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Systemic Optimization Of Microalgae Grown On Swine Wastewater As A Biofuel Feedstock

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Systemic Optimization of Microalgae Grown on Swine Wastewater as a Biofuel Feedstock

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Biographical Sketch

Rifat Hasan was born in 1985 and grew up in the city of Noakhali, Bangladesh. She earned her B.Sc from the Department of Chemical Engineering at Bangladesh University of Engineering and Technology in 2009. During the period of college education, she achieved excellent academic performance while she also involved many campus activities. Rifat was a positive volunteer in serving many non-profit social activities. After graduation she was awarded a research assistantship to pursue her M.S. degree at North Carolina Agricultural and Technical State University. Rifat has been awarded for her excellent academic performance. Her GPA for the master degree program is 3.88.

Rifat is a member of American Society of Agricultural and Biological Engineers (ASABE). She has participated in 2013 ASABE international conference and made an oral presentation on Growth Kinetics of Microalgae on Swine Wastewater. She also participated in 2013 ARD meeting and made an oral presentation on Microalgae Grown on Swine Wastewater as a Biofuel Feedstock.

After finishing her MS studies, Rifat is going to serve as an engineer in chemical and biological engineering.

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Abstract

This research broadens the scope of research on microalgae grown on swine wastewater as it offers a combination of wastewater treatment and biofuel production. Swine wastewater is an enriched source of phosphorus, nitrogen and other organic compounds that are necessary for the growth of microalgae. While growing in swine wastewater, algae consume the nutrients from the wastewater, so there is no need of arable land for their growth. Current biofuel production relies on limited arable lands to supply feedstock making it impossible to meet the global biofuel demands without disrupting food production. Algae can potentially produce 1,000-4,000 gallons of oil/acre/yr which is significantly higher than other oil seed crops that are being used now. In this research, suitable culture conditions (temperature, light intensity etc) were determined for the growth of microalgae in swine wastewater at the farm of the North Carolina Agricultural and Technical State University (NCAT), which is very easy to achieve naturally, and the conditions were optimized to get the maximum removal of nutrients for wastewater treatment. Two commercial microalgae strains of *C. vulgaris* and *C. reinhardtii* were studied and the highest specific growth rate was found to be 1.336 day^{-1} for *C. vulgaris* which were grown in 100% swine wastewater at a temperature of 25°C and light intensity of 600 $\mu molm^{-2}s^{-1}$. A selective strain from NCAT farm was compared with these two commercial strains and was found to be more effective as a feedstock of biofuel.

CHAPTER 1

Introduction

"Neglect in protecting our heritage of natural resources could prove extremely harmful for the human race and for all species that share common space on planet earth. Indeed, there are many lessons in human history which provide adequate warning about the chaos and destruction that could take place if we remain guilty of myopic indifference to the progressive erosion and decline of nature's resources" [1]. In 1988, almost 25 years ago, the United Nations was acutely conscious of the possibility of disaster due to the climate change through increases in sea levels as one of its clauses was significant in having stated, "Noting with concern that the emerging evidence indicates that continued growth in atmospheric concentrations of "greenhouse" gases could produce global warming with an eventual rise in sea levels, the effects of which could be disastrous for mankind if timely steps are not taken at all levels" [1]. The global increase in carbon dioxide concentration is due primarily to fossil fuel use and land use change [1]. Today the climate change on earth provides greater substance to that concern. With these concerns of pollution, global warming, and energy shortages, society is starting to come across to biofuels as a substitute energy source. These biofuels can be produced from plants. At present food crops are widely used to produce biofuels, which seems not economically feasible in a long term.

Any biomass rich in high lipid content can be a good feedstock for biodiesel production, but microalgae are considered as an important energy crop as they offer many technical and economic advantages over other oilseed crops. Algae are capable of producing more oil, sequestering CO_2 from many sources and at the same time need no arable land to cultivate. They can be cultivated in large open ponds or in closed photobioreactors located on non-arable land. They can grow in a wide variety of climate and water condition such as different types of

wastewater. As wastewater contains a large amount of ammonia nitrogen and active phosphorus, it could be a suitable medium for the growth of microalgae. Therefore, algae can spontaneously convert CO_2 as well as nutrients from waste to valuable biomass which in turn can be converted to energy. It is anticipated that the economics will be eventually improved by combining biodiesel feedstock production with wastewater treatment and CO_2 fixation. The research presented here was conducted to determine the feasibility of producing biodiesel feedstock in the form of microalgal biomass grown in swine wastewater from North Carolina Agricultural and Technical State University farm ponds.

However, the evaluation of various culture conditions to grow an algal consortium in wastewater for bioremediation and biofuel/bioenergy applications has received much attention in recent years. Agricultural waste is also becoming recognized as an important environmental problem as the use of high-capacity confined animal farming and intensive plant farming increases. Chemical treatment of these wastewaters is costly, needs more space and produces dry sludge which is more difficult to handle. The use of algae for waste water treatment combined with CO_2 fixation and biofuel production seems more attractive as it overcomes all the challenges of chemical treatment. The production of algae on wastewater is likely to have a much more beneficial carbon balance than feedstocks produced with chemical fertilizers, which require fossil sources in their manufacture. This process is also carbon neutral through the creation of a closed carbon cycle that the CO_2 to be emitted during combustion of the biofuel will be absorbed into the next crop of plants to be grown as the biofuel feedstock.

To increase the production of algal biomass feedstock it is necessary to study the environmental parameters, such as temperature, light intensity and nutrient removal that affect the growth and lipid content of microalgae. Many of the parameters have been studied

individually but the combinative effect of these parameters on the algal growth has not been comprehensively analyzed so far. In this study the effects of four different parameters including temperature, photoperiod, light intensity and nutrient content on the microalgal growth have been analyzed to optimize the growth condition of the selected microalgae in swine wastewater. The algal strain which has the fastest growth was identified. The efficiency of removing the nutrients from swine wastewater by microalgae was one of the major objectives of this research. How the culture conditions affect the wastewater treatment efficiency was also studied intensively. Lipid contents of algae grown at different culture conditions were compared. Finally the algae were characterized for biodiesel production.

This research was conducted to contribute to the development of an integrated algae biofuel and wastewater treatment process. Therefore, the goal of this research was to develop fast growing microalgae strains to assimilate nutrients in wastewater for swine wastewater treatment and bioenergy production.

The specific objectives of this research are:

Objective 1: Screen and select microalgal strains which can grow fast in wastewater

Objective 2: Optimize the growth environment of microalgae in swine wastewater

Objective 3: Determine the microalgae growth kinetics

Objective 4: Determine the removal efficiency of nutrients from swine wastewater by selected microalgae

Objective 5: Characterize the microalgae as a bioenergy source

The ultimate vision for the proposed integrated algae-based treatment production process is shown in Figure 1.

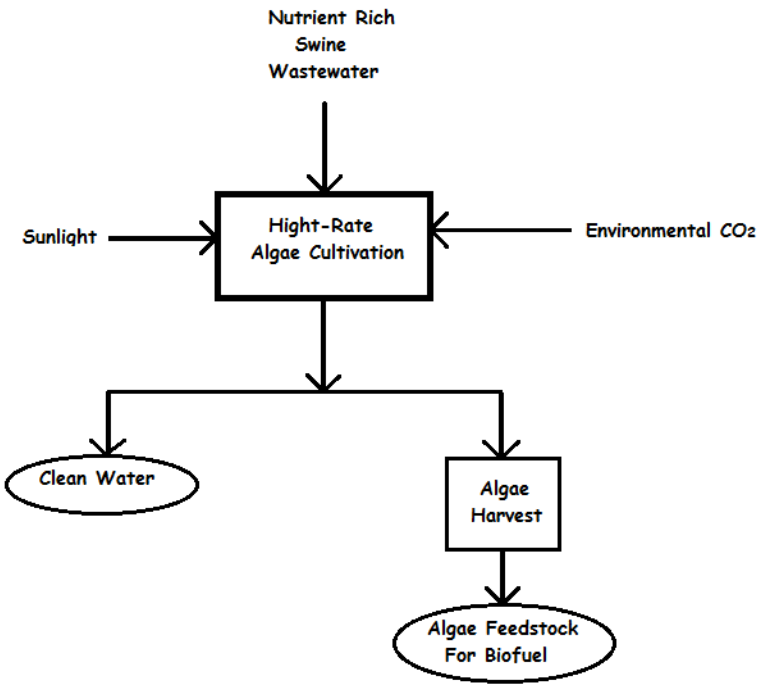

*Figure 1.*Combined swine wastewater treatment and algae-biodiesel feedstock production.

Swine wastewater from NCAT farm was collected and put into a tubular photobio-reactor. CO_2 from environmental air and sunlight accelerates the wastewater treatment and algal growth. The clean water was separated and the algae are then harvested, and the lipids are extracted and converted into biodiesel. The residual algal biomass after lipid extraction can also be used as a fertilizer for crop production.

CHAPTER 2

Literature Review

Microalgae are promising third-generation biofuel feedstocks that offer many potential technical and economic advantages. Algae can use and sequester CO_2 from many sources and may be processed into a broad spectrum of products including biodiesel, green diesel and gasoline replacements, bioethanol, methane, heat, bio-oil and biochar, animal feed and biomaterials, etc. This chapter reviews the microalgae studies for wastewater treatment and biodiesel production. Under suitable conditions microalgae can be cultured in wastewater to reduce nitrate, phosphate and organic matter in the wastewater. These algae that are grown on non-arable lands can meet the demand of feedstock for biofuel production without the disruption of the food production on limited arable lands. With the current requirement for renewable fuels, especially in the transportation sector, there is a need to develop a range of sustainable resources for the production of biofuels, which will be a significant step towards the replacement of fossil fuels.

2.1 Microalgae

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms, and perform oxygenic photosynthesis like higher plants. However, they have a unicellular or simple multicellular structure. Examples of prokaryotic microorganisms are Cyanobacteria (*Cyanophyceae*) and eukaryotic microalgae are green algae (*Chlorophyta*) and diatoms (*Bacillariophyta*) [2]. Algae are essential to global carbon, nitrogen and sulfur cycling. Approximately 45% of photosynthetic carbon assimilation is achieved by algae. Microalgae are presented in all existing habitat where light is available, representing a big variety of species living in a wide range of environmental conditions. Algae have close (sometimes essential)

associations with many other organisms such as lichens, coral and numerous protozoans. It is estimated that more than 50,000 algal species exist, but only a limited number of around 30,000 have been studied and analyzed [3]. Among those, the most widely used microalgae for wastewater treatment as well as biofuel production is *Chlorella* sp.

The first use of microalgae by humans dated back 2000 years to the Chinese, who used Nostoc to survive during famine. However, microalgal biotechnology only began to really develop in the middle of the last century. Nowadays, there are numerous commercial applications of microalgae. For example, (i) microalgae can be used to enhance the nutritional value of food and animal feed owing to their chemical composition, (ii) they play a crucial role in aquaculture, (iii) they can be incorporated into cosmetics, (iv) they can be used in wastewater treatment, and (v) biofuel production.

Generally, they are cultivated as a source of highly valuable molecules. Microalgae in human nutrition are currently marketed in different forms such as tablets, capsules and liquids. They can also be incorporated into pastas, snack foods, candy bars or gums, and beverages. Owing to their diverse chemical properties, they can act as a nutritional supplement or represent a source of natural food colorants [4]. For example, polyunsaturated fatty acid oils are added to infant formulas and nutritional supplements. In addition to its use in human nutrition, microalgae can be incorporated into the feed for a wide variety of animals ranging from fish (aquaculture) to pets and farm animals. In fact, 30% of the current world algal production is used as animal feeds. Microalgae are also refined to different products of aquaculture. Some microalgal species are established in the skin care market, the main ones being *Arthrospira* and *Chlorella*. Some cosmeticians have even invested in their own microalgal production system (LVMH, Paris, France and Daniel Jouvance, Carnac, France). Microalgae extracts can be mainly found in face

and skin care products (e.g., anti-agingcream, refreshing or regenerant care products, emollient and as an anti-irritant in peelers). Microalgae are also represented in sun protection and hair care products. However, pure molecules can also be extracted when their concentrations are sufficiently high. This leads to valuable products like fatty acids, pigments and stable isotope biochemicals [4].

Microalgae have the ability to mitigate CO_2 emission and produce oil with a high productivity, thereby having the potential for applications in producing the third-generation of biofuels. The key technologies for producing microalgal biofuels include the identification of preferable culture conditions for high oil productivity, development of effective and economic microalgae cultivation systems, as well as separation and harvesting of microalgal biomass and oil [5]. In this chapter, we will review these key technologies.

2.2 Microalgae Cultivation

2.2.1 Microalgae culture conditions. The growth characteristics and composition of microalgae are known to significantly depend on the cultivation conditions. There are four major types of cultivation conditions for microalgae: photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic cultivation [6].

2.2.1.1 Phototrophic cultivation. Phototrophic cultivation occurs when the microalgae use light, such as sunlight, as the energy source, and inorganic carbon (e.g., carbon dioxide) as the carbon source to form chemical energy through photosynthesis [7]. This is the most commonly used cultivation condition for microalgae growth [8]. It is found that under phototrophic cultivation, there is a large variation in the lipid content of microalgae, ranging from 5% to 68%, depending on the type of microalgae species and the nutrients in the water. Normally a nitrogen-limiting or nutrient-limiting condition was used to increase the lipid content

in microalgae [9]. As a result, achieving higher lipid content is usually at the expense of lower biomass productivity. Thus, lipid content is not the sole factor determining the oil-producing ability of microalgae. Instead, both lipid content and biomass production need to be considered simultaneously. Hence, lipid productivity, representing the combined effects of oil content and biomass production, is a more suitable performance index to indicate the ability of microalgae with regard to oil production. The highest lipid productivity reported in the literature is about 179 mg/L/d by *Chlorella* sp. under phototrophic cultivation using 2% CO_2 with 0.25 vvm aeration [10]. The major advantage of using autotrophic cultivation to produce microalgal oil is the consumption of CO_2 as a carbon source for the cell growth and oil production. However, when CO_2 is the only carbon source, the microalgae cultivation site should be close to factories or power plants which can supply a large quantity of CO_2 for microalgal growth. Moreover, compared to other types of cultivation, the contamination problem is less severe when using autotrophic growth. Therefore, outdoor scale-up microalgae cultivation systems (such as open ponds and raceway ponds) are usually operated under phototrophic cultivation conditions [9].

2.2.1.2 Heterotrophic cultivation. Some microalgae species can not only grow under phototrophic conditions, but also use organic carbon under dark conditions, just like bacteria. The situation when microalgae use organic carbon as both the energy and carbon source is called heterotrophic cultivation [6]. This type of cultivation could avoid the problem associated with limited light that hinders high cell density in large scale photobioreactors during phototrophic cultivation [7]. Higher biomass production and productivity could be obtained using heterotrophic cultivation. Some microalgae species show higher lipid content during heterotrophic growth, and a 40% increase in lipid content was obtained in *Chlorella protothecoides* by changing the cultivation condition from phototrophic to heterotrophic [11].

2.2.1.3 Mixotrophic cultivation. Mixotrophic cultivation is when microalgae undergo photosynthesis to use both organic compounds and inorganic carbon (CO_2) as carbon sources. This means that the microalgae are able to live under either phototrophic or heterotrophic condition, or both. Microalgae assimilate organic compounds and CO_2 as carbon sources, and the CO_2 released by microalgae via respiration will be trapped and reused under phototrophic cultivation [9]. Compared with phototrophic and heterotrophic cultivation, mixotrophic cultivation is rarely used in microalgal oil production.

2.2.1.4 Photoheterotrophic cultivation. Photoheterotrophic cultivation is that the microalgae require light when using organic compounds as the carbon source. The main difference between mixotrophic and photoheterotrophic cultivation is that the latter requires light as the energy source, while mixotrophic cultivation can use organic compounds to provide energy. Hence, photoheterotrophic cultivation needs both sugars and light at the same time [6]. Although the production of some light-regulated useful metabolites can be enhanced by using photoheterotrophic cultivation [12], it is very rare to use this approach to supply algal lipid for the production of biodiesel, as is the case with mixotrophic cultivation.

2.2.2 Factors that affect algal growth. Microalgal growth rates are affected by a combination of environmental parameters such as light intensity, photoperiod, temperature, CO_2 concentration and nutrient composition etc in the culture system. Table 1 shows those physical, chemical and biological factors that influence the growth rate of microalgae.

Table 1

Factors that influence algal growth in an algal pond [13]

Abiotic factors physical and chemical	Light (quality, quantity)
	Temperature
	Nutrient concentration
	O_2, CO_2
	pH
	Salinity
	Toxic chemicals
Biotic factors	Pathogens (bacteria, fungi, viruses)
	Predation by zooplankton
	Competition between species
Operational factors	Mixing
	Dilution rate
	Depth
	Addition of bicarbonate
	Harvesting frequency

These important parameters have large effect on the growth as well as on the lipid content of microalgae. Several studies are still being accomplished on these parameters [14]. To predict the performance of microalgae under a given set of condition it is necessary to know its potential under an optimum condition.

2.2.2.1 Light Intensity. Illumination factor such as light intensity has an intensive effect on the growth of microalgae. Algae use light as their source of energy for synthesizing cell protoplasm and have light saturation limit around 600 ft. candles [15]. The effects of light intensity on growth and lipid content were studied for different microalgal species. It was found that the effects were different for different microalgae species. The growth of marine *Chlorella* sp. increased with the increase in light intensity up to 8000 lux and a further increase in light intensity did not increase the growth of this strain while a slight decrease was observed when light intensity was increased up to 10,000 lux [16]. The growth of *Nannochloropsis* sp. continuously increased up to the maximum level when increasing light intensity up to a maximum light intensity of 10,000 lux [16]. Here *Chlorella* sp. is facing photo-inhibition which is sometimes important for some microalgae to some extent. Among the environmental factors affecting the growth rates of unicellular microalgae, light is the basic energy source and important factor in photosynthesis and is essential for microalgae photoautotrophic growth [15]. In photosynthetic cultures, the amount of light energy received and stored by the cells has a direct relationship with the carbon fixation capacity, consequently determining the productivity in biomass and cell growth rate as in nature light energy is available in a discontinuous way, since the light varies from day to night [17]. Sometimes, the intensity of the natural light is well above the saturation and may be high enough to inhibit the growth during much of the day. The intensity for saturation and inhibition depends on the suitability of other factors of the environment such as temperature, CO_2 level and nutrient supply. The requirements of light also vary greatly with the culture depth and density of microalgae culture. If the depth and cell concentration are higher, the light intensity must be increased to penetrate through the culture [15].

2.2.2.2 Photoperiod. Among all the environmental factors affecting the growth rates of unicellular algae, photoperiod (light and dark) is frequently at an improper level. This is a prime factor that determines the growth rate of microalgal cultivation [18]. For photoautotrophic culture, the light regime and photoperiod are the critical components in determining the biomass production of a culture [15]. Microalgae need a light/dark regime for productive photosynthesis. It needs light for a photochemical phase to produce Adenosine Triphosphate (ATP) as a cellular energy carrier and Nicotinamide Adenine Dinucleotide Phosphate-oxidase (NADPH) as a cellular electron carrier and also needs dark for biochemical phase to fix carbon dioxide and synthesize essential molecules for growth [16]. The effect of photoperiod has been studied for different microalgae species such as *Haematococcus pluvialis, Chlamydomonas reinhardtii, Nannochloropsis* sp. etc to observe the variation in cell density, cell growth rate and total lipid content towards biodiesel production [15]. Three algae samples were placed in different light conditions (photoperiod and intensity) and a huge difference was found in the growing concentration among them as the maximum biomass was recorded when the algae culture exposed to a photoperiod duration of a16:8 h light/dark period [19]. Research was conducted to evaluate the growth of algae under different light cycles, and the totally dark condition at 24:0 (night: day). A reduction in biomass production was observed in parallel with the reduction in light period duration [17]. It was also demonstrated that very fast alteration between high light intensities and darkness could greatly enhance the photosynthetic efficiency. This is called the flashing light effect and was observed under very short light/dark cycles from less than 40 μs to 1s [20]. Thus light regime analysis is emphasized to produce optimum cell concentration [21].

2.2.2.3 Temperature. Temperature is perhaps the most widely measured environmental variables that affect the algal growth. It is almost invariably measured and controlled in

experimental studies of algal cultivation. It strongly influences cellular chemical composition, the uptake of nutrients, carbon dioxide fixation, and the growth rates for every species of algae. It is known that the growth rate will increase with the increase in temperature up to its optimum and once it reaches its optimum, growth rate will decrease drastically with the further increase in temperature. The growth rate and nutritional content of four tropical Australian microalgal species diatom *Chaetoceros sp.*, two cryptomonads, *Rhodomonas sp*. and *Cryptomonas sp.* and unidentified prymnesiophyte, were studied at five different temperatures and the optimum growth temperature was 25–27 °C for *Rhodomonas sp.* and 27–30 °C for *prymnesiophyte, Cryptomonas sp.*, *Chaetoceros sp*. and *Isochrysis sp.*. Only *Chaetoceros sp.* grew well at a temperature as high as 33 and 35 °C [22]. *Scenedesmus sp*. were studied at temperatures of 15 to 36°C and found at low temperatures its chlorophyll and protein levels were reduced, while the levels of carotenoids, saccharides, and lipid were increased. It was also observed that an increase of 30% of the sugars and lipids at an extreme high temperature of 36°C [23]. Temperature also affects the phosphorus content of wastewater when algae are cultured. It was found that phosphorus content in biomass is higher at a higher temperature (temperature, light intensity and nutrient content) of 25°C than at lower temperatures [24]. For *Chlorella vulgaris*, the optimum temperature ranges from 25 to 30°C. it was reported that lipids would increase from 5.9 to 14.7% when the temperature decreased from 30°C to 25°C [25].

2.2.2.4 CO_2 flow rate. Most algae are capable of using inorganic carbon as a nutrient source. These are referred to as autotrophic. Green microalgae contain chlorophylls that use light to absorb CO_2 from air and are capable of converting hazardous CO_2 into valuable biomass as shown in Figure 2. Various researches have been conducted to determine the effective flow rate and CO_2 concentration that gave optimal microalgae growth. Dry microalgae contain 50%

carbon by mass and carbon is known to be a limiting factor when all other nutrients and environmental conditions are satisfied [26]. *Demodesmus sp* had very low growth rate when only atmospheric air was bubbled at 50 ml/min. With the increase in flow rate and CO_2 concentration of bubbling air, the growth rate of microalgae increased up to a certain level but extremely high flow rates and CO_2 concentrations resulted in reduced growth. This is because CO_2 at a high concentration lowers the pH value of the culture medium significantly as CO_2 forms carbonic acid with water to make the medium acidic and intolerable to the microalgae [26]. Using microalgal photobioreactor as a CO_2 mitigation system is a practical approach for the elimination of CO_2 emission from waste gases. A study showed that the rate of CO_2 reduction using marine microalgae *Chlorella sp* was increased with the increase of CO_2 concentration. Some results showed that air streams containing a high concentration of CO_2 (2-15%) may be introduced directly into a high-density culture of *Chlorella sp*. in a semi-continuous photobioreactor [10]. As microalgae have much higher growth rates and CO_2 fixation abilities compared to conventional forestry, agricultural, and aquatic plants, they could completely recycle CO_2 [27, 28]. They can fix CO_2 from different sources, which can be categorized as (1) CO_2 from the atmosphere, (2) CO_2 from industrial exhaust gases (e.g., flue gas and flaring gas), and (3) fixed CO_2 in the form of soluble carbonates (e.g., $NaHCO_3$ and Na_2CO_3) [27].

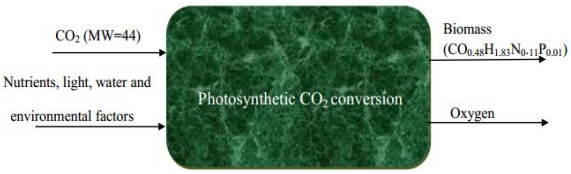

Figure 2. Photosynthetic conversion of CO_2 into microalgae biomass [29].

2.2.2.5 Nutrient composition. Photoautotrophic microalgal growth is mainly dependent on nutrients such as carbon, nitrogen, phosphorus and micronutrients. Any deficiency or excess in these nutritional requirements will limit their growth [3]. Microalgae require nitrogen to grow and build biomass. Nitrogen deficiency in algae results in the alterations in growth, physiological reactions and chemical composition. There is an increase in lipid production when the algal cells are nitrogen-deprived [30]. Microalgae can assimilate inorganic nitrogen forms such as nitrates, ammonia, and inorganic urea, while some species (such as blue-green algae) can fix molecular nitrogen [31]. Ammonium and nitrate salts are the main sources of nitrogen. However, several research reports have indicated that most microalgae do not discriminate different types of nitrogen sources. Increasing the concentration of total nitrogen ions increases both biomass productivity and growth rate [63]. However, nitrogen at a very high concentration slightly reduces growth but does not stop it, probably because of the nitrate regulation of algal cells. In addition, higher nitrate reductase activity can lead to a toxic accumulation of nitrite inside the cells, causing reduced nitrate uptake and inhibition of growth [32].

Phosphorus is another element required for microalgal growth, especially for generating and transforming metabolic energy [33, 34]. Phosphorus is an essential nutrient that constitutes cells, nucleotides and nucleic acids. In natural lakes phosphate concentrations are very low and are therefore at levels that limit microalgal growth [35]. The effects of the concentration of phosphates in the culturing medium on microalgae growth is demonstrated by the more rapid uptake of phosphates in a phosphate-limited environment of a medium containing inadequate phosphates [36].

Different growth parameters of temperature, light intensity and nutrient content have been studied separately for different microalgal strains in literature. So far these parameters have not been studied comprehensively. To optimize the growth and lipid content of microalgae, it is required to combine all growth parameters in a systemic way so that the efficiency of an algal growth system can be maximized. The combination of all these parameters that affect the growth and oil content of different types of microalgae can be optimized.

