MICRO-ALGAE ASSISTED BIOREMEDIATION OF SIMULATED DAIRY EFFLUENT AND VALORIZATION

BY

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ABSTRACT

Dairy industries play a crucial role in improving the economy of a country. Owing to increasing global population, dairy production is expected to continue growing over the coming years. This will lead to an increase in the amount of wastewater that is generated during processing of dairy products. The wastewater generated contains high amounts of organic matter that are detrimental to the environment when left untreated. The problem for most of the dairy processing plants is that the treatment of dairy wastewater adds to their capital and operating costs. This requires the application of treatment procedures that are cost-effective and contribute to water conservation. Current strategies of dairy wastewater treatment involves physical, chemical, mechanical or biological or a combination of any of these methods. Since dairy wastewater contains highly biodegradable organic matter, a biological mode of treatment is the most commonly employed method that is relatively cheap and effective.

Employing microalgae for treating dairy wastewater is one such biological method that has gained popularity as an efficient waste management technology. Projections for the successful commercialization of microalgal technology depends on the availability of a robust consortia that can achieve increased biomass productivity while remediating wastewater. In addition, extraction of value added products from the residual biomass and utilizing it as a biofertilizer could make the process economically feasible. Based on the literature reviewed, *Chlorella* sp. and *Scenedesmus* sp. are considered to be the most widely used microalgae for treating different categories of industrial wastewaters with valorization. Though studies have focused on utilizing different microalgal species for dairy wastewater treatment, the earlier research has focused on utilizing microalgal consortia comprised of specific strains of microalgae that are not specifically targeted for this purpose. The two microalgal strains chosen for this study have the ability to remediate wastewater and are rich in proteins.

Experiment I of this thesis evaluated the effect of media composition including simulated dairy wastewater on the growth, biomass productivity, nutritional and lutein content of an under-reported strain *Chlorella variabilis*. The growth, biomass productivity, nutritional and lutein content was found to be dependent on the media utilized. MN8 medium and simulated dairy wastewater were considered to be the best media for cultivating *Chlorella variabilis* with enhanced growth and biomass productivity of 0.040 g L^{-1} day^{-1} and 0.037 g L^{-1} day^{-1} respectively. The study holds high importance as it reveals for the first time that the algal strain could produce lutein in

significant amounts (9.6 mg g^{-1}) when cultivated in simulated dairy wastewater. Simulated dairy wastewater was utilized for carrying out experiments because they are easy to store and prepare. In addition, the composition of diary wastewater does not vary over time which makes analysis easier and eliminates complications brought about by unknown variables such as biotic components.

Experiment II employed an algal consortia that grew well in simulated dairy wastewater with enhanced biomass productivity, nutritional and lutein contents. The algal consortia containing *Chlorella variabilis* and *Scenedesmus obliquus* were exposed to different light wavelengths in order to investigate its impact on biomass, nutritional and lutein accumulation. Between the varying light treatments employed, cool-white fluorescent light had higher biomass productivity of 0.042 g L^{-1} day^{-1} and lutein content of 7.22 mg g^{-1} when compared to the other treatments. This study reveals for the first time the importance of using amber light for enhancing carbohydrate content in the algal consortia and this can be attributed to the decreased enzyme activity.

Experiment III utilized the microalgal consortia for remediating wastewater with respect to inoculum concentration, time and light intensity under controlled laboratory conditions. A CCD was employed to find the optimal conditions for removing phosphorus, ammoniacal nitrogen, and COD while enhancing biomass productivity and lutein content. This study shows that the microalgal consortia was able to effectively remove the elements with enhanced biomass productivity of 29.13 mg L^{-1} day^{-1} and lutein content of 12.59 mg g^{-1}. This study is highly significant as the lutein content exhibited by the microalgal consortia is higher when compared to other microalgal species and could possibly become a commercial source of lutein in the future.

Experiment IV investigated the effect of utilizing dairy wastewater-grown wet algal biomass as a biofertilizer for cultivating corn and soybean under controlled conditions. The results showed that the microalgal consortia, employed as a biofertilizer, improved the growth performance and nutritional content of both corn and soybean plants.

This study has shown that the microalgal strains of *Chlorella variabilis* and *Scenedesmus obliquus,* when employed as a consortia, can be effectively used for remediating dairy wastewater with enhanced lutein production by the organisms. In addition, the residual algal biomass, along with the treated wastewater can be utilized as a biofertilizer for agricultural crops. This study has therefore revealed for the first time that microalgae assisted bioremediation can be used for efficient dairy wastewater management. This study holds high industrial importance as it makes

the microalgal technology commercially feasible with its multifaceted benefits and functions. Furthermore, this study has opened up the possibility of future research to focus on microalgal consortia employing specific strains for treating complex industrial wastewaters with production of value added products and the possibility of utilizing the wet algal biomass as a biofertilizer.

RÉSUMÉ

Les industries laitières jouent un rôle crucial dans l'amélioration de l'économie d'un pays. En raison de l'augmentation de la population mondiale, la production laitière devrait continuer à croître au fil des ans. Cette production conduira à une augmentation de la quantité d'eaux usées générées lors du traitement des produits laitiers. Les eaux usées ainsi générées contiennent de grandes quantités de polluants organiques qui nuisent à l'environnement lorsqu'ils ne sont pas traités. Le problème pour la plupart des usines laitières est que le traitement de leurs eaux usées augmente leurs coûts opérationnels. Cela nécessite l'application de procédures de traitement rentables et contribuant à la conservation de l'eau. Les stratégies actuelles de traitement des eaux usées de laiterie impliquent des méthodes physiques, chimiques, mécaniques ou biologiques ou une combinaison de ces méthodes. Étant donné que les eaux usées de laiterie contiennent des polluants hautement biodégradables, le mode de traitement biologique est la méthode la plus couramment utilisée, de façons économique et efficace.

L'utilisation de microalgues pour traiter les eaux usées de laiterie est l'une de ces méthodes biologiques qui est devenue une technologie efficace de gestion des eaux usées. Le potentiel de commercialisation de la technologie des microalgues dépend de la mise en place de consortiums robustes capables d'accroître la productivité de la biomasse tout en assainissant les eaux usées. En outre, l'extraction de produits à valeur ajoutée à partir de la biomasse résiduelle et son utilisation en tant que biofertilisant pourraient rendre le procédé rentable. D'après la littérature examinée, *Chlorella* sp. et *Scenedesmus* sp. sont considérées comme les micro-algues les plus largement utilisées pour traiter différentes catégories d'eaux usées industrielles avec valorisation. Bien que les études se soient concentrées sur l'utilisation de différentes espèces de microalgues pour le traitement des eaux usées laitières, les recherches antérieures se sont concentrées sur l'utilisation de consortiums de microalgues composés de souches spécifiques de microalgues qui ne sont pas spécifiquement ciblées à cette fin. Les deux souches de microalgues choisies pour cette étude ont la capacité d'assainir les eaux usées et sont riches en protéines.

L'expérience I de cette thèse a évalué l'effet de la composition du milieu, y compris les eaux usées de laiterie simulées, sur la croissance, la productivité de la biomasse, la teneur nutritionnelle et la teneur en lutéine d'une souche sous-examinée, *Chlorella variabilis*. La croissance, la productivité de la biomasse, la teneur nutritionnelle et la teneur en lutéine ont été trouvées dépendantes du milieu utilisé. Les eaux usées laitières moyennes et simulées MN8 étaient

considérées comme les meilleurs moyens de cultiver *Chlorella variabilis* avec une croissance accrue et une productivité de la biomasse supérieure. L'étude revêt une grande importance car elle révèle pour la première fois que la souche d'algues pourrait produire de la lutéine en quantité significative lorsqu'elle est cultivée dans des eaux usées laitières simulées. Les eaux usées laitières simulées ont été utilisées pour mener des expériences car elles sont faciles à stocker et à préparer. De plus, la composition des eaux usées du journal ne varie pas dans le temps, ce qui facilite l'analyse et élimine les complications provoquées par des variables inconnues telles que les composants biotiques.

L'expérience II a utilisé un consortium qui pourrait bien se développer sur des eaux usées laitières simulées avec une productivité accrue de la biomasse, une teneur nutritionnelle et une teneur en lutéine. Les consortiums d'algues contenant *Chlorella variabilis* et *Scenedesmus obliquus* ont été exposés à différentes longueurs d'onde afin d'examiner ses effets sur la biomasse, la nutrition et l'accumulation de lutéine. Parmi les divers traitements de lumière utilisés, la lumière fluorescente blanche avait une productivité de la biomasse et une teneur en lutéine supérieures à celles des autres traitements. Cette étude révèle pour la première fois l'importance d'utiliser la lumière ambrée pour augmenter la teneur en glucides dans les consortiums d'algues et cela peut être attribué à la diminution de l'activité enzymatique.

L'expérience III a utilisé les consortiums de microalgues pour assainir les eaux usées en ce qui concerne la concentration d'inoculum, le temps et l'intensité de la lumière dans des conditions de laboratoire contrôlées. Une CCD a été utilisée pour trouver les conditions optimales pour éliminer le phosphore, l'azote ammoniacal, la DCO, tout en améliorant la productivité de la biomasse et le contenu en lutéine. Cette étude montre que les consortiums de microalgues ont été en mesure d'éliminer efficacement les polluants avec une productivité accrue de la biomasse et une teneur en lutéine supérieure. Cette étude est hautement significative car la teneur en lutéine présentée par les consortiums de microalgues est plus élevée que celle d'autres espèces de microalgues et pourrait éventuellement être la source commerciale de lutéine.

L'expérience IV a étudié l'effet de l'utilisation de la biomasse d'algues humides cultivée dans les eaux usées laitières comme biofertilisant pour la culture du maïs et du soja dans des conditions contrôlées. Les résultats ont montré que les consortiums de microalgues utilisés en tant que biofertilisant amélioraient les performances de croissance et le contenu nutritionnel du maïs et du soja.

Cette étude a montré que les souches de microalgues de *Chlorella variabilis* et *Scenedesmus obliquus*, utilisées en tant que consortiums, peuvent être utilisées efficacement pour assainir les eaux usées tout en contenant davantage de lutéine. En outre, la biomasse alguale résiduelle des eaux usées peut être utilisée comme biofertilisant pour les cultures agricoles. Cette étude a donc révélé pour la première fois que la biorestauration assistée par microalgues peut être utilisée pour une gestion efficace des eaux usées laitières. Cette étude revêt une grande importance industrielle car elle rend la technologie des microalgues commercialement réalisable avec ses avantages et ses fonctions multiformes. En outre, cette étude a ouvert la possibilité à de futures recherches pour se concentrer sur des consortiums de microalgues utilisant des souches spécifiques pour traiter des eaux usées industrielles complexes avec production de produits à valeur ajoutée, et sur la possibilité d'utiliser la biomasse d'algues humides comme biofertilisant.

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CONTRIBUTION OF AUTHORS

This thesis is a manuscript-based and has been prepared in accordance with the thesis preparation guidelines put forward by the Graduate and Postdoctoral Studies of McGill University and consists of two review manuscripts (published) and four research manuscripts (published/accepted for publication/under revision/review). Chapter I of this thesis contains general introduction and Chapter VIII concentrates on conclusions of the study with future work. Chapter II and Chapter III of this thesis consist of two manuscripts that comprise the literature review and Chapter IV to Chapter VII of this thesis consists of the four research manuscripts. They are:

1. **Bhalamurugan Gatamaneni Loganathan.,** V. Orsat, M. Lefsrud (2018). Factors affecting growth of various microalgal species. Environmental Engineering Science, 35(10), 1037-1048. [Chapter II]
2. **Bhalamurugan Gatamaneni Loganathan.**, V. Orsat, M. Lefsrud (2018). Valuable bioproducts obtained from microalgal biomass and their commercial applications: A review. Environmental Engineering Research, 23(3), 229-241. [Chapter III]
3. **Bhalamurugan Gatamaneni Loganathan.**, V. Orsat, M. Lefsrud (2020). Evaluation and interpretation of growth, biomass productivity and lutein content of *Chlorella variabilis* on various media. Journal of Environmental Chemical Engineering, 8(3), 103750. [Chapter IV]
4. **Bhalamurugan Gatamaneni Loganathan.**, V. Orsat, M. Lefsrud, B.S. Wu (2019). A comprehensive study on the effect of light quality imparted by light emitting diodes (LEDs) on the physiological and biochemical properties of the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* cultivated in dairy wastewater. (Accepted for publication in Bioprocess and Biosystems Engineering) [Chapter V]
5. **Bhalamurugan Gatamaneni Loganathan.**, V. Orsat, M. Lefsrud (2020). Phycoremediation and valorization of synthetic dairy wastewater using microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus*. (Published online on 11 February in "Environmental Technology"; Taylor and Francis) [Chapter VI]
6. **Bhalamurugan Gatamaneni Loganathan.**, V. Orsat, M. Lefsrud (2019). Utilization of microalgal biomass of *Chlorella variabilis* and *Scenedesmus obliquus* produced from treatment of dairy wastewater as biofertilizer. (Under review in Archives of Agronomy and Soil Science) [Chapter VII]

The work reported here was performed by Bhalamurugan Gatamaneni Loganathan under the supervision and guidance of Dr. Valérie Orsat (Supervisor) and Dr. Mark Lefsrud (Co-supervisor), Department of Bioresource Engineering, Macdonald Campus, McGill University, Montreal, Canada. The majority of the research and analytical work were carried out in the Biomass Production Laboratory of the Bioresource Engineering Department of McGill University. Dr. Valérie Orsat provided scientific advice and is directly associated with editing and reviewing of the manuscripts that were published. Dr. Mark Lefsrud provided scientific advice and technical guidance with respect to the experiments that were carried out. Dr. Bo-Sen Wu, Bioresource Engineering Department, McGill University, provided technical support with the lighting experiment. The nitrogen and phosphorus analysis were carried out by Hélène Lalande and Bhalamurugan Gatamaneni Loganathan using Lachat flow injection auto-analyzer in the Environment Chemistry and Soil Nutrient Laboratory, Department of Natural Resource Sciences, McGill University. Estimation of lutein content was performed using high performance liquid chromatography (HPLC) instrument in Dr. Vijaya Raghavan's lab in Macdonald Campus of McGill University, Montreal.

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μ	Specific growth rate (day^{-1})
mM	Millimolar
g L^{-1}	Gram/liter
mg L^{-1}	Milligram/liter
g kg^{-1}	Gram/kilogram
K	Mean daily division rate
mg g^{-1}	Milligram/gram
Λ	Wavelength
w v^{-1}	Weight/volume
Nm	Nanometer
E_c	Economic efficiency
T	Time (days)
P	Power (Watts)
Hz	Hertz
W	Watts
V	Volts

NOMENCLATURE

UNESCO	United Nations Educational Scientific and Cultural Organization
FAO	Food and Agricultural Organization
WHO	World Health Organization
ATP	Adenosine triphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
CCAP	Culture Collection of Algae and Protozoa
ppt	parts per trillion
ppm	parts per million
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
BG-11	Blue-green medium
BBM	Bold basal medium
DHA	Docosahexaneoic acid
EPA	Eicosapentaneoic acid
PUFAs	Polyunsaturated fatty acids
HIV	Human immunodeficiency viruses
FAME	Fatty acid methyl esters
ASTM	American Society for Testing and Materials
PEM	Proton exchange membrane
MBBM	Modified bold basal medium
PPFD	Photosynthetic photon flux density
MZM	Modified zarrouk medium
TCA	Trichloroacetic acid
MANOVA	Multivariate analysis of variance
LED	Light emitting diode
ANOVA	Analysis of variance
HSD	Honestly significant difference

COD	Chemical oxygen demand
CCD	Central composite design
RSM	Response surface methodology
APHA	American Public Health Association
SD	Standard deviation
FC	Folin ciocalteu
GAE	Gallic acid equivalents
QE	Quercetin equivalents
DPPH	2,2-diphenyl-1-picrylhydrazyl
NPK	Nitrogen, phosphorus, potassium

CHAPTER I

INTRODUCTION

Increase of global population has resulted in huge increase of water, food and energy consumption. Water covers nearly 70% of our planet, however only 3% of it is fresh and usable by humans. This results in water scarcity all year round for nearly half a billion people globally. Increased industrial, domestic and agricultural activities have aggravated the situation by consuming more water and it is predicted that by 2025, two-thirds of the global population would suffer from severe water shortages with damage to the eco-system (Mekonnen and Hoekstra, 2016). In addition, the amount of wastewater generated from the industrial and domestic sources has increased rapidly with an enormous negative impact on the environment, economy and the health of the people. As per UNESCO (United Nations Educational, Scientific and Cultural Organization), almost 80% of the wastewater generated is discharged into the environment without proper treatment (Water, 2017) though strict regulations are imposed by environmental agencies.

The composition of wastewater generated varies from one industry to another with water as its principal constituent. For instance, seafood processing industry produces both solid and liquid waste when compared to a dairy industry that primarily produces liquid waste. An estimate predicted that the volume of wastewater produced globally would double by 2025 with a need for the sector in charge of treating to grow by 50% (Water, 2017; Hoornweg and Bhada-Tata, 2012). One of the most logical ways to circumvent this problem is to use minimal water for industrial purposes, which is not always practically feasible. The world must utilize the available resources more effectively and preserve the environment in its current form for future generations. To attain this goal, we require technologies that can effectively treat wastewaters before they are discharged into the environment (Pimentel et al., 2009).

The dairy industry produces enormous quantities of wastewater and have a strong environmental footprint globally. They utilize more water than any other agricultural industry and it has been estimated that for every liter of milk produced approximately three liters of water is required (Dawood et al., 2011). The generated liquid waste contains high levels of organic and inorganic pollutants which need to be treated before being released into the streams. Despite being one of the biggest contributors of wastewater, dairy industries have a positive impact on a nation's economic growth. In Canada alone, dairy industries have a market capitalization of over 6.6 billion dollars with 70% of them located in the provinces of Quebec and Ontario (Centre, 2017; McKenna, 2018). Dairy products, especially milk, have recorded an increase in production and consumption. Globally, the consumption of dairy products is predicted to increase by 13.7% by 2023 (Choi, 2016). If this prediction becomes true, the wastewater generated is expected to increase significantly.

The dairy industries will be challenged in disposal of wastewater generated adding to their operating costs. In addition, with fast evolving eco-friendly government policies to enable sustainable development, the regulations for treating wastewater are going to increase. Therefore, the option of disposing the wastewater without or with minimal treatment is not possible (Britz et al., 2006). Taken together, technologies that can make wastewater safe, or better yet, utilize the wastewater to produce attractive end-products, are urgently needed. Arguably, one of the most efficient ways to address this issue is to use bio-based technologies that are sustainable and environmental friendly. In particular, current and conventional methods of dairy wastewater treatment are done either through physical, chemical, mechanical or biological methods or a combination of any of these methods. Biological methods are by far the most attractive methods as they are cost-effective for removing highly biodegradable contaminants and the residual

biomass could be tapped further for producing value added products (Shete and Shinkar, 2013; Slavov, 2017).

Microalgal-assisted bioremediation of dairy wastewater is considered to be an ideal solution because of its multifaceted benefits. First, the algae production is sustainable as it reduces the environmental pollution by treating the dairy wastewater effectively. Second, studies suggest that microalgae have the ability to sequester carbon due to the presence of bicarbonate in their cells, thereby helping in the reduction of carbon emissions that contribute to global warming and climate change (Sayre, 2010; Pavlik et al., 2017). Third, microalgal cultures, cultivated on wastewater streams, can be effectively used as biofertilizers (Renuka et al., 2016). Finally, microalgal cultures have the capability of producing value added products like lutein or astaxanthin that can be sold at high monetary value in global markets (Bhalamurugan et al., 2018; Singh et al., 2019). Taken together, treatment of dairy wastewater using microalgal cultures can potentially result in efficient waste management while producing commercially attractive biological compounds.

Numerous studies have been carried out for using different species of microalgae as a pollutant load reducer in wastewater streams and have exhibited attractive results (Riaño et al., 2011; Ummalyma et al., 2014; Qin et al., 2016; Bhattacharya et al., 2017; Daneshvar et al., 2019). The effective removal of pollutants from wastewater streams is highly dependent on the algal species/strains selected and the environmental factors affecting their growth (Li et al., 2011; Gatamaneni et al., 2018). Algal cultivation, harvesting, processing and biomass productivity are some of the biggest challenges that need to be optimized, which are currently preventing algal industries from reaching commercialization (Blair et al., 2014). This study serves to incorporate the above-mentioned issues pertaining to commercializing algal technology by developing a robust

consortia that can grow with enhanced biomass, produce value added products, remediate wastewater and act as a biofertilizer. To the best of our knowledge, there have been no reports of using the strains of *Chlorella variabilis* and *Scenedesmus obliquus* for dairy wastewater treatment and subsequent valorization of the residual biomass obtained as a result of the treatment. This study aims to manifest that dairy wastewater could be used as a possible large-scale cultivation medium for growing the microalgal consortia. In addition, the feasibility of utilizing the residual biomass for producing value added products and as a biofertilizer for crop production like corn and soybean is assessed. Briefly, this study is promising from an industrial standpoint as it widens the applicability of using microalgal biomass growth for wastewater remediation in addition to agronomical purposes while enhancing the biomass productivity for extracting value added products thereby providing multi-pronged economic benefits.

1.1 PhD research hypothesis

Dairy industries produce significant amount of wastewater and are considered to be one of the most polluting agricultural industries in terms of volume. The amount of waste generated is almost three times that of the milk processed (Slavov, 2017). Therefore, it has become a necessity to treat it effectively to minimize adverse effects. In spite of the popularity of using microalgae for dairy wastewater treatment, the overall amount of research done on using specific strains for dairy wastewater treatment is quite limited. Although treatment by conventional methods such as electrocoagulation, adsorption and membrane transport has been employed by certain industries to treat dairy wastewater, the effectiveness of such treatment is minimal and also tends to either increase the operating costs or generate secondary pollution through the addition of chemical reagents like aluminum sulfate (Shete and Shinkar, 2013; Melchiors et al., 2016; Slavov, 2017).

Microalgal technology would therefore be an ideal alternative solution yielding multifaceted benefits to address this issue. This thesis is based on the hypothesis that one could exploit a robust microalgal consortia, capable of utilizing simulated dairy wastewater as a cultivation medium, while enhancing the biomass production and subsequently producing lutein as a value added product from the biomass produced. The possibility of using the residual biomass along with treated wastewater as a biofertilizer is assessed. The results from this study will help in evaluating whether the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* has the ability to become the most successful consortia from an industrial standpoint by addressing the issues that prevent microalgal technology from reaching commercial implementation. No ethics certificate or biosafety permit was required for the entire study.

1.2 Overall objective

The overall objective is to remediate and valorize simulated dairy wastewater using a robust microalgal consortia and to effectively use the algal biomass for the extraction of value-added products and use the remainder as a biofertilizer for growing corn and soybean.

1.3 Specific objectives

The specific objectives of this study are as follows:

1) To evaluate and interpret the growth, biomass productivity and nutritional composition and lutein content of the alga *Chlorella variabilis* grown on various media including simulated dairy wastewater.

2) To evaluate the effects of light of varying wavelengths on the growth of the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* and its impact on biomass productivity.
3) To optimize the process parameters that affect the treatment of simulated dairy wastewater while using the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* with focus on biomass productivity and lutein content.
4) To investigate the effect of using the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* as a biofertilizer for enhancing growth and plant quality of a monocot plant (corn).
5) To investigate the effect of using the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* as a biofertilizer for enhancing growth and plant quality of a dicot plant (soybean).

CHAPTER II

REVIEW OF LITERATURE – PART I

FACTORS AFFECTING THE GROWTH OF VARIOUS MICROALGAL SPECIES

2.1 Abstract

The diversity of microalgal species is colossal; however, only a few species are better known and have been investigated relatively well. Lately, microalgae have been garnering great consideration because of their potential to serve as a feedstock for either biofuel or nutraceutical production. They have the capability of producing and storing desired products as cell metabolites and adapting themselves when there is a change in the environmental conditions (pH, temperature, light, carbon dioxide, salinity, and nutrients). The current review focuses on how the environmental conditions, including mixing, affect the growth and biomass productivity of various species of microalgae. This baseline information is important to focus on research efforts for improving biomass productivity to enhance the use of algae as a feedstock for various industries and applications. The optimal environmental conditions for enhancing biomass productivity of various species of microalgae as well as screening and selection of microalgae species are discussed as well.

2.2 Introduction

Potential shortages of food, energy, water, and fuel, as a result of extreme climatic events caused by climate change pose a serious threat to society. These potential shortages directly affect the economic stability of the many facets of society (Stephens et al., 2013). According to the Food and Agricultural Organization (FAO), the number of malnourished people is presumed to be over 815 million, globally (FAO, 2017). In order to meet the food and fuel needs of a globally growing

population, feedstocks obtained from sustainable sources will play a crucial role (Ruiz et al., 2016). Algaculture promises to be one such method, considered to be very economic and may satisfy many of the requirements of the population in a sustainable way (Starckx, 2012). Humans require food for their survival, and for that purpose in the 1950's, fearing the scarcity of food resources in the future, some companies incorporated themselves with the scope of checking the feasibility of microalgal technology in manufacturing food for human consumption (Burlew, 1953). The first industrial development of algal growth for food took place in the 1960's in Taiwan and Japan, where *Chlorella'*s biomass was used as a supplemental human food. The biomass obtained from *Chlorella* was processed in a variety of forms including tablets and powders and sold globally. Another development was the use of *Spirulina* biomass as a food supplement and for the extraction of its phycocyanin content. Certain algae were further investigated for the medicinal properties of their metabolites. For instance, the microalgae *Dunaliella* and *Haematococcus* were found to be rich in antioxidants and were sold in the form of consumable products that could enhance human health through nutrition (Slocombe and Benemann, 2016).

Microalgae, especially diatoms and flagellates established a niche in the aquaculture industry by serving as feedstock for animal and fish production (Slocombe and Benemann, 2016). An estimate from Pulz and Gross (2004) indicated that the retail value of products acquired from microalgae was about US$ 5 – 6.5 billion and this was generated by a diverse range of sectors such as health and food (US$ 1.25 – 2.5 billion), aquaculture (700 million) and through DHA omega-3 production (US$ 1.5 billion) (Carlsson and Bowles, 2007). Thus, in the last few decades, the production of microalgae has expanded commercially, producing products of relatively high value and volume. Until today, DHA omega-3 (docosahexaenoic acid) obtained from the microalga *Crypthecodinium cohnii*, has been leading the sales as the most sold microalgal product. Biomass

productivity is an important aspect when analyzing microalgae, and it is dependent on their gross photosynthetic activity which, in succession, relies on the prevailing environmental conditions (Slocombe and Benemann, 2016). The optimization of the environmental conditions which favour microalgae growth would, therefore, support the potential of microalgae feedstock as source products which are sustainable, ranging from food to fuels in years to come (Chen et al., 2016). However, some of the toxins produced by microalgae may pose a serious threat to public health and the World Health Organization (WHO) recommends countries to monitor this closely (Cardozo et al., 2007). This article aims to provide a critical overview of how environmental conditions, like pH, temperature, light, carbon dioxide, salinity and other factors such as mixing and selection and screening of microalgal strains, affect the growth and biomass productivity of algal species.

2.3 Environmental factors affecting algal growth

2.3.1 Temperature

The average temperature around the globe is increasing rapidly, owing to gaseous imbalances produced by human activities, creating a greenhouse effect on the planet. It is forecasted that before the end of the 21st century, the global average temperature of the sea-surface could increase by 1.4-5.8 °C (Tait and Schiel, 2013). Temperature plays a crucial role in the growth of algae and, in order to optimize growth, it is essential to control the temperature in experiments involving algae (Raven and Geider, 1988). Temperature affects the gross photosynthetic activity of microalgae undergoing cellular division which in turn affects the biomass productivity of microalgae. The cell division occurs due to the increase in enzymatic activities related to the Calvin cycle. Some studies have developed a model for relating growth rate with temperature and the most commonly used

expression is the Arrhenius equation. According to this equation, for every 10 °C increase in temperature, growth doubles until an optimum temperature is reached after which point there will be a decrease in growth. The decrease in growth is due to the heat stress that the algae undergo and this results in the denaturation of proteins and inactivation of enzymes that are involved in the photosynthesis process (Mayo, 1997; Ras et al., 2013). The maximum growth rate with respect to temperature can be estimated by the following Arrhenius expression (μ) (Mayo, 1997).

$$\mu = A' e^{-(\frac{El}{RT})} \quad (2.1)$$

where,

A´ = Constant, day^{-1}

El = Activation energy of the growth limiting reaction, J/mole

R = Universal gas constant

T = Absolute temperature, °K

Depending on the prevailing temperature conditions, microalgal strains should be adequately selected as this enhances the growth of the strain under study (Slocombe and Benemann, 2016). The absorption of nutrients and the chemical composition of cells in microalgae are influenced by changes in temperature. In certain cases, application of temperature stress restricts the nutrient interactions (Chen et al., 2012). In most cases, increasing temperature increases the growth of microalgae up to an optimum value, and then decreases with any further increase in temperature (Cassidy, 2011). Temperatures under 16 °C and over 35 °C are considered to be detrimental for microalgal growth (Pachiappan et al., 2015).

Chinnasamy et al. (2009) reported the optimum temperature for growth of *Chlorella vulgaris* to be between 25 °C to 30 °C (Chinnasamy et al., 2009). A study on unidentified *Chlorella* sp. and *Chaetoceros calcitrans* at temperatures of 20 °C, 25 °C, and 30 °C revealed that the highest

growth rate of the species was achieved at 25 °C (0.35 ± 0.04 day^{-1}) and 30°C (0.27 ± 0.02 day^{-1}) respectively (Adenan et al., 2013). An analysis was carried out on the growth rate of 4 species of microalgae (*Phaeodactylum tricormutum, Tetraselmis gracilis, Chaetoceros* sp. and *Minutocellus polymorphus)* at temperatures ranging from 11 °C to 36 °C. The study revealed that the growth rate of *Phaeodactylum tricormutum* was highest between 16 °C to 26 °C; *Tetraselmis gracilis* showed maximum growth between 11 °C to 16 °C; while *Chaetoceros* sp. and *Minutocellus polymorphus* showed the highest growth at 31°C (Sigaud and Aidar, 1993). A study by Ha (2000) revealed that the most conducive temperature was 28 °C for the growth of *Tetraselmis* sp. and it attained the highest cell density of 196 x 10^4 cells mL^{-1} on day 18. The suitable temperature range for the growth of this microalga was from 22 °C to 31 °C. The growth of the alga began to fall rapidly at 34 °C after the first few days of culturing, indicating that higher temperatures were not suitable for its growth (Ha, 2000). A study showed that *Chlorella zofingiensis* thrived at an ambient temperature of 28 °C (Travieso Córdoba et al., 2008). Kessler (1985) studied the growth rate versus optimal temperatures for 14 different strains of *Chlorella* sp. and revealed that they grew successfully between 26 °C to 36 °C.

An investigation reported that the optimal temperature for the growth of *Scenedesmus almeriensis* was 35 °C and was capable of withstanding up to 48 °C after which cell death occurred (Sánchez et al., 2008a). An analysis found that *Scenedesmus* sp. LX1 could grow within a temperature range of 10 °C to 30 °C (Xin et al., 2011). A study reported that the growth of three strains of *Dunaliella salina* isolated from 60 saline soil samples exhibited the highest growth at 22 °C (Wu et al., 2016). It was found that *Dunaliella* sp. was able to withstand a temperature range between 0 °C to 45 °C. An experiment involving the growth of *Dunaliella antarctica* reported that the micro-alga was able to survive at subzero temperatures. Though *Dunaliella* sp., still flourished

at temperatures above 40 °C, it nonetheless led to a decrease in the microalgal growth, but sequentially led to an increase in the carotenoid content. Hence, the ideal growth of *Dunaliella* sp. was determined at 32 °C with a wide growth temperature span ranging between 25 °C to 35 °C (Hosseini Tafreshi and Shariati, 2009). A study revealed that *Nannochloropsis salina* flourished well at an optimal temperature of 26 °C with no growth detected above 35 °C (Van Wagenen et al., 2012). Another study disclosed that *Nannochloropsis oculata* grew well at a temperature of 20 °C while there was a gradual decrease in growth as the temperature increased (Converti et al., 2009). A study on *Nannochloropsis ocenaica* exhibited that the growth of the species was highest at 20 °C and was incapable of growing at high temperatures of 40 °C to 50 °C (Rai and Rajashekhar, 2014). Experiments conducted on *Nannochloropsis gaditana* showed that the highest cell growth was obtained at a temperature of 25 °C (Al-Adali et al., 2012).

A study on the microalga *Tetraselmis subcordiformis* cultured at 15, 20, 25, 30 and 35 °C indicated that it grew best at 20 °C (Wei et al., 2015). In the case of *Haematococcus pluvalis*, cultivated under different temperature conditions of 20 °C, 23.5 °C, 27 °C, and 30.5 °C, it was reported that the culture growth rate and biomass productivity increased with an increase in temperature to 30.5 °C (Giannelli et al., 2015). The growth of the unicellular micro-alga *Isochrysis galbana* was studied under laboratory conditions at different temperatures of 15, 17, 22, 27, 33 and 35 °C. The optimal temperature for obtaining maximum growth was 27 °C. Temperatures greater than 32 °C or less than 19 °C decreased the growth of the microalga remarkably (Kaplan et al., 1986). Growth responses for *Pithophora oedogonia* and *Spirogyra* sp. at different temperatures indicated that *P. oedogonia* had a maximum growth rate at 35 °C and experience an inhibited growth at 15 °C, indicating that the species was warm stenothermal (ability to withstand only a small range of temperature). Similarly, *Spirogyra* exhibited maximum growth at 25 °C and

showed moderate inhibition at 15 °C and 35 °C, suggesting that this species was eurythermal (ability to withstand wider range of temperatures) over the given temperature range (O'Neal and Lembi, 1995).

2.3.2 Light

Algae absorbs light energy in the presence of light and stores it in the form of, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), which are used for biomass production in the dark reaction (Al-Qasmi et al., 2012; Rastogi et al., 2017). During the light reaction, water is hydrolyzed to form oxygen, while during the dark reaction, carbon dioxide is taken up by the cell components via the Calvin cycle (Al-Qasmi et al., 2012; Rastogi et al., 2017). It is in the dark reaction that the microalgae build up carbohydrates, proteins and lipids (Al-Qasmi et al., 2012; Rastogi et al., 2017). Light impacts the growth of microalgae under any one of the three different light conditions namely light limitation, light saturation, and light inhibition (Chang et al., 2017). When the condition is light limiting, the growth of algae increases with any increase in light intensity. At light saturation, the photosynthetic activity decreases as the photon absorption exceeds the amount of electron turnover, thereby inhibiting photosynthesis. When the light intensity is further increased, irreversible damage can occur to the photosynthetic apparatus, and this process is termed as photo-inhibition (Chang et al., 2017). In addition, certain studies have shown that the photoperiod can have a significant effect on the growth of microalgae. For instance, a study to investigate the effect of photoperiod on the growth of *Dunaliella salina* CCAP 19/30 revealed that longer photoperiods led to an increased growth of microalga with higher cell densities (Xu et al., 2016). Algae grow well under light conditions but tend to divide preferably under dark conditions by binary or multiple fission to produce daughter cells, and this seems to

have a significant implication on the overall productivities of microalgal cultures (Bisova and Zachleder, 2014; Concas et al., 2016b). The duration and intensity of light, therefore, directly affects the growth and photosynthesis of microalgae. A research study disclosed that microalgae tend to flourish under either blue (λ~420-470nm) or red light (λ~660nm) (Schulze et al., 2014). It was observed that red to far-red light accelerated the growth of microalgal cells (Schulze et al., 2014). The kinetic model for photosynthesis in microalgae with relation to light is given by the following equation (Rastogi et al., 2017).

$$\frac{dx_1}{dt} = -\alpha I x_1 + \Upsilon x_2 + \delta x_3 \qquad (2.2)$$

where,

I – Incident light illumination (μE/m^2h)

x_1, x_2, x_3 – Fraction of photosynthetic factory in the open, closed and inhibited state (dimensionless)

α, Υ, δ – Kinetic constants (μE/m^2h)

In an experiment, the light for *Chlorella vulgaris* was regulated at 54 μmol m^{-2} s^{-1} (3,960 lux), 107 μmol m^{-2} s^{-1} (7,920 lux) and 161 μmol m^{-2} s^{-1} (11,920 lux) with light intensity meter and without control over pH. The light/dark period was 12h/12h. It was reported that the maximum growth of cells was observed under 54 μmol m^{-2} s^{-1} (Gong et al., 2014). The effect of light illumination on *Scenedesmus obliquus* for intensities varying from 10 μmol m^{-2} s^{-1} (740 lux) to 1,000 μmol m^{-2} s^{-1} (74,000 lux), indicated that the highest growth was detected at 150 μmol m^{-2} s^{-1} after which the increase in light intensity did not improve the growth rate confirming that the point of saturation for photosynthesis was reached (Sforza et al., 2014). In the case of *Scenedesmus almeriensis*, higher irradiances showed no signs of photo-inhibition even at the maximum tested irradiance of 1,380 μmol m^{-2} s^{-1} (102,250 lux). The biomass productivities were the highest (0.66

g L^{-1} day^{-1}) at this light intensity (Sánchez et al., 2008a). *Dunaliella salina* CCAP 19/30 could modify their photo systems to achieve maximum photosynthesis even when they were exposed to higher light intensities. When the light intensity was increased above 1,000 µmol m^{-2} s^{-1} (74,000 lux), cells displayed photo damage. However, the growth rate increased with increased light intensity (Xu et al., 2016). *Dunaliella bardawil* DCCBC 15 and *Dunaliella salina* CCAP 19/18 were inspected for their growth at light intensities of 50, 100 and 150 µmol $m^{-2}s^{-1}$ (3,700 lux, 7,400 lux and 11,100 lux) and the results showed that the optimal growth of *Dunaliella* sp. was obtained at 50 µmol $m^{-2}s^{-1}$ light intensity and the growth rate decreased with increasing light intensity (Vo and Tran, 2014).

Nannochloropsis salina was exposed to varying intensities of 5, 25, 50, 100, 250 and 850 µmol m^{-2} s^{-1} (370 lux, 1,850 lux, 3,700 lux, 7,400 lux, 18,500 lux and 62,900 lux). The growth rate increased with light intensity and the highest growth rate was achieved at 250 µmol $m^{-2}s^{-1}$; however, photon conversion efficiency decreased for light efficiencies above 50 µmol $m^{-2}s^{-1}$ (Van Wagenen et al., 2012). The growth rate of *Nannochloropsis ocenaica* increased exponentially when exposed to light intensities in the range of 34 to 80 µmol $m^{-2}s^{-1}$ (2,516 to 5,920 lux) reaching a maximum at 80 µmol $m^{-2}s^{-1}$ (Sandnes et al., 2005). It is evident that light influences cultivation of algae and optimal exposure to light is required in order to achieve maximum productivity. In fact, sunlight provides the light required for supporting metabolism, but if present in excess, the light leads to oxidative stress and photo inhibition thereby reducing photosynthetic efficiency (Sforza et al., 2012). A study on the growth of *Odontella aurita* under two light intensities of 150 µmol $m^{-2}s^{-1}$ and 300 µmol $m^{-2}s^{-1}$ (11,100 lux and 22,200 lux) revealed that the micro-alga was able to grow under 150 µmol $m^{-2}s^{-1}$, however, the alga grew faster at early stages under high light (300 µmol $m^{-2}s^{-1}$). This was due to the low cell density at early stages which enabled the cells to receive

an additional amount of irradiance under high light conditions (Xia et al., 2013). The biomass concentration of *Neochloris oleoabudans* HK-129 increased from 1.2 g L^{-1} to 1.7 g L^{-1} when the light intensity was increased from 50 to 200 µmol m^{-2} s^{-1} (3,700 lux to 14,800 lux) (Sun et al., 2014).

