I, Bala P Lingaraju, hereby submit this original work as part of the requirements for the degree of Master of Science in Chemical Engineering.

It is entitled:
Removal of Nitrogen from Wastewater Using Microalgae

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Removal of Nitrogen from Wastewater using Microalgae

A thesis submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Master of Science

in the Department of Chemical Engineering
of the College of Engineering and Applied Science

by

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Dr. Soon-Jai Khang
Dr. Tim C. Keener
Abstract

Presence of residual nitrogen and phosphorus in treated wastewater that is discharged into the rivers and lakes poses a problem to aquatic species by causing “eutrophication”. There is a strong necessity to bring down the levels of nitrogen and phosphorus to as low as 1 ppm so that the nutrient supply to the wild type algae growing uncontrollably can be restricted. In this study, *Chlorella vulgaris* was used to effectively treat wastewater from Mill Creek domestic wastewater treatment plant in Cincinnati, Ohio. Ammonia–nitrogen was the major source of nitrogen. Typically, 8—10 mg/L of ammonia–nitrogen was present in the secondary wastewater effluent. The secondary wastewater effluent was used as a medium to grow *Chlorella vulgaris*. Ammonia–nitrogen reduced by 99% after the batch culture. Effective results were also obtained for orthophosphate removal. The residence time of algae in wastewater required to achieve effective reduction of nutrients was reduced through culture in nitrogen deficient medium. A comparison of suspended and settled algae for their effectiveness in nutrient removal was carried out. Results indicated that suspended algae are better than settled algae. Increasing the cell density also quickens the nutrient removal process.

Lipids were extracted through the well established Bligh and Dyer method and estimated gravimetrically. Additional confirmation of lipids was carried out using Transmission Electron Microscopy (TEM). The motivation behind lipid identification and estimation was to effectively utilize algae that were used to clean wastewater to produce biodiesel at a later stage. Results indicate that lipids can be extracted from *Chlorella vulgaris*. The current setup is a suitable starting point for wastewater treatment
and biodiesel production. However, for practical implementation, further improvements should be made in the maximum density of algae that can be achieved. Increasing the density of algae in the culture medium through elimination of growth limitations can improve the overall efficiency of removal of nitrogen from wastewater and yield of biomass, thereby increasing the yield of lipids. Improvement in the yield of lipids increases the biodiesel yield. Finally, the overall objective of wastewater treatment and renewable energy production can be achieved through improvement in maximum density of algae, for which further research is required.
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Removal of Nitrogen from Wastewater using Microalgae

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Bala P. Lingaraju

November 9th 2011
Date
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INTRODUCTION

Conventional municipal wastewater treatment can partially remove nitrogen and phosphorus concentrations in effluent wastewater [1]. Discharge of untreated water with the presence of residual nitrogen and phosphorus leads to deterioration in water quality and eutrophication of natural water bodies [2-3]. Additional treatments in the municipal wastewater treatment plant can double the cost with the requirement of additional chemicals and energy. Often, tertiary chemical treatment is not preferred due to additional problems from the sludge being contaminated from the by-product of the treatment. For example, sludge can get contaminated by aluminum from the tertiary treatment for phosphorus removal, where sludge disposal raises safety concerns. Microalgal culture was proposed as a solution for wastewater treatment [4-6]. The dual-purpose system is highly attractive since nitrogen and phosphorus can be eliminated and microalgal biomass can be produced in one step. The biomass produced can be harnessed and converted into energy through thermochemical conversion (gasification, direct combustion and pyrolysis), biochemical conversion and transesterification to produce biodiesel [7].

In the current scenario, the transportation sector relies 98% on petroleum, thus any small glitch in the oil supply chain can have rippling effects in many other sectors affecting the progress of the nation. Given the current progress in technology for new unconventional reserves, it is probable that fossil fuel will continue to be available at a
low cost, accompanied with a threat of inconsistent supply due to geo-political developments. In addition to the unsustainability and finiteness of conventional resources, CO$_2$ emissions, and the projected climate change that arises with the use of fossil fuels indicate that there needs to be an alternative to conventional fossil fuels. A possible solution to this problem is replacing fossil fuels by renewable sources, though not completely, but by a small percentage. Congressmen of U.S set the target for renewable energy production in the Energy Bill. According to the Energy Independence and Security Act (Energy Bill) of 2007, 36 billion gallons per year of total renewable fuels must be produced by 2022 [8]. In order to meet this target, there needs to be a four fold increase in the production of renewable fuels. Feedstocks for popular renewable transportation fuel are terrestrial crops such as soy, corn, maize, oil seed crops that need natural resources like land and water. Crops such as corn and soy which are also utilized as food, when used for biofuel generation, can cut into the supply of food and lead to a food vs. fuel debate. Microalgae as a source of renewable fuel has an edge over other renewable sources as it does not interfere with food supply, can grow in water, and harnesses natural sunlight through photosynthesis more effectively than terrestrial plants due to their simple cellular structure. Research conducted by the National Renewable Energy Laboratory (NREL) states that biodiesel produced from algae under controlled conditions can yield 30 times the amount of oil per unit area of land when compared to existing oilseed crops [9]. However, algae culture for renewable energy is deemed uneconomical if fresh water sources are to be used [10-11]. Due to the presence of high percentage of protein in its composition, microalgae need 8-16 tons of Nitrogen/ha [12],
which is uneconomical. Microalgae can utilize the Nitrogen (N) and Phosphorus (P) nutrients in wastewater and eliminate the need for addition of fertilizer. An algae pond can be constructed on barren land which is not suitable for agriculture. In summary, algae can grow in land and water that is not suitable for the growth of any other renewable feedstock, making it a very special source of renewable fuel, which cannot be limited by resources. Biodiesel production from algae can address important needs of the society such as the development of new energy sources, reduction of greenhouse gases and effective management of municipal and agricultural wastes. In some instances, *Chlorella vulgaris* was used directly in an unmodified diesel engine along with transesterified rape seed oil and a surfactant [13]. In addition, *Chlorella vulgaris* can be used as a food supplement. High rate algal ponds (HRP) were identified forty years ago. Lack of sustainable efforts, limited size of scientific faculty involved in the research, lack of integrated work between biologists and engineers resulted in slow progress.

The goal of this research is to contribute to the practical implementation of wastewater treatment and biodiesel production at municipal wastewater treatment plants and animal farms. The process would remove nitrogen and phosphorus from wastewater and generate biomass for biodiesel production simultaneously. Objectives of the current thesis are:

1. Test suitability of wastewater treatment through *Chlorella vulgaris* for secondary effluent from Mill Creek Plant, Cincinnati, Ohio.

2. Test if *Chlorella vulgaris* can uptake nitrogen and phosphorus from secondary effluent from Mill Creek Plant, Cincinnati, Ohio.
3. Improve the kinetics of uptake of nitrogen such that the treatment and residence time can be reduced.

4. Test the possibility of lipid extraction from *Chlorella vulgaris* grown in wastewater.
CHAPTER 1

LITERATURE SURVEY

1.1 Need for Renewable Energy Sources – Role of Algal Biofuel

Atmospheric concentrations of greenhouse gases such as carbon dioxide, methane and nitrous oxide have increased due to human activity [14]. On the other hand, during the year 2007-2008, net petroleum imports were 149 to 153 billion gallons per year (BGY), out of which 71% was allocated for transportation fuel [15]. The demand is only going to increase. Projection for the year 2035 predicts an increase in the share of transportation fuel to 74%. In order to decrease the dependency on foreign oil and to avoid further deterioration of natural resources and global climate, renewable sources were thought of as a promising alternative. U.S policy on renewable biofuels as described in Energy Independence and Security Act, 2007 in the Renewable Fuel Standards (RFS) mandates that 36 billion gallons per year should be produced by renewable source, of which 31 billion gallons per year are from crop based feedstocks, 1 billion gallon from cellulosic feedstocks and 4 billion gallons per year should be produced from advanced biofuels by the year 2022 [15]. There are first, second and third generation of renewable fuels [16]. The first generation renewable fuels are starch and sugar based renewable fuels derived from food sources. Corn, sugarcane, sweet sorghum and vegetable oilseeds such as rapeseed and maize are now being considered for biofuel
production [17-18]. Consideration of food crops for biofuel production creates short supply of grains causing the prices to skyrocket. When an under-nourished population starves for food, burning food grains for luxury automobiles creates a bioethical issue. Currently, 1% of the global arable land is being used for cultivation of energy crops, providing 1% of global transportation fuel, therefore, scaling arable land use for energy crops to 100% is not possible [19]. Excessive cultivation of food crops for biofuel production gives rise to shortage for land, water and creates food vs. fuel debate. Thus emerged second generation of biofuels that have feedstocks which do not include food grains. Second generation of biofuels are cellulosic feedstocks, such as corn stover, sugarcane residue and herbaceous crops which do not interfere with the food supply, but require cultivatable land and water for irrigation, which pose a competition for natural resources. Freshwater resources are under stress. The amount of withdrawal of freshwater was 300 billion gallons per day in 2005, out of which 84% was used for agriculture [20]. Ground water supplies are being withdrawn at unsustainable rates. Nation’s water supplies are limited due to excessive withdrawal to meet the needs of many competing sectors. There are questions about the cost-effectiveness, technological barriers and feedstock collection network problems regarding second generation biofuels. Hence, there is a limitation in terms of resources available for the expansion of first and second generation biofuels to the extent where they can replace significant percentage of conventional fuel requirement. An economically viable biofuel should meet certain conditions; it should be less expensive than petroleum fuel, should not require the use of cultivable land, should use minimum amount of freshwater resources and should
contribute to the enhancement of air quality by reducing emissions and facilitating carbon capture. The third generation of renewable fuel consists of microalgae, which meets almost all the requirements of an economically viable biofuel listed above. Microalgae can be cultivated using the least of the resources. The option to cultivate microalgae in wastewater eliminates the need for freshwater and fertilizer and makes it the most attractive renewable resource available. Algae can be autotrophic, heterotrophic or mixotrophic. Utilizing abundantly available resources such as sunlight, nutrients from wastewater and freely available CO$_2$, algae are able to produce lipids for biofuel production, proteins and carbohydrates which can act as food supplement. Hence, there are high expectations on algae as a suitable biofuel for the future.