2.3 Wastewater Treatment Using Microalgae

Nowadays, it is truism to recognize that the pollution problem is a major concern of a society. Environmental laws are given general applicability and their enforcement has been gradually stricter. So, in terms of health, environment and economy, the battle against pollution has become a major concern [37]. Today, the strategic importance of fresh water and air is universally recognized more than ever before. Issues concerning sustainable water management can be found almost in every agenda all over the world. There are few things invented which can be used to mitigate both water and air pollution. Microalgae are one of them which can be used to reduce these crises as it ensures sustainable management of both air and water.

Without proper treatment, excess nitrogen and phosphorus discharged in wastewater can lead to the damage to ecosystems [38]. The negative effects of such nutrient overloading of receiver systems include low dissolved oxygen concentrations and fish kills, undesirable pH shifts, and cyanotoxin production. Chemical and physical technologies are on hand to remove these nutrients, but they consume significant amounts of energy and chemicals, making them to be costly processes. Chemical treatment often leads to secondary contamination of the sludge byproduct as well, creating additional troubles of safe disposal. The energy and cost required for the treatment of wastewater remain a problem for industries and municipalities. Compared to

physical and chemical treatment processes, algae based treatment can potentially achieve nutrient deduction in a less expensive and ecologically safer way with the added benefits of resource recovery and recycling [39].

The history of the commercial use of algal cultures spans about 75 years with application to wastewater treatment and mass production of different strains such as *Chlorella* and *Dunaliella* [37]. Since the land-space requirements of microalgal wastewater treatment systems are substantial, several efforts are being made to develop wastewater treatment systems based on the use of hyper concentrated algal cultures. Microalgae can treat human sewage, livestock wastes, agro-industrial wastes and industrial wastes. Microalgal systems can also be used for the treatment of other wastes such as piggery effluent, the effluent from food processing factories and other agricultural wastes [37]. Therefore, algae can be used in wastewater treatment for a range of purposes, some of which are used for the removal of coliform bacteria, reduction of chemical and biochemical oxygen demand, removal of N and/or P, and also for the removal of heavy metals [37]. The growth of microalgae for wastewater treatment can further be used to supply feedstock for biofuel production.

2.3.1 Composition of typical wastewater. Watercourses receive pollution from many different sources, which vary both in strength and volume. It is a complex mixture of natural organic and inorganic materials as well as man-made compounds. Three quarters of organic carbon in sewage are present as carbohydrates, fats, proteins, amino acids, and volatile acids. The inorganic constituents include large concentrations of sodium, calcium, potassium, magnesium, chlorine, sulfur, phosphate, bicarbonate, ammonium salts and heavy metals [40]. As wastewater contains high amounts of ammonia nitrogen and active phosphorus, that could be a suitable growth medium for microalgae for the dual purposes of removing nutrients and

obtaining a feedstock for biofuel production. Table 2 shows the nitrogen and phosphorus contents of different types of wastewater. Domestic wastewater treatment plants, confined animal feeding operations (CAFOs), and the other listed industries given in Table 2 are good candidates for algae-based treatment due to the respective wastewater compositions and the existing need to treat these waste streams [39]. Although some of these wastewaters typically contain organics and/or heavy metals, algae-based treatment may also aid in the removal of these constituents[39].

Table 2

Characterization of typical wastewaters with respect to algal nutrients nitrogen and phosphorus [39]

Wastewater type	Nitrogen[a] (mg l^{-1})	Phosphorus[b] (mg l^{-1})
Weak domestic	20[c]	4
Medium domestic	40[c]	8
Strong domestic	85[c]	15
Beef cattle feedlot	63	14
Dairy	185	30
Poultry feedlot	802	50
Swine feedlot	2430	324
Coffee production	85	38[d]
Coke plant	757	0.5[d]
Distillery	2700[c]	680[d]
Paper mill	11[c]	0.6
Tannery	273	21d

Table 2

Cont.

Textile	90	18
Winery	110	52

[a] Total Kjeldahl nitrogen (TKN) unless specified , [b] Total phosphorus unless specified, [c] Total nitrogen, [d] Phosphorus as phosphate (PO_4–P).

2.3.2 Microalgae culture in wastewater. Growing algae requires the consideration of three primary nutrients: carbon, nitrogen, and phosphorus. Micronutrients required in traceable amounts include silica, calcium, magnesium, potassium, iron, manganese, sulfur, zinc, copper, and cobalt, although the supply of these essential micronutrients rarely limits algal growth when wastewater is used. If not already available in the water source, the addition of commercial fertilizers can significantly increase production costs which makes the price of algae derived fuel cost prohibitive. For this reason, wastewater is an attractive resource for algae production [39].

Microalgae can utilize such low quality water as agricultural runoff, municipal/industrial/ agricultural wastewater and/or wastewater effluents as the source of water of growth medium, and of N and P sources, among other minor nutrients [41]. Hence, an additional economic incentive exists due to decreased costs of chemicals for the growth medium and even of freshwater, while providing a pathway for wastewater treatment [42].

Several studies have been conducted to culture different types of microalgae in different types of wastewater to remove the nutrients. A number of researchers have investigated the growth of algae in municipal wastewater treatment effluent (primary, secondary or tertiary) [43-45]. These studies are summarized in Table 3. Various types of bio-reactors are scrutinized

keeping in the view that the main limitation upon the type of usable bioreactors is the enormous volume of water to be treated [46]. The feasibility of growing *Chlorella* sp. in the centrate, a highly concentrated municipal wastewater stream generated from activated sludge thickening process, for simultaneous wastewater treatment and energy production was tested [47]. The results showed that at the end of a 14-day batch culture, algae could remove 93.9% ammonia, 89.1% total nitrogen, 80.9% total phosphorus, and 90.8% chemical oxygen demand (COD), respectively from the raw centrate, and the fatty acid methyl ester (FAME) content was 11.04% of dry biomass providing a biodiesel yield of 0.12 g-biodiesel/L-algae culture solution. In another study, *Chlamydomonas reinhardtii* was grown in wastewaters from three different stages of the treatment process [48]. In another study six microalgal species *Ourococcus multisporus*, *Nitzschia cf. pusilla*, *Chlamydomonas mexicana*, *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Micractinium reisseri* were examined to determine their effectiveness in the coupling of piggery wastewater treatment and the highest removal of nitrogen (62%), phosphorus (28%), and inorganic carbon (29%) were achieved by *C. mexicana* [49]. Freshwater microalgae of *Chlorella zofingiensis* were studied to treat the piggy waste water with six different concentrations and found that it removed 65.81% to 79.84% COD, 68.96% to 82.70% TN and 85.0% to 100% TP, respectively [50].

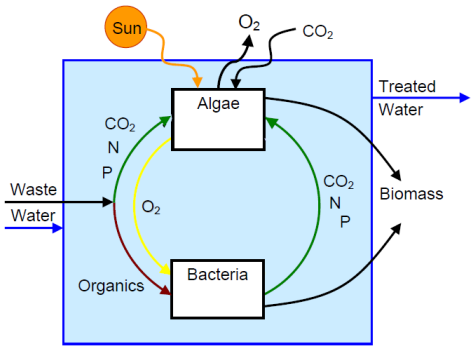

Figure 3. Algae-bacteria symbiosis in wastewater treatment [60].

Recently algae in combination with bacteria were examined to treat wastewater effectively which is shown in Figure 3. Microalgae *Chlorella vulgaris* that are jointly immobilized with *Azospirillum brasilense* as treating agents was used for the secondary treatment of municipal wastewater [51].

Table 3

Published studies on algae cultivation on wastewater

Species	Reactor	Media	Light Intensity ($\mu molm^{-2}s^{-1}$)	Photo Period (h light/dark)	Temperature (°C)	Reference
Chlorella vulgaris and *Botryococcus terribilis*	Batch	Domestic effluent	174	12/12	25 ± 1	[52]
Phormidium bohneri	Batch	Synthetic wastewater	80-350	24/0	15-25	[43]
Spirulina platensis	Airlift	Synthetic wastewater	120	24/0	30	[53]
Rhizoclonium sp.	Algal turf scrubber	Raw and anaerobically digested dairy manure effluents	390	23/1	18-28	[54]
Chlorella zofingiensis	Batch	Anaerobic effluent	N/A [a]	24/0	23-28	[55]
Scenedesmus, Chlorella and etc.	Batch	Dairy farm/municip-al wastewaters	N/A [a]	16/8	23-25	[45]

[a] N/A data were not available

2.4 Biodiesel Production From Microalgae

2.4.1 Biofuel. Since the last few decades, fossil fuels have become an integral part of human daily lives. Specifically, fossil fuels are burned to produce energy for transportation and electricity generation, in which these two sectors have played a vital role in improving human living standard and accelerating advance technological development. However, fossil fuels are non-renewable sources that are limited in supply and will one day be exhausted. In addition, burning fossil fuels have raised numerous environmental concerns, including greenhouse gas (GHG) effects which significantly contribute towards global warming. Apart from that, as energy crisis is beginning to hit almost every part of the world due to rapid industrialization and population growth, the search for renewable energy sources has become the key challenge in this century in order to stimulate a more sustainable energy development for the future [56]. Therefore, discovering and constructing renewable, carbon neutral transportation fuel systems are possibly two of the most vital issues for current society [28].

2.4.1.1 Renewable Energy. Renewable energy is energy that comes from resources which are continually replenished such as sunlight, wind, rain, tides, waves and geothermal heat. The increasing demand of renewable energy can be understood when the current situation in the energy consumption will be studied. According to the EIA U.S., renewable energy consumption grew by 6 percent from 2009 to 2010 [57].

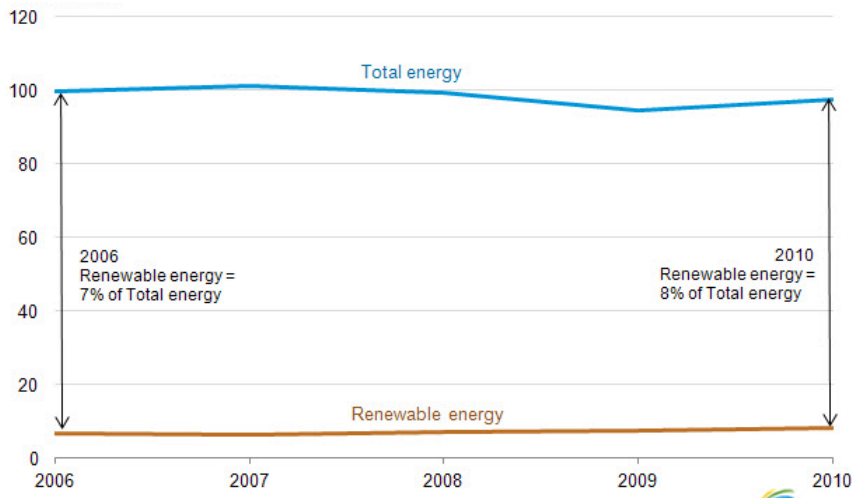

Figure 4. U.S. energy consumption and renewable energy consumption, 2006-2010 [57].

EIA says, the largest portion of our energy is produced from petroleum at 37% followed by natural gas, coal and nuclear power (Figure 5). Of the total amount of renewable energy produced, the largest portion (53%) comes from biomass and only 31% comes from hydroelectric source.

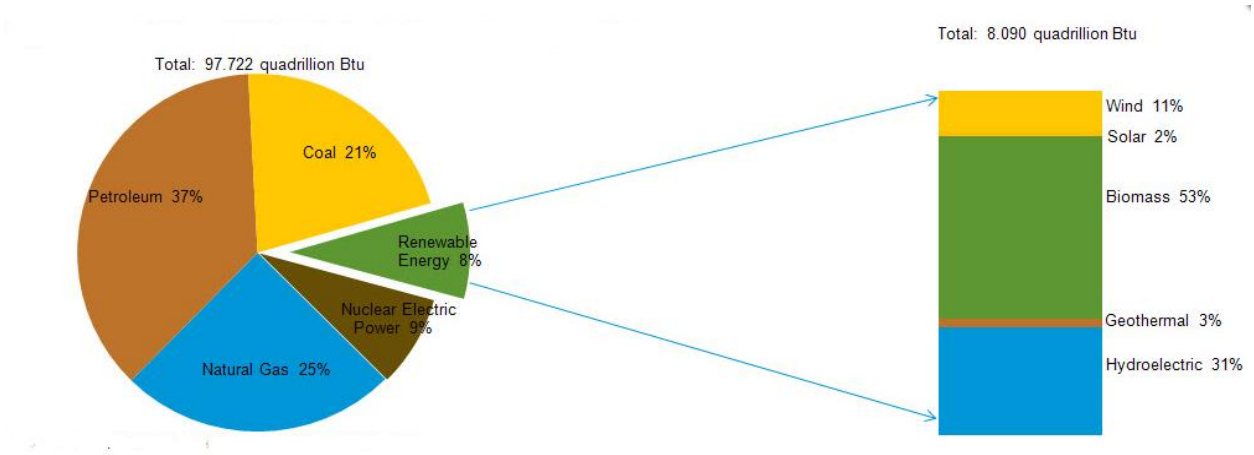

Figure 5. Renewable energy as a share of total primary energy consumption, 2010 [57].

The use of biomass as a renewable energy source increased greatly from 2006-2010.This increase in the use of biomass is putting a huge expectation to biofuel production.

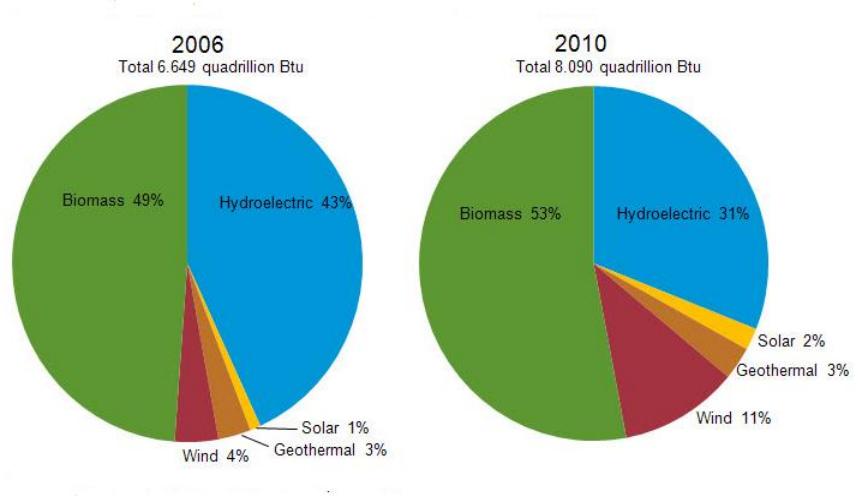

Figure 6. Renewable energy consumption by energy source [57].

Biodiesel, a promising substitute for petroleum fuels, has the potential to address sustainability and energy security issues because it is derived from plant oils or animal fats, and has much lower greenhouse gas emission. Currently, soybean oil is the major feedstock for commercial biodiesel production. Other oil feedstock including canola, corn, jatropha, waste cooking oil, and animal fats are also being tested. While biofuels produced using oil crops and waste oils cannot alone meet the existing demand for fuel. As the capacity and demand for biodiesel production increases, so will the demand for an economic feedstock for biodiesel production as the major cost of biofuel production is the feedstock .

2.4.1.2 Microalgae as biofuel feedstock. Microalgae appear to be a more promising feedstock option as they are known to make far more efficient use of solar energy than conventional agriculture and therefore there is a larger potential for biomass production [28]. Microalgae-based biofuels are an appealing choice [58] to meet these mandates for the production of biofuels because of microalgae's (1) rapid growth rate (cell doubling time of 1–10

days [59]), (2) high lipid content (more than 50% by cell dry weight [60]), (3) sSmaller land usage (15–300 times more oil production than conventional crops on a per-area basis [61]), and (4) high carbon dioxide (CO_2) absorption and uptake rate [62]. Given these advantages, microalgae-based biofuels have been recognized as the ‘‘third-generation of biomass energy” [63] and the ‘‘only current renewable source of oil that could meet the global demand for transport fuels” [59]. To produce a certain amount of biodiesel in indoor system algae needs 1000 times less water than crops.

Table 4

Comparison of crop-dependent biodiesel production from plant oils [59]

Plant source	Biodiesel (L/ha/year)	Area to produce global oil demand (hectares × 10^6)	Area required as percent global land mass
Cotton	325	15,002	100.7
Soybean	446	10,932	73.4
Mustard seed	572	8,524	57.2
Sunflower	952	5,121	34.4
Rapeseed/canola	1,190	4,097	27.5
Jatropha	1,892	2,577	17.3
Oil palm	5,950	819	5.5
Algae (10 $gm^{-2}day^{-1}$ at 30% TAG)	12,000	406	2.7
Algae (50 g m^{-2} day^{-1} at 50% TAG)	98,500	49	0.3

In the recent years, the potential and prospect of microalgae for sustainable energy development have been extensively reviewed and microalgae are foreseen to be the fuel of the future. In fact, microalgae biofuels have been placed globally as one of the leading research fields which can bring enormous benefits to human beings and the environment [56]. Under suitable culture conditions, some microalgal species are able to accumulate up to 50–70% of oil/lipid in their dry mass [28]. The fatty acid profile of microalgal oil is suitable for the synthesis of biodiesel [8]. The major attraction of using microalgal oil for biodiesel is the tremendous oil production capacity by microalgae, as they could produce up to 58,700 L oil per hectare, which is one or two magnitudes higher than that of any other energy crop [28]. However, mass production of microalgal oil faces a number of technical hurdles that render the current development of the algal industry economically unfit. In addition, it is also necessary, but very difficult, to develop cost-effective technologies that would permit efficient biomass harvesting and oil extraction. Nevertheless, since microalgae production is regarded as a feasible approach to mitigate global warming, it is clear that producing oil from microalgal biomass would provide significant benefits, in addition to the fuel [5].

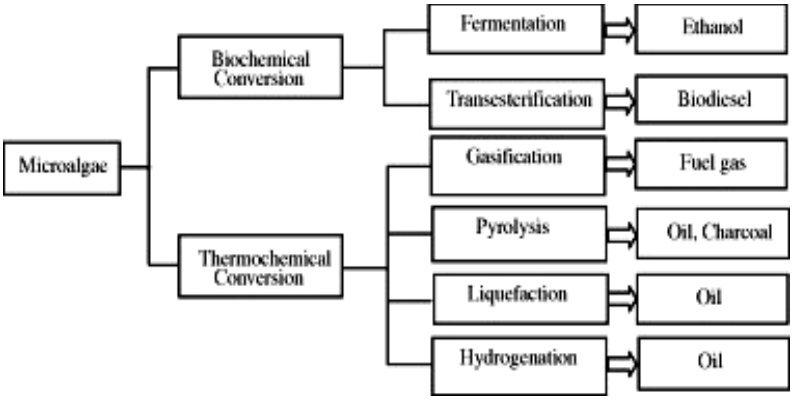

Figure 7. Energy conversion processes from microalgae [64].

The energy conversion reaction of microalgal biomass can be classified into biochemical and thermochemical conversion. Biochemical conversion can be further subdivided into fermentation, anaerobic digestion, bioelectrochemical fuel cells and other fuel producing processes utilizing the metabolism of organisms. Thermochemical conversion can be subdivided into gasification, pyrolysis and liquefaction. Figure 7 shows the energy conversion processes of microalgae [64].

2.4.1.3 Biodiesel Production from microalgae. Biodiesel is a potential substitute for conventional diesel fuel. One of the biotechnological processes that have received increasing interest from companies and researchers is the cultivation of microalgae, which are an excellent source of organic compounds such as fatty acids [65]. The fatty acids that are produced by microalgae can be extracted and converted into biodiesel (Figure 8) [66].

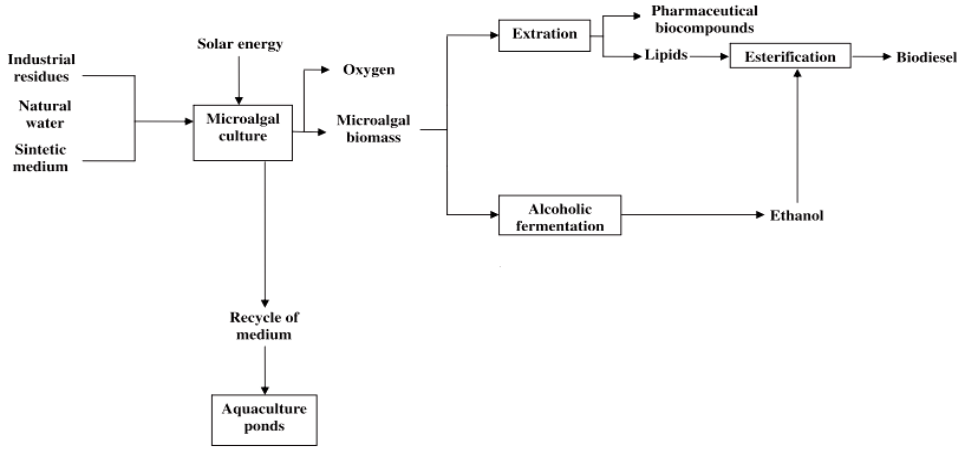

Figure 8. Flow diagram of microalgae biomass for biodiesel production.

Over the past 30 years, microalgal biotechnology for the production of lipids has developed extensively [67]. Microalgae exhibit a great variability in lipid content. Among microalgae species, oil contents can reach up to 80%, and levels of 20–50% are quite common [68]. The microalgae *Chlorella* has up to 50% lipids and *Botryococcus* has 80% lipid. The

variations are due to different growing conditions and methods of extraction of lipids and fatty acids. One of the main factors that influences the lipid and fatty acid content of microalgae in terms of cultivation is the CO_2 concentration. In areas where microalgae are grown for biodiesel production alongside fossil fuel power stations, CO_2 release can be significantly reduced and the lipid content increases [66, 69] .

The carbon and hydrogen contents of microalgal biofuel are greater than those of biofuels produced from other plant materials, even though the oxygen content in microalgal biofuel is lower. The H/C and O/C mean molar ratios of microalgal biofuels were 1.72 and 0.26, while the H/C and O/C molar ratios of plant-based biofuel were 1.38 and 0.37, respectively [70]. Microalgal biofuel is characterized by lower oxygen content and a higher H/C ratio than biofuels from plants, sunflower and cotton [71]. The high hydrogen content of microalgal biofuel is due to chlorophyll and proteins. Microalgal biofuel has a higher calorific value, lower viscosity and lower density than those plant biofuel. These physical properties of microalgae make them more appropriate for biofuel than lignocellulosic materials [71].

The mean annual productivity of microalgal biomass in a tropical climate region is 1.53 kg m^{-3} of a solution with a mean 30.0% of lipids extracted from the biomass, the annual production of a microalgal solution is around 123.0 m^3 ha^{-1} for 90.0% of the 365 days of a year, since the remaining 10.0% of days each year are used for maintenance and cleaning of the bioreactors [70]. Thus, the yield of biodiesel from microalgae is 98.4 m^3 $ha^{-1}year^{-1}$. Therefore, the production of 5.4 billion m^3 of biodiesel requires an area of approximately 5.4 M ha. This represents only 3.0% of the area currently used for the cultivation of plants for biodiesel production. This would be a possible scenario even if the concentration of lipids in the microalgal biomass was 15.0% of dry weight [72].

2.4.1.3.1 Transesterification technologies in the production of biodiesel from microalgae. A widely used process to produce biodiesel from microalgae is transesterification. The viscosities of vegetable and microalgal oils are usually higher than those of diesel oils [73]. Hence, they cannot be applied to engines directly. The transesterification of microalgal oils will greatly reduce the original viscosity and increase the fluidity [7]. Few reports on the production of biodiesel from microalgal oils are available [28]. Nevertheless the technologies for the production of the biodiesel from vegetable oils can be applied to microalgal oils because of their similar physical and chemical properties.

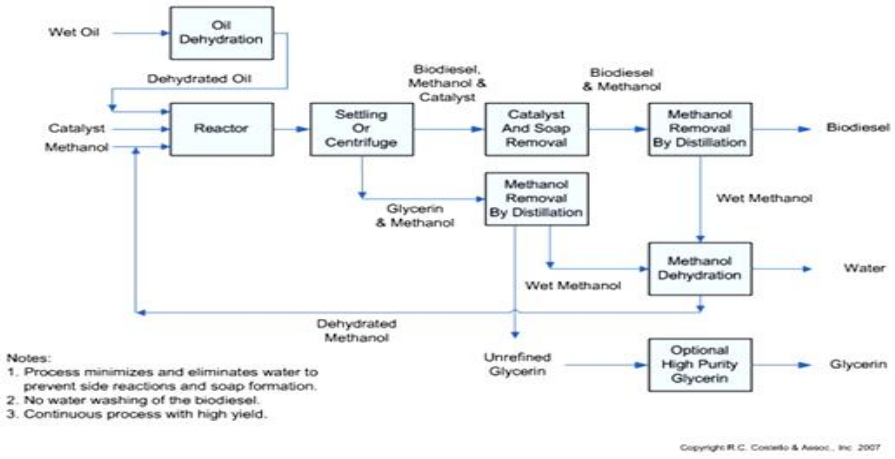

Figure 9. Generic Transesterification Process Diagram [61].

The transesterification reaction involves introducing a triacylglyceride (TAG) from the biomass with an alcohol (typically methanol) to produce a different alcohol (in this case glycerol) and a fatty acid methyl ester (FAME) - more commonly known as biodiesel. In the process of transesterification, alcohols are key substrates in transesterification. The commonly used alcohols are methanol, ethanol, propanol, butanol, and amyl alcohol but methanol is applied more widely because of its low-cost and physical advantages [7]. For the biodiesel production process, this reaction must also be accompanied by multiple pieces of ancillary equipment.

Figure 9 shows a typical process for producing biodiesel via transesterification. The fluent exiting the process has three major streams consisting of mainly methanol, biodiesel, and glycerol. The biodiesel and glycerol are sold as products while, if possible, the methanol is recycled back into the system to improve process efficiency. Alkali, acid, or enzyme catalyzed processes can be applied in transesterification though these processes have their own advantages and disadvantages as shown in Table 5 [7].