The growth of *Chlamydomonas reinhardtii* increased when the light intensities were varied from 60 to 300 µmol $m^{-2}s^{-1}$ (4,440 lux to 22,200 lux). However, there was little difference in growth between 200 µmol m^{-2} s^{-1} (14,800 lux) and 300 µmol m^{-2} s^{-1} (22,200 lux) and it was concluded that the light intensity of 300 µmol m^{-2} s^{-1} was conducive for the growth of the species (Kim et al., 2006). A study on the growth of *Isochrysis* sp. under exposure to varying illumination levels 1,458 µmol m^{-2} s^{-1} (108,000 lux), 1,073 µmol m^{-2} s^{-1} (79,488 lux), 840 µmol m^{-2} s^{-1} (62,208 lux), 423 µmol m^{-2} s^{-1} (31,320 lux) and 0 µmol m^{-2} s^{-1} (100%, 73.6%, 57.6%, 29%, and 0%) of natural sunlight revealed that the maximum growth rate was attained under the illumination exposure of 1,073 µmol m^{-2} s^{-1}. This revealed that for an optimum photosynthetic process, cell growth rate and carbon fixation in microalgae could be achieved by altering both dark and light regimes. Direct exposure of the microalgae to sunlight could potentially damage the cells while unavailability of light negatively impacts the growth of the microalgae (Harun et al., 2014). The growth of four microalgal strains namely *Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa* were studied under various light irradiances 36, 60, 120 and 180 µmol $m^{-2}s^{-1}$ (2,664 lux, 4,440 lux, 8,880 lux and 13,320 lux) with varying light:dark ratios (10:14, 14:10 and 24:0). The results reported that the highest growth rate and biomass productivity for all the species under study was achieved at an irradiance of 180 µmol $m^{-2}s^{-1}$ by continuous illumination for 24 h (Gonçalves et al., 2014). A study using LED lights (red, natural white, warm white and blue) at different light intensities of 50 µmol m^{-2} s^{-1}, 80 µmol m^{-2}

s^{-1} and 110 µmol m^{-2} s^{-1} (3,700 lux, 5,920 lux and 8,140 lux) on the biomass productivity of *Chlorella vulgaris* revealed that warm white light (380-760 nm) with 80 µmol m^{-2} s^{-1} was optimal for enhancing biomass productivity and photosynthetic rate (Khalili et al., 2015). Another study on marine microalgae *Tetraselmis* sp., and *Nannochloropsis* sp., under blue (420-470 nm) and red light (660 nm) of 7,400 lux (100 µmol m^{-2} s^{-1}) with a 24:0 light-dark cycle revealed that both the species grew well under blue light (Teoa et al., 2014). An experiment conducted on red microalga *Pyropia haitanesis* under blue, red, green and fluorescent light of 100 µmol m^{-2} s^{-1} (7,400 lux) with 12:12 light-dark cycle revealed that the highest growth was achieved under fluorescent light (Wu, 2016).

2.3.3 pH

pH is believed to be one of the underlying parameters that controls the cell metabolism and formation of biomass in microalgae. The growth of a majority of microalgal species is known to flourish at neutral pH and all strains of microalgae seem to have a limited optimal range of pH (Lutzu, 2012). Algae consume carbon dioxide during photosynthesis, and at optimal pH, the bicarbonate present in the media is converted into carbon dioxide by the action of the algal enzyme, carbonic anhydrase, with the release of hydroxyl ions which tend to increase the pH (Gerardi and Lytle, 2015).

HCO_3^- ----------Carbonic anhydrase-------> $CO_2 + OH^-$ (2.3)

According to the physiology of microalgae, it is observed that the thylakoid of the chloroplasts carry out the vital functions at a specific pH range, since the media's pH is known to influence the process of photosynthesis in microalgae (Bakuei et al., 2015). Indeed, extremes of pH, that is, high as well as low pH, reduce the rate of photosynthesis. At high pH, the trend of

absorption of the trace metals and nutrients might be altered (Bakuei et al., 2015). Similarly, at low pH, enzyme inhibition occurs in the photosynthetic process and there is a high possibility of the growth media being contaminated by other micro-organisms (Bakuei et al., 2015). At pH 7, the pH increases steadily as carbon dioxide is consumed. The pH influences the availability of nutrients such as iron and organic acids (Lutzu, 2012). Hence, pH is considered to be a major environmental factor that is regulated by carbonate equilibrium both in oceans and inland waters. The optimal pH range for photosynthesis to occur in most of the microalgae is in between 6 to 10, wherein the bicarbonate form is considered to be dominant (Rastogi et al., 2017). The pH in oceans is 8 ± 0.5, however, it fluctuates from < 2 to 12 in natural bodies of water. Low pH water often originates from volcanic regions that receive strong mineral acids, in general, sulfuric acid and hence the pH is often found below pH 4. High pH values can be attributed to lakes that belong to endorheic regions due to the presence of high concentrations of sodium carbonate or sodium bicarbonate (Weisse and Stadler, 2006). Algae have been found to survive at both alkaline and acidic pH (Ying et al., 2014). The effect of pH on *Chlorella vulgaris* species revealed that the microalga exhibited reduced growth at both acidic (3.0 – 6.2) and alkaline (8.3 – 9.0) pH. However, optimal growth was achieved when the pH was between 7.5 and 8.0 (Rachlin and Grosso, 1991). The optimum pH for the growth of *Spirulina platensis* was observed to be between pH 7.0 to pH 9.0. The maximum growth rate for the microalga was observed at pH 8.0, suggesting that moderate alkalinity was necessary for the ideal growth of the microalga (Fagiri et al., 2013). *Scenedesmus almeriensis* grew effectively at a pH of 8.0 with a decrease in growth at higher pH and exhibited tolerance to neutral pH (Sánchez et al., 2008b). *Scenedesmus obliquus* grew well in neutral as well as in weakly alkaline conditions and the maximum growth was observed at a pH of 8.0 (Yang et al., 2016).

The growth of *Scenedesmus* sp. (ADIITEC-II and GUBIOTJT116), at various pH levels ranging from 5.0 to 9.0, showed that the maximum specific growth rate and biomass productivity for the species was achieved at a pH of 7.0. Acidic conditions (pH 5.0 and pH 6.0) did not alter the cell density and demonstrated lower biomass productivity (Difusa et al., 2015). The initial pH for *Scenedesmus* sp. strain R-16 was varied from pH 3.0 to pH 12.0 and it was observed that the alga had strong tolerance to varying pH and grew well at a pH varying between 4.0 to 11.0 (Ren et al., 2013). At pH 3.0 and pH 12.0, the algal cells exhibited poor growth. The micro-alga exhibited the highest biomass productivity at a pH of 7.0 (Ren et al., 2013). A study on *Dunaliella salina* at different pH revealed that the maximum growth occurred at pH 9.18 (4.59×10^6 cells mL^{-1}) (Abu-Rezq et al., 2010). The effect of pH on the growth of *Dunaliella bardawil* and *Chlorella ellipsoidea* over a wide range of pH (pH 4.0 to pH 11.0) showed that the ideal pH for the growth of the species were 7.5 and 10.0 respectively. The growth of both *Dunaliella bardawil* and *Chlorella ellipsoidea* was retarded at a pH over 10.0, as carbonate ion (an important source of inorganic carbon) was not available for the algae (Khalil et al., 2010).

The optimum growth of *Nannochloropsis salina* was observed at a pH of 7.5 to 8.0; however, the micro-alga could grow over a wide range of pH (5.0 to 10.5) (Boussiba et al., 1987). Another study on the growth of *N. salina* at six different pH levels (pH 5, 6, 7, 8, 9 and 10) revealed that highest growth rate was achieved at a pH between 8 and 9 (Bartley et al., 2014). The optimum pH for growth of *Nannochloropsis oculata* was validated using response surface methodology and was found to be 8.4 (Spolaore et al., 2006b). Influence of the media's pH on *Chlorococcum* sp. revealed that the maximum growth of the microalga was observed at a pH of 8.0 and the growth rate was 0.066 h^{-1} (Zhang et al., 1997). The ideal pH for the growth of *Tetraselmis* sp. was observed to be at a pH of 8.5 (Khatoon et al., 2014). A series of experiments to investigate the effect of pH

on the growth of *Nannochloris eucaryotum* revealed that a maximum growth of $9.85\pm0.54\times10^{-4}$ h^{-1} was achieved when the pH was controlled at 6.60±0.67 (Lutzu, 2012). A study on the growth of *Chlamydomonas applanata*, within a pH range of 1.4 to 8.4, showed that no growth was observed at low pH between1.4 to 3.4. Optimum growth was obtained at pH ranging from 5.4 to 8.4, while the maximum growth was observed at pH 7.4 (Visviki and Santikul, 2000). The growth response of *Chlamydomonas acidophila* was examined at pH ranging from 1.4 to 8.4. Analysis of variance showed that the growth was a maximum at pH 7.4 with no growth observed at pH between 1.4 and 2.4 (Visviki and Palladino, 2001). *Euglena mutabilis* exhibited the highest growth between pH 3.4 and pH 5.4 and it was able to survive over a wide range of pH between pH 0.9 and pH 8.2. At pH 0.9, there was reduced growth and within 24 hours all the microalgal cells were dead (Dach, 1943).

2.3.4 Salinity

Each strain of microalgae displays differences in their capacity to adjust to salinity (Asulabh et al., 2012). Stress, from high concentrations of salt, affects the growth of cells and the formation of lipids (Asulabh et al., 2012). It was noted that as salinity increases, the expression of lipids increased but resulted in decreased cell growth. Since the two important traits that researchers look for in selecting a microalgal strain for study is often the ability of the algae to produce both high biomass and lipids, considerable importance is given to microalgae which flourish in a saline environment (Asulabh et al., 2012). Marine microalgae are exceptionally tolerant to alterations in salinity when compared to freshwater species (Blinová et al., 2015).

Spirulina platensis exposed to different concentrations of sodium chloride ranging from 5.84 ppt to 23.37 ppt (0.1 M to 0.4 M) revealed that the growth of micro-alga was higher at lower concentrations of sodium chloride between 5.84 ppt and 11.68 ppt and the growth reduced at higher

concentrations ranging from 17.53 ppt and 23.37 ppt (Sujatha and Nagarajan, 2014). *Chlorella* sp. were exposed to different salinities namely 0 ppt, 30 ppt, 35 ppt and 40 ppt of BG11 (Blue-green medium) with a limited supply of sodium nitrate. The salinity was adjusted to the desired levels using sodium chloride. It was reported that as the salinity increased the growth of the microalgae decreased. The biomass concentration was high (0.09 g L^{-1}) at 0 ppt when compared to 30 ppt (0.045 g L^{-1}), 35 ppt (0.038 g L^{-1}) and 40 ppt (0.04 g L^{-1}) (Andrulevičiūtė et al., 2011). The effect of salinity on growth of *Scenedesmus almeriensis* was studied with different salinities (brackish water (3×10^6 cells mL^{-1}), sea water (7×10^6 cells mL^{-1}) and fresh water. It was found that a higher number of cells were found in fresh water (9.8×10^6 cells mL^{-1}) indicating that the lower the salinity the higher the growth of this micro-alga (Suyono et al., 2015). However, *Scenedesmus almeriensis* showed higher tolerance to medium salt concentrations of 5.844 ppt (0.1 M) sodium chloride and showed higher biomass productivities at 5.844 sodium chloride when compared to productivities observed in freshwater media (Benavente-Valdes et al., 2016).

Measurement of the effect of salinity on growth of *Scenedesmus obliquus* was made at various concentrations of sodium chloride 3.00 ppt, 17.50 ppt, 35.00 ppt, 58.44 ppt, 116.88 ppt and 166.32 ppt (0.05, 0.3, 0.6, 1.0, 2.0 and 3.0 M). The growth of *Scenedesmus obliquus* was inhibited at sodium chloride concentrations above 35 ppt, while there was reduced growth at 17.5 ppt. The highest growth of the microalga was observed at 3 ppt NaCl and it was equivalent to the growth that was obtained in fresh water. The results thus suggested, that low salinities, between 0 M to 0.05 M, were appropriate for the promotion of the growth rate of *Scenedesmus obliquus* (Kaewkannetra et al., 2012). *Dunaliella bardawil* was exposed to salinity levels ranging from 1 M to 3 M. The results revealed that the maximum growth rate was observed at the lowest salinity of 1 M (Gomez et al., 2003). A decrease in cell growth of *Dunaliella tertiolecta* ATCC 30929 was

observed when the concentration of sodium chloride was increased from 58.44 ppt to 116.88 ppt (1.0 M to 2.0 M). Hence, sodium chloride concentration of less than 58.44 ppt was considered to be appropriate for achieving a high cell concentration (Takagi et al., 2006). *Dunaliella salina* is a marine micro-alga that has the ability to tolerate high salinity. *D. salina* CCAP 19/18 was inspected for its growth under different salinities 58.44 ppt, 87.66 ppt and 116.88 ppt (1 M, 1.5 M and 2.0 M). The ideal growth for *Dunaliella* sp. was obtained at 87.66 ppt and 116.88 ppt salinities (Vo and Tran, 2014).

Nannochloropsis salina was exposed to different salinity levels of 10, 22, 34, 46 and 58 ppt. Being a marine micro-alga, *N. salina* exhibited the highest growth rate at 22 ppt and the highest biomass accumulation at salinities of 22 ppt and 34 ppt. *N. salina* exhibited no growth at salinities of 58 ppt and below 10 ppt (Bartley et al., 2013). The effect of salinity on growth of *Nannochloropsis oculata* CS 179 was carried out at various salinities 150 ppt, 250 ppt, 350 ppt, 450 ppt and 550 ppt. The results indicated that the highest biomass was obtained at a salinity of 250 ppt (Gu et al., 2012). The marine micro-alga *Tetraselmis suecica* was capable of tolerating a wider range of salt concentrations. The cultures were grown at 48 different salinity conditions from 0 ppt to 350 ppt. The ideal growth was achieved between 250 ppt and 350 ppt with a maximum cellular density of 1.3×10^{6} cells mL^{-1} (Fabregas et al., 1984). Four species of microalgae *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp., were cultured at 2, 8, 11 and 18 ppt salinity. *Desmodesmus armatus* showed maximum tolerance to salinity growing actively at 18 ppt while *Mesotaenium* sp., was less halotolerant (ability to survive in hyper saline conditions) with the growth rate decreasing as concentration increased past 11 ppt (Von Alvensleben et al., 2016). Therefore, the ideal salinity level for the growth of *Mesotaenium*

sp., was observed to be between 2 ppt to 8 ppt. Both *Scenedesmus quadricauda* and *Tetraedron* sp., grew well at salinity levels of 2 ppt and 8 ppt (Von Alvensleben et al., 2016).

The growth of *Schizochytrium limacinum* OUC88 at various salinities 0 ppt, 0.9 ppt, 1.8 ppt, 2.7 ppt and 3.6 ppt (0, 0.9, 1.8, 2.7 and 3.6% w v^{-1}) was analyzed. The strain performed better and the biomass remained steady with salinity at 1.8 ppt, 2.7 ppt and 3.6 ppt. When there was a decrease in salinity from 0.9 ppt to 0 ppt, there was a significant reduction in the biomass productivity (Zhu et al., 2007). The growth of *Botryococcus braunii* under various salinities 1 ppt, 2 ppt, 3 ppt, 4 ppt and 5 ppt (17 mM, 34 mM, 51 mM, 68 mM and 85 mM) revealed that although the micro-alga was able to grow at all salinity levels, the maximum growth rate was observed at the lowest salinity level of 1 ppt (Rao et al., 2007). A study on the effect of salinity on three microalgal strains, *Crypthecodinium cohnii* ATCC 30556, *C. cohnii* ATCC 50051 and *C. cohnii* RJH revealed that *C. cohnii* ATCC 30556 had its maximum growth rate of 0.090 h^{-1} at a sodium chloride concentration of 9.0 ppt (g L^{-1}) whereas *C. cohnii* ATCC 50051 and *C. cohnii* RJH had their maximum growth rates of 0.049 h^{-1} and 0.067 h^{-1} respectively at a sodium chloride concentration of 5.0 ppt (g L^{-1}). When an optimum salinity was reached, the growth rate decreased with increasing salinity. Almost no growth was observed when the medium did not contain sodium chloride, and at extremely high sodium chloride concentrations, growth was inhibited, and the cells were elongated. The elongation of the cells was attributed to the increase in external ionic concentrations that tend to inhibit cell growth (Jiang and Chen, 1999).

Microalgae have the capability of maintaining a balanced cell composition even if there is a dramatic change in the external environment. When this happens, growth rate can be limited in order to maintain smooth functioning of the cell structures without any changes in the cellular composition. This process is defined as homeostasis. However, there are certain microalgae that

change their cellular composition due to changes in external environment through acclimatation. The conditions that stimulate homeostasis or acclimatation response are currently unknown (Montechiaro et al., 2006). Salinity is considered to be one such factor that will be able to maintain homeostasis in algal cells (Montechiaro et al., 2006). For instance, in *Tetraselmis viridis*, the Na^+ transporting ATPase played an important role in increasing the salt tolerance of this alga by maintaining the cytoplasmic ion homeostasis (Strizh et al., 2004). In short, maintenance of balance in the composition of the cell (homeostasis) holds the key when there is change in salinity or any other external factors (Montechiaro et al., 2006).

2.3.5 Carbon Dioxide

Today about 85% of the world's energy demand is satisfied by burning fossil fuels that emit and concentrate greenhouse gases in our atmosphere. In recent decades, the levels of carbon dioxide in the air have risen from 260 ppm to 380 ppm. Some suggestions have been made and studies have been conducted to minimize the effects of human activities on increasing greenhouse gases (Minillo et al., 2013). The sum of fossil fuels being ignited is directly proportional to the increase of carbon dioxide in the air. The increasing concentration of carbon dioxide in the air is considered to be one of the main causes of global warming. Therefore, fixing carbon dioxide biologically could be considered to help mitigate this problem (Salih, 2011), or the effective removal of carbon dioxide from the point source should be initiated (Li et al., 2012a). Capturing carbon and sequestering it biologically is considered to be safe for reducing environmental carbon dioxide. Microalgae can fix carbon dioxide effectively when compared to terrestrial plants. The selection of microalgal species is important for attaining biological carbon dioxide systems which work and the microalgal species selected depend on the strategy involved in carbon sequestration. The

amount of carbon dioxide in the air plays a major role in the growth of microalgae, that is, the higher the concentration of carbon dioxide more rapid is the growth (Khairy et al., 2014; Salih, 2011). A study on the effect of varied carbon dioxide concentrations, namely a control (absence of carbon dioxide), 280 ppm, 385 ppm, 550 ppm, 750 ppm and 1,050 ppm (Control, 280 µatm, 385 µatm, 550 µatm, 750 µatm and 1,050 µatm) on the growth of *Chlorella gracilis* showed that there was an increase in the cell number up to the carbon dioxide concentration of 385 ppm, followed by a decrease in growth observed at 550 ppm as the micro-alga was not CO_2 tolerant above this limit (Khairy et al., 2014).

A study with *Chlorella vulgaris* ARC1 examined growth under different carbon dioxide concentrations ranging between 350 ppm to 200,000 ppm (0.036% to 20%). The results obtained showed that *Chlorella vulgaris* had the ability to sequester 38.4 ppm (mg L^{-1} day^{-1}) at elevated carbon dioxide concentration of 60,000 ppm (6%) thereby increasing the growth of biomass (Chinnasamy et al., 2009). Another study on *Chlorella vulgaris* showed that the alga had the capability of growing well (0.4 g L^{-1}after 300 hours of cultivation) at carbon dioxide concentrations of 2,000,000 ppm in semi-batch photobioreactor while maintaining low pH values (Cao et al., 2013). An investigation on the growth rate of three species *Chlamydomonas reinhardtii*, *Chlorella pyrenoidosa* and *Scenedesmus obliquus* disclosed that as the concentration of carbon dioxide increased, the growth of microalgae also increased, but attained saturation at 1,320 ppm (30 µM), 4,400 ppm (100 µM) and 2,640 ppm (60 µM) of carbon dioxide respectively (Yang and Gao, 2003). *S. obliquus* showed increased biomass (2.3 g L^{-1}) at 150,000 ppm (15%) carbon dioxide concentration (Singh and Singh, 2014). A study to improve the biomass productivity of filamentous microalgae using carbon dioxide concentrations of 7,480 ppm (170 µM) and 748 ppm

(17 µM) reported that the biomass productivity was higher in the enclosures containing 7,480 ppm of carbon dioxide (Andersen and Andersen, 2006).

The microalgal strain *Botryococcus braunii* LB-572 was exposed to various concentrations of carbon dioxide 0 ppm, 5,000 ppm, 10,000 ppm and 20,000 ppm (0 %, 0.5%, 1% and 2%, v/v) and the growth pattern was studied. The results revealed that at 20,000 ppm of carbon dioxide, the growth of the microalgal strain flourished the most while, the micro-alga also exhibited growth at the other concentrations studied (Ranga Rao et al., 2007). A study on *Dunaliella salina* disclosed that there was no alteration in growth for changes in carbon dioxide concentrations from <230 ppm to 5,100 ppm, thus showing that carbon dioxide had no significant effect on the growth of *D. salina* over that range (King et al., 2015). *Nannochloropsis oculata* NCTU-3 exhibited decreased growth at elevated carbon dioxide concentrations when investigated for its growth at various carbon dioxide concentrations of 20,000 ppm, 50,000 ppm, 100,000 ppm and 150,000 ppm (2%, 5%, 10% and 15%). Microalga exhibited reduced growth at 50,000 ppm, 100,000 ppm and 150,000 ppm of carbon dioxide. However, the growth of microalga was enhanced when aerated with 20,000 ppm of carbon dioxide concentration (Chiu et al., 2009). *Spirulina platensis* was exposed to carbon dioxide concentrations of 0 ppm, 5,000 ppm, 10,000 ppm and 20,000 ppm. The pH decreased with increasing carbon dioxide concentration. The results revealed that the alga grew well at carbon dioxide concentrations of up to 10,000 ppm though the difference in growth was insignificantly small when compared with 20,000 ppm carbon dioxide. The productivity of the microalga was increased to 60% when it was exposed to 10,000 ppm of carbon dioxide (Ravelonandro et al., 2011). The effect of carbon dioxide concentration on *Chlorocuccum littorale* at concentrations of 50,000 ppm, 200,000 ppm, 350,000 ppm and 500,000 ppm (5%, 20%, 35% and 50%) revealed that the growth decreased with increasing carbon dioxide concentration (Ota et al., 2009).

2.4 Nutrients

The growth of algae is directly proportional to the uptake rate of the most limiting nutrients and is described by the Michaelis-Mentis equation as given below.

$$\mu=\mu_{max}[S/S+K] \quad (2.4)$$

where μ is the growth rate, μ_{max} is the maximal growth rate, S is the concentration of the limiting nutrient, and K the concentration which leads to half-maximal growth rate called the half-saturation constant (Titman, 1976). Nitrogen is considered to be a building block for proteins and nucleic acids whereas phosphorus forms parts of phospholipids, DNA and RNA. If these macronutrients are limited, then it tends to shift the metabolic pathways (Juneja et al., 2013). Redfield (2014) had stated that when the ratio of N/P exceeds 16, then phosphorus was considered to be the limiting factor and nitrogen content needs to be controlled to optimize the growing condition of microalgae. The requirement of optimum level of phosphorus was considered to be conducive for the growth of microalgae. Phosphorus content less than 0.045 mg L^{-1}, or greater than 1.65 mg L^{-1}, prohibits the growth of microalgae. The growth of the microalgae is favored when the phosphorus content is equal to 0.2 mg L^{-1} (Redfield, 2014; Ren, 2014).

A study on *Chlorella vulgaris* and *Nannochloropsis oculata* disclosed that if the supply of nitrogen was decreased, the lipid synthesis had increased while no effect on the growth pattern of microalgae was observed (Paes et al., 2016). *Dunaliella* sp. was able to build up a huge volume of carotenoids and astaxanthin when deprived of nitrogen. Unlike for nitrogen, phosphorus was considered to be the main limiting nutrient in the growth of microalga for the expression of value added products (Juneja et al., 2013). Phosphorus limitation in *Scenedesmus* sp. (from 2.0 mg $L^{-1}$to 0.1 mg L^{-1}) led to the increase in lipid content from 23% to 53% (Juneja et al., 2013). For *Scenedesmus species* LX1, nitrogen and phosphorus limitation increased the lipid content but the

growth was low (Xin et al., 2010). Reduction of nitrogen in the medium by 75% for *Nannochloropsis salina* resulted in an increase of lipid content from 34.6% to 59.3% with a significant decrease in growth (Fakhry and El Maghraby, 2015). During Calvin's cycle, inorganic carbon from the liquid media is taken up by the microalgal cell and converted into glyceraldehyde 3-phosphate. The shift of metabolism towards production of storage or functional molecules depends on the ratio between internal carbon and nitrogen. At the initial stage of growth of microalgae, the internal carbon exceeds the C:N ratio due to high photosynthetic rate thereby synthesizing proteins. However, as growth progresses, the nitrogen in the liquid media is consumed and there is a shift of production from proteins to lipids (Concas et al., 2016a).

Trace metals such as iron, manganese, cobalt, zinc, nickel and copper are some of the important trace metals that are required by algae for their metabolic functions. If absent, the growth of algae may be limited (Bruland et al., 1991). A study revealed that the growth of *Nannochloropsis oculata* increased with the addition of trace elements like Fe^{3+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} and EDTA (ethylenediaminetetraacetic acid). Increase in concentration of these trace elements increased the photosynthetic activity of the algae by enhancing the carbon dioxide concentration (Dou et al., 2013).

2.5 Mixing

The effects of light and temperature on microalgae are two important growth parameters that are dependent on each other and simultaneously controlling both can be difficult and costly. As a potential solution, mixing is one of the easiest methods to ensure uniform distribution of light and temperature to all the cells (Rocha et al., 2003). Problems of shading are commonly addressed by seeking mixing solutions, as shading prohibits the microalgae from absorbing light, and mixing is

considered to be a cost-effective solution. Proper mixing can be provided to the microalgae effectively and at a low cost by gas mixing (Ren, 2014). A study on the effect of mixing on *Spirulina platensis* in three ways (mixing with a magnetic agitator inside the column, bubbling air into the column and recirculating through a pump) showed that the growth of the micro-alga was highest when using a bubble column. However, similar values were observed for stirring and mixing as well (0.0122 h^{-1}, 0.009 h^{-1}, and 0.010 h^{-1}) (Ravelonandro et al., 2011). Another study on the mixing of *Chlorella* sp., revealed that continuous mixing (airlift pump) of the culture increased the growth of micro-alga significantly (up to 30%) (Persoone et al., 2000). A study on the influence of mixing (using shaker) on *Desmodesmus communis* revealed that the growth and yield of the micro-alga significantly increased when mixed in comparison with un-mixed cultures (Vanags et al., 2015). The effect of mixing on the biomass productivity of *Scenedesmus obliquus* using three modes namely stirring, aeration and a combination of stirring and aeration revealed that the biomass productivity increased with stirring when compared to the other two methods (Mandal and Mallick, 2012). A study on *Phaeodactylum tricornutum* revealed that mixing, mass transfer and carbon dioxide consumption could be the important factors that limit growth of the microalga (Contreras et al., 1998). Mixing tends to improve when the gas flow rate is increased (Contreras et al., 1998). Subsequently the availability of carbon dioxide increases and so does the growth (Contreras et al., 1998). Depending on biomass concentration, the carbon dioxide consumption was controlled either by carbon-dioxide gradients in the liquid phase or by carbon dioxide transfer from the gas phase (Contreras et al., 1998). However, additional research in this domain should be carried out to uncover a better understanding of the effect of dissolved gases on microalgal growth (Contreras et al., 1998).

2.6 Selection and Screening of Microalgal Strains

The selection of algal strains is carried out through a number of steps. The first step is isolation, which is commonly carried out to acquire pure cultures. The customary isolation technique involves the use of a micropipette and a microscope. The single cell isolation is the most commonly used method today because of its low cost and the applicability of this method to a broad range of samples. The advancement made in this technique as it is used today is called flow cytometry. Flow cytometry is a technique that employs the principle of fluorescence for cell sorting of desired algal strains from an assortment of different algal strains present in water. A novel approach using bioinformatics provides a gateway for exploring new algal isolates however is not commonly recommended due to issues related with approvals and consumer acceptance. The steps commonly involved are DNA/RNA extraction, PCR amplification, and sequencing (Duong et al., 2012). Following isolation, the first level of screening is generally done using Nile Red lipophilic fluorescent dye, for the stability of its fluorescence intensity, and its added benefit of remaining unaffected by changes in dye concentration, duration of staining and organic solvent (Cirulis et al., 2012). The strains that successfully surpass the first level undergo a second level of screening to decide on the medium that should be used for the sustained growth of the microalgae. BG11 (Blue-green medium), BBM (Bold basal medium) and C-media are used as common media for growing freshwater strains while F/2 media is commonly used for marine strains. The second level of screening is mainly done in order to zero down on the most suitable growth medium for the algal strain under study. After selecting the growth medium, the algal strain undergoes the third level of screening which assesses the growth of the strain when subjected to carbon dioxide, constant pH and inhibited oxygen level. Strains that successfully excel in all three levels of screening are considered to be potential candidates for scale-up. Therefore, for future successful

commercial employment of microalgal techniques, implementing strain bioprospecting will be crucial (Slocombe and Benemann, 2016).

2.7 Discussion and Conclusion

Based on the literature reviewed, it is evident that temperature and light are the two most important parameters that influence microalgal growth and are dependent on each other. The optimum temperature for the growth of the majority of the algal species should be between 15 °C to 35 °C although some exceptions exist. The optimal light illumination required for the growth of microalgal species should be between 25 µmol m^{-2} s^{-1} to 200 µmol m^{-2} s^{-1} except for *Scenedesmus almeriensis* which has the capability to withstand higher irradiances. It can be seen that altering the light-dark cycles and the color of light can positively or negatively impact the growth of microalgae but with limited impact (Harun et al., 2014). The optimal pH for growth of most of the algal species should be between pH 7.0 to pH 9.0, although some species that live in both acidic and basic environments also exist (Blinová et al., 2015). Besides this, the amount of major and minor nutrients present in the medium plays a crucial role in the growth of microalgae. The importance of the effect of change in pH, salinity and carbon dioxide on microalgal growth depends on the species (freshwater or marine). Biomass productivity is the major challenge that microalgal industries face for the manufacturing of products of high commercial value.

Microalgae are considered to be a valuable bioresource and are recently receiving great attention. Much progress has been made in understanding the growth of microalgae for the last few decades (Juneja et al., 2013). The present review underlines the different environmental conditions that impact the growth of a number of microalgal species and discusses how mixing and selection of microalgal strains can prove crucial for making microalgal technologies a reality

in the future. The microalgal species discussed in this review provide a scope for future studies as these species have garnered great interest among researchers. Evidence in this review suggests that microalgal growth is directly proportional to the environmental conditions in which the algae are grown (Juneja et al., 2013). Therefore, it becomes important to maintain optimum conditions for microalgae to grow as it directly influences the biomass productivity and in turn, the amount and type of secondary compounds which can be obtained from the produced biomass. Currently algae that belong to the group *Cyanophyceae, Chlorophyceae and Bacillariophyceae* have been investigated more as they are considered to exhibit one or more desirable characteristics (wastewater treatment, carbon dioxide fixation and biofuel production). Discovery of new strains of microalgae, by bioprospecting in novel habitats, may provide a gateway for identifying microalgae which may possess new industrial applications. Optimizing environmental conditions for acquiring maximum growth and biomass productivity from microalgae should be the focus of future research (Slocombe and Benemann, 2016). A summary on the biomass productivity and growth of commonly used microalgae according to varying environmental conditions is provided in Table 2.1. An area that has to be explored more in depth is the conditions of mixing of microalgal strains and the optimal control of nutrients and environmental conditions. Therefore, innovative research for the optimized production of microalgae will make the exploitation of microalgae economically feasible and in rising demand.

Table 2.1 Summary of the impact of environmental factors on growth and biomass productivity of microalgae.

Factor	Organism	Conditions	Biomass productivity ($g\ L^{-1}\ day^{-1}$)	References
Temperature	*Chlorella vulgaris*	30 °C (at elevated carbon dioxide concentration)	0.22	Chinnasamy et al., 2009
	Chaetoceros calcitrans	30 °C	0.27	Adenan et al., 2013
	Tetraselmis sp.	28 °C	-	Ha, 2000
	Scenedesmus almeriensis	35 °C	0.73	Sánchez et al., 2008a
	Dunaliella salina	22 °C	-	Wu et al., 2016
	Nannochloropsis salina	26 °C	-	Van Wagenen et al., 2012
Light	*Chlorella vulgaris*	3,960 lux (with no pH control)	-	Gong et al., 2014
	Scenedesmus obliquus	11,100 lux	0.86	Sforza et al., 2014
	Dunaliella salina	3700 lux	-	Vo and Tran, 2014
	Nannochloropsis salina	18,500 lux	1.30	Van Wagenen et al., 2012
	Chlamydomonas reinhardtii	14,800 lux	-	Kim et al., 2006
	Scenedesmus almeriensis	102,250 lux	0.66	Sánchez et al., 2008a
pH	*Chlorella vulgaris*	7.5-8.0	-	Rachlin and Grosso, 1991

	Scenedesmus almeriensis	8.0	-	Sánchez et al., 2008b
	Scenedesmus obliquus	8.0	-	Yang Li et al., 2016
	Dunaliella salina	9.0	-	Abu-Rezq et al., 2010
	Nannochloropsis salina	7.5-8.0	-	Boussiba et al., 1987
Salinity	*Chlorella* sp.	0 ppt	0.09	Andrulevičiūtė et al., 2011
	Scenedesmus almeriensis	Fresh water	-	Suyono et al., 2015
	Scenedesmus obliquus	3 ppt	-	Kaewkannetra et al., 2012
	Dunaliella salina	88 ppt – 117 ppt	-	Vo and Tran, 2014
	Nannochloropsis salina	34 ppt	-	Bartley et al., 2013
Carbon dioxide	*Chlorella vulgaris*	6%	-	Chinnasamy et al., 2009
	Scenedesmus obliquus	15%	-	Singh and Singh, 2014
	Dunaliella salina	No significant effect on growth	-	King et al., 2015
Mixing	*Chlorella* sp.,	Continuous mixing	Increased growth by 30%	Persoone et al., 2000
	Scenedesmus obliquus	Continuous stirring	-	Mandal and Mallick, 2012
	Spirulina platensis	Bubble column	0.29	Ravelonandro et al., 2011

CONNECTING TEXT

The first part of the literature review (Chapter II) emphasized the environmental conditions, including mixing, that impact the growth and biomass productivity of the algal strains with a significant focus on *Chlorella* and *Scenedesmus* strains. This review showed that temperature, light, pH and nutrients are the most important parameters that govern algal growth and should be optimized for enhancing biomass productivity. Based on the data surveyed, it has been concluded that the algal strains in this study will be grown on an inorganic media with sufficient nutrients at a temperature of 24 ± 2 °C, light intensity of 40 ± 3 µmol $m^{-2}s^{-1}$ with no control over pH on an orbital shaker agitated at 90 rpm. The knowledge of the probable value-added products that can be extracted from the algal strains, in an attempt to valorize the residual biomass, has been discussed in Chapter III.

CHAPTER III

LITERATURE REVIEW – PART II

VALUABLE BIOPRODUCTS OBTAINED FROM MICROALGAL BIOMASS AND THEIR COMMERCIAL APPLICATIONS: A REVIEW

3.1 Abstract

Microalgae are likely to become a part of our everyday diet in the near future as they are considered to be rich in proteins, carbohydrates, and high density lipoproteins. They will play a pivotal role in the food cycle of many people around the globe. Use of microalgae in treating wastewater is one of the disciplines which contributes to a sustainable way of exploiting resources while keeping the environment safe. In addition, microalgal biomass has the potential to be used as a feedstock for producing biofuel, bio fertilizers, pharmaceuticals, nutraceuticals and other bio-based products. This review presents the different value-added products obtained from microalgal biomass and the applicability of these products commercially.

3.2 Introduction

Algae are commonly classified into microalgae and macroalgae depending on their cellular organization (Shivhare et al., 2014). The organizational structure of microalgae defines them as being monocellular; hence they can only be identified using a microscope (Mutanda et al., 2011). The first glimpse of microalgae was reported a long time ago, in oceans where they grew rapidly by utilizing the available carbon dioxide and converting it into oxygen by means of photosynthesis (Sumi, 2009). Classification of microalgae into prokaryotes or eukaryotes is mainly dependent on the organelles present. For instance, prokaryotes have no distinct nucleus whereas eukaryotes possess a distinct nucleus. Microalgae seem to rule both on land and water as they have exhibited

the capability of surviving even in the harshest of environmental conditions (Mata et al., 2010). Microalgae (*Nostoc*) was first utilized some 2,000 years ago by the Chinese for surviving drought, however, it is only lately that innovative technologies using microalgae began to emerge (Priyadarshani and Rath, 2012). Microalgae grow rapidly with small amounts of water and nutrients in comparison with other plants that grow on land. For instance, the amount of water required for producing 1 kg of algal biomass is 333 liters when compared to soy which requires 2,204 liters to produce the same amount (Hannon et al., 2010; Ercin et al., 2012). Another virtue of microalgae is its ability to grow on industrial wastewaters by using their excess nutrients, thereby rendering the wastewater environmentally safe with minimal water utilization. Besides this, microalgae can also sequester the excess carbon dioxide from the atmosphere and flue gasses released by industries. Hence, microalgae have been considered as a sustainable feedstock for the bio-refinery industries of the future (Rashida et al., 2014). Microalgae possess certain medicinal properties, as a function of their composition, which makes them potential candidates for manufacturing bio-based chemicals of pharmaceutical and nutraceutical products. Cultivation of microalgae on a large scale for bio-diesel production was estimated to yield 20 times more bio-diesel than the traditional bio-diesel crops such as soya, rapeseed and jatropha (Schenk et al., 2008). The residual biomass obtained from microalgae, therefore, could be used as a potential feedstock for obtaining value-added products (Rajesh et al., 2014). For algal technology to attain success, researchers need to have a thorough understanding of algal species and their behavior under a variety of conditions in order to carefully choose specific strains based on the desired end products (Pulz and Gross, 2004). The following features make microalgae a source of attraction among researchers. First, microalgae can produce a range of value added products such as proteins, lipids, carbohydrates and pigments which can be enhanced under stressed environmental

conditions (Minhas et al., 2016). Second, they have the ability to introduce stable isotopes (13C and 15N) into products that are manufactured from them (Priyadarshani and Rath, 2012). Third, only a few number of species have been identified to date, making them an interesting domain to be explored (Priyadarshani and Rath, 2012). However, the downstream processing involved in producing these value-added products is high. One of the ways for reducing the cost is to derive multiple products in a single cycle (Biorefinery concept) (Minhas et al., 2016). According to an article published by "Business Insider", it is believed that by 2025, products obtained from microalgae could be produced on a larger scale and in an environmental friendly manner (Baehr, 2017). The current review underlines how the biomass produced from microalgae has been successfully employed for the sourcing of a variety of value added products.

3.3 Value added products obtained from microalgae

The most predominant research that surrounds algae is targeting the production of biofuels, however, the process remains costly and this makes it uneconomical (Misra et al., 2014). Nonetheless, there has been a recent shift in the trend of using microalgae for the production of value-added compounds. A lot of high-value products have already been identified and marketed, however, with the emergence of newer algal growth technologies there is always a possibility of discovering additional products which are of high economic value (Borowitzka, 2013). Some of the most commonly produced value-added products obtained from microalgae are listed below.

3.3.1 Lutein

The carotenoid lutein is predominantly found in almost all fruits and vegetables (Mozaffarieh et al., 2003). However, the most important sources of lutein ingested by humans are from maize and

egg yolk (Mozaffarieh et al., 2003). Most of the lutein produced commercially is extracted from the petals of the marigold flower and is considered to be a high value added product (Fernández-Sevilla et al., 2010). In parallel, microalgae are gaining importance because they show higher productivities of lutein production when compared to the marigold cultivars. Furthermore, the land area and labor involved for cultivating microalgae are less when compared to the cultivation of marigolds (Fernández-Sevilla et al., 2010). The amount of lutein produced by microalgae depends on various environmental conditions like temperature, pH, irradiance, salinity and the quantity of available nitrogen (Guedes et al., 2011). The microalgae commonly used for producing lutein include *Muriellopsis* sp., *Scenedesmus almeriensis*, *Chlorella protothecoides*, *Chlorella zofingiensis*, *Chlorococcum citriforme*, and *Neospongiococcus gelatinosum* (Fernández-Sevilla et al., 2010). For instance, the amount of lutein produced from the microalga *Scenedesmus almeriensis* was 4.77 mg L^{-1} day^{-1}, with 1,000 mg of lutein from algae source costing approximately 2.5 US$ (Sánchez et al., 2008a; Molina et al., 2008).

