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Batch and Fed Batch Cultivation and Harvesting of Nannochloropsis Gaditana for Environmental Applications

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree
in Chemical and Biochemical Engineering

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Abstract

The exhaustion of fossil fuels and climate change are two significant issues of our times. Microalgae are eukaryotic phototrophs or prokaryotic cyanobacteria (blue green algae), which are able to capture CO₂, and thus, can mitigate these challenges. In addition, some microalgae can produce lipids suitable for biodiesel. The potential application of microalgae biomass for biofuel production is a clean and sustainable approach to replace fossil fuels. An important consideration for reducing the cost of biofuel is the economical production of algal biomass with high lipid yields. In this study, the marine microalgae *Nannochloropsis gaditana* were investigated for biomass and lipid production using two wastewater streams, i.e., road salt run-off and vegetable greenhouse industry effluents as the growth media using batch or fed-batch cultivation. The recovery of biomass was also investigated using coagulants or pH change.

In the first phase of the research, the effect of salinity on biomass concentration and lipid production was examined in batch culture. Biomass yields of 1-2 g/L of culture were obtained with the maximum lipid of 22% (g/g) biomass in the road salt media. The crude road salt media provided all the essential micronutrients needed for algal cultivation. The fatty acid composition analysis of the obtained lipid indicated C16 and C18 as the major components (~ 45% of FAME) further confirmed the suitability of biomass generated for biofuel feed-stock. This study has established that the use of road salt containing nitrate and phosphate nutrients will support the growth marine micro algae for remediation of waste water system that are the concern at winter prevalent regions.

The controlled feeding of nitrates in fed-batch cultures can be applied to promote growth and lipid production in microalgae. The fed-batch cultivation system was, therefore, next applied to provide nutrition sufficient, depletion and repletion conditions. In this study, *N. gaditana* was cultured in batch and fed-batch bioreactors. Three modes of nutrient feeding i.e., pulse; continuous and staged were applied under two different light regimes for autotrophic cultivation. Higher nitrates levels negatively affected the lipid production. Increasing the light intensity was found to increase the lipid content in the microalgae for all three fed-batch feeding modes. A maximum of 58.3% lipid fraction of algal dry weight was achieved when

using pulse-fed cultures at an illumination of 200 μ mol photons/m²/and 10mg/d nitrate feeding. This condition also resulted in the maximum lipid productivity of 44.6 mg /L/d. The fatty acid composition of the lipids consisted predominantly of long chain fatty acids (C: 16 and C: 18) and accounted for 70 % of the overall FAME. The other two feeding modes (continuous and staged) were not as effective for lipid and biomass production. This study demonstrates the applicability of pulsed feeding strategies in fed-batch cultures.

In the final phase of the study, the coagulation/flocculation methods were applied to harvest the biomass from the growth media. Cell harvesting was investigated using two approaches: coagulant chemical addition and pH change. The pH value of 11 was identified as the threshold pH for the alkaline flocculation of *Nannochloropsis gaditana* cells in the road salt growth media. The harvesting efficiency increased to 89% at the pH 11 and above. However, the coagulant/flocculant ferric chloride provided the best flocculation with 95% harvesting efficiency and the concentration factor of 23.5. The bio-coagulant chitosan was not effective to coagulate the cells within the 60 minutes of settling time.

This study primarily resulted in three main conclusions. First, the phytoremediation of road salt run-off generates the biomass suitable for biodiesel production. Secondly, the application pulsed fed-batch cultivation with nitrate feeding and increased light intensity simultaneously improves the biomass and lipid production. Finally, the alkaline induced flocculation is an effective harvesting method for the recovery of *Nannochloropsis gaditana* from road salt media. The study provides new directions for the integration of biofuel production with greenhouse vegetable effluent and road salt run-off and the application of marine microalgae for the treatment of hyper saline wastewater.

Keywords

Biodiesel

Biofuel

Biomass

Coagulation

Concentration factor (CF)

Fatty acid methyl esters (FAME)

Fed-batch cultivation

Flocculation

Greenhouse effluents

Harvesting efficiency (HE)

Marine microalgae

Nannochloropsis gaditana

Phycoremediation

Pulsed feeding

Road salt run-off

Co-Authorship Statement

The development of these chapters and manuscripts for submission were written and coauthored, the extent of the collaboration of the co-authors is stated below.

The papers that are being prepared or submitted are given below.

Chapter- 4

| | |
|---|---|
| Paper 1 | Growth kinetics of <i>Nannochloropsis gaditana</i> on road salt media |
| Current status | Submitted to the Journal of Environmental technology |
| Roopa Devasya: Experimental design, laboratory work, analysis of results, and Paper writing. Amarjeet Bassi: Technical and theoretical advisor and corrections of several drafts and | |

Chapter- 5

| | |
|---|---|
| Paper 3 | Investigation of feeding strategies for Fed batch cultivation systems for the biomass and lipid production from <i>Nannochloropsis gaditana</i> |
| Current status | Submitted to the Journal: Biotechnology progress |
| Roopa Devasya: Experimental design, laboratory work, analysis of results, and paper writing. Amarjeet Bassi: Technical and theoretical advisor and corrections of several drafts and | |

Chapter- 6

| | |
|---|---|
| Paper 3 | Investigation of alkaline flocculation for <i>Nannochloropsis gaditana</i> in the phycoremediated road salt run-off media |
| Current status | Prepared for the submission in progress |
| Roopa Devasya: Experimental design, laboratory work, analysis of results, and paper writing. Amarjeet Bassi: Technical and theoretical advisor and corrections of several drafts and | |

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Chapter 1

1 Introduction

The biomass of microalgae contains large amounts of lipids, which have strong potential for the production of renewable biodiesel. While microalgae have the characteristic ability to accumulate lipids photoautotrophically using simple nutrients such as nitrates, phosphates and carbon dioxide, currently, their large-scale cultivation for production of lipids is not economically competitive in particular due to the cost of nutrients. Using wastewaters as a nutrient source can potentially provide for more feasible alternatives for lipid production from microalgae. The removal of nitrogen and phosphorus from wastewater systems using microalgae is referred by the term “phycoremediation” and the process bio transforms the pollutants to biomass with lipids. Thus, phycoremediation offers the opportunity for integrating wastewater treatment with the biofuel production.

This study combined two important wastewater streams, found in Ontario, Canada, namely road salt run-off and vegetable greenhouse industry effluent waters, as the nutrient (nitrate and phosphate) source and cultivation media for the culturing of marine microalgae *Nannochloropsis gaditana* (*N. gaditana*). The manipulation of cultivation conditions and process operation in batch and fed-batch mode was investigated for the improvement of both biomass and lipid production to enhance the lipid productivity. The alkaline and chemical based coagulation/flocculation methods were further investigated to evaluate the harvesting efficiencies for the recovery to the biomass from the growth media.

1.1 Significance of the problem

Road salt run-off and effluent waters from the vegetable greenhouse sector are two examples of wastewaters in Southwestern Ontario region in Canada. A number of commercial vegetable greenhouses populate this region. These hydroponic based industries use liquid fertilizers and generate effluent with residual nitrogen and phosphorus that may exceed provincial water quality guidelines, e.g., an average nitrate to 90.3 mg/L against the standard 3 mg/L, phosphorus to 180 mg/L against standard 0.3mg/l, potassium to 180 mg/L.

<https://www.ontario.ca/page/greenhouse-wastewater-monitoring-project-2010-and-2011> accessed on July 2017).

Road salt is applied as a deicing agent on Canadian roads during the winter. The saline run off increases the alkalinity and chloride levels of the soil and also further deteriorates the water quality of receiving bodies of water such as lakes, rivers and ponds. The low levels of organic carbon and elevated levels of nitrate and phosphates in the effluents pose challenges to the traditional wastewater treatment approach. The disposal of effluents to the water systems causes eutrophication and the production of harmful algal blooms (HABs). Currently there is no treatment method carried out for road salt run-off. Thus this is becoming an effluent of increasing concern for Canadian waters.

Microalgae cultivation in road salt effluent is a potential option for environmental sustainability in Southwestern Ontario. Although the nitrate concentration of the road salt effluent is low, mixing the stream with vegetable greenhouse industry effluents is proposed as a feasible alternative in this study for micro-algal cultivation. The proposed approach can also reduce the water footprint required to produce algae for biofuel production. In particular, *N. gaditana*, a halophilic micro algal species which grows on salt media and produces high levels of lipids was investigated in this study.

1.2 Research goals and structure

The research was divided into three main phases as depicted in Figure 1.1. In the first phase, studies were conducted on the investigation of micro algal biomass production in varying road salt and nitrate concentrations and the effect on biomass; lipid production and fatty acid profiles were evaluated.

In the second phase, studies were conducted in both batch and fed batch mode. The fed batch cultivation strategy was investigated to increase biomass and lipid production and to incorporate the nutrients from greenhouse effluents by investigating the effect of feeding rate and light intensity on the efficiency of production of biomass with the target to optimize the high value metabolites lipid.

Finally, the third phase of the study focused on harvesting the micro algal biomass and downstream wastewater treatment. The chemical coagulants such as ferric chloride and chitosan at various concentrations were investigated. In addition, pH induced auto-flocculation using the NaOH was also investigated.

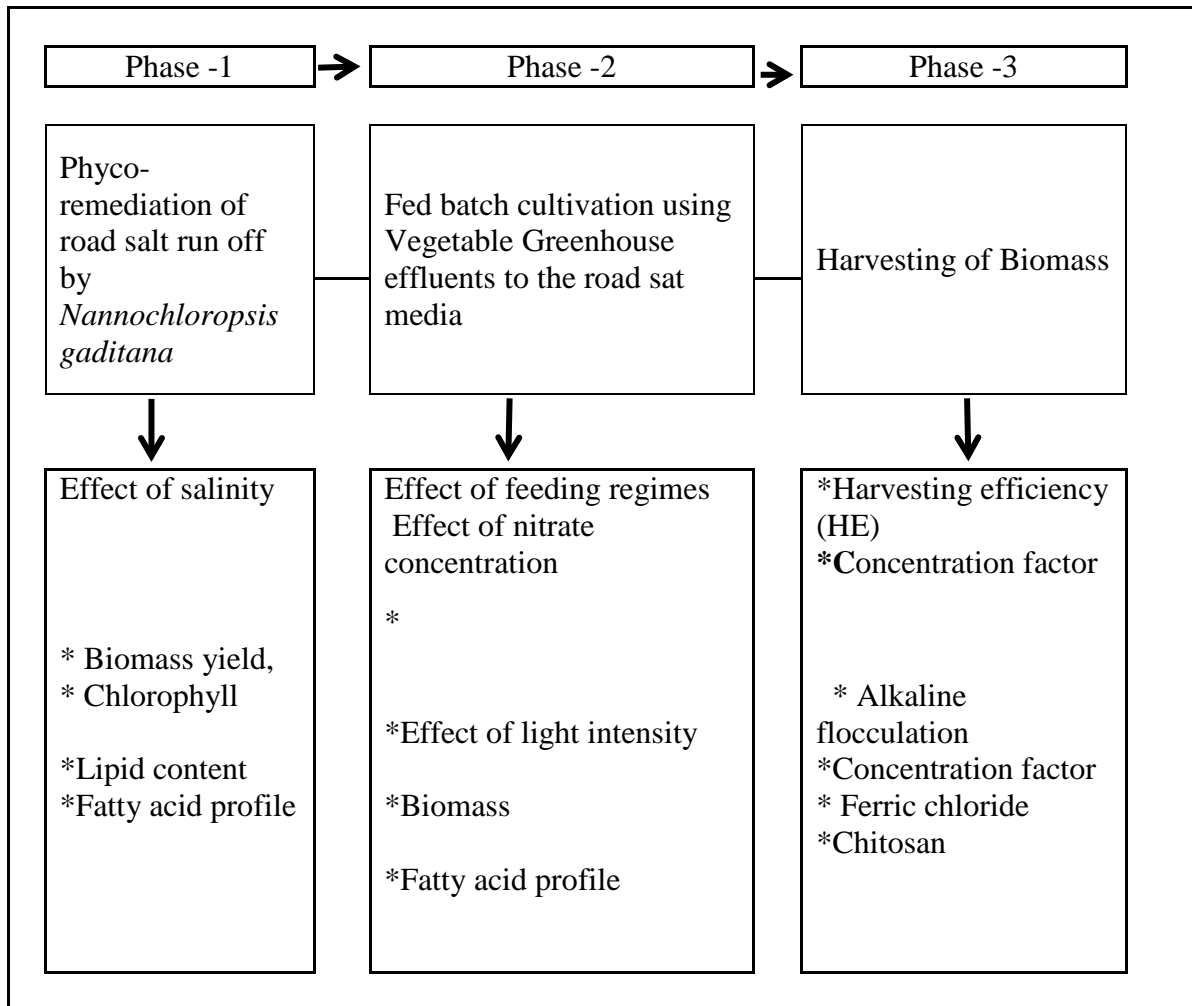


Fig 1.1 General scheme of the thesis structure

1.3 Overall and Specific Objectives

The overall objective and specific objectives are given below.

The overall objective of this study was to demonstrate the applicability of phycoremediation using the marine microalgae *N. gaditana* for the treatment of the effluent streams: road salt run off and greenhouse effluent and to evaluate lipid production.

1.3.1 Specific Objectives

The main objective is achieved by the following four sub objectives.

1.3.2 Specific Objective 1

Phycoremediation of road salt run off using halophilic marine microalgae *N. gaditana*. The effects of salt and nitrate concentration on biomass, lipid productivity and chlorophyll content were studied. The suitability of the growth media for the marine microalgae cultivation was evaluated by compositional analysis and micro algal growth kinetics. The effect of varied salt concentration as a stress factor for the lipid and nitrate for biomass production were studied. The fatty acid profiles of the lipid were investigated for its suitability to biodiesel application.

1.3.3 Specific objective 2

The fed-batch cultivation was investigated to control the supply of nitrate by the nutrient feeding. The feeding of nutrients nitrate using three different feeding regimes of pulsed, continuous and stage was investigated to evaluate its effect on biomass production and to enhance the lipid productivity.

1.3.4 Specific objective 3

The effect of two light intensities (100 and 200 $\mu\text{mol}/\text{m}^2/\text{s}$) on the biomass and lipid production in the fed batch cultivation system was investigated on the above feeding regimes.

Two levels of light intensity and light/ dark cycle were studied to improve the photosynthetic ability in order to increase the biomass and lipid production system in the phycoremediation treatment method systems of road salt runoff.

1.3.5 Specific objective 4

The coagulation and flocculation as a unit operation for harvesting of microalgae from the phycoremediation of effluent streams was investigated.

The effect of the dosages of ferric chloride and chitosan coagulation flocculation agents and media pH was explored for to harvest the biomass of *N. gaditana* generated in the phycoremediation studies.

1.4 Thesis structure

The thesis is divided into 6 chapters: chapter 1 is an introduction to the research and thesis structure, chapter 2 covers the literature review and gives the general background on road salt run off, greenhouse effluent, concept of phycoremediation and its relation to biofuels production from microalgae. The cultivation strategies and harvesting of microalgae are reviewed. Chapter 3 details the material and methods and the experimental set up. Chapter 4 present results on the first phase of experiments regarding the suitability of the media and its effect of salinity on chlorophyll content, biomass and lipid production for the phycoremediation. Chapter 5 describes the effect of feeding rate and light irradiation on biomass and lipid production of *N.gaditana* in fed-batch cultivation system. Chapter 6 shows the results of the flocculation experiments for the development of efficient harvesting of microalgae using flocculation. Finally, chapter7 summarizes the conclusions of the study and provides some recommendations for future work.

1.5 Major contributions

The major contributions of this study are:

Microalgae were cultivated in batch on real effluent streams from road salt run-off and vegetable greenhouse effluents. These studies were used to determine a simulated media composition for further studies.

For the first time, fed-batch cultivation was carried out using three different feeding strategies namely, pulsed, continuous and stage feeding. The applications of operational conditions like the light are efficient to improve the lipid production in combination with the repeated feeding of nitrates to culture systems.

The application of coagulation/flocculation method increases the viability of the rapid recovery of the biomass from the large-scale cultivation systems otherwise it was considered as the bottleneck in the downstream processing. The flocculation using the alkaline method identifies the possibility

of the simple operating methods to pre-concentrate the cells without the application of the toxic chemical flocculants.

1.6 Novelty

The thesis made the following novel contributions:

- For the first time, growth media made up of road salt run-off and vegetable greenhouse effluents was applied to cultivate microalgae and evaluate lipid productivity and fatty acid profiles for the production of biodiesel.
- Batch studies were carried out to evaluate the effect of salt concentrations of road and reef salt in growth media on microalgal growth, lipid production and fatty acid compositions were measured.
- Different feeding regimes were used in a fed batch mode and pulse feeding was identified as an appropriate feeding strategy.
- Simultaneous improvement of biomass and lipid production was obtained using the pulse feeding regime with the controlled nitrate supply techniques for the utilization of the nutrients from effluents streams removed the restriction of lipid production at the cost of biomass.
- Finally, effective harvesting of micro algal cells by flocculants like ferric chloride or alkaline pH change was carried out for the harvesting and concentration of marine microalgae in spent media.

Chapter 2

2 Literature review

Microalgae based biofuel is a potential renewable, carbon neutral green fuel which could address some of the growing needs of energy as the world moves to reduce over-dependence on finite sources of fossil fuels. However, the current available technology is considered unfeasible for the commercial production of microalgae to biofuels due to a competitive, economic disadvantage with lower priced fossil fuels. This chapter describes the background related to the production of microalgae on wastewaters for biodiesel and remediation of nutrients. These approaches are being investigated in many research laboratories to find effective strategies, which could make micro algal processing more competitive by improving both the lipid productivity and efficiency of harvesting of biomass.

2.1 Introduction

A growing demand of energy and over dependence on finite source of fossil fuels and their negative environmental influence including greenhouse gas (GHG) emissions has raised the concerns to seek alternative renewable sources of energy. Currently about 90% of the global energy demand is supplied by fossil fuels. Many countries are developing strategies to reduce dependence on fossil fuels. Previously, the US department of energy (DOE) established the goal for 2030 to use 30% transport fuel or 60 billion gallons per year from renewable sources¹. Biofuels produced from lipid-based biomass of several food crops and oil seeds are considered as the first generation biofuels. Increasing demands of arable land and water led to the controversy of food versus fuel and perceived potential problems to the global food supply and imbalance of ecosystem. Microalgae are considered the third generation feed stock following the second-generation non-food feed stocks (waste oil, and non-food -lignocelluloses)².

Some species of microalgae accumulate up to 70% lipids in their cell body³ and have the photosynthesis rates of 50 times higher than the terrestrial plants. Thus, microalgae offer greater advantages over the other feedstocks. Their high growth rate and environmental tolerance to a wide range of cultivation conditions is a potential advantage. Their ability to grow in non-arable land including on wastewater and carbon dioxide (CO₂) from flue gases and utilize sunlight energy makes their cultivation economical attractive⁴. From an environmental stand-point,

microalgae also can remediate the nutrients from municipal, industrial and agricultural wastewater and could effectively mitigate the problems associated with water pollution and CO₂ emission⁵. The use of biodiesel produced from micro algae formed by glycerol-esterification of fatty acids extracted from the lipid minimizes sulfur emissions contaminants compared to the petroleum based diesel⁶.

The micro-algal biofuel production consists of the upstream process of biomass generation and down process of lipid extraction and conversion. The main upstream production system contains algal species, cultivation and biomass separation systems. The commercial production of biofuel faces challenges due to low productivity and high cost associated with cultivation, harvesting and extraction techniques. The cost of cultivation accounts at least 25% and harvesting around 30% of the total cost of biomass production⁷. In one study, the production cost of biofuel feedstock was estimated to be 10.87-13.32 US\$ per gallon which was considered economically not feasible to the price of fossil fuel⁸.

The application of cost effective and efficient methods is therefore essential for achieving a viable process to overcome the challenges for the sustainable commercial production of microalgae to replace the dependence of fossil fuel. The technological advancements are critical to increase the efficiency of cultivation, harvesting, pretreatment, lipid extraction, and trans-esterification subsystems to reduce the overall production cost⁹.

Growth rate, lipid content and productivity are the main parameters, which determine the potential of micro-algal strains as biodiesel feed stock. Other performance factors accounting for selection of microalgae strain for the suitability to produce biodiesel depends on the lipid content of minimum 20% (w/w) and productivity of 40 mg/L/day¹⁰.

The lipid fraction of biomass represents the triacylglycerides (TAG). The success of biodiesel production depends on the content of TAG and the biomass productivity of microalgal species. The quality and quantity of biodiesel are influenced by the free fatty acid contents and composition in the lipids. These parameters vary between the species of the microalgae. The excessive saturation of fatty acids forms tar by cross linking of fatty acids and increase in polysaturation decreases the oxidative stability of biodiesel¹¹. Genetics of the strains, various

culture conditions, the nutrients, light and environmental stresses are used to improve the quality and quantity of lipids in microalgae¹².

Previous studies on life cycle assessment of biofuel production from microalgae have evaluated the barriers and noted some of the key issues in the cultivation systems. These include:

- Photosynthesis rate of the species,
- Nutrient dependence on biomass productivity
- Nutrient independent biosynthesis and regulation of lipid production systems

Lipid synthesis is improved by selection of algal strains by screening and genetic technology. The biomass production is improved by the development and design and engineered system for large-scale cultivation by suitable nutrient supply and light for culture systems to enhance the photosynthesis rates. The micro algal species or strains with environmental tolerance to high salinity, extreme pH, temperatures and high light intensities, production of by-products with commercial values, and rapid growth in the photo bioreactors are considered favourable for the biodiesel production¹³.

Fresh water supply and nutrients like nitrogen and phosphate are essential to algal production systems but are the primary contributors to the cost of the generation of biomass. Nutrient nitrogen sources alone cost around 80% of the overall nutrient supply¹⁴. Microalgae are aquatic species and require substantial amount of water to grow and proliferate. Nearly one metric ton of water is needed per kg of micro algal biomass production with a water loss by photosynthesis of 5–10 kg per 1.0 kg of dry micro algal biomass¹⁵.

Previously, various municipal, industrial and agricultural wastewaters containing nitrogen and phosphate have been investigated as an alternative and inexpensive sources of nutrients and water^(16–18). However, the dilute concentration of biomass (<2g/L) produced requires the processing of large volumes of culture media could lead to inefficient downstream harvesting operations and processing using conventional approaches such as centrifugation can be prohibitively expensive at large scale. The main possible strategies proposed to reduce the overall cost of biomass for the commercial large-scale cultivation can be attained by, 1) cultivation in suitable nutrient rich wastewater and/or utilization of CO₂ from flue gases, 2) improvement of biomass and bio-refinery

products and 3) development and study of low cost and high efficiency recovery processes for micro algal biomass.

Cultivation in wastewater reduces the cost of nutrients and footprints of fresh water demands. Microalgae have the ability to grow in varied water sources like seawater, brackish and other industrial and domestic wastewater containing nitrogen and phosphorous which would potentially provide the necessary nutrients for large scale cultivation. This strategy integrates the feasibility of wastewater with microalgae cultivation as a source of nutrients for microalgae growth as well as effective remediation technique for the wastewater reduces the cost of the treatment and the generation of biomass.

Improvement of biomass and bio-refinery products approach include the use of the biomass as feedstock for diverse products such as fine chemicals for supplements of nutraceuticals and cosmetic industry⁽¹⁹⁻²²⁾. The production of high value micro algal biochemical compounds such as polyunsaturated fatty acids, docosahexaenoic acid (DHA) and pigments (astaxanthin) have uses in nutraceuticals, medical and cosmetic products that in addition to the use of lipid for the biofuel balances the production cost of the biomass²³. The recovery of micro algal biomass from the culture media is difficult due to the variety of microalgae species with different properties such a size, charge and low density. Highly efficient, low energy processes for the separation of biomass with the generation of recyclable media reduces the cost of harvesting and growth media. Centrifugation, filtration and sedimentation are energy intensive process accounts 20-30% of the total cost of production²⁴. Approaches like auto-flocculation, pH induced flocculation and gravity sedimentation are considered more feasible for the separation of biomass from wastewater based algal cultivation system and require further investigations²⁵.

2.2 Microalgae classification, biochemistry and production systems

Microalgae are unicellular microscopic, polyphyletic, photosynthetic, oxygen evolving microorganisms. One of the oldest living organisms on the planet, they can exist individually or in chains or groups that form the basis of the food chain in oceans and rivers as plankton²³. These microorganisms are responsible for achieving the life promoting current composition of atmospheric oxygen. Microalgae are characterized by being adapted to live in an extremely broad spectrum of environment conditions due to their unicellular or simple multicellular structure and minimum growth requirements²⁶.

There are about 200,000-800,000 species in existence with more than 1000 types of micro algal strains investigated for biodiesel only^{5,27}. Microalgae are taxonomically classified based on the pigmentation, life cycle, and basic cellular structure. They are mostly eukaryotic although the prokaryotic cyanobacteria (blue-green algae) are frequently classified as algae. Algal biomass composition contains three main components carbohydrate, protein and lipids at various concentrations with around 40% of its mass is fatty acids and are extracted and converted into biodiesel. The biochemical compositions of various microalgae are used as basis for its application into many industrial products. The composition of carbohydrate, protein and lipids of various microalgae are shown in the below table 2.1.

Table 2-1 Biochemical composition of microalgae ^[26]

| Microalgae | Protein (%DW) | Carbohydrates (%DW) | Lipid (%DW) |
|---------------------------------|---------------|---------------------|-------------|
| <i>Scenedesmus obliquus</i> | 50–56 | 10–17 | 12–14 |
| <i>Scenedesmus quadricauda</i> | 47 | – | 1.9 |
| <i>Scenedesmus dimorphus</i> | 8 –18 | 21–52 | 16–40 |
| <i>Chlamydomonas reinhardii</i> | 48 | 17 | 21 |
| <i>Chlorella vulgaris</i> | 51–58 | 12–17 | 14–22 |
| <i>Chlorella pyrenoidosa</i> | 57 | 26 | 2 |
| <i>Spirogyra sp.</i> | 6–20 | 33–64 | 11–21 |
| <i>Dunaliella bioculata</i> | 49 | 4 | 8 |
| <i>Dunaliella salina</i> | 57 | 32 | 6 |
| <i>Euglena gracilis</i> | 39–61 | 14–18 | 14–20 |
| <i>Prymnesium parvum</i> | 28–45 | 25–33 | 22–39 |
| <i>Tetraselmis maculata</i> | 52 | 15 | 3 |
| <i>Porphyridium cruentum</i> | 28–39 | 40–57 | 9–14 |
| <i>Spirulina platensis</i> | 46–63 | 8–14 | 4–9 |
| <i>Spirulina maxima</i> | 60–71 | 13–16 | 6–7 |
| <i>Synechoccus sp.</i> | 63 | 15 | 11 |
| <i>Anabaena cylindrical</i> | 43–56 | 25–30 | 4–7 |
| <i>Nannochloropsis sp.</i> | | | 20-70 |

The high carbohydrate contents favour the production of biogas and alcohol based fuels. The higher protein and carbohydrates containing microalgae are used in aquaculture and can be applied to extract essential components for the preparation of human and animal feed supplements^{28–30}. Most of the microalgae contain many carbohydrates and proteins formed at early stage of photosynthesis to serve the structural and metabolic functions and are the starting point for the synthesis of the other biochemical products. Proteins as enzymes are the prime catalysts for cell

metabolism and facilitate growth³¹. While, the lipids are biosynthesised during the adverse growth stage as a secondary metabolite and its content vary between the species and for their suitability to biodiesel production.³²

There are three types of lipid found in the algal cells. Triacylglycerol (TAG) is the storage lipid serves as energy reserve are suitable for the biofuel production. Phospholipids and glycolipids are the structural components of membranes of the cell are not suitable for the biofuel. The TAG proportion of lipid fraction increases as the metabolic rate slows down during the late growth stage and this shift in TAG lipid composition occur through the various phases of growth used as the criteria for enhancing the efficiency lipid productivity. The compositions of three lipids in various microalgae species are listed in the Table 2.2. Almost all listed microalgae species accumulated around 50% of storage lipid with the maximum of up to 68% total lipid noted in blue green algae with an average of 35% lipid content accounting to 1/3rd of the biomass dry weight. However, the lipid content varies with the changes in the nutritional and culture conditions³³.

Table 2-2 Lipid class content as a % of total lipid [31]

| Microalgae | Storage lipids (TAG) | Glyco-lipids | Phospholipids |
|--|----------------------|--------------|---------------|
| Diatoms - <i>Chaetoceros</i> species | 37 ± 16 | 36 ± 8 | 25 ± 8 |
| <i>Phaeodactylum tricornutum</i> | 54 ± 6 | 34 ± 5 | 11 ± 1 |
| Green algae <i>Chlamydomonas</i> species | 48 ± 10 | 44 ± 13 | 6 ± 3 |
| <i>Dunaliella tertiolecta</i> | 7 ± 1 | 67 ± 1 | 25 ± 0 |
| <i>Dunaliella viridis</i> | 13 ± 1 | 44 ± 3 | 42 ± 2 |
| Blue green algae | 11– 68 | 12–41 | 16–50 |
| <i>Nannochloropsis oculata</i> | 22 ± 1 | 39 ± 0 | 38 ± 1 |
| <i>Isochrysis species</i> | 36 ± 3 | | 27 ± 3 |
| Average | 35 ± 3 | 40 ± 2 | 25 ± 2 |

2.3 Industrial application of microalgae

The composition of the algal biomass with regards to lipids, carbohydrates and proteins greatly determine its overall value. Algal lipids are potential substrates used for the biofuel production. High concentration of protein, carbohydrate and long chain fatty acids (Omega-6) present in many species are used for the preparation of functional foods, food additives and nutraceuticals, cosmetics, food, drug and aquaculture application^{19,34}. Long chain polyunsaturated fatty acids (LC-PUFAs) derived from the microalgae are the key components of many of the nutraceutical

and pharmaceutical products and are used for the treatments related to maintenance of mental and cardiovascular health³⁵. In general microalgae can be applied for three types of biotechnological processes.

- 1) Biomass generation for biofuel and/or nutraceutical production³⁶
- 2) Wastewater remediation process^{37,38}
- 3) Carbon dioxide sequestering³⁹

2.3.1 Application of microalgae for biofuel production

Micro algal biomass have been explored as a renewable source of fuel as early as 1950-1970³⁶. But the attention has increased recently due to the increased environmental concerns like GHG emission caused by the fossil fuels⁴⁰. The success of biodiesel production from microalgae as mentioned previously is due to the high oil content (20-50%DW) with high TAG (20-70% total lipid) in the biomass⁴¹. The specific advantages of using microalgae for biofuel include high oil yield (44) demand less land compared to the first and second generation feed stock. The comparison of some sources of feed stocks of biodiesel are given in Table 2.3.

Table 2-3 Comparisons of sources of biodiesel³⁶

| Crop | Oil yield (L/ha) | Land area needed (M ha) | Percent of existing US cropping area |
|----------------------|------------------|-------------------------|--------------------------------------|
| Corn | 172 | 1540 | 846 |
| Soybean | 446 | 594 | 326 |
| Canola | 1190 | 223 | 122 |
| Jatropha | 1892 | 140 | 77 |
| Coconut Oil | 2689 | 99 | 54 |
| Palm | 5950 | 45 | 24 |
| Microalgae (79% oil) | 136,900 | 2 | 1.1 |
| Microalgae (30% oil) | 58,700 | 4.5 | 2.5 |

According to this data, microalgae with 30% lipid content results in 58,700 L/ha of oil yield would occupy only 2.5% of existing US cropping area, while, the production of corn for the biodiesel requires 846% of existing US cropping area. The increase in lipid content drastically reduce the land usage^{11,23}. Many species of microalgae have the lipid content higher than 50% provides the

advantages over the oil seeds of vascular plants for biodiesel production. The higher aerial production yield of lipids from microalgae and its ability to grow in wastewater and seawater, demanding less arable land relaxes the stress of fuel versus feed controversy and the negative impacts on the environments.

2.3.2 Mechanisms of lipid accumulation in microalgae

Microalgae with high lipid productivities are desired for producing biodiesel. Lipid productivity is defined as the mass of lipid produced per unit volume of the micro algal culture per day, depends on the algal growth rate and the lipid content of the biomass. Lipid content in microalgae at an average 20-50%(w/w) is commonly found in many species (Table 2.4) can be used for the sustainable and renewable production of energy.

Table 2-4 Oil content of some microalgae ^[36]

| Microalgae | Lipid content (%dry wt.) |
|----------------------------------|--------------------------|
| <i>Botryococcus braunii</i> | 25–75 |
| <i>Chlorella sp.</i> | 28–32 |
| <i>Cryptocodinium cohnii</i> | 20 |
| <i>Cylindrotheca sp.</i> | 16–37 |
| <i>Dunaliella primolecta</i> | 23 |
| <i>Isochrysis sp.</i> | 25–33 |
| <i>Monallanthus salina</i> | >20 |
| <i>Nannochloris sp.</i> | 20–35 |
| <i>Nannochloropsis sp.</i> | 31–68 |
| <i>Neochloris oleoabundans</i> | 35–54 |
| <i>Nitzschia sp.</i> | 45–47 |
| <i>Phaeodactylum tricornutum</i> | 20–30 |
| <i>Schizochytrium sp.</i> | 50–77 |
| <i>Tetraselmis sueica</i> | 15–23 |

As shown in Table 2.4, *Schizochytrium sp.*, *Nannochloropsis sp.* and *Botryococcus braunii* the highest oil content accounting more than 50% of dry weight (32). The lipid content and productivity differ tremendously because of the vast diversity of microalgae and the levels of lipid accumulation affected by growth conditions like nutrients especially nitrogen deprivation and other environmental stresses ^{28,33,42,43}.

In microalgae, triacylglycerol (TAGs) are the most suitable class of lipid for biodiesel production and are formed under the adverse growth conditions^{44,45}. TAGs are non growth associated products, stored in the cells as the carbon and energy reserve and are not formed during cell cycles and hence are not biomass dependent products⁴⁶. Lipid biosynthesis involves two different metabolic pathways for fatty acid synthesis i.e., photosynthesis and the biochemical pathway converting polysaccharides into lipids (Figure 2.1).

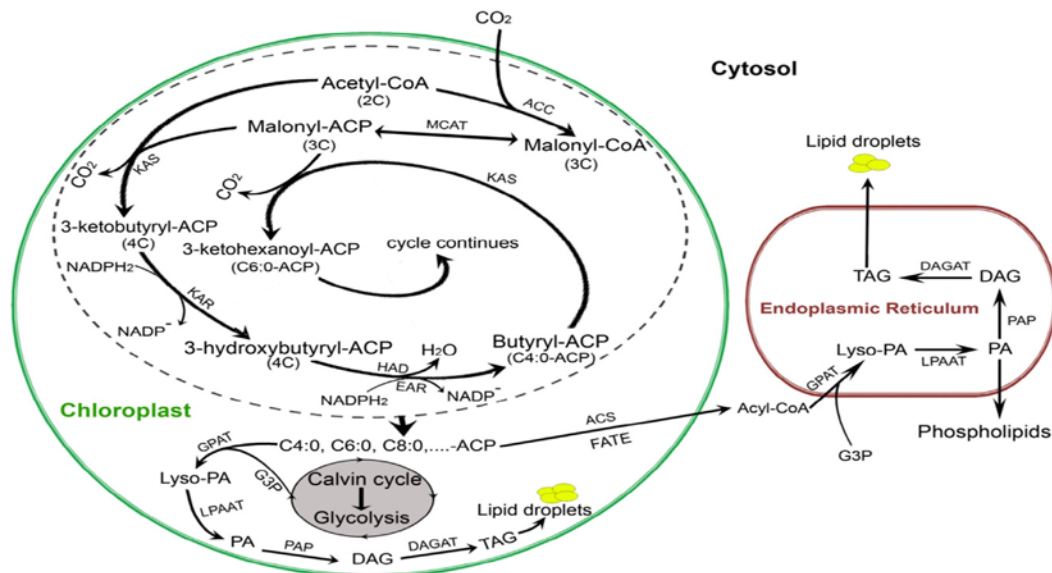


Figure 2:1 Simplified diagram of lipid synthesis process in the microalgae cells⁴⁷

The first pathway is the synthesis of lipids by the photosynthesis process occurs in the plastids (chloroplast). The CO_2 produced in photosynthesis comes from outside the plastid into the Calvin cycle, where glycerol-3-phosphate (G3P) is converted to pyruvate and to acetyl-CoA through a reaction catalysed by the pyruvate dehydrogenase complex. Carboxylation of acetyl-CoA is ATP dependent converts it to malonyl-CoA. Conversion of malonyl-CoA is followed by cycles of decarboxylation, addition of malonyl-CoA to acyl units, and β -reduction, reactions that are catalysed by the fatty acid synthase system (FAS) until production of saturated molecules with 16 (C16) and 18(C18) carbon atoms. Palmitic (C16) and oleic acids (18) are the precursors of polyunsaturated molecules produced by aerobic desaturation and elongation mechanisms^{31,48}.

The heterotrophic cultivation with the supply of sugar as carbon sources follows the second pathway. This pathway uses the glycolysis and citric acid pathway where the citrate is converted to acetyl-CoA and to malonyl-CoA. Malonyl-CoA is transferred to one of the subunits of FAS, enters the fatty acid synthesis cycle^{31,48}.

2.3.3 Conversion of algal lipids into biofuels

Biodiesel from algal lipids is obtained by Trans esterification process of free fatty acid of the TAG in the presence of catalyst (homogeneous or heterogeneous). Figure 2.2 below represents the trans esterification process using NaOH catalyst and methanol for the production of fatty acid methyl ester (biodiesel).

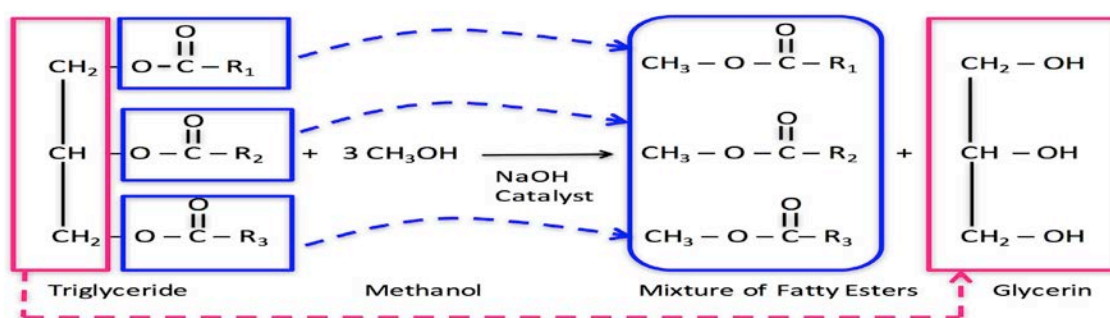


Figure 2:2 Trans esterification reaction of triacylglycerides^[49]

The unit operation of processing for the production of biodiesel from microbial oils involves the three steps such as biomass drying, lipid extraction and trans-esterification. Usually the biomass is dried at 60–70⁰C, to maintain the lipid composition as unchanged. In the trans-esterification process three fatty acid molecules are esterified with a molecule of glycerol in presence of methanol/alkalis as trans- esterification or alcoholysis. Acids, alkalis and lipase enzymes are used as catalytic agents for trans-esterification⁵⁰. Alkali-catalyzed trans esterification is about 4000 times faster than the acid catalyzed reaction⁴⁹ are commonly used in the biodiesel production. The biodiesel obtained from the microalgae should meet the existing standards (ASTM Biodiesel Standard D 6751 in Table 2.5) or European (Standard EN 14214 and if being used for heating oil - Standard EN 14213³⁶). The American Society for Testing and Materials (ASTM) defines biodiesel, as a fuel comprised of mono alkyl esters of long chain fatty acids from vegetable oils or animal fats.

Table 2-5 ASTM Biodiesel Standard D 6751 ^[51]

| Property | Test method | Limits | Units |
|---|-------------|--|--------------------|
| Flash point closed cup | D 93 | 130.0 min | °C |
| Water and sediment | D 2709 | 0.050 max | % Volume |
| Kinematic viscosity, 40°C | D 445 | 1.9–6.0 | mm ² /s |
| Sulfated ash | D 874 | 0.020 max | % mass |
| Sulfur (ppm) | D 5453 | 0.0015 max (S15)* 0.05 max (S500) * | % mass |
| Copper strip corrosion | D 130. | No 3 max number | |
| Cetane no. | D 613 | 47 min | |
| Cloud point | D 2500 | Report | °C |
| Carbon residue | D 4530 | 0.050 max | % mass |
| Acid number | D 664 | 0.50 max | mg KOH/g |
| Free glycerin | D 6584 | 0.020 | % mass |
| Phosphorus content | D 4951 | 0.001 max | % mass |
| Total glycerin | D 6584 | 0.240 | % mass |
| Sodium/potassium | UOP 391 | 5 max. Combined | ppm |
| Distillation temperature, atmospheric equivalent temperature, 90% recovered | D 1160 | 360 max | °C |

*The limits are for Grade S15 and Grade S500 biodiesel, with S15 and S500 refer to maximum sulfur specifications (in ppm)

Biodiesel is the main alternative of diesel fuel in European countries contributes more than three fourths part of overall bio-fuels production ³. Fatty acids and fatty acid methyl esters (FAME) with four or more double bonds found in some vegetable oil are susceptible to oxidation during storage and this reduces their acceptability for use in biodiesel. The microalgae lipid containing higher amount polyunsaturated fatty acids are not suitable for the biodiesel production³⁶.

Even though algal fuel is the best match for the biodiesel production and its commercial cultivation for the effective production of biodiesel depends on the biomass productivity and lipid content. The improvements of oil content and biomass productivity were previously investigated by employing various strategies to reduce the cost of production of algal oil to completely to replace fossil fuels as discussed below.

2.3.3.1 Strategies for improvement of biomass and lipid production

Improvement in lipid productivity is the key factor determines the economic feasibility of micro algal biomass for biodiesel^{52,53}. Optimization of lipid productivity can be approached by increasing the growth rate or lipid content of the algal cells⁵⁴. Lipid productivity is mainly affected by the biological potential (genetics) of the species and the environmental conditions^{48,55}. Growth rate and lipid production are inversely related and are affected by the culture condition and cultivation systems⁴⁴. The highest lipid yields always resulted in the least biomass production and vice versa⁷ and the manipulation of culture conditions for one adversely affects the other. The selection of algae species and the manipulation of physiochemical cultivation parameters are used to determine lipid content. Environmental factors (temperature, light and pH) and availability of nutrients are considered to induce the stress conditions like nitrogen limitation, salinity stress, pH and temperature variation^{52,53,56,57} to improve the overall lipid productivity in microalgae. Among these, nutrient-induced shifts for biomass composition are the most cost-efficient and environmental friendly practices⁵⁸ applied in biotechnology industries by either nutrient limitation or deprivation to optimize the productivity of the desired target compound(s) used in microalgae to achieve the overproduction of metabolites such as lipids, polysaccharides and pigments^{31,58,59}.

2.3.4 Application of microalgae for wastewater treatment

The other emerging application of micro algal applications is for wastewater treatment. Cultivation of microalgae in wastewater reduces the demand of water and supply of nutrients. The large quantity of nitrogen and phosphorus in the wastewater streams can be effectively utilized without the need for additional carbon sources or nutrients by the algae-based technologies. This technique provides the dual benefits of treatment of waste water and generation of biomass for the biofuel production⁶⁰.

The quantity of nutrients at the specific ration is required for the growth of microalgae. Municipal, industrial and agricultural wastewaters contain different amount of total nitrogen (TN) and Phosphorus (TP) and COD with point source treated with the conventional methods. TN and TP of municipal wastewater range between 19-90 and 2- 20 mg/L with the TN/TP ratio of 3.3 and COD but may contain high quantity of heavy metals. Industrial wastewater from textile, winery, tannery, pulp, and paper and oil mill has varied amount of TN (1.1-532 mg/L), TP (0.6-52 mg /L) and its ratios (2.1-13). Agricultural wastewater from livestock production (Beef, poultry, swine

and dairy) are very rich in nitrogen (63- 4165 mg/L), phosphate (14-1195 mg/L) and this ratio ranges from 2 to 7.8 ⁶¹.

