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Cultivation of Microalgae *Chlorella vulgaris* in Photobioreactor for
Biodiesel Production

by

Dorota J. Debska

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Faculty of Graduate and Postdoctoral Studies

In partial fulfillment of the requirements

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Abstract

Microalgae are gaining considerable attention as a feedstock for biodiesel production. They can be grown away from the croplands and hence do not compromise food crop supplies. The ability of microalgae to capture solar energy and fix CO₂ is a promising process for sustainable production of biomass. *Chlorella vulgaris* may be suitable for biodiesel production due to its faster growth and easier cultivation compared to other strains. The effect of media composition and process conditions on biomass productivity of *C. vulgaris* are investigated in a laboratory scale photobioreactor. The results show excellent growth of *C. vulgaris* on 2X Tris-Acetate-Phosphate culture medium, reaching biomass concentrations around 7.7 g/L. The combination of process parameters that result in highest biomass for our system are: agitation at 600 rpm, temperature of 29°C, average light irradiance of 900 $\mu\text{E}/(\text{m}^2 \text{ s})$, and 4% CO₂ in air. The statistical analysis of biomass from fractional factorial experiments confirms that *C. vulgaris* growth in presence of the aforementioned process parameters can give highest biomass for our system from the process parameters that were studied. However, statistical analysis also reveals that increase in irradiance from 450 $\mu\text{E}/(\text{m}^2 \text{ s})$ to 900 $\mu\text{E}/(\text{m}^2 \text{ s})$ in our system did not have significant effect on biomass concentration.

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Résumé

Les micro-algues attirent de plus en plus attention comme le matière premier dans la production de "biodiesel". Ils peuvent être cultivés loin de cultures agricoles traditionnelles et ainsi sans avoir impact négatif sur des productions alimentaires. La capacité des micro-algues de capturer l'énergie solaire et consumer le CO₂ constitue un processus prometteur de production de biomasse. *Chlorella vulgaris* peut convenir à la production de biodiesel grâce à sa vitesse de croissance et la facilité de culture en comparaison avec les autres variations. L'effet de composition de média et condition de processus sur la productivité de biomasse de *C. vulgaris* ont été étudiés dans un photobioreacteur de laboratoire. Nos résultats montrent une excellente vitesse de croissance de *C. vulgaris* ensemencé sur 2X Tris-Acetate-Phosphate, en atteignant la concentration de biomasse autour de 7.7 g/L. La combinaison de paramètres de processus qui donnaient la concentration de biomasse le plus élevé était: agitation à 600 rpm, température à 29°C, illumination moyenne de 900 $\mu\text{E}/(\text{m}^2 \text{ s})$, et 4% de CO₂ dans l'aire. L'analyse statistique de biomasse mesuré (par des "fractional factorial experiments") confirme que la croissance de *C. vulgaris* dans les conditions mentionnées produit la concentration de biomasse le plus élevé parmi tous les cas étudiés. L'analyse statistique montre aussi que l'augmentation d'illumination de 450 $\mu\text{E}/(\text{m}^2 \text{ s})$ à 900 $\mu\text{E}/(\text{m}^2 \text{ s})$ dans notre système n'a pas eu effet visible sur la concentration de biomasse obtenu.

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Chapter 1 Introduction

Fossil fuels are being recognized as unsustainable energy source due to their limited reserves and negative impact on environment. Limited fossil fuel supplies, energy and environment security concerns, coupled with high energy prices have sparked global attention for alternative sources of fuel (Meng et al., 2009). Alternative transport fuel should be renewable and carbon neutral such that it can provide economic and environmental sustainability (Chisti, 2007).

Over the last couple of years biodiesel from oil crops and bioethanol from sugarcane have been produced in increasing amounts as renewable and carbon neutral alternative to fossil fuels. Unfortunately the production of these fuels in large quantities has not been economically and environmentally sustainable (Chisti, 2008). It has been proposed by many authors in the literature that a more promising alternative to fossil fuel might be microalgal based fuel (Groom et al., 2008). In comparison with major terrestrial oil crops, microalgae have higher rates of oil and biomass production (Griffiths and Harrison, 2008) as well they appear to be the only source of renewable biodiesel that can meet global demand for transport fuels (Chisti, 2007).

It is known that maximum rates of oil production occur when microalgae have high rates of biomass production and high oil content (Huntley and Redalje, 2007). When considering microalgal species for biodiesel production it would be most plausible to select species that has high biomass productivity and high lipid content. Unfortunately, microalgae with high lipid content generally show much slower growth, while those

containing low lipid levels can have high growth rates (Huntley and Redalje, 2007; Widjaja et al., 2009).

It has been noted in many literature sources that microalgae produce considerable amount of lipid when exposed to environmental stress such as nitrogen limitation (Illman et al., 2000; Liu et al., 2008; Piorreck et al., 1984). To obtain high oil productivity one can select a relatively rapidly growing strain that can accumulate lipids when exposed to low nitrogen concentrations. The cultivation of this strain would be conducted in a two-stage process. In the first-stage, the strain will be exposed to a nutrient sufficient medium, and because this is a rapidly growing strain high biomass productivity should be achieved. In the second-stage, the high biomass culture will be exposed to a nutrient stress, through nitrogen limitation. During this second-stage microalgae will produce and accumulate lipids. By implementing two-stage process high lipid productivity can be achieved.

Only some types of algae can accumulate lipids in presence of limiting concentrations of nitrogen. In case of green algae their lipid level can be altered by varying nitrogen concentration in the media. In addition, green algae are very good candidates for biodiesel production due to their advantageous characteristics such as relatively fast-growth, ease of isolation, and ability to adapt to diverse natural habitats (Hu et al., 2008). Although the production of biodiesel from microalgae is technically possible, it still has a long way before it can be economically competitive with petroleum derived fuel (Chisti, 2008). The paramount economic hurdle in production of microalgal biodiesel is the biomass productivity (Griffiths and Harrison, 2008). Research and development into the cultivation of microalgae for biodiesel production is necessary to

overcome this hurdle. However, as stated in one literature source, the necessary required improvement in microalgal biodiesel production appears to be attainable (Chisti, 2007).

The focus of our research lays in microalgae *Chlorella vulgaris* and its potential for lipid production by a two-stage process in photobioreactor. As explained previously, the two-stage process involves cultivation of microalgae under nutrient sufficiency to obtain high biomass, followed by exposure of the microalgae to nitrogen limitation in order to induce lipid accumulation. In our research we have decided to work with *C. vulgaris* as it seemed a good candidate for two-stage lipid production due to its high growth rate and ability to accumulate lipids under nitrogen limitation (Griffiths and Harrison, 2008; Piorreck et al., 1984). Although the strategy for the production of lipids involves two stages, in our research we have concentrated our efforts on the first stage. Considerable attention should be given to biomass production stage as it is the bottleneck in economic production of microalgal derived biodiesel (Griffiths and Harrison, 2008).

It is important to note that it is difficult to optimize microalgal growth due to cross-effect of different variables. For this reason a systematic approach should be used such as factorial design.

This thesis is divided into several chapters that we feel are instrumental to give the reader overall outlook on potential of oil production from *Chlorella*. We also hope that this thesis can create interest for further research and development into this area.

Chapter two contains an in-depth literature review that begins with general information on algae and their potential as source of valuable products. Subsequently, different algal large-scale cultivation systems are presented, comparing the various

advantages and disadvantages of the most popular. The focus shifts to fuel production, presenting the problem with petroleum and first-generation biofuels, as well as the potential of microalgal biofuel. Next, the improvement in economics of microalgal biodiesel is discussed, focusing on the integrated system for biodiesel production. Following is the discussion on suitable microalgal species for biodiesel production and the two-stage process that can result in good microalgal oil productivity. Subsequently the focus shifts to *C. vulgaris* and its suitability for biodiesel production by the two-stage process. This discussion is followed by an in-depth review of the culture medium as well as the operating conditions encountered in cultivation of *C. vulgaris* in photobioreactor.

Chapter three contains the experimental methods that were utilized to perform the experiments throughout the study. The subtopics of this chapter include: microalgal strain, culture medium, inoculum, experimental setup, cultivation conditions, analytical methods and the factorial design.

Chapter four contains the results and discussion pertaining to the growth of *C. vulgaris* in the batch mode photobioreactor and study of the culture medium. The first half of the chapter discusses the *C. vulgaris* growth in batch photobioreactor and nutrient availability in the culture medium. The second half of the chapter explores the effect of various concentrations of nitrogen and phosphorus on *C. vulgaris* growth in the flask.

Chapter five presents the results and discussion in regards to the study of the effect of operational conditions on *C. vulgaris* growth in nutrient sufficient fed-batch photobioreactor. The first sub-chapter presents the fractional factorial design used to conduct experiments. The second sub-chapter contains results and discussion of the *C. vulgaris* growth under various process conditions set by the fractional factorial design.

Further, the statistical analysis of the results from fractional factorial design is presented, along with the best process conditions for growth of *C. vulgaris*.

Chapter six contains the conclusions, and the recommendations, specifically, in regards to further examination of oil production from *C. vulgaris*.

Chapter 2 Literature Review

2.1 Algae

Algae are a group of simple organisms that range in size from single celled to multicellular forms. There are two subgroups of algae: microalgae and macroalgae. Most are microscopic; however some forms such as seaweeds can be large reaching lengths up to 50 m. Literature sources report that there are over 40,000 species of algae (Harvey, 2000; Hu et al., 2008), and only a number have been examined comprehensively (Harwood and Guschina, 2008). From the literature it is evident that more research has been done on algae in 1970s and 1990s than in 1980s when the funding for research dried up.

Most algae are photoautotrophs; they convert inorganic carbon, such as CO_2 in presence of light, water, and nutrients to organic molecules and release oxygen. Algal photoautotrophs are more efficient at converting sunlight into biomass than terrestrial plants (Patil et al., 2008). It has been noted in literature that plants can convert solar energy into biomass with photosynthetic efficiency of less than 4%, while light efficiency utilization of algae is around 3 to 9% (Dismukes et al., 2008). In addition, algae are more tolerant of wider range of mean photon flux than terrestrial plants, hence they can live in wider range of different light environments (Dismukes et al., 2008). While most algae utilize photosynthesis, some algal species in addition can also utilize organic carbon source such as glucose to produce organic molecules (Lee Y-K, 2001). Algae that can grow on organic carbon in the absence of light energy are termed heterotrophs. Literature sources report that overall heterotrophic growth is less efficient than phototrophic growth,

because ultimately the organic source required in heterotrophic growth is produced by the photosynthetic crops (Patil et al., 2008). Hence when one looks at the overall picture, in heterotrophic mode energy first must be used to grow the crop, while in photoautotrophic growth energy is used directly for growth of algae.

Algae have adapted to live in wide range of ecosystems ranging from hot springs to snow (Harwood and Guschina, 2008). Some algae live in terrestrial environments; however algae are mostly prominent in bodies of water including freshwater, brackish, marine, and hyper-saline waters (Hu et al., 2008). Algae are classified into the following groups: green algae, cyanobacteria, diatoms, yellow-green algae, golden algae, red algae, brown algae, dinoflagellates, and 'pico-plankton' (Hu et al., 2008). Their ability to survive in large range of environments is reflected in their diverse genetic and biochemical makeup (Faramarzi et al., 2008).

Because algae are very diverse and largely unexplored organisms, there exists a big opportunity for discovery of a wide range of products.

2.2 Large Scale Cultivation Systems

Algae are either grown in open culture systems, or in closed systems such as conventional fermenters or photobioreactors. These cultivation systems do not compromise forest or agricultural land. While most algae require light for their growth, a number of algal species are capable of heterotrophic growth (Gladue and Maxey, 1994).

Heterotrophic organisms are usually cultivated in conventional fermenters that range in volume from 1 to 500,000 L, without the need for light. Heterotrophic fermentor

cultures are cultivated in sterile and highly controlled conditions that can guarantee reproducible results. However, the need for organic carbon might render heterotrophic system economically unfeasible.

Photosynthetic algae can be cultivated in open cultures with natural sunlight, or in closed systems such as photobioreactors that are supplemented with either natural sunlight or artificial illumination (Apt and Behrens, 1999). The proper cultivation system is chosen based on the degree of process control required and on the value of the product. These systems are usually designed to maximize surface to volume ratio in order to supply adequate amount of light.

Open cultures are the most common algal cultivation systems. Two very popular configurations are circular open ponds and raceway open ponds (Lee Y-K, 2001). Circular open ponds, as the name suggests, are circular in shape and have agitator to mix the culture. The largest circular open pond reported is 50 m in length, and the depth of the pond could be as little as 0.05 m (Lee Y-K, 2001). Raceways on the other hand are long channels consisting of single or multiple loops with paddle wheels for agitation (Carvalho et al., 2006). These ponds are typically around 0.3 m deep, and can reach area of 440,000 m² (Chisti, 2007). In raceway ponds, cell concentration of about 0.5 g/L can be maintained with aerial biomass productivity of 25 g/ (m² day), and volumetric biomass productivity of 0.18 to 2.50 g/ (L day) (Lee Y-K, 2001). Open ponds present many drawbacks such as relatively low biomass density, CO₂ and evaporation losses, daily and seasonal variation in temperature and light, and contamination. Contamination issue can be alleviated by culturing algal strains that survive in highly selective conditions in which

contaminating organisms would be unable to survive (Apt and Behrens, 1999). The biggest advantage of open systems is that light energy is supplied by sunshine at no cost.

Closed systems supplemented with natural sunlight or artificial light are known as photobioreactors. These vessels are enclosed in transparent material such as plastic or glass that allows for less contamination in comparison to open systems (Apt and Behrens, 1999). Popular photobioreactor configurations are tubular and flat plate (Lee Y-K, 2001). Relatively high biomass concentration of up to about 20 g/L can be achieved in enclosed tubular and flat plate photobioreactors. The aerial biomass productivity in these types of reactors can be 25 to 130 g/ (m² day), and a volumetric biomass productivity of 0.25 to 4.30 g/ (L day) (Lee Y-K, 2001). Harvesting of biomass is achieved with more ease in photobioreactors than open cultures due to higher biomass densities. Despite many advantages, photobioreactors have high capital and high production cost (Carvalho et al., 2006). Closed systems with artificial light instead of natural sunlight are even more costly, however they can provide better control of temperature and light. To offset the high cost associated with photobioreactors, volumetric biomass productivity needs to be higher (Lee Y-K, 2001). One of the biggest challenges in photobioreactor design is adequate supply of light and CO₂ (Carvalho et al., 2006).

2.3 Biofuel and Microalgae

The driving force for alternative fuel is sparked by limited fossil fuel reserves, and also by the production of CO₂ from combustion and its subsequent emission into the atmosphere (Groom et al., 2008; Patil et al., 2008). Alternative fuel that has potential to provide a sustainable energy system is biofuel (Kalia and Purohit, 2008). The most

popular renewable biofuel currently on the market is bioethanol (Gray et al., 2006) followed closely by biodiesel (Patil et al., 2008). Bioethanol is produced by fermentation of sugars by microorganisms. The raw feedstock for bioethanol production is food crops such as sugarcane or corn among others. Biodiesel on the other hand is produced from oil that is extracted from food crops such as soybean, canola, or palm to name a few.

The arising concerns with bioethanol and biodiesel derived from food crops is the inefficiency and sustainability of these first generation biofuels (Patil et al., 2008). For instance, the oil content from oil crops amounts to miniscule amount of less than 5% on total biomass basis (Chisti, 2007). Hence it requires lots of crops to produce significant amount of oil for biodiesel production. To increase the total amount of biodiesel produced from oil crops, extensive agricultural areas would be required. The development of these areas and subsequent growth of oil crops may have significant negative impact on the environment. Large scale crop production of biofuels requires high pesticide, fertilizer and water use (Groom et al., 2008). For instance, the high nitrogen input into the environment currently results in expansion of hypoxic zone in the Gulf of Mexico (Rabelais et al., 2002). Furthermore, expansion of agricultural lands into more fragile areas would decrease the habitats suited for some of the species and as a consequence lead to decrease in biodiversity (Groom et al., 2008). Groom and co-workers (2008) observe that: “biofuels are sustainable energy source only if feedstocks are grown sustainably”. The sustainability may be achieved only by good environmental practices during the biodiesel life cycle production and by minimizing the land area required for growth of sufficient amount of feedstock.

Unfortunately, the land area required to produce significant amount of biodiesel from terrestrial oil crops is very large and in many cases unattainable as seen in Table 2.1.

Table 2.1: Comparison of some sources of biodiesel.

Crop	Oil yield (L/ha)	Land area needed (M ha) ^a	Percent of existing US cropping area ^a
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae ^b	136,900	2	1.1
Microalgae ^c	58,700	4.5	2.5

^a For meeting 50% of all transport fuel needs of the United States.

^b 70% oil (by wt) in biomass.

^c 30% oil (by wt) in biomass.

(Chisti, 2007)

For instance, to produce enough biodiesel from corn to meet half of all transport fuel needs in the US, a staggering 846% of existing US cropping area would be required. Even in the case of high oil yielding oil palm, it would require a quarter of existing US area for cultivation in order to produce enough biodiesel to meet half of all transport fuel needs of US. Obviously, the amount of biodiesel produced from terrestrial oil crop is only enough for blending with petroleum derived diesel at few percent, but is likely not enough for terrestrial oil crop derived biodiesel to be the main transport fuel in the foreseeable future (Chisti, 2008).

Another concern over first generation biofuel is that it can compromise production of food and fodder from crops (Runge and Senauer, 2007). As a result, rise in

food prices has been partially attributed to increasing global demand for biofuel. As outlined in one literature journal (Song et al., 2008), if Chinese biodiesel was mostly made from terrestrial oil crops, this situation could be a disaster to China that has large population to feed and relatively small agricultural land area. The same scenario could be true in many other geographical areas.

Perhaps a more promising alternative to petroleum derived fuel might be what are termed second generation biofuels rather than food crop derived biofuels. The second generation biofuels such as cellulosic feed stock and microalgae, among others, are derived from non-food feedstock. Cellulosic ethanol is produced from non-edible part of the terrestrial crop such as corn stover or wheat straw, as well as from grass or wood residues (Groom et al., 2008). Production of cellulosic ethanol requires pretreatment step to free up the cellulose before conversion to sugars and subsequent fermentation of sugars to ethanol (Ritter, 2008). Pretreatment of lingo-cellulose is the limiting step to cellulosic ethanol production (Kalia and Purohit, 2008).

Microalgae are a promising source of oil for biodiesel production. In comparison to terrestrial oil crops, microalgae contain more oil and are faster and easier to grow (Hossain et al., 2008). These small organisms (diameter < 2 mm) maintain a simple structure as they utilize energy into photosynthesis, growth, and reproduction rather than into specialized structures (Hossain et al., 2008; Walker T.L. et al., 2005). Consequently, they have higher rates of biomass and oil production than terrestrial crops (Griffiths and Harrison, 2008). Their growth doubling time is usually around 24 hours, and during exponential growth phase they can double their biomass in as short as 3.5 hours (Meng et al., 2009; Song et al., 2008). Microalgal oil composition is similar to that of vegetable

oils (Song et al., 2008), although another literature source states that microalgal oils are richer in polyunsaturated fatty acid with four or more double bonds (Chisti, 2007). Microalgae oil content can vary widely depending on the strain and culture conditions (Song et al., 2008). Typically oil levels of 1 to 26% (dry weight basis) are quite common (Widjaja et al., 2009). However, microalgae can significantly increase their lipid amount by exposure to certain environmental conditions such as abundance of iron (Liu et al., 2008) or limiting nitrogen levels (Yamaberi et al., 1998) in the medium. For instance microalga *C. vulgaris* is able to accumulate 56% (dry weight basis) of lipids under environmental stress (Piorreck et al., 1984). Because microalgae can contain significant lipid levels, they are generally much more productive per unit area than terrestrial oil crop. As seen in Table 2.1, microalgae with 30% of oil on dry biomass basis yields 58,700 L/ha of oil which is around 10 times more than best oil producing terrestrial crop. Of course if the algae contain higher percentage of oil, the difference in oil yield becomes even more pronounced.

It is demonstrated in Table 2.1 that to meet only half of existing US transport fuel needs would require very large cultivation areas for all major terrestrial crops. For crops such as corn, soybean, and canola the required area to meet half of existing US transport fuel needs is larger than the total cropping area of the US. Obviously, these oil crops cannot significantly contribute to replacement of petroleum derived fuel. However, if microalgae are used as source for biodiesel production, only 1 to 3% of total crop growing area of US would be needed to satisfy half of all US transport fuel needs. According to Chisti (2007) the microalgae can possibly completely replace petroleum,

but first the cost of microalgal oil production must decline from about \$ 2.80/L to \$ 0.48/L.

Two major steps are necessary in order to produce biodiesel from microalgae. The first step entails extraction of oil from microalgal cells, while in the second step the extracted oil is transformed through reaction into biodiesel (Chisti, 2007). The extraction of microalgal oil usually occurs by any of the following three methods: ether expeller/press, solvent extraction, or supercritical fluid extraction (“Algae oil extraction.”; Demirbas, 2009). By using a press 70 to 75% of oil can be extracted from dried microalgae. Dried microalgae are utilized as they retain their oil content (“Algae oil extraction.”). Microalgal oil can be also extracted with a chemical solvent; among more popular are hexane, benzene, or ether (“Algae oil extraction.”; Demirbas, 2009). Solvent extraction can be used along with expeller/press method, in which case more than 95% of total microalgal oil can be extracted (“Algae oil extraction.”). The supercritical fluid extraction is more efficient than the other methods already presented, being able to extract 100% of microalgal oil (Demirbas, 2009). In supercritical fluid/CO₂ extraction method liquefied CO₂ fluid is the solvent for oil extraction. The liquefied CO₂ fluid is prepared by liquefying CO₂ under pressure, and heating it to the point that it has properties of both a liquid and a gas (“Algae oil extraction”; Demirbas, 2009).

The oil quantity and quality of microalgae is highly dependent on the environmental conditions (Bertoldi et al., 2006; Hu et al., 2008). It can change with variations in nutrient, temperature, salinity, pH, photoperiod, light intensity and light quality (Dunstan et al., 1993). During the optimal conditions for growth, microalgae synthesize glycerol-based membrane lipids that are mainly composed of various

polyunsaturated fatty acids (Hu et al., 2008). The main purpose of these membrane glycerolipids is to serve a structural role. During stress conditions, such as limitation in nutrients, microalgae shift their lipid biosynthetic pathways and start accumulating large quantities of neutral lipids (Dunstan et al., 1993). The neutral lipids are mostly triglycerides that serve primarily as a storage form of carbon and energy (Hu et al., 2008). They can account for as much as 80% of the total lipid content in the cell (Meng et al., 2009). Generally, microalgal triglycerides contain saturated and monosaturated fatty acids with C₁₆ and C₁₈ profile (Bertoldi et al., 2006; Hu et al., 2008; Meng et al., 2009). Microalgae with higher levels of triacylglycerol should contain higher proportion of saturated and monosaturated fatty acids and lower proportion of polyunsaturated fatty acid (Dunstan et al., 1993). In addition, it is reported that microalgae contain higher variation in fatty acid composition than higher plants (Bertoldi et al., 2006; Hu et al., 2008).

Biodiesel is obtained by transesterification of triglyceride oil or by esterification of fatty acids (Vasudevan and Briggs, 2008). Transesterification is a conventional method of biodiesel production, and it is also used to produce microalgal-derived biodiesel (Chisti, 2007). In this reaction triglyceride (parent oil) reacts with methanol over a catalyst to produce mono-alkyl fatty acid esters (biodiesel) (Hu et al., 2008). Transesterification reaction is shown in Figure 2.1.

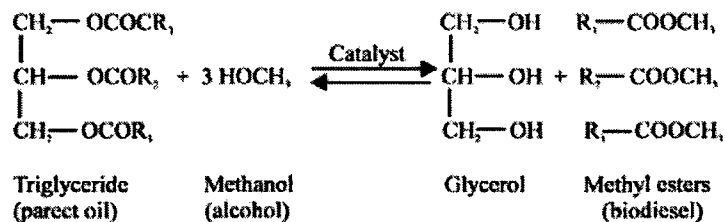


Figure 2.1: Transesterification of oil to biodiesel.

(Note: R₁, R₂, and R₃ are hydrocarbon groups)

(Chisti, 2007; Hossain et al., 2008)

The properties of biodiesel are mainly determined by the hydrocarbon groups that are component of the methyl esters (Hu et al., 2008). These same hydrocarbon groups are also present in fatty acids from which triglyceride oil is composed of. The saturated and monosaturated fatty acids allow for less fuel polymerization during combustion than would be achieved in the presence of polyunsaturated fatty acids (Demirbas, 2009). Polyunsaturated fatty acids tend to decrease stability of the biodiesel, but because of their low melting point the biodiesel can have better cold weather properties (Demirbas, 2009).

Several companies are attempting to commercialize microalgal biodiesel, but at this time it is not on the market (Chisti, 2007; Chisti, 2008). As with most new emerging technologies, production of microalgal derived biodiesel comes with its share of obstacles, and current production strategy is not yet economically feasible. Production of biodiesel from microalgae is 4 to 10 times more expensive than production of petroleum-derived fuels or other biodiesels (Chisti, 2007). Nevertheless, as cited in many literature sources, microalgal biodiesel has a good potential, and through continuous research and development it is bound to become more competitive (Groom et al., 2008; Illman et al.,

2000; Meng et al., 2009). Few pathways have been cited to potentially lead to improvement of economics of microalgal biodiesel: selection of proper strains, genetic and metabolic engineering of strains, proper reactor design, and full use of byproducts (Meng et al., 2009).

2.4 Integrated Process and other Applications

Microalgal biodiesel production could be coupled with other processes to yield a more economic system. The illustration in Figure 2.2 depicts conceptual model for integrated microalgal oil production for biodiesel.

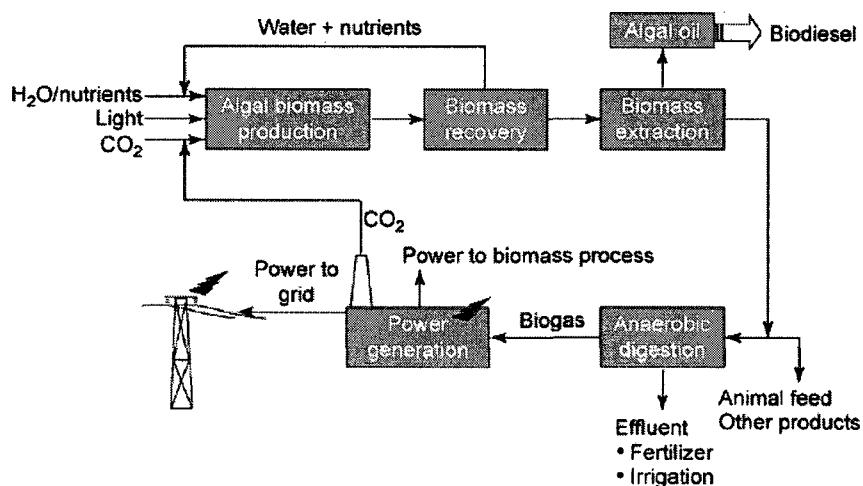


Figure 2.2: A Conceptual process for producing microalgal oil for biodiesel.
(Chisti, 2008)

The first step in biodiesel production is cultivation of microalgal biomass. The inputs to the cultivation vessel include sunlight, CO₂, water, and nutrients. Once sufficient biomass density is reached, the biomass is recovered while water and nutrients are recycled into biomass-cultivation stage. In a facility that produces 100 tons per year of biomass, the cost of cultivation is around \$ 3000 per ton, and as the size of facility increases the cost of cultivation per ton should decrease (Chisti, 2007). The economics of producing microalgal biodiesel has been reviewed briefly by Chisti (2008).