3.3.2 Astaxanthin

Astaxanthin is a carotenoid that belongs to the xanthophyll family and is deemed to be one of the high-value products available in the market today (Panis and Carreon, 2016). Astaxanthin acts as an antioxidant and serves to protect the skin from ultraviolet radiations. The production cost of synthetic astaxanthin is considered to be lower than that obtained from microalgae. This constitutes the greatest drawback for harnessing microalgae in the production of astaxanthin. However, certain microalgae like *Haematococcus pluvialis* (Panis and Carreon, 2016) and *Chlorella zofingiensis* (Guedes et al., 2011) have been successfully employed in producing astaxanthin commercially. For instance, *Haematococcus pluvialis* produced 35 mg g^{-1} of astaxanthin with an approximate

market value of 1.8 US$ for 1,000 mg of astaxanthin (Panis and Carreon, 2016; Shah et al., 2016; Cuellar-Bermudez et al., 2015).

3.3.3 β-carotene

One of the vital carotenoids, β-carotene has been used in industry as a coloring agent, as an antioxidant, and as a vitamin-A supplement. Besides this, it is known to possess antiaging and anticancer properties (Guedes et al., 2011; Pisal and Lele, 2005). The main source of natural β-carotene is the carrot; however, algae have been considered as an alternate natural source for the production of β-carotene (Pisal and Lele, 2005). The most commonly used microalgae for production of β-carotene are *Dunaliella salina*, *Scenedesmus almeriensis*, and *Dunaliella bardawil* (Guedes et al., 2011; Pisal and Lele, 2005). For instance, the amount of β-carotene produced from *Dunaliella bardawil* was 1.65 pg $cell^{-1}$ with an approximate market value of approximately 0.6 US$ per 1,000 mg of β-carotene (Molina et al., 2005; Pisal and Lele, 2005).

3.3.4 Zeaxanthin

Zeaxanthin is generally a yellow colored carotenoid and is typically found in corn, egg yolk, gul mohr, orange, berries, and marigold flowers. Zeaxanthin is mainly used in pharmaceutical, cosmetics and food industry applications (Sajilata et al., 2008). The commonly used microalgae for the production of zeaxanthin are *Scenedesmus almeriensis* and *Nannochloropsis oculata* (Granado-Lorencio et al., 2009; Guillerme et al., 2009). The amount of zeaxanthin produced by *Scenedesmus almeriensis* was 0.34 mg g^{-1} with a market value of approximately 10 US$ per 1,000 mg (Granado-Lorencio et al., 2009; Refinery, 2016).

3.3.5 DHA (Docosahexaneoic acid) and EPA (Eicosapentaneoic acid)

Polyunsaturated fatty acids (PUFAs) such as DHA and EPA have been known to impart good health for humans as part of healthy diets and healthy living (Winwood, 2013). Fish oil, one of the major sources of DHA and EPA has been harnessed successfully and has reached its maximum worldwide production (Winwood, 2013). Environmental hazards are considered to be one of the reason for the limitation and decline in the commercial production of PUFA from fish sources, and hence alternate sources are being investigated (Patil et al., 2005). Another problem of obtaining PUFA from fish is that the oil extracted has an unpleasant "fishy" odor (Patil et al., 2007). Recently, microalgae have been employed in the production of oils rich in DHA and EPA and are now being increasingly used in the food industry (Winwood, 2013; Matos et al., 2017). The most common algae employed for the production of DHA-rich algal oil include *Schizochytrium, Ulkenia, Isochrysis galbana, Chlorella pyrenoidosa, Chlorella ellipsoidea* and *Crypthecodinium*. The important microalgal growth factors affecting the production of DHA and EPA include temperature and salinity (Winwood, 2013; Matos et al., 2017).

3.3.6 Lycopene

Tomatoes are the major source of lycopene. It is considered to be one of the most influential antioxidants and cannot be produced by animals (Agarwal and Rao, 2000). Lycopene is considered to be an effective sunscreen agent (Mourelle et al., 2017). Lycopene is known to have a great impact on human health as it possesses anticarcinogenic and antiatherogenic properties (Agarwal and Rao, 2000). Dietary intake of lycopene by humans, as an antioxidant, reduces the oxidative stress by trapping reactive oxygen species and thereby reduces the risk of chronic diseases like cancer and cardiovascular diseases (Agarwal and Rao, 2000). An epidemiological study showed

that the instance of prostate cancer was reduced with the consumption of foods rich in lycopene (Giovannucci, 1999). The yield of lycopene, as compared to β-carotene, obtained through chemical synthesis was poor (45% and 36% respectively); hence the discovery of an alternate sustainable way is required (Schweiggert and Carle, 2016). An *in vivo* study revealed that algal lycopene obtained from *Chlorella marina* exhibited higher antioxidant and anti-inflammatory effect in high cholesterol fed rats when compared to standard drug lovastatin and trans-lycopene produced from tomatoes (Renju, Kurup et al., 2014). Therefore, production of lycopene from microalgae should be explored and *Blakeslea trispora*, a fungal plant pathogen, is the only lycopene-producing micro-organism (156 mg L^{-1} – 578 mg L^{-1} through fermentation) that has reached commercial production till date (Agarwal and Rao, 2000, Schweiggert and Carle, 2016).

3.3.7 Phycobiliproteins

Phycobiliproteins are formed by bonding water soluble proteins with phycobilins or chromophores during photosynthesis (Markou and Nerantzis, 2013a). Phycobiliproteins are coloured pigments that are mainly found in cyanobacteria and red algae. Based on the UV-visible absorption spectra, four main classes of phycobiliproteins exist namely phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin (Sekar and Chandramohan, 2008). The market value of phycocyanin alone reached between 5-10 million US$ (Odjadjare et al., 2017). The major sources of phycobiliproteins include *Spirulina* sp., *Arthrospira platensis*, and *Amphanizomenon floa-aquae*. Phycobiliproteins are used commercially as natural dyes and fluorescent agents as well as used in pharmaceutical (antioxidant, anti-inflammatory, neuroprotective and hepatoprotective agents) and cosmetic industries (perfumes and eye-make up powders) (Odjadjare et al., 2017; De Jesus Raposo et al., 2013).

3.3.8 Global market of value added compounds obtained from microalgae

The PUFA fatty acids (DHA & EPA) have a global market value of over 700 million US$ per year followed by β-carotene with 261 million US$ per year, followed by astaxanthin with a market value of 240 million US$ per year, closely followed by lutein with a market value of 233 million US$ per year and finally phycobiliproteins with a value of just over 60 million US$ per year (Markou and Nerantzis, 2013a). The global demand for carotenoids was expected to increase further to 1.8 billion US$ and reached this level by the end of 2019 (Research and Markets, 2017).

3.4 Microalgal biomass as human food

Microalgae are rich sources of carbohydrates, proteins, and lipids. They have been used as a source of food for humans in China, Japan, Mexico and in Africa due to their abundant composition in proteins (up to 70% of dry mass), vitamins and essential fatty acids. Until today, *Chlorella* and *Spirulina* have been the most commonly sold food-microalgae because they can grow faster. Nonetheless, the biomass obtained from *Spirulina* has mainly been used for the extraction of phycocyanin (Soletto et al., 2005; Varfolomeev and Wasserman, 2011). A study showed that phycocyanin extracted from *Spirulina platensis* inhibited the growth of human leukemia K562 cells (Liu et al., 2000). *Chlorella* and *Spirulina* have been widely used in the production of tablets, capsules, and liquids and marketed as a source of vitamins and antioxidants. β-1,3 glucan contained in the biomass of *Chlorella* is considered to be one of the major components. This compound is known for stimulating the immune system as well as for lowering the lipid content in the bloodstream. *Spirulina* and *Chlorella* found application as a food colorant (phycocyanin) and in the production of beverages (microalgal health drink, microalgal sour milk and microalgal green tea) (Liang et al., 2004). In addition, *Chlorella*'s content in carotenoids like astaxanthin, lutein and

sulfated polysaccharide β-1,3 glucan, all possessing anticancer effects, are also effective in preventing atherosclerosis and hypercholesterolemia (Soletto et al., 2005; Talero Barrientos et al., 2015). *Dunaliella salina,* being rich in β-carotene, has been used as an orange dye in the food industry and as a vitamin C supplement (Priyadarshani and Rath, 2012). In addition to β-carotene, *Dunaliella* produces α-carotene, lutein and lycopene. β-carotene obtained from *Dunaliella* has been known to inhibit cancer cells (Bishop et al., 2012). *Dunaliella* has been known to impart numerous health benefits in humans (pro vitamin A, anti-inflammatory and anticancer), however, only little data on the risks emphasizing the safety of consuming this alga has been reported. A multigenerational study on rats raised for four generations consuming 10% *Dunaliella* showed no significant differences when tested for their gross pathology. However, histopathological studies revealed that there was a decrease in chronic inflammations between rats fed with algae and control animals with no adverse effects and this indicates that this alga is considered safe for human consumption (Mokady et al., 2014). Similar to other microalgae, *Scenedesmus* exhibited no negative effects and the toxicity assessments conducted using test animals revealed the safety of the alga for human consumption. Nutritional studies conducted on humans concluded that consumption of algae to a certain extent (20 g day^{-1}) had no adverse effects even if the intake was prolonged for longer periods of time. Another study indicated that feeding *Scenedesmus obliquus* to children and adults had no significant hematological effect but an increase in body mass was observed (Gross and Gross, 1978). The same study was carried out for slightly and seriously malnourished infants and the results obtained revealed that there was an increase in body mass for infants fed with an algal diet (27 g day^{-1}) in comparison with infants who had a normal diet, with no adverse effects. It was therefore concluded that algal diet had a significant contribution for improvement of health in humans (Gross and Gross, 1978). *Scenedesmus* species could be used

as a source of antioxidants (astaxanthin) and as a rich vitamin source (C, B_1, and B_2) (Ishaq et al., 2016). *Arthrospira*, another group of microalgal species, has been consumed by humans to reduce hyperlipemia, arterial pressure and to trigger the growth of intestinal *Lactobacillus* (Varfolomeev and Wasserman, 2011).

Haematococcus pluvalis has been known for its rich source of the carotenoid, astaxanthin known to act as an antioxidant or oxygen quencher as well as a powerful radical scavenger (Sousa et al., 2008). *Nannochloropsis* species especially *Nannochloropsis oculata* is considered to be rich in EPA and when administered in rats in the form of an oral suspension, it exhibited no toxic effects (Kagan and Matulka, 2015). A study conducted on *Nannochloropsis oculata* showed that its freeze dried biomass could be used to enrich cookies and pasta with omega-3 fatty acids like EPA and DHA and this promises to offer a niche opportunity for the food industry (Babuskin et al., 2014). A study on *Aphanizomenon flos-aquae* revealed that the alga contains omega-3 alpha-linolenic acid which promotes good overall health (Jensen et al., 2001). A study indicated that oral administration of *Aphanizomenon flos-aquae* (1.5 g day^{-1}) in humans increased the CD3+, CD4+ and CD8+ T cell subsets and CD19+ B cells. This resulted in the increase of immune surveillance. The increase in global production of microalgal species such as *Arthrospira* (3,000 t $year^{-1}$), *Chlorella* (2,000 t $year^{-1}$), *Dunaliella salina* (1,200 t $year^{-1}$) and *Aphanizomenon flos-aquae* (500 t $year^{-1}$) indicates that microalgae have a possibility of becoming an interesting source of food for human population in the years to come (Spolalore et al., 2006). The production is expected to increase further by 27,552 tons by 2024 with a compound annual growth rate of 5.32% (Transparency Market Research, 2016). The other species that have been incorporated as food for humans include *Odontella auriata*, *Tetraselmis chuii*, *Spirogryra* and *Oedigonium* species. One of the major concern of incorporating algae as human feed is that they contain large quantities of

nucleic acid which undergoes metabolic degradation to uric acid and this might result in gout or kidney stones (Garcia et al., 2017).

3.5 Microalgal biomass as fish, animal and poultry feed

The use of microalgal species as feed supplements has grown rapidly in the past few decades. The biomass obtained from microalgae has been used as a feedstock for animals ranging from fish to farm animals (Spolalore et al., 2006). About 30% of the algae biomass produced worldwide is being sold as animal feed (Spolalore et al., 2006). Microalgae is a rich source of protein and has been used as a nutritional supplement for the larvae of crustaceans and molluscs for a particular period of their life cycle (Borowitzka, 1997; Brown et al., 1997). The commonly used species of microalgae for animal feed include *Chlorella, Isochrysis, Phaeodactylum, Chaetoceros, Nannochloropsis, Tetraselmis, Dunaliella, Scenedesmus, Thalassiosira* and *Skeletonema*. The carotenoid astaxanthin, obtained from *Haematococcus* algae, has been endorsed in Canada as a coloring agent in salmonid feed (Spolalore et al., 2006). A study reported that *Haematococcus* has the ability to produce larger amounts of astaxanthin when compared to other algae and has been widely used in aquaculture. The meal produced from *Haematococcus* has been successfully employed as fish feed for its astaxanthin content and has proven to be non-toxic upon consumption (Dore and Cysewski, 2003). *Chlorella,* being rich in protein content could be used as an alternative source as it is less expensive and grows rapidly (Lum et al., 2013). Lutein obtained from *Scenedesmus* species is mostly used as a source for animal nutrition while astaxanthin is mostly used in the field of aquaculture. In addition, *Scenedesmus* species can be considered as a source of monounsaturated, polyunsaturated and saturated fatty acids in animal and fish feeds (Ishaq et al., 2016). *Spirulina* sp. contains nutrients such as proteins, carbohydrates, and vitamins and has the

ability to grow in highly saline and alkaline medium. Hence, these species provide a valuable feed for livestock. A study on the impact of *Spirulina* on growth and pigmentation in broiler chickens showed that there was no change in body mass, however there were slight changes in yellowness and redness of the broiler flesh. The yellowness was possibly due to the accumulation of zeaxanthin (yellow pigment) within the flesh (Yaakob et al., 2014; Toyomizu et al., 2001). The powder form of β-carotene obtained from *Dunaliella* species was used as a coloring agent and as a source of pro-vitamin A for animals and fish (Amotz, 2004). *Nannochloropsis* sp., being rich in EPA have been used as a nutritional supplement in the aquaculture industry (Yaakob et al., 2014). The effect of inclusion of *Nannochloropsis gaditana* in the diet of hens revealed that there was an increase in the content of DHA present in their produced egg yolk and it could be used as an alternative to produce eggs enriched with DHA (Brunel et al., 2013). A study reported that up to 16% of the dried *Spirulina* algae, when introduced into broiler diet, had a significant impact on the performance of the chicks (Evans, Smith et al., 2015).

A study revealed that *Spirulina platensis,* when incorporated into mash starter and finisher diet, increased the chicks' performance in terms of body mass gain and feed conversion ratio (Shanmugapriya et al., 2015). Another study revealed that the incorporation of 2.0% and 2.5% *Spirulina platensis* biomass in the poultry feed has the potential to improve the colour of the egg yolk without any significant effect on production performance (Zahroojian et al., 2013). An investigation to study the impact of dietary supplementation of *Chlorella vulgaris* in poultry feed showed that the inclusion of 1% of fresh liquid *Chlorella* in the diet of chicks improved their growth performance. There was an increase in their intestinal microflora and improvement in the functioning of their immune system (Kang et al., 2013). Fermented *Chlorella vulgaris* biomass incorporated into poultry feed was studied for its effects on egg production, egg quality, liver lipids

and intestinal microflora in laying hens. The results revealed that there was an improvement in egg production, egg yolk colour and positive effect on the contents of hepatic triglycerol and the profiles of cecal microflora (Zheng et al, 2012).

Kotrbáček et al. (2013) studied the effect of incorporation of 1% and 2% of *Chlorella* biomass into the dietary supplementation of poultry feed and revealed that there was an increase in yolk carotenoids, lutein, β-carotene and zeaxanthin with both 1% and 2% inclusions (Kotrbáček et al., 2013). The inclusion of four species of microalgae *Phaeodactylum tricornutum, Nannochloropsis oculata, Isochrysis galbana* and *Chlorella fusca* in the poultry feed concluded that inclusion of 125 mg per 100 g of feed of *Phaeodactylum tricornutum* and *Isochrysis galbana* increased the long chain PUFA content as well as the colour of the egg yolk. Therefore, these two species could be used as an alternative to current sources for enrichment of eggs (Lemahieu et al., 2013). Microalgal biomass, if incorporated into the feed of animals, can positively influence the growth, immune response and gut function (Harel et al., 2004). Therefore, it is evident that microalgal biomass can be used as animal, fish, or poultry feed.

3.6 Microalgal biomass in the field of cosmetics

The current trend in the world of cosmetics is developing products that meet consumers demand for products which provide multiple benefits with little or no efforts. Cosmetic pharmaceuticals, known in short as cosmeceuticals, contain bio-actives that are mainly used to improve the biological skin function, thereby imparting a therapeutic effect (Arora et al., 2012). Recently, microalgae are receiving great attention because they can be effectively used in the treatment of skin disorders like aging, tanning, and problems related with pigmentation (Wang et al., 2015). The commonly used algae employed in cosmetic industries include *Spirulina* sp., *Chlorella* sp.,

and *Arthrospira* sp. (Fabrowska et al., 2015). Conventional methods such as maceration, aqueous extraction, and soxhlet extraction are still the widely used techniques for extracting bioactive compounds from algae (Azmir et al., 2013). Microalgae have been used as skin whitening agents by inhibiting the tyrosinase enzyme which in turn leads to the reduction of melanin pigment. This is considered to be the most common approach for skin whitening as melanin is the compound that is responsible for the colour of the skin, hair and eyes (Wang et al., 2011). Fucoxanthin and phloroglucinol derivates acquired from marine microalgae have the ability to inhibit the formation of melanin. Hence, marine microalgae have been considered to be a promising tool as tyrosinase inhibitor agents (Wang et al., 2011).

Skin wrinkling occurs due to the formation of matrix metalloproteinase that leads to the degradation of skin collagen and this is generally caused by reactive oxygen species due to oxidative stress. The phenolic compounds obtained from marine microalgae have been shown to inhibit the formation of matrix metalloproteinase, thereby preventing skin aging (Thomas and Kim, 2013). Polysaccharides obtained from microalgae have a large number of cosmetic functions. Fucoidans acquired from brown algae, carrageenans obtained from red algae and ulvans obtained from green algae are some of the best examples. They are used as rheology modifiers, hair conditioners, suspending agents and wound-healing agents (Aditya et al., 2016). The extracts obtained from biomass of *Chlorella* and *Spirulina* have been used in the production of creams, lotions, shampoos and sun protection commodities. The Pentapharm Company of Switzerland has found that *Nannochloropsis oculata* could be used for skin elasticity while the Exsymol company of Monaco has used the extract of *Arthrospira* for slowing down skin aging (Varfolomeev and Wasserman, 2011).

The processing of algae in the cosmetic industry starts with the collection of microalgal strain followed by cultivation. Many of the culture conditions have been optimized and the algal biomass has successfully been separated by means of filtration, sedimentation or flocculation. Once produced the biomass is then dewatered and dried. The cells obtained from the culture are further disrupted using milling, grinding or powdering. The last step is the extraction of the desired product from the algal biomass using micronization or extraction techniques (Fabrowska et al., 2015). Microalgal biomass can be a valuable source of extractable compounds of interest to the cosmetic industry because of the diversity of their biological activity. However, in order to commercialize cosmetic products from microalgae there is a need for conducting stability and toxicological studies to ensure their safety and efficacy (Ariede et al., 2017).

3.7 Microalgal biomass in the field of pharmaceuticals

The worldwide market for biopharmaceuticals is rising and algae have been considered to be a prospect in satisfying the increasing demand (Aditya et al., 2016). The primary and secondary metabolites produced by microalgae can be used as ingredients for the pharmaceutical industry (Abedin and Taha, 2008). An increase in the production of bioactive compounds from natural sources (microalgae) has been observed and is considered to be an emerging area of particular interest in the years to come (Herrero et al., 2013). The most commonly employed microalgae for the production of bioactive compounds, of pharmaceutical interest, include *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus* and *Nostoc* sp. The production of vaccines from microalgae that can be administered orally seems to be an interesting aspect for researchers to concentrate on in the future. As per advances in microalgal research, *Chlamydomonas reinhardtii* was found to be one of the most important microalgae that have been employed in the manufacturing of pharmaceutical

proteins (erythropoietin, interferon β insulin and immunoglobin A) (Yan et al., 2016; Scaife et al., 2015). Glycerol, a compound widely used in pharmaceutical industries was produced by *Chlamydomonas reinhardtii* when the alga was deprived of sulfur (Skjanes et al., 2013). Cyanovirin, a bioactive compound obtained from the biomass of *Nostoc* responded positively for treating symptoms of HIV (human immunodeficiency virus) and influenza A (H1N1). Besides this, *Nostoc* produces essential fatty acids and polyunsaturated fatty acids. The biomass of *Chlorella* species is known to be rich in Vitamin B complex especially B_{12}, α-carotene, β-carotene, lutein, ascorbic acid and α-tocopherol and these compounds can help in decreasing the occurrence of certain cancer and in preventing macular degeneration (Bhattacharjee, 2016). *Dunaliella* was considered to be a rich source of bioactive compounds such as enzymes and vitamins. The crude extract obtained from this alga strongly limited the growth of dangerous bacteria, thereby acting as an effective antimicrobial (Bhattacharjee, 2016). Table 3.1 presents many of the value added compounds produced by microalgal species for pharmaceutical purposes (Santhosh et al., 2016).

Table 3.1 Microalgal species producing high-value compounds for pharmaceutical purposes (adapted from Santhosh et al., 2016)

S.No	Name of the microalgae	Product obtained
1	*Chlorella sp.*	Lutein, β-carotene, α-carotene, α-tocopherol
2	*Crypthecodinium cohnii*	Docosahexaneoic acid
3	*Haematococcus pluvalis*	Carotenoids, astaxanthin, lutein
4	*Nannochloropsis gaditana*	Eicosapentaneoic acid
5	*Scenedesmus almeriensis*	Lutein, β-carotene
6	*Chlamydomonas reinhardtii*	Glycerol

3.8 Microalgal biomass in the field of biodiesel production

Biodiesel can be manufactured from oils or lipids obtained from a variety of sources. Triglycerols are considered to be the main component essential for the production of biodiesel. The commonly used sources of fats for the production of biodiesel include pure vegetable oil, animal fats and waste cooking oils. However recently, microalgae have been considered to be a potential source for biodiesel production due to the limited supply of other sources, especially from food-based sources. Microalgae have the ability to multiply at various rates and thrive in a variety of temperatures and environments (Gouveia, 2011) and the oil content they produce can be very high (up to 80% of dry mass) when compared to other traditional feedstocks. Therefore, microalgae are considered to be a key pathway for biodiesel production (Wen and Johnson, 2009). There are different methods available for converting microalgal biomass into biofuels. These include biochemical conversion, thermochemical conversion, chemical reaction and direct combustion. Transesterification is a chemical reaction process that is commonly employed for converting lipids into biodiesel (Wang et al., 2008). The biodiesel produced from microalgae has high caloric value and the viscosity and density of the fuel obtained is lower when compared to biodiesel obtained from other feedstocks. (Mondal et al., 2011). In addition, only 0.3% of the transport fuels are obtained from oil crops and animal fats. The food supply could be affected globally if more arable lands were used for increasing biofuel production. Hence, microalgae are considered to be the only option for producing biodiesel in a sustainable way as they have the ability to grow even on wastewaters and adapt themselves to changing environmental conditions (Gouveia, 2011). A study carried out using *Chlorella sorokiniana* strain under a variety of culture conditions revealed a buildup of target fatty acids that could be used as a feedstock for producing biodiesel (Chader et al., 2011).

An investigative study with *Scenedesmus* sp., for increasing productivity of biodiesel, has shown that *in situ* transesterification process at 70 °C, reaction time of 10 h and biomass to solvent ratio of 1: 15 using a 5% acid catalyst (sulfuric acid) generated pure biodiesel when compared to using a 5% alkaline catalyst (sodium hydroxide) (Kim et al., 2014). A study conducted using *Scenedesmus obliquus* cultivated in simulated brewery effluent showed that production of biodiesel from the oil extracted from *Scenedesmus obliquus* is possible as it contains almost equal proportions of saturated (56.4%) and unsaturated esters (43.6%; Linoleate at 11.42%) (Mata et al., 2013) and this is desirable since saturated esters contribute to the higher stability of the biodiesel whereas unsaturated esters contribute to the lower pour points (temperature below which a liquid loses its flow properties). Cetane number (measure of ignition properties), density and viscosity depend on the ratio of saturated to unsaturated esters. The higher the degree of saturation, the higher the cetane number (time taken for the fuel to ignite), viscosity and density (Moser, 2009). The lipase enzyme extracted from *Scenedesmus dimorphus* could serve as a potential catalyst for transesterification with the help of methanol, ethanol or proponal for producing biodiesel. A yield of 44% of fatty acid methyl esters was obtained after 24 h of reaction with methanol when compared to the other two solvents (24% and 22%) (Shah et al., 2011).

A previous study on *Scenedesmus bijuga* cultivated in food wastewater effluent showed that the alga had a dual potential of treating wastewater as well as for producing biodiesel (Shin et al., 2015). *Scenedesmus* sp. isolated from the coast of Odisha in India, had a high lipid productivity of 24.66 mg L^{-1} day hence, could be used as a potential candidate for biodiesel production (Jena et al., 2012). A study showed that *Scenedesmus acuminatus* cultivated on swine effluent could be used as a potential candidate for biodiesel production. The study revealed that it is possible to produce biodiesel directly using transesterification from wet microalgal biomass in a single step

without the need of drying, thereby making it economically feasible (Unpaprom et al., 2015). A study conducted on the biomass of *Dunaliella* and *Nannochloropsis* sp., showed that direct transesterification of the respective microalgal biomass yielded 66.6% and 68.5% (dry basis) of biodiesel respectively. The amount of palmitic acid, oleic acid and linolenic acid that are considered to be major components for producing biodiesel was found to be high and hence served as a promising feedstock for biodiesel production (Shenbaga Devi et al., 2012). An investigation revealed that *Dunaliella salina* was capable of producing biodiesel as the amount of saturated fatty acids to unsaturated fatty acids was in the proportion of 1:2 (35%:65% dry basis) thereby increasing the cetane number of the produced biodiesel (Eman and El, 2013). Another study on the biodiesel obtained from *Dunaliella salina* revealed that moderate quantity of linolenic and linoleic acid was present during fatty acid methyl esters (FAME) analysis. The presence of both linolenic and linoleic acid could produce biodiesel with higher oxidative stability due to more methylene carbons allylic sites however; very high oxidative stability will decrease its acid value and viscosity. This influences the quality of the fuel obtained. Therefore, *Dunaliella salina* is considered to be a suitable feedstock for biodiesel production due to the presence of high amount of lipids and saturated fatty acids (Abd El Baky et al., 2014).

A study on three marine microalgal species *Isochrysis galbana*, *Pavlova lutheri* and *Dunaliella salina* showed that *P. lutheri* produced more biodiesel than *Isochrysis galbana* and *Dunaliella salina*. The density and the viscosity of the oil were also measured. The study concluded that density of oil (an important fuel quality parameter) obtained from microalgae was high and agreed with ASTM (American Society for Testing and Materials) D941 test standard method when compared to density obtained from other sources (Srinivasakumar, 2013). A study concluded that *Dunaliella salina* is a potential candidate for biodiesel production since it has the

capability to grow in conditions which are not sterile and the amount of lipid produced by this micro-alga is high at 45 mg L^{-1} day. Besides this, *D. salina* can also produce β-carotene as a value-added byproduct and this builds up the feasibility of using *D. salina* for biodiesel production as it brings down the overall cost (Weldy and Huesemann, 2007). A study conducted on 9 *Nannochloropsis* sp., namely *Nannochloropsis gaditana*, *Nannochloropsis salina*, *Nannochloropsis granulata*, *Nannochloropsis limnetica*, *Nannochloropsis ocenaica* and *Nannochloropsis oculata* for biodiesel production showed that the top strain for biodiesel production was *N. ocenaica* because of its higher lipid productivity of 158.76 ±13.83 mg L^{-1} day, cetane number of 54.61 ± 0.25 and lower iodine number of 104.85 ± 2.80 g I_2 as well as low cloud point of 3.45 ± 0.5 °C (Ma et al., 2014). The properties of biodiesel obtained from microalgae seem to be similar to those obtained from oil seed crops and fossil fuels.

Biodiesel from microalgae is considered to be an environmentally friendly and sustainable fuel (Sohi and Eghdami, 2014). Unfortunately, the cost associated with producing biodiesel from microalgae is relatively high. Genetic engineering is an avenue to be explored as it has the scope of increasing the microalgal biomass productivity by producing superior strains and consequently increasing the biodiesel production (Chisti, 2007). If biodiesel from microalgae is commercialized then it would serve as a solution for replacing conventional transport fuels as studies have shown that microalgae have the potential to produce more biodiesel when compared to other oil producing crops (Priyadarshani and Rath, 2012). The use of a biorefinery concept would be an added advantage as the residual biomass obtained after biodiesel extraction could be used as a source of protein for animal feed or in the production of specialty ingredients. This tends to improve the efficiency of the process as well as reduces the cost involved making it technically sustainable (Hariskos and Posten, 2014; Chew et al., 2017).

3.9 Microalgal biomass in the field of biohydrogen production

Microalgae was researched in the 1970's to produce hydrogen using sunlight and water. The microalgae commonly employed in the production of hydrogen include *Chlamydomonas reinhardtii*, *Chlorella fusca*, *Chlorella sorokiniana*, *Scenedesmus obliquus*, *Chlorococcum littorale* and *Platymonas subcordiformis* (Nagarajan et al., 2017). *Scenedesmus obliquus*, a green alga, was the first microalgae reported to produce low quantity of hydrogen in the dark. The production of hydrogen was enhanced when the microalga was exposed to light (Benemann, 2000). The various processes involving biohydrogen production from microalgae include direct biophotolysis, indirect biophotolysis, dark fermentation and photo-fermentation (Shaishav et al., 2013; Saifuddin and Priatharsini, 2016). *Chlamydomonas reinhardtii* has the ability to produce hydrogen and this is dependent on the expression of hydrogenase enzyme. The expression of this enzyme is found to be 10 to 100 fold when compared to other species of microalgae. However, biohydrogen production from *Chlamydomonas reinhardtii* has not yet reached commercial production due to engineering problems related to scale up (Amaro et al., 2013). A study on the strain of *Chlorella sorokiniana* revealed that the micro-alga could produce a high amount of biohydrogen when the amount of sulfur was limited. The biohydrogen produced was then used to power a proton exchange membrane (PEM) fuel cell which showed good performance under ideal conditions (Chader et al., 2011). In another study by Ali et al. (2011), three strains of *Chlorella* sp., (*Chlorella vulgaris* TISTR 8680, *Chlorella ellipsoidea* TISTR 8260 and *Chlorella* sp. TISTR 8262) were studied to determine their capability for producing biohydrogen (Ali et al., 2011). The strains under study were cultured under an anaerobic setup in BG-11 and sulfur-deprived BG-11 medium. The results reported that *Chlorella* sp., TISTR 8262 had the highest biohydrogen production of 13.03%, followed by *Chlorella ellipsoidea* TISTR 8260 which had a yield of 3.05%.

Chlorella *vulgaris* TISTR 8680, did not produce any hydrogen (Ali et al., 2011). A study on marine alga *Platymonas subcordiformis* demonstrated that the alga was able to produce biohydrogen when deprived of sulfur (Guan et al., 2004). Therefore, for biohydrogen production from microalgae to be commercially successful, strains should be genetically modified or strains which are able to survive under sulfur-deprived conditions should be used. The biggest challenge that researchers face today is the inclusion of such traits in a single strain (Kumari et al., 2017).

3.10 Microalgal biomass in the field of bioethanol production

Bioethanol is obtained from various biomass feedstocks through different conversion technologies. The bioethanol obtained from sugar/starch crops through traditional production technologies are generally classified under "First generation biofuels", whereas the bioethanol obtained from lignocellulosic biomass is classified under "Second generation biofuels" (Chiaramonti, 2007). Bioethanol obtained from sugar/starch crops occupies more land and cultivation of such crops for this very purpose creates competition with global food supply (Harun et al., 2010). In this perspective, microalgae are gaining wide attention as a feedstock for bioethanol production (Nigam and Singh, 2011). The most commonly used microalgae for bioethanol production include *Chlorella* sp., *Chlamydomonas reinhardtii*, *Chlorococcum* sp., *Scenedesmus* sp., and *Spirulina fusiformis*. A study on *Chlorella vulgaris* revealed that they could serve as a potential feedstock for production of bioethanol as they have shown the ability to accumulate a high amount of starch (37%) (Hirano et al., 1997). Another study on unicellular marine green alga *Chlorococcum littorale* on starch substrate revealed that dark fermentation led to the decomposition of starch that was accumulated in the cells of the micro-alga. The decomposition of cellular starch in turn led to the formation of ethanol, carbon dioxide and

hydrogen as fermentation products. The study revealed that maximum productivity of ethanol was observed at 30 °C (450 μmol/g) (Ueno et al., 1998). A screen of 200 strains of microalgae from seawater revealed that one particular strain *Chlamydomonas sp.* YA-SH-1 had the ability to convert almost 50% of its 30% starch content (dry basis) into ethanol under dark and anaerobic conditions (Hirayama et al., 1998). *Scenedesmus obliquus* CNW-N was able to produce high amount of carbohydrates (42%) under controlled laboratory conditions. This along with acid hydrolysis with 2% sulfuric acid gave a bioethanol yield of 8.55 g L^{-1} (Ho et al., 2013). A previous study on *Chlorococcum humicola* revealed that the pre-treatment of the alga, with 1% v/v of sulfuric acid for 30 minutes at 140 °C, increased the bioethanol production from 16% to 52% (Harun and Danquah, 2011). A study revealed that *Spirulina platensis* had the ability to produce 16.32% and 16.27% of bioethanol production at 100 °C when treated with 0.5 N nitric acid and 0.5 N sulfuric acid respectively (Markou et al., 2013b). The production of bioethanol from microalgae can therefore be obtained through any one of the following methods: from algal metabolites, dark fermentation and direct production by engineered microalgae. Bioethanol obtained from biomass of microalgae is considered to be a suitable alternative when compared to other traditional technologies but no commercial operation are known at this time (John et al., 2011).

3.11 Microalgal biomass as biofertilizers

Fertilizers contain nutrients that are essential for plant growth. Fertilizers play a vital role in improving agriculture thereby increasing production. Biofertilizers are products that are cheap and contain natural compounds derived from micro-organisms such as algae, bacteria and fungi that can help in improving soil fertility and stimulating plant growth (Abdel-Raouf et al., 2012a).

Arable land is being used continuously for the cultivation of crops and this results in the loss of nutrients that are essential for the optimal growth of plants. The most common nutrients incorporated into fertilizers include nitrogen and phosphorus. The cost of production of chemical fertilizers is rising; hence a suitable alternative is required. The use of biofertilizers produced from microalgae would provide a possible solution (Anand et al., 2015). The majority of cyanobacteria are capable of fixing atmospheric nitrogen and can be effectively used as biofertilizers. They can help build-up soil fertility thereby enhancing crop yield as demonstrated with rice (Song et al., 2005). A study showed that the dry biomass obtained from *Acutodesmus dimorphus* applied as a biofertilizer was able to increase the growth of plant and production of flowers in Roma tomato plants (*Solanum lycopersicum*) (Garcia-Gonzalez and Sommerfield, 2016). The growth parameters in lettuce plant were studied using *Chlorella vulgaris* as biofertilizer. A study revealed that the dry powder of *Chlorella vulgaris* was considered to be a suitable substrate for germinating the seeds of *Lactuca sativa* as it improved germination when compared to unfertilized control. It increased significantly the amount of pigments (chlorophyll a, chlorophyll b and carotenoids) in the lettuce seedlings. Therefore, the study concluded that dry microalgae could be used as a plant fertilizer for improved growth (Faheed and Fattah, 2008). A treatment study containing two formulations i.e., formulation with unicellular microalgae (MC1) and formulation with filamentous microalgae (MC2), applied as a biofertilizer for wheat crop, revealed that the formulation with filamentous microalgae (MC2) increased the nitrogen, phosphorus and potassium in the soil with an improvement in the yield when compared to the formulation with unicellular algae (MC1). This study supports the use of dried microalgal consortia as a biofertilizer for increasing crop yield (Renuka et al., 2016). The impact of two microalgae *Chlorella vulgaris* and *Spirulina platensis* on maize crop was studied with various treatments at the farm of Annamalai University, Tamil Nadu,

India (Dineshkumar et al., 2017). The results concluded that microalgal treatment with cow dung manure + *Spirulina platensis* and cow dung manure + *Chlorella vulgaris* enhanced growth of maize up to 51.1% cm for the treatment time of 60 days (Dineshkumar et al., 2017).

3.12 The Concept of Biorefinery

Biorefining is a process by which biomass is converted into value added products and energy in a sustainable way (Gonzalez-Delgado and Kafarov, 2011). The success of a biorefinery depends largely on the raw material selected and the technologies/processes involved in obtaining the value added products. Microalgae are a raw feedstock that can be processed in a biorefinery. Microalgae, unlike terrestrial plants, lack highly resistant cell wall components with no stem or roots which makes the conversion easier for extraction of value added products. The extraction of the desired value added product from the algae cell largely depends on the algae strain and the cultivation conditions. The algae can be fractionated into different by-products or used as a driving energy for the process. A biorefinery setup should therefore, take into consideration the following aspects such as biomass productivity, strain used along with their biochemical composition and the possibility of extracting value added products (Hariskos and Posten, 2014). A general schema of a microalgal biorefinery is presented in Figure 3.1.

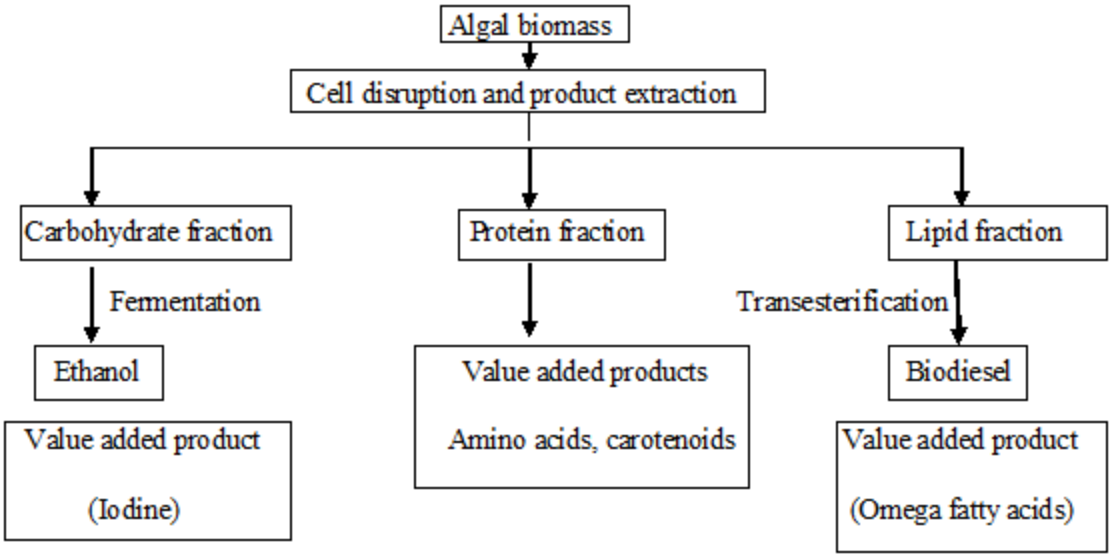


Figure 3.1 General Schema of a microalgal biorefinery (adapted from Chew et al., 2017)

3.13 Conclusion

The microalgal industry is still in its infancy of industrial development, yet it is seen as a possible multibillion dollar industry. Microalgae are considered to be an ecologically safe feedstock for biofuels and for producing products that have high commercial value. Microalgae have been reported to produce more oil for biodiesel development than any other terrestrial plant (soya, rapeseed, palm oil and jatropha). Algae are known to produce highly valued carotenoids like β-carotene and astaxanthin which can be commercialized. Algae also possess the power of converting wastewater into a low environmental impact effluent which in turn could serve as a biofertilizer for plants by improving the fertility of the soil. The need for this sector to be further developed is increasing as there is a demand for innovations that are eco-friendly in order to meet the needs of humans with respect to food, water or fuel. A lot of research has focused on obtaining biodiesel from microalgae over the past few decades, thereby, limiting the exploration of using microalgae in other applications such as pharmaceutical, nutritional and cosmetic industries. For efficient extraction of valuable products from microalgae, large amount of biomass is required and

future research should take this aspect into consideration. The usage of an integrated biorefinery concept should be given high importance as this will allow the processing of microalgae cheaper and competitive when compared with other feedstock. Algal consortium can be used for treating wastewater effectively and promptly. Another avenue for advancement of this technology is to induce certain genes of interest in algae for producing products that are of interest and have high commercial value. Bioprospecting and selection of algal strains combined with genetic engineering holds the key for making this technology successful. The application of microalgae in pharmaceutical, agricultural and cosmetic industries is still in its infancy and further developments will be required to reach its full potential.