Table 5

Application of transesterification technologies [6]

Types of transesterification	Advantages	Disadvantages
Chemical catalysis	1) Reaction condition can be well controlled, large scale production, low cost and high conversion of production 2) Methanol produced can be recycled	1) Reaction temperature is relatively high, process is complex, high energy needed, disposal is complex and pollutes the environment 2) Need an installation for methanol recycle
Enzyme catalysis	Moderate reaction condition, less methanol required, no pollution	Chemical exist in the process of production are poisonous to enzyme
Supercritical fluid techniques	Easy to be controlled, safe, fast and environment friendly.	High temperature and pressure in the reaction condition leads to high cost of production.

2.4.1.4 Economics of biodiesel production. Biodiesel is an alternative that deserves special attention because it has several distinct benefits over other fuels, including oil. Biodiesel can be used to immediately replace conventional diesel in the transportation fuel market, whereas many other alternatives require further research or infrastructural changes in order to improve viability. Biodiesel has many environmental benefits over other fuels that help to reduce the human footprint on the natural world [74].

Biodiesel has the potential to immediately replace a portion of the oil consumed by automobiles because of the existing diesel distribution infrastructure and vehicle fleet. Compression-ignition diesel engines in the transportation sector can operate on biodiesel with little or no costly alterations. The infrastructure for distributing the biodiesel to consumers has already been in place since a regular gas station can be used to dispense the biodiesel. These two benefits will make the transition to biodiesel much simpler than it would be for other alternatives.

Costs of producing microalgal biodiesel can be reduced substantially by using a biorefinery based production strategy, improving capabilities of microalgae through genetic engineering and advances in engineering of photobioreactors [28]. Microalgal oils can potentially substitute petroleum as a source of hydrocarbon feedstock for the petrochemical industry. To achieve this goal, microalgal oil will need to be sourced at a price that is roughly related to the price of crude oil, as follows:

$$C_{algal\ oil} = 0.0069\ C_{petroleum} \qquad (1)$$

where $C_{algal\ oil}$ ($ per liter) is the price of microalgal oil and $C_{petroleum}$ is the price of crude oil in $ per barrel [28]. Eq. (1) assumes that algal oil has roughly 80% of the energy content of crude petroleum.

2.5 Conclusion

In summary, it is promising to use microalgae for waste water treatment and at the same time for biodiesel production. With the favorable conditions and present advanced technology it is economically feasible to reduce greenhouse gas emission by growing algae in wastewater and processing them into biodiesel. Thus the improved climate change will affect the basic elements of human life: water, food, health and the environment and will affect millions of people all the way through famine, drought and floods.

CHAPTER 3

Methodology

3.1 Determination of Kinetics and Nutrient Removal From Wastewater

Although a lot of research has been done to determine the kinetics of different microalgal species cultured in different media, there are still no sufficient information on the kinetics of microalgae grown on swine wastewater and the percentage of nutrients that they can remove from swine wastewater. Different environmental factors that affect the growth of microalgae have been studied widely, but most of the factors have been observed as a single factor. The combined effects of these factors still need to be studied more extensively to determine the optimum condition for the growth of microalgae. In this study, the combined effect of three important factors (temperature, light intensity and nutrient content) has been studied to observe the growth and to determine the performance of microalgae based swine wastewater treatment.

3.1.1 Microalgae strains and pre-cultured conditions. Three different types of microalgae strain were examined in this study. Two of them are *Chlorella vulgaris* (utex #2714) and *Chlamydomonas reinhardtii* (utex # 90) which were collected from UTEX (Austin, TX) grown in proteose medium consists of the following ingredients: $NaNO_3$ (10 ml L^{-1}), $CaCl_2.2H_2O$ (10 ml L^{-1}), $MgSO_4.7H_2O$ (10 ml L^{-1}), K_2HPO_4 (10 ml L^{-1}), KH_2PO_4 (10 ml L^{-1}), NaCl(10 ml L^{-1}), soil water GR + Medium soil extract medium consists of the following ingredients: $NaNO_3$ (10 ml L^{-1}), $CaCl_2.2H_2O$ (10ml L^{-1}), $MgSO_4.7H_2O$ (10 ml L^{-1}), K_2HPO_4 (10ml L^{-1}), KH_2PO_4 (10ml L^{-1}), NaCl(10ml L^{-1}), Proteose Peptone 1 g respectively. The third microalgae strain of select 24 was selected and isolated from some microalgal strains found in NCAT pig farm ponds. This strain has higher lipid content than other isolated microalgal strains from pig farm ponds. Select 24 was grown in swine wastewater collected from those ponds. All

these three selected microalgae were aerated with air with 0.03% CO_2, a surrounding room temperature 20°C and light intensity 606 $\mu molm^{-2}s^{-1}$ continuously.

3.1.2 Pretreatment of swine wastewater from NC A&T farm. Swine wastewater from a NCAT farm near the laboratory was used as a substrate to cultivate the three selected microalgae. Pretreatment was carried out by sedimentation and filtration with a Whatman Quantitative Filter Paper ashless grade 40 with 8 µm pore along with BUCHI vacuum pump V-700 to remove large, non-soluble particulate solids. There are lots of microorganisms in swine wastewater from NCAT farm which might contaminate the microalgae culture. Those needed to be killed in order to get a pure microalgae culture. In order to remove those microorgaisms, two pretreatment processes were studied.

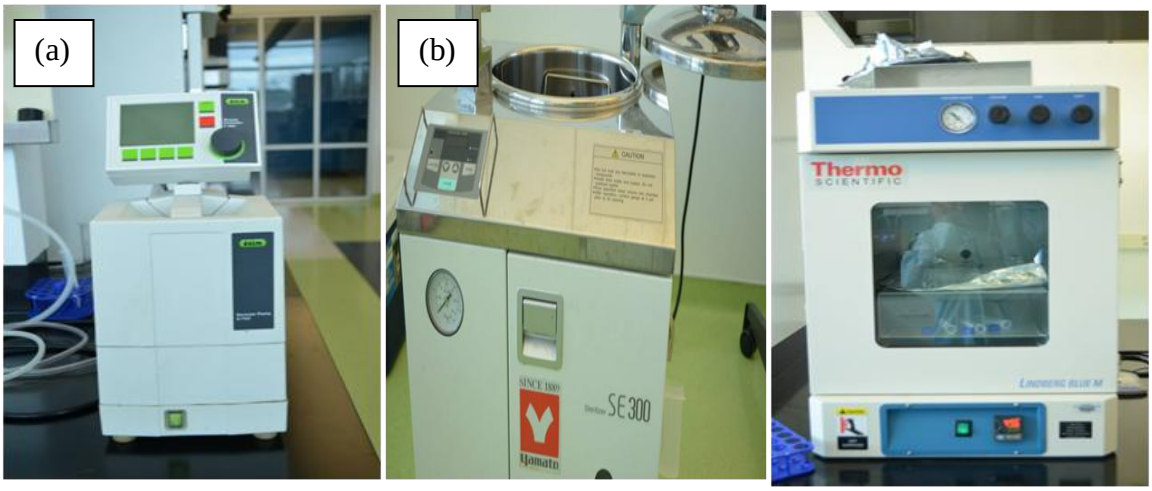

Figure 10. (a) BUCHI vacuum pump V-700, (b) Sterilizer SE 300 autoclave, (c) Oven.

Firstly the wastewater was kept in a sealed bottle without oxygen for one week and secondly it was kept in an oven at an increased temperature of 50°C for 2 days and then both wastewater was used for microalgae culture. Chloromphenicol was used as antibiotic in the waste water. Both cultures were contaminated after the pretreatment processes, so autoclave was done later. After filtration the substrate was autoclaved at 121°C for 15 min using the Sterilizer

SF300. After that the liquid was stored at 4°C in a refrigerator for 2 days for settling any visible particulate solids and the supernatant was used for microalgae growth studies.

3.1.3 Culture of microalgae in swine wastewater. Three growth environment factors 1) nutrient concentration, 2) light intensity and 3) temperature were studied at different levels. The autoclaved supernatant was diluted with distilled water to two different concentrations at a level of 1:0 (wastewater to distilled water) and 1:1 (wastewater to distilled water). The undiluted autoclaved supernatant (1:0) is the control. A volume of 75 ml of swine wastewater with the different concentrations mentioned above were introduced into tbcPBRs.

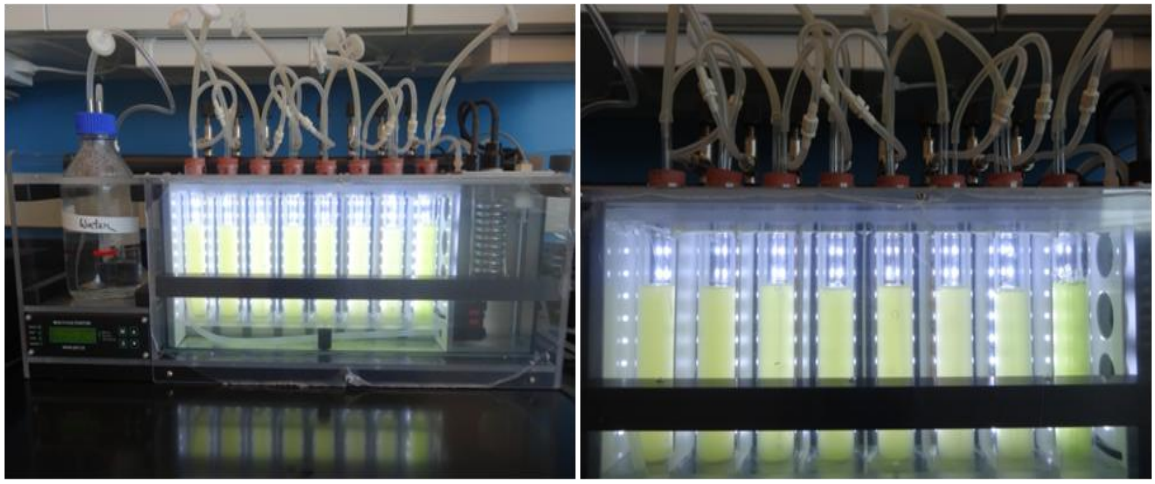

*Figure 11.*Tubular photobioreactor used for microalgae cultivation.

The photobioreactor used for this study was shown in Figure 11. It consists of eight tubular reactors with individual environmental chamber. The temperature of the reactor can be controlled centrally and the light intensity can be controlled in each tubular reactor individually. The photoperiod can also be controlled in each chamber associated with the individual reactors. Air can be passed through each tube and the flow rate can be controlled by the valves located at the top of each tube.

A volume of 5 ml of seed microalgae suspension with an optical density (OD_{680}) of 0.564 A for *chlorella vulgaris* and 0.439 A for *chlamidomonous reinhardtii* was introduced into each photobioreactor. Three different culture temperatures of 20°C, 25°C and 30°C and three optical densities of 300 $\mu molm^{-2}s^{-1}$, 600 $\mu molm^{-2}s^{-1}$ and 900 $\mu molm^{-2}s^{-1}$ were used in this study. The three temperatures were chosen because it is easy to attain outside in North Carolina and most of the period in a year the temperature remains within 20-30°C range. The reason is the same for the selection of three light intensities too. In nature mostly the light intensity remains around 300-900 $\mu molm^{-2}s^{-1}$. Waste water from the NCAT farm might be too concentrated for the growth of microalgae. That is why two concentrations were studied at 1:0 and 1:1 dilution ratio so that if 1:0 ratio is too concentrated for algae, 1:1 concentration would work better. All treatments including a control group were carried out in duplicates. The aerated conditions were identical to that mentioned in Section 3.1.1. In all cases, microalgae were grown for 15 days. The culture conditions are shown in Table 6.

Table 6

Factors & three levels of growth environment

Factors	Nutrient Concentrations (waste water to distilled water)	Light intensity ($\mu molm^{-2}s^{-1}$)	Temperature (°C)
Levels	1:0 and 1:1	300, 600 & 900	20, 25 & 30

3.1.4 Determination of microalgae growth. Microalgal growth was monitored by counting the cell number. A correlation between the optical density of *C. vulgaris* and *C. reinhardtii* at 680 nm spectrum and the cell number was pre-determined. The cell concentration was determined by a Guava easycute HT flow cytometer as shown in Figure 12 and

spectrophotometer. The flow cytometer used to count the cell number in each ml of solution. This flow cytometer can not only count the cells but also give an idea about the lipid content (biodipy and chlorophyll A) of each microalgal strain. Optical density (OD) was measured by the following spectrophotometer. The OD of growth media at 680 nm spectrum were measured every other day using the spectrophotometer.

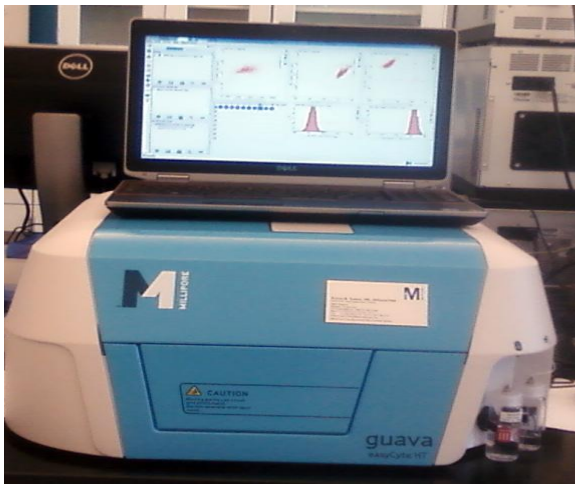

Figure 12. Guava easycute HT flow cytometer.

Figure 13. Spectrophotometer used to measure the optical density at 680 nm spectrum.

The specific growth rate μ in exponential phase of algal growth was expressed as Eq. (2) [15]:

$$\mu = \frac{\ln (N_2 - N_1)}{t_2 - t_1} \quad (2)$$

where, N_1 and N_2 are defined as the cell number concentration (cell/ml) at time t_1 and t_2, respectively. The time required to duplicate the cell number: division rate (k), was calculated by [15]:

$$k = \frac{\mu}{\ln 2} \quad (3)$$

3.1.5 Sampling and nutrients analysis. After *C. vulgaris* and *C. reinhardtii* grew for 15 days, microalgae cells from each tbcPBR were collected and centrifuged at 4000 rpm and 20°C for 15 min using a Sorvall Legend XFR centrifuge.

Figure 14. Sorvall Legend XFR centrifuge.

Supernatants from the centrifuge were separated to check the nutrient removal from wastewater. Those were filtered using a 0.45 mm nylon membrane filter. Then, the filtrates were appropriately diluted and analyzed for COD, ammonia nitrogen and total phosphorus using the LaMotte Smart 3 kit.

Figure 15. LaMotte Smart 3 kit.

To prepare all the samples for LaMotte Smart 3 kit, Fisher scientific digital vortex mixture were used and all the samples were mixed at 1000 rpm.

Figure 16. Fisher scientific digital vortex mixture.

The removal efficiency of nutrients was expressed as:

$$Percent\ removal\ W\% = 100\% \times \frac{C_o - C_i}{C_o} \qquad (4)$$

where C_o and C_i are defined as the mean values of nutrient concentration at initial time t_0 and time t_i, respectively.

All experiments were carried out in duplicate and average values were reported. Results were performed with MS Excel (Microsoft Office Enterprise, 2007).

3.2 Comparison of Lipid Content among the Microalgae Strains

The Guava easycyte flow cytomaeter was used to compare the lipid content among the microalgal strains. A cleaning operation and an easy check were done first to check whether the instrument gave an accurate result or not. If the easy check gave the accurate result then all the samples were put into the cells of the flow cytometer. BIODIPY was used as the dye to generate the lipid plots and the plots generated by the instrument was saved. Those plots give a comparative idea of the lipid content in the different microalgae. It gives plots for two neutron-lipids 1) biodipy and 2) chlorophyll A.

3.3 Extraction of Oil

3.3.1 Sample preparation. C. *vulgaris, C. reinhadtii* and select 24 were grown in six 1-liter bottles for 3 months under room temperature, room light intensity and air. At first 5 ml seeds were cultured from the solid UTEX samples for *C. vulgaris* and *C. reihardtii* and for select 24 from NCAT pig farm. Then these seeds were transferred in 100 ml filtered and autoclaved swine wastewater. Chloromplenical was used as the antibiotic to stop the growth of any bacteria in the growth media. 8 ml of chloromphenicol was used for 100 ml of waste water. The culture were scaled up on 10 days with 300 ml of wastewater until it reached 1000 ml and antibiotic was used regularly with the scale up in the same ratio.

The samples were checked at regular intervals to see if any kind of bacterial and fungal contaminations took place or not using a Ziess microscope.

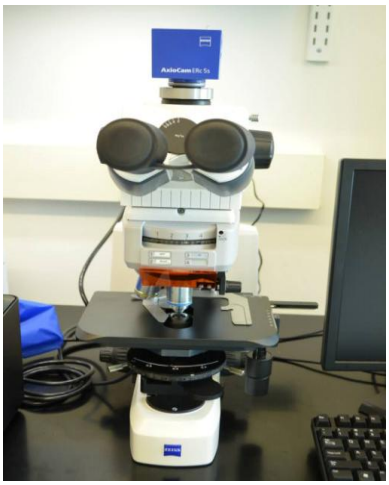

Figure 17. Ziess microscope used to check the bacterial or fungal contamination.

After culturing for 3 months all samples were centrifuged using the Sorvall Legend XFR centrifuge at 4000 rpm at 20°C for 30 min. More centrifuge time was taken due to more samples than those of the previous experiments. The supernatant was discarded and the samples were transferred in six aluminum plates of known weight. An electronic balance with an accuracy of 0.001 mg was used to measure the weight of all samples.

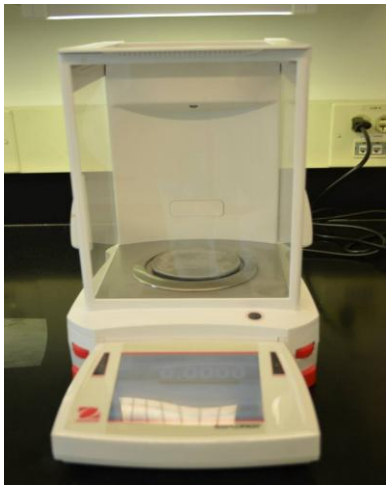

Figure 18. The electronic balance.

All samples were put into an oven at 60°C for two days to measure the dry weight. The weights were check frequently to see whether they have become constant. When the weights became constant, they were recorded and the dry samples were used for oil extraction.

3.3.2 Soxlet extraction of oil. The microalgal paste was dried at 50 °C in an oven for 48 h. Microalgal powder was packed in a cellulose thimble inside the extraction chamber of a 20 ml Soxhlet extractor as shown in Figure 19. Pure n-hexane (10 ml) was used to extract the lipid in the microalgae for 8 h at the rate of 20 refluxes per hour. The temperature was set at 70°C which is the boiling point of the hexane. Following the extraction, the n-hexane containing the extracted lipid was transferred into a 20 ml glass tube.

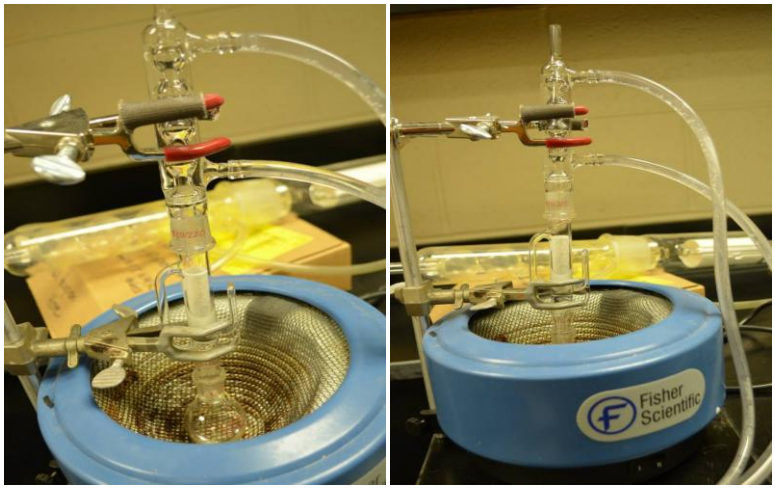

Figure 19. Oil extraction using soxlet extractor from microalgae.

3.3.3 Separation of solvent from oil sample. The mixture was taken out from the extractor and transferred into a test tube of known weight. CO_2 was used to separate the oil from hexane. All the test tubes were put under the fume hood and CO_2 was blown to dry the oil sample and vaporize hexane. The oil samples obtained from microalgae were shown in Figure. 20.

Figure 20. Oil extracted from microalgae samples.

3.3.4 FAME synthesis. Algal oils were weighed into clean, 20 ml screw-top glass tubes, to which 4 ml fresh solution of a mixture of methanol, concentrated sulfuric acid, and chloroform (1.7:0.3:2.0 v/v/v) was added. The bottles were closed tightly with Teflon tape to avoid leakage, and then weighed. For transesterification, tubes were placed inside a heating block at temperatures of 90°C and heated for 60 min. On completion of the reaction, the tubes were cooled down to room temperature and weighed again to determine if there was any leak of the samples. Then, 1 ml distilled water was added into the mixture and thoroughly vortexed for 1 min. After the formation of two phases, the lower phase containing FAME was transferred to a 1.5ml GC vial. Sample were stored in the freezer (-15°C) until GC-MS analysis.

3.3.5 Chemical Analysis. Chemical compositions of the liquid products were identified using an Agilent 7890A gas chromatography /5975c mass spectrometer (GC-MS) with a HP-5MS capillary column. The GC was programmed at 60°C for 4 min and then increased at 10 °C/min to 280°C, and held at the final temperature for 5 min. The injector temperature was 250°C, and the injection size was 1 µl. The flow rate of the carrier gas (helium) was 1 ml/min. The ion source temperature was 230°C for the mass selective detector. The compounds were identified by comparison with the NIST Mass Spectral Database.

Figure 21. GCMS used to analyze the oil extracted from microalgae

CHAPTER 4

Results

4.1 Microalgae Growth Curves

The growth curves of microalgae were determined by measuring the optical density at every other day during the 15 days batch culture. Optical density was measured at 680 nm using a spectrometer. Autoclaved wastewater was used as the growth medium. To analyze the effects of different parameters on the growth of *C. vulgaris* & *C. reinhardtii*, different conditions were set in different experiments to obtain the corresponding growth curves.

4.1.1 Growth of microalgae at 100% waste water concentration

4.1.1.1 The effect of light intensity on growth at 20°C and 100% wastewater. Figure 22 shows the growth curves of two microalgal strains of *C. vulgaries* and *C. reinbardtii* at temperature 20°C, 300 $\mu molm^{-2}s^{-1}$ and 100% wastewater concentration for 15 days.

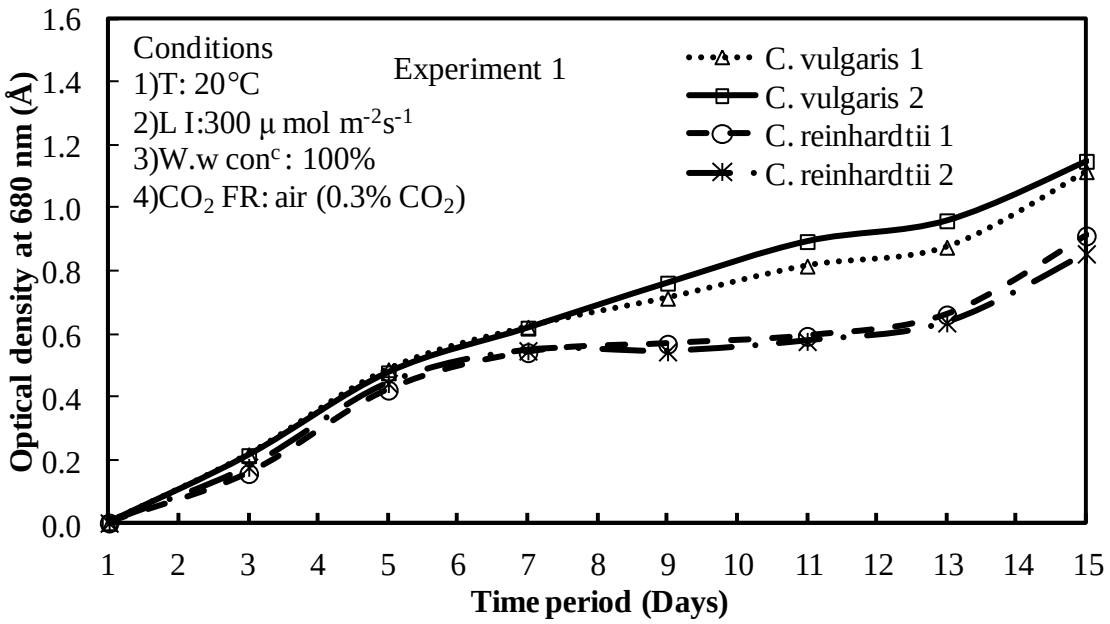

Figure 22. Growth curves of the two selected microalgae grown at 20°C, 300 $\mu molm^{-2}s^{-1}$ and 100% swine wastewater.

The results revealed that the growth curve patterns were different for *C. vulgaris* and *C. reinhardtii*. The lag phase was short for both species according to the curve patterns in this phase. The difference was seen in the exponential phase after day 5. *C. vulgaris* increased more rapidly in this phase than *C. reinhardtii*. After day 13 *C. reinhardtii* shows comparatively high growth rate. The cell densities reached the maximum value on day 15 for both species and the curves were still up-slope on day 15 which indicates that the growth was still in the exponential phases. The experiment was stopped on day 15 in exponential phase for both species.

Figure 23 shows the growth curves that were obtained at temperature 20°C, 600 μmolm^{-2}s^{-1} and 100% waste water concentration. In this experiment the light intensity was doubled compared to the growth curves shown in Figure 22.