One of the ways to reduce biomass production costs and at the same time obtain environmental benefit is to couple emissions control and wastewater management with biomass cultivation (Patil et al., 2008). For instance, microalgal biomass can utilize some of the CO₂ that is released in power plants for its growth (Chiu et al., 2008). The carbon from this source comes at little or almost no cost (Chisti, 2008). It is noted in literature that 100 tons of microalgal biomass fixes around 183 tons of CO₂ (Chisti, 2008). Jeong et al. (2003) are working on a Bioscrubber that incorporates the CO₂ from power plants into algal culture medium. Similarly, using wastewater as source of nutrients for microalgal growth can add additional economic and environmental benefits (Patil et al., 2008). Microalgae grown on wastewater could obtain essential nutrients such as nitrogen and phosphorus, while wastewater could be simultaneously cleaned up.

As seen in Figure 2.2, the biomass recovery step is followed by the extraction of oil from the concentrated biomass slurry. The oil is further utilized for production of biodiesel. Byproduct glycerol from biodiesel production can be transformed into a valuable chemical (Meng et al., 2009). The biomass residue left after extraction of oil is also not discarded, as it holds potential for additional profit. Microalgal biomass is a very good source of proteins, carbohydrates, vitamins and lipids (Khomova et al., 1986). For instance, microalgae could be utilized as protein source in animal feed, health food, and feed supplements (Chisti, 2007; Yamaguchi, 1996). Noda and co-workers (2002) report on microalgal derived protein that showed strong antitumor immuno-activity. Some microalgae are good source of antioxidant astaxanthin, which may be utilized for prevention of certain cancers, and enhancement of immune system (Ip and Chen, 2005). It has been also suggested that biomass residue could be used for the production of

ethanol, as some microalgae are rich in carbohydrates (“Efficient production of”, 2008). According to Chisti (2008), most of the biomass residue from oil extraction is expected to undergo anaerobic digestion in order to produce biogas. This gas will be fed into the power generation station from which the energy will be used for production and processing of the algal biomass. Additional revenue could come by selling excess energy to the grid. The CO₂ effluent from power generation station could be fed as source of carbon to microalgae during its cultivation.

By integrating other processes with biodiesel production, the cost of producing biomass could be reduced. At the same time, the management of CO₂ emissions and wastewater control are also possible.

2.5 Species Selection and Two Stage Process

Maximum rates of oil production occur when algae have high rates of biomass production and high oil content (Huntley and Redalje, 2007). According to Griffifs and Harrison (2008), the lipid productivity is the most desirable characteristic of a species for biodiesel production. Lipid productivity is described as a product of both lipid content and biomass productivity. These authors also observed that biomass productivity was good indicator of lipid productivity, while there was weak correlation between lipid content and lipid productivity. Hence lipid content reported without biomass productivity does not allow for rational species selection (Griffiths and Harrison, 2008). In studies performed by Widjaja and colleagues (2009), the lipid productivity of *C. vulgaris* cultivated for 15 days under nutrient sufficient conditions was around 10 mg/ (L day).

Successful culturing particularly for low-value product such as biodiesel can be achieved with fast-growing microalgae (Griffiths and Harrison, 2008). The cultivation of fast-growing strains can lead to high biomass productivity. The high biomass density achieved in high biomass productive culture leads to higher yield per harvest volume and decrease in cultivation and harvest cost. Furthermore, fast-growing strains can reduce risk of contamination by outgrowing other microalgal strains or microorganisms (Griffiths and Harrison, 2008). To achieve high growth, it is of paramount importance that the medium contains sufficient amount of nutrients, and is not exposed to any environmental stress (Meng et al., 2009). During such conditions the energy is utilized for production of proteins designed for algal growth.

Another very important microalgal characteristic is the type and quantity of lipids produced by microalgae (Griffiths and Harrison, 2008). Xu and co-workers (2006) state that most microalgal species have similar lipid profile, suitable for biodiesel production. According to numerous authors (Chisti, 2007; Huntley and Redalje, 2007; Meng et al., 2009; Rodolfi et al., 2009) microalgal lipid profile and quantity can change drastically with environmental conditions. When microalgae are exposed to environmental stress such as photo-oxidative stress or nutrient limitation, protein production and cell proliferation shift to secondary metabolite production (Meng et al., 2009; Widjaja et al., 2009). The secondary metabolite is a product that is not involved in primary metabolic processes such as photosynthesis and cell respiration. For instance, certain microalgal species exposed to nitrogen limitation shift their metabolism from biomass production and start accumulating lipids (Meng et al., 2009). The lipid content can double or even triple in microalgae exposed to certain nitrogen conditions (Hu et al., 2008). Luckily, the

accumulated lipids are almost exclusively composed of triacylglycerol that is the desired parent oil for production of biodiesel (Chisti, 2007; Hu et al., 2008).

It would seem plausible then to grow species that has high biomass productivity and high amount of lipid. However, microalgae with high lipid content generally show much slower growth (Huntley and Redalje, 2007; Widjaja et al., 2009). To bypass the problem, one can select a relatively rapidly growing strain that can accumulate lipids when exposed to low nitrogen concentrations. The cultivation of this strain will be conducted in two-stage process. In the first-stage, the strain will be exposed to nutrient sufficient medium. Since this is a rapidly growing strain, high biomass productivity should be achieved. In the second-stage, the high biomass culture will be exposed to nutrient stress, through nitrogen limitation. During this second-stage microalgae will produce and accumulate lipids. Huntley and Retanje (2007) report on coupled cultivation system. In this system the microalgae first grow in an enclosed photobioreactor, in a nutrient plentiful medium aimed at biomass growth. Subsequently, the microalgae enter an open raceway pond with nutrient limitations for biosynthesis of oil. Other strains are excluded in this environment. At the end of the two-stage process we have high biomass culture containing relatively high lipid content.

Not all types of algae are able to accumulate lipids when exposed to limiting concentrations of nitrogen. However, in the case of green algae their lipid level can be altered by varying nitrogen concentration in the media. In addition, the green algae are very good candidates for biodiesel production due to their characteristics such as relatively fast-growth, ease of isolation, and the ability to adapt to diverse natural habitats (Hu et al., 2008).

2.6 Genus Chlorella and Species Chlorella vulgaris

Chlorella is a coccoid green algae and also one of the most studied phototrophic eukaryotes (Krienitz et al., 2004). Sometimes it is referred to as a ‘green ball’ due to its green color and somewhat spherical shape. It is single celled, and contains the green photosynthetic pigments chlorophyll *a* and chlorophyll *b* (Pulz and Gross, 2004; Wikipedia). Chlorophyll can comprise up to 4% of dry mass of *C. vulgaris* (Ecke, 2002).

According to Kessler (1976), the genus of *Chlorella* can be classified into twelve species, characterized by biochemical and physiological criteria. Table 2.2 shows the criteria used by Kessler to identify species of *Chlorella*. It must be pointed out that the systematics of *Chlorella* are continuously being revised due to conflicts between morphological and molecular phylogenetic approaches (Krienitz et al., 2004). Hence, the information in Table 2.2 may not be most current; however it can give some general idea of the characteristics of *Chlorella* species.

Table 2.2: Biochemical and physiological characters of 12 *Chlorella* taxa.

(species, hydrogenase, secondary carotenoids, liquefaction of gelatin, acid tolerance, salt tolerance, thermophile, lactic acid fermentation, nitrate reduction, thiamine (B₁) requirement, base composition of DNA (%GC), and number of strains studied.)

Species	hydr.	s. car.	gel. liqu.	pH	NaCl %	therm.	lact. ferm.	NO ₃ red.	B ₁ requ.	DNA % GC	strains
<i>Chl. fusca</i> var. <i>vacuolata</i> SHIHIRA et KRAUSS	+	+	+	3.5	3	—	+	+	—	50	10
<i>Chl. fusca</i> var. <i>fusca</i> SHIHIRA et KRAUSS	+	+	+	4.0	2	—	+	+	—	55	1
<i>Chl. fusca</i> var. <i>rubescens</i> (DANCEARD) KESSLER et al.	+	+	—	4.5	3	—	+	+	—	58	1
<i>Chl. homoeophaera</i> SKUJA	+	+	—	6.0	0	—	—	+	—	60	1
<i>Chl. zoofingensis</i> DÖNZ	—	+	—	5.0	1	—	+	+	—	49	3
<i>Chl. minutissima</i> FORT et NOVÁKOVÁ	—	—	—	5.5	1	—	—	+	—	43	1
<i>Chl. saccharophila</i> (KRÜGER) MIOULA	—	—	—	2.0	4	—	—	+	—	50	6
<i>Chl. luteoviridis</i> CHODAT	—	—	—	3.0	5	—	—	+	—	44	6
<i>Chl. kessleri</i> FORT et NOVÁKOVÁ	+	—	—	3.0	2	—	—	+	—	56	7
<i>Chl. sorokiniana</i> SHIHIRA et KRAUSS (= <i>Chl. vulgaris</i> f. <i>tertia</i> FORT et NOVÁKOVÁ)	+	—	—	4.0	2	+	+	+	—	65	10
<i>Chl. vulgaris</i> BEIJERINCK	—	—	—	4.0	3	—	+	+	—	62	17
<i>Chl. protothecoides</i> KRÜGER	—	—	—	4.0	4	—	+	—	+	62	14

(Kessler, 1976)

Most strains of *Chlorella* grow at temperatures up to 32°C, while only few are thermophiles. One strain of *Chlorella* can grow at pH of 2, however the most sensitive species can only grow at pH greater than 6. Some *Chlorella* species will tolerate salt concentrations of 4 to 5% NaCl, however one species will not tolerate 1% NaCl. The GC content of *Chlorella* species seem to be quite variable.

C. vulgaris is one of the species of genus *Chlorella*. It is about 2 to 10 µm in diameter, has thick cell wall, and usually grows in freshwater (Ecke, 2002; Mandalam and Palsson, 1997; Raja et al., 2008). Figure 2.3 depicts *C. vulgaris* cells.



Figure 2.0.3: *Chlorella vulgaris* cells.

(Bronnenmeier and Markl, 1982)

One of the most striking features of *C. vulgaris* is its high biomass production. Its doubling time has been reported to be less than 20 hours (Griffiths and Harrison, 2008). This species can grow in autotrophic, heterotrophic, or mixotrophic (combination of autotrophic and heterotrophic) conditions (Patino et al., 2007). In addition to chlorophyll *a* and chlorophyll *b*, *C. vulgaris* contains pigments such as carotene and lutein (Maruyama et al., 1997).

The biochemical composition of *C. vulgaris* has been summarized by work of Maruyama and co-workers (1997). These authors have found that *C. vulgaris* is composed of about 55% proteins, 10% lipids, 23% carbohydrate, 6% fiber, and the rest is ash, all based on percentage of biomass. These values can vary from one literature source to the next (Raja et al., 2008).

As shown in Table 2.2, *C. vulgaris* does not contain hydrogenase, an enzyme that catalyzes uptake or production of hydrogen. In addition, under nitrogen-deficient conditions *C. vulgaris* has a pale color as it loses chlorophylls and carotenoid. This

species does not have ability to liquefy gelatin, however it can reduce nitrate and utilize glucose. The products of glucose fermentation for most *C. vulgaris* strains are CO₂, and lactose, and in only few of the strains the products are formic acid, glycerol, or hydrogen (Vinayakumar and Kessler, 1975). In addition, *C. vulgaris* does not have requirement for thiamine, can grow at pH higher than 4, can tolerate 3% NaCl and it is not a thermophile. Strains of *C. vulgaris* have upper limit of temperature tolerance of 28 to 30°C (Kessler, 1985).

It is well known that *C. vulgaris* exposed to low nitrogen medium produces and accumulates lipid (Amotz et al., 1985). Piorreck and co-workers (1984), found that under very low nitrogen levels, *C. vulgaris* could reach 53% lipid by dry weight. Work by Illman and colleagues (2000) reveals that when *C. vulgaris* was grown in low nitrogen medium (203 mg/L (NH₄)₂HPO₄), the lipid content increased from 18 to 40%, while protein level decreased from 29 to 7%. In addition, Liu and associates (2008) report on *C. vulgaris* that has lipid content of 56 % of biomass by dry weight. The average lipid productivity of *C. vulgaris* is around 40 mg/ (L day) (Griffiths and Harrison, 2008).

As can be seen, medium composition is of paramount importance that can dictate the growth rate and secondary metabolite production in microalgae. For this reason extensive literature research has been performed on the effect of medium composition on microalgae, and is presented in the following chapter.

2.7 Medium

Microalgae are simple organisms with plain nutritional requirements (Faramarzi et al., 2008). However, a proper medium design has shown to have large impact on the growth capacity of *C. vulgaris* cultures (Mandalam and Palsson, 1998). Research of literature sources revealed that various medium compositions have been used to cultivate *C. vulgaris* such as; modified Fitzgerald medium (Widjaja et al., 2009), N-8 medium (Mandalam and Palsson, 1997), and Watanbe medium (Illman et al., 2000), among others.

It has been recognized that medium composition for biomass growth can be formulated from the *Chlorella*'s elemental composition (Mandalam and Palsson, 1998), or from cellular and metabolic pathway stoichiometry (Varma and Palsson, 1994). Elemental composition of *Chlorella* species presented by authors Oh-Hama and Miyachi (1988) is shown in Table 2.3. It must be pointed out that the elemental composition of biomass may vary depending on the culture conditions; however the differences cannot be large (Patino et al., 2007).

From the results in Table 2.3 it is apparent that quantitatively carbon is the most important element and accounts for more than a half of *Chlorella* weight. In addition, oxygen and hydrogen also comprise large part of *Chlorella*'s weight. The carbon source for photoautotrophic growth of algae can be obtained from CO₂, while oxygen and hydrogen are derived from water molecules supplied by the medium. Apart from carbon, oxygen and hydrogen, the most important element for algal growth is nitrogen, and as reported by Oh-Hama and Miyachi, *Chlorella* cells contain around 6.2 to 7.7% of

nitrogen by weight. Other source in the literature reports that nitrogen comprises 1 to 10% of cell dry weight (Syrett, 1981).

Table 2.3: Elemental composition of *Chlorella* (by weight).

Element	% Range
Carbon	51.4–72.6
Oxygen	11.6–28.5
Hydrogen	7.0–10.0
Nitrogen	6.2–7.7
Phosphorus	1.0–2.0
Potassium	0.85–1.62
Magnesium	0.36–0.80
Sulfur	0.28–0.39
Iron	0.04–0.55
Calcium	0.005–0.08
Zinc	0.0006–0.005
Copper	0.001–0.004
Manganese	0.002–0.01

*Values obtained from Oh-Hama and Miyachi (1988).

(Oh-Hama and Miyachi, 1988)

Nitrogen is a very important element for the growth of microalgae since it is a building block for the synthesis of proteins. In addition, photosynthetic pigments require nitrogen for their proper functioning (Post, 1993) as low level of nitrogen results in decreased number of photosynthetic pigments (Kolber et al., 1988). Furthermore, nitrogen has a strong influence on metabolism of lipids and fatty acids (Piorreck et al., 1984). Almost all kinds of algae require nitrogen to be supplied in the medium as either inorganic or organic nitrogenous compound or as combination of both. Only blue-green algae can fix gaseous nitrogen and the best known genus with this ability is *Trichodesmium* (Syrett, 1981). The most common inorganic nitrogen sources for algae

include ammonia and nitrate, while common organic nitrogen sources are urea and amino acids.

2.7.1 Inorganic Nitrogen Sources

2.7.1.1 Ammonia

2.7.1.1.1 Ionized and Unionized Ammonia

Ammonia compounds have been utilized as fertilizers, and also as therapeutic agents for many human diseases (Warren, 1962). In many circumstances it seems like ammonia is a relatively non-toxic substance. However the non-toxic appearance of ammonia can change drastically by alteration in hydrogen ion concentration in the medium. At high pH, most of the ammonia is present in its un-ionized form (NH_3) which is toxic, while at lower pH ammonia is much less toxic and mainly exists as ammonium ions (NH_4^+) (Tam and Wong, 1996; Warren, 1962; Wurts, 2003). In addition, the temperature has impact on the degree of ammonia ionization and hence on its toxicity. At the temperature of 25°C ammonia is mostly ionized below pH 8.0, while at pH of 9.0 half of ammonia is unionized (Konig et al., 1987). Sources in the literature state that the ammonia toxicity increases as the temperature in the medium rises (Liu et al., 2008; Wurts, 2003).

The two forms of ammonia have different penetration ability through the cell membrane. Cell membranes are relatively impermeable to the ionized form (Warren,

1962), unlike the unionized form that easily passes across the membrane (Milne et al., 1958). Generally, the unionized ammonia enters the algal cell by diffusion, and only some types of cyanobacteria possess active transport mechanism for ammonia (Post, 1993). The ability of NH_3 to readily cross the hydrophobic cell membrane exists because it is both uncharged and lipid soluble (Milne et al., 1958). A dose of CO_2 delivered into the medium has been noted to enhance the rate of ammonia transfer across the algal membranes (Warren, 1962).

Ammonia is usually supplied to the algal medium as ammonium ion in a compound such as ammonium hydroxide or ammonium chloride, among others. In the literature ammonium hydroxide has been noted as the cheapest and simplest source of nitrogen for growing plants (Jeong et al., 2003; Warren, 1962). However, a solution containing high amounts of ammonium hydroxide at high pH can be very toxic to the cells. High internal ammonium concentration can have detrimental effect on algal photosynthesis. In addition, Azov et al. (1982) point out that the pH has no direct effect on inhibition of photosynthesis, only that it determines the degree of dissociation of nontoxic NH_4^+ to toxic NH_3 form.

2.7.1.1.2 Tolerance to Ammonia

Concentration at which the toxicity of ammonia becomes effective is highly dependent on the algal species and culture conditions (Tam and Wong, 1996). High concentration of nitrogen can lead to toxicity, however low concentration is also undesirable as it can cause nitrogen limitation and consequently poor algal growth. *C. vulgaris* grown in commercial Bristol medium with no pH control and supplemented with

various ammonium-N concentrations ranging from 10 to 1000 mg/L, showed less growth at low concentrations of nitrogen of 10 mg/L or at high concentrations of nitrogen in the range of 750 to 1000 mg/L (Tam and Wong, 1996). Matusiak's (1976) experiments with *C. vulgaris* grown on wastes from nitrogen fertilizer industry revealed that this strain grew well in media containing 600 mg/L ammonium, but was inhibited at concentrations of about 1000 mg/L. In agreement with these studies, König and co-workers (1987) have shown that there was almost no inhibition of growth of *C. vulgaris* in waste stabilization pond with ammonium concentrations of 560 mg/L. On the contrary, some studies report a complete inhibition of growth of *C. vulgaris* at concentrations of ammonia below 1000 mg/L. For instance, Przytocka-Jusiak (1976) found that total inhibition of growth of *C. vulgaris* occurs at concentration of 750 mg/L, while 50% growth inhibition occurs in media containing only 330 mg/L ammonium at pH 7.0.

There are also numerous literature sources studying the effect of nitrogen concentration on other *Chlorella* species. For instance, the optimum concentration of nitrogen in media for *Chlorella sorokiniana* was between 140 to 700 mg/L (Eyster, 1978). In a recent study of nitrogen removal from landfill leachate by *Chlorella pyrenoidosa*, it was determined that the growth of algae was supported at ammonium-N concentration of 135 mg/L, while at higher ammonium-N concentrations of about 400 mg/L, the growth was only apparent in cells previously adapted to high levels of ammonia (Lin L. et al., 2007).

2.7.1.1.3 Adaptation of *Chlorella* to Ammonia

The incubation of *C. vulgaris* with high concentration of ammonium, allows for isolation of cells with developed resistance to high levels of ammonia. In her previous work Przytocka-Jusiak (1976) found that the ammonium-N concentration of 330 mg/L inhibits growth of *C. vulgaris* by 50%. In authors subsequent studies, it was determined that *C. vulgaris* that developed tolerance to high ammonium concentration through adaptation experienced a 50% growth inhibition in media containing 1500 mg/L of ammonium-N (Przytocka-Jusiak et al., 1977). In addition this study also revealed that the ammonia tolerant cells were larger than the parental cells. Hence by adapting the cells to high levels of ammonia, these cells were able to survive in severe conditions for ordinary alga. Similarly, other study has shown that *Chlorella kessleri* can tolerate high concentrations of nitrogen of 1400 mg/L (Lee K. and Lee C., 2002). These results are promising in imploring high nitrogen resistant *Chlorella* for biomass production in wastes containing high nitrogen content.

De-Bashan and co-workers (2008) note that *C. sorokiniana* became tolerant to high temperature of about 40°C and light intensities of 2500 $\mu\text{E}/(\text{m}^2 \text{ s})$ in wastewater pond. The authors also state that when this high tolerant strain is cultivated under similar extreme conditions but in synthetic media, it can remove ammonia with higher efficiency than under temperature of 28°C and light intensity of 60 $\mu\text{E}/(\text{m}^2 \text{ s})$.

As mentioned previously, proper concentration of ammonia and pH are crucial in avoidance of ammonia related toxicity. According to Abeliovich and Azov (1976), ammonia at concentrations of over 2 mM (>34 mg/L) and at pH values over 8.0 inhibits

photosynthesis and growth of *Scenedesmus obliquus*. Some organisms however are naturally resistant to ammonia at high pH. *Spirulina platensis* retained 50% of its maximal photosynthetic capacity at pH 10 and ammonia concentration of 140 mg/L (Belkin and Boussiba, 1991). As suggested by Belkin and Boussiba (1991), the reason for this resistance is because the internal pH of *Spirulina* is relatively high, resulting in small pH gradient across the cell membrane and subsequently low diffusion of ammonia. In the literature it has been noted that genus *Chlorella* is generally quite tolerant to ammonia solution at high pH. As reported in one study, *Chlorella* was able to grow in the media containing 140 mg/L ammonia at pH 9, and temperature of 25°C (Konig et al., 1987).

2.7.1.1.4 Effect of Low pH

Very low pH of the medium is also undesirable for algal growth. According to Bollard (1966), the growth of algae in media containing ammonium nitrate, ammonium sulphate or urea, with the absence of control of pH may result in low pH. The effect of low pH on the deterioration of *C. vulgaris* growth was most pronounced in the presence of ammonium sulphate (Bollard, 1966). Tam and Wong (1996) working with *C. vulgaris* culture in the presence of initial ammonium-N concentration greater than 50 mg/L, has also noted that utilization of ammonia-N from the media was accompanied by rapid pH drop, with lowest pH of 1. The results of rapid pH drop are also noted in other numerous studies (Lewin, 1962; Pratt and Fong, 1940). Trelease and Trelease (1935) proposed a mechanism, shown in Figure 2.4, to explain how a low pH can develop in the media. From Figure 2.4 it can be seen that upon addition of ammonium compound into the medium it dissociates into ammonium ion and into corresponding anion.

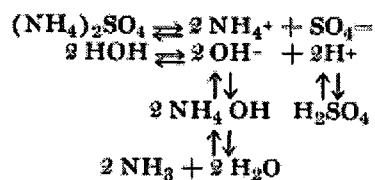


Figure 2.4: Behavior of ammonium sulphate in solution.

(Trelease and Trelease, 1935)

Subsequently ammonium joins with hydroxide, and the resulting compound ammonium hydroxide is utilized by the cells. The sulphuric acid remaining in the medium is responsible for the lowering of the pH. If ammonium sulphate is replaced by ammonium hydroxide, we can predict based on mechanism in Figure 2.4 that instead of sulphuric acid, water molecules would remain in the medium and pH in this case would not change dramatically. In study by Matusiak and co-workers (1976), *C. vulgaris* was grown in wastes containing $(\text{NH}_4)_2\text{CO}_3$ and KNO_3 , and it was observed that the pH dropped sharply from initial value of 7.0 to around 4.5 and maintained around that value. Work by Przytocka-Jusiak and co-workers (1977) shows that medium containing *C. vulgaris* previously adapted to high concentration of ammonia has smaller fluctuations in pH than medium containing parental strain.

2.7.1.1.5 Effect of Nitrogen Concentration in the Medium on Algal Lipid and Protein Content

The degree of growth and lipid accumulation of algal cells is dependent on the level of nitrogen in the medium. In the presence of light and CO_2 , and in N-sufficient

medium, the photosynthetic CO₂ fixation delivers the carbon that acts as a basic building block for the construction of amino acids that are subsequently utilized for production of biomass (Amory et al., 1991). Generally, it has been observed that the higher the initial ammonia nitrogen in the medium the higher the protein concentration per cell (Tam and Wong, 1996). Piorreck and co-workers (1984) have shown that increasing nitrogen levels in the medium led to an increase in the green-algae biomass, in protein content, and in chlorophyll.

However if algal cells are exposed to N-deprived conditions in the presence of light and CO₂, carbon is no longer utilized to produce amino acids and the cell growth diminishes (Rigano et al., 1998). In a nitrogen deficient environment the products of photosynthesis change from protein to carbohydrate and lipid. In addition, the chlorophyll content of the cells is decreased (Mandalam and Palsson, 1998). It is interesting to note that *C. vulgaris* cells are still able to maintain growth after nitrogen deficiency in the medium, most likely because the cells are able to internally accumulate and subsequently utilize the nitrogen reserve (Tam and Wong, 1996). If CO₂ or light is withheld, ammonium utilization is prevented in N-sufficient cells (Rigano et al., 1991).

In N-deprived cells exposed to light and CO₂, ammonium is rapidly utilized (Syrett, 1956). It has been shown that N-deprived algae assimilate ammonium four to five times faster than N-sufficient cells (Lewin, 1962). During nitrogen assimilation in presence of light and CO₂, the nitrogen-starved cells divert the photo-generated electrons from CO₂ fixation and towards nitrogen assimilation (Thomas et al., 1976). Terry (1982) noted that if CO₂ fixation rate is reduced due photosynthetic energy being diverted towards light dependent component of nitrogen uptake, a 1 to 1 mole relationship

between the two rates is expected. It has been noted that during the assimilation of ammonia nitrogen by N-deprived cells the activity of certain enzymes is increased. A nitrate reductase has been shown to appear in N-starved algal cells exposed to ammonium nitrogen (Hipkin et al., 1983). Everest and co-workers (1986) have shown that by increasing nitrogen-deficiency in *Chlorella stigmatophora*, an increase in the level of activity of nitrate reductase, NADPH-glutamate dehydrogenase and glutamine synthetase is observed.

