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 developmental characteristics. *Chlorella sorokiniana* cultures inoculated with inoculum at three different physiological stages (lag, exponential and stationary) were cultivated under three different CO_2 concentrations (0.038%, 5% or 10% CO_2 v/v). Samples inoculated with lag phase inoculum supplied with 5% CO_2 achieved the maximum biomass production whereas samples supplied with 0.038% CO_2 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 wavelengths (λ_{627nm}, λ_{617nm}, λ_{590nm}, λ_{530nm}, λ_{505nm}, λ_{470nm}) and a full spectrum neutral white LED at three different light intensities on the quantum yield of photosystem II (Φ_{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 Φ_{PSII} and light intensity was found across the whole experiment.

As *C. sorokiniana* cell density increased a decrease in Φ_{PSII} values measured under the green light was observed. 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 in the previous experiment, in a second experiment photosynthetic activity and biomass production induced by 4 different LEDs (λ_{470nm}, λ_{530nm}, λ_{655nm}, and white-3000K) were analyzed on high-density cultures of *Scenedesmus bijuga*. 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 during the same period.

INDEX WORDS: microalgae, photosynthesis, biomass, production

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ERICO ROLIM DE MATTOS

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ERICO ROLIM DE MATTOS

Major Professor: Miguel Cabrera

Committee: Keshav Das
Marc van Iersel
Mark Haidekker
Gregory Schmidt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2013

DEDICATION

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

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CHAPTER 1

INTRODUCTION

Microalgae production has 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 successfully 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 the cultivation period, systems capable to optimize environmental factors have being developed to enhance inoculum production efficiency. Inoculum production in photobioreactors (PBR) has been proven to be the best method to avoid external contamination 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 (CO_2) is commonly supplied in PBRs through an air enriched gas flow. Although several studies have been published on the effect of 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 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 PBRs 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 absorption 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 analyzed 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 physiological 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 eukaryotic species, sometimes associated in colonies or in filaments of several hundred 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_2 through photosynthesis and are responsible for up to 90% of the marine photosynthesis (Lourenço, 2006). Intrinsically, microalgae offer the greatest light flux tolerance and photosynthetic efficiency due its lower maintenance energy demand compared to higher plants (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.

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 adapt 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 is a scarce finite resource that has no substitute in food production and cannot be synthesized in laboratory. Its non-gaseous environmental cycle has resulted in no alternative means of production other than mining. One potential solution to the shortage of phosphorus resources lie in recycling it by its recovery from rural and urban wastewater thought 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 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_2 mitigation and biofuels production. Recently, there has been renewed interest in microalgal biofixation as a viable CO_2 sequestration technology (Ono and Cuello, 2006). These processes utilize industrial flue gases and urban or agricultural waste water as a source of CO_2 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 10^8/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_2 emission by human activities from the past 100 years has disturbed the natural equilibrium between sources and sinks of CO_2, accelerating global warming. On May 2013 the concentration of CO_2 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 greenhouse gas (GHG) emissions have been discussed by the industrialized and emerging countries to reduce CO_2 emissions and slow the global warming process. Annual GHG emissions from fossil fuel combustion, primarily CO_2, were estimated at 5,209 million metric tons with 3,490 million metric tons from stationary sources alone (EPA, 2011). The United States is responsible for approximately 25% of global GHG emissions.

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, do not compete with conventional agriculture for arable land or nutrients, can utilize waste and saline waters (Chinnasamy et al., 2010), can recycle carbon from CO_2-rich flue gases, and can be used to produce a variety of fuels and valuable co-products (DOE, 2010).

Photoautotrophic cultivation of microalgae in PBRs 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_2 supply is critical since

atmospheric CO_2 concentration and its low diffusion rate does not support high production rates making CO_2 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_2 for algal cultivation (DOE, 2010). Co-locating algal cultivation with power plants can be a desirable approach that can capture effluent CO_2 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_2 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_2 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_2 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 mixothrophic. Autotrophic algae utilize CO_2 as the carbon source and relay on light energy for photosynthesis, while heterotrophic algae utilize sugars derived from other biomass sources. Mixothrophic has the capability to utilize both, in hybrid systems algae is first cultivated in the sun and then switching it to a boost phase, fed in the dark with organic compounds. 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 normally refers 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_2 whereas the high concentrations of O_2 originated by the photosynthesis process can became an inhibitory condition for microalgae development. 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_2 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 being 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. In these systems, mixing and CO_2-

supply is accomplished by sparging the culture with CO_2-enriched air (Alias et al., 2004; Wang et al., 2005). Among the proposed photobioreactors, the 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 a pump or preferably with an airlift system (Ugwu et al., 2008). The transparent tubing is 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, the 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 light penetration problem resulting in high dark areas 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 photobioreactors with optimized flow regime for the efficient cultivation of microalgae and production of microalgal metabolites (Bergmann et al., 2013), three dimensional architecture bioreactors that utilize available sunlight more efficiently with a smaller footprint (reported on Algaebiodieselinfo), and polymer-based photobioreactors that are designed to float on aquatic acreage (developed by the company Algaesystems) are some of the proposed approaches 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 photo-bioreactors (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^2 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 scaled-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 peak wavelength red light from LED as a light source that allow microalgae production to a cell concentration higher than 2×10^9 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 $l0^9$ 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 are massless particles 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 in oxygenic photosynthetic organisms; 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 molecule, 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 range 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 and microalgae 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 the *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, an electron 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 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 in 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 loose 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 the 'Z'-scheme first described by Hill and Bendall (Hill and Bendall, 1960). During this process, protons released from the splitting of water plus 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 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_2 molecules to hexose in the Calvin-Benson Cycle (dark reactions) in which those intermediates react with CO_2 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 in light reactions can be accepted by other molecules rather than $NADP^+$ (e.g. O_2) 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 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 the xanthophyll cycle. At low lumen pH, the enzyme violaxanthin de-epoxidase removes two epoxides groups from the violaxanthin transforming this pigment into a zeaxanthin, or it removes a single epoxide to make antheraxanthin. (Gilmore et al., 1995). The interaction of zeaxanthin with chlorophyll pigments causes a conformational change in the light harvesting complexes 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. The primary acceptor of electrons from P700 is rapidly re-oxidized, reducing fluorescence emissions at PSI (Falkowski and Kiefer, 1985). Indeed, purified PSI has a very low fluorescence emission even without electron acceptors. 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 of 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 milliseconds. Finally, carbon metabolism within the chloroplast which occurs in second-scales, can also take up to minutes due enzyme activation period (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 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 optimize light delivery, 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 range of the action spectrum of plants and algae due its high reflection and transmission and low absorption by the main photosynthetic pigment present in 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 of strongly absorbed wavelengths in 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). Light intensity of any wavelength decreases exponentially as it moves deeper into microalgae cultures. Kim and Lee (2001) have shown that red light (680nm) penetration

depth in *Chlorella kessleri* cultures decreases exponentially as cell density increases. In contrast, light intensity of weakly absorbed wavelengths that have a slower exponential decay and can penetrate deeper into high density cultures inducing 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 the "**detour effect**" and it could potentially increase the absorbance of weakly absorbed wavelengths in microalgae cultures.

In intact cells present in microalgae culture, photosynthetic pigments are concentrated in 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 effec**t' (also known as the 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). The 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 which contain the green microalgae main pigments chlorophyll *a* and *b*, 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.

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CHAPTER 3

EFFECTS OF INOCULUM PHYSIOLOGICAL STAGE ON THE GROWTH CHARACTERISTICS OF CHLORELLA SOROKINIANA CULTIVATED UNDER DIFFERENT CO_2 CENCENTRATIONS [1]

[1] Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2012. *Applied Biochemistry and Biotechnology* 168(3):519-530
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Abstract

In order to maximize microalgae biomass production and reduce its overall cost, 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_2 concentrations (0.038%, 5% or 10% CO_2 v/v) and growth pattern and biomass production were observed. Samples inoculated with lag phase inoculum supplied with 5% CO_2 achieved maximum biomass production whereas samples supplied with 0.038% CO_2 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, CO2

Introduction

Increased CO_2 emission by human activities over the past 100 years has disturbed the natural equilibrium between sources and sinks of CO_2, accelerating global warming. Restrictive regulations regarding GHG emissions have been discussed by the industrialized and emerging countries to reduce CO_2 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_2 equivalent in 2009. This estimate includes CO_2 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_2, were estimated at 5,209 million metric tons with 3,490 million metric tons from stationary sources alone (EPA, 2011). The majority of CO_2 emission from stationary sources in the US comes from fossil fuel-based power plants, which have flue gas CO_2 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, do 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_2-rich flue gases, and can be used to produce a variety of fuels and valuable co-products (DOE, 2010).