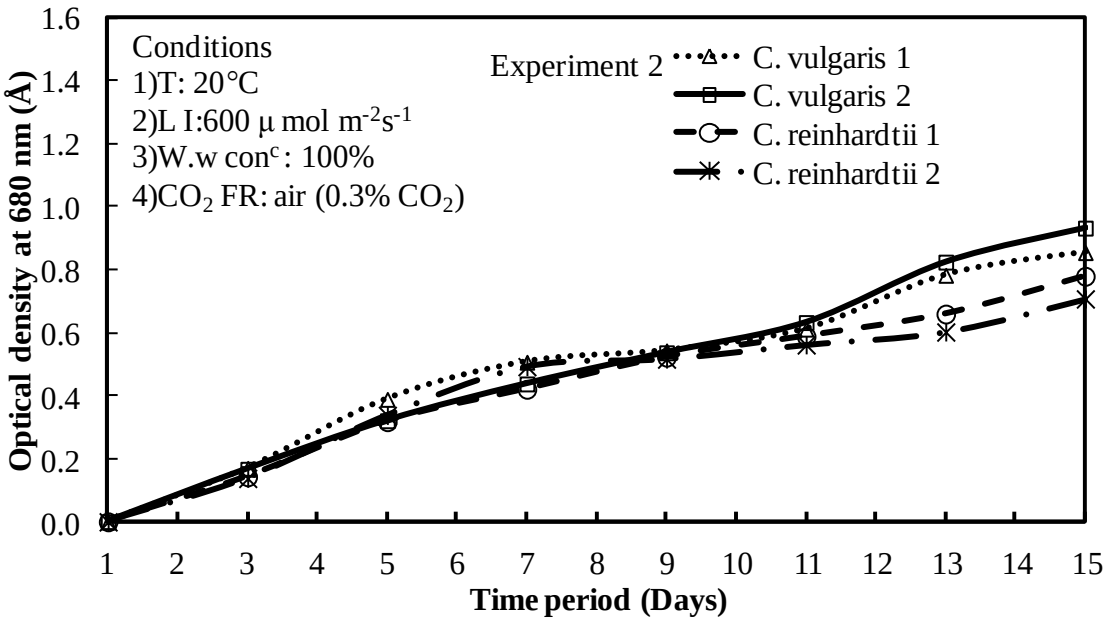

Figure 23. Growth curves of the two selected microalgae grown at 20°C, 600 μmolm^{-2}s^{-1} and 100% swine waste water.

Like the previous growth curves the growth pattern in lag phases were quite similar. In this condition growth rate decreased a little for both species but still *C. vulgaris* had higher

growth rate than *C. reinhardtii*. The difference in the growth rate of *C. reinhardtii* was prominent as a continuous increase was found for 15 days. As light is a basic energy source and at 20°C the increased light intensity from 300 to 600 $\mu molm^{-2}s^{-1}$ slightly inhibits the growth rate. For both species, the growth was still in the exponential phases on day 15.

Figure 24 shows the growth curves at temperature 20°C, 900 $\mu molm^{-2}s^{-1}$ and 100% wastewater concentration.

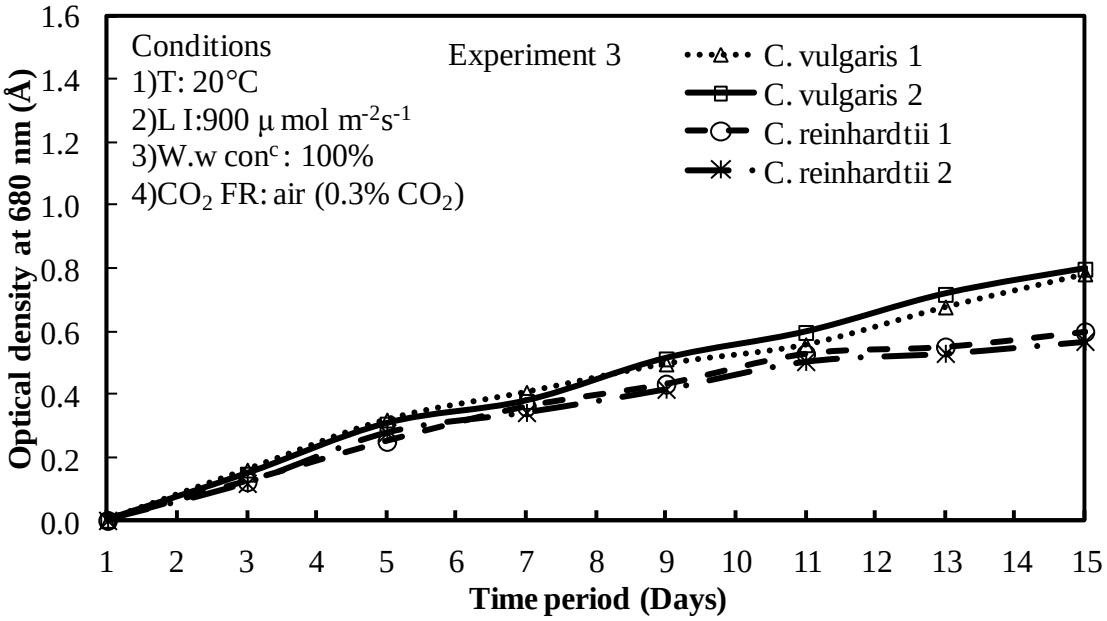

Figure 24. Growth curves of the two selected microalgae grown at 20°C, 900 $\mu molm^{-2}s^{-1}$ and 100% swine wastewater.

These curves show the effects of too intense light intensity (900 $\mu molm^{-2}s^{-1}$) which led to a significant reduced growth rate. If other growth conditions were the same, the growth rates decreased with the increase of the light intensity from 300 to 900 $\mu molm^{-2}s^{-1}$ according to Figures 22-24. Under all three different light conditions the lag phases were very short which were not obviously visible in the growth curves. Exponential phases were obviously observed although the growths were stopped in the middle of the exponential phases. All curves showed

that the two microalgae had prolonged exponential phases when they were grown in the swine waste water from the NCAT farm.

4.1.1.2 The effect of light intensity on growth at 25°C and 100% wastewater. Figure 25 shows the growth curves that were obtained at temperature 25°C, 300 μmolm^{-2}s^{-1} and 100 % wastewater concentration.

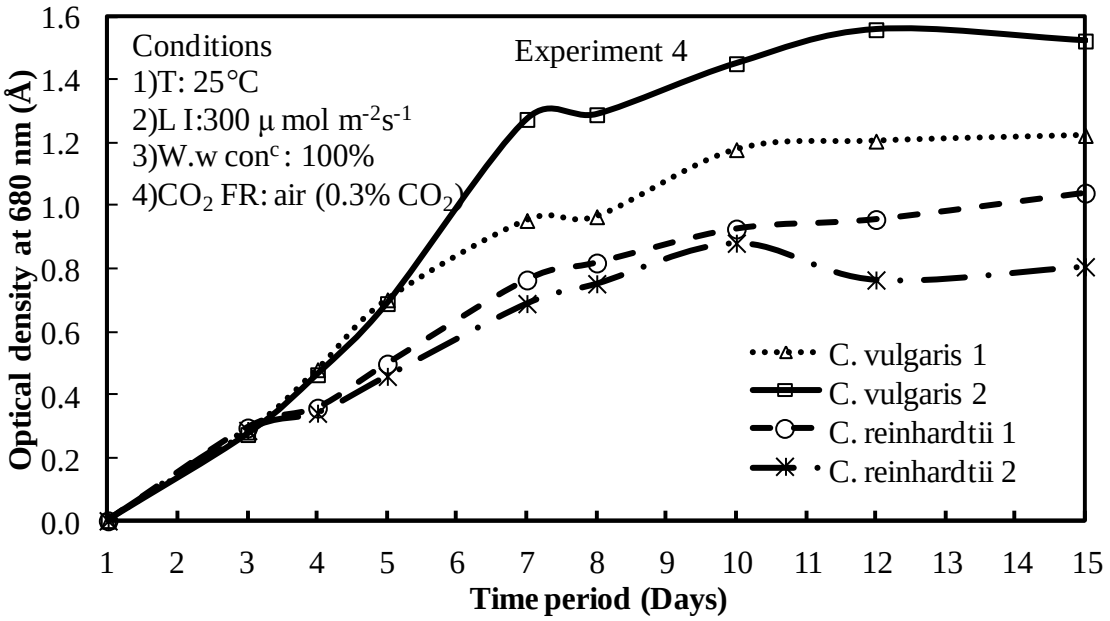

Figure 25. Growth curves of the two selected microalgae grown at 25°C, 300 μmolm^{-2}s^{-1} and 100 % swine wastewater.

The growth rates of both species at 25°C (Figure 25) were higher than those at 20°C (Figure 22) at the same light intensity of 300 μmolm^{-2}s^{-1} and 100% swine wastewater. Some contaminations were found in the repeating experiment of *C. vulgaris* which led to a higher growth rate during the repeating experiment. *C. reinhardtii* showed lower growth rate than *C. vulgaris* at the growth temperature of 25^{o}C again. The lag phase behaviors were similar.

Figure 26 shows the growth curves that obtained at temperature 25°C, 600 μmolm^{-2}s^{-1} and 100 % wastewater concentration.

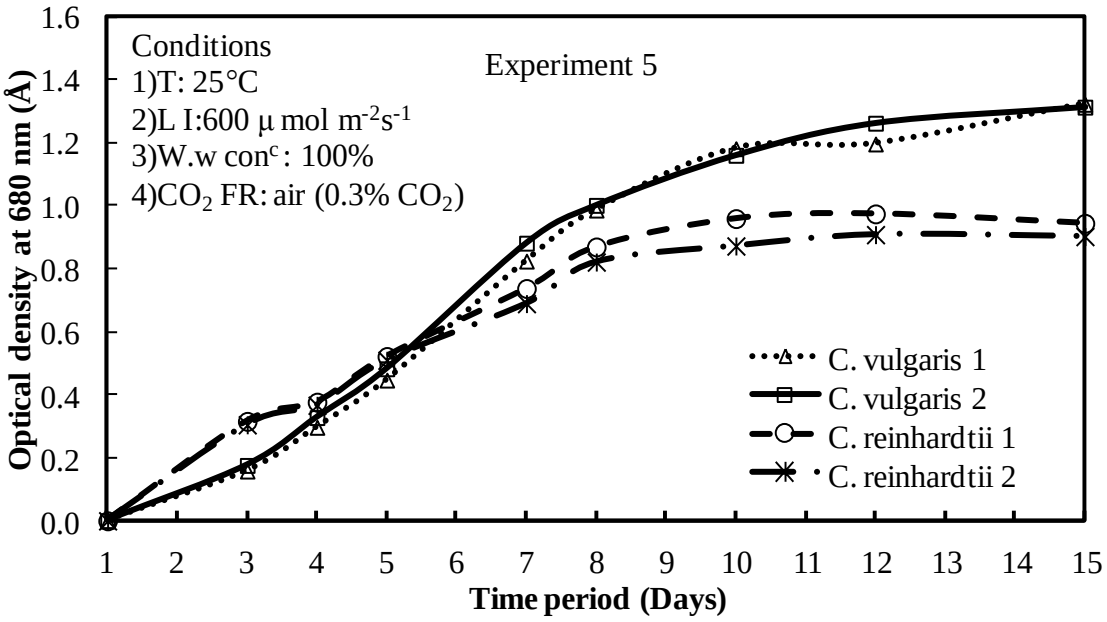

Figure 26. Growth curves of the two selected microalgae grown at 25°C, 600 µmolm^{-2}s^{-1} and 100 % swine wastewater.

In this condition, the growth rate of *C. vulgaris* was higher than all the previous experiment conditions. At the beginning of the experiment, the growth rate of *C. reinhardtii* was higher than that of *C. vulgaris*. On the fifth day, the growth rates were the same for both species. After the 5-day growth, *C. vulgaris* grew much faster than *C. reinhardtii*. The *C. vulgaris* continued to grow during the 15-day period while there was no significant growth for *C. reinhardtii* after 10 days.

Figure 27 illustrates the growth curves that obtained at temperature 25°C, 900 µmolm^{-2}s^{-1} and 100 % wastewater concentration.

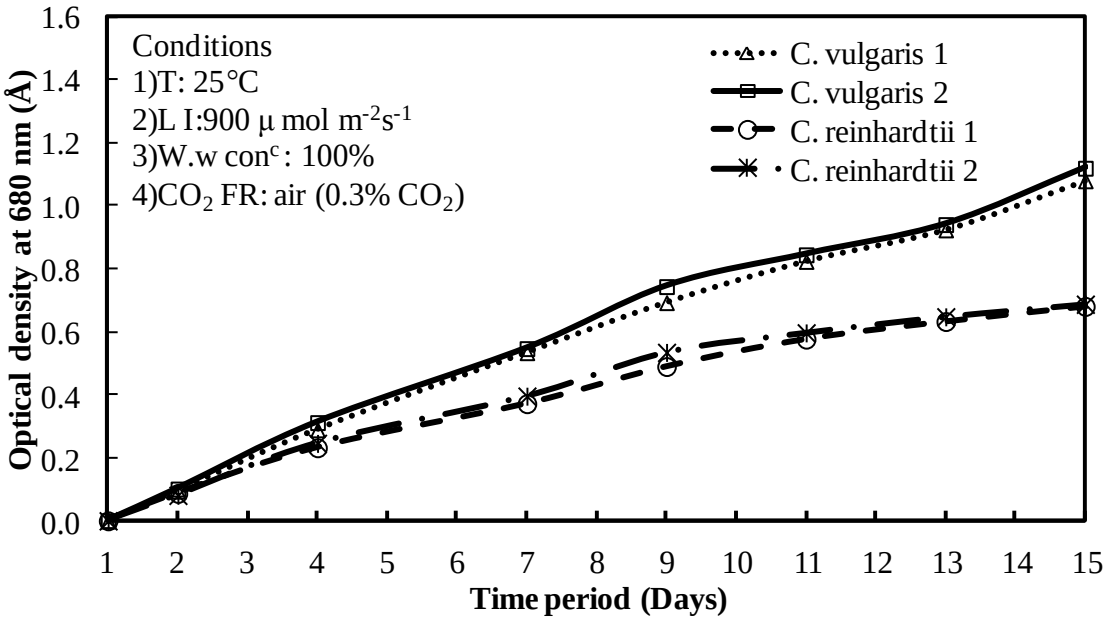

Figure 27. Growth curves of the two selected microalgae grown at 25°C, 900 μmolm^{-2}s^{-1} and 100 % swine wastewater.

Figure 27 further confirmed that high light intensity was not suitable for the growth of microalgae. Both Figures 24 and 27 showed that very high light intensity lowered the growth rates at both 20^{o}C and 25^{o}C. Under the conditions discussed above, the lowest growth rates of both species were found at the highest light intensity of 900 μmolm^{-2}s^{-1} and 25°C. At 25^{o}C, the growth rate of *C reinhardtii* decreased much more than that of *C. vulgaris* with the increase of light intensity. At a very high light intensity of 900 μmolm^{-2}s^{-1}, the growth rates of both species at 25°C were higher than those obtained at 20°C by comparing the growth curves given in Figures 24 and 27.

4.1.1.3 The effect of light intensity on growth at 30°C and 100% wastewater. Figure 28 gives the growth of C. *vulgaris* and *C. reinhardtii* at the temperature 30°C, 300 μmolm^{-2}s^{-1} and 100 % wastewater concentration.

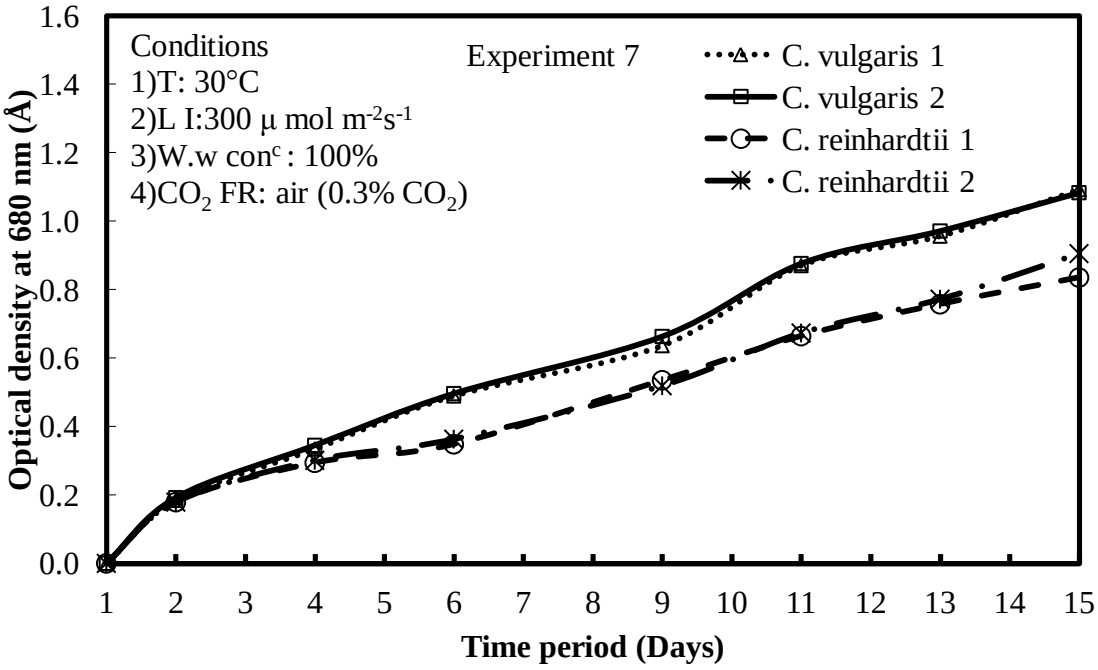

Figure 28. Growth curves of the two selected microalgae grown at 30°C, 300 μmolm^{-2}s^{-1} and 100 % swine wastewater.

From the curves given in Figure 28 it was observed that both species showed a similar growth behavior within the initial 3 days and after that *C. vulgaris* started to grow much faster than *C. reinhardtii*. The growth continued to increase for both species at the end of day 15. According to Figures 23 (temperature: 20^{o}C), 26 (temperature: 25^{o}C), and 29 (temperature: 30^{o}C), at 300 μmolm^{-2}s^{-1} and 100% swine wastewater both species achieved the highest growth rate at 25°C while had the lowest growth rate at 30°C.

Figure 29 gives the growth of *C. vulgaris* and *C. reinhardtii* at temperature 30°C, 600 μmolm^{-2}s^{-1} and 100 % wastewater concentration.

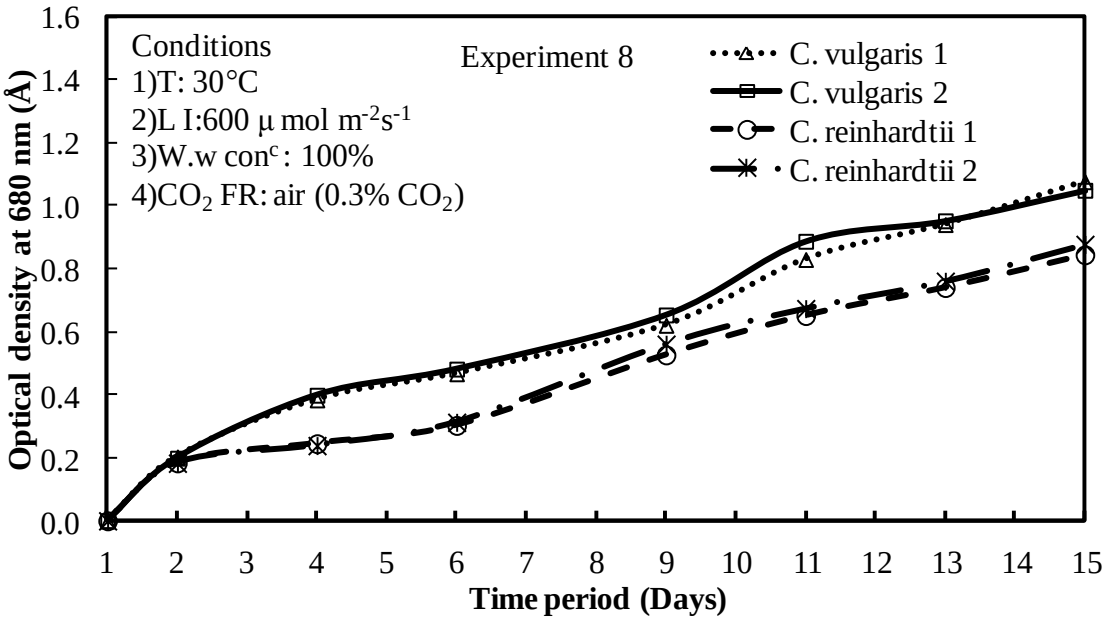

Figure 29. Growth curves of the two selected microalgae grown at 30°C, 600 µmolm^{-2}s^{-1} and 100 % swine wastewater.

Figure 30 shows that at the beginning, the growth rates were the same. However, after 2-day growth, C. *vulgaries* started to grow faster than that of *C. reinhardtii*. The growth rate continued to increase. According to Figures 23 (temperature: 20^{o}C), 26 (temperature: 25^{o}C), and 29 (temperature: 30^{o}C), at 600 µmolm^{-2}s^{-1}, both species also achieved the highest growth rate at 25°C. Both species had a similar growth rates at 20°C and 30°C.

Figure 30 gives the growth of *C. vulgaris* and *C. reinhardtii* at temperature 30°C, 900 µmolm^{-2}s^{-1} and 100 % wastewater concentration.

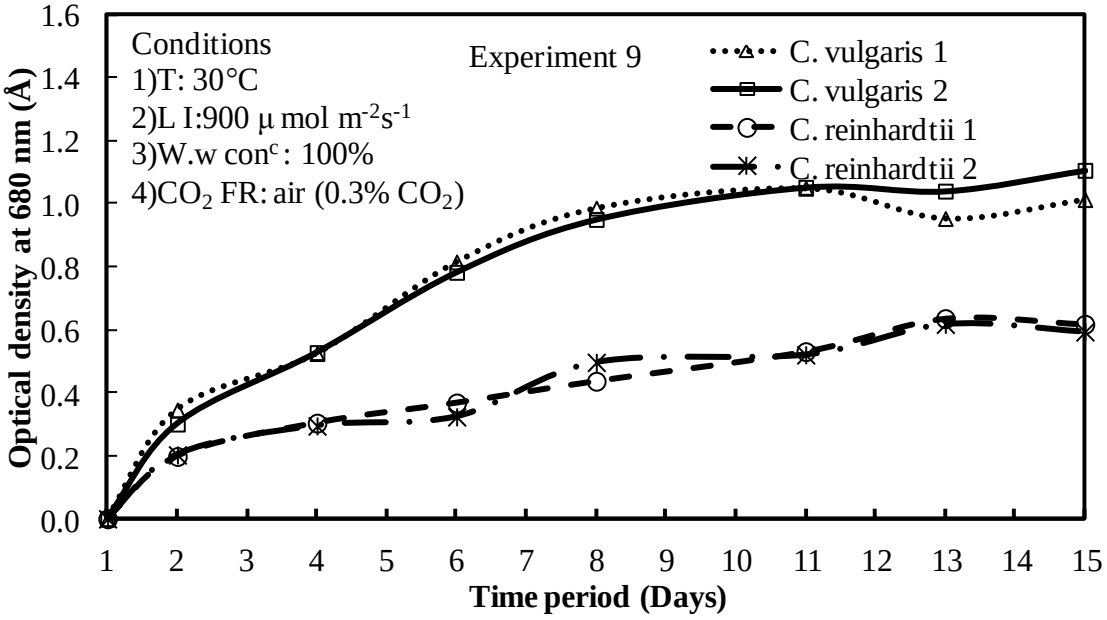

Figure 30. Growth curves of the two selected microalgae grown at 30°C, 900 μmolm^{-2}s^{-1} and 100 % swine wastewater.

At the highest temperature of 30^{o}C and highest light intensity of 900 μmolm^{-2}s^{-1}, the growth rate of *C. vulgaris* was much higher than that of *C. reinhardtii*. At the end of the fifteenth day, the optical density of *C. vulgaris* was almost double that of *C. reinhardtii*. According to Figures 24 (temperature: 20^{o}C), 28 (temperature: 25^{o}C), and 30 (temperature: 30^{o}C), at 900 μmolm^{-2}s^{-1}, both species also achieved the highest growth rate at 25°C. The growth rate of *C. vulgaris* at 30^{o}C was higher than that obtained at 20°C. At the intensity of 900 μmolm^{-2}s^{-1}, the final optical density of *C. reinhardtii* was close at all three temperatures.

4.1.2 Growth kinetics of microalgae on 100 % swine wastewater. Correlations between the optical densities of *C. vulgaris* and *C. reinhardtii* at 680 nm and the cell number were pre-determined. These correlations were used to determine the growth kinetics of both species under different conditions.

The correlation for *C. vulgaris* is

$$\text{Cell Number (cell/ml)} = 8\times10^{6} OD_{680} + 425897 \quad , R^2 = 0.9588 \tag{5}$$

The kinetics for the growth of *C. vulgaris* at different conditions is shown in Table 7.

Table 7

Specific growth rate of C. vulgaris at different temperatures and light intensities in 100% wastewater

Temperature (°C)	Light Intensity ($\mu molm^2s^{-1}$)	Final cell density, × 10^6(cell/ml)	Specific growth rate, μ (day^{-1})	Division rate, k (day^{-1})
20	300	9.48	1.317	1.900
	600	7.58	1.297	1.872
	900	6.75	1.287	1.856
25	300	11.42	1.332	1.921
	600	10.97	1.336	1.928
	900	9.22	1.223	1.764
30	300	9.12	1.214	1.752
	600	8.93	1.211	1.747
	900	8.91	1.199	1.730

The results show that at all three light intensities, the temperature at 25°C gave the highest specific growth rates for *C. vulgaris* in 100% swine wastewater The highest specific growth rate and division rate were found to be 1.336 day^{-1} and 1.928 day^{-1} obtained at 25°C and 600 $\mu molm^{-2}s^{-1}$ in 100% concentrated wastewater. At 25°C the specific growth rate and division

rate obtained at the light intensity at 300 $\mu molm^{-2}s^{-1}$ were 1.332 day^{-1} and 1.921 day^{-1}, which were very close to those obtained at the light intensity at 600 $\mu molm^{-2}s^{-1}$. Therefore, at 25°C, there was no significant change in the specific growth rate and division rate if the light intensity increased from 300 to 600 $\mu molm^{-2}s^{-1}$. If the light intensity was further increased from 600 to 900 $\mu molm^{-2}s^{-1}$, there was significant decrease in both specific growth rate and division rate. The lowest growth rate was found 1.199 day^{-1} obtained at 30°C and 900 $\mu molm^{-2}s^{-1}$.