The wastewater treated by the conventional methods applies primary, secondary and tertiary (advanced) treatment methods. Primary treatment uses mechanical and chemical aids to remove buoyant and non-buoyant suspended materials. Secondary treatment removes dissolved organic and colloidal components by chemical and biological methods. The dissolved inorganic such as N and P are removed by the advanced or the tertiary methods^{5,62,63}.

Conventional treatment demands point source and a supply of organic carbon in the wastewater. Treatment by the chemical processes has inherent disadvantages of increasing the dissolved constituents hinders the reuse of water ³⁷. Biological methods by the microbes are suitable to reduce the organic and inorganic load in the carbon rich only wastewater systems^{61,64}.

Not all the wastewater can be treated with conventional methods. Various industrial processes like sea-food processing, abattoirs, meat processing produce the wastewater with high salt contents ^{63,65,66} and are limited by the biological methods since the presence of salt negatively affects the biological growth⁶⁵. Wastewater effluents with limited carbon sources are also, unable to be treated by biological treatment process. Wastewater generated from some streams may have very low or no organic carbon loading and also may originate from non-point sources, which further limit the conventional treatment strategies. This untreated wastewater causes severe water pollution that disrupts the natural recycling processes such as photosynthesis, respiration, nitrogen fixation, evaporation and precipitation of nutrients in the water systems cause detrimental effect on aquaculture and human beings ⁶⁷. The presence of high levels of pathogenic microorganisms, nutrients and toxic compounds accumulates in rivers and lakes³⁸. Excessive loading of nutrients into natural water systems causes serious risk such as eutrophication leads to an undesirable disturbance in the aquatic ecosystem and accelerates the harmful micro algal growth^{61,68}.

There are two wastewater streams present in Southwestern Ontario that are currently not subjected to the traditional wastewater treatment process, i.e., road salt runoff and vegetable greenhouse industry effluents. These later wastewater streams rich in nitrates, phosphates and other residual nutrients but low in organic carbon loading and the former, the presence of salinity its treatment are limited for the traditional biological process. These wastewater are raising concern on

pollution in Southwestern Ontario^{69,70} and are implicated the concern for the algal blooms in Lake Erie of southwestern Ontario in the recent years^{71,72}.

2.3.5 Road salt runoff

The deicing agent of most highway maintenance departments use common salt, sodium chloride, sometime supplemented by calcium chloride in the colder regions at alarmingly large quantities in every winter^{69,73-75}. The salt is spread on surfaces to melt ice by reducing the freezing point and to act as an abrasive for the road safety. Canada uses on average, 5 million tonnes of road salts per year and approximately 15 million tons of road salts applied to US roads every winter as de-icers on roadways⁷⁶. Much of the salt, however, does not remain on the streets and highways, but gets dissolved in the melt water and high runoff flows into surrounding surface waters⁷⁷. Several studies have also suggested that addition of road salt deicers increase environmental problems such as increased salinity in surface and groundwater and increases the transport of heavy metal into roadside soil in the northern hemisphere. Sodium chloride has variable impact on aquatic environment and aquatic biota⁷⁶. The concentrations ranging up to over 9,000 mg/L of sodium and 13,000 mg/L of chloride in the brine flows on the surface or in the shallow subsurface results in dead vegetation, degradation of fish population in nearby surface streams and significantly affected the contamination of ground water and surface water supply⁷⁸ were also noted. Chloride concentrations in wells and springs exceeded the limit of secondary drinking water standard of 250mg/L (U.S.Environmental Protection Agency-EPA-2002). Sodium is an unregulated contaminant recommended not to exceed 60 mg/L because of adverse effects on taste, and not to exceed 20 mg/L for protection of individuals who are on low-sodium diets⁷⁸.

There are various studies conducted reports the impact of road salt runoff on environment. The urban stream of the large transport route in South-central Ontario, Canada is predicted to cause long-term impact of salt loading on surface and groundwater resources. The chloride of approximately 50% of the total road salt applied to Pine Creek (1700tonnes per winter) removed annually via overland flow with the remainder accumulating in the shallow subsurface results severe degradation of groundwater quality⁶⁹.

Road salt migration is also noted to enhance mineral weathering in shallow aquifers⁷⁶. The road salt runoff of 2004-05 increased the chloride concentrations in the stream to 2000mg/L, and delivered approximately 850 tons of chloride to a shallow (< 3.5m) semi enclosed lagoon on the

shore of Lake Ontario at Frenchman's Bay of 0.85km⁶⁹. Enormous seasonal influx of NaCl, CaCl₂ and other deicing agents in the wetland soils with increased concentrations of calcium, potassium and magnesium in wetland pore waters were reported by the sediment biogeochemistry. The addition of road salt deicers increase trace metal mobility and its bioavailability in soils and sediments lead to additional risk for the contamination of ground-water through mobilization of heavy metals by road salt deicer with increased amount of dissolved Zn, Cu, Cr, Ni and Pb impacted the soils and sediments⁷⁰. Pond bottom with large concentrations of Na (~2,000 ppm), Cl (~3,000 ppm), Fe (up to 100 ppm), and Mn (up to 4 ppm) from road salt were suggested to influence the redox processes in wetland sediments and soils⁷⁹.

2.3.6 Hydroponic greenhouse effluents

Hydroponic vegetable industry greenhouses are clustered in the winter prevalent regions. A majority of such greenhouse systems located in southwestern Ontario with many hydroponic gardens near Niagara and Leamington area. These industries around the regions produce considerable quantity of wastewater with high concentration of inorganic compounds. The residual nutrients in the hydroponic greenhouse effluent has concentrations of nitrates in the range 200-300 mg NO₃⁻/L and phosphorus (30-100 mg PO₄⁻/L)^{80,81}. There are limited treatment methods available for the removal of phosphate and nitrate from these effluents. The predominant modes of treatment for phosphate are crystallization, adsorption and chemical precipitation^{82,83}. Precipitation/flocculation process incorporating addition of lime and a biodegradable flocculants (guar gum, cationic starch, or chitosan) for the removal of phosphorus were noted effective⁸⁴.

The common nitrate removal processes include biological denitrification, chemical methods and anaerobic ammonium oxidation process. Anaerobic ammonium oxidation and heterotrophic denitrification with heterotrophic bacteria converts nitrate-N and nitrite N to nitrogen gas with sufficient organic carbon (as an electron donor) for the complete nitrate removal⁸¹. However, the low carbon availability in these effluents lead to incomplete denitrification and accumulation of denitrification intermediates such as nitrite, nitric oxide and nitrous oxide⁸⁰. Due to the lack of carbon source, there is currently no centralized treatment system for road runoff and hydroponic effluents are available.

Microalgae may offer a useful solution to the treatment of such wastewaters³⁸. Algae can achieve remediation of wastewater in a more environmentally friendly way by assimilating the nutrients using CO₂ and light (photosynthesis) in to the formation of cellular constituents i.e. lipids and carbohydrates⁸⁵. The biochemical pathways of algal cells for the growth to generate biomass with biogenic products have proven ability to assimilate nitrogen, phosphorus and chemical oxygen demand⁵ are referred with the term “Phycoremediation”. The generated biomass can be used for biofuel production and the use of wastewater for cultivation of algae would be economical and environmental sustainable way of employing algae for the production of biodiesel oil and high-value end products.

2.3.7 Phycoremediation

The term “phycoremediation” refers to the combination of words “phyco” and “remediation” introduced for the treatment process carried out by algae. Phycoremediation defined in a broad sense as the use of macro algae or microalgae for the removal or biotransformation of pollutants, including nutrients and xenobiotic from wastewater³⁷. The use of algae for the bioremediation of wastewater has been in vogue for over 40 years to treat the wastewaters with nutrients (nitrogen and phosphorous) and successfully extended to treat effluents from agricultural, agro industrial and industrial wastes^{3,68,86,87}.

The use of beneficial microalgae in the bioremediation of wastewater generates biomass suitable for biofuel production with efficient nutrient recycling. This approach of cultivation of microalgae is considered as one of sustainable and feasible option³⁷ to reduce the cost of the biomass and summarized with the following three advantages,

- Remediation of wastewater nutrients,
- Generation of biomass for biofuel,
- Heavy metal removal.

The use of microalgae were successfully applied for the treatment of various wastewater for the removal of nitrogen and phosphorus from various effluents⁴⁰. Microalgae have the ability to bio remediate the heavy metals and remove the toxic and hazardous compounds like metals, pesticides and recalcitrant from industrial and agricultural wastewater. The functional sites on the cell surface and intracellular ligands like carboxyl, hydroxyl, amino, carbonyl, amide, etc. serves as

bio sorbent assists in binding of metals referred by the term phyco-chelation process to produces reusable effluents ⁶⁸.

2.3.8 Microalgae based wastewater treatment technologies

The efficient growth of microalgae in wastewater depends on composition, the ratio of N, P and C and conditions such as pH, temperature and the availability of light. Microalgae biomass and lipid production results mainly from photosynthesis using the nutrients ammonium and nitrate are the nitrogen sources from the growing media ⁸⁸.

Table 2-6 Microalgae growth and lipid productivity in wastewater ^[87]

| Substrate | Microalgae genera/species | Total nitrogen (mg/L) | Phosphorous (mg/L) | Biomass productivity (mg/L/day) | Lipid productivity (mg/L/day) |
|---|---|-----------------------|--------------------|---------------------------------|-------------------------------|
| Dairy & municipal wastewater | <i>Chlamydomonas reinhardtii</i> | 128.6 | 120.6 | 2000 | 505 |
| Dairy & municipal wastewater | <i>Green algae</i> | 51 | 2.1 | n/a | 17/24 |
| Municipal wastewater before primary settling | <i>Chlorella sp.</i> | 40.65 | 5.66 | n/a | n/a |
| Municipal wastewater after primary settling | <i>Chlorella sp.</i> | 38.95 | 6.86 | n/a | n/a |
| Municipal wastewater after activated sludge tank | <i>Chlorella sp.</i> | 19.1 | 0.32 | | |
| Municipal wastewater after sludge centrifuge: centrate Sea water (50%) & municipal wastewater (50%) | <i>Chlorella sp</i> | 131.5 | 201.5 | | |
| Concentrated municipal wastewater (centrate) | <i>Hindakia sp.</i> 134 | 110.2 | 5.3 | | 109 |
| Concentrated municipal wastewater (centrate) | <i>Chlorella sorokiniana</i> 134 | 134 | 212 | 275 | 78.8 |
| Concentrated municipal wastewater (centrate) | <i>Auxenochlorella</i> | 134 | 212 | 183.3 | 94.8 |
| Concentrated municipal wastewater (centrate) | <i>protothecoides Scenedesmus sp.</i> 134 134 | 134 | 212 | 268.8 | 77.2 |

The stoichiometry analysis of the chemical formula of algal biomass representing the $C_{106}H_{263}O_{110}N_{16}$, indicates 1g of ammonia-nitrogen (NH_3^-N) or nitrate-nitrogen (NO_3^-N) produces about 15.8 g of biomass and consumes 18.1 and 24.34 g of CO_2 in the processes, respectively. Various studies have investigated the treatment of the various industrial effluents using micro algae summarized (Table 2.6) and evaluated the biomass and lipid productivity for the integration to biofuel industry. As seen in the Table 2.6, The total nitrogen ranging from 19 to 128 mg/L and phosphorous from 2.1 to 212 mg/L of sources of wastewater treated using microalgae generated the biomass with the productivity maximum 2of g/l/day and lipid of 0.5g/l/day. Thus wastewater has the potential to generate the biomass to the same level as defined growth media ⁽⁸⁸⁾.

The main challenges in integrating the phycoremediation with biofuel depend on finding the suitable microalgae, which have the ability to survive in different type of wastewater with high nutrient removal efficiency and generation of biomass with high lipid content. Hence the biochemistry and production systems of system of microalgae with the focus on biomass for the biofuel feedstock are discussed in the following sections. The production of the high-density culture is the key factor to reduce the process cost. The cultivation batch, continuous and fed batch have applied for the microalgae. Algal production systems are discussed to improve the biomass and lipid content in the following sessions.

2.4 Microalgae production systems

The whole process of microalgae for the biofuel production consists of strain isolation and selection, cultivation, harvesting, drying, lipid extraction and finally the biodiesel production. The optimization of biomass and lipid contents of micro algal cells during the process depends on various factors. Among them genetics is one of the factors that affect the microalga lipid and biomass productivity. Hence, isolation of robust species of microalgae with highest growth and lipid content is vital for efficient biodiesel production. Diverse environments ranging from fresh water, brackish water, saline and hyper saline environment were used as baseline to select the strains. Species with high grow rate significantly reduces the cultivation time and the cultivation cost, while, high lipid contents attributes to high biodiesel yield. Manipulation of culture system using engineering design and changing the physiological metabolism of micro algal cells have been widely investigated to improve lipid production ^{53,89,90}.

2.4.1 Algal cultivation methods

The nutrient requirements during the growth stage are supplied at the cultivation step. Depending on the energy and carbon source the microalgae can be cultivated by autotrophic, heterotrophic, mixotrophic, or in photo-heterotrophic modes. The impact of stress conditions by the manipulation of the nutritional composition of culture media may be applied to generate specific end products. Among the stress factors nutritional, physical and chemical stresses were known affect the lipid production.

2.4.2 Nutritional and physico-chemical stresses

The nutritional factors are carbon, nitrogen, phosphorus and iron deficiency and physico-chemical stress are operational conditions and external factors like light intensities, temperature, pH, salinity and electromagnetic fields that can affect the microalgae growth and influences the lipid production by the addition, deletion or changing some components of cultivation media. Microalgae in autotrophic growth requires about 30 essential elements in various amounts, these essential nutrients are grouped into two categories: (i) macronutrients and (ii) micronutrients (trace elements) as per the quantity of requirements in the media for the optimal growth are presented in the in Table 2.7.

The nutrient requirement can vary with species of microalgae⁵⁸. Nutrient requirement of freshwater microalgae varies from marine and halotolerant/halophilic species. Sea water is common media for the cultivation of marine microalgae species has a relatively constant composition of major ions (Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Cl^- , SO_4^{2-} , HCO_3^- , CO_3^{2-}) and pH, whereas freshwaters have highly variable compositions^{91,64}.

2.4.3 Nutritional factors: Nitrogen

Nitrogen (N) is an essential element and the key structural component of the most abundant cellular macromolecules such as proteins, nucleic acids and enzymes. The nitrogen content ranges from 1 to 14% of dry weight⁶⁴. Microalgae do not have the ability to convert the atmospheric nitrogen and hence it is supplied in inorganic or organic forms in the media. The inorganic sources like NO_3^- , NO_2^- , NH_4^+ and organic sources like amino acids, urea, uric acid, and dipeptides like xanthine are used by the microalgae. The most widely used nitrogen source for micro algal cultures are nitrate (NO_3^-) and Nitrite (NO_2^-). Nitrogen uptake by the cells depends on the

availability of nitrate and the other nutrients in media. The transport of nitrogen through the cytosol and the chloroplast finally incorporated into carbon skeletons by the glutamine synthetase/glutamate synthase pathway for the amino acid synthesis ⁹².

Nitrogen starvation conditions increase the lipid content but significantly lower the biomass productivity results in lower lipid productivity.

Table 2-7 Essential algal nutrients ^[58]

| Element | Component added to culture medium | Concentration in culture medium (L ⁻¹) | Cell composition (mg/g dry weight) |
|---------|--|--|------------------------------------|
| C | CO ₂ , HCO ₃ ⁻ , CO ₃ | g | 175–650 |
| O | O ₂ , H ₂ O | g | 205–330 |
| H | H ₂ O | g | 29–100 |
| N | NH ₄ ⁺ , NO ₃ ⁻ , NO ₂ ⁻ , urea etc. | g | 10–140 |
| Na | Inorganic salts, i.e. NaCl, Na ₂ SO ₄ , Na ₃ PO ₄ | g | 0.4–47 |
| K | Inorganic salts, i.e. KCl, K ₂ SO ₄ , K ₃ PO ₄ | g | 1–75 |
| Ca | Inorganic salts, i.e. CaCl ₂ , CaCO ₃ | g | 0.0–80 |
| P | Inorganic salts, i.e. Na or K phosphates | g | 0.5–33 |
| S | Inorganic salts, i.e. MgSO ₄ ·7H ₂ O, or amino acids | g | 1.5–16 |
| Mg | Inorganic salts, i.e. Mg sulphates or chlorides | g | 0.5–75 |
| Cl | As Na ⁺ , K ⁺ , Ca ²⁺ or NH ₄ ⁺ salts | g | |
| Fe | In complex with metal ion buffer (e.g. EDTA) | mg | 0.2–34 |
| Zn | Inorganic salts, i.e. ZnSO ₄ , ZnCl ₂ | mg | 0.005–1.0 |
| Mn | Inorganic salts, i.e. MnSO ₄ , MnCl ₂ | mg | 0.02–0.24 |
| Br | As Na ⁺ , K ⁺ , Ca ²⁺ or NH ₄ ⁺ salts | mg | - |
| Si | Na ₃ SiO ₃ ·9H ₂ O | mg | 0–230 |
| B | H ₃ BO ₃ | mg | 0.001–0.25 |
| Mo | Na ⁺ or NH ₄ ⁺ molybdate salts | µg | 0.0002–0.001 |
| V | Na ₃ VO ₄ ·16H ₂ O | µg | - |
| Sr | As sulphates or chlorides | µg | - |
| Al | As sulphates or chlorides | µg | - |
| Rb | As sulphates or chlorides | µg | - |
| Li | As sulphates or chlorides | µg | - |
| Cu | As sulphates or chlorides | µg | 0.006–0.3 |
| Co | Vitamin B12, sulphates or chlorides | µg | 0.0001–0.2 |
| I | As Na ⁺ , K ⁺ , Ca ₂ ⁺ or NH ₄ ⁺ salts | µg | - |
| Se | SeO ₃ ²⁻ , SeO ₄ ²⁻ | ng | - |

The limitation in the cells promotes the production of storage compounds like TAGs and starch that do not contain nitrogen⁶. The overproduction of lipid in the cells serves as an alternate carbon and energy reserve. This phenomenon of nitrogen limitation has been widely applied for the improvement of lipid content of biomass for the biofuel production by applying a two stage

cultivation strategy to ensure high growth rate initially and switching the medium components to trigger the lipid accumulation by the nutrient stress⁹³

2.4.4 Carbon

All the organic compounds of the microalgae cells consist carbon element ranging from 17.5 to 65% of dry weight and varies among the species and culture conditions with majority of species on an average contain about 50% carbon⁶⁴. Inorganic carbon generally from the CO₂ is converted to organic matter through a complex process of photosynthesis through the Calvin cycle forms the three-carbon compound catalyzed by Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). One gram of dried microalgae biomass requires 1.65-1.83 g of CO₂ are from the carbon fixation process. Microalgae utilize the dissolved CO₂ in water in the form of bicarbonate (HCO₃⁻). Aeration and flue gases are major sources of inorganic CO₂ widely used for the cultivation of microalgae but the CO₂ tolerance varies between the species^{60,64}. Microalgal growth via heterotrophic or mixotrophic modes uses organic carbon like monosaccharaides (such as glucose, fructose etc.), starch, volatile fatty acids (such as acetic acid), glycerol and urea^{6,33,94}. Sufficient carbon supply and its bioavailability required for increasing growth and biomass generation⁹⁵.

2.4.5 Phosphorus

Phosphorus (P) is an essential macronutrient necessary for normal growth of all algae and its content varies between 0.5 to 3.3%⁶⁴. Phosphorus is required for nucleic acid and phospholipid biosynthesis, modification of protein function and energy transfer (ATP). Algal biomass usually contains less than 1% of P, but under conditions of “luxury uptake,” its content can exceed 3 % by DW (Powell et al. 2009) and are obtained from non-renewable sources like phosphate rock⁶⁴. P concentration is growth limiting in natural aqueous environments, because it is easily bound to other ions (CO₃²⁻, Fe₃⁺) resulting in its precipitation, and consequently, the nutrient is unavailable for algal uptake.

The major form in which algae acquire P is inorganic phosphate. The transport of P into the micro algal cell is an energy dependent process and its uptake is affected by the culture condition like pH, temperature and salinity. Under P-replete conditions, microalgae form large polyphosphate granules that serve as internal P storage and are metabolised under P deprivation⁴³. This ability exploited in biological P removal from wastewater. Polyphosphate bodies or granules are also

widely distributed amongst many microbial species, where they serve as modulators to overcome stress involved in acclimation to extreme environments, an essential ability for the cell's survival. P limitation lowers the growth rate by reduced concentrations of protein and chlorophylls and decreased level of total adenylates to only 23 % and reduced ADP and ATP levels to 9% and 30%, respectively, as compared to the nutrient sufficient controls were noted ⁹⁶ .

2.4.6 Sulphur

Sulphur (S) is an essential macronutrient required for many key cellular processes. The content of sulphur ranges from 0.15 to 1.6% ⁶⁴. It is required for the synthesis of S-amino acids, membrane sulpho-lipids, cell walls, thiol compounds such as glutathione (GSH) that participate in the stress response, vitamins such as thiamine and biotin, thio-ether and thio-ester compounds such as coenzyme A and S-adenosyl-L-methionine for essential cellular phenomenon is the formation of di-sulphide bonds⁹.

The majority of microalgae use free SO_4^{2-} as the S source for their nutrition, but inorganic SO_4^{2-} is often not the most common form of S in the environment. Deprivation and/or low concentrations of N, P or S increase the carotenoid/ chlorophyll ratio. S deprivation was found to trigger lipid (TAGs) accumulation in cytoplasm of *Chlorella* sp. and *C. reinhardtii* ^{97,98}. Limitation of N, P or S also enables production of algal biomass rich in starch ⁹ .

Besides macronutrients, a healthy micro algal population also requires micronutrients (e.g. Fe, Mn, Zn, Ni, B, V, Co, Cu, Mo, Se) for growth-promoting effect and cell toxicity, as these elements are required in very small amounts of microgram, nanogram or even picogram per liter ^{23,58} . Micronutrients usually serve as cofactors in a variety of metabolic pathways essential for micro algal growth. Cellular requirements for micronutrients influenced by the availability of other essential resources like light, N, P, CO_2 , etc., for regulating the cell's metabolism to induce the production of certain metabolites ⁹⁹ .

2.4.7 Physico-chemical factors: salinity

Salinity, abiotic physical environmental stress factors applied by altering the level of common salt in the growth media are used to maintain the axenic conditions and produce neutral lipids. Increases salt concentration causes biochemical changes in the photosynthesis system and induce pigment and lipid production but decreases the growth in both fresh and marine microalgae

species⁴³. The osmotic changers due the salt stress in combination with nitrogen starvation and low light decreases the photosynthesis ability¹⁰⁰. Unlimited supply of nutrients increases polar lipid (phospho and glycolipid) production under salt stress but the neutral lipids are accumulated during the unfavorable growth condition.⁸⁹

2.4.8 Physico-chemical factors: light intensities

Microalgae can grow under autotrophic, heterotrophic, mixotrophic or photoheterotrophic conditions. However, all microalgae are photoautotrophs and can use light as their only energy source for the synthesis of biomass and metabolites. Natural sun light or artificial light in the range of 400- 700 nm intensity provide the energy for photosynthesis but the intensities varies with the micro algal species⁵³. Photosynthetically active radiation (PAR) is 45% of the light energy of the earth’s surface. Light energy in the form of Photon Flux density (PFD- $\mu\text{mol}/\text{m}^2/\text{s}$) is absorbed by the micro algal pigments are used for the Photosynthesis process. Higher PFD causes photo damages and are regulated by the light dark cycles. Light transfer up to 15 cm depth and light intensity between 76-600 micro $\mu\text{mol}/\text{m}^2 \text{ s}$ can be applied for the cells to stimulate the biochemical reaction¹⁰¹. The photosynthetic process involves two main steps: light-dependent and dark reactions. During the light reactions, electrons donated by water are transferred to two types of photosynthetic units, PSI and PSII³². Along with the cytochrome photons is converted into adenosine triphosphate (ATP) an electron carrier nicotinamide adenine dinucleotide phosphate (NADPH). The products produced by light reactions are then utilized for CO_2 fixation in the Calvin cycle in the subsequent dark reactions¹⁰².

Table 2-8 Characteristics of the major pigments in microalgae [105]

| Pigment group | Color | Ranges of absorption bands (nm) | Pigments |
|---------------|----------------|---------------------------------|--|
| Chlorophylls | Green | 450–475 630-675 | Chlorophyll a Chlorophyll b, c1, c2, d |
| Carotenoids | Yellow, orange | 400–500 | β -Carotene, α -Carotene,Lutein Violaxanthin, Fucoxanthin, |
| Phycobilins | Blue, red | 500-650 | Phycocyanin,Phycoerythrin Allophycocyanin |

Light harvested by light-capturing pigments on light-harvesting antenna complexes. Different pigments absorb specific wavelengths of the solar spectrum and exhibit a distinctive color as shown in Table 2.8. The light intensities of the wide ranges with the objective on the biomass and lipid production were summarized in the Table 2.9.

Table 2-9 Optimal light intensity and light/dark cycles for different species [105]

| Species | Purpose | Optimal light intensity ($\mu\text{mol}/\text{m}^2/\text{s}$) | Light/dark cycle |
|---|---|---|------------------|
| <i>Chlorella vulgaris</i> | Biomass production | 200 | 12/12 |
| <i>C. vulgaris</i> | Biomass and lipid production | 105.41–175.68 | 12/12 |
| <i>C. vulgaris</i> | Wastewater treatment | 2500 | – |
| <i>C. vulgaris</i> | Fatty acid accumulation | 62.5 100 37.5 | 16/8 |
| <i>Chlorella sp.</i> | Biogas upgrading | 2000 | 16/8 |
| <i>Chlorella sp.</i> | Biogas upgrade and biogas effluent nutrient reduction | 350 | 8/16 |
| <i>Nannochloropsis sp.</i> | Biomass production | 100 | – |
| <i>Nannochloropsis salina</i> | Biomass production | 250 | 14/10 |
| <i>Botryococcus spp.</i> | Lipid accumulation | 82.5 | 18/6 |
| <i>Botryococcus braunii</i> KMITL 2 | Lipid accumulation | 87.5 $\mu\text{Em}^{-2} \text{s}^{-1}$ | – |
| <i>B. braunii</i> BOT-22 | Biomass production | 100 | 16/8 |
| <i>Spirulina platensis</i> | Biomass production | 1200 lux | 24/0 |
| <i>S. platensis</i> | Biomass production | 166 | 16/8 |
| <i>Arthrospira platensis</i> | Glycogen production | 700 $\mu\text{Em}^{-2} \text{s}^{-1}$ | – |
| <i>Scenedesmus obliquus</i> CNW- | Biomass and lipid and carbohydrate production | 420 | – |
| <i>N.Scenedesmus sp.</i> 11-1 | Biomass production | 400 | – |
| <i>Desmodesmus sp.</i> | Biomass and lipid production | 600 | – |
| Mixed culture of <i>Chlorella sp.</i> and <i>Saccharomyces cerevisiae</i> | Oil production | 1000 lux | – |

The light intensity of $< 700- \mu\text{mol}/\text{m}^2/\text{s}$ is used in the control media and while the study of the waste treatment used higher light intensity of $2500 \mu\text{mol}/\text{m}^2/\text{s}$ ¹⁰² to promote the penetration in the high turbid media. Short wave length light (blue) has high-energy which causes photo inhibition by striking the peak electrical energy to the photo harvest system.

Higher wavelength (red) light with lower energy inhibits the penetration into deep and high-density cultures¹⁰³. In addition to the type and intensity, the photoperiods with light and dark cycles are essential for the photosynthesis process. The light –dark cycle of 12:12, 12:14, 14:10, 16:8 have reported to have an effect on cell lipid content¹⁰⁴. The higher the duration of the light cycle influence the lipid production and fatty acid composition.

The application sufficient light energy with nutrients supports the cell growth and hence increases the biomass production. Stepwise increments of light intensity were applied to improve the biomass¹⁰⁵.

2.5 Culturing systems

The growth characteristics and composition of microalgae significantly depend on the cultivation conditions¹⁰⁶. The major types of cultivation conditions based on the supply energy and carbon sources are photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic used for microalgae. Microalgae use light, (sunlight, as the energy source), and inorganic carbon (e.g., carbon dioxide) as the carbon source to form chemical energy through photosynthesis in phototrophic cultivation and is the commonly used cultivation method^{104,107}.

The heterotrophic cultivation widely used to improve the productivity of biomass and lipid. The microalgae grow under either phototrophic or heterotrophic conditions, or both. Microalgae use both organic compounds and inorganic carbon (CO₂) as a carbon source for growth in the mixotrophic cultivation. The mixotrophic and photoheterotrophic cultivation are same with the latter requires light as the energy source. Some algal species accumulate large amounts of TAG under nutrient limitation decrease in cell division. The variation of these factors are considered as stress and large number of studies notes the effects of environmental stress on TAG biosynthesis and the change of fatty acid profile due to the decrease or cessation of cell division directs the of excess fixed carbons into storage lipids. Alternatively, inhibition of cell division utilizes less storage lipids while synthesis of new lipids continues. In green microalgae, lipid synthesis shares common carbon precursors with starch synthesis. Blocking of the starch synthesis by inactivation of ADP-glucose pyro phosphorylase lead to remarkable increase of TAG content and lipid droplet formation suggest the shift of carbon precursors from starch to TAG biosynthesis is an efficient strategy for enhancing oil content.¹⁰⁸

2.5.1 Culture systems: batch

High cell density culture is desirable in order to reduce the cost for down- stream processing in commercial production. Culture methods like batch, fed batch and continuous used in the cell cultures are applied for the cultivation of microalgae. In the batch culture, the nutrients and microalgae inoculate added in the beginning of the batch and the cultivation is continued until the

end of the growth period. The growth stage contains lag, log a, stationary and decline stages. During the sufficient availability of nutrient in the beginning of growth time, the cell proliferate at the exponential rate and reaches to stationary phase when the nutrients are exhausted where the cells experience stress and induce the lipid synthesis. Biomass productivity is reduced as the nutrients were depleting during which the lipid production is enhanced. Nutrient limitation evokes change in the physiology and the cells re-program their metabolism to cope with change in nutrient supply or to activate a survival program to outlive sustained periods of starvations.

2.5.2 Culture systems: continues and fed-batch

A batch culture of microbes fed continuously with culture medium is described as a “fed-batch” culture. The reduced biomass productivity compromises the increased lipid productivity enhanced by the feeding of the fresh media or nutrients to increase the cell growth. In fed-batch, cultures may reach a “quasi-steady state” in which the specific growth rate (μ/d) virtually equals the dilution rate, that is, the ratio of medium flow rate to culture volume. In a quasi-steady state the specific growth rate gradually decreases. A unique feature of a fed-batch culture is that it allows continuous reproduction of the transient conditions between two specific growth rates, which can be chosen at will. Fed-batch culture may be used to determine the relation between specific growth rate and the growth-limiting substrate concentration and to determine the maintenance energy¹⁰⁹.

Nutrient-rich medium is used to ensure high growth rate of the micro algal cells, by switching to a different composition to induce the nutrient stress. This can be achieved by the fed-batch techniques¹¹⁰. The fed-batch process is an important strategy because it makes possible to control the growth and the lipid accumulation phased by modifying the feed throughout the cultivation process¹¹¹. During the growth period, the metabolite generated contributes the detrimental effect and to reduce these portions of a “fed-batch culture” is withdrawn at intervals leads to a “repeated fed-batch culture”. Fed-batch cultivation systems have provides better opportunity to increase the biomass generation which has the potential to modify the light intensity¹¹², carbon feeding source⁹² for meeting the growth requirements. Various strategies used in fed-batch to improve the biomass and lipids but the contamination due to the additional feeding lines are the major drawback of the application of fed batch systems for the pure cultures¹¹³.

2.5.3 Nutrient feeding methods

For microalgae, changes in culture conditions and nutrients deficiency affect the cell growth and composition. Light and nitrogen are most reported for their effect on biomass and lipid. Without the nitrogen source, the media is considered nitrate-depleted condition and this lowers the photosynthesis process. While an insufficient supply of nitrate can support the photosynthesis process but the cells produce nitrogen less products like fatty acid and carbohydrates⁴³

In fed batch cultivation, staged feeding, repeated feeding or pulse feeding can be provided to apply the controlled supply of nutrients to maintain cells in stages of growth for either increase in biomass or lipid productivity^{114,115}. Pulse feeding strategy noted as the easier and cheaper method because of the absence of pumping costs, could lead to lower cell growth, productivity and nitrogen-to-cell conversion factor¹¹⁵. The major challenge to the implementation of an integrated algae system in large-scale production of algae is unavailability of cost efficient harvesting process that allows the downstream processing to produce biofuels and other bio-products of value.

2.6 Harvesting techniques

Harvesting of biomass is the important step, which involves the recovery of algal cells from the growth media. This step is considered as one of the major bottlenecks in the production of microalgal-based bulk commodities. Harvesting of microalgae accounts for about 20-65% of the microalgal biomass cost¹⁴ which, translates to 50% of the total biofuel production. Harvesting efficiencies can vary depending on the properties of the growth media and morphology of the strains. Microalgae have wide range of properties including size, shape, specific gravity, surface charge, motility, growth phase, presence of appendages and extracellular organic matter that affect their separation from the media. The challenges of harvesting of microalgae are mainly due to the dilute concentration of microalgae in the bioreactor and the characteristic small size of the algal cells¹¹⁶.

Microalgae harvesting can be a two-step process. The first is thickening in which microalgal biomass is separated from the bulk culture to the solid concentrates of biomass ranging 2–7% dry weight. The second step involves dewatering, the algal slurry to algal cake with solid concentrate of 15-25% dry weight. Thickening is more energy intensive since the large volume of the culture

is handled ¹¹⁷. The selection of harvesting methods depend on many factors namely, 1) value and properties of the end product, 2) Nontoxic, non-contaminated biomass or further processing and 3) generation of recyclable culture medium and 4) reasonable cost and very high degree of recovery rate. Following table (Table 2.10) lists the main advantages and disadvantages of the most commonly used harvesting methods.

Table 2-10 Comparison of micro algal harvesting methods ^[24, 25]

| Harvesting method | Advantages | Disadvantages | Dry solids output concentration (%) |
|------------------------|---|---|-------------------------------------|
| Centrifugation | Fast method, High recovery efficiencies, Can handle most algal types, Recycle of culture media | Expensive method (High capital and operational costs), High energy requirement, Suitable only for the recovery of high value products, possibility of cell damage due to high shear force | 10–22 |
| Filtration | High recovery efficiencies, Recycle of culture media Allows the separation of shear sensitive species, Wide variety of filter and membrane types available | Highly dependent on algal species; Best suited to large algal cells, Clogging or fouling Increasing the operation cost (regular cleaning, membrane replacement and pumping) | 2–27 |
| Ultrafiltration | Can handle delicate cells , Recycle of culture media | High capital and operational costs | 1.5–4 |
| Gravity Sedimentation | Simple and inexpensive method, Low cost, potential for use as a first stage to reduce energy input and cost of subsequent stages Recycle of culture media | Algal species specific, Best suited to dense non-motile cells. Time consuming, Separation can be slow. Possibility of biomass deterioration, Low concentration of algal cake | 0.5–3 |
| Chemical flocculation | Simple and fast method, No energy requirement, Wide range of flocculants available, Price varies although can be low cost | Removal of flocculants, Chemical contamination (toxic) of biomass, Recycle of culture media is restricted | 3–8 |
| Flotation | Feasibility for large scale application, Low cost method, Low space requirement, Short operational times (can be more rapid than sedimentation) Possibility to combine with gaseous transfer, Recycle of culture media | Algal species specific. Unfeasible for marine microalgae harvesting, Generally requires the use of chemical flocculants, surfactants, High capital and op transfer | 7 |
| Electric based process | Applicable to wide variety of microalgae, Do not require the addition of chemical flocculants, | Poorly disseminated, High energy equipment cost | |

Harvesting of micro algal biomass currently uses mechanical, chemical, biological and electrical based methods. These methods can be combined to improve the separation efficiency to reduce the costs. Conventional harvesting methods like filtration and centrifugation require high amounts of energy and are therefore cost-intensive¹¹⁸. Harvesting, such as centrifugation and tangential filtration, can represent 20–57% of the final biomass cost and harvesting cost that they estimated to be \$0.864/L oil.¹¹⁹. The selection of the optimal concentration pathway is essential for the reduction of energy requirements¹²⁰.

2.6.1 Common harvesting techniques

Various techniques like centrifugation, filtration, flocculation/coagulation, electro flocculation, gravity sedimentation or flotation are compared in Table 2.10 are detailed below.

2.6.2 Centrifugation

Centrifugation, a common separation technique used in bioprocessing involves the use of centrifugal forces to separate solids and liquids. The separation is based on the particle size and density difference of the medium components. Centrifugal force accelerates the rate of sedimentation by high-speed rotation of centrifuge that increases the gravity field. The behavior of the smallest particles in the system has the largest effect on separation efficiency¹²¹.

The use of centrifugation for the recovery of microalgae biomass offers advantages of quick process, high recovery rate and non-contaminated recovered biomass²⁹. The physiological properties of most micro algal species and their small size and low concentration in culture result in large energy requirements and operation costs of the centrifugation process¹²². Although centrifugation is a successful dewatering method, the exposure of micro algal cells to high gravitational and shear forces can damage cell structure. Also, the processing of large volumes of culture could be time consuming and costly. The high investment and operating costs required make it not a feasible option for the separation of the micro algal biomass from water¹²³

Studies using laboratory centrifuge (500g–1,000g) showed clarification up to 80%–90% algal cells in 2–5 min. Two forms of high-speed centrifugation with acceleration factors of 6,000g and 13,000g resulted >95% cell recovery. The efficiency of recovery decreased with decreasing acceleration: 60% recovery was achieved for centrifugation at 6000g and 40% recovery for centrifugation at 13,000g¹²⁴.

2.6.3 Filtration

The filtration of micro-algal cells is based on a solid-liquid separation mechanism involving a semi-permeable barrier with small pores that forces the micro algal cells to be retained and harvested. Harvesting biomass via filtration is one of the favorable methods due to its ability to harvest microalgae with low density¹²⁵

Advantage of using membrane filtration for harvesting the microalgae

- Environmental friendly method since it uses few or no chemicals that are harmful to human and environment.
- Water and residual nutrients in the media can be reuse able
- Inclusion of membrane processes will assist in developing continuous production and process system for the algal biomass
- Low operational cost under low trans membrane pressure compared to centrifugation

There are several filtration methods used for microalgae processing including submerged membranes, cross-flow filtration and dynamic microfiltration. Ultrafiltration refers to filters with pores from 0.02 to 0.2 μm and microfiltration refers to filters with pores from 0.1 to 10 μm ²⁵. Microfiltration of pore size of 0.1–0.5 μm is preferable for the most microalgae strains¹²⁶. Dead-end and the tangential (cross) flows are the two process filtration techniques. Dead-end systems consist of cartridge filtration, horizontal filter press, vacuum drum filter, and belt filter are usually conducted in batch modes around 5–37% solid content can be achieved¹²⁷.

Fouling of the filter causes flux reduction and reduces the performance, which increases the processing costs and is the main disadvantage of membrane filtration. Fouling is caused by the formation of layer of cells (cake) and the cake thickness and effect varies by shape, size, and cell wall rigidity. Microalgae with non-spherical shape, large size and rigid cell wall filtered better than without the cell wall or flexible cells¹²⁶

Fouling can be controlled by optimizing the process conditions including pressure, retention time, and cultivation conditions, improving system design like reactor structure and membrane surface modification, and incorporating pretreatment like mechanical washing, chemical cleaning, and chemical coagulation. Back-flushing, supplementation of coagulant, alteration of membrane surface and shear improvement are studied and reported to reduce the fouling¹²⁸.

2.6.4 Sedimentation

Gravity sedimentation is a simple method to separate liquids and/or solids from liquid with different densities. Density, radius of the particle and sedimentation velocity influences the separation efficiency. The process can be slow when the density of the components being separated is similar. Settling velocities can be theoretically calculated through Stokes' Law using the density, radius of the particles, density of liquid in which they are suspended or discharged¹²⁹. The sedimentation rates of micro algal cells are very low (0.1–2.6 cm h⁻¹). Colonial and filamentous algae can be harvested by this method but the singular and motile algae have to flocculate before gravity sedimentation can be applied¹³⁰. The limitation of using gravity settling when the density of media (fresh or salt water around 0.998 and 1.025 g cm⁻³ at 20°C, respectively) is close to the micro algal density and its use to separate cells of *Chlorella sp.*, whose density (1.070 g cm⁻³) is not efficient²⁸

Sedimentation is however a low cost technology to harvest the microalgae, due to the low capital, operating cost and energy input (0.1 kWh m⁻³). This process does not use chemicals and produces contamination free recyclable media into the cultivation system¹²⁹.

2.6.5 Flotation

Flotation is a separation technique used in mineral industries and has been found to be effective for removing algae from suspension. In this process air or gas transformed into bubbles passed into the solid/liquid suspension. The solid particles of the suspension (microalgae) adhere to the gaseous molecules (bubbles) and rise to the surface¹²¹ where they can be collected. This process is faster than sedimentation and requires lower cost.

The flotation efficiency depends on the size and surface characteristics of the microalgae being separated and the size of the air/gas bubbles. The size, charge and hydrophobicity of the micro algal cells will also affect the interaction between the cell and the bubbles.²⁵ A higher surface-to-volume ratio (as the size of the bubbles reduces -diameter of 10–30 micrometer) increases the stability of the bubble¹²². Dissolved air, dispersed air, electrolytic flotation, Jameson cell (high mixing) and dispersed ozone flotation (ozone replaces the air) are the techniques used to separate the microalga and are summarized in the following table (Table 2.11).

Table 2-11 Energy consumption of different flotation systems for microalgae harvesting
[^{131–133}]

| Flotation process | Algal species | Flotation performance (%) | Energy input (kWh/m ³) |
|---------------------------------|---|---------------------------|------------------------------------|
| Dissolved air flotation (DAF) | <i>Chlorella</i> | 83.7 | 7.6 |
| | <i>M. aeruginosa</i> | 87 | Not determined |
| Dispersed air flotation (DiAF) | <i>Chaetoceros</i> sp. | 90 | Not determined |
| | <i>Chlorella</i> and <i>Scenedesmus</i> | 76.6 | 0.003 |
| | <i>D. salina</i> 19/30 | >95 | Not determined |
| Electro flotation (FF) | Mixed microalgae | 99 | 20.7 |
| Jameson cell flotation (JCF) | <i>Tetraselmis</i> sp. M8 | 97.4 | Not determined |
| Dispersed ozone flotation (DOF) | <i>S. obliquus</i> FSP-3 and <i>C. vulgaris</i> | 95 | Not determined |

Flotation in combining the flocculants or surfactants was significantly affected by ionic strength, pH, temperature were reported^{131–133}. However these techniques are yet to be explored for industrialization with extensive studies focusing on the sustainability, optimization of the operating parameters using different microalgae species with use of cheap natural surfactants and collectors¹³⁴. Novel methods such as coagulation-copolymerization where distribution of micro-bubbles and an air release induced microalgae cell flocculate in air bubbles with 94% harvesting efficiency have also been reported¹³⁵.

2.6.6 Coagulation-Flocculation

Flocculation based separation been applied successfully in various industries ranging from mining and brewing to water treatment. Flocculation is well known process for the removal of suspended particles from water to produce potable water¹³⁶. Solute particle in a solution forms an aggregate (floc) when the solute particles collide and adhere to each other in the flocculation process. Floc formation is a physico-chemical process influenced by the mixing speed. Chemicals called flocculants are added to induce flocculation by charge neutralization, electrostatic patch mechanism, bridging polymers and sweeping flocculants^{137, 138}. Flocculation improves the rate of sedimentation by aggregating the dispersed particles into larger particles and thus, increasing the recovery of biomass and the dosage of flocculants increases linearly with cell number^{117,127}.

