *Chlorella sorokiniana* at three different cell densities (OD 0.5, 1.0 and 1.5). An inverted correlation between  $\Phi_{\text{PSII}}$  and light intensity was found across the whole experiment.

Continuously decrease in  $\Phi_{PSII}$  measured under the green light spectrum was observed as *C. sorokiniana* cell density increased whereas NPQ had a noticeable decrease under all light sources as the culture density increased from OD 0.5 to 1.0.

To confirm the indications found on the previously experiment, in a second experiment photosynthetic activity and biomass production induced by 4 different LEDs (blue- $\lambda_{470\text{nm}}$ , green- $\lambda_{530\text{nm}}$ , red- $\lambda_{655\text{nm}}$ , and white-3000K) were analyzed on high-density cultures of *Scenedesmus bijuga*. Light penetration and oxygen evolution were measured on cultures at different cell densities (0.7, 0.8, 1.0 and 1.45 g/L) and final dry biomass production was measured on samples with initial high biomass concentration (2.19 g/L). As culture density increased the weakly absorbed green light became more photosynthetically efficient than the red light, thereby inducing significantly higher oxygen evolution at culture concentration of 1.45 g/L. High-density culture (2.19 g/L) cultivated under the green light showed higher biomass production rate (30 mg/L/d) with a 8.43% dry biomass growth in a 6-day period compared to the red light that induced 4.35% dry biomass growth on the same period.

INDEX WORDS: microalgae, photosynthesis, biomass, production

MICROALGAE BIOPHOTONIC OPTIMIZATION OF PHOTOSYNTHESIS BY  
WEAKLY ABSORBED WAVELENGTHS

by

ERICO ROLIM DE MATTOS

BS, Universidade de Sao Paulo, Brazil, 2008

A Dissertation Submitted to the Graduate Faculty of the University of Georgia in Partial  
Fulfillment of the Requirement for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013

© 2013

Erico Rolim de Mattos

All Rights Reserved

MICROALGAE BIOPHOTONIC OPTIMIZATION OF PHOTOSYNTHESIS BY  
WEAKLY ABSORBED WAVELENGTHS

by

ERICO ROLIM DE MATTOS

Major Professors: Keshav Das & Miguel Cabrera

Committee: Marc van Iersel  
Mark Haidekker  
Gregory Schmidt

Electronic Version Approved:

Maureen Grasso  
Dean of the Graduate School  
The University of Georgia  
June

## DEDICATION

I dedicate this dissertation to my parents Alda and Jose Paulo and my brother Gustavo, who always supported and encourage me.

## ACKNOWLEDGEMENTS

I would like to express my sincerest and profound gratitude to Dr. Keshav Das for such innate and extraordinary mentorship and guidance in my studies and in my life, allowing me to grow up as a person and as a researcher. I will be forever grateful.

I would like to thank Dr. Miguel Cabrera for all the academic guiding and support along my PhD.

I would like to thank Dr. Marisa d'Arce for the constant support and encouragement. This step in my life started because of you. I would also like to thank Ryan Adolphson for his initiative and trust which gave me the opportunity to get here. To my fellow lab mates, especially Eric, Ronnie, Rekha and Ana I would like to acknowledge the constant entertainment, help, knowledge and friendship. And last but not the least, I'd like to thank the faculty of my committee, especially Dr. van Iersel, Dr. Haidekker, and Dr. Schmidt. I have learned so much with all of you.

## TABLE OF CONTENT

	Page
ACKNOWLEDGEMENTS.....	vii
CHAPTER	
1 INTRODUCTION.....	1
2 LITERATURE REVIEW	
Microalgae.....	3
Applications.....	4
Cultivation in Photobioreactors.....	7
Photosynthetic Active Radiation.....	10
Photosynthesis .....	12
Efficiency of Photosynthesis.....	14
Green Light.....	16
3 EFFECTS OF INOCULUM PHYSIOLOGICAL STAGE ON THE GROWTH CHARACTERISTICS OF CHLORELLA SOROKINIANA CULTIVATED UNDER DIFFERENT CO <sub>2</sub> CENCENTRATIONS	
Abstract.....	27
Introduction.....	28
Materials and Methods.....	30
Results & Discussion.....	33
Conclusion.....	39
References.....	41
Tables and Figures.....	43

4	CHANGES IN CHLOROPHYL FLUORESCENCE PARAMETERS IN DIFFERENT CHLORELLA SOROKINIANA CELL DENSITIES	
	Abstract.....	53
	Introduction.....	54
	Materials and Methods.....	57
	Results.....	60
	Discussion.....	62
	Conclusion.....	66
	References.....	67
	Tables and Figures.....	70
5	ENHANCEMENT OF BIOMASS PRODUCTION IN SCENEDESMUS BIJUGA HIGH DENSITY CULTURE USING WEAKLY ABSORBED GREEN LIGHT	
	Abstract.....	74
	Introduction.....	75
	Materials and Methods.....	77
	Results & Discussion.....	82
	Conclusion.....	88
	References.....	89
	Tables and Figures.....	91
6	GENERAL OVERVIEW .....	100

# CHAPTER 1

## INTRODUCTION

Microalgae production has a great potential for carbon mitigation and can produce a vast range of valuable products from waste water and effluent gases. For this reason, most recently it has aroused great interest as a feedstock for biofuels production and biodegradable products (Gunther et al., 2012; Lakaniemi et al., 2011; Wijffels and Barbosa, 2010). However, the high cost of production and downstream process can hold back this potential making microalgae cultivation not feasible for a range of applications. Enhancing biomass production through optimization of cultivation process and optimization of photosynthesis can increase final microalgae cell density and bring the production costs down.

The major drawback to scale up microalgae production is the high cost of harvesting and de-watering due its intrinsic low biomass concentration (Christenson and Sims, 2011). The first step to successful obtain a high biomass concentration microalgae culture is the production of high purity and high density inoculum. In order to increase inoculum density and reduce cultivation period, systems capable to optimize environmental factors have being developed to enhance inoculum production efficiency. Inoculum production in photobioreactors (PBR) have been proved to be the best method to avoid external contaminations and achieve high purity high density cultures in closed environment-controlled ambient. To enhance biomass production and allow the development of high density cultures, the main substrate for microalgae development ( $\text{CO}_2$ ) is commonly supplied in PBRs through a gas flow. Although several studies have been published on the effect of  $\text{CO}_2$  concentration on biomass production and growth rate of algae (de Morais and Costa, 2007a; de Morais and Costa, 2007b; Sung et al., 1999), to the best of our knowledge the correlation of such  $\text{CO}_2$  effects with the physiological stage of algal culture inoculum has never been established. Examining the interaction of environmental and physiological

parameters on microalgae inoculum cultivation can lead to a process efficiency enhancement and contribute to reduce the high cost of microalgae cultivation in PBRs.

To increase microalgae culture cell density, different kinds of closed PBR using artificial illumination systems have been developed capable of achieving high biomass concentrations under controlled environmental conditions (Choi et al., 2003; Fu et al., 2012; Hulatt and Thomas, 2011; Lee and Palsson, 1995). Artificial illumination systems can increase the volumetric productivity and high-density cultures can only be achieved when the cells receive appropriate levels of light energy along the entire culture cycle (Choi et al., 2003).

Light requirements by the photosynthetic microalgae species changes as culture develop and become denser. Exposure of cells to excessive light at early culture stages leads to photodamage and photoinhibition that can cause a decline on photosynthetic capacity and biomass growth, whereas low levels of light energy at later stages become a growth-limiting factor. Providing constant optimal light uptake at different stages of development and allowing individual cells in the whole sample to be continuously exposed to optimal light amounts over the entire culture cycle can significantly increase biomass production (Choi et al., 2003; Lee et al., 2006). Several studies have being done analyzing the effects of light intensity and frequency on microalgae development at different growth stages. Most of these experiments relay on the use of the well-known chlorophyll *a* highly absorbed wavelengths on the blue and red spectrum of light and little attention has being given to the effects of light spectrum on the enhancement of biomass production.

Therefore, the goals of the present research are to improve methods of microalgae biomass production determining the best combination of inoculum stage and carbon dioxide concentration (1) and to increase photosynthetic efficiency and biomass production in photobioreactors at high density microalgae cultures based on light supply (2).

## CHAPTER 2

### LITERATURE REVIEW

#### **Microalgae**

Microalgae are unicellular eukaryotes species, sometimes associated in colonies or in filaments of several hundred cells that do not differentiate into specialized cells. Phytoplankton represents the main component of microalgae and can be defined as the autotrophic part of planktonic community. Photosynthetic microalgae have the capacity to produce carbohydrates from water and inorganic CO<sub>2</sub> through photosynthesis and it is responsible for up to 90% of the marine photosynthesis (Lourenço, 2006). Intrinsically, microalgae offer the greatest flux tolerance and photosynthetic efficiency because they have a minimal internally competitive plant functions (Gordon and Polle, 2007). Microalgae photosynthetic system is based on the same mechanisms of higher plants, but with some advantages over the vascular plants, because all its physiological functions are carried out in a single cell and even in species that grow in colonies and filaments there is no differentiation into specialized cells.

Microalgae are not a well-studied group from a biotechnological point of view. Of the tens of thousands of microalgal species believed to exist, only a few thousand strains are kept in collections around the world, of which only a few hundred have been investigated for their chemical content and only a handful have been cultivated in industrial quantities (tons per year quantities) (Olaizola, 2003). Even though different microalgae species can be adapted to live in a variety of environmental conditions, it is necessary to find the species best-suited to local environments or specific growth characteristics (Mata et al., 2010).

## Applications

Microalgae are fast growing organisms that can be used for a vast range of applications (Mata et al., 2010; Spolaore et al., 2006). Although algae has been used as human food for thousands of years (Borowitzka, 1997) it began to be commercially produced in the 1950s when they were cultivated for food in Japan. Microalgae species like *Chlorella* spp., *Dunaliella* spp., and *Scenedesmus* spp. which have significant amounts of lipid, protein, chlorophyll, carotenoids, vitamins, minerals, and unique pigments are used as nutrient dense food and sources of fine chemicals (Kay, 1991). Comprehensive analyses and nutritional studies have demonstrated that these algal proteins are of high quality and comparable to conventional vegetable proteins (Becker, 2007). Analyses of proteins, fats, carbohydrates, and vitamins indicate that unicellular green algae, especially *Chlorella*, should be excellent sources of these nutrients (Krauss, 1962).

In the 1960s algae were employed in waste water treatment systems (Oswald et al., 1957), and are still successfully used in this capacity. Microalgae can efficiently recover nutrients from residual waters converting it into biomass (Chinnasamy et al., 2010; Martinez et al., 2000; Rhee, 1973) and different methods have being developed to increase removal efficiency (Craggs et al., 1996). Phosphorous which is an essential nutrient for plant growth has no substitute in food production and cannot be synthesized in laboratory. A major opportunity to increase the life expectance of the world's phosphorous resources lie in recycling it by its recovery from rural and urban wastewater through microalgae cultivation (Singh et al., 2011).

In the 1980s the efforts switched towards producing high value compounds (Benemann and Oswald, 1996). Microalgal biotechnology has the potential to produce a vast array of products including pigments, fatty acids, antioxidants, vitamins, industrial chemicals, compounds with therapeutic applications and bioremediation solutions that is attractive to the

private sector (Olaizola, 2003). The productivity and biochemical composition of microalgae depend strongly on the method of cultivation, medium composition, and nutrient profile and special care should be taken to match the necessary physiological requirements to obtain the compounds of interest (Guedes et al., 2011). Due to the increasing interest in valuable products from microalgae several studies were published covering the biotechnologically relevant species of microalgae (Pulz and Gross, 2004) and its bioactive compounds (Singh et al., 2005).

In the 1990s, as a consequence of worsening global warming and accumulating greenhouse gases, great attention was focused on microalgae cultivation for CO<sub>2</sub> mitigation and biofuels production. Recently, there has been renewed interest in microalgal biofixation as a viable CO<sub>2</sub> sequestration technology (Ono and Cuello, 2006). These processes utilize industrial flue gases and urban or agricultural waste water as a source of CO<sub>2</sub> and nutrients to produce renewable fuels. Several reports recently published review the use of microalgae as a source of biofuels investigating the production of a number of different biofuels (Li et al., 2008), approaches for making microalgal biodiesel economically competitive (Chisti, 2007) and reviewing the technologies underpinning microalgae-to-biofuels systems (Brennan and Owende, 2010).

The microalgal biomass market produces ca. 5,000t/yr of dry matter, generating a turnover of approximate US\$ 2,3 x 10<sup>8</sup>/yr. These rates relate only to the biomass as the commercial product. Thus the total global production of microalgal biomass, including processed products, would be rated between 8,000 and 10,000 tonnes (Pulz and Gross, 2004; Posten et al., 2012).

### *Carbon dioxide mitigation*

Increased CO<sub>2</sub> emission by human activities from the past 100 years has disturbed the natural equilibrium between sources and sinks of CO<sub>2</sub>, accelerating global warming. On May

2013 the concentration of CO<sub>2</sub> in the atmosphere surpassed 400 parts per million for the first time since measurements began in 1958 according to the National Oceanic and Atmospheric Administration (NOAA, 2013). Restrictive regulations regarding GHG emissions have been discussed by the industrialized and emerging countries to reduce CO<sub>2</sub> emissions and stop the global warming process. The United States is responsible for approximately 25% of global GHG emissions, which is estimated by the U.S. Environmental Protection Agency (EPA, 2011) to be 6,633 million metric tons CO<sub>2</sub> equivalent in 2009. This estimate includes CO<sub>2</sub> emissions, as well as other GHGs such as methane, nitrous oxide, hydrofluorocarbons, perfluorocarbons and sulfur hexafluoride. Annual GHG emissions from fossil fuel combustion, primarily CO<sub>2</sub>, were estimated at 5,209 million metric tons with 3,490 million metric tons from stationary sources alone (EPA, 2011).

Among the many approaches for carbon capture and storage (e.g. pre-combustion capture, geologic storage) microalgae cultivation is a promising option (Brune et al., 2009). Microalgae can offer high biomass yields, does not compete with conventional agriculture for arable lands or nutrients, can utilize waste and saline waters (Chinnasamy et al., 2010), can recycle carbon from CO<sub>2</sub>-rich flue gases, and can be used to produce a variety of fuels and valuable co-products (DOE, 2010).

Photoautotrophic cultivation of microalgae in photobioreactor is recognized as the best way to reach high rate production while maintaining monocultures without contamination. Although capital costs are currently higher for closed photobioreactors than for open-pond raceways, the former is more efficient in water use, has superior capability for long-term culture maintenance, and provides higher surface area to volume ratio that supports higher volumetric cell densities (Schenk et al., 2008). In these systems, providing supplemental CO<sub>2</sub> supply is critical since the low atmospheric CO<sub>2</sub> concentration and its low diffusion rate does not support high production rates making CO<sub>2</sub> a limiting factor.

The southeast U.S. has the necessary climatic conditions as well as a large number of coal-burning power plants providing ample sources of CO<sub>2</sub> for algal cultivation (DOE, 2010). Co-locating algal cultivation with power plants can be a desirable approach that can capture effluent CO<sub>2</sub> and convert it into biofuels and high value co-products (Brune et al., 2009).

In order to find a feasible and scalable way to convert CO<sub>2</sub> into biomass, studies evaluating the high rate production potential of specific microalgae species cultivated in photobioreactors have identified optimal conditions for culture development under high CO<sub>2</sub> concentrations cultivated in different kinds of waste and fresh media (Keffer and Kleinheinz, 2002; Li et al., 2011; Yewalkar et al., 2011). The effect of CO<sub>2</sub> concentration on biomass production and growth rate of algae has been extensively studied (de Morais and Costa, 2007a; de Morais and Costa, 2007b; Sung et al., 1999).

### **Cultivation in Photobioreactors**

Algal growth is divided into three main classes: autotrophic, heterotrophic, and mixotrophic. Autotrophic algae utilize CO<sub>2</sub> as the carbon source and rely on light energy for photosynthesis process, while heterotrophic algae utilize sugars derived from other biomass sources. Mixotrophic has the capability to utilize both and its cultivation is carried under the light and the media is supplemented with different organic carbon substrates. The autotrophic pathway has generally received more public attention, as attributable to the large number of companies dedicated either in whole or in part to pursuing autotrophic algal biofuels, relative to a smaller number of entities in the heterotrophic field. In order to achieve high biomass concentrations and keep the purity of the culture, autotrophic microalgae cultivation in closed photobioreactors have being vastly investigated.

Photobioreactor is normally referred to closed vessels that allow some type of light source to provide photonic energy input into the reactor. The photobioreactor's function is to provide a suitable environment for the given reaction in terms of process parameters such as

pH and temperature but also to ensure sterility (Bergmann et al., 2013). The advantages of photobioreactors for microalgae cultivation are quite clear: they offer cultivation under environmental controlled conditions and prevent contamination by other microalgae strains and contamination with undesirable microorganism (Posten, 2009).

One of the major practical limiting factors for application of PBRs in algal mass cultures is mass transfer. High density microalgae cultures grown under photoautotrophic method demand a substantial amount of CO<sub>2</sub> whereas the high concentrations of O<sub>2</sub> originate by the photosynthesis process can become an inhibitory condition for microalgae development. Because of the low solubility the oxygen is of most concern. A possibly inhibiting concentration (for some algae >120% air saturation, for others >200%) can occur already after 1 min in a tube without gas exchange (Posten, 2009). To optimize biomass production and allow systems scale up several studies were conducted to enhance mass transfer (Putt et al., 2011), optimize aeration rates (Zhang et al., 2002), and find the optimal amount of CO<sub>2</sub> supply (Grima et al., 1998).

To optimize production and overcome the limitations involved on the use of closed PBRs for microalgae cultivation several designs have been developed. Among the most common PBR designs are the flat-plate, tubular and vertical columns (Ugwu et al., 2008; Janssen et al., 2003). Flat-plate photobioreactors have received much attention for cultivation of photosynthetic microorganisms due to their large illumination surface area. On these systems mixing and CO<sub>2</sub>-supply is accomplished by sparging the culture with CO<sub>2</sub>-enriched air (Alias et al., 2004; Wang et al., 2005). Among the proposed photobioreactors, tubular photobioreactor is one of the most suitable types for outdoor mass cultures. Most tubular photobioreactors are usually constructed with either glass or plastic tube and their cultures are re-circulated either with pump or preferably with airlift system (Ugwu et al., 2008). The transparent tubing are arranged in parallel lines coupled by manifolds, the so-called solar

collector. The single tubes can be straight, they can follow a meandering course either flat on the ground or ordered in panels or coils so called helical reactor (Morita et al., 2001; Hall et al., 2003). Vertical-column photobioreactors are compact, low-cost, and easy to operate monoseptically (Miron et al., 2002). To work with sufficient volume, the diameters of 20 cm and more are higher comparing to tubular reactors. This leads to considerable high dark fraction in the middle of the cylinder (Posten, 2009). To increase axial transport and increase cell exposure to light the airlift principle has been employed (Miron et al., 2000).

New innovative alternatives have also being proposed. Disposable, low-cost, transparent flat panel airlift photobioreactor with optimized flow regime for the efficient cultivation of microalgae and production of microalgal metabolites (Bergmann et al., 2013), three dimensional architecture bioreactor that utilizes available sunlight more efficiently with a smaller footprint (Algaebiodieselinfo), and polymer-based photobioreactors that are designed to float on aquatic acreage (Algaesystems) are some of the proposed approach to reduce production costs.

Although a good number of photobioreactors have been proposed, only a few of them can be practically used for mass production of algae and due to the high technical expenses, this technology is still confined to the production of high value products, e.g. pharmaceuticals (Pulz and Scheibenbogen, 1998).

Production of microalgae biomass is up to now limited to a few thousand tons per year, mostly produced in open ponds. Only a few hundred tons are produced in closed photobioreactors (Posten, 2009). Calculations based on published data shows that that closed systems can out-perform raceway ponds by about 300%, which represents productivities over 100 g dry algal biomass per m<sup>2</sup> per day (Pulz and Scheibenbogen, 1998). Considering indoor systems with internal illumination arrangements, the growth potential can be increased by a further 100%. However, one of the main problems in designing efficient bioreactors that can

be scale-up for photoautotrophic microorganisms production is the inefficient light distribution. Light attenuates exponentially as it penetrates into the culture medium. To overcome this issue several PBRs having artificial illumination systems were designed.

The major advantage of internally-illuminated photobioreactor is that continuously light can be provided along the cultivation cycle by integrating artificial and solar light devices (Ugwu et al., 2008) allowing high density culture production. Lee and Palson (1995) designed a PBR providing 680 nm monochromatic red light from LED as a light source that allow microalgae production to a cell concentration higher than  $2 \times 10^9$  cells/mL (more than 6.6% v/v), cell doubling times as low as 12 h, and an oxygen production rate as high as 10 mmol oxygen/L culture/h. Javanmardian and Palsson (1991) using a fiber-optic based optical transmission system that is coupled to an internal light distribution system that illuminates the culture volume uniformly achieved cell concentration up to  $10^9$  cells/mL [3% (w/v)] for eukaryotic green alga *Chlorella vulgaris*.

## **Photosynthetic Active Radiation**

### *Nature of Light*

Light is electromagnetic radiation that can be produced by a variety of energy conversion process. The spectrum of emitted energy is not continuous, but is delivered in discrete frequencies or energetic packages, called quanta. These mass less particles have an apparent momentum and are called photons (Falkowski and Raven, 2007). There is an inverse relationship between wavelength and quantum energy, the higher the wavelength the lower quantum energy and vice versa. The light spectrum range capable to induce photosynthesis is called Photosynthetic Active Radiation (PAR) and ranges from 400nm to 700nm. Radiation of 700 nm and above has an energy content that is too low to mediate chemical changes; hence, radiant energy absorbed in this range will only appear as thermal effects. Conversely, radiation of 380 nm and below brings about ionizing effects. Between

400 and 700nm, the energy content is sufficient to produce chemical changes in the absorbing molecules, as happens throughout the photosynthetic pathways prevailing in microalgae (Kommareddy and Anderson, 2003).

### *Light Absorption*

Microalgae as any other photosynthetic organism are provided with photosynthetic pigments which are responsible for the capture of light energy during the light-dependent reactions of photosynthesis. These light harvesting pigments are arranged in the antenna pigments located within the thylakoid membrane in the chloroplasts. The antennas permit an organism to increase greatly the absorption cross section for light without having to build an entire reaction center and associated electron transfer system for each pigment, which would be very costly in terms of cellular resources (Blankenship, 1996). Photosynthetic organisms contain an assortment of pigments thereby allowing absorption of a maximum number of wavelengths. The spectrum and quantity of light absorption depends on the photosynthetic pigments composition of each photosynthetic organism (Rabinowitch and Govindjee, 1969). The main pigments found in terrestrial plants, microalgae and cyanobacteria are chlorophyll *a* and *b* with maximum light absorption peaks in the blue and red spectrum of light (Rabinowitch and Govindjee, 1969). Carotenoids with light absorption peaks in the blue and yellow spectrum of light are also present in several photosynthetic organisms as accessory pigments that absorb light energy and pass this on to the reaction center complex where primary electron transfer takes place. The pigments are arranged in three dimensional pigment-protein complexes which determine their function and efficiency of energy transfer. By incorporating many pigments into a single unit, the biosynthetically expensive reaction center and electron transport chain can be used to maximum efficiency (Blankenship, 1996). The wavelength dependence of a photochemical reaction is called an *action spectrum* and the

ratio of the product formed per unit light absorbed is called a *quantum yield* (Falkowski and Raven, 2007).

