HETEROCYSTOUS N₂-FIXING CYANOBACTERIA: MODELING OF CULTURE PROFILES, EFFECT OF RED LIGHT, AND CELL FLOCCULATION STUDY

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HETEROCYSTOUS N₂-FIXING CYANOBACTERIA: MODELING OF CULTURE PROFILES, EFFECT OF RED LIGHT, AND CELL FLOCCULATION STUDY

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Thesis

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ABSTRACT

The concern about the exhaustion of fossil fuels and the harmful effects of their emissions to the environment is guiding efforts to find new energy alternatives. One of the most promising energy resources is hydrogen (H₂). Cyanobacteria are among the most important candidates for photobiological H₂ production. Some filamentous cyanobacteria have evolved to differentiate specialized cells called heterocysts where nitrogen fixation and H₂ production take place.

The complete understanding of the process of heterocyst differentiation is of great importance for photobiological H₂ production. Prior to this study, there were no quantitative culture-level models that describe the effects of cellular activities and cultivation conditions on the heterocyst differentiation. Such a model was developed in this thesis, incorporating photosynthetic growth, heterocyst differentiation, self-shading effect on light penetration, and nitrogen fixation. This first-generation model was found to describe well the experimental results and it was able to predict different culture properties.

Since the productivity and economics of all photosynthesis-related processes are influenced by the light utilization efficiency, the effect of red vs. white light, and their
intensities, on cell growth and heterocyst differentiation was also investigated. Red light was found not only to stimulate the growth of the studied microorganisms but also to give higher heterocyst frequencies.

Gas vesicles were another interesting structure of some cyanobacteria studied in this thesis. Gas vesicles provide cyanobacteria with a mechanism of buoyancy regulation. Purified gas vesicle suspensions had been shown to improve oxygenation in oxygen limited systems. In order to improve the cell-collection efficacy of cyanobacteria with gas vesicles, three different flocculants were tested and compared, i.e. polyethyleneimine (PEI), chitosan and MPE-50 (a commercial product from Ondeo Nalco Center, exact formulation unknown). Even though, they showed similar flocculation efficacy at low cell concentrations, chitosan flocculation ability was greatly increased when the pH of the cell suspension was adjusted to lower values. Chitosan was proved to be useful for cyanobacterial cell collection using a concentration of 50 mg/L and adjusting pH of the cell sample to 5-7. The sensitivity of chitosan to changes in pH may be useful also in the later step of recovery and purification of gas vesicles after cell lysis.
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1.1 Quantitative modeling of culture profiles of *Anabaena flos-aquae*

The progress of our civilization has relied on fossil fuels for many years. However, there are clear evidences not only of their imminent depletion but also of the harmful effects that their emissions are having on the environment. This concern has prompted efforts to find new energy alternatives and technologies capable of satisfying the growing energy demand. One of the most promising energy resources is hydrogen (H₂).

Cyanobacteria are among the most important and fascinating candidates for photobiological hydrogen production [1]. Using sunlight as the source of energy and air as the source of carbon dioxide (CO₂) and nitrogen (N₂), they are capable of performing photosynthesis, splitting water into H₂ and oxygen (O₂). In N₂-fixing cyanobacteria, H₂ is mainly produced as an obligatory byproduct of nitrogen fixation [2]:

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi
\]
With a pure nitrogen atmosphere, at most 75% of the electrons are allocated for nitrogen reduction and at least 25% for proton reduction [3]. Nitrogenase is the enzyme complex that catalyzes this reaction. However, it is sensitive to oxygen. To overcome this obstacle, some filamentous cyanobacteria have evolved to differentiate specialized cells called heterocysts, which possess a protecting cell envelope to limit O₂ penetration and an inactive photosystem II to prevent O₂ evolution. The differentiation allows nitrogen fixation and H₂ production to take place in a microaerobic environment inside the heterocysts [4]. The reaction is, however, highly endergonic, as indicated by the required ATP in the above equation. Heterocysts rely on the surrounding vegetative (photosynthetic) cells for the import of carbohydrates as the energy source. In return, they export the fixed nitrogen to vegetative cells for growth [5]. Quantitative description of the ratio between heterocysts and vegetative cells as well as their symbiotic interaction is of critical importance to the development of H₂ production processes using the heterocystous cyanobacteria.

Heterocyst differentiation is regulated by the N-source condition of the filament [4]. When no external N-source is available, some vegetative cells will start developing into new heterocysts and, when mature heterocysts are formed, the expression of the corresponding nitrogenase will initiate to support the fixed nitrogen needs of the filament.

Some filamentous cyanobacteria can have 5 to 10% of cells as heterocysts [6]. Within a filament, the heterocysts are typically differentiated in positions where they can attain the most efficient production and distribution of fixed nitrogen to the vegetative
cells. During growth the number of vegetative cells is increased by cell division and the space between heterocysts becomes larger. It is generally believed that this process leads to the differentiation of another heterocyst near the center of the newly created chain, to conserve the regular spacing and frequency that was already present [6].

The more mechanistic explanation of the regulation of heterocyst differentiation has generally relied on the concept of a gradient of certain differentiation inhibitor(s) [4]. Initiated at a heterocyst, this compound diffuses from cell to cell along the filament and creates a decreasing concentration gradient that results in the differentiation of a vegetative cell when the concentration of the inhibitor falls below a threshold value. This highly regulated process has been most extensively studied in *Anabaena* and *Nostoc* species [3, 7]. These studies have provided much information about the genes involved in heterocyst function, structure, development, and differentiation. Among them, *ntcA* [8], *hetC* [9], *hetR* [10, 11], *hetN* [12], *patS* [13] and *hetF* [14] are now known to be essential to the differentiation process.

Despite the significant advances in genetic investigation on heterocyst differentiation, there is no quantitative modeling at the system or process level that describes the effects of cellular activities and cultivation conditions on the heterocyst differentiation, and vice versa. Such a model was developed in this study, incorporating photosynthetic growth of vegetative cells, heterocyst differentiation, self-shading effect on light penetration, and nitrogen fixation.
1.2 Effect of red light as an energy source

Another crucial factor not only for H₂ production but in all photobiological processes is the light distribution. The productivity and economics of these processes are usually limited by low light utilization efficiency. This phenomenon is associated with different factors, such as the light saturation effect, the light at wavelengths less effective for photosynthesis, and the culture’s self-shading effect. To efficiently take advantage of the potential of cyanobacteria, a highly efficient light source is desired for growth studies and cultivation. A narrow spectral output that overlaps the photosynthetic absorption spectrum, low heat dissipation, long lifetime, and practical configuration are the most important characteristics of the light source required for photobiological production processes. Other key issues, such as light delivery and its distribution inside the photobioreactor are also of great importance in the successful implementation of the photosynthetic process.

One promising light source is light emitting diodes (LEDs). LEDs have a narrow spectral output, very long life expectancy, and they are highly efficient in converting electricity to light, so that heat generation is minimized. In conventional incandescent lamps, the light-production process involves generating a lot of heat because the filament needs to be warmed. In LEDs, a much higher percentage of the electrical power is going directly to generating light, which reduces considerably electricity demands and maintenance costs.
Studies on the effect of light intensity on growth have not been very frequent in cyanobacteria. Following the fact that chlorophyll $a$, the principal chlorophyll of algae and cyanobacteria, absorbs strongly the red light near 680 nm (Figure 2.2) [15], we propose to examine the use of red LEDs as an alternative light source in cyanobacteria. The effect of this source on growth, as well as on the heterocyst differentiation process and nitrogen fixation kinetics will be discussed.

1.3 Flocculation of cyanobacteria for gas vesicle production

Heterocysts are not the only interesting structure in cyanobacteria. Gas vesicles, which are the components of gas vacuoles, provide buoyancy to cyanobacteria allowing them to position in a place where they take full advantage of the surrounding light conditions. The use of gas vesicles as oxygen carriers have been previously studied in this laboratory[16]. Even though oxygen is essential for growth and maintenance of aerobic cultures, it is often a growth-limiting factor due to its low solubility in aqueous solutions (about 8 mg/L at ambient temperature and pressure). Conventional methods of oxygen delivery such as vigorous sparging of air accompanied with agitation are harmful to certain cells such as animal cells. In order to enhance oxygen transfer in these kind of cultures some researchers have tried non-conventional methods, i.e. in situ generation of molecular oxygen with hydrogen peroxide and catalase, co-immobilization or mixed culture of an oxygen producing photosynthetic microorganism, and introduction of an immiscible phase of hydrocarbon or perfluorocarbons [17]. These approaches still have some problems such as toxicity, chemical incompatibility, increased cost of downstream
processing to remove added chemicals, competition for common nutrients, and complications in bioreactor design and operation.

Sundararajan [16] confirmed that purified gas vesicle suspensions can be used as enhancers for oxygenation in oxygen limited systems. Gas vesicles were harvested from a batch culture of *Anabaena flos-aquae* and then incorporated into a culture of shear sensitive animal cells to determine if there was an increase in oxygenation that resulted in better culture growth. When oxygen became the limiting factor, an improvement of 25% in cell growth was seen in a medium supplemented with 1.8% gas vesicles (v/v). In view of these findings, gas vesicles are thought to be also applicable in the treatment of mammalian cancers, in targeted delivery of biologically active media, and in diagnostic ultrasound [17].

However, there is still a need of an effective production of gas vesicles. Conventional methods of separation like centrifugation and vacuum filtration showed unsatisfactory yield of cell harvesting without compromising the gas vesicles. As a result, Zeleznik [17] investigated the use of polyethyleneimine (PEI) as flocculant aid to improve cell collection/harvesting by studying the cell flotation behavior. It was found that the cells aggregated with the use of PEI as a flocculant aid to enable easy collection and that the effectiveness of the polymer was influenced by the molecular weight as well as the pH of the solution.
Even though PEI was found to improve the collection technique of the cells, an examination of the effects of other flocculants such as chitosan in the recovery of the cells was recommended by Zeleznik [17] to compare the efficacy of PEI. Chitosan has several advantages over other flocculants. It is biodegradable, non-toxic and has been used as coagulating agent of suspended solids in food processing wastes [18]. It is one of the best candidates for flocculation of negatively charged particles due to its cationic nature. In addition, the flocculant action of chitosan is sensitive to pH, which can be helpful in the later step of polymer removal and gas vesicles recovery.

1.4 Research objectives

The main focus of this research is on the formation and activities of cyanobacterial heterocysts. Accordingly, the primary goal is to quantitatively describe, by experimental measurements and by mathematical modeling, the culture profiles of heterocystous cyanobacteria under different cultivation conditions. A secondary goal of this research is to improve the cell-collection efficiency of cyanobacteria with gas vesicles, via the study of cell flocculation under different conditions. The gentle cell collection is essential for the subsequent gas vesicle harvesting.

Accordingly, the specific objectives are summarized below:

1. To examine the effect of different cultivation conditions on cyanobacterial growth and heterocyst frequency.
2. To develop a first generation quantitative culture-level model incorporating photosynthetic growth of vegetative cells, heterocyst differentiation, self-shading effect on light penetration, and nitrogen fixation.

3. To investigate the effect of light intensity and red light on the growth, heterocyst differentiation and nitrogen fixation of cyanobacteria.

4. To study cell collection/harvesting efficiency using three different flocculant agents: chitosan, PEI, and a commercial product (MPE-50).

1.5 Structure of the thesis

Chapter II provides background information and an extensive survey of existing literature on the various topics related to this research. Chapter III describes the materials, cultivation conditions, and analytical methods used during this research to obtain the results. After that, Chapter IV describes the steps taken to develop the quantitative model, explains the equations proposed, and presents a discussion about the significance of the parameters obtained with the fitting process. Chapter V shows the results of the investigation of the effect of light intensities and red light on the growth and heterocyst frequencies of the cyanobacterium *Anabaena sp.* PCC7120. In Chapter VI, flocculation studies with different flocculants are presented and discussed. To finish, Chapter VII completes this thesis with proper conclusions and recommendations for future work.
CHAPTER II
LITERATURE BACKGROUND

2.1 Cyanobacteria

Cyanobacteria, also known as blue-green algae, are Gram-positive bacteria that are widespread and diverse in both freshwaters and the sea [19]. They are found not only as free living cells but also as endosymbionts. The morphologically simplest cyanobacteria are unicellular and the most complex are filamentous. Structurally, the filamentous forms can be classified as branching and non-branching. The non-branching types are again divided into two groups depending on the presence or absence of differentiated cells called heterocysts, which are the sole sites of nitrogen fixation. Cyanobacterial cells range in size from those of typical bacteria (i.e. 0.5-1 µm in diameter) to cells as large as 60 µm in diameter [15].

Cyanobacteria have the simplest nutritional requirements: they can grow in water (source of electrons and reductant) containing simple mineral salts and dissolved air (source of carbon dioxide and nitrogen), using light as the only energy source [2].
Vitamins are not required, and nitrate or ammonia is used as nitrogen source by species which do not fix nitrogen. Most species are obligate phototrophs, being unable to grow in the dark on organic compounds. However, some cyanobacteria, especially the filamentous ones, can use simple organic compounds such as glucose if light is not present.

Even though cyanobacteria are truly prokaryotic in organization, they can be distinguished from all other eubacteria by their capability to perform oxygenic photosynthesis in a manner similar to that of eukaryotic photoautotrophs (i.e. green algae and plants). Fossil evidence suggests that cyanobacteria were present in geographically different regions 2 to more than 3.5 billion years ago, during the Precambrian period. That is why they are considered to be the pioneer oxygenic phototrophs on earth [20]. In fact, photosynthesis is the principal mode of energy metabolism in these organisms [21]. This process is directed by two distinct photosynthetic systems that are working in series (Figure 2.1), using light-driven reactions to split water into hydrogen (H₂) and oxygen (O₂). In cyanobacteria, these reactions involve the hydrogenase and/or nitrogenase enzymes [19]. The first photosynthetic system involves the water splitting and O₂ evolving route (photosystem II, PSII). The second photosystem (PSI) generates the reductant used for carbon dioxide (CO₂) reduction. In this coupled process, two photons (one per photosystem) are used for each electron removed from water and used in CO₂ reduction or H₂ formation (cyanobacteria are able to produce H₂ under certain conditions as described in section 2.7.3). In green plants only CO₂ reduction takes place, as the enzyme that catalyze hydrogen formation, hydrogenase, is absent [22].
Figure 2.1. Electron flow in oxygenic photosynthesis (“Z”-scheme). P680 and P700 are the reaction center chlorophylls of PSII and PSI, respectively. Ph: Pheophytin; Q: quinone; Cyt: cytochrome; PC: plastocyanin; FeS: nonheme iron-sulfur protein; Fd: ferredoxin [15].
In order to be able to perform the process of photosynthesis, cyanobacterial cells possess a complex and highly organized system of internal membranes, in which chlorophyll \textit{a} is the essential photosynthetic pigment [19]. The presence of chlorophyll \textit{a} in cyanobacteria is one of the characteristics that distinguish them from other photosynthetic bacteria such as purple and green bacteria, which possess only bacteriochlorophylls. Accessory and protective pigments such as phycobilins and carotenoids are also part of the photosynthetic systems. They confer extended ability to harvest light for photosynthesis and, in some cases, protection the cells from UV and other light-induced cell damage. One class of phycobilins, phycocyanins, are blue, absorbing light maximally at 625-630 nm (Figure 2.2) and together with the green chlorophyll \textit{a}, are responsible for the \textit{blue-green} color of the bacteria. The light absorbing function of accessory pigments like phycobilins is of obvious advantage to the organism. Light from the sun is distributed over the whole visible range but chlorophylls absorb well in only part of this spectrum. With these accessory pigments, the organism is capable of capturing more of the available energy as shown in Figure 2.2.

2.2 Cyanobacterial structural variations

Cyanobacteria are really interesting not only because they are able to perform photosynthesis but also because some of them have evolved particular structures to survive and take full advantage of their environment. Among them, gas vesicles and heterocysts are two of the most fascinating and potentially useful structures to investigate.
Figure 2.2. Absorption spectrum of cyanobacteria [15]. Phycocyanin is responsible for the peak at 630 nm. The peaks at 680 and 430 nm are due to chlorophyll $\alpha$. 
2.2.1 Gas vesicles

Among the cytoplasmic structures seen in many cyanobacteria are gas vesicles, which are the components of gas vacuoles. Gas vesicles are especially common in species that live in open waters and are responsible for the strongly refractive appearance of the cells under the light microscope. Their function is to provide buoyancy to the organism so that it can position itself at the right depth in a vertical light gradient where their photosynthetic metabolism is optimal and the supply of minerals adequate [21].

Gas vesicles are inert, hollow, gas filled structures formed solely by proteins. A single gas vesicle is a cylinder of approximately 70 nm in diameter and 350 nm in length, closed at both ends by conical caps [21].

The main constituent of gas vesicles is a small hydrophobic protein (GvpA). Many GvpA molecules are arranged in a linear crystalline array to form the “ribs” that make the cylindrical shell and its conical caps. Many molecules of a second protein (GvpC), which has a repeating amino acid sequence, adhere to the outside of the ribs and stabilize the structure [23].

Liquid water is kept out of the vesicles by surface tension at the hydrophobic inner surface. The gas vesicle is a rigid structure that undergoes little decrease in volume when subjected to low pressures but collapses irreversibly at higher pressures [24]. The critical pressure is determined by the mechanical properties of the protein and by the diameter of
the cylindrical structure. The pressures acting on a gas vesicle suspended in water are shown in Figure 2.3. As it is shown in the figure, the vesicle inside a cell is subjected to an extra cell turgor pressure \( (p_t) \) as compared to an isolated vesicle. The gas vesicle breaks when the net pressure \( (p_n) \) exceeds the critical pressure \( (p_c) \).