N-deficient cells exposed to nitrogen source can utilize their carbon reserves for ammonium assimilation and amino acid synthesis in the dark (Thacker and Syrett, 1972). Work with nitrogen-starved *Chlorella* revealed that ammonia was absorbed at similar rate in darkness as in light (Rigano et al., 1998). In the work with flagellate *Heterosigma carterae*, it is shown that the relative difference between uptake rates of nitrogen in light and dark is smaller as the cells become increasingly nitrogen-deprived (Clark and Flynn, 2002).

As already indicated, an alga exposed to N-deficient environment starts to internally accumulate lipids. Lewin (1962) notes that production of carbohydrates proceeds production of lipids. One study revealed that *C. pyrenoidosa* accumulates lipids only 4 to 6 days after nitrogen deficiency, while in *C. vulgaris* lipids have been noted to accumulate much later Lewin (1962). Studies by Piorreck and co-workers (1984) show that *C. vulgaris* is able to accumulate lipids up to 56% of its biomass in presence of a low nitrogen level of 300-600 $\mu\text{mol/L}$ (4.2-8.4 mg/L) in the medium. Most of these lipids were neutral lipids such as triacylglycerols. In contrast, at high nitrogen levels algae contained lipids which amounted to only 20% of the algal biomass. These lipids were

mostly polar containing polyunsaturated C₁₆ and C₁₈ fatty acids. Not all the algae can be manipulated to yield a biomass with desired fatty acid and lipid compositions. Piorreck and colleagues (1984) observed that only lipids of green algae such as *Chlorella* and not blue-green algae can be manipulated in such way. The metabolism of lipids can be also manipulated by nitrogen concentration in red and brown algae. These algae along with green algae are eukaryotes, while blue-green algae are prokaryotes.

2.7.1.2 Nitrate

2.7.1.2.1 Effect of Ammonia on Nitrate

When nitrate (NO₃⁻) is used as nitrogen source it is first reduced to nitrite (NO₂⁻) in a reaction catalyzed by enzyme nitrate reductase. Subsequently, nitrite is reduced to ammonia through reaction catalyzed by nitrite reductase (Converti et al., 2006). Unlike assimilation of ammonium, nitrate reduction requires energy. If energy supply is limited, the growth of algae will be higher on ammonium (Lewin, 1962). According to Lewin (1962), *Chlorella* grows well in 10 mM solutions of nitrates (620 mg NO₃⁻/ L) or ammonium salts (170 mg NH₄⁺/ L). Jeanfils has shown that *C. vulgaris* experienced some inhibition by nitrate at concentration of 97 mM (6015 mg NO₃⁻/L) (Jeanfils et al., 1993). Furthermore, during the uptake of nitrate ions the concentration of the hydrogen ions in the external solution tends to decrease (Pratt and Fong, 1940).

Most studies have shown that *Chlorella* utilize reduced nitrogen such as ammonia or urea in preference to nitrate nitrogen (Converti et al., 2006; Schlee et al., 1985; Syrett and Morris, 1963; Yun et al., 1996). For instance the study by Piorreck and co-workers

(1984) has shown that algae grown on NH_4Cl attained its maximum concentration faster than if grown on KNO_3 . Similarly, Singh and co-workers (2007) work with several microalgal strains has shown that *Chlorella* was most efficient in the uptake of ammoniacal nitrogen. While Clark and Flynn (2002) found that ammonium uptake was faster than that of nitrate. Even though there are lots of data supporting ammonia as a preferable nitrogen source, nitrate is usually the recommended nitrogen in growth media (Schlee et al., 1985). This might be so because ammonium at high concentrations can be very toxic to algae.

When ammonium and nitrate are both added to the medium, the uptake rate of nitrogen decreases in comparison to when each nutrient is added alone (Dortch and Conway, 1984). The work of Pistorius and co-workers (1978) reveals that addition of ammonium results in inhibition of nitrate utilization in *C. vulgaris*. The same conclusion was reached in numerous other studies with different types of algae (Przytocka-Jusiak et al., 1984; Terry, 1982; Tischner and Lorenzen, 1979). Pistorius also speculates that the inhibition of nitrate uptake by ammonium is not mainly due to inactivation of nitrate reductase, however it was noticed that nitrate reductase does become depleted only after prolonged exposure to ammonium. Tischner and Lorenzen (1979) also found that ammonia inactivated nitrate reductase only after prolonged length of time. These same authors suggest that ammonium first interacts with the nitrate uptake and only later with the nitrate reductase. On the contrary, Losada et al. (1970) found that addition of ammonium to *Chlorella* cells grown in presence of nitrate, causes inactivation of nitrate reductase in short amount of time. Ammonium only partially inhibits nitrite assimilation, however if the cells are highly deficient in carbon, ammonium does not inhibit nitrate

uptake at all (Syrett and Morris, 1963). Once almost all of the ammonium is utilized, nitrate uptake takes place (Syrett and Morris, 1963).

2.7.1.2.2 Light and Nitrate Utilization

Results from one study reveal that in the presence of ammonium, nitrate uptake was depressed at all light intensity (Bates, 1976). In a different study the authors state that at low light intensities of $3 \mu\text{E}/(\text{m}^2 \text{ s})$, ammonium did not inhibit nitrate uptake (Yin et al., 1998). In the dark and in the absence of ammonium, the uptake of nitrate by *C. vulgaris* was inhibited, and the rate of decline of the activity of nitrate reductase was much larger than in a case where ammonium was added to nitrate in the light (Pistorius et al., 1978). It is also speculated that even though the enzyme substrate NADH, responsible for reduction of nitrate, is reduced in the dark the reduction could not account for all the effect of inhibition of uptake of nitrate. The inhibition of nitrate by darkness can be relieved by addition of glucose, however a higher nitrate uptake is noted in the light and with glucose (Tischner and Lorenzen, 1979). The type of light has an effect on the nitrate uptake. Blue light has been shown to stimulate nitrate uptake (Ullrich, 1987). Syrett (1981) speculates that blue light induces production of amino acids and protein rather than of carbohydrate with either NO_3 , NH_4 or urea as a nitrogen source.

2.7.1.2.3 Nitrate and Effect of Carbon Source

In the absence of CO_2 , the nitrate uptake and the reduction by *Ankistrodesmus* algal strain was inhibited mostly at low pH (Eisele and Ullrich, 1977). The same authors also found that under these conditions the inhibition of nitrate uptake was relieved by

addition of glucose. The study also revealed that adding 1% CO₂ in air or more to the media, resulted in increase in the nitrate uptake, and the effect was independent of pH. In the absence of carbon source, reduced nitrate in *C. vulgaris* and other algal strains is mostly released to the media as ammonia, while in the presence of carbon dioxide or glucose, ammonia is not accumulated in the media because it is assimilated by the cells (Eisele and Ullrich, 1977; Syrett and Morris, 1963).

2.7.2 Organic Nitrogen Sources

Algae can grow not only on inorganic nitrogen, but also on organic nitrogen as sole source of nitrogen. Study conducted by Birdsey and Lynch (1962) revealed that *C. vulgaris* could grow on organic nitrogen compounds such as urea, uric acid, and xanthine, while it could not grow on allantoin or creatinine. Growth of *Chlorella* on urea has been studied thoroughly in the literature.

2.7.2.1 Urea

Many plants and microorganisms metabolize urea by enzyme urease to ammonia and CO₂ (Hodson and Thompson, 1969). However, urease has not been detected in *C. vulgaris* (Arnold et al., 1953). Instead, it has been shown that *C. vulgaris* is able to metabolize urea to ammonia and CO₂ by activity of two enzymes operating in a sequence (Thompson and Muenster, 1971). The first enzyme, urea carboxylase, acts on urea in the presence of CO₂, biotin, ATP and Mg²⁺ to produce allophanate, this compound is

subsequently decomposed by allophabate lyase to CO_2 and NH_3 (Thompson and Muenster, 1971). Hodson and Thompson (1969) also propose that metabolism of urea by *Chlorella* requires energy.

There is much disapproval in the literature sources whether inorganic or organic source of nitrogen is better for algal growth. Work by Syrett (1981) reveals that microalgae prefer to utilize inorganic instead of organic nitrogen source. However some studies disprove this view. For instance Bollard's (1966) work has shown that growth of *C. vulgaris* on urea was similar to growth either on ammonium sulphate, ammonium nitrate or calcium nitrate. While *S. platensis* actually grew better on urea than on ammonia (Converti et al., 2006). Walker (1953) actually states that urea is the best nitrogen source for obtaining high cell densities, since at high concentrations urea is nontoxic, unlike ammonia or nitrate. In addition, the author goes on to say that pH of the medium is not changed by the urea uptake in comparison to ammonia and nitrate.

According to Przytocka-Jusiak (1976), *C. vulgaris* is the most sensitive to ammonia levels in comparison to nitrate and urea. The author showed that the concentration of ammonia-N, nitrate-N, and urea-N causing 50% of inhibition of growth of *C. vulgaris*, are 330, 1050, and 5000 mg/L, respectively. These studies have also revealed that optimal concentration of urea-N for *C. vulgaris* is 250 mg/L, while that of nitrate-N is 500-700 mg/L. From the experiments conducted with wastes containing around 1300 mg/L ammonium-N and 6000 mg/L urea-N, only *C. vulgaris* previously adapted to ammonia nitrogen grew well (Matusiak, 1976). From these results it seems like the alga was not inhibited by relatively high concentration of urea. Algae grown on nitrogen wastes from nitrogen fertilizer industry show better growth of *C. vulgaris* in

presence of ammonia, nitrate and urea than only in the presence of nitrate and ammonia (Matusiak et al., 1976).

In the presence of nitrate urea is utilized first (Converti et al., 2006). In addition, unlike the effect of blue light on uptake of nitrate, uptake of urea is not influenced by blue light for growing and resting cells and in the presence of nitrate or ammonia (Kamiya and Saitoh, 2002).

2.7.2.2 Amino Acids and other Nitrogen Containing Compounds

Bollard (1966) tested around 170 naturally occurring and synthetic organic nitrogen compounds as the sources of nitrogen for *C. vulgaris*. His work revealed excellent growth of *C. vulgaris* not only on urea, but on several other organic nitrogen compounds such as some protein amino acids (l-arginine, l-glutamine, glycine, l-serine), urea derivatives (1,1-diethylurea and hydantoin), and also compounds derived from purines such as guanidine, and hypoxanthine. However, studies with purines as nitrogen sources are scarce due to purines low solubility in water (Syrett, 1981). Pyrimidines have been noted to be weak nitrogen sources, however uracil has been utilized by *Chlorella fusca* (Knutsen, 1972). *C. pyrenoidosa* grown on nitrogenous compounds showed best growth on amino acids l-arginine, glutamine or cysteine, followed closely on ammonium sulphate, while growth on nitrate was much lower (Ghosh and Burris, 1949). Work by Lynch and Gillmor (1966) shows that *C. pyrenoidosa* grows well on glutamine as well as on urea, and does not utilize glutamic acid as sole external nitrogen source.

When algae are pretreated with glucose, uptake of neutral amino acids glycine, l-alanine, l-proline and l-serine increases more than 100-fold, while uptake of basic amino

acids l-arginine and l-lysine increases by factor of 25-50 (Cho B.H. et al., 1981). The basic amino acids are taken up in the positively charged form, and charge compensation occurs with proton pump, and later by K^+ efflux (Cho B.H. and Komor, 1984). Without pre-treatment the highest rate of uptake is seen with amino acids such as l-lysine, l-arginine, glycine and l-serine (Cho B.H. et al., 1981).

In *C. vulgaris* cells grown in presence of light and CO_2 , glucose induces not only a hexose uptake system, but at the same time it induces amino-acid uptake systems for short-chain neutral amino acids and for basic amino acids (Cho B.H. et al., 1981). The same amino acid uptake systems are also induced in nitrogen depleted cells (Sauer et al., 1983).

2.7.3 Inorganic Phosphorus

Chlorella is comprised of 1 to 2% of phosphorus by weight (Oh-Hama and Miyachi, 1988) and quantitatively it is the most important nutrient after nitrogen. Inorganic phosphate ($H_2PO_4^-$, HPO_4^{2-} , and PO_4^{3-}) is a major source of phosphorus for microalgal cells (Bostroöm et al., 1988; Rose and Morris, 1980). Many microalgae incorporate phosphate into polyphosphate granules that serve as storage site of phosphates and also as source of energy (Kornberg, 1995). The formation of polyphosphates is strongly influenced by environmental stress. For instance, phosphorus starved cells of *C. vulgaris* had a significant increase in polyphosphate content (Eixler et al., 2006).

Concentration of phosphorus in the media is directly proportional to the algal growth rate, unless phosphorus concentration is above critical level (Azad and Borchardt, 1970). Low P levels in the media are associated with declined photosynthetic activity and decreased protein concentrations (Theodorou et al., 1991). During the 7 day cultivation of *C. vulgaris*, the highest OD was achieved with 10 mM (180 mg/L) of ammonia and 1 mM of phosphate (95 mg/L), while a very high concentration of ammonia of 1M (18 g/L) and phosphate of 100 mM (9.5 g/L) inhibited *C. vulgaris* (Jeong et al., 2003). According to Eyster (1978) the optimum concentration of phosphorus for *C. sorokiniana* is between 15.5 to 620 mg P/L. Singh and co-workers (2007) have shown that *C. vulgaris* utilizes available phosphorus efficiently. While the results of Aslan and Kapdan (2006) indicate that *C. vulgaris* effectively utilizes phosphate-P at concentrations of less than 7.7 mg/L in the media, also as the concentration of phosphate-P was increased the utilization was less efficient. *Chlorella* had higher capability of utilizing P from wastewater than *Scenedesmus* strain, and it utilized P from waste with initial concentration of 6.179 mg P/L to 0.572 mg P/L in 13 days (Tam and Wong, 1989).

Phosphate depleted *C. vulgaris* takes up phosphate at much higher rate than phosphate sufficient cells (Aitchison and Butt, 1973; Azad and Borchardt, 1970). Kozłowska-Szerenos and co-workers (2000) report that the growth of *C. vulgaris* in P deficient medium was 30 to 40% slower, however the uptake of P was higher than in P-sufficient medium. The author also notes that cells in P-deficient medium produced glycolate at elevated levels. Studies by Theodorou and co-workers (1991) reveal that P limited culture of green algae *Selenastrum minutum* had lower growth and decreased protein concentrations and chlorophyll amount (Theodorou et al., 1991). During P

limitation, photosynthetic carbon in chloroplast is most likely directed towards starch synthesis (Theodorou et al., 1991). Beardall and co-workers (2005) found that P-limitation had effect of down-regulating CO₂ acquisition by the alga *Chlorella emersonii*. However another group has found that P-limitation up-regulated uptake of CO₂ in *C. vulgaris* (Kozłowska-Szerenos et al., 2000).

Aitchison and Butt (1973) reported high uptake rate of phosphate by *C. vulgaris* in the medium containing some glucose, when cells were illuminated and aerated, however uptake of phosphate also occurs to a lesser degree in presence of darkness and aeration. As light intensity decreases, it is expected to see a decrease in mass growth rate as well as in the efficiency of algae to utilize phosphate from the media (Azad and Borchardt, 1970). This study also showed that as temperature fell below 25°C in the medium, higher phosphorus concentrations were needed to attain same biomass (Azad and Borchardt, 1970).

According to Matusiak (1976) the best growth of *C. vulgaris* on ammonium and nitrogen containing wastes was when the wastes were supplemented not only with phosphorus, but also with magnesium and trace elements

2.7.4 Other Nutrients

Based on the elemental composition of *Chlorella* reported by Oh-Hama and Miyachi (1988), nutrients needed for *Chlorella* growth, apart from N and P include iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), calcium (Ca), potassium (K), manganese (Mg), and sulphur (S). Eyster's (1978) work acknowledges that these nutrients are needed for

the growth of *C. sorokiniana*. Walker (1953) states that with the exception of boron, all inorganic nutrients required by *Chlorella* are identical to those required for higher plants.

Fe along with Mn, Cu, and Zn is involved in some aspect of photosynthesis. Iron is quantitatively the most important trace metal involved in photosynthesis, followed by manganese that plays essential role in O₂ evolution (Raven et al., 1999). Absence of Fe in the medium leads to retardation of algal growth, and reduction of photosynthesis. In addition, the iron deficiency has a negative impact on function of chlorophyll system (Hase et al., 1958; Mandalam and Palsson, 1998), resulting in small cells that are low in chlorophyll content (Meisch et al., 1980). Some authors reported that restriction in iron content of the media increases cellular starch content of the algae (Estevez et al., 2001; Meisch et al., 1980). In addition, it was observed that when the iron is growth limiting, the algal cells adhered to the wall of the flasks (Pirt and Walach, 1978). The results of work by Walach and Pirt (1986) revealed that concentration of trace elements had significant effect on the growth rate of *Chlorella* cells and on the degree of adhesion of the cells to the walls of the vessel. The authors point out that the higher the concentration of trace metals, the smaller the degree of the adhesion of the cells.

The specific growth rate and biomass of *C. vulgaris* increased as the initial concentration of iron was increased in the media up to 0.47 mg/L of FeCl₃6H₂O (Pirt and Walach, 1978). In a different study, addition of iron up to 0.1 mM (5.6 mg Fe/ L) resulted in a slight increase in biomass and in activity of nitrite reductase (Cardenas et al., 1972). Work by Estevez and colleagues (2001) showed that iron in concentrations larger than 0.2 mM (11.2 mg Fe/ L) had negative effect on the growth rate of *C. vulgaris*. Excess iron caused oxidative stress, meaning that the concentration of oxygen radical

intermediates was at a toxic level (Estevez et al., 2001). Furthermore, β -carotene level was not affected by addition of iron; however when iron concentration was above 0.09 mM (5.04 mg Fe/ L), antioxidant (α -tocopherol, ascorbate and thiol) content increased in the cells.

Cu, like Mn and Fe, is also involved in photosynthesis. Cu plays role in photosynthesis by facilitating water dehydrogenation and oxygen evolution (Hankamer and Barber, 1997). According to Knauer and co-workers (1997), optimal concentration of Cu^{2+} for growth of *C. fusca* was in the range of 10^{-13} to 10^{-10} M ($\sim 6.4 \times 10^{-6}$ mg/L), and the studies confirmed that Cu and Zn are required at low concentrations, and can be toxic at higher concentrations. Other study revealed that the optimum concentration of copper for *C. sorokiniana* is between 10^{-8} M ($\sim 6.4 \times 10^{-4}$ mg/L) to 10^{-3} M (64 mg/L). Agitation was performed in this experiment (Eyster, 1978). Absorption of Cu^{2+} by *C. vulgaris* was strongly dependent on pH, with maximum uptake rate at pH of 6 (Mehta et al., 2002). Generally, the binding of metals by algae was higher as pH was changed from 4 to 8 (Lopez-Suarez et al., 2000).

Zn plays role in CO_2 assimilation (Raven et al., 1999). Rachline and Farran (1974) found that concentration of zinc that reduced growth rate of *C. vulgaris* by 50% in 96 hours is 2.4 mg/L. While results by Coleman and co-workers (1971) indicated that zinc concentration of 4.20 mg/L promotes the growth of *C. vulgaris* during the three week experiment, while 8.7 mg/L of zinc retards the growth. A more recent study by Loez and colleagues (1995) revealed that *C. vulgaris* was not inhibited by even 25 mg/L of zinc during three week experiment. Eyster (1978) found that optimum concentration of zinc for *C. sorokiniana* was between 0.66 mg/L to 653 mg/L. Zinc showed to be less

toxic than cadmium to *C. vulgaris* cells (Sandau et al., 1996). These cells died shortly after being treated to additions of Cd at concentration of 10 mg/L.

K is another nutrient required by *Chlorella*. Salts of membrane-permeate acids (ex. acetate) and bases (ex. ammonium chloride) have effect on K movement in and out of *Chlorella* (Tromballa, 1978). Acetate added to *C. fusca* suspension containing KCl, promoted K uptake, while ammonia chloride was found to stimulate release of K (Tromballa, 1978). K movements in and out of *Chlorella* cells are balanced mainly by proton movements (Tromballa, 1978). Mg was needed by cells in synthesis of chlorophyll (Mandalam and Palsson, 1998), while S in cooperation with nitrogen is important for cellular division (Hase et al., 1958). Another author also observed that in the absence of S, cell division is blocked (Mandalam and Palsson, 1998). The addition of sulfite as S source at concentrations of < 1.0 mM (80 mg/L) increased the biomass of *C. vulgaris* (Soldatini et al., 1978).

Results by Walker (1953) confirmed the requirement for Fe, Mn, Zn, Cu and Ca; however he also notes that Mo is needed for the growth of *Chlorella*. Mo was required only in media containing nitrate as the only source of nitrogen, while when urea was used as nitrogen source or together with nitrate, there was no need for Mo (Walker J.B., 1953). Mo and Fe were required more for algal growth when source of nitrogen was NO_3^- instead of NH_4^+ (Raven 1990). It is believed that Mo is an important nutrient in the enzymatic reduction of nitrate (Walker J.B., 1953).

Chlorella is very sensitive to high levels of heavy metals (Wong et al., 1984). A 50% reduction in total *Chlorella* cell volume occurred in the presence of either 50 mg/L Mg, 0.070 mg/L Cu, or 0.700 mg/L Pb (Christensen et al., 1979). In another study

concentration of metal that decreased the cell division by 50% was 0.06 mg/L Cd, 0.18 mg/L Cu, 1.03 mg/L Mg, and 5.1 mg/L Zn (Rosko and Rachlin, 1977). The algae in media containing inhibitory concentration of nutrients exhibited pale green or yellow color during the experiment, and as suggested this result is due to the damage caused to the chlorophyll (Greenfield, 1942). It was found that copper is very toxic to *C. vulgaris*, for instance CuSO₄ was inhibitory even at low concentrations of 10⁻⁷ M (1.6*10⁻² mg/L) (Greenfield, 1942). Less toxic than copper are compounds such as NiSO₄, KCl, H₃BO₃, (NH₄)₂SO₄, and ZnSO₄. These group of substances inhibit photosynthesis at concentrations of around 10⁻¹ M, while MnSO₄, MgSO₄ and KNO₃ inhibit photosynthesis only at higher concentrations. In addition, high concentrations of metals can lead to prolonged lag phase (Rosko and Rachlin, 1977). Furthermore, metals did not have any negative effect on the uptake of NO₃⁻, NH₄⁺, and CO₂ or on enzymes nitrate reductase or urease (Mallick et al., 1990).

2.7.5 Effect of Organic Carbon on Growth

Chlorella is able to utilize both inorganic carbon sources such as CO₂, as well as organic carbon. As noted by Kessler (1972), different strains in genus *Chlorella* exhibit different growth on organic carbon sources.

It is interesting to note that some researchers (Ellner and Steers, 1955) hold the view that urea not only serves as nitrogen source for *C. pyrenoidosa*, but also as a carbon source, while others (Davis, 1951) disprove this view. *Chlorella*, unlike many other algae, is able to assimilate acetate as source of carbon (Martinez et al., 1987). Acetic acid

present in the medium in addition to CO₂ had promoting effect on the growth rate of *C. pyrenoidosa* with maximum growth rate reaching 20 ml/ (L day) (Lin L.P. and Chen, 1994). Post et al. (1994) reports that acetate supported growth of *C. vulgaris* both in light and in dark.

Martinez and Orus (1991) report that maximum growth rate of 3.16 day⁻¹ for *C. vulgaris* can only be obtained in presence of glucose, CO₂ and light. Mixotrophic mode resulted in a higher growth rate of *C. vulgaris* when compared to photoautotrophic and heterotrophic growth (Martinez and Orus, 1991). Martinez and co-workers (1987) shown that *Chlorella* grew best on glucose in mixotrophic conditions with growth rate of 2.30 day⁻¹, however it had also good growth on acetate and sucrose, and less growth on fructose and citrate.

2.8 Operating Conditions

To maximize algal productivities, cultures must be controlled with the light supply, temperature, pH, and nutrient profile, including gaseous substances (Carvalho and Malcata, 2001). In photobioreactors light and CO₂ are the key processing parameters for successful photosynthetic cell cultivation systems, and are most difficult to control (Carvalho and Malcata, 2001; Thomas et al., 1976). According to Flora and co-workers (1995), light and CO₂ are primary factors that can limit algal growth. The greatest scientific and technological challenge in research and development on cultivation of photosynthetic microorganisms stems from inadequate understanding of these important factors (Ogbonna and Tanaka, 2000). Light and CO₂ delivery to the cells is dictated by

mixing characteristics inside the reactor. In turn, mixing characteristics are dependent on reactor geometry and on operating conditions.

2.8.1 Light Supply to the Culture

2.8.1.1 Artificial Light Source and Light Transmission

Different lamps will generate distinct energy spectra (Carvalho et al., 2006), and this energy spectrum is very important, since only light of specific wavelengths is utilized for photosynthesis. The photosynthetically active radiation is limited to the visible light of the energy spectrum, corresponding to wavelengths in the range of 400 to 700 nm. Since chlorophyll *a* and *b*, as well as a limited number of carotenoid pigments absorb light, only certain areas of the white light spectrum can be trapped by these pigments and subsequently used for photosynthesis.

The chlorophyll absorption wavelengths correspond to the blue (475 nm) as well as the red (650 nm) spectral regions (Matthijs et al., 1996). Red light contains energy needed to reach the first excited state of chlorophylls *a* and *b*, while the blue light has more energy and can be elevated to higher excited state (Matthijs et al., 1996). Carotenoids usually absorb in the 480 to 560 nm range, the part of the energy spectrum where the chlorophylls have weak absorption (Larkum et al., 2003).

The light source most commonly used in small scale photobioreactors include light emitting diodes, fluorescent tubes, and metal halide lamp.

2.8.1.1.1 Light-Emitting Diodes

Light-emitting diodes (LEDs) are monochromatic light sources (Shotipruk et al., 1999). Their energy spectrum is composed of a narrow bandwidth of about 20 nm. Figure 2.5 depicts the energy spectrum of red LED, terrestrial solar spectrum and photosynthetic spectral response. LEDs offer many advantages such as high mechanical stability, low temperature sensitivity, long operating life and high electrical efficiency (Matthijs et al., 1996; Shotipruk et al., 1999). They do however emit low intensity light on individual basis.

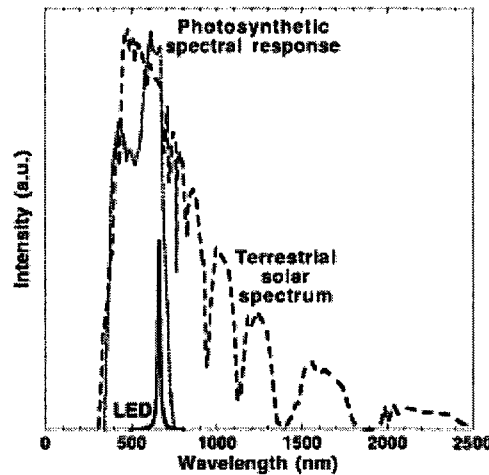


Figure 2.5: Spectral issues in photosynthesis: typical clear-sky terrestrial solar spectrum; photosynthetic spectral response (McCree 1972); and red LED emission.

