CHARACTERISATION OF CYANOBACTERIA CULTIVATION IN A TUBULAR BAFFLED PHOTO BIOREACTOR (TBPBR)

By

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DEDICATED TO THE MARTYRS OF THE SYRIAN

REVOLUTION

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ABSTRACT

Cyanobacteria or blue-green algae are important resources. In some parts of the world, cyanobacteria are used as a staple food and their ability to fix nitrogen has been explored to increase the productivity of many crops and transform a barren soil into a fertile one. An interesting property of cyanobacteria is their ability to absorb nitrogen and inorganic phosphorous, so they have been seen in water purification systems. Most interestingly cyanobacteria produce O_2 and H_2 by the combination of photosynthesis and nitrogen fixing ability; they could potentially become a producer of hydrogen fuel. This project investigates the characterizations of cyanobacteria cultivation in a tubular baffled photo bioreactor (TBPBR).

Many benchmarking experiments were conducted in light boxes in order to understand the reaction kinetics and to examine the effects of the ratio of aeration surface over culture volume, light intensity, light quality, light cycle, mixing, initial cell density and temperature on the growth of *Gloeothece membranacea* and *Oscillatoria amoena.*

Based on the benchmarking results, a tubular baffled photo bioreactor (TBPBR) was designed, constructed and commissioned. Further experiments were conducted using *Gloeothece membranacea* in order to characterize the continuous cultivation of in this novel photobioreactor; examine the effects of the light saturation and the period of light availability

on cell growth and determine the critical cell density for optimal growth. The kinetics information was extracted and compared with that of the benchmarking trials.

The light saturation level for *Gloeothece membranacea* in the TBPBR was 80 μmole m^{-2} sec^{-1}, and the minimum light exposure without affecting the growth was 6 hours, same as that in the light boxes. Also, much higher critical cell density (CCD)g of *Gloeothece membranacea* could be accommodated in the TBPBR than that in the light boxes. Furthermore, the optimum specific growth rate of *Gloeothece membranacea* was obtained at aeration flow rate of 0.08 vvm and Vol CO_2/ Vol air = 6%.

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CHAPTER 1 INTRODUCTION

1.1 Motivation for the Study

Cyanobacteria or blue-green algae are a group of simple photosynthetic micro-organisms that can be found almost everywhere: from marine shores to damp rocks; from salt marshes to trunks of trees and stones. Cyanobacteria are important sources for valuable chemicals, e.g. fatty acids, minerals and pigments, and for health food like proteins and vitamins (Glombitza , 1989; Becker, 1994). For example, *Spirulina* species have very good food value; produce β-carotenes, proteins, amino acids and antiviral polysaccharides; and adsorb toxic minerals (Behera et al., 2007). Also, cyanobacteria are used in wastewater treatment (Chevalier and de la Noüe, 1985, Aziz and Ng, 1992, Yun et al., 1999, Kim et al., 2000, Jin et al., 2003); the removal of phosphorous (Gaffney et al., 2001) and nitrate (Hu et al., 2000) from ground water; and CO_2 bioremediation (De Morais and Costa, 2007). Moreover, cyanobacteria could be used to moderate impacts of thermal effluents by reducing their nutrient content (Weissman et al., 1998). In addition, cyanobacteria produce a variety of secondary metabolites with antibiotic, cytotoxic, immunosuppressive and enzyme inhibiting activities (Mundt et al., 2001). Probably the most beneficial characteristic of cyanobacteria is their nitrogen fixing ability (Bergman et al., 1997), which can help neighbouring plants grow via a symbiotic relationship. This fact has been used to increase the productivity of many crops. Another important application of the nitrogen fixing ability is that cyanobacteria can be used to gradually transform a barren soil into a fertile one (Rao and Burns, 1990, Ariosa et al., 2005).

The combination of photosynthesis and nitrogen fixation in cyanobacteria produces oxygen and hydrogen (Liu et al., 2006). These two gases can be utilized as an energy source for combustion engines and fuel cells. Burning hydrogen would produce energy and water. The water can then go back into the photosynthesis process generating more hydrogen and oxygen. In this way, a clean and totally renewable energy route could be realised.

Clearly, cyanobacteria have many beneficial usages and potentially enormous impact on the generation of the clean and renewable energy of the world tomorrow. The overall process of generating energy via cyanobacteria consists of cultivation, nitrogen fixation and harvesting the generated energy and the disposal of residual cells. This research project however primarily focuses on the characterisation of cultivation of cyanobacteria in a tubular baffled photobioreactor (TBPBR).

1.2 The Objectives of the project

- Characterize the growth profiles and parameters that affect them using the selected cyanobacteria;
- Understand the effects of physical parameters on the growth;
- Implement the learning outcomes into the design of a TBPBR
- Investigate the effects of the novel photobioreactor environment on the interaction with effects of physical parameters, and on the growth;

1.3 Thesis Layout

The structure of the thesis is as follows: after this introduction, the report commences in Chapter 2 with the relevant background of cyanobacteria, including a review of cell biology and growth cycle of population; the two physiological processes: photosynthesis and nitrogen fixation and hydrogen production by cyanobacteria.

In Chapter 3 the different methods of cultivation that have been used to grow cyanobacteria are studied including: open pond systems; vertical, horizontal, flat panel, helical, stirred tank photobioreactors as well as hybrid systems.

In Chapter 4 the species used in this research are presented, the designs of both light boxes and the tubular baffled photobioreactor (CBPBR) are given, and a detailed description of the experimental apparatus and analytical procedure is explained.

The results of the experiments that were carried out in the light boxes and the tubular baffled photobioreactor (TBPBR) are presented and discussed in Chapter 5 and Chapter 6 respectively.

A summary of conclusions that have been drawn from this research is given in Chapter 7, followed by recommendations for future work in Chapter 8.

CHAPTER 2 CYANOBACTERIA

This Chapter surveys the background literature relevant to this work, and is divided into two parts: cyanobacteria and the main metabolic processes which they carry out: photosynthesis, nitrogen fixation and hydrogen production.

2.1 Introduction

Cyanobacteria (formerly known as blue-green algae) are a group of photosynthetic Gram-negative Eubacteria that have cell walls (Holt, 1994), tolerate a wide range of temperatures from 2 °C in the Antarctic saline ponds to 74 °C in hot springs, representing a connection between bacteria and green plants. Cyanobacteria are oxygenic phototrophic microorganisms for the reason that they carry out photosynthesis using light as their energy source, CO_2 as their carbon source and produce oxygen as in green plants, however, some are able to carry out anaerobic, an-oxygenic photo-autotrophy using sulphide, where this inactivates photosystem Π and inhibits oxygenic photosynthesis. Also, cyanobacteria can perform other modes of growth like photo-heterotrophy where other chemicals are used as a source of carbon, as well as aerobic (respiratory) and anaerobic (fermentative) chemo-heterotrophy where other chemicals are employed as sources for both energy and carbon. Yet these modes of growth are slow and occur only when conditions are unfavourable. In addition, they are prokaryotic, which means that the cells lack membrane-bound organelles such as a true nucleus, a chloroplast or a mitochondrion. The genetic material, the photosynthetic and respiratory apparatuses are not therefore separated from the rest of the cell by any means of

internal membranes. However, their diet is the same as that of eukaryotic algae (organisms whose cells are organized into complex structures by internal membranes and cytoskeleton) in green plants. Those microorganisms are very ancient. Fossils of cyanobacteria that are over three or four billion years old (Schopf and Packer, 1987) have been found. At that time, they probably were the first organisms that evolved elemental oxygen and the main producers of organic matter. Although ancient, cyanobacteria are very common and found about everywhere: on oceanic shorelines and the beds of rivers; on moist rocks and saline marshes; or on trunks of trees and pebbles (Fay, 1983).

There are many reasons for growing cyanobacteria. The first is that cyanobacteria have high content of proteins in dry biomass ranging between 33-55 % (González López et al., 2010), which make them probable candidates for new sources of food for animals and human.

The second is that cyanobacteria can undergo nitrogen fixation. Thus, they play a vital role in the natural environment by their ability to initially colonize arid land and produce organic matter. This is because cyanobacteria are able to live in symbiotic relations with other plants and animals, where cyanobacteria provide nitrogen products for those plants or animals in exchange for a place to grow on. Those symbiotic relations could exist in aquatic and terrestrial habitats.

The third is the co-generation of molecular hydrogen and oxygen, which makes cyanobacteria among the most promising agents for biological solar energy systems. Thus,

cyanobacteria could play an important role in the world's most urgent problems of food and energy.

The last, but not the least, reason for the growing interest in cyanobacteria is that they provide a reasonably straightforward model for the understanding of the elementary processes like cell segregation, gene expression and macromolecule synthesis (Fay, 1983).

2.1.1 Cell Biology

Cyanobacteria are a type of microorganism. The chemical composition of the cell wall of cyanobacteria is similar to the wall of the Gram-negative bacteria (Madigan et al., 2000). The wall is composed of two layers:

- The inner layer that is responsible for the mechanical strength of the cell wall
- The outer layer that probably controls the transport of solutes

The space between the two layers has a similar content of lipopolysaccharides and degradative enzymes as in the Gram-negative bacteria. The cell wall is often covered by sheath or capsule which is composed of polysaccharide. This sheath or capsule may promote the attachment of the organisms onto solid substrate. However, the ability to produce the sheath may be lost upon repeated inoculation (Madigan et al., 2000).

Cells are built of chemical compounds and growth occurs when all these chemical compounds increase in amount. The basic substances of a cell come from its environment.

The cell has the ability to transfer those substances into molecules through chemical reactions, then to organize those molecules into specific structures of which the cell is composed. The process by which a cell is built up from simple substances is called biosynthesis or anabolisms (Madigan et al., 2000). However, energy is required for biosynthesis, as well as for transport of nutrients and motility. Most of microorganisms get energy from the oxidation of chemicals (organic or nonorganic), while some microorganisms like cyanobacteria obtain their energy from light. When chemicals are broken down, energy is released and conserved by the cell. This process is called catabolism. The enzyme-catalysed chemical reactions of anabolism and catabolism are collectively referred to as metabolism (Lehninger et al., 2008).

Figure 2.1 summarizes the simplified view of cell metabolism (Madigan et al., 2000).

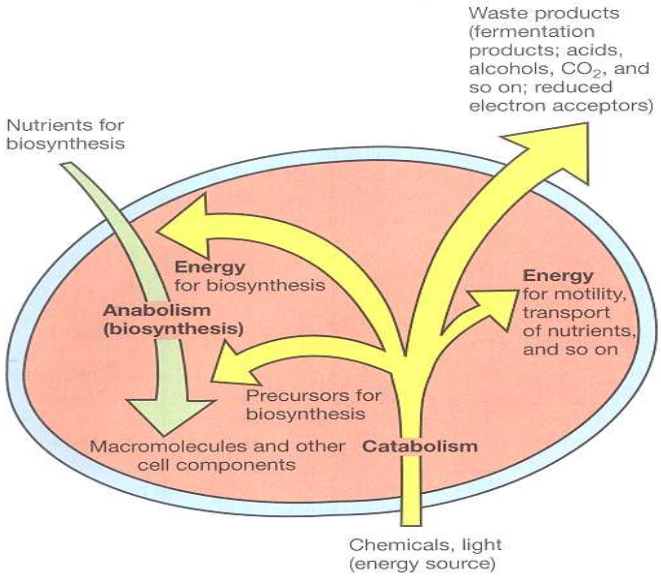

Figure 2.1 A simplified view of cell metabolism (Madigan et al., 2000).

2.1.2 The Prokaryotic Cell

It is useful to speak about prokaryotic cells, as cyanobacteria are prokaryotes. A prokaryotic cell has:

Cytoplasmic membrane**:** This is a critical permeability barrier that separates the cell from its environment. If the membrane is destroyed, the cell dies, as its contents leak into the environment.

The cell wall***:*** This is a rigid structure outside the cytoplasmic membrane. It supports and protects the cell from osmotic lysis.

Ribosomes: They are small particles that are composed of proteins and ribonucleic acid (RNA). The synthesis of proteins takes place on them.

Inclusions: These are storage materials made up of compounds of carbon, nitrogen, sulphur, or phosphorous. Those inclusions are formed when nutrients are in excess. They act as repositories of nutrients.

A nucleoid: This is a single, circular, double-helical molecule of DNA which exist free in the cytoplasm of prokaryotes (Weston, 1997).

Prokaryotes are much smaller than eukaryotes and their small size gives them a big advantage. This is because the rate at which nutrients and waste products pass into and out of a cell is inversely proportional to cell size and these transport rates are to some degree a function of the amount of membrane surface area available, which is relative to cell volume, small cells have more surface available than do large ones (Madigan et al., 2000). Thus, prokaryotes grow much faster than eukaryotes.

Prokaryotic cells have special structures which allow cells to move. This movement gives the cells a selective benefit under certain environmental surroundings. The first structure that prokaryotes may have is gas vesicles, which gives buoyancy to the cells. This characteristic allows cells to float up and down in water in reaction to changes in surroundings factors. The other structure is called a flagellum (plural, flagella), which is made of a single, coiled tube of protein. In the aquatic environment, phytoplankton communities are exposed to a wide range of light regimes ranging from growth-limiting to growth-inhibiting light intensities. Cyanobacteria species have an advantage over most other phytoplankton with their distinctive machinery of buoyancy regulation. By controlling buoyancy, they tend to locate themselves at a depth of most favourable light intensity.

Motility gives prokaryotes the ability to move towards more favourable environmental conditions. These directed movements are called taxis and there are different types of them:

- Chemotaxis: a reaction to chemical changes

- Phototaxis: a reaction to changes in light

- Aerotaxis: a reaction to changes in oxygen concentration

- Osmotaxis: a reaction to changes in ionic strength

2.1.3 The Forms of Cells of Cyanobacteria

Cyanobacterial cells can convert into three different forms: vegetative, heterocysts and akinetes depending on the environmental conditions. The vegetative cells are present when conditions are favourable. They are able to carry out reproduction through photosynthesis.

Heterocysts have modified membranes, generally lack phycobilisomes and oxygenic autotrophic capacity. They have extra wall layers, and work primarily when there are no nitrogen compounds in the environment and the cells have to fix nitrogen. Heterocysts do not grow or divide and their metabolism is primarily directed to supporting N_2 fixation. Their metabolism depends upon the supply of carbohydrate from the two adjacent vegetative cells (Wolk, 1982). The Adenosine-5'-Triphosphate (ATP) and reductant generated by catabolism of this carbohydrate can largely be directed towards N_2 fixation.

Akinetes have very thick cell walls. These structural developments are formed when the environments are unfavourable. They contain large amounts of carbohydrates and work as

reserves. Due to their high density, they sink to the bottom of the lake and stay there till the conditions become favourable again. Then, they act as seeds for the growth of new colonies.

2.1.4 Subgroups of Cyanobacteria

There are two types of cyanobacteria: unicellular and filamentous. The former reproduces either by binary fission where each cell produces two cells identical to the mother cell, or by internal multiple fission where the daughter cells are smaller than half the parent.

The filamentous cells reproduce by binary fission and give either trichomes that are composed of cells that do not differentiate into heterocysts or alkinetes; or trichomes that have one or a few cells which could differentiate into heterocysts, at least when the concentration of nitrogen compounds in the surroundings is low.

In this study both unicellular and filamentous strains will be examined. The candidate for the former is *Gloeothece membranacea* as many studies claimed that *Gloeothece* showed significant nitrogenase activities during dark time (Klipp et al., 2005), and for the latter is *Oscillatoria amoena* as Hiroto stated that *Oscillatoria* displayed higher rates of hydrogen production than *Anabaena cylindrica* that is a very well-studied species for hydrogen production (Hiroto et al., 1995). Members of the genus *Gloeothece* are unicellular, rod shaped cells. Many cells are held together by a distinct sheath. They can undergo nitrogen fixation under aerobic conditions. To grow and flourish they only require photons as an energy source and CO_2 as a carbon source, and this is why they are called photoautotroph

(troph = nourishment). They grow when each cell divides to give two equal size cells. This kind of reproduction is called binary fission (Holt, 1994).

Oscillatoria are filamentous cyanobacteria that divide totally by binary fission. All species of *Oscillatoria* are photoautotrophic. They grow in fresh marine and brackish waters as well as in inland saline lakes, and a few species tolerate temperatures as high as 56-60 °C in some hot springs. Some species form mats in streams (Holt, 1994). Figure 2.2 shows the two types of cyanobacteria used in the current project.

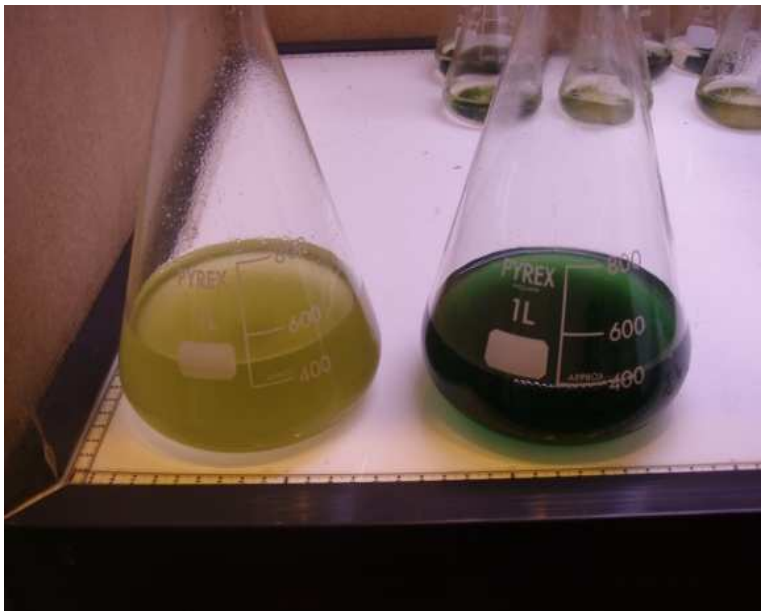

Figure 2.2 *Oscillatoria amoena* on the right and *Gloeothece membranacea* on the left

2.2 Photosynthesis

2.2.1 The Light Harvesting Pigments in Cyanobacteria

Chlorophyll a is the main light harvesting pigment in cyanobacteria, however, these microorganisms have other accessory harvesting systems which absorb wavelengths that chlorophyll a cannot. Thus, they play a vital role in the survival of cyanobacteria in weak light conditions. These accessory pigments are called phycobiliproteins. The phycobiliproteins form organized structures called phycobilisomes. The phycobilisomes are attached to the thylakoid membranes in cyanobacteria; they absorb the light and pass on the photons to photosystem Π which is the photoreaction centre in photosynthesis (Wang et al., 1977).

The phycobiliproteins are water-soluble proteins that are classified into three groups based on their spectroscopic properties: phycoerythrin (PE), λ_{max} = 540-570 nm; phycocyanin (PC), λ_{max} = 610-620 nm; allophycocyanin (APC), λ_{max} = 650-655 nm (Gantt, 1981, Glazer, 1981). There is a fourth type of phycobiliproteins: phycoerythrocyanin (PEC), λ_{max} = 568 nm, 585 nm, which a few species of cyanobacteria use, instead of PE (Bryant et al., 1976). These phycobiliproteins are covalently attached to linear tetrapyrrols by a cysteine bond (Glazer, 1982). According to the absorption characteristics of these tetrapyrrols in aqueous solutions, they are grouped in four types: phcocyanobilin (PCB), λ_{max} = 660 nm; phycobiliviolin (PXB), λ_{max} = 590; phycoerythrobilin, λ_{max} = 555 nm and phycourobilin, λ_{max} = 495 nm (Glazer, 1981, Goodwin, 1976).

Some cyanobacteria species which contain PE have the ability to change the synthesis of PE alone or PE and PC under specific light wavelength; this phenomenon is called complementary chromatic adaptation (Tandeau de Marsac, 1977, Bogorad, 1975). Because of this phenomenon the synthesis of PC under red light and PE under green light is enhanced in some species of cyanobacteria (Gendel et al., 1979, Bennett and Bogorad, 1973, Fujita and Hattori, 1960b, Fujita and Hattori, 1960a).

2.2.2 The Process of Photosynthesis

Photosynthesis is the first stage of cyanobacteria cultivation, where light energy is converted into chemical energy. Light is captured by light harvesting complex proteins, which is known as light harvesting complex I (LHCI) and light harvesting complex Π (LHCΠ) (Figure 2.3). These complex proteins play a vital role in both capturing light and dissipation of excess energy that otherwise would cause photosynthetic inhibition by damaging the photosynthetic reaction centres in particular photosystem Π (PSΠ) (Horton and Ruban, 2005). Through the network of pigments bounds by the LHC, photosystem I (PSI) and photosystem Π (PSΠ) subunits, the excitation energy is passed to the photosynthetic reaction centres of PSI and PSΠ. Photosystem Π uses the energy to split the water into protons, electrons and oxygen as:

$$\mathbf{2H_2O + 2NADP^+ = 2NADPH + 2H^+ + O_2}$$

The electrons pass through the photosynthetic electron transport chain via plastoquinone (PQ), cytochrome b_6f (Cyt b_6f), photosystem I (PSI) and ferredoxin (Fd) and onto NADPH (Figure 2.3). At the same time, PSΠ and PQ/PQH_2 cycles pass proton into the thylakoid membrane. These processes generate a proton gradient, which in turn drives ATP production via ATP synthase. NADPH is produced by recombining the protons and electrons by ferredoxin-$NADP^+$ oxidoreductase (FNR).

The energy molecules ATP and NADPH are used by different biochemical pathways to produce sugars and other chemical compounds that collectively form biomass. These reactions are called Calvin Cycle or Dark Reactions because they happen in the dark. The Calvin cycle includes three steps: carboxylation, reduction and substrate (ribulose-1, 5-bisphosphate RuBP) regeneration. In the first step, CO_2 enters the cycle to react with RuBP. This process is catalysed by ribulose-1, 5-bisphosphate carboxylase/ oxygenase (rubisco). This enzyme is one of the most important proteins, as it constitutes 30% of total proteins in most leaves (Parry et al., 2003). Rubisco has two catalytic functions: as a carboxylase as part of the photosynthetic reduction cycle, and as an oxygenase as part of photorespiration under aerobic conditions. In the first step of the Calvin cycle, rubisco catalyses the formations of two 3-phosphoglycerate molecules from RuBP, CO_2 and H_2O. The forward reaction is strongly favoured by the negative free energy of the process.

The second step is an ATP/NADPH-dependent reduction phase, where these carboxylic acids are reduced to two forms of molecules of glyceraldehydes -3-phosphate dehydrogenase.

The third step consists of a series of reactions, where a quantity of 3-phosphate is converted back to RuBP required to allow the photosynthetic reduction cycle to continue (Lazar, 2003). Figure 2.3 illustrates the process of photosynthesis as described earlier. However, in microalgae and cyanobacteria, the electrons and protons, which are extracted from water, could pass to the hydrogenase enzyme (HydA) via the electron transport chain to drive the photoproduction of hydrogen.

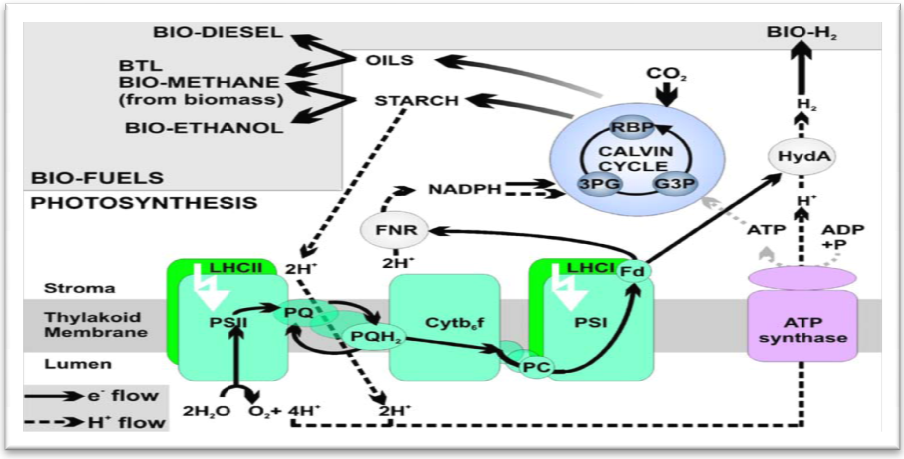

Figure 2.3 The Process of photosynthesis (Schenk et al., 2008)

2.2.3 Photosynthesis in Cyanobacteria

Photosynthesis in cyanobacteria is oxygenic and photoautotrophic. This means that all cyanobacteria have the ability to use light energy, fix CO_2 as a source for carbon, and produce O_2. However some species are also capable of sulphide-dependent, anaerobic, anoxygenic photoautotrophy, when sulphide inhibits photosystem Π. Electrons derived from sulphide entering the photosynthetic electron transport chain closer to photosystem I then results in

CO_2 reduction. Also, some cyanobacteria can also carry out photoheterotrophy where CO_2 is not the source of carbon but other chemicals; aerobic (respiratory) and anaerobic (fermentative) chemoheterotrophy where CO_2 is not the source of carbon nor is light the source of energy. However, cyanobacteria carry out these modes of growth only for maintenance, when environmental conditions are unfavourable. The main characteristic of cyanobacteria that make them different from all other prokaryotes is the fact that they have dual photosystems that allow them to use H_2O as a photoreductant with the liberation of O_2.

2.3 Nitrogen Fixation by Cyanobacteria

Nitrogen is an essential component of cellular compounds; however most organisms cannot absorb it directly because nitrogen is relatively inert, for the reason that it has a triple bond. However, cyanobacteria have the ability to fix atmospheric nitrogen and convert it to other forms such as ammonium ions (NH_4^+) and nitrate ions (NO_3^-), which other organisms can absorb.

Because of this characteristic, cyanobacteria can supply their own need of nitrogen-based nutrient, so they can survive in arid deserts and the frozen Poles. Furthermore, other plants can grow with them through symbiosis.

Cyanobacteria fix N_2 by the reaction:

$$N_2 + 8H^+ + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16Pi$$

This reaction is catalysed by a special enzyme called nitrogenase. Nitrogenase is made up of an iron protein and a molybdenum-iron protein (Sakurai and Masukawa, 2007). The fact is that nitrogenase is very sensitive to oxygen. Where O_2 oxidises iron, it makes nitrogenase inactive. As cyanobacteria produce O_2 during photosynthesis, it is clear why this is a problem.

2.3.1 Nitrogen Fixation by Non-heterocystous Cyanobacteria

Many, though not all, non-heterocystous cyanobacteria, which could be either unicellular or filamentous, could carry out nitrogen fixation. Most of those species fix nitrogen in micro-oxic[1] or anoxic environment, however, a few of them can fix it aerobically[2] (Bergman et al., 1997). Interestingly, this apparently means that those species are able to carry out both oxygenic photosynthesis and O_2-sensitive N_2 fixation in the same cell. This, in turn, implies that those non-heterocystous cyanobacteria have the ability to protect nitrogenase from inactivation because of O_2, which is released as a by-product of photosynthesis. For this reason, the vegetative cells of non-heterocystous cyanobacteria have to produce photosynthate, by CO_2 fixation, to scavenge additional O_2 generated during photosynthesis

[1] *The term micro-oxic refers to environments of cultures grown in the absence of exogenous O_2 but which remain capable of generating O_2 photosynthetically (Bergman et al., 1997).*

[2] *The terms 'oxic' and 'anoxic' refer to environments where O_2 is present or absent, respectively, while the term 'aerobic' is used specifically to describe the presence of O_2 at concentration comparable to that in air (Bergman et al., 1997).*

and to provide energy for cell growth. In many cases, N_2 fixation and photosynthesis in these strains show opposing cyclic fluctuations, with N_2 fixation occurring at dark in most species supported by aerobic respiration, thereby these strains achieve a temporal separation of N_2 fixation and oxygenic photosynthesis (Schneegurt et al., 1994).

2.3.2 Nitrogen Fixation by Heterocystous Cyanobacteria

All heterocystous cyanobacteria fix N_2 aerobically, though only filamentous cyanobacteria can differentiate into heterocysts, and moreover, the filament integrity is very crucial to nitrogen fixation, as filament breakage leads to loss of nitrogenase activity (Lopes Pinto et al., 2002). When there are no nitrogen compounds in the environment, 5-10% of vegetative cells differentiate into specialized cells called heterocysts that present the right circumstances for nitrogenase to catalyse N_2 fixation. Heterocysts do not carry out photosynthesis, thus do not produce O_2 neither do they fix CO_2. They also exhibit a high rate of respiratory O_2 consumption and are surrounded by a thick, laminated cell wall that limits the penetration of oxygen into the cell. Therefore, the internal environment of heterocysts is practically anoxic, which is ideal for nitrogenase, an extremely O_2-sensitive enzyme. In that way, the cells make a spatial separation of N_2 fixation and oxygenic photosynthesis (Zhang et al., 2006), however, the process of N_2 fixation happens exclusively during the light phase of a cycle of alternating light darkness (Khamees et al., 1987). A few strains of non-heterocystous cyanobacteria also fix N_2 during the light time (Ortega-Calvoh and Stal, 1994) using the same approach of spatial separation, where some cells do not photosynthesize. There are many factors that motivate heterocyst differentiation: removing combined nitrogen from the medium, adding

carbon sources, immobilizing cyanobacterial cells in polyvinyl and polyurethane foams, or living with organisms such as the fern *Azolla* in a symbiotic relationship (Hall et al., 1995).

It should be emphasized that the metabolic changes that occur during heterocyst differentiation are permanent, whilst those noted during the progress of N_2 fixation in non-heterocystous cyanobacteria can be inverted following resupply of a nitrogen source such as ammonium. Heterocysts are terminally differentiated cells, specialized in N_2 fixation but lacking the ability to either grow or divide. They do not re-differentiate into vegetative cells following addition of ammonium, though further differentiation of heterocysts is blocked by such treatment and the proportion of pre-existing heterocysts declines as a result of continued division of vegetative cells. In contrast, N_2 -fixing vegetative cells of non-heterocystous cyanobacteria continue to grow and divide and rapidly revert to non-diazotrophic metabolism following addition of ammonium.

2.4 Hydrogen Production

2.4.1 Introduction

Hydrogen is produced in cyanobacteria in two physiological processes:

- **Nitrogen Fixation**: A light-dependant reaction catalysed by nitrogenase

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP = 2NH_3 + H_2 + 16ADP + 16Pi}$$

Happe et. al claimed that *Anabaena variabilis* showed a clear maximum in nitrogenase activity (Happe et al., 2000).

- **Fermentation**: Dark anaerobic conditions catalysed by bidirectional hydrogenase (Stal and Moezelaar, 1997) by the reaction

$$\mathbf{8H^+ + 8e^- + 16ATP = 4H_2 + 16ADP + 16Pi}$$

It has been shown that the specific activity of a bi-directional enzyme from *Synechocystis* is superior to hydrogenases from other cyanobacteria (Schmitz et al., 2002).

2.4.2 Major Enzymes Involved in Hydrogen Production by Cyanobacteria

In total there are three enzymes which are involved in the process of hydrogen metabolism in cyanobacteria:

1. **Nitrogenase**: This enzyme produces hydrogen as a by product through the process of nitrogen fixation. There are three types of nitrogenase: Mo-nitrogenase, V-nitrogenase and Fe-nitrogenase. Tsygankov et al. (1987) claimed that the specific growth rate of *Anabaena variabilis with* Mo-nitrogenase or V-nitrogenase was three times higher than that of the same species with Fe-nitrogenase. However, *Anabaena variabilis* with

V-nitrogenase produced hydrogen at the highest rate of hydrogen (Tsygankov et al., 1997)

2. **Bi-directional hydrogenase**: This enzyme catalyses both reduction of protons to produce hydrogen and oxidation of hydrogen (Tamagnini et al., 2002)

3. **Membrane-bound uptake hydrogenase**: This enzyme re-oxidises hydrogen that is produced by nitrogen fixation

Both nitrogenase and hydrogenase are O_2-sensitive enzymes. They become irreversibly inactive, when O_2 is present. As cyanobacteria produce O_2 during photosynthesis, this is clearly a problem. However, cyanobacteria have adapted several strategies to prevent exposure to O_2. These include avoidance of O_2, physical barriers to its diffusion, and spatial and temporal separation of N_2 fixation and O_2-evoloving photosynthesis (Hansel and Lindblad, 1998). For more details see Nitrogen Fixation (2.3).

All diazotrophic cyanobacteria, which are cyanobacteria that carry out N_2 fixation, possess nitrogenase and uptake hydrogenase, however, some of them have bi-directional hydrogenase as well. On the other hand, non-diazotrophic cyanobacteria only possess bi-directional hydrogenase. Hydrogen uptake is linked to the nitrogenase activity, this can explained by the fact that the re-oxidizing of H_2 is required in order to recover some energy for nitrogen fixation (Böhme, 1998). Figure 2.4 illustrates the interaction of hydrogen production by nitrogenase and hydrogen use by hydrogenase-uptake enzyme.

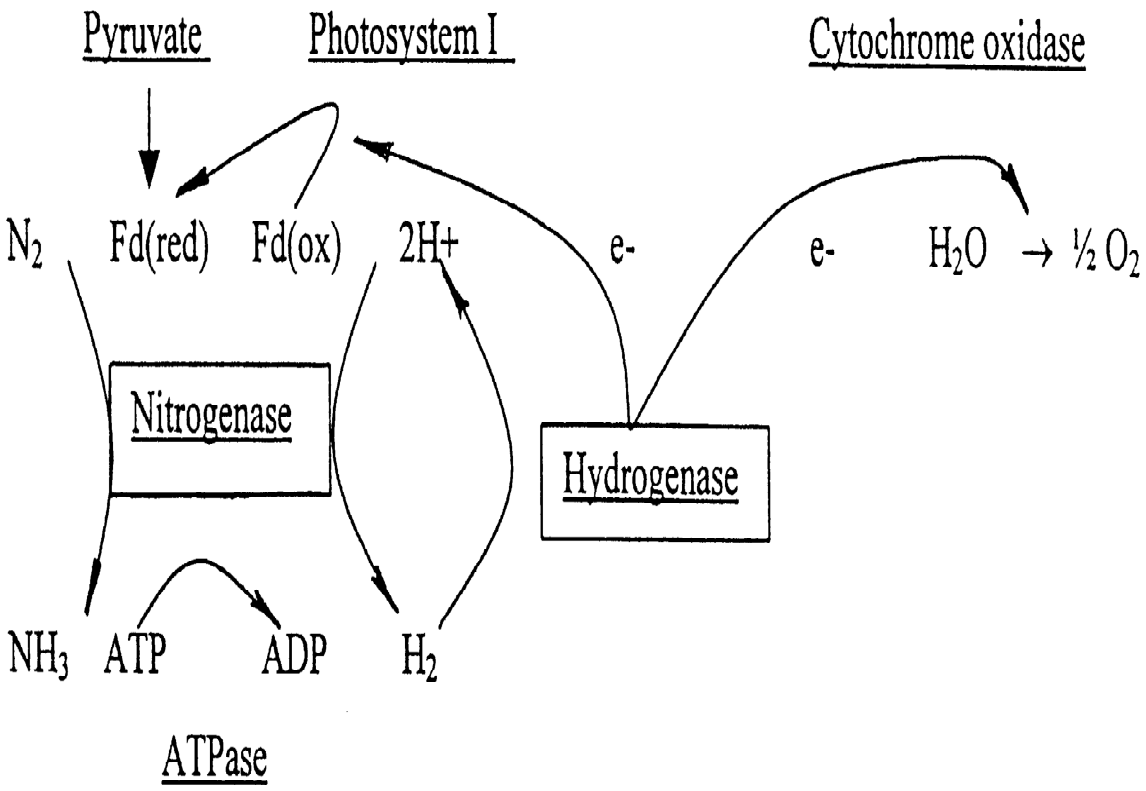

Figure 2.4 Hydrogen production by nitrogenase and hydrogen use by uptake hydrogenase (Das and Veziroglu, 2001)

Whereas the hydrogen evolution activity of the bidirectional hydrogenase is not dependant or even related to diazotrophic growth conditions (Schütz et al., 2004). It is claimed that the rate of hydrogen production by some species of cyanobacteria is comparable to that measured in hydrogenase-based hydrogen production by green algae (Troshina et al., 2002).