The correlation for *C. reinhardtii* is

$$\text{Cell Number (cell/ml)} = 7\times10^{6} OD_{680} + 800979 \qquad R^2 = 0.9591 \qquad (6)$$

Table 8

Specific growth rate of C. reinhardtii in 100% wastewater

Temperature (°C)	Light Intensity $\mu molm^{-2}s^{-1}$	Final cell density $\times 10^6$, (cell/ml)	Specific growth rate, μ (day^{-1})	Division rate, k (day^{-1})
20	300	6.98	1.286	1.854
	600	6.01	1.271	1.834
	900	4.89	1.249	1.868
25	300	7.27	1.272	1.836
	600	7.27	1.275	1.839
	900	5.59	1.173	1.692
30	300	6.89	1.184	1.708
	600	6.83	1.182	1.706
	900	5.04	1.143	1.649

The highest specific growth and division rates of *C. reinhardtii* were found to be 1.275 day^{-1} and 1.839 day^{-1} obtained at 25°C and 600 $\mu molm^{-2}s^{-1}$. At 25°C the specific growth and division rates obtained at the light intensity at 300 $\mu molm^{-2}s^{-1}$ were 1.272 day^{-1} and 1.836 day^{-1}, which were very close to those obtained at the light intensity at 600 $\mu molm^{-2}s^{-1}$. Therefore, at 25^{o}C, there was no significant change in the growth and division rates if the light intensity increased from 300 to 600 $\mu molm^{-2}s^{-1}$. However, if the light intensity was further increased from 600 to 900 $\mu molm^{-2}s^{-1}$, there were significant decreases in both specific growth and division rates. The lowest growth was found at 30°C and 900 $\mu molm^{-2}s^{-1}$ which is 1.143 day^{-1} with a division rate of 1.649 day^{-1}.

4.1.3 Growth of microalgae at 50% waste water concentration

4.1.3.1 The effect of light intensity on growth at 20°C and 50% wastewater. Figures 31-33 give the growth of *C. vulgaris* and *C. reinhardtii* in 50% wastewater and at temperature 20°C, and different light intensities of 300 $\mu molm^{-2}s^{-1}$,600 $\mu molm^{-2}s^{-1}$ and 900 $\mu molm^{-2}s^{-1}$, respectively. As seen from Figures 31-33, at 20^{o}C and in 50% wastewater, the growth rates of both species increased with the increase of the light intensity from 300 $\mu molm^{-2}s^{-1}$ (Figure 31) to 600 $\mu molm^{-2}s^{-1}$ (Figure 32). The further increase of the light intensity to 900 $\mu molm^{-2}s^{-1}$(Figure 33) significantly decreased the growth rates of both species. At the light intensity of 900 $\mu molm^{-2}s^{-1}$, the growth rates of both species were close although the growth rate of *C. vulgaris* was still little higher. Both *C. vulgaris* and *C. reinhardtii* were still in their exponential phases at the end of the fifteenth growth day. The growth rate of both the species decreased significantly by diluting the wastewater to 50%.

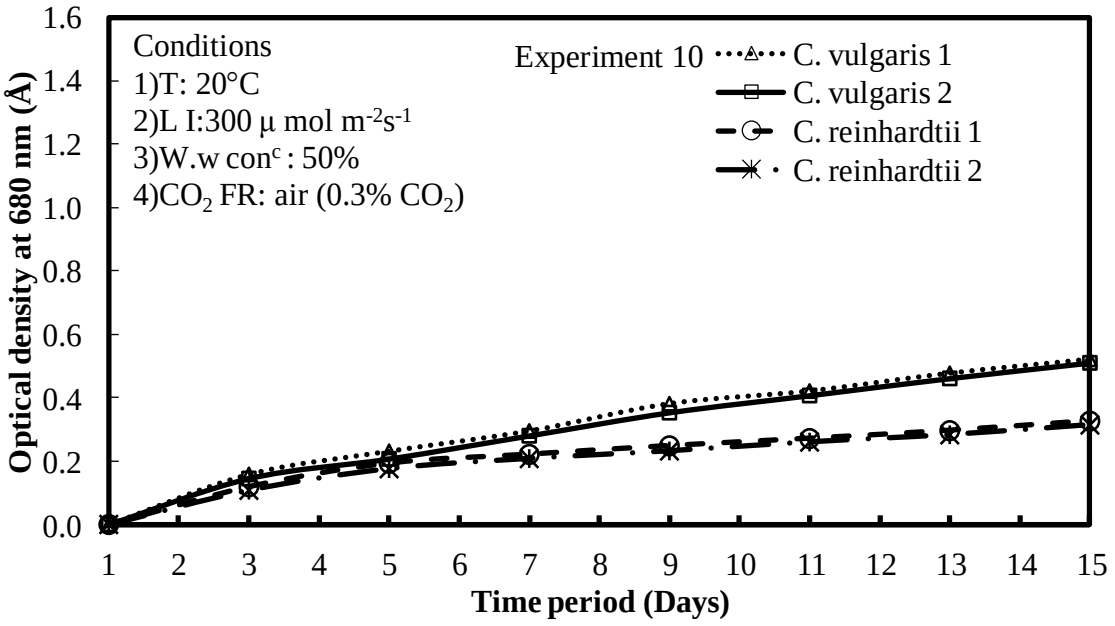

Figure 31. Growth curves of the two selected microalgae grown at 20°C, 300 µmol$m^{-2}s^{-1}$ and 50% swine wastewater.

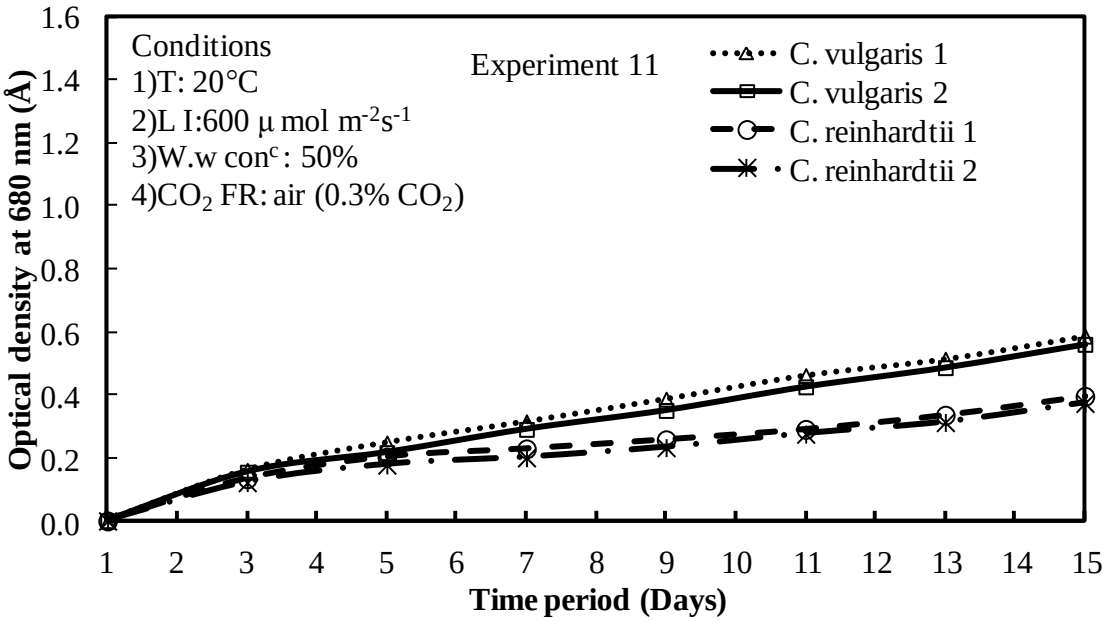

Figure 32. Growth curves of the two selected microalgae grown at 20°C, 600 µmol$m^{-2}s^{-1}$ and 50% swine wastewater.

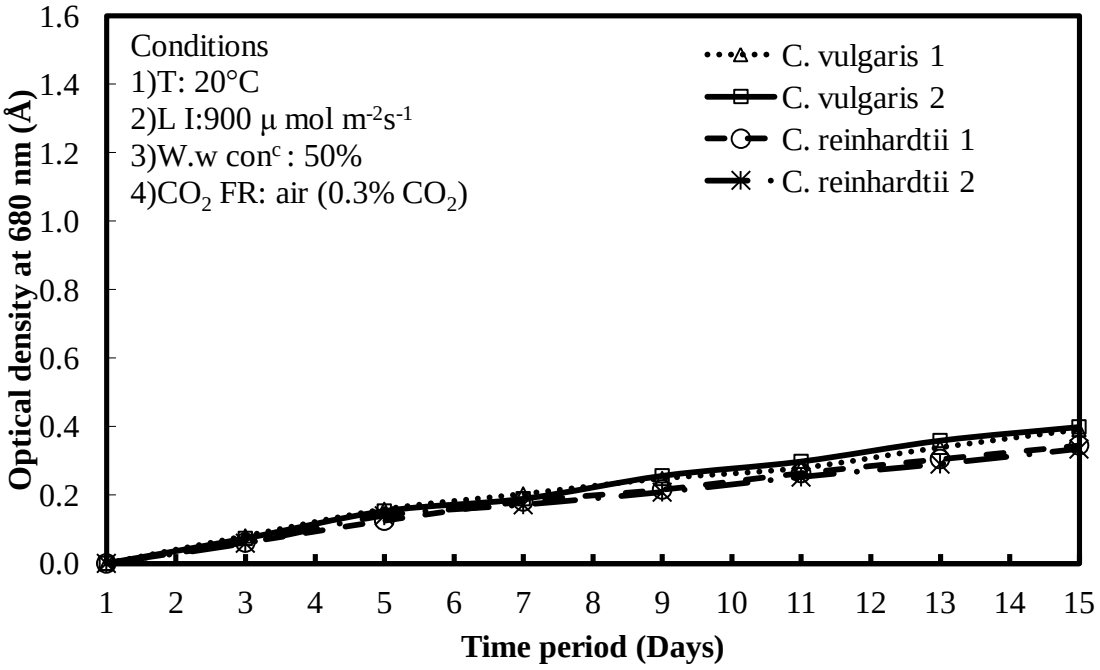

Figure 33. Growth curves of the two selected microalgae grown at 20°C, 900 μmolm^{-2}s^{-1} and 50 % swine wastewater.

4.1.3.2 The effect of light intensity on growth at 25°C and 50% wastewater. Figures 34-36 show the growth curves of *C. vulgaris* & *C. reinhardtii* obtained at temperature of 25°C and in 50% wastewater, and different light intensities of 300, 600 and 900 μmolm^{-2}s^{-1}, respectively. Figures 34-36, at 25^{o}C and 50% wastewater, there was no obvious change in the growth rates of both species if the light intensity increased from 300 μmolm^{-2}s^{-1} (Figure 34) to 600 μmolm^{-2}s^{-1} (Figure 35). However, the further increase of the light intensity to 900 μmolm^{-2}s^{-1} significantly increased the growth rates of both species as shown in Figure 36. It was further found that there was no significant change in the growth rate when both *C. vulgaris* and *C. reinhardtii* were grown in 50% swine wastewater at (1) 25^{o}C and 600 μmolm^{-2}s^{-1} (Figure 35), (2) 25^{o}C and 300 μmolm^{-2}s^{-1} (Figure 34), and (3) 20^{o}C and 300 μmolm^{-2}s^{-1} (Figure 31). However, the growth rate of *C. vulgaris* was higher than that of *C. reinhardtii*. Under those conditions, both species grew very fast during the initial 3 days and then the growth rate became lower.

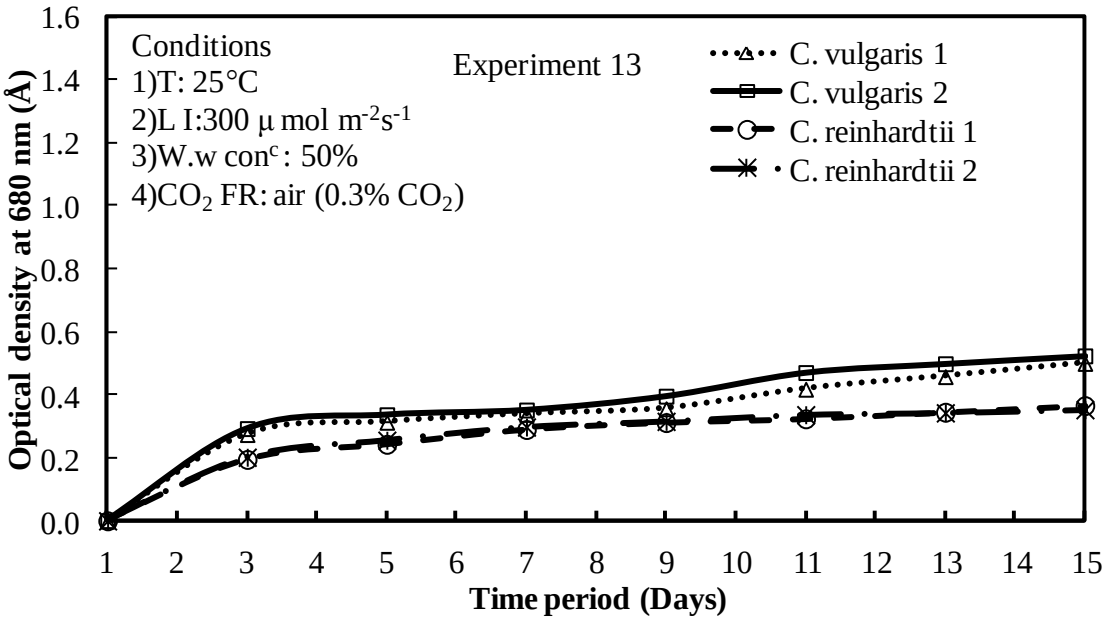

Figure 34. Growth curves of the two selected microalgae grown at 25°C, 300 µmol$m^{-2}s^{-1}$ and 50 % swine wastewater.

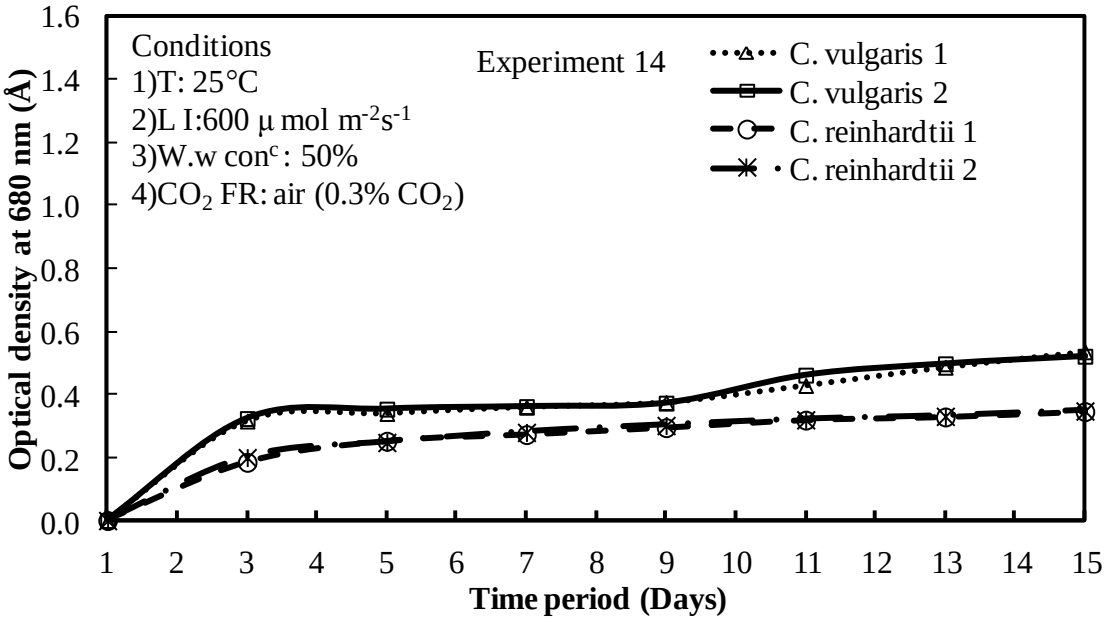

Figure 35. Growth curves of the two selected microalgae grown at 25°C, 600 µmol$m^{-2}s^{-1}$ and 50% swine wastewater.

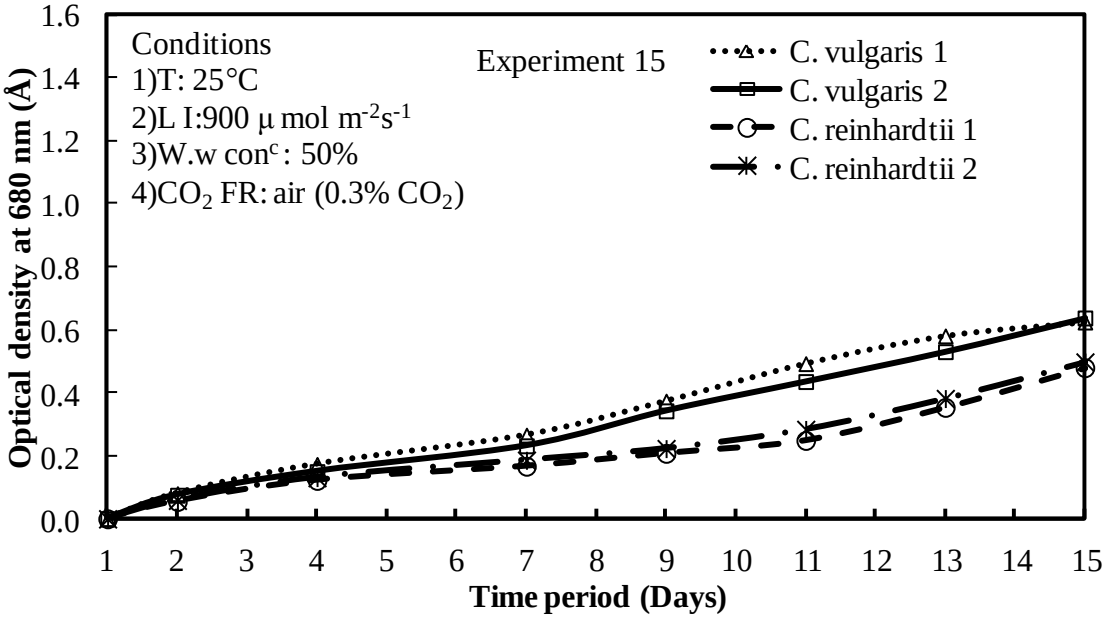

Figure 36. Growth curves of the two selected microalgae grown at 25°C, 900 μmolm^{-2}s^{-1} and 50 % swine wastewater.

4.1.3.3 The effect of light intensity on growth at 30°C and 50% wastewater. Figures 37-39 give the growth curves of the two selected microalgae at 30°C, and in 50% wastewater, and at different light intensities of 300, 600 and 900 μmolm^{-2}s^{-1}, respectively. As seen from Figures 37-39, at 30^{o}C and in 50% wastewater, the growth rates of both species increased with the increase of the light intensity from 300 μmolm^{-2}s^{-1} to 600 μmolm^{-2}s^{-1}. The further increase of the light intensity to 900 μmolm^{-2}s^{-1} significantly decreased the growth rates of both species. The growth of both species was faster during the initial 2 days under different light intensities. If the light was increased beyond the saturation limit, the growth of microalgae becomes inhibited. The light saturation limit depends on the growth temperature.

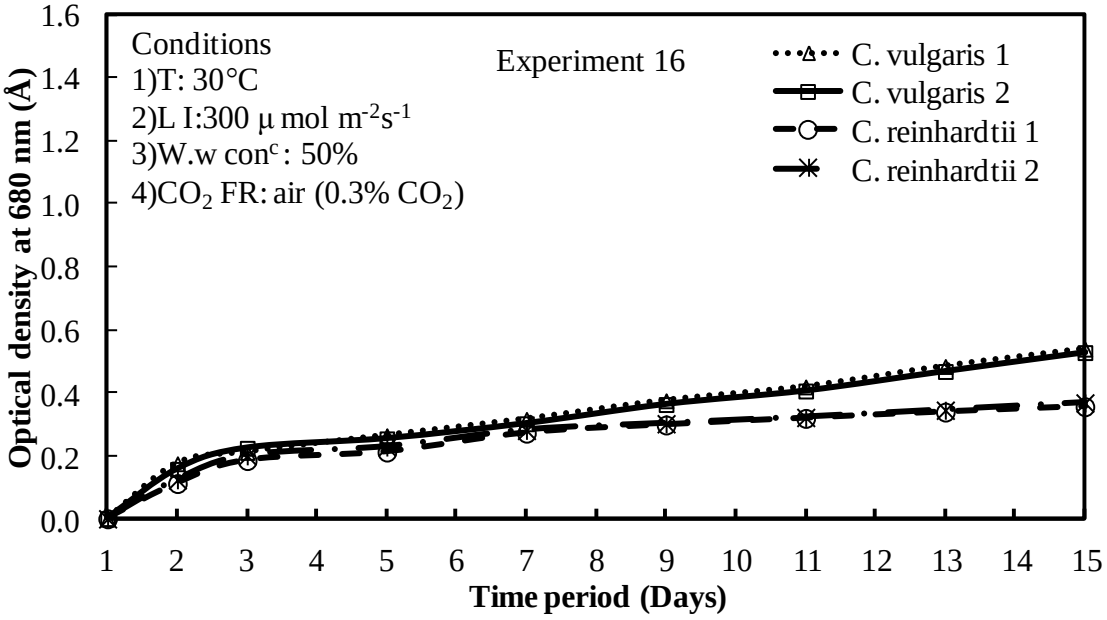

Figure 37. Growth curves of the two selected microalgae grown at 30°C, 300 µmol$m^{-2}s^{-1}$ and 50 % swine wastewater.

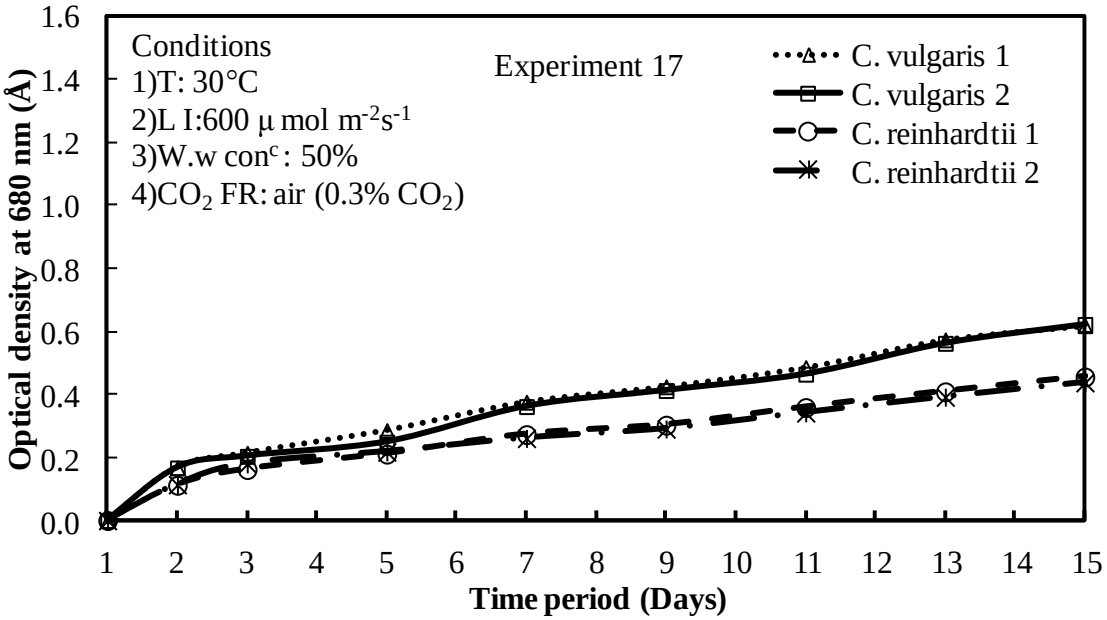

Figure 38. Growth curves of the two selected microalgae grown at 30°C, 600 µmol$m^{-2}s^{-1}$ and 50 % swine wastewater.

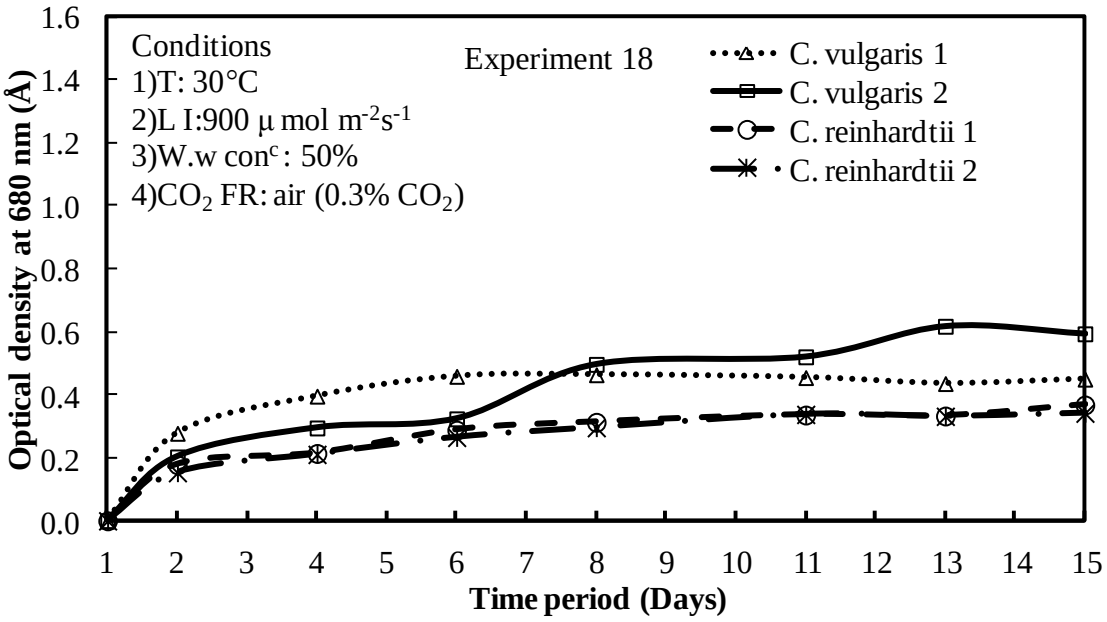

Figure 39. Growth curves of the two selected microalgae grown at 30°C, 900 µmolm^{-2}s^{-1} and 50 % swine wastewater.