1.2 Popular Species of Microalgae and Logic of Strain Selection

Microalgae are eukaryotic. They contain nucleus and other membrane bound organelles unlike prokaryotes. Microalgae have been identified as important extraterrestrial organisms, interest in aquaculture started since 1940s. Microalgae were proposed for the application of wastewater treatment in the 1950s [21-23]. In the 1970s and 80s microalgae were identified for alternative fuel production and production of other fine chemicals and food supplements. After 1980s, there has been research on the mass cultivation of algae in raceway ponds and photobioreactors [24-27]. Microalgae are promising future fuel source, because literature suggests that they can meet 50% of the transportation fuel requirement utilizing as low as 4.5 M ha, which is 2.5% of existing
crop area in U.S., if 30% oil by weight is present in biomass [28]. Table 1 adapted from literature [28] shows the comparison between microalgae and other terrestrial crops.

Table 1. Comparison of Sources of Biodiesel [28]

<table>
<thead>
<tr>
<th>Crop</th>
<th>Oil Yield (L/ha)</th>
<th>Land Area (Mha)</th>
<th>Percentage of existing crop area(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>172</td>
<td>1540</td>
<td>846</td>
</tr>
<tr>
<td>Soybean</td>
<td>446</td>
<td>594</td>
<td>326</td>
</tr>
<tr>
<td>Canola</td>
<td>1190</td>
<td>223</td>
<td>122</td>
</tr>
<tr>
<td>Jatropha</td>
<td>1892</td>
<td>140</td>
<td>77</td>
</tr>
<tr>
<td>Coconut</td>
<td>2689</td>
<td>99</td>
<td>54</td>
</tr>
<tr>
<td>Oil Palm</td>
<td>5950</td>
<td>45</td>
<td>24</td>
</tr>
<tr>
<td>Microalgae(^b)</td>
<td>136,900</td>
<td>2</td>
<td>1.1</td>
</tr>
<tr>
<td>Microalgae(^c)</td>
<td>58,700</td>
<td>4.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^a\)For meeting 50% of the transportation fuel requirement  
\(^b\)70% oil by weight in biomass  
\(^c\)30% oil by weight in biomass

Microalgae utilize photosynthesis to harvest solar energy, they utilize carbon source, light, and essential nutrients such as carbon, nitrogen, phosphorus and other micronutrients to produce lipids, proteins and carbohydrates. Not all kinds of algae are suitable for biofuel production. Microalgae that were popularly used for biofuel production are mentioned in the list with oil content as a percentage of dry weight of biomass in brackets: *Botryococcus braunii* (25-75%) [29], *Chlorella sp* (14-22%) [30], *Dunaliella primolecta* (23%) [31], *Isochrysis sp.* (25-33%) [32], *Monollantus Salina* (>20%) [33], *Nannochloris sp.* (25-35%) [34], *Nitzschia sp.* (45-47%) [35], *Scenedesmus sp.* (8-14%) [36] and *Ankistrodesmus braunii* (18-35%) [37]. Microalgae chosen for lipid production can be divided into two categories: (1) slow cell growth but high lipid productivity, such as *Botryococcus braunii*, which has slow growth rate of 28 mg/L/day
and (2) high doubling time with medium lipid productivity such as Chlorella vulgaris which has a doubling time of 8h, lipid productivity of (14-22%) [30].

Chlorella vulgaris is better suited for applications such as simultaneous wastewater treatment and biofuel production where optimum growth and lipid productivity are required. There are total 8 algae which belong to the species Chlorella [39]: Chlorella conductrix, Chlorella gonglomerata, Chlorella parasitica, Chlorella protothecoides, Chlorella emersonii, Chlorella sorokiniana and Chlorella vulgaris are the common freshwater strains. One strain which can grow in saline water, Chlorella minutissima [40]. Average ash, energy, carbohydrate and lipid contents reported are: ash (5.93%), energy (18.59 MJ/kg), carbohydrate (19.46%), and lipid (28.82%) [41]. Next sections describe growth conditions and important factors for cultivation of Chlorella vulgaris.

1.3 Autotrophic, Heterotrophic and Mixotrophic Growth Conditions

Chlorella vulgaris can be cultivated in autotrophic [42], heterotrophic [43] and mixotrophic [44] growth conditions. Light dependent growth is called autotrophic growth, where Chlorella vulgaris utilizes light from the sun or an external source. The advantage of autotrophic growth condition is that energy from the sun can be converted into valuable products utilizing CO₂ and H₂O. Chlorella vulgaris grown under autotrophic growth conditions is reported to effectively remove nitrogen and phosphorus from wastewater, where, the efficiency of removal of nitrogen is higher than that of phosphorus. However, the limitation is that penetration of light is inversely proportional
to the density of algae. As the algae increase in density, the light penetration decreases resulting in limitation in removal of nitrogen and phosphorus from wastewater in autotrophic growth conditions.

The heterotrophic condition does not require light, organic carbon such as glucose acts as the source of carbon. As light penetration does not limit the growth, high densities can be achieved. Due to high yield in biomass, this growth condition is suitable for lipid production, where high density of biomass is required to achieve high lipid yields. In the current scenario, the intention is to treat wastewater and also extract lipids, hence the mixotrophic growth condition was chosen with alternating 8hr dark and 16 hr light cycles.

1.4 Growth Controlling Factors of Microalgae

The growth of *Chlorella vulgaris* is influenced by four important factors [45-46]. The empirical formula for algal biomass was reported as C_{8.25}H_{14.79}N_{0.02}O_{5.02} [47]. The formula indicates that carbon and nitrogen are important nutrients. If any of the factors is in-sufficient, it will become a rate limiting substrate. The factors are as follows:

1) Light Intensity
2) Carbon
3) Nitrogen (NO\textsubscript{2}^{-}, NO\textsubscript{3}^{-}, NH\textsubscript{3} and NH\textsubscript{4}^{+})
4) Phosphorus (PO\textsubscript{4}^{3-})

The importance of each factor and the role the factor plays in the growth of *Chlorella vulgaris* is as follows:
**Light Intensity:** Light is an important factor for the process of photosynthesis [48]. Literature review indicates that the rate of growth of algae changes with the intensity of the light supplied [49-51]. Though it was reported that *Chlorella vulgaris* can grow in the dark with the supply of organic glucose, it was observed that growth cannot happen when the size of the inoculum is small [52]. Hence, light is an essential and important growth controlling factor. The mixotrophic growth of *Chlorella vulgaris* requires alternate light and dark cycles.

**Carbon:** Carbon source is required for the growth of *Chlorella vulgaris*. Carbon is utilized in the process of photosynthesis, which is essential for the growth and multiplication of biomass. Carbon dioxide is used as the carbon source in many cases. Inorganic carbon can also act as a source of carbon. In the literature, there are studies which report diverse sources of carbon such as glucose, glycerol and acetate [53]. NaHCO$_3$ is used as the carbon source in the current study.

**Nitrogen:** *Chlorella vulgaris* can utilize nitrogen in the form of NO$_2^-$, NO$_3^-$, NH$_3$ and NH$_4^+$. Secondary effluent from the Mill Creek Plant contains only NH$_4^+$. Concentrations of NO$_2^-$ and NO$_3^-$ are insignificant. Hence, when the secondary effluent of Mill Creek Plant or artificial wastewater such as Shuisheseng-4-Medium is used as the growth medium, NH$_4^+$ is the main source of nitrogen in the current study. NH$_4^+$ is taken up by *Chlorella vulgaris* and utilized for the synthesis of amino acids which form proteins later on. Details of assimilation of NH$_4^+$ are described in the next section. The NH$_3$ and NH$_4^+$ equilibrium is dependent on the pH. The graph showing relative percentages of ammonia and ammonium ion is shown in the figure. Above pH 12, the form of nitrogen is NH$_3$. 
Below pH 12, nitrogen exists as varying percentages of NH\textsubscript{3} and NH\textsubscript{4}\textsuperscript{+}. The change in percentages of NH\textsubscript{3} and NH\textsubscript{4}\textsuperscript{+} are shown in Figure 1.

![Figure 1. Distribution of NH\textsubscript{3} and NH\textsubscript{4}\textsuperscript{+} with respect to pH. [54]](image)

*Phosphorus:* There are many forms of phosphorus in water. The forms of phosphorus can be categorized as reactive phosphorus, organic phosphorus and acid-digestible phosphorus. Reactive phosphorus is nothing but orthophosphate. Organic phosphorus consists of organically bound phosphorus, which exists in living matter as a poly-phosphate. Acid-digestible forms of phosphorus like pyrophosphate and meta-phosphate. *Chlorella vulgaris* like all other species of algae can take up soluble reactive phosphorus. Phosphorus is stored in the cell body as polyphosphates for as many as four cell divisions, though there is no supply of phosphorus from the medium.
1.5 Nitrogen, Carbon Assimilation and Fatty Acid Synthesis in Algae

The most important sources of nitrogen for algae are nitrate and ammonium ion. Nitrite is not preferred due to its high toxicity, but can still be utilized by algae. Algae prefer to use ammonium ion first and then utilize nitrate when a combination of nitrate and ammonium ion are present in the growth medium [55]. However, the growth rate is the same for nitrate nitrogen and ammonium nitrogen. The exception to this rule was observed in *Chlamydomonas reinhardtii* only. It was reported that *Chlamydomonas gleopara, C. microsphaerella, C. peterfi*, do not utilize ammonium nitrogen preferentially [56]. Even if nitrate-N is used as a source of nitrogen, it is reduced to ammonium before being incorporated into organic compounds [55]. There is a two-step enzyme catalysis reaction involving the reduction of nitrate to ammonium. The first step involves reduction of nitrate to nitrite by nitrate oxidoreductase, then nitrite to ammonium by nitriteoxidoreductase. There are three well known reactions through which inorganic nitrogen in the form of ammonium ion is assimilated into organic compounds. The first is the reductive amination of ketoacids to amino acids. The second is further amination of amino acids to amides. The third is the reaction involving CO$_2$, ATP and ammonium ion catalyzed by carbamoyl phosphate synthetase to form carbamoyl phosphate. The reactions described above in detail are as follows:
Amidation step is driven by the hydrolysis of ATP. Phosphorylation of the side chain of glutamate results in an acyl-phosphate intermediate. Glutamine synthetase is the enzyme responsible for incorporation of a second ammonium ion resulting in further amidation to form glutamine.