Photoautotrophic cultivation of microalgae in photobioreactors is recognized as the best way to reach high production rate 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_2 supply is critical since the low atmospheric CO_2 concentration and its low diffusion rate does not support high production rates making CO_2 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_2 for algal cultivation (DOE, 2010). Co-locating algal cultivation with power plants can be a desirable approach that can capture effluent CO_2 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_2 into biomass, studies evaluating the 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_2 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_2 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_2 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_2 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_2 concentrations. Therefore, in the present study, we examined the effects of different CO_2

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 ($NaNO_3$, 17.6 mM; K_2HPO_4, 0.22 mM; $MgSO_4 \cdot 7H_2O$, 0.03 mM; $CaCl_2 \cdot 2H_2O$, 0.2 mM; citric acid$\cdot H_2O$, 0.03 mM; ammonium ferric citrate, 0.02 mM; $Na_2EDTA \cdot 2H_2O$, 0.002 mM; Na_2CO_3, 0.18 mM; H_3BO_3, 46 µM; $MnCl_2 \cdot 4H_2O$, 9 µM; $ZnSO_4 \cdot 7H_2O$, 0.77 µM; $Na_2MoO_4 \cdot 2H_2O$, 1.6 µM; $CuSO_4 \cdot 5H_2O$, 0.3 µM; $Co(NO_3)_2 \cdot 6H_2O$, 0.17 µM). The pH of culture medium was adjusted to 7.0±0.2 with 0.1 M hydrochloric acid before inoculation and the algae were maintained in a temperature-controlled, illuminated growth chamber at 25±1^{o}C and 250±10 µmol m^{-2} s^{-1} 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 in the 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^{-2} s^{-1} 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_2 supply

Three concentrations of CO_2 gas supply, namely, 0.038% (atmospheric), 5% and 10% were tested in this experiment. For the 0.038% CO_2 concentration, an air pump was used to bubble atmospheric air into culture flasks. Two premixed gas cylinders with 5% and 10% CO_2 in air (Airgas, USA) were used to supply the enriched CO_2 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^{o}C and 250±10 µmol m^{-2} s^{-1} light intensity provided by cool-white, fluorescent (6500K) T-8 bulbs. Light intensity supply was measured using a Li-Cor (Li-1400) Quantum sensor connected to a datalogger. Flasks were placed on individual triangular pieces of wood to provide an inclined position with CO_2 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 developmental 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_2 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 (µg/mL) showed the same pattern as biomass concentration with a pronounced increase from day 18 to 21 (Fig 3.2). All samples showed a higher 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_2 limitation. Its low diffusion rate and its low concentration in atmospheric air were not sufficient to support higher biomass production. We stoichiometrically 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_2 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_2 concentrations. The experimental design combined 3 inoculum phases (lag, exponential and stationary) and 3 gas supply CO_2 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_2 concentration gas supply (0.038%) showed similar linear growth patterns regardless of the inoculum phase used. Under this condition, CO_2 availability may have been the major limiting factor for culture growth. Samples cultivated with

5 and 10% CO_2 gas supply showed similar growth patterns but different final biomass concentrations depending on the inoculum phase used (Table 3.1). Samples inoculated with lag phase inoculum receiving 5% CO_2 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_2 concentrations. Among samples inoculated with the same inoculum phase the ones supplied with 5% CO_2 achieved higher biomass concentrations (Table 3.1).

Because algae contain 50% carbon (Becker, 1994), to produce 1 g of biomass 1.8 g of CO_2 is necessary. Dry biomass results suggest that cultures inoculated with lag phase inoculum under 5% CO_2 gas supply fixed 1.5 and 4.8 times more CO_2 than cultures under 10% and 0.038% CO_2 gas supply, respectively.

The highest biomass production achieved by the samples under 5% CO_2 indicates that this concentration provides enough CO_2 for microalgae growth. For the set up system here used, the 10% CO_2 gas supply has more CO_2 than the necessary amount to provide optimal growth and as a result a significant portion of CO_2 was probably wasted.

Biomass production and chlorophyll *a* content (Fig 3.3) showed a strong dependence on CO_2 concentration gas supply. All samples supplied with 0.038% CO_2 gas supply showed a linear biomass and chlorophyll *a* production independent of the inoculum used. Samples supplied with 5% CO_2 had the shortest lag phase reaching higher growth rates 3 days earlier (at day 3) than samples supplied with 10% CO_2 (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_2 supply) and the experimental run (CO_2 enriched conditions). At 10% CO_2 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_2 concentrations ranging from 10 to 70% it was found that the lag period increased with higher CO_2 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_2 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_2 concentrations (0.038% and 6%) reached higher biomass concentration (close to 1 g/L) compared to samples grown under high CO_2 concentrations (12% and 18%) which reached maximum biomass concentration close to 0.75 g/L (de Morais and Costa, 2007a).

Effects of CO_2 concentration on medium pH

Bubbling enriched CO_2 gas into the sample results first in CO_2 in the gaseous phase ($CO_{2(gas)}$) being transferred to the liquid phase ($CO_{2(aq)}$), then reacting with water in a hydration reaction generating carbonic acid (H_2CO_3), followed by two dissociation reactions forming bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) (Stumm, 1996). Whereas CO_2 supply reduces pH of the culture medium, photosynthetic activity raises medium pH since CO_2 (acid) removal causes the bicarbonate portion in the water to be proportionately high. These reactions reach equilibrium according to the CO_2 supply/consumption ratio and the major form of available inorganic CO_2 can be assessed 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 CO_2 concentration in the gas supply, independent of the inoculum stage used (Fig 3.5). Under 0.038% CO_2 gas supply, pH immediately increased and stabilized around 10 after 3 days. Under these conditions microalgae CO_2 consumption was higher than CO_2 supply raising the medium pH. At pH 10 no aqueous CO_2 is available and

decreasing HCO_3^- concentration coupled with increasing CO_3^{2-} concentration indicates a CO_2 depleted medium. This supports the hypothesis that at 0.038% CO_2 gas supply, CO_2 is the limiting factor for culture growth. At 5 and 10% CO_2 gas supply the rate of CO_2 supply is higher than the photosynthetic CO_2 consumption, thereby dropping medium pH. Providing 10% CO_2 gas supply caused a greater drop in medium pH, compared to 5% CO_2, which negatively affected *C. sorokiniana* growth due a longer acclimatization period represented by an extended lag phase growth. Samples supplied with 5% CO_2 took 3 days to acclimate to the new environment whereas the same effect was prolonged to 6 days on samples supplied with 10% CO_2 gas supply. After the acclimatization period, both samples stabilized their pH around 7 (5% CO_2) and 6.3 (10% 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_2 and that the optimum pH was between 5.5 to 6.0.

The small pH difference between samples supplied with 5 and 10% CO_2 (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 mass transfer low efficiency, CO_2 mass transfer from the 5% and 10% CO_2 gas supply was able to provide a non-CO_2-limiting condition.

Inoculum characteristics

Among the samples supplied with extra CO_2 (5 and 10% CO_2 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 37th day (OD 1.57) and 23rd day (OD 1.23) were diluted to the same OD as the 14th 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 x 10^6 cells mL^{-1}) was twice that of the 37-day old sample (8.2 x 10^6 cells mL^{-1}) and 1.57 times higher than 23-day old sample (10.6 x 10^6 cells mL^{-1}).

Normal microalgal growth curve is characterized by a lag phase with low number of cells multiplying at a low 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 does 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 µm to 4.0–4.5 µm as the culture matured and got denser going from 53 x 10^9 to 97 x 10^9 cells per liter (Ketchum and Redfield, 1949). The time

required for cells to complete the reproductive 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 µm length x 8 µm width) and *C. ovalis* (10 x 8 µm) had lower specific growth rates of 2 and 1.5 $\mu\ d^{-1}$ (respectively) than the smaller species *C. spaerckii a* (2 x 2 µm) and *C. spaerckii b* (2 x 2 µm) that had specific growth rates of 2.7 and 2.8 $\mu\ d^{-1}$ (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 (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 (Cerveny 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 µg 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 CO_2 gas. The 5% CO_2 gas supply provided enough CO_2 to support high-rate microalgae growth while inducing a short acclimatization phase. Cultures inoculated with lag phase inoculum under 5% 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_2 ambient.

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Tables and Figures

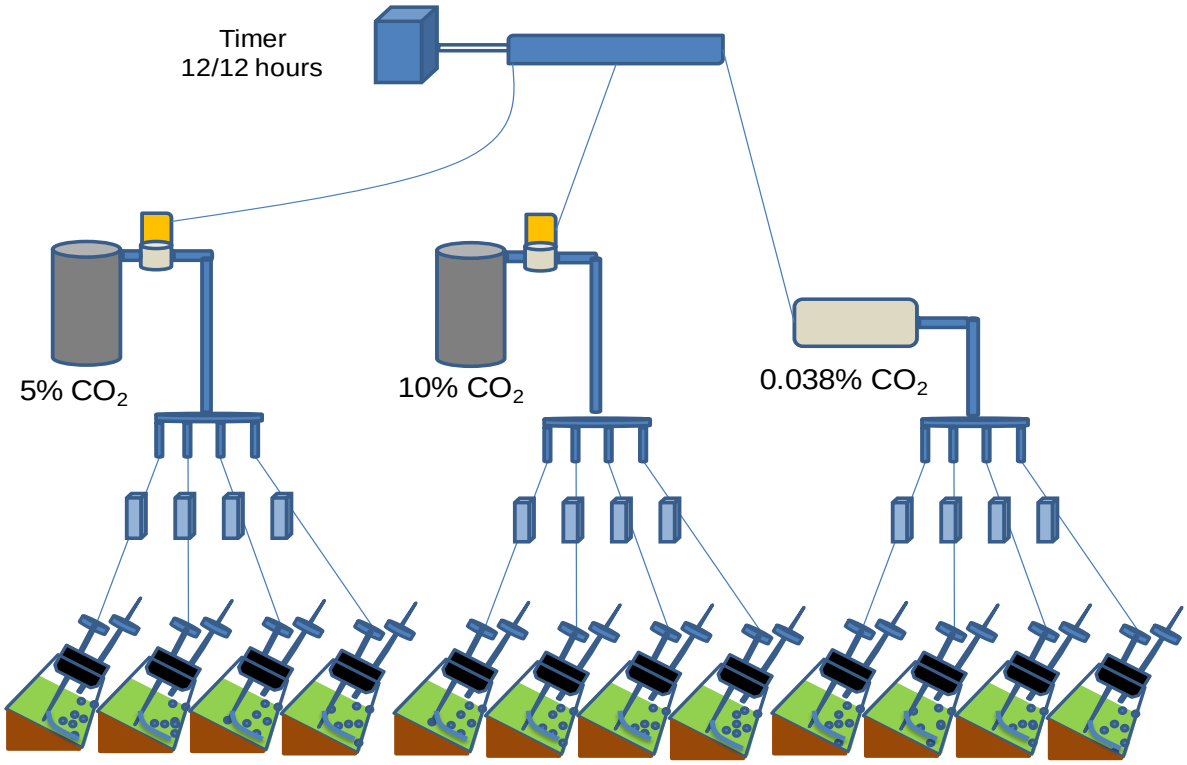

Figure 3.1: Multiple photobioreactor design. 12 microalgae samples are placed on inclined erlenmeyers to allow better mixing inside the flasks promoted by the air bubbling. 4 samples are bubbled with atmospheric air, 4 samples are bubbled with 5% enriched CO_2 (v/v) and 4 samples are bubbled with 10% enriched CO_2 (v/v). Gas flow was adjusted to 45 mL/min (1 scfh) and its supply was controlled by a solenoid valve.

