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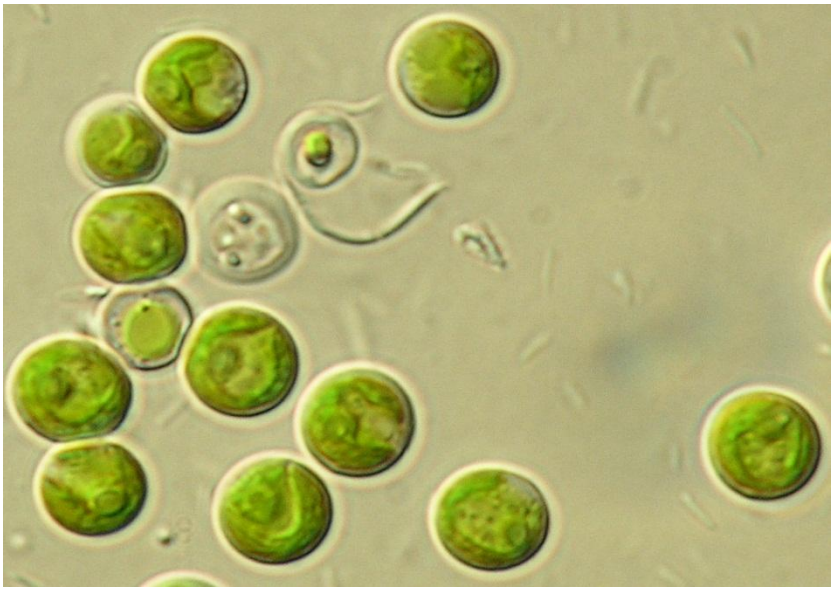

Inexpensive culturing of freshwater algae in a simulated warm environment using chicken manure medium

Master of Science Thesis [in the Master progamme of Biotechnology, CTH, Göteborg]

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"Inexpensive culturing of freshwater algae in a simulated warm environment using chicken manure"

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Preface

This master thesis project was done at SP Technical Research Institute of Sweden. I would like to express my appreciation to the project manager and also my supervisor Susanne Ekendahl for the opportunity to do my master thesis project at SP. SP is a great place to work and I have learned a lot from all the wonderful people and they have always made me feel welcome and appreciated. I would like to thank Susanne for her friendly help and guidance in the whole work. She gave me lots great suggestions to let me have confidence to finish this project. I would also like to thank Catrin Lindblad, Bo-Lennart Anderson, Kjell-Åke Anderson, Gunilla Heineman and other kindly colleagues in SP for their infinite support and help during my work.

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I also want to thank my friends and family who always support me unconditionally.

Yuanjun Li

2011

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Work done at

SP Technical Research Institute of Sweden

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Abstract

In recent years, growing algae for biomass production for different purpose has received large interest around the world, because it provides various potential advantages for biofuel production, carbon dioxide mitigation, wastewater treatment, food production and so on. In this study, the freshwater microalgae *Chlorella vulgaris* and a newly isolated *Scenedesmus* strain named *SIMRIS002* were cultured as cheap and simple as possible aiming at developing an inexpensive method that can be used in developing countries in areas without any facilities. A greenhouse was built which successfully simulated an acceptable warm environment. Plastic bags were manually modified as cheap photobioreactors for algae cultivation. Two volumes, 3L and 5L, were tested and the most suitable volume was found to be 3L for cultivations in plastic bags. One type of waste product (chicken manure) was used as a cheap culture nutrient source in the medium. Compared to previous studies, lower amounts of biomass was produced from the algae in this study when growing them in 2%, 6%, 10% and 14% (dry weight per liter) chicken manure. The reason could be that the low chicken manure level provided low concentrations of carbon, nitrogen and phosphorus, which were not enough for optimal algae growth. In addition, because the plastic bag cultures were placed under natural Swedish summer light conditions, more than 1000 W/m^2 light intensity was reached and therefore the algal growth could be inhibited. Three different gas-exchange methods were investigated during the plastic bag culturing: non-bubbling, manual bubbling and automatic continuous bubbling. Efficient air delivery and good mixing were provided by the bubbles. Thus, growth was clearly improved in automatically bubbled cultures and two times more biomass was found at the end of the cultivations compared to when no bubbling was used. Furthermore, the alkaline environment provided in chicken manure was not suitable for *C. vulgaris* but was preferred by *Scenedesmus SIMRIS002*.

Keywords: freshwater algae, *Chlorella vulgaris*, *Scenedesmus*, chicken manure, greenhouse, plastic bag, bubbling.

Contents

1. Introduction

In recent years, algae production has received large attention all over the world due to the wide utilization of algae. They are potential sources of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical, and fuel industries based on their attractive advantages. These include good growth and formation of large amounts of biomass, non-utilization of arable land, carbon dioxide fixation, high oil accumulation etc. Especially in the biofuel development, algae have been expected to be the most favorable energy "crop" which has the potential to replace fossil fuels in the future. However, algae research on basic biology and species selection and molecular characterization have lasted for a very long time, while the industrial production of algae, also at large scale, for different purposes have only been studied for the last decades. Commercial large-scale algae cultivation has started in the middle of 20^{th} century. In the following decades, large-scale algae production developed very fast in the whole world, mainly for food and nutrients supplement production. Nowadays, with the increased understanding of algal physiology and development of the bioprocess engineering, the research is more focused on the efficient biomass formation for biofuel production. Unfortunately, lots of unwanted problems still challenge scientists. For example, theoretical high lipid productivity has not been validated on a large commercial scale because of the inefficient culturing systems, restricted photosynthetic capability and productivity, and lack advanced technologies for harvesting and oil recovery for an efficient biofuel production [1].

This master thesis project was a part of the algae research within the biofuel development at SP Technical Research Institute of Sweden which studies algae growth in Sweden along the whole chain from algae production to use of algae biofuels. The aim of this master thesis project was to test a simple and cheap method to produce biomass from microscopic algae in specifically modified bioreactors, which can be used for manual algae biomass production in third world countries in areas without any facilities. Plastic bags were manually prepared and used as cheap bioreactors for algae cultivation in this study. A greenhouse was used to simulate the "tropical" conditions of developing countries. To optimize the biomass production, several nutrient media were tested and other growth factors such as temperature, light intensity and pH were also measured. The obtained results will be used for further research within SP and associated partners.

2. Background

2.1 Basic theory of algae

Algae constitute a large and diverse group of eukaryotic microorganisms, ranging from unicellular to multicellular forms [2]. According to this, two main types of algae can be distinguished: 1) macroalgae, also called seaweed that are normally growing in oceans and can reach considerable size (up to about 50 meters); 2) microalgae that are microscopic sized microorganisms, which can be found in marine and fresh waters.

Algae can carry out photosynthesis like other plants, but they lack water transporting tissue, leaves, roots and flowers. This makes them distinct from land plants. Several characteristics, such as their pigmentation, life cycle and basic cellular structure, are used to categorize algae into a variety of classes. The presence of chlorophyll is apparently the most important one. Algae contain chlorophyll and are thus mainly green in color. Some kinds of algae contain other additional pigments, such as xanthophylls and carotenoids, which make them appear brown or red [2].Four divisions have been initially introduced by W.H. Harvey (1811-1866) as green algae (chlorophyta), red algae (rhodophyta), brown algae (heteromontophyta) and diatomaceae [3].

Some algae are autotrophic (photosynthetic) organisms that use light as the energy source and other inorganic compounds such as CO_2 and salts as food. Some are heterotrophic (non-photosynthetic) organisms and require additional organic compounds as nutrient and energy sources (fig. 2.1). In addition, some algae are referred to as mixotrophy that can use a complex spectrum of nutritional strategies, combining photoautotrophy and heterotrophy, *i.e.* they have the ability to both perform photosynthesis and acquire exogenous organic nutrients [4].

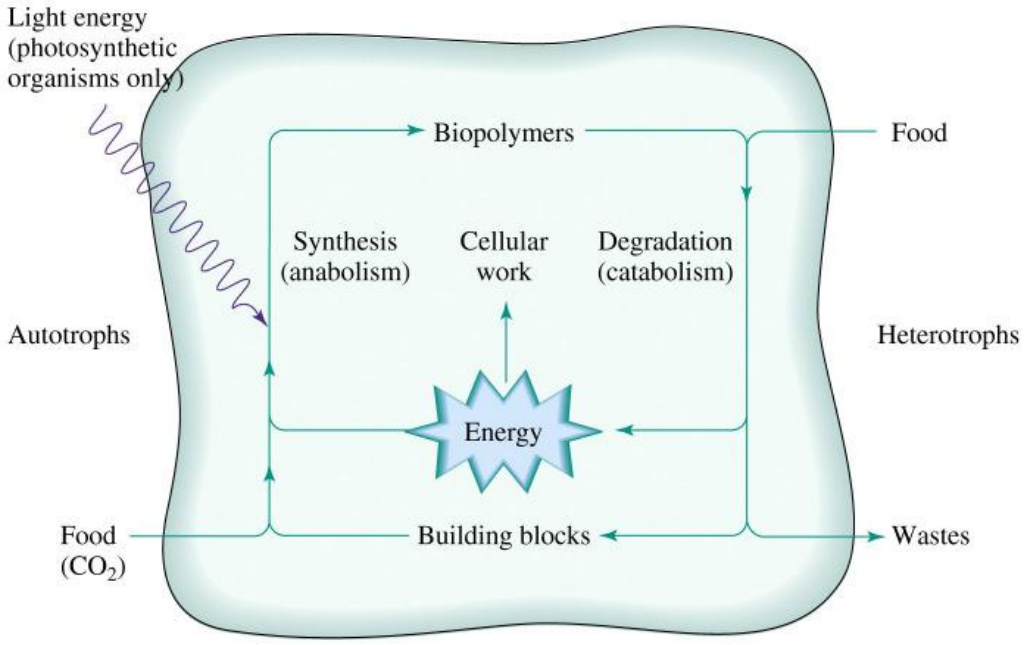

Figure 2.1 Metabolism processes of autotrophs and heterotrophs, taken from [46].

2.2 Economic application of algae

As one of the oldest life-forms on earth, algae have existed for millions of years. The utilization of algae by human beings also has a very long history. The first collection of algae as food appeared 2,500 years ago in China, in where people used edible algae like *Nostoc* to survive. However, the biotechnological investigation of algae is only a few centuries old. Of the 200,000-800,000 algal species that are believed to exist, only a few thousand species have so far been described, only a few hundred have been investigated for chemical content and only a hundred have been cultivated in industrial quantities [5],[6]. With the increased physiological investigation of algae, they have recently been realized as potential sources of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical, and environmental and fuel industries.

2.2.1 Algae for food, nutrients and feeding

In recent years, food insufficiency is becoming more serious with the highly increased world population. Conventional agriculture has not the ability to supply enough food and some new alternative and unconventional food sources have to be searched to feed this much more crowed world. At that time, algae appeared as potential food sources that have the ability to solve the starvation problems. They are growing in a water environment and will potentially not compete for arable land with other conventional food production. Besides the long history of direct utilization as food, algae have been indicated to contain high contents of different chemical compounds, which can be applied in human and animal nutrition. Table 2.1 presents a comparison of the general compositions of human food sources with that of different algae. The high contents of protein, carbohydrates and lipids in various algae species are the important reasons for considering algae as unconventional food sources.

Table 2.1. General composition of human food sources and different algae (% of dry matter) [10]

Commodity	Protein	Carbohydrates	Lipids
Baker's yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirulina maxima	60-71	13-16	6-7
Synechococcus sp.	63	15	11

In addition, it has been indicated that algae contain nearly all important vitamins and are rich in iodine, potassium, iron, magnesium and calcium. That makes algae interesting to develop as nutritional supplements. Feeding animals with algae is also developed for improving the nutritional value of meat products. For example, some researchers discovered that some kinds of algae contain high levels of essential fatty acids, such as the omega-3 fatty acids, DHA and EPA, and could be the original source of these acids in fish oil. Thus, feeding marine fish with special algae is becoming a way to enhance the omega-3 fatty acids content in fish oil [3].

2.2.2 Algae for environmental treatment

During the last century, human activities have caused a lot of environmental problems including high global green house gas emissions and declining water quality. It has been reported that since pre-industrial times global greenhouse gas (GHG) emissions have been increased with 70% between 1970 and 2004, of which especially the CO_2 emissions have increased up to about 80% [7]. A climate change has probably been the result of the increased emissions, and thus this trend will continue to grow over the next few decades. In order to mitigate the climate change many strategies have been suggested, such as change of individual lifestyle, behavioral pattern, and industrial management practices, to reduce energy use and GHG emissions [7]. Besides that, some new techniques are developed to eliminate those gases potentially responsible for global warming. Algae biomass production may be a possible way to reach the purpose due to its ability of CO_2 fixation via photosynthesis. By comparison to conventional forestry, agricultural, and aquatic plants, algae mostly present much higher growth rate and CO_2 fixation abilities, and require small growth areas, energy-consumption and costs. A more profitable system will be made when combining the algal CO_2 bio-mitigation processes with other processes such as wastewater treatment, biofuel production etc. [8]

As mentioned above, another important environmental effect of algae is wastewater treatment. Algae can treat wastewater without adding any chemical additives through a combination of nutrient uptake, elevated pH, and high dissolved oxygen concentration. Thus they can offer a safer, less expensive and more efficient approach to remove nutrients and metals from the wastewater than conventional treatment can do. [9]

2.2.3 Algae for future energy development

The high level of GHG emissions is mainly attributed to the large scale use of fossil fuels for transport, electricity, and thermal energy generation. With the global climate change, it has become increasingly important to develop effective technologies and unconventional renewable biofuels to reduce the fossil fuel usage. The first-generation biofuels is the liquid biofuel production based on sugar and starch crops (for ethanol) and oilseed crops (for biodiesel). But it is restricted due to the negative impact on global food markets like competitive consumption of crops or other food and competitive requirement of arable land. Thus, the currently developed technologies that use lignocellulosic biomass for biofuel production is referred to as the second-generation of biofuels, which can avoid the competition impact of food. The cost of cellulosic feedstock itself is lower than the first-generation feedstocks, however, the conversion technology for converting cellulosic biomass into liquid fuel is difficult and has not yet reached the scales for commercial exploitation [46]. Thus, with many advantages like large oil content (up to 75% of dry weight) [11], low land occupation and efficient photosynthesis, algae have been considered to be an alternative energy resource that can technically and economically overcome the problems associated with production of first and second generation of biofuels, and the liquid biofuels derived from algae is specifically defined as the third generation of biofuels [4]. Especially when combining algae oil production with carbon dioxide elimination, wastewater treatment or other biological processes, there is a great potential that an efficient and economical system can be built for biofuel production [4].