(Gordon and Polle, 2007)

For that reason they are usually incorporated into arrays, as depicted in Figure 2.6, that can emit high intensity light (Lee C. and Palsson, 1996). LED structures with irradiance

of well above $2000 \mu\text{E}/(\text{m}^2 \text{ s})$ have been reported, which corresponds to the irradiance of sunlight (Tennessen et al., 1995). LEDs arrays can be placed inside or outside of the photobioreactor.

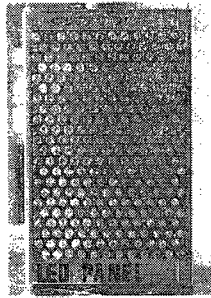


Figure 2.6: LED panel.
(Matthijis et al., 1996)

Lee and Palsson (1996) used red LEDs as the light source for the growth of *C. vulgaris*. When *C. vulgaris* is grown under red LEDs as the sole light source, its cell cycle is shortened so that only one round of replication takes place prior to the cell burst. When compared to full spectrum fluorescent light, the final biomass concentration under red LEDs was similar. It was also found that red LEDs supplemented with blue LEDs did not have improved growth performance when compared to cultivation solely under red LEDs. The authors' overall conclusion is that red LEDs could successfully replace conventional lighting for long-term algal mass cultures.

2.8.1.1.2 Fiber Optics

To obtain more uniform distribution of light than that with sole LEDs, light can be delivered internally through optical-fibers. Javanmardian and Palsson (1991) have used fiber-optic cables inside a cylindrical bioreactor arranged as concentric vertical cylinders. The light source was kept outside of the reactor, protecting the reactor from the lamp waste heat. The schematic of the photobioreactor with fiber optic cables is depicted in Figure 2.7.

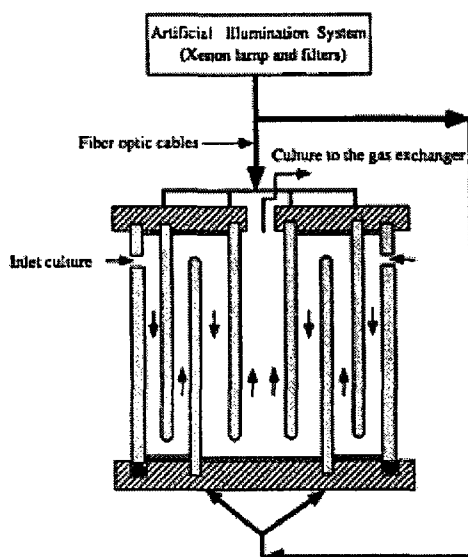


Figure 2.7: Details of prototype photobioreactor unit used in study.

(Javanmardian and Palsson, 1991)

Although the authors have used a xenon lamp source (efficiency of which is only 2 to 3%), they suggest that efficiency can be significantly improved by using LEDs. It is

stated in author's paper that LEDs can reach efficiencies of 80% in converting electrical energy to light at specific wavelengths.

The technical problem with fiber-optic illumination is that large numbers of optical fibers are required to achieve homogenous distribution inside the photobioreactor (Ogbonna and Tanaka, 2000). Mixing in such systems would be a problem, as well as cell sedimentation, adhesion on fiber surfaces, and inadequate lateral diffusivity of light (Ogbonna and Tanaka, 2000). An and Kim (2000) removed the clad material of an optical fiber by physical scratching or through chemical process in order to enhance the lateral diffusivity. They have found however, that physical scratching of optical fiber resulted in cell adhesion and consequently in lateral diffusivity of light. The chemical removal of clad material also presented a problem, whereby the naked fiber was fragile. To solve these problems scratched optical fibers were inserted into Pyrex-glass tubes, such that the optical fiber wasn't in contact with the culture.

2.8.1.1.3 Fluorescent Lights

Fluorescent lights have been utilized as internal and external light sources for small scale photobioreactors. Some more common configurations include fluorescent lamps arranged vertically around the reactor or a fluorescent lamp inside of glass tube fixed to the centre of the reactor, as can be seen in Figure 2.8.

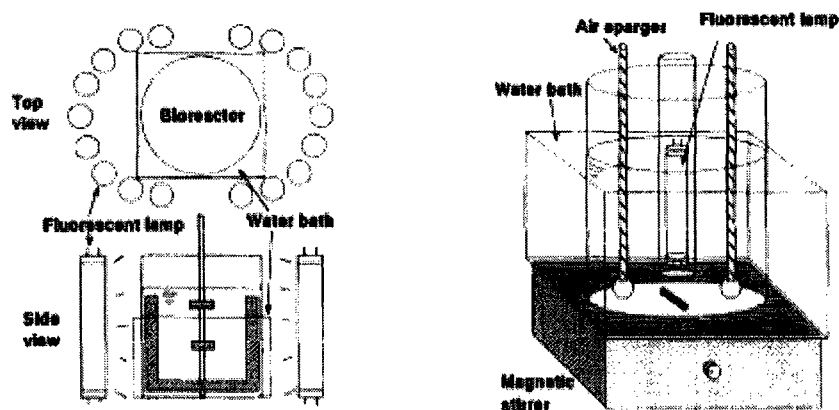


Figure 2.8: Schematic photobioreactors with fluorescent illumination.
(Ogbonna et al., 1995a)

Fluorescent lights usually have higher intensities around a particular wavelength and little intensity at the rest of the light spectrum. To produce light that is similar to sunlight these lights are usually mixed with other types of light.

2.8.1.1.4 Metal Halide

Metal halide lamps have also been used quite extensively to illuminate small scale photobioreactors. As seen in Figure 2.9, these lamps have similar emission spectrum to sunlight. These lamps are strong in the blue area of spectrum, and are well suited for seed-starting and vegetative growth. These lights are more efficient than fluorescent lights; however they are also more costly.

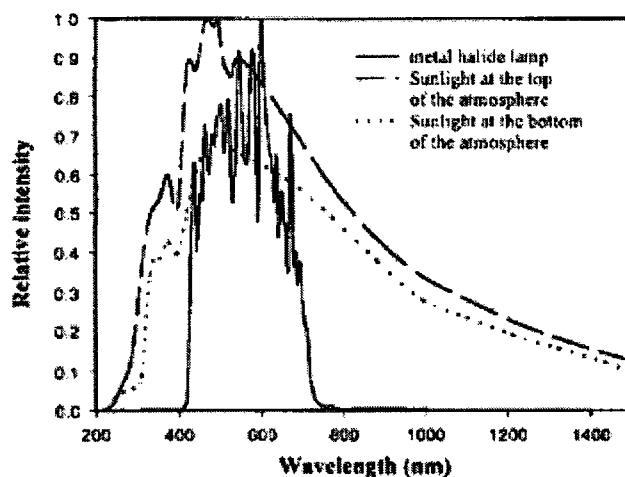


Figure 2.9: Energy spectrum for sunlight and a metal halide lamp.

(Lee K. and Kim, 1998)

It must be noted that it is difficult to compare the performance of the various light systems on biomass concentration presented in research journals, because of the variations in the reported type of cells and cultivation conditions (Ogbonna and Tanaka, 2000).

2.8.1.2 Effect of Light Irradiance on Biomass

2.8.1.2.1 Cell Growth

In a steady state continuous process, the cell concentration is constant and light distribution within the reactor can be assumed constant as well (Ogbonna et al., 1995a). In contrast, the process in batch reactors is unsteady and characterized by changing cell concentration and possibly varying light delivery to each cell. Organisms grown in batch

culture go through lag phase, exponential phase and stationary phase. However, batch growth in photobioreactors may also contain linear phase (Eriksen et al., 2007). Geoghegan (1953) reports that *C. vulgaris* has four distinct stages in the growth cycle: a lag phase, an exponential phase, an approximately linear phase and finally a stationary phase. Linearity may be an indication of limitation of some factor other than algal ability to reproduce (Powell et al., 2009). Limitations such as mass transfer of CO₂ from the air bubbles or light penetration into the media could be responsible for linear growth (Powell et al., 2009).

In the case of light penetration into the media in phototrophic cultures of algae where there is no mutual shading by cells the growth is exponential. As the cell density of algal cultures increases, so does self-shading caused by the sheer crowding of the cells. Shading within the culture limits the light delivery to each cell, resulting in growth that is not solely exponential (Eriksen et al., 2007). During high cell density batch cultivation of photosynthetic cells there are various distinct sequential growth phases (Ogbonna et al., 1995). As the light intensity per cell decreases, cells begin to cycle between illuminated and dark regions in the photobioreactor. As a result specific growth is decreased leading to a linear increase in biomass concentration when growth becomes light-limited (Eriksen et al., 2007). Ogbonna and co-workers (1995a) investigated the growth of *Chlorella* and *Spirulina* cells in various light-limited batch reactors. Their results, depicted in Figure 2.10, showed that the length of each of the growth phases depends on factors such as cell strain and light conditions inside the reactor, and that the linear growth phase lasted longer than the exponential phase in every investigated cultivation condition.

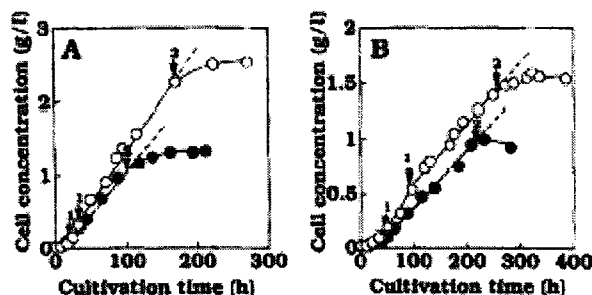


Figure 2.10: Typical growth curves for *Chlorella pyrenoidosa* C-212 (A) and *Spirulina platensis* M-135 (B) cells in cuboidal photobioreactors.

(Note: The Incident light intensities ($\text{mol}/(\text{m}^2 \text{ day})$) were 28.08 and 17.28 for *Chlorella* and *Spirulina*, Respectively, while the depths of the reactors (m) were $\circ$: 0.06 and $\bullet$: 0.08. Arrows 1 and 2 indicate the approximate ends of the exponential and linear growth phases, respectively.

(Ogbonna et al., 1995a)

Ogbonna did not find any correlation between specific growth rates and final cell concentration, but good correlations were found between the linear growth rates and final cell concentrations for both cells regardless of the type and size of the photobioreactor. Overall, Ogbonna concluded that the linear growth rate has a stronger influence on the final cell concentration than the specific growth rate obtained during exponential growth phase. Very recent studies conducted by Janssen and co-workers (2007) have also demonstrated that light-limited photoautotrophic growth in batch reactors is characterized by short exponential growth followed by prolonged linear growth. Similarly, in studies by Contreras and colleagues (1998) algal growth curve was characterized by lag phase, exponential phase, linear phase, and finally stationary phase.

2.8.1.2.2 Light Saturation and Photoinhibition

Due to the light attenuation inside of photobioreactor, light energy limitation is one of the most common problems encountered in practical cultures of photosynthetic cells (Ogbonna et al., 1995b). Therefore efficient supply of light to a photobioreactor is of paramount importance in obtaining high cell productivities.

In optically thin cultures of algae the specific growth rate increases with an increase in light photon flux (Richmond, 2000). Light saturation curve is depicted in Figure 2.11.

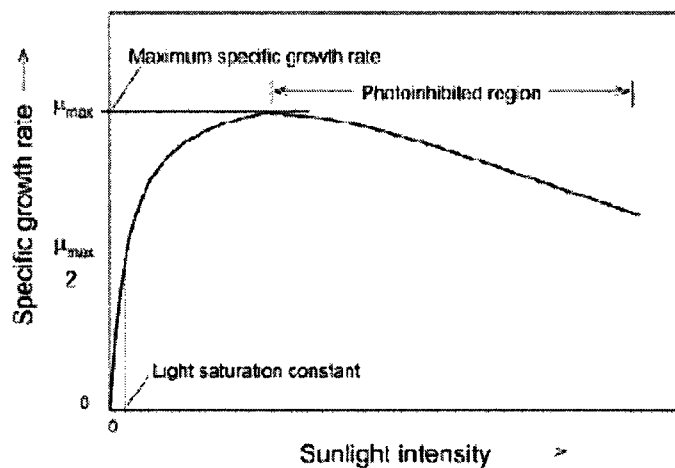


Figure 2.11: Effect of light intensity on specific growth rate of microalgae.
(Chisti, 2007)

As seen in Figure 2.11, light saturation constant is reached when specific growth rate is half of its maximal value. Light saturation constants for microalgae can be around $185\text{-}200 \mu\text{E}/(\text{m}^2 \text{ s})$ (Chisti, 2007). These levels are much smaller than the maximum sunlight levels during midday in equatorial regions which amount to $2000 \mu\text{E}/(\text{m}^2 \text{ s})$.

Once the photosynthetic rate reaches a maximum and does not change with an increase in light intensity, the photosynthetic apparatus becomes fully light saturated. Further increases in light intensity much above the saturation leads to photoinhibition of cells, whereby the photosynthetic apparatus might deteriorate.

From the literature it can be seen that cultures of *C. vulgaris* in photobioreactors are usually irradiated with about 50-300 $\mu\text{E}/(\text{m}^2 \text{ s})$ of light (Chiu et al., 2008; Jeong et al., 2003; Scragg, 2006; Yun et al., 1996). Some literature sources report the effect of high light irradiance of 1000-1500 $\mu\text{E}/(\text{m}^2 \text{ s})$ on the growth of cultures of *C. vulgaris* (De-Bashan et al., 2008; Degen et al., 2001).

In relatively dilute algal cultures, light intensity is the major or only factor that affects photosynthetic rate. However, as the density increases other process factors become important in determining culture productivity (Richmond, 2000).

2.8.2 Agitation and Sparging into the Culture

The degree of agitation inside the reactor determines the cell movement from light to dark regions. While, mass transfer of CO_2 from bubbles to culture medium is facilitated by air bubbling from a sparger, and by the agitation of the culture medium (Rocha et al., 2003). In addition, the bubbling also promotes agitation of the culture. According to Contreras and co-workers (1998) it is required to create intense liquid-mixing and high interfacial turbulence at gas-liquid surface in photobioreactors, especially for systems where CO_2 is the carbon source.

Insufficient mixing may result in high oxygen buildup, and in the case of laminar flow, cell precipitation and growth can take place on the vessel wall (Babcock et al., 2002; Richmond, 2004). Oxygen concentrations above air saturation can result in the inhibition of microalgal photosynthetic apparatus (Carvalho et al., 2006). In addition, an efficient mass transfer of CO₂ is needed as undissolved CO₂ may be lost by outgassing, or CO₂ gradients might be created (Babcock et al., 2002; Contreras et al., 1998). In such case not enough carbon might be available to the cells.

CO₂ must travel from gas phase to liquid phase in order for inorganic carbon to be available to the cells. The efficient CO₂ mass transfer rate could be achieved by considering the air flow rate, and air/water interfacial area that can vary according to the type of the sparger (Rocha et al., 2003). Eriksen et al. (1998) reported on photobioreactor with dual-sparging to obtain good CO₂ mass transfer rates. While Carvalho and Malcata (2001), state that microporous membrane might be more effective in terms of mass transfer than plain bubbling. Plain bubbling through the sparger has drawbacks in that the holes of the sparger might get fouled, and also most CO₂ will get lost to the atmosphere.

Increasing the mixing rates up to certain level might enhance microalgal growth due to enhancement of heat and mass transfer (Contreras et al., 1998). In addition, optimal conditions of light exposure require rapid liquid velocity (Gudin and Chaumont, 1991). However, excessively high mixing rates may result in limited biomass productivity due to cell damage. Microalgae have been noted to be sensitive to shear stress due to their size and in some cases absence of cell walls (Contreras et al., 1998; Gudin and Chaumont, 1991). Microalgal cell damage might be due to pure hydrodynamic forces or due to interactions of cells with bubbles (Contreras et al., 1998). The cell

interaction may occur with bubble generation at the sparger, with rising bubbles, with bubbles coalescing and breaking up in the region of bubble rise, and with bubbles at air-medium interface (Contreras et al., 1998). Gudin and Chaumont (1991) go as far as saying that cell sensitivity to force might be a primary growth-limiting factor. However data from one literature source shows that *C. vulgaris* species is very immune to high hydrodynamic stress (Bronnenmeier and Markl, 1982). Even with agitation speeds of 3000 rpm the growth of *C. vulgaris* in fermentor was unaltered.

Popular agitation speed used in photobioreactors during cultivation of *C. vulgaris* is 300-400 rpm, while a common flux of air/CO₂ gas mixture is 1 to 2 L/min (Patino et al., 2007).

2.8.3 Carbon Dioxide Supply to the Culture

A sufficient amount of CO₂ must be delivered to algal cells to avoid carbon limitation. *C. vulgaris* grows very slowly in presence of air without CO₂ enrichment (Jeong et al., 2003). To increase the amount of carbon delivered to the cells pure air is usually supplemented with pure CO₂ gas. Pure CO₂ is not directly fed to the culture as too much of CO₂ can be detrimental to the cell. Furthermore, CO₂ represents a major operational expense (Rubio et al., 1999).

Variable results were obtained from literature sources regarding the required percentage of CO₂ in air to obtain good biomass growth. One literature source reports that the maximum biomass concentration was achieved in presence of 2% CO₂ (Chiu et al., 2008). Results of Yun and co-workers (1996) indicate that the greatest dry weight of

C. vulgaris was achieved at 6% CO₂ in air. Yet another investigation revealed a better growth in presence of 5% CO₂ rather than 15% CO₂ where growth was somewhat inhibited (Vinayakumar and Kessler, 1975). The inhibition of algal growth at elevated CO₂ levels has been widely reported in the literature. For instance, work by Chiu and colleagues (2008) shows significant inhibition of *C. vulgaris* growth at levels of 10% CO₂ in air and above. According to Yun and his co-workers (1996), inhibition of algal growth by high CO₂ concentrations is not well understood. Meanwhile, Powell and co-workers (2009) report that elevated levels of CO₂ in air inhibit metabolic reactions associated with Krebs cycle.

Some investigators report that there was no inhibition at elevated concentrations of CO₂. For instance findings by Geoghegan (1953) reveal that the growth rates of *C. vulgaris* were identical whether the alga was grown in 5% CO₂ or high CO₂ concentrations such as 20% CO₂ in air. Furthermore, work by Morais and Costa (2007) shows that *C. vulgaris* grown in vertical tubular photobioreactor actually grows better in 12% CO₂ than in 6% CO₂ in air.

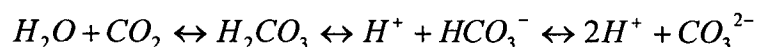
In order for algae to utilize CO₂ from power plants, it needs to withstand concentrations of about 15 to 20% CO₂ in air (Hanagata et al., 1992). Most authors found that at these CO₂ concentrations the growth of *C. vulgaris* is completely or partially inhibited (Chiu et al., 2008). However it has been shown that the inhibition can be alleviated by adaptation to CO₂. The inoculum of *C. vulgaris* adapted to 5% CO₂ resulted in enhanced algal growth in 15% O₂ compared with culture grown by supplying air (Yun et al., 1996). Also, *C. vulgaris* can be adapted to high CO₂ concentration by gradual increases of CO₂ concentration (Yun et al., 1996). Some microalgae are naturally tolerant

to high concentrations of CO₂. According to Papazi and co-workers (2008) *Chlorella minutissima* is extremely tolerant microalgae to high concentrations of CO₂ in air up to 40% under natural temperature and light conditions.

2.8.4 pH and Alkalinity

During algal cultivation the concentration of inorganic and organic species changes in the medium. Changing concentrations of these species occur in the medium due to influx of nutrients from external source and due to uptake or release of nutrients between the cells and the surrounding medium. Changes in concentration of species such as H⁺, and weak electrolytes such as CO₂/HCO₃⁻ and NH₄⁺/NH₃ results in changes of pH and alkalinity of the medium (Granum and Mykkestad, 2002).

The natural buffering system in water occurs between H⁺ and CO₂ species, and the chemical equilibrium between these species is shown in the following equation (Granum and Mykkestad, 2002):



During algal photosynthesis CO₂ is utilized, and the above equation shifts to the left resulting in increase in medium pH. To avoid pH increase that can occur during photosynthesis, CO₂ or HCl can be supplied to the medium.

C. vulgaris was noted in a few literature sources to have relatively broad pH optimum from 6.5 to 8.0 (Khomova et al., 1986; Rachlin and Grosso, 1991). However,

other literature sources report that optimum pH of *Chlorella* is about 6.5 (Mayo, 1997). Furthermore, according to Kessler (1976), *C. vulgaris* can tolerate acidities at around pH 4, and the work by Mayo (1997), reveals that *C. vulgaris* can grow at acidic pH values as low as 3.0. However, *C. vulgaris* is extremely sensitive to high alkaline pH environment (Goldman et al., 1982; Kessler, 1976). Perhaps alkaline conditions outside of the cell cause damage to cytoplasm, exposing the cell internal components to alkaline species.

It has been also noted in the literature that rise in pH triggers phosphorus precipitation and ammonia stripping (Gonzalez et al., 2008). By buffering the media, the pH might change to a lesser degree; however buffering is not a practical option for pH control in large cultivation system (Vinayakumar and Kessler, 1975).

It has been noted from literature that *C. vulgaris* is usually cultivated in photobioreactor at pH ranging from 6.5 to 7.5 (Degen et al., 2001; Patino et al., 2007).

2.8.5 Temperature of the Culture

Temperature controls the rates of chemical reactions in algae hence it is a very important parameter that effects algal growth (Sandnes et al., 2005). However it must be pointed out that in photoautotrophic system light absorption, excitation energy transfer, and photochemistry are independent of temperature (Raven and Geider, 1988). For information on temperature effects on chemical transformations and transport processes involving algae one can consult review paper by Raven and Geider (1988).

According to Chisti (2008), the optimum temperature for most microalgae, including *Chlorella*, is between 20 to 30°C. In studies conducted by Geoghegan (1953)

the optimum temperature for growth of *C. vulgaris* was around 25°C and growth was poor when temperature was either 20°C or 30°C. On the contrary Mayo (1997) found that the optimum temperature for *C. vulgaris* in his study is around 32°C.

Algal cells exposed to 20 to 30°C above their normal growth temperatures lead to inhibition of CO₂ fixation and O₂ evolution (Kanno and Uyama, 2005). For instance, a complete loss of O₂ evolution ability for *C. vulgaris* occurred at temperature of 49°C. While *C. vulgaris* is sensitive to high temperature, it is quite resistant to cold and dark conditions and to some extent even to freezing (Bartosh and Banks, 2007).

It has been noted in the literature that it is difficult to optimize the algal growth in photobioreactor because of various interactions that are taking place between operating parameters. In his work, Ugwu and co-workers (2007) stress the importance of studying interaction between various parameters in optimization of algal growth. Interaction of light intensity and temperature was noted in various literature sources (Miller et al., 1964; Sandnes et al., 2005). In algal cells exposed to low light irradiation, the photosynthesis is proportional to intensity, and not temperature. However at high light irradiation, photosynthesis was found to be highly dependent on temperature (Miller et al., 1964).

2.8.6 Yield of Dry Matter

The growth of *C. vulgaris* in photobioreactors is highly dependent on reactor geometry and operating conditions. The maximum biomass concentrations reported in the literature vary widely as *C. vulgaris* was cultivated under various conditions. For this reason it is difficult to directly compare the maximum biomass concentrations.

Nevertheless, we provide some maximum biomass concentrations that were reported in the literature.

Morais and Costa (2007) cultivated *C. vulgaris* in 2 L Erlenmeyer flask and in vertical tubular photobioreactors with volume of 2 L and 4 L. During a 20 day cultivation period the maximum biomass concentration was about 0.5 to 1.0 g/L. The experiments conducted with *C. vulgaris* grown in tall tubes show that the maximum concentration was 1.9 g/L in 6 days (Geoghegan, 1953). Results of Yun and co-workers (1996) indicate that the maximal dry weight of *C. vulgaris* was around 3 g/L (OD_{750nm} of 3.4) during a 9 day cultivation period. *C. vulgaris* grown in flat panel airlift reactor attained biomass yield of ~4.0 g/L (OD_{625nm} of 27) in 6 days, while when grown in 3L-bubble column for 6 days the maximum biomass was ~3.5 g/L (OD_{625nm} of 23) (Degen et al., 2001).

Most of the literature sources reported maximal biomass concentrations of up to about 5 g/L during the cultivation period (Degen et al., 2001; Geoghegan, 1953; Morais and Costa, 2007; Yun et al., 1996). However some sources reported much higher biomass concentrations. Patino and co-workers (2007) report on biomass yield of *C. vulgaris* grown in a 2 L-cylindrical vessel for number of different experimental conditions. They report that the highest biomass of 9.5 to 9.9 g/L was achieved during 9 day fed-batch autotrophic cultivation.

2.8.7 Lipid Cell Content

There are a couple of different methods for isolation of lipids, as there are different extractants (Khasanova et al., 1978). In the literature it was noted that different lipid isolation techniques give different results. According to Geoghegan (1953), the amount of oil extracted by ether alone from freeze-dried *C. vulgaris* after 6th day of cultivation in photobioreactor was between 1 and 5% (dry wt.), while ether extraction of the same sample after dilute acid hydrolysis or methanol extraction, produced 20 to 25% (dry wt.) of oil. In work by Khasanova and co-workers (1978), more lipids (14% dry wt.) were extracted with chloroform-methanol from fresh cells of *Chlorella* than with diethyl ether from air-dried cells (7.6% dry wt.) after 12 days of cultivation. The authors point out that diethyl ether extracts only weakly bound lipids, while mixture of chloroform-methanol extracts strongly bound lipids. Work by Widjaja and co-workers (2009), examines lipid productivity from *C. vulgaris* under various factors such as CO₂ concentration, nitrogen depletion, harvesting time, and method of extraction.

Chapter 3 Experimental

3.1 Microalgal Strain

The green algae used in the experiments was *C. vulgaris* #8 which was provided by the research group of Dr. Christopher Lan of the Department of Chemical and Biological Engineering at Ottawa University, Ottawa, Canada.

3.2 Culture Medium

Preparation of Tris-Acetate-Phosphate culture medium (Tap) has been described in the literature (“TAP (Tris-Acetate Phosphate)”, 2005). This culture medium is popular for growth of *Chlamydomonas reinhardtii*, but we have used it for growth of *C. vulgaris*. In this report we refer to this medium as 1X Tap culture medium. In experiments conducted in the photobioreactor we have used a modified version of this culture medium denoted as 2X Tap. This culture medium was prepared by doubling the concentrations of all the compounds as appear in the Tap culture medium recipe (“TAP (Tris-Acetate Phosphate)”, 2005).