Suspensions of gas vesicles appear milky white. Intact gas vesicles do not absorb light in the visible spectrum, but they scatter light strongly. The light scattering is mostly due to the gas space rather than the enclosing gas vesicle wall. The amount of light scattered decreases up to 98% or more when gas vesicles are collapsed by pressure [24]. Angular measurements show that gas vesicle suspensions scatter light strongly in forward and backward directions. The minimum intensity of the scattered light occurs at an angle of 105° to the incident beam. The scattered light can be measured directly in a nephelometer to give a relative measure of gas vesicle content.

The unique application of gas vesicles as gas microcarriers in biological systems for purposes such as oxygen supply and carbon dioxide removal in high-density cell and tissue cultures has been demonstrated [16]. Additionally, they have potential medical applications in radiation therapy for cancer treatment and as contrast agents for diagnostic ultrasound. It is known that when mammalian cancers or tumors are present, hypoxic zones are created due to the rapid growth. By increasing the oxygen partial pressure around the tumor area with injection of gas vesicles, radiation therapy could be possible without putting in danger healthy, surrounding tissue. In addition, the incorporation of gas filled vesicles in the formulation for controlled delivery of chemicals, drugs or
Figure 2.3. Pressures acting on gas vesicles suspended in water. (a) Gas vesicle outside a cell. (b) Gas vesicle inside a cell. \( p_f \): gas pressure above the suspension; \( p_h \): hydrostatic pressure; \( p_t \): cell turgor pressure; \( p_n \): net pressure; \( p_g \): gas pressure inside the gas vesicle; and \( p_c \): critical pressure [24].
diagnostic agents could stimulate liposomal release at the targeted site. Gas vesicles can also be used in diagnostic ultrasound, which is a tool for knowing the condition of internal organs of the body. The use of diagnostic ultrasound in the imaging of some metastatic lesions, where a contrast agent is needed to enhance the imaging, is still limited. Gas vesicles could replace polymer coated microbubbles, which have been used with limited success because of their large sizes.

2.2.1.1 Buoyancy regulation

In natural waters, the light intensity decreases exponentially with depth due to the absorption and scattering by the water itself and substances dissolved or suspended in it. Growth of cyanobacteria and other photosynthetic organisms occurs only if they spend sufficient time above the critical depth where photosynthesis only just compensates for respiration losses. The principal function of the gas vesicle is to provide cells with buoyancy [25]. The mechanisms of buoyancy regulation allow microorganisms to position themselves in a particular depth in a body of water or to perform vertical migrations. These mechanisms are not only a consequence of the gas vesicle gene expression and of the destruction of gas vesicle by turgor pressure, but also of the changes in the concentrations of carbohydrate and/or other dense substances. Buoyancy regulation in response to light was first demonstrated in cultures of the cyanobacterium *Anabaena flos-aquae*: buoyancy was active at low light intensities; however, it was lost at high light intensities. Since then, the buoyancy loss at high photon irradiances has been proven in other cyanobacterial species [24, 26].
When *Anabaena flos-aquae* was grown at low light intensities (<13 to 22 µmol·m\(^{-2}·s^{-1}\)) it produced enough gas vesicles to make the cells buoyant, but when transferred to a high irradiance the ability to float was lost [27]. This observation was correlated with a decrease in gas vacuolation and explained by the irreversible collapse of a proportion of the constituent gas vesicles by rising cell turgor pressure. There is evidence that gas vesicle loss due to rising turgor pressure can also occur in *Aphanizomenon flos-aquae* [28] and *Nostoc muscorum* [29].

However, turgor pressures rarely rise above 5.5 bar. That is why in the majority of the cases, the accumulation of carbohydrate ballast alone accounts for buoyancy loss. Cyanobacteria probably stores carbohydrates and byproducts of photosynthesis when grown at high light intensity and deplete these provisions by respiration and conversion to proteins at low light intensity and at night. Therefore, a change in carbohydrate concentration will lead to changes in cell density, due to the fact that carbohydrates are denser than water and proteins. These changes have been quantitatively demonstrated in many studies [28, 30]. Cells lose their ability to float during the first hours of light owing to carbohydrate formation, but buoyancy is recovered by the end of the dark period, when carbohydrates are metabolized.

### 2.2.2 Heterocysts

Another structure of consideration in cyanobacterial research is heterocysts, which are round differentiated cells, usually distributed individually along a filament or at one
end of a filament. Heterocysts arise from differentiation of vegetative (photosynthetic) cells and are the exclusive sites of nitrogen fixation in heterocystous cyanobacteria.

Heterocysts are typically larger than vegetative cells and also possess a thicker envelope with additional layers external to the cell wall. This characteristic limits the diffusion of gases into these specialized cells [31]. Two layers can be differentiated: an inner laminated layer comprising heterocyst specific glycolipids that are derivatives of hexoses with long-chain polyhydroxyalcohols, and an outer layer which contains specific polysaccharides. The outer layer can be further subdivided into an inner homogeneous layer and an external fibrous layer, which is typically the first layer that is formed during the differentiation of a heterocyst. The glycolipid layer appears to be critical in the commitment stage of heterocyst formation, after which these cells are no longer able to revert to vegetative cells.

Heterocysts can be distinguished morphologically from vegetative cells not only by their non-dividing nature but also by the distinct nodules at their polar regions, where they join a vegetative cell. This polar region also contains honeycomb membranes with high respiratory activity to scavenge O2 that enters the heterocyst from adjacent vegetative cells. The pore junction between a heterocyst and a vegetative cell is very narrow compared to the junctions between vegetative cells [32]. Very small channels (microplasmodesmata) are localized in the cytoplasmic membrane that separates adjacent cells. These narrow channels are believed to be the medium to transport metabolites
between cells in a filament. The channels are narrower but larger in number at junctions between heterocysts and vegetative cells.

Heterocysts lack photosystem II activity and ribulose bisphosphate carboxylase. Consequently they cannot reduce CO₂ via the reductive pentose phosphate pathway to provide carbon for the assimilation of fixed nitrogen. On the other hand, photosystem I activity is present, providing energy but not reductant for nitrogen fixation via production of ATP by cyclic photophosphorylation [5]. Because heterocysts cannot fix carbon, it must be supplied to heterocysts from vegetative cells. The carbon compound(s) supplied to heterocysts are not known, but sucrose is a candidate since some of the genes encoding for enzymes that are required for the sucrose metabolism in plants are also present in the genome of *Anabaena sp.* PCC 7120.

Even though heterocysts do not fix carbon, about 43% of their dry weight is carbohydrate and most of it is localized within the heterocyst envelope. Carbohydrates are metabolized in heterocysts mainly through the oxidative pentose phosphate (OPP) pathway, which produces NADPH. The activity of the enzyme that controls carbon flow into the OPP pathway (G6PD) is much higher in heterocysts than in vegetative cells. This enzyme is subject to complex regulation by key metabolites and by the reduction potential in the cell. This could be to prevent useless cycling of the oxidative and reductive pentose phosphate pathway.
Heterocyst differentiation is regulated by the N-source condition of the filament [4]. This process is usually inhibited in cells grown with a source of fixed nitrogen. Concentrations of ammonium in the range of 3-7 µM repress heterocyst differentiation in Anabaena sp. PCC 7120 [33]. The most common sources of fixed nitrogen for cyanobacteria are ammonium, nitrate, nitrite and urea. Transport systems for all these compounds have been identified in cyanobacteria. In Anabaena sp. PCC 7120, the genes involved in the uptake and reduction of nitrate and nitrite include a cluster of nirA (ferredoxin nitrite reductase)-nrtA-nrtB-nrtC-nrtD (four genes encoding an ATP-binding cassette (ABC) transporter)-narB (ferredoxin nitrate reductase). The genes in the nirA-narB cluster are strongly expressed in the absence of ammonium and expression is increased to some extent in the presence of nitrate or nitrite. When ammonium is removed from the medium, cells of Anabaena sp. PCC 7120 greatly increase the transcription of the nirA-narB genes within about 30 minutes [34].

2.3 Heterocyst differentiation and pattern formation

When no external N-source is available, some vegetative cells will start developing into new heterocysts and, when mature heterocysts are formed, the expression of the corresponding nitrogenase will initiate to support the fixed nitrogen needs of the filament. During growth the number of vegetative cells is increased by cell division and the space between heterocysts becomes larger. It is generally believed that this process leads to the differentiation of another heterocyst near the center of the newly extended chain, to conserve the regular spacing and frequency that was already present [6].
The more mechanistic explanation of the regulation of heterocyst differentiation has generally relied on the concept of a gradient of certain differentiation inhibitor(s) [4]. Initiated at a heterocyst, the diffusion of this compound from cell to cell along the filament creates a decreasing concentration gradient that results in the differentiation of a vegetative cell when the concentration of the inhibitor falls below a threshold value. This highly regulated process has been most extensively studied in *Anabaena* and *Nostoc* species [3, 7]. These studies have provided much information about the genes involved in heterocyst function, structure, development, and differentiation. Among them, *ntcA* [8], *hetC* [9], *hetR* [10, 11], *hetN* [12], *pats* [13] and *hetF* [14] are now known to be essential to the differentiation process. In addition, Wolk [35] suggested that 100-200 genes are likely to be dedicated to the process of heterocyst differentiation and nitrogen fixation in *Anabaena sp. PCC 7120*.

**hetR gene**

*hetR* gene has been recognized as one of the most important genes for heterocyst differentiation. Mutations in *hetR* sequence have completely blocked heterocyst differentiation without affecting vegetative growth [36]. Transcription of *hetR* is detectable within 30 min of removing of external nitrogen from the medium (nitrogen step-down). It is the earliest known heterocyst-specific gene to be transcribed. Under nitrogen-fixing conditions, the introduction of extra copies of *hetR* on a plasmid, by genetic engineering, was shown to result in the formation of multiple contiguous heterocysts. Even in the presence of nitrate, the extra copies introduced would result in increased heterocyst development [10]. Expression of the *Anabaena sp. PCC 7120 hetR*
gene from a copper-regulated promoter by the addition of copper to the medium resulted in high frequencies of heterocysts, up to 30% under nitrogen-fixing conditions. Buikema and Haselkorn also found high frequencies of non-fixing heterocysts even in the presence of either ammonia or nitrate [36].

HetR, the product of \textit{hetR}, is a serine protease that shows autodegradation, possibly to regulate the differential accumulation of the protein itself in different types of cells [37]. The degradation could be the cause for loss of the protein in vegetative cells, whereas inhibition of that activity results in its accumulation in heterocysts. In differentiating cells, HetR activates \textit{hetR} expression. On the other hand, in vegetative cells under conditions allowing heterocyst differentiation, \textit{hetR} expression is repressed [36, 38]. Another finding that supported this model of autoproteolysis was made in studies in \textit{N. punctiforme} that used a \textit{hetR} promoter fused to a green fluorescence protein (GFP). Results showed transcription of \textit{hetR} in both cell types, but a higher amount in heterocysts. In addition, a functional HetR-GFP fusion protein was present in heterocysts but was not visible in vegetative cells [14].

\textbf{NtcA protein}

NtcA is a regulatory protein considered to be the main element responsible for nitrogen control in cyanobacteria. It is required for transcription of the \textit{nir-nar} operon for uptake and synthesis of nitrate and nitrite. The rate of ammonium assimilation through the GS-GOGAT (glutamine synthase - 2-oxoglutarate aminotransferase) pathway increases when ammonium is present as nitrogen source. This causes a depletion of 2-
oxoglutarate in the cells which causes inactivation of NtcA. When NtcA is not active, gifA and gifB (GS regulatory genes) are not repressed and NifL, which acts as a negative regulator of nitrogen fixation, is activated. As a consequence, glutamine synthesis and nitrogen fixation do not take place when ammonium is present in the medium.

In the absence of ammonium, NtcA activates transcription of promoters involved in the utilization of alternative nitrogen sources as well as promoters of genes implicated in ammonium assimilation [39]. When nitrate (or nitrite) is present in high concentrations (but ammonium is absent), the levels of glutamine are low causing accumulation of 2-oxoglutarate. This high level of 2-oxoglutarate results in the activation of NtcA, which consequently promotes the conversion of NO$_3^-$/NO$_2^-$ into ammonia. The activation of NtcA also represses gifA and gifB which allows glutamine synthesis to occur.

When neither ammonia nor nitrate are present, the low levels of glutamine cause high 2-oxoglutarate conditions and activation of NtcA. Under nitrogen starvation conditions NtcA induces hetR expression which starts the process of heterocyst differentiation.

**hetC gene**

The product of this gene, the HetC protein, is similar to an ATP-binding cassette (ABC) membrane transport proteins, in particular, to those that export toxins [9, 40]. The hetC gene is activated directly by the NtcA protein. However, HetC is not required for the activation of hetR by NtcA, and HetR is not required for the activation of hetC. A
mutant of hetC fails to differentiate heterocysts. It appears that in developing heterocysts, cell division is not blocked. This fact suggested that HetC may be involved in the activation of the change in the heterocyst to a non-dividing state.

hetF gene

hetF gene has similar characteristics to hetR: It is also expressed early after nitrogen step-down, is essential for heterocyst formation, and leads to multiple contiguous heterocysts when extra copies are included in the plasmid. In contrast to hetR, which is also present in filamentous cyanobacterial strains that do not make heterocysts, hetF has been identified only in heterocyst-forming strains. It is believed that HetF works in combination with HetR in activation of heterocyst formation, either increasing hetR transcription or helping in the autodegradation of HetR, which is associated with loss of HetR in vegetative cells [14].

patS and hetN genes

patS and hetN genes are two negative regulators that encode peptides essential for normal heterocyst pattern formation and maintenance, respectively.

In contrast to hetR, mutants in patS produce multiple contiguous heterocysts, while either extra copies or overexpression of patS results in repression of heterocysts [13]. As a result, the patS peptide seems to act as an inhibitor of heterocyst differentiation. Furthermore, the normal spacing of heterocysts depends not only on PatS but also on its expression in heterocysts at the appropriate time during differentiation [31]. Meeks [41]
proposed a two-stage model for the process of heterocyst differentiation in which the first step is known as the initiation. In this step, only those cells under nitrogen starvation conditions (usually contiguous cells) at a certain stage in the cell cycle are competent to begin differentiation with the transcription of hetR. The second stage describes the relation between HetR and PatS. HetR is thought to induce synthesis of PatS which is released and transported to close cells causing them to regress to vegetative cells. Autoregulation of hetR by HetR results in high concentrations of HetR, which promote heterocyst formation, whereas high concentrations of PatS promote regression. In those cells in which HetR is most abundant more PatS will be made and exported, causing adjacent cells to regress.

Overexpression of hetN prevents up-regulation and patterned expression of hetR after nitrogen step-down. The hetN product is present in vegetative cells grown with external nitrogen, but after nitrogen is removed or depleted, it is located only in mature heterocysts. In studies using Anabaena sp. PCC 7120, the hetN-gene product was found to be important in maintenance of heterocysts spacing. More specifically, HetN acts as a suppressor of heterocyst formation [12]. When the normal promoter for hetN is replaced by the copper inducible promoter petE, expression of hetN in the presence of high concentrations of copper results in complete suppression of heterocyst formation. In the absence of external N, repression of hetN by removal of copper leads in the first 24 hours to a normal heterocyst pattern but later (48 h), it changes to a pattern of multiple contiguous heterocysts. These studies have suggested that HetN may act by either directly or indirectly inhibiting hetR expression. Even in the presence of high
concentrations of HetR, which usually leads to higher heterocyst frequencies, high concentrations of HetN repress heterocyst formation. HetN seems to be required for production of a signal from mature heterocysts that suppresses heterocyst differentiation along the filament. HetN is thought to be responsible for the maintenance, but not the establishment, of the spacing of new heterocysts in a filament [42].

Therefore, PatS and HetN have different but complementary functions in establishment and maintenance of the heterocyst pattern.

2.4 Nitrogen fixation

Nitrogen fixation is the process in which nitrogen gas (N\textsubscript{2}) is used as a source of nitrogen by some prokaryotes. The fixation of atmospheric nitrogen in heterocystous cyanobacteria can be described by the following equation [2]:

\[
N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi
\]  

With a pure nitrogen atmosphere, at most 75% of the electrons are allocated for nitrogen reduction and at least 25% for proton reduction [3]. Nitrogenase is the enzyme complex that catalyzes this reaction. However, it is sensitive to oxygen. To overcome this obstacle, some filamentous cyanobacteria have evolved to differentiate heterocysts, which (as was described in subsection 2.2.2) possess a protecting cell envelope to limit O\textsubscript{2} penetration and an inactive photosystem II to prevent O\textsubscript{2} evolution. The differentiation allows nitrogen fixation to take place in a microaerobic environment inside the heterocysts [4]. The reaction is, however, highly endergonic, as indicated in eq. (1) by the required ATP.
Heterocysts rely on the surrounding vegetative cells for the import of carbohydrates as the energy source. In return, they export the fixed nitrogen to vegetative cells for growth [5].