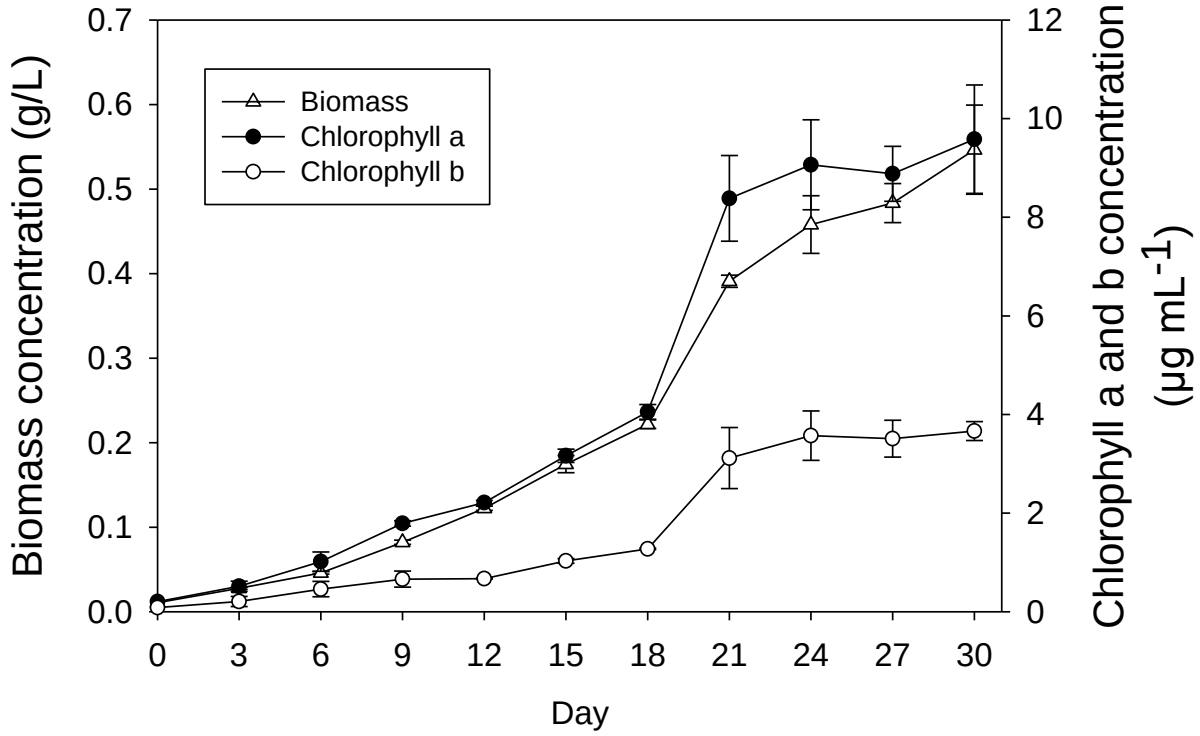

Figure 3.2: Growth curve of *Chlorella sorokiniana* and chlorophyll *a* and *b* concentration as measured in a 30-day growth period.

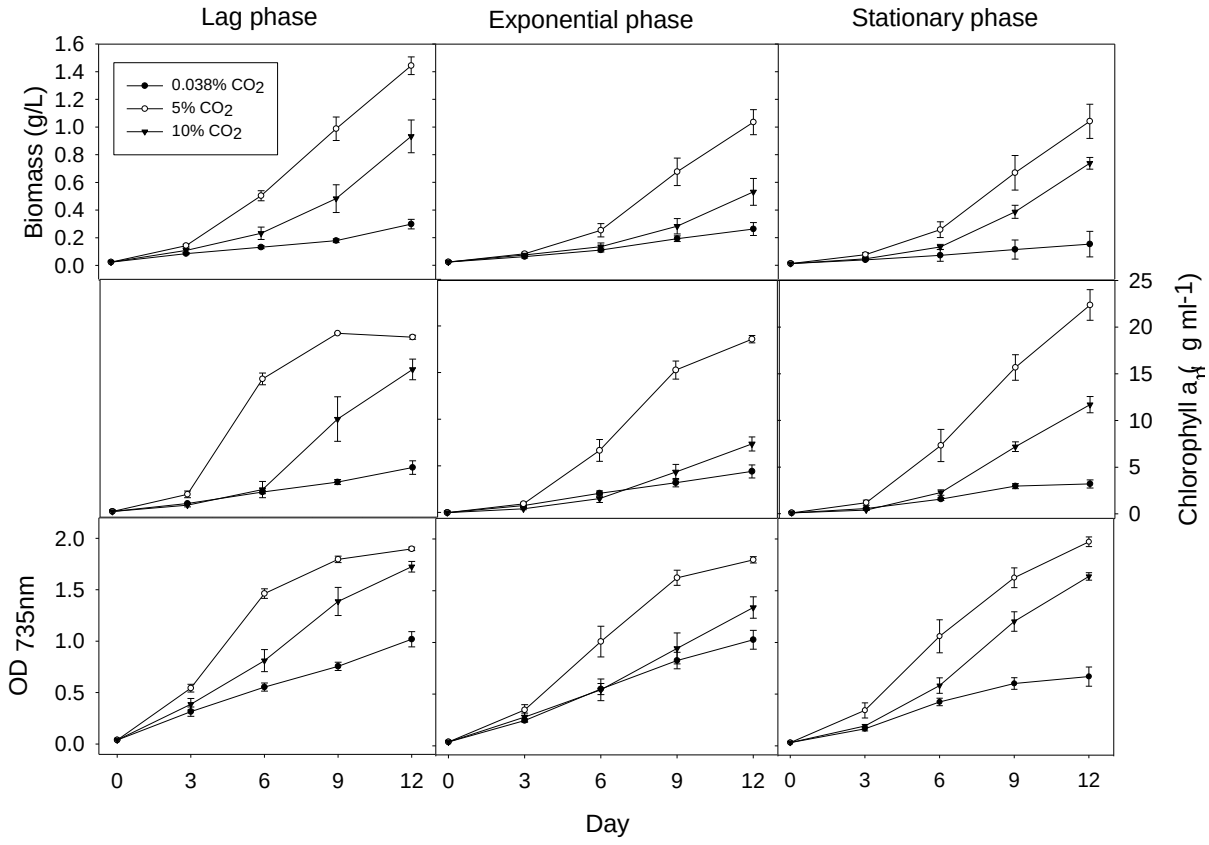

Figure 3.3: *Chlorella sorokiniana* biomass concentration, chlorophyll *a* concentration, and optical density (OD) of samples inoculated with lag, exponential and stationary inoculum phase growth under 0.038, 5 and 10% CO_2 concentration gas supply over 12 days.

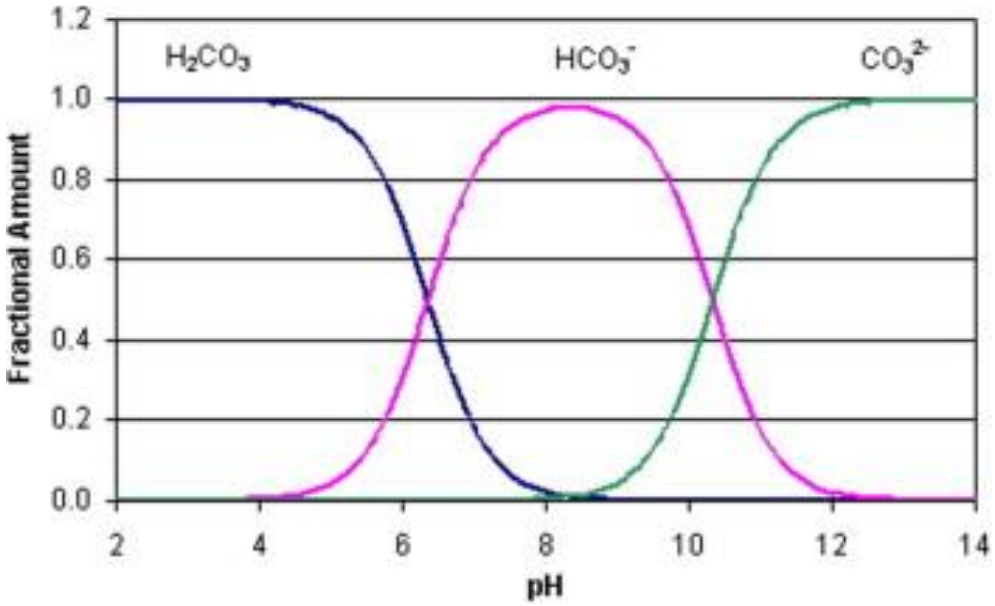

Figure 3.4: Distribution coefficients for CO_2 in water (Carbon Dioxide-Carbonic Acid Equilibrium 2004).

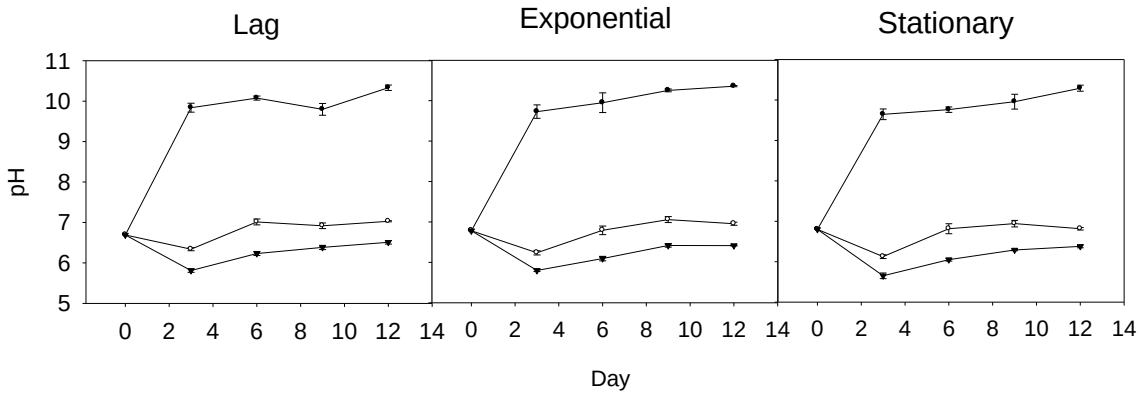

Figure 3.5: pH measurements of samples inoculated with lag, exponential and stationary inoculum phase growth under 0.038% CO_2 (closed circles), 5% CO_2 (open circles) and 10% CO_2 (closed triangles) concentration gas supply over 12 days.