2.4.3 Categorizing Cyanobacteria according to Hydrogen Production

There are five forms of cyanobacteria that produce hydrogen, each of which is described as follows.

2.4.3.1 Non-diazotrophic Unicellular Cyanobacteria

These species do not possess nitrogenase, so they do not fix nitrogen. Hydrogen production is carried out in these species in the dark under anaerobic conditions, catalysed by hydrogenase through a two step process:

- Aerobic photosynthesis is conducted to enable cells to grow. This step occurs in 12h/12h light/dark cycle where the media contain nitrogen compounds and neither nitrogen fixation nor hydrogen production occurs.

$$\mathbf{12H_2O + 6CO_2 = C_6H_{12}O_6 + 6H_2O + 6O_2}$$

$$\mathbf{xC_6H_{12}O_6 = glycogen + yH_2O}$$

- Anaerobic hydrogen production occurs in the dark. The transfer from growing conditions to hydrogen production conditions leads to the loss of oxygen evolution within a day, followed by the loss of the reaction centre in photosystem Π in three

days. This step occurs after 24 h in the dark when the medium is free from nitrogen compounds.

$$\textbf{Glycogen} + \textbf{y}H_2O = \textbf{x}C_6H_{12}O_6$$

$$C_6H_{12}O_6 + 6H_2O = 6CO_2 + 12H_2$$

An example of these species is *Gleocapsa alpicola*. Serebryakova and Tsygankov (2007) stated that, in the first step of hydrogen production from this species, a culture of *Gleocapsa alpicola*, immobilized on a matrix of glass fiber TR-0.3, achieved a density of 37 g cm^{-2} in media with limiting concentrations of nitrate to enhance glycogen accumulation and activate hydrogenase for the second step in the process, where hydrogen was produced in the second step through the fermentation of glycogen in darkness with continuous sparging of Ar and without media flow. The total amount of H_2 produced in one cycle was 957.6 mLL^{-1}_{matrix} (Serebryakova and Tsygankov, 2007).

2.4.3.2 Diazotrophic Unicellular Cyanobacteria

These species possess nitrogenase, so they fix nitrogen. When the media are free from nitrogen compound, hydrogen production is carried out in these species in the dark under aerobic conditions catalysed by nitrogenaze. O_2-evolving photosynthesis is carried out during

the light period of growth, while hydrogen is produced through nitrogen fixation during the dark period. An example of these species is *Gloeothece* (Reade et al., 1999).

However, when the media contain nitrogen compounds and nitrogen fixation is not necessary for growth, hydrogen production occurs in the dark under anaerobic conditions catalysed by hydrogenase through a two step process, the same as hydrogen production by non-diazotrophic unicellular cyanobacteria.

2.4.3.3 Non-diazotrophic, Non-heterocystous Filamentous Cyanobacteria

These species do not possess nitrogenase, so they do not fix nitrogen. Hydrogen production is carried out in these species in the dark under anaerobic conditions catalysed by hydrogenase through two step process, the same as hydrogen production by non-diazotrophic unicellular cyanobacteria. An example of these species is *Spirulina platensis* (Aoyama et al., 1997)

2.4.3.4 Diazotrophic, Non-heterocystous Filamentous Cyanobacteria

These species possess nitrogenase, so they fix nitrogen. When the media are free from nitrogen compounds, hydrogen production is carried out in these species in the light under anaerobic conditions catalysed by nitrogenase through a two step process. The difference from the two step process in hydrogen production from the non-diazotrophic unicellular

cyanobacteria is that the second step in diazotrophic, non-heterocystous filamentous cyanobacteria occurs in the light rather than in the dark. An example of these species is *Oscillatoria* (Mary I. Scranton, 1987).

2.4.3.5 Diazotrophic, Heterocystous Filamentous Cyanobacteria

These species possess nitrogenase, so they fix nitrogen. When the media are free from nitrogen compounds, some cells start to develop structural changes and form heterocysts, which have thick walls (Paumann et al., 2005) and can undergo nitrogen fixation and produce hydrogen in light, and aerobic conditions catalysed by nitrogenaze through a two step process. The difference from the two step process in hydrogen production from the non-diazotrophic unicellular cyanobacteria is that the second step occurs in the light and aerobic conditions rather than in the dark under anaerobic conditions. Examples of these species are *Nostoc* and *Anabaena*. *Nostoc* has the highest nitrogenase activity (Yoshino et al., 2007).

Finally, for hydrogen production by cyanobacteria to be economically attractive, a few considerations should be taken into account:

- Immobilized cells of cyanobacteria are more suitable for the continuous production of biological hydrogen (Markov et al., 1995, Das and Veziroglu, 2001)

- Mutant forms of cyanobacteria lacking uptake hydrogenase enzyme, produce higher rates of hydrogen (Sveshnikov et al., 1997, Tsygankov et al., 1999, Lindblad et al., 2002, Masukawa et al., 2002, Schütz et al., 2004, Liu et al., 2006)

- Photobiological hydrogen production occurs in two stages. Conditions during the growth phase are different from conditions during hydrogen production (Yoon et al., 2002)

- Different bioreactors configurations give different efficiencies for both growth and hydrogen production (Hansel and Lindblad, 1998)

The work presented in this thesis is focused on the cultivation of cyanobacteria, and their possible use as a source of energy, not specifically on hydrogen production.

CHAPTER 3 CULTIVATION OF CYANOBACTERIA

This chapter introduces the type of vessels which have been described in the literature for the growth of cyanobacteria. This includes open ponds, photobioreactors of many different types, and hybrid systems.

3.1.1 Introduction

There are many interrelated parameters that can be limiting the growth of microalgae like cyanobacteria. These factors, which have conflicting and complex effect on optimisation during scale up, include temperature (Cho et al., 2007); mixing (Barbosa et al., 2003a); fluid dynamics and hydrodynamic stress (Barbosa et al., 2003b); gas bubble size and distribution (Poulsen and Iversen, 1999, Barbosa et al., 2004), gas exchange (Eriksen et al., 2007); mass transfer (Molina Grima et al., 1999); light cycle and intensity (Pyo Kim et al., 2006, Perner-Nochta and Posten, 2007); water quality, pH, and salinity, (Abu-Rezq et al., 1999, Ratledge, 2004, Cho et al., 2007, Ranga et al., 2007); mineral and carbon regulation/bioavailability, cell fragility (Gudin and Chaumont, 1991) and cell density and growth inhibition (Benemann, 1994).

The growth of cyanobacteria has simple nutritional requirements: water, mineral salts, air (CO_2 and N_2), with light as the only energy source (Hansel and Lindblad, 1998). The cells grow in a liquid medium and the growth depends upon mass transfer of nutrients to the cells, mass transfer between air and the cells, as well as light availability. To improve the growth

rates, air may be added either continuously from the top of the liquid surface (Burja et al., 2002), or bubbling through the media (Sveshnikov et al., 1997). Bubbling air through the liquid also creates mixing. In this context, bubble columns and airlift photobioreactors have been documented in the literature as being suitable to grow cyanobacteria (Miron et al., 2000).

In photobioreactors, aeration rate, gas hold up and fluid mixing (liquid velocity) affect the availability of light to the cells within the reactors. It has been challenging to attain a uniform light distribution. Previous set ups have used external lights because for most reactors the implementation of light sources within will affect the mixing properties. However, the external lights have two disadvantages compared to internal lights. The first is that external lights do not offer a uniform light distribution, especially when only one is used. The second is that the light intensity decreases with distance from the reactor as the light passes through the reactor wall. In general microalgae are cultivated either in open ponds or in closed photobioreactors.

3.1.2 Cyanobacteria Cultivation in Open Pond Systems

Open ponds have many characteristics that make them favourable for microalgae cultivation, are cheap and easy to build and maintain (Weissman et al., 1988). The raceway pond is the most used design, although there are different shapes and sizes. The open pond is a rectangular grid, where each rectangle has an oval channel; the water flows around continuously. The water depth is 15-50 cm. There is a similar design called a circular pond which is common in Asia and Ukraine (Becker(ed), 1994). In wastewater treatment plants,

algae ponds are built depending on the most suitable shape for the location. These ponds are usually driven by gravity. Melbourne's Werribee wastewater treatment plant is one of the largest of this type (Schenk et al., 2008). Retaining walls or dug trenches are the basis of these ponds, while high flow rates in raceway ponds require more stable structure, as well as the addition of paddle wheels, which make raceway ponds less economic than wastewater treatment plants. However, a lot of materials could be used in constructing open ponds and they are easy to maintain because it is quite straightforward to clear up the bio-film that builds up on surfaces.

The disadvantages of open ponds include difficulties in controlling cultivation conditions; inevitable contamination by other unwanted species; the high level of evaporation of water and the reduced light intensity with increased depth (Costa et al., 2006). An open pond is usually cultivated with the desired microalgae culture, however, over time other unwanted species will inevitably contaminate the media, which reduce the productivities significantly and fight with the cultivated species. Once another species has been introduced into the pond, it is highly difficult to remove them. From 3000 photosynthetic microorganisms in the Aquatic Species Program Collection, no species were found to be able to dominate an open pond and have favourable biofuels characteristics (Sheehan et al., 1998). Usually open ponds have two to six species; however, they grow fast, resist predators and tolerate high concentrations of dissolved oxygen. Though a few species can tolerate and out-compete other species in particular circumstances like high/low acidity or salinity, for example, *Spirulina* can grow at pH 9-11.5 and is the dominant species in soda (Belkin and Boussiba, 1991), and is easy to harvest because of its spiral shape. Another example is *Dunaliella salina* which grows very well in saline ponds because of its high intracellular glycerol content, which

protects cells against osmotic pressure, and moreover, *Dunaliella salina* has valuable carotenoids which protects it against intense light (Borowitzka, 2007).

3.1.3 Cyanobacteria Cultivation in Photobioreactors

Closed photobioreactors have many advantages over open ponds system: the possibility of cultivation of monoseptic culture; prevention of evaporation of water; saving of energy and chemicals; smaller footprints and higher productivities (Barbosa et al., 2003b), where higher productivities would compensate for higher costs of construction (Chisti, 2007). Some closed photobioreactors are given the following subsections:

3.1.3.1 Stirred Tank Photobioreactors

The stirred tank photobioreactor is the most convenient method to cultivate photosynthetic microorganisms. Mixing is provided by impellers of different sizes and shapes. The source of carbon for growth is CO_2-enriched air that is bubbled at the bottom of the photobioreactor. Baffles are used to prevent vortex formation, thus improving mixing (Ugwu et al., 2008). The illumination in this type of photoreactor is externally supplied by fluorescent lamps or optical fibres. However, the drawback of these photobioreactors is the low ratio of surface area over volume which in turn hinders the efficiency of light absorption. To overcome those problems fluorescent lamps are provided internally. The use of optical fibres is another option. However, internal illumination has the disadvantage of hindering the mixing pattern. The unused sparged gas and the produced oxygen during photosynthesis are separated in a

disengagement zone. The main disadvantages of these photobioreactors are the high shear stress imposed as a result of mechanical mixing and the low ratio of surface area over volume which in turn hinders light harvesting efficiency.

3.1.3.2 Vertical Tubular Photobioreactors

These photobioreactors are vertical transparent tubes which allow the transmission of light. At the bottom of the tube a sparger is attached which converts the sparged gas into small bubbles. The process of sparging provides mixing, mass transfer of carbon dioxide to media, and diffusion of oxygen produced by algae through photosynthesis. Vertical tubular photobioreactors are divided according to the mode of liquid flow into the reactors:

a) Bubble column photobioreactors

The bubble column photobioreactors consist of cylinders with a height greater than twice the diameter. The important characteristics of these photobioreactors are: high ratio of surface area over volume, efficient heat and mass transfer, acceptable release of oxygen, no moving parts and low costs (Sánchez Mirón et al., 2000). Bubbling gas mixture through the sparger achieves mixing. Light is provided externally. Light and dark cycle, as the media circulates from central dark zone to external photic zone at high rates, affects gas flow rates, which in turn affects the photosynthetic efficiency. The higher the gas flow rate, the higher the photosynthetic efficiency, as higher gas flow rate leads to shorter light and dark cycle. Photosynthetic efficiency greatly depends on gas flow rate which is influenced by the light

and dark cycle as the liquid circulated regularly from central dark zone to external photic zone at higher gas flow rate.

b) Airlift Photobioreactors

Airlift photobioreactors consist of two connected tubes which are called the riser and the downcomer. In the riser the gas mixture is sparged, while there is no gas addition in the downcomer (Sánchez Mirón et al., 2000). The airlift photobioreactors have two forms: internal loop, where the riser and downcomer are separated by either a draft tube or a split cylinder, and external loop, where the riser and downcomer are physically separated by two different tubes as shown in Figure 3.1. Light is supplied externally.

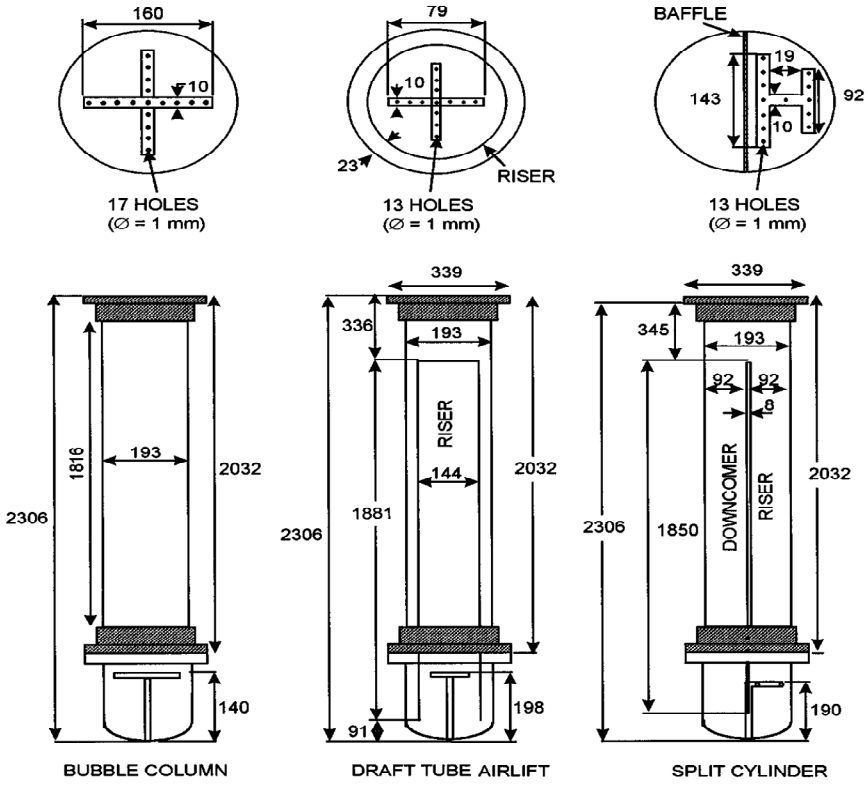

Figure 3.1 Vertical tubular photobioreactor (Miron et al., 2000)

3.1.3.3 Horizontal Tubular Photobioreactor

Horizontal tubular photobioreactors are a parallel set of tubes which are placed horizontally or near horizontally. The advantage of this type of reactors in outdoor cultivation of microalgae is the orientation towards sunlight which leads to improved light conversion efficiency. A special gas system provides CO_2 and releases O_2 which slows down growth (Ugwu et al., 2008). However, the exposure of these reactors directly to sunlight results in heating up the system. In order to cool off the horizontal photobioreactors, different methods have been adapted like spraying water on the system, overlapping of tubes, placing the light harvesting unit inside a pool of temperature controlled water, as it is shown in Figure 3.2 Horizontal airlift-driven-photobioreactor

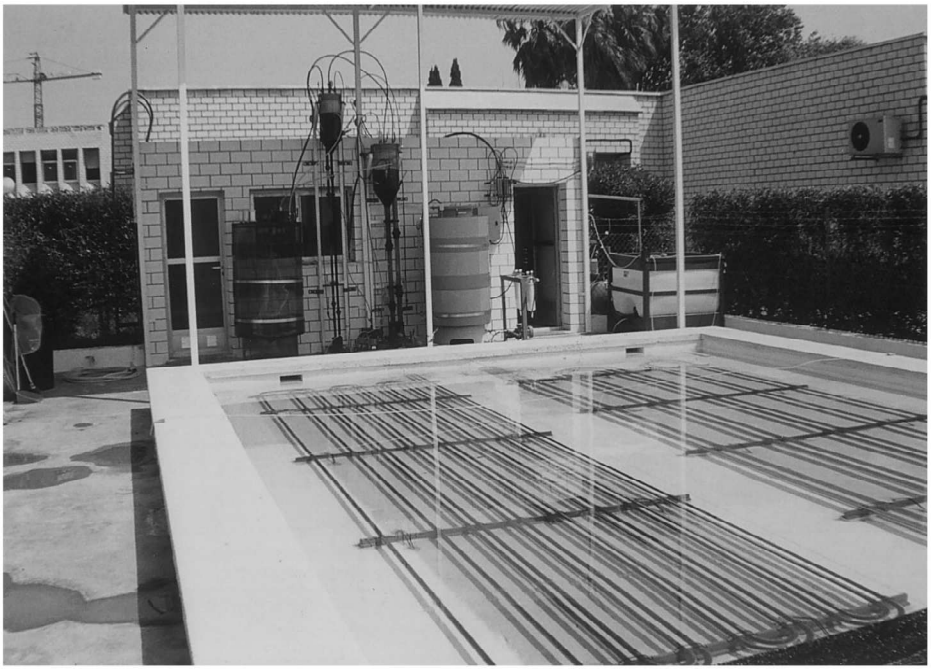

Figure 3.2 Horizontal airlift-driven-photobioreactor (Molina Grima et al., 1999)

Another major disadvantage of horizontal photobioreactors is the high consumption of energy, as high linear liquid velocities are required to achieve turbulent conditions with sufficient short light/dark cycles (Posten, 2009).Near horizontal photobioreactors are inclined towards the sun by a few degrees. This inclination allows more efficient utilisation of sunlight.

3.1.3.4 Flat Panel Photobioreactor

The flat panel reactor consists of cuboids which are made of transparent materials like glass or polycarbonate. The cuboids have a minimal height to give a minimal light path as shown in Figure 3.3.

Figure 3.3 Flat panel airlift bioreactor system (Schenk et al., 2008)

The shape of these photobioreactors gives the advantage of a high surface area over culture volume. Mixing is achieved by either bubbling air from its one side through a perforated tube or rotating it mechanically with a motor. For example,Yuen et al. (2005) built a flat panel photobioreactor from polycarbonate held together in stainless steel. Illumination was achieved by placing 10 fluorescent tubes at one surface. At the bottom of the reactor 17 needles of 0.8 mm diameter were pinched through a piece of silicon to provide the mixture of air and CO_2 (Yuen et al., 2005). Some modifications were made to improve mixing and minimize shear stress.

3.1.3.5 Helical Type Photobioreactor

These photobioreactors are transparent and flexible tubes of small diameter which possess a separate or attached degassing unit. A centrifugal pump is used to drive the culture through a long tube to the degassing unit. Figure 3.4 shows the helical photobioreactor.

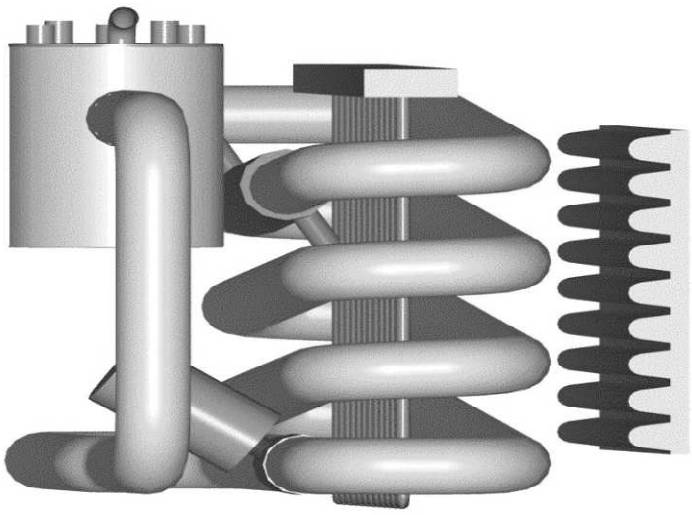

Figure 3.4 A helical photobioreactor (Hai et al., 2000)

However, designing an economically viable photobioreactor, which meets the requirements of growing microorganisms, has many challenges (Weissman et al., 1988). Most microalgae become photo-inhibited at modest light intensities, which means low efficiencies (Melis, 1999, Polle et al., 2002). To overcome this problem, photobioreactors are designed to distribute light evenly over a large surface area in order to provide moderate light intensities for the cells. A fence-like construction, where the fence is oriented in a north-south direction, is the way forward to achieve that purpose as shown in Figure 3.5.

Figure 3.5 A high-end closed bioreactor system (Schenk et al., 2008).

Making the bioreactor surface area ten times larger than the equivalent footprint area maximizes the even distribution of light intensity. The same purpose can be achieved by mounting bubble columns or plate photobioreactors at a defined angle to the sun. However, more transparent surface materials are required. Yet, making the surface to volume ratio as big as possible is the most important principle when designing a photobioreactor, as this results in shorter light path lengths and higher biomass concentrations. Mixing is another essential factor in all photobioreactors as it prevents sedimentation of the cells and supports

distribution of CO_2 and O_2 (Molina Grima et al., 1999). However, culture mixing and light attenuation have complex interactions, as each single algal cell passes through dark and light zones of the reactor in a more or less statistical manner (Barbosa et al., 2003b). Dark zones emerge by self-shading. However, there is an increased interest in the effect of flashing light (Grobbelaar et al., 1996). High light intensities result in photo-inhibition, thus microorganisms have evolved photo-protective mechanisms which dissipate excess energy as fluorescence and heat. Another interesting benefit of mixing is that it allows microorganisms to move between low light and high light regions. This cycle presents the opportunity for the energy in the photosystems to channel into the metabolic reactions during the low light phase. In these cycles the dark period was ten times longer than the light period with frequencies 10 Hz or more (Janssen et al., 2001), which has the same effect as exposing the cells to moderate light intensities (Yoshimoto et al., 2005). Other development include plastic bags mounted as annular reactors or as plate reactors (Richmond, 2004, Tredici, 2007) as well as triangle reactors as shown in Figure 3.6.

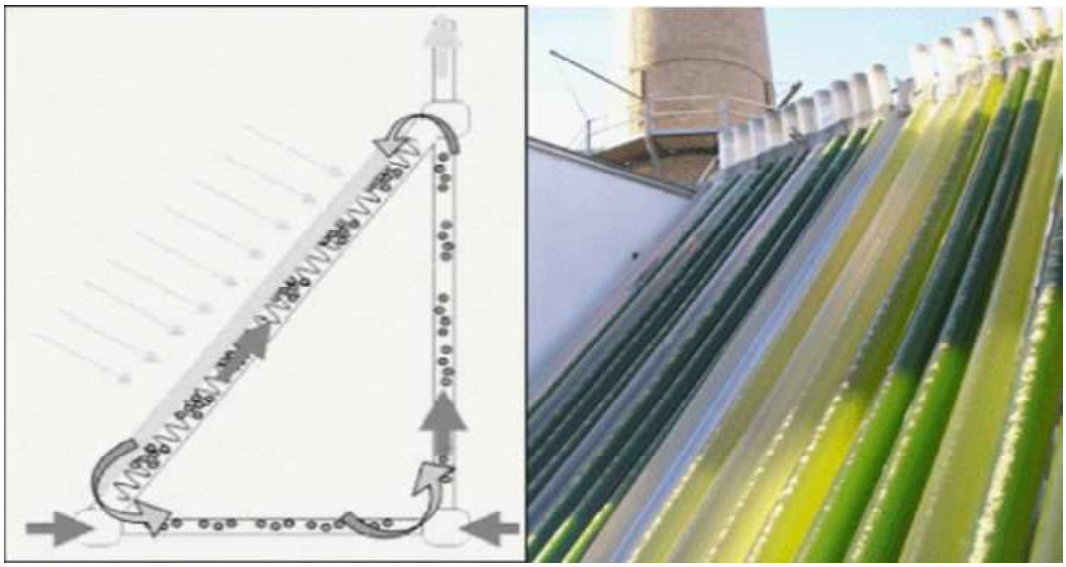

Figure 3.6 Triangle airlift bioreactor systems (Schenk et al., 2008)

Mixing is achieved by in-built static mixers. This system is one of the most productive systems ever built (Pulz, 2007). More work is underway to increase the inner surface area in thin layered reactors which would increase biomass production, decrease energy usage (Rosello Sastre et al., 2007) and enhance gas exchange by diffusion alone without more energy demand for bubbling the system. Another system uses optical fibres to channel light energy from plastic Fresnel lenses to a lump reactor (Schenk et al., 2008).

There is a new technology that proves beneficial in splitting the infra-red from the solar radiation. This development could reduce the problem of overheating in the reactor, by using this wasted heat energy to produce electricity (Schenk et al., 2008). However, this technology still needs more research to be economically available. Four commonly used bioreactor designs are shown in Figure 3.7.

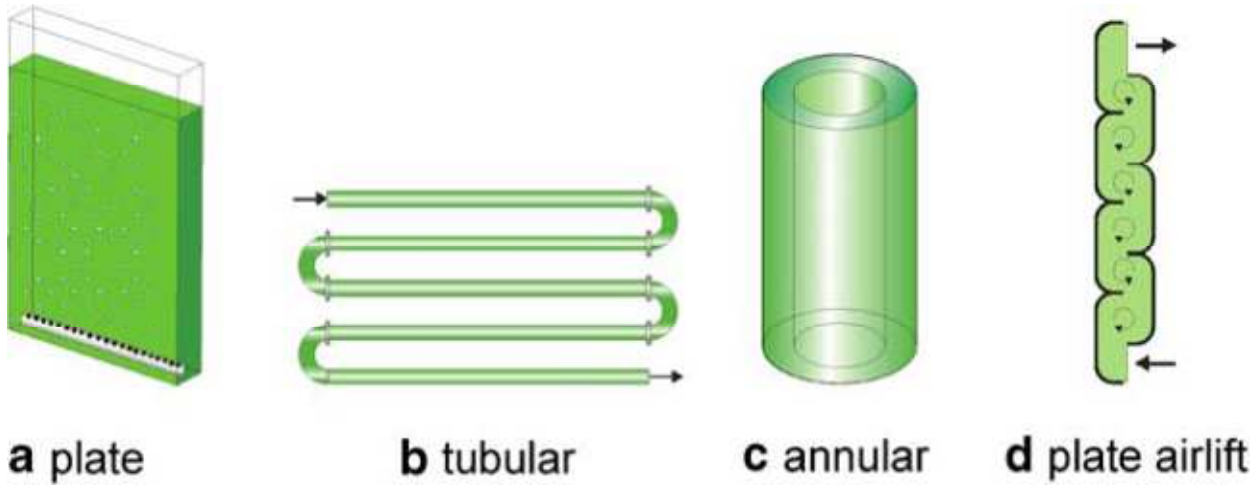

Figure 3.7 Different closed photobioreactor designs (a) plate reactor, (b) tubular reactor, (c) annular reactor, (d) plate airlift reactor with baffles.

3.1.4 Cyanobacteria Cultivation in Hybrid Systems

A combination of open ponds system and photobioreactors is the best possibility for cultivating microalgae. Using this combination would reduce the high cost of using photobioreactors alone, would avoid contamination which occurs in open ponds when used alone. In this combination, open ponds are cultivated with a desired species, which had been initially cultivated in a photobioreactor. One of the most important factors in this process is the size of the inoculum, which would ensure the dominance of the desired species. However, eventually ponds would get contaminated by one or more unwanted species and they have to be cleaned and re-inoculated. This is why hybrid systems are considered as batch cultures, and the cost of cleaning has to be added to the total cost when establishing these systems. *Haematococcus pluvialis* was cultivated in Aquasearch (Hawaii, USA) for the production of astaxanthin. Firstly *Haematococcus pluvialis* was produced in photobioreactors with sufficient nutrients to motivate high cell densities, then a portion of this culture was transferred to open ponds with limited nutrients, as these conditions would motivate the production of astaxanthin. When astaxanthin concentration peaked, *Haematococcus pluvialis* was harvested, and the pond flushed and re-cultivated (Huntley and Redalje, 2007). This approach could be used for the production of biofuels. This is because when transferring the culture into limited-nutrients conditions, microalgae quickly begin to switch solar energy into chemical energy that is stored as lipids, which are very important components for biofuel production. The mechanism of storing chemical energy as lipids enables the survival of microorganisms.

For large scale microalgae biofuel production, a series of photobioreactors of increasing size is to be used, where smaller bioreactors have to be sealed strictly to avoid contamination, however, moving up to bigger reactors, less restrictions are required to avoid contamination, as long as there is a continuous supply of inoculum, which enable re-inoculation if contamination occurs at any stage. As the bioreactors increase in size, the cost should be minimised by reducing the level of complexity.

In this work cyanobacteria will be cultivated in a relatively new photobioreactor, namely the tubular baffled photo bioreactor (TBPBR). The motivations for selecting TBPBR are that it provides uniform and consistent mixing throughout the system, ensuring a constant fluid mechanical condition and environment for cell cultivation; it enables light sources to be evenly planted within the TBPBR, providing a uniform light distribution throughout the reactor; it offers enhanced mass transfer rates due to much smaller and even bubble size distribution and significantly higher gas hold up, facilitating better cell growth; the continuous operation allows the effect of plug flow characteristics on cell growth to be examined.

CHAPTER 4 EXPERIMENTAL SETUP AND PROCEDURES

This chapter describes the cyanobacterial species, media and culture conditions that have been used throughout the project; the design of the light boxes; the design of the tubular baffled photobioreactor (TBPBR); and the experimental and analytical procedures for both systems.

4.1 Microorganisms, Media and Culture Conditions

The two different strains of microorganisms used in this work were *Gloeothece membranacea* (CCAP 1430/3) and *Oscillatoria amoena* (CCAP 1459/39) and were supplied by the Culture Collection of Algae and Protozoa, Scotland, UK (CCAP). The former is unicellular and showed significant nitrogenase activities during dark time (Klipp et al., 2005), and the latter is filamentous and showed higher rates of hydrogen production than *Anabaena cylindrica*, which is a very well-studied species for hydrogen production as stated by Hitoro et al., (1995).

BG11 medium was used to cultivate the cells and consisted of: (gL^{-1}):

$NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.075; $CaCl_2.2H_2O$, 0.036; Citric acid, 0.006; Ammonium Ferric Citrate Green, 0.006; $EDTANa_2$, 0.001; Na_2CO_3, 0.02; Trace metal solution, 1 ml; Distilled water, to 1 L. The pH was adjusted to 7.1 with the addition of NaOH (0.1M).

The trace metal solution contained (g/l^{-1}): H_3BO_3, 2.86; $MnCl_2.4H_2O$, 1.81; $ZnSO_4.7H_2O$, 0.222; $Na_2MoO_4.2H_2O$, 0.390; $CuSO_4.5H_2O$, 0.079; $Co(NO_3)2.6H_2O$, 0.0494.

Cells were cultured in a number of Erlenmeyer flasks, beakers and cylinders with different volumes of BG11 depending on the vessel size. All cultures were incubated at room temperature (25°C) in the light boxes. Media and containers were sterilized by autoclaving at 121°C for 15 mins before inoculating with culture of cyanobacteria. The cultures were mildly shaken by hand on alternative days.

4.2 Light Boxes Design and Measurements

Three light boxes were designed for this study with different light intensities. These were achieved using different cool white fluorescent tubes (Philips) in each light box to give light intensities of 3769, 6697, 9774 Lux respectively. The light intensity was measured using a light sensor (LS-DIN) supplied from Vernier, USA.

The growth of an organism that carries out photosynthesis depends on visible light with wavelengths between 400-700 nm. The photosynthetically active radiation (PAR) could be used as a measurement for the potential growth of cyanobacteria and is defined as the number of moles of photons of visible light available for organisms per squared meter per second. The PAR was measured using a PAR meter (Skye, ref No 98/065)

Each light box was fitted with a fan to make sure that it was well ventilated, a thermometer to measure the temperature, and a timer to control the light cycle.

Figure 4.1 shows the three light boxes with the lowest light intensity on the right (3769 Lux, 100 μmol m^{-2} sec^{-1}), the medium light intensity in the middle (6697 Lux, 250 μmol m^{-2} sec^{-1}) and the highest light intensity on the left (9774 Lux,.520 μmol m^{-2} sec^{-1}).