### **Photosynthesis**

When a photon impacts a chlorophyll *a* molecule it is either reflected, transmitted or absorbed by said molecule. If absorbed, the molecule is excited from a stable ground state (S0) to an excited state (S1) that depends on the energy of the incident wavelength. If the incident wavelength is 450 nm or lower, the chlorophyll molecule will be excited to the second singlet (or excited) state (S2), and consequently will need to lose some energy afterwards, as fluorescence or heat, in order to fall back to S1. The energy lost as heat or fluorescence is not indeed used in photosynthesis, so it contributes unfavorably to light use efficiency. The jump from S0 to S1 requires an energy input close to that conveyed by photons characterized by 680–700 nm, depending on the photosystem at stake (I or II). Once the electron is on S1, it may either: (1) be transferred to a photochemical process; (2) move to the triplet state (a process that entails rearrangement of the excited state, thus leading to spin reversal—and possibly to photooxidation); (3) or lose energy, as heat or fluorescence, and thus move back to S0 (Carvalho et al., 2011).

When the electron is transferred to the photochemical process its energy is directed to the reaction centers P680 (RCII) of Photosystem II (PSII) or P700 of Photosystem I (PSI) and used to drive the light reactions. At RCII water molecules are split releasing electrons to the electron transport chain. Those electrons are conducted through a series of acceptors to PSI where it is excited again and used to produce the reductant molecule NADPH in a process named ‘Z’-scheme first described by Hill and Bendall (Hill and Bendall, 1960). During this process, H<sup>+</sup> protons released from the splitting of water plus H<sup>+</sup> protons pumped across the lumen membrane accumulate inside the lumen creating a proton gradient which drives ATP synthesis. Protons escaping from the thylakoid lumen through a central core of the enzyme

ATP synthase cause conformational changes in the enzyme which catalyzes the phosphorylation of ADP producing ATP on the stromal side (Govindjee, 2004). Both ATP and NADPH molecules are further used to reduce CO<sub>2</sub> molecules to hexose in the Calvin-Benson Cycle (dark reactions) in which those intermediates react with CO<sub>2</sub> to produce glucose — a process that requires a source of electrons and a source of energy: the former is water, whereas the latter is light. The dark reactions are slower than light reactions, so under high light conditions the electron flow is saturated and the electrons transported on light reactions can be accepted by other molecules rather than NADP<sup>+</sup> (e.g. O<sub>2</sub>) producing free radicals (Rabinowitch and Govindjee, 1969). These highly reactive molecules can cause photodamage and photo-oxidation to the photosynthetic apparatus mechanism causing reversible photoinactivation or in severe conditions permanent inactivation of PSII reaction centers, known as photoinhibition (Adir et al., 2003; Krause, 1988) which lowers the efficiency of photosynthesis.

To avoid photodamage microalgae, as all the other photosynthetic organisms, have protective mechanisms that dissipate the excess of absorbed light energy as heat (non-photochemical quenching - NPQ). The most effective and fastest NPQ mechanism is termed high energy state quenching (qE) (Muller et al., 2001). This process is induced by the pH decreasing in the lumen and causes the protonation of PSII proteins activating the xanthophyll carotenoid pigments via xanthophyll cycle. At low lumen pH, the enzyme violaxanthin de-epoxidase removes two epoxides groups from the violaxanthin transforming this pigment into a zeaxanthin (Gilmore et al., 1995). The interaction of zeaxanthin with chlorophyll pigments causes a conformational change that dissipates the excitation energy as heat.

Fluorescence is the re-emission of energy in the form of a photon as an electron returns to ground state from a singlet excited state. As some energy is also given off as heat,

the photon is red-shifted with an emission peak of ~685nm (Cosgrove and Borowitzka, 2010). At room temperature nearly all fluorescence (90-95%) comes from PSII at 685nm. Fluorescence emitted by PSI is very low at normal temperatures and only can be detected at temperature of liquid nitrogen. Indeed, the primary acceptor of electrons from P700 is rapidly re-oxidized, reducing fluorescence emissions at PSI (Falkowski and Kiefer, 1985). Fluorescence can be used to estimate the quantum efficiency of charge separation at RCII (Sugget et al., 2010). The use of fluorescence measurements as a probe of photosynthetic productivity has been vastly investigated due to the simplicity to collect measurements. Many studies correlating photosynthetic carbon fixation and electron transport rate based on changes in fluorescence yield have been reported (Genty et al., 1989; Schreiber et al., 1995; Weis and Berry, 1987). However, the underlying theory and the interpretation of data remains complex.

### **Efficiency of Photosynthesis**

Photosynthesis is a light driven photochemical reaction and for this reason light must be absorbed in order to have any effect. The photosynthetic action spectrum which is the biological effectiveness as a function of wavelength of incident light is highly correlated to the absorption spectra of the main photosynthetic pigments present at a specific organism. It means that higher light absorption at a determined waveband will lead to a more efficient photosynthetic process compared to a waveband with lower absorption (Gorton, 2012). However, microalgae light requirements changes as the culture develop and light penetration decreases as the culture cell density increases.

Exposure of cells to excessive light at early culture stages leads to photodamage and photoinhibition that can cause a decline on photosynthetic capacity and biomass growth, whereas low levels of light energy at later stages become a growth-limiting factor. Providing constant optimal light uptake at different stages of development and allowing individual cells

in the whole sample to be continuously exposed to optimal light amounts over the entire culture cycle can significantly increase biomass production (Choi et al., 2003; Lee et al., 2006).

Two major routes can be devised to increase the level and effectiveness of light utilization by microalgae: action on the receptor via genetic engineering, or action on the source via light engineering (Carvalho et al., 2011). Artificial illumination systems can increase photosynthetic efficiency allowing high volumetric productivity and high-density cultures providing the appropriate levels of light energy along the entire cycle (Choi et al., 2003). Different approaches to optimize lighting systems and light delivery were proved to increase photosynthesis efficiency and maximize biomass production based on light adjustment to match the culture physiological requirements at different stages (Carvalho et al., 2011).

The time scales characterizing photosynthetic processes can be divided in three ranges: primary photochemistry, electron shuttling and carbon metabolism. The former occurs from light harvesting through charge separation in the reaction centers, within pico- to nanosecond-periods. Reactions involving shuttling of electrons between photosystems I and II (dark reactions) are slightly slower—and take micro to millisecond-periods. Finally, carbon metabolism within the chloroplast occurs in second-scales, whereas sucrose metabolism and enzyme activation may take up minutes (Tennessen et al., 1995). The large array of light-absorbing chlorophyll antenna molecules present in photosystems I and II of microalgae allow the photon capture system to be far more efficient than the remaining photosynthetic process; hence, up to 80% of the photons absorbed at high light irradiances may be dissipated afterwards as heat or fluorescence, or even cause photodamage thus decreasing the observed photosynthetic productivity (Carvalho et al., 2011).

Another reason contributing for this inefficiency is that the amount of light energy absorbed by the first layers of cells closer to illuminated surface exceeds the rate at which photosynthesis can occur to fully utilize their energy whereas cells deeper in the culture are exposed to limiting levels of light.

In attempts to surpass the aforementioned limitation, it is necessary to minimize light absorption by individual cells, and concomitantly permit increased transmittance of radiation through the culture, so that cells deeper inside the culture will receive enough light (Carvalho et al., 2011). This can be achieved by adjusting the light spectrum, intensity and frequency. Ultra-high productivities were achieved by tailoring the photonic temporal, spectral and intensity aspects of light (Gordon and Polle, 2007).

### **Green Light**

Green light falls into the low peak of action spectra of plants and algae due its high reflection and low absorption by the main photosynthetic pigment present on these organisms, namely, chlorophyll *a*. For this reason green light is not preferred for artificial illumination systems in greenhouses and photobioreactors which are normally composed by strongly absorbed wavelengths on the red (660nm) and blue spectra (470nm) (Lee and Palsson, 1995). Strongly absorbed wavelengths can indeed stimulate higher photosynthetic activity in low density microalgae cultures (Jeon et al., 2005) and have been shown to induce high biomass production (Fu et al., 2012). However, as the biomass density increases light penetration becomes the main growth limiting factor. In recent studies with higher plants, it was shown that the strongly absorbed wavelengths get completely absorbed by the initial cell layers whereas green light can penetrate further into the leaves (Brodersen and Vogelmann, 2010) and drive carbon fixation more efficiently than red and blue light in cells located deeper in the leaves (Sun et al., 1998). In high density microalgae cultures, the increased number of cells have the same effect as the multiple cell layers present in higher plant leaves

and light penetration becomes limited as the culture gets denser (Suh and Lee, 2003). In high density cultures, auto-shading can significantly reduce cell exposure to light. Photoreceptor molecules shade each other such that strongly absorbed photons would be absorbed close to the illuminated surface while those weakly absorbed photons would penetrate more deeply into the sample reaching more cells (Yun and Park, 2001). Kim and Lee (2001) have shown that red light (680nm) penetration depth in *Chlorella kessleri* cultures decreases exponentially as cell density increases. In contrast, the weakly absorbed wavelengths that can penetrate deeper into high density cultures induce higher photosynthetic efficiency compared to the strongly absorbed wavelengths.

Along with deep penetration, the diffusive nature of the algal culture and the high reflectance index of weakly absorbed wavelengths would increase their pathway inside the culture thus increasing the opportunity for light to encounter chloroplasts, leading to increased absorbance. This phenomenon is described by Terashima et al. (2009) as “**detour effect**” and it can substantially increase the absorbance of weakly absorbed wavelengths in microalgae cultures.

At intact cells present in microalgae culture, pigments are concentrated into chloroplast which decreases the opportunity for light to encounter pigments and generally decreases light absorption per unit pigment in comparison to the same quantity of pigment dispersed in solution. This is referred to as the ‘**package effect**’ (also known as sieve effect) and results in a reduced absorption at the absorption maxima of the pigments, while there is little reduction in absorption at those wavelengths that are weakly absorbed (Kim and Lee, 2001; Terashima et al., 2009). Package effect is a function of cell density, size and pigment content, so as cultures grow and get denser, the magnitude of package effect increases (Morel and Bricaud, 1981).

Although the photosynthetic active spectrum is lower at green wavelengths compared to red and blue at pigments extracted samples, at high density cultures weakly absorbed photons have increased chance to be absorbed, thus increasing its action spectra. These facts point out the relevance of including weakly absorbed light sources in artificial lighting systems to enhance photosynthesis activity in microalgae cultures at high cell density.

## REFERENCES

- Adir, N., H. Zer, S. Shochat, and I. Ohad. 2003. Photoinhibition - a historical perspective. *Photosynthesis Research* 76:343-370.
- Algaebiodieselinfo - <http://www.algaebiodieselinfo.com/tag/algae-pbr-2/>. Accessed on May 20, 2013
- Algaesystems - <http://algaesystems.com/technology/omega/>. Accessed on May 20, 2013
- Alias, C.B., M.C.G.M. Lopez, F.G.A. Fernandez, J.M.G. Sevilla, J.L.G. Sanchez, E.M. Grima. 2004 Influence of power supply in the feasibility of *Phaeodactylum tricornutum* cultures. *Biotechnology and Bioengineering* 87: 723–733
- Becker, E.W. 2007. Micro-algae as a source of protein. *Biotechnology Advances* 25:207-210.
- Benemann, J.R., and W.J. Oswald. 1996. Systems and economic analysis of microalgae ponds for conversion of CO<sub>2</sub> to biomass. . University of California Berkeley, Berkeley.
- Bergman, P. B., P. Ripplinger, L. Beyer, W. Trösch. 2013. Disposable Flat Panel Airlift Photobioreactors. *Chemie Ingenieur Technik* 85 (1-2): 202-205  
*Bioresource Technology* 99(10): 4021-4028
- Blankenship, R.E. 1996. Photosynthetic Antennas and Reaction Centers: Current Understanding and Prospects for Improvement [Online] <http://bioenergy.asu.edu/photosyn/education/antenna.html>.
- Borowitzka, M.A. 1998. Algae as food. In: Wood B.J.B. (ed.), *Microbiology of fermented foods 2*. Blackie Academic & Professional: London. pp. 585-602.
- Brennan, L., and P. Owende. 2010. Biofuels from microalgae - A review of technologies for production, processing, and extractions of biofuels and co-products. *Renewable and Sustainable Energy Reviews* 14:557-577.
- Brodersen, C.R., and T.C. Vogelmann. 2010. Do changes in light direction affect absorption profiles in leaves? *Funct. Plant. Biol.* 37:403-412.
- Brune, D.E., T.J. Lundquist, and J.R. Benemann. 2009. Microalgal Biomass for Greenhouse Gas Reductions: Potential for Replacement of Fossil Fuels and Animal Feeds. *Journal of Environmental Engineering-Asce* 135:1136-1144.
- Carvalho, A.P., S.O. Silva, J.M. Baptista, and F.X. Malcata. 2011. Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. *Applied Microbiology and Biotechnology* 89:1275-1288.
- Chinnasamy, S., A. Bhatnagar, R.W. Hunt, and K.C. Das. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technol* 101:3097-3105.

- Chisti, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances* 25:294-306.
- Choi, S.L., I.S. Suh, and C.G. Lee. 2003. Lumostatic operation of bubble column photobioreactors for *Haematococcus pluvialis* cultures using a specific light uptake rate as a control parameter. *Enzyme Microb Tech* 33:403-409.
- Christenson, L., and R. Sims. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnol. Adv.* 29:686-702.
- Cosgrove, J., and M.A. Borowitzka. 2010. Chlorophyll Fluorescence Terminology: An Introduction, p. pp. 1 - 17., *In* D. J. Suggett, et al., eds. *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications*. Springer, Dordrecht.
- Craggs, R.J., W.H. Adey, K.R. Jenson, M.S. St. John, F.B. Green, and W.J. Oswald. 1996. Phosphorus removal from wastewater using an algal turf scrubber. *Water Science and Technology* 33:191-198.
- de Morais, M.G., and J.A.V. Costa. 2007a. Carbon dioxide fixation by *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp cultivated in flasks and vertical tubular photobioreactors. *Biotechnology Letters* 29:1349-1352.
- de Morais, M.G., and J.A.V. Costa. 2007b. Biofixation of carbon dioxide by *Spirulina* sp and *Scenedesmus obliquus* cultivated in a three-stage serial tubular photobioreactor. *Journal of Biotechnology* 129:439-445.
- DOE. 2010. National Algal Biofuels Technology Roadmap, College Park, Maryland, USA. *Engineering in Life Sciences* 9(3): 165-177
- EPA. 2011. Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990 - 2009, Washington, DC, USA.
- Falkowski, P. and D.A. Kiefer. 1985. Chlorophyll-a fluorescence in phytoplankton – relationship to photosynthesis and biomass. *J. Plankton Res.* 7:715-731.
- Falkowski, P. and J. Raven. 2007. *Aquatic Photosynthesis* (2nd ed.). Princeton : Princeton University Press
- Fu, W.Q., O. Gudmundsson, A.M. Feist, G. Herjolfsson, S. Brynjolfsson, and B.O. Palsson. 2012. Maximizing biomass productivity and cell density of *Chlorella vulgaris* by using light-emitting diode-based photobioreactor. *J Biotechnol* 161:242-249.
- Genty, B., J.M. Briantais, and N.R. Baker. 1989. The Relationship between the Quantum Yield of Photosynthetic Electron-Transport and Quenching of Chlorophyll Fluorescence. *Biochimica et Biophysica Acta* 990:87-92.
- Gilmore, A.M., T.L. Hazlett, and Govindjee. 1995. Xanthophyll cycle – dependent quenching of photosystem-II chlorophyll-a fluorescence – formation of a quenching complex with a short fluorescence life time. *Proceedings of the National Academy of Sciences of the United States of America* 92:2273-2277.

- Gordon, J.M., and J.E.W. Polle. 2007. Ultrahigh bioproductivity from algae. *Appl. Microbiol. Biotechnol.* 76:969-975.
- Gorton, H.L. 2012. Biological action spectra [Online] <http://photobiology.info/Gorton.html> (verified Access on 11/10/2012).
- Govindjee. 2004. Chlorophyll *a* Fluorescence: A Bit of Basics and History, p. pp. 1 - 42, *In* G. C. Papageorgiou and Govindjee, eds. *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. Springer, Dordrecht.
- Grima, E.M., F.G.A. Fernandez, F.G. Camacho, Y. Chisti 1998. Photobioreactors: light regime, mass transfer, and scaleup. *Journal of Biotechnology* 70(1-3): 231-247
- Guedes, A.C., H.M. Amaro, and F.X. Malcata. 2011. Microalgae as sources of high added-value compounds—a brief review of recent work. *Biotechnology Progress* 27:597-613.
- Gunther, A., T. Jakob, R. Goss, S. Konig, D. Spindler, N. Rabiger, S. John, S. Heithoff, M. Fresewinkel, C. Posten, and C. Wilhelm. 2012. Methane production from glycolate excreting algae as a new concept in the production of biofuels. *Bioresource Technol* 121:454-457.
- Hall, D.O., F.G.A. Acien Fernández, E.C. Guerrero, K.K. Rao, E.M. Grima. 2003 Outdoor helical tubular photobioreactors for microalgal production: Modeling of fluid-dynamics and mass transfer and assessment of biomass productivity. *Biotechnology and Bioengineering* 82(1), 62–73.
- Hill, R., and F. Bendall. 1960. Function of the 2 cytochrome components in chloroplasts – working hypothesis. *Nature* 186:136-137.
- Hulatt, C.J., and D.N. Thomas. 2011. Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors. *Bioresource Technol* 102:5775-5787.
- Janssen, M., J. Tramper, L.R. Mur, R.H. Wijffels 2003. Enclosed outdoor photobioreactors: Light regime, photosynthetic efficiency, scale-up, and future prospects. *Biotechnology and Bioengineering* 81(2): 193-210
- Javanmardian, M. and B.O. Palsson 1991. High-density photosutotrophic algal cultures – design, construction, and operation of a novel photobioreactor system. *Biotechnology and Bioengineering* 38(10):1182-1189
- Jeon, Y.C., C.W. Cho, and Y.S. Yun. 2005. Measurement of microalgal photosynthetic activity depending on light intensity and quality. *Biochem Eng J* 27:127-131.
- Kay, R.A. 1991. Microalgae as food and supplement. *Critical Reviews in Food Science and Nutrition* 30:555-573.
- Keffer, J.E., and G.T. Kleinheinz. 2002. Use of *Chlorella vulgaris* for CO<sub>2</sub> mitigation in a photobioreactor. *Journal of Industrial Microbiology & Biotechnology* 29:275-280.

- Kim, N.J., and C.G. Lee. 2001. A Theoretical Consideration on Oxygen Production Rate in Microalgal Cultures. *Biotechnol. Bioprocess Eng.* 6:352-358.
- Kommareddy, A., and G. Anderson. 2003. Study of light as a parameter in the growth of algae in a Photo-Bio-Reactor (PBR). ASAE Annual International Meeting Presentation 034057, Las Vegas, USA.
- Krause, G.H. 1988. Photoinhibition of Photosynthesis - an Evaluation of Damaging and Protective Mechanisms. *Physiologia Plantarum* 74:566-574.
- Krauss, R.W. 1962. Mass culture of algae for food and other organic compounds *American Journal of Botany* 49:425-&.
- Lakaniemi, A.M., C.J. Hulatt, D.N. Thomas, O.H. Tuovinen, and J.A. Puhakka. 2011. Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. *Biotechnol. Biofuels* 4.
- Lee, C.G., and B.O. Palsson. 1995. Light-emitting diode-based algal photobioreactor with external gas-exchange. *J Ferment Bioeng* 79:257-263.
- Lee, H.S., M.W. Seo, Z.H. Kim, and C.G. Lee. 2006. Determining the best specific light uptake rates for the lumostatic cultures in bubble column photobioreactors. *Enzyme and Microbial Technology* 39:447-452.
- Li, Y., M Horsman, N Wu, C.Q. Lan, and N. Dubois-Calero. 2008. Biofuels from microalgae. *Biotechnology Progress* 24(4):815-820
- Li, Y., Y.-F. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, and R. Ruan. 2011. Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology* 102:5138-5144.
- Lourenço, S.O. 2006. *Cultivo de microalgas marinhas: princípios e aplicações*. Rima Editora.
- Martinez, M.E., S. Sanchez, J.M. Jimenez, F. El Yousfi, and L. Munoz. 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. *Bioresource Technology* 73:263-272.
- Mata, T.M., A.A. Martins, and N.S. Caetano. 2010. Microalgae for biodiesel production and other applications: A review. *Renewable & Sustainable Energy Reviews* 14:217-232.
- Miron, A.S., F.G.G. Camacho, A.C. Gomez, E.M. Grima, Y. Chisti. 2000. Bubble-column and airlift photobioreactors for algal culture. *Aiche Journal* 46(9): 1872–1887.
- Miron, A.S., M.C.C. Garcia, F.G. Camacho, E.M. Grima, Y. Chisti 2002. Growth and biochemical characterization of microalgal biomass produced in bubble column and airlift photobioreactors: studies in fed-batch culture. *Enzyme and Microbial Technology* 31: 1015–1023

- Morel, A., and A. Bricaud. 1981. Theoretical results concerning light-absorption in a discrete medium, and application to specific absorption of phytoplankton. *Deep-Sea Research Part a-Oceanographic Research Papers* 28:1375-1393.
- Morita, M., Y. Watanabe, T. Okawa, H. Saiki. 2001. Photosynthetic productivity of conical helical tubular photobioreactors incorporating *Chlorella* sp under various culture medium flow conditions. *Biotechnology and Bioengineering* 74(2), 136–144.
- Muller, P., X.P. Li, and K.K. Niyogi. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiology* 125:1558-1566.
- NOAA. 2013. National Oceanic and Atmospheric Administration [Online] <http://researchmatters.noaa.gov/news/Pages/CarbonDioxideatMaunaLoareaches400ppm.aspx>.
- Olaizola, M. 2003. Commercial development of microalgal biotechnology: from the test tube to the marketplace. *Biomolecular Engineering* 20:459-466.
- Ono, E., and J.L. Cuello. 2006. Feasibility assessment of microalgal carbon dioxide sequestration technology with photobioreactor and solar collector. *Biosystems Engineering* 95:597-606.
- Oswald, W.J., H.B. Gotaas, C.G. Golueke, and W.R. Kellen. 1957. Algae in waste treatment. *Sewage and Industrial Wastes* 29:437-455.
- Oswald, W.J., H.B. Gotaas, H.F. Ludwig, and V. Lynch. 1953. Algae symbiosis in oxidation ponds. 3. Photosynthetic oxygenation. *Sewage Ind Wastes* 25:692-705.
- Posten, C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences* 9(3):165-177
- Posten, C., C. Walter, and I. ebrary. 2012. *Microalgal Biotechnology: Integration and Economy*. Berlin; Boston Web. 31st May 2013.
- Pulz, O. and K. Scheibenbogen. 1998. Photobioreactors: design and performance with respect to light energy input. In: Scheper T (ed) *Bioprocess and algae reactor technology, apoptosis*. Springer: Berlin Heidelberg New York
- Pulz, O. and W. Gross. 2004. Valuable products from biotechnology of microalgae. *Applied Microbiology and Biotechnology* 65:635-648.
- Putt, R., M. Singh, S. Chinnasamy, K.C. Das. 2011 An efficient system for carbonation of high-rate algae pond water to enhance CO<sub>2</sub> mass transfer. *Bioresource Technology*.102(3):3240-3245
- Rabinowitch, E., and Govindjee. 1969. *Photosynthesis* Wiley., New York.
- Rhee, G.Y. 1973. Continuous culture study of phosphate uptake, growth-rate and polyphosphate in *Scenedesmus* sp *Journal of Phycology* 9:495-506.