4.1.4. Growth Kinetics of microalgae on 50% swine wastewater. Table 9 summarizes the effect of different temperature and light intensities on *C. vulgaris* specific growth rates, cell densities and division rates at 50% waste water concentration. The highest final cell density was found 5.48×10^{6} cell/ml which was obtained at the temperature of 25°C, 900 µmolm^{-2}s^{-1} and 50% wastewater. Under these conditions, the highest specific growth rate and division rate were 1.269 day^{-1} and 1.830 day^{-1}, respectively. However, the very low and very high light intensities decreased the growth rate of *C. vulgaris* at temperature both 20°C and 30°C.

Table 9

Specific growth rate of C vulgaris grown in 50% swine wastewater

Temperature (°C)	Light Intensity ($\mu molm^{-2}s^{-1}$)	Final cell density, $\times 10^6$ (cell/ml)	Specific growth rate, μ (day^{-1})	Division rate, k (day^{-1})
20	300	4.55	1.240	1.789
	600	5.02	1.251	1.805
	900	3.58	1.229	1.773
25	300	4.52	1.201	1.733
	600	4.66	1.194	1.723
	900	5.48	1.269	1.830
30	300	4.69	1.228	1.772
	600	5.39	1.251	1.804
	900	4.60	1.199	1.730

Table 10 summarizes the effect of different temperatures and light intensities on specific growth rates, cell densities and division rates of *C. reinhardtii* at 50% wastewater concentration. For *C. reinhardtii* the highest final cell density was found to be 4.22 $\times 10^6$ cell/ml obtained at 25°C and 900 $\mu molm^{-2}s^{-1}$. Under these conditions, the highest specific growth rate and division rate were 1.237 day^{-1} and 1 .784 day^{-1}, respectively.

Table 10

Specific growth rate of C reinhardtii grown in 50% swine wastewater

Temperature (°C)	Light Intensity ($\mu molm^{-2}s^{-1}$)	Final cell density, $\times 10^6$ (cell/ml)	Specific growth rate, μ (day^{-1})	Division rate, k (day^{-1})
20	300	3.04	1.182	1.705
	600	3.49	1.200	1.731
	900	3.18	1.207	1.742
25	300	3.32	1.161	1.675
	600	3.23	1.158	1.670
	900	4.22	1.237	1.784
30	300	3.33	1.165	1.681
	600	3.93	1.206	1.740
	900	3.28	1.160	1.674

4.2 Removal of Nutrients From Swine Wastewater

The nutrient contents of swine wastewater were determined in four different pretreatment conditions. To kill all microorganisms the wastewater was put in a sealed bottle without oxygen for 7 days. The second condition was that the filtered raw wastewater was put in an oven at 50°C for 3 days. The third condition was that the filtered wastewater was autoclaved. Finally air was blown in the autoclaved wastewater for 15 days as autoclaved wastewater was used to determine the growth kinetics of the microalgae. The contents of ammonia nitrogen, COD and total phosphorus were checked and the results are shown in Table 11.

Table 11

Properties of swine wastewater at different pretreatment conditions

Tests	Waste water in a sealed bottle without Oxygen for 7 days	Waste water kept in 50°C for 3 days	Autoclaved Waste Water	Waste Water bubbled with air
Ammonia Nitrogen (ppm)	86.6	84.8	81.5	41.2
COD (mg/L)	2140	2100	2060	1630
pH	8.37	8.72	8.75	8.13
Total Phosphorous(ppm)	182.7	177.85	172.6	144.6

Both *C. vulgaris* and *C. reinhardtii* did not grow in wastewater kept at 50°C for 3 days after 4-day growth. In wastewater that was put in a sealed bottle without oxygen for 7 days both *C. vulgaris* and *C. reinhardtii* grew at the beginning but after 1 week they were contaminated. Autoclaved wastewater worked best for both species, as they grow well in it for more than 3 months without contamination. To calculate the nutrient removal rate, the nutrient contents of the wastewater that was bubbled with air was used. After 15 days of batch culture, wastewater was separated and these four properties including ammonia nitrogen content, COD, total phosphorous content and pH were checked.

4.2.1 Removal of nutrients from 100% wastewater

4.2.1.1 Removal of ammonia nitrogen. Ammonia is a volatile compound, so when only air is blown on wastewater almost 50% removal was achieved. Figure 40 shows the remaining

concentration of ammonia nitrogen in wastewater separated from *C. vulgaris* and *C. reinhardtii* culture after 15 days.

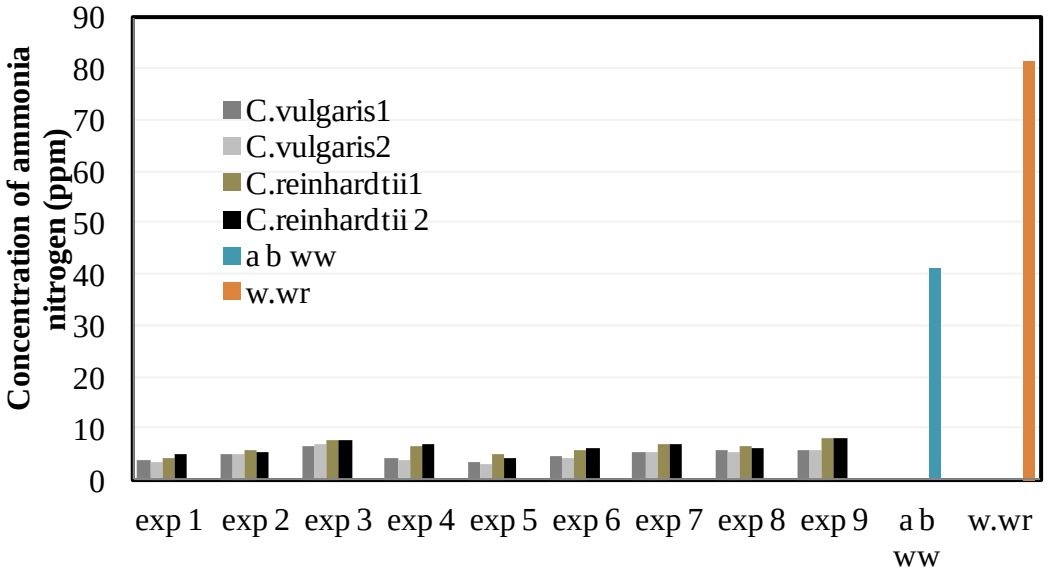

Figure 40. The concentration of ammonia nitrogen in 100% wastewater, in air blown wastewater and in wastewater after 15 days of microalgae culture.

From Figure 40, it was clearly seen that microalgae could remove ammonia nitrogen at a high rate. The removal efficiency of ammonia nitrogen at different conditions can be studied more easily from the Figure 41. As shown in Figure 41, *C. vulgaris* removed more ammonia nitrogen than *C. reinhardtii* under all experimental conditions. For the first three experiments conducted at 20^{o}C, the removal efficiency of ammonia nitrogen decreased with the increase in the light intensity. For the next three experiments conducted at 25^{o}C, the removal efficiency was increased with the increase in light intensity from 300 μmolm^{-2}s^{-1} to 600 μmolm^{-2}s^{-1}, but the further increase in the light intensity decreased the removal efficiency of ammonia nitrogen. The last three experiments conducted at 30^{o}C gave lower removal efficiency. The ammonia nitrogen removal efficiencies were similar in experiment 7 where temperature was 30°C, light intensity was 300 μmolm^{-2}s^{-1} and experiment 8 where temperature was 30°C, light intensity

600 μmolm^{-2}s^{-1}. But at 30°C when the light intensity was the highest the removal efficiency was the lowest as shown in Figure 41. Figure 41 shows that the highest removal efficiency of ammonia nitrogen was achieved in experiment 5 for *C. vulgaris*, which had the temperature of 25°C, light intensity of 600 μmol m^{-2}s^{-1} and 100% wastewater. For *C. reinhardtii* the highest removal efficiency of ammonia nitrogen was achieved in experiment 1 condition, which included the temperature of 20°C, light intensity of 300 μmolm^{-2}s^{-1} and 100% waste water. Table 12 shows the percent removal of ammonia nitrogen in all 9 experiments for both *C. vulgaris* and *C. reinhardtii*.

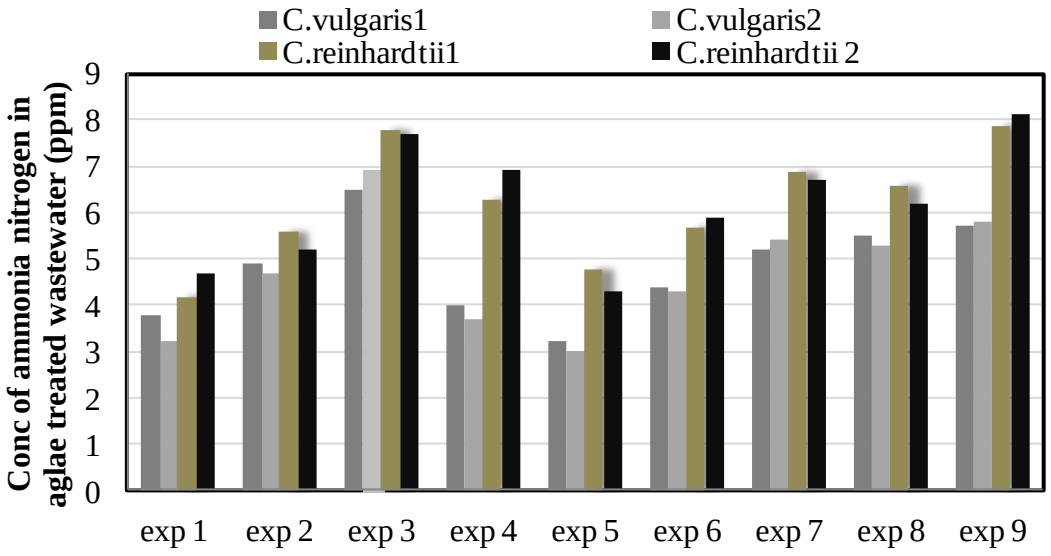

Figure 41. Concentration of ammonia nitrogen in algae treated 100% wastewater.

Table 12

Percent removal of ammonia nitrogen in 100% wastewater treated with algae

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii 1*	*C. reinhardtii 2*
Experiment 1	90.77	92.23	89.80	88.59
Experiment 2	88.11	88.59	86.41	87.38
Experiment 3	84.22	83.25	81.07	81.31
Experiment 4	90.29	91.02	84.71	83.25
Experiment 5	92.23	92.72	88.35	89.56
Experiment 6	89.32	89.56	86.17	85.68
Experiment 7	87.38	86.89	83.25	83.74
Experiment 8	86.65	87.14	83.98	84.95
Experiment 9	86.16	85.92	80.83	80.34

4.2.1.2 Removal of COD. By blowing air COD was also removed by around 20% from the autoclaved wastewater in 15 days. The removal was not as high as ammonia. Figure 42 shows the remaining concentration of COD in the wastewater after 15 days of microalgae culture, in autoclaved wastewater and air blown autoclaved wastewater.

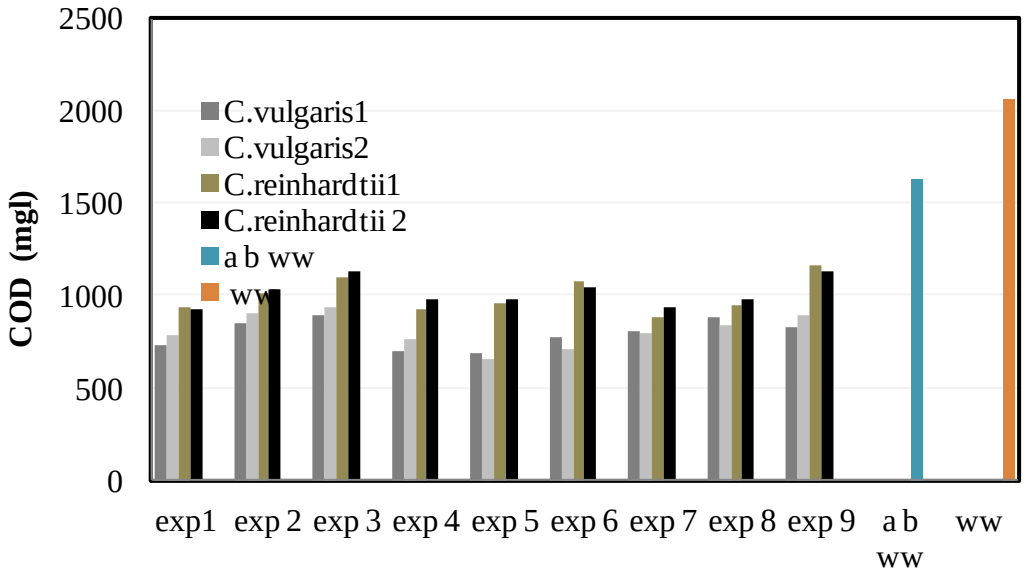

Figure 42. The concentration of COD in 100% wastewater, in air blown wastewater and in wastewater after 15 days of microalgae culture.

Ammonia nitrogen is a part of COD. So by blowing air COD also decreased as ammonia nitrogen was decreased. Like ammonia nitrogen *C. vulgaris* also removed higher COD than *C. reinhardtii* as shown in Figure 43.

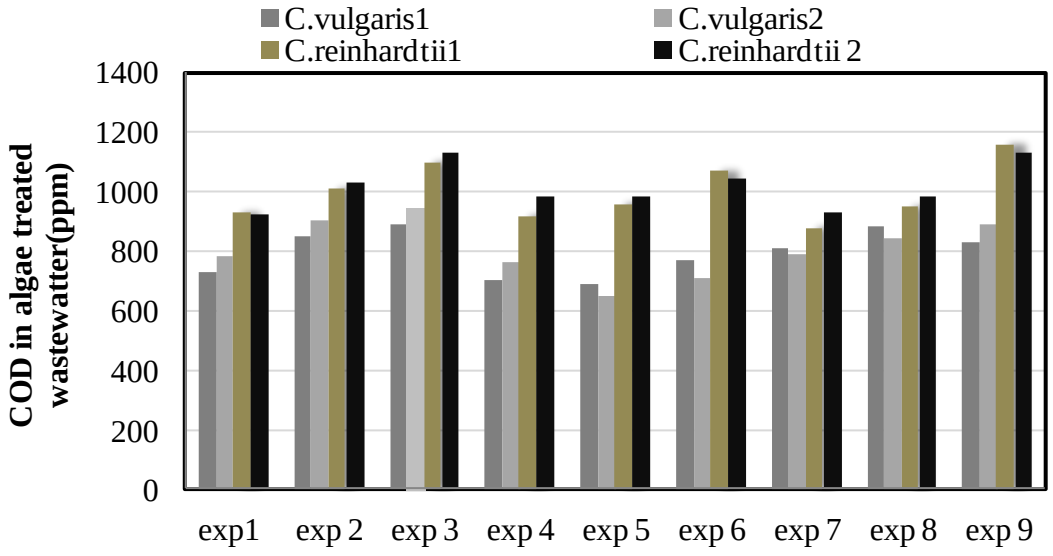

Figure 43. COD concentration in algae treated 100% wastewater.

Table 13 shows the percentage of removal of COD for both *C. vulgaris* and *C. reinhardtii*. Like ammonia nitrogen, the highest COD removal efficiency was 60.12% for *C. vulgaris* which was obtained at the temperature of 25°C, light intensity of 600 $\mu molm^{-2}s^{-1}$ and 100% wastewater. For *C. reinhardtii,* the highest removal efficiency of COD was 46.01% which was achieved at the temperature 20°C, light intensity 300 $\mu molm^{-2}s^{-1}$ and 100% waste water.

Table 13

Percent removal of COD in 100% wastewater treated with algae

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii 1*	*C.reinhardtii 2*
Experiment 1	55.21	52.15	42.94	43.56
Experiment 2	47.85	44.78	38.04	36.81
Experiment 3	45.40	42.33	32.51	30.67
Experiment 4	57.06	53.37	43.56	39.88
Experiment 5	57.67	60.12	41.10	39.87
Experiment 6	52.76	56.44	34.36	36.19
Experiment 7	50.31	51.53	46.01	42.94
Experiment 8	46.01	48.47	41.72	39.87
Experiment 9	49.08	45.39	28.83	30.67

4.2.1.3 Removal of total phosphorus. By blowing air total phosphorus was also removed by around 16% from the autoclaved wastewater in 15 days. The removal efficiency of phosphorus was not as high as that of ammonia nitrogen. Figure 44 shows the remaining concentration of total phosphorus in the wastewater after 15 days of microalgae culture, in autoclaved wastewater and air blown autoclaved wastewater.

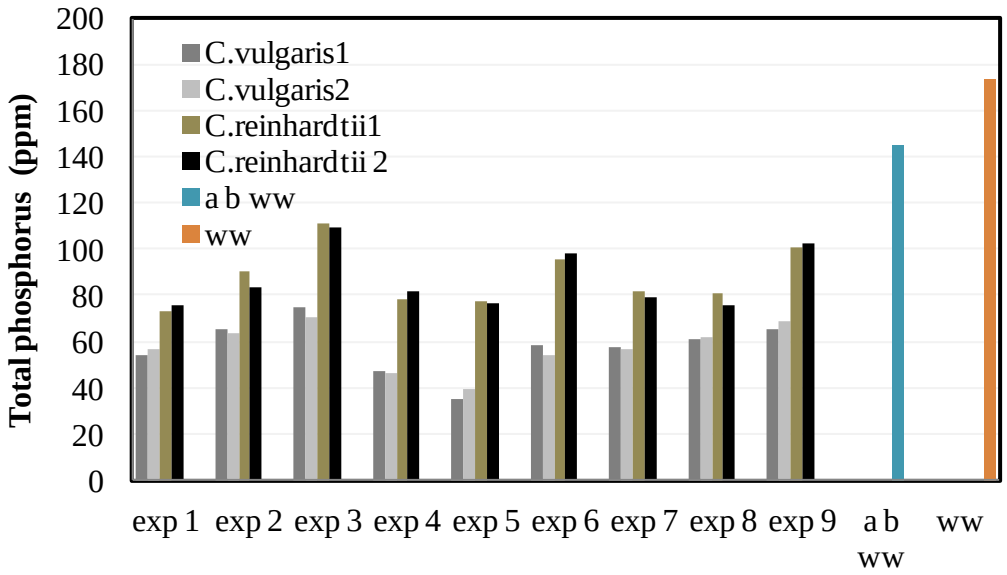

Figure 44. Concentration of total phosphorus in the 100% wastewater after 15 days of microalgae culture, in autoclaved wastewater and air blown autoclaved wastewater.

Figure 45 illustrates that *C. vulgaris* removes more total phosphorus than *C. reinhardtii*. The increase in the light intensity resulted in the decrease of the removal of total phosphorus at all three temperatures (20°C, 25°C and 30°C).

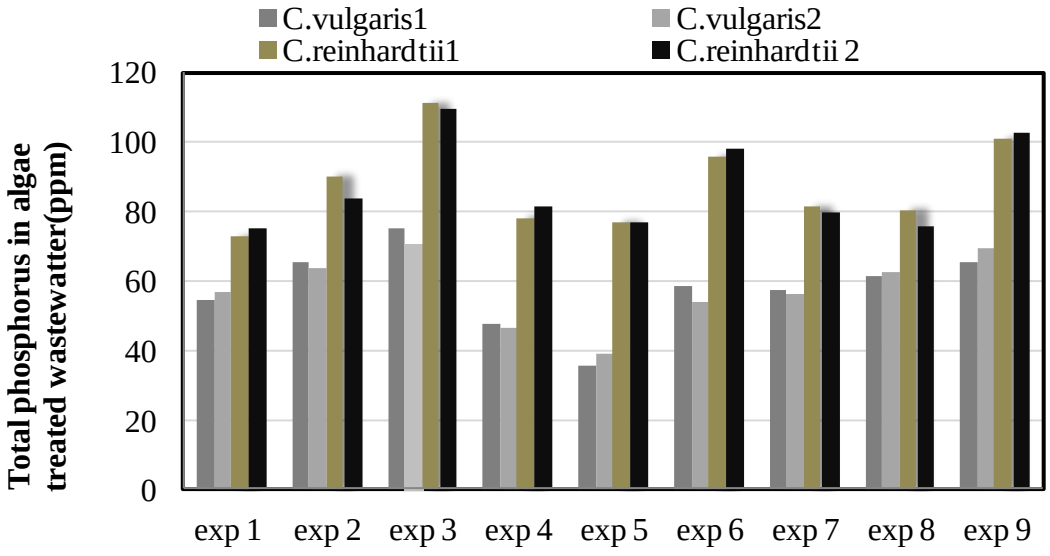

Figure 45. Total phosphorus concentration in microalgae treated 100% wastewater.

Figure 45 shows that *C. vulgaris* has higher removal efficiency of total phosphorus than *C. reinhardtii*. Table 14 shows all the removal rates. The highest removal efficiency of total phosphorus for *C. vulgaris* was 75.55% which was obtained in experiment 5 at the temperature of 25°C, light intensity 600 $\mu molm^{-2}s^{-1}$ and 100% waste water. For *C. reinhardtii* the highest removal efficiency of total phosphorus was 49.58% which was achieved in experiment 1 at the temperature of 20°C, light intensity 300 $\mu molm^{-2}s^{-1}$ and 100% wastewater.

Table 14

Percent removal of total phosphorus in 100% wastewater treated with algae

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii1*	*C.reinhardtii 2*
Experiment 1	62.55	60.75	49.58	47.92
Experiment 2	54.84	55.95	37.55	42.01
Experiment 3	47.99	51.17	23.10	24.20
Experiment 4	67.18	67.81	45.78	43.71
Experiment 5	75.55	73.03	46.68	46.85
Experiment 6	59.47	62.79	33.75	32.09
Experiment 7	60.48	60.99	43.71	45.02
Experiment 8	57.68	57.02	44.19	47.58
Experiment 9	54.98	52.21	30.12	29.18

4.2.2 Removal of nutrients from 50% swine wastewater

4.2.2.1 Removal of Ammonia nitrogen. Figure 46 shows the remaining concentration of ammonia nitrogen in wastewater separated from *C. vulgaris* and *C. reinhardtii* culture after 15 days, 50% wastewater, air blown waste and original wastewater.

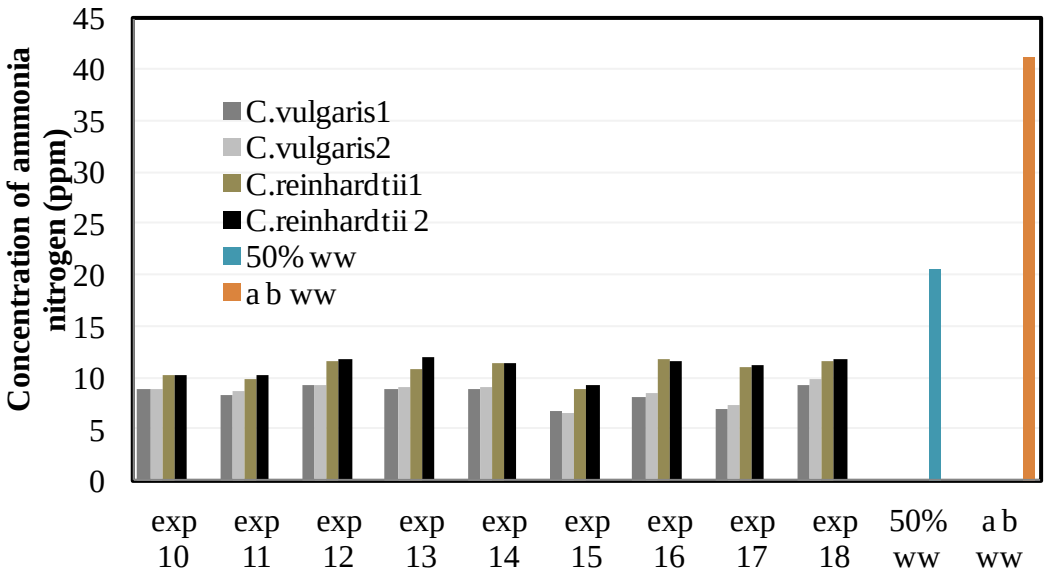

Figure 46. Concentration of ammonia nitrogen in 50% wastewater, air blown wastewater and original wastewater and in wastewater after 15 days of microalgae culture.