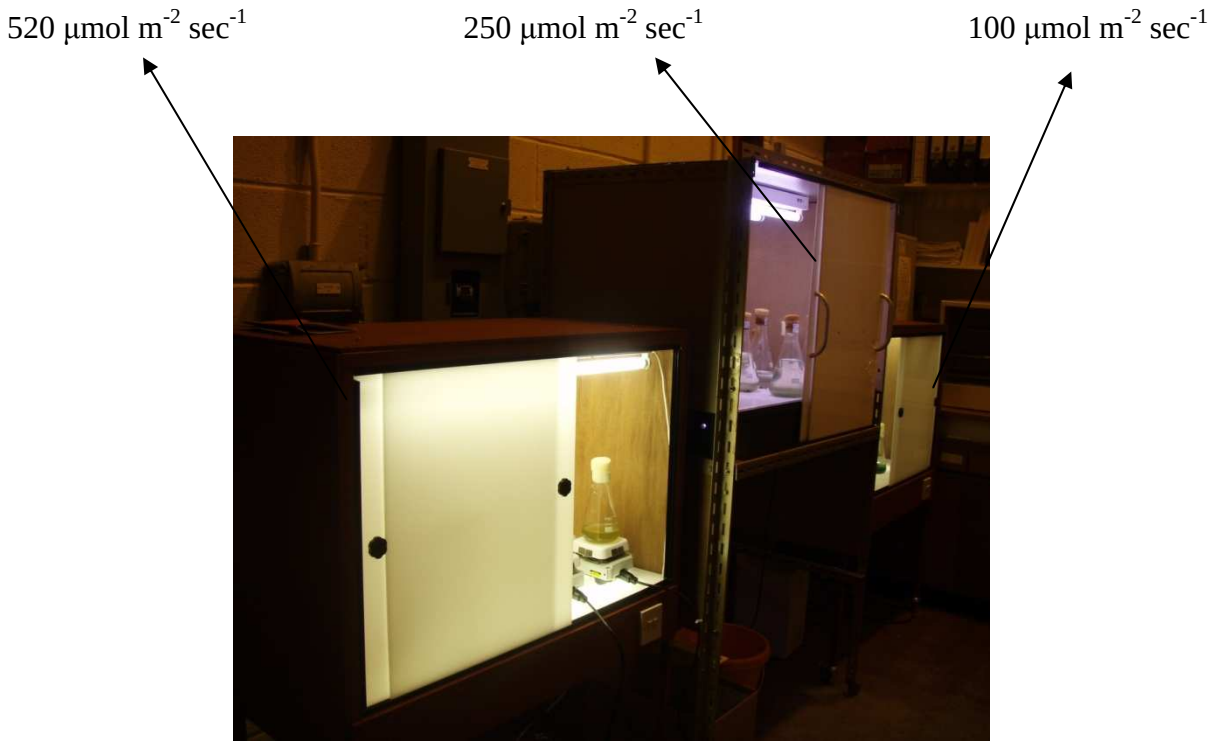

Figure 4.1 The three light boxes used in the project

4.3 Design of the Tubular Baffled Photobioreactor TBPBR

A new tubular baffled photobioreactor (TBPBR) was constructed and consisted of an over 3m long double pass glass tube with equally spaced orifice baffles. The baffles were made of PTFE, spaced 1.8 times the tube diameter with a reduction ratio of 22%. The volume of the TBPBR is 0.0038 m^3.The lights are supplied internally by placing 6 white LEDs regularly around the surface of each baffle. The reason for using internal illumination is that the external solar radiation is much more difficult to control (Molina Grima et al., 1999). White light has been chosen as it gave the highest growth based on the data generated from the experiments in the light boxes. The number of the LEDs was decided after a few experiments had been conducted to measure the PAR that the LEDs delivered (the results are not shown). Initially, two baffles with 10 white LEDs on each were designed; by varying the current (1-20 mA) and measuring the PAR at different positions between the two baffles, six LEDs were found to be optimal for providing light intensities in the range of 20- 200 μmol m^{-2} sec^{-1}, while minimizing the temperature rise of the media within the system.

The design allows the study of the effects of light intensity and light duration on the growth profiles for the species of the microorganisms chosen for the project. The cyanobacteria and the media are pre-mixed in a feed tank of 25 L in volume. A pump is used to deliver the media from the tank into the TBPBR. Flow rates of 1-3 (L min^{-1}) can be achieved (Re=500-1580), giving uniform mixing between the cells and the media without the need of oscillation at such flows (patent from NiTech US2010/0124145). The aqueous media are then returned to the feed vessel, where the removal of air is taking place. The growth will be measured using the spectrophotometer that was also used in the light boxe experiments.

The TBPBR was vertically positioned as it increased the air/CO_2 residence time in the medium, which enhance the carbon dioxide consumption efficiency (Ono, 2004). Table 4.1 shows the specifications which were used in the TBPBR. Aeration in the first runs was only achieved by circulating the medium through the TBPBR, as this process helped in releasing harmful oxygen from the medium, while the big ratio of aeration surface over culture volume in the vessel (97 m^2/ m^3) enhanced CO_2 dissolution in the medium. In the later runs aeration was also achieved by bubbling air through only the right side of the TBPBR. The temperature of the medium in the reactor was controlled using an automatic water bath which was placed underneath the bend of the TBPBR and thermometer was used to monitor the temperature each other day (The water bath and the thermometer are not shown in the photo of the TBPBR)

Table 4.1 The dimensions of the TBPBR

ID (m)	0.04
Length of the bioreactor excluding bends (m)	3
Length of the bend (m)	0.37
Total length of the bioreactor (m)	3.37
The number of the baffles	45
The diameter of the baffle (m)	0.038
The diameter of the orifice (m)	0.02
The distance between two baffles (m)	0.072
The number of LEDs on each baffle	6
The volume of the TBPBR (m^3)	0.0038
The surface area of the TBPBR (m^2)	0.423

Figure 4.2 shows a schematic diagram of the TBPBR set up.

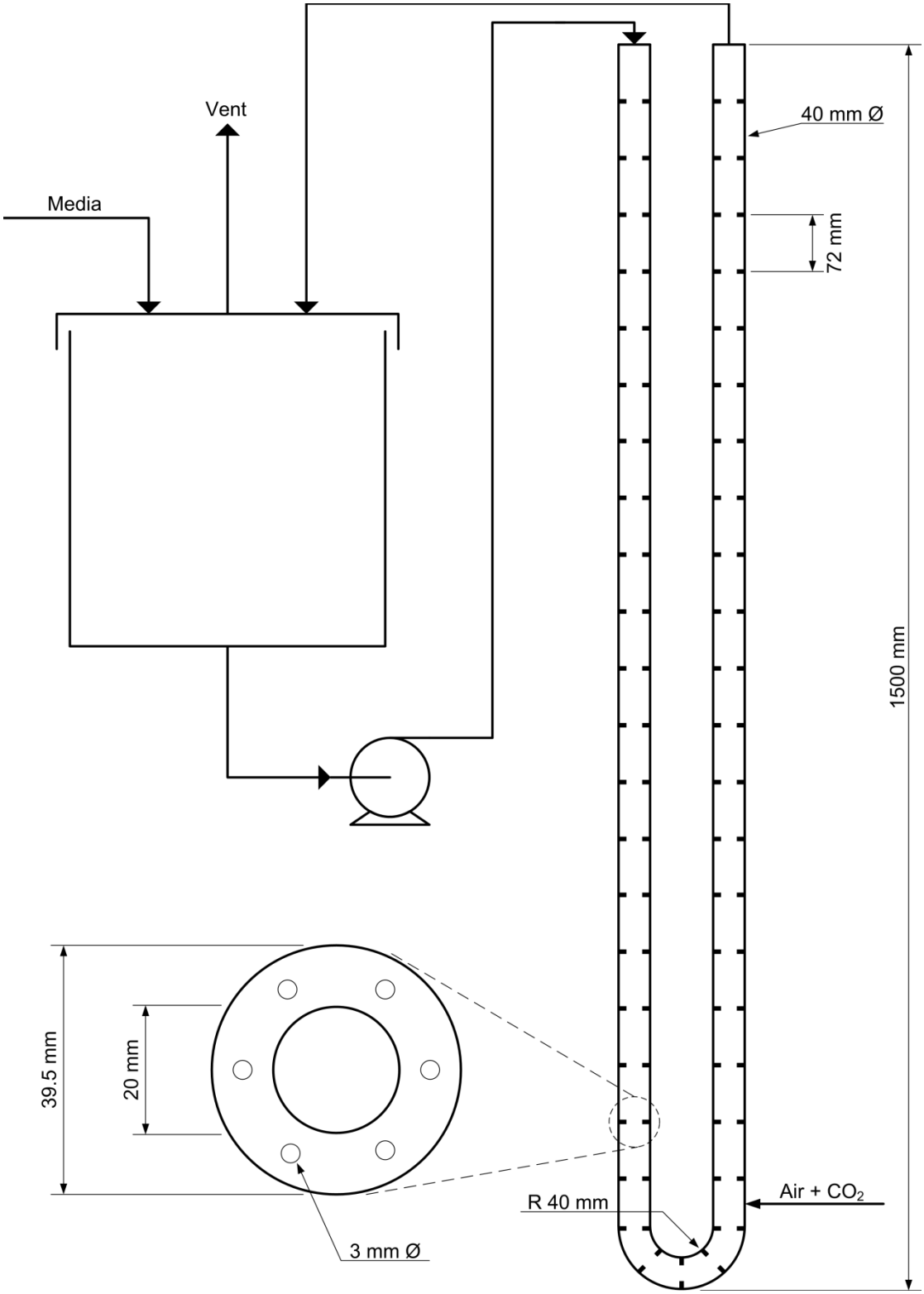

Figure 4.2 The set up of the tubular baffled photo bioreactor (TBPBR) and the baffle with the LEDs (on the top left corner)

Figure 4.3 shows a photo of the TBPBR.

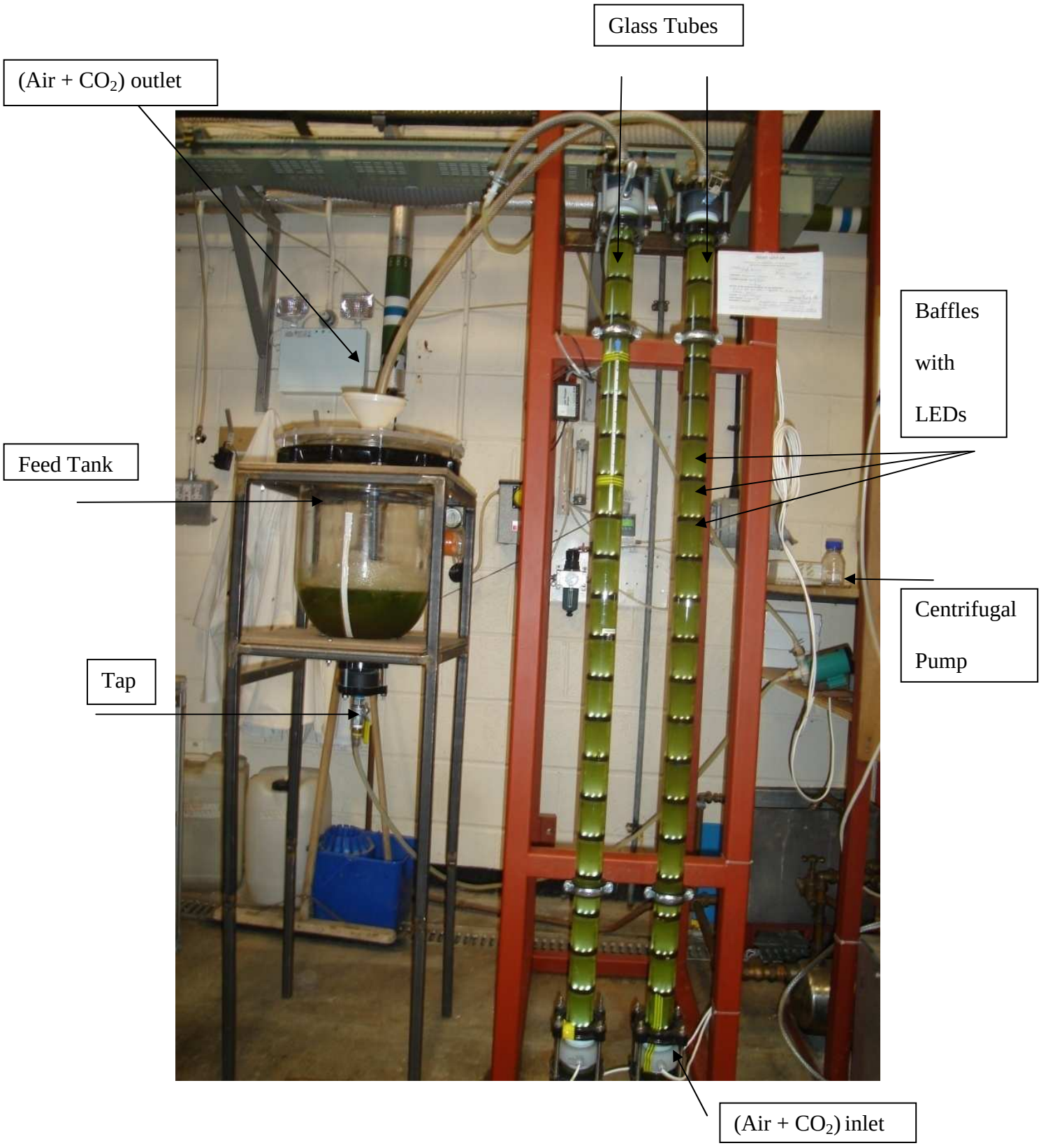

Figure 4.3 The tubular baffled photobioreactor (TBPBR)

4.4 The Experimental and Analytical Procedures

4.4.1 Cultivation of the Microorganisms in the Light Boxes

The procedure for preparing the growth culture is as follows:

- To a suitable container add a desired amount of BG11 medium (suitable containers are those which can be autoclaved);
- Put a sponge stopper at the top of the container and cover it with foil. Stick some autoclave tape on the container and label the tape;
- Wrap several pipette tips in foil;
- Autoclave the containers & pipette tips;
- Allow the medium to cool to ambient temperature, preferably overnight;
- Ignite a Bunsen burner. Whenever a sterile container is opened it is important to pass the Bunsen flame over the opening of the container to stop foreign organisms contaminating the culture;
- Now, use the sterile pipette tips, add some concentrated cyanobacteria culture to the media;
- Measure the optical density of the new culture;
- If a specific optical density is required, more medium or culture can be added and the optical density is measured till the desired one has been obtained;
- Place the new culture in the light box;
- Measure the optical density at regular intervals, typically every 24 hrs.

4.4.2 Cultivation of the Microorganisms in the TBPBR

While there were three light boxes used for batch trials, there was only one TBPBR available for the continuous cultivation. Since the specific growth rate of cyanobacteria in the TBPBR was not quite fast in comparison to that in the light boxes, a run of four weeks was chosen as the cultivation period in this research, which was based on many experimental runs in the light boxes. In order to investigate the effects of each parameter on the growth of cyanobacteria, a total of 12 weeks was needed to cover all operational parameters, as these were done in the light boxes.

It was well noted during the experiments in the light boxes that the cyanobacteria were not easily contaminated. Runs of non-autoclaved and autoclaved cultures were carried out to compare the growth and kinetics of both species. Results showed that the growth rate and generation times were exactly the same (results are not shown). Thus, it was decided that cultures growing in the TBPBR will not be autoclaved. Nevertheless, precautions were taken to prevent any contamination where possible.

The procedure for preparing the growth culture in the TBPBR is as follows:

- Prepare 5L of BG11 media as in 4.1;
- Close the tap underneath the feed tank in the TBPBR, as shown in Figure 4.3;
- Pour the media into the vessel;
- Add some concentrated cyanobacteria culture to the media;

- Shake the vessel well to achieve mixing;
- Measure the optical density of the new culture;
- If a specific optical density is required, more medium or culture can be added and the optical density is measured till the desired one has been obtained;
- After achieving the desired optical density, open the tap underneath the vessel;
- Switch the pump on;
- Put a container underneath the port at the top of the reactor, then open the port to release the air that has been trapped inside the reactor, then close the port;
- Switch on the LED's and set the timer;
- Switch the water bath and set to 25 °C (not shown in Figure 4.3);
- Measure the optical density at regular intervals, typically every 24 hrs for four weeks.

4.4.3 Optical Density Measurement for Cell Mass

The procedure of measuring optical density was based on the work of Yan et al. (1997) and consisted of:

- Shake the culture flask until a uniform green solution has been obtained;
- Take 1 ml sample using a sterilized pipette tip;
- Transfer the sample to a 1.6 ml cuvette;
- Using the spectrophotometer as shown in Figure 4.4 measure the optical density of the sample at 750nm;
- Plot the absorbance vs. time to obtain a growth profile.

4.4.4 Dry Weight measurement

A correlation between absorbance and cell concentration had to be developed by measuring the absorbance of a sample, then measuring the dry weight of a known volume of the sample. The procedure was based on the work by Rand et al. (1976) and is described below:

1) Wash the GF/C 70 mm filter paper with 30 ml distilled water in the vacuum flask apparatus to remove any lose fibres
2) Dry the paper in an oven for 1 hour at 100 °C
3) Remove it and leave it to cool
4) Weigh it using a balance that is accurate to 4 decimal places
5) Filter a known volume of solution through the filter paper as per 1
6) Dry the filter paper as per 2
7) Weigh it as per 3
8) Calculate the cell concentration using the formula below

Cell concentration = Difference in Weight / Volume of sample

4.4.5 Optical Density Measurement

The following is the procedure of using the spectrophotometer CAMLAB DR/4000 U (Figure 4.4) to measure the optical density:

- Switch the spectrophotometer on and wait until it has finished its start-up routine

- Press single λ (the wavelength)
- Next press goto λ;
- Input the desired wavelength and press enter
- press setup;
- Press avg x (this sets the average number of readings to 25 times, considerably increasing the accuracy of readings)
- Input 25 then press enter
- Press exit to leave the setup menu
- Insert the cuvette that contains the blank (distilled water) into the spectrophotometer compartment;
- press zero;
- Next, replace the blank with the sample
- Press read, that will give the measurement of the optical density.

Figure 4.4 The spectrophotometer (CAMLAB DR/4000 U)

4.5 The Calibration Curves of Optical Density versus Dry Weight

Optical density (OD) measurements are usually used to track the growth of light-harvesting and photosynthetic microorganisms. To verify the validity of these measurements and establish the relationship between the measurements (OD@680 nm) and cell concentration (mg l^{-1}), dry weight measurements as described in section 4.4.4 were taken and the calibration curves of the optical density (OD@680 nm) versus dry weight or cell concentration (mg l^{-1}) for both *Gloeothece membranacea* and *Oscillatoria amoena* were obtained. The zero points were taken as BG11 media without cells in the spectrophotometer. Following the procedure in 4.4.3 and 4.4.4 the calibration curves were obtained for *Gloeothece membranacea* (Figure 4.5) and *Oscillatoria amoena* (Figure 4.6).

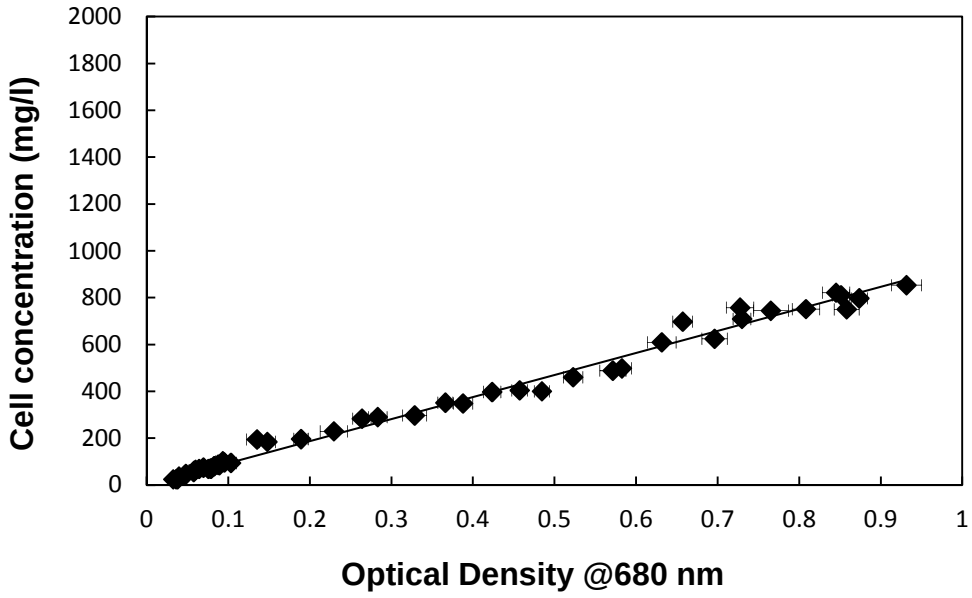

Figure 4.5 The calibration curve of optical density versus cell concentration for *Gloeothece membranacea*

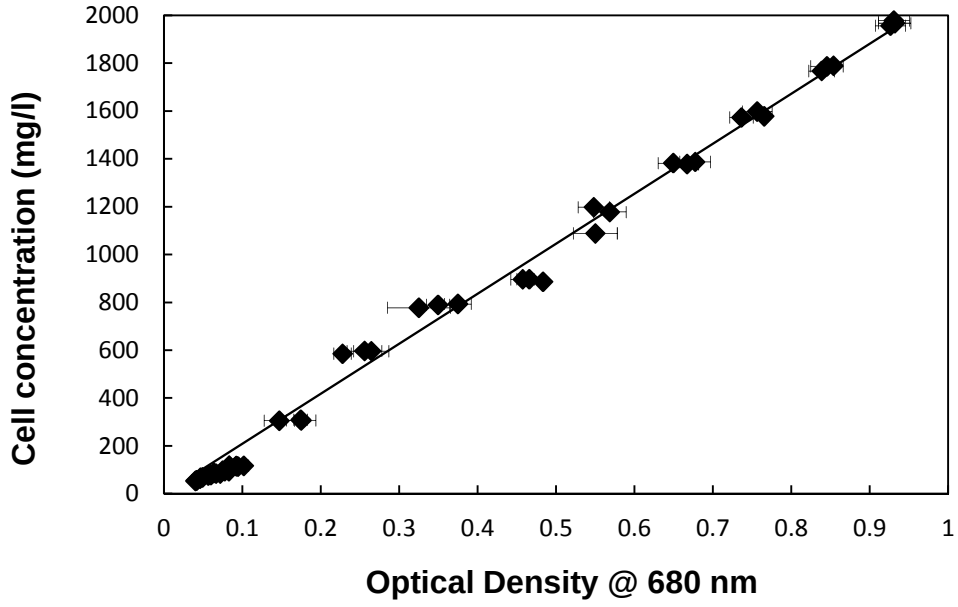

Figure 4.6 The calibration curve of optical density versus cell concentration for *Oscillatoria amoena*

For both *Gloeothece membranacea* (Figure 4.5) and *Oscillatoria amoena* (Figure 4.6), the graphs show a linear relationship between the absorbance and the cell concentration, which is expected. It should be noted that the slope of the curve of *Oscillatoria amoena* (Figure 4.6) is much greater than that of *Gloeothece membranacea* (Figure 4.5). This indicates that the two different species have different abilities to absorb light. At the same cell concentration the unicellular species, *Gloeothece membranacea*, absorbs more light than the filamentous one, *Oscillatoria amoena*. This difference in the ability of absorbing light is comprehensible, agreeable with the literature (Yoon et al., 2002), and could be explained by the fact that different species have different light harvesting pigments, hence different abilities to absorb light according to the original environment of the species.

Nonetheless, having different abilities of light absorbance for the two different species does not affect the accuracy of the method, as the results for each species were analysed independently.

CHAPTER 5 THE CULTIVATION OF *GLOEOTHECE MEMBRANACEA* AND *OSCILLATORIA AMOENA* IN THE LIGHT BOXES

This chapter presents the results obtained and discusses the effects of various operational parameters on the cell growth of *Gloeothece membranacea* (CCAP 1430/3) and *Oscillatoria amoena* (CCAP 1459/39) in the light boxes. These results would be considered as benchmarking data for the experiments in the tubular baffled photobioreactor (TBPBR). It should be noted that the purpose of the trials was to identify the optimal environmental conditions for cultivation of the chosen cyanobacteria, whether they are used as the raw material for either biodiesel production or hydrogen generation.

5.1 Growth Cycle of Populations

There are generally four stages in the growth profile of a batch culture after inoculation into a fresh culture medium:

a) Initial lag phase
b) Exponential phase where growth commences
c) Stationary phase where essential nutrients are depleted or toxic products build up and growth ceases
d) Death phase: if incubation continues, cells may begin to die

Figure 5.1 shows the typical growth curve for a bacterial population (Madigan et al., 2000).

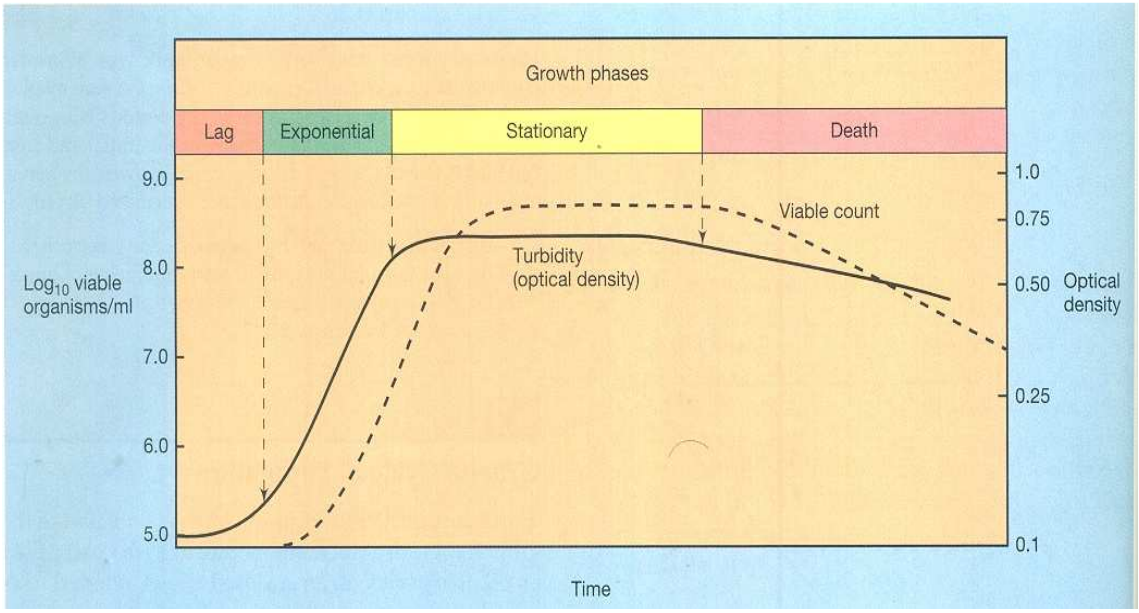

Figure 5.1 Typical growth curve for a bacterial population (Madigan et al., 2000).

5.1.1 Lag Phase

Growth does not usually start instantly when inoculating microorganisms into a fresh medium, but only after a delay in time called lag phase, which may be short or long according to the growth parameters and the background of the culture. If the inoculum is taken from a culture in its stationary phase and inoculated in the same culture, a lag phase generally takes place even if all the cells are viable and able to duplicate. This happens because the cells are usually deprived of some necessary constituents and their re-

synthesising will take time. A lag phase is also observed when the cells are damaged (but not killed) by heat, radiation, or toxic chemicals because time is required for the harm to be repaired. However, if the inoculum is taken from an exponentially growing culture, a lag phase does not occur and exponential growth commences at once.

5.1.2 Exponential Phase

Usually the cells are the healthiest at this stage, thus they are desirable for studies of enzymes, other cell constituents and kinetics. The rate of the exponential growth varies greatly from different species and is affected by surroundings conditions such as temperature, culture medium, as well as by genetic properties of the organism itself.

5.1.3 Stationary Phase

What usually occurs in a batch culture is that either one of the nutrients is deprived or some waste product builds up in the medium. At that point the exponential growth stops and the population has reached the stationary phase. During the stationary phase, growth does not usually occur; however, many cell functions may continue. For some species, some cells grow while others die, the two processes are balanced out so there is no increase or decrease in cell numbers. However, as many bacterial cells in nature are in a non-growing or a very slow growing state, many genes have evolved to deal with conditions.

5.1.4 Death Phase

After the culture reaches the stationary phase, the cells either continue to function and stay alive or die. In some cases cell lysis may occur. Both the growth phase and the death phase are exponential; however, the exponential specific growth rate is much faster than the exponential death rate.

It should be emphasized that the phases of the bacterial growth (lag phase, exponential phase, stationary phase and death phase) are reflections of the events in a population of cells, not in individual cells.

5.2 Growth Profiles of *Gloeothece membranacea* and *Oscillatoria amoena*

The growth and kinetic profiles for both *G. membranacea* and *O. amoena* under the environmental conditions recommended by the Culture Collection of Algae and Protozoa, Scotland, UK (CCAP), the institution where they obtained from, would be considered as benchmarking results through this research. These two experiments were run for 2000 h to give a sufficient amount of time for the study of the growth cycles of these two species.

Figure 5.2 shows a photo of the two species in the light box and Table 5.1 lists the growth conditions for the cultures.

Table 5.1 The growth conditions of the cultures

Strain	*Gloeothece membranacea*, *Oscillatoria amoena*
Light Intensity	520 µmol m^{-2} sec^{-1}
Wavelength	White
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 200 ml
Culture Volume	100 ml
OD	0.030, 0.030
Light Position	External
Gas composition	Air
Aeration S/V 1/m	44.2

Gloeothece membranacea

Oscillatoria amoena

Figure 5.2 The light box with the two species

Figures (5.3 - 5.4) show the growth profiles for *Gloeothece membranacea and Oscillatoria amoena* respectively.

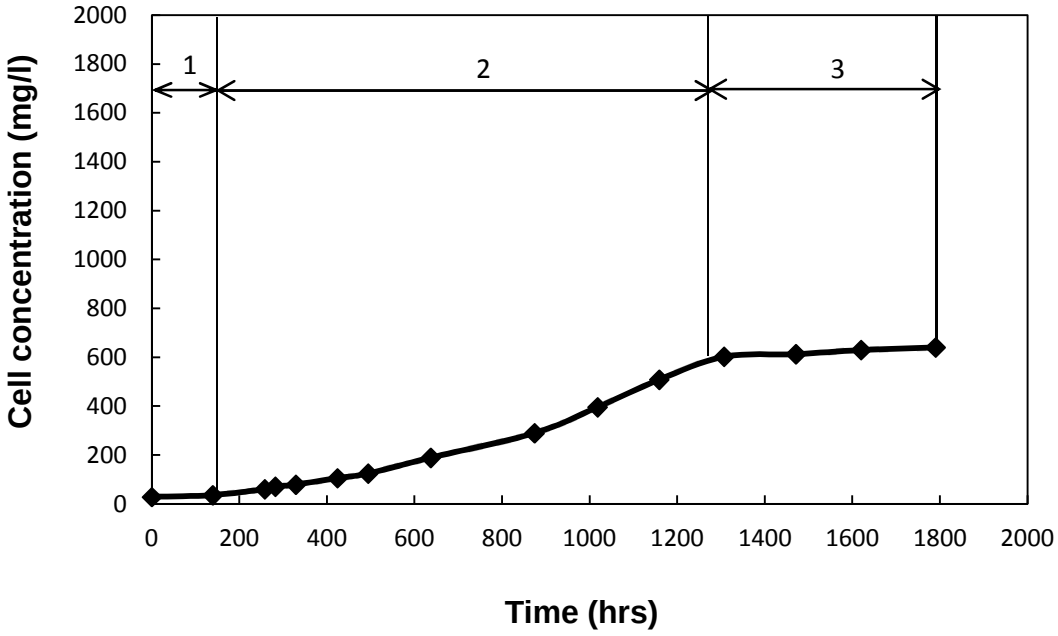

Figure 5.3 Growth profile for *Gloeothece membranacea*

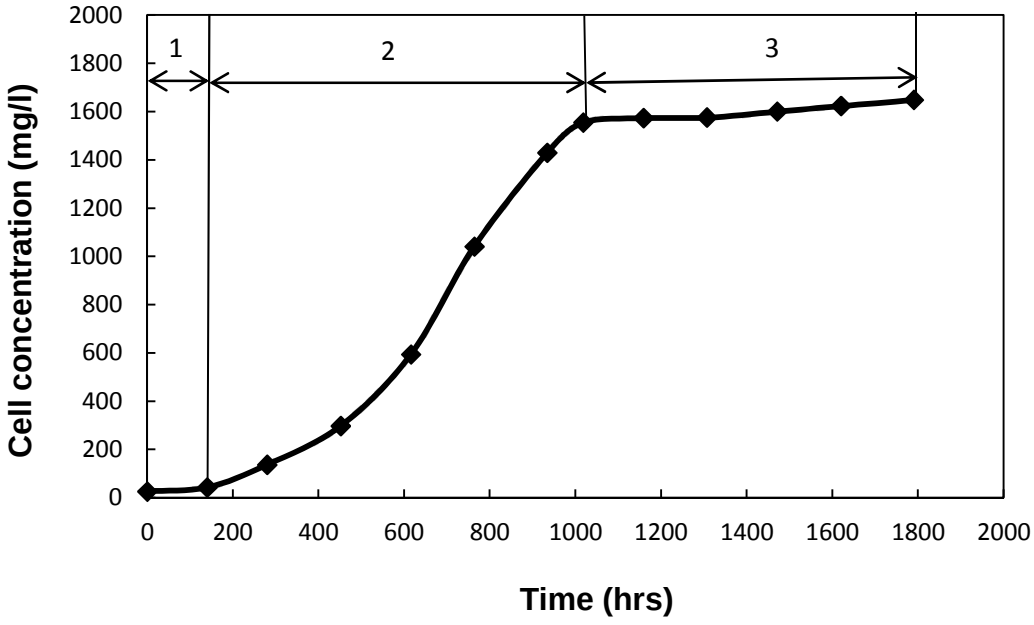

Figure 5.4 Growth profile for *Oscillatoria amoena*

In order to establish a reasonable scientific methodology of differentiating phases, the following procedure was proposed and used throughout this thesis. It should be noted that the method chosen could only be made more accurate by making the interval of data collection smaller. This was not possible during this work. It can be seen that the cell concentrations increased with time for both species, three typical distinguished phases can be identified:

- The lag phase (denoted as 1 in Figures 5.3 - 5.4). During the lag phase cell concentrations do not increase with time. The change in cell concentrations over time between 0 and 140 hrs would be considered as the baseline for the lag phase as there was little change in the cell concentration over that period of time. So the lag phases for both *Gloeothece membranacea* and *Oscillatoria amoena* are around 140 hours.

- The exponential phase (denoted as 2 in Figures 5.3 – 5.4) - where cells start to duplicate. During the exponential phase cell concentrations increase exponentially with time, thus there is a linear relationship between the log of the cell concentration and time. Figure 5.5 illustrates that the cell concentrations of *Gloeothece membranacea* increased exponentially between 140 and 1300 hrs because the relationship between ln C and t is linear, while Figure 5.6 shows that the concentrations of *Oscillatoria amoena* increased exponentially between 140 and 1100 hrs because for the same reason. Therefore, the exponential phases for *Gloeothece membranacea* = 1300 -140 = 1160 (hrs) and for *Oscillatoria amoena* = 1100 – 140 = 960 (hrs).

- The stationary phase (denoted as 3 in Figures 5.3 – 5.4) - where either one of the nutrients is deprived or some waste product builds up in the medium. At that point the exponential growth ends and the population has reached the stationary phase, i.e. during the stationary phase cell concentrations do not increase with time. Figures 5.3 – 5.4 indicate that the cell concentrations of *Gloeothece membranacea* did not increase after 1300 hrs, while the cell concentrations of *Oscillatoria amoena* did not increase after 1100 hrs. This implies that the stationary phases for *Gloeothece membranacea* and *Oscillatoria amoena* are reached after 1300 and 1100 hours respectively.