The overall reaction can be summarized as follows:

\[
\text{α-Ketoglutarate} + \text{NH}_4^+ \rightarrow \text{Glutamate} + \text{NH}_3 + \text{P}
\]

Carbamoyl phosphate synthetase acts as a catalyst in the reaction in which \(\text{NH}_4^+, \text{CO}_2, \text{ATP}\) and \(\text{H}_2\text{O}\) react to form Carbamoyl phosphate.
Important intermediate in fatty acid synthesis, acetyl-coenzyme A (acetyl coA) is generated from amino-acid degradation as shown in the following figures. The link between nitrogen utilization and fatty acid synthesis is that nitrogen is utilized in the form of ammonium ion in the synthesis of amino acids as shown above, then, amino acids degrade to give important intermediates in fatty acid synthesis such as enzyme acetyl-coA as shown below in Figures 5.

Figure 4. Synthesis of Carbamoyl Phosphate using Ammonium Ion. [57]

Figure 5. Synthesis of Acetyl CoA from Leucine. [57]
1.5.1. Utilization of Carbon in Photosynthesis and Fatty Acid Synthesis

Carbon dioxide is utilized in the photosynthesis reaction, more specifically in the Calvin cycle, to produce glucose/starch. Calvin cycle takes place in the stroma of the chloroplast as indicated in Figure 6.

![Figure 6. Utilization of Carbon in Photosynthesis and Production of Triacylglycerol. [58] Synthesis of triacylglycerols is called fatty acid biosynthesis. The reactions involved in the synthesis of fatty acids are as shown stepwise in the following Table 2.](image-url)
Table 2. Steps in the Synthesis of Fatty Acids [57]

<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetyl CoA + HCO₃⁻ + ATP → malonyl CoA + ADP + P₇ + H⁺</td>
<td>Acetyl CoA carboxylase</td>
</tr>
<tr>
<td>2</td>
<td>Acetyl CoA + ACP ↔ acetyl ACP + CoA</td>
<td>Acetyl transacylase</td>
</tr>
<tr>
<td>3</td>
<td>Malonyl CoA + ACP ↔ malonyl ACP + CoA</td>
<td>Malonyl transacylase</td>
</tr>
<tr>
<td>4</td>
<td>Acetyl ACP + malonyl ACP → acetoacetyl ACP + ACP + CO₂</td>
<td>Acyl-malonyl ACP condensing enzyme</td>
</tr>
<tr>
<td>5</td>
<td>Acetoacetyl ACP + NADPH + H⁺ = d-3-hydroxybutyryl ACP + NADP⁺</td>
<td>β-Ketoacyl ACP reductase</td>
</tr>
<tr>
<td>6</td>
<td>d-3-Hydroxybutyryl ACP = crotonyl ACP + H₂O</td>
<td>3-Hydroxyacyl ACP dehydratase</td>
</tr>
<tr>
<td>7</td>
<td>Crotonyl ACP + NADPH + H⁺ → butyryl ACP + NADP⁺</td>
<td>Enol ACP reductase</td>
</tr>
</tbody>
</table>

HCO₃⁻ plays an important role in the synthesis of malonyl CoA, which is an important intermediate. Later on, the CO₂ is released when the following reaction happens:

Acetyl ACP + Malonyl ACP → Acetoacetyl ACP + ACP + CO₂

Fatty acid elongation happens by the addition of 2 carbon atoms at a time. Butyryl-Acyl Carrier Protein (ACP) undergoes condensation reaction with malonyl coenzyme A (CoA) to form C6- β-ketoacyl ACP. Reduction, dehydration followed by another reduction result in C6-acyl-ACP, which continues in the elongation cycle. The elongation cycle continues until fatty acids are produced. The highest number of fatty acid produced differs from one organism to another. Thioesterase is the enzyme that hydrolyzes C16-acyl-ACP form ACP and palmitate, hence the enzyme acts as a ruler in determining the fatty acid chain length.

Summary of Fatty Acid Synthesis:

\[
\text{Acetyl CoA} + 7\text{ malonyl CoA} + 14\text{ NADPH} + 7\text{ H}^+ \rightarrow \text{Palmitate} + 7\text{CO}_2 + 14\text{NADP}^+ + 8\text{CoA} + 6\text{H}_2\text{O}
\]

Malonyl CoA in the above reaction is synthesized by
\[
\begin{align*}
7\text{Acetyl CoA} + 7\text{CO}_2 + 7\text{ATP} & \rightarrow 7\text{Malonyl CoA} + 7\text{ADP} + 7\text{P}_i + 7\text{H}^+ \\
8\text{Acetyl CoA} + 7\text{ATP} + 14\text{NADPH} & \rightarrow \text{Palmitate} + 14\text{NADP}^+ + 8\text{CoA} + 6\text{H}_2\text{O} + 7\text{ADP} + 7\text{P}_i
\end{align*}
\]

The overall reaction is

The above reactions indicate that CO\(_2\) and acetyl CoA play an important role in the fatty acid synthesis.

### 1.6 Algae Culture for Wastewater Treatment

Primary and secondary treatment processes have been introduced in a growing number of places in order to eliminate the easily settled materials (primary treatment) and to oxidize the organic material present in wastewater (secondary treatment). The final result is a clear, apparently clean effluent which is discharged into natural water bodies. This secondary effluent is, however, loaded with inorganic nitrogen and phosphorus, and causes eutrophication and more long-term problems because of refractory organics and heavy metals that are discharged. The use of high rate algae pond (HRAP) is an efficient approach in bioremediation of agro-industrial wastewaters. The system consists of a shallow pond with dense algae cultures aerated with paddle wheels. Apart from removing the pollutants, the algae biomass generated is useful as high-quality animal feed. *Chlorella vulgaris* was used to treat textile water which has strong color, high salinity, variable pH and a high chemical oxygen demand (COD) [59]. Literature shows that both viable and non-viable algae can be useful in wastewater treatment. Dyes present in wastewater can be treated by biosorption and bioconversion. For example,
algae named *Aspergillus niger* and *spyroigira* were used as biosorbents for the removal of a reactive dye called synazol [60]. Rubber effluent and sago starch in wastewater can be treated by *Chlorella vulgaris* and *Spirulina* [61]. Studies were reported by authors working on real wastewater from cattle waste [62], aquaculture [63] and pig farms [64]. In the previous studies variety of strains such as *Chlorella pyrenoidosa* [65], *Scenedesmus acuminatus* [54], *Spirulina maxima* [66], *Haematococcus pluvialis* [67], *Scenedesmus rubescens* [68] and many more. *Chlorella vulgaris* was chosen as a strain, in the current study due to its fast growth (doubling time of 8 hrs) and extensive amount of literature available.

### 1.7 Technologies for Conversion of Algae into Renewable Fuel

Viable conversion technologies of microalgal biomass can be classified into three major categories. They are thermochemical, biochemical conversion and biomass-biodiesel by transesterification [69]. Thermochemical conversion deals with thermal decomposition of organic components of biomass through direct combustion, gasification, thermochemical liquefaction and pyrolysis. Biochemical decomposition can be achieved by anaerobic digestion, alcoholic fermentation, and photobiological hydrogen production. All the technologies are summarized in Figure 7 below and explained in the text thereafter.
Gasification: Biomass is partially oxidized, reacts with oxygen and steam at high temperatures of 800-1,000°C to produce syngas, a mixture of CO, H₂, CO₂, N, CH₄, which has a low calorific value of 4-6 MJ/m³. There are very few studies on gasification of microalgae. Gasification of Chlorella and Spirulina were reported in the literature [70]. The highest yield reported was 0.64 g of methanol from 1 g of biomass. The energy balance is marginally positive due to high energy spent in harvesting algal biomass. Gasification technology requires further research.

Thermochemical Liquefaction: Complex and expensive fuel feed systems are used in thermochemical liquefaction. High pressure (5-20 Mpa) and low temperature (300-350 °C) are used along with a catalyst and hydrogen to produce bio-oil. Important feature of
 thermochemical conversion is that wet biomass can be converted to bio-oils. In the literature, thermochemical liquefaction of *Botryococcus braunii* at 300°C was reported [71]. Yield from *Botryococcus braunii* was 64% dry wt of biomass with a High Heating Value (HHV) of 45.9 MJ/kg.

**Pyrolysis:** Bio-oil, syngas and charcoal are produced from biomass at medium to high temperatures of 350-700°C. The disadvantages of pyrolysis oils are the acidic nature, viscosity, presence of solids and chemically dissolved water. Pyrolysis oils require further hydrogenation and catalytic cracking. *Chlorella prothothecoides* with modified metabolic pathway was reported to have a high record yield of 57.9% dry weight and a HHV of 41 MJ/kg [72]. *Chlorella prothothecoides* and *Mycrocystis aeruginosa* when grown under phototrophic conditions yield 18% and 24% bio-oil with HHV of 30 and 29 MJ/kg, respectively [73]. A noteworthy point is that it was reported that bio-oils obtained from pyrolysis of microalgae are superior in heating value to those obtained from lignocellulosic biomass.