2.6.7 Flocculation phenomenon in microalgae

Most micro algal cells have a size range between 5 and 50 microns. Micro algal cells can form stable suspensions with chemically reactive cellular surfaces that have a net negative surface charge due to the ionization of functional groups. The stability of these micro algal suspensions is dependent on the forces that interact between the particles themselves and the particles and water. The negative charges of micro algal cell walls keep the cells in dispersion in the solution^{119,122,139}. Harvesting marine microalgae requires suitable flocculants to induce the flocculation under marine conditions¹¹⁸.

Bridging phenomenon describes the linking of the cells by the polymers that neutralize the electronegative charges facilitate the binding of the cells^{140,141} induced by applying external chemicals and extra cellular polymeric substances¹⁴². The zeta potential indicates the stabilization of the particles. Particles with >25 mV zeta potential (positive or negative) maintains stable suspensions due to strong electric repulsion. Particles aggregate when the zeta potential is close to zero¹²⁷. The hydrophobicity and micelle properties of microalgae affect the zeta potential and flocculation efficiency. Longer alkyl chains lead to greater hydrophobicity and a lower critical micelle concentration. Surfactants have hydrophobic ends that can bind insert into the hydrophobic cell membranes¹³⁴ enables the separation process.

Charge neutralization occurs due to the interaction of metal and algal cells¹²². Metal hydroxides are formed at high pH, which precipitate the flocs and cause physical linkage between the algae and increases the density of biomass resulting in sedimentation^{122,139,118,140,141 143}.

2.6.8 Flocculants for micro algal harvesting

Many different chemicals have been tested for micro algal flocculation can be classified as inorganic and organic polymer/ polyelectrolyte chemical flocculants^{9,117,119,127}. Various chemical flocculants applied are listed in Table 2.12¹¹⁷ with their advantages and limitations. Inorganic chemical flocculants have binding capabilities, which vary with the dosage, molecular weight and pH. Organic flocculants have high charge densities, are less sensitive to pH and lower dosages are effective to flocculate a wide range of micro algal species. Flocculation followed by sedimentation or filtration consumes less power and can be a cost-effective method for harvesting¹⁴⁴.

Table 2-12 Comparison of inorganic and organic flocculants [119]

| Parameters | Inorganic flocculants | Organic flocculants |
|---|---|--|
| Nature of flocculants | Multivalent salts | Polyelectrolytes/polymers |
| Key characteristics of an effective flocculants | Increasing molecular weight and charge on the polymers has been shown to increase their binding capabilities | Flocculants that have a high charge density are therefore more effective |
| Sensitivity to pH | Coagulation using inorganic coagulants is highly sensitive to pH level | Coagulation using organic coagulants is less sensitive to pH |
| Dosage of flocculants required | A large concentration of inorganic flocculants needed in order to maintain flocculation efficiency thereby producing a large quantity of sludge and may contaminate the end product (for example addition of aluminum and iron salts) | Lower dosages of organic flocculants are required for the flocculation process thus producing less quantity of sludge and lesser contamination probabilities |
| Applicability | Although some coagulants may work for some microalgal species, they do not work for others | Wide range of applications i.e. they can be used for larger number of microalgal species |

Various flocculants were used for the harvesting of microalgae. The application has its own limitation and advantages. The advantageous and the limitation of the specific group flocculation are given in the Table 2.13.

Table 2-13 Overview of highlights and limitations of flocculation ^[144]

| Flocculation | Advantages | Limitations |
|---------------------|--|--|
| Metal coagulants | Reliable, well-established technology | Metal enrichment of biomass, toxicity |
| Biopolymers | Low coagulant dosages, bio-based chemicals | Expensive |
| Magnetic coagulants | Enhanced separation with magnetic force | Expensive, only established on lab scale |
| Electrocoagulation | Reliable, low energy requirement. | Metal enrichment of biomass |
| Ultrasound | No contamination | High power consumption, heating, scalability |
| Bio flocculation | No addition of chemicals, cheap, sustainable | To be confirmed at scale |

2.6.9 Flocculation by organic flocculants

Various flocculants are compared in Table 2.14. Polymeric organic substances have the binding sites to link several colloidal particles that in turn is effective for algal flocculation. Among the organic polymers, natural cationic substances like starch from different sources such as wheat, potato, and corn are effectively applied for the flocculation. Chitosan and synthetic polyacrylamide polymers are also being investigated for their effectiveness to harvest the microalgae¹⁴⁵. These studies have identified the factors such as salinity, pH, biomass concentration, and algal organic matter (AOM) which can affect the efficiency of cationic flocculants¹⁴⁶.

A desirable flocculants should be non-toxic, recyclable, inexpensive, and efficient at low concentrations. Various chemical flocculants like alum (hydrated aluminum potassium sulfate) or alkali cannot be considered for application in harvesting microalgae for biofuel production due to the toxicity and the cost¹¹⁷. The natural polymers are bio degraded to non-toxic compounds and can be used for the production of algae for animal feed or in a bio refinery context¹⁴⁷. Mung bean (*Vigna radiata*) protein extract (MBPE) used for flocculation of *Nannochloropsis* and the dosage of 20 ml/L, With a mixing rate of 300 rpm for 6 min achieved a efficiency of >92% after 2 h of settling time¹⁴⁸.

Chitosan, natural cationic polymer, a derivative of chitin obtained from shrimp shells is quite effective at flocculating microalgae but too expensive for large-scale application^{147,149}. Chitosan has low solubility and increased viscosity upon hydration is major issue. It is not soluble in pure water or organic solvents but is soluble in aqueous solutions of organic or mineral acids under

specific conditions. The properties of the chitosan change with the pH or the solvents used for the solubilisation, impact the flocculation rate and harvesting efficiencies^{150,151}

Table 2-14 Comparison of different chemical flocculants for microalgae harvesting ⁽¹⁵⁴⁾

| Chemical flocculants | Dosage (L ⁻¹) | Classification | Microalgae (Cell density) | FE | settle time | Features |
|---|---------------------------|-------------------|--|--------|-------------|---|
| Al ₂ (SO ₄) ₃ | 0.1 g | Inorganic | <i>C. zoofingiensis</i> (0.5 g L ⁻¹) | >90% | (60 min) | pH depended, and risk of secondary pollution |
| FeCl ₃ | 0.2 g | | <i>Scenedesmus</i> sp. (0.23 g L ⁻¹) | 99% | (60 min) | >High dosage, cell damages, and risk of secondary pollution |
| Fe ₂ (SO ₄) ₃ | 1 g | Inorganic | <i>C. minutissima</i> (2.2-108 m L ⁻¹) | | | |
| Aluminium nitrate sulphate | 5.4 mg | Inorganic | <i>N. salina</i> (10–20 g L ⁻¹) | >95% | (30 min) | Residual aluminium in microalgal biomass |
| Ammonia | 38–120 mM | Inorganic | <i>C. sorokiniana</i> | >85% | (180 min) | Reuse of medium, long flocculating time, and species dependent Chen et al. (2012) |
| Polyacrylamide | 0.05 g | Inorganic polymer | <i>Scenedesmus</i> sp. (0.54 g /L) | 60% | (10 min) | High pH depended, and risk of toxic acrylamide |
| Poly aluminium chloride | 20–40 mg | Inorganic polymer | <i>N. gaditana</i> (132 mg L ⁻¹) | 70–80% | (30 min) | pH sensitive, and risk of secondary pollution |
| Chitosan | 20–30 mg | Organic polymer | <i>C. sorokiniana</i> (2 g L ⁻¹) | 60–99% | (30 min) | Favour pH depended, and high cost of chitosan |
| Cationic starch | 20–40 mg | Organic polymer | <i>C. protothecoides</i> (0.56– 0.77 g L ⁻¹) | 79–90% | (60 min) | pH depended |
| <i>Moringa oleifera</i> seed flour | 1 g | Organic polymer | <i>C.vulgaris</i> (OD730 (0.9– 1.06) | 88% | (120 min) | High dosage, and alkaline pH favoured |
| Polyc-glutamic acid (c-PGA) | 20 mg | Organic polymer | <i>C. protothecoides</i> (0.6 g L ⁻¹) <i>N. oculata</i> (0.6 g /L) <i>P. tricorutum</i> (0.6 g /L) | >90% | (120 min) | Salinity depended, long flocculating time |

Synthetic polyacrylamide polymers form the majority of coagulants-flocculants in commercial use are considered nontoxic, they contain monomer residues that are toxic and are effective to flocculate the microalgae^{152,153}.

2.6.10 Flocculation by pH change

Flocculation induced by pH change has emerged as a viable alternative for large-scale harvesting of algal biomass^{154,155}. Increase in pH obtained by adding lime to sewage, to induce the precipitation of calcium carbonate, calcium ortho-phosphate and magnesium hydroxides were commonly used in the wastewater¹¹⁹. The pH of the culture can increase naturally due to inorganic carbon consumption by the photosynthetic activity of the microalgae¹⁵⁶ and pH can be increased by adding the base like sodium hydroxide which is available at lower cost. After flocculation, the flocculated cells could be re-cultured and the flocculated medium could be reused after neutralizing pH with CO₂ and supplementing nutrients¹⁵⁵.

2.6.11 Mechanism of pH induced flocculation

In natural water, microalgae cells are negatively charged and the repulsive forces impede the cells joining together¹⁵⁷. An increase in pH causes precipitation of inorganic compounds in the culture medium to cause auto- flocculation or charge neutralisation. When sodium hydroxide is added to raise the pH of culture medium a large amount of positive charges are available and charge neutralization occurs leads to disappearance of repulsion forces. During this stage the algal cells tend to coalesce into large aggregates called flocs, which due to its weight, settle down at the bottom by gravity¹⁵⁸.

Both media composition and the salinity play major role in the pH-induced flocculation. The efficiency of the flocculation is increased with the amount of Na, Ca and Mg ions¹⁵⁴. Previously, flocculation induced by pH change for *Scenedesmus obliquus* or *Dunaliella* microalgae harvesting have been reported^{159,160,161}.

2.6.12 Bio/Auto flocculation

Bio flocculation methods are successfully applied in micro algal harvesting and wastewater treatment. Changing the physiology of algae or species in co-culture which is often referred to as bio flocculation¹⁶². Bio flocculation is non-toxic and low-cost harvesting method for microalgae has recently attracted a lot of attention in the field of biofuel research¹⁴⁶. There are three types of methods which are used: 1) microbial bio flocculants-associated bio flocculation, 2) microorganism-associated bio flocculation, and 3) micro algal cell self-flocculation. The mechanism of microbial bio flocculants-associated bio flocculation is due the ability of

production of the Extracellular Polymeric Substances (EPS) by the microalgae. EPS a glyco-protein consists of polysaccharides (57%) and protein (41%) and presence of negatively charged functional groups such as hydroxyl and carboxyl groups stimulates the formation of floc in the presence of metal ions in the media was reported^{146,163}. Bio flocculation depends on multiple factors including the nutrient conditions, pH, and species. Algal–bacterial, aggregation by the interaction between the bacteria and microalgae or by the compounds produced by bacteria are the method that can be applied to harvest the microalgae.

2.6.13 Microalgae/fungal co-palletization

Bio flocculation of algae has been performed by employing suitable microbial partners e.g., algae-algae, algal-bacterial and algal-fungal interaction¹⁶⁴. Microalgae harvesting using microalgae/fungi co-palletization where microalgae cells are attracted to filamentous fungus and form co-pellets during their co-culture is cost effective and sustainable and was influenced by the pH and calcium ion concentration¹⁶⁵. Microbial flocculation had been practiced in processes such as wastewater treatment and fermentation and was also suggested for harvesting technique for microalgae¹⁶⁶. Both microalgae and fungi have low negative zeta-potentials regardless of the pH of the bulk solution (<-10 mV). Fungi have positive electric charge at low pH (pH=3). Different values of pH and the ionic strength of the medium, surface charges of the fungi and microalgae in addition to the magnitude of the zeta potential values influences the co-palletization process.¹⁶⁵. Rapid flocculation of 99% was developed by ration of the algae and fungi, agitation speed and temperature for *Chlorella pyrenoidosa* cells with *Aspergillus fumigatus* within 3 h¹⁶⁴.

2.6.14 Magnetic flocculation

Magnetic separation is typically used for concentrating magnetic materials and for removing magnet stable particles from air and liquid streams. Magnetic separation processes for ferromagnetic materials or ferrous particles have been used extensively in the past¹⁶⁷. This technique has also been successfully applied in solid waste separation and recycling, ultra-purification of many fine chemicals, nano and biotechnological products etc. In addition, various types of magnetic particles have been synthesized and investigated to remove magnetic particle tagged algal and microalgae from lakes based on the intrinsic paramagnetic movement¹⁶⁷.

2.6.15 Electrical based harvesting

Micro algal cells are negatively charged and hence the application of electrical field to culture broth can facilitate the separation process by precipitation on the electrodes (electrophoresis) or by accumulation on the bottom of vessel. Sacrificial and Non sacrificial electrodes used and are considered less toxic than the chemical coagulants^{25,121}.

2.6.16 Flocculation by electromagnetic particles

Electro-coagulation-flotation (ECF) is the technique allows flotation of microalgae cells for surface harvesting¹⁶⁸. ECF process contains successive stages: (i) formation of coagulants by electrolytic oxidation of the “sacrificial electrode”; (ii) destabilization of the contaminants, particulate suspension, and breaking of emulsions; and (iii) aggregation of the destabilized phases to form flocs¹⁵⁹. The application of this technique can lead to microalgae to exhibit a negative charge in the presence of proton-active carboxylic, phosphoric, phospho-diester, hydroxyl, and amine functional groups. Then, the anode and cathode in the ECF release metal ions to neutralize the negativity of microalgae and to produce the flocculated activity of microalgae in water or the culture medium¹⁶⁸.

2.7 Conclusions

Microalgal cells accumulate lipids in the form of triacylglycerol during the adverse growth conditions are used for the biodiesel production. The species and its genetics regulates the lipid biosynthesis are also greatly influenced by the environmental and nutritional conditions. The stresses like pH, temperature and salinity are known to induce the lipid biosynthesis. The application of the stress to induce the lipid production reduces the cell growth cycle resulting in the reduced biomass production. Hence, the nutrient management during the growth stage for the improvement of biomass and lipid production is key to enhance the lipid productivity. Fed batch cultivation has the ability to control the feeding rates of the nutrients and the studies on these directions are important. The energy intensive harvesting techniques increases the biomass cost. The simple rapid coagulation- flocculation process has the ability to separate the algal cells from the media, which depends on the type, dosage and composition of the media.

Chapter 3

3 Materials and Methods

In this chapter, the microalgae species, growth media preparation, culture conditions, experimental set up, analytical methods used for the study are described.

3.1 Growth media

The synthetic media were used for the cultivation of *N. gaditana* in the experiments. Windsor Safe T-Salt (Windsor Salt Ltd. Pointe-Claire (Québec) a common material used for the deicing the driveway was dissolved in the deionized water was used for the preparation of road salt run-off. The artificial seawater is the control media for the marine microalgae was prepared by dissolving the known quantity of reef salt (Instant Ocean salt, Petsmart, London, ON, Canada) in deionized water.

The solutions (3.5 % w/v) of the above noted reef and road salt were filtered through 0.45 μm filters and the 50 ml of filtered samples were submitted to Biotron (Western University, London, Ontario) for the full compositional analysis by inductively coupled plasma atomic absorption spectrometry/mass spectrometry (ICP-MS). The results of the elemental compositions were obtained as mg/L.

3.2 Algal culture

The marine microalgae *N. gaditana* -L526- was used for the study. This culture was obtained from national culture collection of marine phytoplankton (National center for marine microalgae and microbiota NCMA), Mane, USA). The cultures were grown and sub-cultured in synthetic seawater media. The fresh cultures (7 days old) containing the biomass of approximately 500 ± 50 mg/L were used at 10 % (v/v) for the inoculation were used for the experiments

3.3 Algal growth media and cultivation

The two growth media were used for the cultivation of microalgae in the study. The simulated seawater (reef salt water) and road salt run-off media were prepared by dissolving the above reef and road salts at 3.5 % (w/v) in deionized water. Sodium nitrate as nitrogen source (65mg/L) and

potassium phosphate (5mg/L) was added to the media. The F/2 Gaillard's marine water enrichment solution 50x (Sigma Aldrich) as a source of trace minerals and vitamins were added to the reef salt solutions. The initial nitrate concentration was adjusted 100 mg /L in the micro algal growth media for the experiments. The pH of the growth media in all the experiments were adjusted to 8.0 +/-0.05 using 0.1M HCl and or 0.1 M NaOH, the 40 mM Tris-HCl (Sigma Aldrich) were used as the buffer to restrict the changes in the pH of the media. Synthetic greenhouse effluents were prepared by dissolving the sodium nitrate and sodium phosphate as source of nitrate and phosphate were added as 75 mg NaNO₃, and 5.65 mg NaH₂PO₄. 2H₂O. All the media were sterilization by 20 minutes of autoclaving at 15 psig.

The marine microalgae *N. gaditana* -L526- cultures were cultivated using the above synthetic reef salt and road salt media. The 2 L conical flask bioreactors with 1.5L of working volumes equipped with sparger were used for batch cultures. The fresh cultures (7 days old) containing the biomass of approximately 500 ± 50 mg/L were used at the rate of 10 % (v/v) for the inoculation. All the cultivation studies were conducted under photoautotrophic mode by supplying the carbon dioxide at 1 vvm (volume of air /volume of culture /minute) by aeration. The fluorescent light bulbs were used to provide the light of 100±20 micromole photon /m²/sec intensity on the reactor surface to support the cell growth. The light dark cycles of 12:12 hours were maintained and cultivation was continued for the duration of 21 days and was sub-cultured to maintain the viable cell counts in the culture media.

3.3.1 Microalgae growth media for the fed batch studies

The growth media was prepared by dissolving 26 gram/L of salt (Windsor Safe T-Salt (Windsor Salt Ltd. Pointe-Claire (Québec) in deionized water. Sodium nitrate as a nitrogen source (100 mg/L) and potassium phosphate for the phosphate source (5mg/L) was added to the salt media. The pH of the media was adjusted to 8.0+/-0.1 using 0.1M NaOH solutions. For experiments, exploring the effect of feeding used the stock solution of the nitrate solutions were prepared by dissolving 100 mg NaNO₃ and 7.5 mg NaH₂PO₄. 2H₂O (Sigma Aldrich) in the 1 L deionized. All prepared solutions were autoclaved for 20 minutes at 15-psig prior to use and applied for the cultivation.

The NaNO₃ (Sigma Aldrich) solutions were prepared in the deionized water were added at the rate of 50,75 and100 mg/L to the road salt growth media for the initial nitrate concentrations in the

batch culture to simulated the vegetable greenhouse effluents conditions to determine the influence of initial nitrate levels on biomass growth and lipid concentration of *N. gaditana*.

3.3.2 Experimental set up for the batch cultures

Batch cultivation systems used in the study explained below. The simulated seawater (reef salt water) and road salt run-off media were prepared by dissolving various amounts of the above reef and road salts ranging between 2.6, 3.5 % and 4.4 % (w/v) in deionized water. Sodium nitrate as nitrogen source (65mg/L) and potassium phosphate (5mg/L) was added to the media. The F/2 Gaillard's marine water enrichment solution 50x (Sigma Aldrich) as a source of trace minerals and vitamins were added to the reef salt solutions. The initial nitrate concentration were adjusted to 37.5, 75, 100 g /L in the micro algal growth media for the experiments of the investigation of the influence of initial nitrate concentration studies. The pH of the growth media in all the experiments were adjusted to 8.0 +/-0.05 using 0.1M HCl and or 0.1 M NaOH, the 40 mM Tris-HCl (Sigma Aldrich) were used as the buffer to restrict the changes in the pH of the media. Synthetic greenhouse effluents were prepared by dissolving the sodium nitrate and sodium phosphate as source of nitrate and phosphate were added as 75 mg NaNO₃, and 5.65 mg NaH₂PO₄·2H₂O. All the media were sterilization by 20 minutes of autoclaving at 15 psig.

The marine microalgae *N. gaditana* -L526- cultures were cultivated using the above synthetic reef salt and road salt media. The 2 L conical flask bioreactors with 1.5L of working volumes were used for batch cultures. The fresh cultures (7 days old) containing the biomass of approximately 500 ± 50 mg/L were used at the rate of 10 % (v/v) for the inoculation. All the cultivation studies were conducted under photoautotrophic mode by supplying the carbon dioxide at 1 vvm (volume of air /volume of culture /minute) by aeration. The fluorescent light bulbs were used to provide the light of 100±20 micromole photon /m²/sec intensity on the reactor surface to support the cell growth. The light dark cycles of 12:12 hours were maintained and cultivation was continued for the duration of 21 days.

3.3.3 Experimental set up and cultivation conditions for fed-batch cultures

The stock cultures for the study were cultivated in batch in 4 L Erlenmeyer flasks containing 3L of media and the fed batch culture studies were conducted in 6 L Erlenmeyer flasks containing initially 2 L of media under the photoautotrophic growth conditions. Air was injected at 1% vvm to media to provide the carbon dioxide (CO₂) and agitation and the flow was controlled with rotameters (Multi-tube rotameters and gas mixer, Omega, Stamford, USA). The photoperiod of 12 hour light and 12-hour dark were used for the study at room temperature (23±2 C) with the average light intensities of 100±10 and 200±10 μmol photons/m²/s) were provided by white fluorescent lamps.

Fresh microalgae stock cultures containing average 550 ± 50 mg dry weight biomass per liter were used (10 % (v/v) as the inoculum for the fed-batch system. Fed-batch cultivations were initiated by feeding the nutrient feed media to the batch cultures (initial nitrate concentration average 100 mg/L) on the eighth day when the nitrate concentration was decreased below 10 mg/L. Pulse (intermittent) feeding was carried out as follows. On the eight day, a known volume of nitrate feed (pulse) (from 100 mL to 200 mL at 100 mg/L nitrate) was added to the 6 L flask every two days at a fixed time till day 18. Five feeding rates (10, 12.5, 15, 17.5 and 20 mg/day) were investigated for this mode at two different light regimes. Continuous feeding was done by pumping nitrate feed at 10, 12.5, 15, 17.5 and 20 mg/day) till day 18. For staged feeding, known volumes of nitrate feed (500 ml (50 mg/L)) were added to the microalgae culture on Day 5, 10 and 15. This staged feeding mode was repeated for initial nitrate concentrations of 50,75 mg/L. The growth experiments were conducted in duplicates and the analytical methods were applied in triplicates and the averages of the values were used.

3.3.4 Flocculation experiments

The algal culture suspensions from the batch culture were used from the above condition (100mg/L nitrate) was used for the flocculation experiments. The pH induced flocculation methods for the harvesting were conducted by adding 0.1M NaOH (Sigma Aldrich) solutions as flocculating agent to obtain the desired pH in the media.

The micro algal suspensions of 250 ml were placed into each of the 500 mL flasks, and the pH of the media were adjusted to 9.0,9.5, 10,10.5, 11 11.5,12 and 12.5 experiments otherwise the pH was kept at 8.0 +/-0.05

The stock solution of FeCl₃(10g/L) prepared by dissolving (of FeCl₃ (Sigma Aldrich) in deionized water used as inorganic flocculants. The stock solutions of three chitosan (1g/L) prepared by dissolving chitosan in 1% acetic, 1% citric and 1% HCl acid solutions by continuous agitation until a clear solution was obtained was used as the organic flocculants for the treatments. The pHs of the chitosan solutions were approximately below pH 2.

The conductivity of the road salt media was and culture suspension were measures in conductivity meter (even compact 230 conductivity meter, Mettler Toledo, Canada) and the values of 32.7 mS/cm for the culture suspension for the media and 38.7 mS/cm were obtained

3.4 Analytical Methods

3.4.1 Cell count and biomass concentration quantification

The biomass cell densities were measured by cell count using a heamocytometer. Microalgal samples were drawn using syringe every two days and were placed to the counting chamber of the heamocytometer (Hausser-Scientific, Horsham, PA, USA) and counted using microscope (Leica Microsystems, Weizlar, Germany) with 40x magnification. The growth experiments were conducted in duplicates and the analytical methods were applied in triplicates and the averages of the values were used. Biomass dry weights were calculated gravimetrically at the end of the cultivation period, by vacuum filtering the 50 ml volume of the samples using pre-weighed 0.2 μm pore size filters. Filter papers were dried in vacuum oven at 70⁰ C for 24 hours. Mass differences were used to quantify the algal dry weight (m) and biomass concentrations were calculated using the following equation.

$$\text{Biomass X } \left(\frac{\text{g}}{\text{L}}\right) = m \cdot \frac{1000}{50} \quad (3.1)$$

Where m, is the algal cell dry weight (g) in 50 ml of growth media

Specific growth (μ) rate was determined by the slope of the trend line of the plot of the natural logarithm of the cell count versus the cultivation time (days).

$$\mu (/day) = \left(\frac{\ln \frac{X_t}{X_0}}{t_0 - t} \right) \quad (3.2)$$

3.4.2 Nitrate measurements

The nitrate concentrations in the growth media were measured using HACK kits. The Nitrate high range TestTMN tube (0 to 30.0 mg/L NO₃-N) HACH kits and spectrophotometer DR 2800 (HACH, Loveland, Colorado, USA) were used. In this a known volumes of growth media from the cultivation reactors were drawn and the cells were filtered using syringe filters (0.45 μm). The cell free media were analyzed for the residual NO₃-N using HACH kits and HACH spectrophotometer-DR 2800 (HACH company, Loveland, Colorado, USA).

3.4.3 Chlorophyll quantification

Chlorophyll “a” was estimated following the method described by Porra et.all (^{169,170}).In this method 10 ml sample of algal suspensions in the test tubes were centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and 10 ml of 95% methanol was added to the cell pellet. The test tubes were covered with aluminum foil to reduce the photo oxidation and placed in a water bath at 60⁰C for 60minutes to ensure complete chlorophyll extraction. The tubes were then centrifuged again at 5000 rpm for 10 minutes to separate the cell debris. The absorbance of the supernatant containing extracted chlorophyll “a” in methanol was measured at 652 nm and 665 nm using a Varian Cary 50 Bio- UV visible spectrophotometer (Varian, Palo Alto, California). Chlorophyll “a “concentration was calculated using the following formula.

$$\text{Total Chlorophyll } (\mu\text{g/ml}) = \text{Chlorophyll 'a'} (\mu\text{g/ml}) \quad (3.3)$$

$$\text{Chlorophyll 'a'} (\mu\text{g/ml}) = 16.29 \times \text{Ab}_{665} - 8.54 \times \text{Ab}_{652} \quad (3.4)$$

3.4.4 Lipid quantification

Biomass was harvested at the end of the cultivation by centrifugation (Sorvall ST40, Thermo ScientificTM (Life Technologies Inc. Burlington, ON, Canada) for 20 minutes at 3400 rpm and 4°C. Pellets collected were freeze dried using “Free Zone” (Labconco, Kansas City, MO, USA) vacuum freeze dry system. Freeze dried biomass were used for the evaluation of cellular lipid concentration using gravimetrical method¹⁷¹. A known amount of dried biomass was

homogenized with 10 ml of hexane -isopropanol (3:2 v/v) extraction solvent by vortexing for 30 seconds. These mixtures were placed in test tubes covered with aluminum foil and kept overnight for the complete lipid extraction. A known amount of sodium sulphate solution added to remove the phospholipids from the mixtures. The mixtures were then vacuum filtered and the filtrate of the lipid extracts were mixed with 0.2 times its volume of water and allowed to separate into two phases by centrifugation at 3500 rpm for 3 minutes. The upper phase was containing total lipid were carefully removed and placed in pre-weighed aluminum pans for the evaporation of solvent. Lipid content was calculated gravimetrically by the weight differences and the percentage of lipid determined by using the following equation.

$$\text{Lipid \%} = \left(\frac{\text{Lipid mass (g)}}{\text{Dry mass of micro algae (g)}} \right) \times 100 \quad (3.5)$$

3.4.5 GC-FAME analysis

Lipid profiles were estimated by the GC method for the quantification of the triacylglyceride (TAG) by Fatty acid methyl esters (FAME). Lipids extracted from the above methods were subjected for the FAME analysis. This included two steps of process lipid trans-esterification and FAME analysis as described below.

Lipid extracted from microalgae (~100 mg) dissolved in 10 ml of hexane and 100 μl of methanolic 2N KOH (11.2g of KOH in 100 ml methanol) in a test tubes. The samples were mixed for 30 seconds using vortex for the trans- esterification process and the samples were allowed to settle the pigments and non-lipid compounds. 1mL of clear samples from the top was carefully transferred in to GC vials and was stored at -20C to prevent evaporation. FAME was analyzed by injecting 2 μL samples into an Agilent 7890A GC-flame ionization detector equipped with a 30mX0.32mmX0.25 μm J&W HP-5 column. Oven temperature was kept at 80°C for 2 minutes, then heated up to 140°C at the rate of 20°C/min, and then to 260°C at the rate of 4°C/min. Temperature was maintained at 260°C for 10 minutes. Values obtained are used to determine the lipid profile and quantification of TAG¹⁷².

3.5 Determination of flocculation efficiency

Flocculation experiments were conducted using the Jar test in 50 ml Falcon test tubes and 100ml measuring cylinders. In the first step, 250 ml of the algal cultures from the reactors were

distributed in to the 500 ml of flasks for the pH adjustments and addition of various amounts of flocculants. The flasks were agitated rigoursly at 200 rpm for 2 minutes and were subjected for the slow agitation at 100 rpm for another 5minutes. These suspensions (each 45 ml of the mixtures) were immediately transferred to the 50 ml of Falcon test tubes. The 100 ml of the suspensions were transferred to the 100ml measuring cylinders. The suspensions were left to settle for 15, 30, 45 and 60 minutes without agitation in the falcon test tubes and measuring cylinders. At the end of the settling time, the optical density (OD) of the supernatant from half the height of the clarified layer were measured using spectrophotometer (Varian Cary 50 Bio- UV visible spectrophotometer (Varian, Palo Alto, California) at 682nm. Reference blanks were used for every flocculating agent (growth media + used flocculants concentration) in order to take in to the influence of the dissolved flocculants on absorbance.

The harvesting efficiency (HE) was defined as the ratio of the mass of cells recovered to the total mass of cells was calculated using the following equation

$$\text{Harvesting efficiency (HE \%)} = (A - B/A) \times 100 \quad (3.6)$$

Where A represents the OD 682 of the control algal culture without the added flocculants and B is the optical density of the supernatant flocculants after the treatments are calculated below

A= OD 682 of the initial culture

B= OD 682 of the supernatant

The samples of the measuring cylinders were used for measuring the concentration factors. The concentration factor was the ratio of the final product concentration to the initial concentration¹⁷³. Concentration factor were calculated as below.

$$\text{Concentration factor (CF)} = \frac{V_i}{V_i - V_s} \quad (3.7)$$

Where, V_i is the volume of micro algal suspension before flocculation, and V_s is the volume of microalgal supernatant after flocculation.

3.6 Statistical methods

All the experiments were conducted in duplicates and triplicate samples were taken each time for the analysis. The average values and standard deviation were determined and used in the results.

Chapter 4

4 Investigation of phyco-remediation of road salt run-off with marine *N. gaditana*

Summary: Phyco-remediation is an environmental friendly method, which involves the application of beneficial microalgae to treat wastewater-containing pollutants for a diverse range of conditions. Several industrial processes generate hyper saline wastewater, which is a significant challenge for conventional wastewater treatment, and the disposal of saline waters also has a significant negative impact on the environment. Road salt run-off is one such saline wastewater stream not currently treated and one that contributes significant to negatively impacting receiving bodies of water. In the present study, phyco-remediation using the halophilic marine microalgae *N.gaditana* was applied to investigate the removal of nutrients from road salt run-off. The biomass of these algae has high lipid content and they are therefore considered suitable feedstock for the biofuel production. In this study, *N.gaditana* microalgae were able to assimilate >95 % of the nitrates within 8 days in road salt concentrations ranging from 2.6 to 4.4% under photoautotrophic cultivation method. Biomass yield of 1.1 g/L of culture obtained with the maximum lipid of 22% (g/g) biomass in the road salt media. Fatty acid composition analysis of the obtained lipid indicated C16 and C18 as the major components (~ 45% of FAME) further confirmed the suitability of biomass generated could be used for biofuel feedstock. This study established that the use of road salt run off containing nitrate and phosphate nutrients will support the growth marine micro algae for remediation of waste water system that are the concern at winter prevalent regions.

4.1 Introduction

The application of road (de-icing) salts (e.g. sodium chloride) is carried out on roads and highways in certain areas of Canada and northern United States during the winter months. This allows for ice and snow to melt due to lowered freezing points of the mixture. The subsequent brine solution drains as road salt runoff into underlying soil or surface waters or storm water management ponds (SWMPs) and sewer systems. The partial replacement of de-icing salts with biodegradable materials adds COD, nitrates and phosphates to the salt run-off hence may increase eutrophication of receiving bodies of water. A large amount of run-off can end up in catchment

basins and SWMPs. Nitrogen- and phosphorus pollution also arises from urban runoff from roads and highways, residential areas and grassland, vehicular exhaust, or overuse of fertilizers. Phosphorus is often found to be the limiting nutrient for freshwater aquatic ecosystems, but can increase in storm water detention ponds with time. A significant issue with phosphorus accumulation is production of toxins such as microcystins during harmful algal (cyanobacterial) blooms (HABs) which can occur in the SWMPs due to the high phosphorus levels¹⁷⁴. The HAB also lead to eutrophication due to oxygen depletion.

Microalgae are a diverse group of single celled photosynthetic organisms grows in wide range of aquatic environments from fresh to saline water using carbon dioxide and sunlight. Phycoremediation applies beneficial microalgae for the removal of nutrients from the waste water⁵. The produced micro algal biomass is rich in lipid content and has considerable potential for biofuel production or nutraceuticals^{15,29,36,37,175}. Microalgae during photosynthesis utilize solar energy and several essential nutrients (C, N, P, S, K, Fe etc.) to synthesize biomass compounds and to multiply their cells. Typical cell composition of microalgae undergoing photosynthetic growth requires the addition of nutrients in the so-called Redfield ratio of N: P: 16:1^{28,176}. Light and nutrient supply controls the growth kinetics of microalgae¹⁷⁷. Variation in the growth conditions and nutrient shifts induce stress that diverts the metabolic pathways directing the synthesis of high value secondary metabolites like lipid and pigments^{58,95,178}.

N.gaditana a species of marine micro algae has been reported to have an optimal growth in salinity ranging from 0.5 M to 1.0 M NaCl. Biomass of this algae is rich in lipid and pigments like astaxanthin which are of great commercial interest¹⁸. *Nannochloropsis* sp. also exhibit a diverse fatty acid composition due to their physiological and eco-physiological variabilities¹⁷⁹. Marine microalgae are extremophiles are robust organisms producing stable enzymes, and are often able to tolerate changes in environmental conditions¹⁸⁰. If biodiesel is to become an economically viable resource, more efficient novel sources of oil, such as microalgae as well as from extremophiles organisms, need to be researched. The advantage of using extremophile microalgae would be to minimize contamination within the photo bioreactors, which tends to be problematic in outdoor cultures¹⁸¹. Although its cultivation using salt media like road salt run off not previously reported.

Microalgae biomass have low cellulose content compared to the terrestrial crops and thus the nutrient demand is much higher⁶⁴. Mass production of microalgae for biofuel requires massive quantity of nutrients. In large-scale cultivation systems, large amount nutrients like nitrogen, phosphorous and carbon are required to supplement the cultivation medium to the needs of micro algal growth under the specific environmental conditions, in order to achieve high yields. Many studies evaluated the potential of various micro algal monocultures for nutrient removal and biomass production in various types of wastewaters. Availability of the major and minor nutrients affects the cellular biochemical reactions and production of biomass and metabolites were noted. Since, wastewaters differ in their physicochemical characteristics, which directly or indirectly have an effect on algal growth⁶⁴. The present study examined the growth kinetics of *N.gaditana* on synthetically prepared (simulated) road salt run off media and reef salt media (artificial seawater) to investigate its suitability for phyco-remediation. The generated biomass was further evaluated for lipid content to determine its suitability as feedstock for the production of biofuel production by determining the fatty acid profiles.

4.2 Results and Discussion

Road salt run-off is highly variable wastewater and the composition of nutrients (nitrate and phosphate) present in the run-off depends on location, time of year and weather. Various nutrients are required to carry various biochemical reactions to support the growth of microalgae. Nitrogen is the critical nutrient required for the production of nucleic acid, protein, chlorophyll and enzymes. Phosphorous required for the synthesis of energy transfer system of the cells such as ATP, NADPH and phospholipids. Other nutrients like Ca, Mg activates the acetyl CoA and NADPH and fatty acid synthesis¹⁸². Iron (ferric) one of the most important elements required by most microalgae. Ferric ion involves in fundamental enzymatic reactions, photosystem II (PS II), nitrogen consumption and chlorophyll synthesis in the algal cells¹⁸³.

The compositional characteristics of 3.5 % solution of road salt and reef salt solution were first analyzed for the presence of major and trace metals and are shown in Table. 4.1. All elements (major and minor nutrients) found in seawater were identified in the road salt solution with varied concentration. The concentration of sodium ions noted higher in road salt media (5339 mg/L) than in the reef salt media (2229 mg/L). Sodium and chloride are the key salinity providing elements which support the growth of the halophilic microalgae was present in ~2.4x and calcium about

1.4x concentrations in road salt solution. Apart from these, sulphur, magnesium, ferrous and cobalt are also present in a higher concentration. Elemental compositional analysis identified the presence of all most all the ions that would support the growth of marine microalgae in the simulated road salt indicated the feasibility to use road salt run off as base media for the cultivation. Various concentrations of salt and nitrates were used to mimic the field conditions to determine N removal efficient for its application to the phyco-remediation.

Table 4-1 Elemental concentration of reef and road salt media analyzed by the ICP- MS

| Elements in the media (mg/L) | 2.6% Road salt (w/v) | 3.5% Road salt (w/v) | 4.4% Road salt (w/v) | 2.6% Reef salt (w/v) | 3.5% Reef salt (w/v) | 4.4% Reef salt (w/v) |
|------------------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| Na | 3964 | 5336 | 6708 | 1655 | 2228 | 2801 |
| Mg | 5 | 6 | 8 | 4722 | 6357 | 7991 |
| Al | 0.3 | 0.3 | 0.4 | 0.1 | 0.2 | 0.2 |
| Si | <0.01 | <0.01 | <0.010 | <0.01 | <0.01 | <0.01 |
| P | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| S | 32 | 42 | 53 | 167 | 225 | 283 |
| K | 3 | 4 | 5 | 169 | 227 | 285 |
| Ca | 102 | 138 | 173 | 147 | 198 | 249 |
| Mn | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |
| Fe | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 |
| Co | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Cu | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |
| Zn | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |
| As | 0.4 | 0.5 | 0.6 | 0.0 | 0.0 | 0.0 |
| Sr | 2 | 3 | 3 | 6 | 8 | 10 |
| Ba | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |

4.2.1 Effect of media and salt concentration on growth kinetics of *N.gaditana*

The growth kinetics of marine algae was studied by analyzing the cell density and pigment concentration. The cell multiplication time profile was represented by the growth curves. The various stages in growth curves identify the status of the cells in the media, which are specific to species, and are influenced by the environmental and nutrient conditions of the media. The typical growth curve includes the lag, exponential, stationary and decline stages for microalgae. Experiments were conducted to understand the growth kinetics of algal cell production for 21 days

of cultivation time at three salt levels of media. The results are shown for road salt (Figure 4.1) and reef salt (Figure 4.2). It was observed that *N.gaditana* cells rapidly adapted to the simulated growth media as noted by the short lag period at all levels of salt concentrations. The active growth phases (exponential growth) from day two showed a linear increase in cell density. This growth phase continued without reaching the stationary phase (in 21 days) in 2.6% reef salt. In 3.5% reef salt media showed a decline in growth phase on 16th day and maintained the stationary phase (in 21th day).

Although, all these culture media started from the similar concentration of substrate nitrate (65mg/L) and initial cell concentration (~ 35-45 10^5 cells /ml), two noticeable apparent growth phases, an active growth phase and the stationary phase, were observed. The highest cell density (2.93×10^7 /ml) and (3.18×10^7 /ml) in 3.5% reef and in 4.4 % road salt respectively were observed during the active growth on the 14th day of the cultivation.

This variation in the cell density can be attributed that the value of salt concentration particularly the sodium ion in the media affected the algal growth due to the variation in osmotic concentration and its effect on permeability of other elemental compounds to cytoplasm for the cellular biochemical reaction that shifted the cell growth cycles.

Cells started to grow at relatively higher concentration in road salt media than in the reef salt at elevated media concentrations. In general road salt media showed increased cell counts than the reef salt media even though the initial N and P concentration, light and carbon were maintained at the same level in all the experiments. Many species of microalgae exhibit positive growth under nutrient sufficient conditions with optimum environmental conditions like light and carbon dioxide supply were provided. N and P are the key nutrients promote growth significantly¹⁸⁴, Iron, copper were also noted as influencing elements that at specific concentration increases the cell density.

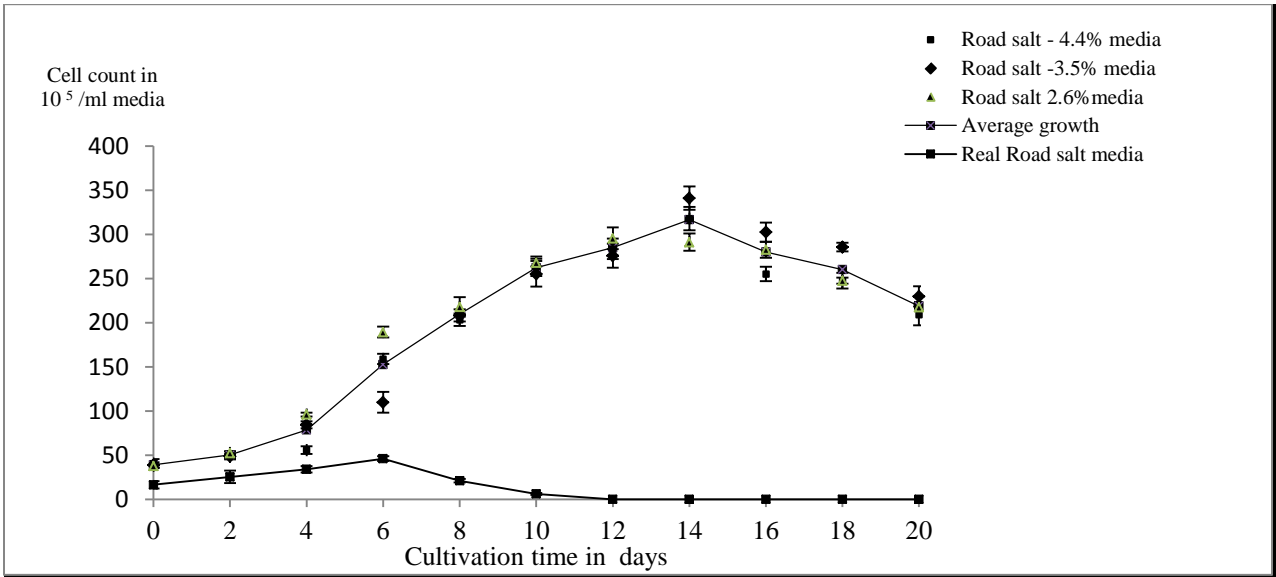


Figure 4:1 Growth curves by the cell count of the *N.gaditana* in road salt media

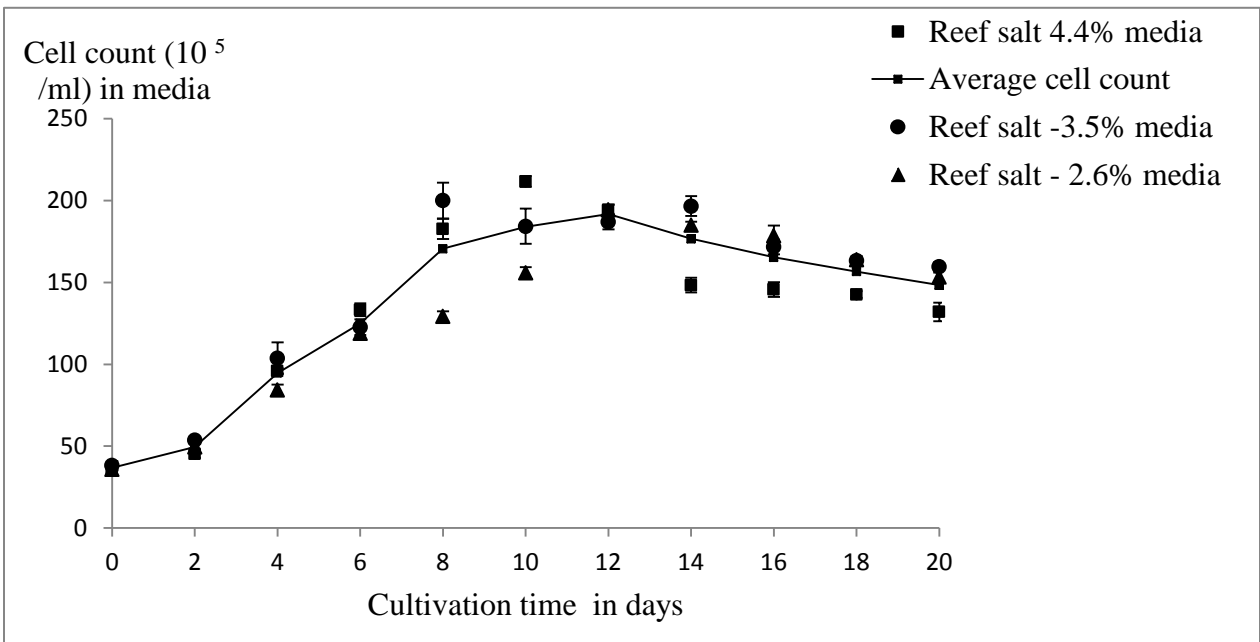


Figure 4:2 Growth curves by the cell count of the *N.gaditana* in reef salt media

Copper was reported to increase the chloroplast and lipid content of the cells ¹²⁶ by promoting esterase activity. Since the amount of trace metals were noted higher concentration in road salt media than in the reef salt media that directly influenced the cellular activity and generations.