As it was described in subsection 2.3, when there is a depletion of external N-source, some vegetative cells will start differentiating into heterocysts expressing nitrogenase to support the nitrogen requirements of the filament. Some filamentous cyanobacteria can have 5 to 10% of cells as heterocysts [6]. Within a filament, the heterocysts are typically differentiated in positions where they can attain the most efficient production and distribution of fixed nitrogen to the vegetative cells. When the number of vegetative cells is increased during growth by cell division, heterocysts are separated from each other even more. This process is believed to cause the differentiation of another heterocyst near the center of the newly created chain. As a result, the regular spacing and frequency would be conserved to provide the filament with the required nitrogen [6].

The major pathway for assimilation of fixed nitrogen in cyanobacteria is via glutamine synthase (GS) and ferredoxin-dependent glutamine: 2-oxoglutarate aminotransferase (GOGAT). Glutamine synthase converts glutamate to glutamine in the presence of ATP and ammonium while GOGAT converts glutamine and oxoglutarate to glutamate (Figure 2.4). Together, these enzymes make glutamate from oxoglutarate and ammonium:
The importance of this pathway for assimilation of N₂ by nitrogen fixation was demonstrated in *Anabaena cylindrica* by the conversion of $^{13}\text{N}_2$ first to $^{13}\text{NH}_4^+$ by nitrogenase and then, through the activities of GS and then GOGAT, to $^{13}\text{N}$-glutamine followed by $^{13}\text{N}$-glutamate [43]. The GS inhibitor, methionine sulfoximine (MSX), inhibited synthesis of both $^{13}\text{N}$-glutamine and $^{13}\text{N}$-glutamate. GOGAT activity is very low or absent in heterocysts, suggesting that glutamate is supplied to heterocysts from adjacent vegetative cells [43]. GS activity is increased after nitrogen step-down. In heterocysts, the ammonium made by nitrogen fixation is assimilated by GS to make glutamine (from glutamate). Glutamine is then transported to vegetative cells where it is converted to glutamate via GOGAT. Figure 2.4 summarizes the major metabolic pathways in heterocysts and vegetative cells related to nitrogen fixation.
Figure 2.4. Major metabolic pathways in heterocysts and vegetative cells related to nitrogen fixation. IDH: isocitrate dehydrogenase; RET: respiratory electron transport; PSI: photosystem I; PSII: photosystem II; glu: glutamate; gln: glutamine; 2-OG: 2-oxoglutarate; OPP: oxidative pentose phosphate pathway; RPP: reductive pentose phosphate pathway; GS: glutamine synthetase; and GOGAT: 2-oxoglutarate aminotransferase [31].
Some studies of nitrogen fixation under natural conditions have indicated that this activity in cyanobacteria is light dependent but may continue also at a decreased rate for short periods in the dark [44].

2.4.1 Nitrogenase

The reduction of N₂ to ammonium is catalyzed by the enzyme complex nitrogenase, which is extremely sensitive to free O₂. It consists of two proteins: the dinitrogenase, which is a MoFe protein, and the dinitrogenase reductase, which is a Fe protein [15]. The dinitrogenase is an \( \alpha_2\beta_2 \) heterotetramer of about 220 to 240 kDa, and the \( \alpha \) and \( \beta \) subunits are encoded by \( \text{nif}D \) and \( \text{nif}K \) genes, respectively. The dinitrogenase reductase is a homodimer of about 60 to 70 kDa, encoded by \( \text{nif}H \) gene. It is in charge of mediating the transfer of electrons from the external donor (a ferredoxin or a flavodoxin) to the dinitrogenase [3]. Mo-containing nitrogenases, V-containing nitrogenases and Fe-containing nitrogenases are some of the alternative nitrogenases that have also been found in different cyanobacterial strains [3]. Figure 2.5 shows the nitrogenase system and the steps in nitrogen fixation of N₂ to NH₃.

The most widely used method for measurement of nitrogenase activity is the acetylene reduction assay [45]. This method relies on the capability of cyanobacteria to reduce compounds with a triple bond. Besides reducing atmospheric N₂ to NH₄⁺, cyanobacteria can also reduce acetylene (C₂H₂) to ethylene (C₂H₄), which can be detected
Figure 2.5. Nitrogenase system and the steps in nitrogen fixation of N₂ to NH₃ [15].
using gas chromatography. This method is easy, inexpensive and provides a rapid and sensitive way of measuring the activity of nitrogen-fixing systems.

2.5 Light requirements

The effect of light is one of the most important variables not only in the cultivation of cyanobacterial strains but also in the photobioreactor design. However, the productivity and economics of photobiological production processes is usually limited by low light utilization efficiency. This phenomenon is associated with different factors, such as the light saturation effect, the light at wavelengths less effective for photosynthesis, and the culture’s self-shading effect.

The light saturation effect limits the light utilization to only a small fraction near the illuminated surface. This event can be described by the Bush equation,

\[ E_s = \frac{I_s}{I_o} \left( \ln\left(\frac{I_o}{I_s}\right) + 1 \right) \]  

(5)

in which \( E_s \) is the fraction of light utilized in photosynthesis, \( I_o \) is the input light intensity, and \( I_s \) is the saturation (maximum) intensity hold by the photosynthetic apparatus. \( I_s \) varies between 50 and 200 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) for most algal groups [46]. These correspond to only 2.5-10% of the incident solar irradiance in daylight hours, which can exceed 2000 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) [47].

On the other hand, the self-shading effect is responsible for the obstruction of the light penetration into the internal portions of the photobioreactor. While a large fraction
of light is wasted as fluorescence and heat near the illuminated surface, the inner portion of the photobioreactor can be in total darkness because of the culture’s self-shading effect. As a consequence, this phenomenon directly affects the volumetric productivity in large-scale photobioreactors, except in shallow ponds of large land commitment. Several groups have been trying to propose alternatives to address these problems by introducing light-diffusing optical fibers [48-50] or using conical, helical, tubular photobioreactors, and using Induced and Diffused Photobioreactors (IDPBRs) which have polyacrylate (PMMA) light-receiving face and modified polyester diffusion sheets [51-54]. These specific designs have worked well for some particular purposes; however, the light delivery equipment in these designs creates additional problems. It occupies a significant fraction of the total reactor volumes (affecting the volumetric productivity), it can also affect the overall homogeneous mixing of the culture medium, and reactor cleaning, maintenance, and repairing becomes a more difficult task. In addition, some of the proposed photobioreactors are efficient at the laboratory scale, but most of them are inefficient when scaled-up. The low mixing in such narrow tube or thin-panel photobioreactors creates cell adhesion to, and growth on, the reactor walls and accumulation of photosynthetically produced oxygen and generated heat inside the photobioreactor. Nevertheless, shallow ponds, having extremely high land requirements and covering costs as well as low productivity, are the primary (if not the only) configuration that has been employed for commercial photosynthetic operations.

Two properties of light energy are important for algal growth and metabolism: spectral quality and intensity. Spectral quality is defined by the absorption spectrum of
the chlorophylls and other photosynthetically active pigments. As shown in Figure 2.2, chlorophyll $a$, the principal chlorophyll of algae and cyanobacteria, absorbs strongly the red light near 680 nm and the blue light near 430 nm.

Studies on the effect of light intensity on growth have not been very frequent in cyanobacteria. The total amount of organic material which can be produced from light energy by growing plant cells increases with the intensity of light up to a certain point (saturation intensity), but beyond that point the amount produced per unit of light decreases rapidly when the intensity of light increases. Light of high intensity apparently stimulates a process of photooxidation which partially offsets some of photosynthesis. As a result, the growth rate shows the expected drop at higher optical densities and increasing the light intensity does not increase the growth rate. Further, higher light intensity normally damages the cells. The term photoinhibition is frequently used for the damage caused by excess light.

2.5.1 Light emitting diodes

Light emitting diodes (LEDs) are based on conventional diodes and consist of two fused semiconductors, the $p$ (dominated by positive electric charges) and $n$ (dominated by negative electric charges) type junctions, operated with a forward bias current. In conventional diodes, movement of electrons from the $n$ to the $p$ type junction results in loss of thermal energy. However, the introduction of large quantities of impurities, called dopants, the largely silicon-based semiconductor, can result in the production of visible
light. The spectral quality of the emitted light depends on the type and the amount of dopants used and the operating temperature.

LEDs have most of the desired properties of an ideal light source. Compared to conventional incandescent lamps, LEDs last much longer since they do not contain a filament that will burn out. Additionally, their small weight and volume characteristics make them not only a lot more durable but easily adapted into any type of photobioreactors. Nevertheless, the main advantage is efficiency. In conventional incandescent lamps, the light-production process involves generating a lot of heat (the filament must be warmed). LEDs generate very little heat. A much higher percentage of the electrical power is going directly to generating light, which reduces considerably electricity demands and maintenance costs. LEDs also have a narrow spectral output. The half power band width is 20 to 30 nm which is less than 0.1% energy outside the LED bandwidth. The light delivery system is simple since light filters to eliminate UV or IR ranges are not necessary. One of the disadvantages of LEDs that prevented them to be used for most lighting applications was that they were built around advanced semiconductor material, which significantly increased their cost. However, the price of semiconductor devices has decreased in the past decade, making LEDs a more cost-effective lighting option.

In this thesis, we examine the use of red LEDs as an alternative light source to study the growth, heterocyst differentiation and nitrogen fixation kinetics of the cyanobacterium *Anabaena sp.* PCC 7120. One clear advantage of red LEDs is that
chlorophyll \(a\), the principal chlorophyll of algae and cyanobacteria, absorbs strongly the red light near 680 nm and the blue light near 430 nm (Figure 2.2). As a result, it could be expected that the absorption efficiency will be higher for red light than other wavelengths.

Another advantage of red comes from the fact that only the number of absorbed photons determines the quantity of photosynthetic products (regardless of the wavelengths of absorb photons). Thus, red photons which carry about 30% less energy per photon than blue photons will have higher photosynthetic efficiency in energy conversion.

2.6 Flocculation

Flocculation is the process whereby suspended particles are destabilized and aggregated using flocculating agents. For biological systems, it is usually used to form cell aggregates before centrifugation, gravity settling or filtration to improve the performance of these separation processes [55]. The use of flocculation is examined in this thesis as a way to complement previous studies made in this laboratory to collect cyanobacterium \textit{Anabaena flos-aquae} cells using PEI (polyethyleneimine) as an initial step in the process of gas vesicle harvesting [17].

2.6.1 PEI as flocculating aid

Flocculation with a polymer flocculant is influenced by ionic strength, pH of the polyelectrolyte solution and the molecular weight of the polymer. For flocculation to occur, PEI is first adsorbed onto the particle surface causing a charge neutralization of the
solids. The electrostatic repulsion then approaches zero which makes the particles able to approach each other close enough for short-range hydrogen bonding forces to cause polymer bridges and agglomeration to take place. When excess polymer is added, however, this adhesive effect can be reversed. Surface adsorption and surface coverage increases causing bridging to be reduced. As the surface charge increases to high positive values, the electrostatic repulsion finally helps to overcome the bridging effect and redispersal to occur [56].

The effectiveness of PEI as a flocculant to aid in the initial biomass concentration of *Anabaena flos-aquae* was thoroughly examined by Zeleznik [17]. Among high, medium and small PEI molecular weights, the medium molecular weight PEI (25,000) was found to provide the quickest and most efficient clearance of a homogeneous cell sample after flocculation and flotation processes. Taking advantage of the special property of PEI to increase the permeability of the outer membrane without direct bactericidal effect, Zeleznik [17] also studied the possibility of gas vesicles release and collection from the cells without any additional aid such as osmotic pressure shock previously used in different studies [16]. PEI is believed to be able to intercalate in the outer membrane and increase the membrane surface area resulting in disruption of the permeability barrier function of the outer membrane. It is possible that PEI interacts with various membrane components such as lipopolysaccharides and proteins. However, when gas vesicle release from the cells was tested just using PEI the process was incomplete and reached just 20% with this system.
Zeleznik [17] recommended not only examining the effect of other flocculants such as chitosan to compare their efficacy to that of PEI but also testing different methods of polymer removal to investigate if the gas vesicles are being fully released from the cells but are just entrapped within flocculated biomass.

2.6.2 Chitosan as flocculating aid

The growing demand of environmentally friendly technologies has also had an impact on using natural flocculants. Chitosan is the deacetylated product of chitin, which is the second most abundant natural polymer (poly-(1→4)-2-acetamido-2-deoxy-β-D-glucose) [57]. Chitin can be found in the exoskeleton of crustaceans such as crabs, shrimps, lobsters and insects. One of the drawbacks of using chitin is that it is insoluble in water and acid; however, chitosan is readily soluble in acetic acid, which makes it easier to use in biological applications. Chitosan is also biodegradable, non-toxic and has been used as coagulating agent of suspended solids in food processing wastes [18].

Chitosan is one of the best candidates for flocculation of negatively charged particles due to its cationic nature. It has been tested as a flocculant agent in several studies. It has showed to be a potential coagulant of kaolin suspensions and for waste water treatment processes [18, 58]. Chitosan was also tested in *E. coli* suspensions resulting in good flocculation, mainly by bridging [59].
2.7 Cyanobacterial applications and products

A number of important advances have occurred in cyanobacterial biotechnology in recent years. These bacteria have been recognized as organisms with potential to be useful to mankind in various ways: in agriculture, as a source of food, as fertilizer, and colorant, and as a fuel resource. However, only a few cyanobacterial strains (including \textit{Spirulina platensis}) have been well-characterized or exploited commercially.

2.7.1 Agricultural applications

Cyanobacteria are used as biofertilizers in fields in which nitrogen is a limited source. These bacteria are currently grown on large scale in India, the Philippines and China, for subsequent dissemination in paddy fields [20, 21]. The fertility of paddy soil is maintained by the activities of heterocystous cyanobacteria which grow spontaneously and richly in the waterlogged field. They provide fixed nitrogen to rice plants through both secretion of nitrogenous substances and on their decay following mineralization of organic substances in the soil.

Cyanobacteria have also been studied as bioremediants. Studies have demonstrated the success of using cyanobacterial mats to treat waste waters and to sequester heavy metals and radionuclides, as well as to degrade recalcitrant toxic organic compounds from polluted waters [60, 61].
The cyanobacterium *Spirulina platensis* is extensively used as food supplement and source of nutrients, such as vitamins, essential amino acids, and essential fatty acids. Some of these acids have proven to be useful in treatment of some diseases such as atopic eczema and hypercholesterolemia via reduction of low density lipoprotein.

### 2.7.2 Fine chemicals

The carotenoids and phycobiliproteins, characteristic of cyanobacteria have high commercial value [20, 62]. They are used as natural food colorants, as food additives, and are also part of the cosmetics and clinical diagnostics industries.

Some other components extracted from cyanobacteria include type II sequence-specific endonucleases, hepatotoxins, and neurotoxins [62]. These toxins have a protective role in the microorganism and could have pharmaceutical applications as well [20].

In addition, cyanobacteria have the ability as photoautotrophs to photosynthetically transform simple labeled compounds such as $^{14}\text{CO}_2$, and $^{13}\text{CO}_2$, among others, to evolve isotopically enriched substances. Isotopically labeled cyanobacterial metabolites such as sugars, lipids and amino acids are commercially available. These labeled components are being used in medical diagnostic tests, such as non-invasive breath tests that assess the metabolic activity of target organs [20, 62].
2.7.3 Cyanobacterial H₂ production

The worldwide concern about the exhaustion of fossil fuels and the harmful effects of their emissions to the environment is guiding efforts to find new energy alternatives. One of the most promising energy resources is hydrogen (H₂): it does not liberate carbon dioxide during its combustion, and because of its properties and reactivity, it has been recognized as the fuel of the future. H₂ is not a greenhouse gas and compared to other gaseous fuels, it is harmless to humans and the environment. Cyanobacteria are among the most important candidates for photobiological hydrogen production [1]. As it was seen in previous subsections, cyanobacteria produce hydrogen from the oxygenic biophotolysis of water. The reaction only requires water and sunshine, being very attractive from the point of view of environmental protection [63]:

\[ 2\text{H}_2\text{O} \rightarrow 2\text{H}^+ + 1/2\text{O}_2 \; ; \; \Delta G = -242 \text{ kJ} \]

Three cyanobacterial enzymes could be directly involved in the process of hydrogen production: nitrogenase(s) catalyzing the production of hydrogen (H₂) concomitantly with the reduction of nitrogen to ammonia, an uptake hydrogenase catalyzing the consumption of hydrogen produced by the nitrogenase, and a bidirectional hydrogenase which has the capacity to both take up and produce hydrogen. The function of hydrogenases may vary according to the necessities of the process. They could be used to dispose an excess of reducing power, or as generator of chemical energy by taking up and oxidizing H₂. It can also be used to maintain a reducing environment for reactions such as fixation of atmospheric N₂.
In N$_2$-fixing cyanobacteria, H$_2$ is mainly produced by nitrogenases, but its partial consumption is quickly catalyzed by a unidirectional uptake hydrogenase. In addition, a bidirectional (reversible) enzyme may also oxidize some of the molecular hydrogen. The same enzyme will under certain conditions evolve H$_2$. These conditions are not well established. On the other hand, H$_2$ evolved under non- N$_2$-fixing would mainly be the product of reversible/bidirectional hydrogenase.