**Direct Combustion:** Biomass from microalgae can be burned directly to yield energy and hot gases. The main drawback of this process is that the moisture content of the feedstock should be less than 50%, which requires drying of algal biomass. The second disadvantage of this process is that after burning, the energy recovered should be used immediately and there is no option for storage. Coal-algae co-firing was reported as an option in the literature but there are many technological barriers involved in practical implementation of direct combustion option for algae [74].
Anaerobic digestion: Anaerobic digestion constitutes of hydrolysis, fermentation and methanogenesis, where organic matter such as algal biomass with 80-90% moisture content is broken down to yield biogas (CH$_4$ and CO$_2$) which has 20-40% less heating value than that of the source biomass. In the hydrolysis step, the algal biomass is broken down into soluble sugars. In the fermentation step, the sugars are converted into alcohol, acetic acid and volatile fatty acids. In the methanogenesis step, methanogens convert products of fermentation step into CH$_4$ (60-70%) and CO$_2$ (30-40%). The drawback involved in using anaerobic digestion technology for algal biomass is that, often algal biomass has high protein content resulting in C/N ratio, which is not suitable for anaerobic digestion. Literature reports that anaerobic digestion can be successful when subjected to co-digestion with another biomass source such as wastepaper and algae biomass [75].

Alcoholic Fermentation: Biomass from microalgae which has high carbohydrate content is suitable for alcoholic fermentation [76]. In this process, the biomass is broken down into starch which is subsequently converted into sugars by a process called saccharification. It is easier to convert biomass from microalgae to sugars compared to lignocellulosic feedstocks [77]. Absence of lignin content in algae makes the process of obtaining sugar from starch very easy. Sugars are then converted into ethanol by fermentative micro-organisms. The ethanol produced requires further refining through distillation. *Chlorella vulgaris* was reported in the literature to be a successful strain for obtaining ethanol with efficiency, as high as 65% through fermentation technology due to its high carbohydrate content of 35% of dry weight of biomass [78].
Photobiological Hydrogen Production: Metabolic and enzymatic characteristics of microalgae are suitable for hydrogen production [79]. Hydrogen (H⁺) and hydroxyl (OH⁻) are produced from water splitting reaction and oxygen is produced in a separate reaction during the process of photosynthesis. Under anaerobic conditions hydrogenase enzyme converts H⁺ ions into H₂ [80]. Continuous production of hydrogen is not possible as this process is limited by the production of oxygen which reduces the activity of hydrogenase enzyme. Spatial separation was suggested in the literature [80] to solve the problem, where algae are grown in the first step and anaerobic conditions are created in the second step by sulfur limitation [81]. An alternative approach is to send H⁺ ions released from oxidation of H₂O into a hydrogen evolution process mediated by hydrogenase. Either of the processes cannot produce hydrogen continuously. The concept is theoretically, very promising.

Biodiesel: Biodiesel is a mixture of mono-alkyl esters of long chain fatty acids (FAME). FAMEs are produced from the trans-esterification reaction between triglycerides (class of lipids) and alcohol in the presence of a catalyst. Example of the reaction is as shown in the Figure 8.

![Figure 8. Transesterification Reaction Using Methanol. [82]](image-url)
Algal cells should be broken down for the extraction of lipids. There are several methods available for algae lysis such as bead beater, french press, sonication, homogenization, freeze thaw method, osmotic shock and supercritical CO\textsubscript{2}. Cell lysis is followed by the extraction of lipids using organic solvents such as chloroform-methanol (2:1, v/v), hexane-isopropanol (3:2, v/v). Solvent-extraction is followed by removal of chlorophyll and isolation of lipids. Mixtures of neutral, phospholipids, glycolipids are obtained which constitute total lipids. Lipids are converted into FAMEs by esterification, which are converted into biodiesel by trans-esterification reaction. Among all the technologies available for conversion of algal biomass into biofuel, conversion to biodiesel is well researched and economically viable. Extraction and conversion of algal lipids into biodiesel is explained in detail in a later section.

1.8 Lipid Extraction from Microalgae

Naturally occurring fats, waxes, sterols and vitamins fall under the category of lipids. Monoglycerides, diglycerides, triglycerides, phospholipids, glycerophospholipids, sphingolipids, and polyketides are the different types of lipids present in algae. Lipids present in microalgae can be classified into two broad categories. One is structural lipids and other is storage lipids. Structural lipids are present in all the membranes in algae, including plasma membrane. Lipid bi-layer plays an important role in the transport of ions from the surrounding environment into microalgae cell. Storage lipids store energy in the form of triglycerides. Microalgae contain straight chain fatty acids containing even numbers of carbon atoms, as a consequence of their biosynthesis. Neutral lipids, in
particular triglycerides, are of interest for the conversion of lipids in fatty acid methyl esters, and then subsequently into biodiesel. Cell disruption, solvent extraction, and esterification are the three important steps involved in the conversion of lipids to biodiesel. In this section literature review of methods used are described.

**Cell Disruption:** In order to get the lipids out of the cell, several cell disruption techniques are used. Popular methods reported are [83]: (1) Autoclaving at 125°C with 1.5 MPa (2) Bead-beating using a bead beater, 0.1mm. (3) Microwaves at 2450 MHz (4) Sonication (5) Osmotic shock with 10% NaCl.

**Solvents for Lipid Extraction:** Organic solvent systems were used for lipid extraction. Exposure to organic solvents dissolves the plasma membrane.

**Chloroform-Methanol:** Chloroform and methanol (2:1, v/v) is reported as the most suitable solvent for lipid extraction from algae, *Chlorella vulgaris* [84-85]. Chloroform phase captures the neutral lipids from algae because of the non-polar nature of the lipids. Chloroform was reported in the literature for extraction of lipids from algae such as: *Chlorella sp* [86], *Rhodomela larix* [87], *Chlamydomonas reinhardtii* [88], *Nannochloropsis oculata* [89], *Isochrysis sp* [90], and *Laurencia Filiformis* [91].

**Hexane-Isoproponol:** Hexane and isopropanol (3:2, v/v) solvent system was used by many authors for the extraction of high triacylglycerol microalgae *Botryococcus Braunii* [92-94]. It was also used for other algae *Chlorococcum* [95], *Nannochloropsis* [96], *Isochrysis sp* [97]. Some studies report that Chloroform-methanol and hexane-isopropanol solvent systems are equally effective [97]. However, it is a less preferred solvent system when compared to chloroform-methanol for *Chlorella vulgaris* [84].
**Supercritical Carbon Dioxide**: Supercritical CO$_2$ was used for extraction of carotenoids and lipids from *Chlorella vulgaris* [98], *Pavlova Sp.*[99] and *Botryococcus braunii* [100] in the literature. Supercritical CO$_2$ has the ability to dissolve lipophilic substances [101], has high diffusivity and low viscosity and can easily penetrate into porous solid materials [102]. Solutes dissolved in supercritical CO$_2$ can be easily separated by de-pressurization [103]. Hence, supercritical CO$_2$ was reported as one of the possible solvents for lipid extraction, but it is not preferred due to requirement of extra unit operations and high cost involved in solvent recovery [104] and high pressure such as 30 MPa [105]. Moreover, supercritical CO$_2$ showed low yield when compared to chloroform-methanol solvent system and higher yield when compared to hexane, when used for extraction from alga *Arthrospira maxima* [106].

**Other solvents**: Acetone/dichloromethane, dichloromethane/ethanol, Methyl-Tertiary Butyl Ether (MTBE), and dimethyl ether are some of the less used solvents reported in few studies for the extraction of lipids from algae [107].
CHAPTER 2

MATERIALS AND METHODS

2.1 Batch Setup

The setup of the experiment is a batch setup. Fluorescent lamps with light intensity of 6,500 K color temperature similar to natural sunlight were used as a source of light, and the incoming light intensity to beakers was set to 6,000 lux by controlling the distance between the beaker and lamp. The light intensity was measured using a light intensity meter (HQRP digital lux meter, LX1010BS, Osprey-Talon Company), and a 16-hr light and 8-hr dark cycle was applied to the culture. Pyrex glass beakers of 4 L volume were used to contain 3.5 L of secondary effluent. The distance between the beakers of volume 3.5 L and the light was 3 inches on either sides. 30 ml of Chlorella vulgaris cultured in shuisheng-4-medium was inoculated into 3.5 L of secondary effluent. To contain ammonia evaporation, the beakers were closed using parafilm or tight lids. The setup of the experiment is shown in Figure 9.
2.2 Growth Medium

A modified Shuisheng-4 medium was used. The composition of Shuisheng-4 medium [108] with some modification, is as follows: which is composed of distilled water and the following chemical ingredients (mg/L): (NH₄)₂SO₄, 200; Ca(H₂PO₄)₂·H₂O, 30; MgSO₄·7H₂O, 80; NaHCO₃, 100; KCl, 25; FeCl₃, 1.5, K₂HPO₄, 10; and 1ml of A5 liquid (chemical ingredients of A5 liquid (g/L) are the following: H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.22; Na₂MoO₄·2H₂O, 0.39; and CuSO₄·5H₂O, 0.079). Nitrogen starved Shuisheng-4-medium is the same as regular Shuisheng-4 medium except that it is deficient of the nitrogen source.
2.3 Biomass Measurement

The cell density of *Chlorella vulgaris* was determined by measuring the optical density of a 15-mL sample at 682 nm [109] for every 24 hours by using UV-vis spectrophotometer (Cary 50, Varian, Inc.). Here, the absorbance of UV spectrophotometer at 682 nm was calibrated by measuring the weight of dried *Chlorella vulgaris*. Then, the weight of dried biomass was obtained from the prepared calibration curve.