Further observation on the varied salt concentration demonstrated that higher salinity resulted in lower cell concentration. Optimum salinity for the growth of the marine microalgae is in the range of seawater but the halophilic microalgae have the varied salt tolerance with the tendency to grow in the wide range of salt concentration. The cell count data of this study showed steady increment of cells reaching the maximum of $(3.18 \times 10^7/\text{ml})$ in 4.4% road salt base media in 14th day as the optimum level. But these reactors noted the salt precipitation indicating the concentration of this level is too high to apply to the field condition where there would be a possibility of evaporation, which might lead to osmotic shock and hinder the cell growth if the cultivation period prolonged to 21 days.

The pigments play vital role in photosynthesis to capture the light energy for the conversation to chemical energy in autotrophs. *Nannochloropsis* species of genera *Eustigmatophyceae* consists only chlorophyll “a” (Chl-“a”) pigment with several kinds of carotenoids¹⁷⁹. The level of Chl-“a” in the algal cells indicate the physiological state of the culture ¹⁰³. In order to explain the difference in the growth profile between the reef and the road salt media the Chl- “a” concentration measured during the various growth stages and the results shown in Figure.4.3 and 4.4

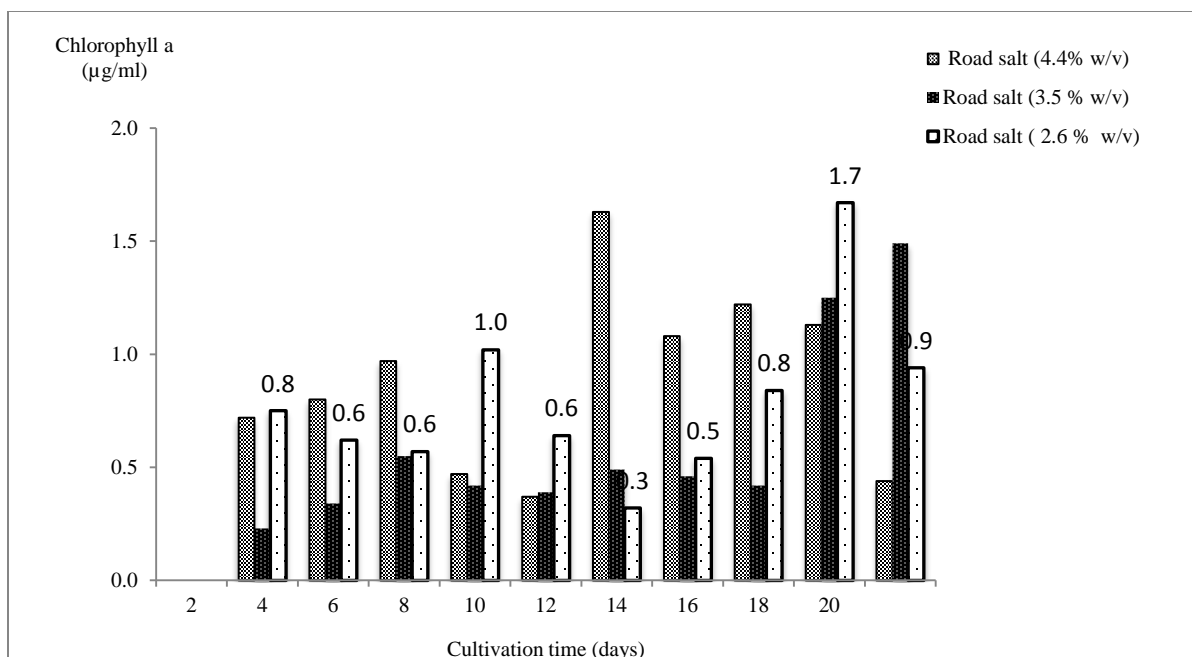


Figure 4:3 Cellular concentration of chlorophyll “a” in biomass of road salt media

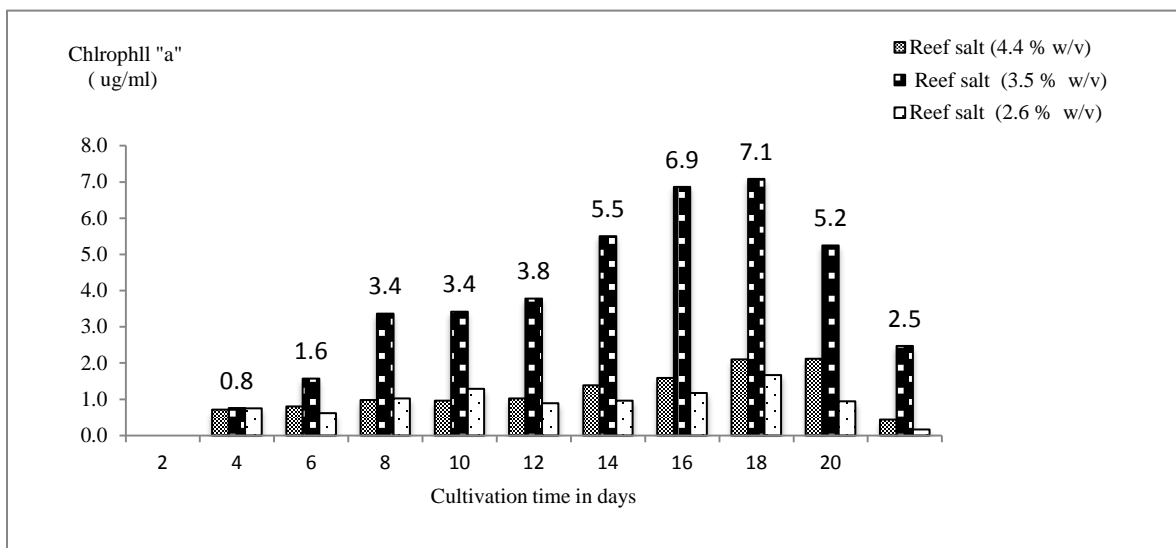


Figure 4:4 Cellular concentration of chlorophyll “a” in biomass of reef salt media

Varied salinity levels affected the Chl-“a” content of cell mass. In general, steady increment of Chl-“a” concentration observed as the cultivation time progressed. The decline in Chl-“a”-content observed when cells reached to the stationary level in all the experimental conditions. The highest concentration of Chl-“a” (7.0µg /ml) noted in 3.5%

reef salt media and the road salt media provided only 2.1 µg /ml (~1/3rd) in 4.4% media. The higher reef salt media concentration (4.4%) resulted in a maximum pigment concentration in day 18. Elevated road salt (2.6% and 4.4 %) resulted in lower pigments of 1.67 and 1.49µg /ml in day 18 and 20 respectively. However, the lowest concentrations of Chl-“a” were detected in both the lower (2.6%) salt levels.

Chlorophyll production is the key indicator of the ability of photosynthesis and cellular growth. Chlorophyll is the green pigment responsible for the transfer of light energy and its conversion in autotrophic green algae and land plants ¹⁷⁹. Chlorophyll structure consists of N and composed of four pyrrole group forming around the magnesium atom¹⁸⁵ indicating the requirement of N and magnesium for the biosynthesis of chlorophyll pigment. Many studies also noted the environmental factors including pH, light and temperature are known to influence cellular physiological characteristics such as the nutrient removal and the yield of pigments ¹⁸⁵.

Meanwhile, Chl-“a” concentration followed the cell growth pattern and was steadily increased when the cells reached to exponential growth phase indicating the photosynthesis correlation of pigment and the cell growth. It is known from the previous studies that the chlorophyll and some carotenoids can absorb light energy and collect in the core reaction of photosynthetic process. The cells attempts to increase the bioenergetics yield by increased biosynthesis of chlorophyll to absorb more light ¹⁸⁶. The total chlorophyll production also always depends on the cellular concentration and nitrogen sources in addition to light energy.

The pigment Chl-“a” concentration in the cells from different cultivation time as function of media concentration, increased as the cultivation period progressed in both media even after the nitrate depletion until the stationary growth stage indicating that the Chl-“a” production is growth dependent. Secondary carotenoid accumulation known to be promoted by nitrogen starvation in aged cultures of many microalgae species, indicating the cessation of chlorophyll for major carotenoid synthesis ¹⁷⁹. The study on *Chlamydomonas* and *Coccomyxa* suggested during the nutrient depletion the turnover of nitrogen-rich compounds such as proteins might provide carbon and energy for TAG

biosynthesis for the nutrient deprived cells¹² where the left over nitrogen from the protein structures further utilized for the generation pigments illustrating the continued chlorophyll production in the nitrate depleted media¹⁰³. Increased cell density during the late stages also limits the light availability for the photosynthesis. In conclusion, variation in the Chl-“a” concentrations of *Nannochloropsis* were observed in the two tested media with the highest in reef salt media than the road salt media due to the compositional variations observed in these media. Cell count and Chl-“a” concentration directly affected by the level and type of base media used were noted in this study.

4.2.2 Effect of type of media and its salt concentration on nitrate consumption of *N. gaditana*

In order to understand the application of phyco-remediation technique to the road salt run off, the growth kinetics of the cells in related to the substrate nitrate, time course profiles of nitrogen concentration in the media during the cultivation period were analyzed. Kinetic profiles of nitrate consumption in the studies determined by analyzing the residual nitrogen-nitrate (N) levels of the cell free media at the fixed time periods during the growth cycle were plotted in the Figures 4.5 and 4.6. Initial N level in all the media were maintained around 65mg /L. Complete consumption of the nitrogen nitrate in culture media recorded when the readings were below the detectable limits of the test method. Concentration of the N level decreased as the cell growth progressed in all the experimental conditions. Faster reduction of nitrate observed in reef salt than in road salt media. Nitrate levels decreased to nil (100% removal) and 5 mg/L (92 % removal) in 3.5% and 4.4% at day 8 respectively, while 2mg/L (97% removal) in 2.6% in 6 days in reef salt media. Nitrate concentration depleted to 3.4 mg/L (95% removal) and 2.7 mg/L (96 % removal) in 2.6% and 3.5% and to 0 mg/L (100% removal) in 4.4% road salt media at 8 and 6 days respectively indicating that *N.gaditana* microalgae were effective for removal of nitrate from the reef and road salt media.

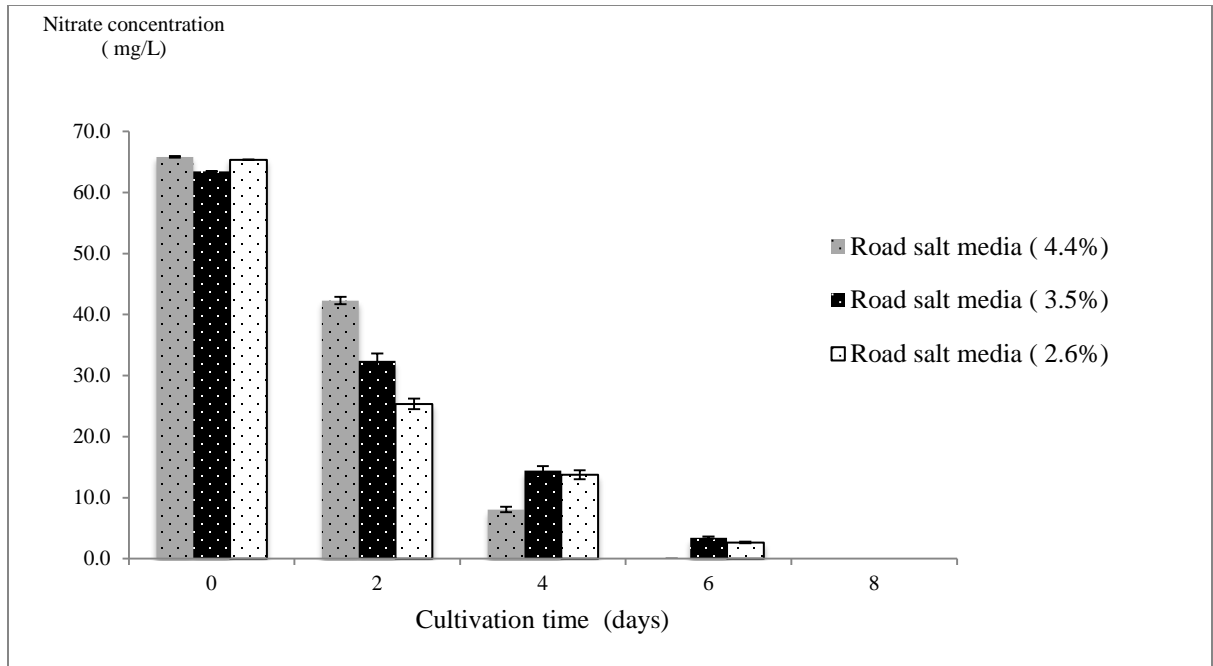


Figure 4:5 Concentration of Nitrate -Nitrogen versus cultivation in road salt media

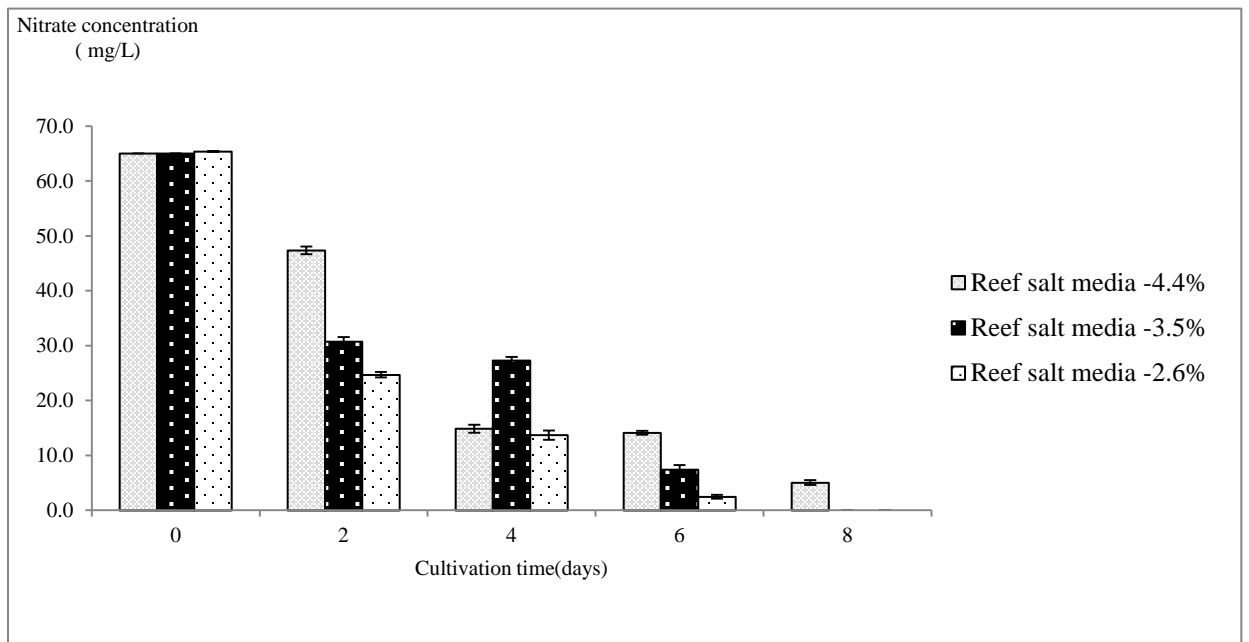


Figure 4:6 Concentration of Nitrate -Nitrogen versus cultivation in reef salt media

Analysis of nitrate in cell free media during the cultivation period confirmed progressive N depletion with increase cell count indicating the consumption of the N by the cell for

the cellular biochemical reaction to generate the biomass. In general, time taken to deplete the N in reef salt media varied from the road salt media due to the concentration of the elemental composition also varied. The highest salt concentration (4.4%) reported longer time duration (8days) for the depletion of nitrate and the fastest depletion to the lowest value (~97%) was observed in day 6 noticed in reef salt media at lowest salt concentration (2.6%). Increased salt concentration decreased the nitrate assimilation rate in both the salt media indicating that the cellular nitrate assimilation further affected by the media type, concentration and composition but supported the cell growth indicating wide range of salt tolerance of the marine halophilic microalgae. Marine microalgae have the optimum growth at the salt level of 3.5 % that is equal to the salinity of seawater. Elevation from this condition induces a stress environment to the growth hence modifies the metabolism rate¹⁸⁷ thus the demands of the nitrate assimilation in to the cytosol. Nitrogen including phosphorus and carbon are the key elements that directly support the cell growth rate. Cells increase metabolism rate during the initial growth stage when the nutrients in the media are in abundant supply. Photosynthetic metabolism of microalgae is also supported by the availability of light and chlorophyll pigment¹⁸⁷. Nitrate consumed at day 8 in all the media during which the cell concentration increased progressively to the exponential growth stage. The work on fresh water micro algae - *Chlorella vulgaris* provided similar trends of nitrate consumption profile at varied salt concentration with the maximum assimilation of nitrates found at 2 to 4 days of cultivation where the cells are in exponential growth phase¹⁸⁸. This result indicates that the increasing salt concentration reduces and nitrate assimilation in reef salt while the optimum reef salt concentration of 3.5 % and 2.6 % road salt provided the fastest rate of nitrate consumption and indicating the optimal media concentration to consider for the phyco-remediation using the marine microalgae. The mechanism behind the effect of N depletion believed to be associated with decrease in intracellular chlorophyll, which play very important role in the cell division rate in addition to the other N containing cellular components. Algae use organic and inorganic N for the synthesis of amino acids and nucleic acid¹⁸⁹ and its demand is highest at the during the active growth stage. Hence the highest N assimilation is noted when the cells were in the exponential growth stage. However, the cell growth continues after the exhaustion of media nitrogen indicating the

nitrogen supply from intracellular storage such as RuBisCo supports the survival of cells. Nutrient starvation triggers the secondary metabolism leading to biosynthesis of lipid was also reported (30).

However the cellular growth rates were affected by the environmental and physical conditions of media. Variation in the salt concentration considered as the stress, which alters the cellular metabolic activities and multiplication rate. Thus the cellular nutrient demands in the stress induced media were different than the optimal growth conditions. In the study varied N depletion rates were noticed. Increased salt concentration showed the reduced N depletion rate and vice versa at the lower salt concentration of the media.

4.2.3 Specific growth rates of *N.gaditana* at various media salt concentration

Specific growth rate determined were presented for the both media at three level of media concentration (Table 4.2). Road salt at 4.4% and 2.6 % provided the highest growth rate with ($\mu =0.26/\text{day}$) while the least growth rate ($\mu=0.13 /\text{day}$) was noted in the lowest reef salt media (2.6%).

Table 4.2: Specific growth rates (μ) and the doubling time of *N.gaditana* in different cultivation media at 2.6, 3.5 and 4.4% (w/v) salt concentrations.

| Growth media | Media salt concentration (% w/v) | Specific growth rate μ (/day) | Doubling time (Td) (days) |
|--------------|----------------------------------|-----------------------------------|---------------------------|
| Road Salt | 4.4 | 0.26 | 2.7 |
| | 3.5 | 0.15 | 4.6 |
| | 2.4 | 0.26 | 2.7 |
| Reef Salt | 4.4 | 0.21 | 3.3 |
| | 3.5 | 0.24 | 2.9 |
| | 2.4 | 0.13 | 5.3 |

Higher specific growth rate resulted in higher biomass production. The change in the external salt concentration disturbing the ion homeostasis in cytoplasm and the varied cell capacity for the regulation of the Na^+ homeostasis during cell adaptation changing the growth rate were reported ¹⁹⁰ and was attributed to the results of in varied specific

growth rates. As noted earlier various environment factors (light and carbon source in autotrophs) and concentration of major and minor nutrients in the growth media and its bioavailability to the cellular reactions were responsible for regulating the rate of cell reproduction and thus shifting the specific growth rate ¹⁹¹

4.2.4 Effect of media, salt and initial media nitrate concentration on biomass production

Determination of biomass production is important in cultivation study of microalgae for its feasibility of its application to biofuel feed stock. Biomass quantities generated were determined at the end of cultivation (Figure 4.7). Changing the growth conditions directly affect the cellular production rate and thus biomass yield. The highest biomass of 1.8 g/L was obtained in the reef salt media at 3.5% salt concentration and lowest of 0.77 g/L biomass was noted in 4.4 % reef salt media. Biomass yield of 0.97, 0.99 and 0.88 g/L were obtained in road salt media at 4.4, 3.5 and 2.6 % salt concentration respectively. The most favourable salt concentration noted was 3.5% reef salt yielded the highest biomass. Increase the salt concentration by 25% to 4.4% reduced the biomass yield by 57% (0.77 g/L) and decrease the media concentration to 2.6% reduced the biomass by 55% and produced 1g/L of biomass. Biomass yield in road salt media obtained was ~ 1 g/L. Increase in the concentration of media increased the osmotic pressure. Osmotic up shocks generally cause severe temporary inhibition of photosynthesis in microalgae, however, osmotic down shocks have little effect on photosynthesis in microalgae with strong cell walls, while wall-less species indicated higher sensitivity. Rapid glycerol synthesis takes place in response to increased external salinity in *Chlamydomonas pulsatilla* were reported (29, 35, 34) indicating the cell protection mechanism than the cell multiplication on the salt stress conditions.

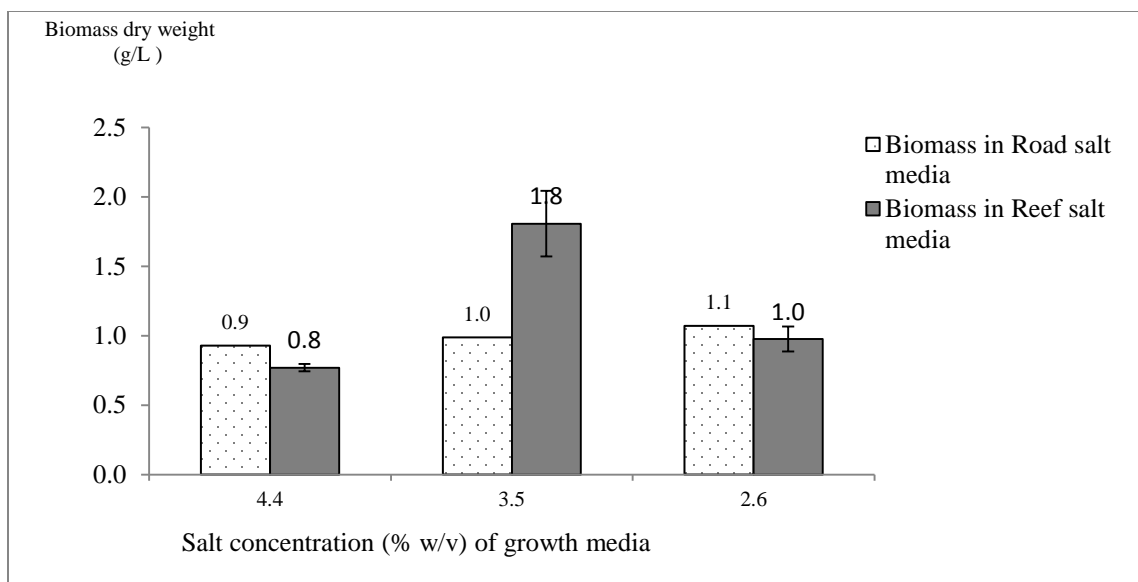


Figure 4:7 Biomass dry weight of *N. gaditana* in road and reef salt growth media

Biomass yield depends on the supply of the major and trace nutrients. In general for biomass growth (consisting of 40–50% carbon) microalgae depend on a sufficient supply of a carbon source and light to carry out photosynthesis²⁹ in addition to the abundant supply of Nitrogen and Phosphorous. *Nannochloropsis sp.* also could adapt to a relatively wide range of salinity and other growth conditions¹⁹² was the reason to obtain the similar level of biomass in all the conditions studied.

4.2.5 Effect of media, salt and nitrate concentration on lipid production and fatty acid profile of *N. gaditana*

The biomass accumulations in microalgae also depended on the availability of sufficient number of nutrients in specific ratio for the cellular activity with the growth supporting environmental conditions. As previously noted the nitrate plays major role in the cellular biochemical reaction. The influence of initial nitrate concentration on biomass production further studied using 2.6% road salt media at three external nitrate concentrations (37.5, 75, 100mg /L) to understand the influence of the substrates N in the on the biomass and lipid production in *N.gaditana* (Table 4.2) with an attempt to mimic the variability of the field condition for the feasibility of the phyco-remediated biomass for the biofuel feedstock production. In general, biomass production rate varied with the

change in the initial media nitrate concentration. Increasing the initial nitrate level in the media from 37.5 to 100mg/L increased the biomass to ~2.8x indicated the biomass generation is substrate dependent. Increase in the initial nitrate concentration to 100mg/L changed the biomass production to 1.78 from 0.7 g/L. The highest biomass of 2.0 g/L obtained at 75 mg/L nitrate containing media. The lowest biomass production noticed at the lowest nitrate concentration (37.5mg/L) indicating this level of nitrate is below the critical limit for the optimum growth. Since the limitation of the other elements like P and C in increased N (100 mg/L) might have resulted in slight reduction of the biomass from the maximum 2.0 to 1.78g/L. This study demonstrated the correlation resulting with variation in the biomass yields in road salt media and to the substrate N level as noted in the previous studied¹⁸⁰. Effect of media, salt and nitrate concentration on lipid production and fatty acid profile of *N. gaditana*

4.2.6 Lipid productivity and salinity

Many microalgae exhibit the ability to enhance lipid content during the stress conditions. Environment and nutrient starvation are the common stress condition. Varying the salt concentration widely applied as cheap stress inducer to trigger the secondary metabolism and to enhance the lipid content of cells¹⁹³. Lipid and biomass to sodium conversion in the tested salt concentration of both growth media were determined to understand the influence of the fluctuations of the salt as stress inducer on the lipid production (Table 4.3). Lipid production of 53.4% and 56.2% (w/w) was observed in the biomass obtained from the 2.6 and 4.4 % and the average of 18.6% (w/w) in 3.5% reef salt media. As the road salt level increased the lipid content in the cells also steadily increased from 17.0 to 21.6% (w/w). Lipid production changed from 23 % (w/w) to 28% (w/w) when the nitrate level elevated from 75 mg/ L to 100 or 37.5 mg/L indicating substrate stress changed lipid production within the tested ranges on nitrate.

The salt stress and substrate starvation affected the lipid production in the study. The lipid content increased from 17% to 38% as the concentration of NaCl increased from 0 to 400mmol/L were noted in previous studies on freshwater microalga *Chlamydomonas mexicana* grown on municipal wastewater with different levels of salinity¹⁹⁴.

Table 4-2 Biomass and lipid to sodium conversation

| <i>Salinity</i> | <i>Media</i> | <i>Na concentration (ICP) g/L</i> | <i>Lipid (% w/w) in biomass</i> | <i>Biomass production (g/L)</i> | <i>Biomass / Na+</i> | <i>Lipid / (biomass / Na+)</i> |
|-----------------|--------------|-----------------------------------|---------------------------------|---------------------------------|----------------------|--------------------------------|
| 4.40% | | 2.8 | 56.2 | 0.8 | 0.3 | 0.16 |
| 3.50% | Reef salt | 2.2 | 18.6 | 1.8 | 0.8 | 0.15 |
| 2.60% | | 1.6 | 53.4 | 1.0 | 0.6 | 0.32 |
| 4.40% | | 6.7 | 21.6 | 0.9 | 0.1 | 0.03 |
| 3.50% | Road salt | 5.3 | 20.9 | 1.0 | 0.2 | 0.04 |
| 2.60% | | 4.0 | 17.0 | 0.9 | 0.2 | 0.04 |

This phenomenon was widely studied and explained noting the cellular lipid production is formed by the secondary metabolism, and is typical considered as the protective mechanism that all microalgae undertake during the various stress conditions¹⁹⁵. During the substrate nitrate source depleted in the media cells derive nitrogen from the cellular storage like protein and leading to increase the cellular carbon levels. This increased level of cellular carbon used for the formation of the fatty acid and thus the lipid formation. Additionally, cells triggers the protective mechanism and the formation of lipid in response to the unfavorable environmental condition like changes in temperature, pH, osmotic level etc.¹⁹⁶ were also reported. Stressing the micro algal cell through salt may cause additional lipid accumulation metabolism than stressing through nutrients limitation were also noted⁸⁹. Through our study we noted cultures experienced increased salt concentration from 2.6% to 4.4% exhibited increased total lipid accumulation (Figure 4.8). The mechanism of increased demand for nitrogen keeps healthy growth of cells under high salinity conditions to prevent cell membranes from being destroyed by changing osmotic pressure also result in lipid accumulation¹⁹⁷. Salt stressing used in the fresh water based microalgae cultivation but is not reported for the marine microalgae. Typically the salinity of the seawater in the ocean ranges due to the evaporation and dilutions from 2.6 to 3.5 %. This fluctuation also anticipated during the open pond cultivation of micro algae. Salinity fluctuation plays a positive trend on cellular lipid accumulation and predator control were also noted¹⁸⁷. Our study suggests marine

microalgae N.gaditana biomass production for biofuel might work best if the algae grown at lower salinity than the higher salinity and this technique allowed for the higher lipid accumulation rates. Varying the type of the base media reduced the biomass yield and lipid content maintained at 20.6% of the biomass and is in consistent to the studies reported of 19.3%¹⁹⁸ for the *Nannochloropsis* species.

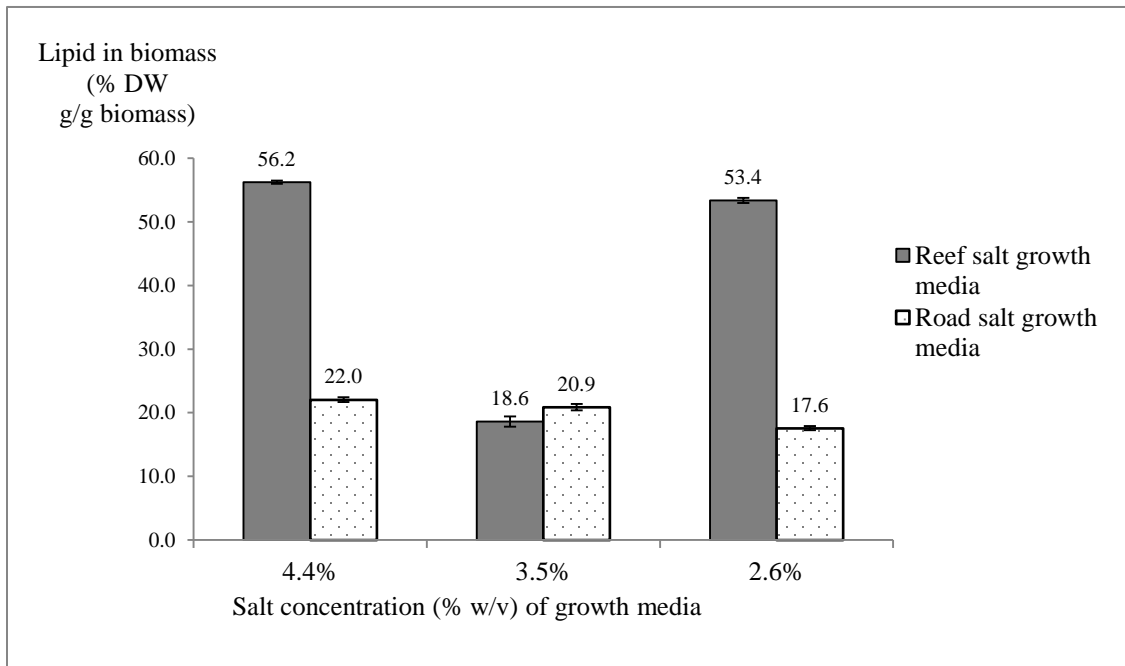


Figure 4:8 Lipid concentrations (%w/w) of *N. gaditana* in the biomass

4.2.7 Effect media, salt and nitrate concentration on fatty Acid profile

Composition of fatty acid in the lipid predominantly determines the quality of biodiesel and its suitability to use as biofuel. Therefore, the lipids extracted from the cells were profiled by using the GC- FAME analysis and the fatty acids identified listed (Figure 4.9)

The highest percentage of FAME (31% and 39% of lipid recovered) observed in 3.5% reef and 4.4% road salt growth media when the biomass yield was highest. Elevating the media concentration from 3.5 to 2.6% decreased the FAME from 31% to approximately 28% in reef salt. While the steadily increased percentage of FAME from 35% to 39% observed as the concentration of road salt media increased from 2.6 to 4.4%. The

predominant fatty acids identified in the analysis were palmitic acid (16:0), palmitoleic acid (16:1) irrespective of the media concentrations. Long chain fatty acid (>C16) accounted over 90% with less than 5% relatively short chain fatty acids (< C14) of the total fatty acid in both media. Over 90 % of C 16 and around 3- 4% of C18 were detected in the biomass obtained by the cultivation in the reef salt media. Around 67% of C16 and around 20 % of C18 were detected in the biomass obtained in the road salt media. Presence of C16 and C18 levels are used as the indicator for the selection of the biomass for the feed stock for biofuel ¹⁹⁹

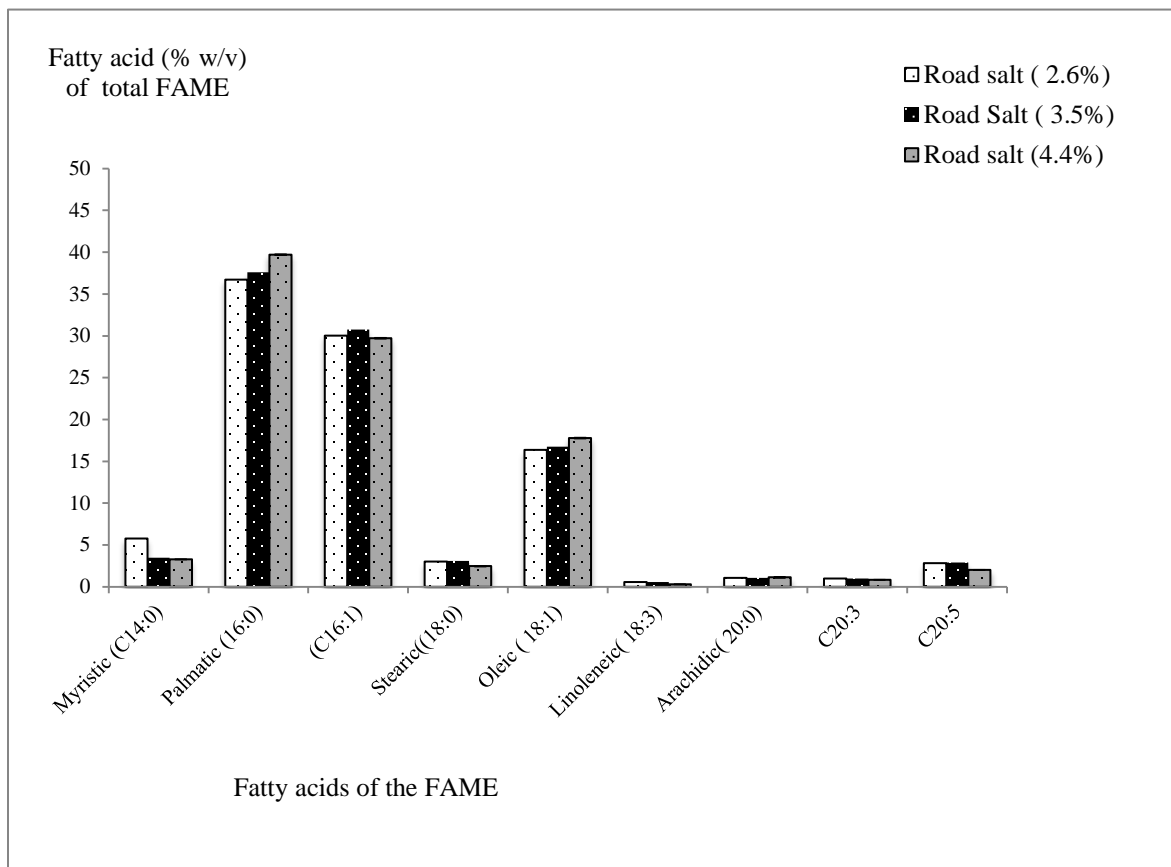


Figure 4:9 Fatty acid profile of FAME in lipids of *N. gaditana*

Percentages of palmitic acid (C16) tended to increase with elevating the media salt concentration from 3.5% in both the growth media; however, arachidic acid (C20) showed the contrary and was observed highest (28%) in 3.5% road salt media. Elevating the salt in media enhanced the percentage of oleic acid and arachidic acid. The road salt

media provided complementary condition for the production of the long chain fatty acid (>C16). Increased the media salt concentration increased the long chain fatty acid in both the media while the road salt accounted overall increased amount of long chain fatty acid and FAME indicating more favorable for the biofuel point of view.

The effect of initial media nitrate concentration on lipid production and fatty acid profile were plotted in Table 6 were evaluated on the biomass obtained from the varied initial nitrate concentration in 2.6 % road salt based media at 37.5, 75 and 100 mg/L sodium nitrate. Lipid profile of these cells illustrated the lower the nitrate level had the higher the long chain (>C16) fatty acid. Overall fatty acid profile comprised 92% of long chain fatty acid in 75 and 100 mg /L of sodium nitrate, while 94% of long chain fatty acid in lowest nitrate (37.5 mg/L) containing medium.

Factors such as temperature, irradiance and nutrient availability affect not only lipid content but also fatty acid composition in many algal species. Large amounts of neutral lipids, mainly including triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), sterol ester were noted in the N-stressed cells often.¹⁰³. Nitrogen deficiency and salt stress induced the accumulation of C18:1 to some extent C20:5 were noted²⁹. The study confirmed the suitability of the generated biomass for the biofuel production and the varied concentration on salt and nitrate resulted with increased level of long chain fatty acids.

4.3 Conclusions

Phycoremediation using marine micro algae addressed in this study to provide the solution to treat the road salt run off efficiently as environmental friendly way with the generation of biomass which were influenced by the concentration of salt and nitrates in the growth media. Road salt run off enriched with leaching of soil fertilizer nutrients could be ideal for the marine microalgae cultivation to mitigate the negative effect of road salt run off on the environment. Nutrient removal of water run-off using marine microalgae will be the cost effective technique, which has the potential to generate biomass that can be further used as feedstock for biofuel. *Nannochloropsis gaditana* exhibited growth in all the tested road salt resulted with the production of ~1g/L biomass

and over 90 % nitrate reduction within the 6 days. Increased level of salt concentration also increased the lipid accumulation and lipid profile. Results indicate that phycoremediation is one of the processing ways that can treat the road salt runoff and can be effectively used to generate valuable biomass in addition to removal of nitrate-based nutrients from the wastewater system that are difficult in the hyper saline wastewater systems. Alternatively, road salt contains sodium chloride, which is the essential salinity provider to replace seawater. The limitations of marine microalgae cultivation only in seawater are eliminated and by introducing viability of these species to utilize in road salt runoff and the biomass production from road salt runoff system will provide economic feasibility of production of biofuel. The two salinity-based approaches in the experimental study provided valuable insight for an ecological approach to overcome the limitation of water for cultivation of microalgae for use in algal biofuel by producing valuable biomass and pigments with removal of pollutants from wastewater treatment system, deeming it as a novel green and sustainable environmental biotechnology.

Chapter 5

5 Investigation of three fed batch feeding strategies for biomass and lipid production of *N.gaditana* in Fed-batch cultivation

Improvement of lipid productivity in microalgae can be enhanced by production of biomass with higher concentration of lipids. Nutrient management in the culture system during the growth period is an important strategy to improve both the biomass and lipid productivity. Nitrogen limitation in the culture medium can increase the lipid synthesis in microalgae but reduces the biomass. The effect of controlled supply of nitrogen in fed-batch cultivation to improve the biomass and lipid production in *N.gaditana* was investigated in this study. Three modes of nutrient feeding in fed-batch cultures i.e., staged, pulse and continuous were investigated for their effects on the cell growth and lipid synthesis. The influence of two average light intensities (100 ± 10 and 200 ± 10 $\mu\text{mol photons/m}^2/\text{s}$) was also investigated in auto phototrophic cultivation. The maximum biomass of 2.08 g/L obtained by the feeding of 20 mg /d of nitrate at light intensity of 100 ± 10 $\mu\text{mol photons/m}^2/\text{s}$ with nitrogen to biomass conversion rate of 8.32 mg nitrate /mg biomass. The supply of nitrate by the continuous feeding mode did not improve the biomass yield in fed batch cultivation. A maximum lipid productivity of 46.84 mg/L/d was obtained when the nitrate supply of 10 mg/L fed at higher light intensity of 200 $\mu\text{mol photons/m}^2/\text{s}$ in fed-batch. The feeding mode influenced the fatty acid compositions and increased the content of two long-chain fatty acids of palmitic acid (C16:0) and palmitoleic acid (C16:1) over the 70 % FAME

5.1 Introduction

The sustainable biofuel production systems from microalgae require high growth rate and lipid yields to replace the fossil fuels^{26,36}. The algal lipid productivity determines the unit cost of the biofuel and is affected by the biological potential of the species and environmental conditions^{31,58}. The choice of species and the design of culture system to provide the correct condition are the key factors plays important role in the cultivation. However the optimization of lipid productivity with the maximized growth rate and lipid

content is challenging due the contrasting effects of nutrients on biomass and lipid production^{58,101,200}.

Microalgae exhibit considerable metabolic versatility and flexibility. The metabolic capability and environmental adaptability of the algal cells are modulated by the availability of nutrients, light, temperature and pH in the growth system. The choice of species and the design of culture systems provide suitable conditions to enhance biomass and lipid production for the biofuel production. Among the many reported studies, two species of green algae, i.e. *Chlorella* and *Nannochloropsis* have been shown to have the capability to produce higher lipid content ranging from 20-60% of the biomass weight and are considered as the most suitable species for the applications to biofuel production^{85,201}.