- Schenk, P.M., S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, and B. Hankamer. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Research* 1:20-43.
- Schreiber, U., H. Hormann, C. Neubauer, and C. Klughammer. 1995. Assessment of photosystem-II photochemical quantum yield by chlorophyll fluorescence quenching analysis. *Aust. J. Plant Physiol.* 22:209-220.
- Singh, M., D.L. Reynolds, and K.C. Das. 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. *Bioresource Technology* 102:10841-10848.
- Singh, S., B.N. Kate, and U.C. Banerjee. 2005. Bioactive Compounds from Cyanobacteria and Microalgae: An Overview. *Critical Reviews in Biotechnology* 25:73-95.
- Spolaore, P., C. Joannis-Cassan, E. Duran, and A.n. Isambert. 2006. Commercial applications of microalgae. *Journal of Bioscience and Bioengineering* 101:87-96.
- Sugget, D.J., C.M. Moore, and R.J. Geider. 2010. Estimating Aquatic productivity from active fluorescence measurements, p. pp. 103 - 127, *In* D. J. Sugget, et al., eds. *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications*. Springer, Dordrecht.
- Suh, I.S., and S.B. Lee. 2003. A light distribution model for an internally radiating photobioreactor. *Biotechnol Bioeng* 82:180-189.
- Sun, J.D., J.N. Nishio, and T.C. Vogelmann. 1998. Green light drives CO<sub>2</sub> fixation deep within leaves. *Plant Cell Physiol* 39:1020-1026.
- Sung, K.-D., J.-S. Lee, C.-S. Shin, S.-C. Park, and M.-J. Choi. 1999. CO<sub>2</sub> fixation by *Chlorella* sp. KR-1 and its cultural characteristics. *Bioresource Technology* 68:269-273.
- Tennessee, D.J., R.J. Bula, and T.D. Sharkey. 1995. Efficiency of photosynthesis in continuous and pulsed-light emitting diode irradiation *Photosynthesis Research* 44:261-269.
- Terashima, I., T. Fujita, T. Inoue, W.S. Chow, and R. Oguchi. 2009. Green Light Drives Leaf Photosynthesis More Efficiently than Red Light in Strong White Light: Revisiting the Enigmatic Question of Why Leaves are Green. *Plant Cell Physiology* 50:684-697.
- Ugwu, C.U., H. Aoyagi, and H. Uchiyama 2008. Photobioreactors for mass cultivation of algae. *Bioresource Technology*. 99(10):4021-4028
- Wang, C. H., Y.Y. Sun, R.L. Xing, L.Q. Sun. 2005. Effect of liquid circulation velocity and cell density on the growth of *Parietochloris incisa* in flat plate photobioreactors. *Biotechnology and Bioprocess Engineering*. **10**(2): 103–108.
- Weis, E., and J.A. Berry. 1987. Quantum efficiency of photosystem-II in relation to energy-dependent quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 894:198-208.

- Wijffels, R.H., and M.J. Barbosa. 2010. An Outlook on Microalgal Biofuels. *Science* 329:796-799.
- Yewalkar, S., B. Li, D. Posarac, and S. Duff. 2011. Potential for CO<sub>2</sub> Fixation by *Chlorella pyrenoidosa* Grown in Oil Sands Tailings Water. *Energy & Fuels* 25:1900-1905.
- Yun, Y.S., and J.M. Park. 2001. Attenuation of monochromatic and polychromatic lights in *Chlorella vulgaris* suspensions. *Appl Microbiol Biot* 55:765-770.
- Zhang, K., N. Kurano, S. Miyachi. 2002 Optimized aeration by carbon dioxide gas for microalgal production and mass transfer characterization in a vertical flat-plate photobioreactor. *Bioprocess and Biosystems Engineering*. 25(2):97-101

**CHAPTER 3**

**EFFECTS OF INOCULUM PHYSIOLOGICAL STAGE ON THE GROWTH  
CHARACTERISTICS OF CHLORELLA SOROKINIANA CULTIVATED UNDER  
DIFFERENT CO<sub>2</sub> CONCENTRATIONS<sup>1</sup>**

---

<sup>1</sup> Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2012. Applied Biochemistry and Biotechnology  
168(3):519-530

## ABSTRACT

In order to maximize microalgae biomass production and reduce its overall costs it is important to optimize inoculum conditions based on its physical and physiological characteristics. *Chlorella sorokiniana* cultures inoculated with inoculum at three different physiological stages (lag, exponential and stationary) diluted to the same optical density were cultivated for 12 days under three different CO<sub>2</sub> concentrations (0.038%, 5% or 10% CO<sub>2</sub> v/v) and growth pattern and biomass production was observed. Samples inoculated with lag phase inoculum supplied with 5% CO<sub>2</sub> achieved the maximum biomass production whereas samples supplied with 0.038% CO<sub>2</sub> never reached exponential growth. The better growth of samples inoculated with lag phase inoculum was attributed to its increased number of cells compared to the other two inocula.

KEYWORDS: microalgae, production, inoculum, CO<sub>2</sub>

## INTRODUCTION

Increased CO<sub>2</sub> emission by human activities from the past 100 years has disturbed the natural equilibrium between sources and sinks of CO<sub>2</sub>, accelerating global warming. Restrictive regulations regarding GHG emissions have been discussed by the industrialized and emerging countries to reduce CO<sub>2</sub> emissions and stop the global warming process. The United States is responsible for approximately 25% of global GHG emissions, which is estimated by the U.S. Environmental Protection Agency (EPA, 2011) to be 6,633 million metric tons CO<sub>2</sub> equivalent in 2009. This estimate includes CO<sub>2</sub> emissions, as well as other GHGs such as methane, nitrous oxide, hydrofluorocarbons, perfluorocarbons and sulfur hexafluoride. Annual GHG emissions from fossil fuel combustion, primarily CO<sub>2</sub>, were estimated at 5,209 million metric tons with 3,490 million metric tons from stationary sources alone (EPA, 2011). The majority of CO<sub>2</sub> emission from stationary sources in the US comes from fossil fuel-based power plants, which have flue gas CO<sub>2</sub> concentrations ranging from 5 to 15%. Among the many approaches for carbon capture and storage (e.g. pre-combustion capture, geologic storage) microalgae cultivation is a promising option (Brune et al., 2009). Microalgae can offer high biomass yields, does not compete with conventional agriculture for arable lands or nutrients, can utilize waste and saline waters (Chinnasamy et al., 2010), can recycle carbon from CO<sub>2</sub>-rich flue gases, and can be used to produce a variety of fuels and valuable co-products (DOE, 2010).

Photoautotrophic cultivation of microalgae in photobioreactor is recognized as the best way to reach high rate production while maintaining monocultures without contamination. Although capital costs are currently higher for closed photobioreactors than for open-pond raceways, the former is more efficient in water use, has superior capability for long-term culture maintenance, and provides higher surface area to volume ratio that supports higher volumetric cell densities (Schenk et al., 2008). Thus it is best suited for high- value

products and/or use in hybrid systems as a breeder/feeder to open raceways, providing high cell density inoculum (Kunjapur and Eldridge, 2010). In these systems, providing supplemental CO<sub>2</sub> supply is critical since the low atmospheric CO<sub>2</sub> concentration and its low diffusion rate does not support high production rates making CO<sub>2</sub> a limiting factor.

The southeast U.S. has the necessary climatic conditions as well as a large number of coal-burning power plants providing ample sources of CO<sub>2</sub> for algal cultivation (DOE, 2010). Co-locating algal cultivation with power plants can be a desirable approach that can capture effluent CO<sub>2</sub> and convert it into biofuels and high value co-products (Brune et al., 2009).

In order to find a feasible and scalable way to convert CO<sub>2</sub> into biomass, studies evaluating the high rate production potential of specific microalgae species cultivated in photobioreactors have been done (Keffer and Kleinheinz, 2002; Li et al., 2011; Yewalkar et al., 2011). These studies have identified optimal conditions for each species for growth under high CO<sub>2</sub> concentrations in different kinds of waste and fresh media. Reaching high biomass concentrations in the shortest time can save significant amount of money and resources. The effect of CO<sub>2</sub> concentration on biomass production and growth rate of algae has been extensively studied (de Morais and Costa, 2007a; de Morais and Costa, 2007b; Sung et al., 1999). However, to the best of our knowledge the correlation of such CO<sub>2</sub> effects with the physiological stage of algal culture inoculum has never been established. As the algal cell physiology varies at different stages of growth (lag, exponential, stationary), it is very likely that such physiological variations may reflect their effect on CO<sub>2</sub> uptake and assimilation by algae. So it is important to evaluate the effects of physiological stages of algal inoculum on biomass production under different CO<sub>2</sub> concentrations. Therefore, in the present study, we examined the effects of different CO<sub>2</sub> concentration (0.038, 5 and 10%) and inoculum physiological stage on biomass production of *C. sorokiniana*, and we evaluated the existing correlation between these parameters.

## MATERIALS AND METHODS

### Algal Culture

*Chlorella sorokiniana* (UTEX 2805) was obtained from UTEX Culture Collections and maintained in BG-11 medium (NaNO<sub>3</sub>, 17.6 mM; K<sub>2</sub>HPO<sub>4</sub>, 0.22 mM; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 mM; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 mM; citric acid·H<sub>2</sub>O, 0.03 mM; ammonium ferric citrate, 0.02 mM; Na<sub>2</sub>EDTA·2H<sub>2</sub>O, 0.002 mM; Na<sub>2</sub>CO<sub>3</sub>, 0.18 mM; H<sub>3</sub>BO<sub>3</sub>, 46 μM; MnCl<sub>2</sub>·4H<sub>2</sub>O, 9 μM; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.77 μM; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1.6 μM; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.3 μM; Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.17 μM). The pH of culture medium was adjusted to 7.0±0.2 with hydrochloric acid 0.1 molar before inoculation and the algae were maintained in a temperature-controlled, illuminated growth chamber at 25±1°C and 250±10 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity provided by cool-white, fluorescent (6500K) T-8 bulbs.

### Physiological growth stage identification

For initial inoculum preparation, 100 mL of *C. sorokiniana* culture at exponential phase was inoculated in 900 mL BG-11 and grown for 10 days. Thirty flasks (500 mL) containing 225 mL BG-11 each were inoculated with 25 mL each of this initial inoculum. Flasks were placed in a growth chamber in a completely randomized manner and the culture was allowed to grow for 30 days in static mode under 250 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity, with no aeration, at 25°C temperature, and a 12-hour light/12-hour dark regime. Every 3 days, 3 flasks were randomly removed and placed in a refrigerator at 4.5°C under dark conditions. After all samples were similarly removed over time, biomass and chlorophyll analyses were conducted on all the flasks. Biomass growth curve and chlorophyll *a* and *b* content curves were developed using these data.

### CO<sub>2</sub> supply

Three concentrations of CO<sub>2</sub> gas supply, namely, 0.038% (atmospheric), 5% and 10% were tested in this experiment. For the 0.038% CO<sub>2</sub> concentration, an air pump was used to

bubble atmospheric air into culture flasks. Two premixed gas cylinders with 5% and 10% CO<sub>2</sub> in air (Airgas, USA) were used to supply the enriched CO<sub>2</sub> gas. Gas flow was adjusted to 45 mL/min (1 scfh). A timer controlling the power supply to the solenoid valves connected to the gas cylinders and the air pump was set to a 12-h cycle of on/off following the lighting regime inside the growth chamber.

### **Photobioreactor design**

The experimental system was composed of 12 2-L Erlenmeyer flasks (Fig 3.1) placed in a temperature-controlled, illuminated growth chamber at 25±1°C and 250±10 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity provided by cool-white, fluorescent (6500K) T-8 bulbs. Light intensity supply was measured using a Li-Cor model Quantum sensor connected to a datalogger (Li-Cor model Li-1400). Flasks were placed on individual triangular pieces of wood to provide an inclined position with CO<sub>2</sub> bubbles coming from the lower edge, thus providing mixing and preventing formation of biofilms which were previously observed when flasks were oriented horizontally. In this system, each gas cylinder and the air pump were attached to a multichannel manifold and gas supplied to each flask was controlled by an individual MR3000 panel-mounted flowmeter with control valve (Key Instruments, Trevose, PA, USA). The gas stream was sterilized by using 0.45-μm Whatman filters. Individual flasks were closed with a rubber stopper containing one gas input, one gas exhaust, and one sample port. The exhaust gas tube was also fitted with a Whatman filter. The whole system was connected using silicon tubing (Tygon 3350, 0.47cm I.D. x 0.79cm O.D.)

### **Inoculum development**

Using similar conditions as mentioned in the physiological growth stage identification section, three 2-L Erlenmeyer flasks designated as lag, exponential and stationary, containing 900 mL BG-11 each were inoculated with 100 mL *C. sorokiniana* in exponential phase and placed in a growth chamber. The lag, exponential and stationary flasks were incubated for 6,

19 and 27 days, respectively, to develop culture inocula that were in the lag, exponential, and stationary phases as desired. The corresponding optical densities (OD) were 0.3, 0.6 and 0.85, respectively, when they were removed, centrifuged to 10% of total volume, and stored in a refrigerator at 4.5°C under dark conditions.

To ensure that inoculation was standardized, a dilution process was used to bring all the three inocula to the same OD. The stored cell concentrates were normalized by diluting the exponential and stationary inocula to the same OD as the lag inoculum. To allow recovery of physiological activity of algae, re-suspended inocula were placed in a growth chamber under static conditions for 24 hours before inoculation. After the recovery period, 12 2-L Erlenmeyer flasks containing 720 mL BG-11 were inoculated with 80 mL inoculum each (4 replicates per inoculum phase). Three separate 12-d runs were conducted, with each run consisting of samples inoculated with the same inoculum phase supplied with three different CO<sub>2</sub> concentrations (0.038%, 5% and 10%).

### **Chlorophyll, Biomass, Optical Density and pH measurements**

For measurements of biomass, chlorophyll, OD and pH, samples were collected through the sample collection port using a 50-mL syringe at days 0, 3, 6, 9 and 12. After harvesting 10 mL of homogenized algal cells by centrifugation (5000 rpm, 10 min), the algal pellet was exhaustively extracted with hot methanol until it was colorless. Chlorophyll (Chl) *a* and *b* content was spectrophotometrically determined with the extinction coefficients in methanol and calculated using the method of Porra et al. (1989). The biomass was determined gravimetrically as described by Chinnasamy et al. (2010).

Optical density was measured at 735 nm using a spectrophotometer (Varian, Inc, Santa Clara, CA, USA) and pH measurements were taken using an accumet® portable AP62 pH/mv Meter (Fisher Scientific, USA)

### **Cell concentration at different ages**

Cell concentration at different culture ages was measured using a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA, USA) and calculated using the following equation:

$$TC = \text{cells} \times 10,000 \quad (1)$$

where TC is the cell concentration (cells per milliliter) and “cells” is in unit cells per square millimeter.

## RESULTS & DISCUSSION

### Physiological growth stage identification

After 30 days of *C. sorokiniana* cultivation in static batch mode, a biomass growth curve was established (Fig 3.2). The characteristic sigmoid shaped curve was divided into three growth phases: initial lag phase; exponential growth phase; and stationary growth phase. The exponential phase was best defined beginning at day 9 and ending at day 24 ( $R^2 = 0.9864$ ). During the initial lag phase, biomass concentration increased 7.6 times from day 0 to 9 with a daily increase of 0.008 g/L. During the exponential growth phase, in 15 days the biomass concentration increased approximately 4.6 times from day 9 to day 24 with a daily increase of 0.025 g/L. In the stationary phase, in 6 days the biomass concentration increased approximately 0.2 times from day 24 to day 30 with a daily increase of 0.014 g/L. Culture chlorophyll *a* and *b* content ( $\mu\text{g/mL}$ ) showed the same pattern as biomass concentration with a pronounced increase from day 18 to 21 (Fig 3.2). All samples showed a constant increased amount of chlorophyll *a* relative to chlorophyll *b*.

Growth limitation after day 24 (at biomass concentration of 0.46g/L) was probably caused by CO<sub>2</sub> limitation. Its low diffusion rate and its low concentration in atmospheric air were not sufficient to support higher biomass production. We verified that BG-11 medium had sufficient amount of nutrients to provide algae biomass accumulation greater than 1.5

g/L. In addition, light penetration and self-shading would not be a limiting factor for biomass production at this biomass concentration.

### **CO<sub>2</sub> concentration vs. inoculum stage**

The 12-flasks photobioreactor setup was operated three separate times, one for each inoculum phase, with 4 replicates for each one of the three CO<sub>2</sub> concentrations. The experimental design combined 3 inoculum phases (lag, exponential and stationary) and 3 gas supply CO<sub>2</sub> concentrations (0.038%, 5% and 10%) with 4 replications resulting in 36 independent measurements of each parameter. The 3 inoculum phases were defined as: lag phase (6 days old); exponential phase (19 days old); and stationary phase (27 days old).

Samples grown under atmospheric CO<sub>2</sub> concentration gas supply (0.038%) showed similar growth pattern regardless of the inoculum phase used. Under this condition, CO<sub>2</sub> availability may have been the major limiting factor for culture growth. Samples cultivated with 5 and 10% CO<sub>2</sub> gas supply showed similar growth patterns but different final biomass concentrations depending on the inoculum phase used (Tab 3.1). Samples inoculated with lag phase inoculum receiving 5% CO<sub>2</sub> gas supply reached the highest biomass production (1.44 g/L). Samples inoculated with lag phase inoculum accumulated more biomass than the others under the 3 different CO<sub>2</sub> concentrations. Among samples inoculated with the same inoculum phase the ones supplied with 5% CO<sub>2</sub> achieved higher biomass concentrations (Tab 3.1).

Because algae contain 50% carbon (Becker, 1994), to produce 1 g of biomass 1.8 g of CO<sub>2</sub> is necessary. Dry biomass results suggest that cultures inoculated with lag phase inoculum under 5% CO<sub>2</sub> gas supply fixed 1.5 and 4.8 times more CO<sub>2</sub> than cultures under 10% and 0.038% CO<sub>2</sub> gas supply, respectively.

The highest biomass production achieved by the samples under 5% CO<sub>2</sub> indicates that this concentration provides enough CO<sub>2</sub> for microalgae growth. For the set up system here

used, the 10% CO<sub>2</sub> gas supply has more CO<sub>2</sub> than the necessary amount to provide optimal growth and as a result a significant portion of CO<sub>2</sub> was probably wasted.

Biomass production and chlorophyll *a* content (Fig 3.3) showed a strong dependence on CO<sub>2</sub> concentration gas supply. All samples supplied with 0.038% CO<sub>2</sub> gas supply showed a linear biomass and chlorophyll *a* production independent of the inoculum used. Samples supplied with 5% CO<sub>2</sub> had the shortest lag phase reaching exponential growth 3 days earlier (at day 3) than samples supplied with 10% CO<sub>2</sub> (at day 6) accumulating the highest biomass and chlorophyll *a* content at day 12 regardless of the inoculum used (Fig 3.3).

The differential lag phase duration can be explained as a result of the different conditions between inoculum development (no CO<sub>2</sub> supply) and the experimental run (CO<sub>2</sub> enriched conditions). At 10% CO<sub>2</sub> gas supply, where the greatest difference existed, a long acclimatization period was seen indicated by the longer lag phase. Comparing *Chlorella* sp. growth under different CO<sub>2</sub> concentrations ranging from 10 to 70% it was found that the lag period increased with higher CO<sub>2</sub> concentrations (Sung et al., 1999). Also, biomass concentration after a 6 days cultivation period decreased from 5.7g/L to 0.71g/L with increasing CO<sub>2</sub> concentration gas supply from 10% to 70% (Sung et al., 1999). In another study conducted with *C. kessleri* in the first 12 days of a 20-day experiment, samples grown under low and moderate CO<sub>2</sub> concentrations (0.038% and 6%) reached higher biomass concentration (close to 1 g/L) compared to samples grown under high CO<sub>2</sub> concentrations (12% and 18%) which reached maximum biomass concentration close to 0.75 g/L (de Morais and Costa, 2007a).

### **Effects of CO<sub>2</sub> concentration on medium pH**

Bubbling enriched CO<sub>2</sub> gas into the sample results first in CO<sub>2</sub> in the gaseous phase (CO<sub>2(gas)</sub>) being transferred to the liquid phase (CO<sub>2(aq)</sub>), then reacting with water in a hydration reaction generating carbonic acid (H<sub>2</sub>CO<sub>3</sub>), followed by two dissociation reactions

forming bicarbonate ( $\text{HCO}_3^-$ ), and carbonate ( $\text{CO}_3^{2-}$ ) (Stumm, 1996). Whereas  $\text{CO}_2$  supply reduces pH of the culture medium, photosynthetic activity raises medium pH since  $\text{CO}_2$  (acid) removal causes the bicarbonate portion in the water to be proportionately high. These reactions reach equilibrium according to the  $\text{CO}_2$  supply/consumption ratio and the major form of available inorganic  $\text{CO}_2$  can be accessed based on predefined pH boundary values (Fig 3.4).

The measured pH of the cultures was constant and very stable during the whole experiment showing a strong dependence on  $\text{CO}_2$  concentration in the gas supply independent of the inoculum stage used (Fig 3.5). Under 0.038%  $\text{CO}_2$  gas supply, pH immediately increased and stabilized around 10 after 3 days. Under these conditions microalgae  $\text{CO}_2$  consumption was higher than  $\text{CO}_2$  supply raising the medium pH. At pH 10 no aqueous  $\text{CO}_2$  is available and decreasing  $\text{HCO}_3^-$  concentration coupled with increasing  $\text{CO}_3^{2-}$  concentration indicates a  $\text{CO}_2$  depleted medium. This supports the hypothesis that at 0.038%  $\text{CO}_2$  gas supply  $\text{CO}_2$  is the limiting factor for culture growth. At 5 and 10%  $\text{CO}_2$  gas supply the rate of  $\text{CO}_2$  supply is higher than the photosynthetic  $\text{CO}_2$  consumption, thereby dropping medium pH. Providing 10%  $\text{CO}_2$  gas supply caused a greater drop in medium pH, compared to 5%  $\text{CO}_2$ , which negatively affected *C. sorokiniana* growth due a longer acclimatization period represented by an extended lag phase growth. Samples supplied with 5%  $\text{CO}_2$  took 3 days to acclimatize to the new environment whereas the same effect was prolonged to 6 days on samples supplied with 10%  $\text{CO}_2$  gas supply. After the acclimatization period, both samples stabilized their pH around 7 (5%  $\text{CO}_2$ ) and 6.3 (10%  $\text{CO}_2$ ) and initiated exponential growth phase. Therefore, these results indicated that *C. sorokiniana* was capable to grow and accumulate biomass at a pH range varying from 6 to 10. This is in agreement with results reported for *C. ellipsoidea* and *Chlorella* sp., which are capable of growing in a pH range of 4–10 and 4–8, respectively (Khalil et al., 2010; Sung et al., 1999). The best growth found at

lower values of pH can be attributed to the greater amount of readily accessible form of inorganic carbon (bicarbonate). It was previously reported by Hirata (1996) that *Chlorella* sp UK001 could grow in an atmosphere containing 0.03 to 40% CO<sub>2</sub> and that the optimum pH was between 5.5 to 6.0.