CONNECTING TEXT

Chapter III has shown that microalgal biomass can be a source of high value added compounds and can be used as a feedstock for various industries. Many research studies have focused on utilizing the microalgal biomass for biofuel production and this has limited the exploration of using microalgae for extracting value added products. Large amounts of biomass is required for extracting such compounds. From the literature review, it is clear that for microalgal technology to be successful, developing a robust consortia with enhanced biomass productivity that can be put to various uses is essential. This can reduce the operating costs and increase feasibility. Based on the data reviewed, it is clear that *Chlorella* sp. and *Scenedesmus* sp. are the most commonly used microalgal species for various industrial applications. However, most of the uses are strain specific. Chapter IV reports the attempt to manifest the use of simulated dairy wastewater as a large scale cultivation medium for growing an under-examined strain *Chlorella variabilis*. First, efforts were made to screen a media for effectively growing the strain and the growth was assessed in terms of dry mass, chlorophyll content, cell count and optical density. Second, the effects of various media on the biochemical composition of the cultivated strain were assessed. Finally, upon identifying the suitable media, the possibility of extracting lutein (high value added product) from the residual biomass was performed.

CHAPTER IV

EVALUATION AND INTERPRETATION OF GROWTH AND BIOMASS PRODUCTIVITY OF *CHLORELLA VARIABILIS* ON VARIOUS MEDIA

4.1 Abstract

Physiological and biochemical changes along with lutein content of an under examined strain *Chlorella variabilis* were studied under controlled laboratory conditions. In this study, four different media, including diluted dairy wastewater prepared artificially, were utilized for optimizing its growth, biomass productivity and lutein content. The isolate of *Chlorella variabilis* was exposed to a continuous light intensity of 40 ± 3 $\mu mol^{-1}\ m^{-2}\ s^{-1}$, temperature of 24 ± 2 °C and agitated on an orbital shaker at 90 rpm. The growth performance was evaluated in terms of optical density, chlorophyll content and cell count for a period of fourteen days and the pH was monitored regularly. In addition, the nutritional value, dry mass (carbohydrate, protein and lipid) and lutein content were evaluated at the end of sixteen days. The results showed that the Modified N8 medium, as well as diluted synthetic dairy wastewater, were the most productive cultures that supported the growth of the microalga *Chlorella variabilis* with enhanced biomass productivity, nutritional value and lutein content when compared to other media, thereby making the algal biomass a potential candidate for use in poultry and aquaculture (~74% of protein) as well as for producing value added products (lutein). Furthermore, the study holds high importance as it reveals for the first time that the algal strain *Chlorella variabilis* could produce a significant amount of lutein of 9.6 mg g^{-1} (9.6 g kg^{-1}).

4.2 Introduction

Almost 2,000 years have passed since the microalga *Nostoc* was first utilized as food by the Chinese for surviving famine. Since then, there was no reported commercial product from microalgae until the early 1950's when microalgal biotechnology started receiving attention to produce biofuels and value-added products (Spolaore et al., 2006a). Microalgae being photosynthetic in nature make use of light, water and carbon dioxide for manufacturing their nutriment and store them as starch and lipids (Yadala et al., 2014). The stored starch and lipids could then be extracted and be utilized as a feedstock for food and fuel industries. The destiny of the algal industry is therefore, dependent on technologies that would improve the biomass yield at a reduced cost in association with the possibility of scaling up commercially (Vonshak et al., 1988). The two probable solutions to this problem would be bioprospecting of microalgae that have desired attributes or inducing genetic modifications that would enhance the biomass yield of microalgae (Hannon et al., 2010) and/or screening of a suitable media for effectively growing the algal strains as it has been observed that the nutrients present in the medium (cultivation) contribute significantly to the growth, biomass productivity and nutritional value of microalgae thereby reducing the overall production costs (Kim et al., 2013a; Blair et al., 2014).

Lutein is a highly valued yellow carotenoid commonly extracted from marigold flowers, but microalgae have recently received great attention for the production of lutein. This is because microalgae are easy to cultivate and possess higher growth rates. However, microalgal lutein production faces major challenges like lower lutein content and the cost associated with harvesting, cell disruption and extraction. Therefore, it is necessary to find species of alga which can produce high lutein content to overcome the associated costs. According to a review by Lin et al. (2015), a strain producing >10 g kg^{-1} is desired for commercial lutein production from microalgae.

Various media are available for the growth of microalgae; however, it varies from one strain to another, so it is worth trying different formulations that are frequently used in literature. In addition, there is a dire need for screening and optimization of the cultivation medium as it could be a limiting factor and holds the key for successful biotechnological application (Raoof et al., 2006). For instance, a study published by Sánchez et al. (2000), showed that culture medium highly influenced the biomass yield and protein content of *Isochrysis galbana*. Another study put forward by Choix et al. (2017) revealed that the nutrient composition of the culture medium improved the biomass productivity of the alga *Scenedesmus obliquus*.

Considering the above, the current work focuses on selecting a suitable medium among Blue green medium (BG11), Modified bold's basal medium (MBBM), Modified zarrouk's medium (MZM), Modified N8 medium (MN8) and diluted synthetic dairy wastewater (10D) with and without yeast extract (10D+Y) for culturing an under-examined strain *Chlorella variabilis* to improve its biomass productivity, lutein content as well as nutritional value and thereby serve as a source for producing value added products and/or animal feed. Furthermore, the ability of *Chlorella variabilis* to produce significant amount of lutein has been reported for the first time in this study. Up to this time, there has only been one research report that has been published with regard to the ability of the strain *Chlorella variabilis* to grow on and remediate an effluent, in this case textile effluent. Besides this, it was reported that it could produce value added products like ϒ-linolenic acid and ϵ-polylysine that could be used in the nutraceutical and pharmaceutical sectors (Bhattacharya et al., 2017).

4.3 Materials and Methods

4.3.1 Culture Maintenance

Axenic culture of microalga *Chlorella variabilis* was obtained from Culture Collection of Algae and Protozoa (CCAP 211/84). Before the start of experiments, the alga was acclimatized to the growth medium to overcome the shock presented by the medium. The stock cultures were maintained in 125 mL Erlenmeyer flasks containing culture medium at 24 ± 2 °C under a 24 h photoperiod. Overhead lighting for the flasks was provided using cool-white fluorescent light placed at ~0.5 m on a wooden board (4200 K, F72T8CW, Osram Sylvania, MA, US) at a photosynthetic photon flux density (PPFD) of 40 ± 3 µmol $m^{-2}s^{-1}$. Lower irradiance was used for reducing the economic costs incurred while using continuous illumination. Sub-culturing was done regularly every 10 days. The purity of the culture, from contamination, was determined at regular intervals by observing the algal culture under an optical microscope. The experimental set-up is presented in Figure 4.1.

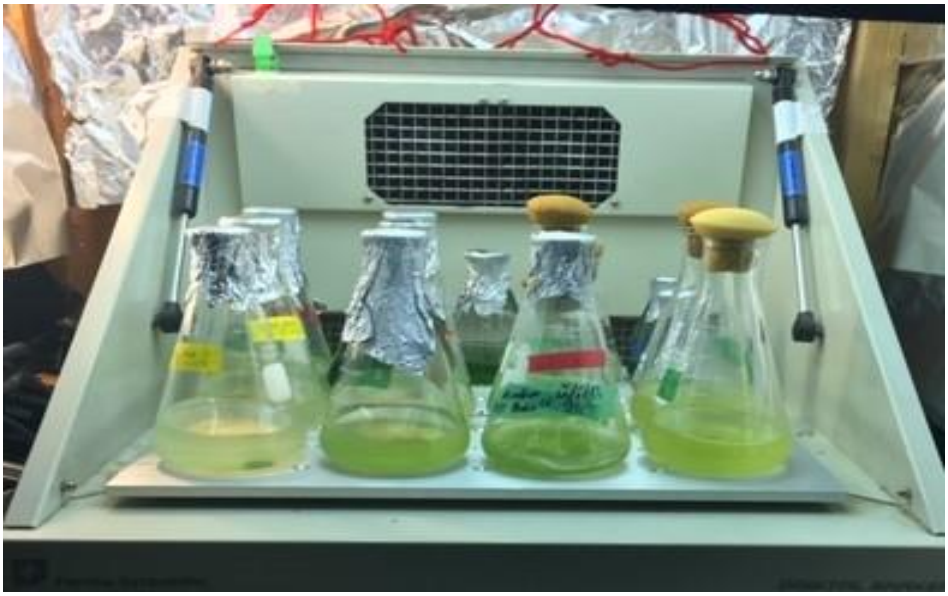


Figure 4.1 Illustration of experimental set-up in the laboratory

4.3.2 Nutrient media utilized for growth

The microalga was inoculated (10% inoculum) into a 500 mL Erlenmeyer flask with a working volume of 200 mL onto four inorganic media and diluted synthetic dairy wastewater of varying composition namely: i) Blue green medium (BG11) (Rippka et al., 1979); ii) Modified bold's basal medium (MBBM) (Agarkova et al., 2008; Kodama and Fujishima, 2015); iii) Modified N8 medium (MN8) (Cheng et al., 2015; Hamedi et al., 2016); iv) Modified zarrouk's medium (MZM) (Delrue et al., 2017; Rajasekaran et al., 2015); v) Diluted synthetic dairy wastewater (10D); and vi) Diluted synthetic dairy wastewater plus 0.1% yeast extract (10D+Y) as presented in Table 4.1. (Vidal et al., 2000). The composition of the media is listed in Table 4.1. The glassware used for culturing the alga was sterilized by pre-heating in an oven at 105°C for 15 minutes. Similarly, the media except diluted synthetic dairy wastewater (10D) were sterilized in an autoclave at 121°C for 30 minutes before inoculation.

Table 4.1 Final composition of BG11, MBBM, MN8, MZM, 10D and 10D+Y.

Blue green medium ($g\ L^{-1}$)	**Modified bold basal medium ($g\ L^{-1}$)**	**Modified N8 medium ($g\ L^{-1}$)**	**Modified zarrouk's medium (MZM) ($g\ L^{-1}$)**	**Non-autoclaved diluted synthetic dairy wastewater ($g\ L^{-1}$)**
$NaNO_3$- 1.5	Part 1	KNO_3- 1	NaCl- 1	Milk powder- 2
K_2HPO_4- 0.04	$NaNO_3$- 25	K_2HPO_4- 0.74	$CaCl_2.2H_2O$- 0.04	NH_4Cl- 2.8
$MgSO_4.7H_2O$- 0.075	K_2HPO_4- 7.5	$MgSO_4.7H_2O$- 0.075	KNO_3- 2.5	$MgSO_4.7H_2O$- 0.1
$CaCl_2.2H_2O$- 0.036	$MgSO_4.7H_2O$- 7.5	$CaCl_2.2H_2O$- 0.013	$FeSO_4.7H_2O$- 0.01	$CaCl_2.H_2O$- 0.076
Citric acid- 0.036	$CaCl_2.2H_2O$- 2.5	$NaH_2PO_4.H_2O$ - 0.26	$EDTANa_2$- 0.08	KH_2PO_4- 2

$C_6H_8FeNO_7$- 0.006	KH_2PO_4- 17.5	FeEDTA- 0.010	K_2SO_4- 1	$NaHCO_3$- 4
$EDTANa_2$- 0.001	NaCl- 2.5	Yeast extract- 1	$NaHCO_3$- 16.8	*Yeast extract – 1
Na_2CO_3-0.02	Part 2	Trace metal mix	$MgSO_4.7H_2O$- 0.2	
Trace metal mix	$EDTANa_2$- 50	$MnCl_2.4H_2O$- 12.98	K_2HPO_4- 0.5	
Boric acid- 2.86	KOH- 31	$ZnSO_4.7H_2O$- 3.2	Trace metal mix ($g\ L^{-1}$)	
$MnCl_2.4H_2O$- 1.81	$FeSO_4.7H_2O$- 4.98	$CuSO_4.5H_2O$- 1.83	Boric acid- 2.86	
$ZnSO_4.7H_2O$- 0.22	H_2SO_4- 1 mL	$KAl(SO_4)_2$- 3.58	$MnCl_2.4H_2O$-1.81	
Na_2MoO_4- 0.039	Part 3		$ZnSO_4.7H_2O$- 0.22	
$CuSO_4.5H_2O$- 0.079	$ZnSO_4.7H_2O$- 8.82		Na_2MoO_4- 0.039	
$Co(NO_3)_2.6H_2O$- 0.049	$MnCl_2.4H_2O$- 1.44		$CuSO_4.5H_2O$- 0.079	
	MoO_3- 0.71			
	$CuSO_4.5H_2O$- 1.57			
	$Co(NO_3)_2.6H_2O$- 0.0s49			
	Part 4			
	Peptone- 1			
	Sucrose- 5			

*Additional ingredient for the preparation of diluted synthetic dairy wastewater plus yeast extract

4.3.3 Estimation of growth and biomass yield

The growth of the microalga in the selected media was assessed by means of optical density, cell count, dry mass and chlorophyll content. Optical density was measured using a UV spectrophotometer at 540 nm (Ultrospec 2100 pro) (Bhattacharya et al., 2016) with the respective media serving as blank. Cell count was performed on a Neubauer haemocytometer utilizing fresh algal cells. Dry mass was determined by filtering a known quantity of biomass through a Whatman filter paper and placing it in an oven at 80 °C for 24 h (Qin et al., 2016). Chlorophyll content was determined using modified Parsons and Strickland method (Parson and Strickland, 1963). The pH of the media was monitored using a Fisher Scientific pH meter. These analyses were carried out every alternate day over a period of two weeks except for dry mass which was carried out once every four days. The experiments were carried out in triplicates and the mean values are reported.

4.3.4 Evaluation of specific growth rate and mean daily division rate

The specific growth rate (μ) was determined by using the following equation,

$$\mu=(\ln (X_2) - \ln(X_1))/(t_2 - t_1) \quad (4.1)$$

where X_2 represents the optical density at time t_2 and X_1 represents the optical density at time t_1.

Mean daily division rate (K) was assessed using the following equation,

$$K = \frac{3.3}{t} * (logOD_t - logOD_0) \quad (4.2)$$

where t represents the days after inoculation, OD_t represents optical density after t days and OD_0 represents optical density on the day of inoculation (Rajasekaran et al., 2015).

4.3.5 Biochemical analysis

After sixteen days, the algal samples were centrifuged at 5,000 rpm for 20 minutes to obtain the leftover biomass. This was followed by freeze drying of the biomass at -55 °C for two days which was then utilized for carbohydrate, protein and lipid analysis by using the methods as described by Waghmare et al. (2016), Slocombe et al. (2013), Folch et al. (1957) respectively.

Carbohydrate analysis was carried out using 5 mg of the freeze-dried biomass and hydrolyzing with 5 mL of 2.5 N hydrochloric acid at 100 °C for 3 h. Sodium carbonate was added to the mixture after cooling and made up to 100 mL with distilled water. The resulting mixture was centrifuged at 4,000 rpm for 10 minutes. The supernatant was subjected to carbohydrate analysis by utilizing the anthrone-sulphuric method published by Waghmare et al. (2016).

Approximately 5 mg of the freeze-dried algal biomass was taken for protein analysis and 0.2 mL of 24% (w v^{-1}) TCA (trichloroacetic acid) was added. The mixture was heated for 15 minutes at 95 °C followed by the addition of 0.6 mL of water after cooling. This was followed by centrifugation at 10,000 rpm for 10 minutes. Lowry reagent D was added to the pellet after discarding the supernatant. After addition of Lowry reagent D, the mixture was again subjected to heating at 55 °C for 60 minutes followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was then subjected to protein analysis, utilizing the Lowry assay method published by Slocombe et al. (2013).

Lipid analysis was carried out by suspending 10 mg of the freeze-dried alga in 5 mL of chloroform and methanol mixture. This was followed by incubation at room temperature for 24 h. The resulting mixture was filtered using a Whatman filter paper grade GF/C and the filtrate was subjected to heating at 50 °C after collecting it in a 50 mL pre-weighed beaker. Total lipids were

calculated by subtracting the mass of the beaker with lipid from the mass of the empty beaker Folch et al. (1957).

4.3.6 Lutein extraction and quantification

Extraction of lutein from the alga was carried out by a method adopted from Inbaraj et al. (2006). In brief, about 10 mg of the alga was treated with 3 mL hexane-ethanol-acetone-toluene in a 10 mL volumetric flask. The treated mixture was then agitated at 100 rpm for 1 h followed by addition of 2 mL of 40% methanolic potassium hydroxide for saponification. The resulting mixture was left in the dark for 16 h. After 16 h, 3 mL of hexane was added to the mixture for partitioning and shaken for 1 min followed by addition of 10% sodium sulfate solution. The solution was then diluted to volume and allowed to stand until the two phases separated clearly. The supernatant containing lutein was collected, evaporated to dryness and re-dissolved in 1 mL methanol-methylene chloride. The extract was finally filtered through a 0.2 μm membrane filter and subjected to HPLC analysis.

4.3.7 HPLC analysis and identification of peaks

The HPLC system used for lutein identification and quantification consists of a quaternary pump with a degasser and a variable wavelength detector. Agilent chromatography computer software (ChemStation version 38) was used for data analysis and reporting. A Discovery C18 column (250 mm × 4.6 mm I.D., 5 μm) was used with two mobile phases (A) consisting of methanol–acetonitrile–water (84:14:2) and (B) consisting of 100% methylene chloride for separation of lutein. The flow rate was set at 0.6 mL/min and a gradient solvent system was utilized for extracting lutein. The injection volume was 20 μL and the response of the peaks was detected at

450 nm. The quantity of lutein was calculated using the following equation put forward by D'Este et al. (2017):

$$\text{Lutein quantity (mg g}^{-1}) = \frac{Lutein\ concentration\left(\frac{mg}{L}\right) * Volume\ of\ solvent(L)}{Dry\ mass(g)} \quad (4.3)$$

4.3.8 Preparation of standard curve for lutein

Commercial lutein standard was obtained from Sigma Aldrich and varying concentrations from 0.5 µg mL^{-1} to 7.5 µg mL^{-1} were prepared and injected into the HPLC. The standard curve was prepared by plotting the concentration against the peak area. A correlation coefficient (R^2) of 0.99 was obtained after performing a regression analysis using Microsoft Excel.

4.3.9 Statistical analysis

The data obtained were expressed with their ± standard deviations. The chlorophyll content, cell count, dry mass and optical density were analyzed statistically using SAS MANOVA and the biochemical characteristics including lutein content were analyzed using one-way ANOVA. The level of significance was set at $p < 0.05$.

4.4 Results

Four inorganic media and diluted synthetic dairy wastewater with and without yeast extract were used in the present study. Figure 4.2 shows the variation of optical density in different media with respect to time. The isolate of *Chlorella variabilis* showed variations in their growth and chlorophyll content in all the media used for the study. The cell count, optical density and dry mass showed that the growth was higher in MN8 medium as compared to other media. Highest optical density of 1.646 ± 0.057 was observed in MN8 after 14 days of inoculation followed by BG11

(1.062 ± 0.045) and 10D (0.565 ± 0.039). However, the microalga did not grow well in MZM, MBBM and 10D+Y and this was supported by the decline in the optical density, cell count, pH and chlorophyll content after day 4 following which the analysis was terminated for these media. Similar trends were observed in terms of cell count, chlorophyll content and dry mass. Highest cell count of 1966 ± 21x10^4 cells mL^{-1} was observed in MN8 on day 6, followed by BG11 and 10D with 1430 ± 5x10^4 cells mL^{-1} and 766 ± 8x10^4 cells mL^{-1} on day 10 and day 12 respectively. The chlorophyll content was highest in MN8 (19.180 ± 1.745 mg L^{-1}) followed by 10D (5.058 ± 0.247 mg L^{-1}) and BG11 (4.312 ± 0.071 mg L^{-1}). The dry mass was highest in MN8 with 647 ± 21 mg L^{-1} followed by 10D and BG11 with 590 ± 20 mg L^{-1} and 440 ± 36 mg L^{-1} respectively after 16 days of inoculation. A maximum volumetric biomass productivity of 0.040 g L^{-1} day^{-1} for the alga was observed in MN8 medium followed by 10D with 0.037 g L^{-1} day^{-1} and BG11 with 0.028 g L^{-1} day^{-1}. The pH of the media on which the alga grew well, appeared to be at near neutral or mildly alkaline pH showing that a pH of 7 to 9 is suitable for the growth of microalgae as put forward by Blinova et al. (2015). Figures 4.3, 4.4 and 4.5 show the variation of cell count, chlorophyll content and dry mass in the three different retained media with respect to time. An overview of pH changes in the media with respect to days is presented in Figure 4.6. An overview of the growth of *Chlorella variabilis* in all the media with respect to days is provided in Table 4.5. The statistical test performed using SAS MANOVA revealed that there was a significant effect of media composition on the growth and biomass productivity of the microalga with respect to days and the statistical analysis results are presented in Tables 4.2, 4.3 and 4.4.

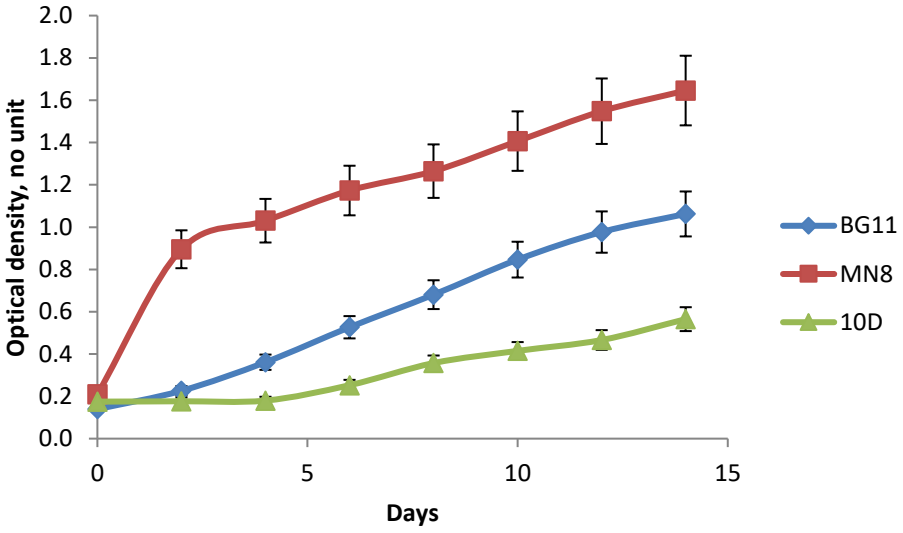


Figure 4.2 Optical density observed in different growth media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

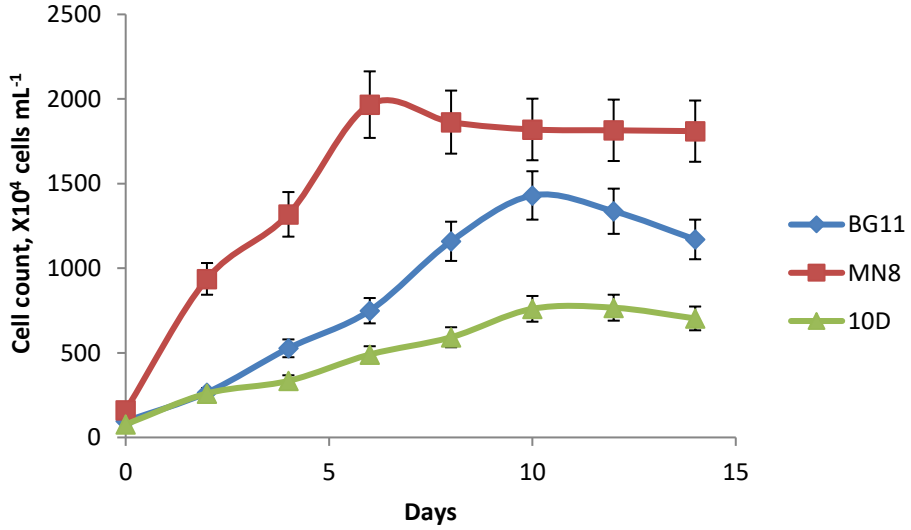


Figure 4.3 Cell count observed in different growth media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

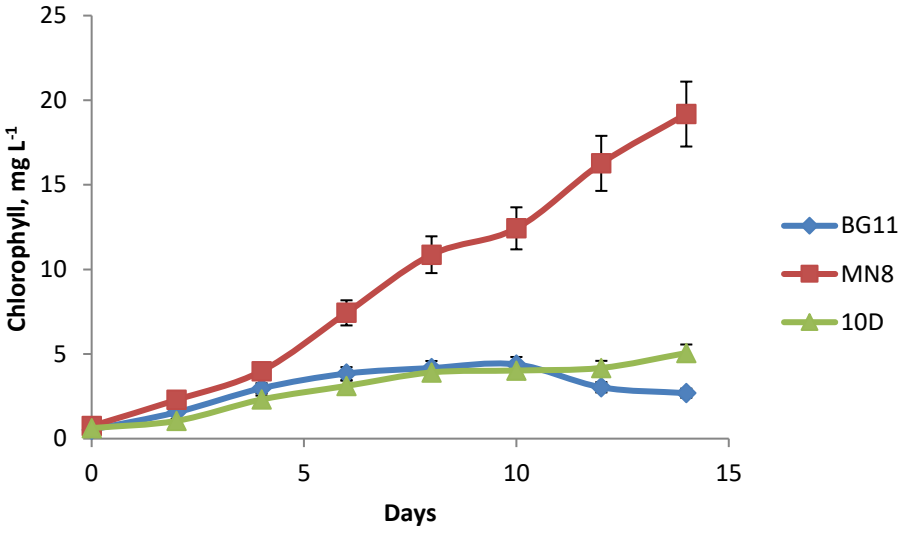


Figure 4.4 Chlorophyll content observed in different growth media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

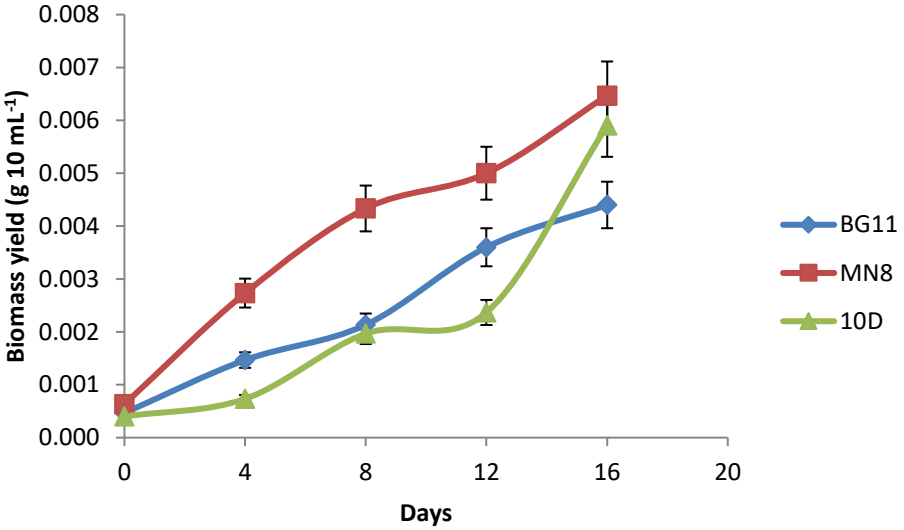


Figure 4.5 Dry mass observed in different growth media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

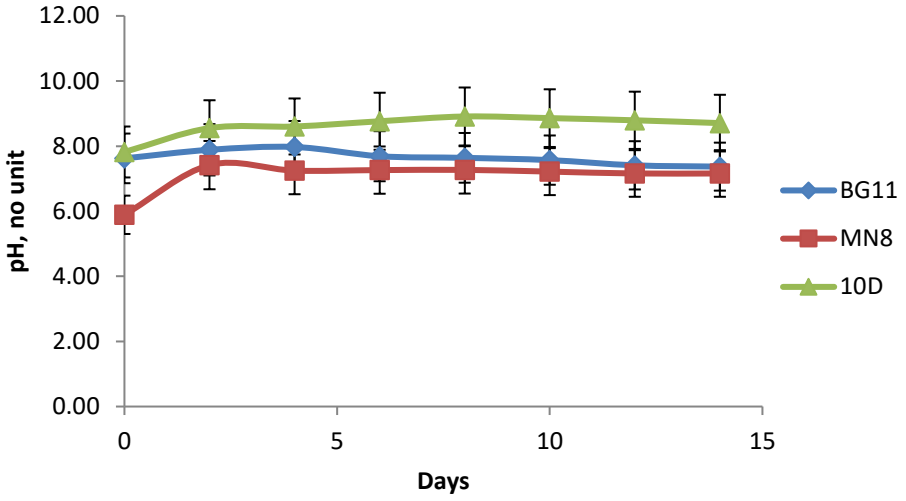


Figure 4.6 pH observed in different growth media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

Table 4.2 MANOVA for overall treatment (media) effects. MANOVA Test Criteria and F Approximations for the Hypothesis of No Overall Media Effect. H = Type III SSCP Matrix for media. E= Error SSCP Matrix

Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	1.99	232.09	12	4	<0.0001
Chlorophyll content	Pillai's Trace	1.96	19.95	12	4	0.0054
Biomass productivity (Dry mass)	Pillai's Trace	1.95	22.98	10	6	0.0005
Cell count	Pillai's Trace	1.99	1072.87	12	4	<0.0001

Table 4.3 MANOVA for days effect. MANOVA Test Criteria and F Approximations for the Hypothesis of No Days Effect. H = Type III SSCP Matrix for days. E= Error SSCP Matrix

Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	0.99	1771.57	5	2	0.0006
Chlorophyll content	Pillai's Trace	0.99	175.06	5	2	0.0057
Biomass productivity (Dry mass)	Pillai's Trace	0.99	521.51	4	3	0.0001
Cell count	Pillai's Trace	0.99	3526.69	5	2	0.0003

Table 4.4 MANOVA for interaction effect (days*media). MANOVA Test Criteria and F Approximations for the Hypothesis of No Days*media Effect. H = Type III SSCP Matrix for days*media. E= Error SSCP Matrix

Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	1.83	6.63	10	6	0.0155
Chlorophyll content	Pillai's Trace	1.86	7.69	10	6	0.0106
Biomass productivity (Dry mass)	Pillai's Trace	1.93	28.74	8	8	<0.0001
Cell count	Pillai's Trace	1.99	1099.65	10	6	<0.0001

Table 4.5 Growth of *Chlorella variabilis* in respective medium with respect to days. Data expressed as mean ± SD. Data is a representation of n=3.

Medium used	Colour observed	Optical Density (no unit) (after 14 days)	Cell count ($x10^4$ cells mL^{-1}) (after 14 days)	Chlorophyll content (mg L^{-1}) (after 14 days)	pH (no unit) (after 14 days)	Dry mass (Biomass yield (mg L^{-1}) (after 16 days)
BG11	Light green colour	1.062 ± 0.045^{c}	1430 ± 5^{c}	2.687 ± 0.101^{c}	7.37 ± 0.02	440 ± 36^{b}
MN8	Dark green colour	1.646 ± 0.057^{a}	1966 ± 21^{a}	19.180 ± 1.745^{a}	7.16 ± 0.11	647 ± 21^{a}
10D	Mild green colour	0.565 ± 0.039^{b}	767 ± 8^{b}	5.058 ± 0.247^{b}	8.71 ± 0.04	590 ± 20^{a}
MZM*	Brown colour	0.058 ± 0.057	28 ± 2	0.003 ± 0.002	10.15 ± 0.01	-
MBBM*	White colour	0.905 ± 0.016	178 ± 26	0.190 ± 0.015	4.33 ± 0.01	-
10D+Y*	Brown colour	0.571 ± 0.010	6 ± 4	1.687 ± 0.286	8.73 ± 0.12	-

*Analysis terminated at day 4 due to poor algal growth

Means with the same letter are not significantly different from each other

4.4.1 Specific growth rate

The specific growth rate with respect to optical density was evaluated and the results demonstrated that the maximum growth was observed in MN8 medium (μ=0.725 d^{-1} on day 2) followed by BG11 (μ=0.243 d^{-1} on day 2) and 10D (μ=0.173 d^{-1} on day 8). The specific growth rates in MBBM, 10D+Y and MZM were the lowest.

4.4.2 Mean daily division rate

The mean daily division rate is the ability of the parent cell to divide into two daughter cells and this was the highest in MN8 with k=1.04 divisions day^{-1} on day 2 followed by BG11 (0.35 divisions day^{-1} on day 2) and 10D (0.13 divisions day^{-1} on day 8).

4.4.3 Effect of growth media on nutritional value

The nutritional value of the alga grown on various media was assessed in terms of carbohydrate, protein and lipid content. It was found that the alga had the highest percentage of protein in MN8 media with 74% followed by 10D with 69% and BG11 with 39%. However, the alga exhibited the highest concentration of carbohydrate and lipid content with BG11 (34%; 22%) followed by 10D (8%; 20%) and MN8 (6%; 17%). The biochemical composition of the alga on MZM, MBBM and 10D+Y was not carried out as the alga did not grow well in these media and the analysis was terminated at day 4. An overview of the biochemical composition of the alga is presented in Figure 4.7.

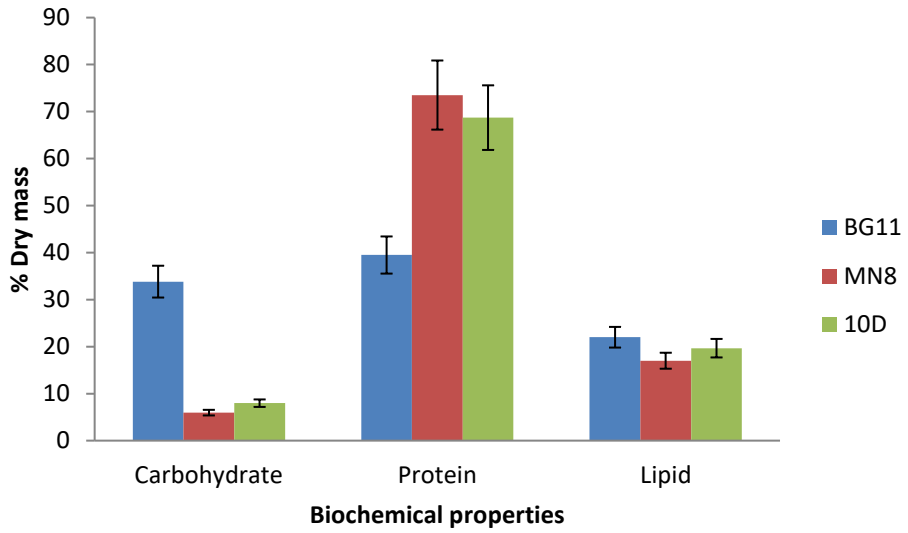
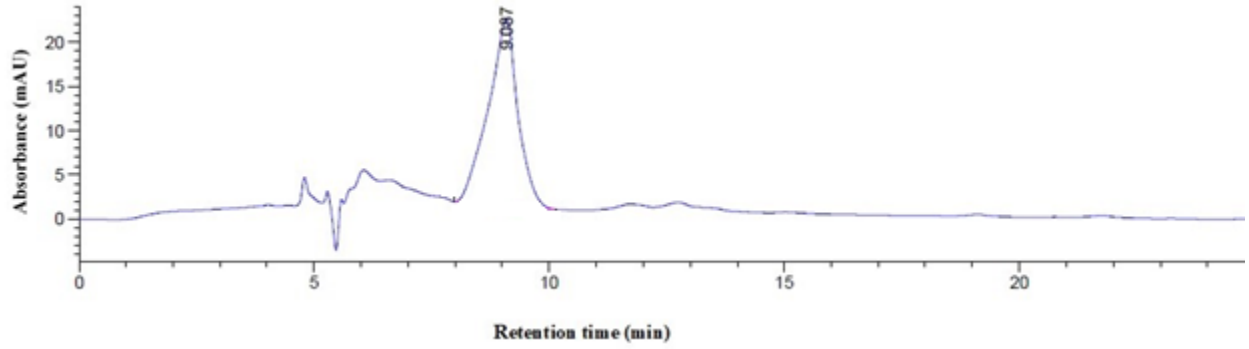


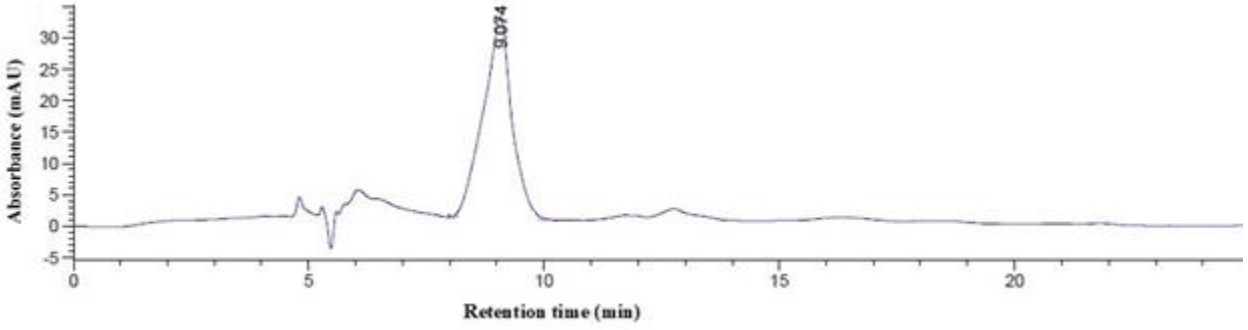
Figure 4.7 Mean percentages of carbohydrate, protein and lipids of *Chlorella variabilis* grown on different media a) BG11; b) MN8; c) 10D. Data is a representation of n=3

4.4.4 Effect of growth media on lutein content

The growth media had a significant effect on lutein content and the results revealed that the alga cultivated on 10D had the highest lutein content of 9.6 mg g^{-1} followed by MN8 with 6.5 mg g^{-1} and BG11 with 0.1 mg g^{-1}. The chromatograms of the crude lutein obtained with 10D and MN8 are presented in Figure 4.8.



a)



b)

Figure 4.8 Chromatogram of crude lutein obtained by extraction after saponification a) MN8; b) 10D

4.5 Discussion

Growth rate and biomass productivity are the two primary factors that have an impact on commercializing microalgal technology. Increasing growth rate and biomass productivity of the microalgae implies selecting a suitable nutrient medium and maintaining optimum environmental conditions. Besides this, the composition of media utilized is known to have a net positive or negative effect on the algal growth rate and biomass productivity and also contributes significantly to the downstream processing cost. Furthermore, providing sufficient nutrients, when it comes to large scale production, poses a serious challenge. Therefore, it becomes a necessity to discover an effective media that would enhance algal growth rate and biomass yield as this paves the way for economic and sustainable algal biomass production. Utilizing wastewater as a growth medium,

along with valorization of the residual biomass, is another technique that could be employed to alleviate production costs (Blair et al., 2014).