The 2X Tap culture media contained the following: Tris base (CH_2OH)₃CNH₂, 4840 mg/L; Glacial acetic acid (CH_3COOH), 2080 mg/L; K_2HPO_4 , 216 mg/L; KH_2PO_4 , 112 mg/L; NH_4Cl , 800 mg/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg/L; CaCl_2 , 76 mg/L; Na_2EDTA , 100 mg/L; BO_3H_3 , 22.8 mg/L; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 44 mg/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 10.1 mg/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 3.2 mg/L; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 3.1 mg/L; and

$\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6 \cdot 4\text{H}_2\text{O}$, 2.2 mg/L. It is important to point out that this medium contained about 2080 mg/L of glacial acetic acid, which serves as the sole organic carbon source.

We have performed experiments both in the 3000 ml photobioreactor and in the 500 ml flasks. All experiments conducted in photobioreactor contained the 2X Tap culture medium. However, for most experiments taking place in the 500 ml flasks a modified 2X Tap culture medium was used. In one set of experiments in the 500 ml flasks, *C. vulgaris* was exposed to various initial concentrations of nitrogen to determine if any of these concentrations were inhibitory to the culture. In this set of experiments modified 2X Tap culture medium was used where the initial concentration of NH_4Cl in this medium was varied accordingly in order to obtain initial nitrogen concentrations of 300 mg/L, 500 mg/L or 1000 mg/L. Only one experiment contained unmodified 2X Tap culture medium where the initial nitrogen concentration is 211 mg/L corresponding to initial NH_4Cl concentration of 800 mg/L.

Experiments testing the effect of different initial concentrations of phosphorus on the growth of *C. vulgaris* also involved the use of modified 2X Tap culture medium. However the initial concentration of K_2HPO_4 in the medium was varied accordingly in order to obtain total phosphorus concentrations of 100 mg P/L, 150 mg P/L, or 300 mg P/L. Note that the initial phosphorus concentration of 64 mg P/L was obtained with unmodified 2X Tap culture medium containing initial K_2HPO_4 concentration of 216 mg/L. The initial concentration of KH_2PO_4 , which was another source of phosphorus in the 2X Tap culture medium, remained unchanged in all of the experiments.

During the fed-batch experiments, K_2HPO_4 and NH_4OH were delivered to the photobioreactor containing 2X Tap culture medium and *C. vulgaris* biomass.

3.3 Inoculum

C. vulgaris was stored on an agar plate supplemented with 1X Tap culture medium at room temperature of $\sim 21^{\circ}\text{C}$. About every month and a half, *C. vulgaris* would be transferred from the old to new agar plate supplemented with 1X Tap culture medium ("TAP (Tris-Acetate Phosphate)", 2005) in order to avoid nutrient limitation.

The inoculum was prepared by first transferring couple of smears of *C. vulgaris* from the agar plates and into a 50 ml Erlenmeyer flask containing 30 ml 1X Tap culture medium. Subsequently, the Erlenmeyer flask containing culture medium and *C. vulgaris* was placed in a light box for duration of three to five days. The conditions in the light box were always maintained at temperature of around 29°C and light irradiance from fluorescent bulbs of about $200 \mu\text{E}/(\text{m}^2 \text{ s})$. The growth of *C. vulgaris* in a 50 ml Erlenmeyer flask constituted the initial biomass build up phase.

Experiments were either performed in 3000 ml photobioreactor or in 500 ml flask. For experiments in the flask, 20 ml of inoculum culture was transferred from the 50 ml Erlenmeyer flask and into a 500 ml flask containing 350 ml of 2X Tap culture medium.

An additional biomass buildup was necessary after initial biomass build up phase for the experiments that were to be conducted in 3000 ml photobioreactor. All of the culture medium with the algae from the initial biomass build up phase was transferred into a 500 ml flask containing about 200 ml of 2X Tap culture medium, and placed into a light box. In addition, 5% CO_2 in air was delivered into the 500 ml flask containing 2X Tap culture medium and *C. vulgaris*. Stir bar inside the flask allowed for the mixing of the biomass. *C. vulgaris* was allowed to grow for approximately two to four days before

inoculation of 150 ml of culture medium with *C. vulgaris* into a 3000 mL photobioreactor containing about 1550 ml of 2X Tap culture media.

3.4 Experimental Setup

In many of our experiments we have used NBS BioFlo 110 3000 mL bioreactor (“Guide to operations”, 2005) for the cultivation of *C. vulgaris*. The schematic of the bioreactor is presented in Figure 3.1.

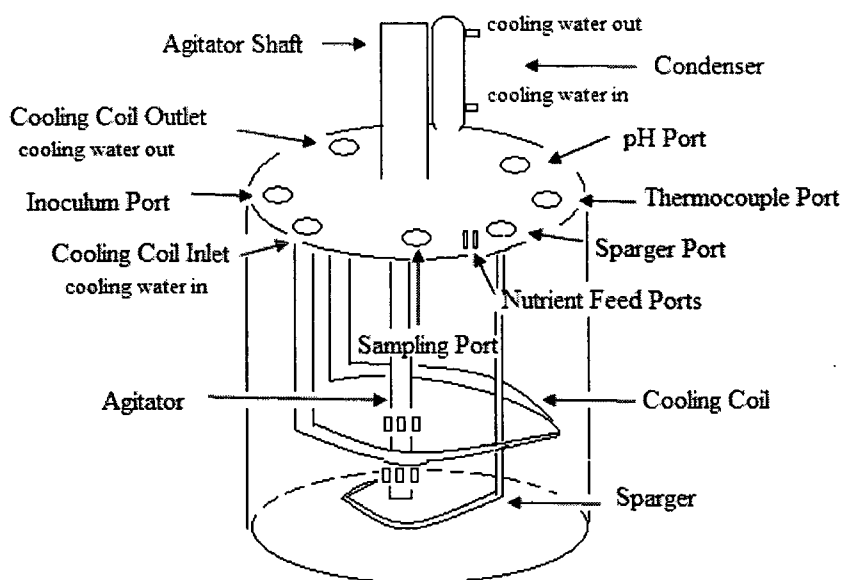


Figure 3.1: Schematic of NBS BioFlo 110 3000 ml bioreactor used for cultivation of *Chlorella vulgaris*.

The bioreactor was a glass vessel with a 3000 mL volume. The area to working volume ratio of this bioreactor was 0.29 cm^{-1} . The bioreactor was composed of an

agitator, thermocouple, cooling coil, condenser, sparger, pH probe, nutrient feed ports, inoculum port and the sampling port. The various ports were located on stainless-steel plate that was fixed on top of the vessel.

The agitation of the culture liquid can create homogeneity in composition and temperature. It was performed with a dual stainless-steel Rushton agitation impellers immersed in the culture liquid. The mechanical motor of the agitator was positioned outside of bioreactor. The agitator motor was connected to the power controller where the desired agitation speed was set on the Primary Control Unit (PCU) using a touch screen. The schematic of the PCU and various control modules used in our experiments are depicted in Figure 3.2.

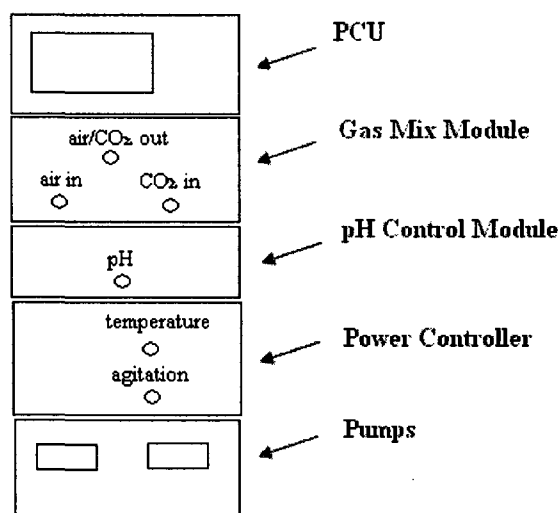


Figure 3.2: Schematic of the primary control unit along with control modules used with NBS BioFlo 110 3000 ml bioreactor.

The temperature of the culture liquid inside the bioreactor was measured by the thermocouple positioned in the housing. The exterior of the housing was directly in contact with the liquid in the bioreactor. Thermocouple was connected to the power controller and the temperature set-point was entered on the PCU. The temperature was controlled by the flow of cold water through the cooling coil immersed in the broth in the bioreactor. As the liquid temperature approached the set-point temperature, the cold-water was diverted from the cooling coil and into the condenser. The condenser was attached to the top of the vessel and allowed for a reduction in water vapor lost due to exhaust.

The algae require that CO₂ gas be present as an inorganic carbon source. Pure CO₂ gas was not directly delivered into the culture liquid as high levels of CO₂ can be detrimental to the cells (Chiu et al., 2008). In addition, pure CO₂ can be quite costly (Doucha et al., 2005). Even delivering pure CO₂ gas at a very small flowrate is unfeasible as too much CO₂ would enter the vessel at a given time. In order to deliver small amounts of CO₂, highly purified CO₂ gas was diluted with the air. The mixing took place in the gas mix module from which a gas stream containing CO₂ and air mixture was delivered into the rotameter (Key Instruments). The mixture out of rotameter was directed towards the sparger port located on top of the vessel. Before entering the sparger port, the gas mixture was first saturated with water by passing the gas through a water bottle, followed by the flow of the hydrated gas mixture through a microfilter. Hydrating of the gas was performed in order to minimize water evaporation from the culture liquid. Hydrating the CO₂ and air mixture was only performed in batch mode experiments. The CO₂ and air mixture was not hydrated during fed-batch experiments. Once the mixture passed through

the filter it was ready to enter the flask that was equipped with a sparger. The sparger was composed of a stainless-steel tube, extending from the port down into the culture liquid. The bottom of the tube contained a horizontal stainless-steel ring perforated with holes from which the CO₂ and air mixture was bubbled upwards directly into the culture liquid. The desired percentage of CO₂ in air could be entered using the PCU touch screen.

A pH probe (Metler Toledo) was inserted to the pH port of the vessel where it extended down into the culture medium. The top part of the probe was connected to the pH control module. The pH in our experiments was not controlled; however the pH of the culture liquid could be monitored on PCU screen.

The nutrients, K₂HPO₄ and NH₄OH, were delivered into the culture liquid through separate streams during the fed-batch operation. These nutrients were pumped from the stock bottles, through the nutrient ports directly into the vessel. The rate of nutrient addition could be controlled by specifying the desired fraction of pumps maximum capacity on PCU screen.

The stainless-steel plate on top of the reactor vessel contained inoculum port with tube extending down into the culture medium. The *C. vulgaris* inoculum was added to the reactor through this tube. The samples were collected by creating a negative pressure in the sampling tubes by the movement of syringe piston.

The bioreactor was surrounded by lights that provided energy for growth of photosynthetic *C. vulgaris* cells. The lights were installed on a adapted part to the NBS BioFlo 110 system. The NBS BioFlo 110 bioreactor with lights was termed as the photobioreactor.

The light source consisted of fourteen 15-W GE Plant & Aquarium linear fluorescent light. The lights were positioned vertically all the way around the reactor and fixed in place on a plastic wall. The schematic of the bioreactor and light system setup is shown in Figure 3.3. These lights could all be 'on' at one time, but also if desired every second light could be 'on' giving different light irradiance.

The radiation spectrum for GE Plant & Aquarium fluorescent lights closely matched absorption spectrum of plants, with high emissions of blue and red light. These lights are very efficient and do not contain much emissions in the infrared and ultraviolet regions of the spectrum.

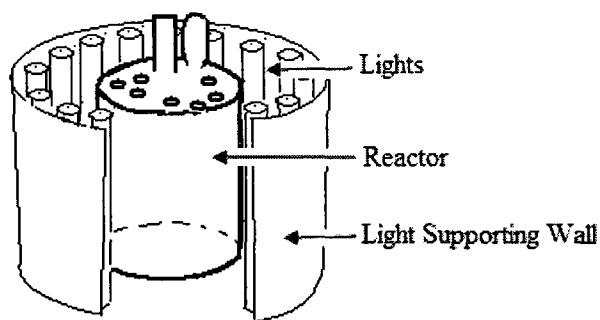


Figure 3.3: Light setup around the NBS BioFlo 110 3000 ml bioreactor.

To make sure that the only light reaching the surface of the vessel was from the GE Plant & Aquarium fluorescent bulbs, the top of the reactor and any gaps on the side of the plastic wall surrounding the reactor were covered in aluminum foil.

While many experiments were performed using the NBS BioFlo 110 system supplemented with lights, some experiments were conducted in 500 ml flasks. The

experiments that involved testing the effect of different initial nutrient concentrations on the growth of *C. vulgaris* were performed in 500 ml flasks inside of the light box. In the light box, up to four flasks could be placed at one time and hence four experiments could be performed in the span of 9 days, unlike the experiments with the photobioreactor where only one experiment could be accomplished during that time. In addition, the set of experiments involving different initial nutrient concentration did not test the effect of various process parameters; hence precise control of these parameters was not critical. In the 3000 ml vessel the control of process parameters was quite precise, unlike the control in the 500 ml flasks; hence it was not absolutely necessary to run these experiments in the 3000 ml vessel.

The area to working volume ratio of the 500 ml flasks was 0.44 cm^{-1} . The flasks were located inside a box with one side of the box being completely exposed to the surroundings. Inside of the box four fluorescent daylight Philips 27 W lights were positioned around the flask.

The experiments conducted in the 500 mL flasks occurred without the control of temperature, agitation speed or pH. The agitation of the culture liquid was accomplished by the combined bubbling of CO_2 and air mixture from the sparger, and by the movement of the magnetic stirrer. In addition, the control of percentage of air delivered to the culture liquid was not very precise. It was determined by the relative volumetric flow of pure air, pure CO_2 , and the combined stream. The combined stream comprised of air and CO_2 gas mixture. Before entering into the flask, the air and CO_2 mixture was first saturated with water by passing the gas through a bottle containing water, and subsequently the hydrated gas mixture was passed through a microfilter. The mixture

entered the flask equipped with a sparger, and subsequently it bubbled into the culture liquid.

3.5 Cultivation Conditions

At the beginning of the experiment, 1550 ml of 2X Tap growth medium was delivered into the 3000 ml reactor. Subsequently, the pH probe was calibrated and inserted into the reactor. The reactor, growth medium, and pH probe were autoclaved for 20 minutes at 121°C. Following the autoclaving, the medium was cooled to the desired set-point temperature of either at 25°C, 28°C or 29°C, by running cooling water through the cooling coils of the reactor. Next, the set-point of the agitator was set either at 300 rpm or 600 rpm, as well as the percentage of CO₂ in air was chosen either at 4%, 5%, or 7%. Before the inoculation of pre-cultured cells the culture medium was agitated and supplied with the CO₂ and air mixture.

About 150 ml of pre-cultured cell suspension was inoculated into the reactor. After the inoculation of cells, the initial optical density in the reactor was around 0.2 to 0.6. The working volume in the reactor was around 1700 ml.

The illumination was provided continuously throughout the duration of the experiment. When all fourteen lights were turned on the average light irradiance at the outer surface of the vessel was around 900 $\mu\text{E}/(\text{m}^2 \text{ s})$, while when every second light was turned on the average light irradiance at the outer surface of the vessel was around 450 $\mu\text{E}/(\text{m}^2 \text{ s})$. Light irradiance was measured with Biospherical Instruments' Laboratory Quantum Scalar Irradiance Meter (QSL-2101). Each experiment was conducted for 8 or 9

days and each day during the cultivation total of around 20 ml of the culture liquid was removed for analytical analysis.

For experiments conducted in fed-batch mode, after 5 hours into the cultivation K_2HPO_4 and NH_4OH stock solutions were fed into the reactor. The concentrations of K_2HPO_4 and NH_4OH in the stock solutions were 9300 mg/L and 6630 mg/L, respectively. The nutrients were fed to the reactor at the rate of 3.1 ml/min. At around 75 hours into the cultivation, the feeding of nutrients was stopped, and experiment continued in a batch mode.

The different experiments conducted in the 3000 ml vessel differed mainly in that they were conducted at various combinations of the following process variables; light irradiation upon surface of the vessel, percentage of CO_2 in air sparged into the culture liquid, temperature in the culture liquid, and agitation speed of the culture liquid.

The first experiment was performed in order to obtain the growth curve of the *C. vulgaris* and to determine which type of nutrients, if any, are depleted during the course of the 8 day long experiment. The experiment was run in batch mode and was conducted at light irradiation of about $900 \mu E / (m^2 s)$, 5% CO_2 in air, temperature of $28^\circ C$, and agitation of 600 rpm.

Other experiments conducted in the 3000 ml vessel are the fractional factorial experiments. Each of the eight fractional factorial experiments lasted 9 days. Part of the experiment was conducted in fed-batch mode, while another part took place in batch-mode. The combination of process variables for the eight fractional factorial experiments is shown in Table 3.1. In this table the high light irradiance refers to $900 \mu E / (m^2 s)$, while low light irradiance is irradiance of $450 \mu E / (m^2 s)$.

Table 3.1: Combination of process parameters for fractional factorial experiments conducted in 3000 ml bioreactor.

Experiment #	CO ₂ in air (%)	Agitation Speed (rpm)	T (°C)	Light Irradiance
1	4	300	25	high
2	7	600	25	high
3	7	300	29	high
4	4	600	29	high
5	7	300	25	low
6	4	600	25	low
7	4	300	29	low
8	7	600	29	low

Experiments testing the effect of nitrogen and phosphorus concentrations in the medium on the growth of *C. vulgaris* were performed in a 500 ml flask with modified 2X Tap culture medium. In addition, one of the experiments was performed in unmodified 2X Tap culture medium.

Prior to the experiments the culture media and the flasks were sterilized in autoclave for 20 minutes at temperature of 121°C. The experiments were conducted in two sets. In the first set of experiments, three flasks were inoculated with 350 ml of modified 2X Tap culture medium containing initially different concentrations of nitrogen ranging from 300 to 1000 mg/L, while a fourth flasks contained unmodified 2X Tap medium containing 211 mg/L of nitrogen. The four flasks were placed in the light box where temperature was around 29°C and light irradiance of about 200 $\mu\text{E}/(\text{m}^2 \text{ s})$. Approximately 5% CO₂ in air was sparged into the flasks, while the magnetic stirrer allowed for mixing of the medium. Subsequently about 20 ml of pre-cultured cell suspension was delivered into each flask. All experiments were conducted in batch mode

for a period of 9 days. Each day a total of 5 ml of the culture liquid was withdrawn from the vessel for analytical analysis. The second set of experiments was conducted under similar culture conditions as the first set of experiments. The only difference was that instead of 2X Tap medium being modified for various initial nitrogen concentrations, in the second set of experiments 2X Tap medium was modified for desired initial phosphorus concentrations. The three flasks contained different initial phosphorus concentrations ranging from 100 to 300 mg P/L.

3.6 Analytical Methods

3.6.1 Biomass Concentration

Off-line biomass concentration was determined by optical density measurement. In addition, for some of the experiments off-line biomass concentrations was obtained by dry weight determination. For optical density, Thermo Electrons' spectrophotometer (GENESYS 10 uv) was used. Three milliliter disposable cuvettes were used, and double distilled water was used as a blank and to dilute the samples. The optical density of the samples was measured at a wavelength of 620 nm. An optical density reading of 0.1 to 0.7 on the spectrophotometer was always ensured by appropriate dilution of the sample.

For the dry weight determination the 16 ml samples of the algal suspension were collected on the daily basis during the duration of the experiment. Each sample was first centrifuged (10,000 rpm, 20 min), washed and centrifuged the second time. The concentrated algal broth was poured into cups and placed into the oven at temperature of

about 80°C for a period of about 24 hours. Afterwards, the cups with algae were weighted periodically until no change in weight was observed. A linear correlation between cell concentration and optical density (OD) at wavelength of 620 nm has been obtained for *C. vulgaris*:

$$\text{Concentration}(g / L) = 0.473 * OD_{620nm} \quad \text{eq. 1}$$

The standard curve correlating cell concentration and optical density is shown in Appendix Section 8.1, Figure 8.1.

3.6.2 Nutrient Concentration

The concentration of acetic acid, and phosphate in the culture medium could be followed off-line during the experiment by high performance liquid chromatography (HPLC). The measurement was performed using automatic system (Agilent Technologies, series 1200) loaded with SH1011 column (8mmID×300mmL, Shodex). The eluent phase comprised of 0.01 N H₂SO₄ aqueous solution with flow rate of 1.0 ml/min. The retention times of the acetic acid and the phosphate were 16.1 and 9.4 minutes, respectively. On the chromatogram, the areas of the peaks were related to the concentration of the compounds by comparison with standard curve that relates concentrations of standard solutions to the area under the peaks. One other metabolite was detected with retention time of 7.4 minutes; however this metabolite has not been identified.

The concentration of ammonium in the samples was determined off-line by an ammonium probe (Fisher Accumet Model 750). The concentration of ammonium in the samples was obtained from the millivolt reading from ammonium probe by comparison with standard solutions previously prepared.

3.6.3 Metal Concentration

The concentration of metal elements in the samples of culture medium were determined by inductively coupled plasma (ICP) spectrometry. The elements analyzed in the 2X Tap medium by ICP technique included: boron, calcium, cobalt, copper, iron, potassium, magnesium, manganese, molybdenum, sodium and zinc. The samples were analyzed by Dr. De Silva from the Department of Earth Sciences at the University of Ottawa in Canada.

3.6.4 Chlorophyll Concentration

The chlorophyll *a* concentration was determined by a method used by Aslan and Kapdan (2006). First, 10 ml of algal suspension was centrifuged at 3000 rpm for 30 min. The pellet was collected, while the supernatant was discarded. Next, the pellet was suspended in 3 ml of methanol, and subsequently it was heated in a water bath (Cole Palmer) to temperature of 40°C for a duration of 5 minutes. After the sample was cooled to room temperature, additional methanol was added so that the total volume of the sample reached 5 ml. The chlorophyll *a* concentration in the extract was determined by

measuring the optical density (OD) at wavelengths of 665 nm and at 650 nm with the spectrophotometer against a water blank by using the following equation:

$$\text{Chlorophyll}(\text{mg} / \text{L}) = 16.5 * OD_{665\text{nm}} - 8.3 * OD_{650\text{nm}} \quad \text{eq.2}$$

(Aslan and Kapdan, 2006)

3.6.5 Lipid Yield

Lipid extraction from *C. vulgaris* cells was performed in Soxhlet extractor. Before the extraction, 30 ml of algae suspension was first collected on the last day of a 9 day cultivation. The suspension was centrifuged at 8,000 rpm for 10 minutes. The pellet was collected and dried in the oven at temperature of approximately 80°C for about 24 hours. The mass of dried algae was recorded as 0.5178 g. The dry biomass was subsequently ground to a powder by using mortar and pestle.

Next, the dry biomass was placed in a thimble made from thick filter paper. The thimble and the dry biomass were subsequently loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor was mounted onto a flask and a condenser was fitted on top of the Soxhlet. The flask was filled with 100 ml of ethyl ether which is the extraction solvent. The solvent was heated to the temperature of 55°C in order for it to reflux. The solvent vapor traveled up a distillation side column and into the condenser. In the condenser the vapor turned to liquid that flowed down into the thimble with dry algae. The lipid dissolved in the warm solvent that flew into the flask. This cycle was repeated many times over for a period of about 12 hours. After extraction was complete, the ethyl

ether in the flask was evaporated at temperature of 55°C. The remaining residue was air dried in the fume hood for about 24 hours. The dried residue was the extracted lipid from *C. vulgaris* cells. The lipid yield was calculated by dividing the mass of dried residue over mass of dried algae and multiplying the expression by one hundred percent.

3.7 Factorial Design

When experiments involve two or more factors we can study their effects on process output through factorial design. Full factorial design involves running experiments with all possible combinations of the levels for all the factors. Factorial design has several advantages over one-factor-at-time experiments in that they are more efficient, take into account interactions that may exist between factors, and allow the effects of a factor to be estimated at several levels of the other factors yielding conclusions over range of experimental conditions. For good description of factorial design one can consult a book by Montgomery (1991).

In full factorial design the total number of runs can be expressed using the notation L^k , where L represents the number of levels of each factor, and k represents the number of factors; we assume that the number of levels is the same for each factor. In the early stages of experimental work when there are most likely many factors to be investigated a useful factorial design to use is 2^k . This special case of the general factorial design contains two levels for each factor and provides the smallest number of runs with k factors in full factorial design. In 2^k factorial design, one of the levels is

denoted by “-1”, and is denoted by “1”. Table 3.2 shows 2^4 factorial design containing the levels “-1” and “1” for four factors (A, B, C and D) and for their interactions.

As can be seen in Table 3.2, column two contains list of treatment combinations. Treatment combination represents combination of levels in each run. For instance, treatment combination denoted as ‘a’, contains level ‘-1’ for all the factors except for factor A which contains level ‘1’, similarly treatment combination ‘b’ contains level ‘-1’ for each factor except for factor B. However, it is important to note that treatment combination (1) contains level ‘-1’ for all the factors. The level for interactions is obtained by multiplying the level notation for corresponding factors. For instance, for treatment combination ‘s’, the level for AB interaction is obtained by multiplying ‘1’ for factor A with ‘-1’ for factor B yielding level ‘-1’.

Table 3.2: Algebraic signs for calculating effects in the 2^4 design.

Run	Treatment Combination	Factorial Effects															
		I	A	B	AB	C	AC	BC	ABC	D	AD	BD	ABD	CD	ACD	BCD	ABCD
1	(1)	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1
2	a	1	1	-1	-1	-1	-1	1	1	-1	-1	1	1	1	1	-1	-1
3	b	1	-1	1	-1	-1	1	-1	1	-1	1	-1	1	1	-1	1	-1
4	ab	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1
5	c	1	-1	-1	1	1	1	-1	1	-1	1	1	-1	-1	1	1	-1
6	ac	1	1	-1	-1	1	-1	-1	-1	-1	-1	1	1	-1	-1	1	1
7	bc	1	-1	1	-1	1	1	1	-1	-1	1	-1	1	-1	1	-1	1
8	abc	1	1	1	1	1	-1	1	1	-1	-1	-1	-1	-1	-1	-1	-1
9	d	1	-1	-1	1	-1	1	1	-1	1	-1	-1	1	-1	1	1	-1
10	ad	1	1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1
11	bd	1	-1	1	-1	-1	1	-1	1	1	-1	1	-1	-1	1	-1	1
12	abd	1	1	1	1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1
13	cd	1	-1	-1	1	1	1	-1	1	1	-1	-1	1	1	-1	-1	1
14	acd	1	1	-1	-1	1	-1	-1	-1	1	1	-1	-1	1	1	-1	-1
15	bcd	1	-1	1	-1	1	1	1	-1	1	-1	1	-1	1	-1	1	-1
16	abcd	1	1	1	1	1	-1	1	1	1	1	1	1	1	1	1	1

(Montgomery, 1991)

In constructing Table 3.2, the first and most crucial point is to list the treatment combinations of column two in what is known as Yates' Order. The Yates' Order for combination treatments of 2-factor, 3-factor, and 4-factor experiments is ("2^k factorial experiment", 2005):

2 factor experiment: (1), a, b, ab

3 factor experiment: (1), a, b, ab, c, ac, bc, abc

4 factor experiment: (1), a, b, ab, c, ac, bc, abc, d, ad, bd, abd, cd, acd, bcd, abcd

Once we have the fractional factorial design we can go ahead with the execution of the experiments. After the experiments have been conducted, one can calculate the factor effects.