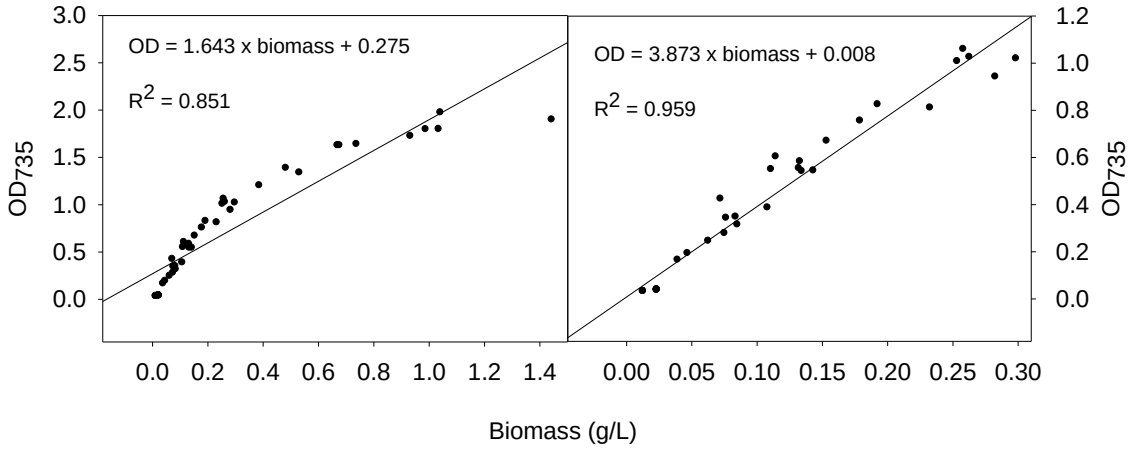

Figure 3.6: Linear correlation, regression equation and R^2 value between optical density at 735 nm (OD_{735}) and biomass concentration (g/L) measurements of *C. sorokiniana*. Left box: correlation for all measurements. Right box: correlation for measurements until OD 1.2.

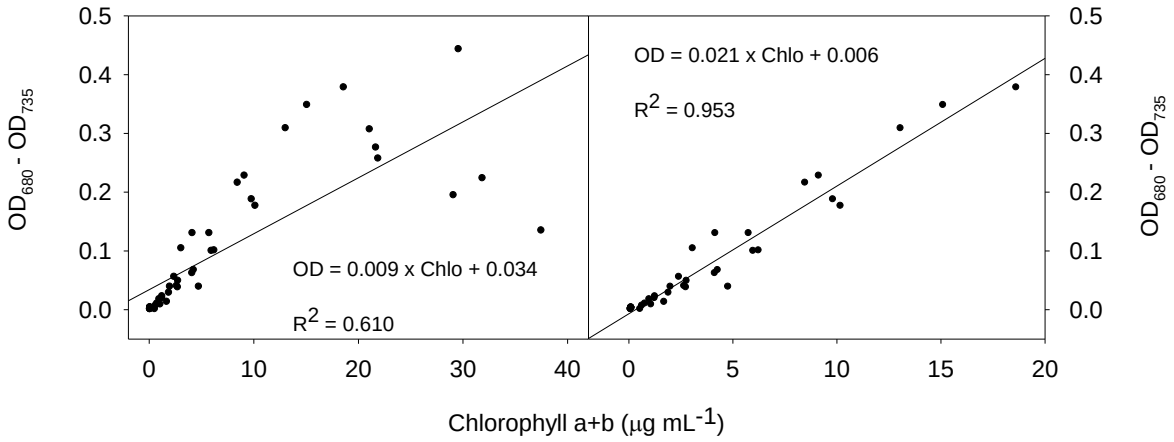

Figure 3.7: Linear correlation, regression equation and R^2 value between $\Delta_{680\text{-}735}$ and chlorophyll concentration of *C. sorokiniana.* Left box: correlation for all measurements. Right box: correlation for measurements until chlorophyll concentration 20 µg mL^{-1}.

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_2 concentrations (0.038, 5 and 10%)

CO_2 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 (µg/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 a (µg/mL)	0.1	0.11	0.09

Addendum

Although the paper "Effects of inoculum developmental stage on growth characteristics of *Chlorella sorokiniana* cultivated under different CO_2 concentrations" has already been published, we have further learned and would like to provide the following discussion for further clarifications.

CO_2 concentration *vs.* inoculum stage

In this experiment three growth trials were conducted over different time periods, one for each inoculum phase. To minimize external environmental effects on the different inocula used and ensure the same inoculum conditions under the different CO_2 concentrations the 3 growth trials conducted at different time periods were placed in the same growth chamber under identical environmental conditions (temperature, light intensity and gas supply).

Physiological growth phase identification

The samples at physiological stages initially identified as lag, exponential and stationary phase should be renamed to initial exponential phase, exponential phase and linear phase (respectively) based on the definition of the growth stages of microalgae. At exponential phase cell density increases as a function of time according to a logarithmic function. Linear phase is defined by the slowdown in cell division after the exponential phase characterized by a linear growth over time (FAO, 1996). Cells at culture-period initially identified as lag phase were actually multiplying at exponential rate. The higher increase in biomass density reported at culture-period identified as exponential phase compared to lag phase was a result of a higher number of cells multiplying at an exponential rate. Cells at the culture-period initially identified as stationary phase were still growing and accumulating biomass at a linear rate.

pH effect on *Chlorella sorokiniana* growth

At low CO_2 concentration environment carbon concentrating mechanisms (CCM) which are present in *Chlorella* species, actively increase the intracellular CO_2 concentration through the expression of carbonic anhydrase (CA) enzyme. The activity of CA causes alkalinization of environment outside the cell (Miller and Colman, 1980) as it transports hydroxide ions outside the cell in association with the capture of H^+ ions for the interiors of the thylakoid membranes (Jacob-Lopes et al, 2008). Medium alkalinization can lead to precipitation of some salts making part of the nutrients not accessible to the microalgae culture thus contributing for the low biomass growth reported at samples cultivated under low CO2 concentration provided by atmospheric air supply. Kumar and Das (2012) found similar rise in pH on *C. sorokiniana* cultures cultivated under atmospheric air supply with subsequent lower biomass production compared to cultures aerated with enriched CO_2 concentrations. However, Chlorella species can grow under high pH values (Khalil and Asker, 2010) and the main growth limiting factor was attributed to carbon availability. *C. sorkiniana* cultures cultivated at higher at CO2 concentrations (5 to 15%) have a maximum specific growth rate 3 times higher than cultures cultivated at atmospheric CO_2 concentration (0.04%) (Morita et al 2000).

Proxy measurements

In this section the coefficient of determination (R^2) value is used to indicate the degree of linear correlation between the two factors analyzed.

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CHAPTER 4

CHANGES IN CHLOROPHYL FLUORESCENCE PARAMETERS IN DIFFERENT CHLORELLA SOROKINIANA CELL DENSITIES

[1] Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2013 – To be submitted to *Applied Biochemistry and Biotechnology*.

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 for 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 LEDs with peak wavelengths at λ_{627nm}, λ_{617nm}, λ_{590nm}, λ_{530nm}, λ_{505nm}, λ_{470nm} and a full spectrum neutral white LED at three different light intensities (100, 250 and 500 µmol m^{-2} s^{-1}) on the photosynthetic parameters quantum yield of photosystem II (Φ_{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 Φ_{PSII} and light intensity was found across the whole experiment and Φ_{PSII} values obtained on samples illuminated by green light decreased as *C. sorokiniana* cell density increased. NPQ values 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 downstream costs related to harvesting and dewatering of low density cultures.

Photobioreactors (PBR) are the best cultivation method to achieve high density biomass production. There are several PBR 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 increased light absorption (Suh and Lee, 2003).

The light spectrum range capable to induce oxygenic photosynthesis is called Photosynthetic Active Radiation (PAR) and ranges from 400nm to 700nm. When incident PAR light reaches a photosynthetic organism it can be absorbed, reflected, or transmitted. The spectrum and quantity of light absorption depends on the photosynthetic pigment composition of each photosynthetic organism (Govindjee, 1969). The main pigments found in terrestrial plants, microalgae and cyanobacteria are chlorophyll *a* and *b* and phycobilins with maximum light absorption peaks in the blue, green and red spectrum of light (Govindjee, 1969). Carotenoids with light absorption peaks in the blue and yellow spectrum of light are also essential structural entities present in the reaction centers and light-harvesting complexes of many photosynthetic organisms

(Plumley and Schmidt, 1987). 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). Monitoring the relaxation time of chlorophyll *a* fluorescence using a chlorophyll fluorometer it 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 antennae complex pigments 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^+ released from the splitting of water plus H^+ 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_2 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 $NADP^+$ (e.g. O_2) 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 one or two epoxide groups from the violaxanthin transforming this pigment into an antheraxanthin or zeaxanthin (Gilmore et al., 1995). The interaction of antheraxanthin or 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 develops and increases 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 it is possible to assess 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 flourometer 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 - Φ_{PSII}) and dissipated as heat (NPQ) are affected under six different wavebands and a full spectrum white light provided by LED's at three different light intensities.