The four culture media MBBM, MZM, BG11 and MN8 including dairy wastewater have been utilized in the past for the growth of various algal species including *Chlorella variabilis*, *Chlorella vulgaris, Scenedesmus dimorphus* and *Scenedesmus quadricauda* (Agarkova et al, 2008; Kodama and Fujishima, 2015; Hamedi et al., 2016; Gour et al., 2018; Choi et al., 2018). However, the aim of the previous studies was to determine the impact of culture media on biomass and lipid productivities. In contrast, the current study involves bioprospecting of an appropriate medium for enhancing the growth, biomass productivity, protein and lutein content for an under-examined strain, *Chlorella variabilis,* for large scale production.

The light and temperature during the experiment were the same for the alga cultivated on all the media. pH plays a significant role in the biomass accumulation of microalgae. Near neutral pH is believed to be the optimum pH for enhancing the growth rate and maximizing biomass productivity of the alga under study. Very high and low pH are detrimental for the growth of the alga. High pH tends to alter the absorption trend of trace metals whereas at low pH enzyme inhibition may occur (Bakuei et al., 2015). In the current study, there was no control over pH. The pH was monitored regularly, and the results revealed that the pH of MN8, BG11 and 10D was maintained except for an initial increase on day 2 and is presented in Figure 4.6. This indicates that the pH of the media (MN8, BG11 and 10D) did not affect the growth or biomass productivity of the alga, leaving us with the culture media composition as the sole reason for the differences observed. However, the pH of MZM, MBBM and 10D+Y decreased/increased drastically after day 4, thereby leading to the death of the alga.

The simplest composition among all the media is 10D and 10D+Y as it contains only macro-nutrients with carbonates and yeast extract. The absence of micro-nutrients makes the selection of this media an interesting option as this can reduce the cost significantly. The MN8 medium differs from the 10D and 10D+Y for the presence of micro-nutrients that are present in the form of trace metals in solution and the absence of carbonates. Furthermore, trace metals such as Zn, Mn, Co and Al, present in optimal amounts in the MN8 medium, induced the maximum growth and chlorophyll content (Ilavarasi et al., 2011). The main reason for choosing yeast extract in media composition is that the alga *Chlorella variabilis* grows poorly as it is unable to fully utilize nitrate as its sole nitrogen source thereby making it a limiting factor (Cheng et al., 2015). The composition of MZM differs from the rest of the media as it contains rich amounts of iron cations, carbonates and sulphates. The BG11 media on the other hand contains iron cations with traces of sulphates. The last medium, MBBM, is much more diverse than all the other media as it contains macro-nutrients, micro-nutrients as well as mineral salts with peptone and sucrose.

The experiment revealed that the culture media had a significant effect on the growth of *Chlorella variabilis* and was evaluated in terms of optical density, cell count, chlorophyll content and dry mass over a period of sixteen days. Algal growth generally progresses in four distinct stages namely lag phase, log phase, stationary phase and death phase (Moazami et al., 2012). Figures 4.2, 4.3, 4.4 and 4.5 showed that the stages of algal growth varied significantly based on the composition of media utilized (MN8, 10D and BG11). In addition, a linear correlation was observed between the optical density and dry mass (biomass yield) (Šoštarič et al., 2009). Maximum specific growth rate and mean division rate were observed in MN8 medium when compared to other media. Optical density, cell count and chlorophyll content were also higher in MN8 medium and 10D when compared to other media. Maximum dry mass and volumetric

biomass productivity were also observed in MN8 medium and 10D. Based on the results obtained, it was concluded that MN8 medium and 10D are the best media for culturing the alga *Chlorella variabilis* on a large scale followed by BG11. Furthermore, assessment of the algal growth on MZM, MBBM and 10D+Y revealed poorer growth.

Phosphorus and nitrogen are the two most essential nutrients for maintaining high growth rate of most of the algal species through protein biosynthesis. Though the sources of nitrogen and phosphorus may vary from one medium to another, the ratio plays a crucial role in determining algal growth. In the current study, the ratio of nitrogen and phosphorus was 1:3 in MN8 whereas in BG11 the ratio was 1:1. Similarly in 10D, the ratio was 1.5:1, while in MBBM it was 1:2.5 and in MZM it was 1:3. From the results obtained, it was observed that the alga exhibited higher growth rates and biomass productivity on media which had nitrogen and phosphorus at 1:3 and 1.5:1 which were MN8 and 10D. This shows that the alga grows well in nitrogen replete media. However, this was not the case in terms of MBBM which had a ratio of 1:2.5 and this was in contradiction to the results obtained by Kodama and Fujishima, (2015) which suggested that the alga could grow well using this ratio. Based on their study, the cell count observed was $1x10^8$ cells mL^{-1} after 6 days of inoculation; however, the current study revealed that the alga achieved only $3.06x10^6$ cells mL^{-1} after 8 days of inoculation in MBBM. Furthermore, on subsequent inoculation, the cell number declined and resulted in the death of the alga after 2 days of inoculation which was observed visually. This variation in results could be due to the difference in culture conditions with respect to light, temperature and agitation used in the study. Similar results were observed in BG11 medium. Though the alga appears to have grown well in terms of optical density and cell count, volumetric biomass productivity and chlorophyll content give a different perspective of the algal growth. From Figure 4.4, it can be seen that the chlorophyll content has decreased after day 10

with a lower volumetric biomass productivity of 0.028 g L^{-1} day^{-1}. The alga could not survive in MZM and 10D+Y due to the ineffectiveness of the yeast extract. The decline in the growth of the alga in MZM was in contradiction with the study put forward by Patidar et al. (2016). This could be because the growth of the strain is highly variable with even minute changes in culture and media conditions and the current study employed 24 h photoperiod when compared to 12 h light and 12 h dark and the light intensity was 40 μmol^{-1} $m^{-2}s^{-1}$ as compared to 67 μmol^{-1} $m^{-2}s^{-1}$.

Carbohydrates, proteins and lipids form the major biochemical components of algae. Growth medium and growth stages are known to affect the biochemical composition and it is evident in this study. The results revealed that the growth of the alga on different media had varied its biochemical composition significantly and this is clearly depicted in Figure 4.7. The alga grown on MN8 and 10D had higher protein and lower carbohydrate content when compared to BG11 and this is characterized by the presence of rapidly growing cells (higher specific growth rate) which tend to produce protein for biosynthesis (Gatenby et al., 2003). Besides this, several studies have suggested that when nitrogen is limited, there is an increase in carbohydrate and lipid content with a decrease in protein content (Kim et al. 2013b; Li et al., 2012b). This is because protein synthesis is suppressed, and the carbon assimilated from photosynthesis is utilized for carbohydrate and lipid synthesis (Cheng et al., 2015). An increase in protein content in this study can be attributed to the nitrogen provided by the media (MN8, 10D) to the alga was abundant and was utilized for protein synthesis.

Production of lutein in microalgae is highly related to the nitrogen concentration available during cultivation of microalgae. A study put forward by Ho et al. (2014) showed that lutein content was enhanced (3.6 mg g^{-1}) along with cell growth in *Scenedesmus obliquus* under nitrogen-rich conditions and decreased with depletion of nitrogen. A similar trend was observed in the case

of Del Campo et al. (2000), who revealed that maximal lutein content was observed in *Muriellopsis* sp., (4.8 mg g^{-1}) when nitrogen was abundant. In this study, lutein content was enhanced (9.6 mg g^{-1}) when the alga was cultivated on diluted dairy wastewater (10D) indicating that nitrogen was higher in dairy wastewater and the ratio of nitrogen to phosphorus (1.5:1) supports the argument. This was followed by MN8 which had a lutein content of 6.5 mg g^{-1} and a nitrogen to phosphorus ratio of 1:3.

4.6 Conclusion

Among the different media tested, the highest biomass, protein and lutein contents were recorded when the microalga was cultivated on MN8 medium and 10D when compared to other media. It revealed that diluted synthetic dairy wastewater, devoid of trace nutrients such as cobalt, manganese, copper and zinc, could be used as a potential substrate for actively growing the strain at a relatively low cost when compared to commercially available inorganic media. The study reveals that the strain *Chlorella variabilis* grows effectively under nitrogen rich conditions and future research employing this strain should focus on this aspect. Furthermore, the addition of yeast extract does influence the growth of alga when used with inorganic media but is ineffective when used with diluted synthetic dairy wastewater. The lutein content obtained from this strain is higher (9.6 mg g^{-1}) when compared to other algal strains and could be considered the microalgal source for lutein production as studies suggest that a microalgal strain with lutein content of >10 mg g^{-1} is desired. Finally, it is concluded that diluted dairy wastewater could be used as a substrate for large scale cultivation of the algal strain *Chlorella variabilis* and this could in turn reduce the overall production costs while the residual biomass could be utilized for lutein production.

CONNECTING TEXT

It is clear from the results of Chapter IV, from the biomass and biochemical characteristics, that MN8 medium is the best medium for the growth of the microalga *Chlorella variabilis* with enhanced biomass productivity and nutritional content. However, *Chlorella variabilis* grown on diluted simulated dairy wastewater showed promising results in terms of biomass productivity, lutein and nutritional content and thus, dairy wastewater, could be used as the cultivation medium for the large scale production of the microalga with huge economic benefits as it is estimated that the cost associated with algal cultivation/harvesting accounts for almost 20-30% of the total production costs. In order to overcome the problems associated with culture crashes and to improve the stability and resistance of the culture with respect to oscillations in environmental conditions, a microalgal consortia would be employed in further studies. Further, utilizing a microalgal consortia is considered to be advantageous as it can enhance the overall uptake of nutrients that occurs due to the co-operative interactions between the co-cultivated microalgae. The next chapter focuses on how light quality imparted by LEDs as well as cool fluorescent light can improve the growth, biomass productivity and nutritional properties of a selected microalgal consortia when cultivated on dairy wastewater. In addition, the study emphasizes the use of amber light for microalgal cultivation which has been less explored.

CHAPTER V

A COMPREHENSIVE STUDY ON THE EFFECT OF LIGHT QUALITY IMPARTED BY LIGHT EMITTING DIODES (LEDS) ON THE PHYSIOLOGICAL AND BIOCHEMICAL PROPERTIES OF THE MICROALGAL CONSORTIA OF *CHLORELLA VARIABILIS* AND *SCENEDESMUS OBLIQUUS* CULTIVATED IN DAIRY WASTEWATER

5.1 Abstract

The effect of light wavelengths using different light sources (cool-white fluorescent, blue, amber, red, and amber+blue LED lights) on the growth, biomass productivity, chlorophyll/lutein content and nutrient composition were evaluated with the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus*. Between different light treatments, cool-white fluorescent light produced the highest biomass of 673 mg L^{-1} with a specific growth rate of 0.75 day^{-1} followed by blue (500 mg L^{-1}; 0.73 day^{-1}), amber+blue (380 mg L^{-1}; 0.037 day^{-1}), red (300 mg L^{-1}; 0.28 day^{-1}) and amber light (280 mg L^{-1}; 0.41 day^{-1}). The chlorophyll content was enhanced under blue light with no significant effect on growth rates (12.9 mg L^{-1}) followed by cool fluorescent light (11.3 mg L^{-1}), whereas the lutein content was enhanced under cool fluorescent light (7.22 mg g^{-1} – highest ever reported for an autotrophic mode of cultivation of microalgae) when compared to other wavelengths of light. Protein content of the microalgal consortia was enhanced under all light treatments with the highest protein accumulation under cool-white fluorescent light (~56% of dry mass), closely followed by amber light (52% of dry mass), whereas the carbohydrate content was higher under amber light (~35% of dry mass), followed by cool-white fluorescent light (~24% of dry mass). The results revealed that the consortia could grow well on diluted dairy wastewater,

thereby reducing the cost of algal production when compared with the use of inorganic media and a two-phase culture process utilizing cool fluorescent and amber light could be employed for maximizing algal biomass and nutrient composition with enhanced lutein production. The study emphasizes the economic efficiency of LED lights in terms of biomass produced based on the modest electricity consumed. Furthermore, the study reveals the importance of using amber light for cultivating microalgae for its nutrient content which has seldom been studied.

5.2 Introduction

Marginal biomass productivity is one of the fundamental constraints preventing algal technology from reaching appreciable commercialization. Various environmental factors are known to influence microalgal growth, and light is considered as one of the primary factors (Palacois et al., 2018). Sunlight is the most cost-effective light source and is mainly used for outdoor microalgal production (raceway ponds); however, electrical lighting is gaining importance as it can provide better control of the lighting intensity and wavelengths for photobioreactors (Schulze et al., 2014). Electrical lighting for microalgal cultivation is generally derived from three major light sources: high intensity discharge lamps, fluorescent lamps and light emitting diodes (LEDs). Among the sources listed, fluorescent lamps are commonly used by researchers for microalgal production as they cover a wide range of wavelengths. In recent years, LEDs have been gaining popularity because of their compactness, longer lifetime, high electrical conversion efficiency and low heat emission (Schulze et al., 2014; Ma et al., 2018).

Microalgae, similar to plants, captures light energy (light-harvesting antennas) and produce electrons in the reaction center of the photosystems. For efficient photosynthesis, preserving an excitation balance between the two photosystems (PSI and PSII) is of prime importance. To serve

the purpose, microalgae possess specific light harvesting antennas for expanding the available wavelength of light. Certain groups of algae contain accessory pigments which help in efficiently harvesting light for photosynthesis (Vadiveloo et al., 2015). Green algae, in particular, possess a chlorophyll-protein complex which is comprised of chlorophyll a and b and carotenoids for carrying out photosynthesis (Ueno et al., 2019). Light quality and intensity highly influence the photosynthetic process in microalgae and induce modifications in their biochemical composition (Krzemińska et al., 2014). Light quality, in particular, has a strong influence on the growth rate in both microalgae and macroalgae (Figueora et al., 1995). Besides this, light quality is known to induce a significantly high amount of mature cells in the culture (Schulze et al., 2016). Red and blue wavelengths in particular are known to induce high photosynthetic machinery due to the high absorbances of photosystems I and II for these wavelengths. Previous studies have reported that blue/red light highly influences the algal growth rate and pigment synthesis (Ra et al., 2018; Wu, 2016). Apart from photosynthesis, blue light also regulates enzyme activation and gene transcription and the cells damaged by exposing the algae to red light could be repaired by exposing them to blue light. Therefore, for achieving maximum growth and biomass productivity, microalgae require light conditions that are ideal and that fall within a narrow band of the spectrum (Atta et al., 2013).

Zhong et al. (2018) reported that the microalgae *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Scenedesmus quadricauda* and *Scenedesmus obliquus*, grown under blue light, have the potential to be used in the production of nutritional supplements for human consumption as well as a biofuel feedstock, due to increased biomass productivity and lipid composition. The authors from the same study concluded that blue light is the best wavelength for enhancing algal growth rates followed by red and white lights. Another study put forward by Teoa et al. (2014) on

the algal species *Nannochloropsis* and *Tetraselmis,* under the mixture of red and blue lights with a 24 h photoperiod, revealed that blue wavelength had a positive effect on the biomass productivity. Another study on *Botryococcus braunii* KMITL 2 showed that a photoperiod of 24 h enhanced the biomass concentration to almost four times when compared to the 12:12 photoperiod. Therefore, longer duration of light increased the specific growth rate and biomass concentration of the algal species and a linear trend was observed with increasing photoperiods (Ruangsomboon et al., 2012). Therefore, adequate selection of a light wavelength can enhance the growth and biomass productivity of microalgal species but this differs not only from species to species but also from one strain to another. This response of microalgae to light quality can be attributed to the variation in light harvesting pigments and membranes (Vadiveloo et al., 2015; Krzemińska et al., 2014; Gatamaneni et al., 2018).

However, the light wavelengths ranging between 500 nm to 600 nm have rarely been studied because of their low electrical conversion and radiation efficiencies (Schulze et al., 2014). The knowledge of how microalgae respond to light quality is still a question that needs to be addressed. The combination of different LEDs with varying wavelengths could promote growth rate and biomass productivity by enhancing the process of photosynthesis but the practice requires further studying (Ra et al., 2018; Wu, 2016). In addition, the impact of light properties on different strains of algae has not been investigated extensively by researchers (Krzemińska et al., 2014). In order to address these gaps, the current study aims to provide a clear insight on how different wavelengths of light from monochromatic LEDs (470, 595, and 655 nm) including cool fluorescent, impact the growth rate, biomass productivity, chlorophyll/lutein contents and biochemical composition for the microalgal consortia composed of *Chlorella variabilis* and *Scenedesmus obliquus,* while keeping the light intensity and temperature at 40 ± 3 µmol $m^{-2}s^{-1}$ and

24 ± 2 °C, respectively, under a 24 h photoperiod agitated at 90 rpm on an orbital shaker. The above strains were chosen as there has been no study reported to date employing this microalgal consortium, composed of *Chlorella variabilis* and *Scenedesmus obliquus*, for enhancing biomass productivity or biochemical composition using varying light treatments. Furthermore, the study on the effect of amber light and amber + blue light (mixture of amber+blue was used in order to improve the electrical and radiation efficiency), on the biomass growth, chlorophyll/lutein contents, biomass productivity and biochemical composition of this microalgal consortia will open a new avenue for researchers to focus on. The study reveals the effect of light quality on the lutein content which is considered to be a high value added product.

5.3 Materials and Methods

5.3.1 Microalgal strains and culture conditions

Axenic algal strains of *Chlorella variabilis* and *Scenedesmus obliquus* were obtained from the Culture Collection of Algae and Protozoa. The stock cultures were maintained in 125 mL Erlenmeyer flasks containing culture medium at 24 ± 2 °C under a 24 h photoperiod. There are several reports on the effect of 24 h photoperiod on the growth and biomass productivity of the algal species. This experiment was accordingly carried out under a 24 h photoperiod with continuous illumination. Lower irradiance of 40 ± 3 µmol $m^{-2}s^{-1}$ was used in order to reduce the economic costs incurred while using continuous illumination. Overhead lighting for the flasks was provided using cool-white fluorescent light (4200 K, F72T8CW, Osram Sylvania, MA, US) at a photosynthetic photon flux density (PPFD) of 40 ± 3 µmol $m^{-2}s^{-1}$. The flasks were agitated on an orbital shaker at 90 rpm. Sub-culturing was done regularly after every 10 days to maintain live algal culture. MN8 medium was used for sub-culturing of *Chlorella variabilis* while Blue green

medium (BG11) was used for sub-culturing of *Scenedesmus obliquus*. The compositions of the media were obtained from Cheng et al. (2015) and Rippka et al. (1979). The glassware for culturing the algal strains was sterilized at 105°C for 15 minutes in a hot air oven. Similarly, the media for sub-culturing the algal strains was autoclaved at 121°C for 30 minutes prior to inoculation.

5.3.2 Cultivation of the microalgal consortia on simulated diluted dairy wastewater under different light wavelengths

The microalgal consortia (20 mL) were inoculated into 500 mL Erlenmeyer flasks containing 180 mL culture medium (diluted dairy wastewater at 10%). The dilution of the dairy wastewater was determined based on the results obtained from a feasibility study that was carried out using undiluted, 25% and 10% wastewater (results not reported here). The LED tubes were installed above the orbital shaker (~0.5 m on a wooden board) and the temperature was maintained at 24 ± 2 °C. The flasks were then exposed to different light wavelengths treatments: blue (450 nm, VanqLED, Shenzhen, Guangdong, China), amber (595 nm, VanqLED), amber + blue (ratio 1:1), red (650 nm, VanqLED) and cool-white fluorescent light (Sylvania Cool White, USA). The spectra of the electrical lighting systems used in this study are presented in Figure 5.1. The flasks were illuminated at 40 ± 3 µmol $m^{-2}s^{-1}$ for sixteen days with a 24 h photoperiod on an orbital shaker agitated at 90 rpm. The photosynthetic photon flux density (PPFD) and spectrum of the lighting systems were measured using a spectroradiometer (PS-300, Apogee, Logan, UT). The temperature was continuously monitored using a temperature sensor (Raytek Minitemp, MT6).

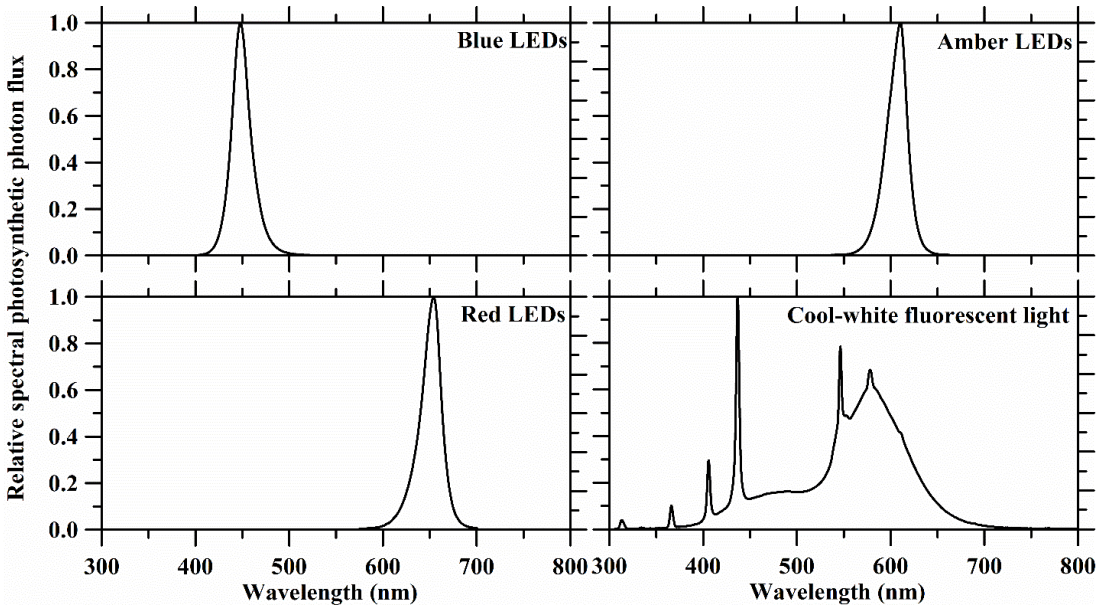


Figure 5.1 Spectral compositions of the lighting systems used in this study.

5.3.3 Estimation of growth rate and biomass productivity

The growth rate of the microalgal consortia was measured in terms of optical density, chlorophyll content and dry mass. Optical density was measured using a UV spectrophotometer (Ultrospec 2100 pro) at 680 nm (Koreivienė et al., 2014). The dry mass was determined by filtering 5 mL of the culture through a Whatman filter paper grade GF/C followed by drying in an oven at 80 °C for 24 h and cooling in a desiccator until a constant mass was achieved (Qin et al., 2016). Chlorophyll content was measured using a procedure put forward by Parsons and Strickland (Parsons and Strickland, 1963). In brief, 5 mL of the algal suspension was taken and centrifuged at 3,500 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 96% methanol followed by vortexing for 2 minutes. The tubes were then kept on a hot plate for 30 minutes at 60 °C. The resulting mixture was centrifuged again at 3,500 rpm for 10 minutes and the supernatant was collected separately. The steps were repeated until the chlorophyll was completely extracted. The absorbance of the supernatant was read at 650 nm and 665 nm and the chlorophyll present was calculated using equation 5.1. The variation in pH was monitored regularly using a Fischer

Scientific pH meter (Accumet Basic, AB15). The analyses were carried out every alternate day for two weeks, except for dry mass which was determined at the end of the sixteen days experiment. Chlorophyll content (CC) was determined using the following Equation 5.1:

$$CC = 0.0255 * A650 + 0.004 * A665 \quad (5.1)$$

where A represents absorbance (Parsons and Strickland, 1963)

Specific growth rate (μ) was determined using the following Equation 5.2:

$$\mu = \frac{ln\left(\frac{X_2}{X_1}\right)}{(t2-t1)} \quad (5.2)$$

where X_2 represents the dry mass at time t_2 and X_1 represents the dry mass at time t_1 (Atta et al., 2013).

Doubling time (D) was determined using the following Equation 5.3:

$$D = \frac{0.693}{\mu} \quad (5.3)$$

where μ represents the specific growth rate (Difusa et al., 2015).

5.3.4 Biochemical analysis

After sixteen days, the algal samples were centrifuged at 5,000 rpm for 20 minutes to assess the biomass yield. This was followed by freeze drying of the biomass at -55 °C for two days which was then utilized for carbohydrate, protein and lipid analyses using modified methods described by Waghmare et al. (2016), Slocombe et al. (2013) and Folch et al. (1957).

In brief, for the carbohydrate analysis, 5 mg of the freeze dried biomass was taken and hydrolyzed with 5 mL of 2.5 N hydrochloric acid at 100 °C for 3 h. After cooling, sodium carbonate was added to the solution until effervescence ceased and made up to 100 mL with distilled water and centrifuged at 4,000 rpm for 10 minutes. To 1 mL of the above solution, 4 mL of anthrone-sulphuric acid reagent was added. The solutions were heated at 100 ºC in a hot water

bath for 10 minutes. The heated solutions were then cooled to room temperature and subjected to spectrophotometric analysis at 630 nm. The observations were noted and the unknown concentration of carbohydrate present was calculated directly from the standard curve (Waghmare et al., 2016).

For protein analysis, about 5 mg of freeze dried algal biomass was taken and 0.2 mL of 24% (w v^{-1}) TCA (Trichloroacetic acid) was added. The samples were then heated at 95 °C for 15 minutes followed by cooling to room temperature. This was followed by addition of 0.6 mL of water and the mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 0.5 mL of Lowry reagant D. The pellet was then heated to 55 °C for 60 minutes and centrifuged again at 10,000 rpm at room temperature for 10 minutes. The pellet was then discarded and the supernatant was retained for further analysis using Lowry assay. In brief, 0.05 mL of sample was taken and 0.95 mL of Lowry reagent D was added and incubated for 10 minutes at room temperature. A 0.1 mL of Folin's reagent was added and vortexed immediately and the absorbance of the sample was read at 600 nm after 30 minutes. The unknown concentration of protein was obtained directly from the standard curve (Slocombe et al., 2013).

For lipid analysis, 10 mg of freeze dried algae was taken and suspended in 5 mL of chloroform and methanol (2:1). The mixture was incubated at room temperature after closing the opening with aluminum foil for 24 h. The mixture was then filtered using a Whatman filter paper grade GF/C. The filtrate was collected in a 50 mL pre-weighed beaker which was then kept on a hot plate at 50 °C. The chloroform methanol mixture was evaporated leaving a residue at the bottom of the beaker. The total lipid was calculated by subtracting the mass of the beaker with lipid from the mass of the empty beaker (Folch et al., 1957).

5.3.5 Lutein extraction and quantification

Lutein was extracted from the algal consortia by adopting a method described by Inbaraj et al. (2006). In short, 10 mg of the alga was treated with a 3 mL mixture of hexane–ethanol–acetone–toluene (10:6:7:7, v/v) in a 10 mL volumetric flask and shaken for 1 h. To the contents, 2 mL of 40% methanolic potassium hydroxide was added for saponification at 25 °C in the dark for 16 h. After saponification, 3 mL of hexane was added for partitioning, shaken for 1 min and 10% sodium sulfate solution was added and diluted to volume. The mixture was allowed to stand until two phases separated clearly. The upper layer containing lutein was collected. The extracts of upper layer were evaporated to dryness, re-dissolved in 1 mL methanol–methylene chloride (50:50, v/v), and filtered through a 0.2 μm membrane filter for HPLC analysis.

5.3.6 HPLC analysis and identification of peak

The separation, identification, and quantification of lutein was made by using high-performance liquid chromatography. The Agilent HPLC system consists of a quaternary pump with a degasser, variable wavelength detector equipped with Agilent chromatography computer software (ChemStation version 38). For separation of lutein, a Discovery C18 column (250 mm × 4.6 mm I.D., 5 μm) was used. The mobile phase (A) consisted of methanol–acetonitrile–water (84:14:2) and the mobile phase (B) consisted of methylene chloride (100%), with a flow rate set as 0.6 mL/min. A gradient solvent system with 100% A and 0% B in the beginning, decreased to 95% A and 5% B in 8 minutes, 75% A and 25% B in 25 minutes, 100% A and 0% B in 25 minutes was utilized for extracting lutein. Extract injection volume was 20 μL and the response of the peak was detected at 450 nm. The identification of lutein was made by comparing the retention time of the

standard with that of the sample. The lutein quantity for each sample was calculated using the following equation (D'Este et al., 2017):

$$\text{Lutein quantity (mg g}^{-1}) = \frac{Lutein\ concentration\left(\frac{mg}{L}\right) * Volume\ of\ solvent(L)}{Dry\ mass(g)} \quad (5.4)$$

5.3.7 Preparation of standard curve for lutein

Commercial lutein obtained from Sigma Aldrich was used as the analytical standard. Concentrations of lutein varying from 0.5 µg mL^{-1} to 7.5 µg mL^{-1} were injected and the standard curve was prepared by plotting concentration against peak area. The regression analysis was done using Microsoft Excel and a correlation coefficient (R^2) of 0.99 was obtained.

5.3.8 Statistical analysis

All the experiments were carried out in triplicates to ensure accuracy and consistency. The values obtained were expressed as means of triplicate values ± standard deviation. The optical density, chlorophyll content and dry mass were analyzed statistically using SAS MANOVA and the biochemical characteristics were analyzed using ANOVA followed by Tukey's HSD test. The statistical analyses revealed that there was a significant effect of light treatments on the physiological and biochemical properties of the microalgal consortia with respect to days of growth at a significance level of $p<0.05$.

5.4 Results

5.4.1 Effect of LED light wavelengths on the growth and biomass productivity of the microalgal consortia

The microalgal consortia was cultured on diluted dairy wastewater and exposed to varying wavelengths of light for a period of sixteen days. The culture growth of the microalgal consortia differed significantly and was dependent on the wavelength of light applied. Continuous illumination was provided to stimulate the growth of the microalgal consortia. The growth and biomass productivity of the microalgal consortia under cool-white fluorescent light and LED light treatments are presented in Figure 5.2. The wavelengths of light had a crucial effect on the microalgal biomass yield (dry biomass obtained after 16 days of inoculation) and productivity (dry biomass obtained per day with respect to the day zero). Among the wavelengths of light used for this study, a maximum biomass yield of 673 mg L^{-1} was observed under cool-white fluorescent light followed by blue, amber+blue, red and amber light with 500 mg L^{-1}, 380 mg L^{-1}, 300 mg L^{-1}, 280 mg L^{-1} and a biomass productivity of 0.042 g L^{-1} day^{-1}, 0.031 g L^{-1} day^{-1}, 0.024 g L^{-1} day^{-1}, 0.019 g L^{-1} day^{-1}, 0.018 g L^{-1} day^{-1} respectively. This suggests that cool-white fluorescent light was the best light source for maximal growth and biomass productivity of this microalgal consortium followed by blue, amber+blue, amber and red. The results for statistical analyses show that the light treatment had a significant effect on growth rate and biomass productivity and are presented in Tables 5.1, 5.2 and 5.3.

Table 5.1 MANOVA for overall treatment (light) effect. MANOVA Test Criteria and F Approximations for the Hypothesis of No Overall Light Effect. H = Type III SSCP Matrix for light. E= Error SSCP Matrix

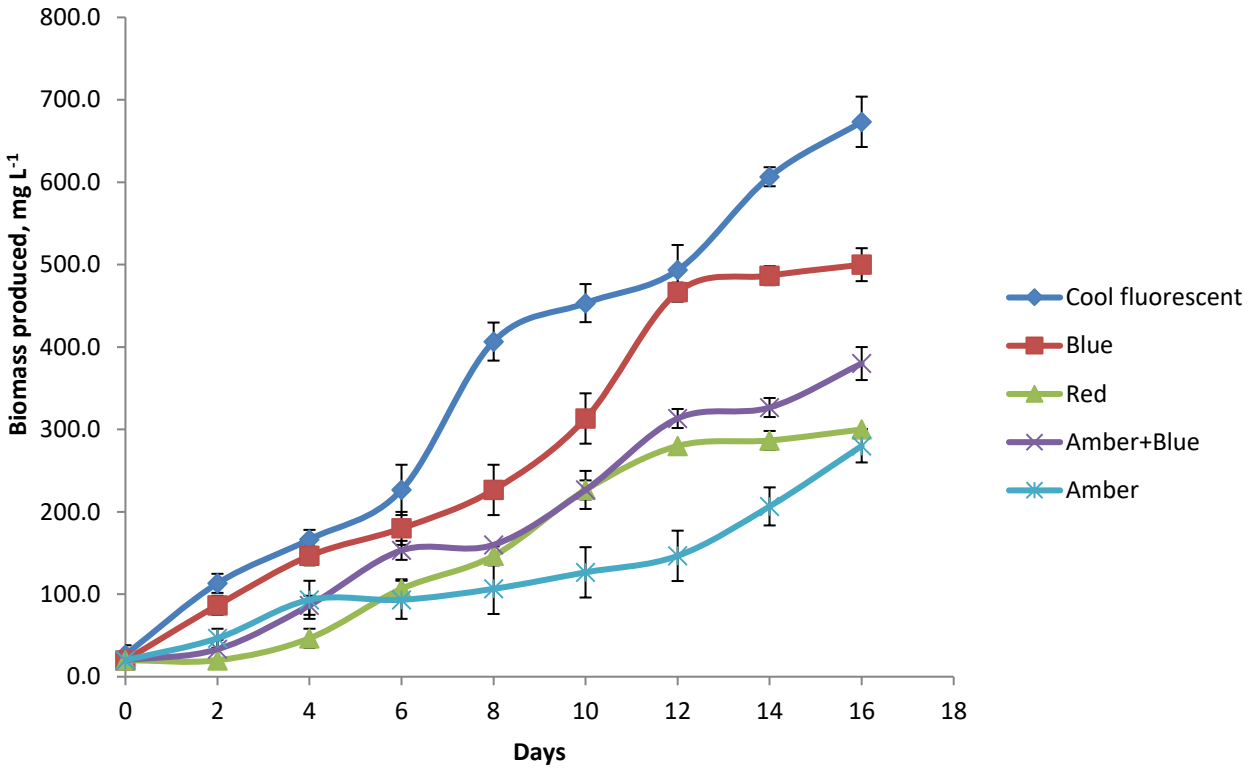
Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	3.53	5.59	32	24	<0.0001
Chlorophyll content	Pillai's Trace	3.05	2.42	32	24	<0.0142
Biomass productivity (Dry mass)	Pillai's Trace	3.48	5.06	32	24	<0.0001

Table 5.2 MANOVA for days effect. MANOVA Test Criteria and F Approximations for the Hypothesis of No Days Effect. H = Type III SSCP Matrix for days. E= Error SSCP Matrix

Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	0.99	575.04	7	4	<0.0001
Chlorophyll content	Pillai's Trace	0.99	407.00	7	4	<0.0001
Biomass productivity (Dry mass)	Pillai's Trace	0.99	1083.59	7	4	<0.0001

Table 5.3 MANOVA for interaction effect (days*light). MANOVA Test Criteria and F Approximations for the Hypothesis of No Days*light Effect. H = Type III SSCP Matrix for days*light. E= Error SSCP Matrix

Parameter	Statistic	Value	F Value	Num DF	Den DF	Pr>F
Growth (Optical density)	Pillai's Trace	3.48	6.68	28	28	<0.0001
Chlorophyll content	Pillai's Trace	2.98	2.92	28	28	<0.0030
Biomass productivity (Dry mass)	Pillai's Trace	3.40	5.62	28	28	<0.0001



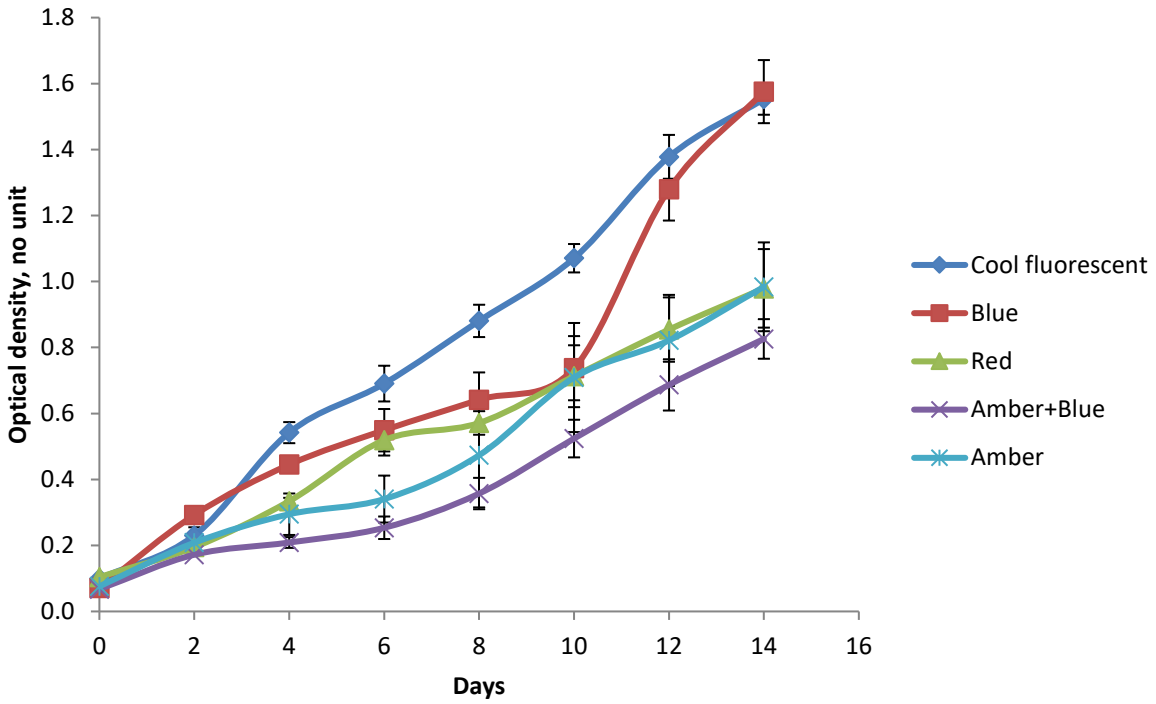


Figure 5.2 Growth curves (optical density and dry mass) for microalgal consortia under Cool fluorescent; Blue; Red; Amber+Blue; Amber. Data expressed as mean ± SD. Data is a representation of n=3.

The specific growth rate was divided into two phases namely phase I (days 0-4) and phase II (days 4-16), under different light treatments is presented in Figure 5.3. The specific growth rate of the microalgal consortia under cool-white fluorescent and blue light in the growth phase (up to 4 days) was higher (μ = 0.75 and 0.73 day^{-1}) than that of amber, amber+blue and red (0.41 day^{-1}, 0.37 day^{-1} and 0.21 day^{-1}, respectively). It is observed that in the second culture phase (up to 16 days), the growth rates of the microalgal consortia under cool, blue and amber+blue were higher (0.37 day^{-1}, 0.37 day^{-1}, 0.34 day^{-1}, respectively) when compared to the other wavelengths of light under study. The results revealed that the biomass doubling time was highly influenced by the wavelength of light under study. The biomass doubling time of the microalgal consortia was 22.2 h under cool fluorescent light followed by blue (22.8 h), amber (40.6 h), amber+blue (45.0 h) and red (79.2 h).