It should be noted that the death phase was not reached in these experiments as obviously this would have taken much longer period of time, which was not feasible during the length of the project. The durations of growth phases for both species are summarised in Table 5.2.

Table 5.2 The duration of the growth cycles of the two species

Growth Phase (hrs)	Lag Phase	Exponential Phase	Stationary Phase
Gloeothece membranacea	140	1300-140 = 1160	1300
Oscillatoria amoena	140	1100-140 = 960	1100

5.3 Kinetic Parameters of *Gloeothece membranacea* and *Oscillatoria amoena*

The specific growth rates can be determined using the experimental data obtained in the exponential phase only. Sandnes et al. (2005) assumed that in the exponential multiplication stage, the cell number is given by:

$$N_t = N_0 \exp(\mu t) \quad (5.1)$$

Where N_t is the cell number at time t, N_0 the initial number of cells, and μ the specific specific growth rate ($hour^{-1}$) (Sandnes et al., 2005). Normally cell concentration ($mg\ l^{-1}$) is the preferred terminology, was obtained by dividing the cell number by volume of the bioreactor at both sides of equation (5.1),

$$N_t/V = (N_0/V) \exp(\mu t) \quad (5.2)$$

Then,

$$C_t = C_0 \exp(\mu t) \quad (5.3)$$

Where C_t is the cell concentration at time t, C_0 the initial cell concentration. The specific growth rate is obtained from a linear fit in a semi-logarithmic plot of cell concentration against time, as

$$\ln C_t = \ln C_0 + \mu t \quad (5.4)$$

The specific growth rate ($hour^{-1}$) is constant and at its maximal value for as long as the culture remains in the exponential growth phase (Sandnes et al., 2005). Thus, the specific growth rates are the slopes of Figures 5.5-5.4, the intercept at y-axis is $\ln C_0$.

The time required to duplicate the cell number, i.e. the mean generation time (t_d), is given by the following equation:

$$t_d = \ln (2 / \mu) \qquad (5.5)$$

It should be emphasised though that the kinetics were derived using the data from the exponential phases only as the Equations 5.4-5.5 are solely valid in that phase as explained earlier in the literature review. Equations 5.4 -5.5 were used to calculate the specific growth rates and the generation times of the two species throughout the thesis. By using those equations, it is assumed that the reactions which lead to the growth of the species is first order (Sandnes et al., 2005), however Figures 5.5 – 5.6 prove that the assumption is acceptable, as the results fit Equation 5.4. The results are summarized in Table 5.3 and Figures 5.5 – 5.6 displays the kinetic profiles for the two species.

Table 5.3 The kinetic parameters of the two species

	μ (hour^{-1})	t_d (hrs)	C_0 (mg l^{-1}) $_{experiment}$	C_0 (mg l^{-1})$_{graph}$	ΔC_0 (mg l^{-1})
Gloeothece membranacea	0.0025	277	28.2	27.1	1.1
Oscillatoria amoena	0.0049	141	62.7	60.3	2.4

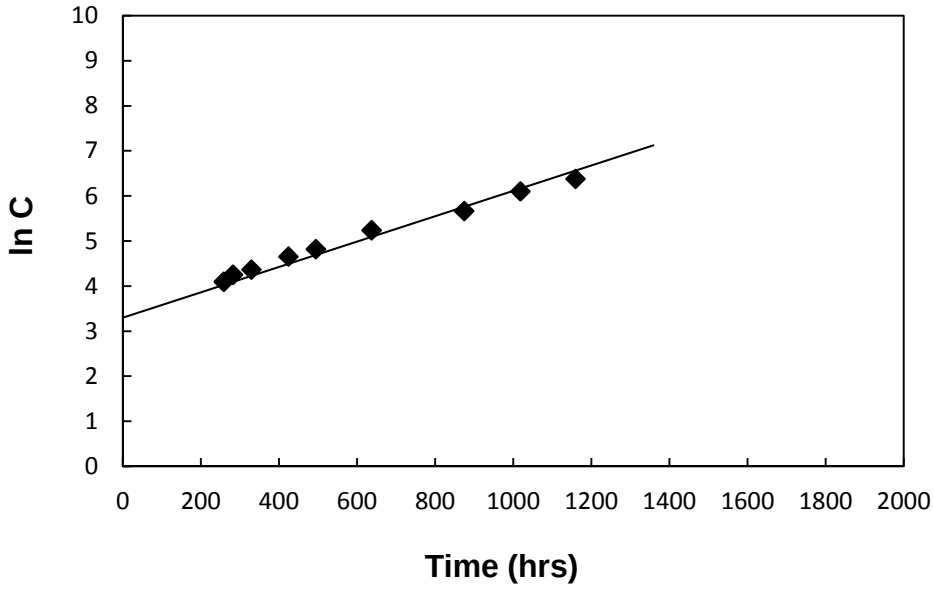

Figure 5.5 Kinetic profile for *Gloeothece membranacea*

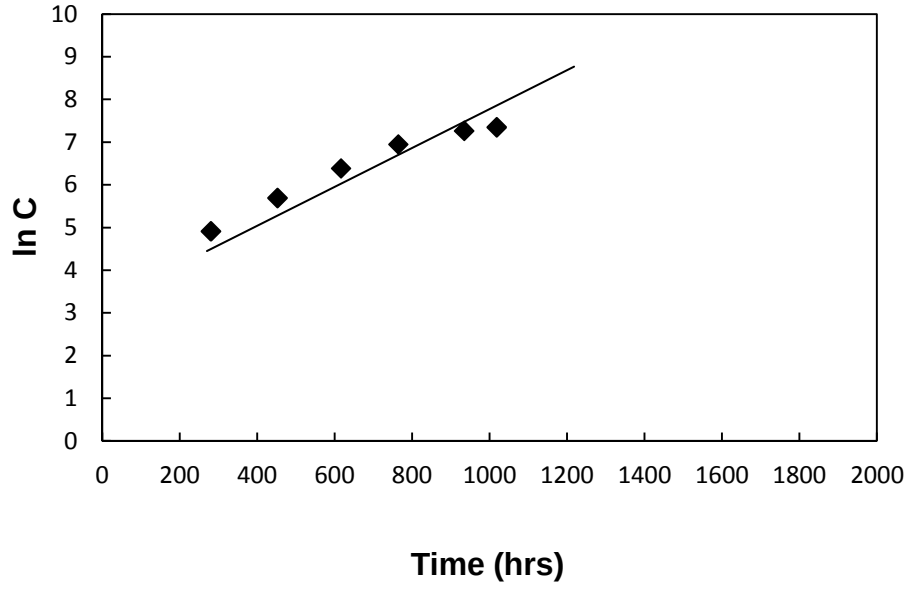

Figure 5.6 Kinetic profile for *Oscillatoria amoena*

It is clear that the filamentous species, *Oscillatoria amoena*, is growing faster than the unicellular one, *Gloeothece membranacea*, as the specific growth rate and doubling time for the former is 0.0049 $hour^{-1}$, 141 hours respectively, and for the latter is 0.0025 $hour^{-1}$, 277 hours respectively. Moreover, the lag phases for both *Oscillatoria amoena* and *Gloeothece membranacea* is 140 hours (Table 5.2), this further confirms the more rapid growth for the former than for the latter species.

When a lag phase is bypassed by taking the inoculums from an exponentially growing culture, exponential growth commences at once. This method is applied to shorten experiments periods. This methodology was adopted throughout this thesis. So based on the times of the exponential phases of the two species (1160 hours for *Gloeothece membranacea* and 960 hours for *Oscillatoria amoena*), 1000 hours were set for both the experiments in the light boxes and the tubular baffled photo bioreactor later on.

5.4 Effect of the Ratio of Aeration Surface over Culture Volume

Gas-liquid mass transfer and the subsequent cell growth rate are affected by the ratio of aeration surface over culture volume for the reason that the transport rate is a function of the amount of surface area available (Madigan et al., 2000). The bigger the ratio the more surface is accessible for the mass transfer between the air and liquid and the faster CO_2 transferred across the surface of the culture and to the microorganisms in the medium. In turn, there is a shorter diffusion route for gases which are produced as by-products like O_2. Note that O_2 has a negative impact on nitrogen-fixation processes, when O_2 is accumulated in great amounts it inactivates the nitrogenase enzyme, which is responsible for the nitrogen fixation process in species of nitrogen-fixing cyanobacteria. To examine the effect of this factor on the cell growth of *Gloeothece membranacea* and *Oscillatoria amoena*, experiments were carried out with three different surface-to-volume ratios of 9.6, 38.5, 44.2 (m^2 m^{-3}) using different containers and culture volumes. Table 5.4 lists the growth conditions for the cultures.

Table 5.4 The growth conditions for the cultures
of different ratios of aeration surface over culture volume

Strain	*Gloeothece membranacea*, *Oscillatoria amoena*
PAR	250 μmol m^{-2} sec^{-1}
Wavelength	All visible light
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Cylinder250 ml, Flask 1 L, Flask 250 ml
Culture Volume	100 ml, 500 ml, 100 ml
OD	0.030
Light Position	External
Gas composition	Air
S/V L/m	9.6,19,44.2

Figures 5.7 -5.10 show the growth and kinetic profiles for *Gloeothece membranacea and Oscillatoria amoena* respectively. Note that the results represent the exponential phases only.

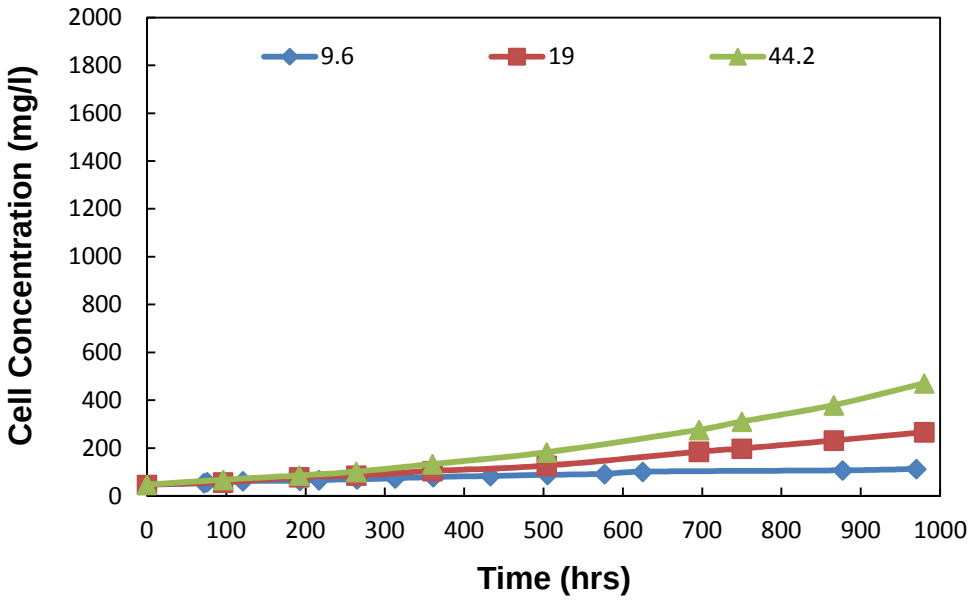

Figure 5.7 Growth profile for *Gloeothece membranacea* with different ratios of aeration surface to culture volume

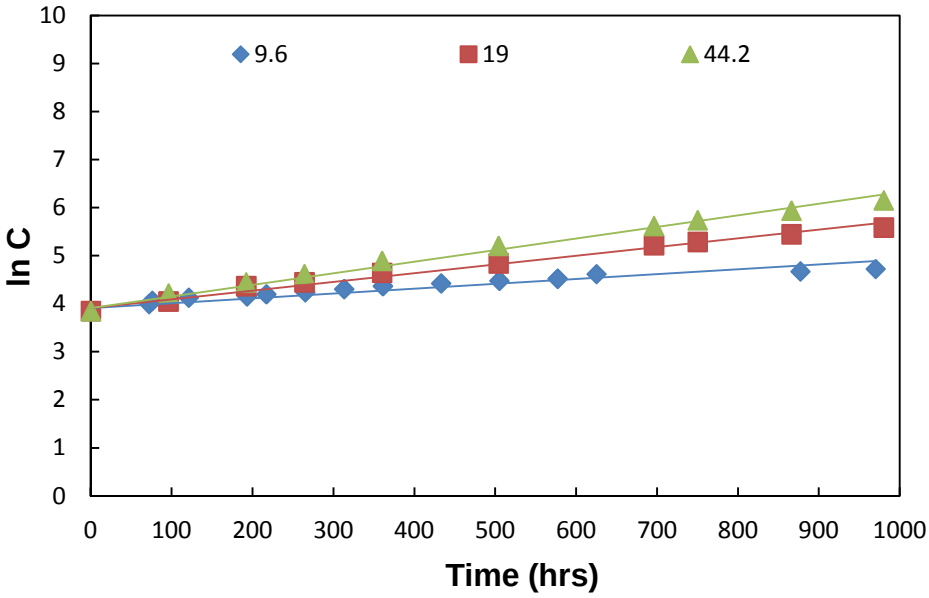

Figure 5.8 Kinetic profile for *Gloeothece membranacea* with different ratios of aeration surface to culture volume

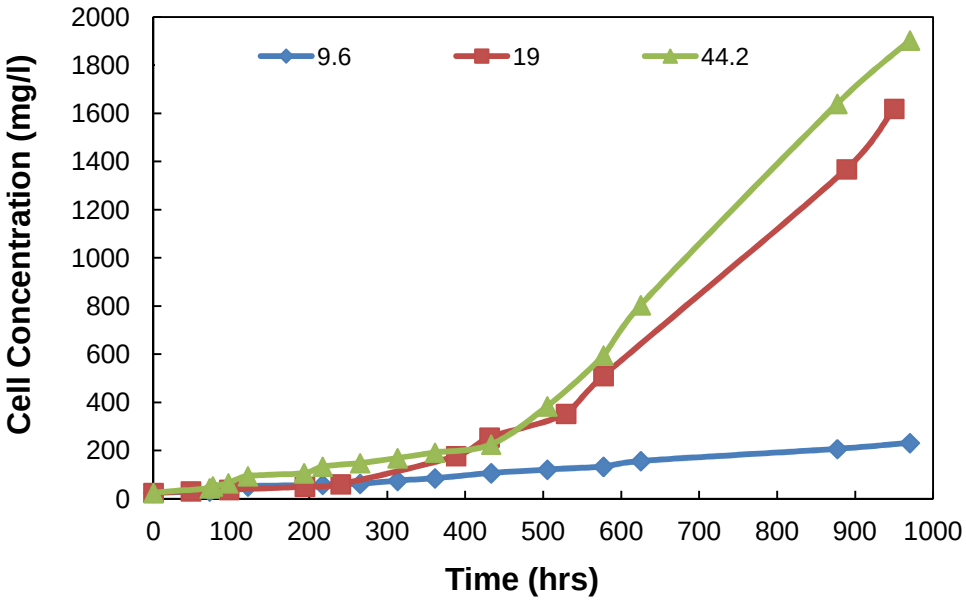

Figure 5.9 Growth profile for *Oscillatoria amoena* with different ratios of aeration surface to culture volume

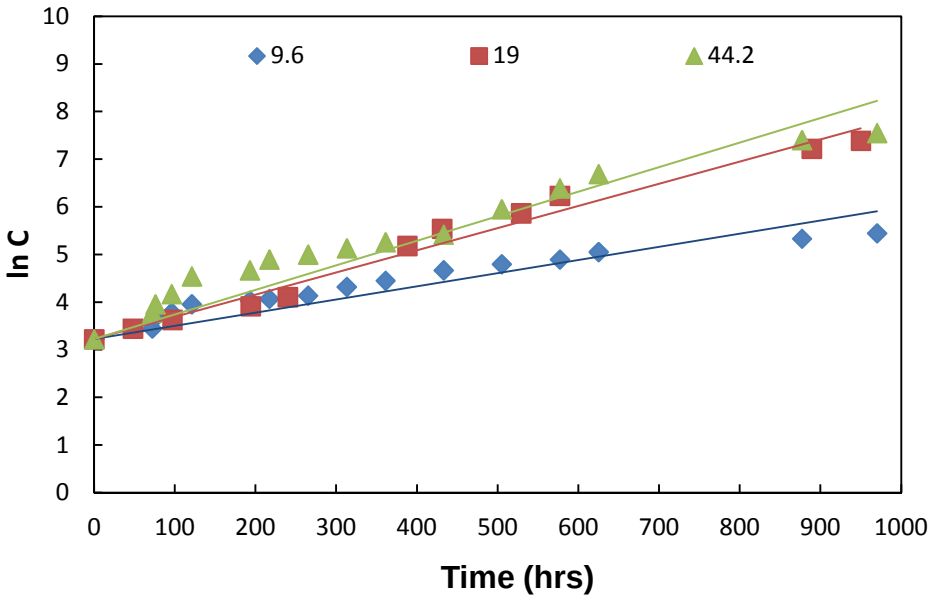

Figure 5.10 Kinetic profile for *Oscillatoria amoena* with different ratios of aeration surface to culture volume

Equations 5.4 -5.5 were used to calculate the growth rates and the generation times of the two species and the results are summarized in Table 5.5.

Table 5.5 The kinetic parameters of the two species with different ratios of surface area over culture volume

	Gloeothece membranacea			*Oscillatoria amoena*		
S/v ($m^2 m^{-3}$)	9.6	19	44.2	9.6	19	44.2
μ ($hour^{-1}$)	0.0010	0.0018	0.0024	0.0028	0.0047	0.0052
T_d (hours)	693	385	289	248	147	133

The growth and kinetic profiles for *Gloeothece membranacea* in Figures 5.7 – 5.8 and Table 5.5 suggest that the growth was better with bigger ratios of aeration surface over culture volume, as cultures with ratios of 9.6, 19, 44.2 have growth rates of 0.0010, 0.0018, 0.0024 $hour^{-1}$, and the generation times of 693, 385, 289 hours, respectively. The same result can be concluded for *Oscillatoria amoena* from Figures 5.9 – 5.10 and Table 5.5, as cultures with ratios 9.6, 19, 44.2 have growth rates of 0.0028, 0.0047, 0.0052 $hour^{-1}$, and the generation times of 248, 147, 133 hours, respectively. The results are as expected.

It is apparent that the effect of the ratio of aeration surface over culture volume on cell growth is more pronounced with *Oscillatoria amoena* than *Gloeothece membranacea*, as the doubling time, which is a distinctive character for each strain of microorganisms and it is related to the genetics of that particular strain and its natural environment, of *Oscillatoria*

amoena (133 hours) is shorter than that of *Gloeothece membranacea* (289 hours). This could be due to the fact that *Oscillatoria amoena* is filamentous while *Gloeothece membranacea* is unicellular; the ratio of aeration surface over culture volume has more impact on filamentous species than on unicellular ones.

The outcome of the experiments indicates that the bigger the ratio of surface over volume, the faster the cell growth. The ratio of the surface area over culture volume in the TBPBR is 97 (m^2 m^{-3}), which is superior to the ratios used in the light boxes. This in turn supports the idea of using the TBPBR to grow these two species.

5.5 Effect of Light Intensity

Light provides the energy source to the growing culture, and is crucial to the photoautotrophic process. Increasing light intensity has been shown to affect nutrients uptake, which in turn affects the photosynthesis (Hu et al., 2000). Live vegetative cells and heterocysts show a peak, either in numbers or as a percentage of the total cells, at the saturation light intensity and decrease at lower and higher intensities (Lee and Rhee, 1999). On the other hand, too much light intensity can cause photo inhibition (Ibelings, 1996). Photo-oxidative stress is introduced in cyanobacteria due to the absorption of excess light that cannot be used productively for photosynthesis. These photo-oxidative conditions lead to deactivation of superoxide dismutase (SOD) that absorbs harmful oxygen.

To examine the effect of this factor on the cell growth of *Gloeothece membranacea* and *Oscillatoria amoena*, experiments were carried out at three light intensities:

The lowest light intensity = 3769 Lux, PAR=100 μmol m^{-2} sec^{-1};

The medium light intensity = 6697 Lux, PAR 250 μmol m^{-2} sec^{-1};

The highest light intensity = 9774 Lux, PAR 520 μmol m^{-2} sec^{-1}.

Table 5.6summarizes the growth conditions for the cultures of different light intensities, and Figure 5.11 shows the light boxes with the cultures of different light intensities. Note that the culture volumes remain constant.

Table 5.6 The growth conditions for the cultures of different light intensities

Strain	*Gloeothece membranacea, Oscillatoria amoena*
Light Intensity	100, 250, 520 μmol.m^{-2}.sec^{-1}
Wavelength	All visible light
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 250 ml
Culture Volume	100 ml
OD	0.050
Light Position	External
Gas composition	Air
S/V l/m	44.2

Figure 5.11 Light boxes with the cultures at different light intensities

Figures 5.12 – 5.15 show the growth and kinetics profiles for *Gloeothece membranacea and Oscillatoria amoena* respectively. Note that the results represented the exponential phases only.

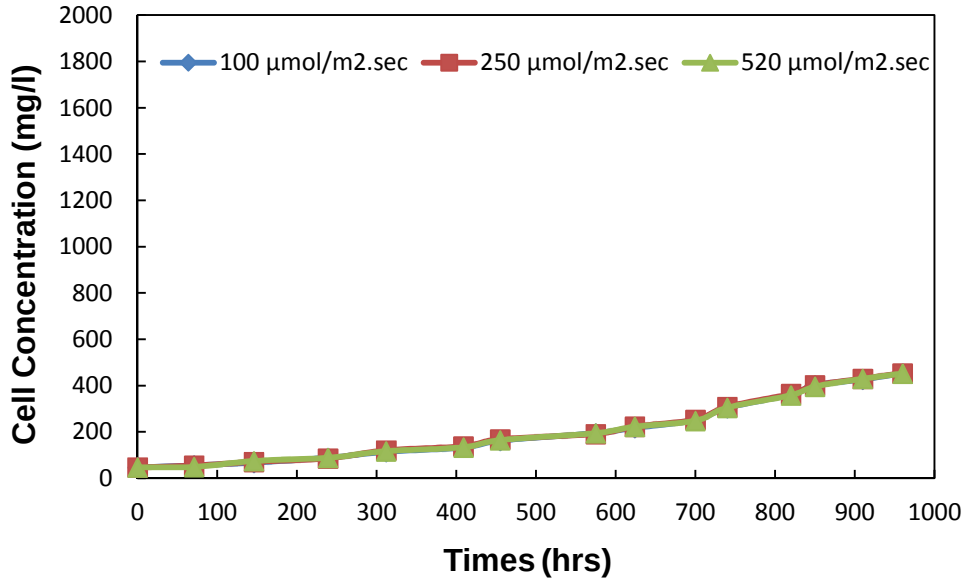

Figure 5.12 Growth profile for *Gloeothece membranacea* with different light intensities

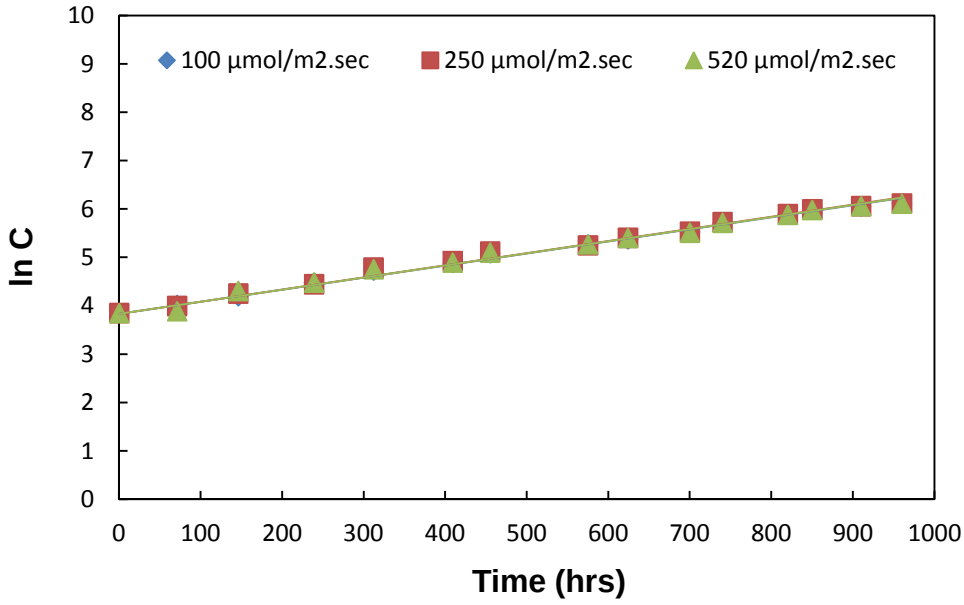

Figure 5.13 Kinetic profile for *Gloeothece membranacea* with different light intensities

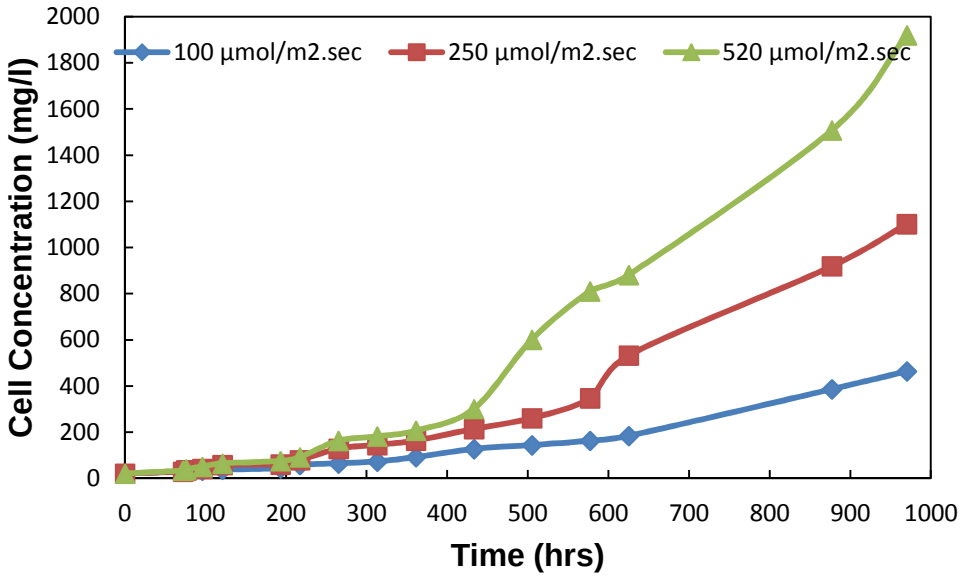

Figure 5.14 Growth profile for *Oscillatoria amoena* with different light intensities

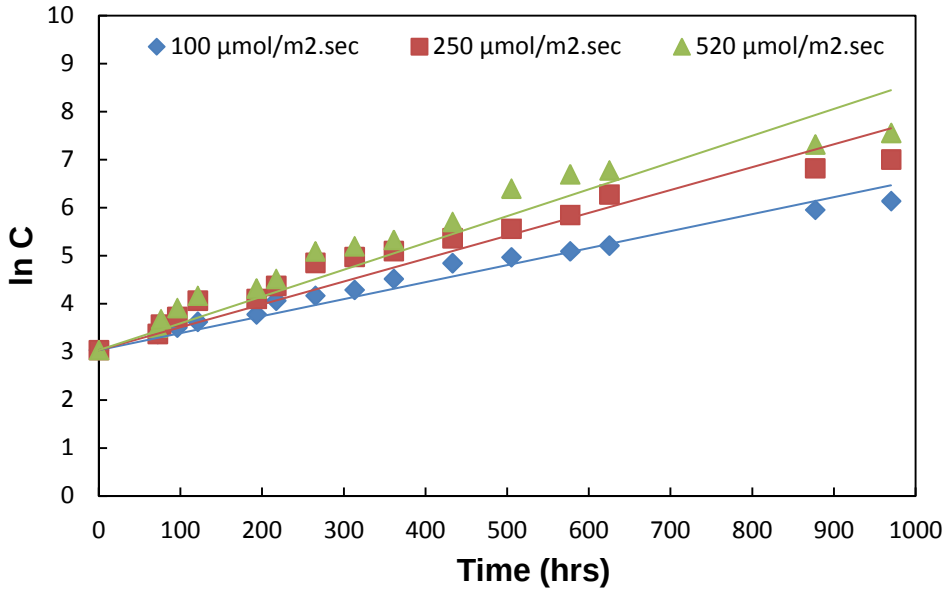

Figure 5.15 Kinetic profile for *Oscillatoria amoena* with different light intensities

Equations 5.4 -5.5 were used to calculate the growth rates and the generation times of the two species and the results are summarized in Table 5.7.

Table 5.7 The kinetics parameters of the two species with different light intensities

	Gloeothece membranacea			*Oscillatoria amoena*		
PAR (μmole m^{-2} sec^{-1})	100	250	520	100	250	520
μ ($hour^{-1}$)	0.0025	0.0025	0.0025	0.0035	0.0048	0.056
T_d (hours)	277	277	277	198	144	125

For *Gloeothece membranacea*, the light intensity did not seem to have any impact on the growth, as all three curves overlapped in Figures 5.12 -5.13 with the same specific specific growth rate of 0.0025 $hour^{-1}$ and the same generation time of 277 hours, as shown in Table 5.7. This could be due to the light intensities available in these experiments were in excess for this particular species. Thus it could be concluded that light intensity ≥ 100 μmol m^{-2} sec^{-1} is higher than the optimum light intensity of *Gloeothece membranacea*, as high light intensities overload the phtotsystems with photons which lead to the alteration and interruption of the synthesis and degradation of the light harvesting systems (Kumar et al., 2011).

However, Table 5.7 indicates that for *Oscillatoria amoena*, increasing the light intensity enhanced the growth, as cultures with light intensities of 100, 250, 520 (μmol m^{-2} sec^{-1}) gave growth rates of 0.0035, 0.0048, 0.0056 $hour^{-1}$, and the generation times of 198, 144, 124 hours, respectively. This supports that the light intensity available catered for the energy needed for the maintenance of this species only, thus the specific growth rate was proportional to light intensity (Merchuk et al., 2007). The difference in results could be

explained by the fact that the effect of light intensity and the efficient manipulation of photosynthetic irradiation differ significantly for different species of cyanobacteria (Yoon et al., 2002). The design of the TBPBR would give a wide range of light intensities to further investigate the findings in the light boxes.

5.6 Effect of Light Wavelength

The light is absorbed mainly by chlorophyll a within a cell, which receives the blue_ violet and the red regions of the visible light spectrum, reflects green light and this is why cyanobacteria look green.

As well as chlorophyll a, cyanobacteria contain other pigments with a range of colours including yellow, red, violet, green and blue. These super molecular assemblies are called phycobilisomes (PBS), which are attached to the surface of photosystem Π core in the thylakoid membrane. Each phycobilisome consists of a group of brilliantly coloured "phycobiliproteins" (PBP), each of which in turn contains covalently bonded pigments or chromophores called "phycobilins" (PB). The primary function of this remarkable light harvesting apparatus is to allow the organism to survive in weak light conditions. Phycobilisomes possess the particular ability to absorb photons in spectral regions where light is only weakly absorbed by chlorophyll, and funnels this absorbed energy to photosystem Π (PSΠ) reaction centres with an efficiency greater than 95% (Malkin, 2005). As the super molecules structure of phycobilisome is formed, the absorption in the visible

region is further enhanced. Apparently, the absorption is influenced by interaction of the chromophore with the protein environment (Malkin, 2005).

Chromatic adaptation occurs in some cyanobacteria, which means that these microorganisms can adapt to varying light colours. This is done by cascading energy. The energy is carried from higher levels down to lower levels. Violet, blue and green lights have the shortest wavelength. This means that these lights have higher frequency and therefore higher energy than yellow, orange and red. If violet, blue, or green lights are present the cyanobacteria produce high energy carriers whereas when yellow, orange or red light is present they produce only low energy carriers.

Phycobilisomes from cells of different species can have quite different absorption spectra (Malkin, 2005), moreover, light quality has an impact on the components of phycobilisome (Babu et al., 1991). For those reasons further experiments were carried out using three different colours of light (red, yellow, and blue). That was achieved by placing coloured transparent films over the flasks to act as filters as shown in Figure 5.16. Those three colours were chosen to be tested because LEDs, which were going to be used in the design of the TBPBR, are only available in red light or blue and yellow. So it was important to see the effect of these three colours on the growth of *Gloeothece membranacea* and *Oscillatoria amoena*. Table 5.8 lists the growth conditions for the cultures of different light qualities. Note that the culture volumes remain constant. All the cultures were placed in the same light box to ensure the same light intensity.

Table 5.8 The growth conditions for the cultures of different light qualities

Strain	*Gloeothece membranacea*, *Oscillatoria amoena*
Light Intensity	520 μmol m^{-2} sec^{-1}
Wavelength	White, Blue, Orange, Red
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 200 ml
Culture Volume	100 ml
OD	0.030, 0.030
Light Position	External
Gas composition	Air
Aeration S/V 1/m	44.2

Figure 5.16 The light box with the cultures at different light qualities

Figures 5.17 – 5.20 show the growth and kinetics profiles for *Gloeothece membranacea* and *Oscillatoria amoena* respectively.