Materials & Methods

Algal Culture

Chlorella sorokiniana (UTEX 2805) was obtained from UTEX Culture Collections and maintained in BG11 medium ($NaNO_3$, 17.6 mM; K_2HPO_4, 0.22 mM; $MgSO_4 \cdot 7H_2O$, 0.03 mM; $CaCl_2 \cdot 2H_2O$, 0.2 mM; citric acid$\cdot H_2O$, 0.03 mM; ammonium ferric citrate, 0.02 mM; $Na_2EDTA \cdot 2H_2O$, 0.002 mM; Na_2CO_3, 0.18 mM; H_3BO_3, 46 µM; $MnCl_2 \cdot 4H_2O$, 9 µM; $ZnSO_4 \cdot 7H_2O$, 0.77 µM; $Na_2MoO_4 \cdot 2H_2O$, 1.6 µM; $CuSO_4 \cdot 5H_2O$, 0.3 µM; $Co(NO_3)_2 \cdot 6H_2O$, 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^{o}C and 100±10 µmoles/m$^{2/}$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 at 25±1^{o}C and 250±10 µmoles/m$^{2/}$s. Samples from both flasks were taken daily for optical density measurements at 750 nm. The culture was grown for 30 days until reaching the highest optical density OD 1.5. Samples were collected for measurements at day 7 (OD 0.5), day 15 (OD 1.0) and day 30 (OD 1.5).

Optical System

Using aluminum heat sink, an LED panel was built containing five Luxeon® III Star LED's: red-λ_{627nm} (min $\lambda_{620.5nm}$/max λ_{645nm}), red-orange-λ_{617nm} (min $\lambda_{613.5nm}$/max $\lambda_{620.5nm}$), amber-λ_{590nm} (min $\lambda_{584.5nm}$/max λ_{597nm}), green-λ_{530nm} (min λ_{520nm}/max λ_{550nm}), cyan-λ_{505nm} (min λ_{490nm}/max λ_{520nm}), and two Luxeon® K2 Emitter LEDs: blue-λ_{470nm} (min λ_{460nm}/max λ_{490nm}) and neutral white (5000K). Each LED was used to illuminate the microalgae samples through a ¼ inch diameter fiber-optic light guide positioned 1 mm away from the surface of the 10 mm path-length cuvette. A LED condenser light cone 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 µmol m^{-2} s^{-1}

using a quantum sensor (LI-COR, Quantum Sensor LI-190, and Datalogger, LI-1400 Lincoln, NE, USA).

Analytical Methods

Light absorbance spectrum from 400-800 nm and culture optical density at 750 nm was measured using a Varian Cary 50 UV/Visible spectrophotometer (Varian, Inc, Santa Clara, CA, USA). The chlorophyll *a* fluorescence parameters, quantum yield of Photosystem II ($\mathbf{\Phi_{PSII}}$) 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 μmol m^{-2} s^{-1}). Each treatment had a fresh 3 ml of algal culture injected into the sample cuvette and Φ_{PSII} and NPQ measurements were recorded for 5 minutes. Following the process described by Maxwell and Johnson (Maxwell and Johnson, 2000) both Φ_{PSII} (Eq. 1) and NPQ (Eq.2) were calculated as:

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

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

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^o_m is the maximum fluorescence value in dark-adapted state. In order to record F^o_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 μmol m^{-2} 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 Φ_{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 Φ_{PSII} data was analyzed as a Factorial Design within each OD and NPQ was analyzed as a Factorial Design within each LED wavelength.

Results

Light absorption spectra curves

Measurements of light spectrum absorbance were taken on *C. sorokiniana* samples at OD_{750} 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 λ_{470nm} which overlaps the absorbance spectrum of carotenoids and chlorophyll showed constant higher absorbance than the wavelengths from the other LEDs used. A valley between 550 nm and 650 nm 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_{750} 1.5 (30 days) and OD_{750} 1.0 (15 days) were diluted to same OD_{750} as the youngest sample (0.5) and its light absorption profile was compared. The

older samples showed slightly less absorption within PAR spectrum than the young sample (Fig 4.1b).

Quantum Yield

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

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

Within the wavelengths, λ_{530nm} and λ_{505nm} exhibited higher Φ_{PSII} on samples with OD 0.5. As samples OD increased, the Φ_{PSII} measured at λ_{530nm} and λ_{505nm} decreased and at the highest OD (1.5) it was lower than the Φ_{PSII} measured at λ_{590nm}, λ_{617nm} and λ_{627nm}. At the red spectrum (λ_{617nm}; λ_{627nm}), Φ_{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 λ_{590nm} the change in Φ_{PSII} as OD increases from 1.0 to 1.5 is very small at the two higher light intensities (250 and 500 µmol m^{-2} s^{-1}).

Non-Photochemical Quenching

Non-photochemical quenching (NPQ) is a protective mechanism by which microalgae cells dissipate the excess of absorbed energy as heat. It is measured by the quenching of chlorophyll fluorescence after a short bright light pulse that saturate photochemical quenching thus removing its contribution from the observed quenching (Horton et al., 1994). Although NPQ does not show any behavior pattern 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 wavelengths and the control white LED under all light intensities. At λ_{470nm} an interaction between the factors: light intensity and OD, was observed. No significant changes in NPQ values were noticed as culture OD further increased from 1.0 to 1.5.

Discussion

Light absorption

Light absorbance increased across the full spectrum of light as culture cell density increased as a result of increasing samples opaqueness and higher light absorbance and light scattering by the *in vivo* cells present in the analyzed samples (Fig 4.1a). When incident light encounters intact entire microalgae cells part of the full spectrum of incident light may be absorbed, most 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 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 (λ_{470nm}) is attributed to an overlap in primary chlorophylls and carotenoid absorption peaks (Biehler et al.2010). Although the two red spectrum LEDs λ_{617nm} and λ_{627nm} 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 λ_{680nm} and λ_{660nm}, respectively and for this reason this two wavelengths are relatively weakly absorbed by the samples at the three culture densities.

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). β-carotene (not listed in table 4.3), another carotenoid pigment present in *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 β-carotene and lutein showed peak absorptions at 510nm and 495nm (Deroche and Briantai.Jm, 1974).

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 older cultures diluted to the same optical density as younger cultures does not have the same number of cells (Mattos et al., 2012), 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 per cell.

Yield

Light intensity

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

$$\Phi_{PSII}' = \Phi_P' \times \Phi_{NPQ}'$$

These measurements are taken under actinic light illumination, indicated by the apostrophe. Φ_P' estimates the efficiency of charge separation in RCII and Φ_{NPQ}' is defined as the efficiency of excitation energy transfer from antenna pigments to the RCII. Any NPQ of PSII will reduce Φ_{PSII}'. Providing algae with higher light intensities, more light is absorbed and more RCII will be reduced causing a decrease in Φ_P' value with a consequently dropping of the Φ_{PSII} value. As defined, yield is the part of light absorbed that is used to initiate electron transport process. 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 Φ_{PSII}'. Analyzing the individual parameters F'_m and F_t (used to calculate Φ_{PSII} on Eq. 1) 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 µmol m^{-2} s^{-1}, which leads to an overall Φ_{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 Φ_{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 λ_{470nm}, showed the lowest yield on samples at all OD's tested (Tab 4.1). Less than 1% of the blue light is transmitted through the chloroplast, thus in high density cultures, almost all λ_{470nm} light emitted on microalgae culture is readily absorbed and/or scattered by the cell layers at the surface of illumination on the cuvette. On the other hand, wavelengths that are less absorbed by the chloroplast are able to penetrate deeper into high density culture. Comparing monochromatic light saturation curves for photosynthesis in Chlorella, Pickett and Myers (1966) found different monochromatic light saturation points for wavelengths on blue, red and green spectrum. Because of the difference in light absorption, it was necessary to provide 5 times more light intensity at the weakly absorbed band λ_{630nm} to reach the same photosynthetic rate obtained using the strongly absorbed band at λ_{450nm} (Pickett and Myers, 1966).

Providing different wavelengths at the same light intensity will result in a differential absorption of energy that leads to different Φ_{PSII}. The higher Φ_{PSII} values found for λ_{530nm} and λ_{505nm} compared to the other wavelengths at samples with OD 0.5 indicate that a relative smaller amount of energy is absorbed by the former wavelengths. 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 Φ_{PSII}.

The wavelengths on the red spectrum λ_{590nm} and λ_{627nm} did not induce further drop on ϕ_{PSII} as culture density increased from OD 1.0 to 1.5 whereas ϕ_{PSII} of λ_{505nm} and λ_{530nm} which are not absorbed by chlorophyll pigments but are absorbed by carotenoids pigments showed a continuously Φ_{PSII} decrease as the culture density increased, switching positions with λ_{590nm} and λ_{627nm} at the highest culture density (OD1.5).

Emerson and Lewis (1943) illuminated a thick layer of *Chlorella* cells with wavelengths in the blue (λ_{450nm}), green (λ_{550nm}) and red (λ_{600nm}) spectra and found scatter 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 assess NPQ (qE) is induced by a pH decrease in the lumen as a response of cells to over exposure to light (Muller et al., 2001). The rate of thermal dissipation increases with cells exposure to increasing light intensity (Demmig-Adams, 1996). 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 were exposed to a lower flux of light leading to an overall more efficient energy absorption resulting in lower levels of NPQ. It is not clear why both quantum yield of PSII (Φ_{PSII}) and NPQ decrease with increasing OD and that this needs further investigation

Conclusion

Light intensity and the quantum yield of PSII (Φ_{PSII}) showed an inverted correlation. As culture density increases from OD1.0 to OD1.5 wavelengths absorbed by carotenoids induced extra Φ_{PSII} drop whereas the wavelengths weakly absorbed exclusively by chlorophylls (λ_{590nm}, λ_{617nm}, λ_{627nm}) were no longer capable to induce extra Φ_{PSII} drop. The continuous decrease in (Φ_{PSII}) measured under green light at increasing samples OD indicates a constant increase in light absorption as culture density increases.