However, after several decades of investigations it is still not possible to produce algae biofuels in an economically efficient way compared to using fossil fuels. Lots of challenges still exist, such as lack economical and efficient culture system, no standard harvesting

techniques, and restricted oil recovery process that caused by the oxidation reactions of presented polyunsaturated fatty acids (PUFAs) and high moisture content of algal feedstock. In order to solve those problems, the most important things to work on are the development of screening techniques for selecting better algae strain, as well as the investigation of optimal culture conditions and production systems. Furthermore, some studies have suggested that the harvesting technologies already used in the food or wastewater treatment sector may suitably be used as possible solutions for the harvesting problems in biofuel production, and thermochemical liquefaction and pyrolysis may also be used as the most technically and feasible methods for conversion of algal biomass to biofuels [4].

2.3 Algae strains used in this study

In this study, three kinds of freshwater algae were investigated, *Botryococcus braunii*, *Chlorella vulgaris* and *Scenedesmus SIMRIS-002*. *Botryococcus braunii* (fig. 2.1 right) is a well-studied freshwater green alga. Its high ability of producing carbohydrates up to 75% of the dry weight makes it interesting to use as a renewable source in biofuel production [13]. In addition to carbohydrates, *B. braunii* also synthesizes classic lipids such as fatty acids, triacylglycerols and sterols [14]. Like all photosynthetic algae, *B. braunii* can grow by using light as energy source as well as CO_2 and other organic nutrients as carbon source. It has been indicated that the optimal growth conditions for *B. braunii* are a temperature of 23 ℃, a light intensity of 30-60 W/m ^{2}in a period of 12 hours per day, and a salinity of 0.15 M NaCl [13].

As one of the fastest growing microalgae, *Chlorella vulgaris* (fig. 2.2 left) belongs to a genus of unicellular green algae containing the green photosynthetic pigments chlorophyll-a, and -b. It has a spherical shape with 2-10 µm diameter and no flagella. *C. vulgaris* also requires CO_2, light, inorganic nutrients for growth. *C. vulgaris* has been used as a widely available microalgae species in the commercial applications for food and nutritional purposes. Recent research shows that *C. vulgaris* also has the capability of producing lipids at very high rate giving a high fuel quality which makes it a potential bioenergy producer. It has been reported that the growth of *C. vulgaris* can be optimized at the optimum growth conditions of 10% CO_2 (v/v) in air and with an applied 8h dark phase. The best temperature range was determined as 20 ℃ to 30 ℃ [16].

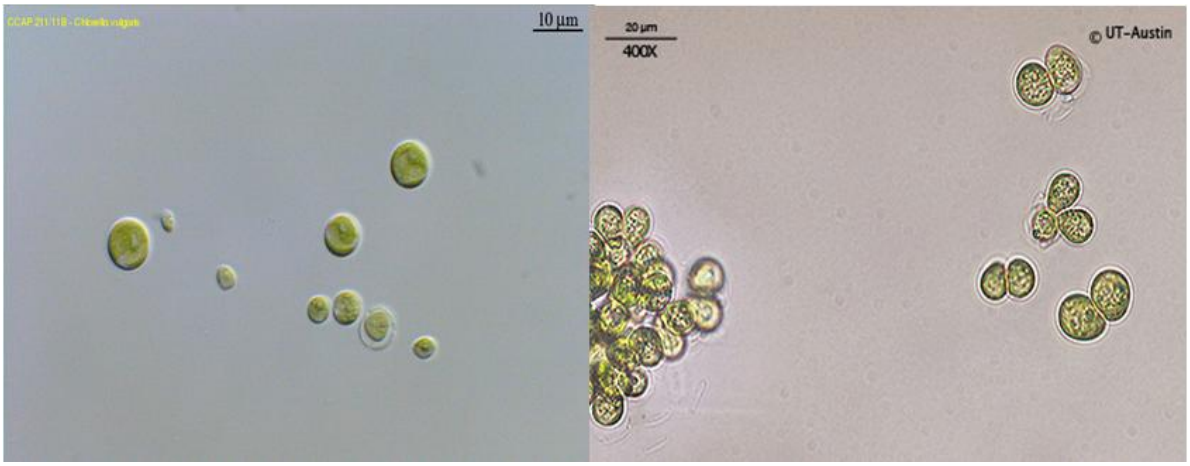

Figure 2.2. Image of *Chlorella vulgaris* (left) [15] and *Botryococcus braunii* (right) [36].

Another special freshwater alga genus used in this study was *Scenedesmus*, which is a small, nonmotile unicellular green alga with cylindrical, lunate or fusiform shape. Four or more cells are often aligned in a flat plate as colonies [18]. *Scenedesmus* is also one of the favorites of the high-yield oil formation for biodiesel production due to its high lipid content of 16-40% by dry weight. In addition, it can grow in high temperature environments with the optimal growth temperature between 30-35 ℃. Therefore it can grow well in tropical conditions that were simulated in this study [18]. The strain *Scenedesmus SIMRIS002* (fig. 2.3) used in this study was recently isolated from an artificial pond built for cleaning of industrial water in Linköping.

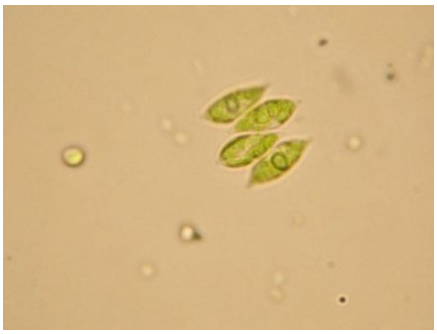

Figure 2.3. Image of *Scenedesmus SIMRIS002*. [Gustav Knutsson, SimrisAlg]

2.4Cultivationof algae

2.4.1 Culture medium used for algae cultivation

The correct maintenance of algal strains is dependent on the choice of growth media and culture parameters. It is important to consider the natural habitat and environment requirements of the algae species when choosing a culture medium. The freshwater green algal strains used in this study are characterized by a high specific growth rate, autotrophic metabolism, and a wide environmental plasticity [17]. Thus, two general freshwater media were selected for this study: Bristol medium and BG-11 medium which both are minimal defined media. Bristol medium is a well-known freshwater medium, which is a modification of the Bristol's recipe done by H.C. Bold [19], commonly used for growing photosynthetic green algae due to its lack of carbon-containing compounds.BG-11 medium is a modification done by Stanier R.Y. of theG-11medium of Hughes, Gorham, and Zehnderto to get a low phosphate content and a poor buffering character. This medium is considered as a general culture medium for unicellular strains [20].

The suitability of animal waste, *i.e.* chicken manure, as source of nutrients has been investigated and it was found that it could serve as nutrient enrichment giving a high yield of cells, high protein content and a lower level of various metals in the algae [40]. In this study, the low cost of chicken manure was another attractive benefit and encouraged us to choose it as a simple nutrient source of the culture medium.

2.4.2 Bioreactors used for algae cultivation

Bioreactors are used for culturing algae in a well-controlled environment to optimize the growth conditions and get the highest biomass production. Because of the large scale, simple design and low cost, open ponds are the most common bioreactors for algal cultivation in present commercial production [12]. However, some undesired situations like high risk of

contamination, evaporation of water and difficulties to control temperatures significantly limit the number of algal species that can be cultivated in open ponds and resulting low biomass. Thus, the photobioreactors offered as closed systems have been developed to overcome those problems [12].

Photobioreactors are culture systems generally incorporated with some light sources and have no direct exchange of gases and contaminants between reactors and the environment. So they result in better biomass productivity because better growth condition can be provided even though the cost is much higher than in open pond systems. Based on the design terms, photobioreactors can be classified into three major types: tubular photobioreactors, flat-plate photobioreactors and air-lift photobioreactors [18]. The advantages and disadvantages of different photobioreactors are summarized in table 2.2.

The flat-plate photobioreactors have been developed for decades and a lot of research has been performed. They are made of transparent flat plates in which algae are cultivated. Because of the transparent material used for the plates and the large surface area, a high utilization of solar energy can be reached. Thus, flat photobioreactors can provide higher photosynthetic efficiency compared to tubular versions [18].

Tubular photobioreactors consist of multiple tubular units and are formed as a flat loop. They provide a very large surface area and therefore are frequently built for outdoor cultivation. However, the scale of tubular photobioreactors is restricted by the limited lengths of the tubes [9].

Air-lift bioreactors have a low level and homogeneous distribution of hydrodynamic shear and therefore can potentially be used for industrial bioprocesses [18].

Table2.2. The characteristics of different photobioreactors.

Photobioreactors	**Photographs [21][22][23]**	**Advantages [18]**	**Disadvantages [18]**
Flat-plate photobioreactors		• Maximum utilization of solar light. • Has large illumination surface area. • Oxygen accumulation is low compared to that in tubular photobioreactors.	• Difficult to scale up • Difficult to control temperatures
Tubular photobioreactors		• It has a large illumination surface area. • High productivity. • Suitable for outdoor mass cultivation.	• Photoinhibition is a common occurrence. • Difficult to control temperature. • When scaled up, mass transfer becomes a problem and light distribution is not very effective.
Air-lift photobioreactors		• Low level and homogeneous distribution of hydrodynamic shear. • Great potential for industrial bioprocesses	• Length limitation.

Column photobioreactors (fig. 2.4) can be considered as one type of tubular photobioreactors. It has the highest volumetric mass transfer rates and the best controllable growth conditions. It achieves the highest effective for biomass production due to the mixing possibility by injecting compressed air from the bottom. In this study, one kind of column photobioreactors, polyethylene sleeves, were used for our outdoor cultivations, because these reactors are easy to produce and inexpensive to replace.

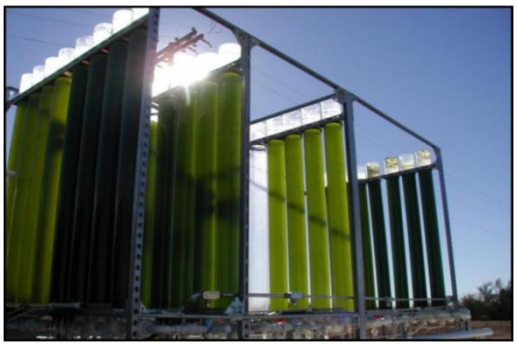

Figure2.4. Photograph of column photobioreactors. [41]

2.5 Environment control of algae cultivation

2.5.1 Temperature control

A suitable environmental temperature should be reached in order to get a high specific growth rate and biomass productivity of algae. When growing algae in a natural way the temperature is depending on the actual variations of the climate in the specific geographic area and hard to control manually. This is especially important to take into consideration in this study, in which the purpose was to develop an efficient and cheap way to grow algae in developing countries, which mostly are located in tropical areas. In such areas the average temperature will be greater than 18 ℃during all months of the year, especially the tropical wet climate areas in where the monthly temperature averages vary from 24 ℃ to 30 ℃ [42].Figure2.5 shows the temperature in different areas of Sweden in summer (June) and winter (December), which ranges from -2 ℃ to 16 ℃ in June and -16 ℃ to 4 ℃ in December [24]. According to this, it can be considered that winter is not a suitable season in Sweden for growing algae. Even in summer, the temperature in Sweden is still not enough to reach the perfect conditions for growth of the selected algae strains in this study (as described in 2.3, the optimal temperature conditions for all strains are higher than 20 ℃). Thus, a greenhouse was built for this study to simulate a high temperature environment and an air conditioner was provided to keep it constant.

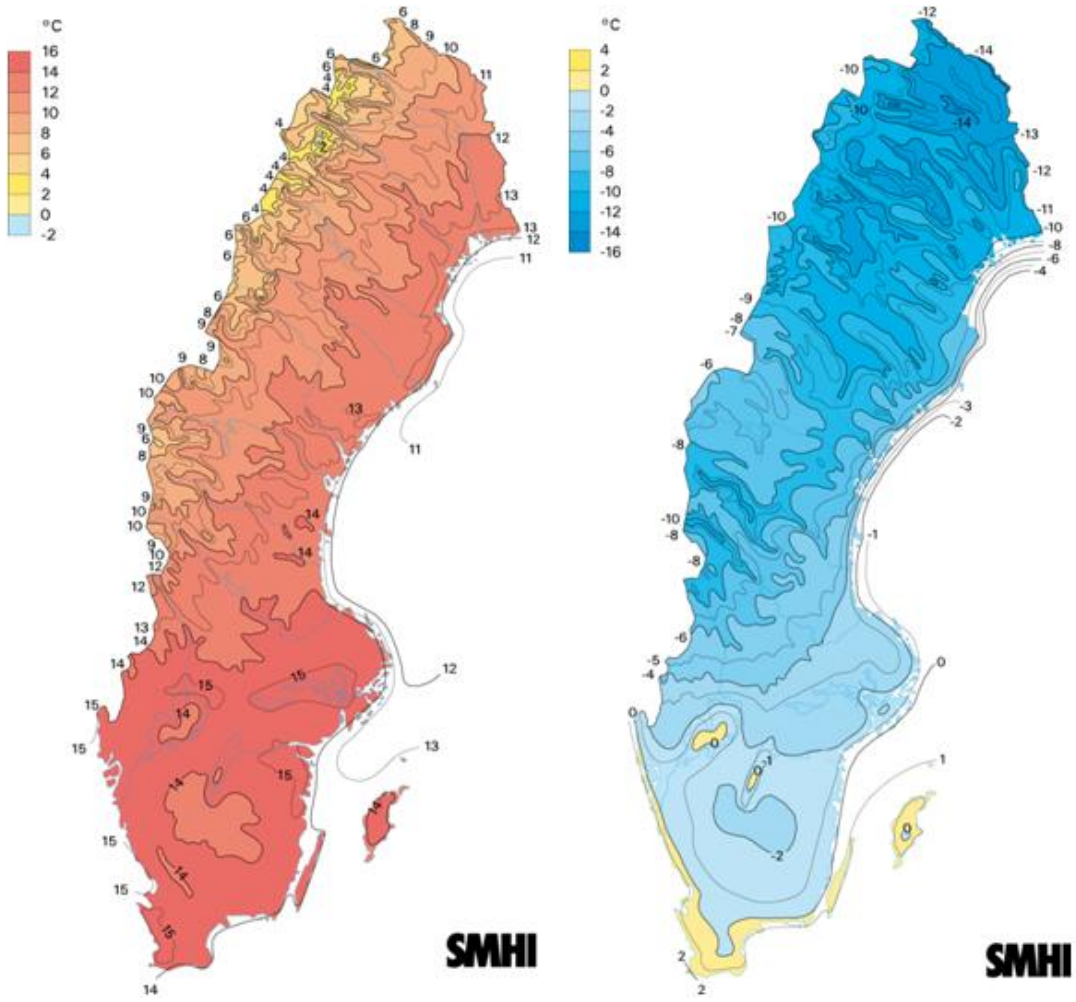

Figure 2.5. The variation of temperature in Sweden in June (left) and December (right) in the period from 1961 to 1990. [24]

2.5.2 Light conditions

As the energy source of photosynthesis is light, it is a necessary growth parameter for autotrophic algae. When growing algae outdoors, free natural sunlight is mainly used for lowering the cost of production and for taking advantage of the sun's energy. But, relative to its economic benefit, natural light has several unfavorable restrictions that could significantly influence the cultivation efficiency.