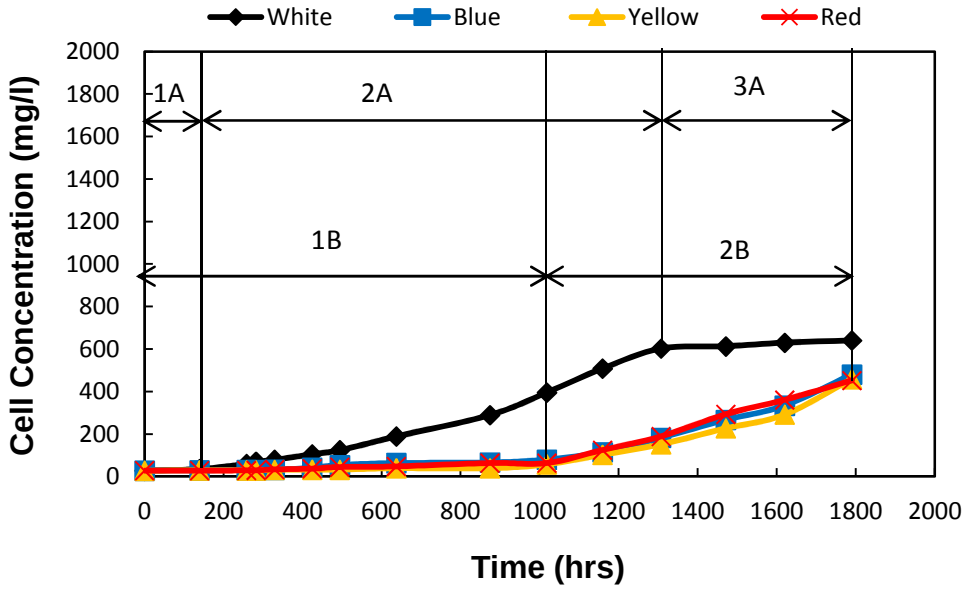

Figure 5.17 Growth profile for *Gloeothece membranacea* with different colours of light

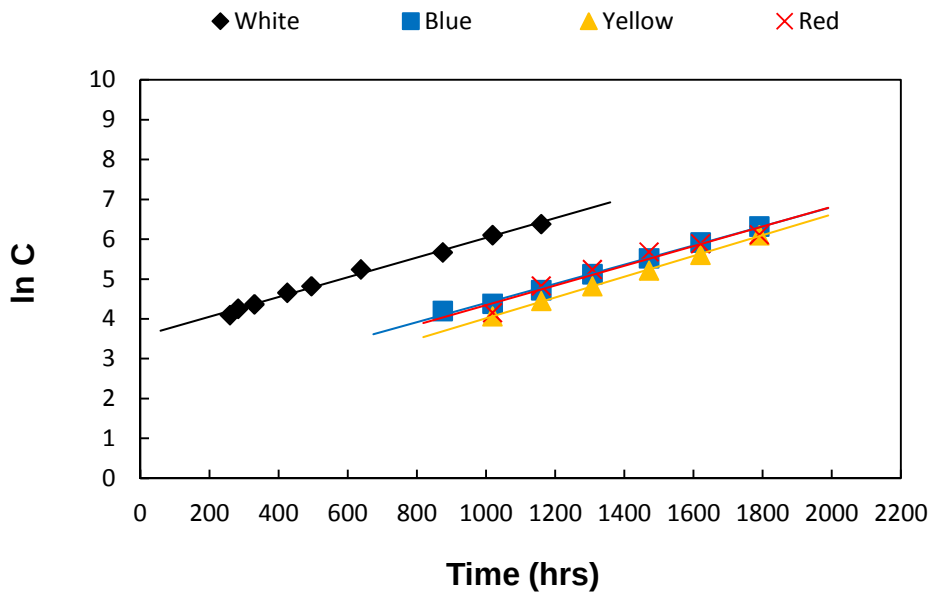

Figure 5.18 Kinetic profile for *Gloeothece membranacea* with different colours of light

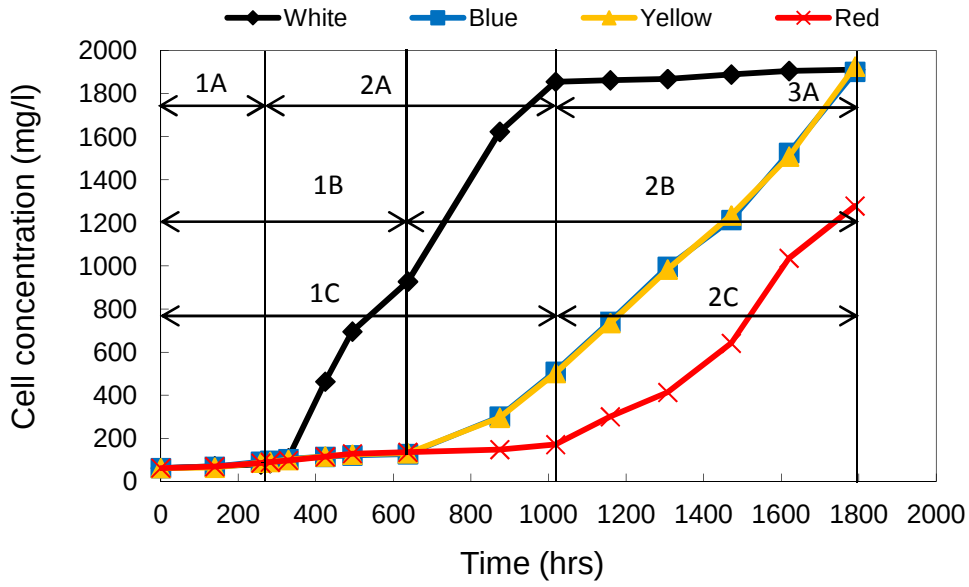

Figure 5.19 Growth profile for *Oscillatoria amoena* with different colours of light

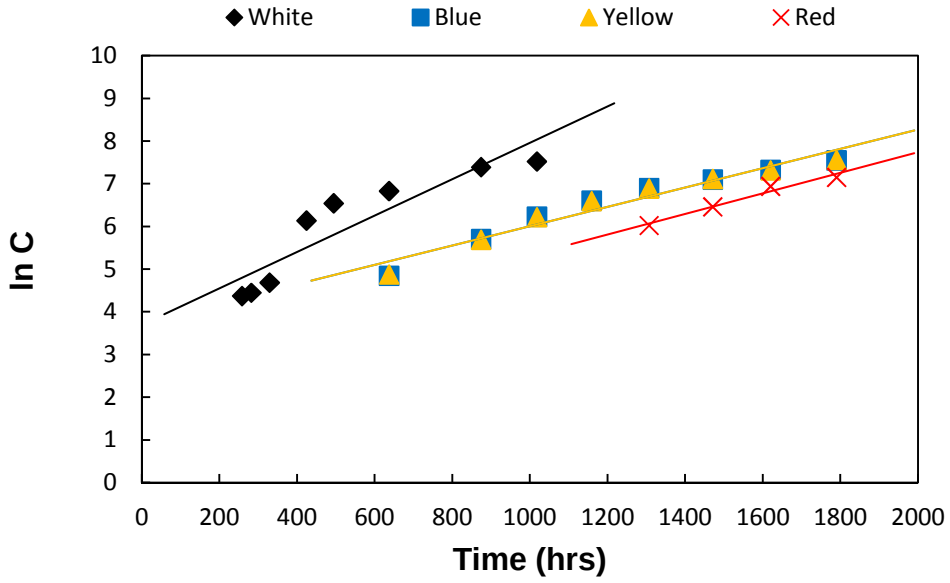

Figure 5.20 Kinetic profile for *Oscillatoria amoena* with different colours of light

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Equations 5.4 -5.5 were used to calculate the specific growth rates and the generation times of the two species and the results are summarized in Table 5.9 .

Table 5.9 The kinetics parameters of the two species with different colour of light

	Gloeothece membranacea				*Oscillatoria amoena*			
Light Colour	White	Blue	Yellow	Red	White	Blue	Yellow	Red
μ (hour^{-1})	0.0025	0.0025	0.0026	0.0024	0.0049	0.0023	0.0023	0.0024
T_d (hours)	277	277	266	288	141	301	301	289

The same approach which had been used in (5.2) was used here in specifying the growth phases. From a brief look at both Figures 5.17 and 5.19, the white light gave the highest measurements. However, for *Gloeothece membranacea*, the growth of the three cultures with blue, yellow and red lights looked more or less the same, with the lag phases (denoted as 1B on Figure 5.17), where cell concentrations hardly changed over time, being 1160 hrs, comparing to 140 hrs for the white light as shown in Table 5.10.

Table 5.10 The lag phases of *Gloeothece membranacea* subject to different light colours

Growth Phase (hrs)	Lag Phase
White	140
Blue	1160
Yellow	1160
Red	1160

The high specific growth rate of the culture with the white light was expected as the culture received the highest light intensity, as placing the transparent films around the other cultures reduced the light intensity. Nevertheless in the exponential phases (denoted as 2A and 2B in Figure 5.17) where cell concentrations increase exponentially over time, the cultures with

white, blue, yellow, red had more or less the same growth rates of 0.0025, 0.0025, 0.0026, 0.0024 $hour^{-1}$, and the same generation times of 277, 277, 266, 288 hours, respectively.

For *Oscillatoria amoena* the growth profiles were slightly different for each of the light wavelengths. First of all, the lag phases (denoted as 1A, 1B, and 1C in Figure 5.19) were different, e.g. 260 hrs for the white light, 640 hrs for yellow and blue lights, and 1020 hrs for red light as shown in Table 5.11.

Table 5.11 The lag phases of *Oscillatoria amoena* subject to different light colours

Growth Phase (hrs)	Lag Phase
White	260
Blue	640
Yellow	640
Red	1020

However in the exponential phases (denoted as 2A, 2B and 2C in Figure 5.19) where cell concentrations increased exponentially over time and, the cultures with white, blue, yellow, red had different growth rates of 0.0049, 0.0023, 0.0023, 0.0024 $hour^{-1}$ and different generation times of 141, 301, 301, 289 hours, respectively as it is summarized in Table 5.9. The growth of culture with white light was the best with the shortest generation time 141 hours, while the growth of the other cultures was more or less the same with the generation time between 298 and 301 hours.

The difference in growth in response to the different wavelengths between the unicellular species (*Gloeothece amoena*) and the filamentous one (*Oscillatoria amoena*) is expected for the reason that different species have different light harvesting pigments and different phycobiliproteins, and consequently different abilities of chromatic adaptation.

The initial results in these experiments show that *Gloeothece membranacea* would grow in the yellow, blue and yellow lights at more or less the same rate as in white light, however, *Oscillatoria amoena* would grow at much slower specific growth rate in red, yellow, blue lights than in white light.

5.7 Effect of Photoperiod

Light availability is a critical factor in cyanobacteria cultivation; however, the time period that would provide enough light energy for cells to commence photosynthesis has not been fully investigated. Because of the high efficiency of pigments, they absorb all light energy available for them, although they cannot utilise all. This excess energy is lost as heat; it could cause photo-damage to cells, which results in time delay for the cells to repair. This is why it is believed that making light available for shorter periods would be better. This in turn would save energy consumption. Moreover, mutual shading is generated when cell densities are very high, i.e. the cells at the lit surface would absorb all the photons available and shield the other cells within the cultures from receiving light energy. The investigation in the following experiments aimed to study the effect of different light cycles on the growth of *Gloeothece membranacea* and *Oscillatoria amoena.* With that purpose in mind, three different light cycles for each species were achieved by employing a timer to leave the light on for 24 hrs at the first run, 12 hrs on and 12 hrs off at the second run, and 6 hrs on and 18 hrs off at the third run. The same light box was used to assure the same light intensity. Table 5.12 summarizes

the growth conditions for the cultures of different light-cycles. Note that a constant culture volume was used.

Table 5.12 The growth conditions for the cultures of different light cycles

Strain	*Gloeothece membranacea*, *Oscillatoria amoena*
Light Intensity	250 μmol m^{-2} sec^{-1}
Wavelength	All visible light
Light Dark Cycle	24 hrs light, 12/12 Light/Dark, 6/18 light/dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 400 ml
Culture Volume	200 ml
OD	0.060, 0.050
Light Position	External
Gas composition	Air
Aeration S/V 1/m	28.4

Figures 5.21 – 5.24 displays the growth and kinetics profiles for *Gloeothece membranacea* and *Oscillatoria amoena* respectively. Note that the results represent the exponential phases only.

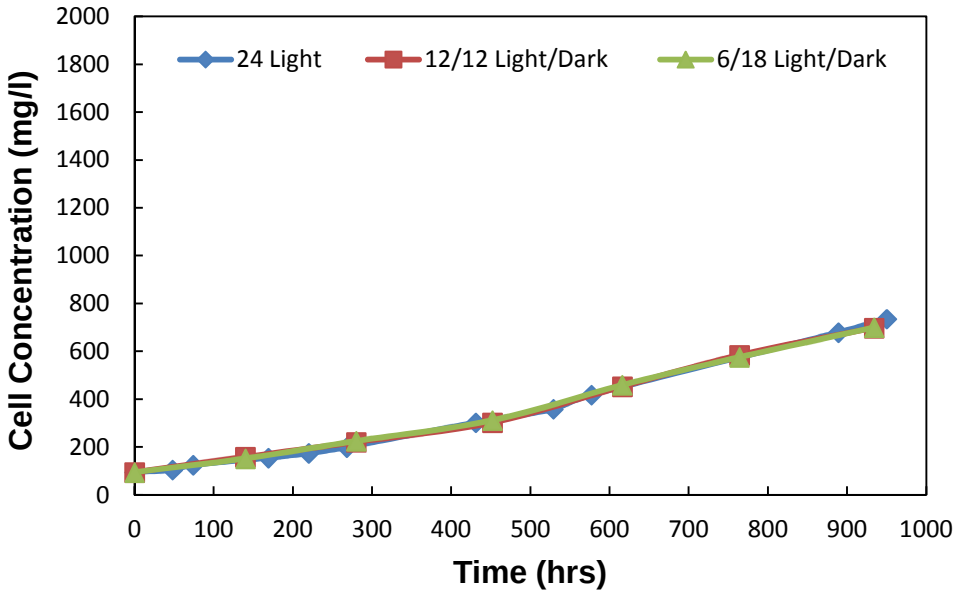


Figure 5.21 Growth profile for *Gloeothece membranacea* with different light cycles

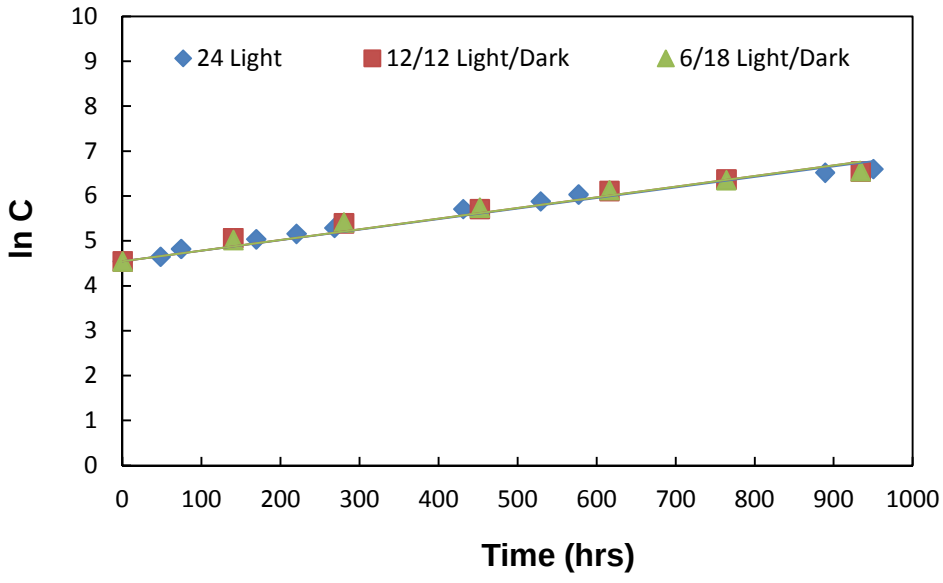


Figure 5.22 Kinetic profile for *Gloeothece membranacea* with different light cycles

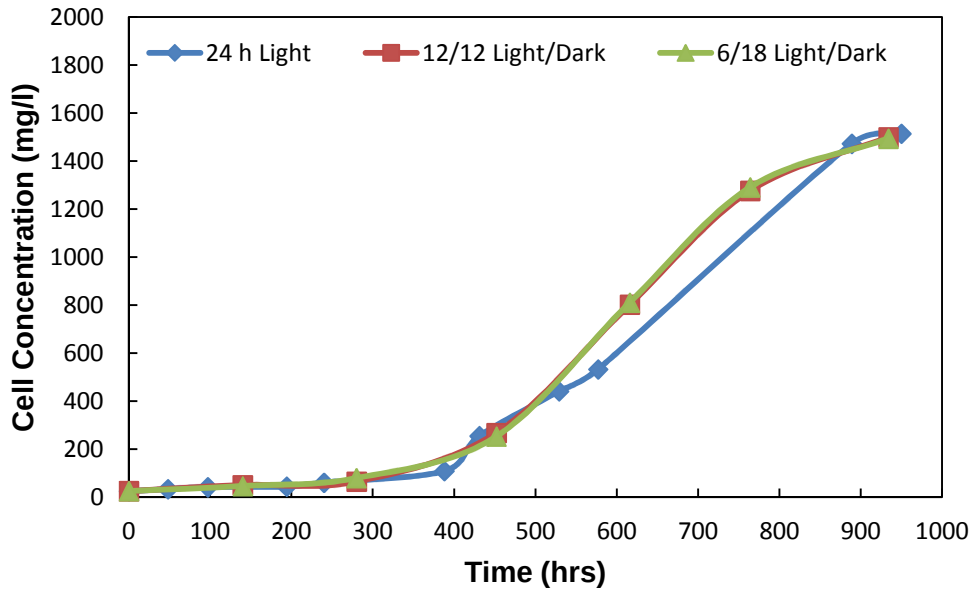


Figure 5.23 Growth profile for *Oscillatoria amoena* with different light cycles

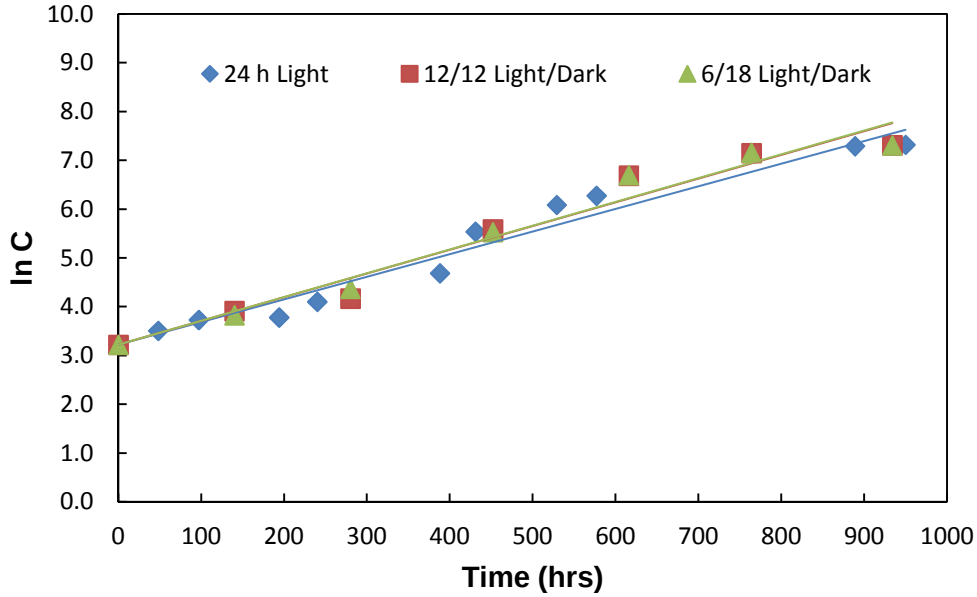


Figure 5.24 Kinetic profile for *Oscillatoria amoena* with different light cycles

Equations 5.4 -5.5 were used to calculate the growth rates and the generation times of the two species and the results are summarized in Table 5.13.

Table 5.13 The kinetics parameters of the two species with different light cycles.

	Gloeothece membranacea			*Oscillatoria amoena*		
Light/Dark	24/0	12/12	6/18	24/0	12/12	6/18
μ ($hour^{-1}$)	0.0024	0.0024	0.0024	0.0048	0.0048	0.0048
T_d (hours)	289	289	289	144	144	144

For both *Gloeothece membranacea* and *Oscillatoria amoena*, it is apparent that making the light available for 24 hrs did not improve the growth, as *Gloeothece membranacea* with different light cycles gave the same specific growth rate of 0.0024 $hour^{-1}$ and the same generation time of 289 hours, and *Oscillatoria amoena* with different light cycles also gave the same specific growth rate of 0.0048 $hour^{-1}$and the same generation time of 144 hours.

The most interesting results are that the same growth kinetic as the other two light cycles were obtained with only 6 hrs light duration for both *Gloeothece membranacea and Oscillatoria amoena*. This could be explained by the fact that once a cell has enough light energy to commence photosynthesis, excess light is redundant. The results here are very encouraging because making the light period shorter would mean less energy consumption. However, due to the shortage of time, what would be the minimum light exposure without affecting the growth is still to be determined. In the TBPBR, a 6 hrs on and 18 hrs off would be used as the operational parameters. Note that culture that had the light on all the time

turned yellow. This would indicate that the conditions were adverse for the cells, since there is evidence that when environmental conditions become unfavourable, the culture starts forming resistant spores, and when this happens the green coloured culture turns yellow (Thiel and Pratte, 2001, Thiel and Wolk, 1983).

5.8 Effect of Mixing

Mixing is one of the most important factors in microorganism growth, as it has a vital influence on light availability; reduces mutual shading in high cell densities cultures; mimics the effect of flashing light in cultures with densities over the critical cell density; makes more air available for the cells (Hu et al., 2000); affects mass transfer rate between cells and media, and between cells and air; prevent sedimentation of cells. Moreover, mixing helps to release the evolved oxygen from the media that is produced during photosynthesis, as too much dissolved oxygen will oxidise photosystem Π and inhibit photosynthesis, which in turn could lead to severe growth inhibition (Wang et al., 2012). However, violent mixing might damage microorganisms and prevent metabolism, which in turn might be detrimental to cell growth. To study the effect of mixing on the growth, two different mixing rates were used as well as without mixing. Table 5.14 displays growth conditions for cultures with different mixing conditions and Figure 5.25 shows the light box with the cultures with different mixing conditions. Note that the culture volumes remain the same.

Table 5.14 The growth conditions for the cultures of different mixing conditions

Strain	*Gloeothece membranacea, Oscillatoria amoena*
Light Intensity	520 μmol.m^{-2}.sec^{-1}
Wavelength	All visible light
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 400 ml
Culture Volume	200 ml
OD	0.053, 0.053
Light Position	External
Gas composition	Air
Aeration S/V 1/m	28.4

Figure 5.25 The light box with the cultures at different mixing conditions

Figures 5.26 – 5.29 show the growth and kinetics profiles for *Gloeothece membranacea* and *Oscillatoria amoena* respectively. Note that the results represented the exponential phases only.

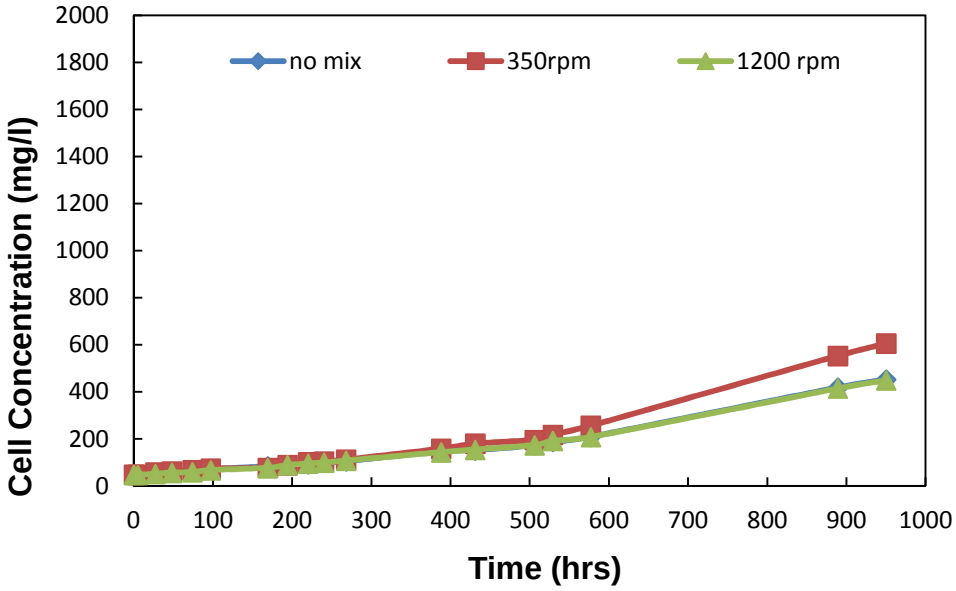


Figure 5.26 Growth profile for *Gloeothece membranacea* with and without mixing

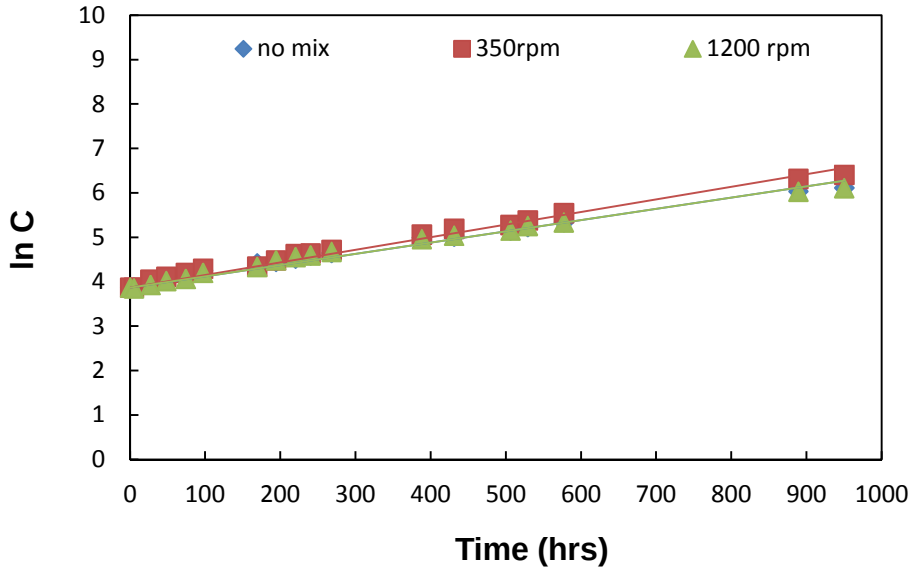


Figure 5.27 Kinetic profile for *Gloeothece membranacea* with and without mixing

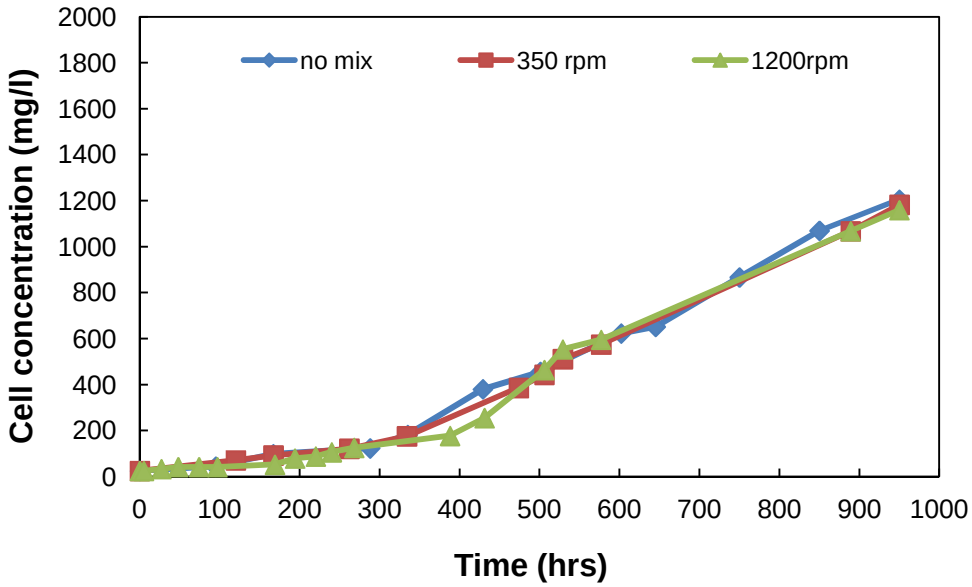


Figure 5.28 Growth Profile for *Oscillatoria amoena* with and without mixing

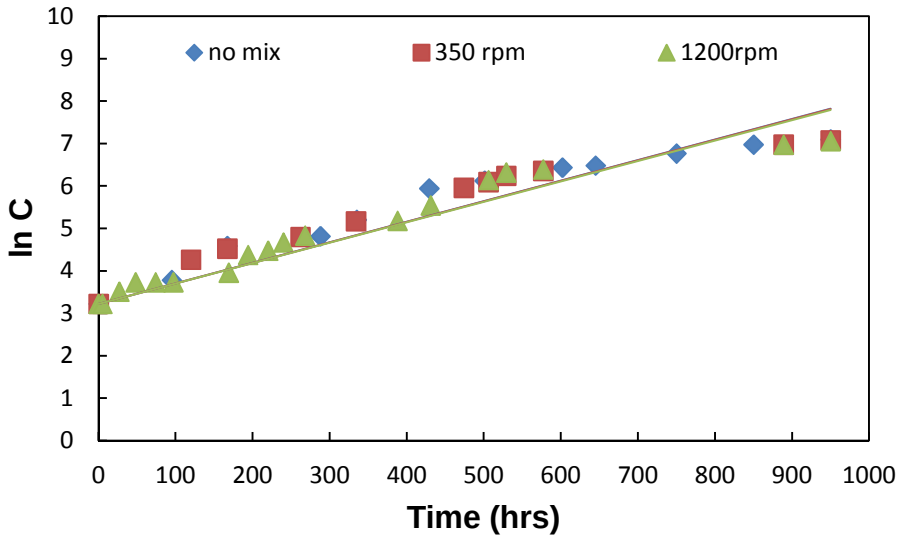


Figure 5.29 Kinetic profile for *Oscillatoria amoena* with and without mixing

Equations 5.4 -5.5 were used to calculate the growth rates and the generation times of the two species and the results are summarized in Table 5.15.

Table 5.15 The kinetics parameters of the two species with different mixing intensity

	Gloeothece membranacea			*Oscillatoria amoena*		
Re	0	40,000	160,000	0	40,000	160,000
μ (hour^{-1})	0.0024	0.0029	0.0024	0.0048	0.0048	0.0048
T_d (hours)	289	239	289	144	144	144

The growth and kinetic profiles in Figures 5.26 -5.27 and the summarized results in Table 5.15 reveal that mixing at 350 rpm improved the growth of *Gloeothece membranacea*, as it had a higher specific growth rate of 0.0029 hour^{-1} and a shorter generation time of 239 hours. Without mixing it had a specific growth rate of 0.0024 hour^{-1} and a generation time of 289 hours. However, this was not the case with mixing at 1200 rpm, as the culture at the highest mixing rate had the same specific growth rate of 0.0024 hour^{-1} and the same generation time of 289 hours as a culture with no mixing. This maybe due to the high shear experienced in the later condition, which had adverse effect on cell integrity.

For *Oscillatoria amoena* mixing had little effect on the cell growth, as the cultures had the same specific growth rate of 0.0048 hour^{-1} and the same generation time of 144 hours (Figures 5.28 -5.29). This is may be due to that *Oscillatoria amoena* is filamentous and mixing broke down the filaments and may have resulted in the same severe effect it had on the growth of *Gloeothece membranacea* at 1200 rpm, as vigorous mixing might prevent cell

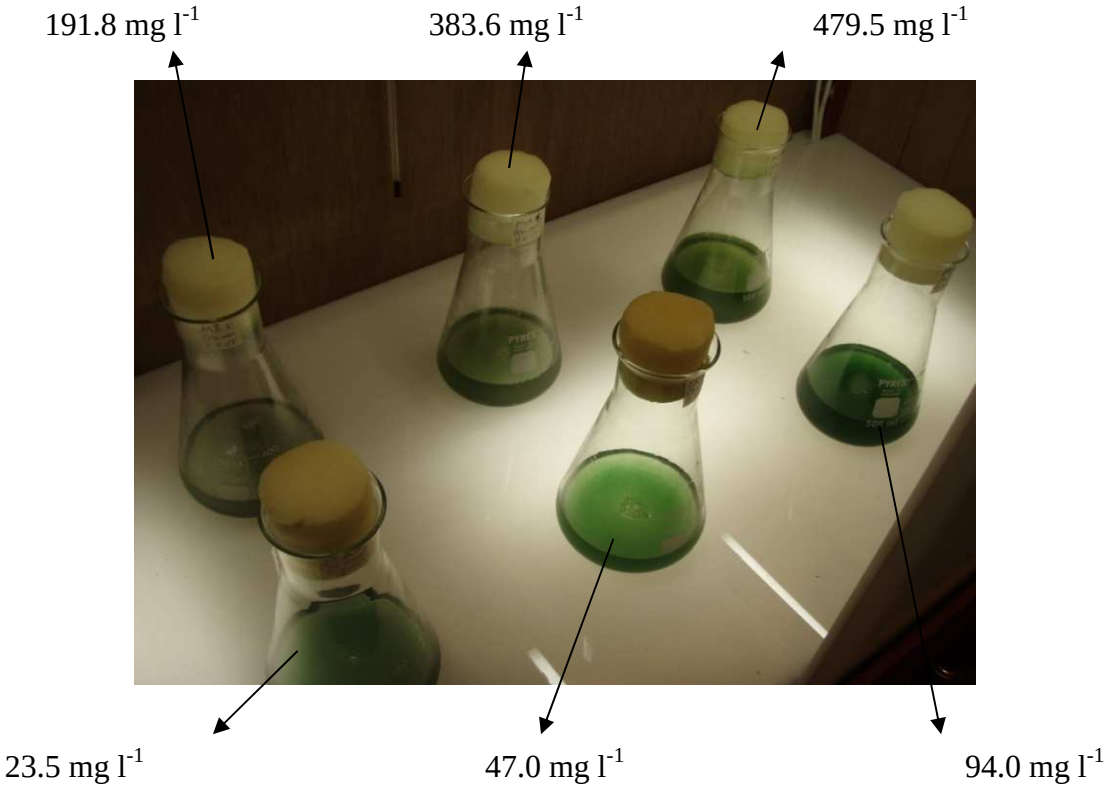
growth as a result of hydrodynamic stress on the microalgal cells (Vunjak-Novakovic et al., 2005). In the design of the TBPBR, only moderate mixing (Re=500-1580) will be applied offering an enhanced mass transfer rate due to much small and even bubble size distribution, facilitating better cell growth.

5.9 Effect of Cell Density

. Available light intensity decreases as cell density increases. Hence, a lesser cell density culture will be exposed to stronger light intensities. However, as cell densities increases, mutual shading would have more effect on cell growth. The cell density of a culture which grows without mutual shading is called the critical cell density (CCD). This new parameter has increasingly been used in the design of photobioreactors. To study the effect of the initial cell density on the growth, find out the critical cell densities of the two species in this research, six different initial cell densities were used for each species. Table 5.16 tabulates the growth conditions for the cultures of different initial cell densities, and Figure 5.30 shows the light box with the cultures of different initial cell densities. Note that the culture volume remains the same.

Table 5.16 The growth conditions with the cultures of different cell densities

Strain	*Gloeothece membranacea, Oscillatoria amoena*
Light Intensity	100 μmol m^{-2} sec^{-1}
Wavelength	All visible light
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C
Container	Flask 400 ml
Culture Volume	200 ml
OD	Varied
Light Position	External
Gas composition	Air
S/V L/m	44.2



The numbers indicate the optical densities at the start of the experiments.

Figure 5.30 The light box with the cultures of different initial cell densities

Figures 5.31 -5.34 show growth and kinetics profiles for *Gloeothece membranacea* and *Oscillatoria amoena* respectively. Note that the results represented the exponential phases only.