The main objective of factorial design is to estimate factor effect (denoted as l_i , where i represents certain factor or factors) on the process response. For instance, the estimate of the effect of factor A on output response is denoted as l_A , while for instance l_{AB} denotes the effect of interaction between factors A and B on output response. The factor effects for 4-factor design can be calculated by referring to Table 3.2 and by using the process outputs for each run. The formula to calculate the effect of factor A on process output for 4-factor design experiment is:

$$l_A = \frac{1}{4}(-1) + a - b + ab - c + ac - bc + abc - d + ad - bd + abd - cd + acd - bcd + abcd$$

This formula contains treatment combination listed in Yates' Order. The sign in front of each treatment combination is obtained from the column for factor A in Table 3.2. In calculating factor effect, the treatment combinations are substituted with corresponding value of process output. Similarly, mathematical expressions can be obtained for other factor effects. Generally $\frac{1}{k}$ instead of $\frac{1}{4}$ is used in the beginning of the formula.

If we have experiments in which many factors are involved it may not be practical to perform the complete factorial design due to the large number of runs, and instead only a fraction of the complete factorial experiment may be considered. Fractional factorial designs can render our experiments more economical and efficient. Running a fraction of a full factorial design will give us information on main-effects and two-order interactions, while higher-order interactions (third-order and higher) are assumed to be negligible. Fractional factorial design can be expressed using notation L^{k-p} , where L represents the number of levels of each factor, k represents the number of factors, and $\frac{1}{L^p}$ describes the size of the fraction of the full factorial.

A fractional design with two levels for each factor is denoted as 2^{k-p} . A one-half fraction of full fractional experiments is often used if we cannot afford to run full design. One-half fraction of 2^k design is denoted as 2^{k-1} . For instance, a factorial design with 4 factors each with 2-levels renders $2^4 = 16$ runs, while the one-half fraction of this design results in $2^{4-1} = 8$ runs. To construct a matrix with '+1' and '-1' signs for 2^{k-1} design, we can select the appropriate rows from the matrix of 2^k design. For the case with $k = 4$, the 2^{4-1} design is formed by selecting only treatment combinations that have a plus sign in the ABCD column of the matrix for 2^4 design of Table 3.2 (Montgomery 1991). In

this case, ABCD is termed the generator. Furthermore, the identity column I has always plus sign, hence we can equate I to ABCD to give $I=ABCD$ for 2^{4-1} design. This expression is called the defining relation. The 2^{4-1} design matrix with ABCD generator is depicted in Table 3.3.

Table 3.3: Algebraic signs for calculating effects in the 2^{4-1} design.

Run	Treatment Combination	Factorial Effects															
		I	A	B	AB	C	AC	BC	ABC	D	AD	BD	ABD	CD	ACD	BCD	ABCD
1	(1)	1	-1	-1	1	-1	1	1	-1	-1	1	1	-1	1	-1	-1	1
2	ab	1	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	1
3	ac	1	1	-1	-1	1	-1	-1	-1	-1	-1	1	1	-1	-1	1	1
4	bc	1	-1	1	-1	1	1	1	-1	-1	1	-1	1	-1	1	-1	1
5	ad	1	1	-1	-1	-1	-1	1	1	1	1	-1	-1	-1	-1	1	1
6	bd	1	-1	1	-1	-1	1	-1	1	1	-1	1	-1	-1	1	-1	1
7	cd	1	-1	-1	1	1	1	-1	1	1	-1	-1	1	1	-1	-1	1
8	abcd	1	1	1	1	1	-1	1	1	1	1	1	1	1	1	1	1

Using the defining relation we can generate the alias structure for that particular design. By multiplying any column by the defining relation we obtain the aliases (Montgomery, 1991). For example in order to determine alias of A, we multiply both sides of defining relation by 'A' to yield:

$$A * I = A = A * ABCD = A^2 BCD = BCD$$

$$\text{note : } A^2 = 1$$

Similarly, we can obtain aliases of other factors. The aliasing relationships for 2^{4-1} factorial experiment are presented in Table 3.4.

Table 3.4: Aliasing relationships for 2^{4-1} factorial experiment.

Alias Relationships
A=BCD
B=ACD
C=ABD
D=ABC
AB=CD
AC=BD
BC=AD

(Montgomery, 1991)

We note from Table 3.4 that each main factor is aliased with a three-factor interaction and every two-factor interaction is aliased with another two-factor interaction. Furthermore, the four main factors and three two-factor interactions account for seven degrees of freedom for the design.

The estimates of factor effects for 2^{4-1} design can be obtained from the following relationships derived from Table 3.3:

$$l_A = \frac{1}{4}(-1) + ab + ac - bc + ad - bd - cd + abcd$$

$$l_B = \frac{1}{4}(-1) + ab - ac + bc - ad + bd - cd + abcd$$

$$l_C = \frac{1}{4}(-1) - ab + ac + bc - ad - bd + cd + abcd$$

$$l_D = \frac{1}{4}(-1) - ab - ac - bc + ad + bd + cd + abcd$$

$$l_{AB} = \frac{1}{4}((1) + ab - ac - bc - ad - bd + cd + abcd)$$

$$l_{AC} = \frac{1}{4}((1) - ab + ac - bc - ad + bd - cd + abcd)$$

$$l_{BC} = \frac{1}{4}((1) - ab - ac + bc + ad - bd - cd + abcd)$$

The important information about the factor effect is its magnitude as well as its direction. A higher absolute value of the effect means that the factor responsible for it affects output to a significant degree. A negative value of effect means that if the level of a factor responsible for that effect is increased, the magnitude of output of the process will decrease.

Major use of fractional factorials is in screening experiments that are usually performed in the early stages of experimentation. Once important factors have been identified, they may be investigated in more detail in subsequent experiments, while the non-important factors may be dropped from the design.

Chapter 4 Effect of Medium Composition on Growth of *Chlorella vulgaris*

4 Results and Discussion

4.1 Growth and Nutrient Utilization of Chlorella vulgaris in 2X Tap Culture Medium in Batch Photobioreactor

Figure 4.1 depicts the growth curve of *C. vulgaris* in the photobioreactor, as well as a profile of acetic acid concentration (Table 8.1 in the Appendix contains raw data). Acetic acid was the only organic source of carbon in the medium, while CO₂ was the only inorganic source of carbon. During about the first 30 hours into the experiment the cells appear to go through exponential phase. Next, it appears that the cells go through a linear phase until about 120 hours of experiment. Subsequently the cell growth rate decreases, and reaches plateau. According to sources in the literature the linear phase might be an indication of a limitation such as mass transfer of CO₂ from air bubbles or light penetration into the media (Powell et al., 2009). As seen in Figure 4.1, the acetic acid in the photobioreactor was utilized within the first 20 hours.

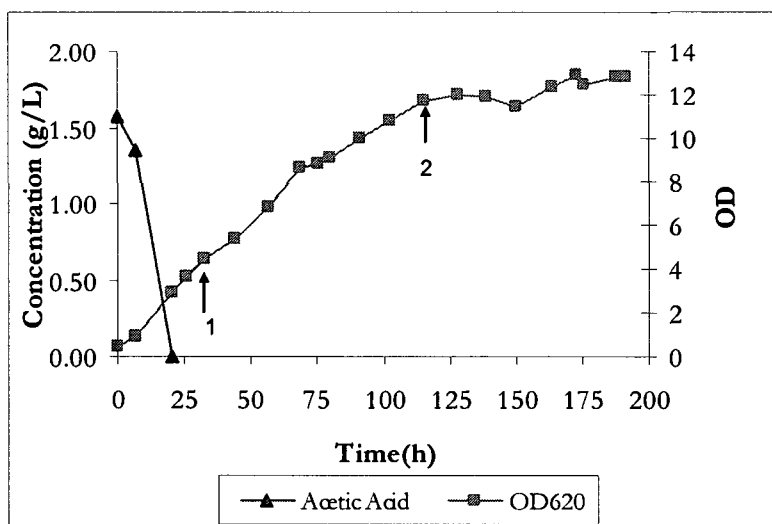


Figure 4.0.1: Growth curve of *Chlorella vulgaris* and depletion of acetic acid from the medium during batch cultivation in photobioreactor

(Note: Operational conditions are 28°C, 600 rpm, 5% CO₂ in air, 900 μE/ (m² s). Arrows 1 and 2 indicate the approximate ends of the exponential and linear growth phases, respectively).

The maximum optical density reached during 8 days of cultivation of *C. vulgaris* was about 13, which amounted to biomass concentration of approximately 6 g/L. The correlation between optical density and biomass concentration was determined to follow equation 1 found in Section 3.6.1. Batch studies by Patino and co-workers (2007), conducted in cylindrical photobioreactor for period of 8 days, showed that highest *C. vulgaris* concentration achieved was 5.5 g/L at initial glucose concentration of 12 g/L, giving yield of biomass to glucose of 0.46 g/g. Our experiment was conducted with initial acetic acid concentration of about 2.0 g/L, and the biomass the yield of biomass to acetate

was 2.75 g/g. It is desirable to maximize the yield of the biomass to the organic carbon source in order to improve the economics of the process.

The pH profile during the cultivation of *C. vulgaris* is depicted in Figure 4.2. Initially the pH in the medium was around 6.4; however in a span of around 20 hours the pH jumped to about 7.1 where it remained relatively unchanged until the end of cultivation. As pointed out in Figure 4.1, the first 20 hours into the *C. vulgaris* cultivation also marks the time when acetic acid was depleted.

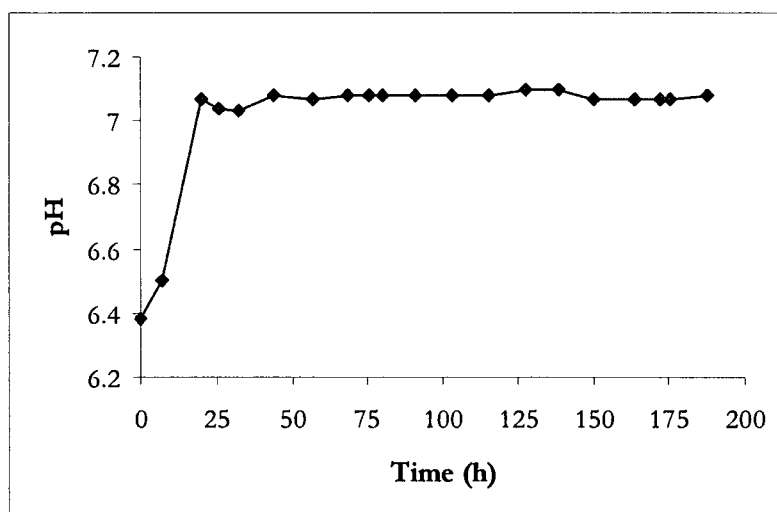


Figure 4.2: pH of the medium during batch cultivation of *Chlorella vulgaris* in photobioreactor.

When acetic acid is removed from the medium the pH of the medium should rise. In addition, the uptake of CO₂ by algae from the medium also causes a rise in the pH (Granum and Myklestad, 2002). However, in this experiment CO₂ was continually added to the medium at constant rate. We speculate that it is unlikely that a rise in algal biomass

during initial 20 hours of cultivation was responsible for a net lowering in CO₂ concentration such that it had influence on the sharp rise in pH.

After 20 hours of cultivation the pH remained approximately constant for the duration of the experiment. Perhaps this constancy was the result of a balance achieved by the uptake of CO₂ that caused the pH rise, and by the uptake of ammonium hydroxide that caused the decrease in pH (Pratt and Fong, 1940).

Figure 4.3 shows the same growth curve as in Figure 4.1, and in addition it also depicts the depletion of ammonium-N and phosphate-P from the medium (Table 8.1 in the Appendix contains raw data) .

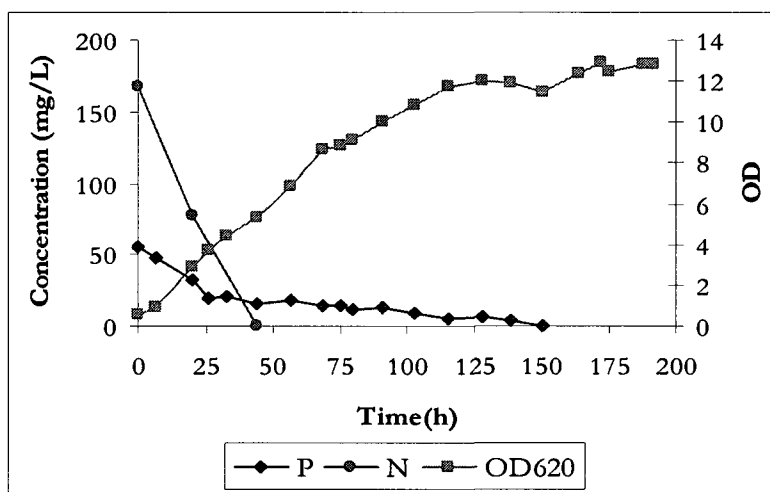


Figure 4.3: Growth curve of *Chlorella vulgaris* and depletion of ammonium-N and phosphate-P from the medium during batch cultivation in photobioreactor.

As seen in Figure 4.3, the nitrogen was completely consumed within the first 44 hours. The phosphate-P was utilized at much smaller rate than ammonium-N. This result

was consistent with the work by Patino and co-workers (2007). It is also interesting to compare the curve of phosphate-P utilization in Figure 4.3 and the curve of acetic acid utilization in Figure 4.1. It can be seen that the rate of phosphate-P removal was highest when acetic acid was being taken up by the cells that occurred during the first 20 hours of cultivation. Once acetic acid was completely consumed the removal rate of phosphorus-P decreased quite drastically. The interesting point was that even after the depletion of ammonium-N early in the cultivation the cells were still able to grow. Studies of Tam and Wong (1996) have also shown that cell growth continued after ammonia-N depletion in the medium. These authors claim that *C. vulgaris* cells can utilize their internal reserves of nitrogen, hence maintain growth after external source of nitrogen is depleted. Also, at time of around 140 hours when phosphate-P was extremely scarce in the medium, the cells reached a declining phase; however after depletion of phosphate-P at around 150 hours the cells continued to grow, even if for short time.

From the results it is evident that phosphorus was consumed by *C. vulgaris* at higher rates when in presence of acetic acid plus CO₂, rather than in the presence of CO₂ and absence of acetic acid. During the utilization of acetic acid the growth rate of *C. vulgaris* was higher (0.15 h⁻¹) than during any other time of cultivation. For comparison, the growth rate of *C. vulgaris* grown on glucose in mixotrophic mode was reported as 0.198 to 0.131 h⁻¹ in literature (Lee Y-K, 2001). Perhaps the decrease in growth rate shortly after acetic acid depletion was mainly due to absence of acetic acid. According to Lin and Chen (1994) the highest growth rate of *Chlorella* was achieved in presence of both acetic acid and CO₂ supplemented air, while a lower growth rate occurred in the absence of acetic acid when the only source of carbon was CO₂ supplemented air. On the

other hand, perhaps the decrease in growth rate was mainly due to ammonium-N depletion that also occurred shortly after acetic acid depletion.

Nitrogen and phosphorus are one of the most important nutrients for algal growth (Mandalam and Palsson, 1998). In comparison to other required nutrients, these two nutrients are required in largest concentrations for algal growth. Because both ammonium-N and phosphate-P are depleted before the end of cultivation (before day 9), they are both considered limiting nutrients in the medium. Other required elements for algal growth were also examined in the course of the cultivation and their concentration profile in the medium is depicted in Figure 4.4 (Table 8.2 in the Appendix contains raw data). After nitrogen and phosphorus, the most important element is potassium (Oh-Hama and Miyachi, 1988). From Figure 4.4 it is evident that potassium was not a limiting nutrient in the medium.

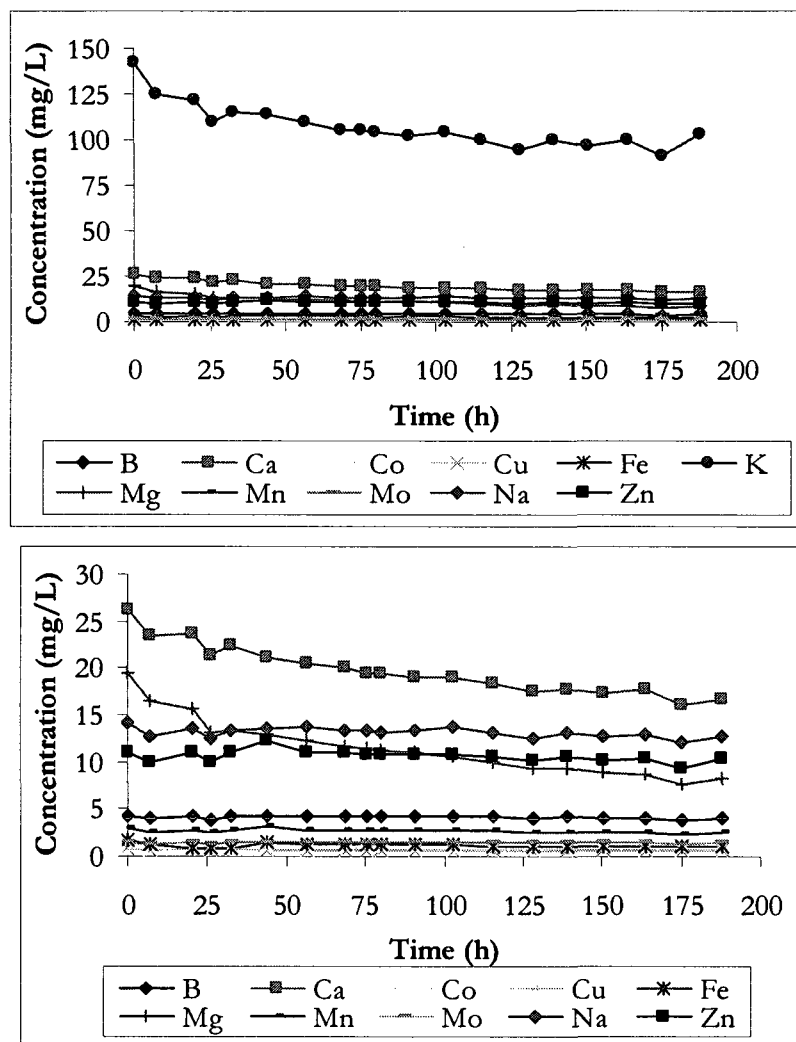


Figure 4.4: Concentration profiles of elements present in the culture medium during batch cultivation of *Chlorella vulgaris* in photobioreactor.

In the inset one can see more clearly the curves depicting the concentration profile of all the other required elements during the cultivation period. It can be observed that calcium and magnesium experienced relatively high utilization rates. Upon closer inspection we see that none of these elements were limiting.

By analyzing the medium during the course of algal cultivation, we have discovered that nitrogen and phosphorus were limiting nutrients. Next step in the research was to obtain the medium such that all the required nutrients for growth of *C. vulgaris* were in sufficient amount in order to improve on biomass production. But before we constructed such a medium, first we wanted to find the effect of various initial concentrations of nitrogen and phosphorus on *C. vulgaris*, such that we had an upper limit on how much of limiting nutrients could be added before they can be detrimental to cell growth.

4.2 Effect of Initial Concentrations of Nutrients on Batch Growth of *Chlorella vulgaris* in 2X Tap Culture Medium in 500 ml Flasks

4.2.1 Effect of Ammonium-N Concentration

The effect of concentration of nitrogen on algal cell growth is dependent on culture conditions (Tam and Wong, 1996). Even though a couple of studies have been performed by other authors on the effect of nitrogen and phosphorus concentration on growth of *C. vulgaris*, we wanted to find the effect of concentrations of these nutrients on growth of *C. vulgaris* in our culture conditions. Previously we had performed a batch photobioreactor experiment; results are shown in Figure 4.1 and Figure 4.3, where the initial ammonium-N concentration was near 211 mg/L. In that experiment ammonium-N was depleted quite early in the cultivation. In the next set of experiments the growth of *C. vulgaris* in batch flasks was monitored at initial ammonium-N concentrations ranging from 211 to 1000 mg/L. Figure 4.5 shows growth curves of *C. vulgaris* under different initial nitrogen concentrations (Table 8.3 in the Appendix contains raw data).

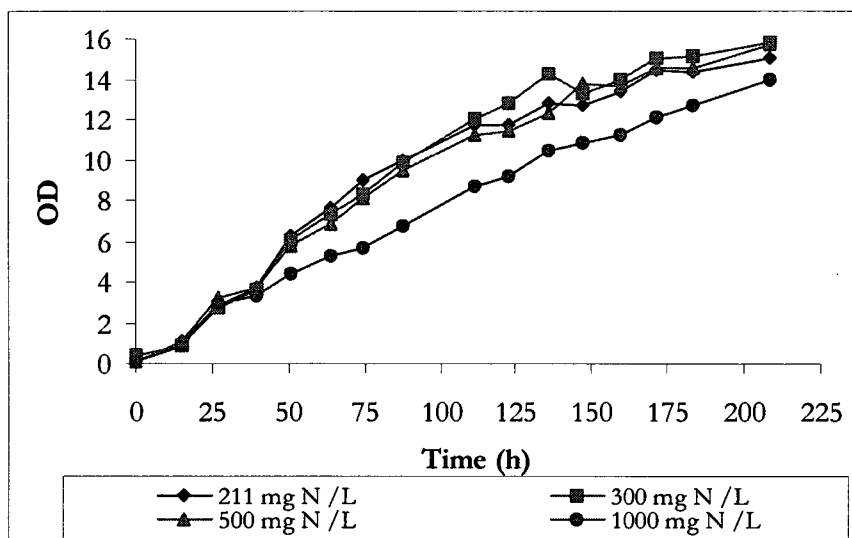


Figure 4.5: Growth curves of *Chlorella vulgaris* grown in flask under initial ammonium-N concentrations of 211 mg/L, 300 mg/L, 500 mg/L, and 1000 mg/L.
(Note: The initial concentration of phosphate-P is 64 mg/L.)

It is evident from Figure 4.5 that lower growth occurred at initial ammonium-N concentration of 1000 mg/L. When initial ammonium-N was 211 mg/L, 300 mg/L or 500 mg/L, no differences in growth curves were observed. These results are consistent with literature data, where usually the inhibitory concentration of ammonium-N to *C. vulgaris* was around 750 to 1000 mg/L (Matusiak, 1976; Tam and Wong, 1996). Although our experiment studying the effect of ammonium-N concentrations on algal growth were performed in flasks, we fully realize that this effect could be different in photobioreactor where we have different culture conditions than in flasks. Nevertheless, the results from flask experiments gave us some idea of inhibitory concentration of ammonium-N on *C. vulgaris* that we could expect to achieve in photobioreactor.

The pH during the course of this set of experiments was also monitored and the results are shown in Figure 4.6.

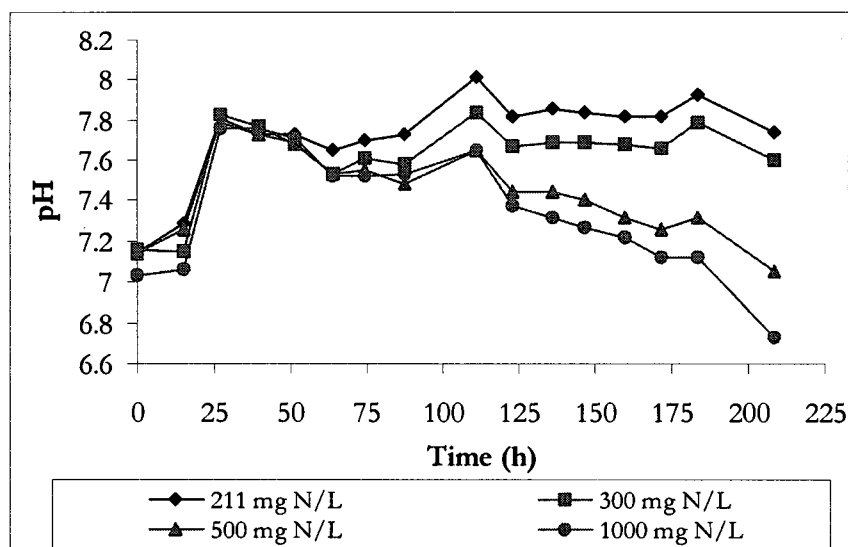


Figure 4.6: pH change in the medium during batch cultivation of *Chlorella vulgaris* in flask under initial ammonium-N concentrations of 211 mg/L, 300 mg/L, 500 mg/L and 1000 mg/L.

(Note: The initial concentration of phosphate-P is 64 mg/L.)

The pH increased from around 7-7.3 to 7.8 from about 20th to 25th hours of cultivation. After that the pH was relatively constant for experiments when the initial concentration of ammonium-N in the medium was either 211 or 300 mg/L. On the contrary, when the initial ammonium-N concentration was 500 or 1000 mg/L the pH dropped after 25 hours into the cultivation. These results were expected. According to the mechanism proposed by Trelease and Trelease (1935), the ammonium compound and water molecules dissociate into their respective anion and cation pairs. According to this mechanism the cation from the ammonium compound, which is NH_4^+ , binds with OH^-

that came from the water molecule, forming ammonium hydroxide that is taken up by the algal cells. What is left in the medium is the H^+ from water molecule and the anion from dissociated ammonium compound. In our set of experiments the source of nitrogen was ammonium chloride. Hence the H^+ ion combined with Cl^- ion to form HCl, and as result the pH in the medium was reduced. Algal cells uptake more ammonium-N resulting in more drastic pH drop when there is a higher concentration of ammonium chloride in the medium (Tam and Wong, 1996).

By comparing the curves representing the experiment where the initial ammonium-N concentration is 1000 mg/L, in Figure 4.5 and Figure 4.6, one might be tempted to conclude that the algal growth was inhibited because of the sharp drop in pH, resulting in low pH. It should be pointed out that *C. vulgaris* has very broad optimum pH, ranging from 6.5 to 8.0, and it will tolerate acidities at or above pH of 4 (Kessler, 1976). Thus it was unlikely that the inhibition of growth was due to low pH of the medium. Perhaps the inhibition occurred due to high internal concentration of ammonium in *C. vulgaris* cells, and as noted by Schuldiner and colleagues (1972) such internal cell conditions can have detrimental effect on algal photosynthesis. In addition, the salt in the medium may play role in creating osmotic pressure that could affect cell viability.