Natural light can theoretically be divided into several components on the basis of the wavelength, such as radio waves, visible light, gamma and X-rays. Only the visible light with the wavelengths of 380 nm to 750 nm can be utilized in photosynthesis. Thus, only a small portion of the total light energy can be captured through photosynthesis [24]. Furthermore, the availability of natural light is extremely depending on the climate of the area in which algae are growing. Both daily and seasonal variations in light intensity and the available hours of light can affect the algal production.

As already mentioned this study aims at developing culturing methods for tropical areas. In these areas the average of sun light in whole year range from 200-500 W/m^2 [43]. In Sweden, the numbers of hours with available sunlight differ a lot between summer and winter. As shown in figure 2.6, the range of sunlight time is from 160 to 340 hours in June, and only from 0 to 60 hours in December. Figure 2.7 shows the variations in mean light intensity,

which is between 140 to 200 kWh/m^2 in June and 0 to 20 kWh/m^2 in December [24]. These data confirm that both sunlight hours and light intensity of winter in Sweden is not suitable for this investigation. So this study was processed in summer time from April to September.

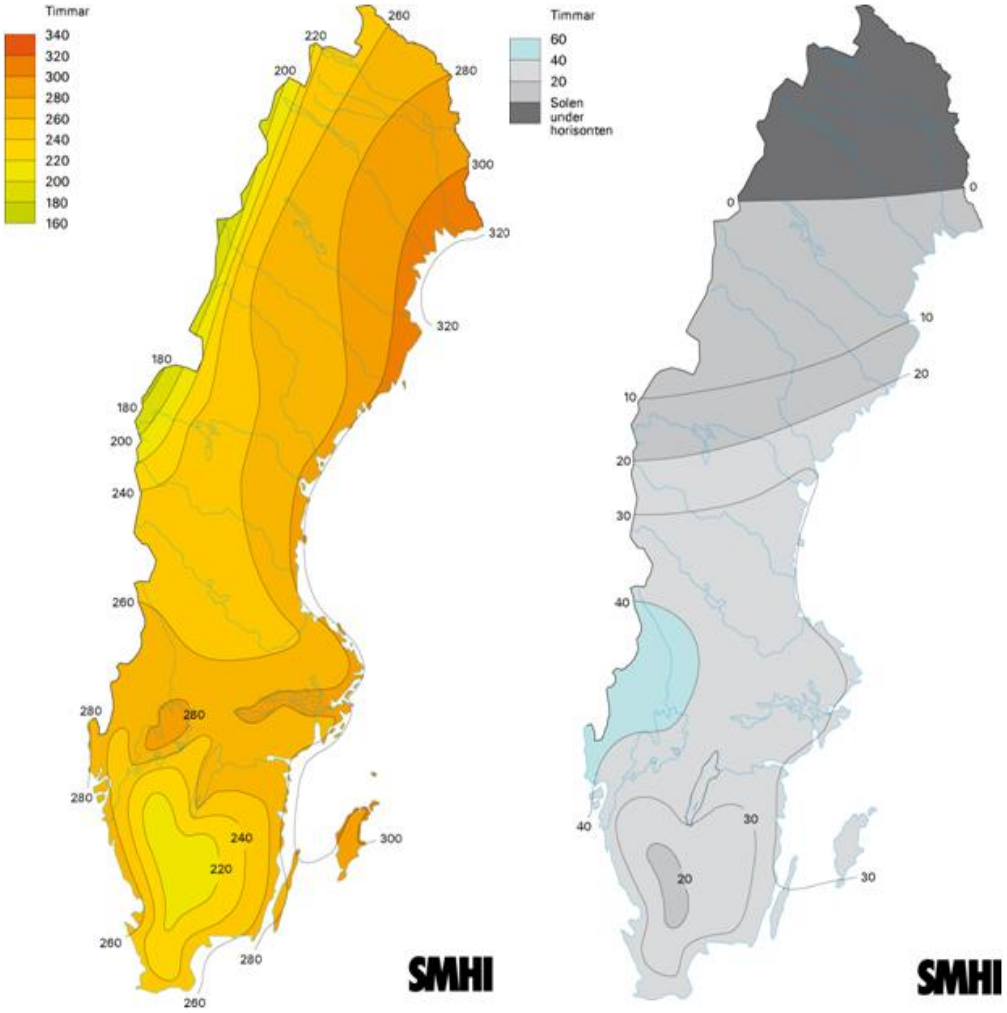

Figure 2.6. The hours of available light of Sweden in June (left) and December (right) in the period from 1961 to 1990. [24]

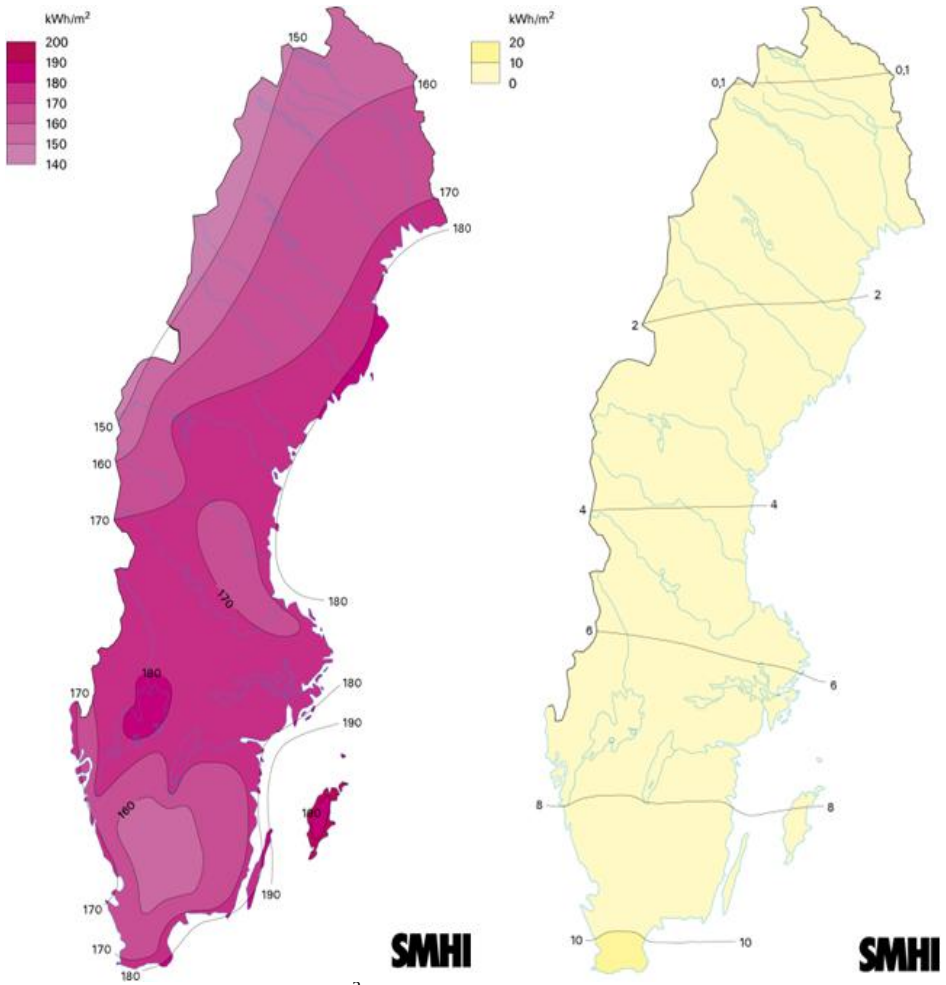

Figure 2.7. The mean sun intensities (kWh/m^2) of Sweden in June (left) and December (right) in the period from 1961 to 1990. [24]

2.5.3 Greenhouse used for cultivation

A greenhouse is a structure generally built for growing plants or other photosynthetic microorganisms. It consists of transparent materials, frequently glass or plastic, to allow the solar radiation from the sun getting inside. During daytime, the roof and walls of the greenhouse work as a barrier to lower air exchange with the outside environment. Therefore the air inside the building can be heated up quickly by the heat remaining inside. In addition, the roof and walls can also reflect the thermal energy which is re-radiated from those heated structures or plants as a mirror to increase the inside temperature. But, without sunshine, the inside temperature of a greenhouse drops down immediately during the night [25].

Actually, a greenhouse provides a controllable individual environment that could be easily changed based on different requirements. For example, a cooling or heating system could be built in order to maintain a constant temperature. Different weather conditions can also be simulated in a greenhouse.

2.6 Growth measurement of algae cultivation

2.6.1 Cell counting

The growth properties of algae can be determined by measuring the cell number per unit volume of cell suspension. Thus, the number of cells in the algal suspension should be counted to calculate the cell concentration by using for example a device called haemocytometer counting chamber. In the past, the haemocytometer was developed for counting cells in blood samples. But now it is widely used for determining the growth of algae and other microorganisms. It consists of a mirror-like polished surface, which has a grid etched upon it, and a special thick cover slip. When counting cells, the cover slip is placed on top of the surface giving a defined height and a drop of well-mixed algal suspension is introduced beneath the cover slip by using a pipette. Then, the chamber is placed under a microscope and the counting grid is brought into focus at low power [26].

In this study, one type of haemocytometer named Bürker counting chamber was used (fig. 2.8). The etched grid consists of 9 gridded fields shown as A-box. Each A-box has 16 smaller squares marked as B and, each B includes C-box, D-box and E-box. The areas of all these boxes are well-defined [44] and since the height of the Bürker chamber is 0.1 mm the volume counted can be calculated. In this study, 15 of the E-boxes were used for counting and they have each a volume of 1/250 µl.

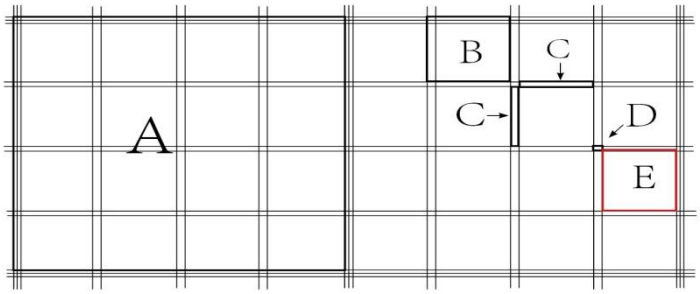

Figure 2.8. Etched grid of Bürker counting chamber.

2.6.2 Biomass measurement

The biomass concentration of algae can be determined by measuring either dry mass or wet mass. However wet mass is not frequently used due to the lower accuracy in the results resulting from the water content. Dry mass is given as total dry weight of biomass per volume of culture. Cell separation should be done by membrane filtration or centrifugation. The filter membrane or centrifuge tubes should be pre-weighed. After separation, the filter membrane or centrifuge tubes are dried at high temperature around 100 ℃, and then weighed immediately to avoid air-moisture absorption [12].

2.6.3 pH measurement

pH is another important growth factor for algae. Different algal species have different favorite pH ranges and outside the optimal range the growth is affected resulting in slower specific growth rates. Both *Chlorella* and *Botryococcus* are growing very well at around pH 6. *Scenedesmus* prefer alkaline medium between pH 9 to 10. In addition, algae can also change the pH of the medium during cultivation. When using CO_2 as carbon source, rapid growth of algae can cause the pH to rise due to photosynthetic uptake of inorganic C.

3. Material and methods

In this study, three kinds of freshwater green algal strains were selected. *Chlorella vulgaris* (UTEX 259) [35] and *Botryococcus braunii* (UTEX 572)[36] were both purchased from UTEX the Culture Collection of Algae, Austin, Texas, USA. Both of them were obtained as agar slants. The *Scenedesmus SIMRIS002* strain was kindly obtained as a 50 ml liquid sample from SIMRIS Alg AB, Sweden [37]. Before used, all algae strains were pre-cultured in 100ml culture media (Bristol and BG-11) in 250 ml glass bottles in the laboratory under 16/8 h light-dark cycles using 18 W fluorescent lamps and that they were mixed manually by shaking the bottles daily. These bottles were restarted every 15 days to always have fresh cultures available.

3.1 Growth media

C. vulgaris was grown in Bristol medium which was prepared according to the recipe given by UTEX [38]. The concentrations of nutrients in this medium (mg/l of sterile water) were: 250 $NaNO_3$, 25 $CaCl_2{\cdot}2H_2O$, 75 $MgSO_4{\cdot}7H_2O$, 75 K_2HPO_4, 175 KH_2PO_4 and 25 NaCl. *B. braunii* was grown in BG-11 medium which was prepared according to the recipe of ATCC medium 616 [39]. The concentrations of nutrients in this medium (mg/l of sterile water) were: 1500 $NaNO_3$, 40 K_2HPO_4, 75 $MgSO_4{\cdot}7H_2O$, 6 Citric acid, 6 Ferric ammonium citrate, 1 EDTA, and 20 Na_2CO_3.

Two other types of media were used to compare with the general culture media. For the first medium the waste product chicken manure was selected to be the source of nutrient and thus to stimulate culturing algae in a natural way. Chicken manure is also easily available in developing countries. The product was obtained as Hönsgödsel from Weibulls, Sweden. According to the content given by the manufacturer (appendix, table 8.3), suitable amount of dry chicken manure products was weighted up in diluted in sterile water to reach a similar content of nutrients as in Bristol medium. The concentrations of nutrients (mg/l of sterile water) were: 280 organic nitrogen (N), 210 phosphorus (P_2O_5), 175 potassium (K_2O), 70 magnesium (MgO), 70 sulphur (S), and 630 calcium (CaO). For the second medium liquid plant nutrients (VitaGro from Bayergarden, Sweden) was added as nutrient source. It was prepared as described in the information sheet of the manufacture (appendix, table 8.4) and the concentrations of nutrients in this medium (µg/l sterile water) were: 168 N, 60 P, 213 K, and 0.3 MgO.

All prepared media were sterilized in an autoclave at 121 ℃ for 20 min and cooled to room temperature (23 ℃) prior to use.

3.2 Chicken manure pre-test in the laboratory

Before tests in larger scale with chicken manure, a small test was done in the laboratory to compare algal growth in different media. Two freshwater green algae strains: *Chlorella vulgaris* and *Botryococcus braunii* were selected for this test. Autoclaved chicken manure suspension as described in 3.1 was used as stock solution and diluted to give 10% and 100% solutions, respectively, for the first part. *C. vulgaris* was cultured in 100 ml of Bristol medium, liquid plants nutrients medium, 10% and 100% chicken manure medium respectively. *B. braunii* was cultured in100ml of BG-11 medium, liquid plants nutrients medium, 10% and

100% chicken manure medium, respectively. All cultivations were done in 250ml glass bottles at room temperature (23 ℃) and placed under light that was applied by 18W fluorescent lamps (OSRAM L18w/77) with a light and dark cycle of 16:8 hours. Cell concentration of each sample was measured by counting the cell number approximately twice per week during the test.