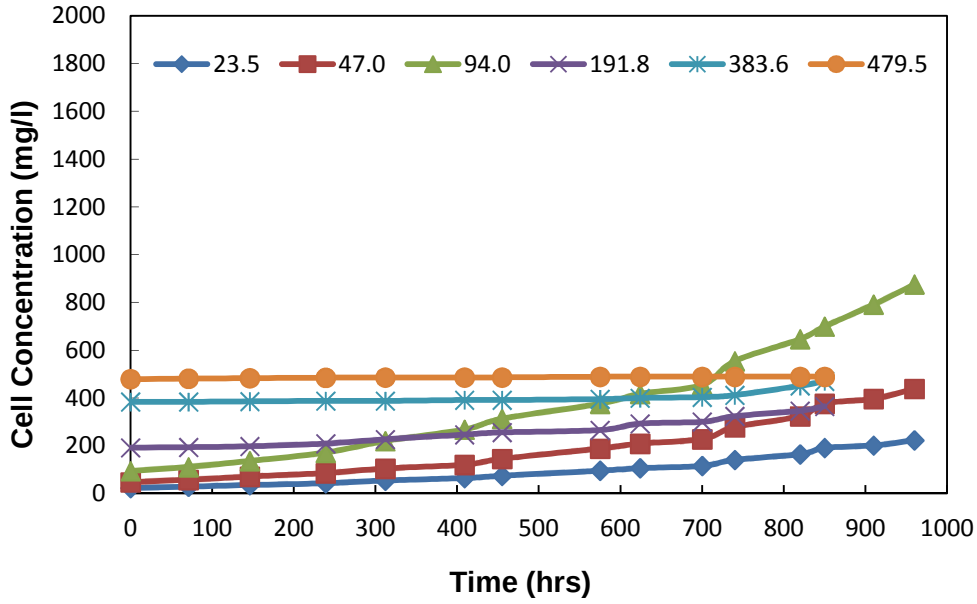


Figure 5.31 Growth profile for *Gloeothece membranacea* with different cell densities

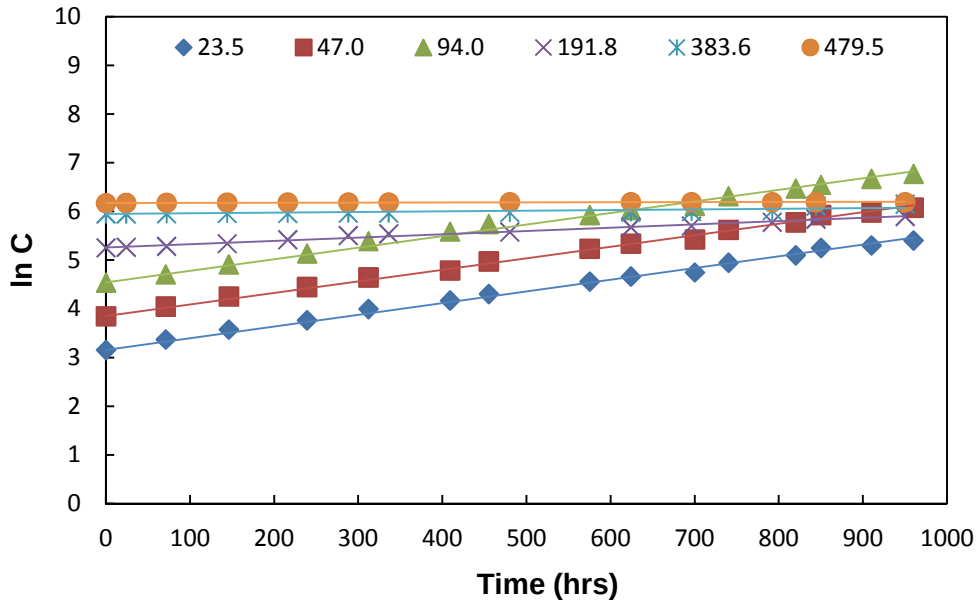


Figure 5.32 Kinetic profile for *Gloeothece membranacea* with different cell densities

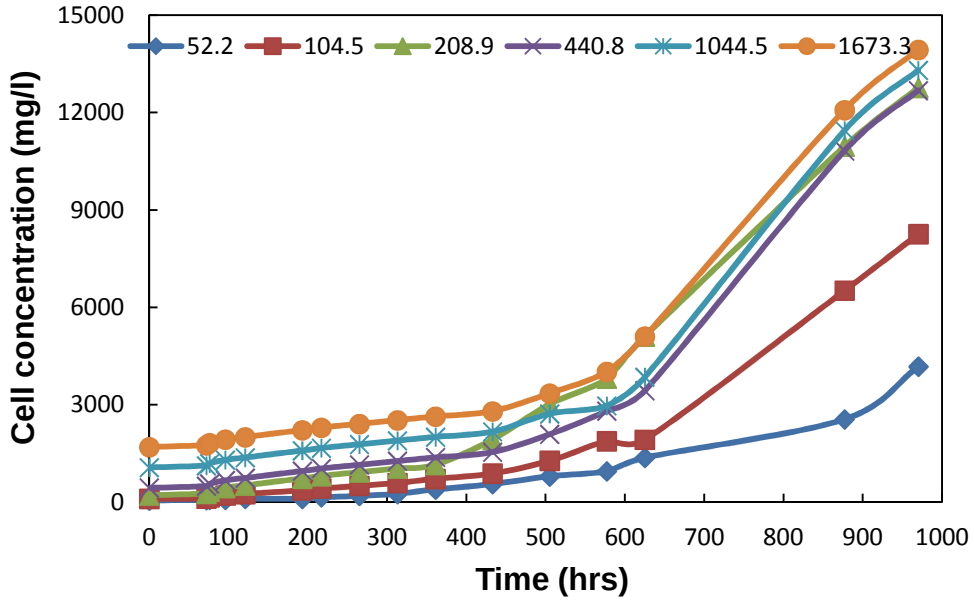


Figure 5.33 Growth profile for *Oscillatoria amoena* with different cell densities

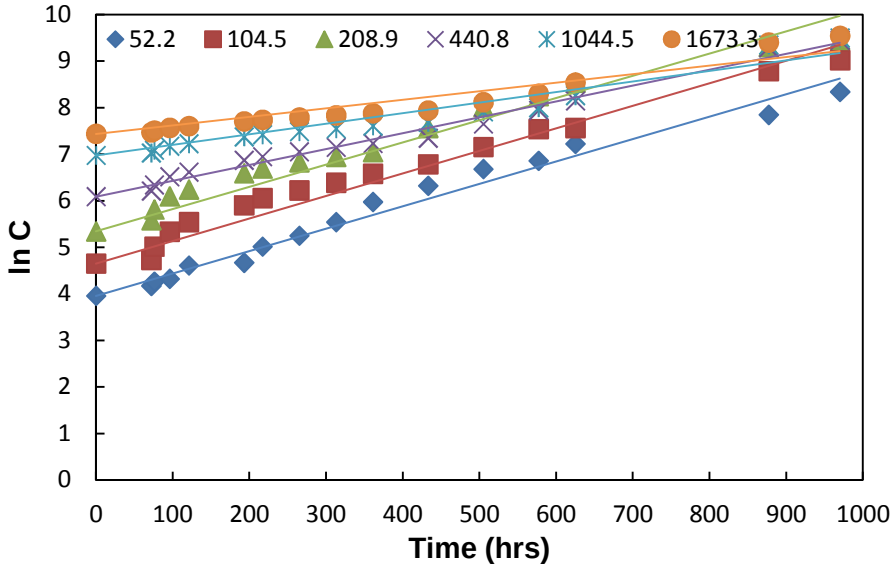


Figure 5.34 Kinetic profile for *Oscillatoria amoena* with different cell densities

The results in Figures 5.31 – 5.32 and Table 5.17 show that the growth rates for *Gloeothece membranacea* at the initial cell densities of 23.5, 47.0 and 94.0 mg l^{-1} were the same of 0.0024 $hour^{-1}$ as with the same slope, and the generation times were the same too of 289 hours. However, the specific growth rate and the generation time for the culture of the initial cell density of 191.8 mg l^{-1} were 0.0007 $hour^{-1}$ and 990 hours which were much longer than that of the previous cell densities. There was hardly any growth at all for the cultures of the initial cell densities of 383.6 and 479.5 mg l^{-1}, where the growth rates were 0.0001 and 0.00003 $hour^{-1}$, and the generation times were 6931 and 23105 hours, respectively. It should be noted that there is a gap in the growth rates and the generation times between the cell densities 94.0 and 191.8 mg l^{-1}, and due to time constraint these experiments were not carried out in this thesis work. Based on available results, it could be concluded that the critical cell density for *G. membranacea* (CCD) is about 94.0 mg l^{-1}, which means that cultures with CD ≤ 94.0 $mg.l^{-1}$ would grow without mutual shading, while cultures with CD > 94.0 $mg.l^{-1}$ would grow with mutual shading. All results are summarised in Table 5.17.

Table 5.17 Kinetics parameters of *Gloeothece membranacea* at different cell densities

	Gloeothece membranacea					
C (mg l^{-1})	23.5	47.0	94.0	191.8	383.6	479.5
μ ($hour^{-1}$)	0.0024	0.0024	0.0024	0.007	0.0001	0.00003
T_d (hours)	289	289	289	990	6931	23105

The same trend applies for *Oscillatoria amoena*, as the cultures of the initial cell densities of 52.2, 104.5, and 208.9 mg l^{-1} had the same growth rates and generation times of 0.0048 $hour^{-1}$ and 144 hours respectively. While the growth rates and generation times of the cultures of the initial cell densities of 440.8, 1044.5, 1673.3 mg l^{-1} were of 0.0034, 0.0023 and 0.0018

hour^{-1}, 204, 301 and 385 hours respectively. Once again there is a gap in the growth rates and generation times between the cell densities 208.9 and 440.8 mg l^{-1}, and due to time constraint these experiments were not able to be carried out in this thesis work. Based on the data obtained, the critical cell density for *Oscillatoria amoena* (CCD) was found to be around about 208.9 mg.l^{-1}, which means that cultures with CD ≤ 208.9 mg.l^{-1} would grow without mutual shading; cultures with CD > 288.9 mg.l^{-1} would grow with mutual shading. Also, further investigation is required as there is a gap. These critical cell densities will be further investigated at the TBPBR trials. All results are summarized in Table 5.18.

Table 5.18 Kinetics parameters of *Oscillatoria amoena* at different cell densities

	Oscillatoria amoena					
C (mg l^{-1})	52.2	104.5	208.9	440.8	1044.5	1673.3
μ (hour^{-1})	0.0048	0.0048	0.0048	0.0034	0.0023	0.0018
T_d (hours)	144	144	144	204	301	385

5.10 Effect of Temperature

From chemical reaction engineering viewpoint, the reaction rate is a function of temperature. The effect of temperature on cell cultures relates to both the temperature dependence of the structural components of the cells (particularly lipids and proteins) and the temperature coefficient of reaction rates. A consequence of these primary effects are the significant changes in metabolic regulatory mechanics, specificity of enzyme reactions, permeability and cell composition (Richmond, 1986). To examine the effect of temperature on microbial growth, experiments were conducted at three temperatures (25 °C, 30 °C and 38

°C). These were achieved by using hot plates to control the temperature. Table 5.19 lists the growth conditions for the cultures of different temperatures, and Figure 5.35 is the light box with the cultures of different temperatures. Note that the culture volume remains constant.

Table 5.19 The growth conditions for the cultures different temperatures

Strain	*Gloeothece membranacea, Oscillatoria amoena*
Light Intensity	520 µmol m^{-2} sec^{-1}
Wavelength	All visible light
Light Dark Cycle	12/12 Light/Dark
Growth Medium	BG11
Temperature	25 °C,30 °C, 38 °C
Container	Flask 400 ml
Culture Volume	200 ml
OD	0.102, 0.012
Light Position	External
Gas composition	Air
Aeration S/V 1/m	44.2

Figure 5.35 The light box with cultures at different temperatures

Figures 5.36 -5.39 show the growth and kinetics profiles for *Gloeothece membranacea* and *Oscillatoria amoena* respectively. Note that the results represent the lag and the exponential phases.

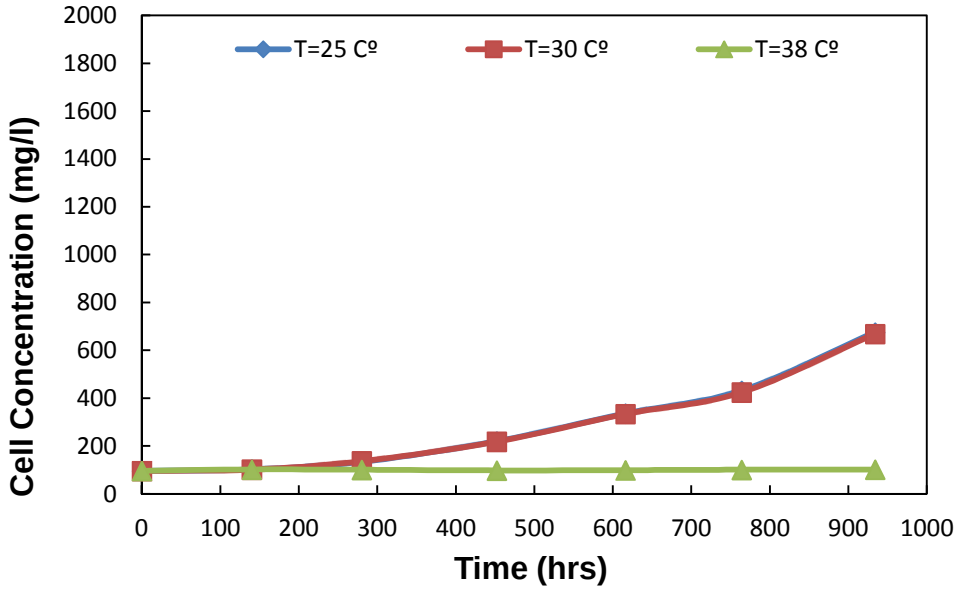


Figure 5.36 Growth profile for *Gloeothece membranacea* with different temperatures

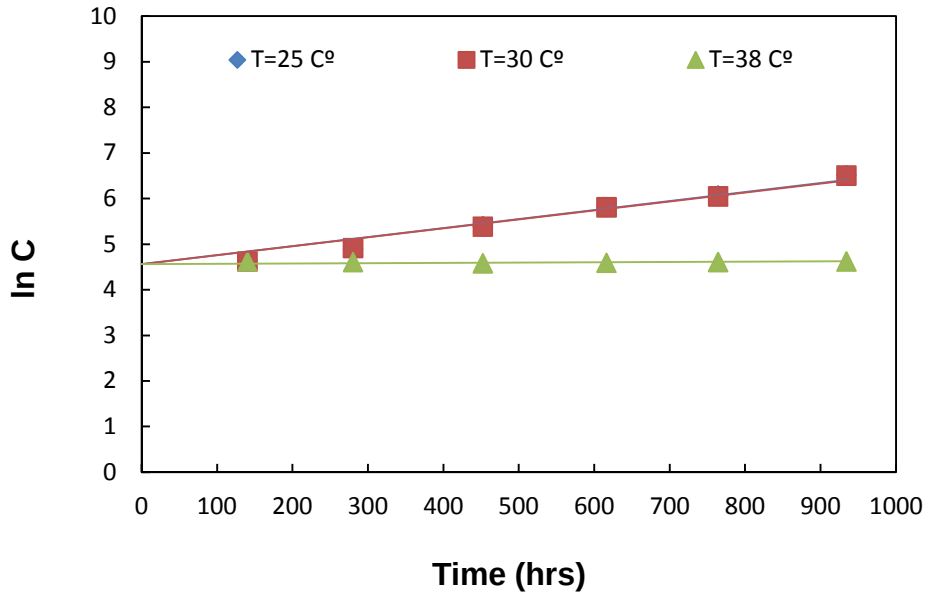


Figure 5.37 Kinetic profile for *Gloeothece membranacea* with different temperatures

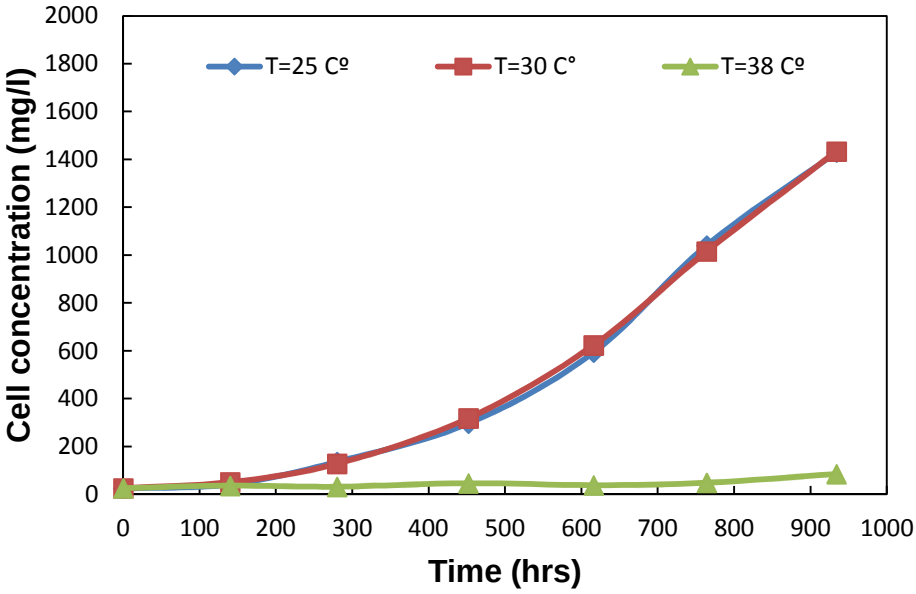


Figure 5.38 Growth profile for *Oscillatoria amoena* with different temperatures

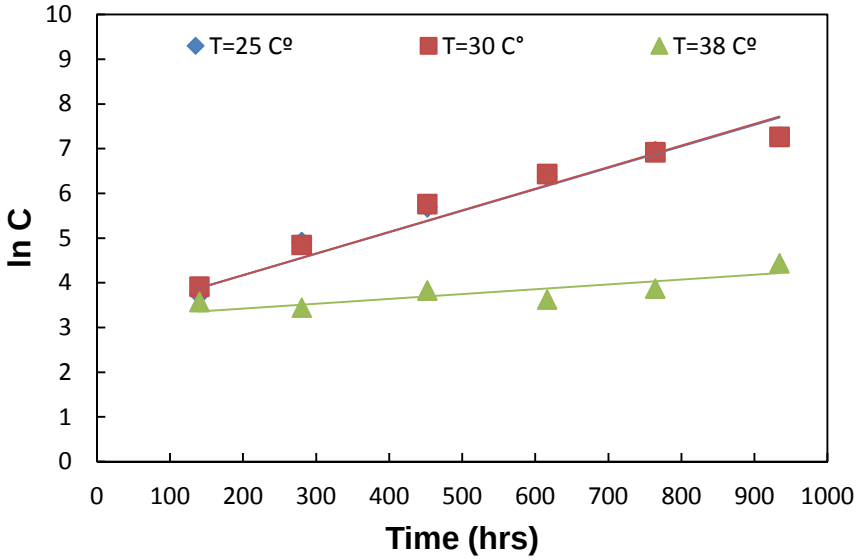


Figure 5.39 Kinetic profile for *Oscillatoria amoena* with different temperatures

Equations 5.4 -5.5 were used to calculate the growth rates and the generation times of the two species and the results are summarized in Table 5.20.

Table 5.20 The kinetic parameters of the two species with different temperatures

	Gloeothece membranacea			*Oscillatoria amoena*		
T (°C)	25	30	38	25	30	38
μ (hour^{-1})	0.0024	0.0024	0.0006	0.0048	0.0048	0.0005
T_d (hours)	289	289	11552	144	144	1386

For both *Gloeothece membranacea* and *Oscillatoria amoena*, the growth at 38 °C seems to be stopped, as the growth rates were much slower of 0.00006 and 0.0005 hour^{-1}and the generation times much longer of 11552 and 1386 hours respectively, compared to growth rates of 0.0024 and 0.0048 hour^{-1} and the generation times of 289 and 144 hours at 25 °C. This could be explained by the fact that above a certain temperature, particular proteins and lipids may irreversibly be damaged (Madigan et al., 2000). Moreover, the reaction rates in micro algal cells are significantly affected by the environmental temperature (Sandnes et al., 2005). It can further be noted that the temperature had a more adverse effect on the unicellular species than that on the filamentous one. This could be explained by the fact that filamentous species cells are closely connected to each other in each filament which in turn makes them more protective.

Furthermore, the growth and kinetic profiles are identical for 25 °C and 30 °C for both the species studied, this could imply that the temperature range of 25-30 °C is the optimum one for both *Gloeothece membranacea* and *Oscillatoria amoena* as the growth rates were 0.0024

and 0.0048 $hour^{-1}$ and the generation times 289 and 144 hours. However, more experiments are required at temperatures less than 25 °C in order to further confirm the optimum temperature range. Due to the time constraint, these tests were not undertaken. As a result, 25 °C will be the baseline temperature in the design of TBPBR.

CHAPTER 6 THE CULTIVATION OF *GLOEOTHECE MEMBRANACE* IN THE TUBULAR BAFFLED PHOTO BIOREACTOR (TBPBR)

In general photo-bioreactors are classified according to the shape of the devices: tubular or flat panel; the orientation of the device; the means of flow of the culture; the method used to supply the light; and the type of gas exchange system. The most important factors that need to be addressed are: the availability and efficient use of both light and CO_2, and the handling of the release of O_2 which is produced by photosynthesis, as it inhibits metabolism and eventually prevents cells from growth, if it is allowed to accumulate. Productivity is dependent on the environmental conditions of the micro-algal species chosen for the study and is determined by the growth rate. However, more research is still required for the optimum design of a photo bioreactor on an industrial level with many technical and economical challenges (Sforza et al., 2012).

In an oscillatory baffled bioreactor, the mass transfer coefficient k_La was 75% higher than that in a stirred tank fermenter and six times higher than that in bubble columns (Sforza et al., 2012, Ruiz-Marin et al., 2010, Ni and Gao, 1996, Gaidhani et al., 2003, Yuen et al., 2005), and the shear rate is lower than that in an equivalent stirred tank reactor (Posten, 2009) The uniform mixing with excellent mass transfer in this type of bioreactors would lead to satisfactory contribution of nutrients to microorganisms and efficient removal of gases and other by-products of catabolism from the microenvironment of the cells. Moreover, the scale-up correlation of oscillatory baffled reactors is linear (Reijnders, 2013, Wang et al., 2012,

Vega-Estrada et al., 2005, Vunjak-Novakovic et al., 2005), enabling direct transfer of knowledge learnt from laboratory to full scale production. The motivation of applying this type of bioreactor was stemmed from those previous studies. In continuous oscillatory baffled bioreactors, the uniform mixing is achieved by the combination of fluid oscillation with the presence of baffles. Plug flow characteristics are obtained under laminar flow conditions. A variation of this type of devices is the tubular baffled reactor (NiTech Patent US2010/0124145). The mixing is achieved by the combination of the net flow with the presence of orifice baffles. The advantage of this variation is that the need for oscillation is removed, this brings up simplicity and robustness to the operation, in particular, to this project where each experiments has a typical duration of 4 weeks. The downside of this is the compromise on the residence time, since the net flow Reynolds number is in the range of 500+ in comparison to 150 with oscillation. This shortcoming was overcome by the design of operating it in a loop in order to accommodate the duration of the experiment.

Only *Gloeothece membranacea* was chosen to be studied in the TBPBR for the following reasons:

1) From the batch data it was concluded that mixing improved the growth of *Gloeothece membranacea,* but not *Oscillatoria amoena,* which is a filamentous species and mixing might have caused the breaking down of the filaments;
2) *Gloeothece membranacea* grew as fast with blue and yellow LEDs as with white LEDs, while *Oscillatoria amoena* grew very poorly in blue and yellow light;
3) The cells of *Oscillatoria amoena* turned yellow when mixing was applied and when growing with blue and yellow LEDs. This would indicate that the conditions were adverse for the cells, since there is an evidence that when environmental conditions

become unfavourable, the culture starts forming climate resistant spores, and when this happens the green coloured culture turns yellow (Thiel and Pratte, 2001, Thiel and Wolk, 1983).

The objectives of these planned experiments were to

a) Confirm and verify some of the data obtained in the light boxes;
b) Extend the findings from the light boxes;
c) Identify the operational conditions for continuous culture of cyanobacteria in the TBPBR, whether these could be used as the raw materials for either biodiesel production or hydrogen generation.

6.1 The Cultivation Parameters in the TBPBR

The outcome of the batch work in the light boxes indicated that the bigger the ratio of surface area over volume, the faster the cell growth. That ratio in the TBPBR was 97 m^{-1}, which is superior to the ratios used in the light boxes.

It was also noted that light intensities ≥ 100 µmol m^{-2} sec^{-1} did not have any impact on the growth of the species chosen for this study (Figure 5.12), so 100 µmol m^{-2} sec^{-1} was the baseline light intensity, however other light intensities in the range of 20-200 µmol m^{-2} sec^{-1} were tested in the bioreactor in order to find out if the parameters of cultivation of *G.*

membranacea would change between the light boxes and the TBPBR, while other process parameters remain the same.

As LEDs were only available in white (which has blue and yellow wavelength but looks white to the human eye) or in red, and *G. membranacea* grew in yellow and blue light as fast as in white light (Figure 5.17), and the effects of these two light colours on cell growth were rarely, if at all, studied in the literature, the same white LEDs were used in the TBPBR.

The batch data also indicated that exposing the cells of *G. membranacea* for only 6 hours a day allowed the species to grow as fast as exposing it to 12 or 24 hours of light a day (Figure 5.21). Thus it was decided that the light cycle of 6/18 light/dark was the baseline for the experiments in the TBPBR. However, other light cycles were also investigated to verify and expand the results of light cycles in the light boxes and the TBPBR.

To achieve uniform mixing between the cells and the media, the flow rates of 1-3 L min^{-1} with Reynolds numbers of 500-1580 were applied in the TBPBR. The data can be compared and extended with respect to the benchmarking data.

It was concluded that the critical cell density of *G. membranacea* (CCD) was $\geq$ 94.0 mg/l, which means that cultures with CD $\leq$ 94.0 $mg.l^{-1}$ would grow without self shading, while cultures with CD > 94.0 $mg.l^{-1}$ would grow with self shading (Figure 5.31). It was decided that the the baseline of the cell density used in the TBPBR would be 94.0 mg l^{-1}.

Temperature experiments suggested that the optimum range for the growth of *G. membranacea* is 25-30 °C (Figure 5.36), so 25 °C was the baseline temperature for the operation in the TBPBR. All growth conditions of the species are summarized in Table 6.1.

Table 6.1 The growth conditions of *Gloeothece membranacea* in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	100 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	6/18 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94 (mg l^{-1})
Light Position	Internal
Gas composition	Air
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97 m^2 m^{-3}

The same methods of evaluating growth kinetics in Chapter 5 are used here. Also, culture preparation was the same as in the light boxes to ensure the same culture conditions.

6.2 The Light Saturation Level

Mass culture growth is limited by light availability and micro-algal photosynthesis kinetics are greatly affected by light because it is easily absorbed and scattered by cells (Jeon et al., 2005). However excessive light intensities inhibit photosynthesis, cause photo-oxidation of chlorophyll, and enhance photo-respiration (Smith et al., 1980). A large lit surface area over volume is very desirable in photobioreactors. There is evidence in the literature that 7.3 μmol/m^2.sec light intensity was enough to maintain photosynthesis (Ogbonna and Tanaka, 1997). This must depend on the devise used to grow the culture. In the light boxes the results indicated that light intensities equal or higher than 100 μmol m^{-2} sec^{-1} did not improve the growth for *Gloeothece membranacea*, which might imply that light saturation level for this particular species is equal or less than 100 μmol m^{-2} sec^{-1}. In order to investigate the light saturation level for this species 5 different levels of light intensities were applied within the TBPBR as shown in Table 6.2. Figure 6.1 shows the set-up of the tubular baffled photo bio-reactor for the light saturation level experiments.

Table 6.2 The growth conditions of the cultures of different light intensities in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	20,40,80,120,160 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	6/18 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94 (mg l^{-1})
Light Position	Internal
Gas composition	Air
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97

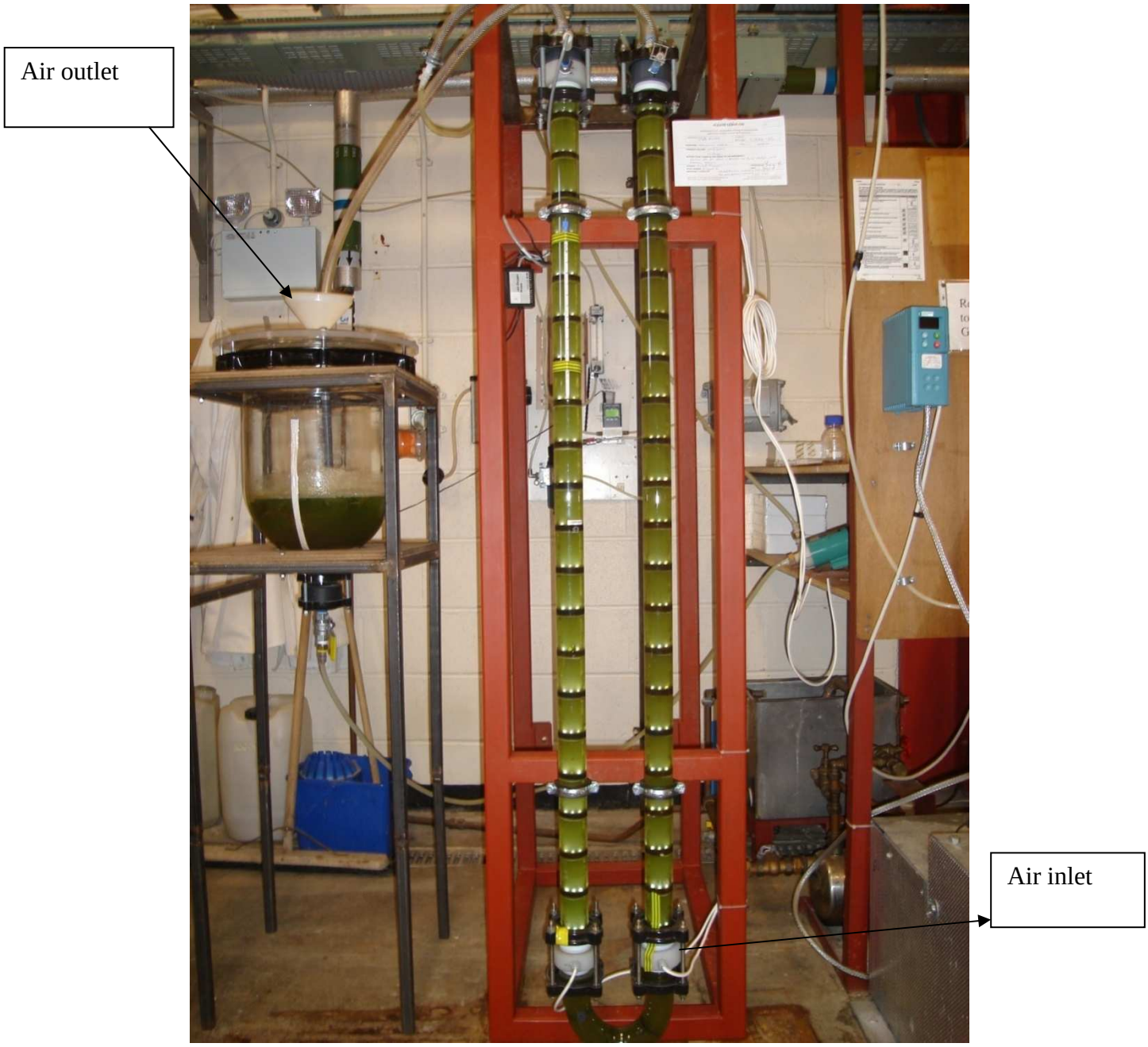


Figure 6.1 The set-up of the TBPBR for the light saturation level experiments

Figures 6.2 – 6.3 show the growth and kinetics profiles for *Gloeothece membranacea* in the TBPBR. Note that the results represent the exponential phases only.

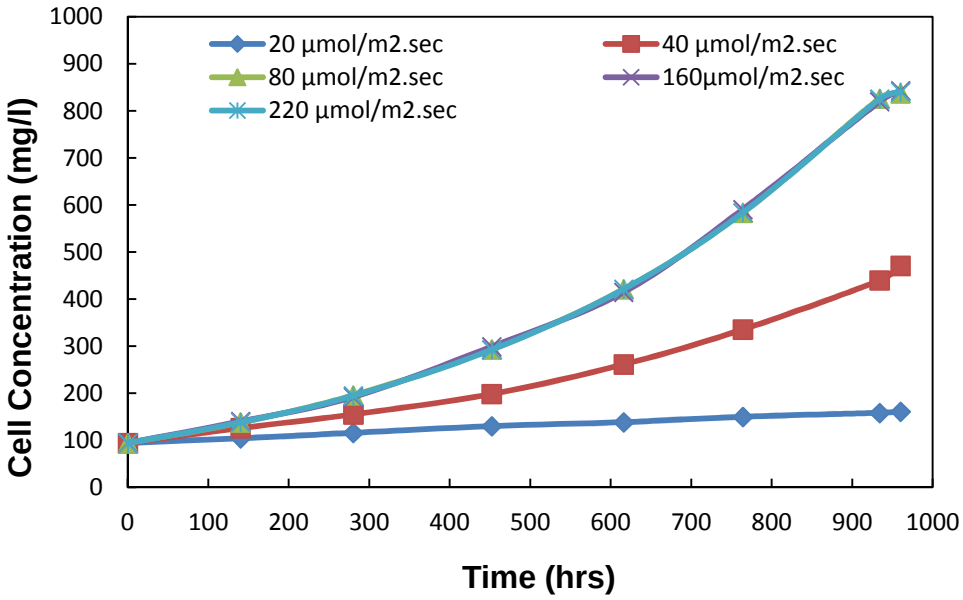


Figure 6.2 The Growth profile of *Gloeothece membranacea* in the TBPBR with different light intensities

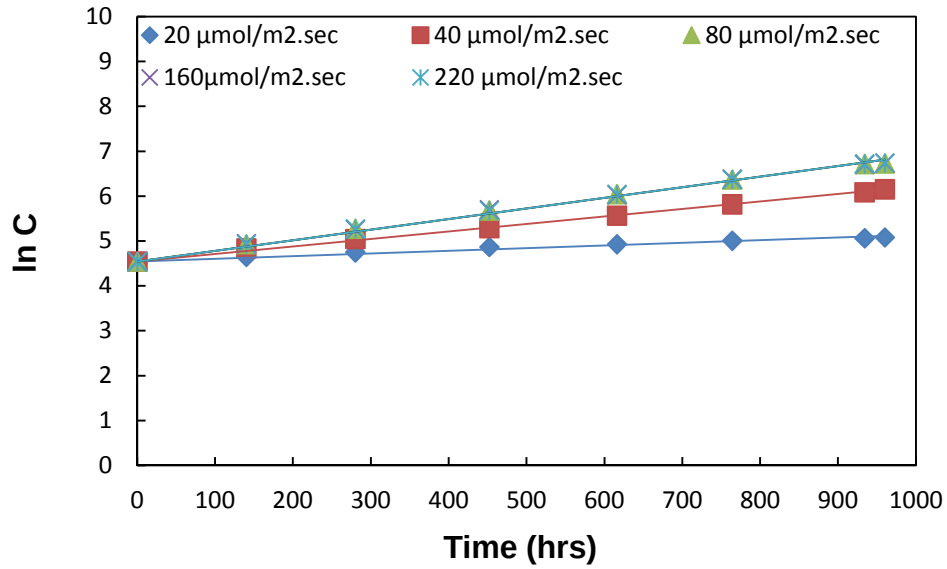


Figure 6.3 The kinetic profile of *Gloeothece membranacea* in the TBPBR with different light intensities

Equations 5.4 -5.5 were used to calculate the specific growth rate and the generation time of *Gloeothece membranacea*, and the results are summarized in Table 6.3.