2.4 Determination of Total Inorganic Carbon Concentration

An acid-base titration method [110] was used to determine the inorganic carbon species in the aqueous phase. This titration method determines total inorganic carbon concentration (carbonate (*CO$_3^{2-}$*), bicarbonate (*HCO$_3^-$*) and aqueous carbon dioxide (*CO$_2$ (aq)*)) using a 0.01 N hydrochloric acid solution as a titrant.

2.5 Determination of Nitrogen Concentration in the Form of NH$_3$/NH$_4^+$

Nitrogen concentrations in the form of ammonia and ammonium ion present in the wastewater were measured using a commonly used ammonia probe (Model: 9512HPBNWP Orion Thermo Scientific) [111-112]. All ammonium ions were converted into ammonia by raising the pH of the sample solution of the culture medium (i.e. wastewater effluent) above 12 while monitoring its pH (Oakton pH 11 series pH meter),
and the resultant ammonia concentration is determined by the ammonia probe. Then the concentration distribution of ammonia and ammonium ion is determined by the equilibrium at the original pH of the sample solution. The standard ammonia solution required for the calibration of the ammonia probe was prepared according to the EPA Method 350.3 [113]. 10N NaOH (reagent grade, Fischer Scientific) was used for raising the pH of the solution above 12. Distilled and deionized water from a MilliQ water purification system was used for preparing the standard and NaOH solutions.

The probe was re-calibrated every 5 samples according to EPA Method 350.3 [113] over a range of 1,000 mg/L to 1 mg/L of NH₄Cl solution. 25 mL of a sample was taken in a container with an equal surface to volume ratio as recommended by the EPA Method 350.3. Then the sample was stirred using a flat micro-magnetic stirrer placed on a magnetic stirrer base (Fischer Scientific magnetic stirrer base). The probe was immersed in the sample at an inclination angle of 45 degrees with continuous stirring. A strong base solution (10 N NaOH) was added to reach the pH higher than 12. A reading was taken after the voltage signal in mV reached a constant value after a 1-min time interval. After each measurement, the probe was washed thoroughly using deionized water and wiped with a soft tissue. When it was not in use, the ammonia probe was stored in a 0.1 M ammonium chloride solution.

A 25-mL sample was withdrawn from the batch culture at a 24-hour interval. The sample was filtered out using a syringe filter (0.45 µm nominal pore with 25 mm diameter, Whatman filter) in order to avoid potential blockage of the membrane of the
ammonia probe. The ammonia measurement was carried out using an ammonia probe. All the measurements were carried out in triplicate to ensure the validity of the data.

2.6 Determination of Orthophosphate

Orthophosphate is measured using Phosver® 3 phosphate reagent available with the HACH Model PO-19 reactive phosphorus measurement kit. First, a standard curve was constructed using the orthophosphate standard solution from Ricca Chemical Company. 1000 mg/L Orthophosphate standard was diluted to known concentrations of 100, 10, 1 and 1-0.1mg/L. 20 ml of sample was taken in a beaker and one pillow of Phosver® 3 phosphate reagent was added to it. After a time interval of 10 min, due to the presence of phosphorus, blue color appeared, the intensity of which is proportional to the amount of orthophosphate concentration in the solution. The color intensity is measured using a UV-Visible spectrophotometer at 880nm. A standard curve was constructed with known concentrations of orthophosphate and the corresponding intensity of color measured from the spectrophotometer. The unknown sample was taken and the same procedure was repeated. The value of orthophosphate was read from the standard curve.

2.7 Extraction and Gravimetric Estimation of Lipids

*Chlorella vulgaris* was cultivated in waste water. After a culture time of 15 days, the algal suspension was centrifuged at 2,500 rpm for 15 mins. Algal pellet obtained at the bottom of the centrifuge tube was collected and dried under vacuum for 24 hours at room temperature. The vacuum applied quickened the process of drying and additional
temperature was not applied to avoid thermal decomposition. Dried algae powder was recovered and stored in an air-tight bag. Lipids were extracted and measured gravimetrically using a modified Bligh and Dyer (1959) method [114].

Step 1: 1 gram of dried algae powder was weighed on a micro balance after necessary calibration of the balance.

Step 2: Dried algae powder was subjected to solvent extraction using 2:1 ratio of chloroform and methanol (20 mL chloroform and 10 mL methanol) per gram of algae. The mixture was added to a 150 ml beaker with a magnetic stirrer and stirred along with the solvent mixture for 24 hours. During this process the cell wall of algae was broken and the lipids were extracted into the solvent mixture.

Step 3: Algae powder and solvent mixture were separated using vacuum filtration. The filtrate was collected and again passed through 0.45 micron syringe filter so that all the algae powder was removed and solvent with lipids was recovered. The solvent had a thick green color.

Step 4: Chloroform fraction contained the desired total lipids, (neutral, phospholipids, and glycolipids) hence the chloroform fraction and methanol fraction which were miscible were separated into two phases in a conical flask by the addition of water. Water was miscible with methanol and not with chloroform. The chloroform phase was high in density compared to the methanol-water phase. Hence, the chloroform phase was at the bottom and the methanol-water phase was at the top. The conical flask was shaken vigorously and the phases were allowed to separate. Chloroform phase was recovered by
opening the knob at the bottom of the conical flask. Water washing step was repeated 5-7 times in order to remove the methanol from chloroform.

Step 5: Total lipids along with the chlorophyll were separated from chloroform using a rotary-vacuum evaporator. Chloroform phase was kept in a round-bottom flask immersed in a water bath heated to a temperature of 40°C and a vacuum pressure of 15 psi was applied. The pressure and temperature were determined from the phase equilibrium data of chloroform to ensure that chloroform evaporates completely at this temperature and pressure. The rotary vacuum evaporator had provision to circulate cold water and collect the evaporated chloroform in a collection flask. The residue left at the bottom of the round-bottomed flask had total lipids and chlorophyll. Difference in weight of the empty round-bottomed flask and round-bottomed flask with residue gives the weight of the residue. Residue containing chlorophyll and total lipids was re-dissolved in chloroform.

Step 6: Silica gel purification step was performed to remove chlorophyll from total lipids and separate the total lipids into individual fractions of neutral, phospho-lipids, and glyco-lipids. Silica gel of 60-200mesh size was used to prepare a dry packed silica gel column. 300 grams of silica gel was used for each of the 100 grams of lipid expected. Literature suggests that 14-22% of total lipids could be present in each gram of algae of Chlorella vulgaris [30]. Hence 180 mg of total lipids were expected from 1,000 mg of algae powder and a minimum of 540 mg of silica gel should be used for purification of total lipids. In the current experiment, 1,080 mg of silica gel was used to be on the safer side. The chloroform containing chlorophyll and total lipids was passed through the
6 volumes of chloroform were used for each volume of sample eluted through the silica gel column to recover neutral lipids. Further elution will recover other lipids such as glycolipids and phospholipids but in the current study, the fraction of interest is neutral lipids, the major fraction of which is composed of triglycerides.

Step 7: Eluent was collected and evaporated in a vacuum chamber. Chloroform was evaporated and the residue was weighed for the gravimetric estimation of total lipids.

### 2.8 Preparation of Algae Samples for TEM and SEM Image Analysis

Cells from a mixed culture of *Chlorella vulgaris* and *Scenedesmus dimorphus* were analyzed using TEM to observe the lipid droplets inside algae cells following the procedure suggested in the literature [115].

1. Cells were taken in a 2mL vial, washed 3-4 times with distilled water, followed by 2 min of centrifugation at 15 rpm in a mini centrifuge.

2. Cells were suspended in 1 mL of 2.5% gluteraldehyde solution (Electron Microscopy Sciences, PA, USA) to kill the cells. Glutaraldehyde is a cross-linking agent, it cross-links the lipid dissolving enzymes that are released and dissolve the plasma membrane after the cells are killed. Thus, even after the biological activity is stopped, organelles are preserved as it is inside the cells. Incubation in gluteraldehyde was carried out for 90 min at 4 °C.

3. After 90 min, cells were washed with 0.1 M phosphate buffer at pH 7.2. Cells were post-fixed for another 90 min at 4 °C in 1% osmium tetroxide (Electron Microscopy Sciences, PA, USA) to a final concentration of 2% (v/v). Osmium tetroxide stains the
cells, gives contrast to the images observed using TEM. Again, the cells were washed with 0.1 M phosphate buffer and centrifuged.

(4) Melted agar at 60 °C was added to the cells in order to encapsulate the cells followed by washing with 0.1 M phosphate buffer.

(5) Dehydration by incubation is performed in a graded series of 30, 50, 70, 80, 90, 95, 100% ethanol (10 min each) with continuous shaking.

(6) Ethanol was replaced by an embedding solution of propylene oxide and epoxy resin (2:1, v/v), incubated for 40 min.

(7) Final samples were placed in the embedding molds, placed in a drying oven overnight at 70 °C.

(8) Ultra-thin sections of 50 µm were performed using a microtome.

(9) Ultra-thin sections were placed on copper grids and observed using TEM.

Procedure for SEM is the same except that osmium tetroxide staining in step 3 and all the steps after step 6 are eliminated. Algae samples are gold coated for SEM.
CHAPTER 3

EXPERIMENT DESIGN, RESULTS AND DISCUSSION

3.1 Preliminary Study

3.1.1 Objective

Check whether cultures of *Chlorella vulgaris* can take up nitrogen from secondary effluent from Mill Creek Plant, Cincinnati.

3.1.2 Experimental Method

(1) Wastewater medium preparation: Secondary effluent was collected from Mill Creek wastewater treatment plant. Two beakers of volume 3 liters containing wastewater from Mill Creek plant were set-up. The beakers were covered with air-tight lids. A magnetic stirrer was added to each beaker to stir before sampling. There was no continuous stirring. Initially, Orthophosphate was 0.9 mg/L, orthophosphate concentration was fortified to make the final concentration 4.14 mg/L.