The small pH difference between samples supplied with 5 and 10% CO<sub>2</sub> (Fig 3.5) was probably a result of low mass transfer caused by the short residence time (<0.5 sec) of gas bubbles inside the culture (Putt et al., 2011). Despite the system low efficiency, CO<sub>2</sub> mass transfer from the 5% and 10% CO<sub>2</sub> gas supply was able to provide a non-CO<sub>2</sub>-limiting condition.

### **Inoculum characteristics**

Among the samples supplied with extra CO<sub>2</sub> (5 and 10% CO<sub>2</sub> concentration) the ones inoculated with lag phase inoculum showed the best performance with a pronounced exponential growth phase and higher final biomass concentrations. Initially, all the inocula were diluted to the same OD before inoculation, and all the samples showed very similar biomass and chlorophyll *a* content after inoculation at day 0 (Tab 3.2). It is believed that the lag phase inoculum contained a higher number of smaller individual cells compared to exponential and stationary inocula which likely contains a smaller number of larger individual cells.

### **Evaluation of cell concentration at different ages**

Using a hemocytometer, the hypothesis that there are fewer cells present in older samples diluted to the same OD as younger samples was investigated. In a further experiment *C. sorokiniana* samples were grown to the age of 14, 23 and 37 days. Samples from the 37<sup>th</sup> day (OD 1.57) and 23<sup>rd</sup> day (OD 1.23) were diluted to the same OD as the 14<sup>th</sup> day (OD 0.85) and cells were counted in these samples. At the same OD 0.85, the 14-day old sample cell

concentration ( $16.7 \times 10^6$  cells  $\text{mL}^{-1}$ ) was twice as the 37-day old sample ( $8.2 \times 10^6$  cells  $\text{mL}^{-1}$ ) and 1.57 times higher than 23-day old sample ( $10.6 \times 10^6$  cells  $\text{mL}^{-1}$ ).

Normal microalgal growth curve is characterized by a lag phase with low number of cells multiplying at a high rate followed by exponential phase with moderate number of cells multiplying at a high rate and finally a stationary phase with high number of cells multiplying (or not) at a low rate (Ketchum et al., 1949). As the algae culture matures and gets older, not only does the cell amount and metabolism change but so is the cells size, which increases with age. *Chlorella pyrenoidosa* cultures had its medium-sized cells (dominant member of the population) average diameter increased from 3.0–3.5  $\mu\text{m}$  to 4.0–4.5  $\mu\text{m}$  as the culture matured and got denser going from  $53 \times 10^9$  to  $97 \times 10^9$  cells per liter (Ketchum and Redfield, 1949). The time required for cells to complete the reproduction cycle is normally longer for larger cells compared to smaller cells. Comparing different *Chlorella* species it was found that in the larger species *C. marina* (10  $\mu\text{m}$  length x 8  $\mu\text{m}$  width) and *C. ovalis* (10 x 8  $\mu\text{m}$ ) had lower specific growth rates of 2 and 1.5  $\mu \text{d}^{-1}$  (respectively) than the smaller species *C. spaerckii a* (2 x 2  $\mu\text{m}$ ) and *C. spaerckii b* (2 x 2  $\mu\text{m}$ ) that had specific growth rates of 2.7 and 2.8  $\mu \text{d}^{-1}$  (respectively) (Benamotz and Gilboa, 1980).

Physiological processes involved in cell reproduction are also affected by culture age, with slowing reproduction cycle as the culture gets older. Studying growth and reproduction of *Chlorella* species Agrawal (Agrawal, 2007) reported a dropping from 19:1 to 2:1 in the ratio of vegetative cells to autospore mother cells over 120 d cultivation of *C. vulgaris* and *C. variegata* cultures.

Normally, exponential phase inoculum is used to inoculate microalgae samples due its natural advantage of having an increased amount of medium size cells multiplying at a high rate. After diluting exponential and stationary phase samples to the same OD as the lag phase inoculum, the exponential inoculum lost its primary advantage of having more cells

multiplying at a high rate compared to lag phase inoculum which had a greater number of individual cells multiplying at a high rate. The increased number of young cells present in samples inoculated with the lag phase inoculum multiplying at a faster rate than the fewer older cells present in samples inoculated with exponential and stationary phase inocula reached higher final biomass concentrations as a consequence of a more pronounced exponential phase.

### **Proxy measurements**

Optical density measurements can be used as a proxy estimate of green algae cell density and chlorophyll content (Cervený et al., 2009). Plotting optical density measurements at 735 nm ( $OD_{735}$ ) versus dry biomass concentration a linear correlation was observed until  $OD_{735}$  1.2 (0.3 g/L) with a  $R^2=0.959$  (Fig 3.6). After that point  $OD_{735}$  measurements begin to plateau whereas biomass concentrations continues to increase exponentially (Fig 3.3) and the correlation between  $OD_{735}$  and biomass reduces ( $R^2=0.851$ ). Comparing  $\Delta_{680-735}$  (calculated as  $OD_{680}-OD_{735}$ ) and chlorophyll content (chlorophyll  $a + b$ ), a strong correlation was found until a chlorophyll content of  $20 \mu\text{g mL}^{-1}$  (Fig 3.7) with  $R^2=0.953$ . When higher values of chlorophyll content are used, the correlation between  $\Delta_{680-735}$  and chlorophyll gets weaker ( $R^2=0.610$ ).

## **CONCLUSION**

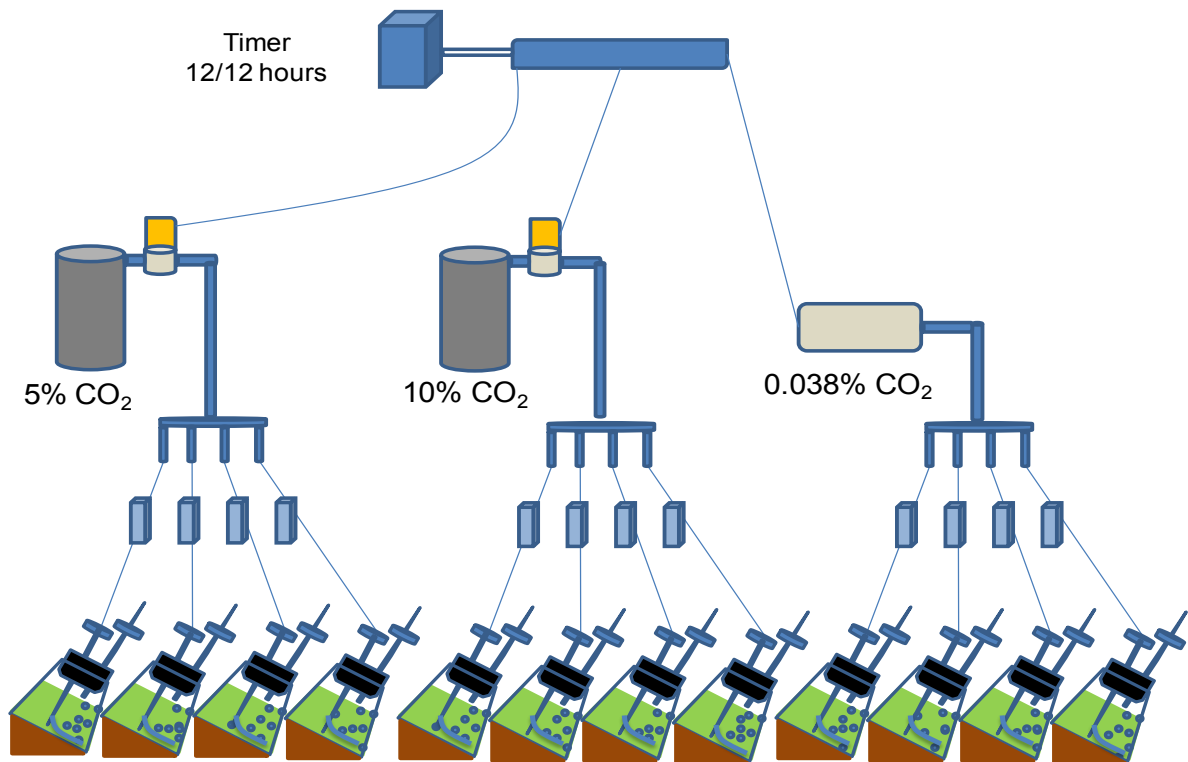
*Chlorella sorokiniana* samples inoculated with lag phase inoculum showed a pronounced exponential growth when supplied with enriched  $\text{CO}_2$  gas. The 5%  $\text{CO}_2$  gas supply provided enough  $\text{CO}_2$  to support high-rate microalgae growth while inducing a short acclimatization phase. Cultures inoculated with lag phase inoculum under 5%  $\text{CO}_2$  showed the maximum biomass production. The dilution process of samples at different stages to the same optical density, containing the same biomass and chlorophyll content, did not ensure the

same amount of cells. Among microalgae inoculums at different physiological stages diluted to the same OD the youngest ones containing increased amount of cells are more likely to grow better in enriched CO<sub>2</sub> ambient.

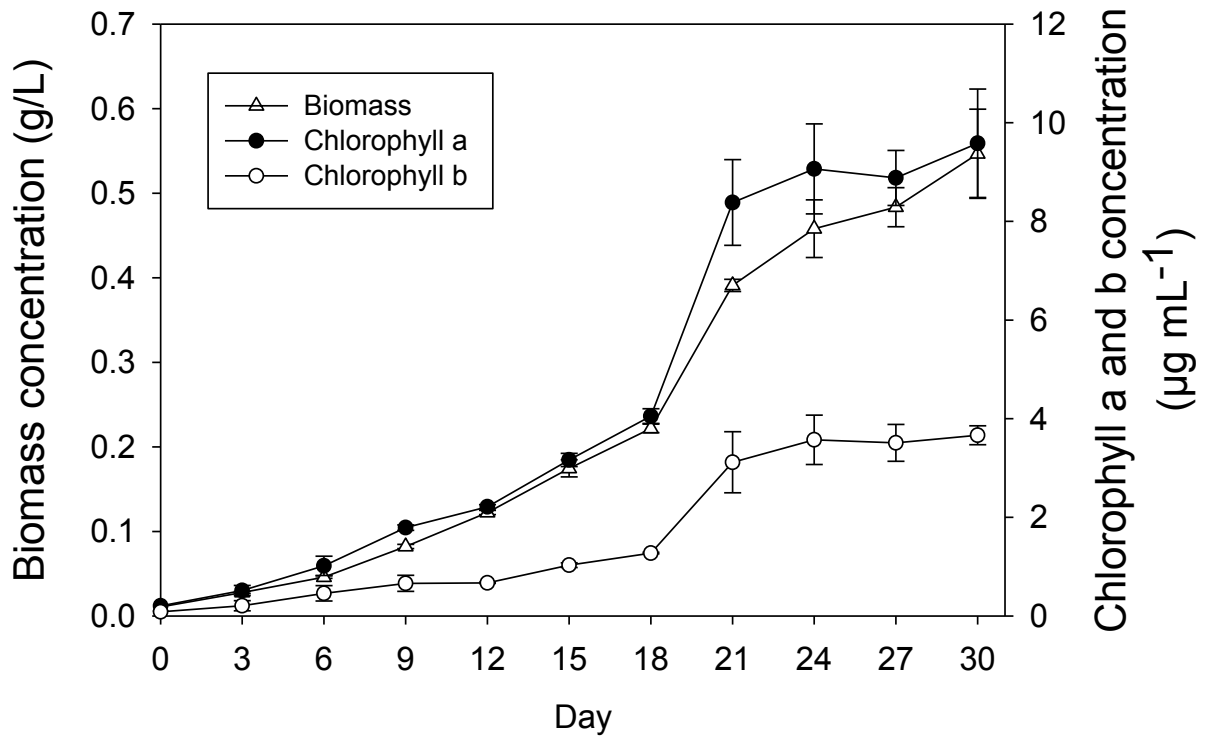
## REFERENCES

- Agrawal, S.C., Manisha. 2007. Growth, survival and reproduction in *Chlorella vulgaris* and *C. variegata* with respect to culture age and under different chemical factors. *Folia Microbiol (Praha)* 52.
- Becker, E.W. 1994. *Microalgae: biotechnology and microbiology*, New York, NY, USA.
- Benamotz, A., and A. Gilboa. 1980. Cryopreservation of marine unicellular algae: 1. A survey of algae with regard to size, culture age, photosynthetic activity and chlorophyll-to-cell ratio *Marine Ecology Progress Series* 2:157-161.
- Brune, D.E., T.J. Lundquist, and J.R. Benemann. 2009. Microalgal Biomass for Greenhouse Gas Reductions: Potential for Replacement of Fossil Fuels and Animal Feeds. *Journal of Environmental Engineering-Asce* 135:1136-1144.
- Carbon Dioxide-Carbonic Acid Equilibrium 2004. Available from:  
<http://ion.chem.usu.edu/~sbialkow/Classes/3600/Overheads/Carbonate/CO2.html>.  
Accessed November 14, 2011
- Cerveny, J., I. Setlik, M. Trtilek, and L. Nedbal. 2009. Photobioreactor for cultivation and real-time, in-situ measurement of O<sub>2</sub> and CO<sub>2</sub> exchange rates, growth dynamics, and of chlorophyll fluorescence emission of photoautotrophic microorganisms. *Engineering in Life Sciences* 9:247-253.
- Chinnasamy, S., A. Bhatnagar, R.W. Hunt, and K.C. Das. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technol* 101:3097-3105.
- de Morais, M.G., and J.A.V. Costa. 2007a. Carbon dioxide fixation by *Chlorella kessleri*, *C. vulgaris*, *Scenedesmus obliquus* and *Spirulina* sp cultivated in flasks and vertical tubular photobioreactors. *Biotechnology Letters* 29:1349-1352.
- de Morais, M.G., and J.A.V. Costa. 2007b. Biofixation of carbon dioxide by *Spirulina* sp and *Scenedesmus obliquus* cultivated in a three-stage serial tubular photobioreactor. *Journal of Biotechnology* 129:439-445.
- DOE. 2010. *National Algal Biofuels Technology Roadmap*, College Park, Maryland, USA.
- EPA. 2011. *Inventory of U.S. Greenhouse Gas Emissions and Sinks: 1990 - 2009*, Washington, DC, USA.
- Hirata, S., M. Hayashitani, M. Taya, and S. Tone. 1996. Carbon dioxide fixation in batch culture of *Chlorella* sp using a photobioreactor with a sunlight-collection device. *Journal of Fermentation and Bioengineering* 81:470-472.
- Keffer, J.E., and G.T. Kleinheinz. 2002. Use of *Chlorella vulgaris* for CO<sub>2</sub> mitigation in a photobioreactor. *Journal of Industrial Microbiology & Biotechnology* 29:275-280.

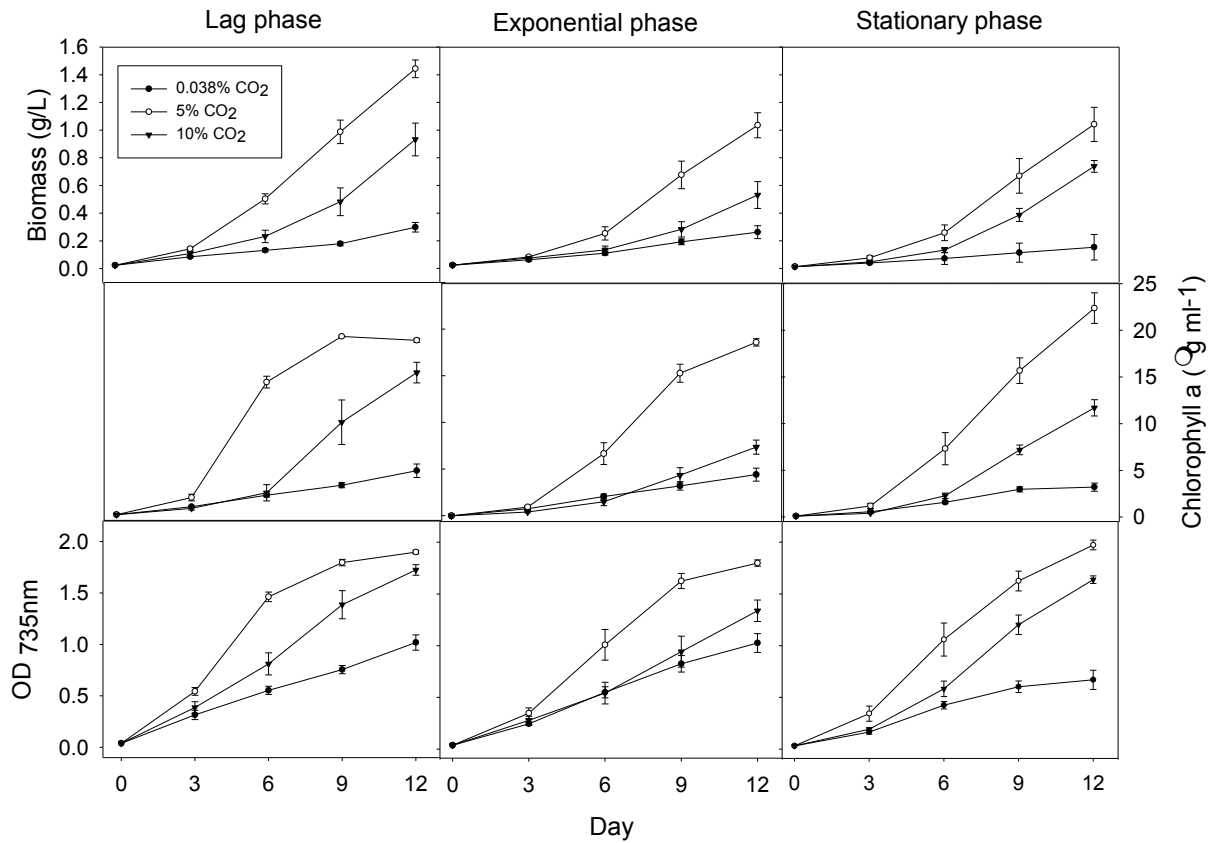
- Ketchum, B.H., and A.C. Redfield. 1949. Some physical and chemical characteristics of algae growth in mass culture. *Journal of Cellular and Comparative Physiology* 33:281-299.
- Ketchum, B.H., L. Lillick, and A.C. Redfield. 1949. The growth and optimum yields of unicellular algae in mass culture. *Journal of Cellular and Comparative Physiology* 33:267-279.
- Khalil, Z.I., M.M.S. Asker, S. El-Sayed, and I.A. Kobbia. 2010. Effect of pH on growth and biochemical responses of *Dunaliella bardawil* and *Chlorella ellipsoidea*. *World Journal of Microbiology & Biotechnology* 26:1225-1231.
- Kunjapur, A.M., and R.B. Eldridge. 2010. Photobioreactor Design for Commercial Biofuel Production from Microalgae. *Industrial & Engineering Chemistry Research* 49:3516-3526.
- Li, Y., Y.-F. Chen, P. Chen, M. Min, W. Zhou, B. Martinez, J. Zhu, and R. Ruan. 2011. Characterization of a microalga *Chlorella* sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production. *Bioresource Technology* 102:5138-5144.
- Porra, R.J., W.A. Thompson, and P.E. Kriedemann. 1989. Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-a and chlorophyll-b extracted with 4 different solvents - verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy *Biochim. Biophys. Acta* 975:384-394.
- Putt, R., M. Singh, S. Chinnasamy, and K.C. Das. 2011. An efficient system for carbonation of high-rate algae pond water to enhance CO<sub>2</sub> mass transfer. *Bioresource Technology* 102:3240-3245.
- Schenk, P.M., S.R. Thomas-Hall, E. Stephens, U.C. Marx, J.H. Mussgnug, C. Posten, O. Kruse, and B. Hankamer. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Research* 1:20-43.
- Stumm, W. 1996. *Aquatic chemistry chemical equilibria and rates in natural waters*, NY, USA.
- Sung, K.-D., J.-S. Lee, C.-S. Shin, S.-C. Park, and M.-J. Choi. 1999. CO<sub>2</sub> fixation by *Chlorella* sp. KR-1 and its cultural characteristics. *Bioresource Technology* 68:269-273.
- Yewalkar, S., B. Li, D. Posarac, and S. Duff. 2011. Potential for CO<sub>2</sub> Fixation by *Chlorella pyrenoidosa* Grown in Oil Sands Tailings Water. *Energy & Fuels* 25:1900-1905.



**Figure 3.1:** Multiple photobioreactor design. 12 microalgae samples are placed on inclined erlenmiers to allow better mixing inside the flasks promoted by the air bubbling. 4 samples are bubbled with atmospheric air, 4 samples are bubbled with 5% enriched CO<sub>2</sub> (v/v) and 4 samples are bubbled with 10% enriched CO<sub>2</sub> (v/v). Gas flow was adjusted to 45 mL/min (1 scfh) and its supply was controlled by a solenoid valve.



**Figure 3.2:** Growth curve of *Chlorella sorokiniana* and chlorophyll *a* and *b* concentration as measured in a 30-day static mode.



**Figure 3.3:** *Chlorella sorokiniana* biomass concentration, chlorophyll *a* concentration, and optical density (OD) of samples inoculated with lag, exponential and stationary inoculum phase growth under 0.038, 5 and 10% CO<sub>2</sub> concentration gas supply over 12 days.