Microalgae cultivation can be carried out in photoautotrophic, heterotrophic and mixotrophic modes based on the energy and carbon supply. Light and nutrient mediated growth enhancement for biomass productivity in photoautotrophic mode is considered as the most inexpensive strategy suitable for large-scale outdoor cultivation⁵³. The demand of light energy and nutrients varies with algal growth stages. A lower level of light intensity is adequate during the initial stages of growth, whereas, a higher level of light is needed for moderate-high density cultures to avoid mutual shading of cells and to enhance photosynthesis efficiency¹⁰¹.

Nutrient nitrogen (N), phosphate (P) and carbon(C) are required at the specific ratio to the growth, biomass generation and lipid synthesis²⁰². Nitrogen is a major component of proteins, enzymes, chlorophyll and pigments which regulates the cellular reaction and its concentration is regarded as the key factor to control the biosynthesis of lipids and its manipulation can leads to remarkable changes in lipid content and the fatty acid profile of microalgae²⁰³. Thus, a positive correlation exists between the nitrogen concentration of the growth media and the production of biomass which results in the reduced lipid accumulation⁹³. The nitrogen limitation decreases the production of the growth promoting components and the cell utilizes nitrogen rich cellular components like proteins and carotenoids for the nitrogen supply.

Higher concentration of the nutrients in the initial growth phases therefore increases the biomass generation and the decreased nutrient levels at the later growth phase shifts metabolic cycles for the synthesis of the secondary metabolites such as lipids²⁰⁴. The microalgae cells respond to deprivation and limitation of nutrients by decrease in cell division, photosynthesis and respiration and an increased accumulation of storage compounds (starch, oil) and/or secondary metabolites^{58,203}. Even though, nutrient deprivation conditions promotes the lipid accumulation in micro algal cells the reduced growth rate, reduces the overall lipid yield.^{9,204,95}.

In microalgae, the structural membrane lipids are phospholipids synthesized during the active growth stage and these serve as carbon stocks during the limitation of nutrients in the growth stages³¹. The storage lipids are the neutral lipids which are accumulated as triacylglycerol (TAG) and are composed of long chain fatty acids suitable for biofuel(biodiesel)production. These are synthesized in the late growth stage using the carbon stocks from the structural lipids and other degraded biogenic components (Protein, chlorophyll, etc.,)^{36,95}. Production of TAG is enhanced due to stress by adverse growth conditions⁵³. Single variable substrate nitrogen control has been shown previously to manipulate the biomass and lipid production in autotrophic microalgae³³. The supply of nutrient to provide different nutritional situation like repletion, sufficiency, limitation and deprivation conditions varies the composition of biomass with the balanced growth^{42,58,202}.

A fed-batch cultivation system has the flexibility to control the chosen nutrient concentration by varying the flow rate and addition regimes using intermittent or continuous feeding during the growth stage to support the requirements of micro algal growth^{109,205}. The continuous and pulse feedings of urea as a nitrogen source in fed-batch cultivation were used for the cultivation of *Spirulina platensis* provided the 1231 ± 86 mg/l) and 1145 ± 52 mg/l biomass¹¹⁵. By employing low feed rates, the fed-batch system can operate with cells in the stationary phase of growth. Thus, a quasi-steady state culture system can be developed and these conditions support growth and trigger lipid production in the fed- batch cultivation system^{36,105,109}. Fed-batch systems can operate using diverse nutrient strategies, such as periodic, continuous or pulsing nutrient addition.

Only limited studies have been carried out on different feeding modes and their effect on lipid accumulation in microalgae. The study proposes for the first time to investigate alternative feeding strategies for fed-batch cultivation of *N. gaditana*.

5.2 Results and Discussion

Improvement of lipid productivity in the microalgae can be enhanced by maximized production of biomass with higher concentration of lipid. Nutrient management in the culture system during the growth period is essential to improve the biomass and lipid productivity.

5.2.1 Effect of cultivation methods and feeding rates nitrate conditions for media on nitrate uptake and biomass

5.2.1.1 Batch cultivation

The nitrate uptake process by the cells during the growth time were determined by the residual media nitrate concentration (Figure 5.1 and 5.2) to identify the nitrate-depleted states in the media at two light intensities of 100 ± 10 and $200 \pm 10 \mu\text{mol. photons/ m}^2 / \text{s}$ for the production of biomass and lipid. Microalgae are predominantly known for their capability of rapid assimilation of nitrogen even at low concentration. Nitrate consumption by the cells progressively increased during the growth period with the reduction to $< 10 \text{ mg/L}$ in the media within the 8 days of cultivation. Increased supply of light of $200 \mu\text{mol. photons/ m}^2 / \text{s}$ improved the nitrate utilization rate reaching the 100% depletion in the media. However increasing the initial nitrogen concentration affected the nitrogen uptake rate by the cells and the total removal of nitrogen were observed in the reactors with lower nitrate concentration (75 and 50 mg/L) at $200 \mu\text{mol. photons/ m}^2 / \text{s}$ light intensity. The batch culture cultivation continued for 20 days after the nitrate depleted conditions to investigate the impact of the nitrate depleted condition on the biomass and lipid production.

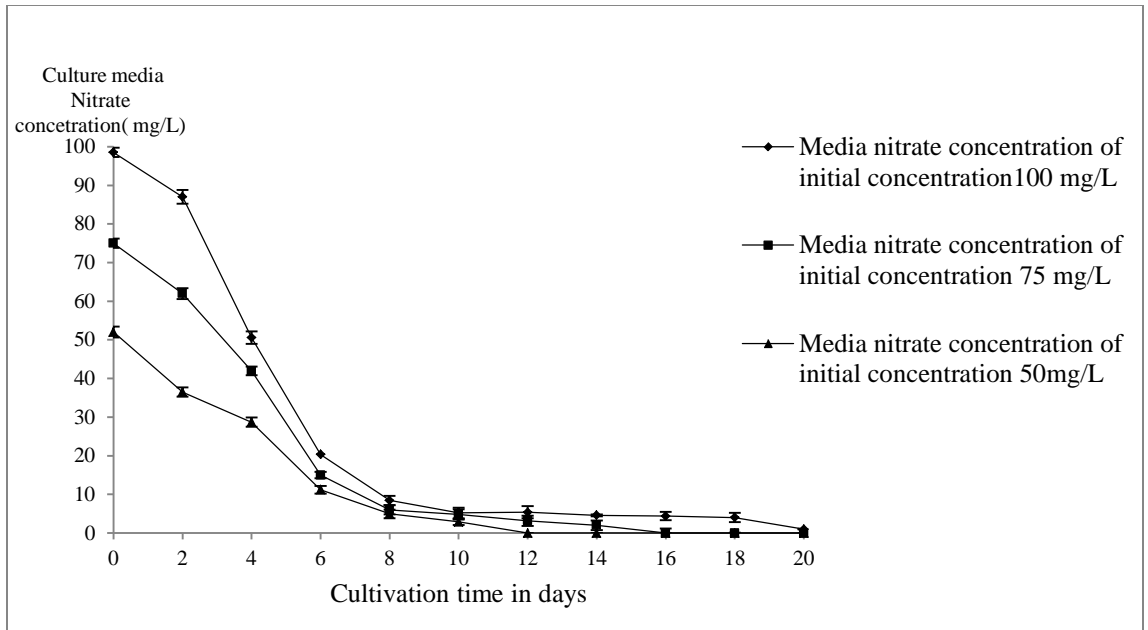


Figure 5:1 Media nitrate concentration in batch culture media at $100 \mu\text{mol photons}/\text{m}^2/\text{s}$ light intensity

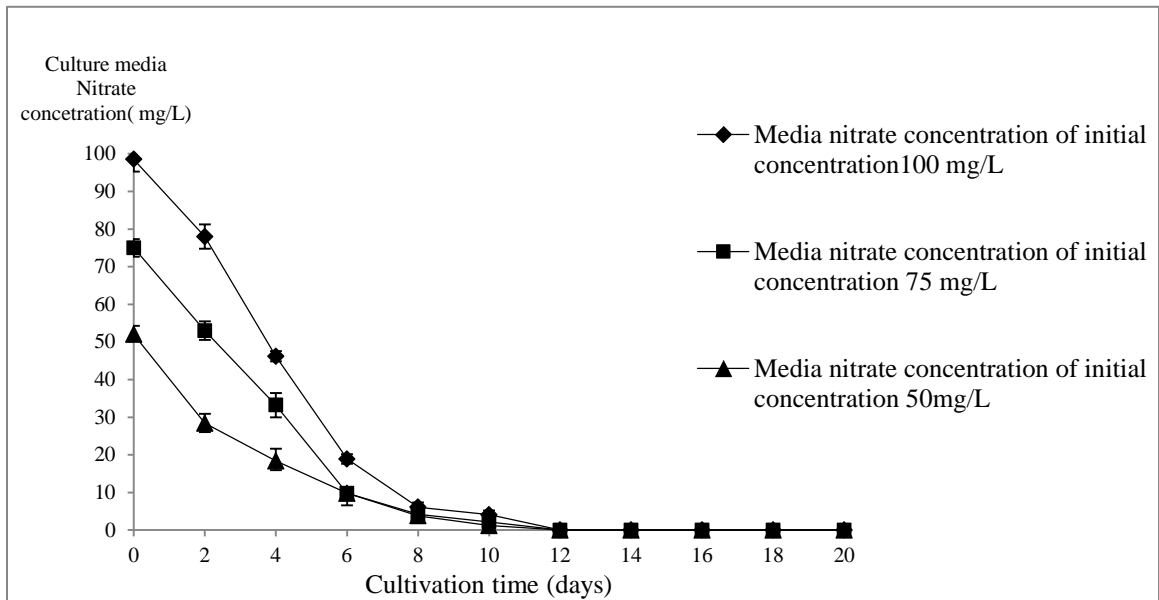


Figure 5:2 Media nitrate concentration batch culture media at $200 \mu\text{mol photons}/\text{m}^2/\text{s}$ light intensity

The effects of different nitrate concentration in the batch culture on the production of biomass were investigated by determining the biomass dry weight (DW) during the growth (Figure 5.3 and 5.4).

The increases in the biomass observed from the day 2 of the cultivation time indicated the increased assimilation of nitrates supported the cellular growth. The growth curves showed a very short lag phase and followed the dynamic growth between 4 to 15 days in all initial nitrate levels. The continued productions of biomass were observed even after the nitrate depletion conditions (<10 mg/L). The maximum biomass of 1.39, 1.17, and 1.045 g/L at the end of the cultivation were obtained in the media with 100, 75, 50 mg/L initial nitrate N concentration respectively under the 100 light intensities. The increased light intensity (200) in the batch cultivation increased the biomass to 1.59 and 1.275g/L in the media with 75 and 100mg/L initial nitrate concentration. The lowest biomass of 0.90 g/L obtained in reactor with lowest nitrate N concentration (50mg /L) at the highest light intensity 200 $\mu\text{mol. photons/ m}^2 \text{/s}$.

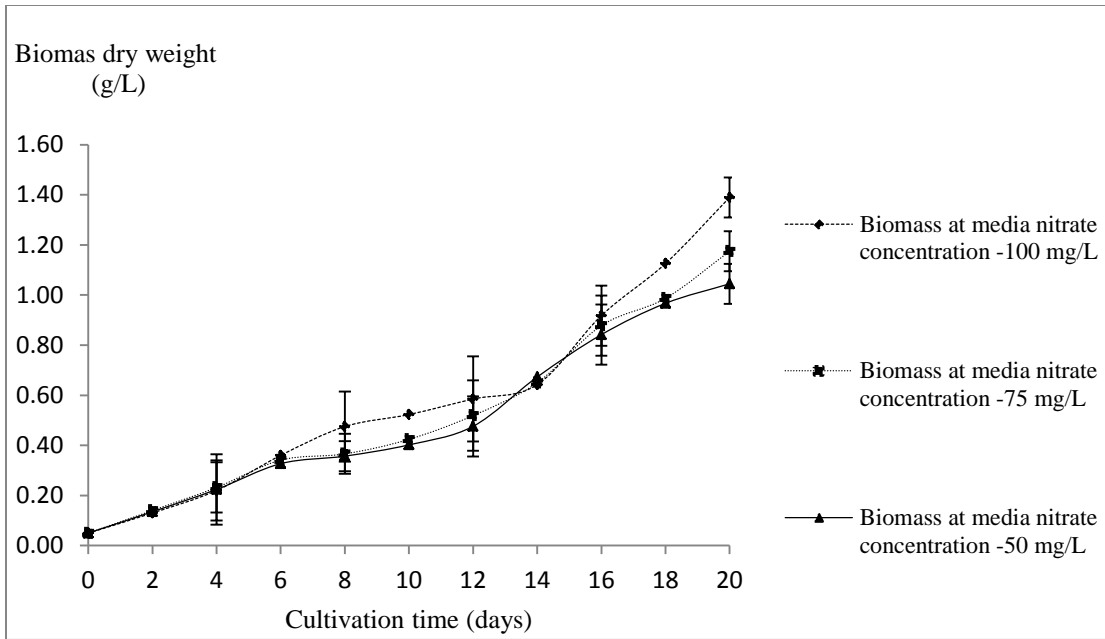


Figure 5:3 Biomass concentrations of 100- $\mu\text{mol photons/m}^2/\text{s}$ light intensity in batch culture

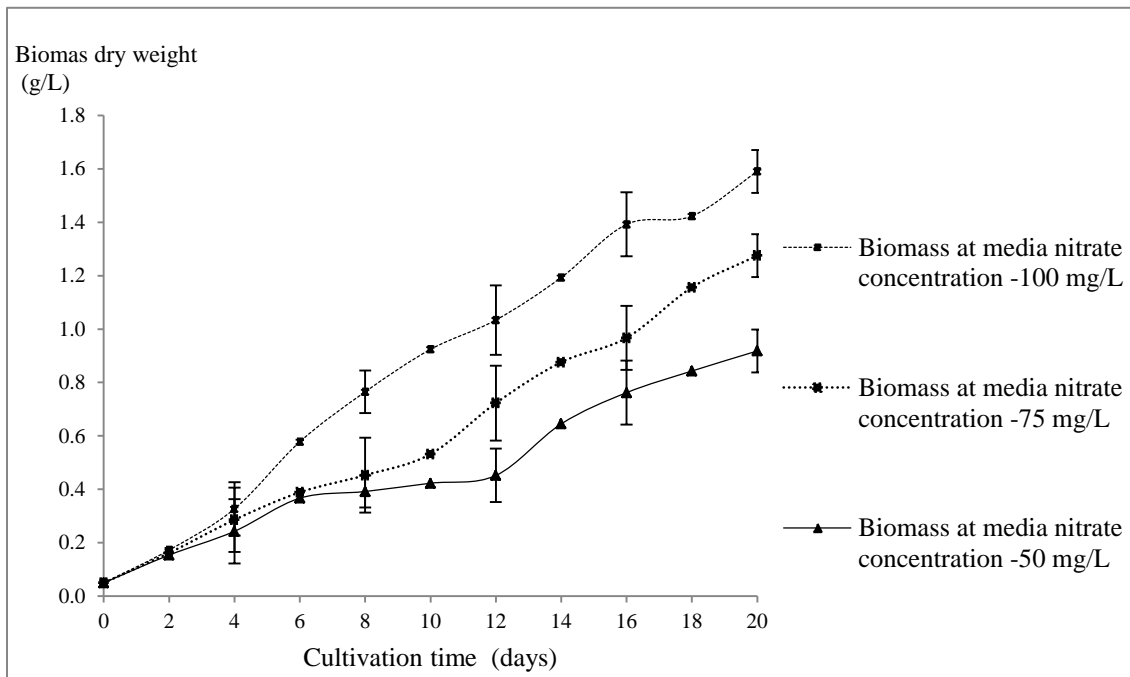


Figure 5:4 Biomass concentrations of 200 $\mu\text{mol photons/m}^2/\text{s}$ light intensity in batch culture

The increases in the biomass observed from the day 2 of the cultivation time indicated the increased assimilation of nitrates supported the cellular growth. The growth curves showed a very short lag phase and followed the dynamic growth between 4 to 15 days in all initial nitrate levels. The continued productions of biomass were observed even after the nitrate depletion conditions (<10 mg/L). The maximum biomass of 1.39, 1.17, and 1.045 g/L at the end of the cultivation were obtained in the media with 100, 75, 50 mg/L initial nitrate N concentration respectively under the 100 light intensities. The increased light intensity (200) in the batch cultivation increased the biomass to 1.59 and 1.275g/L in the media with 75 and 100mg/L initial nitrate concentration. The lowest biomass of 0.90 g/L obtained in reactor with lowest nitrate N concentration (50mg /L) at the highest light intensity 200 $\mu\text{mol. photons/ m}^2 /\text{s}$. The Lipid content of the biomasses was analyzed at the end of the cultivation. The lower initial nitrate increased the lipid content but the lower light intensity resulted with the lower lipid contents in the cells. The lipid of 17, 20.6 and 22.0 and 35.7, 39.7 and 42.7(%DW) were obtained in 100, 75 and 50 mg/L of initial nitrate concentration at 100 and 200 $\mu\text{mol. photons/ m}^2 /\text{s}$ respectively. The increased light intensity doubled the cellular lipid concentration.

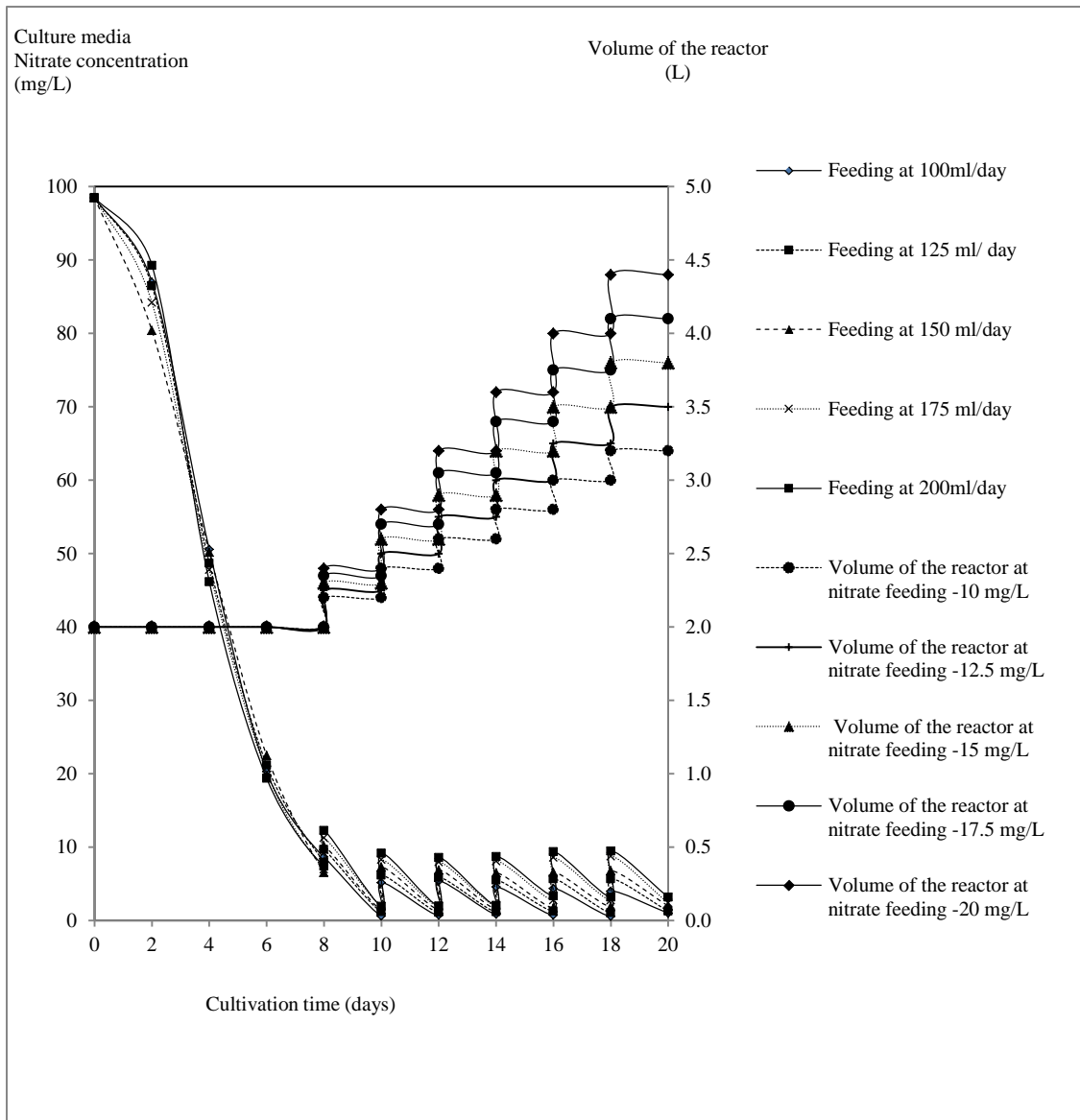
The nitrogen is the key nutrient for the production of various biogenic components to support the growth in microalgae. Three Nitrogen treatments with nitrate concentration levels (50, 75 and 100mg/L) of greenhouse effluents to provide the nitrate depletion conditions for *Nannochloropsis gaditana* growth model were studied first in the batch culture using road salt run off.

5.2.1.2 Fed batch cultivation Microalgae Culture and media

5.2.1.2.1 Pulse feeding Fed batch cultures

Nitrate consumption by the cells of *N. gaditana* was observed as the concentration of the nitrate in the media nitrate decreased rapidly from day one. The reduction of 99% nitrate observed in day 8 in batch cultures were used to initiate the Fed-batch culture systems. On day 8th the nitrate repletion conditions were introduced to the growth media by the feeding of nitrates. The pulse feeding of the nitrates were operated in two sets of 100 and 200 $\mu\text{mol. photons/ m}^2 /\text{s}$ light intensities in five 6L photo bioreactors with steady level of

initial nitrate concentration (100mg/L) at 5 feeding rates of nitrate concentration ranging from 10, 12.5, 15, 17.5 and 20 mg/day using stock solution of 100 mg/L Nitrate N containing stimulated greenhouse effluents by following the alternative days of feeding regimes. The nitrate consumption rates were evaluated (Figure 5.5 and 5.6 by analyzing the residual nitrate N concentration of the growth media.



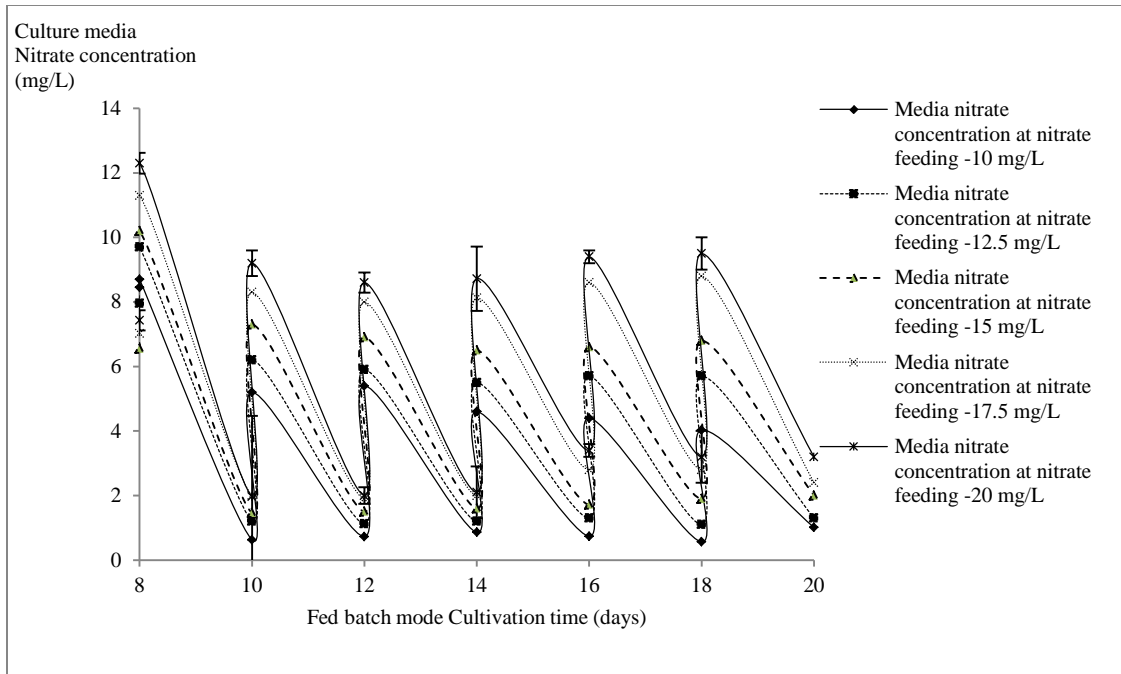
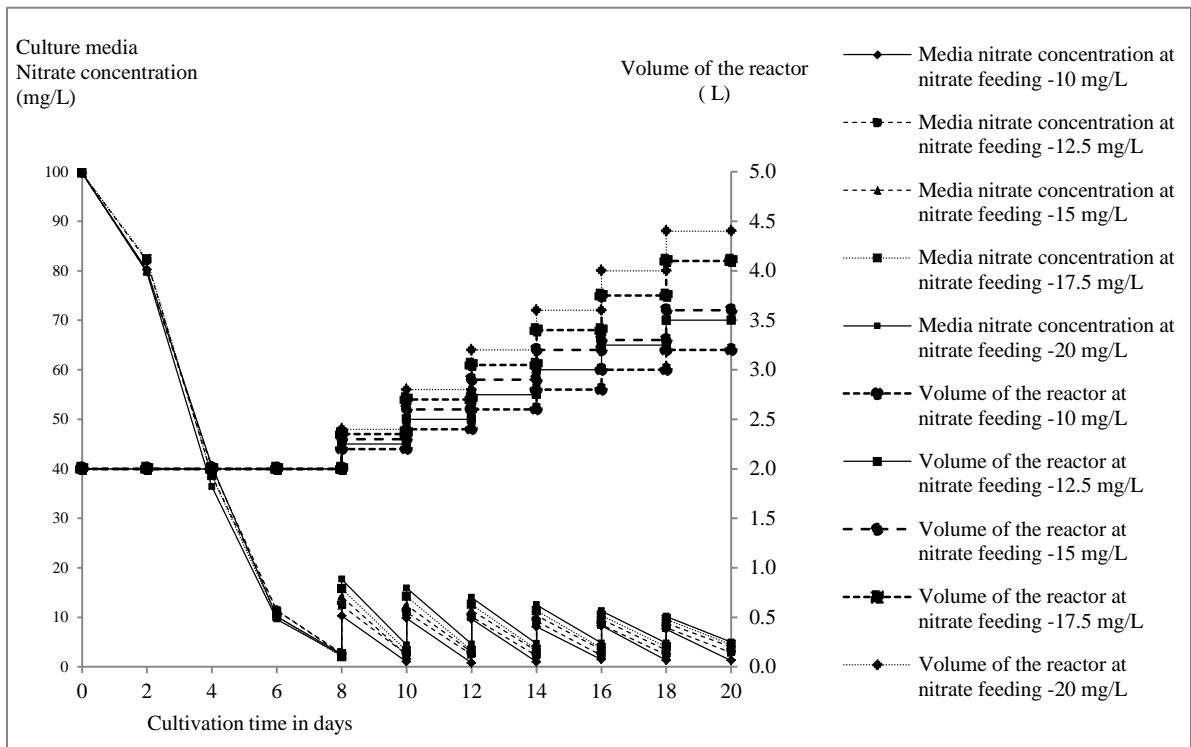


Figure 5:5 Nitrate concentrations in pulse feeding fed batch culture at $100 \mu\text{mol photons/m}^2/\text{s}$ light intensity



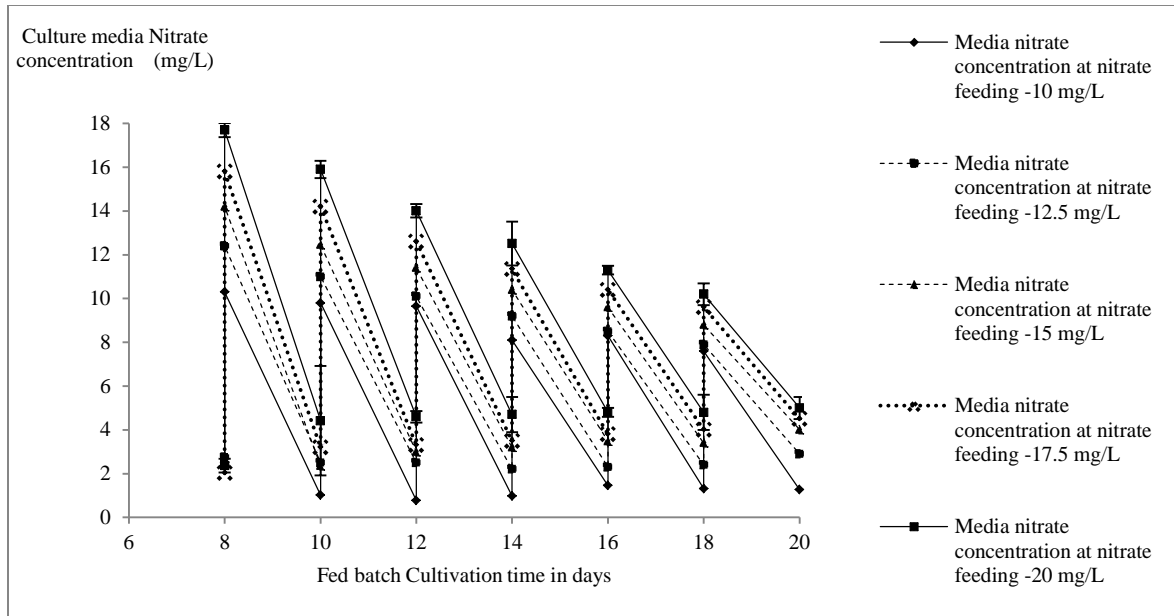


Figure 5:6 Nitrate concentrations in pulse feeding fed batch culture at 200 $\mu\text{mol photons/m}^2/\text{s}$ light intensity

Switching the batch reactors to the pulse feeding mode resupplied nitrates to the nitrate depleted media and established the nitrate replete conditions to enhance the biomass production. The increased nitrate N concentration approximately to 8-12 mg/L provided nitrate replete conditions. The oscillating of the nitrate N level in the media were observed with the feeding time indicated the continued nitrate assimilation process by the starved cells with the rapid decline in the nitrate concentration prior to the pulse feeding. The increased supply of light intensity of from 100 to 200 $\mu\text{mol photons/m}^2/\text{s}$ further improved the process of nitrate assimilation processes in the cells with an approximate nitrates levels oscillated between <10 mg/L. The introduction of pulse feeding to maintain the nitrogen replete condition promoted the biomass growth (Figure 5.7 and 5.8).

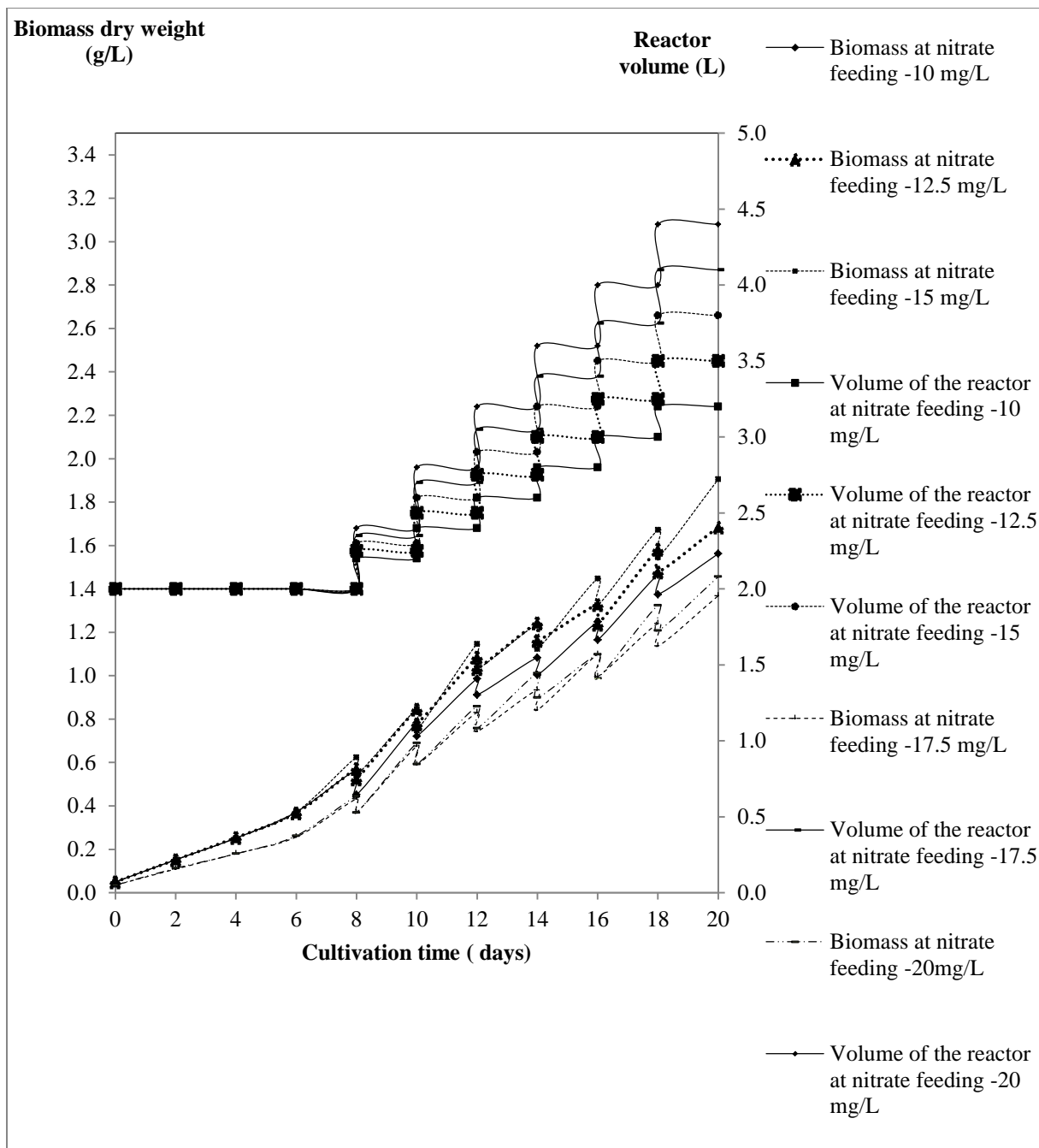


Figure 5:7 Biomass concentrations in pulse feeding at $100\mu\text{mol photons/m}^2/\text{s}$ light intensity

The increasing the rate of pulse feeding increased the nitrate levels in the growth media increased the cellular growth and the biomass of 1.56,1.69,1.91,1.96 and 2.08mg/L were obtained at $100\mu\text{mol photons/ m}^2 /\text{s}^1$. The increased supply of light generated slightly lower biomass 1.365, 1.445, 1.625, 1.88 and 1.925 g/L biomass at $200\mu\text{mol photons/ m}^2$

/s¹ at 10, 12.5, 15, 17.5 and 20 mg/day feeding rates of Nitrate N concentration. The increase in the feeding rate increased the biomass but the increased light of 200 $\mu\text{mol photons/m}^2/\text{s}$ resulted the lower total biomass in comparison to the biomass at 100 $\mu\text{mol photons/m}^2/\text{s}$ at the entire nitrate feeding rates.

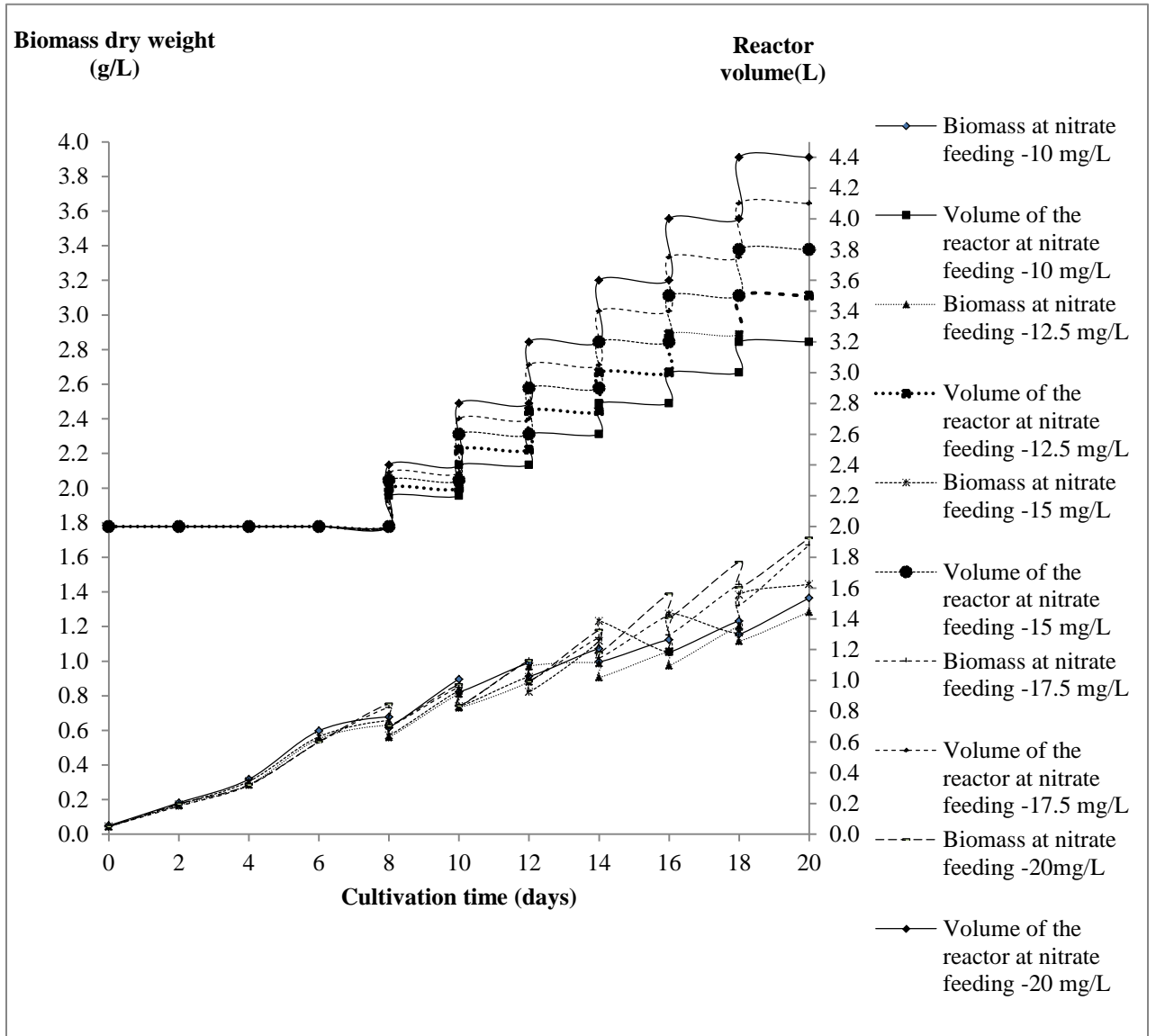


Figure 5:8 Biomass concentration in pulse feeding fed batch culture at 200 $\mu\text{mol photons/m}^2/\text{s}$ light intensity

Lipid contents of 25.0,22.9,21.36,20.6, 20.2(%DW) were obtained at 100 light $\mu\text{mol photons/m}^2/\text{s}$ at 10,12.5,15,17.5 and 20 mg/day feeding rates of Nitrate N concentration. The increased light supply of 200 light with introduction of pulse feeding increased the

lipid content to approximately 2 times with total of 58.3,52.5,50.4,49.8 and 45.1(%DW) were obtained 10,12.5,15,17.5 and 20 mg/day feeding rates of Nitrate N concentration (Table 5.4).

5.2.1.2.2 Continuous feeding fed batch cultures

In the third set of experiments under continuous feeding fed batch cultivation induced the nitrate sufficient conditions. The experiments in continuous mode at above feeding rates ranging were performed by operating two sets of reactors with average light intensities of 100 and 200 $\mu\text{mol.photons/ m}^2 /\text{s}^1$ in five reactors simultaneously to provide the nitrate sufficient conditions. The continuous feeding of the stock solutions was introduced at 8th day similar to the pulse feeding when the nitrate concentrations were diminished to < 10 mg/L. The concentrations of nitrogen in alternative days (Fig 5.9 and 5.10) were analyzed to identify the nitrate sufficient condition in the growth media. The nitrate concentration in the media continuously decreased as the cultivation time progressed in all the feeding rates. The concentrations below 10mg/L rate were noted in all the reactors.

The higher residual nitrate concentrations were observed in the reactors that are supplier with higher light in comparison to the reactors with the low light intensity. The residual nitrate concentration of <10 mg/L observed at the end of the 20 day cultivation indicating there were steady nitrate sufficient conditions were maintained for the cells.

The nitrate concentration in the media continuously decreased in all the feeding rates. The concentrations below x rate were noted in all the reactors. The higher residual nitrate concentrations were observed at the higher light supply in comparison to the reactors with the light intensity. The residual nitrate concentration of <10 mg/L observed at the end of the 20 day cultivation indicating there were steady nitrate sufficient conditions were maintained for the cells.'