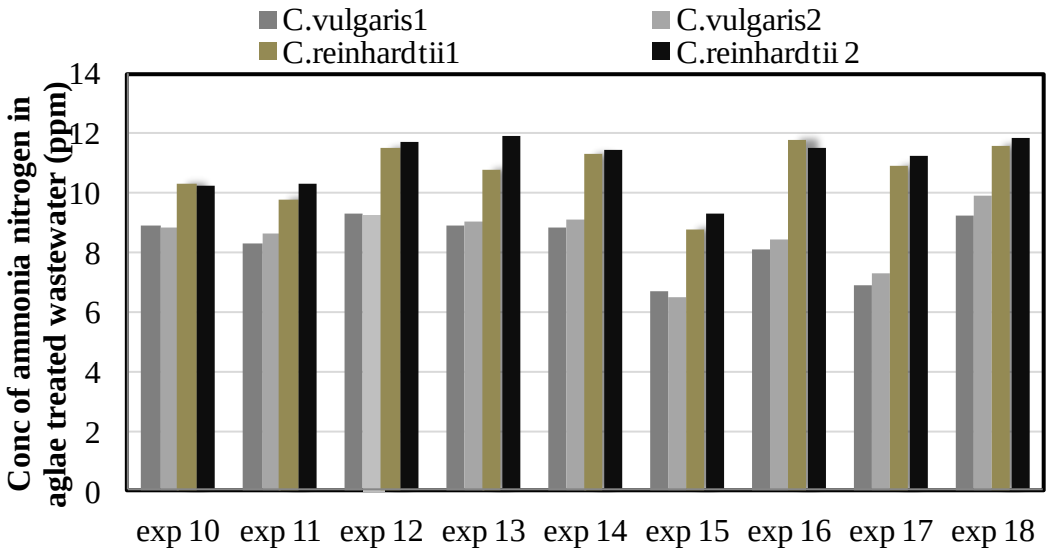

Figure 47. Ammonia nitrogen concentration in microalgae treated 50% wastewater.

When the concentration of wastewater was lowered to 50% the removal rate of ammonia nitrogen also decreased for both species. In the first two experiments the removal rates were quite similar. With the increase in light intensity the removal efficiency was decreased for both

species. The highest removal for both the species were found at experiment 15 conditions where temperature was 25°C and light intensity 900 $\mu molm^{-2}s^{-1}$.

Table 15 shows the percent removal of ammonia nitrogen in all 3 experiments. *C. vulgaris* can remove more ammonia nitrogen than *C. reinhardtii*. The highest removal efficiency of ammonia nitrogen was 68.45% which was obtained at 25°C and 900 $\mu molm^{-2}s^{-1}$ in 50% wastewater. For *C. reinhardtii* the highest removal rate was 57.28% obtained at 25°C and 900 $\mu molm^{-2}s^{-1}$.

Table 15

Percent removal of ammonia nitrogen in algae treated 50% wastewater

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii 1*	*C.reinhardtii 2*
Experiment 10	56.79	57.28	50	50.48
Experiment 11	59.701	58.25	52.43	50
Experiment 12	54.85	55.34	44.17	43.20
Experiment 13	56.79	56.31	47.57	42.23
Experiment 14	57.28	55.82	45.15	44.66
Experiment 15	67.48	68.45	57.28	54.85
Experiment 16	60.67	59.22	42.72	44.17
Experiment 17	66.50	64.56	47.09	45.63
Experiment 18	55.34	51.94	43.69	42.72

4.2.2.2 Removal of COD. Figure 48 shows the remaining concentration of ammonia nitrogen in wastewater separated from *C. vulgaris* and *C. reinhardtii* culture after 15 days of microalgae culture, 50% wastewater, air blown wastewater.

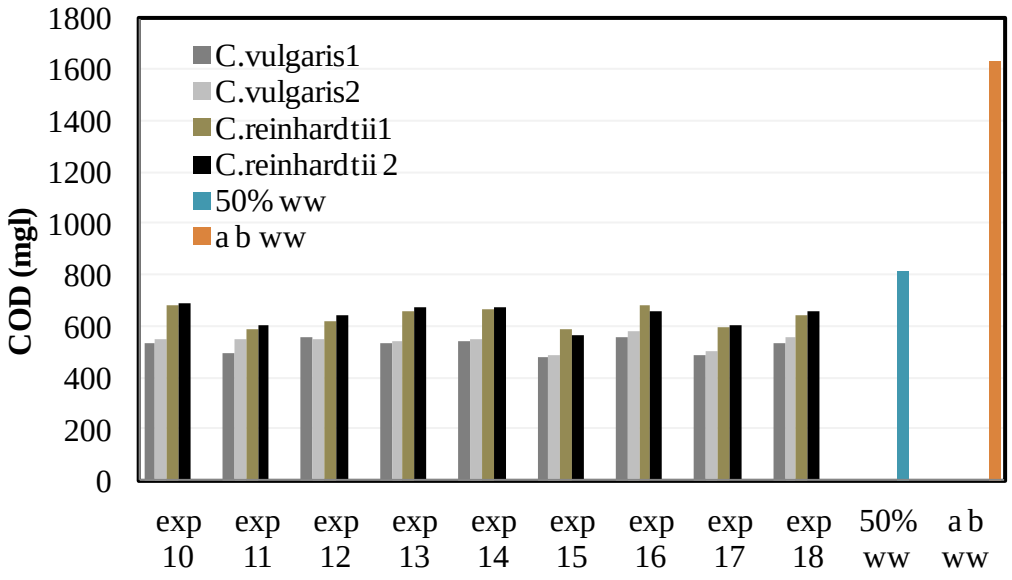

Figure 48. Concentration of COD in 50% wastewater, air blown wastewater and original wastewater and in wastewater after 15 days of microalgae culture.

Compared to the 50% wastewater the removal of COD by *C. reinhardtii* was not significant. Figure 49 illustrates the performance of COD removal between the two selected microalgae strain at different growth conditions.

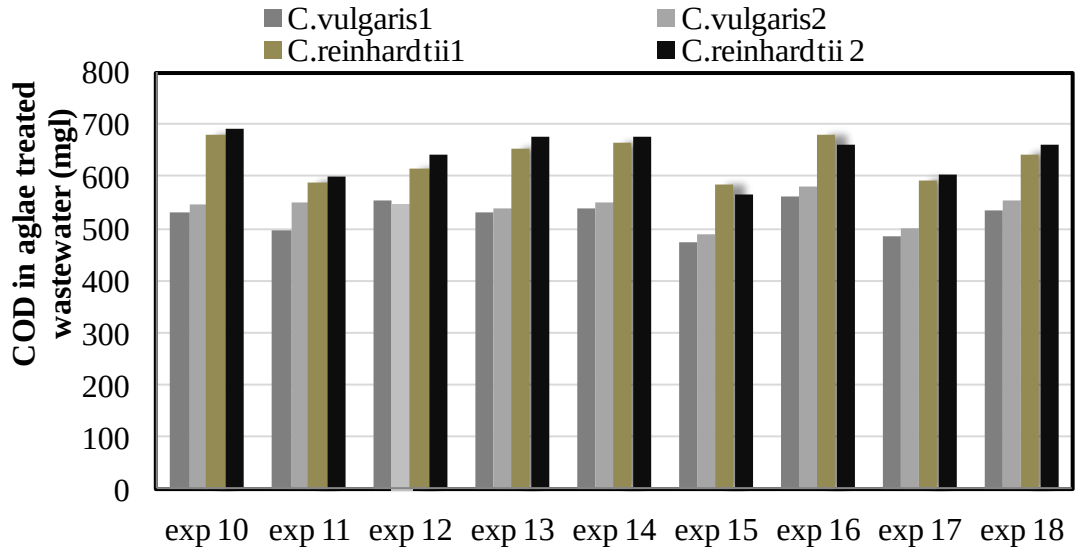

Figure 49. COD concentration of algae treated 50% wastewater.

The highest removal efficiency of COD was obtained at experiment 15 for both *C. vulgaris* and *C. reinhardtii* at the temperature of 25°C and light intensity of 900 $\mu molm^{-2}s^{-1}$. The highest COD removal rate for *C. vulgaris* and *C. reinhardtii* were 41.72% and 30.67%, respectively. The following table shows the percentage removal of COD in all experiment conditions as shown in Table 16.

Table 16

Percent removal of COD in 50% wastewater treated by algae

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii 1*	*C.reinhardtii 2*
Experiment 10	34.97	33.13	16.56	15.34
Experiment 11	39.26	32.51	27.61	26.38
Experiment 12	31.90	33.13	24.54	21.47
Experiment 13	34.96	33.74	19.63	17.17
Experiment 14	33.74	32.51	18.40	17.18
Experiment 15	41.72	39.88	28.22	30.67
Experiment 16	31.29	28.83	16.56	19.02
Experiment 17	40.49	38.65	26.99	25.77
Experiment 18	34.35	31.90	20.86	19.02

4.2.2.3 Removal of total phosphorus. Figure 50 shows the remaining concentration of total phosphorus in wastewater separated from *C. vulgaris* and *C. reinhardtii* culture after 15 days, 50% wastewater, air blown wastewater and original wastewater.

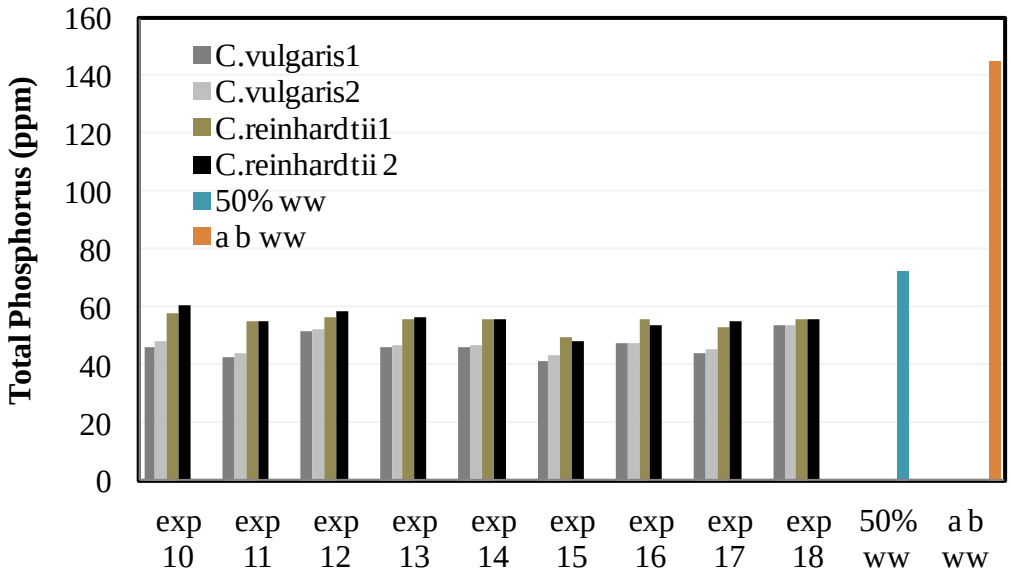

Figure 50. Concentration of total phosphorus in 50% wastewater, air blown wastewater and original wastewater and in wastewater after 15 days of microalgae culture.

C. vulgaris again shows higher removal of total phosphorus than the *C. reinhardtii*. To study the performances of both microalgae, all the results were compared to the total phosphorus concentration of 50% wastewater.

The highest removal of total phosphorus was obtained at experiment 15 for both *C. vulgaris* and *C. reinhardtii* at the temperature of 25°C and light intensity of 900 $\mu molm^{-2}s^{-1}$. Figure 51 shows the concentration of total phosphorus after 15 days of microalgae culture in all experiment conditions.

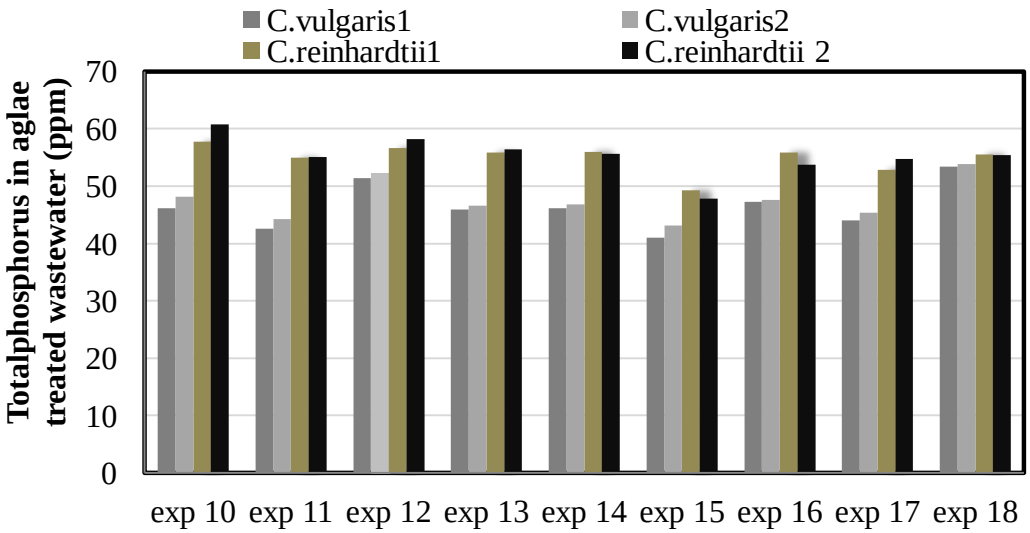

Figure 51. Total phosphorus concentration of algae treated 50% wastewater after 15 days of culture.

Table 17 shows the percentage removal of total phosphorus. The highest removal of total phosphorus was obtained at experiment 15 for both *C. vulgaris* and *C. reinhardtii* where the temperature was 25°C and 900 $\mu molm^{-2}s^{-1.}$ The highest total phosphorus removal rate for *C. vulgaris* and *C. reinhardtii* were 43.19% and 33.78%, respectively.

Table 17

Percent removal of total phosphorus in 50% wastewater treated by microalgae

	C.vulgaris 1	*C.vulgaris 2*	*C.reinhardtii 1*	*C.reinhardtii 2*
Experiment 10	36.24	33.33	20.18	15.90
Experiment 11	41.08	38.83	24.03	23.82
Experiment 12	28.97	27.66	21.70	19.49
Experiment 13	36.41	35.55	22.75	21.99
Experiment 14	36.17	35.30	22.54	23.03
Experiment 15	43.19	40.32	31.88	33.78

Table 17

Cont.

Experiment 16	34.62	34.19	22.67	25.66
Experiment 17	39.04	37.21	26.83	24.34
Experiment 18	26.14	25.48	23.2	23.3

4.3 Comparison of Lipid Content Between *C. vulgaris* and *C. reinhardtii*

Two types of lipids were checked for both species, one is Biodipy and the other is Chlorophyll A. Biodipy content of *C. vulgaris* was found to be higher than that of *C. reinhardtii* in all the experiments whereas chlorophyll A content was almost the same for both species. Figure 52 shows the biodipy plot for both species in experiment 5 which contributed to the highest growth rate in 100% swine wastewater.

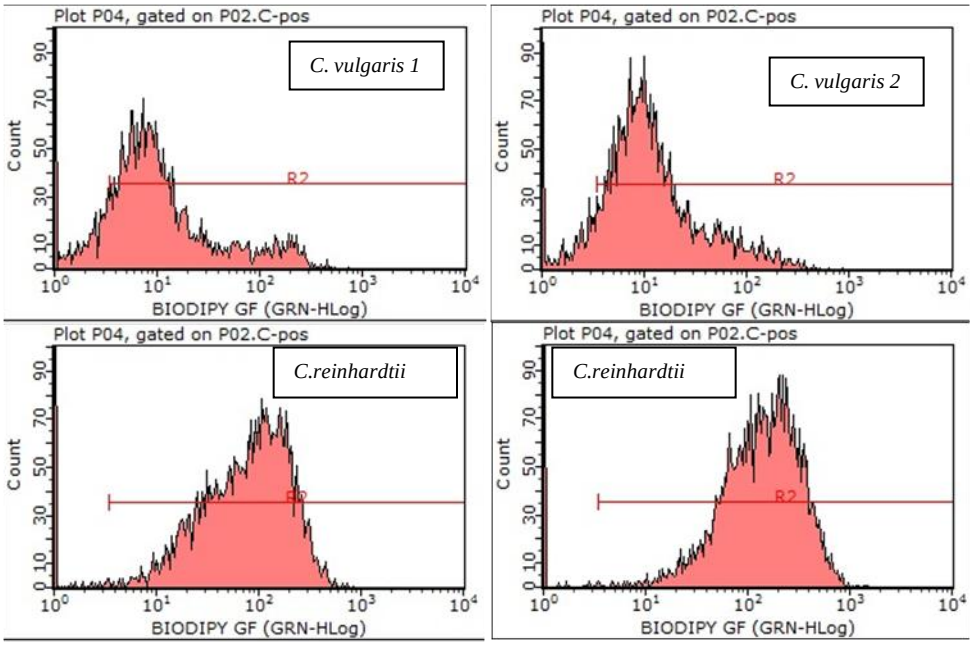

Figure 52. Biodipy plot for *C. vulgaris* and *C. reinhardtii* at experiment 5 conditions.

Figure 53 shows the Chlophyll A plot for both the species in the same experiment conditions.

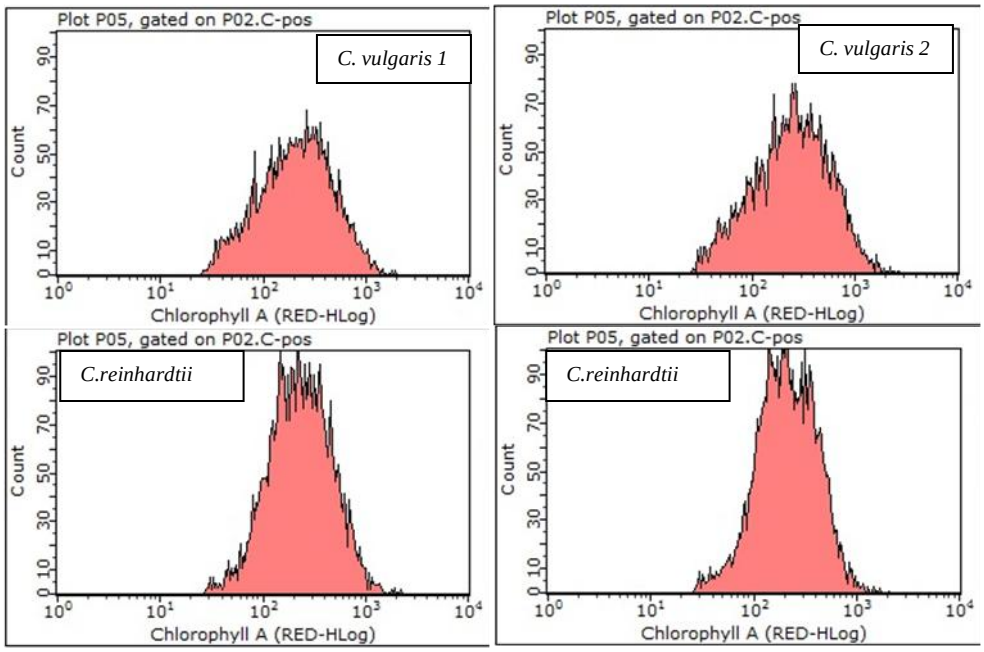

Figure 53. Chlorophyll A plot for *C. vulgaris* and *C. reinhardtii* at experiment 5 conditions.

Figure 53 illustrates that there was no significant variation in Chlorophyll A range for both species. The other plots for all the experiments were given in appendix.

4.4 Comparison of Select 24 with C. *vulgaris* & *C. reinhardtii*

Three different types of microalgae 1) *C. vulgaris* 2) *C. reinhardtii* & 3) Select 24 were cultured in wastewater from the NCAT pig farm. Select 24 was chosen from NCAT pig farm and two experiments were performed in the optimum condition found in the 1st part of the research to compare this algal strain with the other two commercial algae strains from UTEX. The wastewater used for these two experiments were collected from the NCAT farm at two different times (in June 2012 & September 2012). So the compositions of wastewater were different in two different experiments.

4.4.1 First experiment to compare select 24 with *C. vulgaris* and *C. reinhardtii*. In the first experiment the temperature was maintained at 25℃, light intensity 600 $\mu molm^{-2}s^{-1}$ and the wastewater concentration 100%. The dry weight was measured some discrepancies were found. So the experiment was repeated.

4.4.1.1 Nutrients removal. To study how select 24 can change the nutrients from swine wastewater three tests (ammonia nitrogen, COD and total phosphorus) were done. The pH of the wastewater solution after 15 days of microalgae culture was also measured and all the results as shown in Table 18 were compared with *C. vulgaris* and *C. reinhardtii*.

Table 18

Properties of wastewater, air blown wastewater and wastewater after 15 days of 3 selected microalgae culture

Nutrients	Pure waste water	Air blown waste water	Select 24	*C. vulgaris*	*C. reinhardtii*
Total P in ppm	149	115.2	83.8	62.925	66.3
pH	8.69	8.54	8.26	8.77	8.38
Ammonia Nitrogen in ppm	75.5	36.5	9.9	8.65	9
COD in mgl	1870	1155	895	687.5	957.5

C. vulgaris removes the higher amount of nutrients among the three algal strains. Select 24 showed better performance in removing COD from swine wastewater than *C. reinhardtii*. In case of ammonia nitrogen and total p the removal efficiencies of Select 24 were lower than those

of the two commercial strains. The pH was found to be a little lower in the wastewater treated by select 24 than the other two species.

The change in nutrients can be seen easily from the figure 54.

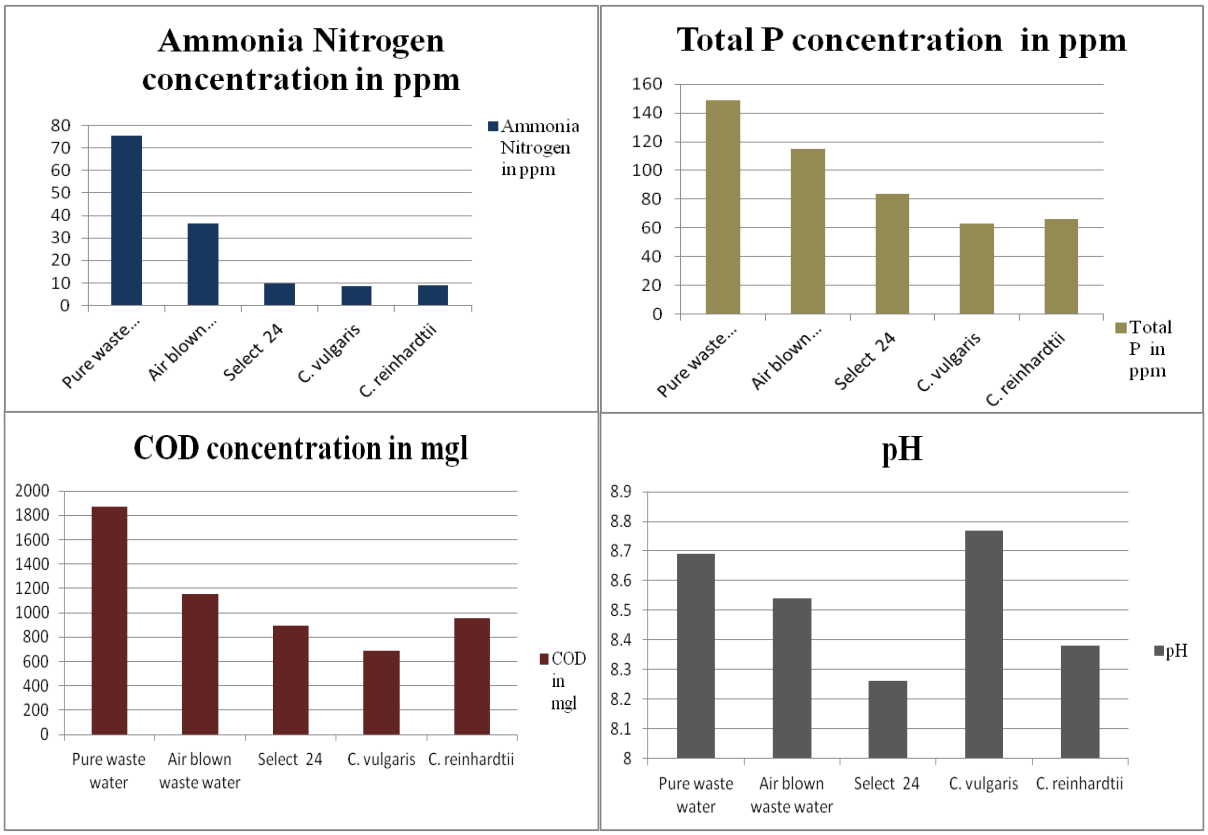

Figure 54. Change in nutrients in wastewater treated by select 24, *C.vulgaris* and *C. reinhardtii* in first experiment.

4.4.2 Second experiment to compare select 24 with *C. vulgaris* and *C. reinhardtii*. As the growth curve could not be achieved from the first experiment it was repeated. The conditions were same. Average was taken to generate the growth curves for three different algae strains. Figure 55 shows the growth curves for *C. vulgaris*, *C. reinhardtii* and select 24.

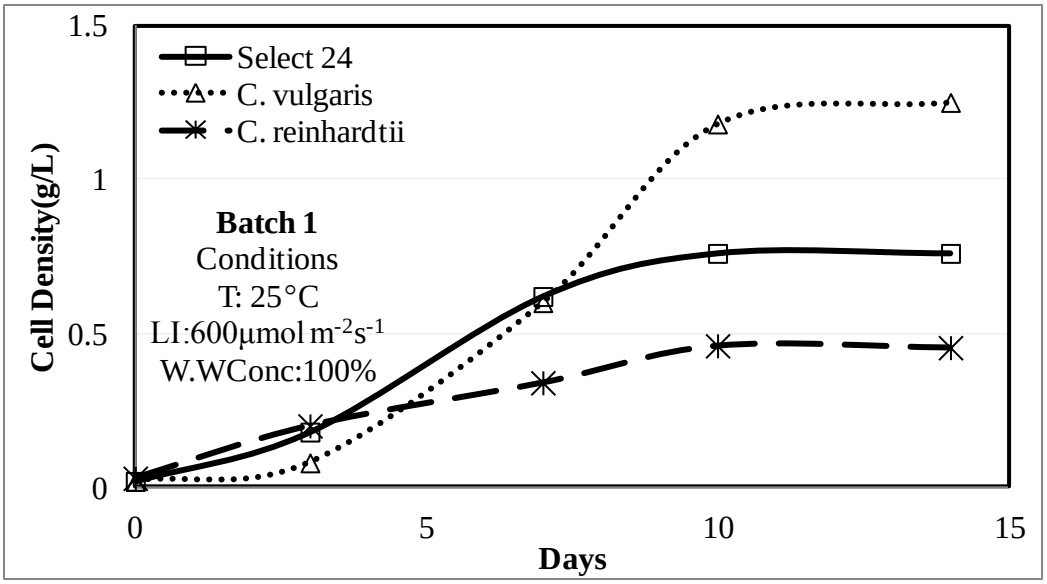

Figure 55. Comparison of growth among the three different algal srtains.