After the small test described above, a second test was done to compare the effects of different chicken manure concentrations. The original chicken manure stock solution was diluted to give 2%, 6%, 10% and 14% chicken manure media for this test. *C. vulgaris* was cultivated in these solutions respectively. Since *B. Braunii* exhibited slow growth in the chicken manure media of the first test, this strain was excluded from further experiments. Thus, the algae strain *Scenedesmus SIMRIS002* was used for comparison with *C. vulgaris*. All cultivations were done in the same way as described above. Duplicate cultures were prepared for each alga in every medium and the whole set of cultures were repeated one more time. Cell counting was done every day and the biomass content at the end of cultivations was measured in both replicates, but the pH of initial media and at the end of each cultivation was only measured in the second set of cultures.

3.3 Plastic bag cultivation in the greenhouse

A greenhouse was built at SP to simulate the tropical temperature conditions for algae cultivation (fig. 3.1). Before doing plastic bag cultivation, *Chlorella vulgaris* and *Scenedesmus SIMRIS002* were first cultured in aquaria with a total volume of 10L in the greenhouse to check their heat-tolerance properties. After this testing, an air conditioner was established to keep a constant temperature of 28±1 ℃ inside the greenhouse.

Figure 3.1. Photograph of the greenhouse built at SP.

Plastic bags were modified to contain two different volumes for the plastic bag cultivations in this study: 3L and 5L. The material for the plastic bags was obtained from Rigef Plastemballage AB, Sweden (fig. 3.2). *Chlorella vulgaris* and *Scenedesmus SIMRIS002* were grown in 10% chicken manure medium in the plastic bag cultivations.

Figure 3.2. Photograph of the plastic bag modified for algae cultivation.

Two tests were done during the plastic bag culturing. Test 1 (fig. 3.3a) was a pre-test for adjusting the properties of the plastic bags, which cannot be described further in this report. Two gas-exchange methods were involved in test 1: non-bubbling and automatic bubbling. Automatic continuous bubbling was achieved by using ELITE Air pumps to continuously pump air into the algae suspension to form bubbles. The ELITE Air pumps (Art. # A-799) were obtained from Rolf C. Hagen Inc, Canada. Test 2 (fig. 3.3b) mainly investigated the effects of different gas-exchange methods. Besides the non-bubbling and automatic bubbling methods as in test 1, manual bubbling was additionally used as a more economical bubbling method compared with the automatic bubbling method. Manual bubbling was achieved by manually blowing air bubbles into the algae suspension once per day. The same air pumps were used as for automatic bubbling. Besides that, the non-bubbled plastic bag cultures were mixed by shaking manually every day. Duplicate cultivations were prepared for every condition in test 2. Biomass of each culture was measured during the experiments as well as the pH value of the suspension at the end of cultivation was measured too. The temperature inside and outside the greenhouse and the light intensity were measure every minute during culturing.

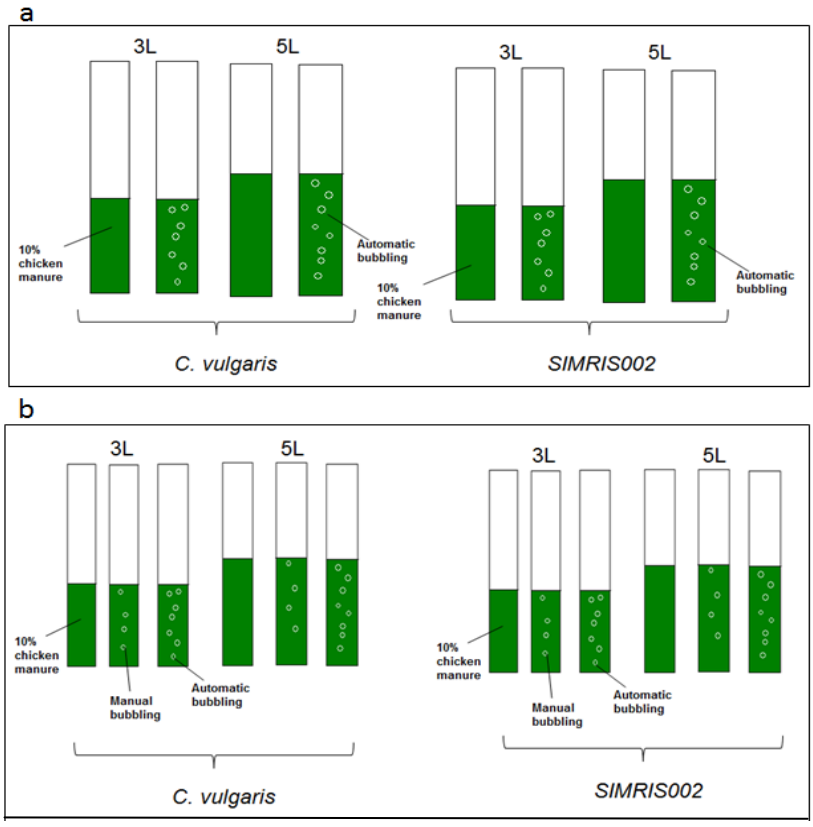

Figure3.3. Process image of plastic bags cultivation test 1 (a) and test 2 (b).

3.4 Sampling and analysis of biomass

The growth of algae was measured by cell counting as described in 2.6.1. If the cell number per square was over 30, the algal suspension was diluted to make the cell number low enough for counting.

For drawing the growth curves, the cell concentration of each sample was calculated according to the following formula:

Cell concentration (n/ml) = total cell number / squares $\times 4 \times 10^6$

The biomass of the entire cultures was measured by weighing dried sample of the culture suspensions. Filterpapers (Whatmann GF/C 1.2µm, 55mm in diameter) were stored in a constant room with controlled temperature (23±2 ℃) and moisture content (50±5%) and were pre-weighed. Then, samples of 30ml of each alga culture were filtered through the filterpapers. They were dried over night at 100 ℃ and then weighed again at the same room temperature and moisture. The biomass content was calculated as follows:

Biomass (g/l) = (Total weight (g) – Filterpaper weight (g)) $\times$ 1000ml / 30ml

3.5 pH, temperature and light intensity measurements

In this study, the Sym**pH**ony SP80PD equipment (VWR, Sweden) was used for pH measurement. The pH value in the initial culture medium was measured before cultivation and after culturing, the pH in the final suspension was measured again. The temperature inside the greenhouse was measured every minute by the equipment, Vaisala Weather Transmitter WXT510 (Vaisala, Finland). Sun radiation was also measured minutely by a Pyranometer equipment (Kipp & Zonen, USA).

4. Results

4.1 Effect of initial culture media

In the first part of this study, both *Chlorella vulgaris* and *Botryococcus braunii* were cultured in the laboratory in the general liquid plant nutrients media and chicken manure media, respectively. The images of the flask cultures in fig.4.1 show that *C. vulgaris* (b) grew apparently better than *B. braunii* (a) in all media, especially in 10% chicken manure.

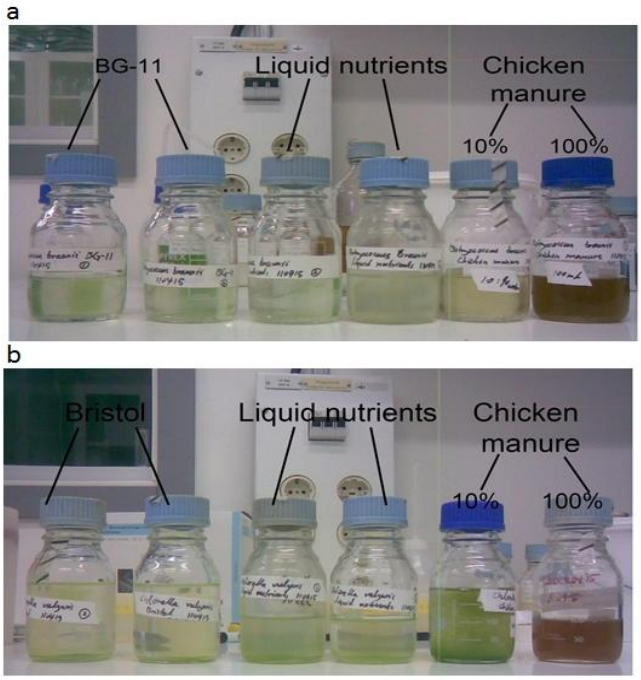

Figure 4.1. Photographs of algae flask cultivation in different culture media. (a) *B. braunii* cultivation in BG-11, liquid plant nutrients medium and 10% and 100% chicken manure media; (b) *C. vulgaris* cultivation in Bristol, liquid plant nutrients medium and 10% and 100% chicken manure media.

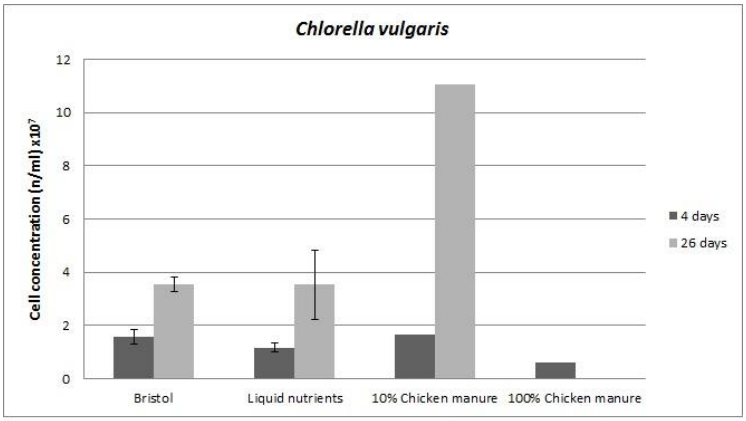

Figure 4.2. Cell concentrations of *C. vulgaris* when cultured in Bristol, liquid plant nutrients medium and 10% and 100% chicken manure media in the beginning (4 days) and the end (26days) of the culture. Data shown are average from duplicate cultures with standard deviation as error bars.

Cell numbers were counted for *C. vulgaris* to calculate the cell concentration in each medium (fig. 4.2). The growth was similar in 10% chicken manure and in Bristol media at the beginning, but the growth in chicken manure medium was significantly improved as seen

from the almost three times higher cell numbers after 26 days of cultivation in this medium as compared to the Bristol medium. In contrast, algae grew slower in 100% chicken manure and were even dead at the end of the test.

4.2 Effect of chicken manure concentration

As the 10% chicken manure medium provided a better growth for the algae investigated, different concentrations of chicken manure was used to test the effects on algae growth. In this experiment, *B. braunii* wasn't used due to its slow growth that appeared in the first test. Another algae genus, *Scenedesmus*, was selected based on its ability of growing fast at high nutrients contents and its good heat-tolerance, which would be suitable for further cultivation at tropical weather conditions. Four chicken manure concentrations were prepared as 2%, 6%, 10% and 14% of the stock solution. It was clear that the response of the algal strains to the chicken manure concentration was variable both in cell and biomass concentrations. The cell concentration (n/ml) of *C. vulgaris* was decreased with the increase in chicken manure level (fig. 4.3a). However, when investigated under the microscope, the cells in 2% chicken manure medium were much smaller than in the 14% chicken manure medium, and therefore the biomass concentration (g/l) of *C. vulgaris* was lower at low chicken manure concentrations (fig. 4.3b). A visible difference was observed from the gradual color change in the culture flasks (fig. 4.4). Furthermore, another situation should be mentioned. The cells were gathered as colonies in 10% and 14% chicken manure and thus the cell numbers were difficult to count under the microscope and might cause incorrectly low cell concentrations.

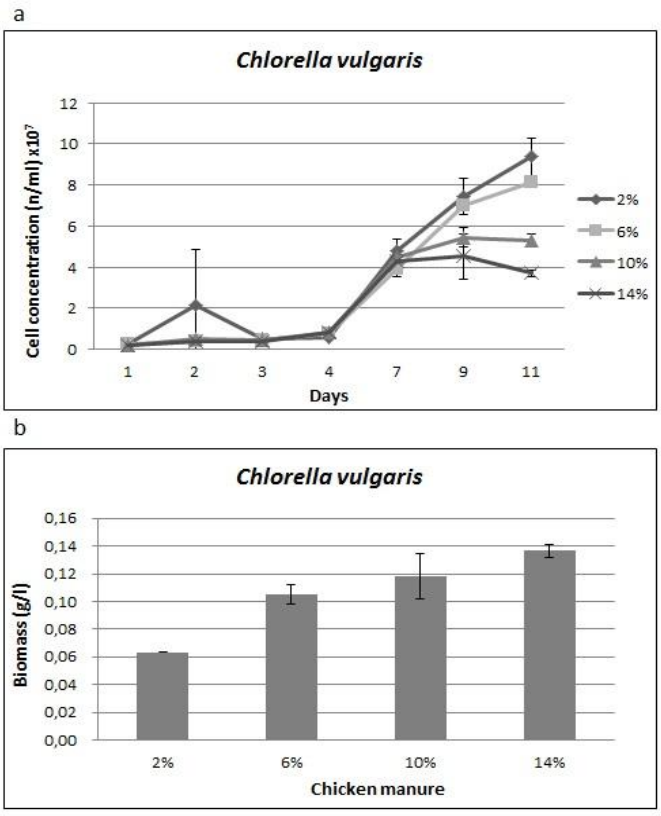

Figure 4.3. (a) Cell concentrations (n/ml) of *C. vulgaris* at different chicken manure concentrations; (b) Biomass concentrations (g/l) of *C. vulgaris* at different chicken manure concentrations at the end of cultivations. Data shown are average from duplicate cultures with standard deviation as error bars.

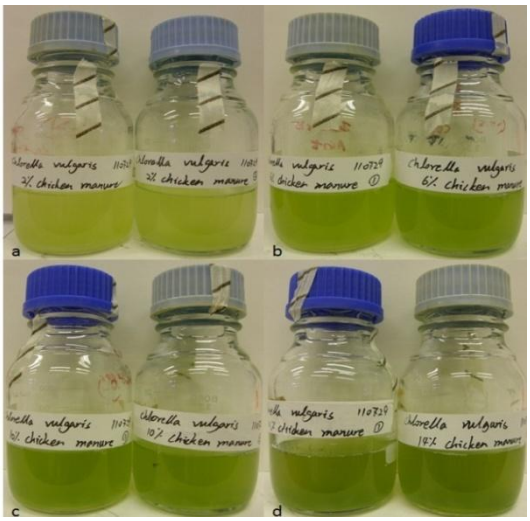

Figure 4.4. Photographs of *C. vulgaris* culture flasks with (a) 2%, (b) 6%, (c) 10% and (d) 14% chicken manure media at the end of cultivations. A gradual color change from light green to dark green was seen with the increasing concentrations from 2% to 14%.