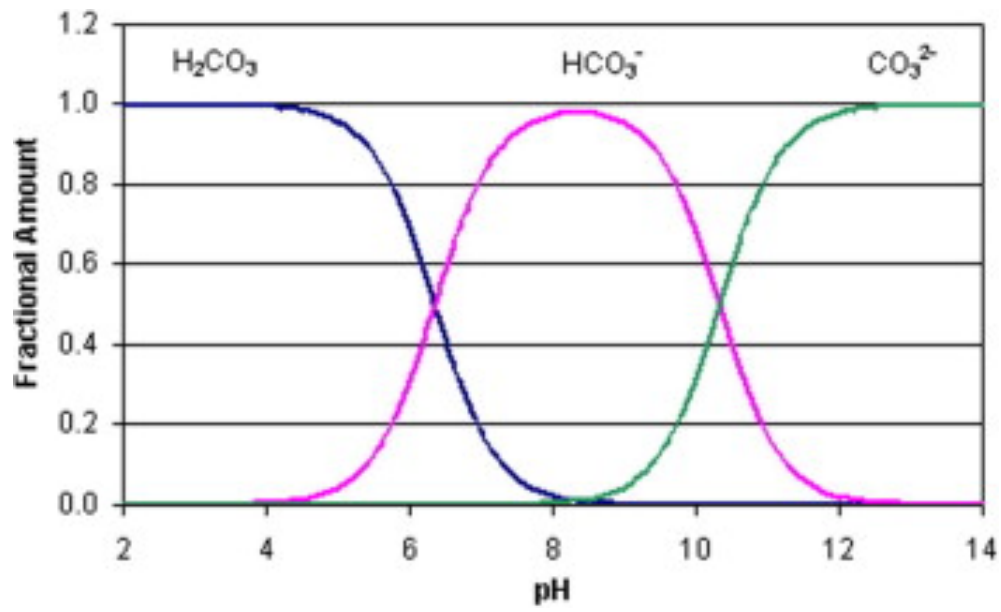
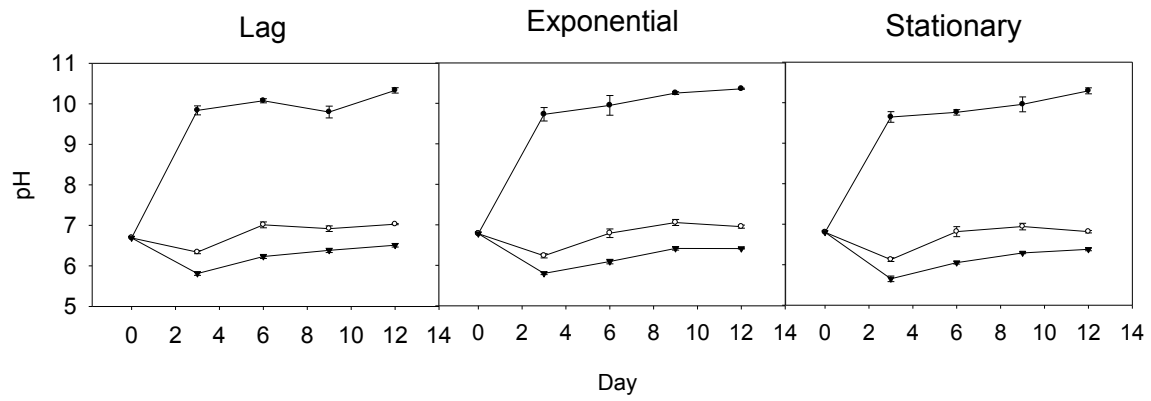
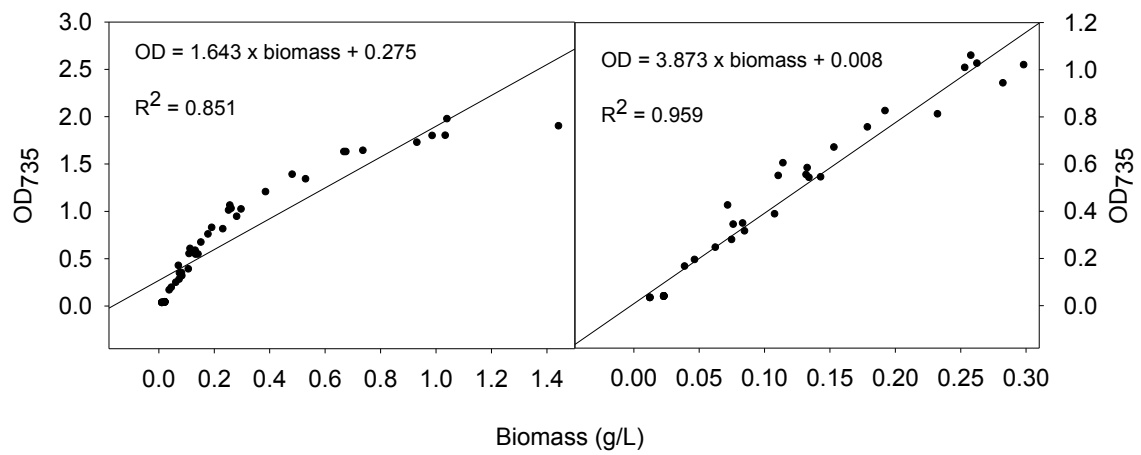


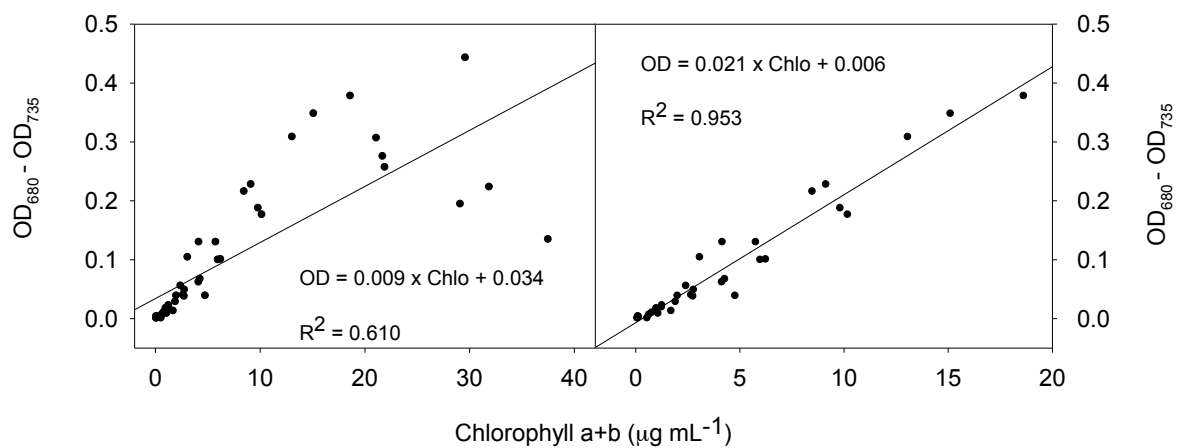
Figure 3.4: Distribution coefficients for  $\text{CO}_2$  in water.



**Figure 3.5:** pH measurements of samples inoculated with lag, exponential and stationary inoculum phase growth under 0.038% CO<sub>2</sub> (closed circles), 5% CO<sub>2</sub> (open circles) and 10% CO<sub>2</sub> (closed triangles) concentration gas supply over 12 days.



**Figure 3.6:** Linear correlation, regression equation and  $R^2$  value between optical density at 735 nm ( $OD_{735}$ ) and biomass concentration (g/L) measurements of *C. sorokiniana*. Left box: correlation for all measurements. Right box: correlation for measurements until OD 1.2.



**Figure 3.7:** Linear correlation, regression equation and  $R^2$  value between  $\Delta_{680-735}$  and chlorophyll concentration ( $\mu\text{g/mL}$ ) of *C. sorokiniana*. Left box: correlation for all measurements. Right box: correlation for measurements until chlorophyll concentration  $20 \mu\text{g mL}^{-1}$ .

**Table 3.1.** Final biomass accumulation in g/L at day 12 of samples inoculated at three different physiological stages (lag, exponential, and stationary) under three different CO<sub>2</sub> concentrations (0.038, 5 and 10%)

CO <sub>2</sub> concentration	Biomass (g/L)		
	Lag	Exponential	Stationary
0.038%	0.2985	0.26275	0.15345
5%	1.44375	1.03525	1.04125
10%	0.93275	0.53125	0.738

**Table 3.2.** Initial optical density (OD), biomass concentration (g/L), and chlorophyll *a* content ( $\mu\text{g/mL}$ ) at day 0, from samples inoculated with inoculum at lag, exponential and stationary phase.

Parameter measured	Inoculum phase		
	lag	exponential	stationary
OD	0.038	0.037	0.032
Biomass (g/L)	0.023	0.023	0.021
Chl <i>a</i> ( $\mu\text{g/mL}$ )	0.1	0.11	0.09

**CHAPTER 4**

**CHANGES IN CHLOROPHYL FLUORESCENCE PARAMETERS IN DIFFERENT**

**CHLORELLA SOROKINIANA CELL DENSITIES**

---

<sup>1</sup> Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2013 – To be submitted

## ABSTRACT

In microalgae cultivation high density biomass production is expected to overcome the high costs of cultivation and to reduce the costs of downstream processing. Light penetration is the main limiting factor on high density microalgae culture cultivation. Chlorophyll fluorescence measurements can be used as an indicator of light use efficiency by photosynthetic microalgae. This real time measurement can be useful to optimize light delivery and increase biomass production. Using chlorophyll fluorescence measurements we evaluated the effects of 6 monochromatic wavelengths ( $\lambda_{627\text{nm}}$ ,  $\lambda_{617\text{nm}}$ ,  $\lambda_{590\text{nm}}$ ,  $\lambda_{530\text{nm}}$ ,  $\lambda_{505\text{nm}}$ ,  $\lambda_{470\text{nm}}$ ) and a full spectrum neutral white LED at three different light intensities (100, 250 and 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) on the photosynthetic parameters quantum yield of photosystem II ( $\Phi_{\text{PSII}}$ ) and non-photochemical quenching (NPQ) in the microalgae *Chlorella sorokiniana* at three different cell densities (OD 0.5, 1.0 and 1.5). An inverted correlation between  $\Phi_{\text{PSII}}$  and light intensity was found across the whole experiment. Continuously decrease in  $\Phi_{\text{PSII}}$  measured under the green light spectrum was observed as *C. sorokiniana* cell density increased whereas NPQ had a noticeable decrease under all light sources as the culture density increased from OD 0.5 to 1.0.

KEYWORDS: microalgae, chlorophyll fluorescence, wavelengths

## INTRODUCTION

Microalgae biomass is a potential source of bioenergy and can be used to produce a vast range of biofuels (Amin, 2009). However, the petroleum industry exhibits competitive pricing and market fluctuations, which make it hard for potential microalgae producers to compete for low-value transportation fuels. The development of methods capable to maximize biomass production and sustain high density culture is of paramount importance to reduce the price of the final product and overcome the higher costs of culture cultivation.

Photobioreactors (PBR) are the best alternative to achieve high density biomass production. There are several PBR's designs which attempt to optimize mass transfer, light exposure, and the culture environmental control (Chiu et al., 2009; Singh and Sharma, 2012; Ugwu et al., 2008). Even in these optimized systems light irradiance onto the cells is one of the major growth limiting factors. As the culture achieves high biomass density, light penetration substantially decreases due to physical shading and physiological parameters (Suh and Lee, 2003).

The light spectrum range capable to induce photosynthesis is called Photosynthetic Active Radiation (PAR) and ranges from 400nm to 700nm. Always PAR light reaches a photosynthetic organism it can be absorbed, reflected, or transmitted. The spectrum and quantity of light absorption depends on the photosynthetic pigments composition of each photosynthetic organism (Govindjee, 1969). The main pigments found in terrestrial plants, microalgae and cyanobacteria are chlorophyll *a* and *b* with maximum light absorption peaks in the blue and red spectrum of light (Govindjee, 1969). Carotenoids with light absorption peaks in the blue and yellow spectrum of light are also present in several photosynthetic organisms as auxiliary pigments. The pigments are arranged in three dimensional pigment-protein complexes which determine their function and efficiency of energy transfer.

The absorbed light has three fundamental fates: it is either used to drive photosynthesis (photochemical quenching - qP); dissipated as heat (non-photochemical quenching - NPQ); or re-emitted as fluorescence (Cosgrove and Borowitzka, 2010). Measuring chlorophyll *a* fluorescence is possible to make assumptions on how the other portion of absorbed light is being used by the plants (Maxwell and Johnson, 2000).

The photosynthetic process begins at light absorption by the chloroplast. Absorbed photons are trapped by chlorophyll and other pigment's antennae complex embedded in the thylakoid membrane of the chloroplasts. The energy is directed to the reaction centers P680 (RCII) of Photosystem II (PSII) and P700 of Photosystem I (PSI) and used to drive the light reactions. At RCII water molecules are split releasing electrons to the electron transport chain. Those electrons are conducted through a series of acceptors to PSI where it is excited again and used to produce the reductant molecule NADPH in a process named 'Z'-scheme first described by Hill and Bendall (Hill and Bendall, 1960). During this process, H<sup>+</sup> protons released from the splitting of water plus H<sup>+</sup> protons pumped across the lumen membrane accumulate inside the lumen creating a proton gradient which drives ATP synthesis. Protons escaping from the thylakoid lumen through a central core of the enzyme ATP synthase cause conformational changes in the enzyme which catalyzes the phosphorylation of ADP producing ATP on the stromal side (Govindjee, 2004). Both ATP and NADPH molecules are further used to reduce CO<sub>2</sub> molecules to hexose in the Calvin-Benson Cycle (dark reactions). The dark reactions are slower than light reactions, so under high light conditions the electron flow is saturated and the electrons transported on light reactions can be accepted by other molecules rather than NADP<sup>+</sup> (e.g. O<sub>2</sub>) producing free radicals (Rabinowitch and Govindjee, 1969). These highly reactive molecules can cause photodamage and photo-oxidation to the photosynthetic apparatus mechanism causing reversible photoinactivation or in severe

conditions permanent inactivation of PSII reaction centers, known as photoinhibition (Adir et al., 2003; Krause, 1988) which lowers the efficiency of photosynthesis.

To avoid photodamage microalgae, as all the other photosynthetic organisms, have protective mechanisms that dissipate the excess of absorbed light energy as heat (NPQ). The most effective and fastest NPQ mechanism is termed high energy state quenching (qE) (Muller et al., 2001). This process is induced by the pH decreasing in the lumen and causes the protonation of PSII proteins activating the xanthophyll carotenoids pigments via xanthophyll cycle. At low lumen pH, the enzyme violaxanthin de-epoxidase removes two epoxides groups from the violaxanthin transforming this pigment into a zeaxanthin (Gilmore et al., 1995). The interaction of zeaxanthin with chlorophyll pigments causes a conformational change that dissipates the excitation energy as heat.

Fluorescence is the re-emission of energy in the form of a photon as an electron returns to ground state from a singlet excited state. As some energy is also given off as heat, the photon is red-shifted with an emission peak of ~685nm (Cosgrove and Borowitzka, 2010). At room temperature nearly all fluorescence (90-95%) comes from PSII at 685nm. Fluorescence emitted by PSI is very low at normal temperatures and only can be detected at temperature of liquid nitrogen. Indeed, the primary acceptor of electrons from P700 is rapidly re-oxidized, reducing fluorescence emissions at PSI (Falkowski and Kiefer, 1985). Fluorescence can be used to estimate the quantum efficiency of charge separation at RCII (Sugget et al., 2010). The use of fluorescence measurements as a probe of photosynthetic productivity has been vastly investigated due to the simplicity to collect measurements. Many studies correlating photosynthetic carbon fixation and electron transport rate based on changes in fluorescence yield have been reported (Genty et al., 1989; Schreiber et al., 1995; Weis and Berry, 1987). However, the underlying theory and the interpretation of data remains complex.

Microalgae light requirements changes as the culture develop and increase its cell density. Different approaches to optimize lighting systems and light delivery were proved to increase the biomass production and maximize photosynthesis efficiency based on light adjustment to matching the culture requirements at different stages (Carvalho et al.). Using chlorophyll fluorescence is possible to access the microalgae light use efficiency to gather important data to optimize artificial lighting systems.

In this contribution, we measured fluorescence re-emissions using a PAM fluorometer from the microalgae *C. sorokiniana* at different cell densities to analyze how the proportion of light absorbed by pigments associated with PSII used to drive photosynthesis (quantum yield of Photosystem II -  $\Phi_{\text{PSII}}$ ) and dissipated as heat (NPQ) are affected under six different monochromatic wavelengths and a full spectrum white light provided by LED's at three different light intensities.

## MATERIALS AND METHODS

### Algal Culture

*Chlorella sorokiniana* (UTEX 2805) was obtained from UTEX Culture Collections and maintained in BG11 medium (NaNO<sub>3</sub>, 17.6 mM; K<sub>2</sub>HPO<sub>4</sub>, 0.22 mM; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 mM; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 mM; citric acid·H<sub>2</sub>O, 0.03 mM; ammonium ferric citrate, 0.02 mM; Na<sub>2</sub>EDTA·2H<sub>2</sub>O, 0.002 mM; Na<sub>2</sub>CO<sub>3</sub>, 0.18 mM; H<sub>3</sub>BO<sub>3</sub>, 46 μM; MnCl<sub>2</sub>·4H<sub>2</sub>O, 9 μM; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.77 μM; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1.6 μM; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.3 μM; Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.17 μM). The pH value of culture medium was adjusted to 7.0 ± 0.2 before inoculation and the algae were maintained in a temperature controlled illuminated growth chamber at 25±1°C and 100±10 μmoles/m<sup>2</sup>/s light intensity provided by cool white fluorescent (6500K) T-8 bulbs.

## **Samples growth**

The microalgae cultures were grown in BG11 nutrient media. For the preparation of the mother culture, two flasks were filled with 270 mL of BG11 media and inoculated with 30 mL's of *C. sorokiniana* (10% v/v) in exponential phase. The flasks were placed into a temperature controlled illuminated growth chamber. Samples from both flasks were taken daily for optical density measurements at 750 nm. The culture was grown for 30 days until reach the highest optical density OD 1.5. Samples were collected at day 15 (OD 1.0) and day 7 (OD 0.5).

## **Optical System**

Using aluminum heat sink, an LED panel was built containing five Luxeon® III Star LED's: red ( $\lambda_{627\text{nm}}$ ), red-orange ( $\lambda_{617\text{nm}}$ ), amber ( $\lambda_{590\text{nm}}$ ), green ( $\lambda_{530\text{nm}}$ ), cyan ( $\lambda_{505\text{nm}}$ ), and two Luxeon® K2 Emitter LEDs: blue ( $\lambda_{470\text{nm}}$ ) and neutral white (5000K). Each LED was used to illuminate the microalgae samples through a  $\frac{1}{4}$  inch diameter fiber-optic light guide positioned 1 mm away from the surface of the 10 mm path-length cuvette. A light condenser was attached from the flat end of the fiber optic cable to the LED to collect and direct the LED output through the light guide. Each LED was driven by a single Luxdrive™ 1000mA Buckpuck 3021–D-E-1000. The Buckpuck driver was powered by an Agilent power supply (Agilent Technologies, Santa Clara, CA, USA) which enables voltage and current regulation to adjust light intensity output. Radiometric light intensity measurements were performed at tip of the fiber optic light guide and reported in  $\mu\text{mol m}^{-2} \text{s}^{-1}$  using a quantum sensor (LI-COR, Quantum Sensor LI-190, and Datalogger, LI-1400 Lincoln, NE, USA).

## **Analytical Methods**

Absorbance spectrum from 400-800 nm was measured and the culture optical density was recorded at 750 nm using a Varian Cary 50 UV/Visible spectrophotometer (Varian, Inc, Santa Clara, CA, USA). The chlorophyll *a* fluorescence parameters, quantum yield of

Photosystem II ( $\Phi_{PSII}$ ) and non-photochemical quenching (NPQ), were determined by a Pulse Amplitude Modulation (PAM) fluorometer Walz Mini-PAM (Heinz Walz GmbH, Effeltrich, Germany). The microalgae samples were analyzed inside a 5.0 mL cuvette with 10 mm path-length. Two fiber optic light guides were positioned on the surface of the cuvette at right angles. One light guide was connected to the LED panel for illumination and the second light guide was connected to the Mini-PAM Fluorometer for fluorescence measurements. The cuvette and light guides were contained inside a hinged-top dark enclosure (12.5 cm x 15.5 cm x 16.5 cm) custom made out of delrin plastic.

### Experimental procedure

Individual samples at three different optical densities were illuminated by one of the seven LED's at three different light intensities (100, 250 and 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Each treatment had a fresh 3ml of algal culture injected into the sample cuvette and  $\Phi_{PSII}$  and NPQ measurements were recorded for 5 minutes. Following the process described by Maxwell and Johnson (Maxwell and Johnson, 2000) both  $\Phi_{PSII}$  (Eq. 1) and NPQ (Eq.2) were calculated as:

$$\Phi_{PSII} = (F'_m - F_t) / F'_m \quad \text{Eq. 1}$$

$$\text{NPQ} = (F^0_m - F'_m) / F'_m \quad \text{Eq. 2}$$

where  $F'_m$  is the fluorescence maximum in the light;  $F_t$  is the steady state value of fluorescence immediately prior to the flash and  $F^0_m$  is the maximum fluorescence value in dark-adapted state. In order to record  $F^0_m$ , samples were dark adapted for 30 minutes prior to fluorescence analysis to allow total NPQ relaxation. The saturating light pulses were provided by the PAM's internal halogen bulb from 1.5 seconds at 10,000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity and were used to produce a transient closure of the PSII photochemical reaction center every 20 seconds.

The average of the last three measurements of  $\Phi_{\text{PSII}}$  and NPQ was taken as the final equilibrium values for each treatment. The final results reported in the tables were calculated from the average of duplicate runs per treatment. Statistical analysis was performed using SAS 9.2. The  $\Phi_{\text{PSII}}$  data was analyzed as a Factorial Design within each ODs and NPQ was analyzed as a Factorial Design within each LED color.

## RESULTS

### Light absorption spectra curves

Measurements of light spectrum absorbance were taken on *C. sorokiniana* samples at OD 0.5, 1.0 and 1.5. All wavelengths demonstrated higher absorption at denser cultures (Fig 4.1a). Apart from variations in amplitude, the wavelength  $\lambda_{470\text{nm}}$  which overlaps the absorbance spectrum of carotenoids and chlorophyll showed constant higher absorbance than the other wavelengths used here. A valley between 550nm and 650nm and a peak at 680 nm can be observed (Fig 4.1a). To compare the spectrum absorption of samples at the three different ages, the older samples at OD 1.5 (30 days) and OD 1.0 (15 days) were diluted to same OD as the youngest sample (0.5) and its light absorption profile was compared. The older samples showed slight less absorption within PAR spectrum than the young sample (Fig 4.1b).

### Quantum Yield

In general, as the light intensity increases, a decrease in  $\Phi_{\text{PSII}}$  values was observed (Tab 4.1). Orderly interaction was observed between the factors light intensity and color at OD 1.0 and 1.5. Despite difference in the magnitude between levels of light intensity change from monochromatic wavelengths and white LED the order of means for levels of light intensity is always the same. Thus the main effects of wavelength and light intensity are meaningful.

At the low density culture (OD 0.5)  $\lambda_{470\text{nm}}$  had lower  $\Phi_{\text{PSII}}$  and  $\lambda_{505\text{nm}}$  had higher  $\Phi_{\text{PSII}}$  compared to the control white LED whereas all the other wavelengths showed no significant difference. At the medium density (OD 1.0)  $\lambda_{470\text{nm}}$  and  $\lambda_{617\text{nm}}$  had lower  $\Phi_{\text{PSII}}$  compared to control white LED which had no significant difference to  $\lambda_{627\text{nm}}$ . All the other wavelengths had a higher  $\Phi_{\text{PSII}}$  compared to control white LED. At higher culture density (OD 1.5)  $\lambda_{470\text{nm}}$  and control white LED showed the lowest  $\Phi_{\text{PSII}}$  with no significant difference whereas all the other wavelengths had higher  $\Phi_{\text{PSII}}$ .

Within the monochromatic wavelengths,  $\lambda_{530\text{nm}}$  and  $\lambda_{505\text{nm}}$  exhibited higher  $\Phi_{\text{PSII}}$  on samples with OD 0.5, whereas on samples with OD 1.0 showed a gradual drop in  $\Phi_{\text{PSII}}$  switching position with  $\lambda_{590\text{nm}}$ ,  $\lambda_{617\text{nm}}$  and  $\lambda_{627\text{nm}}$  on samples with OD 1.5. At the red spectrum ( $\lambda_{617\text{nm}}$ ;  $\lambda_{627\text{nm}}$ ),  $\Phi_{\text{PSII}}$  does not drop as the samples OD increase from 1.0 to 1.5 in any of the 3 light intensities (shaded area on Tab 4.1). For  $\lambda_{590\text{nm}}$  the variation in  $\Phi_{\text{PSII}}$  as OD increases from 1.0 to 1.5 is very small.