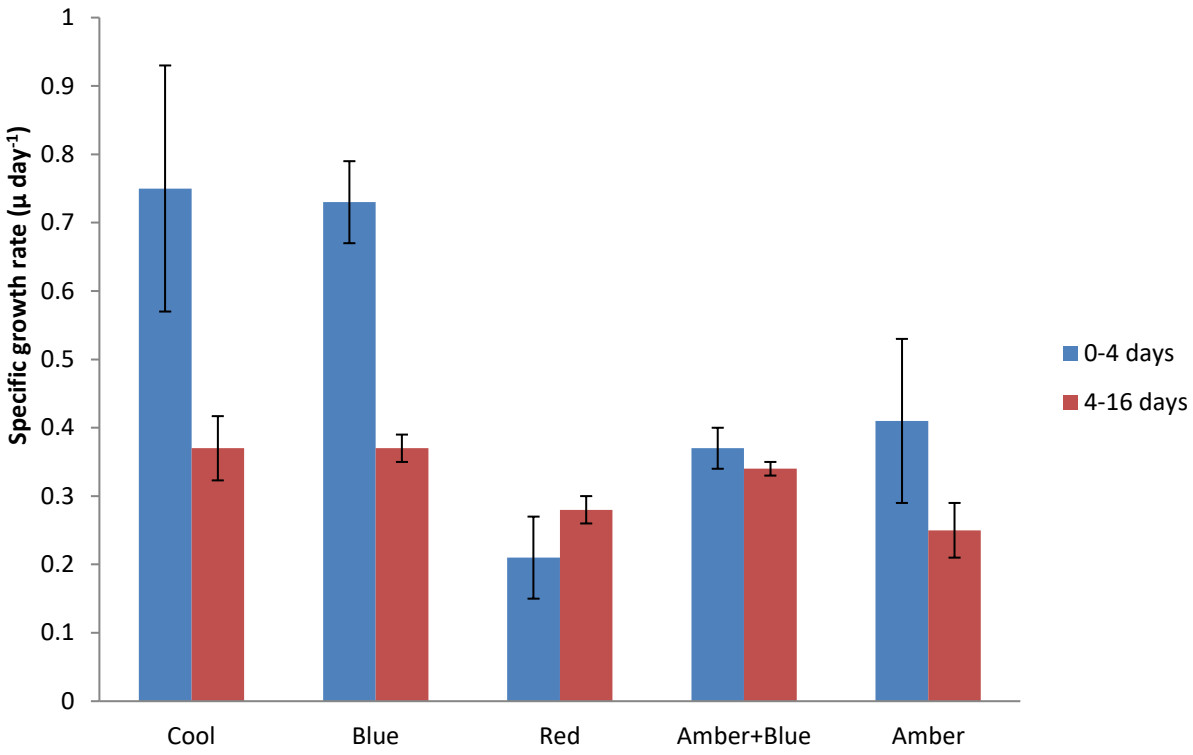


Figure 5.3 Specific growth rate of the microalgal consortia within two phases under Cool-white fluorescent; Blue; Red; Amber+Blue; and Amber lights.

5.4.2 Chlorophyll content of the microalgal consortia with respect to light treatments

An increase in chlorophyll content was observed under blue LED (12.9 mg L^{-1}), followed by cool-white fluorescent (11.3 mg L^{-1}), amber (5.6 mg L^{-1}), red (5.4 mg L^{-1}) and amber+blue (5.3 mg L^{-1}). The results thus revealed that the microalgal consortia behaved differently under different wavelengths of light as presented in Figure 5.4 and the statistical analyses for chlorophyll content seems to be significant at $p<0.05$ (Tables 5.1, 5.2 and 5.3). A positive linear correlation was observed between chlorophyll content and dry mass for each light treatment up to 14 days and is presented in Figure 5.5 with the following equations.

For Cool fluorescent; $y=52.142x + 36.079$ ($R^2 = 0.90$) (5.5)

For Blue; $y=39.176x + 36.994$ ($R^2 = 0.95$) (5.6)

For Red; y=67.316x – 47.343 (R^2 = 0.93) (5.7)

For Amber+Blue; y=65.439x + 30.816 (R^2 = 0.83) (5.8)

For Amber; y=29.379x + 25.408 (R^2 = 0.92) (5.9)

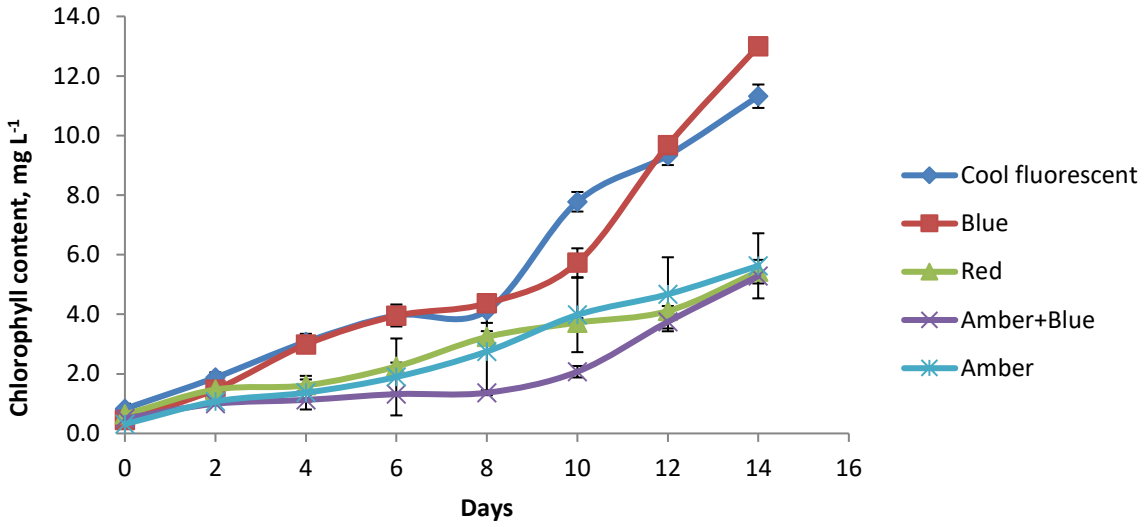


Figure 5.4 Chlorophyll content of the microalgal consortia under Cool fluorescent; Blue; Red; Amber+Blue; Amber. Data expressed as mean ± SD, n=3.

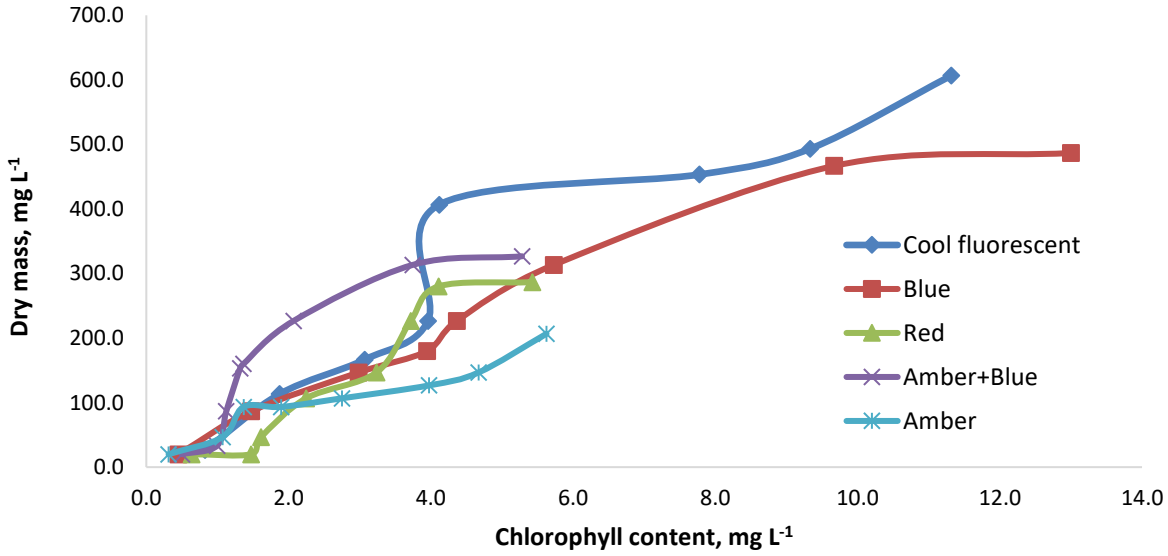


Figure 5.5 Chlorophyll content vs Dry mass of the microalgal consortia under Cool fluorescent; Blue; Red; Amber+Blue; Amber.

5.4.3 Effect of light wavelengths on the nutritional composition of the microalgal consortia

The effects of varying wavelengths of light on the composition of the microalgal biomass are presented in Figure 5.6. Cool-white fluorescent light had the best nutritional composition in term of proteins followed by amber, blue, red and amber+blue lights. To the contrary, carbohydrate composition was higher under amber light whereas the lipid composition was higher under red light. The results revealed that the protein composition of the microalgal consortia was higher under all wavelengths of light when compared to the carbohydrate and lipid compositions thereby indicating the potential use of the microalgal biomass as a protein source for animal feed and/or as an ingredient for the production of value added products.

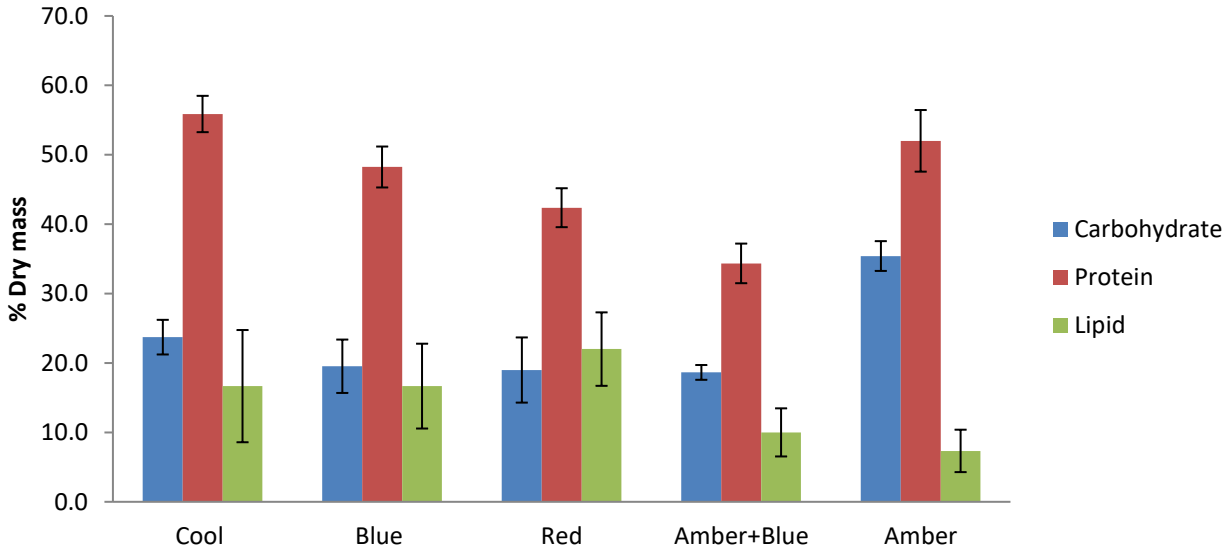


Figure 5.6 Nutritional composition of the microalgal consortia under Cool fluorescent; Blue; Red; Amber+Blue and Amber lights. Data expressed as mean ± SD, n=3

5.4.4 Economic efficiency of energy consumption related to biomass production

The economic efficiencies of different light sources were evaluated based on the cost of energy consumption and the amount of biomass produced. The equation to assess the economic efficiency of energy used to produce biomass (E_c) is as follows:

$$Ec = \frac{DMn - DMo}{k * T * P} \quad (5.5)$$

where DMn and DMo are the dry mass at day n and at day zero, k is the cost per unit of electricity consumed by the light source ($/W), T is the time (days) and P is the power consumed by the light source (W) (Wang et al., 2007). The results revealed that blue light had the highest energy to biomass efficiencies (12.5 g L^{-1} $\$^{-1}$) as compared to other light treatments and the efficiencies are presented in Figure 5.7. The amber light had the lowest energy to biomass efficiency (2.26 g L^{-1} $\$^{-1}$) and this can be attributed to the lower electrical conversion efficiency of amber light.

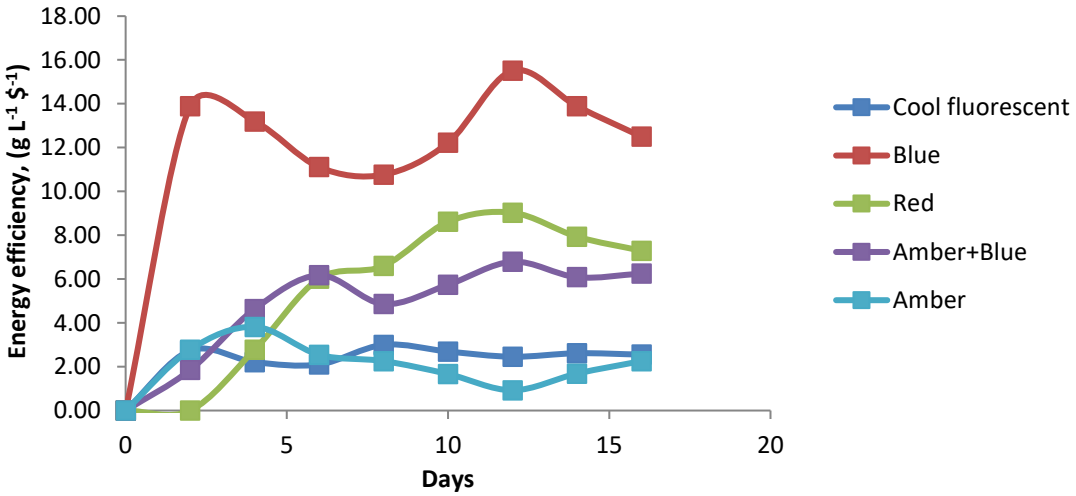


Figure 5.7 Economic efficiencies for various light sources under Cool fluorescent; Blue; Red; Amber+Blue; Amber.

5.4.5 Effect of light quality on lutein concentration

The effect of light quality on lutein concentration was significant and the results revealed that cool fluorescent light had the highest lutein content of (7.22 mg g^{-1}) followed by amber+blue (6.50 mg g^{-1}), amber (5.96 mg g^{-1}), blue (1.82 mg g^{-1}) and red (1.68 mg g^{-1}). The lutein content reported in

this study is the highest lutein concentration reported for any microalgal consortia grown under an autotrophic mode of cultivation on dairy wastewater till date. For optimum separation of a compound, the k (retention factor) value should be between 1 to 10 and in case of a complex mixtures it should be between 2 to 10. The retention factor indicates the ratio of retention time of the analyte on the column to the retention time of a non-retained compound. So, higher k value indicates that the desired compound spent significant amount of time with the stationary phase with higher retention. Since this study has only one compound eluting at a retention time of 9.6 as shown in Figure 5.8, the k value of the lutein peak obtained was calculated to be approximately 1 indicating that optimum separation of the compound was achieved and the strength of the solvent used for preparing mobile phase was acceptable (Gupta et al., 2015).

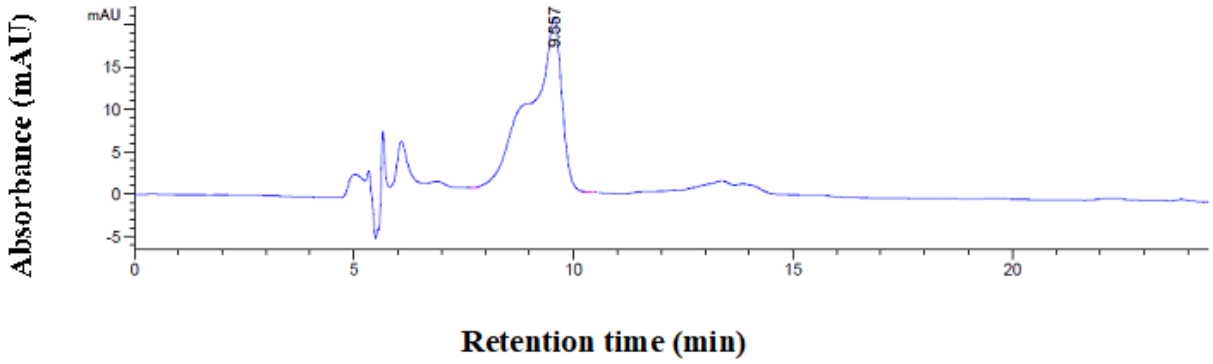


Figure 5.8 Chromatogram of the crude lutein obtained by extraction after saponification under cool fluorescent light.

5.5 Discussion

Different wavelengths of light highly influence the synthesis of chlorophyll and pigments in algae (Wu, 2016). In addition, light wavelengths along with light intensity and photoperiod are considered to be the major driving factors in enhancing the growth and biomass productivity of

algal species (Atta et al., 2013; Amini Khoeyi et al., 2012). Shorter wavelengths of light possess higher energy when compared to light of longer wavelengths under the same PAR values. This implies that blue light, with its shorter wavelength, has more energy and photosynthetic efficiency and could enhance biomass production when compared to other light wavelengths within the spectrum (Das et al., 2011).

In this study, a higher growth rate and biomass productivity were observed with cool-white fluorescent light followed by blue, amber+blue, amber and red at about 40 µmol $m^{-2}s^{-1}$. This implies that the microalgal consortia utilized cool-white fluorescent light better than any other wavelength treatments, and this can be explained that cool-white fluorescent light had a balanced mix of blue and red lights as shown in Figure 5.1. Though there are several reports of the impact of light wavelengths on the productivity and growth rate of *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Scenedesmus obliquus, Scenedesmus quadricauda, Nannochloropsis Phaeodactylum tricornutum*, *Isochrysis galbana*, *Nannochloropsis salina* and *Nannochloropsis oceanica*, no study using the consortium of algal strains *Chlorella variabilis* and *Scenedesmus obliquus* has been reported to date (Ma et al., 2018; Ra et al., 2018; Zhong et al., 2018). The results obtained in this study indicate that the microalgal consortia grown under cool-white fluorescent light can generate more biomass when compared to other wavelengths of light.

The chlorophyll content, however, was higher under blue light when compared to other wavelengths of light tested. This could be attributed to the increase in the number/size of light-harvesting units under light-limiting conditions when the consortium was exposed to blue light. The higher chlorophyll content obtained under blue light could be associated with a mechanism known as chromatic adaptation which helps the algal species to maximize their photosynthetic ability by utilizing the available light efficiently under light limiting conditions (Ahluwalia et al.,

1980). The outcome obtained from the study was in accordance with the results published by Mercado et al. (2004), wherein blue light exhibited higher chlorophyll content when compared to white light in four out of the five strains of algae with no significant effects on the growth rates. In addition, the absorption bands of chlorophyll were significantly present in the blue wavelength of light favoring photosynthesis (Wang et al., 2007). A positive correlation between chlorophyll content and dry mass shows that chlorophyll content could be used as an indicator to measure dry mass or vice versa. The outcome obtained from this study was in accordance with the results published by Canfield et al. (1985) wherein a significant correlation was observed between chlorophyll a concentration and phytoplankton biomass. The study also concluded that chlorophyll measurements could be used for estimating phytoplankton biomass as the latter is more labor-intensive and costly compared to the former.

Numerous studies have reported that the response of algae to varying light wavelengths is species dependent, or more specifically strain dependent due to the variation in their light harvesting mechanisms/membranes. As a result, manipulating light wavelengths can alter the biochemical composition of algae (Vadiveloo et al., 2015). Based on the results obtained here, it can be seen that the protein content of the algal consortia was higher under all wavelengths of light with white light exhibiting the highest concentration. This increase in protein content could be attributed to the enhancement of the structural protein of PSII (has not been demonstrated conclusively) and the amount of nitrogen present in the wastewater. Availability of nitrogen, in excess, results in higher protein content (Safafar et al., 2016; Marchetti et al., 2013). The lipid content was enhanced under red light and this could be explained by the increase in enzyme activity of carbonic anhydrase, Rubisco and respiratory enzymes. The accumulation of triglycerides is highly dependent on the activity of enzymes i.e., the higher the enzyme activity, the higher the

amount of triglycerides produced and vice versa. The carbohydrate content was enhanced under the amber wavelength of light and this could signify a decrease in enzyme activity as the concentration and activity of enzymes is directly proportional to the rate of breakdown of carbohydrates. In this study, the microalgal consortia grown under all the other light wavelengths (red, amber+blue, blue and cool fluorescent), except amber, had a reduction in carbohydrate content owing to the increase in enzyme activity (more catabolism of carbohydrates) (Vadiveloo et al., 2015; Atta et al., 2013)].

Taking into consideration the amount of biomass produced per unit of electricity consumed, it can be clearly seen that blue light had a biomass production (500 mg L^{-1}) with higher economic efficiency of 12.5 g L^{-1} $\$^{-1}$ when compared to the other light treatments after 16 days of inoculation. Though cool fluorescent light had the highest biomass productivity, it was found to have the least economic efficiency owing to the needed increased electricity consumption to produce the required intensity of light (Wang et al., 2007).

Some microalgae have been known in recent years for producing interesting quantities of lutein. For instance, the optimal conditions for producing lutein from *Scenedesmus almeriensis* has been studied and was considered to produce much higher lutein content (~5 mg g^{-1}) than the traditional source from marigold flowers (~1 mg g^{-1}), thereby making microalgae a potential source for the extraction of lutein (Sánchez et al., 2008). Similarly, another strain of microalga *Chlorella vulgaris* produced approximately 0.7 mg $g^{-1}$of lutein (D'Este et al., 2017). In the current study, it was revealed that the microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* had a maximal lutein content of 7.22 mg g^{-1} as compared to around 5 mg g^{-1} for *Scenedesmus almeriensis* and 0.7 mg g^{-1} for *Chlorella variabilis*, thereby making it a promising consortia for

biotechnological purposes. Finally, based on our results, tailoring light quality can significantly alter the biomass productivity and biochemical composition including lutein content.

5.6 Conclusion

Light wavelengths have a profound impact on the growth, biomass productivity, chlorophyll/lutein and nutrient contents of the microalgal consortia composed of *Chlorella variabilis* and *Scenedesmus obliquus* and the data obtained from this study supports this claim. This work clearly states that the microalgal consortia could grow well on diluted (10%) dairy effluent and the most effective light for enhancing the growth and biomass productivity is white light (380-700 nm). However, blue light had the highest energy to biomass conversion (highest economic efficiency) as compared to white light which could mean that blue light can also serve as a light source for enhancing growth rate and biomass productivity. The most effective light for enhancing chlorophyll content is blue light (470 nm) with no significant effect on growth rate, while for optimized nutrient content, amber light (595 nm) is the best. Thus, a two-phase culture process of cultivating the microalgal consortia under cool-white fluorescent light, followed by exposure to amber light could maximize the benefits in terms of enhancing biomass productivity and nutrient content with higher lutein content within sixteen days of growth. The biomass produced could be used as animal feed for its high protein (30-80% of protein) as well as for bio-ethanol production due to its high carbohydrate content. Furthermore, this study opens up a new avenue of research for the targeted cultivation of algae for nutraceutical production (lutein) under amber light wavelength (500- 630 nm) which has long been neglected by researchers.

CONNECTING TEXT

Though many environmental factors influence microalgal growth, light and temperature are considered to be the most crucial ones. In particular, studies have revealed that the impact of light on microalgal growth can be detrimental to the growth of microalgae. In the previous study, the impact of light quality on microalgal growth, biomass productivity, nutritional and lutein content was evaluated. The results indicated that the microalgal consortia cultivated on simulated dairy wastewater could follow a two-phase culture process and utilize cool fluorescent light followed by amber light for maximizing their growth, biomass productivity, nutritional and lutein contents. In addition, evaluating the efficiency of amber light for microalgal consortia cultivation of *Chlorella variabilis* and *Scenedesmus obliquus* has been carried out for the first time in this study. In particular, the previous chapter focused on improving the biomass productivity of the microalgal consortia with respect to light quality. Chapter VI deals with the optimization of process parameters to predict the bioremediation potential as well as the optimal biomass productivity and lutein content for the microalgal consortia grown on simulated dairy wastewater.

CHAPTER VI

PHYCOREMEDIATION AND VALORIZATION OF SYNTHETIC DAIRY WASTEWATER USING THE MICROALGAL CONSORTIA OF *CHLORELLA VARIABILIS* AND *SCENEDESMUS OBLIQUUS*

6.1 Abstract

Microalgae are known to grow on wastewater utilizing their available nutrients. The residual algal biomass thus obtained could be used for producing value-added products thereby making it an economically viable and sustainable option for the dairy industry. The present study evaluates the ability of the microalgal consortia composed of *Chlorella variabilis* and *Scenedesmus obliquus* to treat and valorize diluted synthetic dairy wastewater under controlled laboratory conditions. The effect of time, inoculum concentration and light intensity on five responses, namely phosphate removal, ammoniacal nitrogen removal, COD reduction, biomass productivity and lutein content, are studied by response surface methodology utilizing central composite design. The quadratic models are found to be suitable for phosphate removal, ammoniacal nitrogen removal, COD reduction and biomass productivity. At optimized experimental conditions, the microalgal consortia exhibited phosphate removal of 70.2%, ammoniacal nitrogen removal of 86.2%, COD reduction of 54.7%, biomass productivity of 29.13 mg L^{-1} day^{-1} and lutein content of 12.59 mg g^{-1} respectively. This study is of high importance as the lutein content exhibited by the microalgal consortia is higher when compared to other reported microalgal species and could be considered in the future as a commercial source of lutein.

6.2 Introduction

The consumption of dairy products by humans has been on the rise over the past few years. According to the Food and Agricultural Organization (FAO), it is predicted that by 2023, the global consumption of dairy products is expected to increase by 13.7% (Choi, 2016). The dairy industry utilizes large quantities of water in all the cleaning steps throughout the process line. The wastewater thus generated is composed of phosphates, ammoniacal nitrogen, biological oxygen demand (BOD), chemical oxygen demand (COD), fats, oils and either suspended solids or dissolved solids (Sarkar et al., 2006). With the composition of the wastewater, treatment is necessary prior to disposal and has become a requirement as it poses a serious threat to the environment and public health (Trevor et al., 2006).

Dairy wastewater is generally treated using physical, chemical or biological methods based on the composition of the effluent. Biological methods are given high importance due to their inexpensiveness and eco-friendly nature when compared to other modes of treatment. Recently, microalgae have received great attention because of their ability to effectively treat waste generated from agriculture, livestock, household and food processing industries by removing their organic and inorganic compounds (Delrue et al., 2016; Abdel-Raouf et al., 2012b). The culturing of algae on wastewaters provides multifaceted benefits of treating wastewater while producing residual algal biomass which can be exploited further for producing value added products, biofuels and biofertilizer (Yadavalli et al., 2013). Furthermore, microalgal treatment of wastewater using high-rate algal ponds was able to reduce 660 kg of carbon dioxide per million liters of wastewater in contrast to activated sludge facility which produced 550 kg of carbon dioxide per million liters (De Francisci et al., 2018; Woertz et al., 2009).

Microalgal growth depends on various environmental factors and is strain specific. In particular, light and temperature are considered to be the primary factors for achieving the maximum growth rate of microalgae. Selection of algal strains is pivotal for achieving desired outputs. A study on *Chlorella vulgaris* and *Pseudomonas putida* showed that the elements and organic matter removal (phosphate, ammonium and COD) from municipal wastewater was enhanced while using a co-culture consortium as compared to the axenic culture of *Chlorella variabilis* or *Pseudomonas putida* independently (Mujtaba et al., 2017). Another study on mixed culture of two *Scenedesmus* species showed that 60% microalgal consortium concentration was optimum for maximum removal of pollutants (phosphates, nitrogen, BOD and COD) (Usha et al., 2016). Utilizing co-cultures for wastewater treatment could prevent possible culture crashes which may occur while using monocultures. Maintaining monocultures has proved to be expensive as well as labor intensive. Besides, algal consortia are resilient to adverse conditions and can resist invasion from other species better when compared to monocultures (Padmaperuma et al., 2018).

According to a review by Delrue et al. 2016, *Chlorella* sp. and *Scenedesmus* sp. have been widely used for treating a majority of wastewater streams effectively. Previous studies have shown that these microalgal consortia can treat dairy wastewater while enhancing lipid production (Hena et al., 2015; Wang et al., 2010; Ding et al., 2015; Ummalyma et al., 2014). To the best of our knowledge, there have been no previous reports about culturing microalgal consortia specifically *Chlorella variabilis* and *Scenedesmus obliquus* for treating diluted dairy wastewater while enhancing biomass and lutein production. Furthermore, optimization of the culture conditions is required for economical biomass and lutein production. Response surface methodology (RSM) using a central composite design (CCD) is one such tool that can help in optimizing the response

parameters using minimal experimental runs with varying dependent and independent variables (Prasad et al., 2018).

The current study aims to develop an eco-friendly and economical approach for treating dairy wastewater using a microalgal consortia. The experiments were carried out indoor using an artificial lighting system in order to minimize the variabilities of outdoor conditions in terms of light, temperature and photoperiod. The optimizing of biomass productivity and lutein content with concurrent removal of phosphate, ammoniacal nitrogen and chemical oxygen demand (COD) by varying time, inoculum concentration and light intensity was performed. In addition, the effects of operating conditions such as inoculum concentration, time and light intensity on biomass productivity and lutein content as well as phosphate, ammoniacal nitrogen and COD removal have been explored using RSM.

6.3 Materials and Methods

6.3.1 Experimental organisms and culture conditions

Axenic strains of microalgae *Chlorella variabilis* (CCAP 211/84) and *Scenedesmus obliquus* (CCAP 276/3C) were procured from the Culture Collection of Algae and Protozoa (CCAP). The two algal strains were cultured in MN8 and BG11 media respectively under sterile conditions. The MN8 medium contained 1 g L^{-1} KNO_3, 0.74 g L^{-1} K_2HPO_4, 0.075 g L^{-1} $MgSO_4.7H_2O$, 0.013 g L^{-1} $CaCl_2.2H_2O$, 0.26 g L^{-1} $NaH_2PO_4.H_2O$, 0.010 g L^{-1} FeEDTA, 1 g L^{-1} yeast extract, 12.98 g L^{-1} $MnCl_2.4H_2O$, 3.2 g L^{-1} $ZnSO_4.7H_2O$, 1.83 g L^{-1} $CuSO_4.5H_2O$ and 3.58 g L^{-1} $KAl(SO_4)_2$ (Cheng et al., 2015). Similarly, BG11 medium contained 1.5 g L^{-1} $NaNO_3$, 0.04 g L^{-1} K_2HPO_4, 0.075 g L^{-1} $MgSO_4.7H_2O$, 0.036 g L^{-1} $CaCl_2.2H_2O$, 0.036 g L^{-1} citric acid, 0.006 g L^{-1} $C_6H_8FeNO_7$, 0.001 g L^{-1} $EDTANa_2$, 0.02 g L^{-1} Na_2CO_3, 2.86 g L^{-1} boric acid, 1.81 g L^{-1} $MnCl_2.4H_2O$, 0.22 g L^{-1}

$ZnSO_4.7H_2O$, 0.039 g L^{-1} Na_2MoO_4, 0.079 g L^{-1} $CuSO_4.5H_2O$ and 0.049 g L^{-1} $Co(NO_3)_2.6H_2O$ (Rippka et al., 1979). The cultures were grown autotropically in 125 mL Erlenmeyer flasks with a working volume of 50 mL under batch conditions and placed on an orbital shaker agitated at 90 rpm, light intensity of 40 ± 3 µmol $m^{-2}s^{-1}$ and temperature of 24 ± 2 °C with no control over pH. The artificial lighting to the flasks was provided using cool-white fluorescent light (4200 K, F72T8CW, Osram Sylvania, MA, US).

6.3.2 Composition of synthetic dairy wastewater

The synthetic dairy wastewater was prepared using 2 g L^{-1} dried milk powder, 2.8 g L^{-1} NH_4Cl, 0.1 g L^{-1} $MgSO_4.7H_2O$, 0.076 g L^{-1} $CaCl_2.H_2O$, 2 g L^{-1} KH_2PO_4 and 4 g L^{-1} $NaHCO_3$ (Modified from Vidal et al., 2000). Due to high turbidity, the dairy wastewater was diluted 10 times with distilled water and the final concentration of wastewater used was at 10%. The dilution rate was determined based on a feasibility study which experimented with two different concentrations (25% and 10%) including undiluted dairy wastewater (Results not reported here). The dairy wastewater was not sterilized prior to cultivation of the microalgal consortia.

6.3.3 Experimental setup

The experiments were carried out in 500 mL Erlenmeyer flasks with a working volume of 200 mL. The microalgal consortia were cultured into flasks containing dairy wastewater and placed in a completely randomized fashion on an orbital shaker. The cool-white fluorescent light was installed at 0.5 m on a wooden board placed above the orbital shaker. The flasks were then exposed to varying light intensities of 40 µmol $m^{-2}s^{-1}$, 25 µmol $m^{-2}s^{-1}$ and 10 µmol $m^{-2}s^{-1}$ respectively. The

light intensity was measured using a spectroradiometer (PS-300, Apogee, Logan, UT) and the temperature was continuously monitored using a temperature sensor (Raytek Minitemp, MT6).

6.3.4 Estimation of microalgal growth, nutrient removal rate and biomass productivity

The growth of the microalgal consortia was measured as optical density using a UV spectrophotometer (Ultrospec 2100 pro) at 680 nm (Koreivienė et al., 2014). The biomass productivity was determined by filtering 5 mL of the culture through a Whatmann filter paper and placing it in an oven at 80 ℃ for 24 h. This was followed by cooling in a desiccator to achieve a constant mass (Qin et al., 2016). Biomass productivity (P, mg L^{-1} day^{-1}) was calculated using the Equation 6.1 given below:

$$\text{Biomass productivity (P)} = \frac{(X2-X1)}{t} \quad (6.1)$$

where X_1 and X_2 are defined as the initial and final dry biomass (mg L^{-1}) at time (day) t respectively.

Nutrient removal rates (S) were calculated using the Equation 6.2 given below:

$$S = \frac{(S0-S1)}{S0} \quad (6.2)$$

where S_0 and S_1 are the initial and final nutrient concentrations of phosphates, ammoniacal nitrogen and COD respectively (Ding et al., 2015).

6.3.5 Nutrient analysis and determination of lutein content

The treated samples were centrifuged to separate the microalgal biomass and the supernatant was used for phosphorus, ammoniacal nitrogen and COD analysis. Phosphates were analyzed by colorimetric method and ammoniacal nitrogen determination was conducted by the phenate method analyses following procedures put forward by the American Public Health Association

(APHA) (APHA, 2002). COD was determined by using the Environmental Protection Agency (EPA) approved kits (Accu-TEST COD system) obtained from Bioscience ranging from 20-4500 mg L^{-1}.

For phosphates, 7 mL of the sample was taken and 2 mL of vandate-molybdate reagent was added. This was followed by diluting the solution to 10 mL with water. The obtained sample was then subjected to spectrophotometric analysis at 470 nm. Similarly, ammoniacal nitrogen was determined by taking 5 mL of the sample into an Erlenmeyer flask. To this solution, 0.2 mL of phenol, 0.2 mL of sodium nitroprusside and 0.5 mL of oxidizing solution were added and swirled. The resultant solution was left aside for an hour and subjected to spectrophotometric analysis at 640 nm. For COD analysis, either 2.5 mL or 0.5 mL of sample was added to the reagents in the vial depending on the range of COD kit utilized (20-900 mg L^{-1}or 100-4500 mg L^{-1}). The prepared vials were then placed in a COD heater block preheated to 130 ℃ for 2 h after shaking thoroughly. The vials were then removed and allowed to cool for 30 min. The absorbance of the sample was measured at 600 nm using a UV-spectrophotometer. The amount of phosphates, ammoniacal nitrogen and COD present in the sample were obtained by comparison with their respective standard calibration curves.

The obtained residual biomass, after centrifugation, was freeze-dried and used for determination of lutein content using high performance liquid chromatography (HPLC). Lutein was extracted from the algal consortia using a method described by Inbaraj et al. (2006). Briefly, 2 to 10 mg of the freeze-dried algal biomass was taken and treated with 3 mL of hexane-ethanol-acetone-toluene (10:6:7:7) in a 10 mL volumetric flask and shaken for 1 h. To the resulting solution, 2 mL of 40% methanolic KOH was added for saponification at 25 ℃ in the dark for 16 h. This was followed by addition of 3 mL of hexane for partitioning and shaken for 1 min. A 10%

sodium sulfate solution was added and diluted to volume (10 mL). The resulting mixture was allowed to stand until the two phases separated clearly. The upper layer containing lutein was collected and evaporated to dryness. Finally, the extract was re-dissolved in 1 mL methanol-methylene chloride (50:50, v/v), and filtered through 0.2 µm membrane filter for HPLC analysis.

6.3.6 HPLC analysis and peak identification

The Agilent HPLC system was made up of a quaternary pump with a degasser and a variable wavelength detector (VWD) with Agilent chromatography software (ChemStation version 38). A Discovery C18 column (250 mm × 4.6 mm I.D., 5 µm) was used as the stationary phase and methanol-acetonitrile-water (84:14:2) was used as mobile phase (A) with 100% methylene chloride used as mobile phase (B). The flow rate was set at 0.6 mL/min with a gradient solvent system. The injection volume was 20 µL and the response of the peak was detected at 450 nm. The amount of lutein present in the sample was calculated using the following Equation 6.3 (D'Este et al., 2017):

$$\text{Lutein quantity (mg g}^{-1}) = \frac{\text{Lutein concentration}\left(\frac{\text{mg}}{\text{L}}\right) * \text{Volume of solvent(L)}}{\text{Dry mass(g)}} \quad (6.3)$$

Commercial lutein obtained from Sigma Aldrich was used as the analytical standard and a standard curve was prepared by plotting varying concentrations against peak area. Regression analysis was performed using Microsoft Excel and a correlation coefficient of 0.99 was obtained.

6.3.7 Optimization of process parameters

Optimization of process parameters was performed using JMP version 14.1. A CCD model for three factors (light intensity, time and inoculum concentration) with three levels each was used to determine the experimental conditions. The levels set for the factors were as follows: light intensity

(40 µmol $m^{-2}s^{-1}$, 25 µmol $m^{-2}s^{-1}$, 10 µmol $m^{-2}s^{-1}$), time (6, 10, 14 days), inoculum concentration (20%, 40%, 60%). The experimental runs were performed under different conditions as outlined in Table 6.1 and the optimum conditions for lutein content, biomass productivity as well as phosphate removal, ammoniacal nitrogen removal and COD removal were determined through regression analysis using JMP software. A total of 18 experimental runs were carried out with 4 experimental runs repeated at the center point of the experimental design.

Table 6.1 CCD for light intensity, inoculum concentration and time.

Experimental run	Factor 1 A: Light intensity (µmol $m^{-2}s^{-1}$)	Factor 2 B: Inoculum concentration (%)	Factor 3 C: Time (days)
1	40	20	6
2	40	60	6
3	40	40	10
4	40	20	14
5	40	60	14
6	10	20	6
7	10	60	6
8	10	40	10
9	10	20	14
10	10	60	14
11	25	40	6
12	25	20	10
13	25	40	10
14	25	40	10
15	25	40	10
16	25	40	10
17	25	60	10
18	25	40	14

6.4 Results and Discussion

6.4.1 Effect of process parameters and model development

The effect of light intensity, inoculum concentration and time on pollutant removal, biomass productivity and lutein content were studied using CCD and the actual and predicted values are presented in Table 6.2. The quadratic regression model was developed to evaluate the interaction effect of process parameters on response variables and is described below. The characteristics of synthetic dairy wastewater before and after treatment are presented in Table 6.4.

6.4.2 Effect of process variables on phosphate removal and model development

Phosphate removal with respect to light intensity and inoculum after 10 days of consortia growth is shown in Figure 6.1 (a). Orthophosphate is the predominant source of phosphorus for cultivation of microalgae and is responsible for the development of the cell membrane, polyunsaturated fatty acids, nucleic acids and transfer of energy in the cell (Chen and Chen, 2006). Microalgae remove phosphorus from wastewater through various mechanisms such as assimilation, precipitation or adsorption (Craggs et al., 1996). For maximum removal of phosphates in a shorter period of time, a greater concentration of inoculum is required. Inoculum concentration is known to affect the biomass productivity as well as the metabolite production of microalgae. Ammonia inhibition is higher at lower inoculum concentrations while at higher inoculum concentrations there is an inhibition of cell reproduction due to limited nutrients and light. Hence, optimum inoculum concentration is required for achieving higher biomass productivity and pollutant removal (Li et al., 2017). Figure 6.1 (a) shows that phosphate removal is maximum at 20% inoculum concentration when compared to 40% and 60% at a light intensity of 25 µmol $m^{-2}s^{-1}$ following a 10 days process (Figure 6.1 c). The effect of light intensity and time on phosphate removal is

shown in Figure 6.1 (b). Maximum phosphate removal of 76.77% is observed at 25 µmol $m^{-2}s^{-1}$ light intensity and a process time of 10 days. A study by Prasad et al. (2018) reported that phosphate removal was maximum at 6% inoculum concentration when compared to 3% and 4.5% and varies with respect to days of inoculum growth. These results are similar to the present study, where phosphate removal was maximum at an inoculum concentration of 20% at 10 days of process.