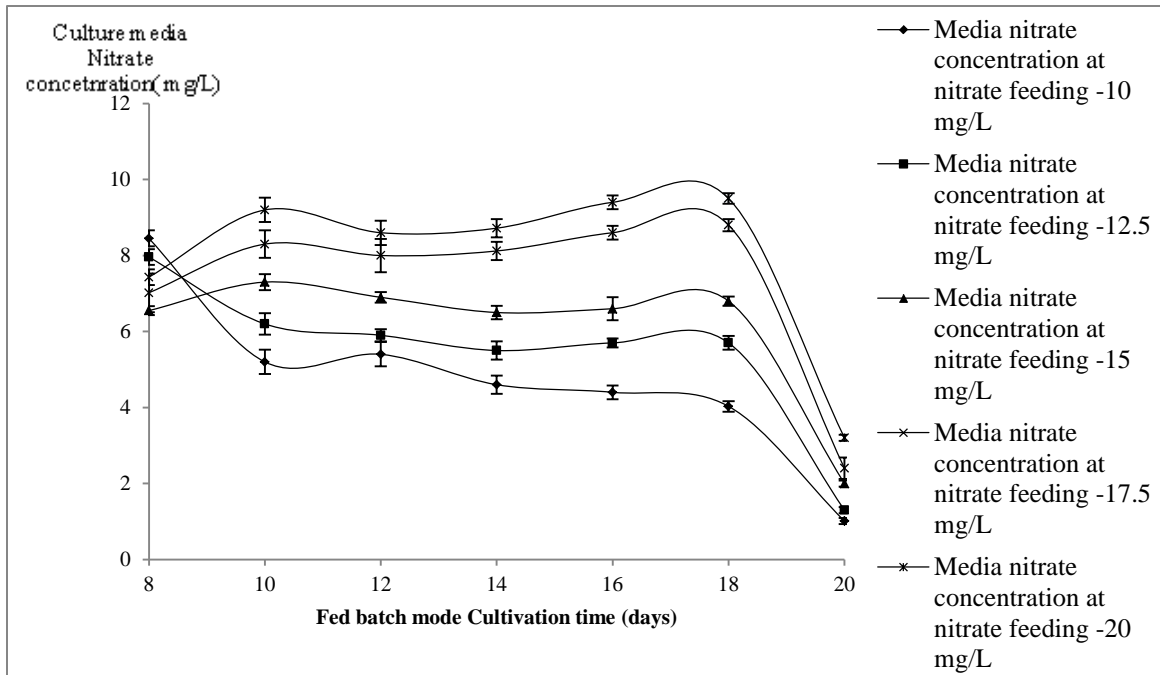
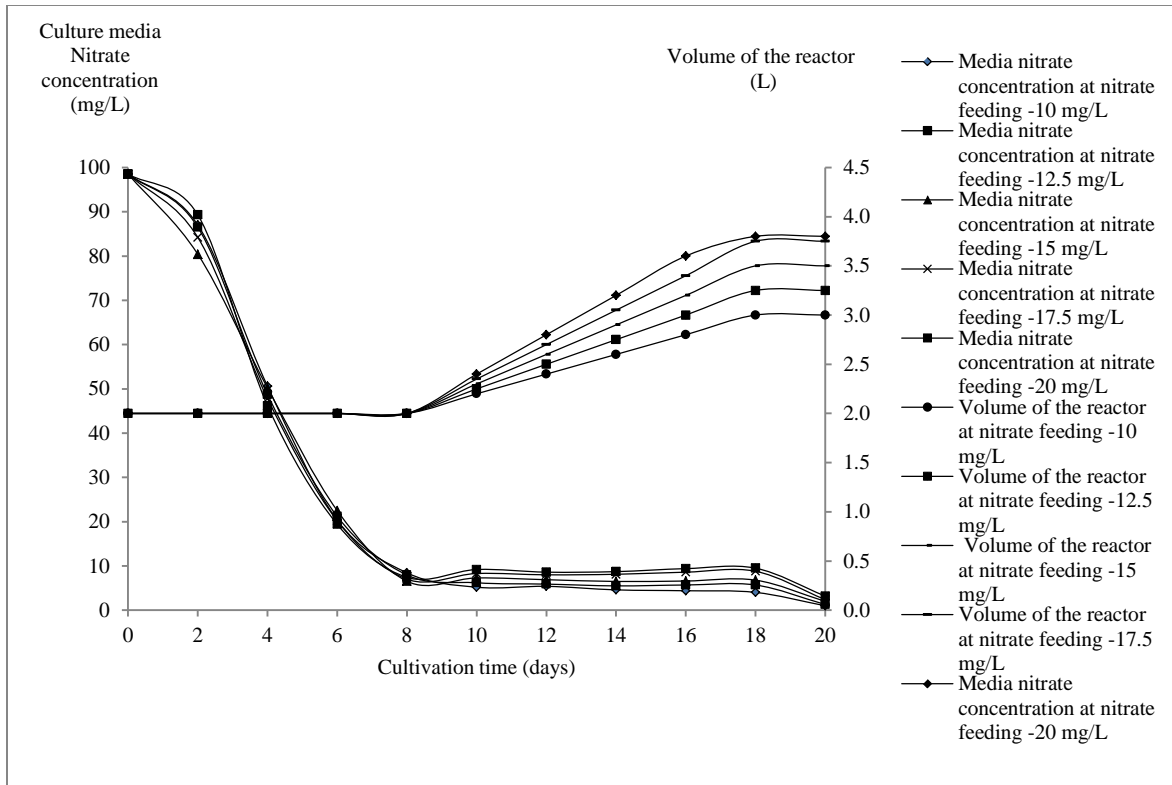
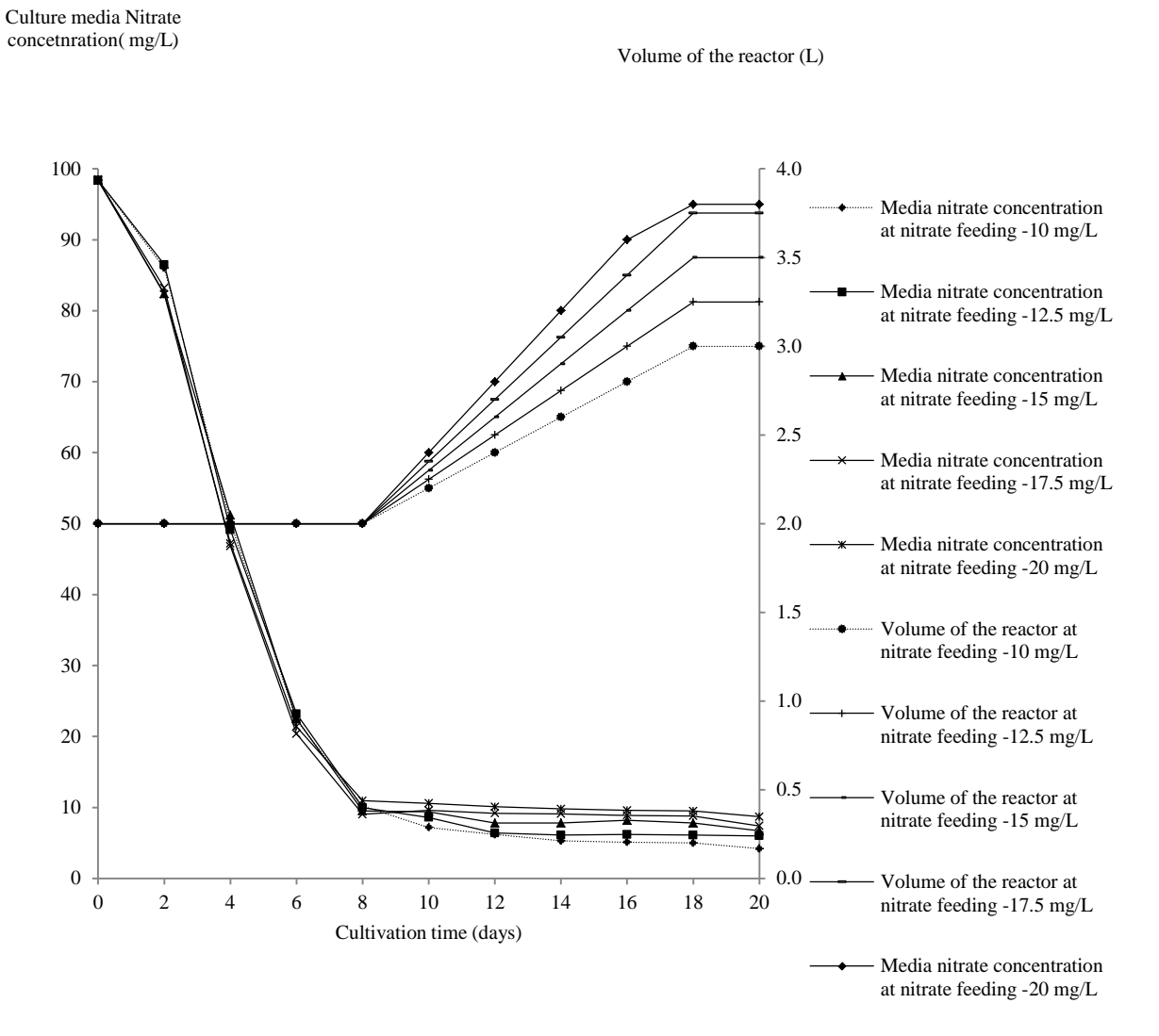


Figure 5:9 Growth media nitrate concentration in continuous fed FB culture at $100 \mu\text{mol photons/m}^2/\text{s}$ light intensity



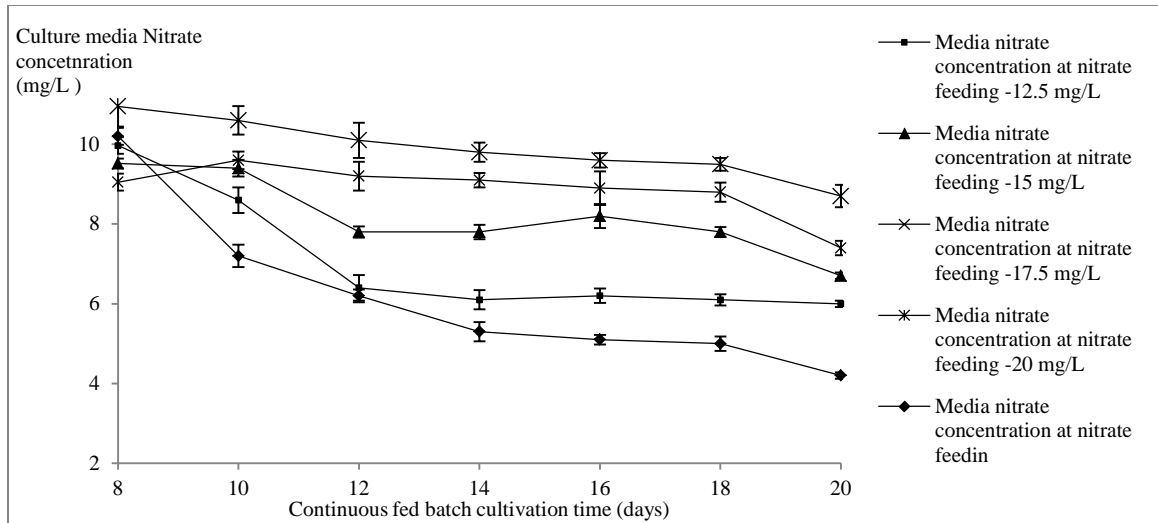


Figure 5:10 Nitrate concentration during the continuous fed FB batch culture at 200 $\mu\text{mol photons/ m}^2 / \text{s}$ light intensity

The generation of biomass decreased as the feeding rates increased in the continuous feeding fed batch cultures and with increased light intensities (Figure 5.11 and 5.12). The highest biomass of 1.42 g/L generated at the lowest feeding rate (10 mg/d) and with 100 $\mu\text{mol photons/ m}^2 / \text{s}$. The increased light to 200 $\mu\text{mol photons/ m}^2 / \text{s}$ resulted in the slight reduction of biomass to 1.39 g/L.

The increased supply of nitrate with feeding increased the biomass levels were observed at the lower light irradiation. The lowest values of biomasses of 1.1 g/L at 17.5 g/d feeding rates at the lower supply of light and at 20 mg/L with higher supply light were observed.

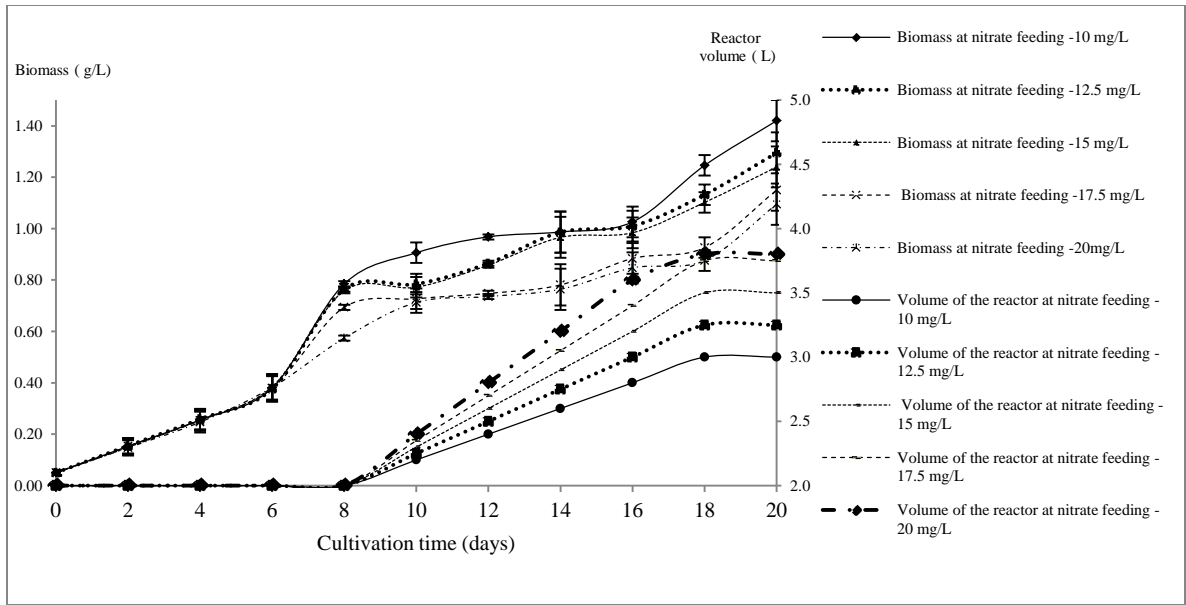


Figure 5:11 Biomass concentration in continuous fed FB batch culture at $100 \mu\text{mol photons/m}^2/\text{s}$ light intensity

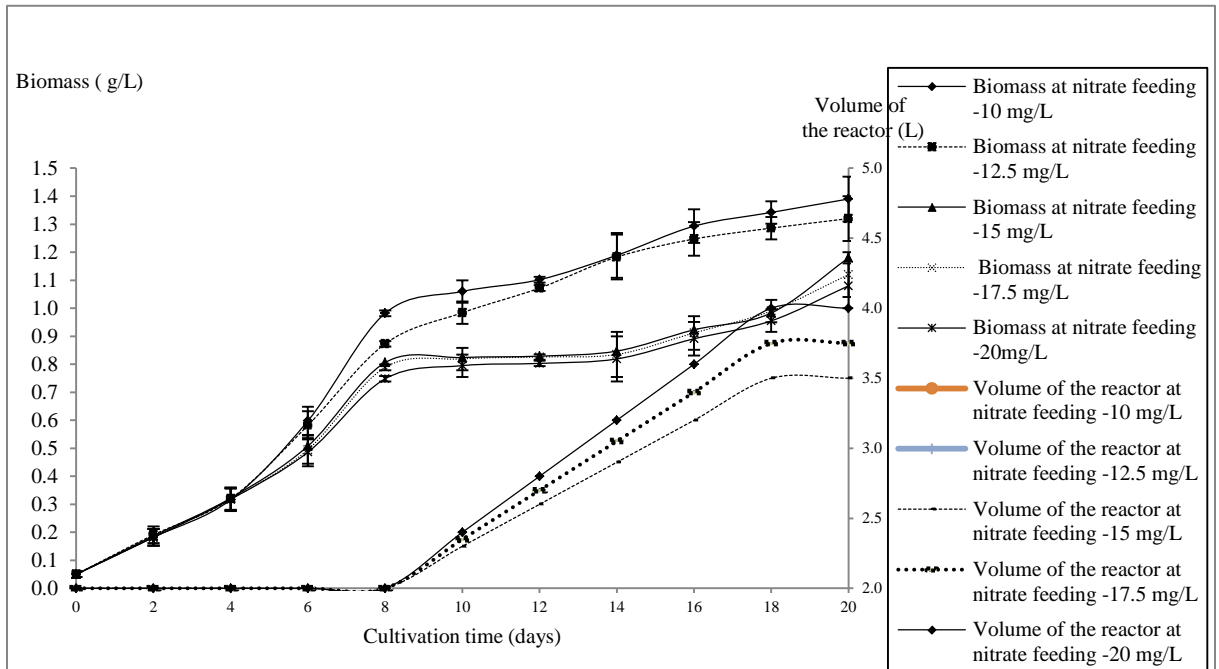


Figure 5:12 Biomass concentration in continuous fed FB batch culture at $200 \mu\text{mol photons/m}^2/\text{s}$ light intensity

The nitrates dose of 17.5 mg/ L resulted with the lowest (1.09 mg/L) generation of biomass, which was below the total biomass concentration obtained in the batch cultivation. The lipid concentration in the cells reduced from 18.6% to 9.62 % as the feeding rates increased from 10 to 20 mg/L by the continuous feeding modes (Table 5.4).

5.2.1.2.3 Stage feeding fed batch culture

In the last part of this study, a stage feeding strategy for the fed batch was performed for *N. gaditana* cells to induce the prolonged conditions of nitrate depletion prior to the nitrate replete conditions to influence the lipid production. This experiment was performed in the two sets with average impinging irradiance 100 to 200 $\mu\text{mol. photons/m}^2 \text{/s}^1$ in three 6L photo bioreactors with initial volume of 2L with feeding 500 ml of stock solution containing 100 mg/L nitrate at 5,10 and 15 days. The reactors set up with three initial nitrate concentrations of 100, 75 and 50 mg/ L similar to the levels of the batch cultivation reactors. The initial levels of nitrate concentration and supplementation with stage feeding affected the availability nitrogen to the cells in the growth media. Nitrate concentrations of the media are noted in the Figure 5.13 and 5.14

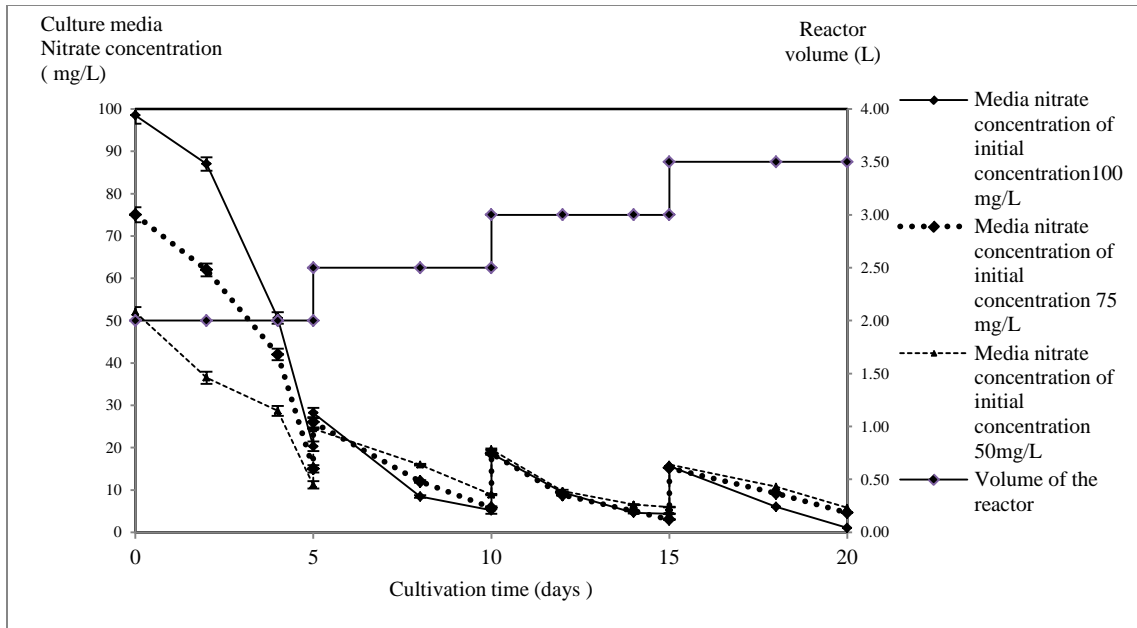


Figure 5:13 Media nitrate concentration in stage fed FB batch culture at 100 $\mu\text{mol photons/m}^2/\text{s}$ light intensity

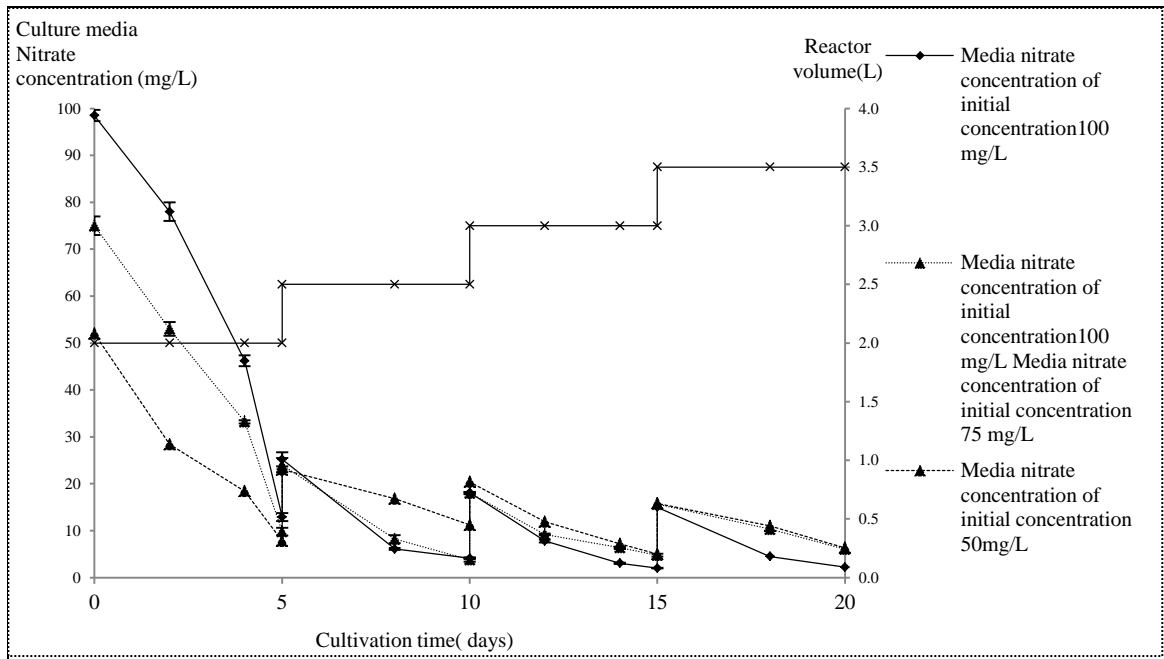


Figure 5:14 Media nitrate concentration in stage fed FB batch culture at 200 $\mu\text{mol photons/m}^2/\text{s}$ light intensity

The average residual nitrate concentration of <2 mg/L were noted prior to the stage feeding at day 10 and 15 and the concentration bounced to 7- 10 mg/L levels indicating the nitrate depleted and replete conditions were established in the growth media.

The reactor that supplied low light intensity resulted slow nitrate intake rates and the low initial nitrate N concentration further reduced the uptake rate with the residual nitrate concentration of 4-5 mg/L remained at the end of the cultivation in the reactor with <10mg/L . The stage feeding to the batch cultivated *N.gaditana* at three initial nitrates levels of 50, 75and100 mg/L in the media reintroduced the nitrates.

The increased biomass productions with the growth were observed (Figure 5.15 and 5.16). The stage feeding accompanied by the lower light intensity resulted the maximum production of biomass (1.5 g/L) when the initial nitrate concentration was at highest level (100mg/L). The increased light intensity reduced the biomass and the lowest of 0.7g/L obtained at lowest (50 mg/L) initial nitrate concentration.

The reintroduction of nitrates at the various growth stages influenced the lipid production when the nitrate concentration was higher (100mg/L) at the low light intensity. The other sets of the reactor not showed in the improvements in the lipid concentrations except in the initial nitrate 75 and 50 mg/L slightly decreased lipid concentrations observed with the introduction of the stage feeding to the nitrate depleted cells.

The lipid concentration varied from 18-18.5 (%DW) in the low supplied light and lipid content increased with the increased light to 35-42 (%DW) at all the initial nitrate concentrations (Table 5.4). Improvement of lipid productivity in the microalgae can be enhanced by maximized production of biomass with higher concentration of lipid. Nutrient management in the culture system during the growth period is essential to improve the biomass and lipid productivity.

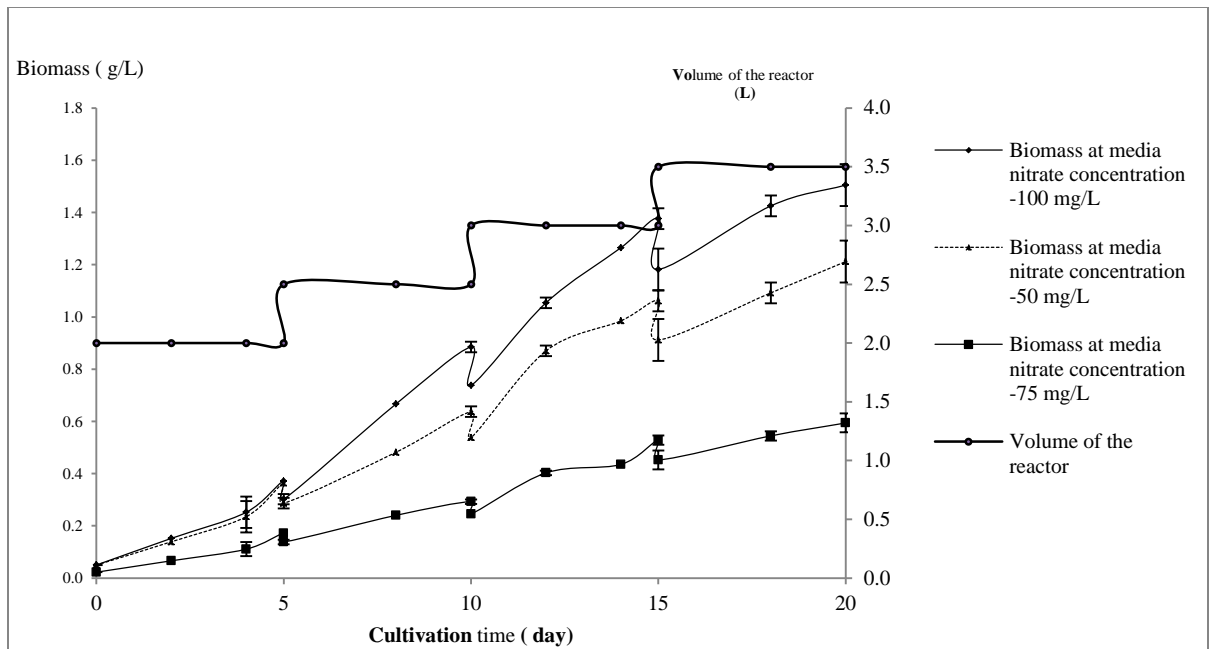


Figure 5:15 Biomass concentration in stage fed batch FB culture at 100- $\mu\text{mol photons/m}^2/\text{s}$ light intensity

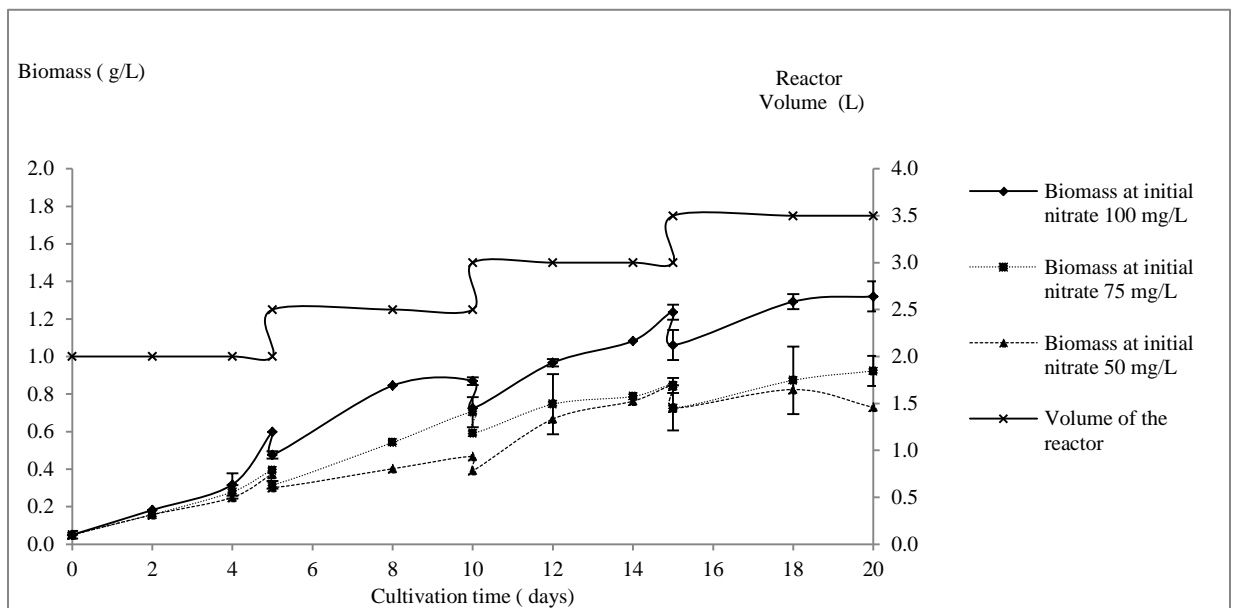


Figure 5:16 Biomass concentration in stage fed FB culture at 200- $\mu\text{mol photons/m}^2/\text{s}$ light intensity

5.2.2 Effect of culture conditions on biomass

The goal of the study was to optimize the biomass productivity with increased lipid contents by managing the nitrogen concentration in the growth media using various culture systems. The change in the supply of light and the initial nitrate concentrations provide the varied the nitrate conditions in the growth media during the growth period. The continued reduction of nitrate in all the conditions can be attributed to the requirement of nitrogen by the cells for synthesis of various biogenic compounds to support the cell replication process to enhance the growth. The nitrate assimilation processes for the use age of cellular activities were noticed in all the cultures. Nitrogen contributes around 10% of microalgal cell dry weight⁹⁶. The process of nitrogen uptake are influenced by the various factors like, cell physiology, metabolic flux, concentration of the available nitrate and the light irradiation in the photoautotrophic cultivation system. The stoichiometry ratio of N/P and N/C in the growth media significantly affects the growth.

Based on the trends displayed in the batch cultivation, the biomass growth continued after the cells experienced the nitrate depletion in the media indicating the use of containing components are utilized as cellular survival mechanism. The rate of nitrate depletion in the media with the increased supply of the light intensity in the autotrophic cultivation mode increased the production of biomass confirms the positive correlation of cellular nitrate and the biomass growth. The nitrate-depleted condition attained at day 8 and the concentration of 100 mg/L in the batch culture was considered as optimum levels were used further for the investigating the feeding strategies.

In general maximum biomass concentration as dry weight achieved in fed-batch was higher than in batch culture mode, as shown on Table 5.2. In terms of feeding strategy pulse feeding in fed-batch system resulted in the maximum biomass of 2.08 g/L. However stage feeding was efficient in achieving the biomass of 1.505 g/l and was further depended on the higher initial nitrate concentrations and the reduction in the initial nitrate concentration (50 mg/l) generated the lowest biomass of 0.7 g/L below than the batch culture mode at the higher (200 $\mu\text{mol. photons/ m}^2 \text{/s}$) light intensity.

Increase in the nitrate concentration and light intensity increased the biomass production in the batch cultivation only. Increasing the light intensity increased the cell mass to 1.14 fold and the 1.6 fold increment observed with increasing the initial nitrate concentration from 50 to 100 mg/L indicating both the nitrate and lights were efficient in promoting the cell growth. Light is the source of energy enhances the photosynthetic carbon fixation in the autotrophic organisms with the abundant supply of N for the syntheses of biogenic compounds. The reduced the initial nitrate concentration to 50 mg/L produced the lowest biomass and the influence of increased light intensity was not efficient due to the low availability of nitrogen limited to support the synthesis of components to improve the biomass production. The insufficient availability nitrate resulted in inefficient use of light to promote the cell growth. Unavailability of sufficient nitrogen concentration in the cytosol hindered the biochemical reactions to produce the nitrogen containing auxiliary cellular light harvesting pigments like chlorophyll to harvest the light energy might be the reason for the reduced generation of biomass.

The trends of increased biomasses were attained with the introduction of pulsed feeding fed batch cultivation. The increased feeding levels in parallel increased the biomass with maximum biomass of 2.08 g/L were obtained at the 20 mg/day feeding rate of nitrate. The influence of light intensity was not as efficient in the fed batch cultivation as in the batch cultivation since the dilution of the culture media by the pulse feeding improved the light availability to the cells. The continuous feeding strategy with the increased feeding rate reduced the biomass concentration in both light intensities. The decrease in the biomass concentration can be attributed to the continued increment in the dilution of the media resulting in the overall reduction in the cell density. And the dilution was sufficient to enhance the light penetration to the cells to carry the photosynthesis the increased light intensity did not resulted with increased cell growth. Similarly, stage-feeding strategy resulted with trends of reduced biomass with increased light. However, the increased initial nitrate concentration promoted the cell growth and biomass production.

In these studies, the higher amounts of biomasses were produced when the media fed with higher nitrogen concentration. This indicates that the application of nitrate nitrogen

by the fed-batch culture mode with promoted the cell divisions and biomass accumulation. Among fed-batch treatments, pulsed feeding result the highest biomass concentration. Results could indicate that a good cultivation strategy to obtain high biomass concentration will be to use pulsed feeding to induce nutrient replete conditions and feeding of 20 mg /L should be aimed to return nutrients like nitrogen to maintain the cell growth.

5.2.3 Effect of feeding on nitrogen to cell conversion factor

To understand the effect of nutrient feeding in the *N.gaditana*, cell to nitrogen conversion factor ($Y=X/N$) were calculated. The results presented in Table 5.1 indicate that highest cell to nitrogen conversion factor ($Y=X/N$) of 20.9 where accounted when lower total mass of nitrate (50 mg/L) were added per unit volume values and at high light intensities in the batch cultivation.

In other words, the cell to nitrogen conversion factor increased with the increase in the light intensity and decreased with the increase in the total mass of nitrate. In general, the increased supply of light with decreased amount of nitrate feeding reduced the generation of cell mass. While, in batch cultures, at low light intensity, the increase in the nitrate led to a decreased cell to nitrogen conversion because the cellular growth is limited by the light intensity. The overall cell to nitrogen conversion factors were low in the fed batch cultivation systems since the feeding regimes increased the total mass of the nitrate added which did not increase the biomass proportionately. The cell to nitrogen conversion factor ranged from 6 to 8.5 in the pulse fed batch, while the 4 -6 and 6-8.5 were obtained in the continuous and stage fed batch respectively.

Table 5-1 Biomass to substrate nitrogen conversion rates in the batch and fed batch cultivations at light 200 $\mu\text{mol photons/ m}^2 / \text{s}$

| Cultivation mode | Feeding rate (ml/day) | Initial nitrate concentration (mg/L) | Total nitrate (mg/L)(N) | Biomass (g/L)(X) | Biomass to substrate conversion ratio (Y=X/N) |
|--------------------------------|-----------------------|--------------------------------------|-------------------------|------------------|---|
| Batch | | 100 | 100 | 1.39 | 13.90 |
| | | 75 | 75 | 1.18 | 15.67 |
| | | 50 | 50 | 1.05 | 20.90 |
| Pulse- Feeding Fed batch | 100 | 100 | 233.33 | 1.56 | 6.69 |
| | 125 | 100 | 238.46 | 1.69 | 7.07 |
| | 150 | 100 | 242.86 | 1.91 | 7.84 |
| | 175 | 100 | 246.67 | 1.96 | 7.93 |
| | 200 | 100 | 250 | 2.08 | 8.32 |
| Continuous feeding fed batch | 100 | 100 | 233.33 | 1.42 | 6.09 |
| | 125 | 100 | 238.46 | 1.30 | 5.43 |
| | 150 | 100 | 242.86 | 1.24 | 5.11 |
| | 175 | 100 | 246.67 | 1.10 | 4.44 |
| | 200 | 100 | 250 | 1.15 | 4.60 |
| Stage feeding * (500ml/5 days) | 100* | 100 | 242.86 | 1.51 | 6.20 |
| | 100* | 75 | 192.86 | 1.30 | 6.74 |
| | 100* | 50 | 142.86 | 1.21 | 8.47 |

5.2.3.1 Specific growth rates in batch and fed-batch cultures

Specific growth rate is a useful index to evaluate culturing methods based on the initial and terminal cell biomass. *Nannochloropsis* has a very slow growth rate. The specific growth rates were ranged from 0.03- 0.09/day in the studied growth conditions (Table 5.2). Batch cultivation with increased nitrate concentration and light intensity resulted with maximum specific growth rates of 0.08/day and pulse fed batch with maximum of 0.12 /d in increased feeding rate of 20mg/L of nitrate concentration in the lower light intensity. Similarly increased nitrate feeding rate with increased light resulted with reduction of the specific growth rates in continuous and stage feeding fed-batch cultivation. In the stage feeding the initial nitrate concentration is important to increase the specific growth rate. The maximum specific growth rate of 0.7 /d at the 100 mg/L

nitrate concentration and the least of 0.03 /d were obtained in the 50 mg/L initial nitrate concentration.

Table 5-2 Specific growth rate and Biomass concentration in various cultivation methods

| | | | Light intensity -100 μ mol photons/ m ² /s | | Light intensity - 200 μ mol photons/ m ² /s | |
|--------------------------------|-----------------------|---------------------------------------|---|--------------------------------|--|--------------------------------|
| Cultivation mode | Feeding rate (ml/day) | Initial nitrate concentration (mg/L) | Biomass (g/L) | Specific growth rate (per day) | Biomass (g/L) | Specific growth rate (per day) |
| Batch | | 100 | 1.39 | 0.07 | 1.59 | 0.08 |
| | | 75 | 1.18 | 0.06 | 1.28 | 0.06 |
| | | 50 | 1.05 | 0.05 | 0.90 | 0.04 |
| Pulse- Feeding Fed batch | 100 | 100 | 1.56 | 0.08 | 1.37 | 0.06 |
| | 125 | 100 | 1.69 | 0.09 | 1.45 | 0.06 |
| | 150 | 100 | 1.91 | 0.11 | 1.63 | 0.07 |
| | 175 | 100 | 1.96 | 0.11 | 1.88 | 0.09 |
| | 200 | 100 | 2.08 | 0.12 | 1.93 | 0.09 |
| Continuous feeding fed batch | 100 | 100 | 1.42 | 0.05 | 1.39 | 0.03 |
| | 125 | 100 | 1.30 | 0.04 | 1.32 | 0.04 |
| | 150 | 100 | 1.24 | 0.04 | 1.18 | 0.03 |
| | 175 | 100 | 1.10 | 0.03 | 1.12 | 0.03 |
| | 200 | 100 | 1.15 | 0.05 | 1.08 | 0.03 |
| Stage feeding * (500ml/5 days) | 100* | 100 | 1.51 | 0.07 | 1.32 | 0.06 |
| | 100* | 75 | 1.30 | 0.06 | 0.90 | 0.04 |
| | 100* | 50 | 1.21 | 0.06 | 0.70 | 0.03 |

Increased light supply did not correlate with the increasing of the specific growth rates in all the experiments. The specific growth rates were reduced in the pulse, continuous and stage fed FB cultivation with increased light supply as these treatments also provided lower biomass. In most of the studies thus far, the reported specific rates were usually below 0.4/day for the *Nannochloropsis* species and the studied treatments were provided the lower specific growth rates. The ample supply of light and nitrate might be an effective way to boost photosynthetic efficiency to promote the weight of the biomass accumulation but the not the cell reproduction (daughter cells) as the specific growth rates were derived by the cell counts. In batch cultivation, the decreasing light intensity available per cell as a result of the increase in cellular biomass often leads to light limitation for algal growth. However, when cells are grown in continuous cultivation,

growth can be controlled by the fixed operational conditions that provide a steady supply of nutrients and allow for continuous cell density and light distribution. The dilution factors directly reduce the cell densities of the culture media, which increases the incident lights to the cell surfaces. The higher light intensity cause the photo inhibition due to the damages to the photo systems thereby the cell growth is reduced.

The amount and timing of N feeding in fed batch culture had significant effect on the biomass productivity. The nitrate supply increased the biomass production in the pulse fed batch cultures when compared to the batch. The process of photosynthesis in auto phototrophic microalgae can explain the influence of nitrogen supply on the biomass growth. The cellular responses vary with the availability nitrogen. Nitrogen is an essential nutrient and is critical for the synthesis of proteins, nucleic acids, and chlorophyll molecules. The green pigment chlorophyll “a” is the only pigment in *Nannochloropsis* species plays a central role in photosynthesis for absorbing and transferring light energy. Chlorophyll accumulation was higher in autotrophic cultivation and lower in heterotrophic cultivation explained as the more degree of chlorophyll needed for efficient light absorption when light is the sole energy source. Since the treatments in the cultivation provided sufficient supply of nitrogen and the synthesis of pigments were not disturbed. The sufficient nitrogen conditions in the all the cultivation systems improved the use of light energy and enhanced the growth and biomass generation.

Although light is important for algal growth, levels that are too low or too high limit or inhibit the photosynthetic process. Production of chlorophyll depends on the availability of the nutrients especially the nitrogen in the media. Nitrogen deficiency reduces the chlorophyll contents in microalgae which declines the growth rate and chlorophyll content were reported for *C. vulgaris*¹⁷⁸.

The nitrogen-starved cells responses by the cessation synthesis or gradual degradation of chlorophyll during the nitrogen depleted conditions as noticed in the batch cultures. Apart from the supply of nitrogen, the availability of micronutrients and phosphorus affect the chlorophyll concentration, since the phosphorous is essential nutrient for many phosphorylation-dependent reactions like ATP synthesis and the Calvin cycle and its

deficiency affects chlorophyll synthesis and cell growth and metabolism. The reduction in chlorophyll content of N-starved micro algal cultures associated with the lack of nitrogen atoms that form part of the chlorophyll structure, which is composed of four pyrrole groups forming a ring around a magnesium atom, which is stabilized by four nitrogen atoms⁹⁸.

5.2.4 Effect of cultivation methods on lipid production

It is possible to change the biochemical composition of microalga by modifying the cultivation mode. Lipid production kinetics determined with the growth at day 10, 15 and 20 days of batch cultivation in the biomass obtained from the initial nitrate concentration of 100 mg/L (Table 5.3). As noted in the table 5.3, the lipid content increased as the growth progressed from 12.30 to 27.05 (%DW) in the cells, as the nitrate-depleted conditions were prevalent in the media in the batch cultures.

The increased light intensity enhanced the lipid production during the growth stage (Table 5.4). Fed batch cultures were supplied with nitrate feeding below 20 mg/din all the treatments in order to maintain the nitrate depleted conditions (<10 m/L) to promote the lipid synthesis process.

Table 5-3 Lipid and biomass production in various cultivation stages in the batch culture with starting nitrate concentration of 100 mg/L

| Cultivation days | Light | Light | | |
|------------------|------------------------------------|----------------|----------------|-------------|
| | 100µmol photons/ m ² /s | Biomass (mg/L) | Biomass (mg/L) | Lipid (%DW) |
| Day 10 | 0.52 | 12.3 | 0.88 | 17.65 |
| Day 15 | 0.88 | 17.39 | 1.09 | 23.79 |
| Day 20 | 1.36 | 27.05 | 1.59 | 35.73 |

In order to determine the effects of cultivation and feeding regimes on lipid productivity the biomass (X), lipid content (P), volumetric lipid concentration (P_{Vol}) and Lipid productivity (Q_P) and lipid yield on nitrate (Table 5.4) were compared.

The lower intensity of light resulted with lower concentration of the cellular lipids in all the cultivation modes. The increased light intensity improved the lipid content in the cells irrespective of nitrogen availability.

The maximum concentration of lipid ranging from 45 to 58 (%DW) obtained under the pulse feeding in combination with increased light. The pulse fed-batch cultures with increased light yielded the highest volumetric lipid concentration ranging from 796 to 937mg/l was able to increase the lipid productivity to the ranges of 38 to 47 mg/L/day since the lipid contents in these treatments increased 2 fold. All the other culture treatments were showed the lipid productivity below 25 mg/L/day. The trends of overall increased lipid concentration were also observed as the availability of nitrates was reduced severely in the culture media except the continuous feeding fed batch cultures where the lowest biomasses were produced.

The continuous feeding fed batch cultures maintained the lipid concentration at 19-20(%DW) at the low supply of light and varied to 18- 35 (%DW) at the higher light intensity. The timing of nitrate feeding by the stage feeding (during log, linear and decline phase) in the fed batch culture system affected the lipid content that depended on the starting concentration of the nitrates with lipid yields and productivities were in comparable to the continuous fed batch cultures.

The yield of lipid on nitrate (g/g) ranged from 0.2 to 0.8 in the fed batch cultures and the yield of lipid on nitrate were reached the maximum of 7.68 in the batch cultures when the light supply was highest due to the total nitrate input to the culture system was at the lowest levels in the batch cultures.