Scenedesmus SIMRIS002 provided a much higher cell concentration (n/ml) grown in 14% chicken manure medium, more than two times higher than at 2% chicken manure medium as shown in fig. 4.5a. The biomass concentration (g/l) was relatively increased with the increase of chicken manure concentration (fig. 4.5b). The microscopic observations of *SIMRIS002* were similar as for *C. vulgaris* in that almost only small round-shaped cells were obtained in 2% chicken manure medium while many large fusiform shaped cells were present in 14% chicken manure medium. However, there were no innumerable big colonies present in the *SIMRIS002* cultivations. A similar color change from light green to dark green as for *C. vulgaris* was also observed in the culture flasks of *SIMRIS002* (fig. 4.6).

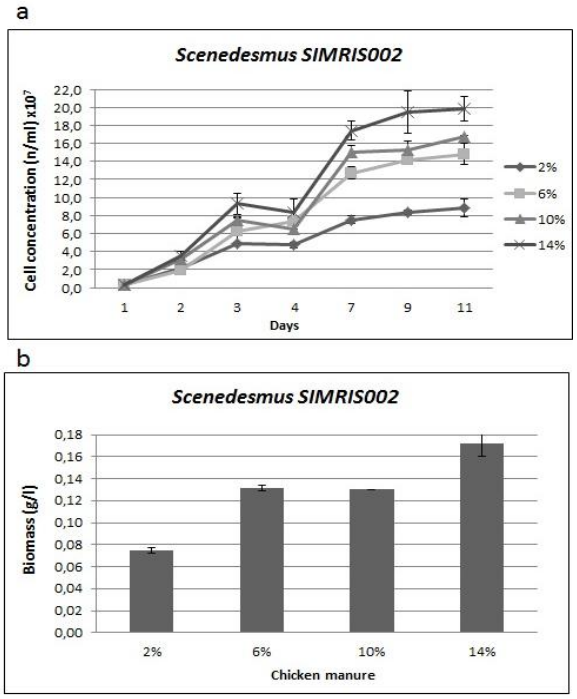

Figure 4.5. (a) Cell concentrations (n/ml) of *SIMRIS002* in different chicken manure concentrations; (b) Biomass concentrations (g/l) of *SIMRIS002* at different chicken manure concentrations at the end of cultivations. Data shown are average from duplicate cultures with standard deviation as error bars.

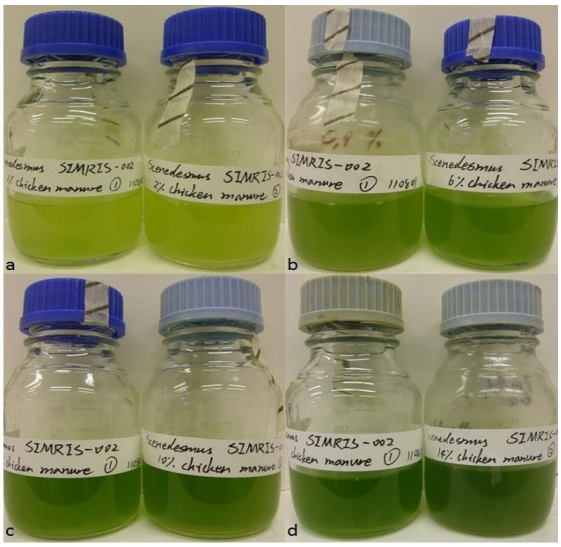

Figure 4.6. Photograph of *SIMRIS002* culture flasks with (a) 2%, (b) 6%. (c) 10% and (d) 14% chicken manure media at the end of the cultivations. A gradual color change from light green to dark green was seen with the increasing concentration from 2% to 14%.

4.3 Effect of temperature and gas exchange

The purpose of this study was to produce biomass in an economical way, which could be used in third world countries in tropical areas. Thus, besides the weather simulating greenhouse establishment, it was required that the algae species we investigated possessed heat-tolerance properties. According to previous research which has reported that *Chlorella* species can grow in a temperature range from 20 ℃ to 30 ℃ [17] and that *Scenedesmus* species can exist at a high temperature (up to 35 ℃) [18], we assumed the species used, *Chlorella vulgaris* and *Scenedesmus SIMRIS002*, can grow in a relatively high temperature environment. So, before the biomass production testing in plastic bags, heat-tolerance testing was done by culturing both *C. vulgaris* and *SIMRIS002* in aquaria in the greenhouse. During these cultivations, the temperature inside the greenhouse was initially influenced by the outside weather conditions, and during day-time inside the greenhouse the temperature could reach +40 ℃ due to direct sunshine.

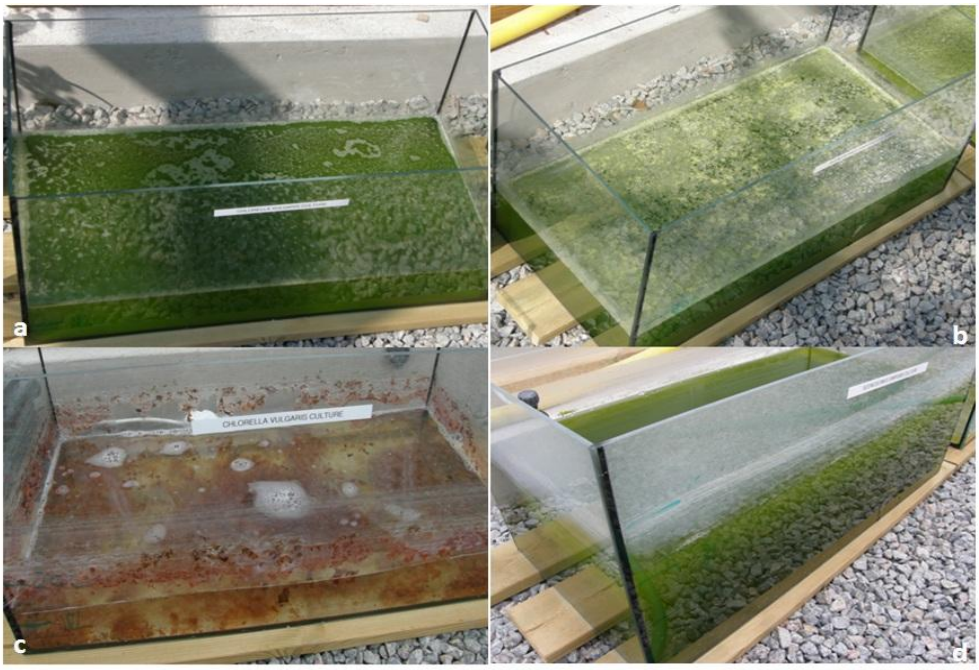

Figure 4.7. (a), (c): photograph of culture aquaria of *C. vulgaris* in the greenhouse; (b), (d): photograph of culture aquaria of *SIMRIS002* in the greenhouse.

Fig. 4.7 is a photograph of the aquarium cultures of *C. vulgaris* and *SIMRIS002* in the greenhouse and as seen at the beginning, both strains were growing very well with a pleasant green color (a, b). But, *C. vulgaris* died at the end of this cultivation with a visible color change from green to brown (fig. 4.7c) while *SIMRIS002* still grew very well (fig. 4.7d). This result matched the results that have been reported by Attilio Converti et al. (2009) in where *C. vulgaris* died at 38℃ [28]. Therefore, before the cultivation in plastic bags started, an air conditioner was established in the greenhouse to control the inside temperature within a reasonable tropical range (average temperature was 28±1℃) to protect *C. vulgaris*.

After the temperature level was under control, the plastic bag cultivations were started in the greenhouse. In the case of test 1, algae were cultured in two volumes of plastic bags under non-bubbling and automatic bubbling conditions. During the cultivation, one of the 5L plastic bags was broken, but all 3L plastic bags worked very well. Furthermore, automatic bubbling was obviously beneficial for the algae growth of both strains (appendix8.4). For confirmation of these results, the second test was performed.

In test 2, as shown in figure 4.8, significant higher biomass content was reached for both *C. vulgaris* (a) and *SIMRIS002* (b) in 3L plastic bags when air was automatically bubbled into the bioreactors. Manual air support could not significantly improve the biomass formation as compared to using the non-bubbling method.

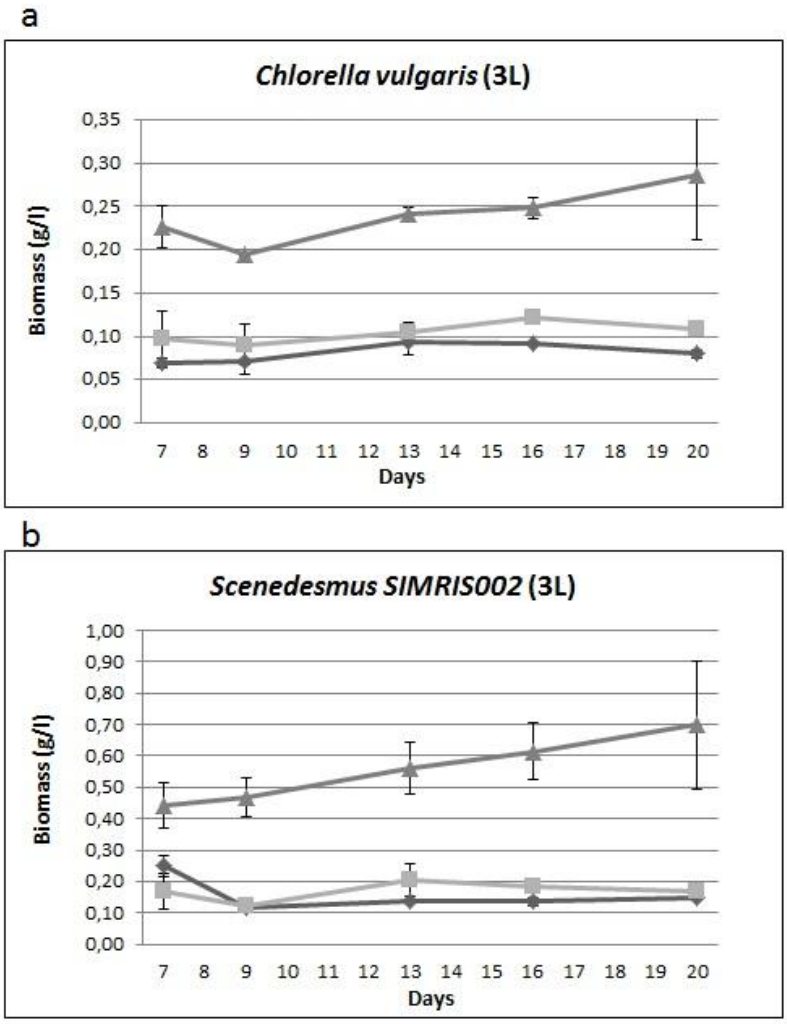

Figure 4.8. (a) Biomass (g/l) of *C. vulgaris* with different gas-exchange methods in 3L plastic bags; (b) Biomass concentrations (g/l) of *SIMRIS002*with different gas-exchange methods in 3L plastic bags. ▲ automatic bubbling; ■ manual bubbling; ◆ non-bubbling. Data shown are average from duplicate cultures with standard deviation as error bars.

Similar results were seen from the cultivations in 5L plastic bags as shown below (fig. 4.9). Higher biomass content was reached in automatic bubbled samples of both selected algae. However, an increasing effect of manual bubbling in this volume was indicated for *SIMRIS002*but was not clearly statistically significant.

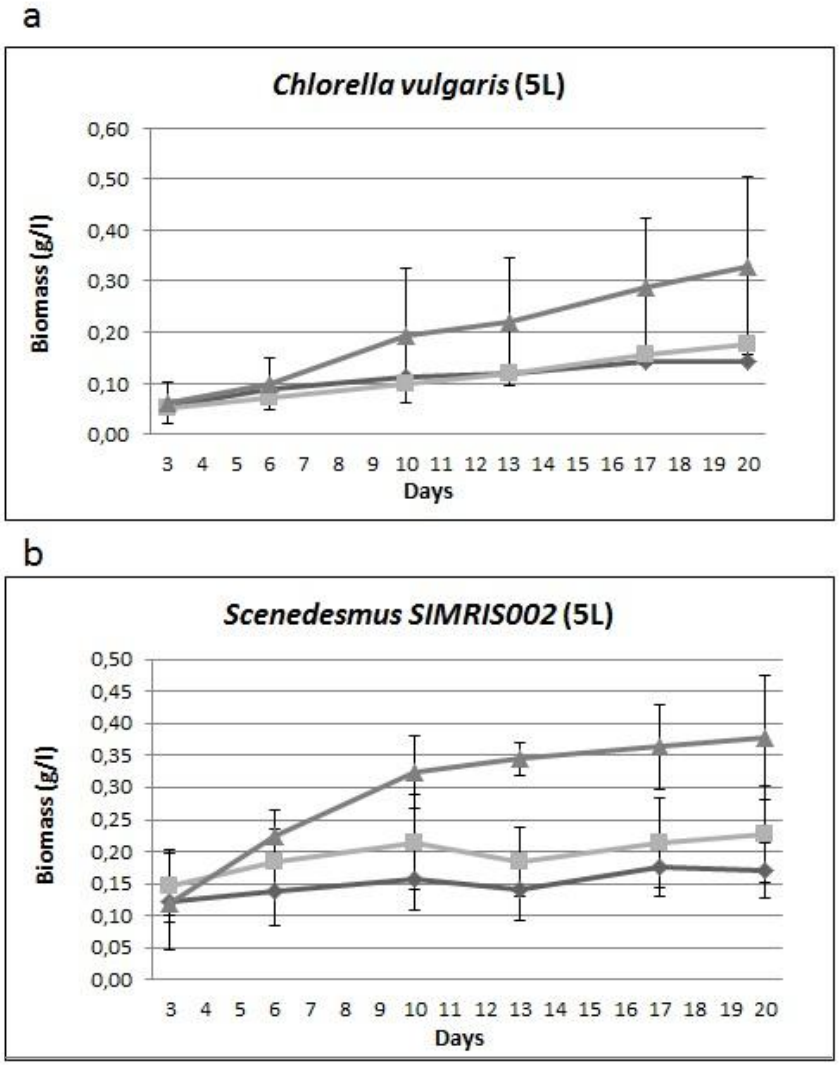

Figure 4.9. (a) Biomass (g/l) of *C. vulgaris* with different gas-exchange methods in 5L plastic bags; (b) Biomass concentrations (g/l) of *SIMRIS002* with different gas-exchange methods in 5L plastic bags. ▲ automatic bubbling; ■ manual bubbling; ◆ non-bubbling. Data shown are averages from duplicate cultures with standard deviation as error bars.