4.2.2 Effects of Phosphate-P Concentrations

Previously we have performed a batch photobioreactor experiment, of which results are depicted in Figure 4.1 and Figure 4.3, where the initial phosphate-P concentration was around 64 mg/L. Because the phosphorus was limiting in our medium,

we wanted to create a nutrient sufficient medium. Before we added more phosphorus, first we wanted to find out the effect of various higher concentrations of this element on *C. vulgaris*. A batch study was performed in flasks to assess the growth curves of *C. vulgaris* exposed to different initial phosphate-P concentrations ranging from 64 mg/L up to 300 mg/L. The algal growth curves for this set of experiments are shown in Figure 4.7.

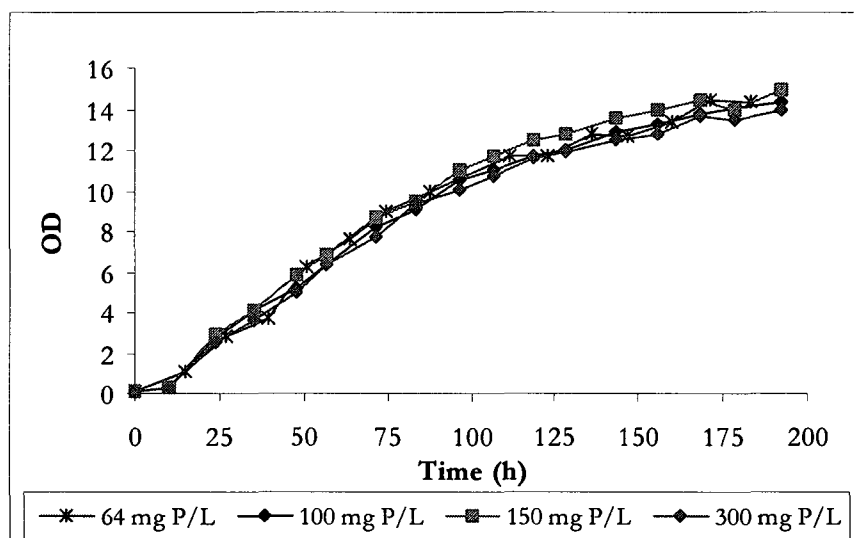


Figure 4.7: Growth curves of *Chlorella vulgaris* grown in flask under initial phosphate-P concentrations of 64 mg/L, 100 mg/L, 150 mg/L, and 300 mg/L.

(Note: The initial concentration of ammonium-N is 211 mg/L.)

It can be seen from Figure 4.7 that at the specified initial concentrations of phosphate-P none of these concentrations was inhibitive to the growth of *C. vulgaris*. From the researched literature none of the journal papers described effects of different concentrations of phosphorus on *C. vulgaris* growth under the range of concentrations that we have tested. However, according to Eyster (1978) the optimal concentration of

phosphorus for *C. sorokiniana* is between 20 to 600 mg/L. The concentrations of phosphate-P that we have tested fall within this range.

The pH of the medium during the course of the cultivations was approximately the same for the experiments with different initial phosphate-P concentrations as indicated in Figure 4.8. The pH increased drastically in the first 25 hours of cultivation, and after that time it remained relatively constant (Table 8.4 in the Appendix contains raw data). The source of phosphorus in our experiments was phosphate. Increasing phosphate concentration did not have effect on pH perhaps because phosphates have high buffering capacity.

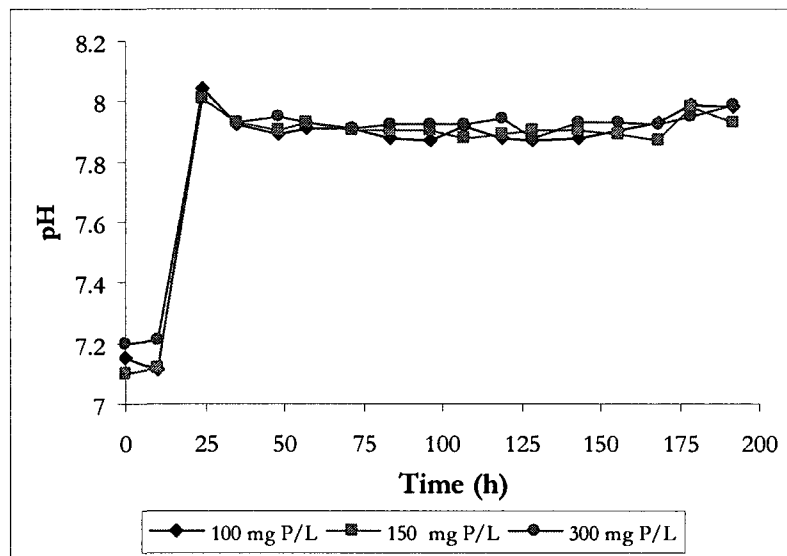


Figure 4.1: pH change in the medium during batch cultivation of *Chlorella vulgaris* in flask under initial phosphate-P concentrations of 100 mg/L, 150 mg/L, and 300 mg/L.

(Note: The initial concentration of ammonium-N is 211 mg/L)

Chapter 5 Effect of Operational Conditions on Growth of *Chlorella vulgaris*

Algal biomass productivity can be increased by proper choice of operational conditions. Important processing parameters in phototrophic algal growth include: percent of CO₂ in air delivered to culture medium, agitation speed of culture medium, temperature of culture medium, light irradiance at surface of photobioreactor, and pH of culture medium.

5.1 Design of Fractional Factorial Experiments

The purpose of the fractional factorial design is to determine the important factors that have an effect on output variable. In optimizing the *Chlorella* growth, fractional factorial approach has been found to be very scarce in the literature sources. Work by Cho S.H. and co-workers (2007) is the only one of the few examples where fractional factorial design is utilized to optimize *Chlorella* growth.

In our study of the effect of operating conditions on biomass concentration, we have chosen to work with four factors; percent of CO₂ in air delivered to culture medium, agitation speed of culture medium, temperature of culture medium, and the light irradiance at the outer surface of photobioreactor. The pH of culture medium was not controlled in any of the experiments as preliminary results revealed that pH stays within acceptable levels for the growth of *C. vulgaris*. In Table 5.1 we have the four factors, upper and lower value for each factor, along with corresponding level notation. We have

chosen the indicated lower and upper level for each factor based on the levels that were chosen in the literature. In the literature, generally the highest biomass is achieved with levels close to what we have chosen in our research.

Table 5.1: Fractional factorial design; the variables and their levels along with the level notation.

Variables	Levels	Level Notation
CO ₂ in Air (%)	4	-1
	7	1
Agitation Speed (rpm)	300	-1
	600	1
Temperature (°C)	25	-1
	29	1
Light Irradiance ($\mu\text{Em}^{-2}\text{s}^{-1}$)	900	-1
	450	1

By utilizing the 2^{4-1} fractional factorial design presented in Table 3.3, we can determine the combination of parameters for each experiment. We can achieve this task by combining all the columns from the 2^{4-1} fractional factorial design that correspond to the main factors into a new table. Columns two to five in Table 5.2 represent the levels of main factors.

Table 5.2: Fractional factorial design with level notation and the corresponding combination of process parameters for each run.

Run	Factorial Effect				CO ₂ in air	Agitation Speed	T	Light Irradiance
	A	B	C	D	(%)	(rpm)	(°C)	
1	-1	-1	-1	-1	4	300	25	high
2	1	1	-1	-1	7	600	25	high
3	1	-1	1	-1	7	300	29	high
4	-1	1	1	-1	4	600	29	high
5	1	-1	-1	1	7	300	25	low
6	-1	1	-1	1	4	600	25	low
7	-1	-1	1	1	4	300	29	low
8	1	1	1	1	7	600	29	low

Factors A, B, C, and D, correspond to the four process variables; CO₂ in air, agitation speed, temperature, and light irradiance, respectively. Columns six to nine in Table 5.2 denote the combination of parameters for each experiment. For instance, experiment number one will be conducted with 4% CO₂ in air, agitation speed of 300 rpm, temperature of 25°C, and light irradiance set at high.

5.2 Results and Discussion

5.2.1 Growth of *Chlorella vulgaris* in Photobioreactor in 2X Tap Culture Medium under Various Process Conditions

The growth curves of *C. vulgaris*, for the eight fractional factorial runs are depicted in Figure 5.1. For details of each of the eight runs please consult Table 5.2. The data used to construct the growth curves can be found in Appendix in Table 8.5 and Table 8.6.

As seen from Figure 5.1, the best algal growth occurred during run 4 where the culture medium was maintained at temperature of 29°C, sparged with 4% CO₂, agitated at 600 rpm, and irradiated with 900 $\mu\text{E}/(\text{m}^2 \text{ s})$ of fluorescent light.

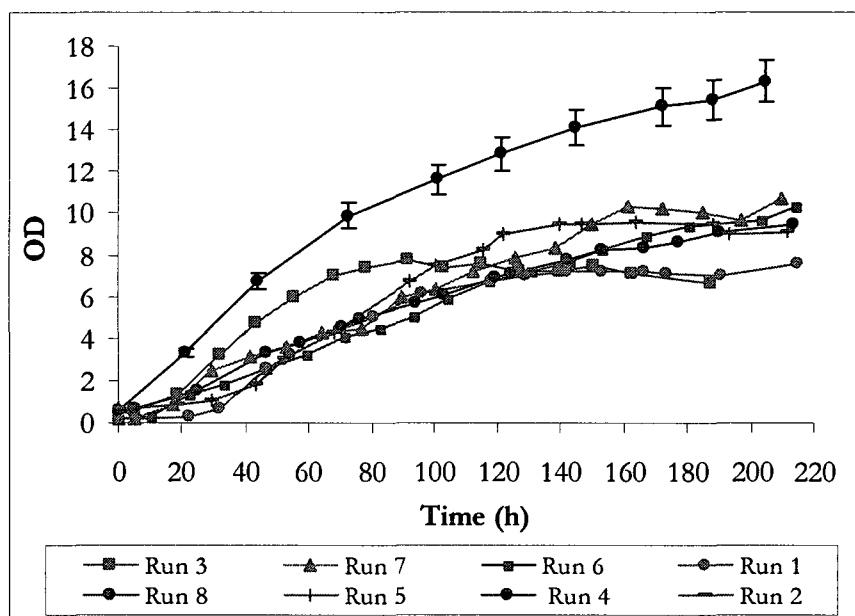


Figure 5.1: Growth curves of *Chlorella vulgaris* for the fractional factorial runs performed in photobioreactor.

(Note: Refer to Table 5.2 for the details of each run.)

The experiment specified as run 4 was performed three times. The vertical lines for that experiment presented in Figure 5.1 represent error bars. Through close examination, we can see that the growth curves in the above figure generally go through a lag phase, an exponential phase, a linear phase, and in some cases a stationary and declining phase. It is difficult to describe shape of curve for run 4 as small number of samples was collected. The occurrence of these growth phases for *C. vulgaris* grown in photobioreactor has been also observed in the literature (Geoghegan, 1953). Similarly, in

studies of Ogbonna and co-workers (1995a) *C. vulgaris* grown in photobioreactor went through a lag phase, an exponential phase and a linear phase. It is interesting to note that these growth curves contain an exponential phase that is always shorter than the linear phase. This trend has been also observed in the literature (Amory et al., 1991).

The onset of exponential and linear phases and in some cases stationary phase, generally occurred at different times for different runs. The biggest difference can be seen in growth curve of run 3. The growth curve of run 3 shows relatively rapid algal growth, compared to the other six growth curves. In addition, the stationary phase occurred relatively early in the growth curve of run 3, while the algal growth in other runs during this time was more or less still increasing.

Run 3 was performed in presence of relatively high CO₂ concentration, a high temperature, and in relatively high light intensity. Perhaps the cells grew well at the early stages of cultivation because light and carbon were not limiting. However the exposure to relatively high light and high carbon concentration coupled with relatively small agitation speed was responsible for the quick onset of stationary and declining phase. The small agitation speed is less capable of providing sufficient changes of illumination for cells, as result some cells could have been exposed to too much light while others to too little.

However regardless of the operating conditions during the fractional factorial runs, growth curves always exhibited exponential growth followed by a linear growth. It has been stated in the literature that onset of linear growth is the result of limitation of some process factor (Powell et al., 2009). Any limitation in growth process for the fractional factorial runs depicted in Figure 5.1 are most likely due to CO₂ and/or light, as other nutrients were likely supplied in excess. As noted in one literature source (Gaudin

and Chaumont, 1991), it is also possible that the growth limitation is due to the degree of cell fragility to shear stress imposed by agitation. However according to Bronnenmeier and Markl (1982) *C. vulgaris* is extremely stable against hydrodynamic stress, and thus it is very unlikely that growth limitation factor is shear stress. An experimental method necessary to determine if limitation was due to CO₂ or to the photosynthetically active irradiance has been noted in the literature (Contreras et al., 1998).

Figure 5.1 not only gives us the information about the shape of the growth curves, but more importantly it gives us the information about the maximum optical density achieved in each run at specific time. The tabulated values of maximum optical density and the corresponding biomass for each fractional factorial run can be seen in Table 5.3.

Table 5.3: Combination of process parameters for each fractional factorial run, the maximum optical density achieved in each run and the corresponding maximum biomass concentration, listed in descending order of maximum optical density.

Run	CO ₂ in air (%)	Agitation Speed (rpm)	T (°C)	Light Irradiance	Maximum OD (λ=620 nm)	Maximum Biomass (g/L)
4	4	600	29	high	16.3*	7.7**
7	4	300	29	low	10.7	5.1
6	4	600	25	low	10.3	4.9
8	7	600	29	low	9.5	4.5
2	7	600	25	high	9.5	4.5
5	7	300	25	low	9.1	4.3
3	7	300	29	high	7.8	3.7
1	4	300	25	high	7.6	3.6

Note: * average maximum OD from three runs

** average final biomass from three runs

The maximal biomass concentration from all the runs ranged from 7.7 to 3.6 g/L. The maximal biomass of 7.7 g/L with standard deviation of 0.8 g/L was achieved for run 4 on the last day of a 9 day cultivation period.

The factorial runs were performed in fed-batch mode. The ammonium-N and phosphate-P were supplied to the vessel at the beginning of the experiment for during the first 70 hours of cultivation. As stated in the literature, the advantage of fed-batch is that one can control the levels of nutrients that can inhibit cell growth (Carvalho et al., 2004). Feeding of ammonium semi-continuously to *Spirulina* cells has been noted in the literature source (Carvalho et al., 2004), however fed-batch of nitrogen or phosphorus to *C. vulgaris* was not found in the literature. For that reason we were unable to directly compare the biomass concentrations achieved in our experiments to others for fed-batch of nitrogen or phosphorus to *C. vulgaris*.

Nevertheless, we can compare the maximum biomass concentration that we have achieved to that reported in the literature for growth of *C. vulgaris* in photobioreactor disregarding the mode of cultivation and process variables. Compared to the maximal concentrations of *C. vulgaris* biomass reported in the literature, the 7.7 g/L concentration achieved in our lab is considered high. Most of the maximal concentrations reported in the literature were up to 5 g/L (Degen et al., 2001; Geoghegan, 1953; Morais and Costa, 2007; Yun et al., 1996), although Patino and co-workers (2007) reported *C. vulgaris* biomass concentration of 9.5 to 9.9 g/L.

The pH during the course of the fractional factorial runs is depicted in Figure 5.2. Please note that pH curve for run 4 is depicted only for one of the three trials as it is

similar in the other two trials as seen in Appendix section Table 8.5. The raw data of the pH for other runs is shown in Table 8.7 of Appendix.

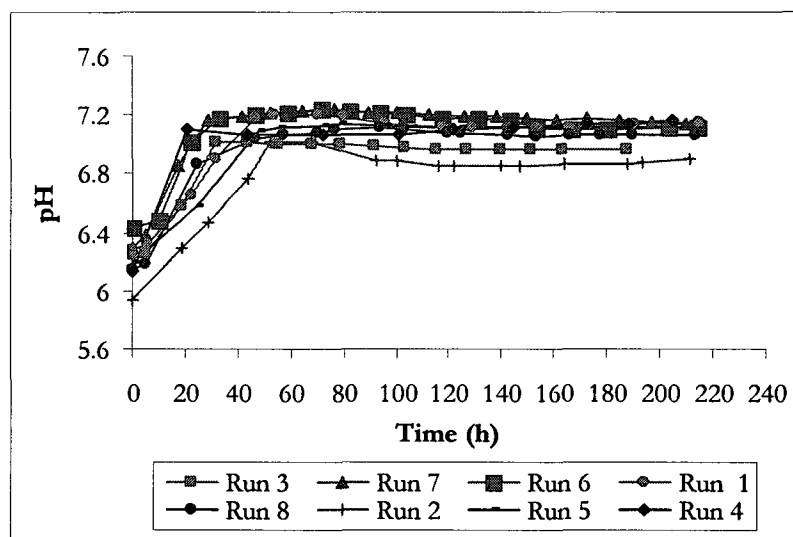


Figure 5.2: pH change in the medium during various fractional factorial runs.

At the beginning of the experiment the pH in the medium rose for all of the fractional factorial runs. Subsequently the pH plateaued, however at different times for all the runs. For run 4, the run where the highest final biomass was achieved, the pH rose only for the first 20 hours of the cultivation. For all the other runs it took longer time for the pH to become stable. In Figure 4.1 it was shown the growth curve of *C. vulgaris* and the utilization of acetic acid from the medium. In that batch experiment the process parameters were closest in value to those of run 4 of fractional factorial designs. In both of these experiments, batch and fed-batch, the stable pH of approximately 7.1 was reached around 20 hours into cultivation. In the batch experiment the first 20 hours into the cultivation also corresponded to the utilization of acetic acid from the medium. Perhaps the acetic acid utilization was slower in all the other fractional factorial runs,

resulting in longer time for pH plateau. In such case we could predict when acetic acid is completely removed based on the time the pH plateaus.

For the fractional factorial run that gave the highest biomass concentration (run 4) *C. vulgaris* chlorophyll *a* concentration was determined during the experiment. The initial total chlorophyll *a* content was 34 mg/L, and total chlorophyll *a* content increased during the cultivation period. This is to be expected because the biomass increases during the course of experiment. Literature sources report that total chlorophyll content increased with incubation time (Tam and Wong, 1996). Figure 5.3 shows total chlorophyll *a* content of cells during the course of run 4.

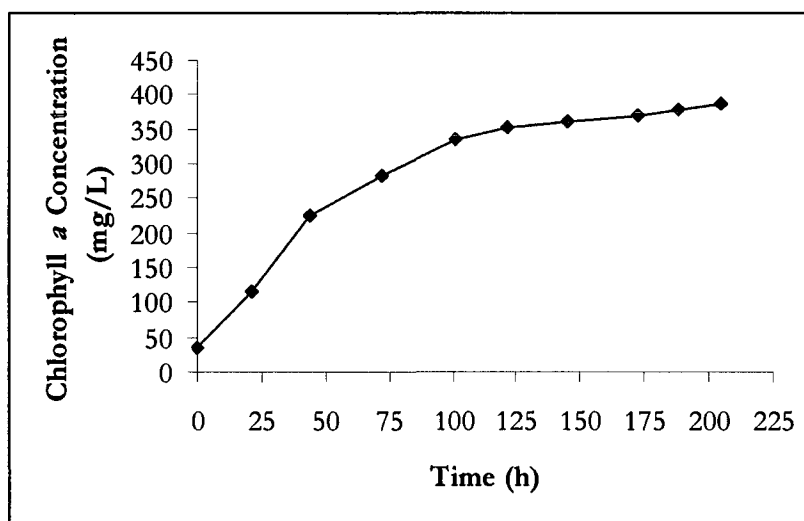


Figure 5.3: Total chlorophyll *a* content for *Chlorella vulgaris* during run 4 (4 % CO₂ in air, 600 rpm, 29°C, 900μE/ (m² s)) of fractional factorial experiments.

From Figure 5.3 we see that rate of total chlorophyll *a* content production decreased during cultivation period, with highest total chlorophyll *a* content production during the initial stages of experiment. This is an expected result because the highest growth rate of *C. vulgaris* was also during the initial stages of experiment. The total

chlorophyll *a* content of *C. vulgaris* at the end of 9 day cultivation period is around 390 mg/L. Study by Mandalam and Palsson (1998) shows that during cultivation of *C. vulgaris* the highest total chlorophyll content of around 310 mg/L occurs on day 8 and day 9 of cultivation period.

As mentioned previously, microalgal lipid production can be achieved by a two-stage process. The first-stage involves optimization of biomass production, while the second stage involves accumulation of lipids by imposing environmental stress on microalgae. Our research has focused on the first-stage and before any research on second-stage of the lipid-production process can be performed, it is important to find out the lipid content of microalgae at the end of first-stage. This information along with lipid content at the end of second-stage, will allow one to determine the amount of accumulated lipids in the second-stage of lipid production.

The *C. vulgaris* sample that was analyzed for lipids was obtained at the end of 9 day cultivation period in the fractional factorial experiment designated as run 4. It was decided to obtain lipid analysis of this sample as this run gave the highest final biomass concentration. The lipid content of dry *C. vulgaris* cells as determined by Soxhlet extractor method was about 3.7%. According to literature sources, lipid extraction from *C. vulgaris* by ether resulted in 1 to 5 % by dry weight (Geoghegan, 1953), while in another study it resulted in 7.6% by dry weight (Khasanova et al., 1978). It is important to note that the amount of extracted lipid from algae can vary depending on the extraction method utilized (Geoghegan, 1953; Khasanova et al., 1978).

Lipid productivity is a major factor that determines if a microalga species is suitable for biodiesel production (Griffiths and Harrison, 2008). This factor is a function

of both microalgal lipid content and biomass productivity. Since only the first-stage of lipid process was performed, we can only calculate the lipid productivity during nutrient sufficient conditions. The lipid productivity of *C. vulgaris* cultivated for 9 days in the photobioreactor under nutrient sufficient conditions was about 32 mg/ (L day). In comparison, one literature study reports that lipid productivity of *C. vulgaris* cultivated for 15 days under nutrient sufficient conditions is around 10 mg/ (L day) (Widjaja et al., 2009).

5.2.2 Statistical Analysis of Chlorella vulgaris Biomass from Fractional Factorial Experiments

In order to determine the process factors that have highest effects on biomass concentration, one needs to determine the factor effects. The estimates of factor effects for the 2^{4-1} design were calculated from equations shown in Section 3.7. Table 5.4 depicts the factors, their aliases and the corresponding estimates of effects. For sample calculation of factor effects please consult Appendix Section 8.2.1.

Table 5.4: The factors and corresponding estimates of factor effect.

Factors	Factor Effect
A+BCD	-2.3
B+ACD	2.6
C+ABD	2.0
D+ABC	-0.4
AB+CD	-1.6
AC+BD	-2.6
BC+CD	1.1

The high absolute values of factor effects in Table 5.4 correspond to factors that have high effect on the process output. The important main factors are CO₂ in air (factor A), agitation (factor B) and temperature (factor C). In Table 5.4 we can see that light irradiance (factor D) has very small absolute value of effect relative to other effects, and hence it can be considered negligible. In addition there are also high absolute values of factor effects for interactions corresponding to CO₂ in air/agitation (AB interaction) and its alias temperature/ light irradiance (CD interaction), CO₂ in air/ temperature (AC interaction) and its alias agitation/light irradiance (BD interaction), and agitation/temperature (BC interaction) and its alias temperature/light irradiance (CD interaction). Because irradiance (factor D) is insignificant on the process output we have chosen interaction CO₂ in air/ agitation (AB interaction) to be more important than temperature/ light irradiance (CD interaction), as well as the interaction CO₂ in air/temperature (AC interaction) to be more important than temperature/ light irradiance (CD interaction), and interaction agitation/ temperature (BC interaction) to be more important than temperature/light intensity (CD interaction). The important main effects and interactions are indicated in bold in Table 5.4. In summary, the important main effects in our system are percent of CO₂ in air, agitation speed and temperature, while the important interactions are percent of CO₂ in air on agitation speed, percent of CO₂ in air on temperature, and agitation speed on temperature.

Furthermore, the sign of the factor effect in Table 5.4 can tell us whether increasing or decreasing the value of the factor will result in increase of biomass concentration of *C. vulgaris*. Since decreasing value of CO₂ in air (factor A), increasing

value of agitation (factor B) and increasing value of temperature (factor C) increases the process output, the main effects in our system that increase biomass concentration are 4% CO₂ in air, agitation speed of 600 rpm, and temperature of 29°C.

To determine the process parameters for the interactions CO₂ in air/agitation (AB interaction), CO₂ in air/temperature (AC interaction)) and agitation/temperature (BC interaction) that increase process out put we need to construct graphs as depicted in Figure 5.4, Figure 5.5 and Figure 5.5.

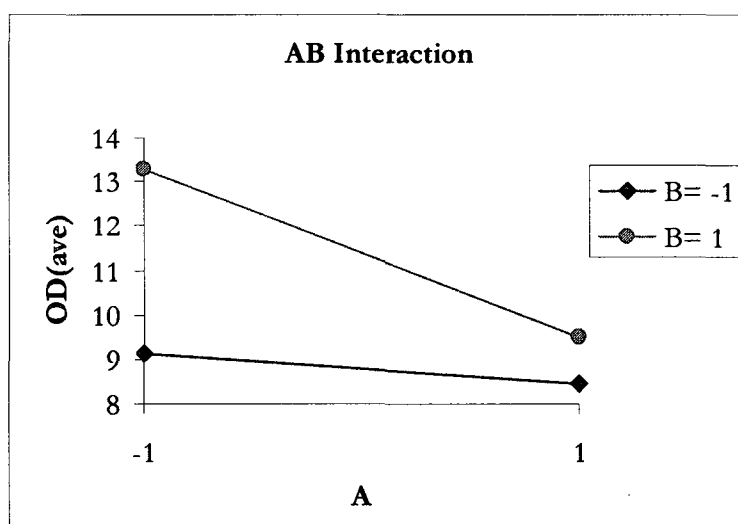


Figure 5.4: Effect of interaction of factors A (% CO₂ in air) and B (agitation speed) on the optical density of *Chlorella vulgaris* cells.

From Figure 5.4 we see that the highest optical density can be achieved when factor A (% CO₂ in air) has level ‘-1’ and factor B (agitation speed) has level ‘1’. By consulting Table 5.1, the AB interaction corresponds to 4% CO₂ in air and agitation speed of 600 rpm (Appendix section 8.2.2 contains the explanation on how Figure 5.4 was constructed).

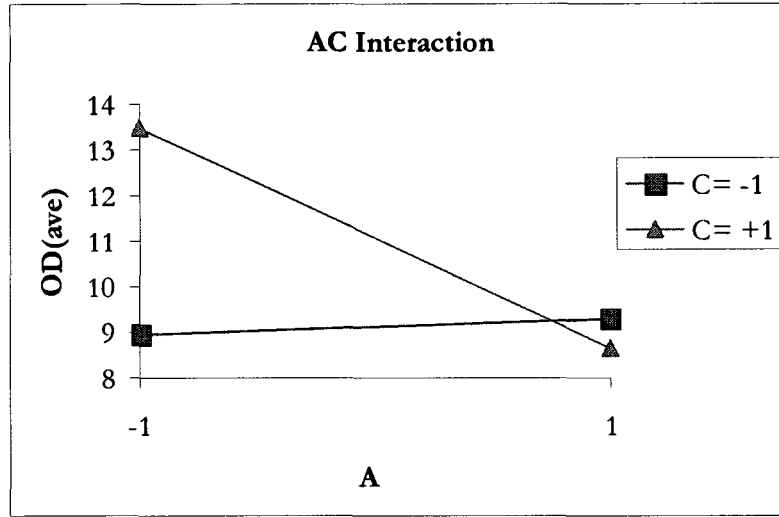


Figure 5.5: Effect of interaction of factors A (% CO₂ in air) and C (temperature) on the optical density of *Chlorella vulgaris* cells.