### **Non-Photochemical Quenching**

Non-Photochemical Quenching (NPQ) refers to the difference between the initial, dark-adapted maximum level of fluorescence and that recorded after a period of illumination (Horton et al., 1994). Although NPQ does not show any logically behavior when analyzed within each OD either as a light intensity or wavelength response (model p-value > 0.05), a relation was found when it was analyzed within wavelengths as culture OD response (Tab 4.2). As the culture increased its OD from 0.5 to 1.0 NPQ had a significant decrease at all monochromatic wavelengths and the control white LED under all light intensities. At  $\lambda_{470\text{nm}}$  an interaction between the factors: light intensity and OD, was observed. Using T comparison lines for least square means of the interaction OD\*light intensity we identified the same significant drop in NPQ from OD 0.5 to 1.0 (shaded area Tab 4.2).

## DISCUSSION

### Light absorption

Light absorbance curves increased across the full spectrum of light as culture cell density increased as a result of light absorbance and light scattering by the *in vivo* cells present on the analyzed samples (Fig 4.1a). When incident light encounter intact entire microalgae cells part of the full spectrum of incident light may be absorbed, part is scattered and part is transmitted. Light absorbance measurements obtained with the spectrophotometer is a result of the difference in total light transmittance through a blank sample less the light transmittance through the microalgae samples. This obtained value is then the sum of light absorption by the photosynthetic pigments and light scattering by the larger sub cellular structures (Latimer, 1982). Similar shift in light absorption curves is reported by (Charney and Brackett, 1961) comparing results obtained from light absorption measurements obtained from a spectrophotometer plotted together with the spectral absorption curve of the same microalgae cell suspension taken with an integrating sphere, which can accurately measure the light absorption independently of the light scattering.

The higher absorbance in the blue spectrum of PAR is attributed to an overlap in primary chlorophylls and carotenoid absorption peaks (Biehler et al.). Although the two red spectrum LEDs  $\lambda_{617\text{nm}}$  and  $\lambda_{627\text{nm}}$  are absorbed by the main photosynthetic pigments present on *C. sorokiniana* (chlorophyll *a* and *b*), these two wavelengths fall out of their main absorbance peak at  $\lambda_{680\text{nm}}$  and  $\lambda_{660\text{nm}}$ , respectively and for this reason this two wavelengths are relatively weakly absorbed by the samples at the three culture densities.

Different age samples diluted to the same OD showed differential absorption at PAR spectrum of light (Fig 4.1b) resulting in less absorption by the older samples. Total carotenoids in the batch cultivation of microalgae follow the same pattern as the biomass productivity, showing growth at an exponential rate and reaching the steady state along with

cell biomass indicating that the pigment content is tight to the number of cells. However, the carotenoid content measured in the individual cells decreases with time (Del Campo et al., 2004). The same pattern is observed for chlorophyll *a* and *b* pigments in batch cultures growth. Considering that younger cultures have more individual cells than older cultures diluted to the same culture density (Mattos et al., 2012) based on OD measurements it is expected that the older samples containing less cells with a lower pigment content per cell absorb less light than younger samples containing more cells with higher pigment content.

## **Yield**

### *Light intensity*

In this research, we found an inverse correlation between light intensity and  $\Phi_{\text{PSII}}$ . The overall decrease in  $\Phi_{\text{PSII}}$  is associated to more efficient use of energy at low light irradiations. Second Sugget et al. (Sugget et al., 2010),  $\Phi_{\text{PSII}}$  can be de-composed into:

$$\Phi_{\text{PSII}}' = \Phi_{\text{P}}' \times \Phi_{\text{NPQ}}'$$

These measurements are taken under actinic light illumination, indicated by the apostrophe.  $\Phi_{\text{P}}'$  estimates the efficiency of charge separation in RCII and  $\Phi_{\text{NPQ}}'$  is defined as the efficiency of excitation energy transfer from antenna pigments to the RCII. Any NPQ of PSII will reduce  $\Phi_{\text{PSII}}'$ . Providing algae with higher light intensities more light is absorbed and more RCII will be reduced causing a decrease in  $\Phi_{\text{P}}'$  value with a consequently dropping on  $\Phi_{\text{PSII}}$  value. As defined, yield is the part of light absorbed that promotes photosynthesis. Increasing the total amount of light absorbed cause a natural decrease in efficiency charge separation on RCII promoting more NPQ leading to a decrease on  $\Phi_{\text{PSII}}'$ . Analyzing the individual parameters  $F_m'$  and  $F_t$  (used to calculate  $\Phi_{\text{PSII}}$  on Eq. 1) Genty et al. (Genty et al., 1989) demonstrated larger drops in  $F_m'$  compared to the relatively stable values of  $F_t$  during increasing light intensities from 0 to 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , which leads to an overall  $\Phi_{\text{PSII}}$  decreases.

Fluorescence analysis conducted on phytoplankton samples before and after incubation at fixed depth showed a maximum reduction in operational quantum yield of 34% on samples exposed to full solar irradiance (water surface) whereas 2 meter depth samples showed an increase of 23% when compared to their correspond values before incubation (Gilbert et al., 2000). Exposing the samples to higher PPFD caused a decrease on  $\Phi_{PSII}$  as a consequence of more light absorption by the whole culture, reducing more RCII decreasing the efficiency of photochemical quenching under saturating light pulses.

### **Wavelengths**

The strongest absorbed wavelength  $\lambda_{470nm}$ , showed the lowest yield on samples at all OD's tested (Tab 4.1). In high density cultures, less than 1% of the blue light is transmitted through the chloroplast, thus all  $\lambda_{470nm}$  light emitted on microalgae culture is readily absorbed by the cell layers at the surface of illumination on the cuvette, which is different from the other wavelengths tested that can have more reflection than absorption. Pickett and Myers (Pickett and Myers, 1966) comparing monochromatic light saturation curves for photosynthesis in *Chlorella* found different monochromatic light saturation points for wavelengths on blue, red and green spectrum. Despite the fact that the maximum rate of photosynthesis is independent of wavelength within PAR spectrum to reach the same photosynthetic rate at  $\lambda_{630nm}$  (weakly absorbed band) it was necessary to provide 5 times the light intensity used at  $\lambda_{450nm}$  (strongly absorbed band) due differential amount of light absorbed.

Providing different wavelengths at the same light intensity will cause a differential absorption of energy that leads to different  $\Phi_{PSII}$ . The higher  $\Phi_{PSII}$  values found  $\lambda_{530nm}$  and  $\lambda_{505nm}$  compared to the other wavelengths indicate that a relative smaller amount of energy is absorbed at OD 0.5. This energy is efficiently drained by the available oxidized reaction

centers whereas the higher amounts of energy absorbed under the other wavelengths reduces more reaction centers naturally resulting in a lower  $\Phi_{\text{PSII}}$ .

The wavelengths on the red spectrum  $\lambda_{590\text{nm}}$  and  $\lambda_{627\text{nm}}$  did not induce further drop on  $\Phi_{\text{PSII}}$  as culture density increased from OD 1.0 to 1.5 whereas  $\Phi_{\text{PSII}}$  of  $\lambda_{505\text{nm}}$  and  $\lambda_{530\text{nm}}$  which are highly reflected by chlorophyll pigments and absorbed by carotenoids pigments showed a continuously  $\Phi_{\text{PSII}}$  decrease as the culture density increased, switching positions with  $\lambda_{590\text{nm}}$  and  $\lambda_{627\text{nm}}$  at the highest culture density (OD1.5) indicating an increase in light absorption in the green spectrum over the red spectrum of light as the culture density increased.

*Chlorella sorokiniana* is rich in carotenoid pigments (0.69% of dry matter) that can absorb light in the blue and green spectrum (400nm to 530nm) of PAR. Lutein is the primary carotenoid pigment found in *C. sorokiniana* representing 60% of the total carotenoids (Matsukawa et al., 2000) (Tab 4.3).  $\beta$ -carotene (not listed in table 4.3) is another carotenoid pigment present on *C. sorokiniana* (Matsukawa et al., 2000) was reported to have peak absorption spectrum at 538nm on spinach lamellae (Ji et al., 1968) and parsley leaves (Nishimura and Takamatsu, 1957). In freeze-dried chloroplasts from wheat leaves  $\beta$ -carotene and lutein showed peak absorptions at 510nm and 495nm (Deroche and Briantai.Jm, 1974).

Emerson and Lewis (Emerson and Charlton, 1943) illuminated a thick layer of *Chlorella* cells with wavelengths in the blue ( $\lambda_{450\text{nm}}$ ), green ( $\lambda_{550\text{nm}}$ ) and red ( $\lambda_{600\text{nm}}$ ) and found scattered indices by green light 2 times higher than blue and 1.65 times higher than red. Thus, wavelengths in the green spectrum of light are more likely to be homogeneously distributed throughout the whole culture than red and blue light in high density cultures, resulting in a relative higher light absorbance increase than wavelengths on red and blue spectrum.

## NPQ

NPQ was significantly affected by cell density, independent of the wavelength and light intensity. The drop in NPQ from samples at OD 0.5 to OD 1.0 was a response to the reduced light exposure of individual cells caused by the higher cell density and increased light attenuation through the sample. The parameter used to access NPQ (qE) is induced by a pH decreasing in the lumen as a response to cell over exposure to light (Muller et al., 2001). The cells present at the lower cell concentration samples (OD 0.5) were exposed to a high photosynthetic photon flux density that induced high levels of NPQ whereas the cells present at the higher cell density samples (OD 1.0 and 1.5) exposed to the same levels of incident light achieved an overall more efficient energy absorption resulting in lower levels of NPQ.

## CONCLUSION

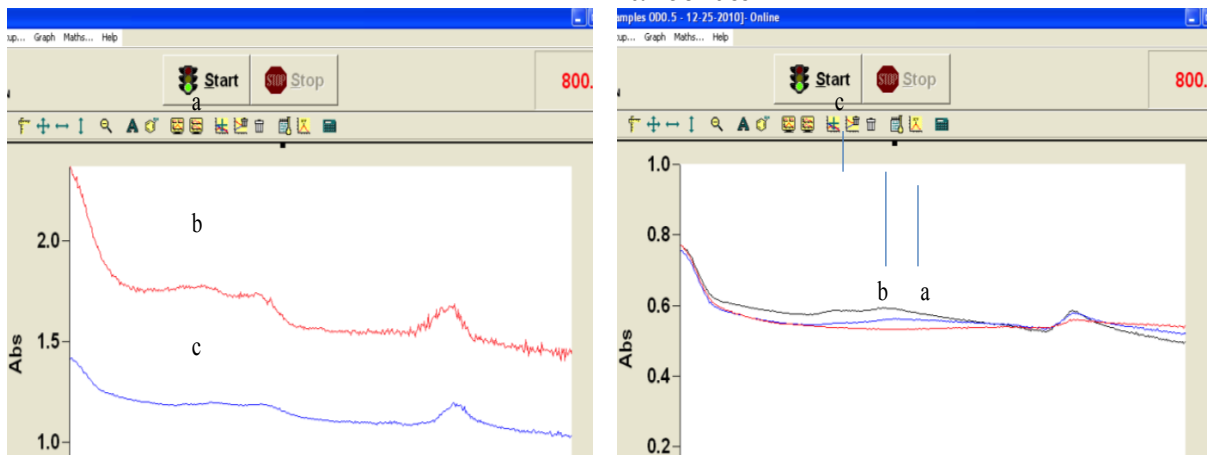
Light intensity and chlorophyll *a* fluorescence yield ( $\Phi_{\text{PSII}}$ ) showed an inverted correlation. As culture density increases from OD1.0 to OD1.5 wavelengths absorbed by carotenoids induced extra  $\Phi_{\text{PSII}}$  drop whereas the wavelengths weakly absorbed exclusively by chlorophylls ( $\lambda_{590\text{nm}}$ ,  $\lambda_{617\text{nm}}$ ,  $\lambda_{627\text{nm}}$ ) were no longer capable to induce extra  $\Phi_{\text{PSII}}$  drop. The continuously decrease in ( $\Phi_{\text{PSII}}$ ) measured under green light indicates a constant increase in light absorption as culture density increases. NPQ was found to have a direct relation with cell density.

## REFERENCES

- Adir, N., H. Zer, S. Shochat, and I. Ohad. 2003. Photoinhibition - a historical perspective. *Photosynthesis Research* 76:343-370.
- Amin, S. 2009. Review on biofuel oil and gas production processes from microalgae. *Energy Conversion and Management* 50:1834-1840.
- Biehler, E., F. Mayer, L. Hoffmann, E. Krause, and T. Bohn. Comparison of 3 Spectrophotometric Methods for Carotenoid Determination in Frequently Consumed Fruits and Vegetables. *Journal of Food Science* 75:C55-C61.
- Carvalho, A.P., S.O. Silva, J.M. Baptista, and F.X. Malcata. 2011. Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. *Applied Microbiology and Biotechnology* 89:1275-1288.
- Charney, E., and F.S. Brackett. 1961. Spectral dependence of scattering from a spherical alga and its implications for state of organization of light-accepting pigments. *Archives of Biochemistry and Biophysics* 92:1-12.
- Chiu, S.Y., M.T. Tsai, C.Y. Kao, S.C. Ong, and C.S. Lin. 2009. The air-lift photobioreactors with flow patterning for high-density cultures of microalgae and carbon dioxide removal. *Engineering in Life Sciences* 9:254-260.
- Cosgrove, J., and M.A. Borowitzka. 2010. Chlorophyll Fluorescence Terminology: An Introduction, p. pp. 1 - 17., *In* D. J. Suggett, et al., eds. *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications*. Springer, Dordrecht.
- Del Campo, J.A., H. Rodriguez, J. Moreno, M.A. Vargas, J. Rivas, and M.G. Guerrero. 2004. Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol.* 64:848-854.
- Deroche, M.E., and Briantai.Jm. 1974. Absorption-Spectra of Chlorophyll Forms, Beta-Carotene and Lutein in Freeze-Dried Chloroplasts. *Photochem. Photobiol.* 19:233-240.
- Emerson, R., and M.L. Charlton. 1943. The Dependence of the Quantum Yield of *Chlorella* Photosynthesis on Wavelength of Light. *Am. J. Bot.* 30:165-178.
- Falkowski, P., and D.A. Kiefer. 1985. Chlorophyll-a fluorescence in phytoplankton – relationship to photosynthesis and biomass. *J. Plankton Res.* 7:715-731.
- Genty, B., J.M. Briantais, and N.R. Baker. 1989. The Relationship between the Quantum Yield of Photosynthetic Electron-Transport and Quenching of Chlorophyll Fluorescence. *Biochimica et Biophysica Acta* 990:87-92.
- Gilbert, M., A. Domin, A. Becker, and C. Wilhelm. 2000. Estimation of primary productivity by chlorophyll a in vivo fluorescence in freshwater phytoplankton. *Photosynthetica* 38:111-126.

- Gilmore, A.M., T.L. Hazlett, and Govindjee. 1995. Xanthophyll cycle – dependent quenching of photosystem-II chlorophyll-a fluorescence – formation of a quenching complex with a short fluorescence life time. *Proceedings of the National Academy of Sciences of the United States of America* 92:2273-2277.
- Govindjee. 2004. Chlorophyll *a* Fluorescence: A Bit of Basics and History, p. pp. 1 - 42, *In* G. C. Papageorgiou and Govindjee, eds. *Chlorophyll a Fluorescence: A Signature of Photosynthesis*. Springer, Dordrecht.
- Hill, R., and F. Bendall. 1960. FUNCTION OF THE 2 CYTOCHROME COMPONENTS IN CHLOROPLASTS - WORKING HYOTHESIS. *Nature* 186:136-137.
- Horton, P., A.V. Ruban, and R.G. Walters. 1994. Regulation of light-harvesting in green plants – indication by nonphotochemical quenching of chlorophyll fluorescence. *Plant Physiology* 106:415-420.
- Ji, T.H., J.L. Hess, and A.A. Benson. 1968. Studies on chloroplast membrane structure. I. Association of pigments with chloroplast lamellar protein. *Biochim. Biophys. Acta* 150:676-685.
- Krause, G.H. 1988. Photoinhibition of Photosynthesis - an Evaluation of Damaging and Protective Mechanisms. *Physiologia Plantarum* 74:566-574.
- Latimer, P. 1982. Light-scattering and absorption as methods of studying cell-population parameters. *Annual Review of Biophysics and Bioengineering* 11:129-150.
- Matsukawa, R., M. Hotta, Y. Masuda, M. Chihara, and I. Karube. 2000. Antioxidants from carbon dioxide fixing *Chlorella sorokiniana*. *J. Appl. Phycol.* 12:263-267.
- Mattos, E.R., M. Singh, M.L. Cabrera, and K.C. Das. 2012. Effects of Inoculum Physiological Stage on the Growth Characteristics of *Chlorella sorokiniana* Cultivated Under Different CO<sub>2</sub> Concentrations. *Appl Biochem Biotech* 168:519-530.
- Maxwell, K., and G.N. Johnson. 2000. Chlorophyll fluorescence - a practical guide. *J. Exp. Bot.* 51:659-668.
- Muller, P., X.P. Li, and K.K. Niyogi. 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiology* 125:1558-1566.
- Nishimura, M., and K. Takamatsu. 1957. Carotene-protein complex isolated from green leaves. *Nature* 180:699-700.
- Pickett, J.M., and J. Myers. 1966. Monochromatic light saturation curves for photosynthesis in *chlorella*. *Plant Physiology* 41:90-98.
- Rabinowitch, E., and Govindjee. 1969. *Photosynthesis* Wiley., New York.
- Schreiber, U., H. Hormann, C. Neubauer, and C. Klughammer. 1995. Assessment of photosystem-II photochemical quantum yield by chlorophyll fluorescence quenching analysis. *Aust. J. Plant Physiol.* 22:209-220.

- Singh, R.N., and S. Sharma. 2012. Development of suitable photobioreactor for algae production - A review. *Renewable & Sustainable Energy Reviews* 16:2347-2353.
- Sugget, D.J., C.M. Moore, and R.J. Geider. 2010. Estimating Aquatic productivity from active fluorescence measurements, p. pp. 103 - 127, *In* D. J. Sugget, et al., eds. *Chlorophyll a Fluorescence in Aquatic Sciences: Methods and Applications*. Springer, Dordrecht.
- Suh, I.S., and S.B. Lee. 2003. A light distribution model for an internally radiating photobioreactor. *Biotechnol Bioeng* 82:180-189.
- Ugwu, C.U., H. Aoyagi, and H. Uchiyama. 2008. Photobioreactors for mass cultivation of algae. *Bioresour. Technol.* 99:4021-4028.
- Weis, E., and J.A. Berry. 1987. Quantum efficiency of photosystem-II in relation to energy-dependent quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 894:198-208.



**Figure 4.1:** Visible light absorbance (a) of *Chlorella sorokiniana* at three OD's: 0.5 (a), 1.0 (b), and 1.5 (c). (b) of different age *Chlorella sorokiniana* at 7 days (a), 15 days (b), and 30 days (c) diluted to the same OD 0.55

**Table 4.1:** Results for quantum yield of Photosystem II ( $\Phi_{PSII}$ ) of *C. sorokiniana* at three Optical Densities (OD) under interactive Wavelength ( $\lambda$ ) and Light intensity. \*refers to orderly interaction. Shaded area indicate the constant ( $\Phi_{PSII}$ ) values.

		$\lambda$ (nm)						
		470	505	530	590	617	627	White
<i><math>\Phi_{PSII}</math></i>								
<u>OD</u>	<u>Light Intensity</u> ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )							
	100	0.4387	0.6388	0.5575	0.5485	0.4910	0.5357	0.5492
0.5	250	0.3700	0.6378	0.5428	0.4513	0.4592	0.4763	0.4815
	500	0.3255	0.5352	0.4995	0.4398	0.3612	0.4452	0.4433
	100	0.3208	0.3770	0.3983	0.3540	0.2981	0.3080	0.3700
1	250	0.2340	0.3012	0.3590	0.2853	0.2477	0.2476	0.2940
	500	0.1365	0.2286	0.3136	0.2198	0.1643	0.1966	0.2041
	100	0.1995	0.2252	0.2495	0.3055	0.3028	0.3202	0.2097
1.5	250	0.1402	0.1718	0.2228	0.2568	0.2445	0.2717	0.1587
	500	0.0793	0.1345	0.1893	0.2102	0.1585	0.2183	0.1150

*Significant Effects (P<0.001)*

OD	Light Intensity	color	Light Intensity x color
0.5	25.37	29.38	n. s.
1.0	838.48	174.44	11.96*
1.5	428.44	234.43	8.98*

**Table 4.2:** General Linear Model for Non-photochemical quenching (NPQ) of *C. sorokiniana* at six monochromatic wavelengths ( $\lambda$ ) and full spectrum white LEDs under interactive Optical Density (OD) and Light intensity. \* refers to significance at  $P < 0.05$ ; \*\* to significance at  $P < 0.01$ ; \*\*\* to significance at  $P < 0.001$ . Shaded area indicates the significant different NPQ values.

		$\lambda$ (nm)						
		470	505	530	590	617	627	White
<i>NPQ</i>								
<u>OD</u>	<u>Light Intensity</u> ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )							
0.5	100	0.2393	0.2490	0.1932	0.3463	0.1853	0.2383	0.1685
	250	0.2935	0.1992	0.2103	0.2388	0.1663	0.2220	0.2033
	500	0.3858	0.3102	0.1672	0.2380	0.2412	0.1842	0.1890
1	100	0.0703	0.0360	0.0633	0.0477	0.1342	0.1435	0.0580
	250	0.1432	0.0605	0.0715	0.0772	0.1645	0.1480	0.0983
	500	0.1078	0.1363	0.0950	0.1315	0.1732	0.1113	0.1432
1.5	100	0.0932	0.0692	0.0638	0.0990	0.0447	0.0893	0.1103
	250	0.0923	0.0705	0.0757	0.0847	0.0430	0.0810	0.1133
	500	0.0863	0.0950	0.0685	0.0715	0.1432	0.0723	0.1260
<i>Significant Effects</i>								
OD		108.34***	31.8***	50.37***	13.5**	26.87***	26.36***	15.53**
Light Intensity		6.95*	4.64*	n.s.	n.s.	9.39**	n.s.	n.s.
Light Intensity*OD		5.08*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

**Table 4.3:** *Chlorella sorokiniana* carotenoid pigments content and its maximum absorbance wavelength corrected for acetone ( $\lambda_{ad}$ ).