ANOVA results for phosphate removal are shown in Table 6.3. The quadratic regression model for phosphate removal is represented in the form of equation 6.4. The model is significant and this is supported by an *F*-value of 73.06. The R^2 and the adjusted R^2 values for phosphate removal are 0.988 and 0.974 respectively. Higher R^2 value and lower *p*-value of <0.0001 show that the model predicted successfully the responses and is significant. The predicted values for phosphate removal are obtained from the quadratic model equation presented in Equation 6.4. Figure 6.1 (d) shows the plot between predicted and actual values with the data points spread homogenously on either side of the zero line for the response on phosphate removal. The Lack of Fit (Prob>*F* = 0.2175) for phosphate removal presented in Table 6.3 suggested that the fitted model is adequate. All the main effects of inoculum concentration and days of process, except light intensity, are statistically significant at <0.0001 and 0.0458 respectively.

$$\text{Phosphate removal} = 70.194 + 0.225 \times A - 8.982 \times B + 0.918 \times C + 0.001 \times AB - 0.201 \times AC - 0.241 \times BC - 6.315 \times A^2 - 0.750 \times B^2 + 0.860 \times C^2 \quad (6.4)$$

Table 6.2 Actual and predicted data of biomass productivity, phosphate removal, ammoniacal nitrogen removal, COD removal and lutein content for CCD.

	Biomass productivity (mg L^{-1} day^{-1})			Phosphate removal (%)			Ammoniacal nitrogen (%)			COD removal (%)			Lutein content (mg g^{-1})		
Exp. run	**Actual value**	**Predicted value**	**Error (%)**	**Actual value**	**Predicted value**	**Error (%)**	**Actual value**	**Predicted value**	**Error (%)**	**Actual value**	**Predicted Value**	**Error (%)**	**Actual value**	**Predicted value**	**Error (%)**
1	56.67	47.00	17.06	73.06	72.24	1.12	91.67	90.36	1.43	57.26	63.21	-10.39	9.34	8.85	5.22
2	46.67	58.52	-25.39	54.52	54.76	-0.44	82.71	83.86	-1.39	43.02	40.43	6.02	6.09	5.32	12.65
3	68.00	59.53	12.46	64.03	64.1	-0.11	87.96	86.51	1.65	28.77	17.78	38.20	4.41	5.66	-28.22
4	30.00	43.10	-43.67	74.19	74.15	0.05	91.11	93.65	-2.79	71.51	68.92	3.62	9.54	9.37	1.83
5	81.43	74.61	8.38	55.16	55.71	-1.00	92.53	91.60	1.01	14.53	24.75	-70.34	6.35	6.53	-2.87
6	13.33	21.71	-62.87	71.94	71.39	0.76	79.82	81.37	-1.94	85.75	76.03	11.34	6.91	6.98	-0.95
7	43.33	31.80	26.61	53.87	53.9	-0.06	86.21	84.30	2.22	43.02	46.12	-7.21	7.93	8.36	-5.45
8	28.00	30.20	-7.86	63.71	63.65	0.09	82.72	81.67	1.27	14.53	23.48	-61.60	8.77	6.49	25.96
9	21.43	11.15	47.97	74.35	74.11	0.32	84.06	83.54	0.62	71.51	74.61	-4.34	6.96	7.99	-14.85
10	30.00	41.23	-37.43	54.84	55.66	-1.50	88.98	90.91	-2.17	28.77	23.33	18.91	9.31	10.05	-7.92
11	33.33	34.29	-2.88	69.03	70.14	-1.61	86.94	87.46	-0.60	71.51	74.77	-4.56	12.59	13.35	-6.04
12	10.00	8.47	15.30	76.77	78.43	-2.16	87.90	85.64	2.57	85.75	89.01	-3.80	13.96	13.51	3.24
13	24.00	29.13	-21.38	70.65	70.19	0.65	85.92	86.22	-0.35	43.02	54.72	-27.20	12.29	12.59	-2.46
14	20.00	29.13	-45.65	70.97	70.19	1.10	82.97	86.22	-3.92	57.26	54.72	4.44	11.54	12.59	-9.11
15	30.00	29.13	2.90	69.03	70.19	-1.68	85.00	86.22	-0.29	57.26	54.72	4.44	12.59	12.59	0.00
16	30.00	29.13	2.90	70.16	70.19	-0.04	85.97	86.22	-0.29	57.26	54.72	4.44	11.91	12.59	-5.72
17	34.00	29.27	13.91	62.10	60.46	2.64	86.31	86.07	0.28	57.26	51.97	9.24	13.35	12.78	4.29
18	44.29	37.06	16.32	73.06	71.97	1.49	95.43	92.41	3.16	71.51	66.22	7.40	16.23	14.45	10.98

Table 6.3 ANOVA result for phosphate removal, ammoniacal nitrogen removal, phosphate removal, biomass productivity and lutein content.

Source	Sum of squares	df	Mean square	F-value	p-value	Remark
Phosphate removal						
Model	992.95	9	110.328	73.06	<.0001	Significant
Lack of fit	9.91	5	1.982	2.75	0.2175	Not significant
R^2=0.988, Adj R^2=0.974						
Ammoniacal nitrogen removal						
Model	215.95	9	23.99	3.81	0.0364	Significant
Lack of fit	44.46	5	8.89	4.52	0.1222	Not significant
R^2=0.811, Adj R^2=0.598						
COD reduction						
Model	7306.59	9	811.84	8.89	0.0026	Significant
Lack of fit	578.86	5	115.77	2.28	0.2642	Not significant
R^2=0.909, Adj R^2=0.807						
Biomass productivity						
Model	4619.29	9	513.25	3.56	0.0438	Significant
Lack of fit	1080.80	5	216.16	9.01	0.0501	Not significant
R^2=0.800, Adj R^2=0.576						
Lutein content						
Model	160.58	9	17.84	9.28	0.0023	Significant
Lack of fit	14.76	5	2.95	14.18	0.0268	Significant
R^2=0.913, Adj R^2=0.814						

Note: df=degree of freedom

Table 6.4 Characteristics of synthetic dairy wastewater before and after treatment

Parameter	Units	Synthetic dairy wastewater (undiluted)	Synthetic dairy wastewater (after dilution)	Synthetic dairy wastewater after microalgal treatment at 10 days, 25 µmol $m^{-2}s^{-1}$, 40%
NH3-N	mg L^{-1}	295	30	4
TP	mg L^{-1}	500	50	15
COD	mg L^{-1}	1170	117	50

6.4.3 Effect of process variables on ammoniacal nitrogen removal and model development

The ammoniacal nitrogen removal, with respect to light intensity and inoculum concentration for a 10 days process is shown in Figure 6.2 (a). Nitrogen is considered to be an essential element, after carbon, for cell growth and is utilized in various metabolic activities. The amount of nitrogen present in a typical microalga is around 10% (Prasad et al., 2018). Assimilation of nitrogen by microalgae is more rapid than phosphorus and takes place in the presence of light in the chloroplast. Maximum removal of nitrogen (95.43%) was observed at light intensity of 25 µmol $m^{-2}s^{-1}$, inoculum concentration of 40% and process time of 14 days (Table 6.2). Increasing inoculum concentration and time shows maximum nitrogen removal as presented in Figure 6.2 (c). These results are similar to a study put forward by Prasad et al. (2018), where maximum nitrate removal was observed at 6% inoculum concentration and a process time of 27 days. Another study by Lau et al. (1995) on high, medium and low concentration algal cultures showed that the nitrogen removal was higher in concentrated cultures during the first 6 days and this was attributed to the large cell numbers. However, the removal efficiency of the concentrated culture decreased after day 6 onwards to 89.7% as compared to the medium concentration (98%) which supports the

results put forward in this study where ammoniacal nitrogen removal was maximum at a medium concentration of 40% as compared to 20% and 60%.

The ANOVA results for nitrogen removal are presented in Table 6.3. The quadratic regression model for nitrogen removal is presented in Equation 6.5. The model is significant and this is supported by the *F*-value of 3.81. The R^2 and the adjusted R^2 values for nitrogen removal are 0.811 and 0.598 respectively. Higher R^2 value and lower *p*-value of 0.0364 show that the model successfully predicted responses and is significant. The predicted values for nitrogen removal are obtained from the quadratic model in Equation 6.5. Figure 6.2 (d) shows the plot between predicted and actual values with the data points spread homogenously on either side of the zero line for responses on nitrogen removal. The Lack of Fit (Prob>*F* = 0.1222) for nitrogen removal in Table 6.3. suggested that the fitted model is adequate. All the main effects including light intensity and process days, except for inoculum concentrations, are statistically significant at <0.0158 and 0.0142 respectively.

$$\text{Nitrogen removal} = 86.215 + 2.419 \times A + 0.218 \times B + 2.476 \times C - 2.357 \times AB + 0.281 \times AC + 1.114 \times BC - 2.125 \times A^2 - 0.360 \times B^2 + 3.720 \times C^2 \quad (6.5)$$

6.4.4 Effect of process variables on COD removal and model development

The variations in COD content with respect to inoculum concentration, light intensity and time are shown in Figure 6.3 (a-c). The response surfaces show that maximum COD removal of 85.75% was achieved when the inoculum concentration was at 40%, light intensity at 25 µmol $m^{-2}s^{-1}$ and process time of 10 days. The COD removal increased as inoculum concentration, light intensity and time increased up to an optimum and on further increase of the factors the COD removal rate reduced. A study on the mixed culture of *Chlorella vulgaris* showed that 88% COD removal

occurred for an initial COD concentration of 250 mg L^{-1} at 8 days and is similar to the removal rate in this study (Travieso et al., 2006). Another study on *Chlorella kessleri* and *Chlorella protothecoide* showed that the COD removal rate was maximum at 30 µmol $m^{-2}s^{-1}$ light intensity and is almost similar to the results observed in this study (Li et al., 2012c). COD is considered to be an indirect measure of the carbon content in wastewater. The COD removal suggests that the microalgal consortia is tolerant to COD and that the consortia utilized organic carbon as their energy source for cell growth instead of carbon dioxide (Ding et al., 2015).

The ANOVA results for COD removal are presented in Table 6.3. The quadratic regression model for COD removal is presented in Equation 6.6. The model is significant and this is supported by the *F*-value of 8.89. The R^2 and the adjusted R^2 values for COD removal are 0.909 and 0.807 respectively. Higher R^2 value and lower *p*-value of 0.0026 show that the model successfully predicted responses and is significant. The predicted values for COD removal are obtained from the quadratic model equation. Figure 6.3 (d) shows the plot between predicted and actual values with the data points spread homogenously on either side of the zero line for response on COD removal. The Lack of Fit (Prob>F = 0.2642) for COD removal presented in Table 6.3 suggested that the fitted model is adequate. The main effect (inoculum concentration) is statistically significant at 0.0003.

$$\text{COD removal} = 54.718 - 2.849 \times A - 18.518 \times B - 4.273 \times C + 1.781 \times AB + 1.781 \times AC - 5.343 \times BC - 34.056 \times A^2 + 15.769 \times B^2 + 15.774 \times C^2 \quad (6.6)$$

6.4.5 Effect of process variables on biomass productivity and model development

The response surfaces for biomass productivity with respect to inoculum concentration, light intensity and process time are shown in Figure 6.4 (a-c). The biomass productivity increases with

an increase in inoculum concentration and light intensity as observed in Figure 6.4 (a). The biomass productivity is seen to increase with an increase in inoculum concentration and time as observed in Figure 6.4 (b). Microalgal cells require time to adapt themselves to the given conditions of light intensity. Light intensity greatly influences microalgal photosynthesis, cell composition and metabolic pathways. A study on the microalgal polyculture showed that the light intensity had a significant effect on biomass productivity when it increased from 20 – 100 µmol $m^{-2}s^{-1}$ as compared to nutrient concentrations (Isaimone et al., 2018). Another study on the microalgal species of *Scenedesmus* showed that biomass productivity increased as the light intensity increased from 27 µmol $m^{-2}s^{-1}$ to 67.5 µmol $m^{-2}s^{-1}$ (Difusa et al., 2015). The results observed in this study are similar to the previously reported studies wherein the biomass productivity of the microalgal consortia increased with an increase in light intensity from 10 µmol $m^{-2}s^{-1}$ to 40 µmol $m^{-2}s^{-1}$. The variation of biomass productivity with respect to inoculum concentration and process days is presented in Figure 6.4 (c). Higher inoculum concentrations resulted in higher cell division and varied as time progressed. A study on *Desmodesmus abundans* showed that the biomass productivity increased as the inoculum concentration increased from 3% to 6% and attained a maximum at 27 days (Prasad et al., 2018). Similar results were obtained in this study where the biomass productivity increased from 20% to 60% and attained a maximum after 14 days of process.

The ANOVA results for biomass productivity removal are presented in Table 6.3. The quadratic regression model for biomass productivity is presented in Equation 6.7. The model is significant and this is supported by the *F*-value of 3.56. The R^2 and the adjusted R^2 values for biomass productivity are 0.800 and 0.576 respectively. Higher R^2 value and lower *p*-value of 0.0438 shows that the model successfully predicted responses and is significant. The predicted

values for biomass productivity are obtained from the quadratic model equation. Figure 6.4 (d) shows the plot between predicted and actual values with the data points spread homogenously on either side of the zero line for responses on biomass productivity. The Lack of Fit (Prob>*F* = 0.0501) for biomass productivity found in Table 6.3 suggested that the fitted model is adequate. All the main effects of light intensity and inoculum concentrations, except for process time, are statistically significant at 0.0048 and 0.0255 respectively.

$$\text{Biomass productivity} = 29.134 + 14.668 \times A + 10.400 \times B + 1.382 \times C + 0.358 \times AB + 1.665 \times AC + 5.000 \times BC + 15.732 \times A^2 - 10.268 \times B^2 + 6.542 \times C^2 \quad (6.7)$$

6.4.6 Effect of process variables on lutein content and model development

The variations in lutein content with respect to inoculum concentration, light intensity and process days are presented in Figure 6.5 (a-c). The response surfaces show that the maximum lutein content of 16.23 mg g^{-1} was obtained at the inoculum concentration of 40%, light intensity of 25 µmol $m^{-2}s^{-1}$ and process time of 14 days. Optimization of lutein content is dependent on several factors that include carbon dioxide content, inoculum concentration, light intensity, temperature, pH, nitrogen source and availability, salinity and growth rate (Molino et al., 2019). A study on *Chlorella zofingiensis* showed that the lutein production was enhanced at lower light intensity of 90 µmol $m^{-2}s^{-1}$ when compared to higher light intensities of 460 µmol $m^{-2}s^{-1}$ and 920 µmol $m^{-2}s^{-1}$ and decreased as time progressed leading to the accumulation of astaxanthin (Del campo et al., 2004). Similarly, a study on *Murielopsis* showed that light intensities from 184 to 460 µmol $m^{-2}s^{-1}$ had a positive effect on lutein content, whereas light intensities from 460 to 1,625 µmol $m^{-2}s^{-1}$ had a negative effect (Del campo et al., 2001, Fernández-Sevilla et al., 2010) and varied with respect to

process days. The current study presented similar results in terms of light intensity with the lutein content increasing up to optimum values around 25 µmol $m^{-2}s^{-1}$ and then decreasing with the increasing light intensity of 40 µmol $m^{-2}s^{-1}$. The effect of inoculum concentration on lutein content has not been reported yet. Figure 6.5 (c) shows that the lutein content was maximum at the edges but it is not the case as while plotting the figure using JMP the axis of inoculum concentration was altered. In this study, lutein content was maximum at the inoculum concentration of 40% showing that an optimal inoculum concentration is required for optimal accumulation of lutein. Figure 6.6 (a and b) presents the maximum and minimum lutein content obtained at varying inoculum concentrations, light intensities and process times.

The ANOVA results for lutein content are presented in Table 6.3. The quadratic regression model for lutein content is presented in Equation 6.8. The model is significant and this is supported by the *F*-value of 9.28. The R^2 and the adjusted R^2 values for lutein content are 0.913 and 0.814 respectively. Higher R^2 value and lower *p*-value of 0.0023 show that the model successfully predicted responses and is significant. The predicted values for lutein content are obtained from the quadratic model equation. Figure 6.5 (d) shows the plot between predicted and actual values with the data points spread homogenously on either side of the zero line for responses on lutein content. The interaction terms which were significant include light intensity*inoculum concentration (AB) and light intensity*light intensity (A^2).

$$\text{Lutein content} = 12.594 - 0.415 \times A - 0.368 \times B + 0.553 \times C - 1.226 \times AB - 0.121 \times AC + 0.174 \times BC - 6.516 \times A^2 + 0.549 \times B^2 + 1.304 \times C^2 \quad (6.8)$$

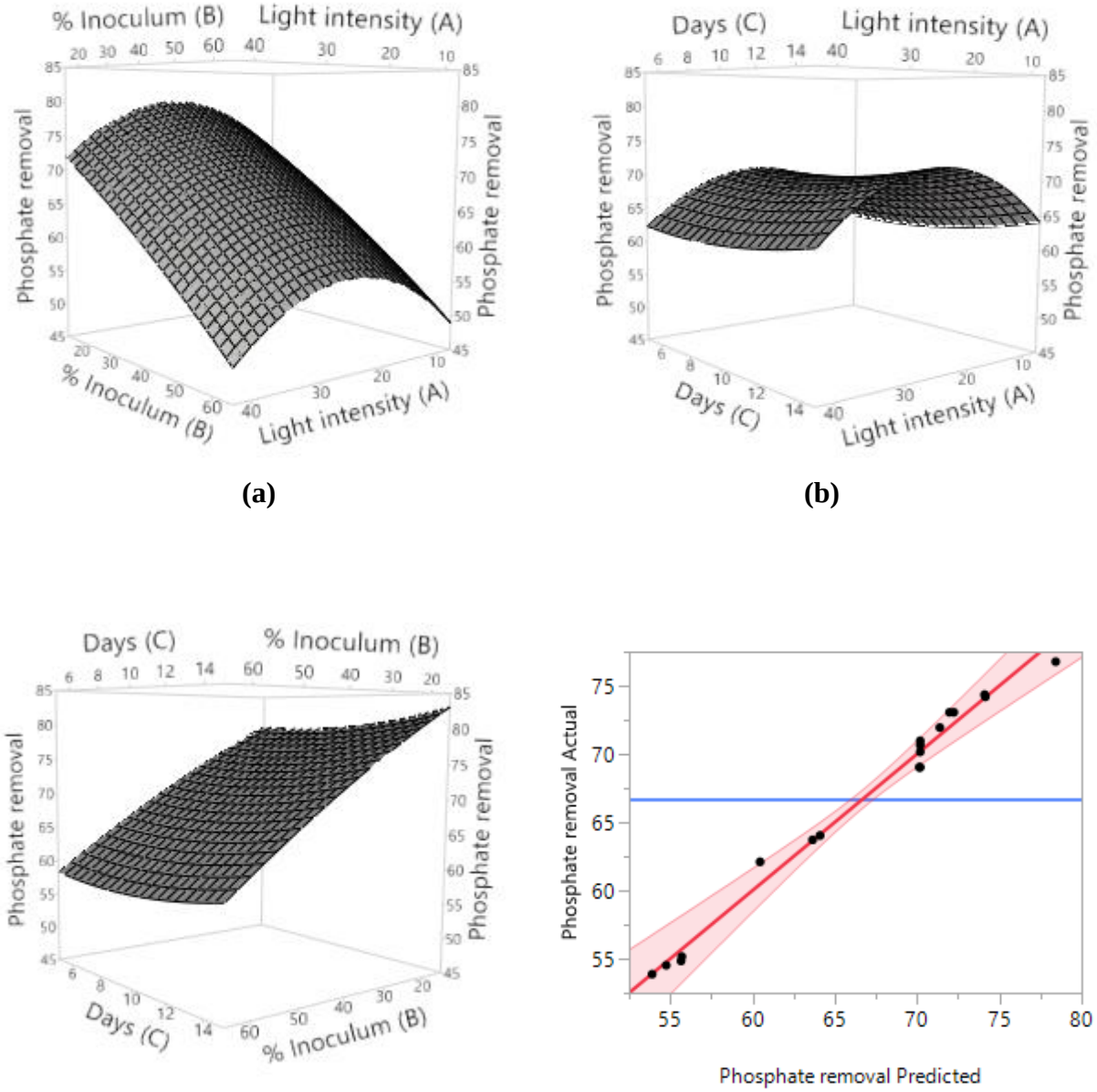


Figure 6.1 Response surfaces for correlating phosphate removal with (a) light intensity and inoculum concentration at time of 10 days (b) light intensity and time at inoculum concentration of 40% (c) inoculum concentration and time at light intensity of 25 µmol $m^{-2}s^{-1}$ (d) plot between predicted and actual values for phosphate removal.

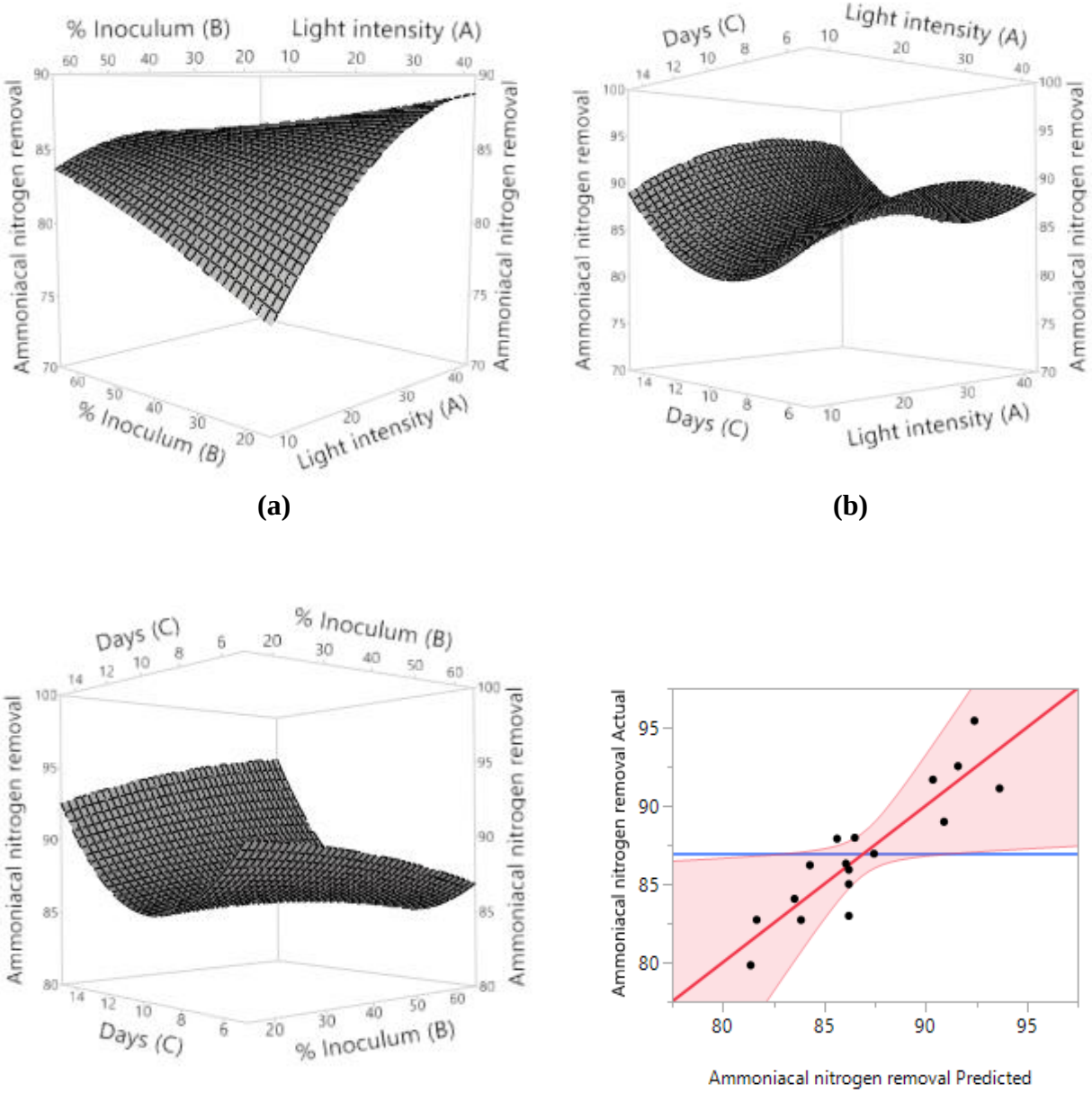
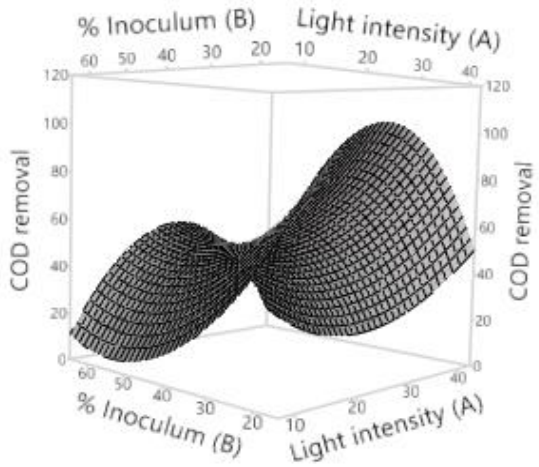
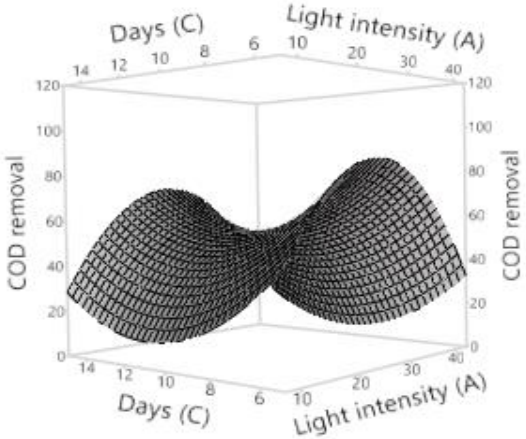


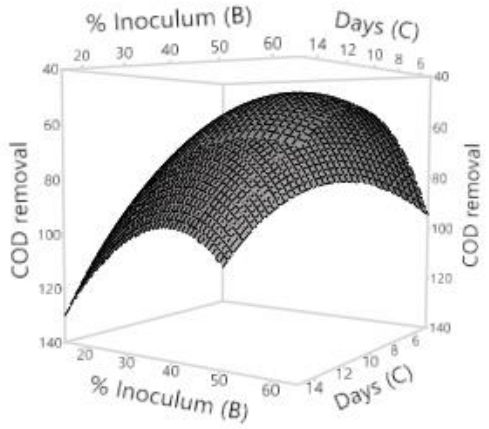
Figure 6.2 Response surfaces for correlating ammoniacal nitrogen removal with (a) light intensity and inoculum concentration at time of 10 days (b) light intensity and time at inoculum concentration of 40% (c) inoculum concentration and time at light intensity of 25 µmol $m^{-2}s^{-1}$ (d) plot between predicted and actual values for ammoniacal nitrogen removal.



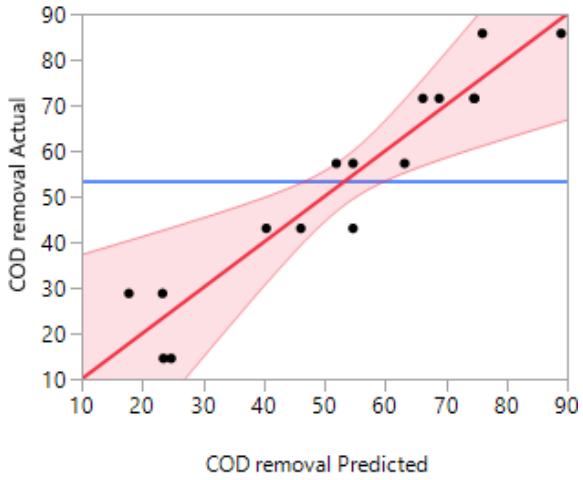
(a)



(b)

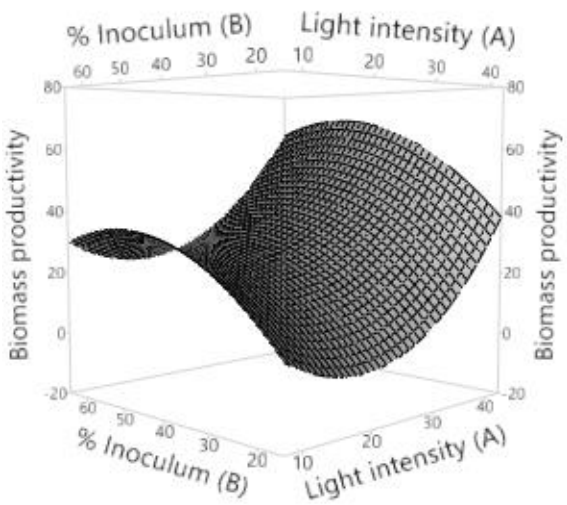


(c)

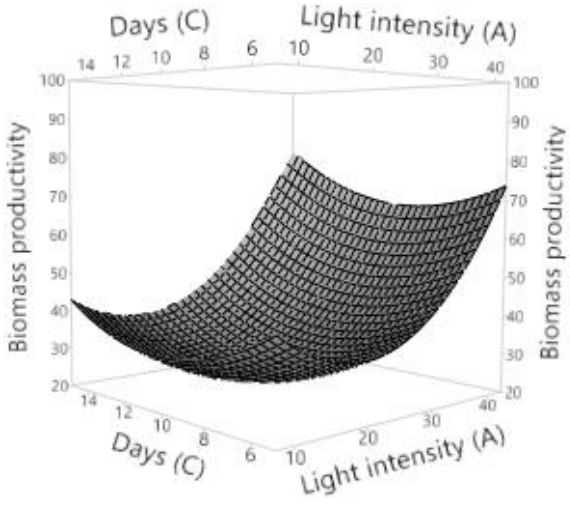


(d)

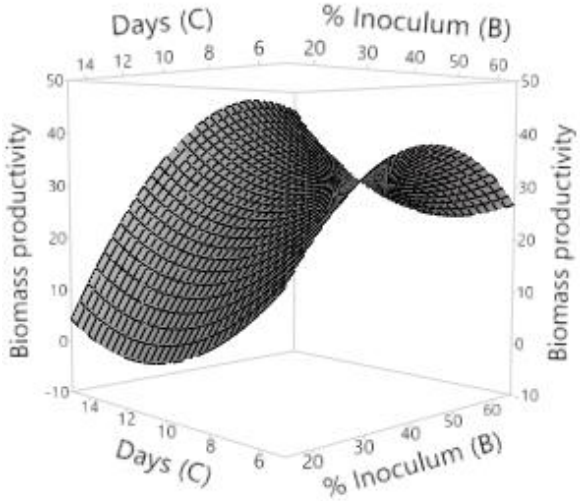
Figure 6.3 Response surfaces for correlating COD reduction with (a) light intensity and inoculum concentration at time of 10 days (b) light intensity and time at inoculum concentration of 40% (c) inoculum concentration and time at light intensity of 25 µmol $m^{-2}s^{-1}$ (d) plot between predicted and actual values for COD removal.



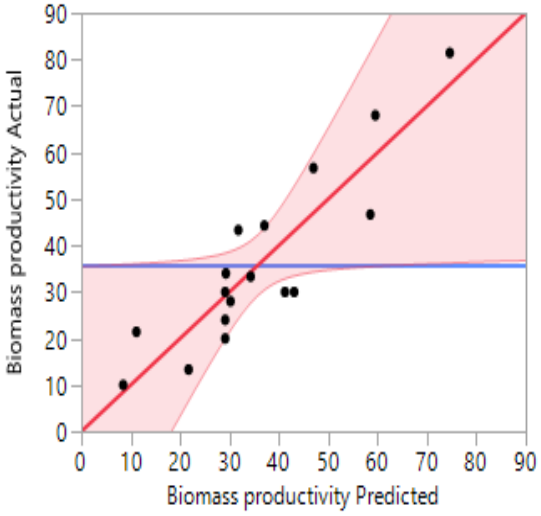
(a)



(b)



(c)



(d)

Figure 6.4 Response surfaces for correlating biomass productivity with (a) light intensity and inoculum concentration at time of 10 days (b) light intensity and time at inoculum concentration of 40% (c) inoculum concentration and time at light intensity of 25 µmol $m^{-2}s^{-1}$ (d) plot between predicted and actual values for biomass productivity.

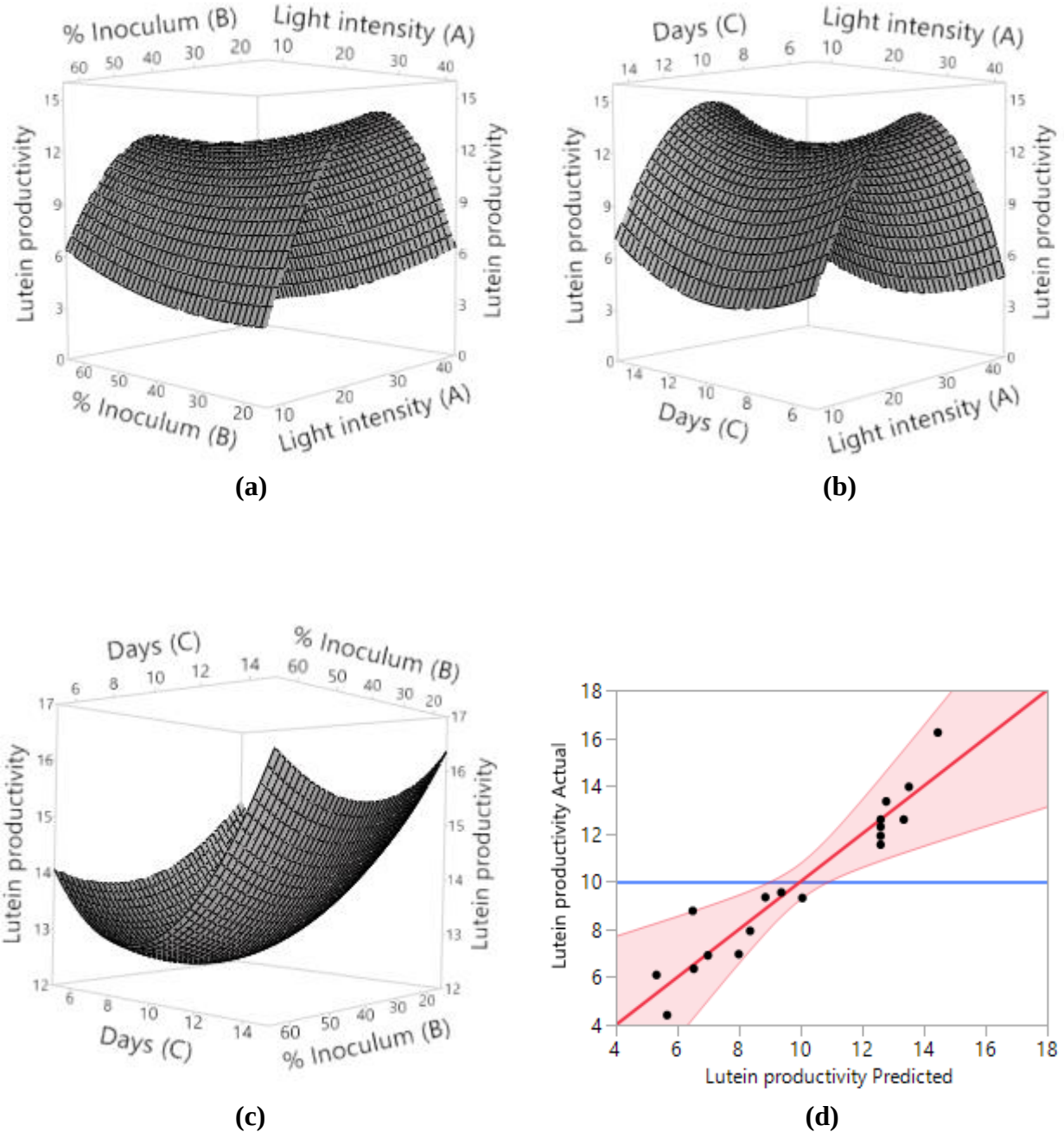
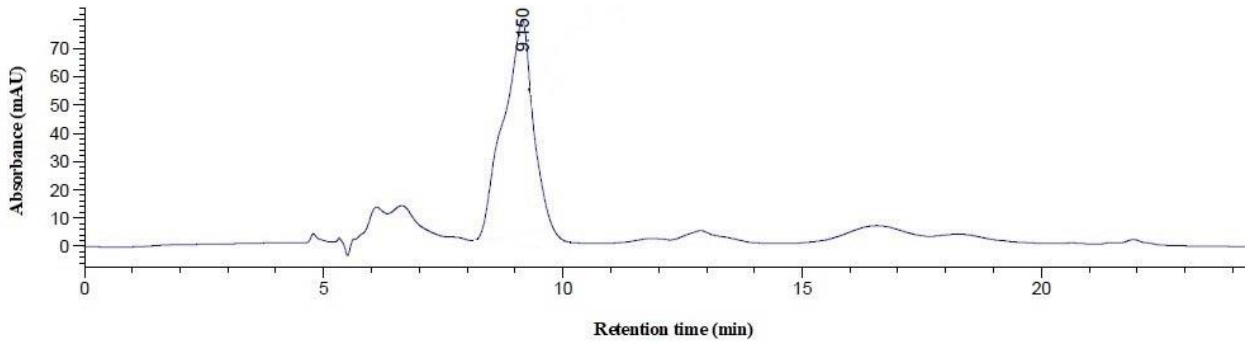
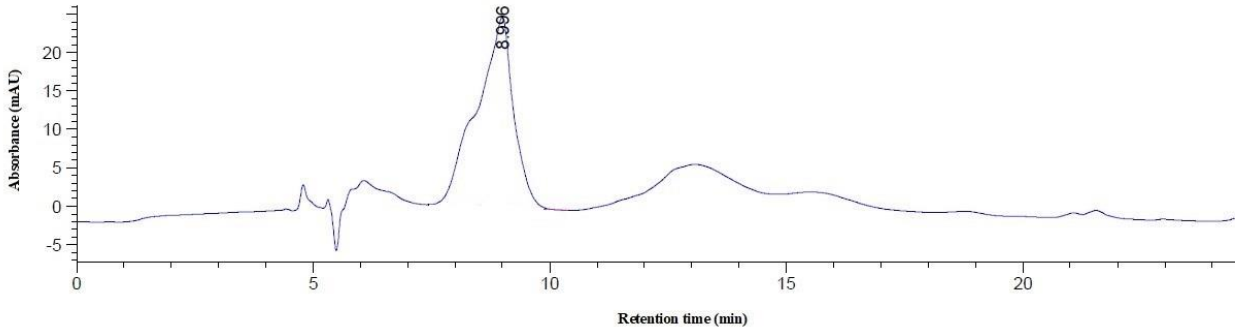


Figure 6.5 Response surfaces for correlating lutein content with (a) light intensity and inoculum concentration at time of 10 days (b) light intensity and time at inoculum concentration of 40% (c) inoculum concentration and time at light intensity of 25 µmol $m^{-2}s^{-1}$ (d) plot between predicted and actual values for lutein content.



(a)



(b)

Figure 6.6 Chromatogram of crude lutein obtained by extraction after saponification (a) Maximum lutein content (b) Minimum lutein content

6.5 Conclusion

The current study exhibited that the microalgal consortia *Chlorella variabilis* and *Scenedesmus obliquus* could effectively treat dairy wastewater with higher algal biomass productivity, lutein content and pollutant reduction. At optimized experimental conditions, the algal consortia showed lutein content of 12.59 mg g^{-1}, biomass productivity of 29.13 mg L^{-1} day^{-1}, phosphate reduction of 70.19%, ammoniacal nitrogen reduction of 86.22% and COD reduction of 54.72% after 10 days, when the light intensity was 25 µmol $m^{-2}s^{-1}$ and inoculum concentration of 40%. The lutein content of 16.23 mg g^{-1}, obtained at time of 14 days, inoculum concentration of 40% and light intensity of 25 µmol $m^{-2}s^{-1}$ which is reported in this study is the highest ever lutein content found in microalgae.