Some cyanobacteria can produce hydrogen using the hydrogenase-based system in a manner similar to green algae. However, this system has a serious problem since oxygen is simultaneously evolved during biophotolysis of water and hydrogenases are highly sensitive to oxygen inactivation. Alternatively, the O$_2$-producing water splitting and O$_2$-sensitive hydrogenase reactions could be temporally separated using an indirect process. In this process the microorganism undergo cycles of CO$_2$ fixation into storage carbohydrates followed by their conversion to H$_2$ by endogenous metabolic processes, first in the dark to create an anoxic environment for hydrogenase induction, and then, briefly, in the light. However, dark anaerobic incubation of microalgae results in little H$_2$ production [64]. When light is applied, only a transient period of active H$_2$ production occurs, before becoming inhibited by O$_2$.

On the other hand, hydrogen is mainly produced as an obligatory byproduct of the process of nitrogen fixation in nitrogen-fixing cyanobacteria using the nitrogenase complex as was described in subsection 2.4. Nitrogenase is sensitive to oxygen as well. However, to overcome this obstacle, some filamentous cyanobacteria have evolved to
differentiate heterocysts, which possess a protecting cell envelope to limit O₂ penetration and an inactive photosystem II to prevent O₂ evolution. The differentiation allows H₂ production to take place in a microaerobic environment inside the heterocysts [4]. The reaction is, however, highly endergonic, as indicated in eq. (1) by the required ATP. The theoretical energy efficiency for hydrogen production mediated by nitrogenase is therefore lower than that by hydrogenase. The hydrogen produced may also be recycled by uptake hydrogenases, a group of enzymes found in all nitrogen-fixing cyanobacteria examined so far [3]. In spite of all these disadvantages, hydrogen production by N₂-fixing cyanobacteria has the advantage of physical mechanisms evolved by cyanobacteria to isolate the nitrogenase systems in differentiated heterocysts, which protect the enzymes from the photosynthetically produced O₂ [64].
3.1 Microorganisms and media

The filamentous cyanobacterium *Anabaena flos-aquae* CCAP 1403/13f was obtained from the Institute of Freshwater Ecology, Windermere, UK. The culture was maintained by regular subculturing in loosely capped (40-mL) glass vials with the medium of Walsby and Booker [65] shown in Table 3.1. The medium was supplemented with different concentrations of NaNO₃ (0.4 or 0.6 g/L) when used for experiments with external fixed nitrogen. No ammonium was added in the media. The routine maintenance was performed in static cultures at room temperature (22 ± 1°C) under constant illumination of about 715 lx (i.e., 13 µE/m²-s).

Additionally, the filamentous cyanobacterium *Anabaena* sp. PCC 7120 (*Nostoc* sp. ATCC® 27893) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). This culture was also maintained by regular subculturing in loosely capped (40-mL) glass vials with the ATCC medium #616 Broth: BG-11 for blue-
Table 3.1 Composition of medium for cultivation of *Anabaena flos-aquae*

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>39</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>5</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>37</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>9</td>
</tr>
<tr>
<td>FeNaEDTA</td>
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</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>6.34</td>
</tr>
<tr>
<td>NaNO$_3$(optional)</td>
<td>400</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
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</tr>
<tr>
<td>MnSO$_4$·4H$_2$O</td>
<td>0.51</td>
</tr>
<tr>
<td>(NH$_4$)$_6$Mo$<em>7$O$</em>{24}$·4H$_2$O</td>
<td>0.046</td>
</tr>
<tr>
<td>H$_3$BO$_3$</td>
<td>0.71</td>
</tr>
<tr>
<td>ZnSO$_4$·7H$_2$O</td>
<td>0.55</td>
</tr>
<tr>
<td>CuSO$_4$·5H$_2$O</td>
<td>0.020</td>
</tr>
<tr>
<td>CoSO$_4$</td>
<td>0.012</td>
</tr>
</tbody>
</table>
green algae. This medium preparation is presented in Table 3.2. NaNO₃ was withheld from the medium when used for experiments without external nitrogen. As well, the routine maintenance of this strain was performed in static cultures at 25 ± 1°C under constant illumination of about 715 lx (i.e., 13 µE/m²-s).

The inoculum for each experiment for both strains was prepared with identical medium composition as that of the specific system being tested. The light intensity used for inoculum preparation was around 900 lx (i.e., 17 µE/m²-s).

3.2 Cell cultivation

Batch culture experiments with *Anabaena flos-aquae* were conducted at room temperature in 250-mL Pyrex Erlenmeyer flasks, with working volume of 80 mL. Each flask was inoculated at 5%, i.e., with 4 mL of the prepared inoculum. Typical experiments were carried out in pairs with the flasks being agitated by stirring bars using a magnetic multi-stirrer (Bellstir Multistir 4, Bellco Glass Inc., Vineland, New Jersey, USA) for direct comparison of the effect of medium composition and cultivation conditions on the heterocyst frequency. To assure the same light conditions in all the experiments, the flasks were placed at exactly the same distance from the light source, and at the same height. The agitation speed was 265 rpm in all agitated systems. A layer of cheesecloth-wrapped cotton was used to cover the flask while allowing gas transfer. Gas exchange between the liquid broth and headspace took place through the medium surface.
Table 3.2 Composition of medium BG-11 for cultivation of *Anabaena sp.* PCC 7120

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>1.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.04</td>
</tr>
<tr>
<td>MgSO₄ · 7H₂O</td>
<td>0.075</td>
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<tr>
<td>CaCl₂ · 2H₂O</td>
<td>0.036</td>
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<tr>
<td>Citric acid</td>
<td>0.006</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.006</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.001</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace Metal Mix A5 (see below)</td>
<td>1 (mL)</td>
</tr>
<tr>
<td>Agar noble (if needed)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Trace Metal Mix A5</strong></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2.86</td>
</tr>
<tr>
<td>MnCl₂ · 4H₂O</td>
<td>1.81</td>
</tr>
<tr>
<td>ZnSO₄ · 7H₂O</td>
<td>0.222</td>
</tr>
<tr>
<td>Na₂MoO₄ · 2H₂O</td>
<td>0.39</td>
</tr>
<tr>
<td>CuSO₄ · 5H₂O</td>
<td>0.079</td>
</tr>
<tr>
<td>Co(NO₃)₂ · 6H₂O</td>
<td>0.0494</td>
</tr>
</tbody>
</table>
Fluorescent lamps (Sylvania 20-W Cool White) were placed around the flasks to provide the light energy necessary for photosynthesis. The average light intensity at the surface of the bioreactor was measured using a Lutron LX-101 lux meter (Lutron Electronics Company Inc., Coopersburg, PA). It was maintained at 900 lx (i.e., 17 \( \mu \text{E/m}^2\text{-s} \)) in the experiments conducted in this work, but was varied in a wide range (2, 5, 10, 20 and 50 \( \mu \text{E/m}^2\text{-s} \)) in Spencer’s study [66], whose results where used for modeling purposes as described in Chapter IV. The pH of the medium was initially adjusted to 7.1 and followed periodically during cultivation. It increased gradually to 8.4–8.8 in different systems. pH was not controlled because pH in the range of 7.5–9.0 had been reported to have no unfavorable effects to the culture [67].

Experiments with the cyanobacterium *Anabaena sp.* PCC7120 were carried out in batch systems conducted in 600 mL polystyrene Corning flasks with gas vented caps that permitted gas exchange in the bioreactors while preventing contamination. These flasks allowed free light penetration without distortion and their narrow width (3.5cm) minimized light attenuation from one side of the flask to the other. Light penetration and attenuation were measured using a Lutron LX-101 lux meter (Lutron Electronics Company Inc., Coopersburg, PA). In order to maximize the surface area of the cells in contact with the light and to prevent as much as possible the culture’s self shading effect, the flasks were accommodated horizontally inside a gyratory water bath shaker (New Brunswick Scientific Co, Inc; Model G76). The shaker assured gently mixing of the bacteria and the water bath (Refrigerated bath, Fisher Scientific Company, Pittsburgh, PA; Model 90) was used to maintain a constant temperature of 25±1°C in all the experiments.
The flasks were continuously illuminated with either LED panels (described below) or with fluorescent lamps (Sylvania 20-W Cool White), according to the experiment.

Each LED panel had 100 red LEDs (Kingbright, City of Industry, CA, USA) arranged in a flat surface of 15 X 15 cm. The panels were coated with fiber glass epoxy resin to make them functional under water. They were positioned directly under the flasks. Both the panel and the flask were immersed in the water bath up to about the same level as the culture inside the flask, to maintain the culture temperature. The power for the panels was provided by a Protek Dual DC Power supply (Model 1825) that permitted us to work with different light intensities. For the studies of higher light intensity, two panels had to be used for each culture flask, one under the flask and the other on top of it. A precaution was taken to keep a small distance (15 mm) between the top panel and the upper flask surface to avoid overheating the culture. To make sure that the cells were exposed only to the red light provided, the shaker was enclosed with a specially made cover that did not allow any ambient light to penetrate. The average light intensity at the upper surface of the bioreactor was measured using a Lutron LX-101 lux meter (Lutron Electronics Company Inc., Coopersburg, PA).

3.3 Analytical methods

This section describes the analytical methods used in all parts of this study using heterocystous N₂-fixing cyanobacteria.
3.3.1 Heterocyst frequency determination

Cell dry-weight concentrations as well as the ratios between vegetative cells and heterocysts were followed in the samples taken periodically from the flasks. The ratio between vegetative cells and heterocysts was determined by observing the freshly taken sample under a 400X magnification on an Olympus BX60 compound light microscope. Heterocysts and vegetative cells could be easily distinguished and counted separately under the microscope, taking no less than 500 cells in each sample. The heterocyst frequency was calculated as its averaged percentage in the total number of cells.

3.3.2 Cell concentration measurement

The cell dry-weight concentration was determined from the optical density measurement made with a Shimatzu UV/Vis spectrophotometer (Model 1601), using a wavelength of 685 nm. A calibration curve between the optical density and the cell dry-weight concentration was first developed for this purpose. For calibration, the dry cell weight was determined after centrifuging a 30-mL sample, removing the supernatant, washing once with distilled water, and drying the washed cells at 90 °C for 24 h.

Assuming that vegetative cells and heterocysts had about the same dry weight per cell, the heterocyst frequency determined microscopically (as described in Section 3.3.1) was used to calculate the dry-weight concentrations of vegetative cells and heterocysts, respectively, in each sample.
3.3.3 Intracellular protein concentration

The intracellular protein concentration was measured with the Lowry method on cell lysate [68], using the protein assay dye reagent and the assay protocol from Bio-Rad laboratories Inc. (Hercules, CA, USA). Samples were centrifuged and washed twice to avoid interferences from extracellular proteins. Cells lysis was done by adding sodium hydroxide to the pellet and heating the solution to 100°C for 20 min. The protein content was determined at 595 nm using a Shimatzu UV/Vis spectrophotometer (Model 1601). A bovine γ-globulin standard from Bio-Rad laboratories Inc. (Hercules, CA, USA) was used for establishing the calibration curve.

3.3.4 Flocculation with PEI, Chitosan and MPE-50

Batch culture experiments with *Anabaena flos-aquae* were conducted in a manner similar to that described in subsection 3.2 in 250-mL Pyrex Erlenmeyer flasks, with a working volume of 100 mL. Before flocculation, a 2-mL sample was taken from the photobioreactor and the optical density of the culture was determined using the spectrophotometer at a wavelength of 685 nm. The measured optical density was used to determine the cell concentration according to the calibration curve established, as described in section 3.3.2. The sample was then equally divided into several test tubes or 40-mL vials for studying the effects of different types and concentrations of flocculants, i.e., PEI, chitosan, and the commercial product MPE-50 from Ondeo Nalco Center (Naperville, IL).
PEI of medium molecular weight (25,000) was used through this study since a previous study in this laboratory [17] had shown that PEI of this molecular weight provided the quickest and most complete flocculation of *A. flos-aquae* culture. Accordingly, stock solutions of PEI of medium molecular weight (Aldrich Chemical Company, Inc, Milwaukee, WI), chitosan (Klarify 201, Vanson and Halo Source, Redmond, WA) and MPE-50 (Ondeo Nalco Center, Naperville, IL; exact formulation unknown) were made at a concentration of 1 g/L. After the initial optical density of the culture was determined, different doses of these three flocculants were added into the vials to reach the desired final concentrations. The vials were gently mixed and then left undisturbed during the test period.

After flocculation, a sample was taken from the flocculation-cleared zone in each vial and the optical density of the sample was determined at 685 nm using a Shimatzu UV/Vis spectrophotometer (Model 1601). For the vials where the cell mass floated to the top surface due to the presence of intracellular gas vesicles, the sample for OD measurement was taken with careful penetration of the floated cell mass using a Pasteur pipette. The flocculation efficiency was calculated as \((\text{OD}_{\text{initial}} - \text{OD}_{\text{final}})/\text{OD}_{\text{initial}}\).

### 3.3.5 Nitrate concentration

Nitrate concentrations were measured according to the ultraviolet spectrophotometric screening method described by Franson et al [69]. A nitrate calibration curve was developed for every batch of analysis using potassium nitrate.
solutions (prepared with the fresh culture medium) as the standards. The calibration curve was found to follow Beer’s law (i.e. linear correlation between absorbance and concentration) up to the concentration of 11 mg/L of NO$_3^-$-N. The standards were treated in the same manner as the samples. In this method, absorbance was measured at both wavelengths of 220 nm and 275 nm, to obtain NO$_3^-$ readings and to determine the interference due to dissolved organic matter, respectively. The correction value for dissolved organic matter was calculated substracting two times the absorbance reading at 275 nm from the reading at 220 nm to obtain absorbance due to NO$_3^-$. This absorbance value was converted to NO$_3^-$-N from the standard curve. The culture samples taken from the bioreactor were centrifuged twice to remove traces of biomass. The adequacy of the 275 nm reading was established using the standard nitrate solutions prepared with the culture medium.

3.3.6 Nitrogenase activity

Nitrogen fixation was estimated using the acetylene (C$_2$H$_2$) reduction assay [70]. Five-mL sample aliquots from the tissue culture flask were placed in 14-mL Pyrex test tubes and tightly sealed with rubber septa. The gas phase in the tubes was replaced by 15% C$_2$H$_2$ (v/v). The tubes were then incubated for 3 hours under the same light conditions used in the photobioreactor. The gas phase was sampled using a 500 µL gas tight syringe and analyzed using a gas chromatograph (Model 8610C, SRI Instruments, INC. Las Vegas, NV) equipped with a flame ionization detector and a Carbon Molecular Sieve column (Carbosieve S-II, 100/120 mesh and 10-ft long).
CHAPTER IV

MODELING CULTURE PROFILES OF HETEROCYSTOUS N₂-FIXING CYANOBACTERIUM ANABAENA FLOS-AQUAE

Heterocyst differentiation is a unique quality of nitrogen-fixing cyanobacteria, potentially important for photobiological hydrogen production. Despite the significant advances in genetic investigation on heterocyst differentiation, there are no quantitative culture-level models that describe the effects of cellular activities and cultivation conditions on the heterocyst differentiation. Such a model is developed in this chapter, incorporating photosynthetic growth of vegetative cells, heterocyst differentiation, self-shading effect on light penetration, and nitrogen fixation. The heterocystous cyanobacteria Anabaena flos-aquae CCAP 1403/13f was used in this study (Figure 4.1). The particular strain had been studied earlier in this laboratory for its production of intracellular gas vesicles [71-73]. Other researchers had reported some results on the heterocyst frequency of this species [66, 74, 75]. Particularly, when investigating the roles of light, carbon dioxide, and N-source in the regulation of cell buoyancy and growth, Spencer [66] included heterocyst frequency as one of the monitored parameters in most of the experiments [66]. In the reported study, A. flos-aquae CCAP 1403/13f
Figure 4.1 Vegetative (photosynthetic cells) and heterocysts cells of *Anabaena flos-aquae*. Arrows point to heterocysts.
cells were grown at different light intensities with or without addition of ammonium as external N-source. The ammonium-supplemented systems were maintained with multiple additions but, unfortunately, the addition schedule and amounts were not described in detail. The effect of external N-source could not be modeled quantitatively using the results from these systems. Nonetheless, the results from the purely nitrogen-fixing systems were directly useful to the purpose of this current work.

Accordingly, the current study was designed to complement that of Spencer [66], with most experiments being conducted in media containing different concentrations of ammonium and/or nitrate. All of the pertinent results of this and Spencer’s studies (reported in Table 4.1) were used to develop the intended model and to obtain the best-fit model parameters for A. flos-aquae CCAP 1403/13f. The insights gained from the obtained model parameters are also discussed in this chapter.