(2) Schematic representation of the experimental set-up:
(3) Light intensity was adjusted to 6,500 lux before beginning the experiment.

(4) Inoculation: *Chlorella vulgaris* cultivated in Shuisheng-4 medium was used for inoculation. Uniform cell density of 30 mg/L was maintained in inoculums in both the beakers. The inoculums used were centrifuged in an Eppendorf tube at 2,500 rpm for 15 minutes and the pellet was re-suspended using mediums from respective beakers. Pelletization and re-suspension of inoculums was performed in order to minimize the carry over of nutrients from the growth medium to experimental medium.

(5) Daily Observations: Biomass, nitrogen, total inorganic carbon, pH were taken everyday in triplicate. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.

(6) Duration: The observations were taken till the growth of biomass, take up of nitrogen and orthophosphate reached saturation.

Figure 10. Schematic representation of the set-up for the experiment - Preliminary Study.
3.1.3 Results

Figure 11. Growth of Chlorella vulgaris and Uptake of Total Inorganic Carbon.

Figure 12. Increase in pH with the Uptake of Total Inorganic Carbon.
Figure 13. Removal of Nitrogen from Wastewater using *Chlorella vulgaris*.

Figure 14. Removal of Orthophosphate from Wastewater using *Chlorella vulgaris*.
3.1.4 Discussion

Wastewater collected from the Mill Creek wastewater treatment plant in Cincinnati contained essential nutrients such as inorganic carbon and nitrogen in the form of ammonia (NH$_3$)/ammonium ion (NH$_4^+$). The initial concentrations of total inorganic carbon and nitrogen in Mill Creek Plant were 58.6±0.28 mg/L and 7.7±0.19 mg/L respectively. The pH was 7. Figure 11 shows the growth of biomass, with removal of total inorganic carbon. Microalgae use carbon source in the process of photosynthesis, which is represented by the equation, 6CO$_2$ + 12 H$_2$O + light energy $\rightarrow$ C$_6$H$_{12}$O$_6$ + 6O$_2$ + 6H$_2$O. Growth of biomass and consumption of inorganic carbon are mirror images of each other, since biomass increase occurs due to the uptake of inorganic carbon. Growth of biomass follows standard phases: Lag, log or exponential phase, stationary phase and death phase. In Figure 11, it can be observed that there was a short lag phase of 24 hours. Increase in the pH with the uptake of total inorganic carbon can be observed in Figure 12. When inorganic carbon is taken by microalgae, the following reaction occurs: HCO$_3^-$ $\rightarrow$ CO$_2$ + OH$^-$ inside the algal cell and results in the release of OH$^-$ ions into the medium. The release of OH$^-$ ions increased with time. The pH of the medium, which was 7 initially increased to 10.2 at the end of 216 hrs of cultivation. During the lag phase which lasted for the first 24 hours, there was no significant uptake of nitrogen. The uptake of nitrogen was maximum during the exponential growth phase as shown in Figure 13 which starts at 25 hrs and ends at 96 hrs, nitrogen concentration steeply decreased from 7.6 mg/L to 3.4 mg/L, rapid decrease in the total inorganic carbon was also observed.
during exponential growth phase. Removal of orthophosphate was shown in Figure 14. There was an initial lag phase in the removal of orthophosphate, similar to removal of nitrogen and total inorganic carbon, the decrease in orthophosphate was maximum in the exponential growth phase of microalgae i.e., after 25 hrs but the steep decrease extends till 120 hrs, whereas in case of nitrogen and total inorganic carbon, steep decrease stops at 96 hrs. After 120 hrs, the decrease in orthophosphate was slow. Between 120 hrs and 216 hrs, orthophosphate concentration changed from 1.52 mg/L to 0.5 mg/L. Decrease in the total inorganic carbon was not significant after 96 hrs, but nitrogen and orthophosphate continue to decrease. The uptake of inorganic carbon in Figure 12 matched with the increase in the biomass in Figure 11, which serves as indirect indication that carbon contributes directly to the growth and is translated proportionally into biomass. It is interesting to note that there is a direct quantitative relation between the disappearance of total inorganic carbon, and biomass. The amount of nitrogen and orthophosphate at the end of 216 hrs of cultivation were reduced to 1.7 mg/L and 0.9 mg/L, respectively. The amount of nitrogen and orthophosphate could not be removed completely down to zero because the pH reached 10.2, which was not suitable for the growth of *Chlorella vulgaris*. If the pH of the medium can be controlled and adequate nutrients are available, nitrogen and phosphorus concentrations can be reduced to almost zero. The hypothesis of pH control of the medium being able to reduce the concentrations of nitrogen and phosphorus to zero is tested in the next experiment.
3.2 Effects of pH Control on Uptake of Nitrogen by Algae

3.2.1 Objective

Check the uptake of nitrogen by *Chlorella vulgaris* when a pH of 7 was maintained.

3.2.2 Background

During the growth of algae, the pH of the medium increases continuously due to a metabolic by-product released and due to the release of OH\(^-\) ion after bicarbonate is converted to CO\(_2\) and OH\(^-\). It was found that pH 7 is the most suitable and anything higher or lower than that is not very suitable. Though nutrients are available for growth, the biomass stops growing. Thus, there is no uptake of nitrogen. In order to prolong the growth phase of biomass and make algae consume maximum possible amounts of nitrogen, pH was being controlled. A comparison of the uptake with and without pH control will throw light on how much improvement can be achieved with pH control in terms of nitrogen consumption compared to the uptake without pH control.

3.2.3 Experimental Method

1. Wastewater medium preparation: Two batch reactors, each containing 3 liters of shuisheng-4-medium were prepared; one was pH controlled and the other without pH control. A magnetic stirrer was added to each beaker for stirring before sampling. There was no continuous mixing.

2. Light intensity was adjusted to 6,500 lux before beginning the experiment.

3. Inoculation: *Chlorella vulgaris* cultivated in Shuisheng-4 medium was used for inoculation. Uniform cell density 30 mg/L was maintained in inoculums of both of the
batch reactors. The inoculums used were centrifuged in an eppendorf tube at 2,500 rpm for 15 minutes and the pellet was re-suspended using mediums from respective beakers. Pelletization and re-suspension of inoculums was performed in order to minimize the carry over of nutrients from the growth medium to the experimental medium.

(4) Daily Observations: Nitrogen and orthophosphate were taken everyday in triplicate. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.

(5) pH Control: pH was adjusted to 7 once everyday after the samples were collected. 1M HCl was used for pH control.

(6) Duration: The observations were taken till the growth of biomass, uptake of nitrogen and orthophosphate reached saturation.

3.2.4 Results

Figure 15. Removal of Nitrogen from Wastewater with and without pH Control.
3.2.5 Discussion

Utilization of bicarbonate ion by microalgae releases OH\(^-\) during the reaction, HCO\(_3\)^- \(\rightarrow\) CO\(_2\) + OH\(^-\). Carbon dioxide released is utilized in the process of photosynthesis. Release of OH\(^-\) ions increased the pH of the solution continuously as shown in the preliminary study experiment 3.1. In Figures 15 and 16, it can be observed that nitrogen from NH\(_3\)/NH\(_4\)^+ and orthophosphate could not be removed completely due to the unfavorable growth conditions created due to high pH. When pH was adjusted everyday to 7, it was possible to remove the nitrogen and orthophosphate concentrations to less than 1 ppm. Thus, with pH control, nitrogen and orthophosphate can be removed from initial concentrations of 60.2 mg/L to 0 mg/L where as without pH control, it could be removed only up to 10.5 mg/L. Similarly, with pH control, orthophosphate concentration could be reduced from 4.14 mg/L to 0 mg/L in 192 hrs where as it could be reduced up to 0.3
mg/L in the same time without pH control. Without pH control, the concentration of orthophosphate could not be reduced beyond 0.3 mg/L whereas it could be removed completely with pH control.

3.3: Effect of Cell Density on Uptake of Nitrogen by Algae

3.3.1 Objective

Check the reduction in time required to take up nitrogen, by increasing the cell density of algae Chlorella vulgaris.

3.3.2 Experimental Method

(1) Wastewater medium preparation: Five batch reactors, each containing 2 liters of shuisheng-4-medium were prepared. A magnetic stirrer was added to each beaker for stirring before sampling.

(2) Light intensity was adjusted to 6,500 lux before beginning the experiment.

(3) Inoculation: Chlorella vulgaris cultivated in Shuisheng-4-medium was used for inoculation. Mixture of suspended and settled Chlorella vulgaris was used for inoculation. Cell densities of 10, 20, 100, 200, and 300 mg/L were inoculated to test the effect of increase in the density of algae. The inoculums used were centrifuged in an eppendorf tube at 2,500 rpm for 15 minutes and the pellet was re-suspended using mediums from respective beakers. Pelletization and re-suspension of inoculums was performed in order to minimize carry over of nutrients from the growth medium to experimental medium.
(4) Daily Observations: Nitrogen observations were taken everyday in triplicate. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.

(5) pH Control: pH was adjusted to 7 once everyday after the samples were collected. 1M HCl was used for pH control.

(6) Duration: The observations were taken till uptake of nitrogen did not change or until it reached almost zero.

3.3.3 Results

![Graph showing the removal of nitrogen with different initial concentrations of algae.](image)

Figure 17. Removal of Nitrogen with Different Initial Concentrations of Algae.