Table 6.3 The kinetics parameters in the light boxes and the TBPBR with different light intensities

	The TBPBR					The Light Boxes		
PAR	20	40	80	160	220	100	250	520
μ (hour^{-1})	0.0006	0.0017	0.0024	0.0024	0.0024	0.0025	0.0025	0.0025
T_d (hours)	1155	408	289	289	289	277	277	277

The growth and kinetic profiles of *Gloeothece membranacea* in Figures 6.2 – 6.3 and the summarized results in Table 6.3 show that increasing the light intensity up to 80 μmol m^{-2} sec^{-1} in the TBPBR enhanced the growth, as these light intensities gave higher growth rates of 0.0006, 0.0017, 0.0024 hour^{-1} and shorter generation times of 1155, 408, 289 hours. However, light intensities higher than 80 μmol m^{-2} sec^{-1} did not improve the growth, as the cultures with light intensities of 80, 160, 220 μmol m^{-2} sec^{-1} had the same specific growth rate of 0.0024 hour^{-1} and the same generation time of 289 hours. This may be due to the fact that the high light intensities caused photo-inhibition, which in turn caused the inactivation of other oxygen evolving systems and electron carriers (Kumar et al., 2011). The results in the light boxes also showed that the culture of *Gloeothece membranacea* at light intensities of 100, 250, 520 μmol m^{-2} sec^{-1} gave the same specific growth rate 0.0025 of hour^{-1} and the same generation time of 277 hours. The findings in the light boxes and the TBPBR might imply that the light saturation level for the growth of *Gloeothece membranacea* is 80 μmol m^{-2} sec^{-1} .

6.3 The Period of Light Availability

Light availability is one of the most important limiting factors in cyanobacterial growth, as it controls the rate of photosynthesis and productivity. The tests in the light boxes indicate that the light period of 6 hrs on and 18 hrs off gave the same specific growth rate as that when the light period was 24 hrs. In order to validate and confirm the findings and to find out the minimum light exposure, the effect of varing light periods were further examined in the TBPBR. Table 6.4 displays the growth conditions for the cultures of different light cycles inside the TBPBR and Figure 6.4 shows the set-up of the tubular baffled photo bio-reactor for the light on and off for the light availability experiments.

Table 6.4 The growth conditions of the cultures of different light cycles in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	80 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	2/22,4/20,6/18,12/12, 24/0 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94 (mg l^{-1})
Light Position	Internal
Gas composition	Air
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97

Figure 6.4 The tubular baffled photo bio-reactor with the lights on (on the left) and off (on the right).

Figures 6.5 - 6.6 show the growth and kinetics profiles for *Gloeothece membranacea* in the TBPBR. Note that the results represent the exponential phases only.

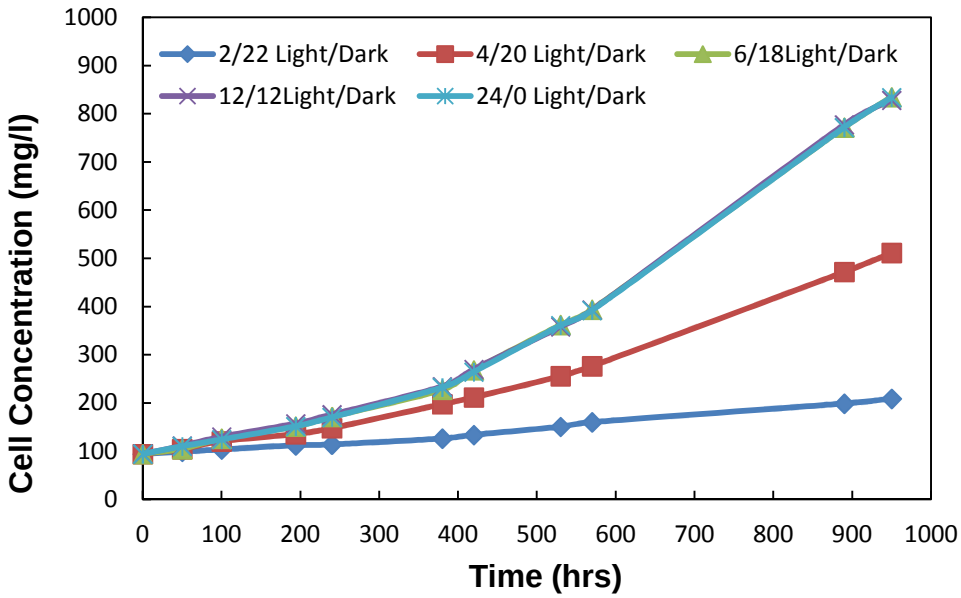


Figure 6.5 The growth profile for *Gloeothece membranacea* in the TBPBR with different light cycles

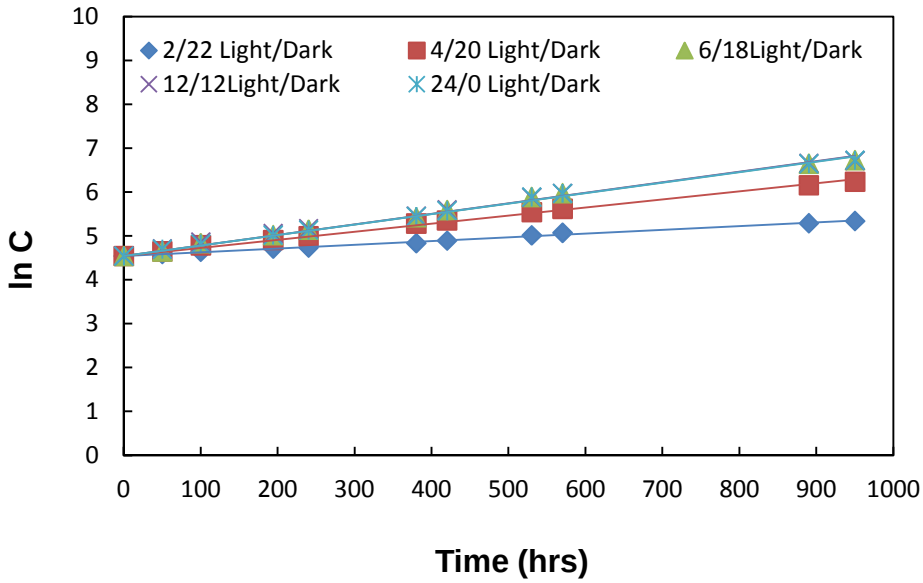


Figure 6.6 Kinetic profile for *Gloeothece membranacea* in the TBPBR with different light cycles

Equations 5.4 -5.5 were used to calculate the specific growth rate and the generation time of *Gloeothece membranacea* and the results are summarized in Table 6.5

Table 6.5 The kinetics parameters in the light boxes and the TBPBR with different photoperiods

	The TBPBR					The Light Boxes		
Light/Dark	2/22	4/20	6/18	12/12	24/0	6/18	12/12	24/0
μ (hour^{-1})	0.0009	0.0018	0.0024	0.0024	0.0024	0.0024	0.0024	0.0024
T_d (hours)	770	385	289	289	289	289	289	289

The growth and kinetic profiles for *Gloeothece membranacea* in Figures 6.5 - 6.6 and Table 6.5 reveal that making the light available for 6, 12, or 24 out of 24 hours did not enhance the growth, as cultures subjected to these conditions had the same specific growth rate and the generation time of 0.0024 hour^{-1} and 289 hours respectively. However, decreasing the period of light availability decreased the specific growth rate and increased the generation time, as cultures subjected to 4 or 2 out of 24 hours had growth rates of 0.0018 and 0.0009 hour^{-1} respectively, and generation times of 385 and 770 hours respectively. The results indicate that only 6 hours of light in 24 hours is sufficient for the growth of *Gloeothece membranacea* in the TBPBR. This further validates the results in the light boxes, which also suggest that exposing this species for only 6 hours of light is enough for the growth as shown in Table 6.5.

6.4 The Critical Cell Density CCD

In high density cultures, light availability is affected by self-shading. However fluid dynamics plays a major role, not only in minimizing the effect of self-shading in dense cultures, but also in mass transfer and transport mechanism between CO_2, other nutrients and cells. In the light boxes the critical cell density (CCD) of *Gloeothece membranacea* was 94.0 mg l^{-1}, which means that cell densities higher than 94.0 mg l^{-1} would grow with self shading. In order to evaluate and validate the findings and to find out the desirable CCD in the TBPBR without mutual shading, further experiments were undertaken. Table 6.6 displays the growth conditions for the cultures of different cell densities inside the TBPBR.

Table 6.6 The growth conditions of the cultures of different initial cell densities in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	80 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	6/18 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94.0,188.0,282.0,376.1,470.1 (mg l^{-1})
Light Position	Internal
Gas composition	Air
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97

Figures 6.7 – 6.8 show the growth and kinetics profiles for *Gloeothece membranacea* in the TBPBR. Note that the results represent the exponential phases only.

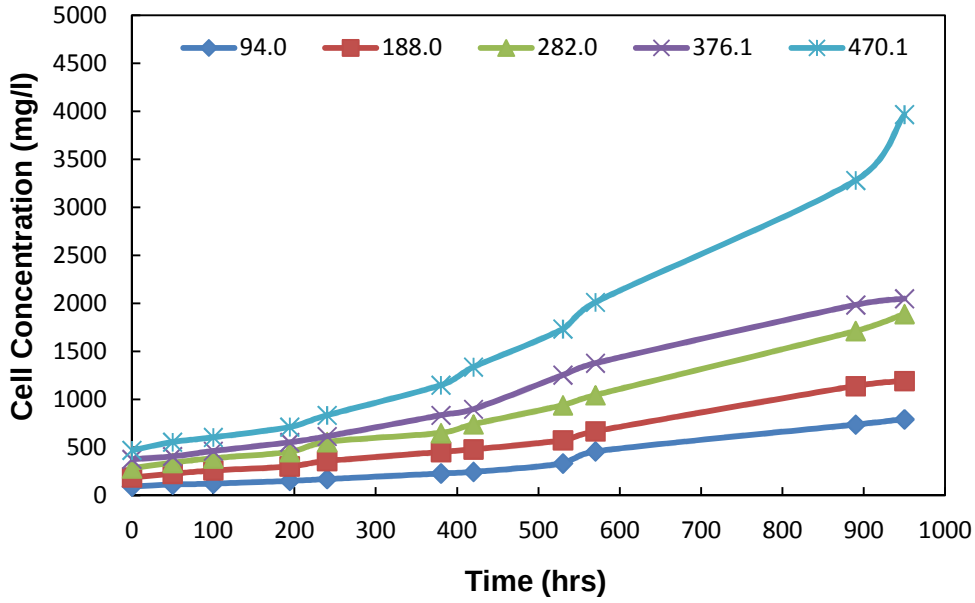


Figure 6.7 Growth profile for *Gloeothece membranacea* with different initial cell densities

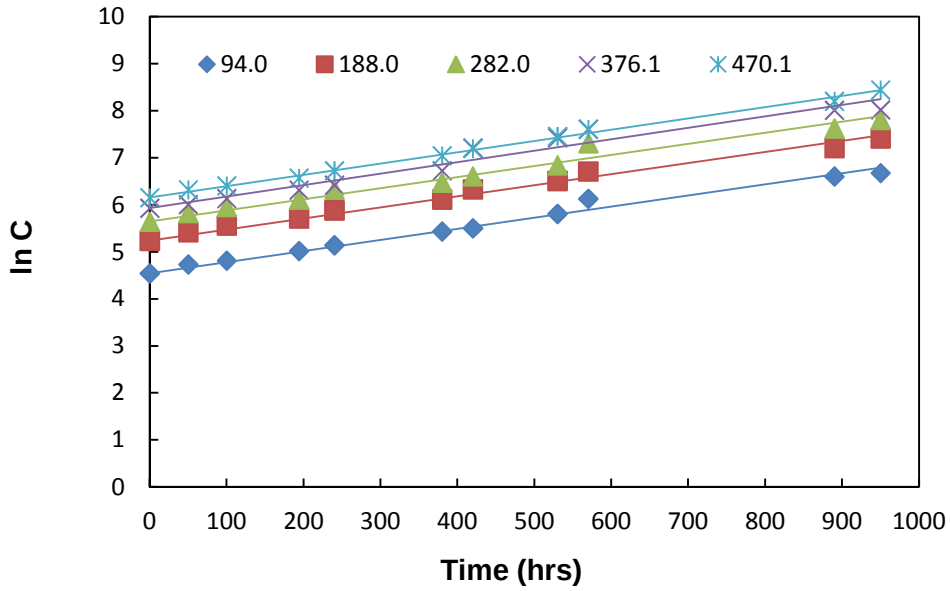


Figure 6.8 Kinetic profile for *Gloeothece membranacea* with different initial cell densities

Equations 5.4 -5.5 were used to calculate the specific growth rate and the generation time of *Gloeothece membranacea* and the results are summarized in Table 6.7.

Table 6.7 The kinetics parameters in the light boxes and the TBPBR with different cell densities

	TBPBR					LIGHT BOXES					
C (mg l^{-1})	94.0	188.0	282.0	376.0	470.0	23.5	47	94	191.8	383.6	479.5
μ ($hour^{-1}$)	0.0024	0.0024	0.0024	0.0024	0.0024	0.0024	0.0024	0.0024	0.0007	0.0001	0.0003
T_d (hours)	289	289	289	289	289	289	289	289	990	6931	23105

Table 6.7 demonstrates that cultures with the initial cell densities 94.0, 188.0, 282.0, 376.0 and 470.0 mg l^{-1} had the same specific growth rate and the generation times of 0.0024 $hour^{-1}$ and 289 hours respectively. The results could be explained by the efficiency of mixing in the TBPBR, which may resulted in higher efficiency of light availabilities at higher cell densities, as improving mixing is one of the most tailored strategies to develop light delivery (Wang et al., 2012).

Comparing the results in the TBPBR to that in the light boxes reveals that the growth was improved in the TBPBR as the cell density of 94.0 mg l^{-1} was no longer the critical cell density for *Gloeothece membranacea* in the TBPBR as higher cell densities up to 470 mg l^{-1} grew at the same specific growth rate and had the same generation time, which means that this species could be cultivated in the light boxes of density up to 94.0 mg ^{l}, while up to 470 mg l^{-1} in the TBPBR without affecting the growth, as light intensities higher than the

optimum values lead to higher percentage of the cells to stay in the dark due to self shading (Kumar et al., 2011).

6.5 Air Addition

High velocities and small bubbles enhance mixing, as in traditional stirred tanks air velocity affects bubble size and bubble diameter affects transfer coefficients (Anderson et al., 2002). Bubbling gas through the bottom of the photo bioreactor increased the efficiency of consuming CO_2 and released the inhibitory accumulated O_2 (Wang et al., 2012). However, it is practically challenging to have both high velocities and small bubbles. To investigate the effect of bubbling air through the bottom of the TBPBR, further tests were conducted where air at different flow rates was added only at the bottom of the right side of the loop, i.e. there are air bubbles in the baffled tube on the right hand side of Figure 6.1, however, there are no air bubbles at all in the baffled tube on the left hand side of the same figure. The purpose of this arrangement was to compare the effect of culture with and without air on the growth as well as cleanliness. Table 6.8 shows the growth conditions for the cultures with different air velocity inside the TBPBR.

Table 6.8 The growth conditions of the cultures of different air flow rates in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	80 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	6/18 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94 (mg l^{-1})
Light Position	Internal
Gas composition	Air
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97

Figures 6.9 – 6.10 show the growth and kinetics profiles for *Gloeothece membranacea* in the TBPBR. Note that the results represented the exponential phases only.

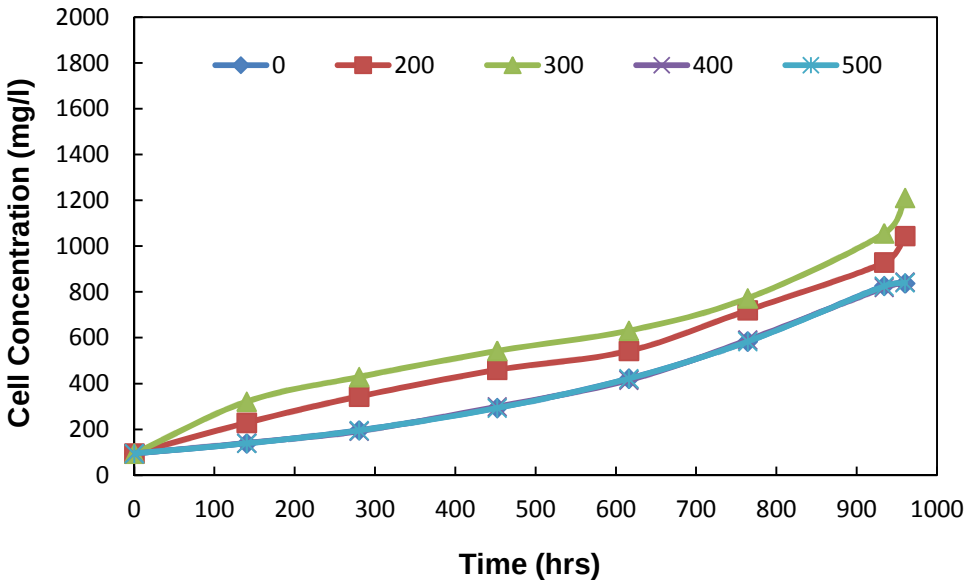


Figure 6.9 Growth profile for *Gloeothece membranacea* with different air flow rates

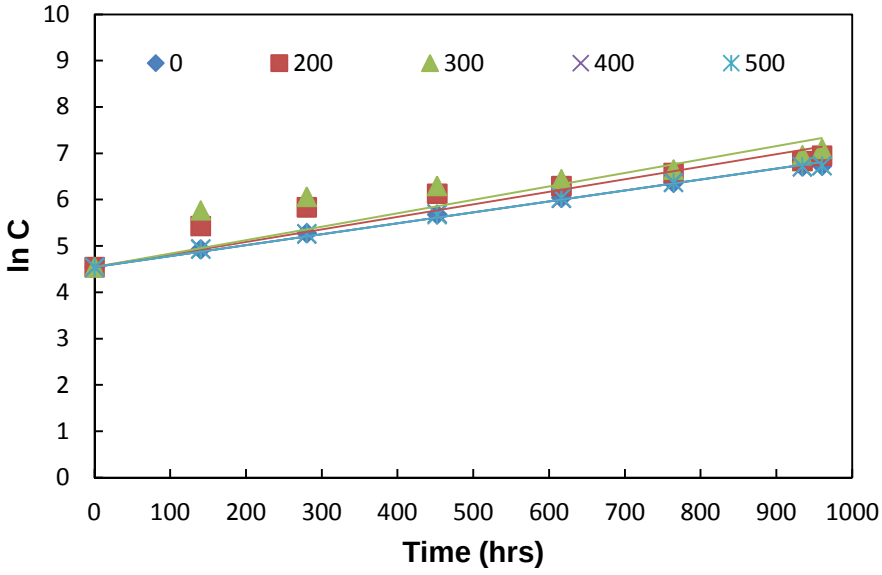


Figure 6.10 Kinetic profile for *Gloeothece membranacea* with different air flow rates

Equations 5.4 -5.5 were used to calculate the specific growth rate and the generation time of *Gloeothece membranacea* and the results are summarized in Table 6.9

Table 6.9 The kinetics parameters in the TBPBR with different air flow rates

Air Flow (ml min^{-1})	0	200	300	400	500
Aeration rate (vvm)	0	0.04	0.06	0.08	0.10
μ ($hour^{-1}$)	0.0024	0.0027	0.0029	0.0024	0.0024
T_d (hours)	289	257	239	289	289

The growth and kinetic profile in Figures 6.9 – 6.10 and the summarized results in Table 6.9 indicate that the specific growth rate of *G. membranacea* with different air flow rates went through a peak, as increasing the flow rates of air from 0 – 400 ml min^{-1} increased the specific growth rate from 0.0024 to 0.0029 $hour^{-1}$ and decreased the generation times from 289 to 239 hrs. This might be due to the removal of the accumulated oxygen, which has negative impact on cell growth. The incoming air (Wang et al., 2012), and the higher the flow rates of air removed greater amounts of the oxygen. Also, the presence of baffles promotes narrower bubble size distribution, as baffles lead to bubble breakage as well as bubbles trapping underneath them. The effect of breakage and trapping of bubbles was to simultaneously increase the number of both small bubbles and gas hold-up (Oliveira et al., 2003, Oliveira and Ni, 2004). The higher the superficial gas velocity, the higher the gas hold-up. However, increasing the air flow rates further from 400 – 600 ml min^{-1} decrease the growth rates from 0.0029 to 0.0024 $hour^{-1}$ and increase the generation times from 289 to 239 hrs as extreme gas velocities damage cells and prevent growth (Vega-Estrada et al., 2005).

Furthermore, bubbling air through the bottom of the right side of baffled tube can improve the cleanliness of the TBPBR, as air bubbles break loose and carry away cyanobacteria attached onto the baffles and tube surfaces, leading to far cleaner baffles and brighter lights in the side of the TBPBR where the air was added as shown in Figure 6.11. The close-up shows that adding air prevented the microorganisms from accumulating on the baffles which could eventually lead to blockage.

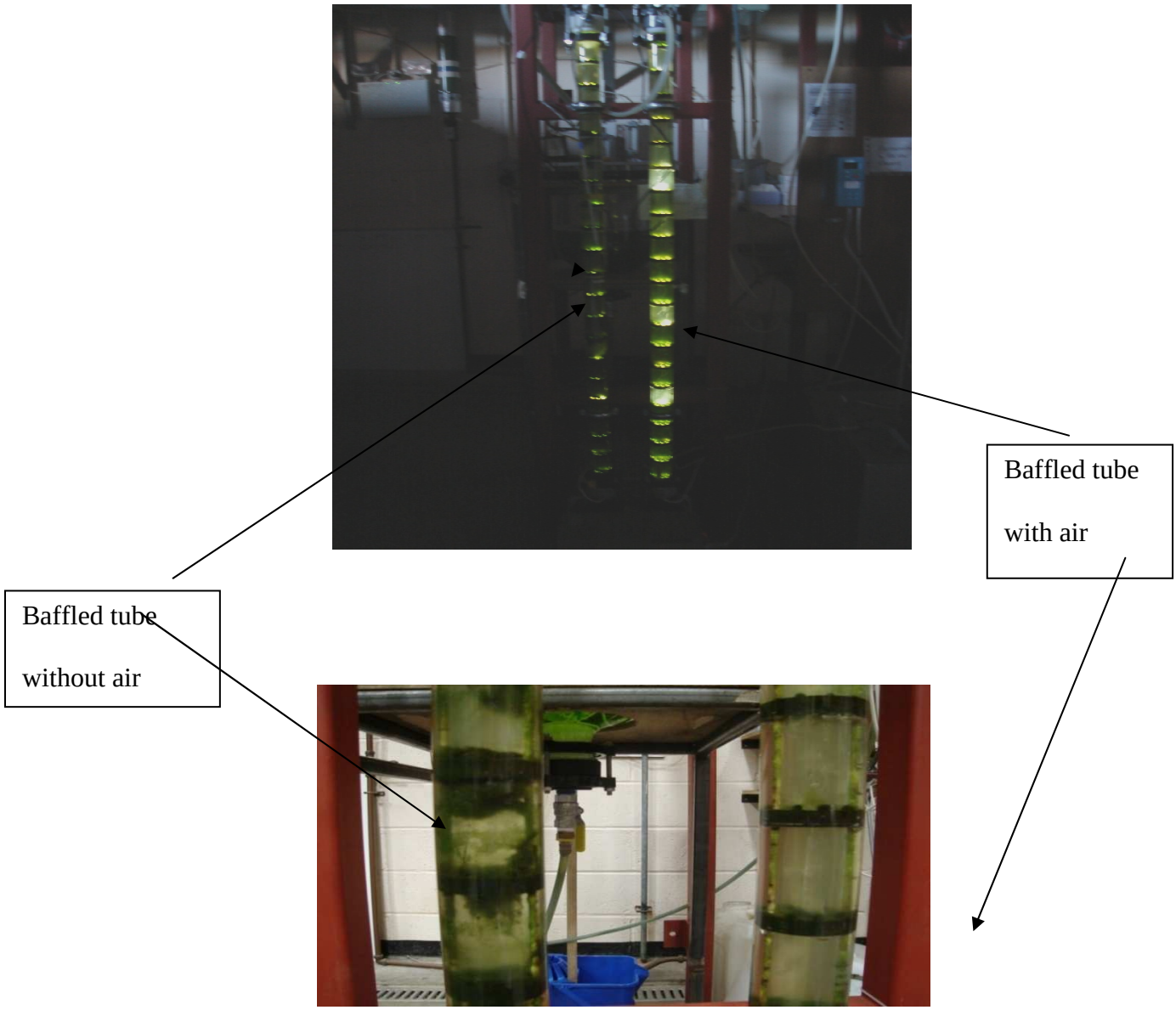


Figure 6.11 The effect of adding air on the fouling in the TBPBR.

6.6 Addition of Carbon dioxide

Cyanobacteria have the distinctive advantage of using CO_2 in the air as their carbon source to produce cellular substances, hence reduce the effect of this green house gas in the environment. In fact, CO_2 is a limiting factor in cyanobacteria cultures. The cultivation of cyanobacteria for bio-fixation of CO_2 not only would reduce the cost of production, but also would moderate carbon discharge (De Morais and Costa, 2007). In order to investigate the effect of CO_2 concentration on growth, a number of experiments were conducted under a continuous feed of enriched CO_2. The gas was added through the air flow duct containing different CO_2 concentrations, as a minimum of 3% of mole fraction in the gas phase must be added (Sforza et al., 2012). Table 6.10 displays the growth conditions for *Gloeothece membranacea* in the TBPBR with different CO_2 concentrations.

Table 6.10 shows the growth conditions for the cultures with different CO_2 concentrations in the TBPBR

Strain	*Gloeothece membranacea*
Light Intensity	80 (μmol m^{-2} sec^{-1})
Wavelength	Yellow, Blue
Light Dark Cycle	6/18 Light/Dark
Growth Medium	BG11
T	25 Cº
Container	TBPBR
Culture Volume	5L
Cell Density	94 (mg l^{-1})
Light Position	Internal
Gas composition	Air/CO_2
Flow Rate	1-3 (l min^{-1})
Re	500-1580
S/V l/m	97

Figures 6.12- 6.13 show the growth and kinetics profiles for *Gloeothece membranacea* in the TBPBR. Note that the results represent the exponential phases only.

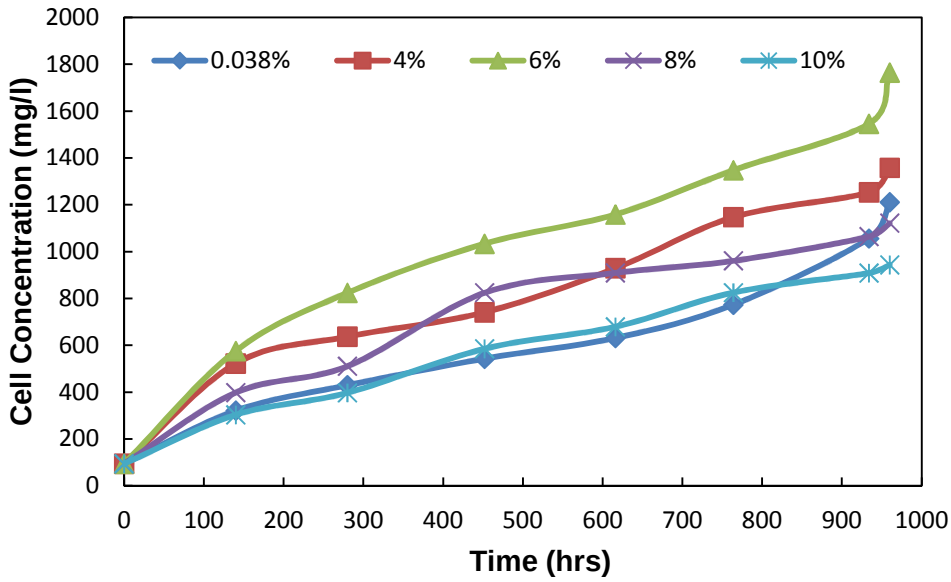


Figure 6.12 Growth profile for *Gloeothece membranacea* with different CO_2 concentrations

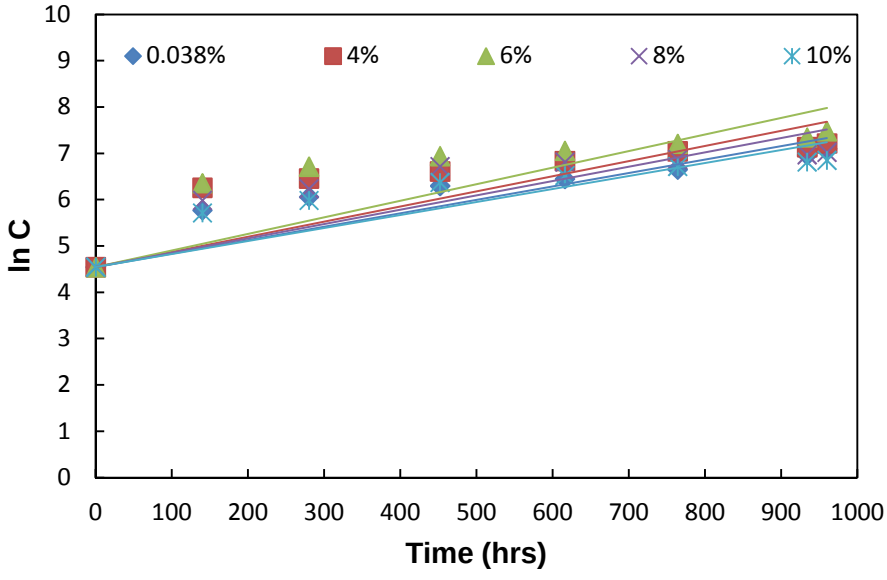


Figure 6.13 Kinetic profile for *Gloeothece membranacea* with different CO_2 concentrations

Equations 5.4 -5.5 were used to calculate the specific growth rate and the generation time of *Gloeothece membranacea* and the results are summarized in Table 6.11.

Table 6.11 The kinetics parameters in the TBPBR with different CO_2 concentrations

(V_{CO2}/ V_{Air})%	0.038	4	6	8	10
μ (hour^{-1})	0.0029	0.0033	0.0036	0.0031	0.0028
T_d (hours)	239	210	193	224	248

The growth and kinetic profiles in Figures 6.12 – 6.13 and the summarized results in Table 6.11 indicate that the specific growth rate of *G. membranacea* increased with increasing the concentration of CO_2 in the gas flow up until 6%. The growth rates increased from 0.0029 to 0.0036 hour^{-1} and the generation times decreased from 239 to 193 hrs, when increasing CO_2 concentrations from atmospheric level to 6%. This could be explained by the fact that when CO_2 concentration is liming, the specific growth rate is reduced because photosynthetic activity is slowed down (Sforza et al., 2012), as CO_2 is a limiting factor for the growth of microalgae if its concentration is low (Wang et al., 2012). However, increasing CO_2 concentrations further decreased the growth rates and increased the generation times. This may be due to the fact that higher CO_2 concentrations cause excess acidification (Ruiz-Marin et al., 2010). This was confirmed by the results as the pH values decreased from 8 to 5.7, when CO_2 concentrations increased from atmospheric level to 10% (results not shown). The concentrations of dissolved CO_2 becomes the dominant factor of the pH of a culture in high density cultures with air enriched with CO_2, it is important to highlight that the maximum value of dissolved CO_2 which can be consumed by cyanobacteria depends on strain, pH, and light availability (Kumar et al., 2011)

CHAPTER 7 CONCLUSIONS

A large number of experiments have been carried out to characterize the growth of *Gloeothece membranacea* and *Oscillatoria amoena* in light boxes as the benchmarking data for the design and comparison of the TBPBR, as well as the growth of *Gloeothece membranacea* in the TBPBR. The results in the light boxes can generally be summarized as

- The bigger the ratio of surface area over culture volume, the faster the growth rate;

- Increasing the light intensity in the range of 100-550 μmole m^{-2} sec^{-1} did not enhance the growth of *Gloeothece membranacea*, though, the higher the light intensity, the faster the growth of *Oscillatoria amoena*;

- The results of light quality clarify that different species had different absorption spectra, as *Gloeothece membranacea* growth in yellow, blue, or red lights was as fast as that in the white light, however the growth of *Oscillatoria amoena* in the coloured lights was much slower than the that in the white light;

- On the effect of the light cycle, making the light available for 6 hrs gave the same specific growth rate as that for 12 and 24 hrs;

- The effect of mixing on growth is that there was an optimal intensity of mixing for the unicellular species *Gloeothece membranacea*, while mixing did not show any effect on the growth of the filamentous species *Oscillatoria amoena*;

- The critical cell densities for both *G. membranacea* and *O. amoena* were identified as 0.100 and 0.222 $mg.l^{-1}$ respectively;

- The growth ceased at 38 °C, and the optimum range of temperature for both the species was 25-30 °C;

- It has been notified that the growth of the filamentous strain *Oscillatoria amoena* was faster than that of the unicellular strain *Gloeothece membranacea*;

- The specific growth rate and the generation time for *Gloeothece membranacea* are 0.0025 $hour^{-1}$ and 277 hours respectively, the specific growth rate and the generation time for *Oscillatoria amoena* are 0.0049 $hour^{-1}$ and 141 hours respectively under the conditions summarized in the following Table:

Strain	*Gloeothece membranacea*	*Oscillatoria amoena*
Aeration S/V (m^{-2} m^{-3})	44.2	44.2
Light intensity	520 µmol m^{-2} sec^{-1}	520 µmol m^{-2} sec^{-1}
Light colour	white	white
Light cycle (light/dark) (h/h)	6/18	6/18
Mixing (rpm)	350	0
CCD mg l^{-1}	94.0	208.0
T (°C)	25	25

Using the data from these benchmarking experiments in the light boxes, the tubular baffled photobioreactor (TBPBR) was designed, built and commissioned and further experiments with *Gloeothece membranacea* only were undertaken. The following general conclusions can be drawn:

- The light saturation level for *Gloeothece membranacea* was about 80 µmole m^{-2} sec^{-1}, and light intensity less than this value became a limiting factor for growth;

- On the period of light availability, the minimum light exposure without affecting the growth in the TBPBR and the light boxes was 6 hours;

- Much higher cell densities of *Gloeothece membranacea* can be accommodated in the TBPBR;

- Increasing the flow rate of air up to 0.08 vvm increased the specific growth rate of *Gloeothece membranacea*, however further increase in flow rate of air decreased the growth rate;

- The higher the concentrations of CO_2 in air up to 6% (Vol CO_2/ Vol air), the higher the specific growth rate of *Gloeothece membranacea*, however higher concentrations had a negative effect of the growth.