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Tables and Figures

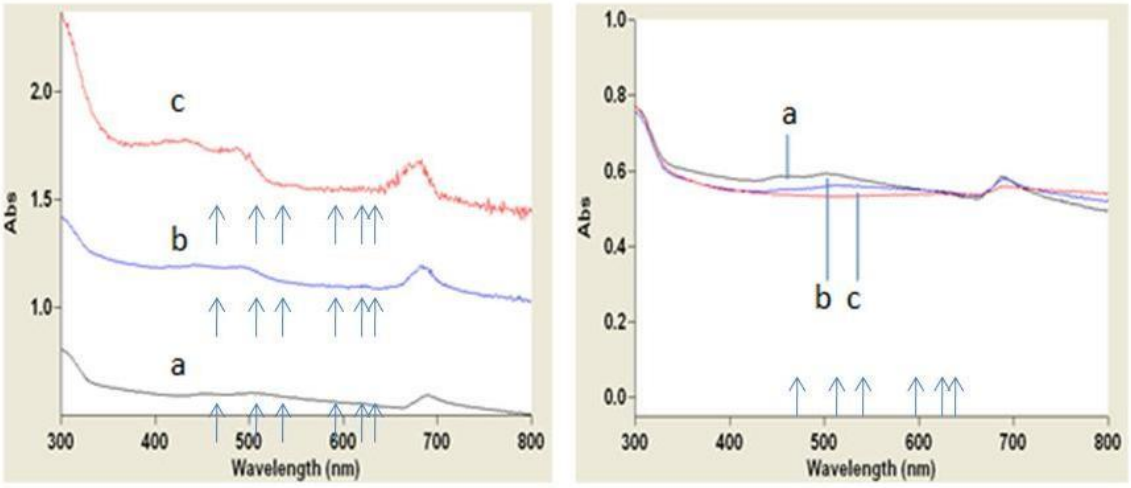

Figure 4.1: Visible light absorbance (a) of *Chlorella sorokiniana* at three OD's: 0.5 (a), 1.0 (b), and 1.5 (c). (b) of different age *Chlorella sorokiniana* at 7 days (a), 15 days (b), and 30 days (c) diluted to the same OD 0.55. Specific LEDs wavelengths (λ_{627nm}, λ_{617nm}, λ_{590nm}, λ_{530nm}, λ_{505nm}, λ_{470nm}) are indicated by arrows .

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

		λ (nm)						
		470	505	530	590	617	627	White
ϕ_{PSII}								
OD	Light Intensity (μmol m^{-2} s^{-1})							
0.5	100	0.4387	0.6388	0.5575	0.5485	0.4910	0.5357	0.5492
	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
1	100	0.3208	0.3770	0.3983	0.3540	0.2981	0.3080	0.3700
	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
1.5	100	0.1995	0.2252	0.2495	0.3055	0.3028	0.3202	0.2097
	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	wavelength	Light Intensity x wavelength
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: Results for Non-photochemical quenching (NPQ) of *C. sorokiniana* at six wavelengths (λ) 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 signifance at P<0.001.

		λ (nm)						
		470	505	530	590	617	627	White
NPQ								
OD	Light Intensity ($\mu mol\ m^{-2}\ s^{-1}$)							
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
Significant Effects								
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 their maximum absorbance wavelength (λ_{ad}). Coefficients are given for their maximal absorbance wavelength (nm) and corrected by own spectrophotometric data for acetone if the solvent in literature was different.

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

[a] taken from Matsukawa et al (2000); [b] taken from Biehler et al (2010**).**

CHAPTER 5

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

[1] Mattos, E. R., M. Singh, M. L. Cabrera, and K. C. Das. 2012. Submitted to *Algal Research* 10/05/13

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 overcome light limitation conditions and sustain photosynthetic activity and biomass production in high biomass concentration cultures inducing an extra growth. Photosynthetic activity and biomass production induced by 4 different LEDs (blue-λ470 nm, green-λ530 nm, red-λ655 nm, and white-3000 K) were analyzed on high-density cultures of *Scenedesmus bijuga.* As culture density increased the weakly absorbed green light (λ530nm) became more photosynthetically efficient than the red light, thereby inducing 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 same quantum flux of 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 in photosynthetic capacity and biomass growth, whereas low levels of light energy at later stages become a growth-limiting factor. Providing constant optimal light absorption 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 a high scattering coefficient and a low absorbance coefficient (Yun and Park, 2001) can penetrate deeper into high-density cultures and can be more 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 (2009) as the "detour effect" and it can possibly increase the absorbance of weakly absorbed wavelengths in microalgae cultures. Also, in intact cells present in microalgae culture, pigments are concentrated into chloroplasts, which decrease the opportunity for light to encounter pigments. This is referred to as the '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 limiting 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 may be 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 wavelengths (red-λ655 nm; blue-λ470 nm; green-λ530 nm) as well as a full spectrum neutral white light (3000 K) on dissolved oxygen evolution and biomass production of the microalga *Scenedesmus bijuga* at high biomass concentrations.

Materials & Methods

Algal Culture

Scenedesmus bijuga was previously isolated from waste water (Chinnasamy et al., 2010) and maintained in modified BG-11 medium ($NaNO_3$, 17.6 mM; K_2HPO_4, 0.22 mM; $MgSO_4 \cdot 7H_2O$, 0.03 mM; $CaCl_2 \cdot 2H_2O$, 0.2 mM; citric acid$\cdot H_2O$, 0.03 mM; ammonium ferric citrate, 0.02 mM; $Na_2EDTA \cdot 2H_2O$, 0.002 mM; Na_2CO_3, 0.18 mM; H_3BO_3, 46 μM; $MnCl_2 \cdot 4H_2O$, 9 μM; $ZnSO_4 \cdot 7H_2O$, 0.77 μM; $Na_2MoO_4 \cdot 2H_2O$, 1.6 μM; $CuSO_4 \cdot 5H_2O$, 0.3 μM; and $Co(NO_3)_2 \cdot 6H_2O$, 0.17 μM). The pH of culture medium was adjusted to 7.0 +/- 0.2 with 0.1M hydrochloric acid before inoculation, and the algae were maintained in a temperature-controlled, illuminated growth chamber at 25 +/- 1°C and 250 μmol m^{-2} s^{-1} light intensity provided 12 hours a day by cool-white, fluorescent (6500K) T-8 bulbs.

Chlorophyll extraction and light absorbance

For chlorophyll extraction and light absorbance measurements, 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. 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 (λ530 nm), two Luxeon® K2 Emitter LED: blue (λ470 nm) and neutral white (3000 K), and a Luxeon® Rebel Star LED: red (λ655 nm). Each LED was used to illuminate the microalgae samples through a 6.35 mm (¼ 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 to 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* in 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 μmol m^{-2} s^{-1} light intensity (T-8 bulbs), at 25°C temperature, and a 12-h light/12-h dark regime. Enriched CO_2 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^{th} to 11^{th} 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 LEDs at 300 μmol m^{-2} s^{-1}. For each different light color, dissolved oxygen evolution was measured for 15 minutes using a Clarke type dissolved oxygen micro 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 the light path was 6 cm. For all measurements, agitation was provided by a micro stir bar. Dissolved oxygen consumption was measured in control samples under 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_2 per liter per hour. 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: Luxeon® III Star green-λ_{530nm} (min λ_{520nm}/max λ_{550nm}); Luxeon® III Star blue-λ_{470nm} (min λ_{460nm}/max λ_{490nm}); Luxeon® K2 Emitter LEDs neutral white (3000K); and Luxeon® Rebel Star LED red-λ_{655nm} (min λ_{650nm}/max λ_{670nm}). 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* in 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 $\mu mol\ m^{-2}\ s^{-1}$ light intensity (T-8 bulbs), at 25 °C temperature, and a 12-h light/12-h dark regime. To ensure satisfactory conditions, samples were collected every 2 days and the concentrations of the two main nutrients necessary for algae growth, nitrogen and phosphorous, were periodically monitored and adjusted to its original concentration (176 and 10.8 mg/L, respectively) when the 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_2 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 (2012).

Biomass Production

On the 28th 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 in 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 µmol m^{-2} 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

Culture media 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 NO_3^--N. Culture media phosphorous content was measured as reactive phosphorous (orthophosphate) using the molybdovanadote method using HACH Test'N tube™ kits (HACH, USA) and results were expressed as mg/L PO_4^{3-}.

Experimental Design

The flaks containing microalgae high biomass density culture were randomly assigned to the spots on the LED table. Treatments were carried in triplicates and final biomass production

for each treatment was reported as the final average and standard deviation obtained from the 3 replicates.

Results & Discussion

Light absorption and penetration

Light absorbance by the extracted chlorophyll content of *S. bijuga* samples showed a pronounced increase at the blue (λ_{470nm}) and red (λ_{655nm}) spectrum with a minor increase at the green (λ_{530nm}) spectrum as the culture biomass concentration increased (Fig 5.1). Attenuation of red and blue light penetration in green microalgae is mostly 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 in 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 (2001) have shown that light penetration depth of red LED (λ_{680nm}) in *Chlorella kessleri* cultures decreases exponentially as cell density increases.

Suh and Lee (2003) working with *Synechococcus* sp. showed that light emitted at 600 μmol m^{-2} 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 wavelengths from a fluorescent lamp 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 λ_{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. NADH) required for biosynthesis and cellular maintenance are generated by dark respiration in a regular manner through an oxygen consumption process. As expected, total dissolved oxygen consumption increased as culture density increased. 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_2/L/hr at the lowest biomass concentration (0.7 g/L) to 4.23 mg O_2/L/hr at the highest biomass concentration (1.45 g/L). When the dissolved oxygen consumption is expressed as a function of dry biomass (mg O_2/g-dry-matter/hr) the oxygen consumption by gram of microalgae increases from cell density 0.7 to 1.0 g/L then decreases at the cell density (1.45 g/L) (Fig 5.3). At the highest cell density (1.45 g/L) the increased amount of cells are competing for the same amount of oxygen diluted on the sample, resulting in lower oxygen consumption by individual cells.