3.3.4 Discussion

Microalgae such as *Chlorella vulgaris* consume nitrogen for the synthesis of proteins. Nitrogen is present in all the amino acids. When more biomass is present, the
consumption of nitrogen also increased as expected. Highest density of algae tested was 300 mg/L. When 300 mg/L of initial density of algae was used, the initial nitrogen concentration of 7.46 mg/L of nitrogen could be removed completely in 72 hrs, where as when 10 mg/L of biomass was inoculated, it took 144 hrs as shown in Figure 17. Increase in the inoculums by 30 times from 10 mg/L to 300 mg/L reduced the time by 72 hrs. As shown in Figure 17. The performance of the beaker with 20 mg/L inoculum was also similar to that of 10 mg/L. Interestingly, no lag phase was observed. During the first 24 hours, the beakers containing 10, 20, 100, 200 and 300 mg/L could remove 30, 40, 59, 49 and 75% of the initial value. It may be required that when such a process is practically implemented, the residence time should be as minimum as possible. If high density of algae is used, the time required for the removal of nitrogen can be reduced.

3.4 Uptake of Nitrogen by Settled and Suspended Algae (Mixed Culture)

3.4.1 Objective

Check the uptake of nitrogen, orthophosphate by settled and suspended algae, *Chlorella vulgaris* and *Scenedesmus dimorphus*.

3.4.2 Background

It was observed that when algae grow, a portion of the algae remains suspended and rest of it settles down at the bottom of the batch reactor. This experiment was designed to find which portion plays a crucial role in the uptake of nitrogen, what percentage of total nitrogen consumed is taken up by suspended and what percentage is taken up by settled
algae. In order to facilitate the faster uptake of nitrogen, increasing the density of algae is being proposed as one of the alternatives.

3.4.3 Experimental Method

(1) Three batch reactors containing 2 liters of shuiesheng-4 medium were prepared. A magnetic stirrer was added to each beaker for stirring before sampling. There is no continuous mixing. Light intensity was adjusted to 6,500 lux before beginning the experiment.

(2) Inoculation: Mixed culture of *Chlorella vulgaris* and *Scenedesmus dimorphus* was cultured for 15 days which is a typical time required to complete one growth cycle. The suspended portion of mixed strains of *Chlorella vulgaris* and *Scenedesmus dimorphus* with an initial cell density of 34.5 mg/L was inoculated in one of the batch reactors. The settled portion having initial cell density of 302.4 mg/L was inoculated in the second batch reactor. In the third batch reactor, settled algae of the same initial concentration as suspended algae, i.e., 34.5mg/L was used for comparison of performance of settled and suspended algae of same initial concentration. Pelletization and re-suspension of inoculums was performed in order to minimize carry over of nutrients from the growth medium to experimental medium.

(3) Daily Observation: Nitrogen measurements were taken everyday in triplicate. Biomass measurement was taken at the beginning and at the end of the experiment. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.
(4) pH Control: pH was adjusted to 7 once everyday after the samples were collected. 1M HCl was used for pH control.

3.4.4 Results

![Diagram showing the removal of nitrogen from wastewater by settle and suspended algae](image)

Figure 18. Removal of Nitrogen from Wastewater by Settled and Suspended Algae (Chlorella vulgaris and Scenedesmus dimorphus).
3.4.5 Discussion

According to the Figure 18, in the first 12 hours of growth, settled algae of initial concentration 34.5 mg/L removed 39% of the initial concentration of 7.57 mg/L whereas suspended algae of the same concentration removed 53% of the initial nitrogen, showing better performance. The effect is even more pronounced after 24 hours, where settled algae of 34.5 mg/L removed 69% of the initial nitrogen, whereas suspended algae of the same concentration removed 86% of the initial nitrogen. A high initial density of suspended algae of 302.4 mg/L was used. High density of suspended algae could remove 74% of the initial concentration of nitrogen in the first 12 hours and 90% removal of nitrogen in 24 hours. After 36 hours, there is no significant difference in the performance of settled, suspended and high density settled algae. Figure 19 shows increase in the biomass. The final biomass concentration of settled algae is 108 mg/L which is close to 3
times the initial biomass inoculated, where as suspended algae reached 178 mg/L which is close to 6 times the original concentration. The high density suspended algae increased from 302.4 mg/L to 455 mg/L which can be noted as very slow growth. Two important things of practical significance can be observed from the current experiment. One is that suspended algae, being young, perform better compared to the same concentration of suspended algae. Second point is that, increase in the density of algae, even though it is settled can result in impressive removal of nitrogen as seen in the current experiment. Hence, algae density can be increased for reducing the residence time of wastewater when algae treatment for wastewater is practically implemented. Second option to reduce holding time is to maintain highest possible density of suspended algae. A combination of high total density (suspended and settled) and maximum suspended density would result in optimal reduction of holding time and improve the kinetics of nitrogen removal.

### 3.5 Uptake of Nitrogen by Settled and Suspended Algae

#### 3.5.1 Objective

Check the uptake of nitrogen and orthophosphate by settled and suspended algae, *Chlorella vulgaris*.

#### 3.5.2 Experimental Method

Experimental method is very similar to the previous experiment 3.4, except for three aspects. (1) Batch reactor of volume 2 liters was selected. (2) Shuisheng-4-medium was autoclaved for 70 minutes at 120°C and 20 psig. (3) Suspended culture of *Chlorella*
*vulgaris* of initial cell density 153.6 mg/L was used for inoculation in two batch reactors, from one of the reactors, the algae that settled down was removed everyday. High density settled algae inoculation was done using 461.3 mg/L of settled *Chlorella vulgaris*.

### 3.5.3 Results

![Figure 20. Removal of Nitrogen from Wastewater by Settled and Suspended Algae](Chlorella vulgaris).
3.5.4 Discussion

According to Figure 21, during the first 24 hours, lag phase was observed. High density settled algae could remove 16% and suspended algae could remove 27% of initial concentration of nitrogen. Exponential growth phase for suspended algae was observed from 25 hrs to 96 hrs as observed in Figure 21. Between 25 hrs and 96 hrs, nitrogen was removed completely by suspended algae, whereas the medium with high density settled algae which has three times the biomass, still has 1.47 mg/L of nitrogen left at the end of 96 hrs. Suspended algae were able to remove nitrogen completely by 96 hrs whereas settled algae took 144 hrs for complete removal of nitrogen as shown in Figure 20. Result of the current experiment is in agreement with the previous result, it is confirmed that suspended algae are better than settled algae. In another batch reactor, where suspended algae was inoculated and the algae that settled were removed on a daily basis,
the performance was better at 48 hrs than settled algae and suspended algae where it was inoculated and settle. Due to the removal of settled biomass on a daily basis, though the suspended algae’s activity was slightly enhanced, due to the total amount of biomass being low, the performance at the end of 96 hours was almost the same as suspended algae inoculum which was allowed to grow and settle. Valuable observation from this experiment is that metabolites and polymer like material secreted by algae, which make the algae settle down, might hinder the ability of microalgae to consume nitrogen. Instead of removing the settled biomass completely, partial removal of biomass, from time to time can enhance the removal of nitrogen, improve kinetics and at the same time provide biomass for biofuel production. However, the amount of suspended algae that should be left behind so that there is enough biomass to consume nitrogen should be optimized.

3.6 Uptake of Nitrogen by Starved Algae Phase I

3.6.1 Objective

Check the uptake of nitrogen by *Chlorella vulgaris* which was starved in a nitrogen deficient shuiesheng-4 medium for 48 hrs before inoculation, in comparison with the inoculum that was grown in nutrient sufficient shuiesheng-4 medium.

3.6.2 Background

It was reported that if algae were starved of a particular nutrient and exposed to nutrient sufficient condition, uptake would be faster. It was also reported that starved algae accumulate lipids. Accumulation of lipids improves the yield of biodiesel, which is a
useful by-product of waste treatment using algae. If starvation of algae can improve nitrogen uptake and lipid yield, the benefit can be doubled in terms of reduction in time required for water treatment and revenues from biodiesel produced due to improved lipid yield.

3.6.3 Experimental Method

(1) Three sets of batch reactors containing 3 liters of shuiesheng-4 medium were prepared. The nitrogen concentration in Shuiesheng-4 medium was adjusted to 10 mg/L which was the typical concentration in wastewater from Mill Creek Plant. Set 1, 2 and 3 were intended for blank, starved algae and algae grown in nutrient-sufficient Shuiesheng-4 medium respectively. A magnetic stirrer was added to each beaker for stirring before sampling. There is no continuous mixing. Light intensity was adjusted to 6,500 lux before beginning the experiment.

(2) Starvation: *Chlorella vulgaris* was inoculated into Shuiesheng-4 medium without any nitrogen source and allowed to stay in the nitrogen deficient medium for 48 hrs.

(3) Inoculation: One set of batch reactors were inoculated with the starved strain of *Chlorella vulgaris* and another set were inoculated with *Chlorella vulgaris* grown in nutrient sufficient Shuiesheng-4 medium. The cell density of biomass inoculated was 30 mg/L in all the batch reactors. Pelletization and re-suspension of inoculums was performed in order to minimize carry over of nutrients from the growth medium to the experimental medium.
(4) Daily Observation: Nitrogen measurements were taken everyday in triplicate. Biomass measurement was taken at the beginning and at the end of the experiment. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.

3.6.4 Results

![Graph showing Nitrogen Removal by Nitrogen Sufficient and Nitrogen Deficient Inoculums of Algae (Chlorella vulgaris).]

Figure 22. Nitrogen Removal by Nitrogen Sufficient and Nitrogen Deficient Inoculums of Algae (Chlorella vulgaris).

3.6.5 Discussion

Inoculum from nitrogen deficient medium when exposed to nitrogen sufficient medium took up nitrogen as observed in Figure 22. After 48 hours, inoculum from nitrogen deficient medium could remove 77% of the initial nitrogen; whereas, the inoculum that continued to grow in nitrogen sufficient medium could remove 41%. Complete removal
of nitrogen was achieved at the end of 96 hours by inoculum from nitrogen deficient medium, where as for inoculum from nitrogen sufficient medium, it took 192 hours. The time taken for complete removal was reduced by half. The result is valuable as starvation of nitrogen can improve the kinetics and reduce the time required to remove nitrogen.