Table 5-4 Comparison of difference cultivation regimes for biomass (X), Lipid content (P), volumetric lipid concentration (Pvol.) and lipid productivity (QP) and yield per g of nitrate

| Cultivation | Light ($\mu\text{mol photons}/\text{m}^2/\text{s}$) | Biomass (X)(g/L) | Lipid (P) (%DW) | P _{vol} (mg/L) | Q _P (mg/L/d ay) | Yield of Lipid on nitrate (g/g) |
|--|--|---------------------|-----------------------|----------------------------|----------------------------------|--|
| Batch at 100 mg/L Nitrate | 100 | 1.39 | 17 | 236 | 12 | 2.36 |
| Batch at 75 mg/L Nitrate | 100 | 1.18 | 21 | 242 | 12 | 3.22 |
| Batch at 50 mg/L Nitrate | 100 | 1.05 | 22 | 230 | 12 | 4.61 |
| Batch at 100 mg/L Nitrate | 200 | 1.59 | 36 | 568 | 8 | 5.68 |
| Batch at 75 mg/L Nitrate | 200 | 1.28 | 40 | 506 | 25 | 6.75 |
| Batch at 50 mg/L Nitrate | 200 | 0.9 | 43 | 384 | 19 | 7.68 |
| Fed batch -pulse-10mg/day nitrate | 100 | 1.57 | 25 | 391 | 20 | 0.39 |
| Fed batch -pulse-12.5mg/day nitrate | 100 | 1.69 | 23 | 386 | 19 | 0.31 |
| Fed batch -pulse-15mg/day nitrate | 100 | 1.91 | 21 | 407 | 20 | 0.27 |
| Fed batch -pulse-17.5mg/day nitrate | 100 | 1.96 | 21 | 402 | 20 | 0.23 |
| Fed batch -pulse-20mg/day nitrate | 100 | 2.08 | 20 | 420 | 21 | 0.14 |
| Fed batch -pulse-10mg/day nitrate | 200 | 1.37 | 58 | 796 | 40 | 0.80 |
| Fed batch -pulse-12.5mg/day nitrate | 200 | 1.45 | 53 | 759 | 38 | 0.61 |
| Fed batch -pulse-15mg/day nitrate | 200 | 1.63 | 50 | 819 | 41 | 0.55 |
| Fed batch -pulse-17.5mg/day nitrate | 200 | 1.88 | 50 | 937 | 47 | 0.54 |
| Fed batch -pulse-20mg/day nitrate | 200 | 1.93 | 45 | 868 | 43 | 0.29 |
| Fed batch -continuous-10 mg/day nitrate | 100 | 1.42 | 19 | 263 | 13 | 0.26 |
| Fed batch -continuous-12.5mg/day nitrate | 100 | 1.30 | 20 | 261 | 13 | 0.21 |
| Fed batch -continuous-15 mg/day nitrate | 100 | 1.24 | 19 | 241 | 12 | 0.16 |
| Fed batch -continuous-17.5mg/day nitrate | 100 | 1.10 | 19 | 205 | 10 | 0.12 |
| Fed batch -continuous-20mg/day nitrate | 200 | 1.15 | 18 | 202 | 10 | 0.10 |
| Fed batch -continuous-10 mg/day nitrate | 200 | 1.39 | 32 | 440 | 22 | 0.44 |
| Fed batch -continuous-12.5mg/day nitrate | 200 | 1.32 | 34 | 454 | 23 | 0.36 |
| Fed batch -continuous-15 mg/day nitrate | 200 | 1.18 | 35 | 416 | 21 | 0.28 |
| Fed batch -continuous-17.5mg/day nitrate | 200 | 1.12 | 30 | 331 | 17 | 0.19 |
| Fed batch -continuous-20mg/day nitrate | 200 | 1.08 | 27 | 294 | 15 | 0.15 |
| Fed batch - stage feeding (3x 50 mg/L and initial 100 mg/L nitrate) | 100 | 1.51 | 25 | 377 | 19 | 0.24 |
| Fed batch - stage feeding (3x 50 mg/L and initial 75mg/L nitrate) | 100 | 1.20 | 29 | 348 | 17 | 0.22 |
| Fed batch - stage feeding (3x 50 mg/L and initial 50 mg/L nitrate) | 100 | 1.30 | 32 | 416 | 21 | 0.26 |
| Fed batch - stage feeding (3X50 mg/L and initial 100 mg/L nitrate) | 200 | 1.32 | 35 | 463 | 23 | 0.29 |
| Fed batch - stage feeding (3 x 50 mg/L and initial 75mg/L nitrate) | 200 | 0.90 | 38 | 342 | 17 | 0.21 |
| Fed batch - stage feeding (x 50 mg/L and initial 50 mg/L nitrate) | 200 | 0.70 | 42 | 294 | 15 | 0.18 |

5.2.4.1 Lipid productivity

The best culture strategy in terms of volumetric lipid productivity (P_{Vol}) and yield (Q_P) and yield on nitrate (table 5.5) was pulsed fed batch culture. The light intensity of 200 $\mu\text{mol photons/ m}^2 \text{/s}$ provided the highest productivity, yield and yield of lipid on nitrate and the light intensity of 100 $\mu\text{mol photons/ m}^2 \text{/s}$ in the same cultivation mode provided the second best condition. The batch cultures provided the highest yield of lipid on nitrate with the lowest lipid productivity (P_{Vol}) and yield (Q_P) values. N replete condition maintaining by the continuous feeding with lower nitrate feeding and lower intensity were not efficient cultivation methods to increase the lipid productivity (P_{Vol}) and yield (Q_P) and yield on nitrate despite of having the moderately higher biomass, the continuous supply of nitrate to the media did not promote the conditions for the lipid synthesis.

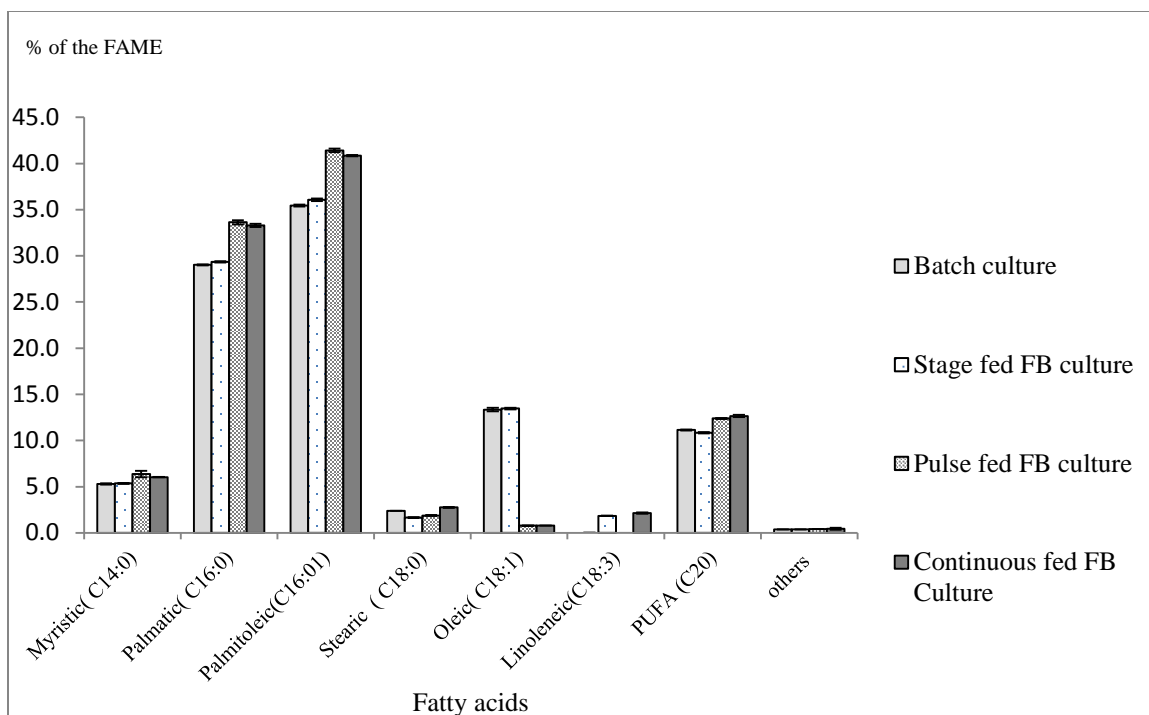
The increased trend of lipid production observed with increased supply of light intensity. The excessive light energy promotes the lipid production to avoid photo-oxidative damage in the algal cells were reported⁴¹. The optimal light intensity for algal growth, product accumulation, and other applications varies significantly among different algal species ranges from 62.5 to 2000- $\mu\text{mol m}^2 \text{/s}$. The study conducted by increasing the light intensity to 200 $\mu\text{mol m}^2 \text{s}^{-1}$ in the reactors noticed the influence able impact on the lipid concentration in *N. gaditana*. Algal light absorption and conversion efficiency is species specific and can significantly vary due to inherent properties such as the pigment profile, cellular architecture and chloroplast arrangements. Various factors such as light spectra, irradiance level and the nature of microalgal photochemical machinery affect the photosynthetic responses and the accumulation of organic matter in various microalgae. These variables govern the biomass production and overall biochemical concentration of algal cells. The lipid biosynthesis is the secondary metabolisms that are triggered during the adverse growth conditions as the survival mechanism to protect the cells from light and nutrient starvation. Since the lipid synthesis reaction consumes higher NADPH (58), which serve as electron sink which relaxes the electron transport fluxes that were occurred by the increased supply of light as electron. Also, the lipid molecules serve as energy and carbon reserve to the cells during the adverse growth conditions.

The phenomenon of increased lipid content related to the ratio of the carbon/nitrogen were reported in the studies were not provided in the treatments. Only the light and the nitrates were studied since the increased carbon supply generally switches the metabolic pathways to starch synthesis which increases the carbohydrate contents of the cells but not the lipid. Fed-batch cultures with high glucose concentrations (50g/L) and intermittent nitrate feeding promoted specific carbohydrate accumulation. There are theories on the shifts of the metabolic pathway indicating the enzyme potentially hydrolyzes starch chains to channel the carbon flow to lipid synthesis in *N.oleoabundans* cultivation under prolonged nitrogen starvation conditions ¹⁸⁹ were the possible reason for the increased concentration of the lipid in the study.

Secondly the studies reported were noted, depending on the intracellular N concentration, the enzymes modulate the activities. Nitrogen-limited conditions trigger the overproduction of proteins to scavenge internal and external nitrogen ¹⁸⁹ and to activate the lipid synthesis pathways were can be explained to increased lipid production in the treatments.

5.2.4.2 Fatty acid composition of micro algal lipid

The study aimed to utilize the biomass of *N. gaditana* to the production of biofuel. The biomass obtained in the various feeding rates of fed batch cultures were collectively further analyzed for the fatty acid profiles. The lipids extracted from biomass from four cultivation strategies were trans esterified to fatty acid methyl ester (FAME) and analyzed by gas chromatography. The compositions of the fatty acids are summarized in Figure 5.17.



5:17 Percentage of fatty acids in FAME of the lipids

The total FAME content varied between 31- 39% of the total lipids. The major constituents were the two long-chain fatty acids of palmitic acid (C16:0) and palmitoleic acid (C16:1) and these two fatty acids contributed to more than 60% of FAME. The concentration of palmitic and palmitoleic acid accounted highest of 75 and 77.4% in the FAME of the lipid obtained from the biomass of pulse feeding and continuous feeding fed batch respectively. Interestingly the concentration (>35% of FAME) of the palmitoleic acid (C16: 1) was noted in the lipids and this fatty acid is known as omega 7 fatty acid which is considered to have the benefits also in human health for maintaining health cholesterol and blood sugar. The increased amount of the C16 indicates the continuous / intermittent supply of the nitrate exposes the cells to the nitrate deplete conditions and continuously triggers the lipid synthesis process.

These results are consistent with other reported studied that *Nannochloropsis* sp. accumulated a high proportion of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) to >70% of the total lipid content. Compared to the commonly used soybean oil and rapeseed oil as feed stocks for biodiesel, the biodiesel derived from

microalgal lipid in this study contained SFA and MUFA which are more saturated and hence will provide a higher cetane number (CN), lower NO_x emissions, shorter ignition delay time, and higher oxidative stability

5.3 Conclusions

Three fed batch-feeding regimes were compared in this study to see the effects of different nitrate dose in combinations with light on the productions of biomass and lipid. Overall, it can be safely summarized that fed batch culture treatments were efficient in enhancing the growth and lipid yield of *N. gaditana*. In this study the combination of the higher light intensity and nitrates efficiently improved the production of biomass. The data obtained from different fed batch cultivation imply the biomass production efficiency was highest in the pulse feeding. The biomass could be improved by developing pulse feeding culturing method to target the nitrate concentration in the growth media with sufficient light intensity. The continuous feeding data indicated the increased concentration of nitrate concentration has the very little influence on the biomass production.

The maximum biomass of 2.08 g/L obtained using the pulse feeding fed batch culture with the specific growth rate of 0.12 per day achieved was superior to the other cultivation. Furthermore the use of feeding regime and fed batch cultivation can be used to enhance the biomass and lipid production. Marine species of *N. gaditana* are capable of producing higher lipid when the pulse feeding was applied with sufficient light supply the lipid content increased to >50%DW and the higher light was found to have more effectiveness for lipid production. The lipid contents were doubled with increased light in all the treatments. The biomass contained the lipids that were rich with palmitic acid (C16:0) and palmitoleic acid (C16: 1).

The method developed to use the nutrient nitrate rich vegetable greenhouse effluents has the potential to integrate with road salt run off for the application to generate the biomass which would reduce the nutrient and water footprint of the biofuel production.

Chapter 6

6 Investigation of harvesting efficiency of *N. gaditana* from phyco-remediated road salt media by flocculation and settling

Harvesting of microalgae is a necessary step where the microalgae are physically separated from the spent cell growth media. Investigations were conducted to evaluate various flocculation methods to separate microalgae from spent growth media and these results are described in this chapter.

The recovery and harvesting of biomass is the major hurdle in the production of bio-fuel from the microalgae. The rapid, efficient, and economic technologies for harvesting microalgae are currently a subject of research and are essential for successful applications. This study aims to investigate the harvesting of marine microalgae from the phycoremediated road salt media by using flocculation as a unit operation. Chemical based flocculation using inorganic metal FeCl_3 ; organic polymer chitosan or pH change by alkali addition was examined and compared. The optimal coagulant dosage, sedimentation time and the effectiveness of pH on both the harvesting efficiency and concentration factor of algal suspensions were determined.

The results of the study showed that increasing the pH of the road salt-based growth media helped to induce the aggregation and sedimentation of *N.gaditana* cells. An increase of pH above 10.5 led to rapid flocculation of cells with harvesting efficiency of 89.2% at pH 11 and 91.5% at pH 11.5 with the rapid settling in less than 15 min. Raising the pH to 11 (noted as a critical point) promotes the flocculation of the cells by the alkaline flocculation method. In the chemical flocculation study, the inorganic salt FeCl_3 was the most efficient as a coagulant/flocculant and showed the 95% harvesting efficiency at the higher dosage of 80 mg/L. However, the settling rate was slower (60 minutes to achieve the complete (95%) harvesting efficiency). The concentrations of cells using alkaline induced flocculation were lower than the FeCl_3 method. A 16.2 concentration factor was obtained at pH 11.5 and a concentration factor of 23.6 was

found when the FeCl_3 were used at the dosage of 60 mg/L at the end of 60 minutes of settling time

The use of a bio coagulant, i.e., chitosan polymer for the application of flocculation was not found to be efficient. A solution of chitosan in 1 % HCl at the dosage of 10-100 mg/L only provided below 5% of the flocculation efficiency. Thus, the alkaline and FeCl_3 methods for the flocculation are more appropriate for the pre-concentration of the cells.

6.1 Introduction

The use of microalgae for the biofuels is a promising alternative to replace the fossil fuels for the production of sustainable, renewable clean energy. Microalgae have the ability to fix carbon dioxide by photosynthesis and utilize nutrients from wastewater^{60,206}. The micro algal systems for the biofuel productions are preferred due to their characteristic of high growth rate, the shorter growth time than the terrestrial plants and demands for less land usage^{36,100,101}. However, the high energy input for the production and recovery of the biomass in the downstream processing is a key limitation for the economical commercial cultivation^{29,207}. Harvesting of biomass is an important factor and the major bottleneck for the commercial use of algal biomass^{25,139}. The cost of the dewatering process has been estimated around 25-65 % of the algal biomass cost itself^{4,139,208}. The development of methods for the recovery of biomass from the growth media are challenged by the smaller size, negative surface charges of the microalgae, low cell concentration and the similarity in density of algal cells to the growth media^{32,209-211}.

The methods applied for the harvesting of the biomass included the traditional separating techniques like filtration and centrifugation. Both of these techniques have disadvantages including high energy input, fouling of membranes (filtration) and hence are not considered economically viable at the present time (17–20). Thus, more effective, simple, environmentally friendly harvesting methods would be important for commercial biodiesel production from microalgae.

Flocculation techniques are applied in the waste water treatment as, low cost, effective and convenient process that could be potentially applied for the treatment of large

volumes of microalgal cultures²¹³. Flocculation of cells attained by the use of physical, chemical and biological (microbial) methods conducted by the two –step process contain coagulation/sedimentation and dewatering²⁴.

Depending on the pH, microalgae cell surfaces are negatively charged and are suspended in the media by electrostatic repulsion forces. The application of flocculants aggregates the cells by destabilizing the net negative charges by charge neutralization, electrostatic patches (sweeping) and by the bridging mechanisms (netting). The aggregated cells sediment by gravity as sludge and the sludge removed or dewatered by filtration²¹⁴.

Inorganic coagulants or flocculants like chloride and sulphates of ferric and aluminum and organic polymers (chitosan, starch, moringa flour etc.) are used to harvest microalgae^{173,215}. The polyvalent metal, electro coagulant and polymers have been investigated for their efficiency to sediment microalgae to determine the optimal conditions to harvest the biomass from various culture media^{164,215,216}. Among these, chitosan, i.e., d-glucosamine and N-acetyl-d-glucosamine deacetylated from chitin (the second- most-abundant natural polymer in the world), contain primary amino groups is one of the promising flocculants emerged to use it in the harvesting of microalgae²¹⁷. However, the practical application of chitosan is limited due to its insolubility in water at neutral or higher pH²¹⁸.

The high dosage of organic based coagulants like starch and chitosan are required for the flocculation adds cost to the treatment^{148,219}. The dosage of chemical coagulants contaminate the biomass with metals limit the application of recovered biomass in the environment²²⁰. The other reported techniques include physical flocculation using ultrasound, electro-flocculation. These are cleaner processes but consume more energy and requires specialized equipment and may be difficult to scale up²²¹. Bio-based purified bio flocculants and co-flocculation using microbes are considered more safe and environmentally friendly, but the cost is higher in comparison to the chemical flocculants¹⁶².

Another approach to destabilize the negative charges is by adjusting the pH of the media leading to cell self-flocculation was reported recently for the harvesting of microalgae¹⁴⁶. The cell self-flocculation and the auto-flocculation by increasing the pH can be more

economical. Especially if the use of increased pH can be achieved by the depletion of CO₂ by the photosynthetic activity of the cells itself then this could be a simple method that can be easily adopted to the culture systems¹²¹.

The negatively charged carboxyl and/or sulphate groups of cell surface stabilizes the microalgal suspensions¹⁴². The fact of flocculation of microalgae at a high pH is therefore surprising, since the surface charge of micro algal cells are expected to become more negative at a high pH and thus flocculation should be inhibited. But in contrast to the stabilization, the increase in pH above 10, algae are reported to auto flocculate due to electrostatic interactions between anionic algae and divalent cations with the formation of calcium or magnesium precipitates^{25,127,222-224}. Some reports noted that the auto-flocculation did not occur in waters poor in calcium and magnesium and addition of lime or with high concentration of phosphates induce the flocculation^{154,223}.

Several questions have emerged which applying flocculation as a unit operation for microalgae. First, the majority of work done on microalgae flocculation has been reported mostly on freshwater species. The reports noted the inhibition of flocculation efficiency of polymers and the requirement of increased dosage of inorganic flocculants in the high ionic strength media¹²¹. Secondly, the effect of salinity and ionic strength of the growth media for the microalgae grown in a high ionic strength marine environment on the flocculation efficiency were not reported for the all the growth media and species. Each growth medium has unique electrical charge properties depending on types of ions and composition; therefore, even identical species being cultured in different culture medium might also have a specific threshold pH that triggers the flocculation. Finally, the intrinsic characteristics of the media and algal strains impacts on the harvesting efficiency with varied pH ranges are not reported for marine cultures cultivated in the high saline waste water systems.

Since the marine microalgae in the study is cultivated in road salt containing media, the ionic strength of the media is high with relatively higher concentration of metal ions and sodium, it was hypothesized that the increasing pH of the media would potentially induce the destabilization of the algal surface charges and promote the cell aggregation.

However, the high ionic could pose difficulties in harvesting the biomass with the use of chemical induced coagulation. Hence this study investigated the harvesting efficiencies (HE %) (See equation 3.6) using three separate approaches: inorganic ferric chloride (chemical), chitosan (bio coagulant) and pH to determine the effectiveness on *N. gaditana* flocculation on spent media. The degrees of pre-concentration were determined by applying the concentration factors as parameters (See Equation 3.7).

6.2 Results and Discussion

The flocculation experiments were performed in three sets using the above-mentioned three flocculants. The spent media culture suspension of *N. gaditana* cells are obtained from the growth experiments at end of the growth stage (21 days). Chitosan was prepared as in the materials and methods 1 % acetic, 1% citric and 1% hydro chloric acids were used. The flocculation efficiency (FE) referred in the study as harvesting efficiency (HE) expressed by the percentage of the sediment of algal sludge to the initial algal cell mass.

6.2.1 Effect of pH on sedimentation of *N.gaditana* cells with alkaline flocculation

The growth media of cultures was at pH (8+/-0.05) at the end of the growth stage. Hence the harvesting efficiency was studied using 0.1M NaOH to increase the pH of media. The previous studies reported noted that the potassium hydroxide and calcium hydroxide induced flocculation at the same pH as sodium hydroxide but at higher concentrations²²⁴.

The harvesting efficiencies (%HE) were determined in four sedimentation time intervals (15, 30, 45 and 60 minutes) for the microalgal cells by increasing the pH of the media by 0.5 units from 9 to 12.5. The increase in the pH of the suspension with the addition of sodium hydroxide caused the settling of cells to the bottom of the test tubes with the formation of visibly noticeable interface between the clarified solution and the concentrated algae cells.

Figures 6.1 and 6.2 show the harvesting efficiencies of the *N.gaditana* with four settling times. As shown in the figures, the two separate harvesting efficiency (HE) conditions

were noted with the increased pH of media as a function of time. In the first condition, the slower settling of cells observed at pH below 11.

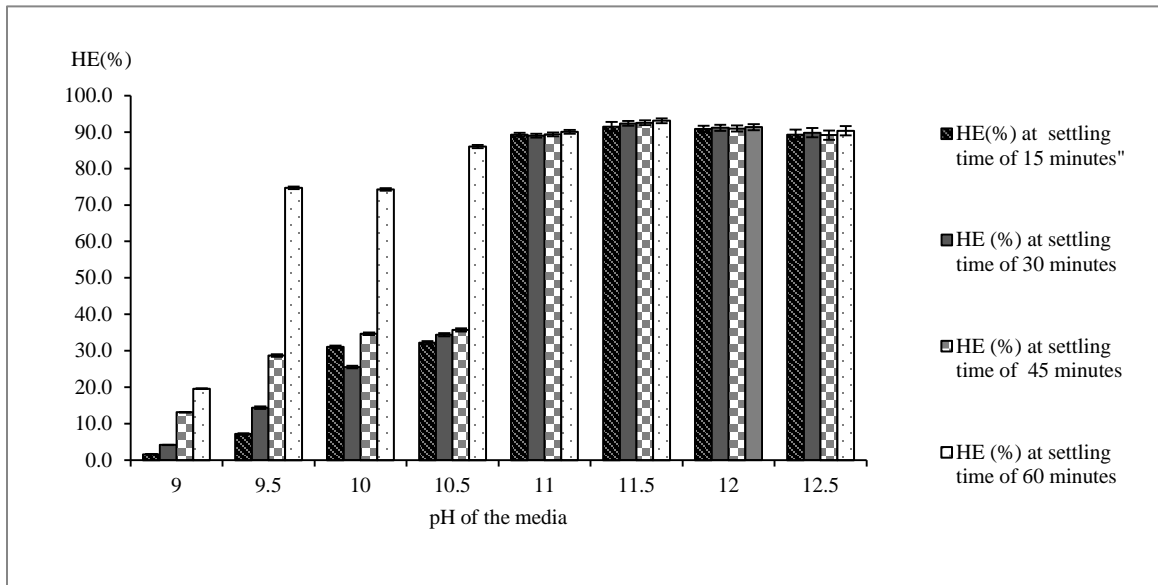


Figure 6:1 Harvesting efficiency versus the pH of media at various settling time

The HE was below <32% until the 45 minutes of settling time in the pH range of 9.5- to 11 were observed. And the second conditions at Above 11 pH showed rapid HE of 90%. The zones of the cell aggregation observed in the pH ranges were further showed by the harvesting efficiency versus the settling time at four time intervals (15 minutes increment) to investigate the effect of pH on the settling of the cells as a function of time. The harvesting efficiency increased as the settling time progressed from 15 minutes 60 minutes and with the increased of the pH of algal suspension media from 9 to 10.5 ranges. The settling time of 45 minutes were required to flocculate <40% of the cells and the 60 minutes flocculated 70 to 86 % of the cells at the pH 9.5 10.5 range.

In the pH 11 and above settling of the cells were faster and maximum cells were sedimented to the bottom of the test tubes with the HE of above 85% within the 15 minutes were obtained when the cells were exposed to higher pH by the increasing media pH. The two clear zones of settling rates of cells with pH were obtained. The Figure 6.2 was depicted to see the differences by plotting the percentage HE as a function of time. Hence the pH 11 was considered as threshold pH in the media, which differentiated the

settling speed of the algal cells in the experiments. The Figure 6.3 and 6.4 were plotted further to differentiate the two zones of pH to understand the sedimentation process during the increased pH of the media.

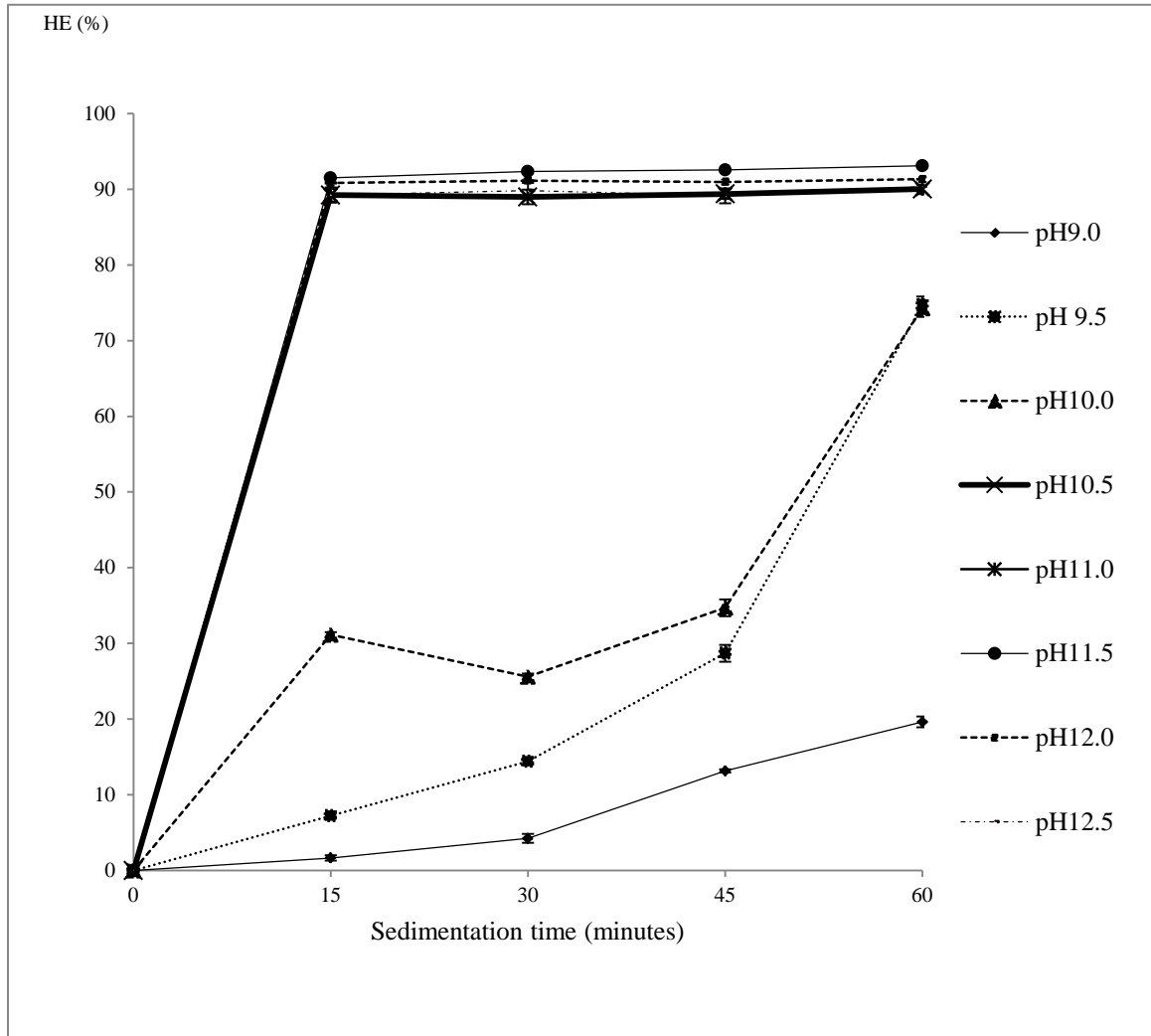


Figure 6:2 Harvesting efficiency as a function of settling time at various pH of media

As seen in Figure 6.2, the lower settling rate was noted below the threshold pH (pH=11) a. At settling times of 60 minutes in region of pH of 9.5, 10, and 10.5 provided the harvesting efficiency was 19.6%, 74.7%, 74.2% and 86% for the cells respectively. The further increased the pH by 0.5 units from 10.5 to 11 caused rapid settling of the cells resulting with 1.8x increased harvesting efficiency from 32.3 % to 89.2% noticed with in

the 15 minutes of settling time. This zone is referred as the fastest settling pH range (second zone $\text{pH} > 11$). Above pH 11 no increase in HE is noted at the end of 60 minutes. The harvesting efficiency above 90% was achieved by increasing the pH from the threshold ($\text{pH}=11$) to pH 11.5 and 12.0 at the 15 minutes of settling time. Further increase in the pH to 12.5 showed slight decrease to 89% in the harvesting efficiency. The trends of the HE were further described by the linear equation of the trend line obtained.

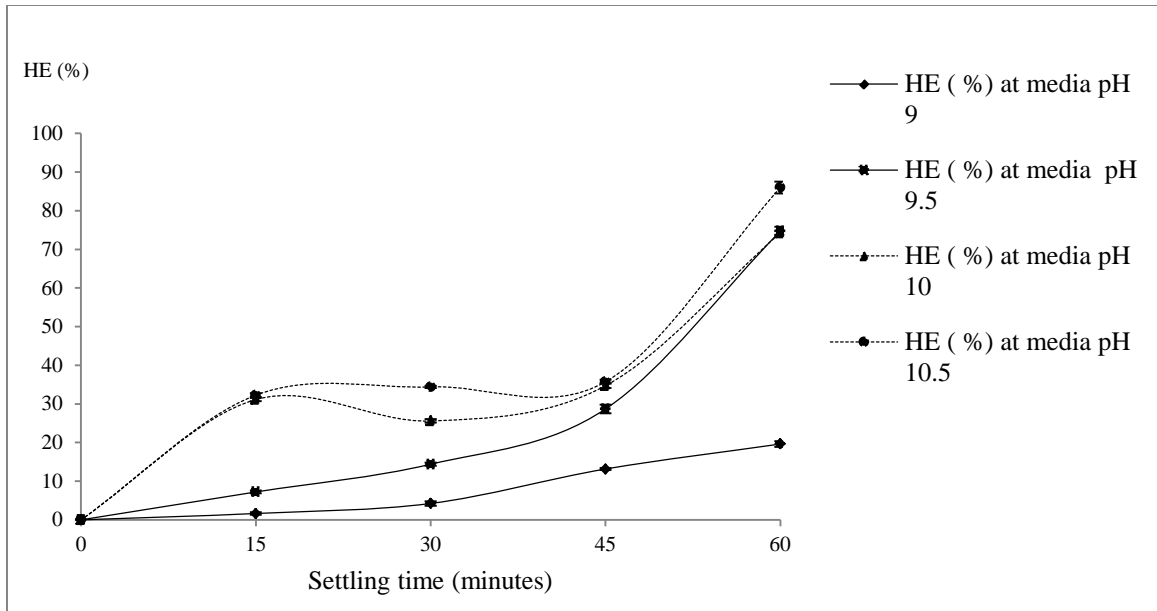


Figure 6:3 Harvesting efficiency of *N.gaditana* as a function of time in the pH ranges (9 to 10.5)

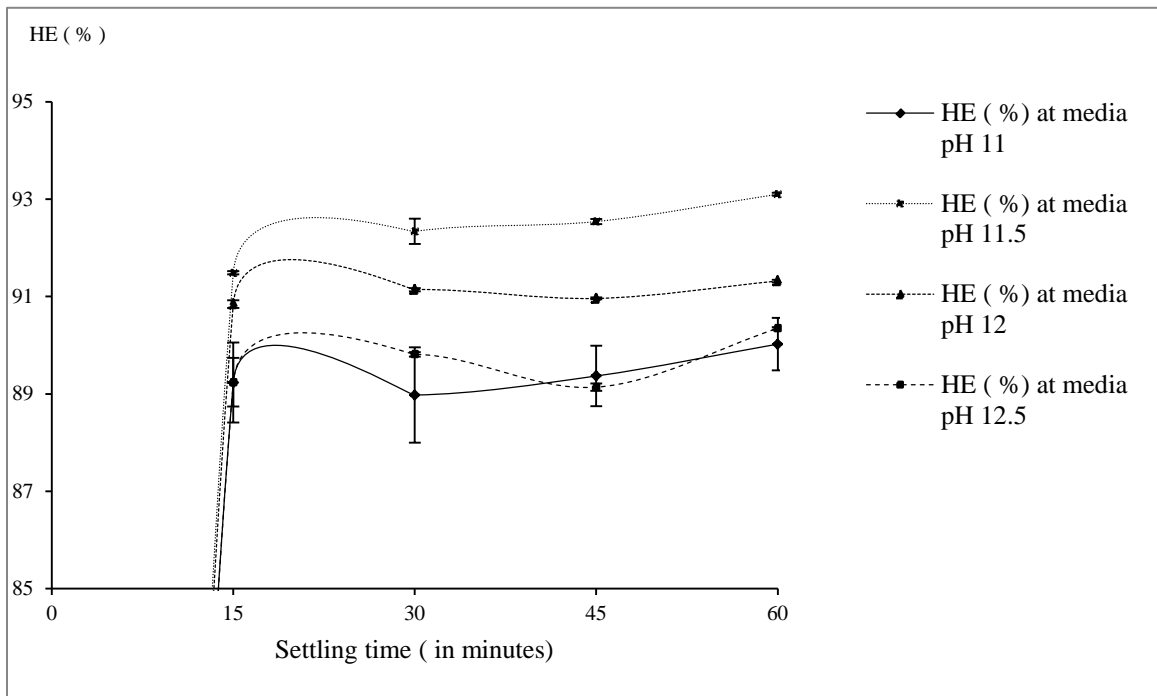


Figure 6:4 Harvesting efficiency of *N. gaditana* as a function of time in the pH ranges (11 to 12.5)

An empirical linear relationship in the following equation were observed between the harvesting efficiency versus the settling time (0-60 minutes) (see the below equations 6.4 to 6.7)

$$\text{HE \%} = 0.34 x + 2.4 (R^2 = 0.92)\text{at pH 9} \quad (6.4)$$

$$\text{HE \%} = 1.17x + 2.4 (R^2 = 0.92)\text{at pH 9.5} \quad (6.5)$$

$$\text{HE \%} = 1.23 x + 2.4 (R^2 = 0.81)\text{at pH 10} \quad (6.6)$$

$$\text{HE \%} = 1.17 x + 2.6 (R^2 = 0.81)\text{at pH 10.5} \quad (6.7)$$

Where x is the sedimentation time and the HE (%) is the harvesting efficiency as per the equation 3.6.

The increasing the pH of the media from 9 to 10.5 with the NaOH was provided the linear relationship between the harvesting efficiency (HE %) and settling time. The flocculation and settling of the cells were increased with the time (0-60 minutes) .The settling of the cells was steadily increased when the cells were exposed to the pH longer time.

But these trends were not observed when the pH of the media increased from 11 and above. The increased pH in the range of 11 to 12.5 provided the increased harvesting efficiency (HE %) and the faster settling of the cells. The harvesting efficiency of 89-91.5% was obtained at the 15 minutes of the settling time at pH 11, 11.5,12 and 12.5 respectively. The harvesting efficiency (%HE) were 90, 93.1, 91.3 and 90.3% were obtained at pH11, 11.5,12 and 12.5 respectively at exposure of the cells for 60 minutes to these alkaline pH.

Since the majority of the cells experienced the immediate flocculation, the increasing the sedimentation time did not further increased the harvesting efficiency. The steady values between the 90 -93% were obtained at the end of the 60 minutes sedimentation time. The harvesting efficiency (%HE) as function of time were not maintained the linear relationship, since the maximum settling of the cells were completed within the first

15minutes of the sedimentation time. The settling of the cells in the 15 minutes were 5.95x, 6.1 x, 6.1 x, 5.95x at pH 11,11.5,12 and 12.5 were obtained when the trend line of the harvesting efficiency (%HE) versus the settling time further implying the settling rate is

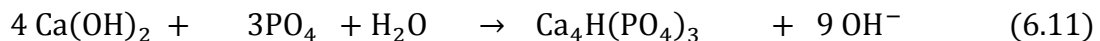
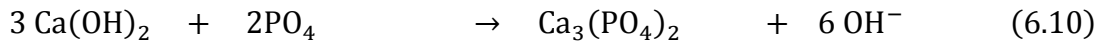
In this study, a pH-induced flocculation method was applied to harvest marine *N. gaditana* and the degree of the cell aggregation was determined by the flocculation efficiency. The flocculation efficiency was presented as the harvesting efficiency versus the pH values.

The steady increased settling of the cells increasing the harvesting efficiency (%HE) were observed in the 9 to 10.5 pH range with the 70 to 86 % of HE at 60minutes of the sedimentation time. When the pH increased above 10.5, the cell flocculation experienced immediate effect with the change in the pH of the media. The rapid settling of 90% cells noted at the first interval of settling time (15 minutes). But the progressing the settling time did not able to flocculate 100% of the cells since the increased settling time reached an asymptote (Figure 6.4). The pH 11 and above provided the maximum settling of cells with highest HE was noted at the pH of 11.5. Hence the pH of 11 was considered the critical threshold pH that changes the settling behavior of the cells. The low flocculation of the cells at below the pH 10.5 might be accounted to the inherent buffering capacity of salt media.

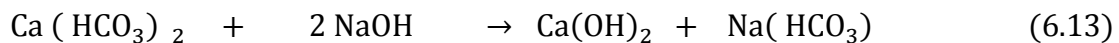
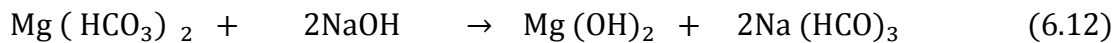
The effect of flocculation efficiency with change in pH were previously noted in several studies and it has been suggested that calcium, magnesium, phosphate may have a role. Since the cultivation was conducted using the road salt media which has the initial concentration of Mg of 4.6 mg/L and Calcium of 102 mg/L (see Table 4.1 materials previously measured by the ICP-MS). The role of calcium and phosphate precipitation at different pH ranges were noted in the waste water system were previously provided²²³ described below for the explanation to the threshold pH and maximum settling. The formation of calcium carbonate precipitates out at pH 9.1- to 9.5 (Equation 6.8 and 6.9) and entraps the suspended and colloidal particles by sweep coagulation as per the below equation.



The precipitation of Phosphate takes place at the pH of 10.5 to 11 as per the below equation (Equation 6.10 and 6.12). The microalgae consume the phosphate for the growth and hence the effect of phosphate precipitation will not be applied for the microalgae.



Since the sodium hydroxide is used as base to increase the alkalinity of the media, the increased pH precipitates the calcium and magnesium as per the below equation.



Since the media contains magnesium, the precipitation magnesium begins at approximately pH 9.5, becomes significant above pH 10.5, and is essentially complete at pH 11.0–11.5 were noted in the previous studies²²³ will be applicable to the media. The metal hydroxide formed above during the increased pH acts as “weighting agent “for the suspending particles induce the settling and described in the waste water treatment process²²². The increased flocculation at the pH 11 and above is in the agreement of previous reports of *Chlorella* cultivated in fresh water media provided a noticeably increased flocculation exceeding 95% at pH 11.5 and 12, ranges²²⁴ were further explained. The charge neutralization at increased pH was explained by the formation of magnesium hydroxide, which will be most likely mechanism induced the flocculation of the *N. gaditana* cells. The explanation provided previously as the trivalent aluminum and ferric ions if present in the media replaces bivalent magnesium in the crystal structure of magnesium hydroxide provides net positive charges to neutralize the negative surface charges. The effect of cell density and the effectiveness harvesting using the pH were also explained for the marine species defined the amount of alkaline required for the bulk harvesting using different bases²²⁵.

6.2.2 Harvesting efficiency of *N. gaditana* cells with ferric chloride

The harvesting efficiency of *N. gaditana* was determined using the inorganic flocculants ferric chloride at the concentration ranging from 10-to100 mg/L. The harvesting efficiencies were plotted for the four settling times in the figure 6.5. As shown in the figure the increasing the dosage of ferric chloride and settling time increased the harvesting efficiency.

The dosage of 50 mg/L and above could start the immediate aggregation the cells were noticed at 30 minutes. The dosage of 50 mg/L resulted 89 % HE at the end of 30 minutes of settling time. The dosage of 100 mg/l resulted with cell aggregation provided the harvesting efficiency of 45% at the end of 15 minutes accounting approximately for the 50% of the total harvesting efficiency obtained by the study. The dosage of 60 mg /L and above of ferric chloride provided the >90 % harvesting efficiency at the 30 minutes of settling time with maximum of 94.7% harvesting efficiency obtained at the 100 mg /L dosage.

The increased settling time to 45 minutes increased the harvesting efficiency over 90% for the samples added with dosage of 20 mg/L and above ferric chloride. In general at the end of the 60 minutes the harvesting efficiency of 95% obtained in all the samples that are added with 70 mg/L and above ferric chloride. Further increasing the settling time had very minimum effect on the flocculation. The dosage of the ferric chloride varies between the culture conditions were reported previously²¹⁵.