4.1 Model

The changes of the (dry-weight) concentrations of vegetative cells (V, g/L) and heterocysts (H, g/L) are described by the following equations:

$$\frac{dV}{dt} = \mu V - (r_{V \rightarrow H}) V - k_d V$$

(1)

$$\frac{dH}{dt} = (r_{V \rightarrow H}) V - k_d H$$

(2)

Only the vegetative cells are capable of growth via fission, as described by the first term on the right-hand side (RHS) of eq. (1), with the specific growth rate of $\mu$ (h$^{-1}$). The
Table 4.1 Systems with respective experimental conditions, and vegetative and heterocysts cell concentration experimental results

<table>
<thead>
<tr>
<th>System</th>
<th>Medium</th>
<th>Light intensity</th>
<th>Time (h)</th>
<th>Vegetative cell conc. (g/L)</th>
<th>Heterocyst cell conc. (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Without N supplementation</td>
<td>2 µE/m²s</td>
<td>0</td>
<td>0.017</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
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<td>120</td>
<td>0.021</td>
<td>0.0013</td>
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<td></td>
<td>192</td>
<td>0.016</td>
<td>0.0016</td>
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<td></td>
<td></td>
<td></td>
<td>264</td>
<td>0.018</td>
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<td></td>
<td></td>
<td>480</td>
<td>0.008</td>
<td>0.0008</td>
</tr>
<tr>
<td>2</td>
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<td>5 µE/m²s</td>
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<td>336</td>
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<td>384</td>
<td>0.051</td>
<td>0.0052</td>
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<tr>
<td>3</td>
<td>Without N supplementation</td>
<td>10 µE/m²s</td>
<td>0</td>
<td>0.010</td>
<td>0.0005</td>
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<td>0.011</td>
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<td>0.0075</td>
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<td>Without N supplementation</td>
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<td>0.012</td>
<td>0.0006</td>
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<td>192</td>
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<td>5</td>
<td>Without N supplementation</td>
<td>17 µE/m²s</td>
<td>0</td>
<td>0.007</td>
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</tr>
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<td></td>
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<td>15</td>
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<td>31</td>
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<td>50</td>
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<td>80</td>
<td>0.036</td>
<td>0.0019</td>
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Table 4.1 Systems with respective experimental conditions, and vegetative and heterocysts cell concentration experimental results (Continued)

<table>
<thead>
<tr>
<th>System</th>
<th>Medium</th>
<th>Light intensity</th>
<th>Time (h)</th>
<th>Vegetative cell conc. (g/L)</th>
<th>Heterocyst cell conc. (g/L)</th>
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<tbody>
<tr>
<td>6</td>
<td>Without N supplementation</td>
<td>20 µE/m²s</td>
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<td>0.010</td>
<td>0.0006</td>
</tr>
<tr>
<td></td>
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<td>24</td>
<td>0.017</td>
<td>0.0011</td>
</tr>
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<td>48</td>
<td>0.021</td>
<td>0.0017</td>
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<td>84</td>
<td>0.041</td>
<td>0.0043</td>
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<td>108</td>
<td>0.062</td>
<td>0.0061</td>
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<td>7</td>
<td>Without N supplementation</td>
<td>50 µE/m²s</td>
<td>0</td>
<td>0.010</td>
<td>0.0006</td>
</tr>
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<td>12</td>
<td>0.012</td>
<td>0.0008</td>
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<td>0.0044</td>
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<td>84</td>
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<td>0.0052</td>
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<td>8</td>
<td>With NO₃⁻-N supplementation (0.4g/L NaNO₃)</td>
<td>17 µE/m²s</td>
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<td>0.014</td>
<td>0.0007</td>
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<td>0.0013</td>
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<td>72</td>
<td>0.057</td>
<td>0.0021</td>
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<td></td>
<td>144</td>
<td>0.176</td>
<td>0.0032</td>
</tr>
<tr>
<td>9</td>
<td>With NO₃⁻-N supplementation (0.6g/L NaNO₃)</td>
<td>17 µE/m²s</td>
<td>0</td>
<td>0.010</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24</td>
<td>0.017</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48</td>
<td>0.065</td>
<td>0.0026</td>
</tr>
<tr>
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<td></td>
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<td>96</td>
<td>0.104</td>
<td>0.0027</td>
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<td>120</td>
<td>0.137</td>
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<td>0.0081</td>
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<td>336</td>
<td>0.389</td>
<td>0.0084</td>
</tr>
<tr>
<td>10</td>
<td>With NO₃⁻-N and NH₃-N</td>
<td>17 µE/m²s</td>
<td>0</td>
<td>0.011</td>
<td>0.0005</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>15</td>
<td>0.016</td>
<td>0.0007</td>
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<td></td>
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<td>26</td>
<td>0.015</td>
<td>0.0005</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>31</td>
<td>0.023</td>
<td>0.0008</td>
</tr>
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<td></td>
<td>80</td>
<td>0.058</td>
<td>0.0018</td>
</tr>
</tbody>
</table>
increase in H is possible only by the transformation of vegetative cells to heterocysts, as described by the (negative) second RHS term of eq. (1) and the (positive) first left-hand side (LHS) term of eq. (2). In both terms, the specific V-to-H transformation rate is denoted as \( r_{V \rightarrow H} \) (h\(^{-1}\)). Both vegetative cells and heterocysts are assumed to have constant specific decay rates (h\(^{-1}\)), i.e., \( k_{dV} \) and \( k_{dH} \), respectively.

4.1.1 Vegetative Cell Growth

For nitrogen (N)-limited growth, the specific growth rate (\( \mu \)) is modeled to have contributions from both the growth with external N sources (NH\(_3\)-N, NO\(_3\)-N, and the N released from decaying cells) and the growth with the fixed N, i.e.,

\[
\mu = \mu_{\text{max}} \left( \frac{N}{K_N + N} \right) \left( \frac{I}{K_I + I} \right) + (r_{NF})(Y_{X/N})(\frac{H}{V + H})
\] (3)

The growth dependent on external N-source (the first term on RHS) assumes the common Monod-type dependency on both N concentration and the available light intensity (I, lx), with \( \mu_{\text{max}} \) (h\(^{-1}\)) referring to the maximum specific growth rate and \( K_N \) (g/L) and \( K_I \) (lx) to the Monod constants for N and I, respectively. The specific rate of growth dependent on N\(_2\) fixation (the second term on RHS) is postulated to be directly proportional to the specific rate of N\(_2\) fixation (\( r_{NF}, \) g N/g H-h). The proportionality is governed stoichiometrically by the cell yield from N (\( Y_{X/N}, \) g cells/g N) and the heterocyst fraction in the culture [H/(V + H)]. For the same culture growing on nitrate as the N-
source, a previous study in this laboratory has determined that $Y_{X/N} = 17 \pm 2 \text{ g cells/g N}$ [16]. The fixation-dependent growth is also affected by the light intensity, which effect is incorporated in the changing $r_{NF}$ with $I$, as described in the following subsection.

4.1.2 Nitrogen Fixation

Analyzed typically by the rate of acetylene reduction [70, 76], the $N_2$ fixation rate by cyanobacteria has been reported to have both light-dependent and light-independent components [77]. In addition, it is well known that $N_2$ fixation is inhibited by the presence of external N-sources [67]. Accordingly, the specific rate of $N_2$ fixation in the heterocysts ($r_{NF}$, g N/g H-h) is modeled by the following equation:

$$r_{NF} = r_{NF}^m \left( \frac{I}{K_I + I} \right) + r_{NF}^c \left( 1 + \frac{N}{K_{NF}^I} \right)$$

(4)

The expression inside the brackets is consistent with that proposed by Staal et al. [77], who compared four different models for the light dependency of $N_2$ fixation in heterocystous cyanobacteria and concluded that the above form gave the best fit to their experimental results. $r_{NF}^m$ is the “maximum” rate of the light-dependent portion of $N_2$ fixation, and $r_{NF}^c$ is the “constitutive” rate of the light-independent portion of $N_2$ fixation. For simplicity, the inhibition by external N-sources is described by the general noncompetitive inhibition form ([55]) involving only one modeling parameter, i.e., the inhibition constant of external N to $N_2$ fixation, $K_{NF}^I$ (g/L).
4.1.3 Heterocyst Differentiation

The genetic and metabolic regulation of heterocyst differentiation has been studied quite extensively [31]. The current knowledge suggests the involvement of multi-leveled complex regulatory mechanisms [4, 31, 78, 79]. As a first-generation model, a simple culture-level approach is taken here to describe the transformation of vegetative cells to heterocysts, as described in the following equation:

\[ r_{V \rightarrow H} = \left( k_{V \rightarrow H} \right) \mu \left( \frac{\mu_{\text{max}}}{Y_{X/N}} \frac{I}{K_I + I} - \frac{H}{V} r_{NF} \right) + \mu \left( \frac{H}{V + H} \right) \left( 1 + \frac{N}{K_H} \right) \]  

(5)

At the culture level, it is reasonable to assume that the “driving force” for accelerating heterocyst differentiation is the difference between the following two rates: (1) the required specific (per unit V) N-generation rate for supporting the maximal growth under the present light intensity, i.e., \( \left( \frac{\mu_{\text{max}}}{Y_{X/N}} \right) \times \left[ \frac{I}{(K_I + I)} \right] \), and (2) the actual specific N-generation rate by the existent heterocysts (via N₂ fixation), i.e., \( r_{NF} \times \left( \frac{H}{V} \right) \).

When the driving force is zero, the culture grows with balanced N generation and consumption and, presumably, tends to maintain the same H/V ratio by having \( r_{V \rightarrow H} = \mu \times \left[ \frac{H}{(V + H)} \right] \). With a positive driving force (i.e., more N deficit), the culture is expected to raise the H/V ratio by increasing the heterocyst differentiation rate \( r_{V \rightarrow H} \). In addition, the increase in \( r_{V \rightarrow H} \) should be proportional to \( \mu \) so that the H/V ratio may actually increase, without the increasing H being countered (diluted) by the increasing V due to vegetative growth. Further, it is generally believed that heterocyst differentiation is
regulated by the presence of external N-sources [31]. Accordingly, in eq. (5), the extent of actual realization of the above “potential” for heterocyst differentiation is modeled with the general noncompetitive inhibition by the existent external-N concentration.

4.1.4 Mass Balance of External N-Sources

As described in eq. (3), the external N-sources and the fixed N are treated separately in this model. The separate treatment avoids the difficulties in measuring the fixed-N concentration. Therefore, only the vegetative growth on external N is considered in the following mass balance equation for external N sources:

\[
\frac{dN}{dt} = \frac{\mu_{\text{max}} V}{Y_{X/N}} \left(\frac{N}{K_N + N}\right) \left(\frac{I}{K_I + I}\right)
\]

(6)

A constant cell yield \(Y_{X/N}\) is assumed in this model for simplicity, although \(Y_{X/N}\) may vary slightly at different metabolic stages and under different light intensities. For example, in our earlier studies, the \(A. flos-aquae\) CCAP 1403/13f culture was found to have an approximately constant specific content of the proteinaceous gas vesicles (~ 370 \(\mu\text{L}\) per gram of dry cells) but have increasing carbohydrate contents as the growth rate slowed down, due to the diminishing light penetration caused by the increasing self-shading at higher cell concentrations [73].
In addition, at very low light or complete darkness, the culture consumed the carbohydrates as food reserve [73]. The more detailed model to account for these (and other) metabolic activities is beyond the scope of this current work.

4.1.5 Effects of Light Intensity and Self Shading

The common Monod-type dependency on light intensity has been assumed for both cell growth and N\textsubscript{2} fixation, as described in eqs. (3) and (4). \(K_I\) is the Monod constant for light. The well-known self-shading effect, which reduces the light penetration as the cell concentration (\(X = V + H\)) increases, is described in the following equation [80]:

\[
I = I_0 e^{-K_X (V + H)}
\]  

(7)

\(I_0\) (lx) is the incident light intensity measured at the surface of cultivation vessel, and \(K_X\) is the empirical self-shading constant.

4.2. Model Fitting

For clarity, the variables and kinetic parameters involved in the model are summarized in Table 4.2.

The model was fit with experimental results using Excel and the built-in Solver program. All of the above equations (1-7) were coded in Excel’s Visual Basics
Table 4.2 Kinetic parameters and variables involved in the model

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V$</td>
<td>concentration of vegetative cells (g/L)</td>
</tr>
<tr>
<td>$H$</td>
<td>concentration of heterocysts (g/L)</td>
</tr>
<tr>
<td>$\mu$</td>
<td>specific growth rate (h$^{-1}$)</td>
</tr>
<tr>
<td>$r_{V\rightarrow H}$</td>
<td>specific V-to-H differentiation rate (h$^{-1}$)</td>
</tr>
<tr>
<td>$k_{dV}$</td>
<td>specific decay rate of vegetative cells (h$^{-1}$)</td>
</tr>
<tr>
<td>$k_{dH}$</td>
<td>specific decay rate of heterocysts (h$^{-1}$)</td>
</tr>
<tr>
<td>$I$</td>
<td>light intensity (lx)</td>
</tr>
<tr>
<td>$\mu_{max}$</td>
<td>maximum specific growth rate (h$^{-1}$)</td>
</tr>
<tr>
<td>$K_N$</td>
<td>Monod constant for N (g/L)</td>
</tr>
<tr>
<td>$K_I$</td>
<td>Monod constant for light (lx)</td>
</tr>
<tr>
<td>$r_{NF}$</td>
<td>specific rate of N$_2$ fixation (g N/g H-h)</td>
</tr>
<tr>
<td>$Y_{X/N}$</td>
<td>cell yield (g cells/g N)</td>
</tr>
<tr>
<td>$r_{NF}^m$</td>
<td>maximum rate of light-dependent portion of N$_2$ fixation (g N/g H-h)</td>
</tr>
<tr>
<td>$r_{NF}^c$</td>
<td>constitutive rate of light-independent portion of N$_2$ fixation (g N/g H-h)</td>
</tr>
<tr>
<td>$K_{NF}^i$</td>
<td>inhibition constant of external N to N$_2$ fixation (g/L)</td>
</tr>
<tr>
<td>$k_{V-H}$</td>
<td>time constant associated with accelerated heterocyst differentiation (h)</td>
</tr>
<tr>
<td>$K_{H}^i$</td>
<td>inhibition constant of external N to heterocyst differentiation (g/L)</td>
</tr>
<tr>
<td>$I_0$</td>
<td>incident light intensity (lx)</td>
</tr>
<tr>
<td>$K_X$</td>
<td>empirical self-shading constant (L/g)</td>
</tr>
</tbody>
</table>
Applications, with the three simultaneous ordinary differential equations, i.e., (1), (2) and (6), being solved numerically using the fourth-order Runge-Kutta method [81].

The fitting errors for V, H, and (H/X) were calculated as the sums of squares of the “fractional” difference between calculated and experimental values; e.g., \([\frac{(V_{cal} - V_{expt})}{V_{expt}}]^2\). The Solver program was set up to find the best-fit parameters that give the minimal error. Different initial values of these parameters were tried to obtain the corresponding “local” minimal errors. The global minimum was then identified from the collection of local minimums. The values of variables giving the global minimum were taken as the best-fit values.

To improve the efficiency in managing the large number of parameters involved, the model fitting was performed in four steps: Step 1 – Estimate the values of \(\frac{Y_{X/N}}{N}\), \(\mu_{max}\) and \(r_{NF}^m\) from experimental results; Step 2 – Fix \(\frac{Y_{X/N}}{N}\), \(\mu_{max}\) and \(r_{NF}^m\) at the estimated values and fit the experimental results of V and H in Systems 1-7 (without external N sources) to get estimates of other kinetic parameters except those associated with external-N concentration, i.e., \(K_N\), \(K_{NF}^l\) and \(K_H^l\); Step 3 – Fit the results of V and H in Systems 8-10 (with external N sources) using the above estimates as initial values, to obtain the best-fit values of \(K_N\), \(K_{NF}^l\) and \(K_H^l\) (as well as to adjust the other kinetic parameters); and Step 4 – Fine-tune the above best-fit values, without fixing \(\frac{Y_{X/N}}{N}\), \(\mu_{max}\) and \(r_{NF}^m\), by refitting the results of all systems considered.
4.3 Results

4.3.1 Estimation of $Y_{X/N}$, $\mu_{\text{max}}$ and $r_{NF}^m$

As mentioned earlier, $Y_{X/N}$ for *A. flos-aquae* CCAP 1403/13f growing on nitrate as N source was determined at 17 ± 2 (g cells/g N) in a previous study[16]. Note that common heterotrophic bacteria have approximately (12-14)% of N content and, accordingly, $Y_{X/N}$ ~ 7 (g cells/g N) ([15, 55]). Intracellular proteins constitute (40-50)% of the cell dry weight in the common bacteria [82] but only about 20% in this culture, measured in this study using the common Lowry method on cell lysate [68]. The finding of much lower intracellular protein contents in the cyanobacterium supports the larger $Y_{X/N}$ determined previously. $Y_{X/N}$ was fixed at 17 (g cells/g N) in the fitting at Steps 2 and 3.

Using the experimental results from Systems 8-10 (with external N sources), $\mu_{\text{max}}$ was estimated by plotting the value of ($X/X_0$) against time (t) in a semi-logarithmic plot (Figure 4.2), where $X$ is the cell concentration at any time and $X_0$ is the cell concentration at $t = 0$ (right after inoculation). The initial (maximal) slope of the curve for each system was estimated and used to calculate the $\mu_{\text{max}}$ in that system, as given in Figure 4.2. The maximum value, $3.8 \times 10^{-2}$ (h$^{-1}$), was chosen as the initial input value of $\mu_{\text{max}}$ in the subsequent Step-2 fitting.