4.4 Effect of pH in culture medium

In order to study the effect of pH on algae growth, the chicken manure test was done twice, as described in chapter 3.2. The pH of the initial culture medium before cultivation and of final suspension after the cultivation was ended is listed in table 4.1. As can be seen, in the chicken manure medium there was a slightly alkaline medium and the pH values were increased with increased concentration of manure. After the culturing was finished, the pH was significantly increased in the culture broth for both algae species, especially for *SIMRIS002*. The cell (n/ml) and biomass (g/l) concentrations of all cultivations were measured for both *C. vulgaris* and *SIMRIS002* (table 4.2 and table4.3). As described in chapter 2, *Scenedesmus* algae prefer a higher pH environment than *Chlorella*, and therefore, in this experiment, *SIMRIS002* indeed provided both faster growth (higher cell concentration) and higher biomass productivities than *C. vulgaris*. This effect was mostly pronounced at higher manure concentrations in the

medium, which provided a higher pH environment. Besides that, at higher pH in high chicken manure concentration a flocculation effect was induced for *C. vulgaris*, which made the cell numbers difficult to count.

Table 4.1. The pH measured in initial medium and final cultured suspension of *C. vulgaris* and *SIMRIS002* in different concentrations of chicken manure media performed in laboratory. The averages from duplicate cultivations with standard deviation as ± value are shown.

Chicken manure	**2%**	**6%**	**10%**	**14%**
Initial medium	7,71	8,16	8,65	8,69
Chlorella vulgaris	9,03±0,19	9,94±0,21	10,39±0,15	10,29±0,03
Scenedesmus SIMRIS002	10,09±0,05	10,64±0,02	10,87±0,02	10,90±0,03

Table 4.2. The averages of cell concentrations ($\times 10^7$n/ml) of *C. vulgaris* and *SIMRIS002* in different chicken manure concentrations performed in laboratory. The averages from duplicate cultivations with standard deviation as ± value are shown.

Chicken manure	**2% $\times 10^7$**	**6% $\times 10^7$**	**10% $\times 10^7$**	**14% $\times 10^7$**
Chlorella vulgaris	8,9±0,3	10,0±0,2	5,8±0,8	5,5±0,5
Scenedesmus SIMRIS002	9,6±0,6	21,9±3,7	27,5±2,4	30,5±2,4

Table 4.3. The biomass concentrations (g/l) of *C. vulgaris* and *SIMRIS002* in different chicken manure concentrations performed in laboratory. The averages from duplicate cultivations with standard deviation as ± value are shown.

Chicken manure	**2%**	**6%**	**10%**	**14%**
Chlorella vulgaris	0,11±0,01	0,18±0,00	0,20±0,04	0,21±0,01
Scenedesmus SIMRIS002	0,11±0,01	0,18±0,00	0,24±0,00	0,29±0,00

For the plastic bag culturing test, 10% chicken manure was used as the nutrient level of the initial medium for all cultivations. Table 4.4 shows the pH values measured from the samples of both 3L volume and 5L volume cultures. A similar situation as described for the lab culturing was seen in this experiment, pH was largely increased during the proceeding of the cultures, and this effect was especially seen for *SIMRIS002*. From the difference seen when comparing the cultures treated with different bubbling methods, a decrease in pH was obtained in automatic bubbled cultures and the highest potential for the decreasing effect was seen with *SIMRIS002*.

Table 4.4. The pH measured in initial 10% chicken manure medium and final cultured suspension of *C. vulgaris* and *SIMRIS002* in two plastic bag culture volumes performed in the greenhouse. The averages from duplicate cultivations with standard deviation as ± value are shown.

	10% chicken manure	***Chlorella vulgaris***		***Scenedesmus SIMRIS002***	
		3L	5L	3L	5L
Non bubbling	8,65	10,79±0,75	11,37±0,00	11,29±0,01	11,37±0,01
Manual bubbling	8,65	9,74±0,04	11,39±0,29	11,27±0,02	11,38±0,01
Automatic bubbling	8,65	9,52±0,12	11,34±0,05	9,68±0,13	7,81±0,02

Another interesting result was only observed for *SIMRIS002*. The automatic bubbling obviously decreased the pH values in both volumes from the 10th day of culturing (figure 4.10). Accompanying this pH change a clear visible color change from green to yellow from the 10th day and it became pink at the end. When observing the samples under a microscope, there was a pink colored "membrane" formed outside the cell (data not shown). But *C. vulgaris* did not show similar results.

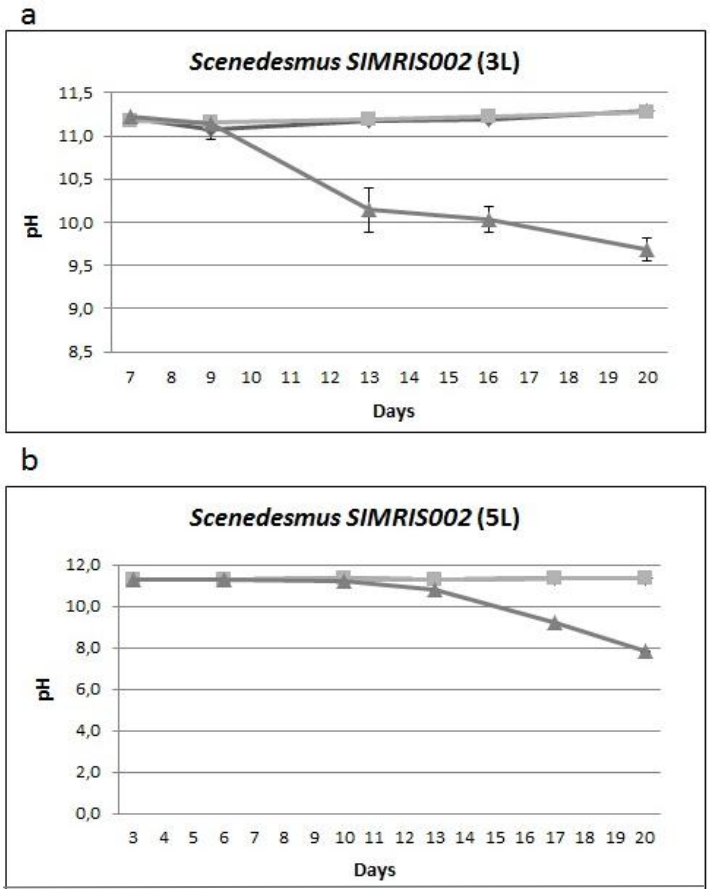

Figure 4.10. pH in *SIMRIS002* cultures done with different gas-exchange methods in (a)3Land (b)5L plastic bags. ▲automatic bubbling; ■ manual bubbling; ◆ non-bubbling. Data shown are averages from duplicate cultures with standard deviation as error bars.

5. Discussion

The photosynthesis of algae is driven by the energy absorbed from light. Thus, factors regarding light, like intensity, spectral quality, and photo period, need to be considered when culturing algae. Light intensity is a very important growth factor for algae culturing, but the optimal range vary largely depending on the algae species, culture depth and the density of the algal culture. A photoinhibition effect can be caused by too high light intensities, *i.e.* direct sunlight during cultivation [17]. In this study, all chicken manure tests were processed in the laboratory under acceptable light intensities supplied by fluorescent lamps. Thus, the light intensity and quality were under control and an optimal light/dark cycle of 16:8 was also supplied. However, the plastic bag cultivations were all placed in the greenhouse under natural Swedish light conditions. No controllable constant light intensity could be obtained during the cultivation. Thus, the light intensity could become limiting factors for algal growth.

In previous algae research done at SP, the biomass concentration of *C. vulgaris* after 13 days has reached a level up to 1,2 g/l when cultured in Bristol medium with 10% CO_2 as carbon source at 25℃ under simulated Swedish summer light conditions [29]. It has also been indicated that favorable Swedish light intensity for *C. vulgaris* is 100 W/m^2 [29], which is naturally provided during cloudy days. Furthermore, the growth of *C. vulgaris* can be inhibited by the high light intensity of 300W/m^2, which is the average light intensity during sunny days in Swedish summer. According to the light intensity measurement of our study, the average light intensity was 190 W/m^2 per day and during noon a high intensity more than 1000 W/m^2 was reached in sunny days. Thus, the growth of *C. vulgaris* could be inhibited by these high light intensities. Furthermore, the low initial algae density (30 ml of algae inoculums in 3L medium and 50 ml in 5L) could make algae cells more sensitive to the light intensity at the beginning and therefore further enhance the photoinhibition effect. This is probably the reason for the low biomass produced in all cultures done in plastic bags at natural Swedish summer light conditions (table 5.1).

Table 5.1. The biomass concentrations (g/l) of *C. vulgaris* and *SIMRIS002* in plastic bag culturing at the 20th day. The averages from duplicate cultivations with standard deviation as ± value are shown.

	Plastic bag					
	3L			5L		
	Non bubbling	Manual bubbling	Automatic bubbling	Non bubbling	Manual bubbling	Automatic bubbling
Chlorella vulgaris	0,08±0,01	0,11±0,01	0,29±0,08	0,14±0,00	0,17±0,00	0,33±0,17
Scenedesmus SIMRIS002	0,15±0,00	0,17±0,00	0,70±0,21	0,17±0,04	0,23±0,08	0,38±0,10

Culture medium is another factor that can influence the algae growth. Carbon, nitrogen and phosphorus are the most important nutrients mediating the production of biomass. In this study, chicken manure was used as a source of important nutrients to grow algae as in a natural medium environment. It provided organic and inorganic components for algae growth. Table 5.2 summarizes the initial content of carbon, nitrogen and phosphorus in different chicken manure medium concentrations.

Table 5.2.Theconcentrations of different limiting nutrients in chicken manure media compared with reported optimal conditions for *C. vulgaris* and *S. dimorphus.*

	Chicken manure					**Optimal conditions for *C. vulgaris* [30][31]**	**Optimal conditions for *S. dimorphus* [34]**
	2%	**6%**	**10%**	**14%**	**100%**		
Available nitrogen content (mg/l)	5,6	16,8	28,0	39,2	280,0	70-137	14-43
Available phosphorus content (mg/l)	1,8	5,5	9,2	12,8	91,7	23-45	2-6
Organic carbon content (mg/l)	50,4	151,2	252,0	352,8	2 520,0	4000	-
N-P ratio (mgN mgP^{-1})	3	3	3	3	3	≥3	5-20

To optimize the growth of *C. vulgaris*, one study has suggested that the initial nitrogen concentration should not be lower than 70 mg/l, the initial phosphorus concentration range should be 23 mg/l to 45 mg/l and the ratio of N: P in mgN mgP^{-1} should not be lower than 3:1 [30]. In this study, 10% chicken manure was used for plastic bag culturing in the greenhouse. The available nitrogen concentration in 10% chicken manure was only 28 mg/l (table 5.2), which was much lower than the optimal level. The phosphorus content was also very low (only 9,17 mg/l in 10% chicken manure).The N-P ratio is used to indicate which nutrient is the limiting factor for algal growth, since when the ratio is low nitrogen will be the limiting nutrient and when the ratio is high phosphorus will be the limiting nutrient. In the chicken manure media we used, both nitrogen and phosphorus were limiting nutrients due to their much lower concentrations, although the N-P ratio was enough for optimal growth. A low suboptimal nitrogen and phosphorus concentration was not only present in the 10% chicken manure medium but also in all the other three levels of chicken manure tested (up to 14%). Consequently, the biomass formation in the flask culturing (described before in table 4.3) was also low. The algae were found dead when cultured in 100% chicken manure. This may be caused by that in the 100% chicken manure medium both the nitrogen and phosphorus concentration were much higher than the optimal level, which could become toxic for algae. Also some unknown compounds could cause a toxic effect. Further investigations should be done to find the most suitable chicken manure concentration for optimal algal growth.

Another important limiting factor is the carbon source. The carbon present at different chicken manure concentrations is also listed in table 5.2. A research study has reported that the carbon source from 1% glucose (w/v) can provide an optimal growth for *C. vulgaris* [31]. This means that the optimal carbon content for *C. vulgaris* should be 4000 mg/l which is much higher than the carbon present in the chicken manure media (the highest concentration was 2520 mg/l in 100% chicken manure medium). Thus, some inorganic components like $NaHCO_3$ could be added to increase the carbon concentration and extra carbon source like CO_2 as done with the bubbling could also be applied to increase the algal growth.

In addition, the optimal pH of *C. vulgaris* has been reported to be 6,8 [16]. Thus, the inhibition effect could also be caused by the high initial pH (more than 8) in the chicken manure medium used in this study. However, the high pH caused additionally a flocculation effect that is beneficial for algae harvesting.

The second algae species, *Scenedesmus SIMRIS002*, used in this study is a newly isolated algae strain and has not yet been well characterized. Since the taxonomic identification on species level has not been done for this strain, in this discussion, a special freshwater strain named *Scenedesmus dimorphus*is considered to assess the culturing conditions of *SIMRIS002*. Because of that the optimal light intensity for *S. dimorphus*has been reported to be 60 W/m^2 [33], it could be indicated that Swedish light condition was too high also for good growth of *Scenedesmus* in batch culture (plastic bags) with low initial cell concentration and low mixing. For the culture medium conditions used (table 5.2), as compared to the reported optimal ratio range from 5 to 20 [34], the low N-P ratios were probably also resulting in the low biomass content in the cultures shown in table 5.1. The low N-P ratio indicated that the limiting nutrient for *Scenedesmus* growth was nitrogen. Additional nitrogen containing components like urea could be added to increase the nitrogen concentration [32]. However, *SIMRIS002* showed a faster growth and higher biomass content than *C. vulgaris* probably because *Scenedesmus* algae prefer higher temperature and alkaline environment.

Furthermore, there are some other conditions that should be mentioned. One study has been indicated that the continuously supplied large bubbles in dual sparging bubble column photobioreactor can perform better mixing effect and highly increase the CO_2 transfer rate and efficiencies [45]. In this study, from the results described earlier in fig. 4.8 and fig. 4.9, automatic continuous bubbling could indeed help increase biomass production both for *C. vulgaris* and *SIMRIS002*. The reason could be a sufficient delivery of air with CO_2, which could be extra carbon source, into the algae suspensions and a better mixing of the cell suspension caused by the bubbles. For the different volumes of cultivations done in the plastic bags, only a little increase in biomass concentration appeared in the higher volume of 5L for *C. vulgaris* (table 5.1). In the 5L *SIMRIS002* automatic bubbling cultures, the biomass level was even decreased. A reason for these findings may be that the high volume suspensions were not mixed very well by neither manual bubbling nor by automatic bubbling. Additionally, the weight of the 5L suspension was too heavy for the plastic bags and made it break occasionally during cultivation. Thus, we could consider that a high volume didn't give a better effect and the 3L volume was more suitable in this study.