At the beginning the growth of *C. vulgaris* was lower than those of the other two strains. After 3 day culture, the growth rate of *C. vulgaris* became faster than those of the other two strains. The growth of select 24 was higher than *C. reinhardtii* but lower than *C. vulgaris*.

4.4.2.1 Nutrients removal. Total phosphorous, ammonia nitrogen and COD concentrations were tested from the waste water after 15 days of culture. Again air was blown in wastewater to check how these nutrient contents changed as different wastewater was used in this experiment. The growth conditions were temperature of 25°C, light intensity of 600 $\mu molm^{-2}s^{-1}$ and waste water concentration was 100%. Table 19 shows the results.

Table 19

Properties of wastewater, air blown wastewater and wastewater after 15 days of 3 selected microalgae culture

Nutrients	Pure waste water	Air blown waste water	Select 24	*C. vulgaris*	*C. reinhardtii*
Total P in ppm	163.2	123.0	94.0	33.8	81.325
pH	9.29	8.85	8.47	8.62	8.76
Ammonia Nitrogen in ppm	75	23.6	8.7	7.05	10.7
COD in mgl	2200	1320	1085	1065	1095

From Table 19, some differences were found compared to the first experiment. The COD removal efficiency of select 24 was lower than *C. reinhardtii* while the removal efficiency of ammonia nitrogen was higher. Figure 56 shows the difference in ammonia nitrogen, total P, and COD concentration in raw wastewater, air blown wastewater and the wastewater after 15-day culture of select 24, *C. vulgaris* and *C. reinhardtii* culture. *C. vulragis* gave the highest removal efficiency among the three strains. By blowing air in the wastewater ammonia nitrogen was removed by 68.5%. Select 24 removed 63.1% ammonia nitrogen, 23.6% total P and around 17% COD from swine wastewater.

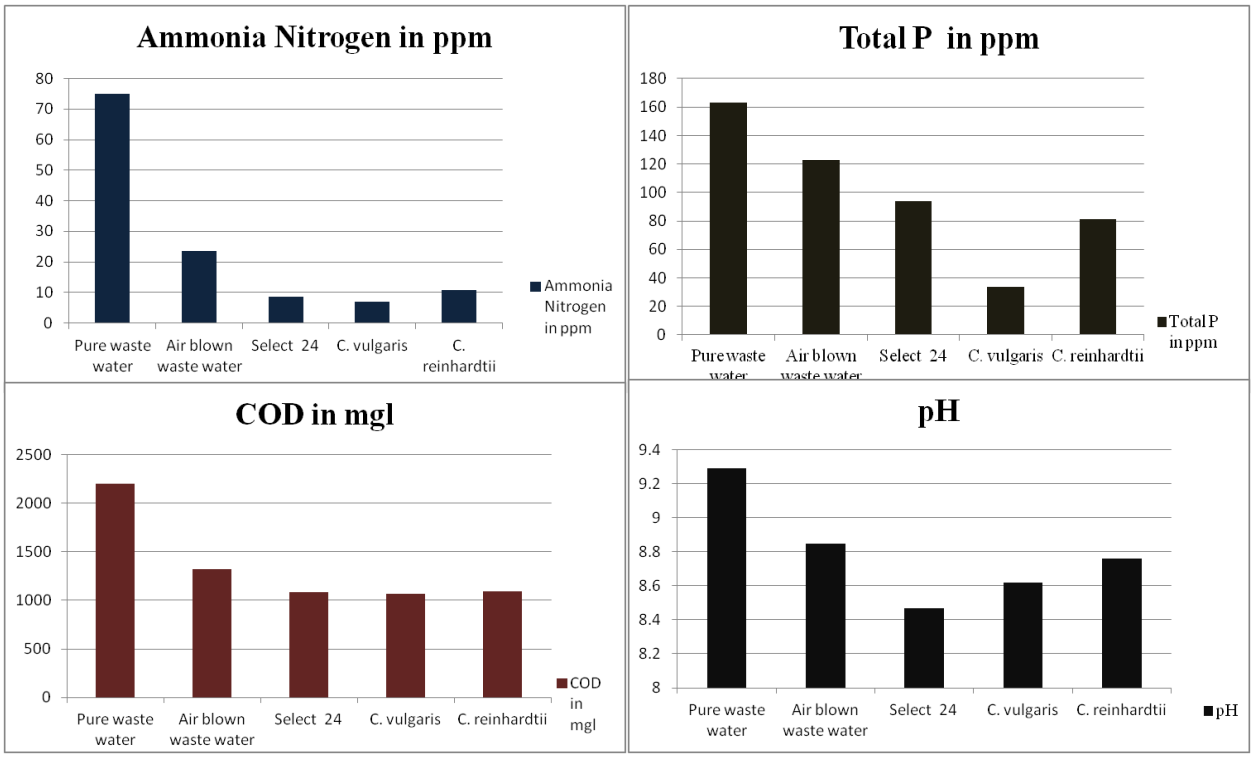

Figure 56. Change in nutrients in wastewater treated by select 24, *C.vulgaris* and *C. reinhardtii* in the second experiment.

4.4.3 Third experiment to compare select 24 at three different light intensities. In the third experiment studied select 24 at 25°C at three different light intensities 300, 600 and 900 $\mu molm^{-2}s^{-1}$. Figure 57 shows 1st batch of select 24 at different light intensities at 25°C. At the beginning,higher growth rate was achieved at lower light intensity 300 $\mu molm^{-2}s^{-1}$. On day 9 the cell density was almost close for all the three different light intensities. On day 13 the growth of select 24 subjected to 900 $\mu molm^{-2}s^{-1}$ was increased faster than the other two, but lowered at the end of the experiment. The highest cell density was achieved at both 300 $\mu molm^{-2}s^{-1}$ and 600 $\mu molm^{-2}s^{-1}$ at the end of the experiment.

In the second run as shown in Figure 58, a prolonged lag phase was observed at the three light intensities. On day 8 the cell densities were almost the same. After that the growth of select 24 subjected to 300 $\mu molm^{-2}s^{-1}$ light intensity was increased faster than the other two. The final

cell density was also higher in the algae strain subjected to 300 $\mu molm^{-2}s^{-1}$. From these two batches it was observed that at 25°C lower light intensity 300 $\mu molm^{-2}s^{-1}$ is more suitable for select 24 strains than the other two light intensities.

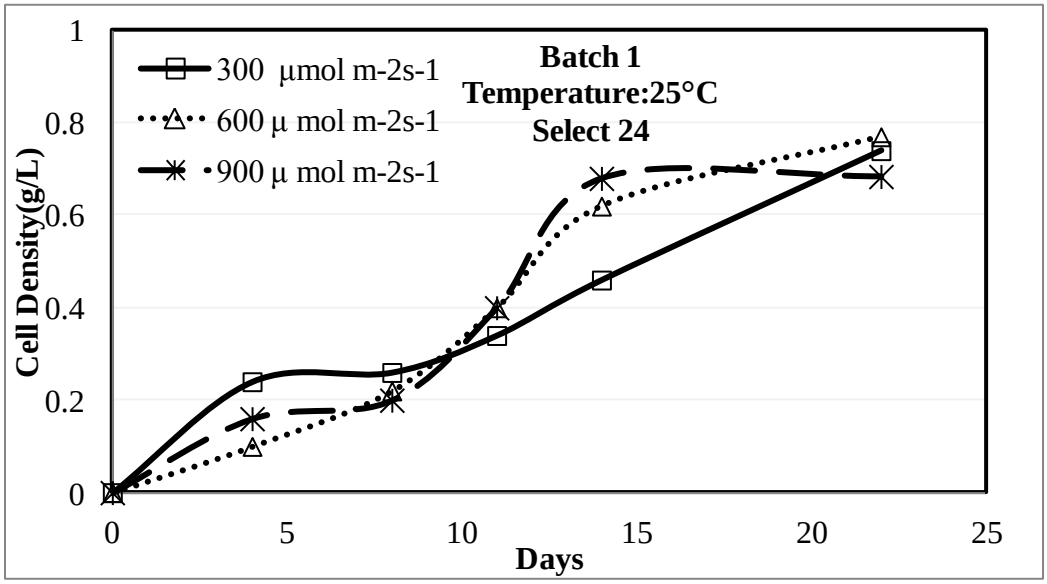

Figure 57. 1st batch of select 24 at 300, 600 & 900 $\mu molm^{-2}s^{-1}$ light intensities.

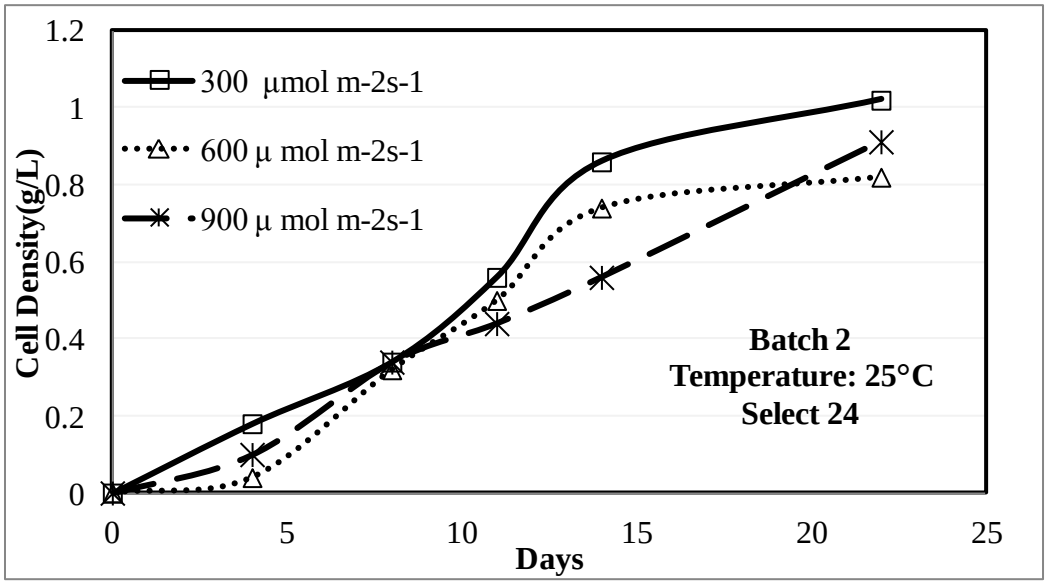

Figure 58. 2nd batch of select 24 at 300, 600 & 900 $\mu molm^{-2}s^{-1}$ light intensities.

4.4.3.1 Removal of nutrients from swine waste water by select 24 at three different light intensities. Table 20 and 21 shows the removal of nutrients by select 24 cultured at 300, 600 and 900 $\mu molm^{-2}s^{-1}$ light intensities in batch 1 and batch 2, respectively.

Table 20

Removal of nutrients from swine wastewater after 15 days of select 24 cultures at 300, 600 and 900 $\mu molm^{-1}s^{-1}$ light intensities for batch 1

Nutrients	Pure waste water	Air blown waste water	Select 24 300 $\mu molm^{-2}s^{-1}$	Select 24 600 $\mu molm^{-2}s^{-1}$	Select 24 900 $\mu molm^{-2}s^{-1}$
Total P in ppm	163.2	123	48.55	53.05	63.3
pH	9.29	8.85	8.54	8.74	8.75
Ammonia Nitrogen in ppm	75	23.6	8.4	8.9	9.8
COD in mgl	2200	1320	1045	895	970

Table 21

Removal of nutrients from swine wastewater after 15 days of select 24 cultures at 300, 600 and 900 $\mu molm^{-2}s^{-1}$ light intensities for batch 2

Nutrients	Pure waste water	Air blown waste water	Select 24 300 $\mu molm^{-2}s^{-1}$	Select 24 600 $\mu molm^{-2}s^{-1}$	Select 24 900 $\mu molm^{-2}s^{-1}$
Total P in ppm	163.2	123	53.25	51.2	56.75
pH	9.29	8.85	8.24	8.77	8.56

Table 21

Cont.

Ammonia Nitrogen in ppm	75	23.6	6.3	6.7	5.9
COD in mgl	2200	1320	820	955	917

4.5 Effect of Photo-periods in the Three Selected Microalgae in Removing the Nutrients From Swine Wastewater

Two different photo periods 14:10 h L/D and 16:8 h L/D were studied for the three selected microalgae at 25°C and 600 $\mu molm^{-2}s^{-1}$ light intensities and in 100% concentrated wastewater. Table 22 shows the concentration of the nutrients in wastewater, air blown wastewater and wastewater after 15 days of three selected microalgae culture with photoperiods 14:10 h L/D and 16: 8 h L/D. It can be seen from Table 22 that with the increase in light period the removal of nutrients were increased by all the three species. *C. vulgaris* removes more nutrients in both photoperiod conditions than the other two algae.

Table 22

The concentration of the nutrients in wastewater, air blown wastewater and wastewater after 15 days of three selected microalgae culture at 25°C and 600 $\mu molm^{-2}s^{-1}$ light intensities at 14:10h L/D and 16: 8 h L/D

Nutrients	Pure waste water	Air blown waste water	14:10h L/D			16: 8 h L/D		
			Select 24	*C. vulgaris*	*C. reinhardtii*	Select 24	*C. vulgaris*	*C. reinhardtii*
Total P in ppm	163.2	123	87	66.45	81.65	70.71	60.84	67.86
Ammonia Nitrogen in ppm	75	23.6	12.8	8.95	10.2	10.25	8.8	9.15
COD in mgl	2200	1320	930	760	880	820	742.5	752.5
pH	9.29	8.85	8.23	7.98	8.11	8.495	8.94	8.65

4.6 Oil Extraction and Characterization

4.6.1 Oil extraction from three different microalgal strains. Oil was extracted from *C. vulgaris, C. reinhardtii* and Select 24. The extracted oil was characterized to see which algal strain is a better feedstock among the three for biodiesel production. The percentage of oil extracted from dry microalgae is shown in Table 23.

Table 23

Oil extracted from C. vulgaris, C. reinhardtii and select 24

Algae Stranis	Dry weight	Oil extracted	Percentage of oil extracted from dry algae
C. vulgaris	0.3692g	0.0012g	0.325
C. reinhardtii	0.3909g	0.0013g	0.332
Select 24	0.4413g	0.0032g	0.725

4.6.2 Characterization of the oil extracted.

Gas chromatography of oil extracted from *C. vulgaris*, *C. reinhardtii* and Select 24 was done to separate and analyze the compounds that are present in the oil samples. Figure 59 shows the GC for *C. vulgaris* oil.

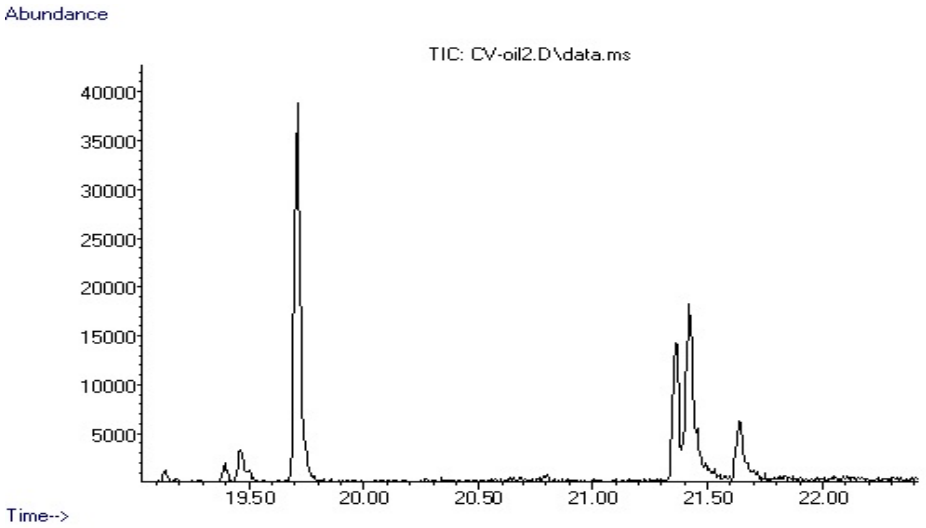

Figure 59. Gas Cromatography of *C. vulgaris* oil.

Four important peaks were found at retention times 19.722min, 21.364 min, 21.421 min and 21.639 min where four different fatty acid methyl esters were found for *C. vulgaris* oil. To identify those compounds FAME analysis was done. The results are listed in Table 24.

Table 24

FAME analysis for C. vulgaris oil

Retention Time (min)	Formula	Molecular weight	Name
19.722	$C_{17}H_{34}O_2$	270	Hexadecanoic Acid, methyl ester
21.364	$C_{19}H_{34}O_2$ (2 double bond)	294	9, 12-Octadecadienonic Acid, methyl ester
21.421	$C_{19}H_{32}O_2$ (3 double bond)	292	9, 12,13-Octadecatrienoic Acid, methyl ester
21.639	$C_{19}H_{38}O_2$	298	Methyl stearate

At 19.722 min retention time C_{16} fatty acid (Hexadecanoic Acid) was found. At times 21.364, 21.421 and 21.639 min three different C_{18} fatty acid were found. One is 9, 12-Octadecadienonic Acid that has 2 double bonds and is known as Linoleic acid which is an unsaturated omega-6 fatty acid. The second one is 9, 12, 13-Octadecatrienoic Acid that has 3 double bonds and is known as Linolenic acid which is also an omega three fatty acid. The third C_{18} fatty acid is Methyl stearate that has no double bond.

Figure 60 shows the gas chromatography *for C. reinhardtii* oil.

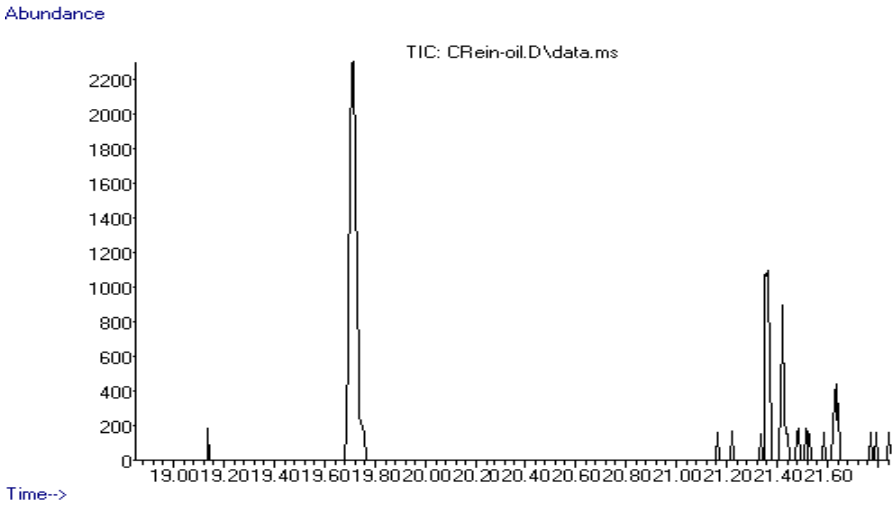

Figure 60. Gas cromatography for *C. reinhardtii* oil.

For *C. reinhardtii* four important peaks were also found at around the same retention times. At retention time 19.716 min C_{16} acid was found. At times 21. 37 min it was C_{18} fatty acid-Linoleic acid, 21.416 min another C_{18} fatty acid-Linolenic acid and 21.627 min Octadecanoic acid was found for *C. reinhrdtii* too. Table 25 shows the results from FAME analysis.

Table 25

FAME analysis for C. reinhardtii oil

Retention Time (min)	Formula	Molecular weight	Name
19.716	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester
21.370	$C_{19}H_{34}O_2$ (2 double bond)	294	Linoleic acid, methyl ester

Table 25

Cont.

21.416	$C_{19}H_{32}O_2$ (3 double bond)	292	Linolenic acid, methyl ester
21.627	$C_{19}H_{38}O_2$	298	Octadecanoic acid, methyl ester

Figure 61 shows the gas chromatography *for* Select 24 oil.

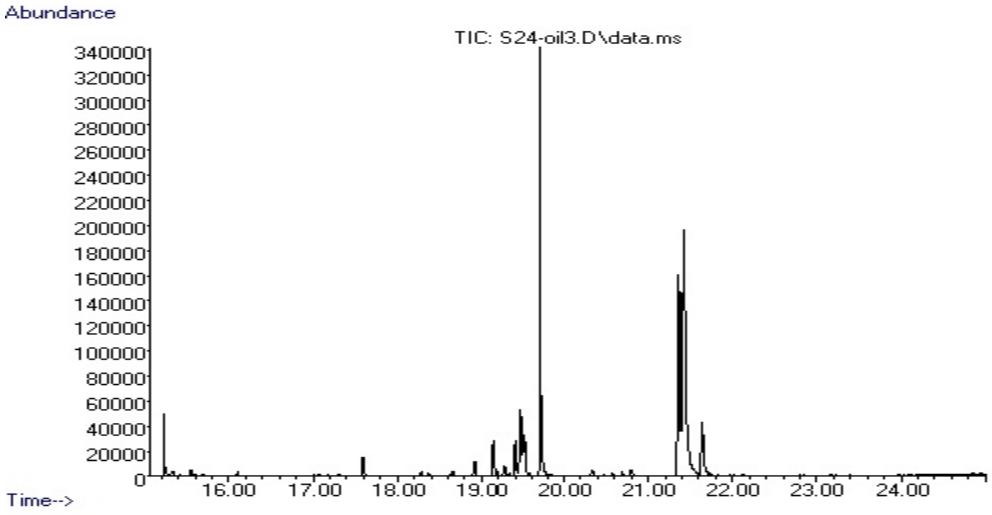

Figure 61. Gas cromatography for Select 24 oil.

For select 24 the previous four fatty acids were also found. From the gas chromatography of Select 24 oil it is observed that there are many small peaks in the earlier retention times. Table 26 shows the FAME analysis of Select 24. Mass spectrometer results are very impressive for Select 24. At time 15.235 min C_{12} fatty acid Dadecanoic acid was found, this low carbon fatty acid is a good source for the production of biodiesel. At retention time 17.582 min C_{14} Methyl tetradecanoate, at 18.286 min C_{15} Tetradecanoic acid, at 19.396 min C_{16} 7,10-Hexadecandienoic

acid and at 19.396 and 19.716 min two C_{16} fatty acids were found which can be used as biodiesel.

Table 26

FAME analysis of Select 24 oil

Retention Time (min)	Formula	Molecular weight	Name
15.235	$C_{13}H_{26}O_2$	214	Dadecanoic acid, methyl ester
17.582	$C_{15}H_{30}O_2$	242	Methyl tetradecanoate
18.286	$C_{16}H_{32}O_2$	256	Tetradecanoic acid, 12-methyl-methyl ester
19.396	$C_{17}H_{30}O_2$	266	7,10-Hexadecandienoic acid, methyl ester
19.716	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester
20.334	$C_{18}H_{36}O_2$	284	Hexadecanoic acid 15-methyl-methyl ester
21.370	$C_{19}H_{34}O_2$	294	Linoleic acid, methyl ester
21.427	$C_{19}H_{32}O_2$	292	Linolenic acid, methyl ester
21.645	$C_{19}H_{38}O_2$	298	Octadecanoic acid, methyl ester

CHAPTER 5

Conclusions and Future Research

A combination of biological treatment of swine wastewater and biofuel production could be the most effective approach to sustainably produce bioenergy and treat wastewater. Microalgae can be used to produce biofuels, treat wastewater and sequester CO_2. This research was to optimize the conditions for the growth of microalgae in swine wastewater to obtain the maximum use of the microalgae for the treatment of swine wastewater and the production of biofuels.

Commercial microalgae strains of *Chlorella vulgaris* and *Clamidomonus reinhardtii* were chosen to grow in the swine wastewater from the NCAT farm after a preliminary screening. The highest specific growth rates was 1.336 day^{-1} with a division rate of 1.928 day^{-1} for *C. vulgaris* and 1.275 day^{-1} with a division rate of 1.839 day^{-1} for *C. reinhardtii* obtained at a temperature of 25°C and light intensity of 600 $\mu molm^{-2}s^{-1}$ when they were grown in 100% autoclaved swine wastewater. The highest removal efficiencies of nutrients in the wastewater were also obtained at the same growth condition. For *C. vulgaris*, the highest removal efficiencies of ammonia nitrogen were 92.72%, 60.12% for COD and 75.55% for total phosphorus. For *C. reinhardtii* the highest removal of ammonia nitrogen was 89.8%, 42.94% for COD and 46.85% for total phosphorus. The performance of the selected strain 24 was also compared with those of the two commercial strains under the optimum growth condition. It was found that the growth rate of selected 24 was lower than the other two species *C. vulgaris* and *C. reinhardtii* and so as the removal of nutrients from swine wastewater. The change in pH in wastewater after 15 days of microalgae culture was in the range between 8 and 9.8.

Selected strain 24 gives higher percentage of oil compared to the two commercial microalgae strains grown on swine wastewater. The GC-MS analysis of extracted oil shows that select 24 will be a good feedstock for biodiesel production than *C. vulgaris* and *C. reinhardtii* as Select 24 oil has C_{12}-C_{17} fatty acids which are desirable fatty acids for the production of biodiesel. The three oil samples extracted from these three different algal strains have three different C_{18} fatty acids found in fish oil as omega three fatty acids.

Response surface methodology will be used analyze the effect of all the parameters involved in the growth of microalgae. So there is a huge scope of further exploration of the research on microalgae culture in swine wastewater. Contamination is a very common problem in microalgae culture in wastewater. So it is recommended to perform some pretreatment of wastewater e.g. chemical pretreatment, thermal pretreatment before using it as the growth medium of microalgae. Air can be replaced with CO_2 along with N_2 in culture environment as algae can sequester this greenhouse gas as a carbon source for their growth.

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