Similarly, $r_{NF}^m$ can be estimated from the maximum specific growth rate supported by nitrogen fixation in the systems without external N supplementation. Another set of
Figure 4.2 Maximum specific growth rate of systems containing external N (i.e. Systems 8-10). System 8 (◊), 9 (□), and 10 (△).
semi-logarithmic plots of \((X/X_0)\) versus time was therefore generated from the results of Systems 1-7 (Figure 4.3). The specific growth rate supported by nitrogen fixation under a specific light intensity \((I)\) was again determined from the initial slope of the corresponding curve (values given in Figure 4.3). The specific growth rate increased with increasing \(I\) but appeared to level off at \(I \geq 17\mu E\ m^{-2} s^{-1}\). The highest value estimated, i.e., \(2.9 \times 10^{-2}\) (h\(^{-1}\)), was taken as the maximum specific growth rate supportable by nitrogen fixation. This value was then used to estimate the maximum rate of light-dependent portion of N\(_2\) fixation \((r_{NF}^m)\), considering only the contribution to growth from N-fixation, i.e., the second RHS term in Equation (3):

\[
\text{Maximum Specific Growth Rate by N Fixation} = (r_{NF}^m)(Y_{X/N})(\frac{H}{V + H})
\]  

(8)

The value of \(r_{NF}^m\) was thus estimated to be \(3.0 \times 10^{-2}\) (g N/g H-h), i.e., \((2.9 \times 10^{-2})/(17)/(0.057)\), where \(Y_{X/N}\) was estimated at 17 g cells/g N and the heterocyst fraction, \(H/(V + H)\), was estimated at 5.7\% from the experimental data. Note that the above estimation for \(\mu_{max}\) and \(r_{NF}^m\) did not take into account of the decay constants, \(k_d^V\) and \(k_d^H\). While likely being underestimated, these values should be in the correct orders of magnitude.

4.3.2 Best-Fit Kinetic Parameters and Growth Profiles

All of the best-fit values of the parameters are summarized in Table 4.3, as well as the initial value ranges tried in the model fitting. Some justifications and/or implications
Figure 4.3 Maximum specific growth rate of systems without N supplementation (i.e. Systems 1-7) at different light intensities ($I_o = 2, 5, 10, 17, 20, 50 \mu E m^{-2} s^{-1}$, respectively). System 1(▲), 2(×), 3(●), 4(■), 5(♦), 6(−), and 7(+).
Table 4.3 Best-fit values and fitting restrictions for model parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Best-fit values</th>
<th>Fitting restriction ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{V-H}$</td>
<td>37.95 (h)</td>
<td>1 - 100</td>
</tr>
<tr>
<td>$\mu_{max}$</td>
<td>$4.67 \times 10^{-2}$ (h$^{-1}$)</td>
<td>$1 \times 10^{-3}$ - 0.1</td>
</tr>
<tr>
<td>$r_{NF}^m$</td>
<td>$3.55 \times 10^{-2}$ (g N/g H-h)</td>
<td>$1 \times 10^{-4}$ - 0.1</td>
</tr>
<tr>
<td>$r_{NF}^c$</td>
<td>0 (g N/g H-h)</td>
<td>0 - 0.1</td>
</tr>
<tr>
<td>$K_I$</td>
<td>$5.77 (\mbox{J m}^{-2}\mbox{s}^{-1})$</td>
<td>0.1 - 20</td>
</tr>
<tr>
<td>$K_N$</td>
<td>$1.45 \times 10^{-2}$ (g/L)</td>
<td>$1 \times 10^{-6}$ - 1</td>
</tr>
<tr>
<td>$K_H^i$</td>
<td>$2.56 \times 10^{-2}$ (g/L)</td>
<td>$1 \times 10^{-5}$ - 0.1</td>
</tr>
<tr>
<td>$K_{NF}^i$</td>
<td>$5.78 \times 10^{-3}$ (g/L)</td>
<td>$1 \times 10^{-7}$ - $1 \times 10^{-2}$</td>
</tr>
<tr>
<td>$k_d^V$</td>
<td>$8.47 \times 10^{-3}$ (h$^{-1}$)</td>
<td>$1 \times 10^{-4}$ - $1 \times 10^{-2}$</td>
</tr>
<tr>
<td>$k_d^H$</td>
<td>$6.80 \times 10^{-3}$ (h$^{-1}$)</td>
<td>$1 \times 10^{-4}$ - $1 \times 10^{-2}$</td>
</tr>
<tr>
<td>$Y_{X/N}$</td>
<td>17 (g cells/g N)</td>
<td>14 - 20</td>
</tr>
<tr>
<td>$K_X$</td>
<td>4.5 (L/g)</td>
<td>1 - 100</td>
</tr>
<tr>
<td>$K_{X-2}$</td>
<td>20.7 (L/g)</td>
<td>1 - 100</td>
</tr>
</tbody>
</table>
of the best-fit values obtained are given later in Discussion. For comparison, profiles of heterocyst and vegetative cell concentrations for Systems 4 and 10 are shown in Figure 4.4, where the symbols represent the experimental data while the lines are the results of model prediction using the best-fit parameters. These two systems (4 and 10) are chosen as representatives of the two growth conditions studied in this work: System 4 for the cyanobacterial growth on nitrogen fixation and System 10 for the growth with externally supplemented N sources, respectively. Among the systems without external N supplementation, System 4 had an intermediate level of light intensity (i.e., 10µE m\(^{-2}\) s\(^{-1}\)) in the range investigated (i.e., 2-50µE m\(^{-2}\) s\(^{-1}\)). The current model is shown to describe the cell growth profiles fairly well for both culture conditions.

4.3.3 Model-Predicted Profiles of \(N, I, H/V, \mu, r_{V \rightarrow H}\) and \(r_{NF}\)

Using the model and the best-fit parameters obtained, the profiles of various culture properties can be computed for different conditions. For example, the profiles of \(N, I, H/V, \mu, r_{V \rightarrow H}\) and \(r_{NF}\) for growth on different external N concentrations at a fixed \(I_o (=17\mu\text{E m}^{-2}\text{ s}^{-1})\) are summarized in Figure 4.5, 4.6 and 4.7, respectively. (All parameters are taken from Table 4.3 and the values \(V_o=0.014\ g\ /L\) and \(H_o=6.9 \times 10^{-4}\) are the vegetative and heterocyst cell initial concentrations of a representative system with external N supplementation, such as System 8.)

As expected, the light intensity \(I\) and the external N concentration decrease along with cell growth. Owing to the ability of nitrogen fixation, the growth is eventually
Figure 4.4 Experimental results of heterocyst (□) and vegetative cell concentration (♦) of two representative systems: System 4 (A) and System 10 (B). Model best-fitted profiles of heterocyst and vegetative cell concentration are presented as discontinuous and continuous lines respectively.
Figure 4.5 Model predicted profiles of nitrogen (A), cell concentration and light intensity (B) for growth on different external N concentrations: $1 \times 10^{-4}$ ($♦$), 0.01($■$), 0.07($▲$), 0.15($×$), 0.25($+$) g/L at a fixed $I_o=17\mu E\ m^{-2}\ s^{-1}$. All parameters are taken from Table 4.3 and the values $V_o=0.014\ g/L$ and $H_o=6.9 \times 10^{-4}$ are the vegetative and heterocyst cell initial concentrations of a representative system with external N supplementation.
Figure 4.6 Model predicted profiles of H/V (A), and specific growth rate (B) for growth on different external N concentrations: $1 \times 10^{-4}$ (♦), 0.01(■), 0.07(▲), 0.15(×), 0.25(+) \text{g/L}$ at a fixed $I_0=17\mu\text{E m}^{-2}\text{s}^{-1}$. All parameters are taken from Table 4.3 and the values $V_o=0.014 \text{ g/L}$ and $H_o=6.9 \times 10^{-4}$ are the vegetative and heterocyst cell initial concentrations of a representative system with external N supplementation.
Figure 4.7 Model predicted profiles of nitrogen $r_{NF}$ (A) and $r_{V\rightarrow H}$ (B) for growth on different external N concentrations: $1 \times 10^{-4}$ (♦), 0.01(■), 0.07(▲), 0.15(×), 0.25(+) g/L at a fixed $I_o=17\mu E \ m^{-2} \ s^{-1}$. All parameters are taken from Table 4.3 and the values $V_o=0.014$ g/L and $H_o=6.9 \times 10^{-4}$ are the vegetative and heterocyst cell initial concentrations of a representative system with external N supplementation.
limited by $I$ as a result of self-shading effect. With the assumed initial $H/V = 5\%$, $H/V$ decreases with time in systems with high external N concentrations, caused by the inhibited heterocyst differentiation. However, in systems with low or no external N concentrations, $H/V$ increases initially and then levels off. The initial increase in $H/V$ corresponds to accelerated growth while $H/V$ levels off when the specific growth rate starts to decrease (due to decreasing $I$). The profiles of specific growth rate are particularly interesting. For the system without any external N sources, the profile corresponds to the growth supported completely by nitrogen fixation. For the systems with high external N concentrations, the profiles show the growth on external N only. For the system with initial external N concentration of 0.01 g/L, the profile indicates that the current model successfully simulates the expected smooth transition under the combined growth on external N and nitrogen fixation.

4.4 Discussion

Some justifications and/or implications of the best-fit kinetic parameters obtained in this study are discussed below:

4.4.1 $\mu_{\text{max}}, r_{NF}^m, r_{NF}^c$ and $Y_{X/N}$

The estimated values of $Y_{X/N}$, $\mu_{\text{max}}$ and $r_{NF}^m$ were 17 (g cells/g N), $3.8 \times 10^{-2}$ (h$^{-1}$) and $3.0 \times 10^{-2}$ (g N/g H-h), respectively. The final best-fit value for $Y_{X/N}$ did not change (dropped insignificantly to 16.96). The values for $\mu_{\text{max}}$ and $r_{NF}^m$ increased to $4.67 \times 10^{-2}$
(h⁻¹) and 3.55×10⁻² (g N/g H-h), respectively, primarily compensating for the underestimation due to the ignored decay constant \(k_d = 8.5 \times 10^{-3} \text{ h}^{-1}\).

The rate of N fixation was originally modeled in a form similar to that reported by Staal et al. [77], including both light-dependent and light-independent \(r_{NF_c}\) components. The values of \(r_{NF_m}\) and \(r_{NF_c}\) were calculated to be 4.16 (± 3.85) × 10⁻⁴ (g N/g cells-h) and 2.50 (± 2.02) × 10⁻⁴ (g N/g cells-h) from the data reported by Staal et al. [77]. (Note that Staal et al. evaluated the rate of N-fixation according to that of acetylene (C₂H₂) conversion to ethylene (C₂H₄), in the unit of \(\mu\text{mol C}_2\text{H}_4/\text{mg Chla-h}\), where Chla stands for chlorophyll \(a\) [77]. The above values were converted using the following correlations: (1) 3 moles of ethylene produced are equivalent to one mole of N₂ fixed [66], and (2) 1 g of cell dry weight is equivalent to ~1.93 mg of Chla [83]. However, Staal et al. [77] did not report the heterocyst concentrations in their environmental samples. The above values could not be further converted to the unit used in this model, (g N/g H-h), for direct numerical comparison.) While \(r_{NF_c}\) was ~ 60% of \(r_{NF_m}\) in the literature report, \(r_{NF_c}\) consistently reduced to zero (or very small values) during the model fitting in this study. The difference may be well explained by the different experimental conditions employed. Staal et al. [77] cultivated the cells under 40 µE m⁻² s⁻¹ in a photobioreactor. Samples were then taken at different growth stages for evaluation of the N-fixation rates under different light intensities, ranging 0-1600 µE m⁻² s⁻¹. In effect, the experiments evaluated the response to different light intensities of the cells established at the same high-light cultivation condition, and the finding of a high \(r_{NF_c}\) value merely indicated that the N-fixing machinery already present in the high-light-grown culture could function at a
significant level even at completely dark conditions. On the other hand, in this work the N-fixation rate was modeled with the cells cultivated at different light intensities. The negligible value of $r_{NF}$ observed in this work indicated the reasonable adjustment of the culture to its growth conditions, i.e., the culture substantially limited the expense of resources for maintaining the N-fixing machinery that was useless anyway in the darkness.

4.4.2 Time constant associated with accelerated rate of heterocyst differentiation, $k_{V\rightarrow H}$

$k_{V\rightarrow H}$ is a time constant in eq. (5), associated with the accelerated rate of heterocyst differentiation when there is a driving force caused by the shortage in N for growth. The best-fit value of $k_{V\rightarrow H}$ was found as 38.0 h. In eq. (5), the accelerated differentiation is assumed to be proportional to $\mu$, and the maximum proportionality constant $\sim (k_{V\rightarrow H})(\mu_{max})/Y_{X/N} = 0.10$. The heterocyst differentiation is therefore conservatively regulated, with $r_{V\rightarrow H}$ never too large a fraction of $\mu$. This conservative nature of differentiation is very reasonable considering that the differentiation is irreversible.

4.4.3 Monod constant for light, $K_I$

The “saturation” light intensity for $A. flos-aquae$ was reported to be approximately 22µE m$^{-2}$ s$^{-1}$ (~1,200 lx) [84]. The best-fit value of $K_I$ was found 5.77µE m$^{-2}$ s$^{-1}$ (367 lx) in this study, which, at 22µE m$^{-2}$ s$^{-1}$, corresponds to the culture performance at 77% [=
\[ I/(K_I + I) \] of its maximum. The observations of the two studies were therefore in fairly good agreement.

4.4.4 \( K_N, K_{NF}^l \) and \( K_{HI}^l \)

The three N-related constants had comparable values: \( K_N = 1.45 \times 10^{-2} \) g/L, \( K_{NF}^l = 5.78 \times 10^{-3} \) g/L, and \( K_{HI}^l = 2.56 \times 10^{-2} \) g/L. The finding of comparable \( K_N \) and \( K_{NF}^l \) was reasonable; otherwise, there would have been no or minimal growth at some intermediate external N concentrations where they were too low to support growth but high enough to inhibit growth by nitrogen fixation. The finding of appreciably smaller \( K_{NF}^l \) than \( K_{HI}^l \) reflected that the N-fixation activity of existent heterocysts was much more sensitive to the presence of external N sources than the heterocyst differentiation. This difference in sensitivity is reasonable, indicating that the culture relies on the direct inhibition on nitrogenase for immediate, short-term response to the presence of N while modifying its heterocyst content through differentiation via a less sensitive, longer-term approach.

It should be pointed out that all these N-related constants are determined from experiments with nitrate as the external N source. The constants can have very different values if ammonium or other N-species are used as the external N source. For example, the \( K_{HI}^l \) value obtained in this study for \( A. \) flos-aquae CCAP 1403/13f was significantly larger than that implied by a literature report for \( Anabaena \) sp. PCC 7120 [33]. In the cited report, the addition of ammonium at the concentrations of \((4-10) \times 10^{-5} \) g/L of N was shown to repress the culture’s heterocyst differentiation. The different observations
were definitely not due to inadequacy of the current model, because if $K_{i,j}$ were in the range of $(4-10) \times 10^{-5} \text{ g/L}$ of N in this study, the heterocyst differentiation would have been completely repressed and the heterocyst concentrations would have never increased during the cultivation in Systems 8-10 where the N concentrations were $\geq 5 \times 10^{-2} \text{ g/L}$ of N.

4.4.5 Decay rates of vegetative cells and heterocysts, $k_{d}^{V}$ and $k_{d}^{H}$

The best-fit death constants, $k_{d}^{V}$ and $k_{d}^{H}$, were $8.47 \times 10^{-3}$ and $6.80 \times 10^{-3} \text{ (h^{-1})}$, corresponding to $\sim 18\%$ and $15\%$ of $\mu_{\text{max}}$, respectively. The percentages were appreciably higher than the percentage, $\sim 5\%$, seen in typical heterogeneous bacteria and yeast ([55, 85]) but closer to (and lower than) that, $\sim 20\%$, reported for the mold *Cephalosporium acremonium* ([86]). Studies with more cyanobacterial species are needed to assess if the percentage is representative among cyanobacteria.

4.4.6 Self-shading constants, $K_{X}$ and $K_{X-2}$

$K_{X}$, the empirical self-shading constant, can be considered as the product of the characteristic extinction coefficient ($K_a$) and the path length ($p$) traveled by the light ray in the cultivation system [80]. While the experiments carried out in this work were done in small 250-mL Erlenmeyer flasks as previously described in the Materials section, Spencer’s [66] microcosm experiments were carried out in 600-mL polystyrene Corning flasks continuously illuminated from only one side with 40-W Sylvania fluorescent lamps.
These Corning flasks are flat walled with a width of 3.5 cm, and were placed at approximately 20 cm away from the lights. In the current model, two different $K_X$ were considered to account for the different experimental setups, and the best-fit values were $K_X = 4.48$ L/g for this work and $K_{X-2} = 20.7$ L/g for Spencer’s. $K_a$ for microalgal biomass was reported to be between $0.01-0.08$ m$^2$/g [80]. This range multiplied by the path length ($p \sim 40-50$ cm) provided a range of approximately 1-50 L/g for $K_X$. The values obtained in the model were therefore in agreement with the above range.

Even though the model developed in this chapter describes fairly well the experimental results and culture profiles of the cyanobacterium *Anabaena flos-aquae*, a second stage model that incorporates carbon transport, the effect of different light wavelengths, and $H_2$ production kinetics is required. This second step is currently being developed in this laboratory using the cyanobacterium *Anabaena sp. PCC7120*, which has been reported to have high $H_2$ production rates [76].
The role of heterocysts as sole sites of nitrogen fixation in cyanobacteria and the importance of understanding the process of heterocyst differentiation were described and studied in previous chapters. It was also recognized that the light quality and intensity were key factors to all the activities in these microorganisms since light is the energy source for both photosynthetic systems. Accordingly, the productivity and economics of all photosynthesis-related processes is influenced by the light utilization efficiency. However, the efficiency is usually low. This is the result of factors such as the light saturation effect and the culture’s self-shading effect. Additionally, the use of light at wavelengths less effective for photosynthesis could be another factor affecting the efficiency of these processes. To overcome this problem, a highly efficient light source is desired for growth studies and cultivation. Other key issues, such as light delivery and its distribution inside the photobioreactor are also of great importance in the successful implementation of the photosynthetic process.
One promising light source is light emitting diodes (LEDs). LEDs have narrow spectral output, very long life expectancy, and they are highly efficient in converting electricity to light, so that heat generation is minimized. Noting the fact that chlorophyll $a$, the principal chlorophyll of algae and cyanobacteria, absorbs strongly the red light near 680 nm (Figure 2.1) [15], we proposed to examine in this chapter the use of red LEDs as an alternative light source for cyanobacteria. The effects of red vs. white light, and their intensities, on cell growth and heterocyst differentiation will be discussed.