6. Conclusions

This study successfully simulated a tropical environment under Swedish light conditions by establishing a greenhouse. The temperature inside was constantly kept at around 28 ℃ by an air conditioner. Two possibly heat-tolerant algae species were selected, *Chlorella vulgaris* and *Scenedesmus (SIMRIS002).*PE plastic bags were modified as models for economic photobioreactors for algae cultivation in developing countries. A waste product (chicken manure) was used as a cheap nutrient source in the culture medium. Compared to previous studies, a lower biomass production in the plastic bags was found in this study, the possible reasons being the high Swedish light intensity and a poor mixing system. To avoid this problem, a shade could be used to decrease the light intensity during cultivation and a swirling system could be built for mixing. The low chicken manure concentration might be another limiting factor due to its lower nitrogen, phosphorus and carbon concentrations than reported optimal conditions. Besides that, chicken manure provided an alkaline environment which probably inhibited the growth of *C. vulgaris* and caused a flocculation effect. Thus, some other local alkaline tolerant species should be selected that are tolerant to the high pH environment. However, the high pH was preferred by *SIMRIS002*. To enhance the algal growth, the nutrient content in the chicken manure medium should be adjusted to reach the optimal levels by increasing the initial concentration of chicken manure for optimal growth of *C. vulgaris*. Further investigation should be done to find the most suitable chicken manure concentration. For *SIMRIS002*, additional components like urea could be added to increase the nitrogen concentration and thereby increase the N-P ratio. Besides that, CO_2 could be applied as additional carbon source by an efficient gas-exchange system.

During the cultivation, automatic bubbling was an efficient way for optimal algae growth because of the sufficient air delivery and good mixing. An interesting situation appeared as the *SIMRIS002* changed color during cultivation under automatic bubbling conditions with a pink “membrane” outside the cells. A large drop in pH was also found in the cultures with automatic bubbling. Further measurement should be done to find out if there was something produced by *SIMRIS002* that result in this drop in pH. For the plastic bags we used, 3L was an acceptable volume for this kind of hanging plastic bags.

7. References

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8. Appendix

8.1 Medium preparation

Before preparation of Bristol medium, the stock solutions were prepared by first weighing each component as described in table 8.1and then diluting in 1L dH_2O. Then, 20ml of each stock solution was added in a 2L volume flask and filled up to 2L with dH_2O. Prepared solutions were autoclaved at 121 ℃ for 20min and then cooled to room temperature (23 ℃).

Table 8.1. Components in Bristol medium. [38]

	Component	Quantity used/L	Stock Solution Concentration	Final Concentration
1	$NaNO_3$	20 ml	25 g/L dH_2O	2,94 mM
2	$CaCl_2 2H_2O$	20 ml	2,5 g/L dH_2O	0,17 mM
3	$MgSO_4 7H_2O$	20 ml	7,5 g/L dH_2O	0,3 mM
4	K_2HPO_4	20 ml	7,5 g/L dH_2O	0,43 mM
5	KH_2PO_4	20 ml	17,5 g/L dH_2O	1,29 mM
6	NaCl	20 ml	2,5 g/L dH_2O	0,43 mM

For BG-11 medium, each component was weighed as described in table 8.2 and diluted in 2L dH_2O. The media was then autoclaved at 121 ℃ for 20 min and then cooled to room temperature (23 ℃).

Table 8.2. Components in BG-11 medium. [39]

Component	Quantity used/2L	Concentration
NaNO3	3 g	1,5 g/L dH_2O
K2HPO4	0,08 g	0,04 g/L dH_2O
$MgSO_4 \cdot 7H2O$	0,15 g	0,075 g/L dH_2O
Citric acid	0,012 g	0,006 g/L dH_2O
Ferric ammonium citrate	0,012 g	0,006 g/L dH_2O
EDTA	0,002 g	0,001 g/L dH_2O
Na_2CO3	0,04 g	0,02 g/L dH_2O

For the stock solution of chicken manure, 14g dry product was diluted in 2L dH_2O. The concentration of each component in this solution is summarized in table 8.3. The suspension was autoclaved at 121 ℃ for 20min and then cooled to room temperature (23 ℃). The

autoclaved stock solution was diluted with dH_2O to get 2%, 6%, 10% and 14% chicken manure media.

Table 8.3. Components in chicken manure stock solution according to manufacturer description.

Component	% on total product	Concentration
Nitrogen (N) total	4,0%	0,56 g/2L
Phosphorus (P_2O_5) total	3,0%	0,42 g/2L
Potassium (K_2O)	2,5%	0,35 g/2L
Magnesium (MgO) total	1,0%	0,14 g/2L
Sulphur (S) total	1,0%	0,14 g/2L
Calcium (CaO)	9,0%	1,26 g/2L
Carbon (C/N=9)	36,0%	5,04 g/2L

The fourth medium used in this study was liquid plant nutrients. 6ml of the product was diluted in 2L dH_2O to get the final solution. The concentration of each component in this solution is listed in table 8.4. It was also autoclaved under 121 ℃ for 20 min and then cooled to room temperature (23 ℃).

Table 8.4. Components in liquid plant nutrient according to manufacturer description.

Component	% on total product	Concentration
Nitrogen (N)	5,6%	0,336 ml/2L
Phosphorus (P)	2%	0,120 ml/2L
Calcium (K)	7,1%	0,426 ml/2L
Magnesium (MgO)	0,01%	0,001 ml/2L

8.2 Data from initial media test in laboratory

The cell concentrations of *C. vulgaris* in different media at the 4^{th} day and the 20^{th} day are shown in table 8.5.

Table 8.5. The cell concentrations (n/ml) of *C. vulgaris* in different medium at the 4th day and the 20th day. Only one sample was prepared for chicken manure. The averages from duplicate cultivations with standard deviation as ± value are shown.

Days	Bristol media $\times 10^7$ n/ml	Liquid plant nutrient $\times 10^7$ n/ml	10% chicken manure $\times 10^7$ n/ml	100% chicken manure $\times 10^7$ n/ml
4th day	1,6 ±0,3	1,2 ±0,2	1,7	0,6
20th day	3,6 ±0,3	3,5 ±1,3	11,0	Dead

8.3 Data from chicken manure tests in laboratory

The chicken manure test was done in two sets of cultivations in this study to improve accuracy. Firstly, both algal strains were cultured in 2%, 6%, 10% and 14% chicken manure medium respectively (test one). Duplicate cultures (replicate 1 and 2) were prepared for each medium. The cell number of every culture was counted once per day and the cell concentration was then calculated to draw the growth curve. The growth curves for *C. vulgaris* and *SIMRIS002* in 2%, 6%, 10% and 14% chicken manure medium are shown in below.

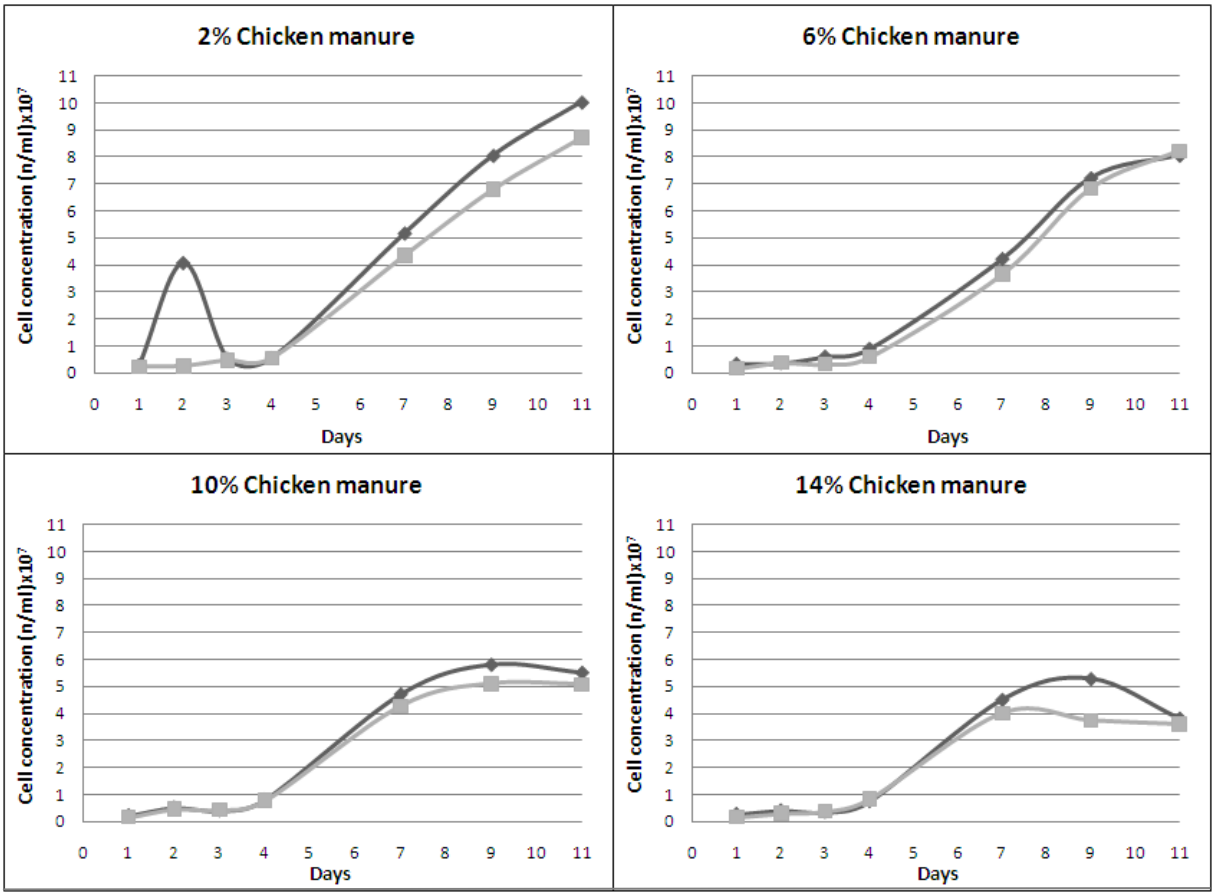

Figure 8.1. First set of cultivations. Cell concentrations (n/ml) of *C. vulgaris* cultures with different chicken manure concentrations in test one, replicate 1 (◆), replicate 2 (■).

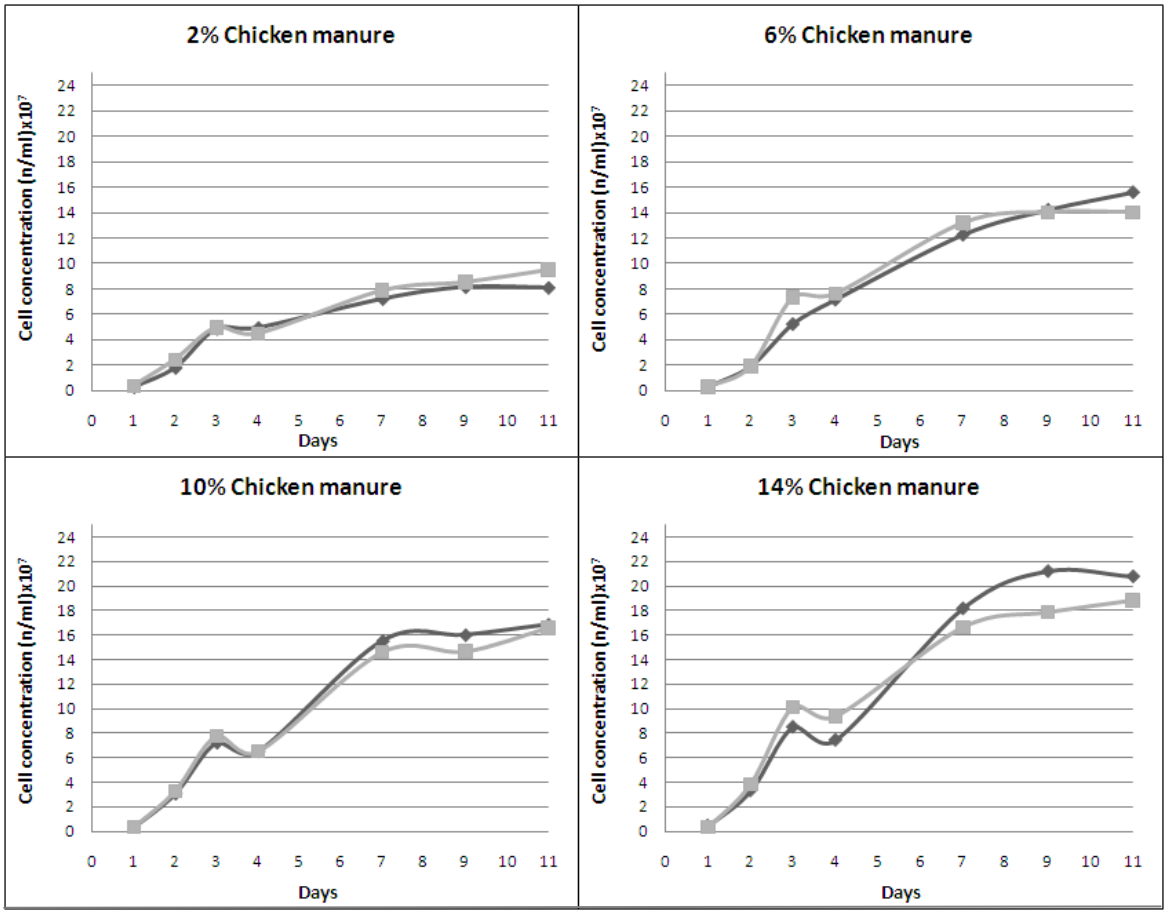

Figure8.2. First set of cultivations. Cell concentrations (n/ml) of *SIMRIS002*cultures with different chicken manure concentrations in test one, replicate 1 (◆), replicate 2 (■).

The biomass content of every culture was measured at the end of the cultivation. Every filterpaper was first weighed in constant room with controlled temperature (23±2 ℃) and moisture content (50±5%). 30ml suspension for every sample was filtered and dried at 100 ℃ over night. Then the filterpaper was weighed again to calculate the dry weight (g/l) as the biomass content. The biomass content of *C. vulgaris* and *SIMRIS002* in 2%, 6%, 10% and 14% chicken manure medium are shown in the following tables.

Table 8.6. The biomass contents (g/l) of *C. vulgaris* grown at different chicken manure concentrations.