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 from 0.7 to 1.0 g/L. 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_2 evolved/g-biomass/hr for four different biomass concentrations under the four LED lights at 300 µmol m^{-2} 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 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 in photosynthetic efficiency can be explained by the fact that in low density cultures both strongly and weakly absorbed wavelengths can easily penetrate into the culture,

however strongly absorbed wavelength can induce higher photosynthetic activity as a consequence of higher 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 0.215 g/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 µmol m^{-2} 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 not only by light intensity, but also by light penetration and distribution (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 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 (Fig 5.9) 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 in 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.*

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Tables and Figures

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

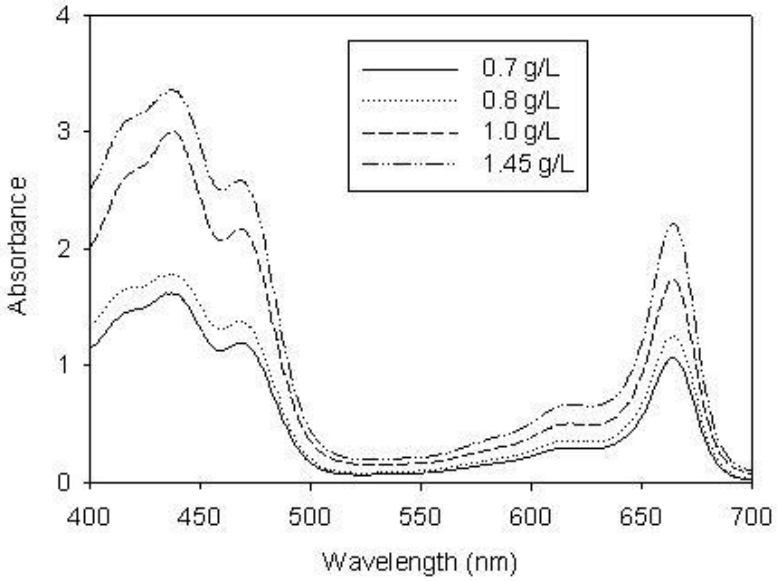


Figure 5.2: Light penetration at *Scenedesmus bijuga* samples at different biomass concentrations illuminated by four LED light sources (white-3000K; red-λ655nm; green-λ530nm; blue-λ470nm) at 300 µmol m^{-2} s^{-1} light intensity.

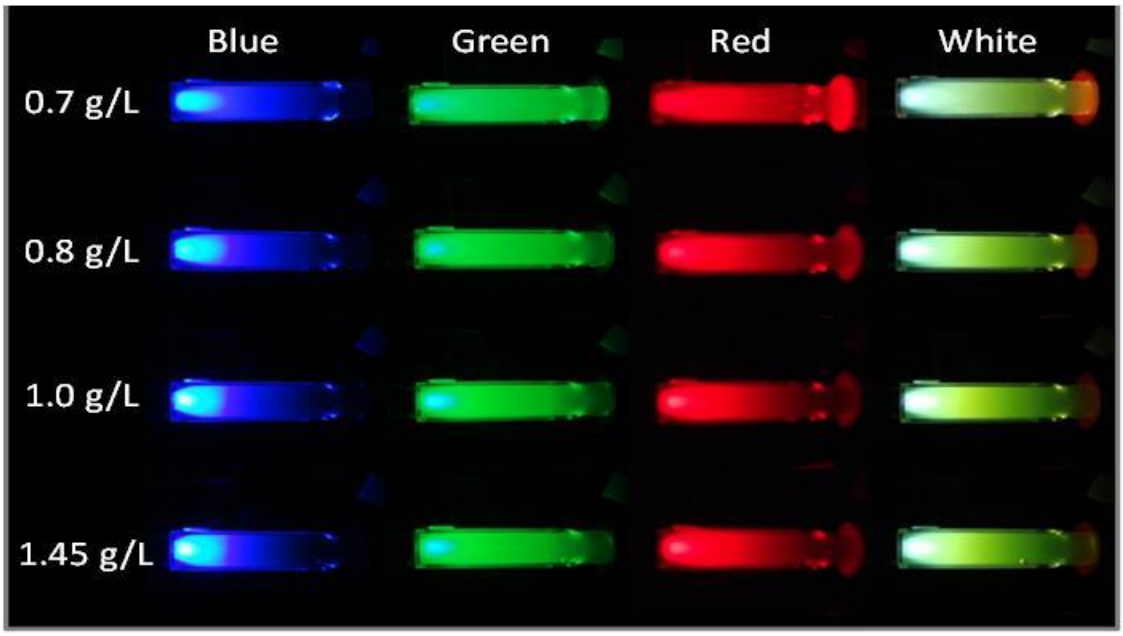


Figure 5.3: Dissolved oxygen consumption by *Scenedesmus bijuga* cultures at different biomass concentrations in darkness.

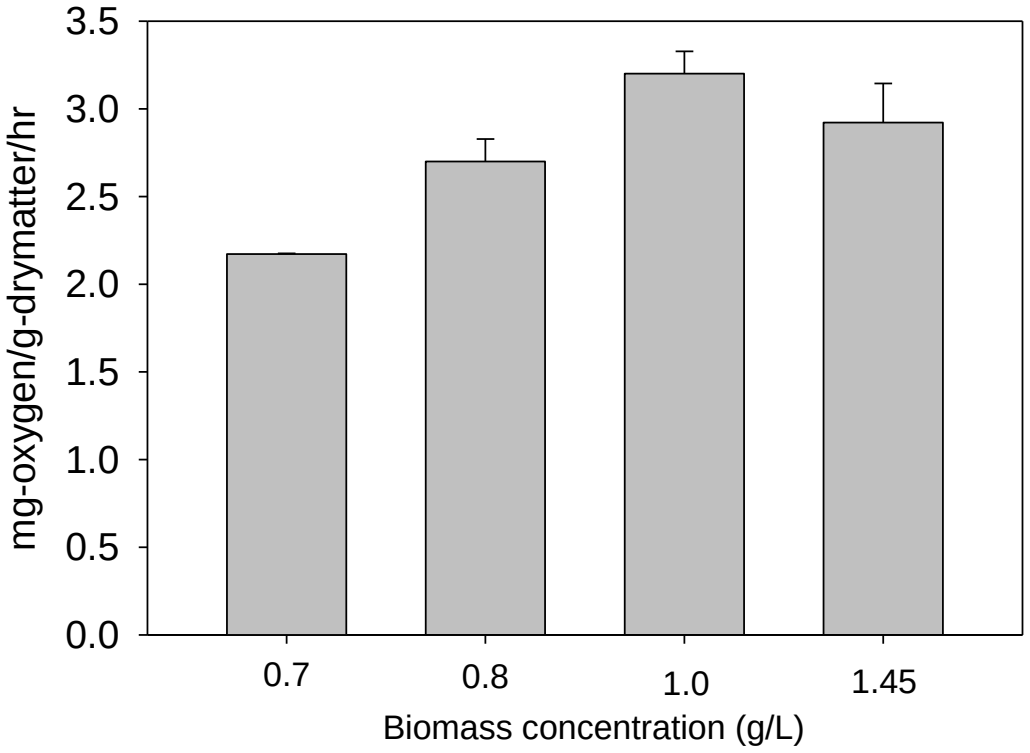


Figure 5.4: Net dissolved oxygen evolution by *Scenedesmus bijuga* cultures at different biomass concentrations induced by four LED light sources (white-3000K; red-λ655nm; green-λ530nm; blue-λ470nm) at 300 µmol m^{-2} s^{-1} light intensity.

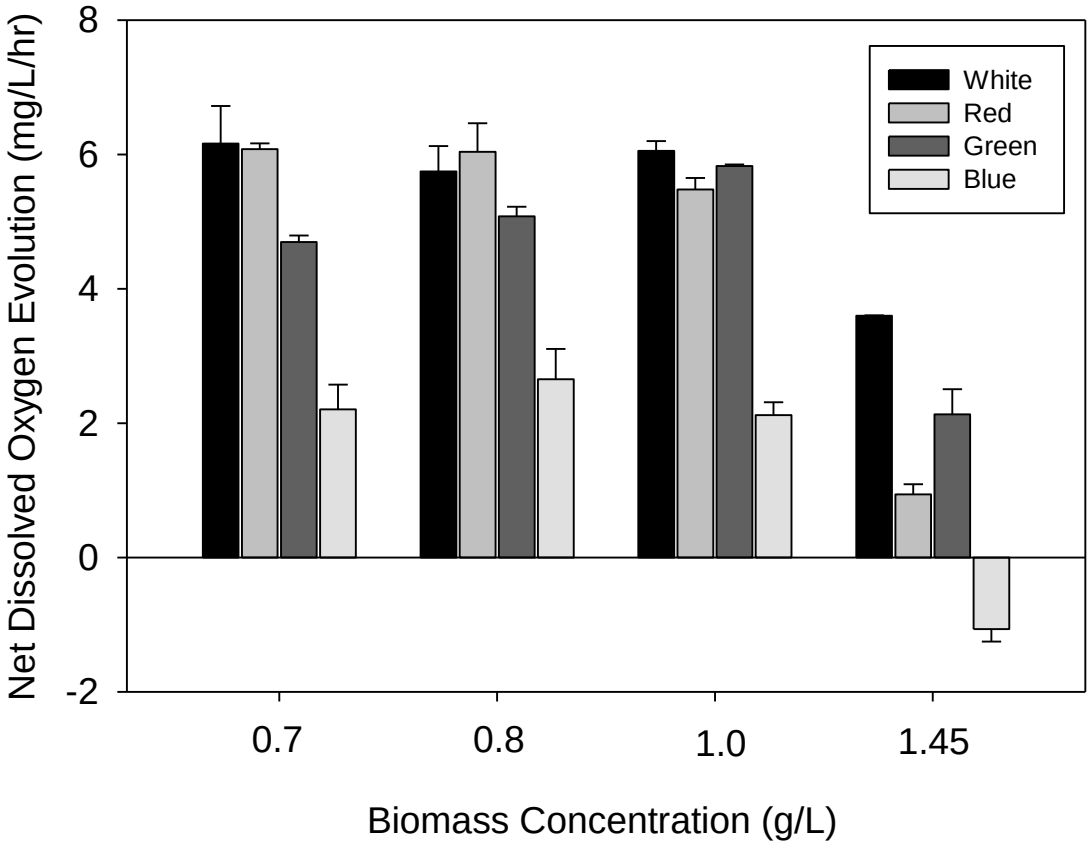


Figure 5.5: Photosynthetic capacity of *Scenedesmus bijuga* cultures at different biomass concentrations induced by four LED light sources (white-3000K; red-λ655nm; green-λ530nm; blue-λ470nm) at 300 µmol m^{-2} s^{-1} light intensity.