5.1 Cultivation

The recent sequencing of the genome of the filamentous heterocystous cyanobacterium *Anabaena sp. PCC7120* is an important step towards understanding the process of heterocyst differentiation. Accordingly, this strain has been the focus of much research [10, 12, 37, 76, 78], including the work on nitrogenase and H$_2$ production rates. For the same reason, *Anabaena sp. PCC7120* was chosen as the strain to be used in this part of the research to take advantage of the available information.

In order to minimize the light limitation that was observed in the experiments conducted in regular Erlenmeyer flasks (described below), and to increase the surface illumination area, it was decided to use tissue culture flasks (T-flasks of 13 x 3.5 x 16 cm). Its narrow width (3.5 cm) minimized light attenuation from one side to the other and allowed light penetration without distortion. These Corning flasks were equipped with gas venting caps that permitted gas exchange without contamination. In addition, to
maximize the surface area of the cells in contact with the light and to minimize the
culture’s self shading effect, the flask was placed horizontally on the gyratorily shaken
plate immersed slightly in the water bath. The flask was continuously illuminated with
either red LED panels or with white-light fluorescent lamps, according to the light
conditions for each batch experiment.

5.2 Culturing *Anabaena sp. PCC 7120* in tissue culture flasks

Typical batch experiments were carried out in both 250-mL Erlenmeyer flasks and
in the Corning tissue culture flasks to compare the growth profiles of the cyanobacterium
*Anabaena sp. PCC 7120* in these reactor configurations. Two different medium
compositions, with and without nitrate, were used. For this comparison study, only white
light lamps were used as the light source. The average light intensity for these
experiments was measured to be ~1000 lux (~20 µE/m² s). Samples were withdrawn
aseptically from each flask and the cell concentration was determined as previously
described in Chapter III.

Figure 5.1 shows a plot of (X/Xo) against time for all of the systems examined. In
the figure, X is the cell concentration at any time and Xo is the cell concentration at t = 0
(right after inoculation). The cells grew faster in the tissue culture flasks than in the
Erlenmeyer flasks in both media with and without external N supplementation. This
result suggests that the light limitation effect is stronger in the Erlenmeyer flasks because
of the differences in configuration. Corning flasks have the same geometry along their
Figure 5.1 Batch cultivation of *Anabaena sp.* PCC 7120 in tissue culture flasks (T-flasks) and 250-mL Erlenmeyer flask with and without nitrate supplementation.
length and they have the additional advantage that they can be used horizontally. This allows a shallow volume to be used and the cells to be exposed to similar light intensity which also prevents the culture’s self-shading effect to be present early. Gentle shear conditions as well as a uniform light intensity throughout the system in the tissue culture flasks could be promoting favorable conditions for growing. Corning tissue flasks were used for the experiments performed in this part of the research.

Additionally, a low % of inoculation was used to prevent even further the self-shading effect to take place early in the experiments. Each flask was inoculated at no more than 3% of the prepared inoculum.

The light intensity from one side of the flask to the other was tested to see if there was attenuation produced from the wall of the tissue culture flasks. For this purpose, the conditions inside the shaker were simulated in the room to easily make the measurements with the detectors. A red LED panel was first positioned on top of a tissue culture flask containing water and the light intensity was measured immediately outside the bottom wall of the flask. Then, the flask was removed and the intensity was measured conserving the same distance. Without the flask the average light intensity was 1550 lux; with the flask it was 1450 lux. After that, the attenuation was also tested using white light lamps. These results were similar: without the flask the intensity was 1470 lux and with the flask it was 1300 lux. Even though these measurements were not exactly the same, the small difference between them was present with the light going through two walls of the flask.
However, in the experiments the light only has to go through one of the walls to reach the cells, which makes this difference smaller.

5.2.1 Effects of Light Intensity and Nitrate Supplementation

To compare the effect of different light intensities on the growth and heterocyst differentiation of the cyanobacterium *Anabaena sp.* PCC 7120, several batch experiments were carried out in tissue culture flasks. Three different light intensities were studied for white light experiments: 500 lux (9.1 µE/m² s), 1200 lx (21.9 µE/m² s), and 3000 lx (54.6 µE/m² s), thereafter referred to as low, medium and high light intensities, respectively. The illumination was provided from the top of the shaker for medium and high light intensities experiments. On the other hand, to test the effect of low light intensity, the shaker was covered with a plastic lid that decreased the contribution from ambient light and provided the low-intensity conditions. For each of the intensities, two medium compositions were used in terms of nitrogen source, i.e. with nitrate supplementation and without nitrate supplementation.

For clarity, Figure 5.2-A shows a typical growth profile obtained for the experiments conducted with varying light intensities. The figure represents a profile for two different systems grown under high light intensity: a system containing nitrate and a system without nitrate supplementation. The differences in lag phase and exponential growth phase for each system are identified in the figure as discontinuous lines. The maximum growth rate for each experiment was calculated from the slope at the
Figure 5.2 Typical growth profiles of *Anabaena* sp. PCC 7120 under high light intensity and different nitrogen supplementation.
exponential growth phase of a semi-logarithmic plot of the same profile (Figure 5.2-B). These experiments were repeated three times for the same cultivation conditions. The maximum growth rate for the experiments performed under the same light intensity and nitrogen supplementation conditions was averaged and the results were summarized in Figure 5.3.

Comparison of the growth in the two media shows that the presence of nitrate shortened the lag phase and increased the growth rate during the exponential growth phase. As expected, the maximum growth rate increased with increasing light intensity in both media. This is the result of the growth dependency on light intensity. The systems relying on nitrogen fixation (without nitrate supplementation) showed that the growth was ultimately limited by the light intensity since the ability to fix nitrogen assures that nitrogen is not the limiting factor. The growth rate curves at high light intensity reached higher cell concentration in response to the higher specific growth rate in these systems. In comparison, the cell concentration attained in the low light intensity systems was considerably lower.

In the systems with nitrate supplementation, the NO₃⁻-N concentration decreased along with cell growth. Figure 5.4 shows NO₃⁻-N consumption profiles of the systems supplemented with nitrate at different light intensities. The profiles shown in the figure indicated that nitrate was not depleted in any of the experiments performed in this study. The limitation in these systems was most likely due to the diminishing light penetration as a result of self-shading effect when high cell concentrations were obtained.
Figure 5.3 Maximum growth rates of *Anabaena* sp. PCC 7120 grown under white light at different light intensities: low, medium and high intensities (500, 1200, and 3000 lx). Nitrate supplemented systems (○) and non-nitrate supplemented systems (□) are shown.
Figure 5.4 Nitrate consumption and growth profiles of *Anabaena sp.* PCC 7120 under different light intensities for nitrate supplemented systems.
The averaged maximum cell concentration attained at the end of each run (around 200h) at the same cultivation conditions for the systems supplemented with nitrate at low, medium and high light intensities was 0.015 g/L, 0.18 g/L, and 0.3 g/L, respectively. On the other hand, for the systems without nitrate supplementation, these values were dramatically lower, i.e., 0.009 g/L, 0.037 g/L, and 0.04 g/L, respectively. The differences observed between the systems with and without nitrate supplementation were clearly related to the consumption of metabolic energy of the cells for carrying out the reduction of N₂ to NH₃ during the process of nitrogen fixation, which consumes 16 ATP molecules per mol of N₂ fixed as described in section 2.4 of this thesis.

It is important to note that the growth curves at high intensities in the systems with nitrate supplementation also showed an earlier leveling off in the last 25-50 hours of the experiments. There was a possibility of CO₂ limitation that might have been caused by the small area available for gas exchange through the vent holes on the cap of the tissue culture flask. However, this was not considered a significant drawback since the main purpose of this study was not to get high cell concentrations, but to obtain the “maximum” specific cell growth rates when the cells were exposed to similar, non-limiting light intensity.

Even though there is a proportional relation between the surface illumination intensity and the growth rate and maximum cell concentration in this kind of photobioreactors, high light intensities may not be the optimal condition for heterocyst differentiation. The effect of light intensity as well as the effect of nitrate
supplementation on heterocyst frequencies was also examined in these experiments. The averaged heterocyst frequencies for similar culture conditions at different light intensities for both systems, with and without nitrate supplementation, were calculated. For comparison purposes, the deviation from the average value is presented in Figure 5.5. This range represents the variation of the heterocyst frequency in response to the changing culture conditions during each experiment.

The highest heterocyst frequencies for the systems containing nitrate in the media were obtained at the low light intensity. The highest frequency reached in these systems was 4.1%. In contrast, 7.7% was the highest heterocyst frequency reached in the systems without nitrate supplementation at medium light intensity.

The systems relying only on nitrogen fixation resulted in higher frequencies. This is the result of the relation between the process of heterocyst differentiation and the nitrogen condition of the filament. When external nitrogen is present in the medium, the process of differentiation of vegetative cells into heterocysts is believed to be inhibited [4]. However, our results showed that under nitrate supplementation conditions the process of differentiation and maintenance of the pattern was not completely repressed. Even though, concentrations of ammonium in the range of 3-7 µM have been reported to repress heterocyst differentiation in Anabaena sp. PCC 7120 [33], there is also evidence that heterocyst can differentiate in the presence of external nitrogen. For instance, Anabaena variabilis grown on glutamine as the sole nitrogen source was also reported to contain heterocysts [87]. The presence of heterocysts under nitrate conditions supports
Figure 5.5. Heterocyst frequency ranges of *Anabaena* sp. PCC 7120 grown under white light at different light intensities: low, medium and high intensities (500, 1200, and 3000 lx). Nitrate supplemented systems (- -) and non-nitrate supplemented systems (—) are shown.
the fact that while heterocysts prevent the differentiation of nearby vegetative cells, nitrate does not have a direct function in heterocyst differentiation or pattern formation.

In both nitrogen conditions studied in these experiments, high light intensity conditions showed the lowest heterocyst frequencies. Since the cell concentrations attained at high light intensity were higher than in medium and low intensities, it was expected that the heterocyst frequency was also going to be higher to fulfill the needs of the cells for nitrogen supply. The heterocyst concentration profiles at low and medium light intensity appeared to increase smoothly and continuously along with the growth. On the other hand, at high light intensity, a leveling off of the heterocyst concentration was observed when the stationary phase was reached. This could explain the difference in heterocyst frequencies since the stationary phase was reached sooner in most cases at high light intensity than at the other intensities studied. Part A of Figure 5.6 illustrates these observations with a comparison between the heterocyst concentration profiles at high and medium light intensities in nitrate supplemented systems.

Additionally, Part B of Figure 5.6 shows the heterocyst frequency profile of the same systems used for illustration of the heterocyst concentration profiles. This figure points out the variations of heterocyst frequencies along the periods studied for each experiment. This figure serves to demonstrate that the large ranges reported in Figure 5.5 are not due to experimental error but to the intrinsic differences in frequencies related to the changing culture conditions during the time of each run.
Figure 5.6. Heterocyst concentration (A) and heterocyst frequency profiles (B) of *Anabaena sp.* PCC 7120 for nitrate supplemented systems grown under white light.
5.2.2 Effect of red LEDs as light source in photobioreactors

Red LED panels were constructed to provide light to the culture systems and examine the effect of red light on growth and heterocyst differentiation. Two light intensities were tested in these experiments: low light intensity, which was measured to be 500 lux (9.1 µE/m² s), and high light intensity which was 3000 lux (54.6 µE/m² s). The medium compositions that were tested in the experiments using white light were also tested under red light, i.e. with and without nitrate supplementation.

*Anabaena sp.* PCC 7120 was found to be able to grow under red LEDs as light source. The relation between growth rate and light intensity for the systems with nitrate supplementation was similar to the one found in white light experiments: cells did not seem to grow under the low light intensity; however, they seemed to have enough energy for maintenance. On the other hand, the cells grew better under the high light intensity, with a higher specific growth rate. The maximum growth rate in nitrate-containing medium was 7.8 x 10⁻³ and 0.039 h⁻¹ for low and high light intensities, respectively.

In contrast, the systems relying only on nitrogen fixation (without nitrate supplementation) showed similar growth rates in both light intensities. The maximum growth rate for these systems was 0.038 and 0.035 h⁻¹ at low and high light intensities, respectively.
For comparison, the values of maximum growth rate attained under white light conditions and without nitrate supplementation were 0.01 and 0.031 h\(^{-1}\) at low and high intensities, respectively. For the systems containing nitrate grown with white lamps as light source, these values were 0.015 and 0.054 h\(^{-1}\) at low and high intensities, respectively.

These results suggest that red LEDs as light source are stimulating the growth of *Anabaena* sp. especially under nitrogen fixation conditions. This finding could be the result that cyanobacteria absorb strongly the red light near 680 nm. The content of red light in white light lamps is smaller than the content of red light in red LEDs, which could result in an obvious advantage for the cells. In addition, the presence of red light as the only light source might be leading to the expression of different genes related with the pigments involved in the mechanisms of absorption of light. However, it is not clear why it had a stronger effect on the growth without nitrate supplementation.

Another interesting finding that could support the idea of different genes being expressed under red light conditions is that the color of the cells was different from the color of cells grown under white light conditions. While under white light the cells showed their characteristic blue-green color, under red light the color was green-yellow (Figure 5.7). The culture color under red light may be naturally different because the cells do not need the pigments for harvesting or blocking light at other wavelengths present in the white light lamps.
Figure 5.7. Difference in color of *Anabaena sp.* PCC 7120 grown under white lamps (A) and red LED panels (B) as light source.
The heterocysts frequency was also determined for these red light experiments. As Figure 5.5, Figure 5.8 includes the range of frequencies of the systems with and without nitrate supplementation under low and high light intensities. These results were different from the ones obtained with white light. The light intensity seems to have no significant effect on the heterocysts content. Moreover, the clear distinction that was observed in the frequencies of white light experiments with respect to the nitrogen supplementation factor was not observed under red light. The frequencies in the nitrate supplemented systems were not significantly different from the frequencies determined for the systems relying only on nitrogen fixation. Interestingly, the heterocyst frequencies under red light were considerably higher for both systems compared to those under white light conditions.

These findings suggest that red light also has an effect on the process of heterocyst differentiation and pattern formation, possibly in the expression and regulation of different genes.

This part of the research is on-going, with the focus on examining the effects of both the higher heterocyst content under red light conditions and the presence of heterocyst in nitrate supplemented medium on the nitrogenase activity of *Anabaena sp.* PCC 7120. Preliminary results showed that the nitrogenase activity in the heterocyst formed under nitrate conditions is being repressed, which was probably a consequence of nonfunctional heterocysts. These findings and future nitrogenase activity determinations will be examined closely in the continuation of this research in this laboratory.
Figure 5.8 Heterocyst frequencies of *Anabaena* sp. PCC 7120 grown under red light at different light intensities: low and high intensities (500 and 3000 lx). Nitrate supplemented systems (---) and non-nitrate supplemented systems (—) are shown.
Gas vesicles, which are the components of gas vacuoles, provide cyanobacteria with a mechanism of buoyancy regulation. As a result, cyanobacteria are able to direct their position in a body of water to find the required light conditions for growth and maintenance. In a previous work in this laboratory, it was found that purified gas vesicle suspensions improved oxygenation in oxygen limited systems [16]. Gas vesicles were harvested from a batch culture of *Anabaena flos-aquae* and then incorporated into a culture of shear sensitive animal cells to determine if there was an increase in oxygenation. When oxygen became the limiting factor, an improvement of 25% in cell growth was seen in a medium supplemented with 1.8% gas vesicles (v/v).

These findings recognized the need of an effective production of gas vesicles. The gentle cell collection is essential for the subsequent gas vesicle harvesting. Zeleznik [17] investigated the use of polyethylenimine (PEI) as flocculation aid to improve cell collection/harvesting. It was established that the cells flocculated with the addition of PEI
and the flocculation enabled easy cell collection. In addition, the PEI molecular weight and the solution pH were found to influence the effectiveness of the polymer.

Even though PEI improved the collection of cells, it caused the release of cellular component(s), indicated by the development of blue color during the flocculation process, and it resulted in poor recovery of gas vesicles in the subsequent step of cell rupture by osmotic shock [17]. It is therefore desirable to investigate the feasibility of using other flocculants such as chitosan for cell recovery. As a result, this chapter presents a study to improve the cell-collection efficiency of cyanobacteria with gas vesicles, via the study of cell flocculation under different conditions.

6.1 Cultivation

Batch cultures of the heterocystous cyanobacterium *Anabaena flos-aquae* CCAP 1403/13f were used in this study. The cells were grown in 250-mL Pyrex Erlenmeyer flasks at room temperature and with an average light intensity of 900 lx (i.e., 17 µE/m²-s). The agitation was provided by stirring bars using a magnetic stirrer. The flasks were covered with a layer of cheesecloth-wrapped cotton that also allowed gas transfer to take place.