Chicken manure	**2%**		**6%**		**10%**		**14%**	
Sample no.	1	2	1	2	1	2	1	2
Filterpaper (g) (before filter)	0,1223	0,1199	0,1228	0,1209	0,1240	0,1213	0,1207	0,1215
Filterpaper (g) (after filter)	0,1242	0,1218	0,1258	0,1242	0,1279	0,1245	0,1247	0,1257
Biomass (g/l)	0,0633	0,0633	0,1000	0,1100	0,1300	0,1067	0,1333	0,1400

Table8.7. The biomass contents (g/l) of *SIMRIS002* grown at different chicken manure concentrations.

Chicken manure	**2%**		**6%**		**10%**		**14%**	
Sample no.	1	2	1	2	1	2	1	2
Filterpaper (g) (before filter)	0,1211	0,1241	0,1221	0,1216	0,1242	0,1226	0,1235	0,1208
Filterpaper (g) (after filter)	0,1233	0,1264	0,1261	0,1255	0,1281	0,1265	0,1289	0,1257
Biomass (g/l)	0,0733	0,0767	0,1333	0,1300	0,1300	0,1300	0,1800	0,1633

After test one was finished, new cultivations were started (test two) by newly culturing both algal strains in 2%, 6%, 10% and 14% chicken manure medium again as done in test one. Duplicate cultures (replicate 1 and 2) were also prepared for each condition. In this time, the cell number of each culture was counted twice per day. The growth curves for *C. vulgaris* and *SIMRIS002* are shown below.

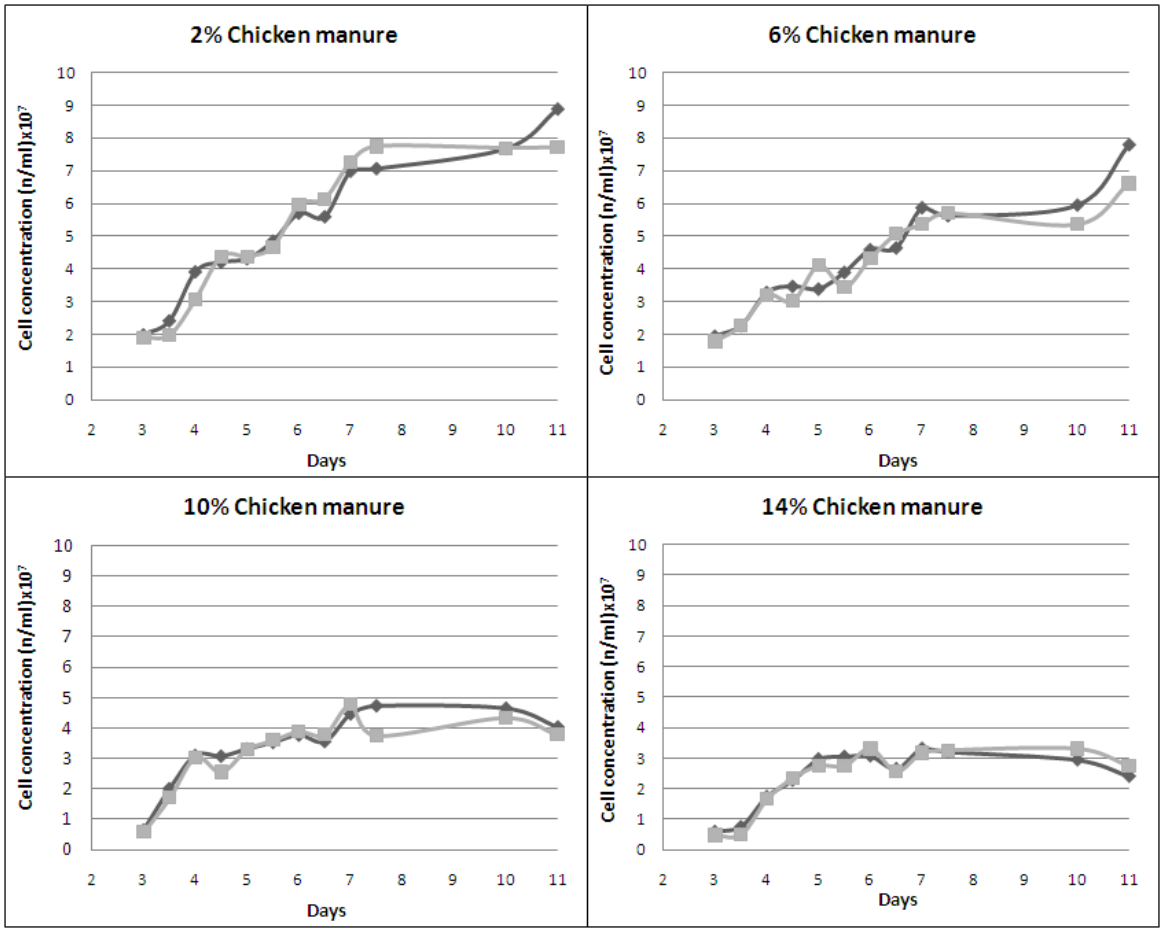

Figure 8.3. Second set of cultivations. Cell concentrations (n/ml) of *C. vulgaris* cultures with different chicken manure concentrations in test two, replicate 1 (◆), replicate 2 (■).

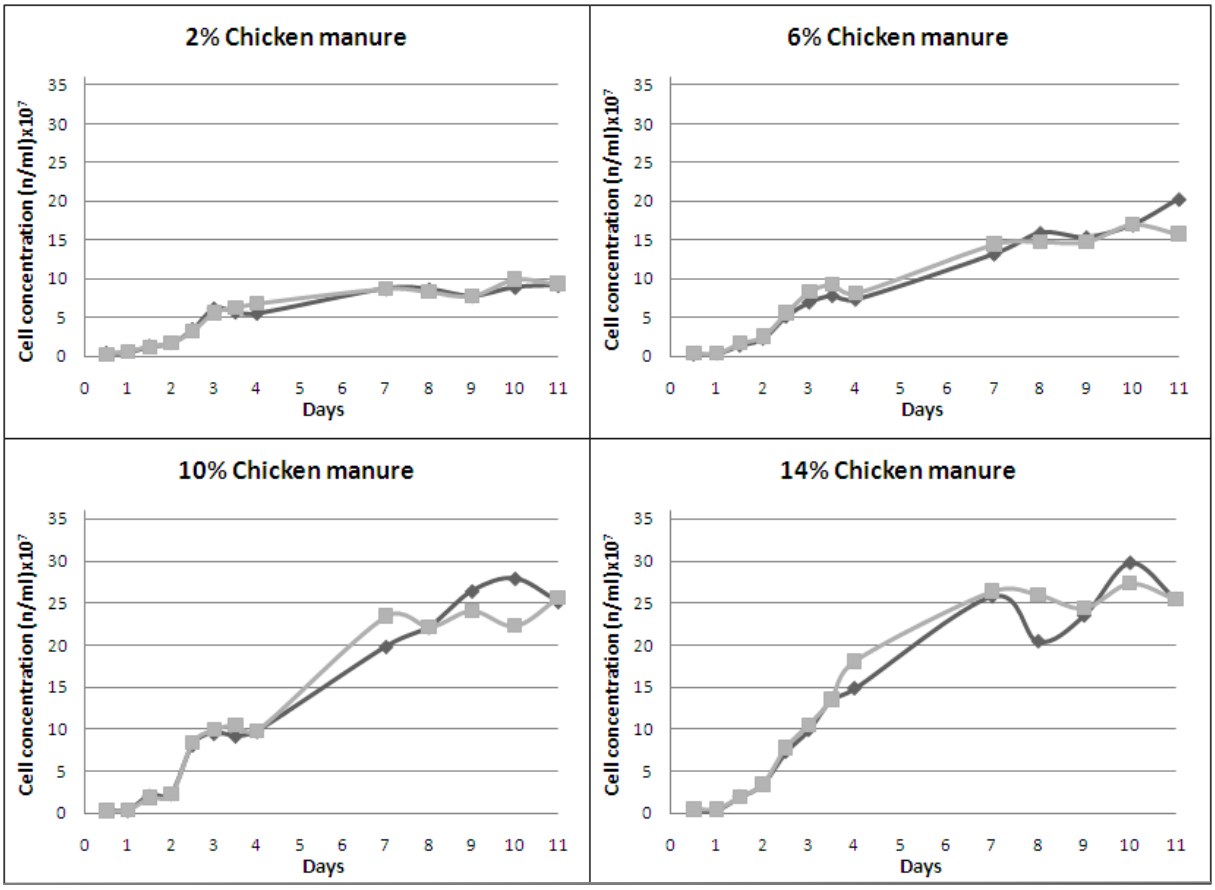

Figure 8.4. Second set of cultivations. Cell concentrations (n/ml) of *SIMRIS002*cultures with different chicken manure concentrations in test two, replicate 1 (◆), replicate 2 (■).

The biomass content of every culture in test two was measured at the end of cultivation as well. The biomass contents of *C. vulgaris* and *SIMRIS002* in 2%, 6%, 10% and 14% chicken manure medium are shown in the following tables.

Table 8.8. The biomass contents (g/l) of *C. vulgaris* grown at different chicken manure concentrations.

Chicken manure	**2%**		**6%**		**10%**		**14%**	
Sample no.	1	2	1	2	1	2	1	2
Filterpaper (g) (before filter)	0,1210	0,1224	0,1220	0,1234	0,1245	0,1221	0,1203	0,1218
Filterpaper (g) (after filter)	0,1244	0,1255	0,1276	0,1288	0,1314	0,1273	0,1265	0,1284
Biomass (g/l)	0,1133	0,1033	0,1866	0,1800	0,2300	0,1733	0,2066	0,2200

Table 8.9. The biomass contents (g/l) of *SIMRIS002* grown at different chicken manure concentrations.

Chicken manure	**2%**		**6%**		**10%**		**14%**	
Sample no.	1	2	1	2	1	2	1	2
Filterpaper (g) (before filter)	0,1235	0,1229	0,1221	0,1234	0,1226	0,1227	0,1218	0,1217
Filterpaper (g) (after filter)	0,1265	0,1263	0,1274	0,1286	0,1298	0,1299	0,1306	0,1305
Biomass (g/l)	0,1000	0,1133	0,1766	0,1733	0,2400	0,2400	0,2933	0,2933

The pH of every sample was measured in test two after the cultivation was ended as shown in below.

Table 8.10. The pH measured in final cultured suspensions of *C. vulgaris* and *SIMRIS002*grown with different concentrations of chicken manure.

Chicken manure	**2%**		**6%**		**10%**		**14%**	
Sample no.	1	2	1	2	1	2	1	2
Chlorella vulgaris	8,89	9,16	9,79	10,08	10,29	10,50	10,26	10,31
Scenedesmus SIMRIS002	10,05	10,13	10,65	10,63	10,86	10,88	10,88	10,92

8.4 Data from plastic bag cultivation in greenhouse in test one

Two tests were done for the plastic bag culturing. Test one was done for around 60 days to adjust the plastic bags' properties. Two gas-exchange methods were used: non-bubbling and automatic bubbling. One culture was prepared for each method. The biomass concentrations of all cultures were measured once per week during cultivation (fig. 9.5). There is no data for one of the 5L cultivation with *C. Vulgaris* after the 26th day, because the 5L volume was too heavy for the plastic bag and the bag was broken at the 26th day.

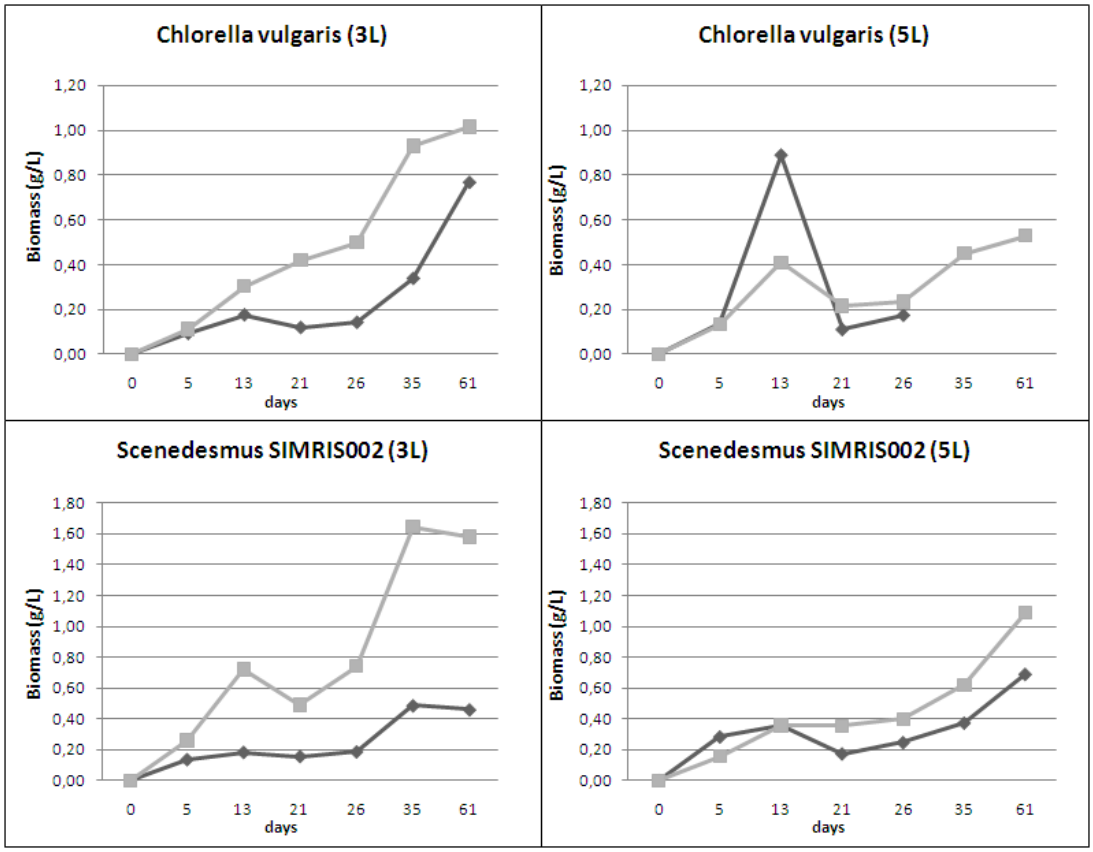

Figure 8.5. The biomass contents (g/l) of *C. vulgaris* and *SIMRIS002* grown in plastic bags in test one. ◆ is non-bubbling; ■ is automatic bubbling.

8.5 Temperature and light intensity data

The temperature inside the greenhouse and the natural light intensity were measured each minute during plastic bag culturing. The average of temperature and light intensity per week was calculated and listed in table 8.11.

Table8.11. The inside temperature and light intensity during cultivation.

	Inside temperature average (℃)	**Light intensity average (W/m^2)**	**Maximum light intensity (W/m^2)**
Week 27	29	161	1 015
Week 28	28	149	1 054
Week 29	29	133	1 007
Week 30	28	132	1 049
Week 31	29	170	1 005
Week 32	27	63	701