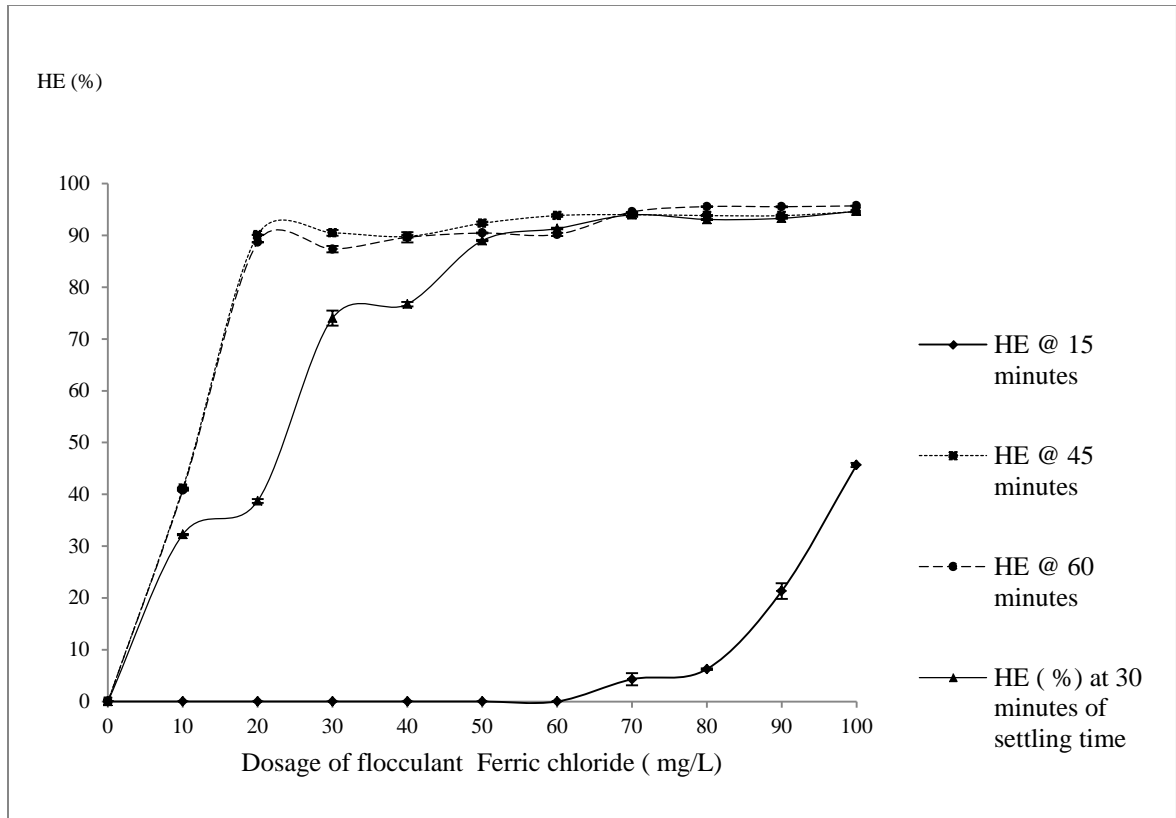


Figure 6:5 Harvesting efficiencies at various settling time versus ferric chloride dosage

To understand the combined effect of change in the pH of the culture system pH=9,10 and 6 on the flocculation of efficiency of ferric chloride were studied at 10,20,30, 40 and 50 mg/L dosages rates with the settling time of 30 minutes. The acidic pH (pH=6) reduced the flocculation efficiency of the *N.gaditana* cells as noticed in the figure 6.6, since the decreased pH to 6 reduced harvesting efficiency and the higher dosage was required higher to flocculate the cells. The 50mg/L dosage provided the HE of 80 % at 30 minutes of settling time. But the increased pH to 9 and 10 showed the increased harvesting efficiency with the low dosage of ferric chloride. The maximum of harvesting efficiency of 90% were obtained at pH 9 and 10 with the dosage of 50 mg/L of ferric chloride flocculants.

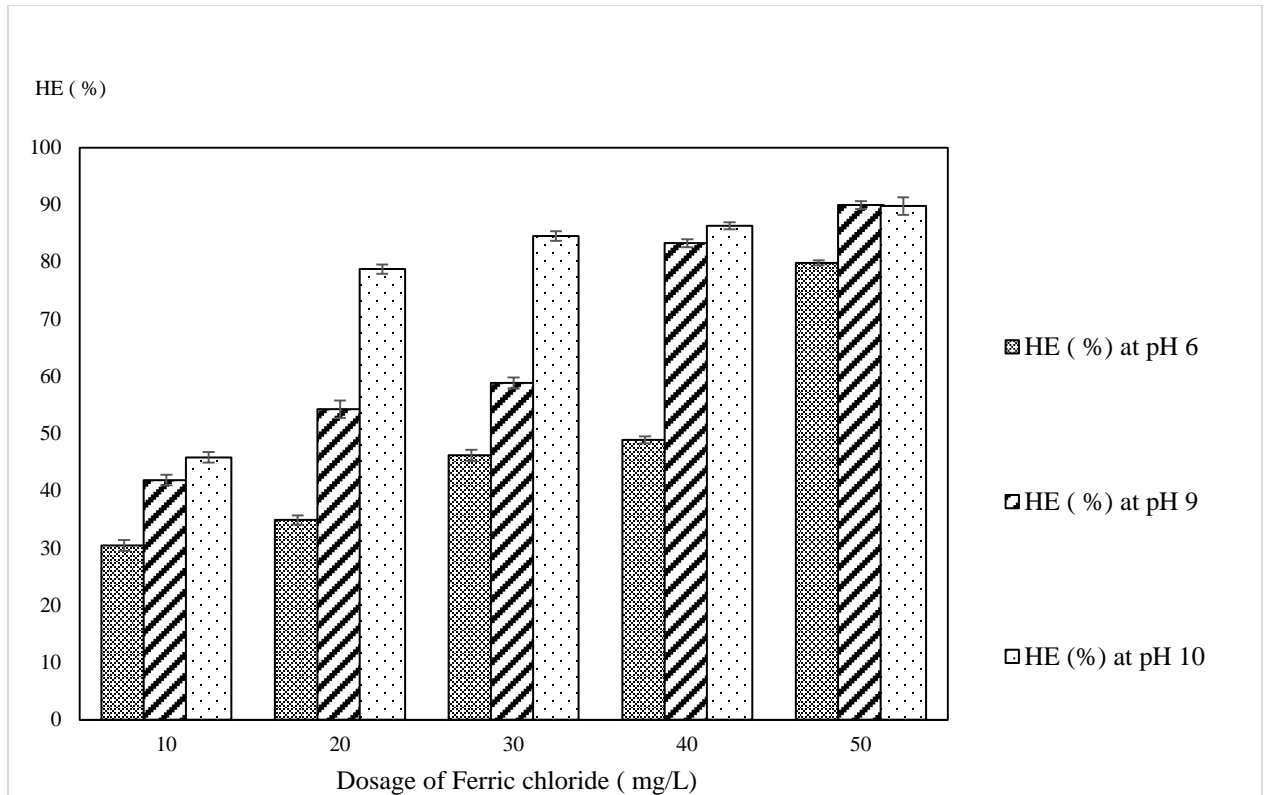


Figure 6:6 Harvesting efficiencies with ferric chloride and change in the media pH

The dissociation and availability of ferric ions to neutralize the surface charges of the cells vary with the media composition. Generally, ferric ions can react with hydroxyl to form ferric hydroxides, even at low pH. The change in the pH changes to the alkaline conditions induces the sweeping flocculation by the metal hydroxide as noticed in the alkaline induced treatments. The chemical flocculation by the ferric chloride takes place by the charge neutralization. The combined effect of sweeping and charge neutralization showed a positive impact on the cell flocculation. The amount of flocculants required is highly dependent on the number of charges that need to be neutralized, which are in turn a function of the charge density of the cell surface as well as the surface to volume ratio of the cells, parameters that differ strongly between species were noted²²⁶. But the slight increased pH assisted in reduction of dosage of coagulant/ flocculant ferric chloride in the study.

There were several mechanisms causes flocculation due the reduction in the energy barrier between the cells by increased ionic strength of the media, reduction of negative

charges of cell surface or charge neutralization. The multivalent metal cations, such as Mg^{2+} , Ca^{2+} and Fe^{3+} play important roles in destabilizing the negative surface charges in microalgae to promote the flocculation process at higher pH. The hydroxyl ions at the higher pH combine with metal form metal hydroxides ions. The positive charges of the metal hydroxides neutralize the negative surface charges of microalgae. The increased flocculation efficiency hence will be related to the charge neutralization. The increase the pH by adding NaOH ferric hydroxides are formed neutralizes the surface charges of the microalgal cells enhances the rate of the cell aggregation and settling. Formation of ferric hydroxides increases with the increase in pH or dosage of NaOH results in higher availability of ionic form of metal hydroxide to enhance the flocculation process.

The culture parameters define the flocculation properties of the ferric chloride. The flocculation properties of ferric chlorides explained by the formation of ferric hydroxide and interaction of ferric with released polysaccharides from the cells^{155,227}. The inhibitory influence of released polysaccharides (RPS) on the recovery of halophilic microalgae the cyanobacterium *A. halophytica GR02* with ferric chloride were reported that the attachment of ferric ions to cell surfaces were reduced with the presence of RPS²²⁷. But in the study the inhibition of the flocculation were not observed.

6.2.3 Harvesting efficiency of *N. gaditana* cells with chitosan

Chitosan is soluble in acidic pH and the solution (10 mg/ml) of chitosan was prepared in acetic, citric and HCl were added at 10-100mg/L concentrations to the algal suspension to investigate the effectiveness of flocculation of the marine microalgae. The chitosan is soluble in acidic pH and its application to the marine microalgae growth media provided the lower efficiencies. Since chitosan is soluble in acidic solution, the effects of three acids solvents were used for the preparation of chitosan flocculants solution. The influence of chitosan on flocculation efficiency was failed to provide the sedimentation of the cells as noticed in the previous two treatments. The clear separations of cells from the media were not observed and the tested conditions provided the HE were less than 10% efficiency.(fig 6.7). Among the three-tested solution of the chitosan solution, the chitosan solubilized with HCl provided the flocculation of 5.9%, followed by the chitosan in the citric acid solution (HE=4.9%). The optical density of the top layer fluctuated in the algal

suspension when the chitosan solution prepared with acetic acids were added and the least HE (>3.5%) based on the optical density were observed at 10-100 mg/L dosage levels of flocculants chitosan.

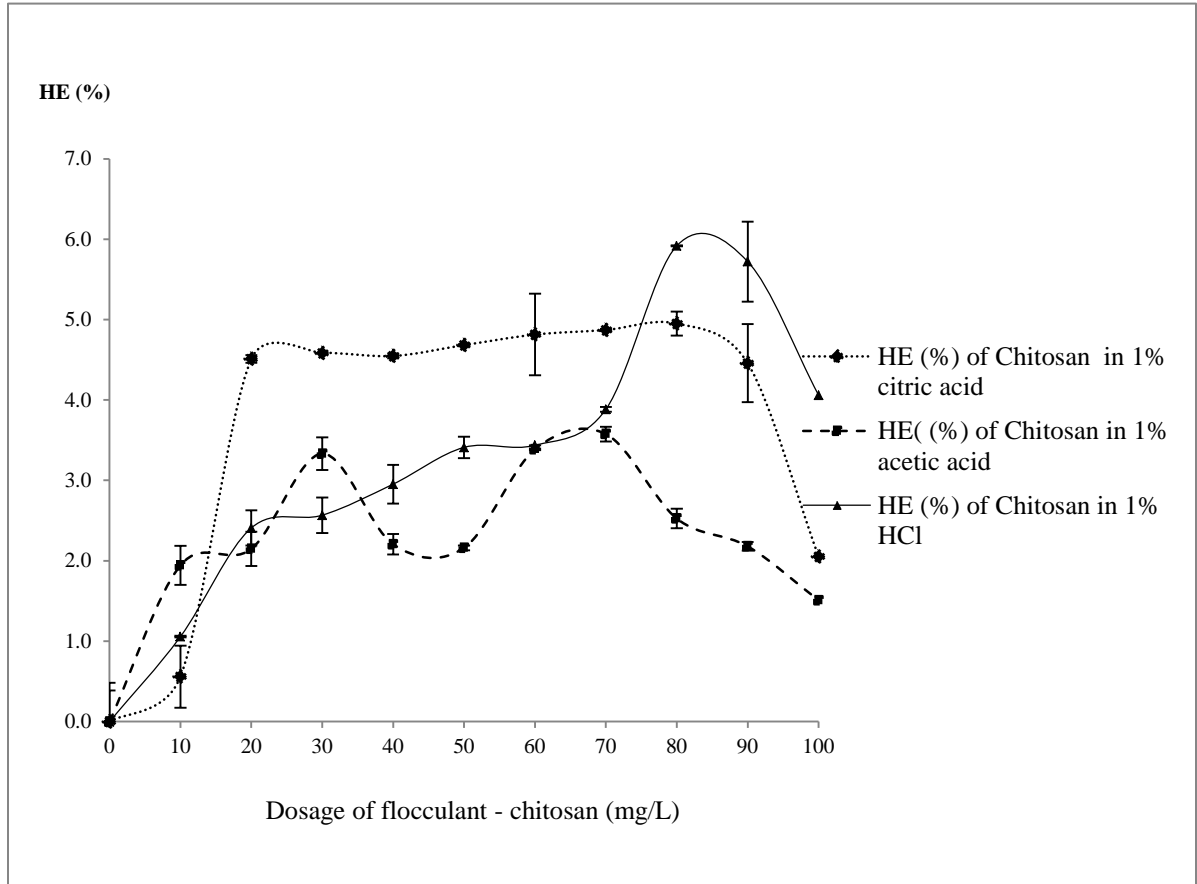


Figure 6:7 Harvesting efficiency of chitosan in acetic and citric acid solution at various dosage rates

The optimal working pH for chitosan flocculation is pH 6 or lower and is more efficient cationic flocculants in a weak acidic environment. The chitosan contains a poly-glucosamine backbone and has an isoelectric point around 6.5. More parts of the polymeric chains are positively charged at pH 6 and below than at pH 7 or higher. The decrease of pH below 7 reported to increase the chitosan activity and harvesting efficiency due to the decreasing in the viscosity and the mean surface charge of algal cells²²⁸.

Among all the tested flocculants, chitosan showed the lowest performance for the flocculation of biomass even at very high dosages (100 mg/L). This was possibly due to the pH of the road salt media were around 8. It is clear that chitosan flocculation conditions depend on the pH, which influences the availability of the active sites of the chitosan for the charge neutralization. The maximum harvesting observed in the HCl based chitosan solution than the citric acid. However the acetic acid showed the lowest flocculation efficiency and the increased inclusion of chitosan did not influence the performance of chitosan on cell flocculation as no clear separation of the cell/sludge formation were noted in the testing tubes.

The mechanism of operating in chitosan–algal cell flocculation described by the reports suggests the combination of partial charge neutralization, static patch effects, and bridging (netting). The structure of the chitosan consist positively charged deacetylated units that are spread along the whole backbone of chitosan chains. This extended arrangement of the polymer maintained by the repulsive forces. The decrease or the no effect in flocculation efficiency of chitosan in the road salt media might be due the high ion concentration of the environment is probably the large amounts of ions in a high ionic medium shield the repulsion of positive charges on chitosan chains, thus causing a reduction in their ability to cause flocculation.

The DLVO theory states that suspensions are stabilized by surface charges of the particles. The balance between the electrostatic repulsion and the Van der Waals attraction can be shifted towards attraction, causing coagulation and flocculation through several mechanism.^{229,230} The alkaline flocculation mechanism is by the sweeping mechanisms while the chemical coagulants is by charge neutralization were previously reported were effectively flocculated the cells.^{127,137,215,231,232} Flocculation induced by high pH is a potentially useful method to pre-concentrate microalgal biomass. However, the method depends on sufficiently increased high pH (>10.5) using sodium hydroxide can be attained over >90 % cell aggregation within 15 minutes.

6.2.4 Concentration factors of algal suspension

Flocculation and concentration factor are the two important factors determine the harvesting efficiencies. The concentration factor (CF) is important factor to evaluate the degree of harvest and estimates the pre concentration of the biomass. The volumes of the clarified media obtained in the flocculation experiments were used to determine the concentration factors. Clarification factor of the pH induced, chemical and chitosan flocculation were plotted in figure 6.8, 6.8 and 6.10. As shown in the figures the concentration factor increased with settling time provides the clear indication of increased sedimentation of the algal cells. The maximum concentration factor of 16.2 ± 0.3 obtained with pH 11.5 at the 60 minutes sedimentation time in the pH induced flocculation treatments, which corresponds to the maximum harvesting efficiency.

The flocculants ferric chloride provided the maximum concentration factor of 23.56 ± 0.3 at end of the settling time 60 minutes with the dosage of 60 mg/L of flocculants. Among the tested flocculation methods application of chitosan was failed to separate the cells and hence the lowest concentration factors were noted.

However this highest value of the ferric chloride based flocculation does not corresponds to the highest harvesting efficiency. The highest harvesting efficiency of 95.7 % obtained at the dosage of 100 mg/l of the ferric chloride provided the 12.51 concentration factor. The concentration factors were further compared to the ratios of biomass obtained by the dry weight (Table 6.1). The concentration factor of the biomass obtained does not by the volume differences were noted slightly higher than the dry weight, since the dry weight removes the moisture and did not directly translated to the concentration factor of the volume reduction

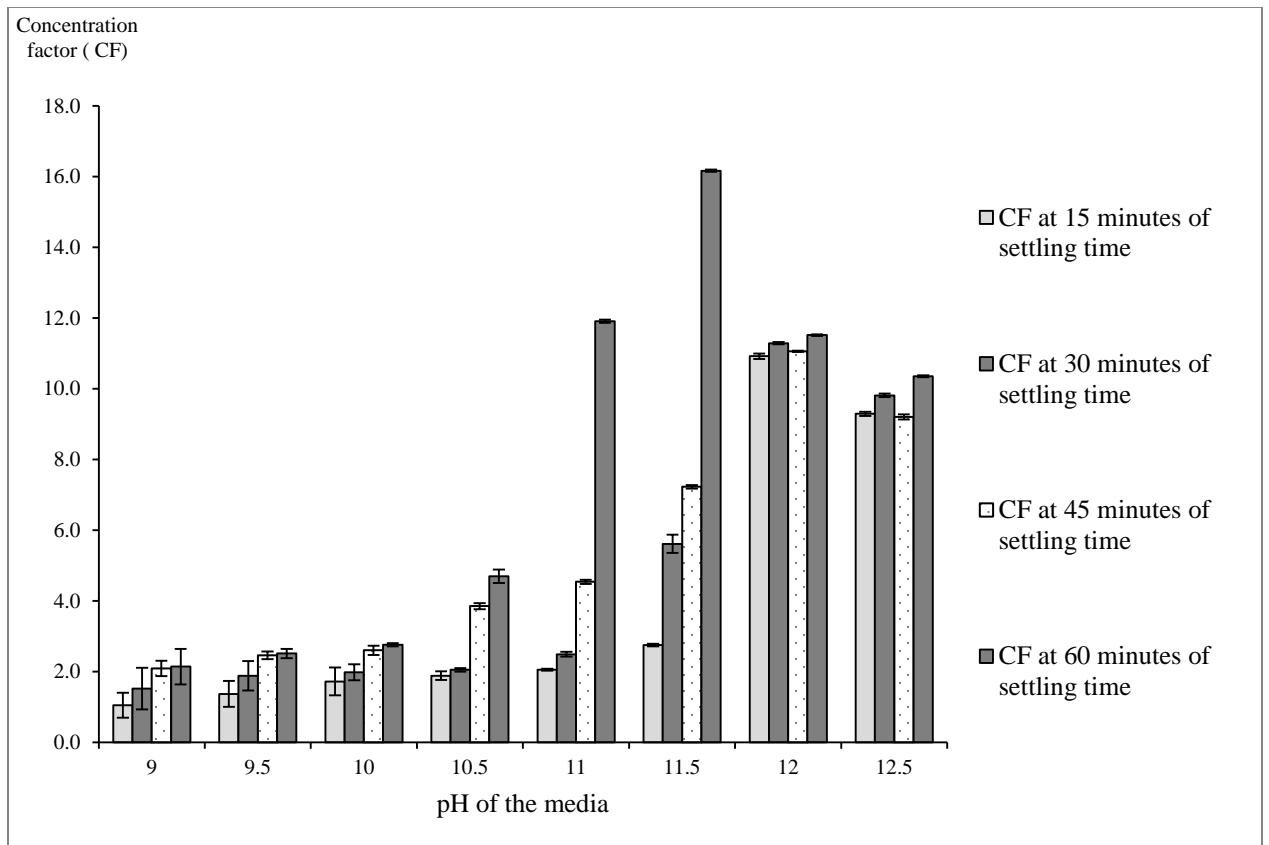


Figure 6:8 Concentration factor by pH induced flocculation at 4 sedimentation times

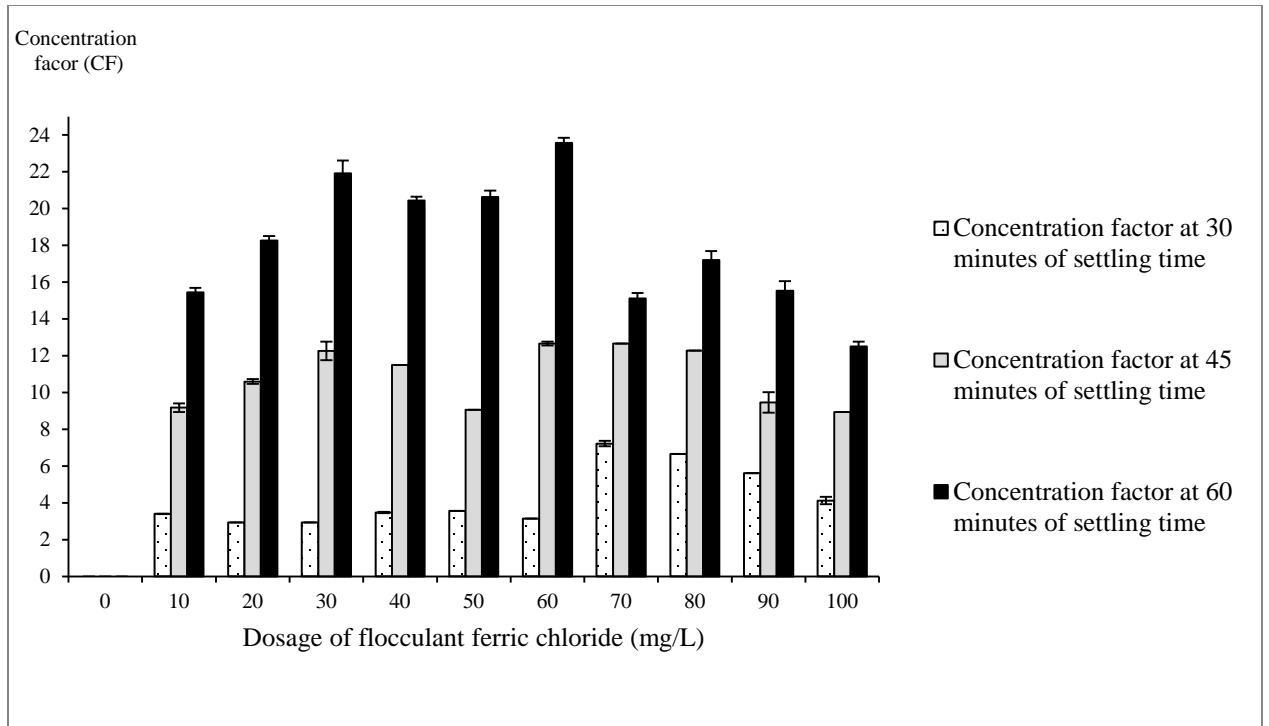


Figure 6:9 Concentration factor with ferric chloride at 3 sedimentation times

The concentration to 20x were reported previously in *Chlorella* species with the dosage of 5mM of NaOH High flocculation efficiency of 90% ¹⁵⁵ and on marine diatom *Phaeodactylum tricornutum* with the alkaline ²³³ were noted in the previous studies . However the present on the *N. gaditana* by the alkaline pH were achieved the maximum of 15.36 concentration factor. But the application of ferric chloride resulted above 20x concentration factor in the study. The pre concentration of this level by the flocculation techniques provided opportunity to apply the flocculation methods for harvesting microalgae from the growth media.

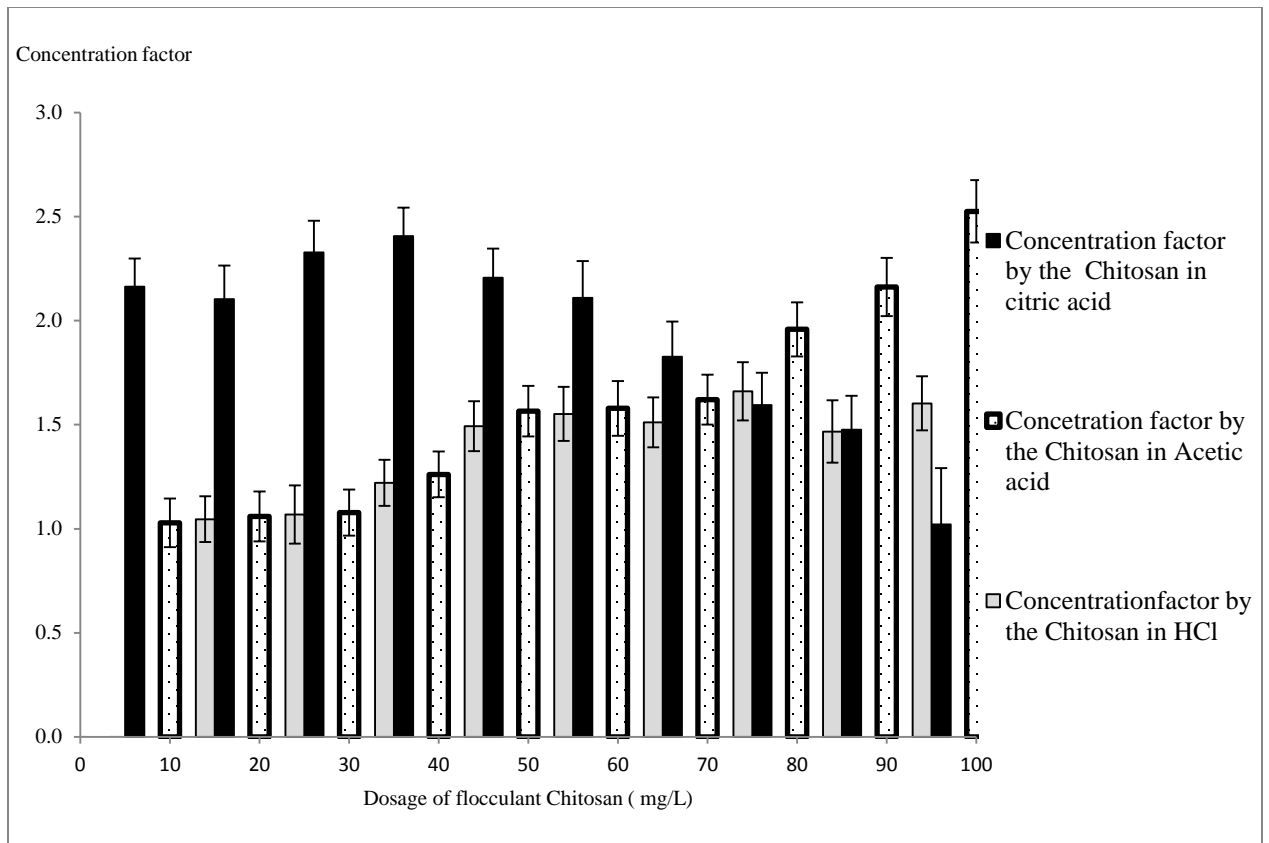


Figure 6:10 Concentration factor with chitosan at 60 minutes of settling

6.2.5 Comparison of concentration factors

While the harvesting efficiency was defined as the ratio of the mass of cells recovered to the total mass of cells and the concentration factor was the ratio of the final product concentration to the initial concentration²³⁴. The increased concentration factor means to the decreased amount of the sludge, which provided increased quantity of the clarified media in the flocculation methods. The higher the concentration factor lesser the growth media in the sludge, which reduces the further, drying process in the downstream processing. Concentration factor of the three methods were further analyzed by the cell dry weight Table 6.1. Flocculants are attached to the cells, when the flocculation is achieved by the use of flocculants, the amount of the ions attached would contribute to the weight of the biomass.

Table 6-1 Comparison of Concentration factor dry weight method and flocculation volume reduction methods

| Treatment method | Initial biomass concentration (g/L) | Biomass concentration by the dry weight method (g/L) | CF by the mass difference by dry weight method | CF by the volume difference of flocculation |
|---|-------------------------------------|--|--|---|
| pH 11.5 at 60 minutes sedimentation | 1.25±0.05 | 19.2±0.23 | 15.36 | 16.2±0.03 |
| Ferric chloride @ 60mg/L dosage at 60minutes of sedimentation | 1.27±0.05 | 27.6±0.043 | 21.73 | 23.56±0.29 |
| Ferric chloride @ 100 mg/L dosage at 60minutes of sedimentation | 1.27±0.05 | 15.2±0.217 | 11.97 | 12.51±0.26 |

6.3 Conclusions

In conclusion, three chemical flocculants namely alkaline and ferric chloride and chitosan were applied to harvest the cells of *N. gaditana* from road salt run off media for the biomass recovery. The alkaline based flocculation provided the two different settling patterns of the cell, The harvesting efficiencies were in the liner relationship with the setline time at the pH range of 9-10.5 and the rapid settling of the cells were observed the pH range of 11-12.5. Over 89 % of harvesting efficiencies were obtained at the pH 11 and above.

The chemically induced flocculation using ferric chloride is the most effective method for the harvesting the marine microalgae *N. gaditaa* and was dosage dependent. The highest

harvesting efficiency of 95% at the settling time of 60 minutes were obtained when the dosage of 80 to 100 mg /L were applied to the spent media. Averages of 90 % harvesting efficiencies were obtained at the dosage of 50mg/L ferric chloride. The increased pH reduces the dosage of ferric chloride to obtain the >90 % of harvesting efficiency.

The last method of flocculation with the organic flocculant chitosan provided very poor performances of cell harvesting and was considered not effective to harvest the marine microalgae in the road salt media. Chitosan is soluble in acid and the change in the acid solution (citric, acetic, HCl) did not improve the harvesting efficiencies. The harvesting efficiencies obtained were below 6% at the tested dosage range of 10-100 mg/L of chitosan.

Therefore, the alkaline-based flocculation method has important advantages, such as high harvesting efficiency, environment friendly, and low cost, which could be more applicable for harvesting microalgae for biofuel production than other methods.

The concentration factor (CF) is important factor to evaluate the degree of harvest and estimate for the pre concentration of the biomass. The alkaline flocculation at pH 11 and above provided the CF of above 10 and the maximum of 16.2 concentration factor. The ferric chloride was the most efficient method, which provided the maximum concentration of the cells (CF=23.6) in the sludge.

Chapter 7

7 Conclusions and recommendations

The research study investigated phycoremediation using marine microalgae cultivated on simulated media representing road-salt and vegetable Greenhouse industry effluents and the potential to use in biofuel application. Here the overall conclusions and recommendations are provided.

7.1 Conclusions

In this study, a review of the wastewater systems that are limited for the traditional treatment in the local area identified the road salt run off and green house industry effluents as candidates for the phycoremediation processes. The use of low cost/zero cost effluents was chosen to provide a competitive advantage to biofuel production in microalgae. *Nannochloropsis* was the microalgal species of choice as it is halophilic and accumulates high content of lipids. First batch growth studies were carried out to evaluate a range of parameters including salt concentrations and nitrate loading on cell growth. Simulated nutrient media, which represented road-salt effluent mixed with vegetable Greenhouse effluents, were investigated in batch and fed-batch bioreactors under different light regimes. Overall it is concluded that the study showed that phycoremediation is a useful unit operation, which can add value to the bioprocess by generating lipids for applications as biofuels.

The investigation of fed batch cultivation methods for the continuous supply of the nitrate nutrients to the growth media is an effective method for simultaneously increasing the biomass yield and lipid content. This method was efficient was able to increase the biomass yield to 2.08 g/L and lipid content to 58% DW with the application of light production. The results of these studies are summarized with the nitrogen to cell conversation rate and volumetric lipid yield (mg/L) and the lipid yield on nitrate, which can be used for the cost efficiencies

Finally the approaches of flocculation methods to harvest the biomass were investigated using the alkaline, chemical flocculants ferric chloride and chitosan and the results showed the increased the pH of the media induce the flocculation of the cells in the road salt media .The effective threshold pH 11 and above provided the 90% of the cell aggregation and sedimentation . The ferric chloride is the most efficient flocculants resulted with h 95 % of harvesting efficiency at the dosage of 70mg/L with the settling time of 60 minutes. The chitosan solution were solublised using three acids were failed to coagulate the cells in road salt media with in the settling time of 60 minutes

The biomasses obtained were subjected for the fatty acid analysis. The fatty acid profile of the lipid defines the suitability for use as biofuel to meet the standards of ASTM. The fatty acid profile of the lipids extracted from the biomass dominated the C16 and C18 indicated the monounsaturated and saturated lipid were contributed to76 % of the FAME

7.2 Recommendations

From the experience obtained after the completion of this thesis the following suggestions are done for future work:

- Chapter 4: The vegetable greenhouse were used for the study, but it would be interesting to conduct growth study to on other types of wastewaters and industrial effluents to analyze the biomass productivity and lipid yield for the field conditions and to examine productivities in other marine and halophilic microalgae species..
- Chapter 5. The details study of the feeding regimes on three-fed batch cultivation for the microalgae cultivation provided the direction of its efficiency on increasing the biomass and lipid yield. Further studies could focus on scale up on a larger scale under field conditions.
- In chapter 6 briefly flocculation/harvesting approaches were compared for algal removal from spent media. This approach could be further compared with other

traditional and non-traditional methods. Methods for the recovery of the cells from the growth media. It will be useful to discuss and explore other types of bio and chemical coagulants and also determine economic and life cycle analyses of the processes.

- Care should however be taken that the high pH does not destroy the microalgal cells, as this may result in loss of useful bio products from the biomass. The chemical flocculation and the pH-induced method are sustainable. Further research regarding the impact of pH induced flocculation on cell viability and recycling of growth media and effect on the biomass and upstream processing like lipid extraction and fatty acid ethyl ester are needed for the for the consideration of application of biomass for the biofuel production.

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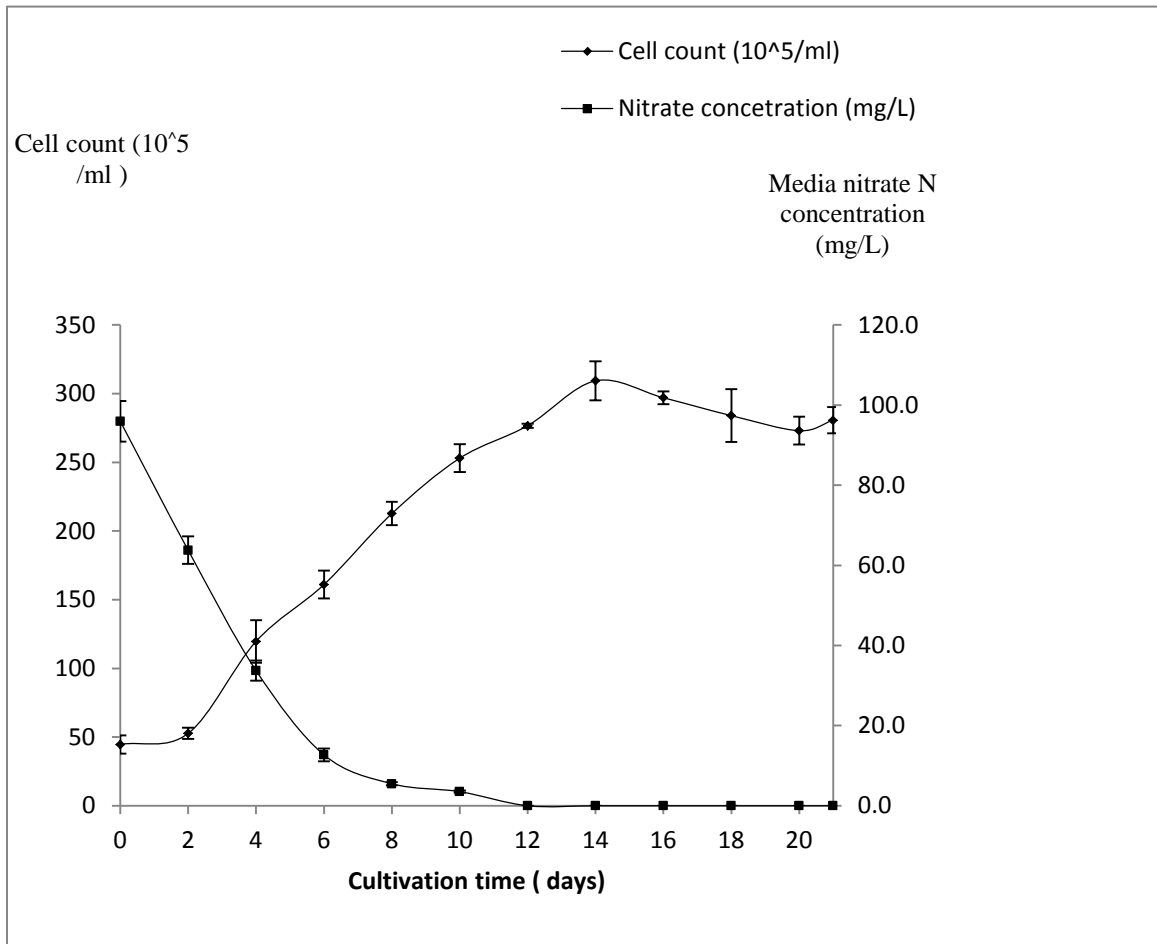
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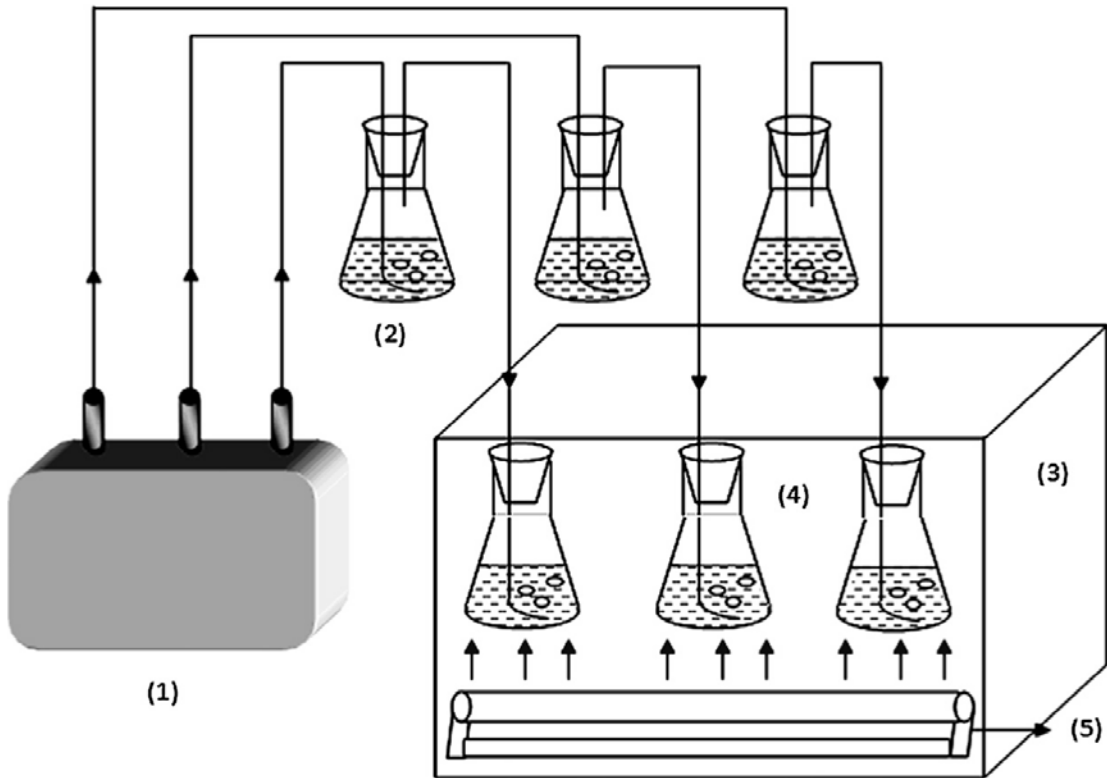
Appendices

Appendix A Growth kinetics and nitrate uptake profile of *N. gaditana* in real vegetable greenhouse effluents



Appendix B Elemental composition of the road and reef salt

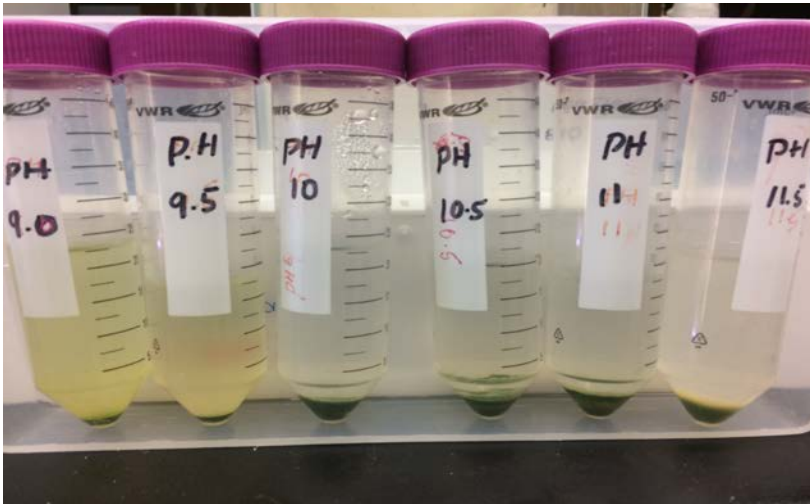
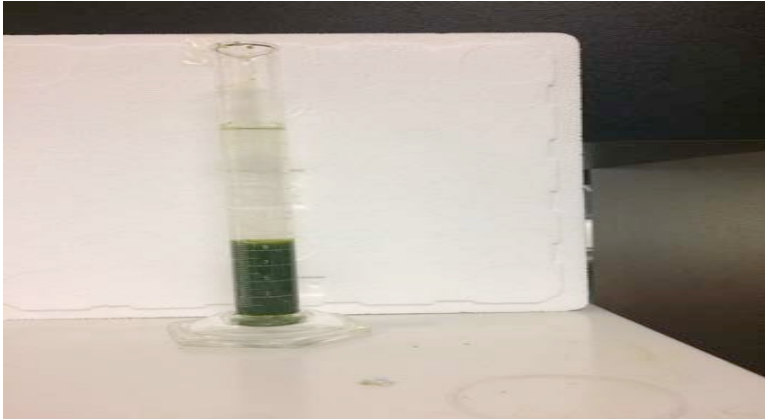
| Element | 2.6% Road salt (mg/L) | 3.5% Road salt (mg/L) | 4.4% Road salt (mg/L) | 2.6% Reef salt (mg/L) | 3.5% Reef salt (mg/L) | 4.4% Reef salt (mg/L) | F/2 media (mg/L) |
|---------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------|
| Na | 3963.811 | 5335.900 | 6707.989 | 1655.309 | 2228.300 | 2801.291 | 4893.617 |
| Mg | 4.576 | 6.160 | 7.744 | 4721.971 | 6356.500 | 7991.029 | 4418.188 |
| Al | 0.250 | 0.336 | 0.422 | 0.131 | 0.176 | 0.221 | 0.507 |
| Si | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 | |
| P | 0.016 | 0.022 | 0.027 | 0.002 | 0.003 | 0.004 | |
| S | 31.564 | 42.490 | 53.416 | 166.957 | 224.750 | 282.543 | 107.142 |
| K | 2.674 | 3.600 | 4.526 | 168.629 | 227.000 | 285.371 | 383.235 |
| Ca | 102.143 | 137.500 | 172.857 | 147.011 | 197.900 | 248.789 | 388.349 |
| V | 0.006 | 0.008 | 0.010 | 0.004 | 0.006 | 0.007 | |
| Cr | 0.001 | 0.002 | 0.002 | 0.001 | 0.001 | 0.001 | |
| Mn | 0.037 | 0.050 | 0.063 | 0.012 | 0.017 | 0.021 | |
| Fe | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 | <0.010 | |
| Co | 0.003 | 0.004 | 0.004 | 0.005 | 0.006 | 0.008 | |
| Cu | 0.071 | 0.095 | 0.119 | 0.028 | 0.038 | 0.048 | |
| Zn | 0.063 | 0.085 | 0.107 | 0.003 | 0.004 | 0.005 | |
| As | 0.374 | 0.503 | 0.632 | 0.022 | 0.030 | 0.037 | |
| Sr | 1.998 | 2.690 | 3.382 | 5.861 | 7.890 | 9.919 | |
| Ba | 0.040 | 0.054 | 0.068 | 0.008 | 0.011 | 0.014 | |



Appendix: C Experimental set up

1: air pump, 2 Moisturizing sterilized air, 3- stands to hold the reactors, 4 microalgae reactors 5) Fluorescent lights

Appendix: D Photo of the jar experiment



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26–29 June 2016