The pH of the medium was initially adjusted to 7.1. pH values in the range of 7.5–9.0 had been reported to have no unfavorable effects to the culture [67]. Consequently,
the pH was not controlled since previous experiments conducted in this laboratory with *A. flos-aquae* have shown that the pH increased to 8.4–8.8 in all experiments.

6.2 Flocculation

The overall collection efficiency was monitored using spectrophotometric optical density measurements. A sample was taken from the flasks to determine the initial optical density. This measurement was converted to cell dry weight concentration using a calibration curve previously established for this purpose. After that, the whole content of the culture flask was equally divided into several 40-mL vials and the studied doses of flocculant were added. The tubes were gently mixed and then left undisturbed for the remainder of the experiment. When there was no further flocculation and the cells either floated or sank, a sample from the flocculation-cleared zone was taken from the tube to determine the final optical density. When the flocculation resulted in flotation of the cells, the biomass layer was carefully penetrated to a depth of around one inch with a Pasteur pipette to collect the subnate. On the other hand, when the cells sank after flocculation, the supernatant was recovered instead using also a Pasteur pipette. The flocculation efficacy was calculated from the initial and final optical density measurements:

\[
\frac{(OD_{\text{initial}} - OD_{\text{final}})}{OD_{\text{initial}}}.
\]

It was originally expected that the cells were going to float after flocculation and it was proposed to follow the rates of enhanced flotation by monitoring the speed at which the cell mass floated. However, in this study, a high percentage of the cells did not float
after flocculation within the tubes. As a consequence, the rates of enhanced flotation were not examined and the efficacy of flocculation was just evaluated using the percentage reduction of optical density as described above.

Zeleznik [17] reported that after flocculation, cyanobacterial cells floated to the top of the tubes. The difference in flotation behavior between these two studies may be explained by the different experimental conditions employed. Zeleznik [17] used a bubble column photobioreactor while the mechanically agitated 250-mL Erlenmeyer flasks were used as photobioreactors in this study. The narrower diameter of the bubble column reactor allowed the cells to have less self-shading effect and consequently higher growth rates. Cells collected at this stage will have high buoyancy. Nonetheless, this aspect is not critical for this study, which was focused on cell flocculation instead of intracellular gas vesicle content.

6.2.1 Flocculation with PEI, chitosan and MPE-50

Three different flocculants were studied; they were PEI, chitosan and MPE-50, the last being a commercially available product from Ondeo Nalco Center. Different concentrations of the flocculants were tested to find the optimal doses that would provide high flocculation efficacy.

Initial flocculation tests were performed using 14-mL test tubes with a diameter of around 1.2 cm. However, the results performed in this type of tubes indicated that there
was a wall effect taking place probably caused by the small diameter of the tubes. Three different concentrations of the flocculants were tested: 10, 30 and 50 mg/L. A tube without flocculant was included in the experiment as a control tube.

The cells of the tube that did not contain flocculant remained dispersed along the tube without flotation or settlement during the experiment. In contrast, the cells in the other test tubes agglomerated into small flocs, some of which sank to the bottom while some remained in the suspension or attached to tube wall. The only flocculant-added system that did not show significant flocculation was the one added with 10 mg/L of chitosan. The cell flocculation with MPE-50, at all of the studied concentrations, became apparent to naked eyes approximately 20 minutes after the flocculant addition. On the other hand, the cell flocculation with PEI and chitosan was not noticeable until 35 and 40 minutes, respectively. The calculated results of overall efficacy are given in Table 6.1. It is interesting to note that while the commercial MPE-50 caused faster flocculation, PEI at all studied concentrations gave higher final values of flocculation efficacy. Furthermore, with MPE-50 the flocculation efficacy was about the same at all concentrations while the flocculation improved with increasing concentrations of PEI and, particularly, chitosan.

PEI and MPE-50 appeared to require lower concentrations than chitosan in flocculation of *A. flos-aquae* cells. However, there were two important factors to be considered in these results. The cell concentration was low and small changes in the
<table>
<thead>
<tr>
<th>Flocculant agent</th>
<th>Concentration (mg/L)</th>
<th>OD initial</th>
<th>OD final</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0.055</td>
<td>0.057</td>
<td>-0.036</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.055</td>
<td>0.017</td>
<td>0.691</td>
</tr>
<tr>
<td>PEI</td>
<td>30</td>
<td>0.055</td>
<td>0.014</td>
<td>0.745</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.055</td>
<td>0.015</td>
<td>0.727</td>
</tr>
<tr>
<td>MPE-50</td>
<td>10</td>
<td>0.055</td>
<td>0.018</td>
<td>0.673</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.055</td>
<td>0.019</td>
<td>0.655</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.055</td>
<td>0.019</td>
<td>0.655</td>
</tr>
<tr>
<td>Chitosan</td>
<td>10</td>
<td>0.055</td>
<td>0.038</td>
<td>0.309</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.055</td>
<td>0.025</td>
<td>0.545</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.055</td>
<td>0.015</td>
<td>0.727</td>
</tr>
</tbody>
</table>
optical density had a marked influence in the efficacy calculations. In addition, a considerable amount of flocs remained in the suspension or attached to tube wall which did not allow the formation of a clear zone (either subnate or supernatant). The wall was thought to be slowing down or even preventing flotation of cell flocs by providing the surface for floc adsorption or attachment. The higher flocculant concentrations might have caused tighter packing and reduced the wall effect. In order to confirm that the wall was having a significant effect on the flocculation results, a comparative experiment was carried out using test tubes (diameter of 1.2 cm) and 40-mL vials with a diameter of 2.5 cm.

The results of this comparison are presented in Table 6.2. Cells with higher optical density were used in this experiment to better perceive the changes in optical densities. Following the previous experiment, PEI and MPE-50 were added to a final concentration of 30 mg/L since they resulted in higher efficacy. In addition, Zeleznik [17] reported that concentrations above 30 mg/L did not show an increase in product collection. The concentration used for chitosan was 50 mg/L since it also resulted in higher efficacy in the previous experiment.

Even though the overall efficacy among flocculants was not found to be considerably different, the test tubes for all cases showed lower efficacy compared to the vials. In addition, the formation of suspended flocs was not observed in the vials, which supports the idea that the smaller the diameter, the higher the wall effect on flocculation and flotation.
Table 6.2 Wall effect on cell flocculation with PEI, chitosan and MPE-50

<table>
<thead>
<tr>
<th>Flocculant agent</th>
<th>Column</th>
<th>OD initial</th>
<th>OD final</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Vial</td>
<td>0.134</td>
<td>0.128</td>
<td>0.045</td>
</tr>
<tr>
<td></td>
<td>Test tube</td>
<td>0.134</td>
<td>0.116</td>
<td>0.134</td>
</tr>
<tr>
<td>30 mg/L PEI</td>
<td>Vial</td>
<td>0.134</td>
<td>0.014</td>
<td>0.896</td>
</tr>
<tr>
<td></td>
<td>Test tube</td>
<td>0.134</td>
<td>0.032</td>
<td>0.761</td>
</tr>
<tr>
<td>30 mg/L MPE-50</td>
<td>Vial</td>
<td>0.134</td>
<td>0.019</td>
<td>0.858</td>
</tr>
<tr>
<td></td>
<td>Test tube</td>
<td>0.134</td>
<td>0.023</td>
<td>0.828</td>
</tr>
<tr>
<td>50 mg/L Chitosan</td>
<td>Vial</td>
<td>0.134</td>
<td>0.017</td>
<td>0.873</td>
</tr>
<tr>
<td></td>
<td>Test tube</td>
<td>0.134</td>
<td>0.033</td>
<td>0.754</td>
</tr>
</tbody>
</table>
Chitosan was found to have comparable efficacy at 50 mg/L to that of PEI and MPE-50 at 30 mg/L at lower initial optical densities (Figure 6.1). However, at higher initial optical densities (> 0.3), both PEI and chitosan did not work well compared to their performance at lower cell concentrations. In contrast, MPE-50 started visible flocculation very fast (~15 min after addition of the flocculants) and it was the only vial that showed flotation of the mass of cells to the top. MPE-50 had an efficacy of 0.909 when applied to higher cell concentrations. The flocculation efficacy of PEI and chitosan at the same initial optical densities was 0.118 and 0.312, respectively. These results are also shown in Figure 6.1.

The reason for the low results of efficacy of PEI and chitosan at higher optical densities is not clear. However, Zeleznik [17] reported an efficacy for PEI of 0.888 with an initial optical density of 1.04, which is considerably higher than the initial optical density used in this study.

Figure 6.1 also shows the change in efficacy of chitosan when the pH of the cell suspension is adjusted to 7.0 prior flocculation. In a previous study conducted in this laboratory for the flocculation of microorganisms in a wastewater treatment process, it was established that chitosan was not effective at pH higher than 8 [88]. The pH of the culture used in this study was found to gradually increase to 8.4–9.0 in different systems, which could have been causing the chitosan to lose solubility at high pH.
Figure 6.1 Flocculation efficacies at low and high initial optical densities for 30 mg/L PEI, 30 mg/L MPE-50, and 50 mg/L chitosan. The efficacy of chitosan for a cell suspension with high optical density and pH adjusted prior flocculation is also shown.
The cell suspension was adjusted to a pH of about 7 and the flocculation efficacy was calculated for a system with high initial optical density. The efficacy of chitosan clearly improved under this condition. It was not only higher than for the system in which the pH was not adjusted but also higher than MPE-50 that was giving the highest efficacy under high initial cell concentrations. This result confirmed that the ability of chitosan to flocculate negatively charged particles such as cells is dependent on changes of pH.

6.2.2 Flocculation ability of chitosan at different pH

In order to determine the optimal pH of the cell sample for flocculation using chitosan, pH ranges from 5 to 10 were tested. Lower pH ranges were not included since it has been reported that the cyanobacterial mass is destroyed in high acid content conditions (pH < 4) and the gas vesicles proteins are denatured [17].

The results of the flocculation efficacy at different pH of the cell suspension are reported in Table 6.3. The control vials, to which chitosan was not added, did not flocculate or float at the entire pH range tested. In contrast, the vials containing chitosan started visible flocculation at about 15 minutes after addition of the flocculant. After that, the cell mass in those tubes that had good efficacy tended to migrate to the bottom of the vial. It was found that the flocculation ability of chitosan is better at pH from 5 to 7. At pH 8 the efficacy decreased significantly and decreased further as the pH increased. Even though the efficacy of chitosan at pH 6 was a little bit lower than at pH 5 and 7, it is important to note that it flocculated faster and the clear zone at the top of the vial was
Table 6.3 Cell flocculation efficacy using chitosan at different pH adjustments

<table>
<thead>
<tr>
<th>pH</th>
<th>OD initial</th>
<th>OD final</th>
<th>Efficacy</th>
<th>OD initial</th>
<th>OD final</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>not adjusted</td>
<td>0.325</td>
<td>0.323</td>
<td>0.0062</td>
<td>0.325</td>
<td>0.27</td>
<td>0.169</td>
</tr>
<tr>
<td>5</td>
<td>0.325</td>
<td>0.179</td>
<td>0.4492</td>
<td>0.325</td>
<td>0.015</td>
<td>0.954</td>
</tr>
<tr>
<td>6</td>
<td>0.325</td>
<td>0.312</td>
<td>0.04</td>
<td>0.325</td>
<td>0.018</td>
<td>0.945</td>
</tr>
<tr>
<td>7</td>
<td>0.325</td>
<td>0.323</td>
<td>0.0062</td>
<td>0.325</td>
<td>0.015</td>
<td>0.954</td>
</tr>
<tr>
<td>8</td>
<td>0.325</td>
<td>0.312</td>
<td>0.04</td>
<td>0.325</td>
<td>0.209</td>
<td>0.357</td>
</tr>
<tr>
<td>9</td>
<td>0.325</td>
<td>0.325</td>
<td>0</td>
<td>0.325</td>
<td>0.23</td>
<td>0.292</td>
</tr>
<tr>
<td>10</td>
<td>0.325</td>
<td>0.329</td>
<td>-0.0123</td>
<td>0.325</td>
<td>0.278</td>
<td>0.145</td>
</tr>
</tbody>
</table>
obtained also sooner than in the other two vials. It was also seen that the vial containing chitosan without pH adjustment flocculated poorly but a little bit better than the one with chitosan at pH 10, which is consistent with the pH tendency observed since the pH of the cell sample was 9.3.

The pH dependency of the flocculation ability of chitosan was also clear in this experiment. Chitosan is a cationic polyamine with a pKa of 6.5. At pH < pKa it is positively charged, which promotes the binding with negatively charged surfaces such as the cell membrane. On the other, at pH > pKa, chitosan is neutral, which causes less tendency to bind with cells and at the same time becomes less water soluble.

According to the results of this study, chitosan ability as a flocculant can be successfully used for cyanobacterial cell collection prior gas vesicle harvesting using a concentration of 50 mg/L and adjusting pH of the cell sample to a range around 5 to 7. In addition, the sensitivity of chitosan to changes in pH can be useful in the later step of recovery of gas vesicles after cell lysis. Investigation of this process is currently being performed in this laboratory since the conventional methods of separation such as centrifugation and filtration have shown to break the shear sensitive gas vesicles.
CHAPTER VII
CONCLUSIONS

7.1 Conclusions

The conclusions drawn as a result of meeting the specific research objectives are summarized as follows:

The effect of different cultivation conditions on cyanobacterial growth and heterocyst frequency was examined. A first-generation, quantitative, culture-level model was developed from the studies conducted under different light intensities and nitrate supplementation conditions. The model proposed in this thesis incorporated photosynthetic growth of vegetative cells, heterocyst differentiation, self-shading effect on light penetration, and nitrogen fixation. The current model was shown to describe the cell growth profiles fairly well for both cultivation conditions: with and without external nitrogen supplementation. Additionally, the best-fit values for the parameters included in the model were within physically meaningful ranges.
Owing to the ability of nitrogen fixation, the growth was found to be eventually limited by the light intensity as a result of the self-shading effect. Additionally, the ratio between heterocyst and vegetative cell concentration decreased with time in systems with high external nitrogen concentrations, caused by the inhibited heterocyst differentiation. On the other hand, in systems with low or no external nitrogen concentrations, this ratio increased initially and then leveled off. The initial increase corresponded to accelerated growth while the leveling off appeared when the specific growth rate started to decrease due to the decreasing light intensity.

From the simulation of the specific growth rate under different initial nitrate concentrations, the model was also found to successfully describe the smooth transition from the growth on external nitrogen to that on nitrogen fixation.

For both strains studied in this thesis, i.e. *Anabaena flos-aquae* and *Anabaena sp. PCC 7120*, the differences in growth rates observed between the systems with and without nitrate supplementation were related to the process of nitrogen fixation. The consumption of metabolic energy in the cells that carry out nitrogen fixation is considerably higher than that in the cells that do not need to fix atmospheric nitrogen.

Heterocyst frequency results from the cells grown under white light showed that the process of differentiation and maintenance of the pattern was not completely repressed under nitrate supplementation conditions. The highest frequency reached in these systems was 4.1%. In contrast, 7.7% was the highest heterocyst frequency reached in the systems
without nitrate supplementation. The presence of heterocysts in the filaments grown with nitrate supports the fact that while heterocysts prevent the differentiation of nearby vegetative cells, nitrate does not have a direct function in heterocyst differentiation or pattern formation.

Anabaena sp. PCC 7120 was able to grow under red LEDs as light source. Interestingly, the heterocyst frequencies under red light (8.44 and 8.48% for the systems with and without nitrate supplementation, respectively) were considerably higher than those under white light. These findings suggest that red light also has an effect on the process of heterocyst differentiation and pattern formation, possibly in the expression and regulation of different genes.

Finally, for the flocculation study of the cell suspension prior to gas vesicle production, chitosan was found to have comparable efficacy at 50 mg/L to that of PEI and MPE-50 at 30 mg/L at lower initial optical densities. Chitosan ability as a flocculant was found to be greatly improved with adjustment of pH of the cell suspension. It was determined that chitosan can be successfully used for cyanobacterial cell collection using a concentration of 50 mg/L and adjusting pH of the cell sample to a range from 5 to 7.

7.2 Recommendations for future research

The model developed in this thesis is a first-generation quantitative model that describes fairly well the experimental results and culture profiles of the cyanobacterium
Anabaena flos-aquae. It is nonetheless desirable to improve the model by incorporating other aspects such as carbon transport, the effect of different light wavelengths, and H₂ production kinetics. This improved model is currently being developed in this laboratory using the cyanobacterium Anabaena sp. PCC7120, which has been reported to have higher H₂ production rates. The outcome of the study under red light conditions as well as the nitrogenase activity results under different cultivation conditions will also be included.

The fact that overexpression of the hetR gene leads to increased heterocyst frequency in Anabaena sp. PCC 7120 can be taken under consideration to create a recombinant strain with variable and controllable heterocyst frequency. Anabaena sp. PCC7120 is currently being transformed and screened. Once obtained, the strain will be used to study the effect of controllable heterocyst frequency on the condition of the cells and on H₂ production rates. The outcome of this study will supply the necessary information to complete the quantitative model that will provide the optimal conditions for photobiological production of H₂ via N₂-fixing cyanobacteria.

Finally, and for the gas vesicle production, the sensitivity of chitosan to changes in pH can be useful in the later step of recovery of gas vesicles after cell lysis. Investigation of this process is recommended since the conventional methods of separation such as centrifugation and filtration have been shown to break the shear sensitive gas vesicles.
REFERENCES


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