CHAPTER 8 RECOMMENDATIONS FOR THE FUTURE

A large number of batch and continuous experiments have been carried out in this thesis work, covering a variety of key process parameters, however, some areas were left out, some new aspects emerged based on the results obtained. The following is a list, but not exhaustive one, of future work to be recommended:

- Based on the available data in the light boxes, it is assumed that the critical cell density of *Gloeothece membranacea* CCD is about 94.0 mg l^{-1}, however there is a gap in the growth rates and the generation times between the cell densities 94.0 and 191.8 mg l^{-1}, so trials of cell densities between 94.0 and 191.8 mg l^{-1} should be carried out in the light boxes. The same argument applies for *Oscillatoria amoena*, as it is assumed that the critical cell density of that species CCD is about 208.9 mg l^{-1} , however, also there is a gap in the growth and generation times between the cell densities 208.9 and 440.8 mg l^{-1}, so trials of cell densities between 208.9 and 440.8 mg l^{-1} should be carried out in the light boxes;

- The temperature experiments could imply that the temperature range of 25-30 °C is the optimum one for both *Gloeothece membranacea* and *Oscillatoria amoena* as the growth rates were 0.0024 and 0.0048 $hour^{-1}$ and the generation times 289 and 144 hours. However, more experiments are required at temperatures less than 25 °C in order to further confirm the optimum temperature range;

- Appling the principle of airlift photo bioreactor in the TBPBR to achieve air circulation in both baffled tubes, i.e. one acts as the riser, another as the downcomer, is recommended. Circulating air through both tubes would not only help with mixing, but also eliminate O_2, which would harm the cells and eventually causes termination of growth, if it is allowed to build up.

- The experiments on the critical cell densities in the TBPBR suggested that cultures with cell concentrations up to 470.0 mg l^{-1} could grow without mutual shading. However due to time constraint of the experiments only 5 different cell densities were examined in these experiments. Exploration of cultures with cell concentrations higher than 470.0 mg l^{-1} should be undertaken in order to find out the maximum cell concentrations that is viable in the TBPBR without self shading. Higher critical cell densities are advantageous as higher densities mean higher productivity.

- Investigating the growth of other unicellular species of cyanobacteria as well as filamentous ones in the TBPBR would be recommended. For example *Synechococus (Cyanothece 7822)* as it interestingly produces H_2 and does not carry out hydrogen uptake activity, which is the major drawback in most hydrogen producing species (Hiroto et al., 1995). However trials should be carried out in the light boxes in order to understand if the species could grow at the wavelengths of the LEDs available and whether the process is commercially viable.

- The methodologies and procedures for cleaning and harvesting should be considered to investigate the feasibility of commercial usage.

- The energy consumption for the batch and continuous operations should be studied.

REFERENCES

ABU-REZQ, T. S., AL-MUSALLAM, L., AL-SHIMMARI, J. & DIAS, P. 1999. Optimum production conditions for different high-quality marine algae. *Hydrobiologia,* 403**,** 97-107.

ANDERSON, G. A., KOMMAREDDY, A. & SCHIPULL, M. A. 2002. Photobioreactor Design. *The Society for engineering in agriculture,food, and biological systems* (ASAE/CSAE Meeting Presentation).

AOYAMA, K., UEMURA, I., MIYAKE, J. & ASADA, Y. 1997. Fermentative metabolism to produce hydrogen gas and organic compounds in a cyanobacterium, *Spirulina platensis*. *Journal of Fermentation and Bioengineering,* 83**,** 17-20.

ARIOSA, Y., CARRASCO, D., LEGANÉS, F., QUESADA, A. & FERNÁNDEZ-VALIENTE, E. 2005. Development of cyanobacterial blooms in Valencian rice fields. *Biology and Fertility of Soils,* 41**,** 129-133.

AZIZ, M. A. & NG, W. J. 1992. Feasibility of wastewater treatment using the activated-algae process. *Bioresources Technolgy,* 40**,** 205-208.

BABU, T. S., KUMAR, A. & VARMA, A. K. 1991. Effect of light quality on phycobilisome components of the cyanobacterium *Spirulina platensis*. *Plant Physiology,* 95**,** 492-497.

BARBOSA, M., JANSSEN, M., HAM, N., TRAMPER, J. & WIJFFELS, R. 2003a. Microalgae cultivation in air-lift reactors: Modeling biomass yield and specific growth rate as a function of mixing frequency. *Biotechnology and Bioengineering,* 82**,** 170-179.

BARBOSA, M. J., ALBRECHT, M. & WIJFFELS, R. H. 2003b. Hydrodynamic stress and lethal events in sparged microalgae cultures. *Biotechnology and Bioengineering,* 83**,** 112-120.

BARBOSA, M. J., HADIYANTO & WIJFFELS, R. H. 2004. Overcoming shear stress of microalgae cultures in sparged photobioreactors. *Biotechnology and Bioengineering,* 85**,** 78-85.

BECKER, E. W. 1994. *Microalgal Biotechnology and Microbiology,* Cambridge.

BEHERA, B. K., BALASUNDARAM, R., GADGIL, K. & SHARMA, D. K. 2007. Photobiological production of hydrogen from *Spirulina* for fueling fuel cells. *Energy Sources Part a-Recovery Utilization and Environmental Effects,* 29**,** 761-767.

BELKIN, S. & BOUSSIBA, S. 1991. Resistance of *Spirulina platensis* to ammonia at high pH values. *Plant and Cell Physiology,* 32**,** 953-958.

BENEMANN, J. R. 1994. Systems and economic analysis of microalgae ponds for conversion of CO_2 to biomass. 4th Quarterly technical progress report. *Conference: US/Japan meeting on coal energy research, Albuquerque, NM (United States), 26-30 Sep 1994; Other Information: PBD: 28 Dec 1994.*

BENNETT, A. & BOGORAD, L. 1973. Complementary chromatic adaptation in a filamentous blue-green Alga. *The Journal of cell Biology,* 58**,** 419-35.

BERGMAN, B., GALLON, J. R., RAI, A. N. & STAL, L. J. 1997. N_2 Fixation by non-heterocystous cyanobacteria. *FEMS Microbiology Reviews,* 19**,** 139-185.

BOGORAD, L. 1975. Phycobiliproteins and complementary chromatic adaptation. *Annual Review of Plant Physiology,* 26**,** 369-401.

BÖHME, H. 1998. Regulation of nitrogen fixation in heterocyst-forming cyanobacteria. *Trends in Plant Science,* 3**,** 346-351.

BOROWITZKA, M. A. H., GM 2007. Economic importance of algae. *In:* MCCARTHY, P. M. & (ED), A. E. O. (eds.) *Algae of Australia.*

BRYANT, D. A., GLAZER, A. N. & EISERLING, F. A. 1976. Characterization and structural properties of the major biliproteins of *Anabaena sp*. *Archives of Microbiology,* 110**,** 61-75.

BURJA, A. M., ABOU-MANSOUR, E., BANAIGS, B., PAYRI, C., BURGESS, J. G. & WRIGHT, P. C. 2002. Culture of the marine cyanobacterium, *Lyngbya majuscula (Oscillatoriaceae),* for bioprocess intensified production of cyclic and linear lipopeptides. *Journal of Microbiological Methods,* 48**,** 207-219.

CHEVALIER, P. & DE LA NOÜE, J. 1985. Wastewater nutrient removal with microalgae immobilized in carrageenan. *Enzyme and Microbial Technology,* 7**,** 621-624.

CHISTI, Y. 2007. Biodiesel from microalgae. *Biotechnology Advances,* 25**,** 294-306.

CHO, S. H., JI, S.-C., HUR, S. B., BAE, J., PARK, I.-S. & SONG, Y.-C. 2007. Optimum temperature and salinity conditions for growth of green algae *Chlorella ellipsoidea* and *Nannochloris oculata*. *Fisheries Science,* 73**,** 1050-1056.

COSTA, J. A. V., DE MORAIS, M. G., DALCANTON, F., REICHERT, C. D. & DURANTE, A. J. 2006. Simultaneous cultivation of *Spirulina platensis* and the toxigenic cyanobacteria *Microcystis aeruginosa*. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences,* 61**,** 105-110.

DAS, D. & VEZIROGLU, T. N. 2001. Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy,* 26**,** 13-28.

DE MORAIS, M. G. & COSTA, J. A. V. 2007. Biofixation of carbon dioxide by *Spirulina sp.* and *Scenedesmus obliquus* cultivated in a three-stage serial tubular photobioreactor. *Journal of Biotechnology,* 129**,** 439-445.

ERIKSEN, N., RIISGÅRD, F., GUNTHER, W. & LØNSMANN IVERSEN, J. 2007. On-line estimation of O_2 production, CO_2 uptake, and growth kinetics of microalgal cultures in a gas-tight photobioreactor. *Journal of Applied Phycology,* 19**,** 161-174.

FAY, P. 1983. *The Blue-Greens : (Cyanophyta-Cyanobacteria) / Peter Fay,* London ; Baltimore, Md., U.S.A. :, E. Arnold.

FUJITA, Y. & HATTORI, A. 1960a. Effect of chromatic lights on phycobilin formation in a blue-green alga, *Tolypothrix Tenuis*. *Plant & Cell Physiol,* 1**,** 293—303.

FUJITA, Y. & HATTORI, A. 1960b. Formation of phycoerythrinin pre-illuminated cells of *Tolypothrix tenuis* with special reference to nitrogen metabolism. *Plant & Cell Physiology,* 1**,** 281-292.

GAFFNEY, A. M., MARKOV, S. A. & GUNASEKARAN, M. 2001. Utilization of cyanobacteria in photobioreactors for orthophosphate removal from water. *Applied Biochemistry and Biotechnology,* 91-3**,** 185-193.

GAIDHANI, H. K., MCNEIL, B. & NI, X.-W. 2003. Production of pullulan using an oscillatory baffled bioreactor. *Journal of Chemical Technology & Biotechnology,* 78**,** 260-264.

GANTT, E. 1981. Phycobilisomes. *Annual Review of Plant Physiology,* 32**,** 327-347.

GENDEL, S., OHAD, I. & BOGORAD, L. 1979. Control of phycoerythrin synthesis during chromatic adaptation. *Plant Physiology,* 64**,** 786-790.

GLAZER, A. 1981. *The Biochemistry of Plants,* New York.

GLAZER, A. N. 1982. Phycobilisomes: structure and dynamics. *Annual Review of Microbiology,* 36**,** 173-198.

GLOMBITZA K. M. and Koch M (1989). Secondary metabolites of pharmaceutical potential. *Algal and Cyanobacterial Biotechnology* (eds. R. C. Cresswell, T. A. V. Rees and M. Shah), pp. 161-238. Harlow: Longman Scientific and Technical.

GONZÁLEZ LÓPEZ, C. V., GARCÍA, M. D. C. C., FERNÁNDEZ, F. G. A., BUSTOS, C. S., CHISTI, Y. & SEVILLA, J. M. F. 2010. Protein measurements of microalgal and cyanobacterial biomass. *Bioresource Technology,* 101**,** 7587-7591.

GOODWIN, T. W. 1976. *Chemistry and Biochemistry of Plant Pigments,* London; New York, Academic Press.

GROBBELAAR, J., NEDBAL, L. & TICHÝ, V. 1996. Influence of high frequency light/dark fluctuations on photosynthetic characteristics of microalgae photoacclimated to different light intensities and implications for mass algal cultivation. *Journal of Applied Phycology,* 8**,** 335-343.

GUDIN, C. & CHAUMONT, D. 1991. Cell fragility - The key problem of microalgae mass production in closed photobioreactors. *Bioresource Technology,* 38**,** 145-151.

HAI, T., AHLERS, H., GORENFLO, V. & STEINBÜCHEL, A. 2000. Axenic cultivation of anoxygenic phototrophic bacteria, cyanobacteria, and microalgae in a new closed tubular glass photobioreactor. *Applied Microbiology and Biotechnology,* 53**,** 383-389.

HALL, D. O., MARKOV, S. A., WATANABE, Y. & KRISHNA RAO, K. 1995. The potential applications of cyanobacterial photosynthesis for clean technologies. *Photosynthesis Research,* 46**,** 159-167.

HANSEL, A. & LINDBLAD, P. 1998. Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Applied Microbiology and Biotechnology,* 50**,** 153-160.

HAPPE, T., SCHUTZ, K. & BOHME, H. 2000. Transcriptional and mutational analysis of the uptake hydrogenase of the filamentous cyanobacterium *Anabaena variabilis* ATCC 29413. *J. Bacteriol.,* 182**,** 1624-1631.

HIROTO, M., NISHIMURA, H., KODERA, Y., KAWAI, F., RUSIN, P., EHRLICH, H., LEE, S., CHANG, H., MARKOV, S., BAZIN, M., HALL, D., GUTMAN, A., SHAPIRA, M., INADA, Y., MATSUSHIMA, A., MARKOV, S., BAZIN, M. & HALL, D., (ed A. FIECHTER), 1995. The potential of using cyanobacteria in photobioreactors for hydrogen production. *Microbial and Eznymatic Bioproducts.* Springer Berlin / Heidelberg.

HOLT, J. G. 1994. *Bergey's Manual of Determinative Bacteriology*, Baltimore, Williams & Wilkins.

HORTON, P. & RUBAN, A. 2005. Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. *Journal of Experimental Botany,* 56**,** 365-373.

HU, Q., WESTERHOFF, P. & VERMAAS, W. 2000. Removal of nitrate from groundwater by cyanobacteria: Quantitative assessment of factors influencing nitrate uptake. *Applied and Environmental Microbiology,* 66**,** 133-139.

HUNTLEY, M. & REDALJE, D. 2007. CO_2 mitigation and renewable oil from photosynthetic microbes: A New Appraisal. *Mitigation and Adaptation Strategies for Global Change,* 12**,** 573-608.

IBELINGS, B. W. 1996. Changes in photosynthesis in response to combined irradiance and temperature stress in cyanobacterial surface waterblooms. *Journal of Phycology,* 32**,** 549-557.

JANSSEN, M., SLENDERS, P., TRAMPER, J., MUR, L. R. & WIJFFELS, R. 2001. Photosynthetic efficiency of *Dunaliella tertiolecta* under short light/dark cycles. *Enzyme and Microbial Technology,* 29**,** 298-305.

JEON, Y. C., CHO, C. W. & YUN, Y. S. 2005. Measurement of microalgal photosynthetic activity depending on light intensity and quality. *Biochemical Engineering Journal,* 27**,** 127-131.

JIN, E., POLLE, J. E. W., LEE, H. K., HYUN, S. M. & CHANG, M. 2003. Xanthophylls in microalgae: From biosynthesis to biotechnological mass production and application. *Journal of Microbiology and Biotechnology,* 13**,** 165-174.

KHAMEES, H, S., GALLON, J, R., CHAPLIN & A, E. 1987. *The pattern of acetylene reduction by Cyanobacteria grown under alternating light and darkness,* London, ROYAUME-UNI, Academic Press.

KIM, M. H., CHUNG, W. T., LEE, M. K., LEE, J. Y., OHH, S. J., LEE, J. H., PARK, D. H., KIM, D. J. & LEE, H. Y. 2000. Kinetics of removing nitrogenous and phosphorus compounds from swine waste by growth of microalga, *Spirulina platensis*. *Journal of Microbiology and Biotechnology,* 10**,** 455-461.

KLIPP, W., MASEPOHL, B., GALLON, J., NEWTON, W. & GALLON, J. 2005. N_2 fixation by non-heterocystous cyanobacteria. *In:* DILWORTH, M. J., JAMES, E. K.,

SPRENT, J. I. & NEWTON, W. E. (eds.) *Genetics and Regulation of Nitrogen fixation in Free-Living Bacteria.* Springer Netherlands.

KUMAR, K., DASGUPTA, C. N., NAYAK, B., LINDBLAD, P. & DAS, D. 2011. Development of suitable photobioreactors for CO_2 sequestration addressing global warming using green algae and cyanobacteria. *Bioresource Technology,* 102**,** 4945-4953.

LAZAR, T. 2003. Taiz, L. and Zeiger, *E. Plant physiology. 3rd edn*.

LEE, D. Y. & RHEE, G. Y. 1999. Kinetics of growth and death in *Anabaena flos-aquae* (cyanobacteria) under light limitation and supersaturation. *Journal of Phycology,* 35**,** 700-709.

LEHNINGER, A., NELSON, D. & COX, M. 2008. *Lehninger Principles of Biochemistry*, W. H. Freeman.

LINDBLAD, P., CHRISTENSSON, K., LINDBERG, P., FEDOROV, A., PINTO, F. & TSYGANKOV, A. 2002. Photoproduction of H_2 by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: from laboratory experiments to outdoor culture. *International Journal of Hydrogen Energy,* 27**,** 1271-1281.

LIU, J., BUKATIN, V. E. & TSYGANKOV, A. A. 2006. Light energy conversion into H_2 by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations. *International Journal of Hydrogen Energy,* 31**,** 1591-1596.

LOPES PINTO, F. A., TROSHINA, O. & LINDBLAD, P. 2002. A brief look at three decades of research on cyanobacterial hydrogen evolution. *International Journal of Hydrogen Energy,* 27**,** 1209-1215.

MADIGAN, M., MARTINKO, J. & PARKER, J. 2000. *Brock Biology of Microorganisms*, Prentice Hall.

MALKIN, R. 2005. Book review: Photosynthesis – Photobiochemistry and Photobiophysics. Advances in Photosynthesis and Respiration, Vol. 10. Bacon Ke, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2001, 763 pp, ISBN 0-7923-6334-5. *Photosynthesis Research,* 83**,** 97-98.

MARKOV, S. A., BAZIN, M. J. & HALL, D. O. 1995. Hydrogen photoproduction and carbon dioxide uptake by immobilized *Anabaena variabilis* in a hollow-fiber photobioreactor. *Enzyme and Microbial Technology,* 17**,** 306-310.

MARY I. SCRANTON, P. C. N., ANTHONY MICHAELS, SARAH G. HORRIGAN AND EDWARD J. CARPENTER 1987. Hydrogen production and nitrogen fixation by *Oscillatoria thiebautii* during in situ incubations *Limnology and Oceanography,* 32**,** 998-1006.

MASUKAWA, H., MOCHIMARU, M. & SAKURAI, H. 2002. Hydrogenases and photobiological hydrogen production utilizing nitrogenase system in cyanobacteria. *International Journal of Hydrogen Energy,* 27**,** 1471-1474.

MELIS, A. 1999. Photosystem-II damage and repair cycle in chloroplasts: what modulates the rate of photodamage in vivo. *Trends in Plant Science,* 4**,** 130-135.

MERCHUK, J. C., GARCIA-CAMACHO, F. & MOLINA-GRIMA, E. 2007. Photobioreactor design and fluid dynamics. *Chemical and Biochemical Engineering Quarterly,* 21**,** 345-355.

MIRON, A. S., CAMACHO, F. G., GOMEZ, A. C., GRIMA, E. M. & CHISTI, Y. 2000. Bubble-column and airlift photobioreactors for algal culture. *AIChE Journal,* 46**,** 1872-1886.

MOLINA GRIMA, E., FERNÁNDEZ, F. G. A., GARCÍA CAMACHO, F. & CHISTI, Y. 1999. Photobioreactors: light regime, mass transfer, and scaleup. *Journal of Biotechnology,* 70**,** 231-247.

MUNDT, S., KREITLOW, S., NOWOTNY, A. & EFFMERT, U. 2001. Biochemical and pharmacological investigations of selected cyanobacteria. *International Journal of Hygiene and Environmental Health,* 203**,** 327-334.

NI, X. & GAO, S. 1996. Scale-up correlation for mass transfer coefficients in pulsed baffled reactors. *The Chemical Engineering Journal and the Biochemical Engineering Journal,* 63**,** 157-166.

OGBONNA, J. C. & TANAKA, H. 1997. Industrial-size photobioreactors. *CHEMTECH,* 27**,** 43-49.

OLIVEIRA, M. S. N., FITCH, A. W. & NI, X.-W. 2003. A study of velocity and residence time of single bubbles in a gassed oscillatory baffled column: effect of oscillation amplitude. *Journal of Chemical Technology & Biotechnology,* 78**,** 220-226.

OLIVEIRA, M. S. N. & NI, X.-W. 2004. Characterization of a gas-liquid OBC: Bubble size and gas holdup. *AIChE Journal,* 50**,** 3019-3033.

ONO, E. 2004. Design parameters of solar concentrating systems for CO_2 mitigating algal photobioreactors. *6th International Conference on Greenhouse Gas Control Technologies,* 29**,** 1651-1657.

ORTEGA-CALVOH, J.-J. & STAL, L. J. 1994. Sulphate-limited growth in the N_2 fixing unicellular cyanobacterium *Gloeothece (Nägeli) sp*. PCC 6909. *New Phytologist,* 128**,** 273-281.

PARRY, M. A. J., ANDRALOJC, P. J., MITCHELL, R. A. C., MADGWICK, P. J. & KEYS, A. J. 2003. Manipulation of Rubisco: the amount, activity, function and regulation. *Journal of Experimental Botany,* 54**,** 1321-1333.

PAUMANN, M., REGELSBERGER, G., OBINGER, C. & PESCHEK, G. A. 2005. The bioenergetic role of dioxygen and the terminal oxidase(s) in cyanobacteria. *Biochimica et Biophysica Acta - Bioenergetics,* 1707**,** 231-253.

PERNER-NOCHTA, I. & POSTEN, C. 2007. Simulations of light intensity variation in photobioreactors. *Journal of Biotechnology,* 131**,** 276-285.

POLLE, J. E. W., KANAKAGIRI, S., JIN, E., MASUDA, T. & MELIS, A. 2002. Truncated chlorophyll antenna size of the photosystems--a practical method to improve microalgal productivity and hydrogen production in mass culture. *International Journal of Hydrogen Energy,* 27**,** 1257-1264.

POSTEN, C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences,* 9**,** 165-177.

POULSEN, B. R. & IVERSEN, J. J. L. 1999. Membrane sparger in bubble column, airlift, and combined membrane–ring sparger bioreactors. *Biotechnology and Bioengineering,* 64**,** 452-458.

PULZ, O. 2007. Performance summary report: Evaluation of GreenFuel's 3D Matrix Algae Growth Engineering Scale Unit.

PYO KIM, J., DUK KANG, C., HYUN PARK, T., SUN KIM, M. & JUN SIM, S. 2006. Enhanced hydrogen production by controlling light intensity in sulfur-deprived *Chlamydomonas reinhardtii* culture. *International Journal of Hydrogen Energy,* 31**,** 1585-1590.

RAND, M. C., GREENBERG, A. E. & TARAS, M. J. 1976. *Standard methods for the examination of water and wastewater,* Washington, American Public Health Association, The American Water Works Association, and the Water Environment Federation.

RANGA, R. A. O., A, SARADA, R, RAVISHANKAR & G, A. 2007. Influence of CO_2 on growth and hydrocarbon production in *Botryococcus braunii,* Seoul, COREE, REPUBLIQUE DE, Korean Society for Applied Microbiology.

RAO, D. L. N. & BURNS, R. G. 1990. Use of blue-green algae and bryophyte biomass as a source of nitrogen for oil-seed rape. *Biology and Fertility of Soils,* 10**,** 61-64.

RATLEDGE, C. 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. *Biochimie,* 86**,** 807-815.

READE, J. P. H., DOUGHERTY, L. J., ROGERS, L. J. & GALLON, J. R. 1999. Synthesis and proteolytic degradation of nitrogenase in cultures of the unicellular cyanobacterium *Gloeothece* strain ATCC 27152. *Microbiology,* 145**,** 1749-1758.

REIJNDERS, L. 2013. Lipid-based liquid biofuels from autotrophic microalgae: energetic and environmental performance. *Wiley Interdisciplinary Reviews: Energy and Environment,* 2**,** 73-85.

RICHMOND, A. 1986. *Cell Response to Enviromental Factors,* Boca Raton, Fl, CRS.

RICHMOND, A. 2004. Principles for attaining maximal microalgal productivity in photobioreactors: an overview. *Hydrobiologia,* 512**,** 33-37.

ROSELLO SASTRE, R., CSÖGÖR, Z., PERNER-NOCHTA, I., FLECK-SCHNEIDER, P. & POSTEN, C. 2007. Scale-down of microalgae cultivations in tubular photo-bioreactors. A conceptual approach. *Journal of Biotechnology,* 132**,** 127-133.

RUIZ-MARIN, A., MENDOZA-ESPINOSA, L. G. & STEPHENSON, T. 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating real wastewater. *Bioresource Technology,* 101**,** 58-64.

SAKURAI, H. & MASUKAWA, H. 2007. Promoting R&D in photobiological hydrogen production utilizing mariculture-raised cyanobacteria. *Marine Biotechnology,* 9**,** 128-145.

SÁNCHEZ MIRÓN, A., GARCÍA CAMACHO, F., CONTRERAS GÓMEZ, A., GRIMA, E. M. & CHISTI, Y. 2000. Bubble-column and airlift photobioreactors for algal culture. AIChE Journal, 46, 1872-1887.

SANDNES, J. M., KALLQVIST, T., WENNER, D. & GISLEROD, H. R. 2005. Combined influence of light and temperature on growth rates of *Nannochloropsis oceanica*: linking cellular responses to large-scale biomass production. *Journal of Applied Phycology,* 17**,** 515-525.

SCHENK, P., THOMAS-HALL, S., STEPHENS, E., MARX, U., MUSSGNUG, J., POSTEN, C., KRUSE, O. & HANKAMER, B. 2008. Second generation biofuels: high-efficiency microalgae for biodiesel production. *BioEnergy Research,* 1**,** 20-43.

SCHMITZ, O., BOISON, G., SALZMANN, H., BOTHE, H., SCHÜTZ, K., WANG, S.-H. & HAPPE, T. 2002. HoxE-a subunit specific for the pentameric bidirectional hydrogenase complex (HoxEFUYH) of cyanobacteria. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1554**,** 66-74.

SCHNEEGURT, M. A., SHERMAN, D. M., NAYAR, S. & SHERMAN, L. A. 1994. Oscillating behavior of carbohydrate granule formation and dinitrogen fixation in the cyanobacterium *Cyanothece sp*. strain ATCC 51142. *J. Bacteriol.*, 176**,** 1586-1597.

SCHOPF, J. W. & PACKER, B. M. 1987. Early Archean (3.3-billion to 3.5-billion-year-old) microfossils from Warrawoona Group, Australia. *Science,* 237**,** 70-73.

SCHÜTZ, K., HAPPE, T., TROSHINA, O., LINDBLAD, P., LEITÃO, E., OLIVEIRA, P. & TAMAGNINI, P. 2004. Cyanobacterial H_2 production — a comparative analysis. *Planta,* 218**,** 350-359.

SEREBRYAKOVA, L. T. & TSYGANKOV, A. A. 2007. Two-stage system for hydrogen production by immobilized cyanobacterium *Gloeocapsa alpicola* CALU 743. *Biotechnology Progress,* 23**,** 1106-1110.

SFORZA, E., BERTUCCO, A., MOROSINOTTO, T. & GIACOMETTI, G. M. 2012. Photobioreactors for microalgal growth and oil production with *Nannochloropsis salina*: From lab-scale experiments to large-scale design. *Chemical Engineering Research and Design,* 90**,** 1151-1158.

SHEEHAN, J., DUNAHAY, T., BENEMANN, J. & ROESSLER, P. 1998. A look back at the U.S. department of energy's aquatic species program biodiesel from algae. National Renewable Energy Laboratory.

SMITH, R. C., BAKER, K. S., HOLM-HANSEN, O. & OLSON, R. 1980. Photoinhibition of photosynthesis in natural waters. *Photochemistry and Photobiology,* 31**,** 585-592.

STAL, L. J. & MOEZELAAR, R. 1997. Fermentation in cyanobacteria. *FEMS Microbiology Reviews,* 21**,** 179-211.

SVESHNIKOV, D. A., SVESHNIKOVA, N. V., RAO, K. K. & HALL, D. O. 1997. Hydrogen metabolism of mutant forms of *Anabaena variabilis* in continuous cultures and under nutritional stress. *FEMS Microbiology Letters,* 147**,** 297-301.

TAMAGNINI, P., AXELSSON, R., LINDBERG, P., OXELFELT, F., WUNSCHIERS, R. & LINDBLAD, P. 2002. Hydrogenases and hydrogen metabolism of cyanobacteria. *Microbiology and Molecular Biology Reviews.,* 66**,** 1-20.

TANDEAU DE MARSAC, N. 1977. Occurrence and nature of chromatic adaptation in cyanobacteria. *J. Bacteriology.,* 130**,** 82-91.

THIEL, T. & PRATTE, B. 2001. Effect on heterocyst differentiation of nitrogen fixation in vegetative cells of the cyanobacterium *Anabaena variabilis* ATCC 29413. *Journal of Bacteriology,* 183**,** 280–286.

THIEL, T. & WOLK, C. P. 1983. Metabolic activities of isolated akinetes of the cyanobacterium *Nostoc spongiaeforme*. *Journal Of Bacteriology,* 156**,** 369-374.

TREDICI, M. R. 2007. *Mass Production of Microalgae: Photobioreactors,* Blackwell Publishing Ltd.

TROSHINA, O., SEREBRYAKOVA, L., SHEREMETIEVA, M. & LINDBLAD, P. 2002. Production of H_2 by the unicellular cyanobacterium *Gloeocapsa alpicola* CALU 743 during fermentation. *International Journal of Hydrogen Energy,* 27**,** 1283-1289.

TSYGANKOV, A. A., BORODIN, V. B., RAO, K. K. & HALL, D. O. 1999. H2 photoproduction by batch culture of *Anabaena variabilis* ATCC 29413 and its mutant PK84 in a photobioreactor. *Biotechnology and Bioengineering,* 64**,** 709-715.

TSYGANKOV, A. S., SEREBRYAKOVA, L. T., SVESHNIKOV, D. A., RAO, K. K., GOGOTOV, I. N. & HALL, D. O. 1997. Hydrogen photoproduction by three different nitrogenases in whole cells of *Anabaena variabilis* and the dependence on pH. *International Journal of Hydrogen Energy,* 22**,** 859-867.

UGWU, C. U., AOYAGI, H. & UCHIYAMA, H. 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology, 99, 4021-4028.

VEGA-ESTRADA, J., MONTES-HORCASITAS, M. C., DOMÍNGUEZ-BOCANEGRA, A. R. & CAÑIZARES-VILLANUEVA, R. O. 2005. *Haematococcus pluvialis* cultivation in split-cylinder internal-loop airlift photobioreactor under aeration conditions avoiding cell damage. *Applied Microbiology and Biotechnology,* 68**,** 31-35.

VUNJAK-NOVAKOVIC, G., KIM, Y., WU, X., BERZIN, I. & MERCHUK, J. C. 2005. Air-lift bioreactors for algal growth on flue gas: mathematical modeling and pilot-plant studies. *Industrial & Engineering Chemistry Research,* 44**,** 6154-6163.

WANG, B., LAN, C. Q. & HORSMAN, M. 2012. Closed photobioreactors for production of microalgal biomasses. *Biotechnology Advances,* 30**,** 904-912.

WANG, R. T., STEVENS, C. L. R. & MYERS, J. 1977. Action spectra for photoreactions I and II of photosynthesis in the blue-green alga *Anacystis Nidulans*. *Photochemistry and Photobiology,* 25**,** 103-108.

WEISSMAN, J. C., GOEBEL, R. P. & BENEMANN, J. R. 1988. Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation. *Biotechnology and Bioengineering,* 31**,** 336-344.

WEISSMAN, J. C., RADWAY, J. C., WILDE, E. W. & BENEMANN, J. R. 1998. Growth and production of thermophilic cyanobacteria in a simulated thermal mitigation process. *Bioresource Technology,* 65**,** 87-95.

WESTON, G. 1997. Cell biology. Second edition: Edited by C A Smith and E J Wood. pp 540. Chapman and Hall, London. 1996. £24.99 ISBN 0-412-631504. *Biochemical Education,* 25**,** 179-179.

WOLK, C. P. 1982. Heterocysts. *In:* CARR, N. G. & B.A., W. (eds.) *The Biology of Cyanobacteria.* Oxford: Blackwell.

YAN, G. A., YAN, X. & WU, W. 1997. Effects of the Herbicide Molinate on Mixotrophic growth, photosynthetic pigments, and protein content of *Anabaena*. *Ecotoxicology and Environmental Safety,* 38**,** 144-149.

YOON, J. H., SIM, S. J., KIM, M.-S. & PARK, T. H. 2002. High cell density culture of *Anabaena variabilis* using repeated injections of carbon dioxide for the production of hydrogen. *International Journal of Hydrogen Energy,* 27**,** 1265-1270.

YOSHIMOTO, N., SATO, T. & KONDO, Y. 2005. Dynamic discrete model of flashing light effect in photosynthesis of microalgae. *Journal of Applied Phycology,* 17**,** 207-214.

YOSHINO, F., IKEDA, H., MASUKAWA, H. & SAKURAI, H. 2007. High photobiological hydrogen production activity of a *Nostoc sp*. PCC 7422 uptake hydrogenase-deficient mutant with high nitrogenase activity. *Marine Biotechnology,* 9**,** 101-112.

YUEN, H., CORNDORF, E., BARBOSA, G. & KUMAR, P. 2005. Barbosa et al. Reply. *Physical Review Letters,* 94**,** 048902.

YUN, Y.-S., PARK, J. M. & VOLESKY, B. 1999. Simultaneous treatment of carbon dioxide and ammonia by microalgal culture. *Korean J. Biotechnology and Bioengineering,* 44**,** 328–336.

ZHANG, C.-C., LAURENT, S., SAKR, S., PENG, L. & BÉDU, S. 2006. Heterocyst differentiation and pattern formation in cyanobacteria: a chorus of signals. *Molecular Microbiology,* 59**,** 367-375.