Compound	<sup>a</sup> Content ( $\mu\text{g g}^{-1}$ dry weight)	<sup>b</sup> $\lambda_{ad}$ [nm]
Carotene	600	452
Cryptoxanthin	36	452
Lutein	4300	448
Zeaxanthin	140	452
Total Carotenoids	6900	

<sup>a</sup> taken from Matsukawa et al (2000); <sup>b</sup> taken from Biehler et al (2010)

## CHAPTER 5

### ENHANCEMENT OF BIOMASS PRODUCTION IN SCENEDESMUS BIJUGA HIGH DENSITY CULTURE USING WEAKLY ABSORBED GREEN LIGHT

---

<sup>1</sup> Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2012. Algal Research – under review

## ABSTRACT

Light penetration is the main growth-limiting factor in high-density microalgae cultures. Weakly absorbed light, which can penetrate deeper into high-density cultures, can be used as an alternative to sustain photosynthetic activity and biomass production providing extra growth to high biomass concentration cultures. Photosynthetic activity and biomass production induced by 4 different LEDs (blue- $\lambda$ 470nm, green- $\lambda$ 530nm, red- $\lambda$ 655nm, and white-3000K) were analyzed on high-density cultures of *Scenedesmus bijuga*. As culture density increased the weakly absorbed green light ( $\lambda$ 530nm) became more photosynthetically efficient than the red light, thereby inducing significantly higher oxygen evolution at culture concentration of 1.45 g/L. High-density culture (2.19 g/L) cultivated under the green light showed higher biomass production rate (30 mg/L/d) with a 8.43% biomass growth in a 6-day period compared to the red light that induced 4.35% biomass growth on the same period.

KEYWORDS: *Scenedesmus*, green, light, biomass, photosynthesis, photobioreactor

## INTRODUCTION

Microalgae cultivation has been used since 1960 for water treatment and high-value compounds commercialization (Oswald et al., 1953). Most recently, it has aroused great interest as a feedstock for biofuels production and biodegradable products (Gunther et al., 2012; Lakaniemi et al., 2011; Wijffels and Barbosa, 2010). However, the high cost of harvesting and de-watering due its intrinsic low biomass concentration is still a major drawback to scale up (Christenson and Sims, 2011). To increase microalgae cultures cell density, different kinds of closed photobioreactors using artificial illumination systems have been developed capable of achieving higher biomass concentrations under controlled environmental conditions (Choi et al., 2003; Fu et al., 2012; Hulatt and Thomas, 2011; Lee and Palsson, 1995). Artificial illumination systems can increase the volumetric productivity and high-density cultures can only be achieved when the cells receive appropriate levels of light energy along the entire cycle (Choi et al., 2003). Exposure of cells to excessive light at early culture stages leads to photodamage and photoinhibition that can cause a decline on photosynthetic capacity and biomass growth, whereas low levels of light energy at later stages become a growth-limiting factor. Providing constant optimal light uptake at different stages of development and allowing individual cells in the whole sample to be continuously exposed to optimal light amounts over the entire culture cycle can significantly increase biomass production (Choi et al., 2003; Lee et al., 2006). To optimize light supply, illumination systems have been designed to match microalgae absorbance spectrum based on its main pigments – chlorophyll *a* and *b*. These systems are normally composed of strongly absorbed wavelengths at blue and red spectrum and do not include the weakly absorbed wavelengths at green and orange spectrum (Lee and Palsson, 1995). Strongly absorbed wavelengths can indeed stimulate higher photosynthetic activity in low-density microalgae

cultures (Jeon et al., 2005) and have been shown to induce high biomass production (Fu et al., 2012). However, in high cell density cultures, light penetration is limited (Suh and Lee, 2003) and these strongly absorbed wavelengths are mostly absorbed by the layers of cells close to the illuminated surface resulting in a large fraction of its energy dissipated as heat by non-photochemical quenching mechanisms, whereas cells at the center of the sample may not receive sufficient light energy for photosynthesis. On the other hand, weakly absorbed wavelengths which have high scattering coefficient and low absorbance coefficient (Yun and Park, 2001) can penetrate deeper into high-density cultures and be homogeneously distributed among the cells as well as within the chloroplasts of individual cells. Along with deep penetration, the diffusive nature of the algal culture and the high reflectance index of weakly absorbed wavelengths would increase their pathway inside the culture thus increasing the opportunity for light to encounter chloroplasts, leading to increased absorbance. This phenomenon is described by Terashima et al (Terashima et al., 2009) as “detour effect” and it can substantially increase the absorbance of weakly absorbed wavelengths in microalgae cultures. Also, at intact cells present in microalgae culture, pigments are concentrated into chloroplasts, which decrease the opportunity for light to encounter pigments. This is referred as ‘package effect’ (also known as sieve effect) and results in a reduced light absorption at the absorption maxima of the pigments, while there is little reduction in absorption at those wavelengths that are weakly absorbed by the pigments (Kim and Lee, 2001; Terashima et al., 2009). Package effect is a function of cell density, size and pigment content and its magnitude becomes more severe as cultures cell density increases (Morel and Bricaud, 1981).

Recent studies with higher plants show that the strongly absorbed wavelengths get completely absorbed by the initial cell layers whereas green light can penetrate further into the

leaves (Brodersen and Vogelmann, 2010) and drive carbon fixation more efficiently than red and blue light in cells located deeper in the leaves (Sun et al., 1998). In high-density microalgae cultures, the increased number of cells have the same effect as the multiple cell layers present in higher plant leaves and light penetration becomes limited as the culture gets denser (Suh and Lee, 2003). By switching light spectrum from strongly absorbed wavelengths to weakly absorbed wavelengths at higher cell densities, it is possible to achieve deeper light penetration and homogeneous light distribution in high-density algae culture, promoting extra biomass growth. In the present study we compared the effect of different monochromatic wavelengths (red- $\lambda$ 655nm; blue- $\lambda$ 470nm; green- $\lambda$ 530nm) as well as a full spectrum neutral white light (3000K) on dissolved oxygen evolution and biomass production of the microalga *Scenedesmus bijuga* at high biomass concentrations.

## MATERIALS AND METHODS

### Algal Culture

*Scenedesmus bijuga* was previously isolated from waste water (Chinnasamy et al., 2010) and maintained in modified BG-11 medium (NaNO<sub>3</sub>, 17.6 mM; K<sub>2</sub>HPO<sub>4</sub>, 0.22 mM; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 mM; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.2 mM; citric acid·H<sub>2</sub>O, 0.03 mM; ammonium ferric citrate, 0.02 mM; Na<sub>2</sub>EDTA·2H<sub>2</sub>O, 0.002 mM; Na<sub>2</sub>CO<sub>3</sub>, 0.18 mM; H<sub>3</sub>BO<sub>3</sub>, 46  $\mu$ M; MnCl<sub>2</sub>·4H<sub>2</sub>O, 9  $\mu$ M; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.77  $\mu$ M; Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 1.6  $\mu$ M; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.3  $\mu$ M; and Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.17  $\mu$ M). The pH of culture medium was adjusted to 7.0 +/- 0.2 with hydrochloric acid 0.1M before inoculation, and the algae were maintained in a temperature-controlled, illuminated growth chamber at 25 +/- 1°C and 250  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light intensity provided by cool-white, fluorescent (6500K) T-8 bulbs.

## **Chlorophyll extraction and light absorbance**

For chlorophyll pigments extraction and light absorbance measurement, 10 mL of homogenized algal cells suspension was collected and centrifuged (5,000 rpm, 10 min). The supernatant was removed and re-suspended with the same amount of 95% methanol solution. The new solution was placed on hot water (60°C) for 30 min it was colorless. After further centrifugation the supernatant was collected and analyzed. Absorbance spectrum from 300-700 nm was measured using a Varian Cary 50 UV/Visible spectrophotometer (Varian, Inc, Santa Clara, CA, USA).

## ***Oxygen Evolution Experiment***

### **Light system**

An LED panel was built above an aluminum heat sink containing one Luxeon® III Star LED: green ( $\lambda$ 530nm), two Luxeon® K2 Emitter LED: blue ( $\lambda$ 470nm) and neutral white (3000K), and a Luxeon® Rebel Star LED: red ( $\lambda$ 655nm). Each LED was used to illuminate the microalgae samples through a 6.35 mm ( $\frac{1}{4}$  inch) diameter fiber-optic light guide positioned 1 mm away from the surface of the 10 mm path-length cuvette. A light condenser was attached from the flat end of the fiber optic cable to the LED to collect and direct the LED output through the light guide. Each LED was powered by an Agilent power supply (Agilent Technologies, Santa Clara, CA, USA) which enables voltage and current regulation to adjust light intensity output. Radiometric light intensity measurements were performed at the tip of the fiber optic light guide using a quantum sensor (LI-COR, Quantum Sensor LI-190, and Datalogger, LI-1400 Lincoln, NE, USA).

## **Culture cultivation**

A 100-mL sample of *S. bijuga* at its exponential phase of growth was inoculated in 900 mL BG-11 medium and cultivated for 12 days in a controlled-environment growth chamber under  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity (T-8 bulbs), at 25°C temperature, and a 12-h light/12-h dark regime. Enriched CO<sub>2</sub> gas supply (5% v/v) was supplied 12-h per day following the light regime. The set up was the same as described in Mattos et al (Mattos et al., 2012).

## **Dissolved oxygen evolution**

Measurements of dissolved oxygen were conducted for four days. Samples were collected at the exponential growth phase (8<sup>th</sup> to 11<sup>th</sup> d) and its cell suspension adjusted to the lower concentrations (0.7 and 0.8 g/L) by dilution with fresh BG-11 medium and to the higher concentrations (1.0 and 1.45 g/L) through centrifugation at room temperature (5,000 rpm, 10 min). After adjustment, samples were kept in the dark to bring the dissolved oxygen concentration down. Aliquots of 5 mL of *S. bijuga* were illuminated by the four different monochromatic LEDs at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . For each different light color, dissolved oxygen evolution was measured for 15 minutes using a Clarke type micro dissolved oxygen electrode (LAZAR, DO-166MT-1, USA) attached to the top of a 5-mL cuvette in a closed system isolated from external illumination. The cuvette was placed in a horizontal position so that light path was 6 cm. For all measurements agitation was provided by a micro stir bar. Dissolved oxygen consumption was measured in control samples on dark conditions. To minimize the differences in cultures between experiments, samples at the same biomass concentration were analyzed on the same day. Results for dissolved oxygen evolution and consumption were reported in mg O<sub>2</sub> per liter. All measurements were conducted in triplicates.

## ***Biomass Production Experiment***

### **LED table**

An LED panel containing 12 LEDs was built above an aluminum heat sink (30 x 30cm). The LEDs were dispersed in 4 rows of 3 LEDs connected in series. Each row was composed by the same color LEDs: green- $\lambda$ 530nm (Luxeon® III Star); blue-  $\lambda$ 470nm (Luxeon® K2 Emitter); neutral white (3000K) (Luxeon® K2 Emitter); and red- $\lambda$ 655nm (Luxeon® Rebel Star). Each LED row was controlled by potentiometers that enable regulation of light intensity. Each potentiometer was connected to a Luxdrive™ 1000mA Buckpucks 3021–D-E-1000 that were all connected to a single power transformer (12V). O-rings were mounted at the LED table to keep the erlenmeyer flasks 1 cm above from the LEDs and radiometric light intensity measurements were performed at the flasks illuminated surface using a quantum sensor (LI-COR, Quantum Sensor LI-190, and Datalogger, LI-1400 Lincoln, NE, USA).

### **Culture cultivation**

A 300-mL sample of *S. bijuga* at its exponential phase of growth was inoculated in 2,700 mL nutrient enriched BG-11 and cultivated for 28 days in a 4-L erlenmeyer flask in a controlled-environment growth chamber under  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity (T-8 bulbs), at 25 °C temperature, and a 12-h light/12-h dark regime until it reached light limited condition. To ensure satisfactory conditions concentrations of the two main nutrients necessary for algae development nitrogen and phosphorous were constantly monitored and adjusted to its original concentration (176 and 10.8 mg/L, respectively) when level of one of them dropped under 10% of its original concentration. Micronutrients had their concentration adjusted following the stoichiometrically calculated nitrogen adjustment. Enriched CO<sub>2</sub> gas (5% v/v) was supplied 12-h per day following

the light regime. To avoid algal sedimentation, the culture flask was positioned in an inclined position as described in Mattos et al (Mattos et al., 2012).

### **Biomass Production**

On the 28<sup>th</sup> day, 2.0 L of sample collected at biomass concentration of 1.8 g/L was concentrated by centrifugation at room temperature (5,000 rpm, 10 min) to a higher biomass concentration of 2.19 g/L and samples were transferred to the LED plate to grow above the different LED lighting sources for 6 additional days. Dissolved oxygen evolution was measured at the culture before it was transferred to the LED plate. Aliquots of 100-mL samples were placed in 12, 125-mL erlenmeyer flasks that were placed above the LEDs. The flasks were coated black to avoid external illumination and ensure that the light provided by the LED was the sole light source. Light intensity was adjusted to  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ . To avoid algae sedimentation the LED plate was placed on an orbital shaker at a 100 rpm.

### **Biomass and pH**

The biomass concentration of cultures was determined gravimetrically as described by Chinnasamy et al. (Chinnasamy et al., 2010) and pH measurements were taken using an accumet® portable AP62 pH/mv Meter (Fisher Scientific, USA).

### **Nitrogen and Phosphorous**

Nitrogen content was measured as nitrate using chromotropic acid method using HACH Test'N tube™ kits (HACH, USA) and results were expressed as mg/L  $\text{NO}_3^-$ -N. Phosphorous content was measured as reactive phosphorous (orthophosphate) using molybdovanadote method using HACH Test'N tube™ kits (HACH, USA) and results were expressed as mg/L  $\text{PO}_4^{3-}$ .

### **Algae Pictures**

Digital photographs were taken using a Sony Cybershot camera model DSC-W120

## RESULTS AND DISCUSSION

### Light absorption and penetration

Light absorbance by the extracted chlorophyll content of *S. bijuga* samples showed a pronounced increase at the blue ( $\lambda 470\text{nm}$ ) and red ( $\lambda 655\text{nm}$ ) spectrum with a discrete increase at the green ( $\lambda 530\text{nm}$ ) spectrum as the culture biomass concentration increased (Fig 5.1). Attenuation of red and blue light penetration in green microalgae is mostly caused due to light absorption by chlorophyll pigments which have higher absorption coefficients in these wavebands whereas green light attenuation is caused by light scattering (Yun and Park, 2001). The difference in light absorption is reflected on light penetration pictures where the decrease in light penetration is more noticeable in samples illuminated with red and blue light than in samples illuminated with green and white light as the culture increases its biomass concentration from 0.7 g/L to 1.45 g/L (Fig 5.2). Interestingly, the visualization of 'green effect' in whole spectrum set (white light) indicated that green waveband was least absorbed and was able to penetrate to the other end of solution as indicated by the dominant color (Fig 5.2). Kim and Lee (Kim and Lee, 2001) have shown that light penetration depth of red LED ( $\lambda 680\text{nm}$ ) in *Chlorella kessleri* cultures decreases exponentially as cell density increases.

Suh and Lee (Suh and Lee, 2003) working with *Synechococcus* sp. showed that light emitted at  $600 \mu\text{mol m}^{-2} \text{s}^{-1}$  had a pronounced penetration decrease as biomass concentration increased from 0 – 4.0 g/L. At high culture concentrations, light transmittance of all monochromatic wavelengths was near zero. Yun and Park (Yun and Park, 2001) working with *C. vulgaris* found that the transmitted spectral Photosynthetic Flux Density declined with increasing cell concentration and eventually reached zero when cell concentration was more than 0.855 g/L. In agreement with the light absorption results observed in this study (Fig 5.1), Yun and Park

(Yun and Park, 2001) reported that light attenuation was higher for the strongly absorbed  $\lambda 450$  and  $\lambda 650$  nm light than for the weakly absorbed  $\lambda 550$  nm light which could penetrate further into the algal suspension.

### **Oxygen Consumption**

Dark respiration which is the non-photorespiratory mitochondrial respiration is vital for growth and survival of microalgae. Much of the usable energy (ATP), and reducing power (e.g. NADPH and NADH) required for biosynthesis and cellular maintenance are generated by dark respiration in a regular manner through an oxygen consumption process. As expected, dissolved oxygen consumption increased as culture density increased (Fig 5.3). The higher amount of cells present at high biomass concentrations consumed more oxygen compared to samples with lower biomass concentrations going from 1.52 mg O<sub>2</sub>/L/hr at the lowest biomass concentration (0.7 g/L) to 4.23 mg O<sub>2</sub>/L/hr at the highest biomass concentration (1.45 g/L) (Fig 5.3).

### **Oxygen Evolution**

Light energy harvested by the antenna pigment of photosystem II is directed to the reaction centers where charge separation takes place and light energy is used to split a water molecule providing electrons for the electron flow process. During this process free oxygen is generated as a sub product and can be measured to estimate photosynthetic activity. This is a light-dependent process and for this reason is conducive to evaluate the effect of different light sources on photosynthetic activity. At the four biomass concentrations selected, the full spectrum white LED induced the highest dissolved oxygen evolution whereas blue light always induced the lowest (Fig 5.4). At this specific combination of light intensity and biomass concentrations, the full spectrum white light could provide the benefits of the strongly absorbed wavelengths inducing high rates of photosynthesis at the cells close to the illuminated surface of high-density

cultures while providing deep light penetration by the weakly absorbed wavelengths thus inducing photosynthesis at the cells further from the illumination source. For the other 3 lower biomass concentrations, the strongly absorbed blue light had the poorest light penetration as it gets completely absorbed by a limited amount of cells closer to the illuminated surface resulting in the lowest oxygen evolution rates compared to the other light colors. At the highest biomass concentration blue light penetration was most severely limited and the total amount of oxygen produced by the photosynthetic process was not enough to compensate the amount of oxygen consumed by the respiration process resulting in no net oxygen evolution (Fig 5.4).

A comparative analysis on the effect of red and green light illumination on oxygen evolution rate of various cell densities of algal cultures was conducted based on data presented in Fig 5.4. At lower biomass concentration (0.7 g/L), oxygen evolution induced by the strongly absorbed red light ( $\lambda 655$  nm) was 23% higher than what was induced by the weakly absorbed green light ( $\lambda 530$  nm). However, as the cell density increased to 1.0 g/L, oxygen evolution was adversely affected in red light illuminated culture where a 11% decrease in oxygen evolution was observed relative to that in the 0.7 g/L culture. In contrast, cultures illuminated by weakly absorbed green light showed linearly proportional increase ( $R^2$  0.9672) in oxygen evolution when plotted against increasing cell density. About 24% increase in oxygen evolution was observed when biomass concentration was increased from 0.7 to 1.0 g/L (Fig 5.4). At the highest biomass concentration tested (1.45 g/L), green light induced 126% more oxygen evolution than red light. This is a clear indication that in high-density cultures the weakly absorbed green light can penetrate deeper into the sample and be more homogeneously distributed and absorbed by the culture inducing an overall higher oxygen evolution than the strongly absorbed red light.

At biomass concentration 1.45 g/L, a decrease in net oxygen evolution (Fig 5.4) by all light colors was observed and is attributed to the high oxygen consumption due to respiration (Fig 5.3). In microalgae cultures, as cell concentration increases the specific photosynthetic activity decreases whereas the volumetric photosynthetic activity increases as a consequence of higher amount of pigments present in the culture and limited light penetration (Lee and Palsson, 1995). Photosynthetic capacity of *S. bijuga* was calculated as mg-O<sub>2</sub> evolved/g-biomass/hr for four different biomass concentrations under the four LED lights at 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The results indicated that overall photosynthetic capacity of *S. bijuga* decreased as culture density increases (Fig. 5.5).

However, the decrease for green light illuminated culture was relatively less prominent than what was observed for red light illuminated culture, indicating that weakly absorbed light (green) may contribute to sustained higher photosynthetic capacity of algae at higher cell densities. This is evident from results presented in Fig 5.5 where the photosynthetic capacity of red light compared to green light was higher in lower density cultures (0.7 and 0.8 g/L) whereas at the higher density cultures (1.0 and 1.45 g/L) the weakly absorbed green light showed higher photosynthetic capacity. This shift on photosynthetic efficiency can be explained by the fact that in low density cultures strongly absorbed wavelengths can induce photosynthesis activity more efficiently than weakly absorbed wavelengths as they can easily penetrate into the culture and induce high photosynthesis activity as a consequence of high light absorption by the majority of the cells. Jeon et al (Jeon et al., 2005) reported higher photosynthetic activity in low biomass concentration cultures (0.123 and 0.215g/L) of green algae *Haematococcus pluvialis* in red light compared to green light. However, in high-density cultures only a small fraction of superficial cell layers are exposed to light levels higher than the compensation point and an even smaller

fraction can have sufficient light exposure to induce their maximal photosynthetic activity (Kim and Lee, 2001). Light penetration attenuation and reduction in photosynthetic activity in high density culture is more severe considering a strongly absorbed source of light than a weakly absorbed one.

### ***Biomass Production Experiment***

#### **Culture cultivation**

At day 12, nitrogen and phosphorous contents were at 50 and 7.5% of their respective initial concentrations, and 20% of sample was harvested and replaced with the same volume of concentrated BG-11. At day 24, nitrogen and phosphorous level were at 46 and 8% of their respective original concentrations and the same procedure was repeated. However, the volume of replacement was increased to 40%, which caused the biomass concentration drop to 1.08 g/L (day 26) (Fig 5.6). At day 28, biomass concentration was fast growing again when samples were harvested and concentrated to 2.19 g/L for the biomass production experiment.

#### **Biomass Production**

Before samples were placed on the LED table, oxygen evolution measurements were taken. At  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, the samples at a biomass concentration of 2.19 g/L showed an oxygen evolution of 11.07 mg/L/hr under green light, followed by 10.67, 5.74 and 1.44 mg/L/hr under white, red and blue illumination, respectively (Fig 5.7). The photosynthetic activity and oxygen production rates of high density microalgae culture are determined by light penetration and distribution rather than light intensity (Kim and Lee, 2001). Green and white light were able to penetrate deeper into the samples inducing the highest oxygen evolution and overcoming the two strongly absorbed wavelengths red and blue. The overall oxygen evolution for all the monochromatic wavelengths and the full spectrum white LED measured here was

higher than that observed in earlier experiments as explained under the section “3.3 Oxygen Evolution” due to higher light intensity provided.

After 6 days on the LED plate, samples illuminated with weakly absorbed green light had the highest biomass production (0.18 g/L) going from 2.19 g/L on day 0 to 2.37 g/L on day 6 (Fig 5.8). At high cell density, the weakly absorbed wavelength promoted an additional growth of 8.43% of its initial biomass concentration, followed by the white, red and blue light that promoted an additional growth of 6.33, 4.35 and 2.21%, respectively. The growth rate induced by the weakly absorbed green light (30 mg/L/d) was twice that of the strongly absorbed red light (15 mg/L/d). In microalgae cultivation, biomass growth rate is proportional to the amount of light absorbed by the cells (Choi et al., 2003; Lee et al., 2006) Assuming light penetration is the primary limiting factor to cell exposure to light, and considering that the only variable in the current study was the wavelength of light supplied to the algae culture, a correlation between biomass growth rate light penetration can be established in which the green light was the most efficient light source.

The higher biomass production induced by the green light compared to the other wavelengths is in line with the results found in the oxygen evolution experiment confirming the hypothesis that the weakly absorbed wavelength is photosynthetically more efficient than the strongly absorbed wavelengths promoting higher biomass production on high-density *S. bijuga* cultures under the conditions provided. Previous studies have shown that optimizing the light supply might be an efficient means of achieving high biomass productivity and a profitable biomass yield on light (Choi et al., 2003; Gordon and Polle, 2007b; Hu et al., 1998; Lee et al., 2006). In line with these studies and considering the results found on this study we suggest that artificial lighting systems should also have weakly absorbed wavelengths to induce more

efficient illumination and enhance biomass production at the final stages of cultivation by switching the output light spectrum composition towards weakly absorbed wavelengths at a certain high density set point.