3.7 Uptake of Nitrogen by Starved Algae Phase II

3.7.1 Objective

Check if the improvement achieved in the nitrogen uptake by starved strain can be sustained.

3.7.2 Background

The time required by *Chlorella vulgaris* for the uptake of nitrogen could be reduced by using a strain that was starved in nitrogen deficient Shuiesheng-4 medium as demonstrated by the results of the experiment 3.6-Uptake of nitrogen by starved algae phase I. Current experiment is designed to test if the effect of starvation i.e., improvement in the time required to uptake nitrogen could be sustained. Algae from bioreactors which had starved inoculum were collected after they completed one growth cycle and used as inoculum in the current experiment. Comparison was done with *Chlorella vulgaris* strain that was never subjected to starvation.

3.7.3 Experimental Method

(1) Three sets of batch reactors containing 3 liters of shuiesheng-4 medium were prepared. The nitrogen concentration in Shuiesheng-4 medium was adjusted to 4.5 mg/L.
(2) Inoculation: Algae in the batch reactors in the phase I experiment were collected by centrifugation and used as inoculum in the current experiment. The density of biomass inoculated in all the batch reactors was 30 mg/L.

(3) Daily Observation: Nitrogen measurements were taken everyday in triplicate. Biomass measurement was taken at the beginning and at the end of the experiment. The medium was mixed thoroughly before taking each sample. A new syringe/tip was used every time to avoid cross-contamination.

3.7.4 Results

![Figure 23. Nitrogen Removal by Nitrogen Sufficient and Nitrogen Deficient Inoculums of Algae which Completed One Growth Cycle (Chlorella vulgaris).](image)

Set 1 for blank, Set 2 for inoculum from starved algae in Phase I experiment, and Set 3 for algae which were never subjected to starvation. A magnetic stirrer was added to each beaker for stirring before sampling. There is no continuous mixing. Light intensity was adjusted to 6,500 lux before beginning the experiment.
3.7.5 Discussion

Exposing algae cells to nitrogen deficiency could improve the take up of nitrogen, immediately after the cells grown in nitrogen deficient culture were exposed to regular medium, but the effect of starvation could not be sustained beyond one growth cycle. Observation from the Figure 23 indicates that there is almost no difference between the performances of algae after the culture completes one growth cycle. It can be observed that the algae adapt themselves to nitrogen sufficient culture in one growth cycle. If improvement in kinetics of nitrogen uptake by starvation by exposing the algae to nitrogen deficient conditions should be sustained, then after every growth cycle, they should be periodically starved. It may require starvation tanks of very large area, to accommodate to the high flow rate of water through a wastewater treatment plant. Hence, starvation as a method for improving the kinetics of nitrogen uptake cannot be practically implemented.

3.8 Lipid Extraction from Microalgae

3.8.1 Objective

To extract total lipids from microalgae.

3.8.2 Experimental Procedure

Experimental procedure as described in section 2.8.
3.8.3 Results

Table 3. Total Lipid Estimation from Different Strains

<table>
<thead>
<tr>
<th>S. No</th>
<th>Strain</th>
<th>Initial Dry Weight in Grams</th>
<th>Weight in Grams Before Silica Gel Purification</th>
<th>Weight in Grams After Silica Gel Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>C. Vulgaris + Scenedesmus (WW)*</td>
<td>1000</td>
<td>364.18</td>
<td>178.29</td>
</tr>
<tr>
<td>Trial 2</td>
<td>C. Vulgaris + Scenedesmus (WW)*</td>
<td>1000</td>
<td>372.44</td>
<td>190.42</td>
</tr>
<tr>
<td>Trial 3</td>
<td>C. Vulgaris + Scenedesmus (WW)*</td>
<td>1000</td>
<td>360.32</td>
<td>185.63</td>
</tr>
<tr>
<td>Trial 4</td>
<td>C. Vulgaris + Scenedesmus (SS)†</td>
<td>1000</td>
<td>358.11</td>
<td>182.92</td>
</tr>
<tr>
<td>Trial 5</td>
<td>C. Vulgaris + Scenedesmus (SS)†</td>
<td>1000</td>
<td>350.58</td>
<td>161.26</td>
</tr>
<tr>
<td>Trial 6</td>
<td>C. Vulgaris + Scenedesmus (SS)†</td>
<td>1000</td>
<td>356.23</td>
<td>152.41</td>
</tr>
<tr>
<td>Trial 1</td>
<td>Ankistrodesmus braunii§</td>
<td>1000</td>
<td>290.51</td>
<td>127.82</td>
</tr>
<tr>
<td>Trial 2</td>
<td>Ankistrodesmus braunii§</td>
<td>1000</td>
<td>264.18</td>
<td>108.31</td>
</tr>
<tr>
<td>Trial 3</td>
<td>Ankistrodesmus braunii§</td>
<td>1000</td>
<td>287.34</td>
<td>137.92</td>
</tr>
<tr>
<td>Trial 1</td>
<td>C. vulgaris ss</td>
<td>200</td>
<td>126.18</td>
<td>43.15</td>
</tr>
<tr>
<td>Trial 2</td>
<td>C. vulgaris ss (At Inoculation)</td>
<td>60</td>
<td>32.14</td>
<td>12.28</td>
</tr>
<tr>
<td>Trial 3</td>
<td>C. vulgaris ss (After finishing growth)</td>
<td>60</td>
<td>32.69</td>
<td>14.12</td>
</tr>
</tbody>
</table>

WW*- Algae cultured in secondary effluent wastewater from Mill Creek Plant
(SS)†- Algae cultured in Shuisheng-4-Medium.
§-Dried powder obtained from Algaeventure
s-Settled Algae
ss-Suspended Algae

3.8.4 Discussion

During the culture of Chlorella vulgaris, it was contaminated by the strain Scenedesmus dimorphus. The contamination cannot be prevented when the culture was in wastewater obtained from Mill Creek wastewater treatment plant. Mixed culture of Chlorella vulgaris and Scenedesmus dimorphus could yield total lipids of 15-19% of the initial dry weight when measured according to the Bligh and Dyer method. Dried powder of Ankistrodesmus braunii obtained from Algaeventure, a company in Columbus, Ohio
yielded total lipids of 10-13%. *Chlorella vulgaris* cultured in Shuisheng-4-Medium resulted in a higher lipid yield than other algae of 20% at inoculation, after completion of the growth cycle, the yield slightly increased to 23%. There was an excessive loss during the silica gel purification process. Extraction of lipids and total lipid measurement proves that algae grown in wastewater can be utilized for lipid extraction and subsequently, for biofuel production.

3.9 Transmission Electron Microscopy and Scanning Electron Microscopy

3.9.1 Objective

Use transmission electron microscopy to observe the organelles inside and scanning electron microscopy to observe the surface.

3.9.2 Experimental Procedure

Experimental procedure as described in section 2.9.
3.9.3 Results

Figure 24. Transmission Electron Microscopy of *Scenedesmus dimorphus*. 
3.9.4 Discussion

Transmission electron microscopy image, Figure 24 indicates that there are lipid droplets inside the chloroplast. It can also be observed that cells were divided and the autospores stick to the mother cell without being released. This phenomenon of autospores aggregating with the mother cell was suggested in literature [116] as one of the reasons...
for settling of algae. From the scanning electron microscopy image Figure 25, it can be observed that the surfaces of the cells were ruptured due to treatment by organic solvent.

3.10 Conclusion

Current study indicates that *Chlorella vulgaris* can be used successfully to remove nitrogen and orthophosphate removal from wastewater in Mill Creek Plant, Cincinnati. In order to reduce the residence time and to facilitate the practical implementation of algae treatment for nitrogen removal, several methods were tried. Summary of those results are:

1. pH control helps in complete removal of nitrogen without leaving any residue. Complete removal of nitrogen is possible as the algae grow with a delayed negative growth phase, without being hindered by high pH, but it does not reduce the time required for removal of nitrogen significantly.

2. Increase in cell density of algae reduces the time required for removal of nitrogen by 72 hours, by increasing the algae density 30 times from 10 mg/L to 300 mg/L as indicated in Figure 17. Increasing the density of algae can be noted as one of the viable options for improving the nitrogen uptake kinetics.

3. Suspended algae play an important role in the uptake of nitrogen. Suspended algae grow twice as fast as settled algae and are primarily responsible for the uptake of nitrogen, though settled algae also remove nitrogen to some extent as indicated in Figure 19. In a performance time of 24 hours, which is the typical residence time in the wastewater treatment plant, settled algae remove 69% of the nitrogen where as suspended
algae remove 89% of the initial concentration of nitrogen. Hence maintaining maximum suspended algae concentration helps in the improvement of nitrogen uptake kinetics. Polymer by-product released by algae during the growth makes the algae settle down. Removing part of the settled algae from time to time also reduces the time required for nitrogen removal.

(4) Exposing algae to nitrogen deficient conditions reduces the time required for nitrogen uptake, but the effect cannot be sustained beyond one growth cycle, hence starvation cannot be practically implemented.

(5) Lipids can be extracted from algae grown in wastewater, hence biomass can be successfully generated for production of biofuel from the lipids extracted, in addition to remediation of wastewater. Gravimetric yield of lipids is between 15-23% of the initial dry weight of biomass.

In conclusion, *Chlorella vulgaris* is a good candidate for waste water remediation and biomass production for lipid extraction and subsequent biofuel production. Further research is required for practical implementation of algae treatment option for polishing the wastewater. Increasing the cell density, pH control, maintaining maximum suspended algae density, partially removing settled algae are some of the methods for improving the kinetics of nitrogen removal.
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