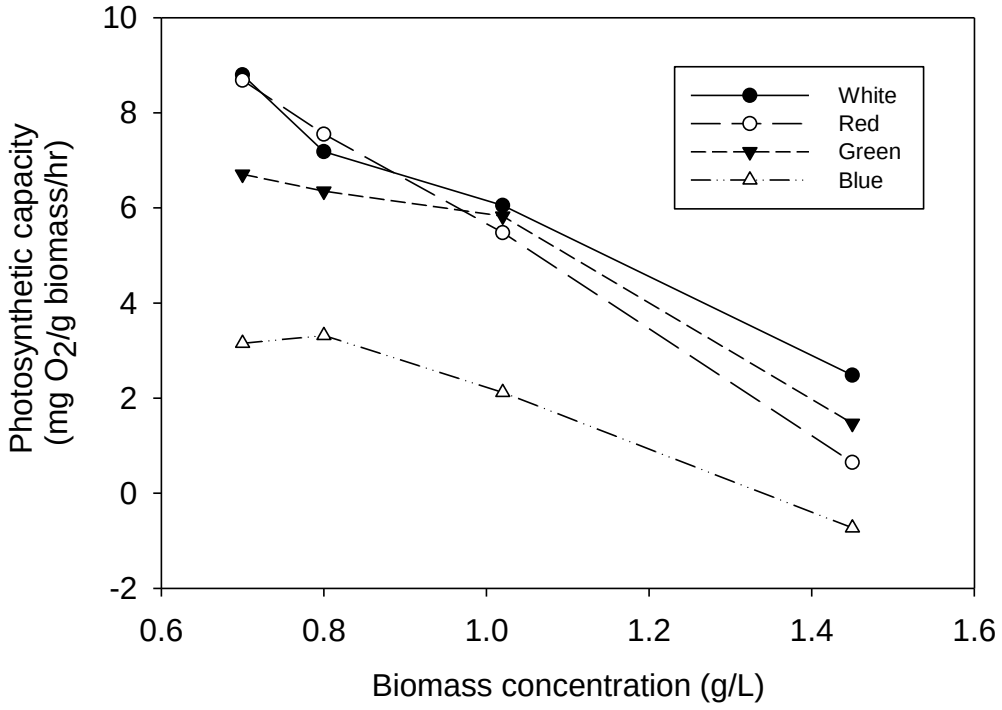


Figure 5.6: *Scenedesmus bijuga* biomass concentration during a 28d cultivation period.

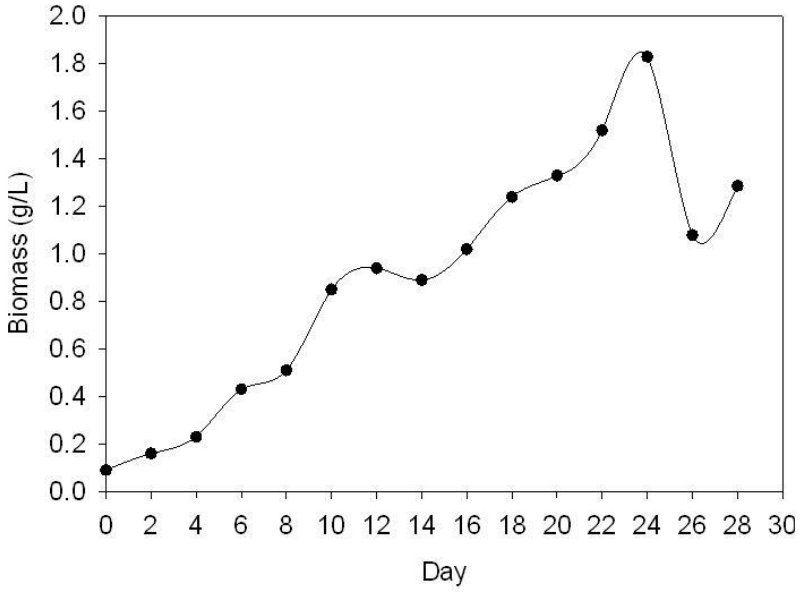


Figure 5.7: Net dissolved oxygen evolution by *Scenedesmus bijuga* cultures on day 0 at biomass concentration of 2.19 g/L induced by four LED light sources (white-3000K; red-λ655nm; green-λ530nm; blue-λ470nm) at 500 µmol m^{-2} s^{-1} light intensity.

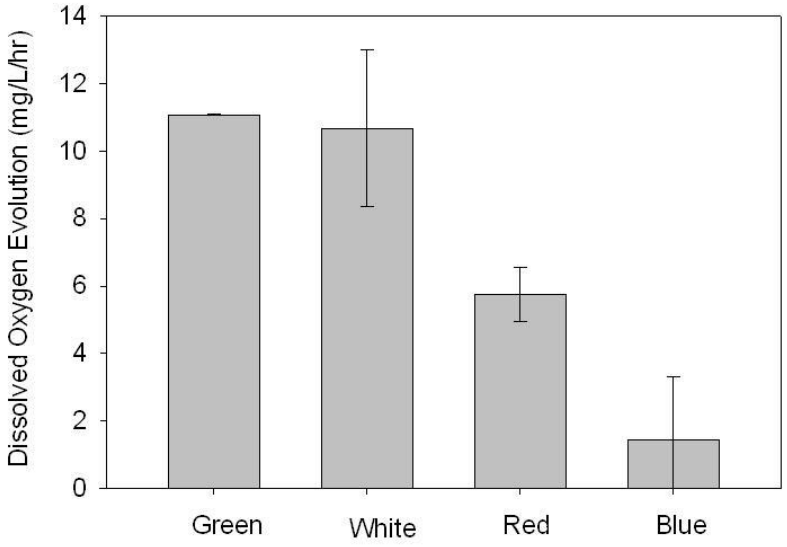


Figure 5.8: *Scenedesmus bijuga* biomass increase induced by four LED light sources (white-3000K; red-λ655nm; green-λ530nm; blue-λ470nm) during a 6 days cultivation period.

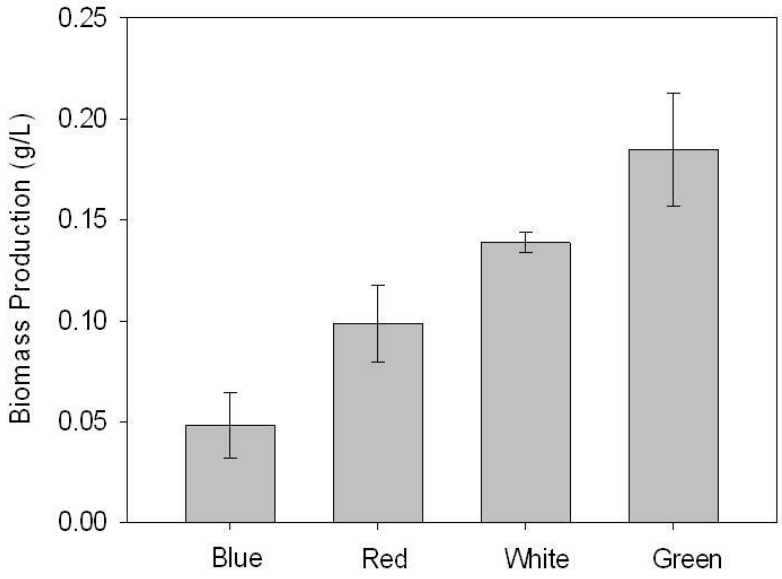
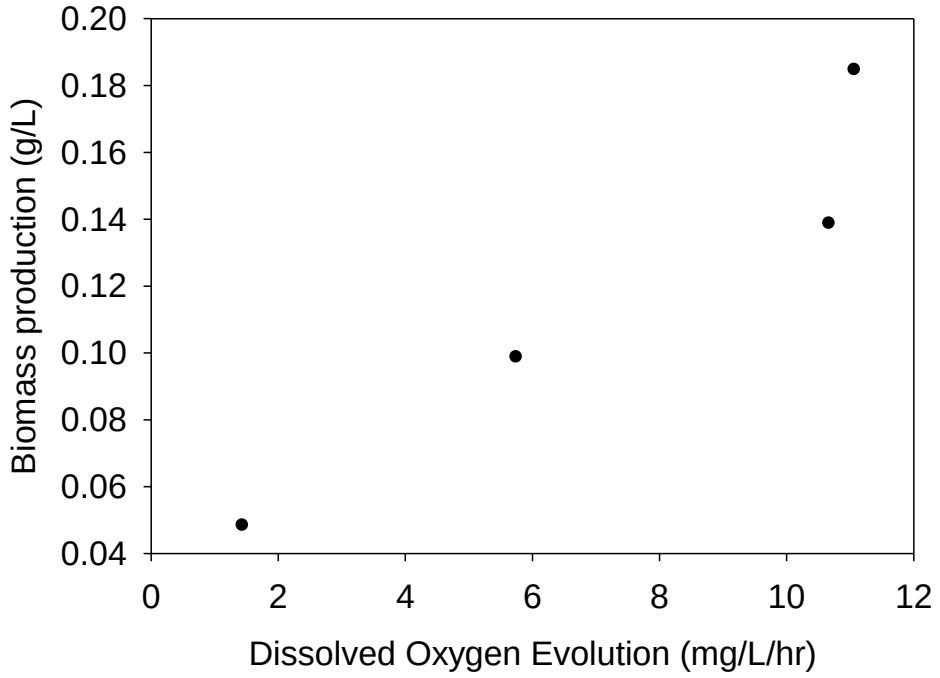


Figure 5.9: Results of initial dissolved oxygen evolution measured at day 0 plotted against the final biomass accumulation after 6 days of cultivation on the LED plate. Each individual point was obtained under different wavelengths.



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_2 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 alkalinization induced by photosynthetic activity and acidification of the medium by the CO_2 supply. Low CO_2 supply can hold back culture development while excessive CO_2 supply can induce a longer acclimation 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 been 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

that 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_2 ambient is mainly affected by the initial number of cells present in each inoculum. 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.