In Figure 5.5, the highest optical density occurs when factor A (% CO₂ in air) has level of '-1' and factor C (temperature) has level of '1'. By looking back at Table 5.1, the AC interaction corresponds to 4% CO₂ in air and temperature of 29°C.

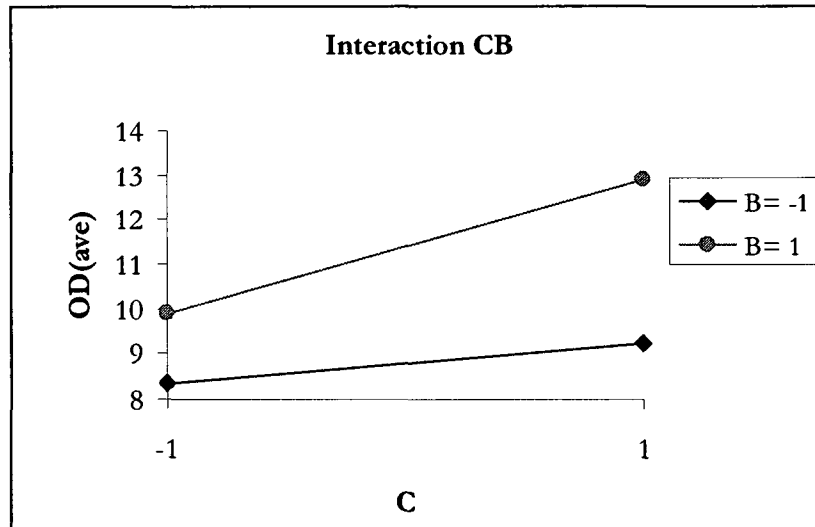


Figure 5.6: Effect of interaction of factors C (temperature) and B (agitation speed) on the optical density of *Chlorella vulgaris* cells.

By looking at Figure 5.6 we see that the highest optical density occurs when factor C (temperature) has level of '1' and factor B (agitation speed) has level of '1'. By looking at Table 5.1, the BC interaction corresponds to agitation 600 rpm and temperature of 29°C.

Considering the important main effects and important interactions, the best conditions for our system from the process variables studied are 4% CO₂ in air, temperature of 29°C, agitation speed of 600 rpm, and average light irradiance on the surface of the vessel of 450 $\mu\text{E}/(\text{m}^2 \text{ s})$. Since the light irradiance in our system did not have significant effect on the biomass concentration; we have chosen the lower irradiance as the cost of supplying light with lower irradiance would be less.

Light irradiance is of paramount importance in growth of photoautotrophic algae. The photosynthetic rate of algae increases with increase in light intensity until photosynthetic apparatus becomes fully saturated (Richmond, 2000). During saturation,

the photosynthetic rate is at the maximum and it doesn't change with increase in light intensity. However the increase of light intensity much above saturation may lead to cell inhibition (Chisti, 2007). The results from the fractional factorial experiments indicate that increasing light irradiance from $450 \mu\text{E}/(\text{m}^2 \text{ s})$ to $900 \mu\text{E}/(\text{m}^2 \text{ s})$ had no effect on the *C. vulgaris* biomass concentration. The reason for such result might be that light irradiance of $450 \mu\text{E}/(\text{m}^2 \text{ s})$ or $900 \mu\text{E}/(\text{m}^2 \text{ s})$ causes saturation of photosynthetic apparatus, however perhaps is not strong enough to cause cell inhibition. In such scenario the photosynthetic rate does not change and consequently the rate of biomass production due to change in irradiance does not change either. It is important to note that the results derived in these set of factorial experiments may not be applicable to different systems. For instance, experiments conducted under different process conditions can yield interdependence of light irradiance and temperature as presented in literature sources (Dauta et al., 1990; Miller et al., 1964; Sandnes et al., 2005).

In the literature, the authors stress that it is very important to study the interaction between various parameters for the optimization of the biomass (Ugwu et al., 2007). One of the important interactions in our system is 4% CO_2 in air and the agitation speed of 600 rpm. Perhaps at this combination of percent CO_2 in air and agitation speed we have a sufficient amount of CO_2 transferred to cells. Meanwhile the cells were not exposed to concentration levels that would cause inhibition (Yun et al., 1996). In addition, perhaps the sufficient agitation speed did not cause oxygen buildup in the vessel that could be detrimental to cell growth (Carvalho et al., 2006) or other gradients that can exist during conditions of insufficient mixing. Another important interaction in our system is 4% CO_2 in air and temperature of 29°C . Possibly at lower temperature and 4% CO_2 in air, the CO_2

would not diffuse fast enough to reach all the cells, and we would have mass transfer limitation resulting in lower biomass (Bird et al., 1960). Finally, the third important interaction in our system is the agitation speed of 600 rpm and temperature of 29°C. The relatively high agitation speed and high temperature allow for movement of the cells and allow more uniform distribution of nutrients.

Chapter 6 Conclusion and Future Work

As pointed out in the literature, currently it is the microalgal biomass production that is a bottle neck in cost effective production of lipids for biodiesel (Griffiths and Harrison, 2008). For this reason it was instrumental to devote time for research into microalgal biomass production.

The growth of *C. vulgaris* in batch photobioreactor showed various growth phases such as exponential phase, linear phase, stationary and declining phases. This result was in-line with those reported in the literature.

Acetic acid, which served as the only organic carbon, was used up by *C. vulgaris* very quickly during batch cultivation in photobioreactor. The initial acetic acid concentration of around 1.5 g/L was completely depleted within the first 20 hours of cultivation. In addition, during acetic acid utilization, the pH of the culture medium increased sharply and upon its depletion the pH became constant. Furthermore, phosphate-P utilization rate was the highest during acetic acid uptake.

From the literature it was seen that Tap culture medium was popular for growth of green microalgae *C. reinhardtii*, and none of the literature sources examined reported growth of *C. vulgaris* on this culture medium. We have found that *C. vulgaris* not only survived on 2X Tap culture medium, but it actually grew very rapidly. The batch growth of *C. vulgaris* in photobioreactor resulted in biomass concentration of about 6 g/L at end of 8-day cultivation period. *C. vulgaris* continued to grow after nitrogen depletion in the medium. This result showed that *C. vulgaris* can utilize its internal reserves of nitrogen.

C. vulgaris showed decreased growth when initial ammonium nitrogen concentration in the medium was 1000 mg/L, while when in presence of smaller concentrations of ammonium nitrogen, no inhibition was observed. This result shows that elevated concentrations of ammonium nitrogen can have detrimental effect to *C. vulgaris* growth. In addition, when initial ammonium nitrogen concentration was 500 mg/L or 1000 mg/L the pH dropped during the cultivation period. Increasing phosphate-P concentrations from 64 to 300 mg/L did not result in any alteration in *C. vulgaris* growth and pH never dropped during cultivation.

From eight fractional factorial experiments, best algal growth occurred during experiment when operational conditions were; temperature of 29 °C, sparging of 4% CO₂, agitation of 600 rpm, and irradiation with 900 $\mu\text{E}/(\text{m}^2 \text{ s})$ of fluorescent light. The maximum OD_{620nm} of 16.3 and corresponding biomass of 7.7 g/L was achieved on the last day of 9-day cultivation period. This is a relatively high concentration when compared to literature results.

In addition, this result showed better growth of *C. vulgaris* during fed-batch mode rather than during batch mode, with operational conditions being approximately the same for these two experiments. In addition, the lipid content of *C. vulgaris* at the end of this experiment was 3.7% on dry wt. cell basis, and lipid productivity was higher than that recorded in literature for *C. vulgaris* growth during nitrogen sufficient phase.

Statistical analysis from fractional factorial experiments revealed that change in light irradiance from 450 $\mu\text{E}/(\text{m}^2\text{s})$ to 900 $\mu\text{E}/(\text{m}^2\text{s})$ had no effect on biomass productivity. The best process conditions for growth of *C. vulgaris* as revealed by

statistical analysis were; temperature of 29 °C, sparging of 4% CO₂, agitation of 600 rpm, and irradiation with 450 μE/ (m² s) of fluorescent light.

Overall, we conclude that growth of *C. vulgaris* on 2X Tap culture medium in photobioreactor proved to be a promising way of biomass production. As pointed out previously, our research has focused on first-stage of two-stage process for lipid production. We have found the process conditions that can give high biomass productivity.

Next step in the research would be to perform experiment at best process conditions as determined from statistical analysis to verify the validity of statistical analysis. Subsequently, second-stage of two-stage process for lipid production can be performed. In this second-stage, *C. vulgaris* biomass that accumulated during first-stage would be exposed to nitrogen depleted culture medium. Lipid content of *C. vulgaris* should be tracked during this stage.

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Chapter 8 Appendix

8.1 Standard Curve

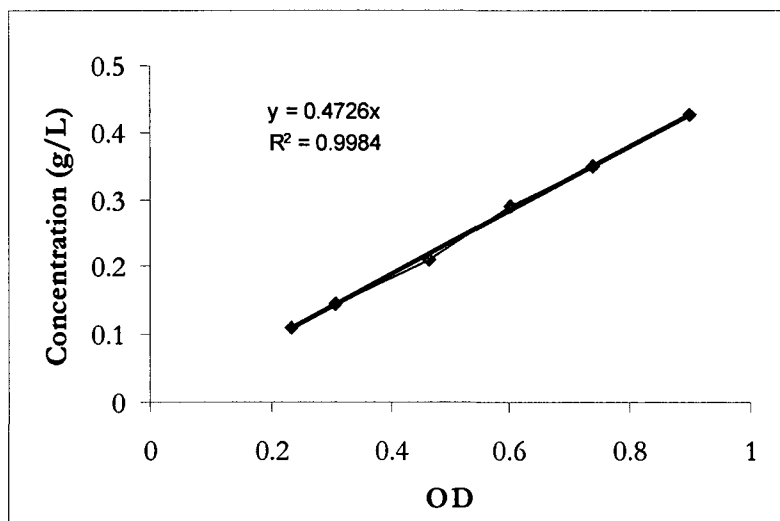


Figure 8.1: Standard curve relating optical density to concentration of *Chlorella vulgaris*.

8.2 Sample Calculations

8.2.1 Calculating Factor Effects

As previously pointed out in Factorial Design Section 3.7 of this report, factor effect l_A , corresponding to percentage CO₂ in air, can be estimated by the following relationship;

$$l_A = \frac{1}{4}(-1) + ab + ac - bc + ad - bd - cd + abcd)$$

Treatment combinations (eg. (1), ab, ac ...) in the above relationship correspond to the factor outcome (maximum OD) of Table 5.3 in the following way;

(1) corresponds to maximum OD of run 1

ab corresponds to maximum OD of run 2

ac corresponds to maximum OD of run 3

bc corresponds to maximum OD of run 4

ad corresponds to maximum OD of run 5

bd corresponds to maximum OD of run 6

cd corresponds to maximum OD of run 7

abcd corresponds to maximum OD of run 8

Hence, by consulting Table 5.3, estimate of effect of factor A is;

$$l_A = \frac{1}{4}(-7.6 + 9.5 + 7.8 - 16.3 + 9.1 - 10.3 - 10.7 + 9.5) = -2.3$$

8.2.2 Calculating Average Optical Density that Corresponds to Interaction

To find levels for interaction between factor A (% CO₂ in air) and B (agitation) that give highest *C. vulgaris* optical density, one creates graph as depicted in Figure 5.5 of this report. To construct this graph one needs to find four points as indicated in this figure. Since both factor A and factor B have each two possible levels, there are four possible interactions between A and B. Each one of these interactions has impact on *C. vulgaris*' optical density. For instance, to find the optical density (OD) resulting from interaction of factor A at level '-1' (4% CO₂ in air) and factor B at level '1' (agitation of

600 rpm) one must calculate the average OD resulting from two OD values determined at 4% CO₂ in air and agitation of 600 rpm. From Table 5.3 of this report one can see that these two OD values are 16.3 and 10.3, and by taking average of these OD's we obtain value of 13.3. Hence, interaction of 4% CO₂ in air and agitation of 600 rpm correspond to average OD value of 13.3. This is one of the four points as shown in Figure 5.5, the other three points are found in similar way. The purpose of this figure is to find interaction that gives highest OD of the four possible values.

8.3 Raw Data

Table 8.1: Results from a batch experiment in photobioreactor at 28°C, 600 rpm, 5% CO₂ in air, and 900 µE/ (m² s).

Time (h)	OD ₆₂₀	Acetic Acid (g/L)	pH	Ammonium-N (mg/L)	Phosphate-P (mg/L)
0.0	0.50	1.570	6.38	167.97	55.83
7.0	0.89	1.349	6.50		48.17
20.0	2.88	0.000	7.07	77.23	31.87
26.0	3.67		7.04		19.95
32.5	4.46		7.03		20.33
44.0	5.37		7.08	0.00	15.69
56.5	6.82		7.07		17.96
68.5	8.66		7.08		13.88
75.5	8.86		7.08		13.88
80.0	9.12		7.08		11.56
91.0	10.00		7.08		12.66
103.0	10.84		7.08		8.43
115.5	11.74		7.08		5.35
128.0	12.04		7.10		6.44
139.0	11.90		7.10		4.34
150.5	11.46		7.07		0.00
164.0	12.40		7.07		
172.5	12.88		7.07		
175.5	12.44		7.07		
188.0	12.82		7.08		
192.0	12.82		7.08		

Table 8.2: Results of ICP analysis for a batch experiment in photobioreactor at 28°C, 600 rpm, 5% CO₂ in air, and 900 µE/ (m² s).

Time (h)	B (mg/L)	Ca (mg/L)	Co (mg/L)	Cu (mg/L)	Fe (mg/L)	K (mg/L)	Mg (mg/L)	Mn (mg/L)	Mo (mg/L)	Na (mg/L)	Zn (mg/L)
0.0	4.31	26.23	0.912	0.867	1.607	142.5	19.46	2.89	1.48	14.22	10.94
7.0	3.91	23.42	0.815	0.692	1.236	124.7	16.43	2.59	1.36	12.67	10.00
20.0	4.32	23.67	0.886	0.797	0.879	122.0	15.56	2.81	1.52	13.50	11.01
26.0	3.91	21.25	0.803	0.700	0.914	109.5	13.09	2.52	1.35	12.44	9.94
32.5	4.32	22.46	0.892	0.741	0.877	115.6	13.40	2.74	1.52	13.35	10.94
44.0	4.27	21.15	0.966	0.726	1.389	114.0	12.87	3.23	1.51	13.53	12.33
56.5	4.26	20.51	0.918	0.737	1.252	109.7	12.27	2.79	1.50	13.74	10.94
68.5	4.23	20.09	0.890	0.708	1.198	106.0	11.67	2.77	1.51	13.39	10.93
75.5	4.21	19.41	0.882	0.703	1.187	105.0	11.35	2.74	1.50	13.24	10.87
80.0	4.18	19.37	0.864	0.669	1.171	104.3	11.09	2.70	1.50	13.05	10.72
91.0	4.18	18.95	0.925	0.727	1.230	102.1	10.92	2.76	1.49	13.25	10.82
103.0	4.21	18.96	0.892	0.689	1.197	104.4	10.63	2.72	1.48	13.76	10.80
115.5	4.14	18.36	0.858	0.660	1.159	100.5	10.02	2.67	1.47	13.03	10.59
128.0	3.97	17.64	0.825	0.617	1.101	95.1	9.21	2.56	1.42	12.51	10.25
139.0	4.13	17.68	0.849	0.636	1.130	100.4	9.35	2.63	1.46	13.00	10.53
150.5	3.97	17.34	0.873	0.661	1.146	96.5	8.81	2.59	1.42	12.61	10.20
164.0	4.09	17.68	0.856	0.648	1.133	100.3	8.74	2.61	1.44	12.98	10.40
175.5	3.71	15.97	0.764	0.570	1.012	91.2	7.62	2.35	1.32	12.02	9.40
188.0	4.06	16.71	0.835	0.607	1.110	103.2	8.20	2.57	1.45	12.75	10.28

Table 8.3: Results from flask experiments at various initial nitrogen concentrations. Algal culture was exposed to $\sim 29^{\circ}\text{C}$, $\sim 5\%$ CO_2 in air, and $\sim 200 \mu\text{E}/(\text{m}^2 \text{ s})$.

Time (h)	OD ₆₂₀				pH			
	211 mg/L	300 mg/L	500 mg/L	1000 mg/L	211 mg/L	300 mg/L	500 mg/L	1000 mg/L
0.0	0.12	0.37	0.13	0.13	7.14	7.16	7.14	7.03
15.0	1.11	0.90	1.08	0.86	7.29	7.15	7.26	7.06
27.0	2.84	2.71	3.21	2.94	7.81	7.83	7.79	7.76
39.5	3.70	3.57	3.74	3.31	7.73	7.77	7.73	7.76
51.0	6.27	6.04	5.73	4.36	7.73	7.68	7.69	7.71
64.0	7.57	7.30	6.87	5.23	7.65	7.53	7.53	7.52
74.5	8.98	8.30	8.06	5.66	7.70	7.61	7.55	7.52
87.5	9.94	9.84	9.42	6.78	7.73	7.58	7.48	7.53
111.5	11.68	12.04	11.18	8.68	8.01	7.84	7.65	7.65
123.0	11.74	12.76	11.46	9.20	7.82	7.67	7.44	7.38
136.0	12.80	14.24	12.34	10.48	7.86	7.69	7.44	7.32
147.0	12.72	13.24	13.72	10.82	7.84	7.69	7.40	7.27
160.0	13.40	13.92	13.68	11.24	7.82	7.68	7.32	7.22
171.5	14.46	15.06	14.50	12.08	7.82	7.66	7.26	7.12
183.5	14.30	15.10	14.52	12.64	7.93	7.79	7.32	7.12
208.5	15.02	15.78	15.68	13.98	7.74	7.60	7.05	6.73

Table 8.4: Results from flask experiments at various initial phosphorus concentrations. Algal culture was exposed to ~ 29°C, ~ 5% CO₂ in air, and ~ 200 µE/ (m² s).

Time (h)	OD ₆₂₀			pH			Time (h)	OD ₆₂₀		pH
	100 mg/L	150 mg/L	300 mg/L	100 mg/L	150 mg/L	300 mg/L		64 mg/L	64 mg/L	
0.0	0.08	0.08	0.12	7.27	7.34	7.45	0.0	0.12	7.14	
10.0	0.29	0.27	0.27	7.11	7.12	7.21	15.0	1.11	7.29	
24.0	2.74	2.92	2.56	8.04	8.01	8.01	27.0	2.84	7.81	
35.0	4.12	4.10	3.60	7.92	7.93	7.93	39.5	3.70	7.73	
48.0	5.18	5.84	4.94	7.89	7.90	7.95	51.0	6.27	7.73	
57.0	6.30	6.86	6.30	7.91	7.93	7.93	64.0	7.57	7.65	
71.5	8.22	8.72	7.72	7.91	7.90	7.91	74.5	8.98	7.70	
83.5	9.10	9.50	9.40	7.88	7.90	7.92	87.5	9.94	7.73	
96.5	10.56	11.04	10.06	7.87	7.90	7.92	111.5	11.68	8.01	
107.0	10.98	11.68	10.72	7.92	7.88	7.92	123.0	11.74	7.82	
119.0	11.68	12.44	11.60	7.88	7.89	7.94	136.0	12.80	7.86	
128.5	12.00	12.80	11.88	7.87	7.90	7.88	147.0	12.72	7.84	
143.0	12.90	13.54	12.48	7.88	7.90	7.93	160.0	13.40	7.82	
156.0	13.22	13.96	12.82	7.90	7.89	7.93	171.5	14.46	7.82	
168.5	13.76	14.42	13.70	7.93	7.87	7.92	183.5	14.30	7.93	
178.5	14.04	14.00	13.42	7.99	7.98	7.95	208.5	15.02	7.74	
192.0	14.38	14.90	14.00	7.98	7.93	7.99				

Table 8.5: Results for fractional factorial run 4.

Trial #1			Trial #2				Trial #3		
Time (h)	OD ₆₂₀	pH	Time (h)	OD ₆₂₀	pH	chl a (mg/L)	Time (h)	OD ₆₂₀	pH
0.0	0.47	6.19	0.0	0.63	6.14	34.20	0.0	0.53	6.26
5.0	0.56	6.28	21.0	3.79	7.10	114.14	44.0	7.02	7.22
17.0	1.49	6.68	44.0	6.54	7.07	225.49	117.5	13.96	7.24
28.5	3.84	7.06	72.5	9.58	7.06	281.27	164.0	16.00	7.27
41.0	6.63	7.07	101.0	11.24	7.07	334.53	212.0	17.80	7.25
52.5	7.20	7.07	121.5	13.14	7.10	352.70			
65.0	8.90	7.08	145.0	14.44	7.11	360.00			
75.5	10.12	7.10	172.5	15.80	7.14	370.00			
89.0	10.02	7.10	188.5	16.35	7.14	378.59			
101.0	10.94	7.09	205.0	17.04	7.17	385.86			
113.0	10.22	7.08							
126.0	11.44	7.07							
150.0	12.84	7.05							
162.5	12.90	7.03							
173.5	13.12	7.02							
185.5	13.10	7.01							
197.5	13.94	7.01							
209.0	14.41	7.06							

Run 1			Run 2		Run 3		Run 5		Run 6		Run 7		Run 8		
Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀	Time (h)	OD ₆₂₀
0.0	0.22	0.0	0.64	0.0	0.48	0.0	0.58	0.0	0.23	0.0	0.18	0.0	0.56		
6.0	0.16	18.5	0.82	5.0	0.60	24.0	1.00	10.0	0.28	5.0	0.20	5.0	0.68		
22.5	0.26	29.0	1.04	18.5	1.37	48.0	3.19	22.5	1.28	17.0	0.83	24.5	1.50		
31.5	0.67	43.5	1.83	31.5	3.26	56.0	3.75	33.0	1.79	29.0	2.47	46.5	3.34		
46.5	2.60	52.0	3.01	43.5	4.73	72.0	4.63	46.5	2.58	41.5	3.16	57.0	3.76		
54.0	3.24	68.0	4.30	55.0	5.96	79.0	5.05	59.0	3.24	52.5	3.58	70.5	4.55		
70.5	4.49	92.5	6.76	68.0	7.00	96.0	5.76	71.5	4.06	64.5	4.28	76.5	4.90		
80.5	5.04	100.5	7.45	78.5	7.38	106.0	6.10	82.5	4.50	77.0	4.44	94.0	5.70		
96.0	6.15	116.0	8.25	91.5	7.78	120.0	6.59	93.5	5.02	89.5	6.00	103.0	6.10		
118.0	6.72	122.0	9.00	103.0	7.42	124.5	6.73	104.0	5.84	100.5	6.34	119.5	6.91		
129.0	6.97	140.0	9.48	115.0	7.58	144.0	7.49	117.5	6.68	112.5	7.16	124.5	7.13		
142.5	7.20	147.0	9.46	127.0	7.16	157.0	7.88	131.0	7.18	126.0	7.86	142.5	7.77		
153.5	7.20	164.0	9.54	140.0	7.18	161.5	8.02	143.0	7.70	138.5	8.30	153.0	8.25		
166.5	7.18	188.0	9.46	151.0	7.50	186.5	8.87	153.5	8.24	150.0	9.50	166.5	8.36		
173.5	7.06	193.5	8.96	163.0	7.06	204.5	9.10	167.0	8.86	161.5	10.32	177.5	8.60		
190.5	7.02	211.5	9.12	187.5	6.64			180.5	9.40	172.5	10.22	190.0	9.05		
215.0	7.56							203.5	9.62	185.0	10.00	213.5	9.46		
								214.5	10.30	197.0	9.68				
										210.0	10.70				

Table 8.7: The pH for fractional factorial runs (except run 4).

Run 1			Run 2			Run 3			Run 5			Run 6			Run 7			Run 8		
Time (h)	pH		Time (h)	pH		Time (h)	pH		Time (h)	pH		Time (h)	pH		Time (h)	pH		Time (h)	pH	
0.0	6.28		0.0	5.93		0.0	6.24		0.0	6.20		0.0	6.43		0.0	6.29		0.0	6.15	
6.0	6.32		18.5	6.29		5.0	6.26		24.0	6.58		10.0	6.48		5.0	6.38		5.0	6.18	
22.5	6.66		29.0	6.47		18.5	6.58		48.0	7.09		22.5	7.02		17.0	6.85		24.5	6.87	
31.5	6.91		43.5	6.77		31.5	7.01		56.0	7.11		33.0	7.18		29.0	7.17		46.5	7.04	
46.5	7.18		52.0	7.01		43.5	7.01		72.0	7.13		46.5	7.20		41.5	7.19		57.0	7.07	
54.0	7.20		68.0	7.01		55.0	7.00		79.0	7.14		59.0	7.22		52.5	7.21		70.5	7.08	
70.5	7.20		92.5	6.89		68.0	7.00		96.0	7.13		71.5	7.24		64.5	7.23		76.5	7.10	
80.5	7.19		100.5	6.89		78.5	7.00		106.0	7.12		82.5	7.23		77.0	7.24		94.0	7.12	
96.0	7.14		116.0	6.86		91.5	6.99		120.0	7.12		93.5	7.21		89.5	7.22		103.0	7.10	
118.0	7.12		122.0	6.86		103.0	6.98		124.5	7.11		104.0	7.20		100.5	7.22		119.5	7.08	
129.0	7.11		140.0	6.86		115.0	6.97		144.0	7.11		117.5	7.18		112.5	7.20		124.5	7.08	
142.5	7.11		147.0	6.86		127.0	6.97		157.0	7.11		131.0	7.18		126.0	7.19		142.5	7.06	
153.5	7.11		164.0	6.87		140.0	6.97		161.5	7.11		143.0	7.17		138.5	7.19		153.0	7.05	
166.5	7.12		188.0	6.87		151.0	6.97		186.5	7.11		153.5	7.11		150.0	7.18		166.5	7.06	
173.5	7.13		193.5	6.88		163.0	6.97		204.5	7.13		167.0	7.10		161.5	7.17		177.5	7.06	
190.5	7.14		211.5	6.90		187.5	6.97					180.5	7.10		172.5	7.18		190.0	7.06	
215.0	7.15											203.5	7.11		185.0	7.16		213.5	7.07	
												214.5	7.11		197.0	7.15				
															210.0	7.14				