## CONCLUSION

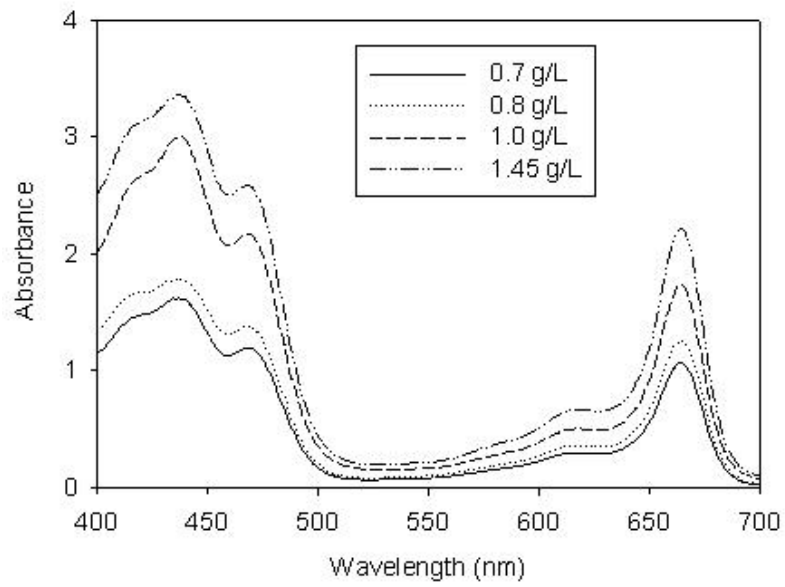
At high culture densities of *Scenedesmus bijuga* the weakly absorbed green light ( $\lambda$ 530 nm) penetrated deeper into the samples than the strongly absorbed red ( $\lambda$ 655 nm) and blue ( $\lambda$ 470 nm) lights. As *S. bijuga* culture density increased, the weakly absorbed green wavelength became photosynthetically more efficient than the strongly absorbed red wavelength. Green light was the most effective to induce oxygen evolution and biomass production in high density cultures of *S. bijuga*.

## REFERENCE

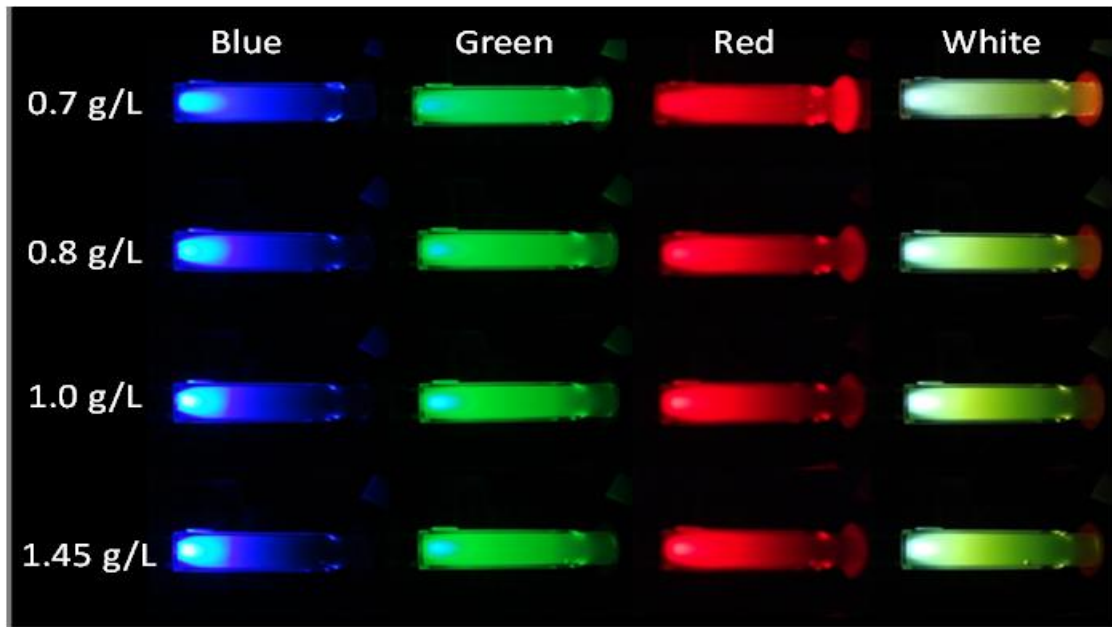
- Brodersen, C.R., and T.C. Vogelmann. 2010. Do changes in light direction affect absorption profiles in leaves? *Funct. Plant. Biol.* 37:403-412.
- Chinnasamy, S., A. Bhatnagar, R.W. Hunt, and K.C. Das. 2010. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. *Bioresource Technol* 101:3097-3105
- Choi, S.L., I.S. Suh, and C.G. Lee. 2003. Lumostatic operation of bubble column photobioreactors for *Haematococcus pluvialis* cultures using a specific light uptake rate as a control parameter. *Enzyme Microb Tech* 33:403-409.
- Christenson, L., and R. Sims. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnol. Adv.* 29:686-702.
- Fu, W.Q., O. Gudmundsson, A.M. Feist, G. Herjolfsson, S. Brynjolfsson, and B.O. Palsson. 2012. Maximizing biomass productivity and cell density of *Chlorella vulgaris* by using light-emitting diode-based photobioreactor. *J Biotechnol* 161:242-249.
- Gordon, J.M., and J.E.W. Polle. 2007b. Ultrahigh bioproductivity from algae. *Appl Microbiol Biot* 76:969-975.
- Gunther, A., T. Jakob, R. Goss, S. Konig, D. Spindler, N. Rabiger, S. John, S. Heithoff, M. Fresewinkel, C. Posten, and C. Wilhelm. 2012. Methane production from glycolate excreting algae as a new concept in the production of biofuels. *Bioresource Technol* 121:454-457.
- Hu, Q., N. Kurano, M. Kawachi, I. Iwasaki, and S. Miyachi. 1998. Ultrahigh-cell-density culture of a marine green alga *Chlorococcum littorale* in a flat-plate photobioreactor. *Appl Microbiol Biot* 49:655-662.
- Hulatt, C.J., and D.N. Thomas. 2011. Productivity, carbon dioxide uptake and net energy return of microalgal bubble column photobioreactors. *Bioresource Technol* 102:5775-5787.
- Jeon, Y.C., C.W. Cho, and Y.S. Yun. 2005. Measurement of microalgal photosynthetic activity depending on light intensity and quality. *Biochem Eng J* 27:127-131.
- Kim, N.-J., and C.-G. Lee. 2001. A Theoretical Consideration on Oxygen Production Rate in Microalgal Cultures. *Biotechnol. Bioprocess Eng.* 6:352-358.
- Lakaniemi, A.M., C.J. Hulatt, D.N. Thomas, O.H. Tuovinen, and J.A. Puhakka. 2011. Biogenic hydrogen and methane production from *Chlorella vulgaris* and *Dunaliella tertiolecta* biomass. *Biotechnol. Biofuels* 4:34.

- Lee, C.G., and B.O. Palsson. 1995. Light-emitting diode-based algal photobioreactor with external gas-exchange. *J Ferment Bioeng* 79:257-263.
- Lee, H.S., M.W. Seo, Z.H. Kim, and C.G. Lee. 2006. Determining the best specific light uptake rates for the lumostatic cultures in bubble column photobioreactors. *Enzyme Microb Tech* 39:447-452
- Mattos, E.R., M. Singh, M.L. Cabrera, and K.C. Das. 2012. Effects of Inoculum Physiological Stage on the Growth Characteristics of *Chlorella sorokiniana* Cultivated Under Different CO<sub>2</sub> Concentrations. *Appl Biochem Biotech* 168:519-530.
- Morel, A., and A. Bricaud. 1981. Theoretical results concerning light-absorption in a discrete medium, and application to specific absorption of phytoplankton. *Deep-Sea Research Part a-Oceanographic Research Papers* 28:1375-1393.
- Oswald, W.J., H.B. Gotaas, H.F. Ludwig, and V. Lynch. 1953. Algae symbiosis in oxidation ponds. 3. Photosynthetic oxygenation. *Sewage Ind Wastes* 25:692-705.
- Suh, I.S., and S.B. Lee. 2003. A light distribution model for an internally radiating photobioreactor. *Biotechnol Bioeng* 82:180-189.
- Sun, J.D., J.N. Nishio, and T.C. Vogelmann. 1998. Green light drives CO<sub>2</sub> fixation deep within leaves. *Plant Cell Physiol* 39:1020-1026.
- Terashima, I., T. Fujita, T. Inoue, W.S. Chow, and R. Oguchi. 2009. Green Light Drives Leaf Photosynthesis More Efficiently than Red Light in Strong White Light: Revisiting the Enigmatic Question of Why Leaves are Green. *Plant Cell Physiol* 50:684-697.
- Wijffels, R.H., and M.J. Barbosa. 2010. An Outlook on Microalgal Biofuels. *Science* 329:796-799.
- Yun, Y.S., and J.M. Park. 2001. Attenuation of monochromatic and polychromatic lights in *Chlorella vulgaris* suspensions. *Appl Microbiol Biot* 55:765-770.

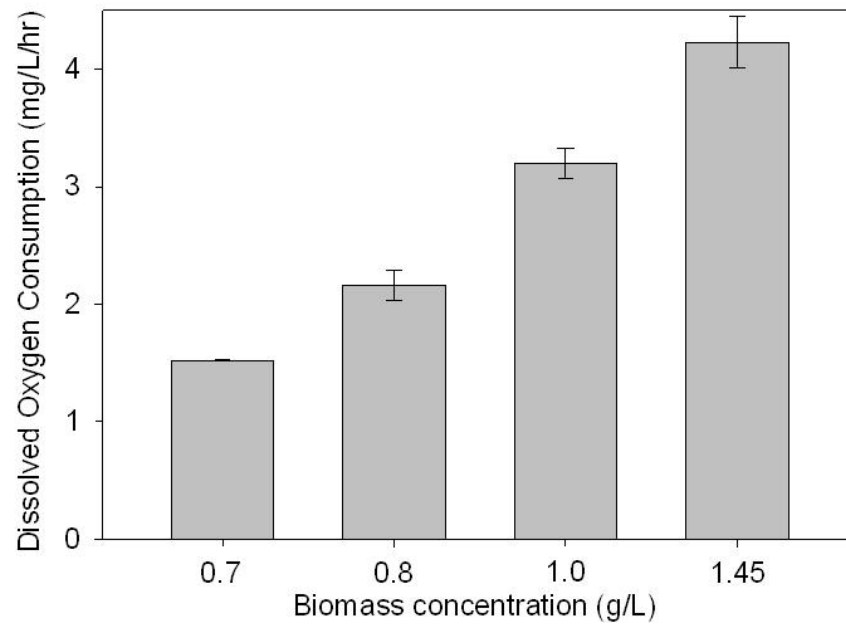
**Figure 5.1:** Light absorbance of *Scenedesmus bijuga* cultures at different biomass concentrations.



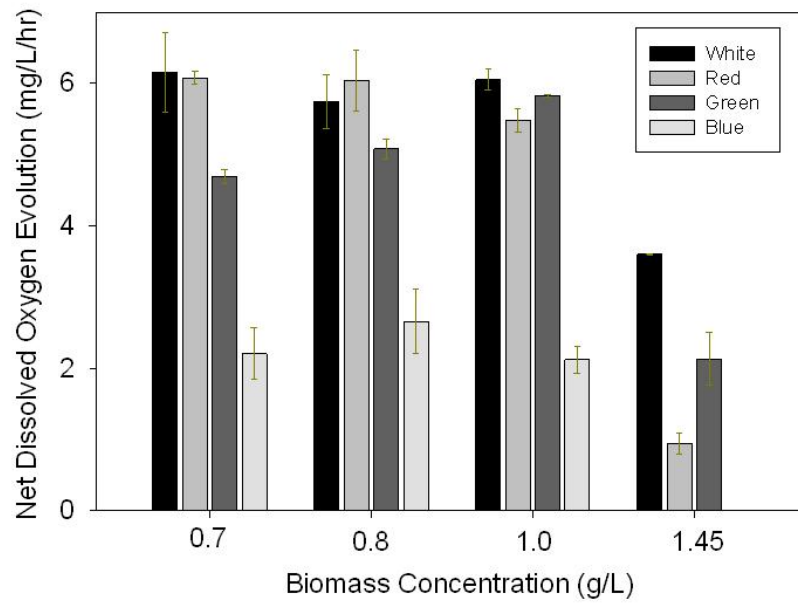
**Figure 5.2:** Monochromatic light penetration at *Scenedesmus bijuga* samples at different biomass concentrations illuminated by four LED light sources (white-3000K; red- $\lambda$ 655nm; green- $\lambda$ 530nm; blue- $\lambda$ 470nm) at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.



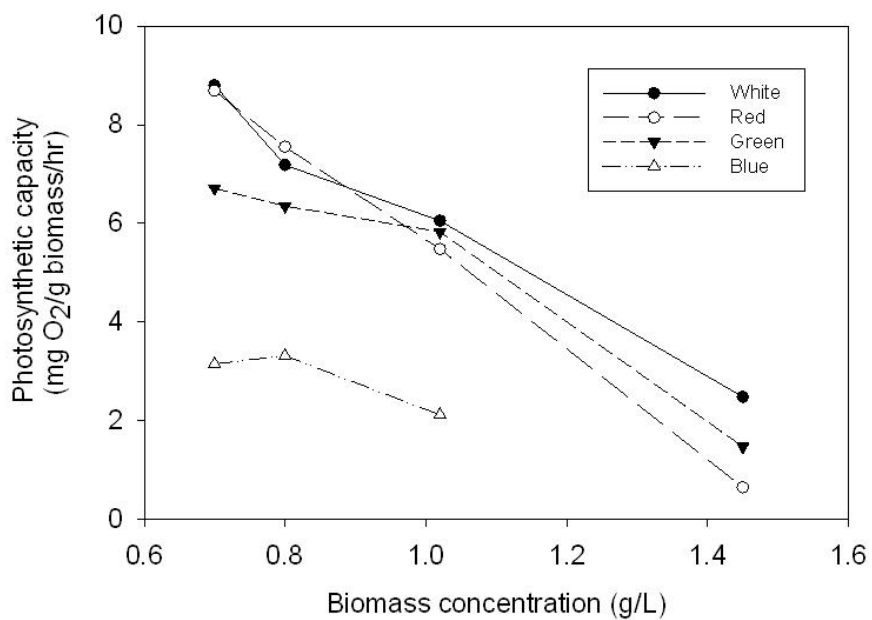
**Figure 5.3:** Dissolved oxygen consumption by *Scenedesmus bijuga* cultures at different biomass concentrations.



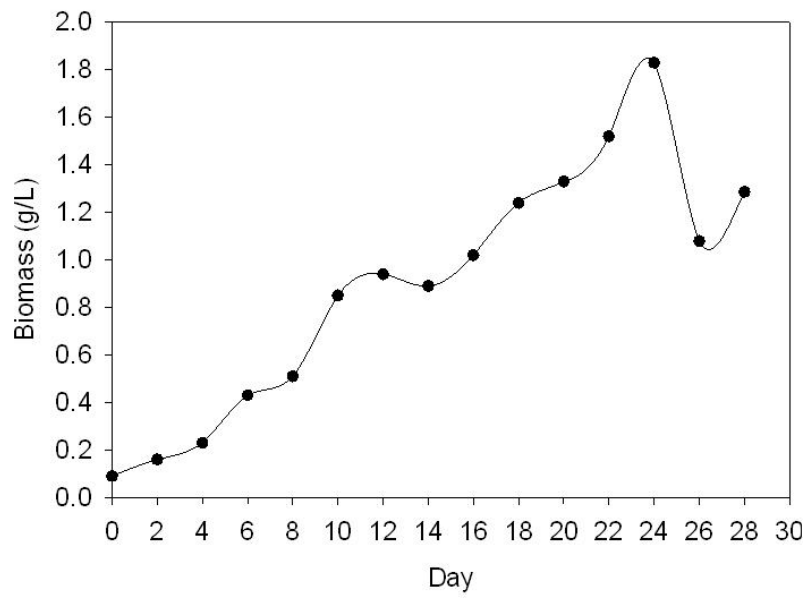
**Figure 5.4:** Net dissolved oxygen evolution by *Scenedesmus bijuga* cultures at different biomass concentrations induced by four LED light sources (white-3000K; red- $\lambda$ 655nm; green- $\lambda$ 530nm; blue- $\lambda$ 470nm) at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.



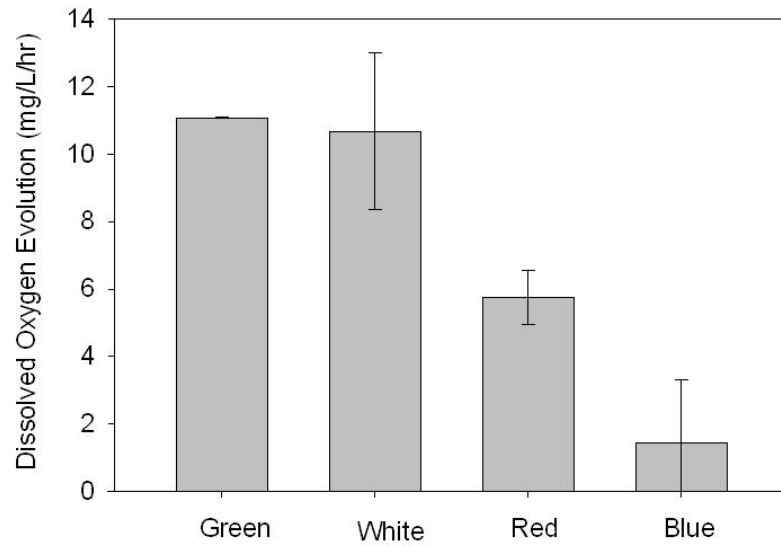
**Figure 5.5:** Photosynthetic capacity of *Scenedesmus bijuga* cultures at different biomass concentrations induced by four LED light sources (white-3000K; red- $\lambda$ 655nm; green- $\lambda$ 530nm; blue- $\lambda$ 470nm) at  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.



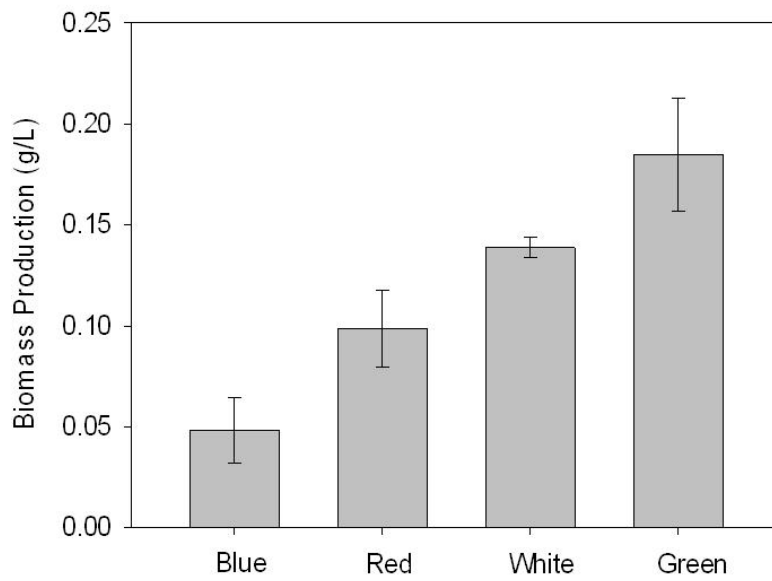
**Figure 5.6:** *Scenedesmus bijuga* biomass concentration during a 28d cultivation period.



**Figure 5.7:** Net dissolved oxygen evolution by *Scenedesmus bijuga* cultures on day 0 at biomass concentration of 2.19 g/L induced by four LED light sources (white-3000K; red- $\lambda$ 655nm; green- $\lambda$ 530nm; blue- $\lambda$ 470nm) at  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity.



**Figure 5.8:** *Scenedesmus bijuga* biomass increase induced by four LED light sources (white-3000K; red- $\lambda$ 655nm; green- $\lambda$ 530nm; blue- $\lambda$ 470nm) during a 6 days cultivation period.



## GENERAL OVERVIEW

The necessity to optimize microalgae production methods and increase microalgae final biomass density is readily evident given that the high cost of downstream processing due the intrinsic nature of low biomass concentration is the main factor avoiding the scale up of microalgae production systems. Methods to increase inoculum density and optimize photosynthetic efficiency can reduce the time of cultivation and increase final cell density in photobioreactors.

In order to maximize microalgae biomass production and reduce its overall costs it is important to optimize inoculum conditions based on its physical and physiological characteristics. Reaching high biomass concentrations in the shortest time can save significant amount of money and resources. Bubbling microalgae culture with CO<sub>2</sub> enriched air can provide the necessary amount of carbon to sustain exponential growth. The number of cells present at the initial inoculum cultivation is an important parameters ruling the balance between alkalization induced by photosynthetic activity and acidification of the medium by the CO<sub>2</sub> supply. Low CO<sub>2</sub> supply can hold back culture development while excessive CO<sub>2</sub> supply can induce a longer lag growth phase.

For the cultivation of autotrophic microalgae species, photobioreactors have demonstrated higher biomass productivities than open ponds. However, light penetration is the main issue for biomass production in high density cultures. Several artificial illumination systems have being developed to overcome this limitation and increase biomass productivity. Past studies regarding light spectrum have demonstrated that wavelengths strongly absorbed by the microalgae main photosynthetic pigment chlorophyll *a* can drive photosynthesis more efficiently than the highly reflected green light. For this reason, among the several studies

recently published related to photosynthetic optimization, the vast majority is about the effects of light frequency and intensity. However, these weakly absorbed wavelengths can penetrate deeper into microalgae cultures at high cell density and bring benefits at the final stages of microalgae production.

The focus of this work was to optimize inoculum production and photosynthetic light use efficiency to maximize biomass production. The current research indicates that the differential biomass production of algae cultures inoculated at different physiological stages under enriched CO<sub>2</sub> ambient is mainly affected by the initial number of cells present at each inocula. This is important considering that the common practice to measure microalgae cell density is optical density which does not indicate the number of cells present at the analyzed sample.

The current research also indicates that weakly absorbed wavelengths can induce photosynthesis more efficiently than strongly absorbed wavelengths at high density microalgae cultures. Weakly absorbed green light was proved to penetrate deeper into the culture and become more photosynthetic efficient than strongly absorbed blue and red light as culture density achieve high concentration inducing higher biomass productivity at high density cultures. These findings add to the vast studies aimed to increase photosynthetic efficiency based mainly on light intensity and light frequency.

In conclusion, photosynthetic optimization is necessary to increase microalgae biomass production in photobioreactors. However, the methods to accomplish light delivery optimization to maximize biomass and provide always the perfect amount of light energy can be and should be further refined to increase cell density using the minimum energy to reduce overall costs and achieve profitability and sustainability of the industry. The inclusion of weakly absorbed

wavelengths to conventional lighting systems should be considered as an alternative to further increase final biomass density resulting in higher biomass productivity.