Good lutein content, biomass productivity and efficient reduction of pollutant load from wastewater make the microalgal consortia a viable source for commercial lutein production as well as a cost-effective and sustainable technology for treating dairy wastewater.

CONNECTING TEXT

The previous chapters have focused on the potential of using microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus* for addressing two major objectives of improving biomass productivity and bioremediation with valorization. However, in order to further improve the economic benefits of microalgal technology, utilizing the microalgal biomass as a biofertilizer holds the key. The emphasis in this study was to evaluate the relationship between varying concentrations of microalgal consortia cultivated on dairy wastewater with plant growth. In addition, the effect on the composition of the macronutrients (N and P) and the secondary metabolites were assessed.

CHAPTER VII

UTILIZING THE MICROALGAL BIOMASS OF *CHLORELLA VARIABILIS* AND *SCENEDESMUS OBLIQUUS* PRODUCED FROM THE TREATMENT OF DAIRY WASTEWATER AS A BIOFERTILIZER

7.1 Abstract

Microalgae are known to have higher growth rates than terrestrial plants and are garnering great attention among researchers as they can produce commodities of high commercial value. Remediating wastewater with microalgae and utilizing it as a biofertilizer could significantly improve the economic standpoint of algal production. In this study, varying concentrations (0%, 40% and 60%) of aqueous cell extracts of *Chlorella variabilis* and *Scenedesmus obliquus* cultivated on dairy wastewater were used as a biofertilizer to evaluate plant growth of corn (*Zea mays)* and soybean (*Glycine max)*. The treatments that employed the microalgal consortia as a biofertilizer exhibited increased growth rates, total phenolic content, total flavonoid content, antioxidant activity and mineral content when compared to the control group for both corn and soybean.

7.2 Introduction

Increasing global population and climate change effects are threatening mankind and forcing the scientific community to look for innovative technologies that could improve agricultural production to increase yields (food security) and reduce environmental impacts. Although use of synthetic fertilizers has provided an interim solution of increasing production yields, it has in turn resulted in soil infertility, eutrophication, environmental pollution and biodiversity loss. A sustainable alternative to address this issue is to utilize biofertilizers instead of synthetic fertilizers

(Garcia-Gonzalez and Sommerfeld, 2016). Biofertilizers, as the name suggests, are products that are derived from living organisms (such as bacteria, algae and fungi) to promote plant growth and improve soil fertility by providing essential nutrients such as nitrogen, potassium and phosphorus. Biofertilizers are naturally occurring, considered to be environmentally benign and cost effective. In recent years, biofertilizers from microalgae are garnering great interest as they tend to increase agricultural production while reducing the environmental footprint and can survive in highly concentrated wastewater streams that prove fatal to most other living organisms (Win et al., 2018). Microalgae are invaluable as the biomass they produce during their cultivation can be utilized as food for humans, fuel for vehicles, feed for animals and fertilizers for improving the chemical and biological properties of agricultural soils (Ronga et al., 2019). The commercialization of algal technologies will only become feasible if there are associated economic benefits. Biomass production from microalgae grown on wastewaters could hold the key for availing such economic benefits as the microalgae can remove the pollutant load from the wastewater, and the residual biomass produced can be utilized for extracting value added products, while the residues from extraction can be used as solid fertilizers. In addition, the discharged effluent after remediation can be used as a low-nutrient irrigation water for growing crops (Wuang et al., 2016).

Corn (*Zea mays)* and soybean (*Glycine max*) are considered to be among the most popular plant foods consumed by both humans and animals worldwide. In Canada, corn and soybean constitute the third and fourth most valuable crops with the majority of production concentrated in the provinces of Ontario and Quebec (Statistics Canada, 2015; Soy Canada, 2017; Farm Credit Canada, 2017). Since the demand for the crops are consistently rising, there is a need to improve their growth and yield without posing a threat to the environment. In addition, they have become an integral part of our daily diet and are known to be healthy food choices to help prevent diseases

such as atherosclerosis, diabetes and cancer by providing protection against oxidative damage. The health benefits associated with the consumption of corn and soybean can be attributed to the presence of phenols and flavonoids which contribute to their potent antioxidant activity (Peiretti et al., 2019; Zhang et al., 2017). The presence of such secondary metabolites is highly influenced by the amount of macronutrients present in the plants (Du et al., 2011).

Microalgae are known to contain macronutrients and micronutrients vital for plant growth at a higher level. Several studies have showed that both residual biomass and growth medium can be effectively used as a biofertilizer for enhancing plant growth and secondary metabolites (Garcia-Gonzalez and Sommerfeld, 2016; Wuang et al., 2016; Dineshkumar et al., 2018a; Dineshkumar et al., 2019). A study on rice plants grown on amended soils with blue-green algae showed significant increases in rice yield (Tripathi et al., 2008). Similarly, another study by Saadatnia and Riahi (2009) showed that the rice plants amended with wet inoculum of blue-green algae as a biofertilizer had a significant increase in plant height, root length, shoot fresh and dry mass, root fresh and dry mass when compared to the control. Renuka et al. (2016) showed that the wet algal biomass obtained from microalgal consortia grown on sewage improved the crop yield of wheat when applied to the soil under controlled conditions. A study by Yadavalli et al. (2013) showed that the algal biomass of *Chlorella pyrenoidosa*, obtained after treating dairy wastewater, improved the growth of rice plants with a 30% increase in terms of root and shoot lengths. In addition, the ability of blue-green algae to fix atmospheric nitrogen has been demonstrated by various studies (Tripathi et al., 2008; Saadatnia and Riahi, 2009). However, the ability of green algae to fix atmospheric nitrogen has been less explored. To the best of our knowledge, there has been no study on the impact of using *Chlorella variabilis* and *Scenedesmus obliquus* strains grown on dairy wastewater as a biofertilizer for improving the crop yield of corn and soybean. The current

study evaluated the effect of varying concentrations of *Chlorella variabilis* and *Scenedesmus obliquus* as a biofertilizer on growth performance, total phenolic content, total flavonoid content and total antioxidant activity at the mid-vegetative stage of corn and soybean plants. In addition, the germination potential of corn and soybean seeds under microalgal treatments was assessed.

7.3 Materials and Methods

7.3.1 Materials

The microalgae *Chlorella variabilis* (CCAP 211/84) and *Scenedesmus obliquus* (CCAP 276/3C) were obtained from the Culture Collection of Algae and Protozoa (CCAP) and cultured periodically in MN8 (Cheng et al., 2015) and BG11 (Rippka et al., 1979) media respectively. Hoagland complete medium was purchased from Plant Media (BioWorld) while the corn (*Zea mays* cv. DKC46-17RIB) and the soybean (*Glycine max* cv. Absolute RR) were obtained from Blue River Hybrid, Iowa, USA and Genuity, Saint Louis, USA respectively.

7.3.2 Cultivation of *Chlorella variabilis* and *Scenedesmus obliquus*

The synthetic dairy wastewater was prepared artificially in the laboratory using 2 g L^{-1} dried milk powder, 2.8 g L^{-1} NH_4Cl, 0.1 g L^{-1} $MgSO_4.7H_2O$, 0.076 g L^{-1} $CaCl_2.H_2O$, 2 g L^{-1} KH_2PO_4 and 4 g L^{-1} $NaHCO_3$ (Vidal et al., 2000). Batch cultivation of the varying concentrations of the microalgal consortia (40% and 60%) were performed in 2 L Erlenmeyer flasks containing dairy wastewater and placed on an orbital shaker agitating at 90 rpm with no aeration. Illumination of 25±3 µmol $m^{-2}s^{-1}$ was provided to the flasks by fluorescent lamps (4200 K, F72T8CW, Osram Sylvania, MA, US) and the temperature was maintained at 24±2 °C. The light intensity was measured at the beginning and end of the experiment using a quantum meter (Apogee instruments,

USA). Temperature was measured periodically with a temperature sensor (Raytek Minitemp, MT6). There was no control of the pH for the algal strains cultivated on dairy wastewater but it was monitored periodically.

7.3.3 Experimental set-up

The plant growth experiments were carried out in 6 inch pots containing vermiculite, planted with corn and soybean under controlled conditions in a growth chamber (Conviron, Model E15, Winnipeg, Canada). The temperature was set at 25 ℃ during the day and 22 ℃ at night with a photoperiod of 14:10, light intensity of 250 µmol $m^{-2}s^{-1}$ light and 40% relative humidity. The treatments chosen in this study were based on a germination test carried out at 20%, 40% and 60% algal concentrations. The results for 20% algal concentration is not reported here due to its lower germination rate. For each plant, five trials were conducted – T1, (control treatment – Tap water + 150 mL Hoagland), T2 (T2 treatment – Dairy wastewater + 150 mL Hoagland), T3 (T3 treatment – 30 mL of 40% algal consortia grown on dairy wastewater + 150 mL Hoagland), T4 (T4 treatment – 30 mL of 60% algal consortia grown on dairy wastewater + 150 mL Hoagland), T5 (T5 treatment – 30 mL of treated wastewater obtained after centrifugation of T3 + 150 mL Hoagland) and T6 (T6 treatment – 30 mL of treated wastewater obtained after centrifugation of T4 + 150 mL Hoagland). Treatments T5 and T6 consist of only dairy wastewater treated by the 40% and 60% microalgal consortia without the residual biomass as compared to T3 and T4. This was done mainly to find the effects of the microalgal consortia as a biofertilizer on the plant growth of corn and soybean as opposed to only dairy wastewater. The elemental level for each treatment is presented in Table 7.1. The duration of the experiments was set at 28 days and the trials were performed in triplicates.

Table 7.1 Elemental composition of treatments under study

Treatments	Nitrogen (mg L^{-1})	Phosphorus (mg L^{-1})
T1 (Control)	211	31
T2 (Dairy wastewater)	241	81
T3 (40% microalgal consortia)	241	81
T4 (60% microalgal consortia)	241	81
T5 (40% treated dairy wastewater)	215	46
T6 (60% treated dairy wastewater)	215	50

7.3.4 Growth and Nutritional analysis

At the end of 28 days, the plants were harvested and the plant height, leaf area, leaf number, chlorophyll content, total phenolics, total flavonoids, total antioxidant activity, nitrogen content, phosphorus content, root and shoot dry mass were determined. Leaf area was measured using the LI-COR LI-3100C area meter and the chlorophyll content was measured using the chlorophyll meter SPAD 502 (Konica Minolta, Japan).

7.3.5 Seed germination study

The seed germination study was performed for both corn and soybean using 50 seeds per treatment (5 replicates * 10 seeds) for 6 treatments; T1 to T6 containing varying concentrations of microalgal consortia as specified in section *7.3.3*. The seeds were placed in petri dishes containing Whatman filter paper grade 4 soaked in the respective treatments. Duplicate runs of 50 seeds (5 replicates * 10 seeds) per run were performed for each treatment group and the average results were reported. The germination of the seeds were continuously monitored from 0 h to 48 h. Germination rate was determined based on the number of seeds that germinate over a 2-day period.

7.3.6 Analyses of nitrogen and phosphorus

The harvested plant biomass was ground using a coffee grinder (Smart Grind, Black and Decker) and was passed through a sieve of 40-micron to obtain uniform particle size. The resulting tissue was digested in a mixture of sulfuric acid and peroxide with the addition of catalysts (lithium and selenium) at 340 ℃ for about three hours. The content was then diluted to 100 mL using double distilled water and analyzed colorimetrically for nitrogen and phosphorus using a flow injection instrument (FIA Lachat QuickChem 8000, USA) at 660 nm and 880 nm respectively (Parkinson and Allen, 1975).

7.3.7 Sample preparation for determination of total phenolic content, total flavonoid content and DPPH assay

A SCP Science miniWAVE oven (115 V – 60 Hz, 15 A, 1000 W, Quebec, Canada) equipped with 75 mL quartz vessels and infrared sensors for temperature control was used for microwave assisted extraction (MAE) for sample preparation. Briefly, 0.1 g of sample was mixed with 25 mL of 50% aqueous-ethanol. The resulting mixture was then placed in the microwave and the extraction was performed at 1000 W at 50 ℃ for 10 minutes. The samples were then filtered through Whatman filter paper grade 4 and the supernatant was retained at -20 ℃ for further analysis. All extractions were performed in triplicates (Gallo et al., 2010).

7.3.7.1 Determination of total phenolic content

The modified Folin Ciocalteu (FC) colorimetric method described by Zhang et al. (2017) was used for estimation of total phenolic content of samples. Briefly, about 1 mL of the obtained extract was taken and mixed with 5 mL of Folin's reagent (1:10). After 4 minutes, 4 mL of 7.5% sodium

carbonate was added and kept in the dark for 60 minutes at room temperature. The absorbance was read at 765 nm using an Ultrospec pro 2100 spectrophotometer (Biochrom, Cambridge, England). The standard used was gallic acid and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per 100 g of sample.

7.3.7.2 Determination of total flavonoid content

The total flavonoid content was determined by using a modified method adopted from Zhang et al. (2017). Briefly, 1 mL of extract was taken and mixed with 4 mL of distilled water. This was followed by addition of 0.3 mL of 5% sodium nitrite. After 5 minutes, 0.3 mL of 10% aluminium chloride was added. To the resulting mixture, 2 mL of 1 M sodium hydroxide was added after 5 minutes and diluted to 10 mL using distilled water. The absorbance was read at 510 nm using an Ultrospec Pro 2100 spectrophotometer. Quercetin was used as the standard and the total flavonoid content was expressed as milligrams of quercetin equivalents (QE) per 100 g of sample.

7.3.7.3 Determination of total antioxidant activity using DPPH assay

The total antioxidant activity was determined by DPPH assay using a modified method from Zhang et al. (2017). Briefly, 0.1 mL of sample was added to 3.9 mL of DPPH methanol solution. After vigorous shaking, the mixture was placed in the dark for 30 minutes. A control sample was prepared by adding 0.1 mL of methanol with 3.9 mL of DPPH methanol solution. The absorbance of the samples and control was determined using an Ultrospec Pro 2100 spectrophotometer at 517 nm. The antioxidant activity was expressed in terms of inhibition percentage (I%) and calculated using the following equation (7.1):

$$I\% = \frac{(Absorbance\ (control) - Absorbance\ (sample))}{Absorbance\ (control)} * 100 \qquad (7.1)$$

7.3.8 Statistical analysis

The results were analyzed statistically for their significance using JMP 14.0. The experiments were performed in triplicates except for the germination rate (duplicates) and the data were presented as means of replicate values ± standard deviation. The experimental design was a complete randomized design and Analysis of variance (ANOVA) was utilized to determine difference between various treatments. The comparisons among different means were evaluated using Student's t test at the $P<0.05$ level. The comparison of each treatment with the control was carried out using Dunnett's test at the $P<0.05$ level.

7.4 Results and Discussion

7.4.1 Efficacy of the microalgal consortia as a biofertilizer at mid-vegetative growth stage of corn and soybean

The algal biofertilizers comprising of *Chlorella variabilis* and *Scenedesmus obliquus* have shown positive results in enhancing the growth of corn and soybean as illustrated in Tables 7.2 and 7.3. For corn, the positive performances in terms of shoot dry mass (g), root dry mass (g), leaf area (cm^2), plant height (cm), number of leaves and chlorophyll content (CCI) were observed in all the treatments when compared to the control. Statistically significant changes were highlighted with 15.8% increase in plant height, 9.5% increase in number of leaves, 70.7% increase in shoot dry mass, 51.8% increase in root dry mass and 36.9% increase in leaf area while utilizing 40% algal consortia, as treatment (T3), when compared to the control. Similarly, statistically significant changes were observed in terms of plant height, shoot dry mass and root dry mass when applying treatment T4; number of leaves, plant height and root dry mass when applying treatment T5 and

only chlorophyll content when applying treatment T6. The results obtained agreed with the previously reported studies (Dineshkumar et al., 2018a; Tripathi et al., 2008; Saadatnia et al., 2009; Dineshkumar et al., 2018b) which mainly focused on comparing the efficacy of utilizing either wet or dry microalgal biomass as a biofertilizer compared with a control without fertilizer. In this study, the efficacy of using the biofertilizer produced from microalgal consortia was compared to the raw dairy wastewater (T2). Corn cultivated with raw dairy wastewater (T2) showed no significant differences in terms of leaf area, number of leaves, chlorophyll content and root and shoot dry mass when compared to the control (T1). The root dry mass in treatment T3 had significant differences when compared to T1, whereas no significant differences were observed between T3 and the other treatments (T2, T4, T5 and T6). Statistically, 40% algal consortia resulted as the best treatment for enhancing the growth of corn.

Soybean plants enriched with 40% algal consortia (T3) showed a 9.5% increase in plant height (cm), 6.6% increase in number of leaves, 5.1% increase in chlorophyll content (CCI), 20.0% increase in shoot dry mass (g), 17.1% increase in root dry mass (g) and a 9.7% increase in leaf area (cm^2) in comparison with the control. In this study, the plants cultivated using raw dairy wastewater (T2) showed no comparable increase in all growth parameters as compared to the control (p value of 1.000 for shoot dry mass; 0.735 for root dry mass; 0.834 for plant height; 0.699 for leaf area; 0.662 for number of leaves and 0.630 for chlorophyll content). Statistically significant differences were obtained in terms of plant height, number of leaves and shoot dry mass when applying treatment T3 as compared to the control. Similarly, significant differences were observed in terms of plant height, shoot dry mass and number of leaves when applying treatments T4 and T5 as compared to the control. The shoot dry masses obtained in T3, T4, T5 and T6 were significantly different from the control. Statistically, the best treatment is T3 (p value of 0.018),

however, T4 (p value of 0.0361) and T5 (p value of 0.0299) could also be recommended for bio-fertilizing for enhancing soybean growth (Table 7.3). The published work on using different concentrations of algae grown on dairy wastewater as biofertilizer is very limited. The results in this study suggest that the 40% microalgal based biofertilizer produced from *Chlorella variabilis* and *Scenedesmus obliquus* can be effectively utilized for enhancing the plant growth of corn and soybean.

The efficacy of the effect of biofertilizers on plant growth could be attributed to the increased amount of nutrients available for protein synthesis which in turn leads to higher plant growth (Dineshkumar et al., 2018b; Marschner, 1995). Table 7.1 shows the elemental composition of all the treatments under study. It can be seen that all the treatments have a higher nitrogen and phosphorus content as compared to the control. This can be attributed to the uptake of the nutrients by the algal consortia when cultivated on dairy effluent. Tables 7.4 and 7.5 show the effect of microalgal treatments on the content of nitrogen and phosphorus in both corn and soybean. For corn, nitrogen content was significantly different between treatment T3 and the control group and other treatments. An increase of 42.5% nitrogen content in the plant biomass was observed when applying treatment T3 as compared to control. This could be attributed to the algae having the ability to fix the available nitrogen which in turn leads to higher plant biomass with enhanced uptake of essential nutrients (Fogg, 1956; Han and Lee, 2006). No statistical difference was observed in the phosphorus content in the corn plants with respect to most treatments and the control except for the T3 treatment that increased by 25% over the control. For soybean plants, nitrogen content was significantly different between treatments T4 and T5 when compared to other treatments. The phosphorus content was significantly different between the control T1 and treatments T3, T4, T5 and T6. An increase of 7.7% in nitrogen content and a decrease of 17.6% in

phosphorus content was observed when applying treatment T5 in comparison with the control and all other treatments which reportedly decreased.

The use of microalgal biofertilizers could reduce the application of chemical fertilizers, thereby reducing the environmental footprint of agricultural production (Grzesik et al., 2017). A large number of studies on algal biofertilizers have focused on using cyanobacteria or macroalgae as the former can fix atmospheric nitrogen and the latter are ubiquitous and easier to process in comparison with microalgae. The evidence of using live microalgal culture/cell extracts from green algae as a potential biofertilizer has been less explored by researchers. Previous studies have revealed that microalgal extracts could enhance plant growth and this could be attributed to the presence of plant growth regulators (Garcia-Gonzalez and Sommerfield, 2016; Tarakhovskaya et al., 2007). The results from this study revealed that the plant growth was enhanced for both corn and soybean. The increase in shoot dry mass (biomass), plant height, leaf area and number of leaves of both corn and soybean can be attributed to the presence of auxins, gibberellic acid, cytokinins, macronutrients (N, P), micronutrients and other secondary metabolites produced by the green algae. In addition, the uptake of nitrogen by the green algae under study could be another reason for the observed increase in plant growth. Furthermore, the enhanced plant growth could be due to the green algae being able to provide nutrients that can be easily absorbed by the plants (Grzesik et al., 2017). The increase in chlorophyll content is likely due to the higher amount of nitrogen present in the plant tissues and this can be clearly seen in Tables 7.4 and 7.5 for both corn and soybean. This is in accordance with the studies put forward by Khan et al. (2012) and Spinelli et al. (2009), where foliar application of seaweed extract of *Ascophillum nodosum,* when applied as a biofertilizer, increased the chlorophyll content (higher N content) in leaves by 19% and 12% respectively for both grapes and apples.

Table 7.2 Efficacy of the microalgal consortia in enhancing corn growth

Plant parameter	Control (T1)	T2	T3	T4	T5	T6
Corn (28 days at harvest)						
Shoot dry mass (g)	0.41 ± 0.11^{c}	0.46 ± 0.06^{bc}	0.70 ± 0.07$^{\#,a}$	0.62 ± 0.09^{ab}	0.58 ± 0.16^{abc}	0.53 ± 0.11^{abc}
Root dry mass (g)	0.27 ± 0.05^{b}	0.36 ± 0.06^{ab}	0.41 ± 0.05^{a}	0.38 ± 0.06^{a}	0.39 ± 0.06^{a}	0.37 ± 0.07^{ab}
Leaf area (cm^2)	48.45 ± 7.57^{b}	51.95 ± 2.52^{ab}	66.34 ± 6.18^{a}	62.84 ± 8.90^{ab}	63.72 ± 14.88^{ab}	62.92 ± 13.94^{ab}
Plant height (cm)	43.97 ± 0.40^{c}	45.17 ± 2.83^{bc}	50.90 ± 1.65$^{\#,a}$	48.63 ± 3.31^{ab}	48.37 ± 2.07^{ab}	45.30 ± 1.77^{bc}
Number of leaves	4.20 ± 0.00^{bc}	4.13 ± 0.23^{c}	4.60 ± 0.20^{a}	4.53 ± 0.23^{ab}	4.67 ± 0.23^{a}	4.40 ± 0.20^{abc}
Chlorophyll content	29.00 ± 4.37^{b}	31.10 ± 3.15^{b}	31.70 ± 2.62^{ab}	29.97 ± 1.82^{b}	30.73 ± 2.60^{b}	32.00 ± 6.64^{a}

$^{\#}$ denotes statistical significance with control group

Means not connected by the same letter are significantly different

Table 7.3 Efficacy of the microalgal consortia in enhancing soybean growth

Plant parameter	Control (T1)	T2	T3	T4	T5	T6
Soybean (28 days at harvest)						
Shoot dry mass (g)	0.65 ± 0.03^{b}	0.65 ± 0.04^{b}	0.78 ± 0.02$^{\#,a}$	0.72 ± 0.03^{a}	0.73 ± 0.06^{a}	0.72 ± 0.04^{a}
Root dry mass (g)	0.29 ± 0.04^{a}	0.30 ± 0.01^{a}	0.35 ± 0.05^{a}	0.35 ± 0.06^{a}	0.34 ± 0.05^{a}	0.37 ± 0.07^{a}
Leaf area (cm^2)	104.82 ± 9.53^{a}	102.29 ± 1.71^{a}	116.05 ± 7.39^{a}	112.70 ± 4.18^{a}	107.65 ± 9.00^{a}	106.77 ± 11.01^{a}

Plant height (cm)	25.50 ± 0.53[c]	25.57 ± 0.50[c]	27.93 ± 0.25[#,a]	26.73 ± 0.31[#,b]	26.83 ± 0.31[#,b]	26.33 ± 0.31[b]
Number of leaves	8.07 ± 0.12[b]	8.00 ± 0.00[b]	8.60 ± 0.35[#,a]	8.60 ± 0.20[#,a]	8.53 ± 0.12[a]	8.07 ± 0.12[b]
Chlorophyll content	35.57 ± 2.21[a]	36.17 ± 1.25[a]	37.37 ± 0.85[a]	37.17 ± 1.50[a]	37.87 ± 1.66[a]	37.63 ± 1.08[a]

[#] denotes statistical significance with control group

Means not connected by the same letter are significantly different

Table 7.4 Effect of microalgal treatments on the nutrient composition of corn

Treatment group	**N-content (%) (Whole plant)**	**P-content (%) (Whole plant)**
T1 (Control)	1.06 ± 0.04[b]	0.16 ± 0.02[ab]
T2 (Dairy wastewater)	1.14 ± 0.05[b]	0.16 ± 0.02[ab]
T3 (40% microalgal consortia)	1.51 ± 0.12 [#,a]	0.20 ± 0.04[a]
T4 (60% microalgal consortia)	1.28 ± 0.31[ab]	0.19 ± 0.04[a]
T5 (40% treated dairy wastewater)	1.08 ± 0.04[b]	0.13 ± 0.02[b]
T6 (60% treated dairy wastewater)	1.23 ± 0.30[ab]	0.16 ± 0.02[ab]

[#] denotes statistical significance with control group

Means not connected by the same letter are significantly different

Table 7.5 Effect of microalgal treatments on the nutrient composition of soybean

Treatment group	N-content (%) (Whole plant)	P-content (%) (Whole plant)
T1 (Control)	1.55 ± 0.13^{ab}	0.17 ± 0.00^{a}
T2 (Dairy wastewater)	1.50 ± 0.02^{abc}	0.17 ± 0.02^{a}
T3 (40% microalgal consortia)	1.52 ± 0.24^{ab}	$0.13 \pm 0.01^{\#,b}$
T4 (60% microalgal consortia)	1.29 ± 0.10^{c}	$0.14 \pm 0.01^{\#,b}$
T5 (40% treated dairy wastewater)	1.67 ± 0.03^{a}	$0.14 \pm 0.02^{\#,b}$
T6 (60% treated dairy wastewater)	1.39 ± 0.09^{bc}	$0.12 \pm 0.00^{\#,b}$

$^{\#}$ denotes statistical significance with control group

Means not connected by the same letter are significantly different

7.4.2 Efficacy of the microalgal consortia on seed germination of corn and soybean

Many studies have showed that microalgae such as *Spirulina platensis*, *Chlorella vulgaris* and blue-green algae have increased the germination percentage of crops like corn and leafy vegetables when applied as a biofertilizer (Wuang et al., 2016; Dineshkumar et al., 2019; Saadatnia and Riahi, 2009). A study carried out by Wuang et al. (2016) exhibited a germination rate of 100% for Chinese cabbage and 81.7% for Kai-lan (Chinese kale/broccoli) when the concentration of *Spirulina platensis* was at 10 g L^{-1} as compared to the control. Similarly, another study by Dineshkumar et al. 2019 showed that the germination percentage of maize was higher when incorporated with cow dung + *Spirulina platensis* followed by cow dung + *Chlorella vulgaris* when compared to the control. Phytohormones such as gibberellic acid and abscisic acid, play a crucial role in the germination of seeds by either mobilizing or demobilizing the reserves. The mobilization and demobilization of the reserves tend to vary between crops (Han et al., 2013). Tables 7.6 and 7.7 report on the performance of the microalgal consortia on seed germination of

corn and soybean. The obtained results showed that the germination rate was higher as the concentration of the microalgal consortia increased for soybean with a maximum of 79% achieved for T6 as compared to the control which was at 69% after 2 days of treatment. For corn, the highest germination percentage of 100% was observed in T3 as compared to the control with 98% germination after 2 days. The results show that the germination of the seeds could have been affected by the levels of gibberellic acid or abscisic acid present. In addition, the results suggest that the microalgal consortia holds high significance for germination of soybean when compared to corn as the efficacy of the biofertilizer is considered to vary between different plant species (Wuang et al., 2016).

Table 7.6 Efficacy of microalgal consortia on the seed germination of corn

Treatment group	Germination percentage
T1 (Control)	98.00 ± 0.00
T2 (Dairy wastewater)	100.00 ± 0.00
T3 (40% microalgal consortia)	100.00 ± 0.00
T4 (60% microalgal consortia)	99.00 ± 1.41
T5 (40% treated dairy wastewater)	97.00 ± 1.41
T6 (60% treated dairy wastewater)	96.00 ± 5.66

Table 7.7 Efficacy of microalgal consortia on the seed germination of soybean

Treatment group	Germination percentage
T1 (Control)	69.00 ± 9.90
T2 (Dairy wastewater)	68.00 ± 11.31
T3 (40% microalgal consortia)	73.00 ± 9.90
T4 (60% microalgal consortia)	74.00 ± 8.49
T5 (40% treated dairy wastewater)	76.00 ± 5.66
T6 (60% treated dairy wastewater)	79.00 ± 4.24

7.4.3 Efficacy of the microalgal consortia on total phenolic content, total flavonoid content and radical scavenging activity for corn and soybean

Phenolics are secondary metabolites that are synthesized by plants during growth. Phenolics have the capability of increasing the antioxidant activity by improving the redox potential and also have the ability to scavenge excessive free radicals through various mechanisms (Zhang et al., 2017). With regard to the total phenolic content, corn treated with 40% microalgal consortia (T3) exhibited higher phenolics of 59.2 mg GAE $100g^{-1}$ when compared to the control (T1) which had a phenolic content of 56.2 mg GAE $100g^{-1}$ (Table 7.8). Statistically, no significant differences were observed between the treatments and the control, however, an increase of 5.4% in the total phenolic content was exhibited by treatment T3 as compared to the control. Similarly, flavonoids which are also considered to be a part of the phenolics family, are comprised of 15 classes of compounds including isoflavones, flavones, flavanones and chalcones (Josipović et al., 2016). Higher flavonoid content was measured under different treatments. Corn cultivated using microalgal treatment T3 showed a maximum flavonoid content of 133.3 mg QE $100g^{-1}$ with control exhibiting 125.2 mg QE $100g^{-1}$ (Table 7.8). Statistically, no significant differences were observed between treatments and the control, however, an increase of 6.5% in the total flavonoid content was exhibited by treatment T3 as compared to the control. DPPH assay was used to evaluate the % DPPH radical scavenging activity and the results obtained exhibited that lower scavenging activity is a depiction of higher antioxidant activity. For corn, treatment T3 had the lowest scavenging activity of 30.2% followed by T4 with 30.4%, T6 with 31.2% and T5 with 32.0% as compared to the control with 33.3% which means that treatment T3 had the highest antioxidant activity. Statistically, no significant differences were observed between the treatments and the control for antioxidant activity.

The total phenolic content of the soybeans treated with 40% microalgal consortia (T3) exhibited higher phenolics of 59.0 mg GAE $100g^{-1}$ when compared to control (T1) which had a phenolic content of 56.3 mg GAE $100g^{-1}$ (Table 7.9). Statistically, no significant differences were observed between the treatments and the control, however, an increase of 4.7% in the total phenolic content was exhibited by treatment T3 as compared to the control. Similarly, soybean cultivated using microalgal treatment T3 showed a maximum flavonoid content of 118.5 mg QE $100g^{-1}$ with control exhibiting 109.0 mg QE $100g^{-1}$ (Table 7.9). Statistically, no significant differences were observed between treatments and the control, however, an increase of 8.7% in the total flavonoid content was exhibited by treatment T3 and 10.9% by T5 as compared to control. For soybean, treatment T3 had the lowest scavenging activity of 25.3% followed by T5 with 26.1%, T6 with 26.4% and T4 with 26.9% as compared to the control with 27.7%, which means that treatment T3 had the highest antioxidant activity. Statistically, no significant differences were observed between the treatments and the control for antioxidant activity.

Several studies have reported a relationship between the macronutrients and the level of phenolics and flavonoids (Malenčić et al., 2007; Salehi et al., 2019; Oliveira et al., 2013; Ibrahim et al., 2011; Ibrahim et al., 2012). The plants grown under nitrogen deprived and phosphorus rich conditions are found to contain more secondary metabolites such as phenolics and flavonoids when compared to crops that grow in a nitrogen rich or phosphorus deficient environment. However, according to Growth Differentiation Balance Hypothesis, nitrogen or phosphorus availability are not the only factors that influence the production of secondary metabolites. A study by Aina et al. (2019) showed that the tomato fruit exhibited higher antioxidant activity when amended with cow dung in comparison to inorganic NPK fertilizer or chicken droppings. A study on *Labisia pumila* revealed that the total phenolics and flavonoid contents were significantly increased when the

concentration of nitrogen was lower (Ibrahim et al., 2011). Similarly, Du et al. (2011), working on American ginseng, reported that the total phenolic compounds increased significantly under nitrogen deficient and phosphorus rich conditions. This supports the result from this study for corn and soybean, as the total phenolic content, total flavonoid content and total antioxidant activity were not significantly different from each other between treatments and the control except for a minimal increase or decrease with respect to nitrogen and phosphorus contents. Another reason for the increase in the secondary metabolites, while applying treatments, maybe due to the higher photosynthetic rate of the algal consortia that was introduced with the treatment. This was in accordance with the study put forward by Dineshkumar et al. (2018a) where the application of microalgae as a biofertilizer, for improving onion cultivation, exhibited higher amount of phenols as compared to the control due to higher photosynthetic activity as a result of higher content of photosynthetic pigments.

Table 7.8 Content of total phenolics, total flavonoids and percentage DPPH inhibition for different treatments of corn

Treatment group	Total phenolic content (mg GAE $100g^{-1}$)	Total flavonoid content (mg QE $100g^{-1}$)	% DPPH inhibition
T1 (Control)	56.15 ± 2.39	125.19 ± 7.19	33.27 ± 8.71
T2 (Dairy wastewater)	55.54 ± 6.71	126.14 ± 8.69	31.30 ± 9.74
T3 (40% microalgal consortia)	59.20 ± 2.08	133.29 ± 9.37	30.20 ± 5.63
T4 (60% microalgal consortia)	58.13 ± 8.60	129.00 ± 7.95	30.38 ± 6.91
T5 (40% treated dairy wastewater)	57.74 ± 1.96	126.14 ± 14.07	31.98 ± 9.40
T6 (60% treated dairy wastewater)	58.04 ± 6.48	126.62 ± 8.37	31.24 ± 6.51

Table 7.9 Content of total phenolics, total flavonoids and percentage DPPH inhibition for different treatments of soybean

Treatment group	Total phenolic content (mg GAE $100g^{-1}$)	Total flavonoid content (mg QE $100g^{-1}$)	% DPPH inhibition
T1 (Control)	56.27 ± 2.98	109.00 ± 8.57	27.68 ± 1.69
T2 (Dairy wastewater)	57.92 ± 8.37	107.57 ± 10.00	29.46 ± 3.76
T3 (40% microalgal consortia)	58.96 ± 9.30	118.52 ± 8.61	25.34 ± 3.14
T4 (60% microalgal consortia)	54.98 ± 6.52	102.81 ± 10.91	26.88 ± 6.95
T5 (40% treated dairy wastewater)	57.00 ± 7.21	120.90 ± 9.29	26.14 ± 6.56
T6 (60% treated dairy wastewater)	52.48 ± 10.22	99.95 ± 7.87	26.38 ± 5.45

7.5 Conclusion

The current study demonstrated the effect of dairy wastewater-grown biomass, from microalgal consortia of *Chlorella variabilis* and *Scenedesmus obliquus*, on the growth of corn and soybean. Utilizing microalgal consortia as a biofertilizer enhanced the growth parameters, total phenolic content, total flavonoid content, antioxidant activity and minerals for both corn and soybean when compared to the control. Seed germination rate was improved for both corn and soybean cultivated under microalgal treatments. From these results, the best treatment for effectively growing corn and soybean is 40% microalgal consortia grown on dairy wastewater (T3). This work highlighted the importance of dairy wastewater-grown microalgal consortia as a potential low cost and sustainable biofertilizer for cultivation of both corn and soybean. Future work should focus on

evaluating the effect of dairy wastewater-grown microalgal biomass as a biofertilizer for field level studies for corn and soybean.

CHAPTER VIII

OVERALL SUMMARY, CONCLUSIONS, CONTRIBUTION TO KNOWLEDGE AND FUTURE WORK RECOMMENDATIONS

8.1 Overall summary and conclusions

The primary objective of this thesis was to develop a robust consortia that has the potential to be utilized for a wide variety of functions (bioremediation, valorization and biofertilization) for successful commercialization of microalgal technology. The literature review showed that various environmental factors are responsible for affecting the growth and biomass productivity of microalgae. Among the factors listed, light and nutrients are considered to be the most crucial factors that influence microalgal growth. In addition, the biomass produced could be used for extracting a range of value added products that have a high commercial value and serve as a feedstock for various industries (Chapter II and Chapter III). The strains that would make-up the microalgal consortia were determined after conducting an extensive literature survey of a wide variety of microalgal strains. It is quite clear that the strain *Chlorella variabilis* has not been studied extensively by researchers for the purpose mentioned (bioremediation, valorization and biofertilization).

Chapter IV of this thesis focused on finding a suitable media for effectively growing the under-examined *Chlorella variabilis* strain before investigating its ability to remediate wastewater. The selected experimental design utilized lower light intensity, a 24 h photoperiod and agitation on an orbital shaker at 90 rpm, with no carbon dioxide supplementation. MN8 medium and simulated dairy wastewater were identified as the best cultivation media for enhancing biomass productivity with significant nutritional and lutein contents. For large scale production of the algal strain, dairy wastewater could be utilized as the cultivation medium. This significantly brings down

the cost of algal cultivation that might be incurred when using inorganic media. The presence of lutein as a value added product in the algal biomass further improves the economic consideration of the concept.

Chapter V reported the results of the effect of light wavelengths on biomass productivity, nutritional and lutein contents of the microalgal consortia *Chlorella variabilis* and *Scenedesmus obliquus*. The microalgal consortia was cultivated on simulated dairy wastewater in batch mode using 125 mL Erlenmeyer flasks over 14 days under continuous illumination of varying wavelengths of light (cool-white fluorescent, blue, red, amber and amber+blue). Remarkably, light treatments had significant effects on biomass productivity, nutritional and lutein contents. Cool-white fluorescent light exhibited enhanced biomass productivity, protein and lutein contents when compared to other light treatments. In addition, the microalgal consortia cultivated under amber light exhibited higher carbohydrate content when compared to the other light treatments.

In Chapter VI, the potential of the microalgal consortia to remediate simulated dairy wastewater was assessed using RSM. The effects of inoculum concentration, time and light intensity on the pollutant removal efficiency, while improving its biomass productivity and lutein content, were investigated under controlled laboratory conditions. The optimized reduction of phosphorus, ammoniacal nitrogen and COD was observed at a time of 10 days, light intensity of 25 µmol $m^{-2}s^{-1}$ with 40% algal concentration. The biomass productivity was also higher with 29.13 mg L^{-1} day^{-1} and a lutein content of 12.59 mg g^{-1}.

Finally, in Chapter VII, the potential of the dairy wastewater-grown microalgal consortia as a biofertilizer for enhancing the growth of corn and soybean was investigated. The results showed that the plants treated with the microalgal consortia performed better in terms of growth

performance when compared to the control. In addition, an increase in the amount of secondary metabolites and macronutrients (N and P) was observed.

Overall, this thesis demonstrated how the robust consortia studied had the ability to perform multiple functions such as bioremediation of simulated dairy wastewater, valorization of the residual biomass to produce lutein and act as a biofertilizer while enhancing overall biomass productivity. In addition, the study showed that this microalgal consortia could be a successful candidate when incorporated into a biorefinery. The proposed schematic of a biorefinery for this study is depicted in Figure 8.1. This thesis contributes to knowledge on how amber light could be employed for algal cultivation which has been less explored by researchers. It is evident that this microalgal consortia could be exploited as a commercial source of microalgal lutein in the future.

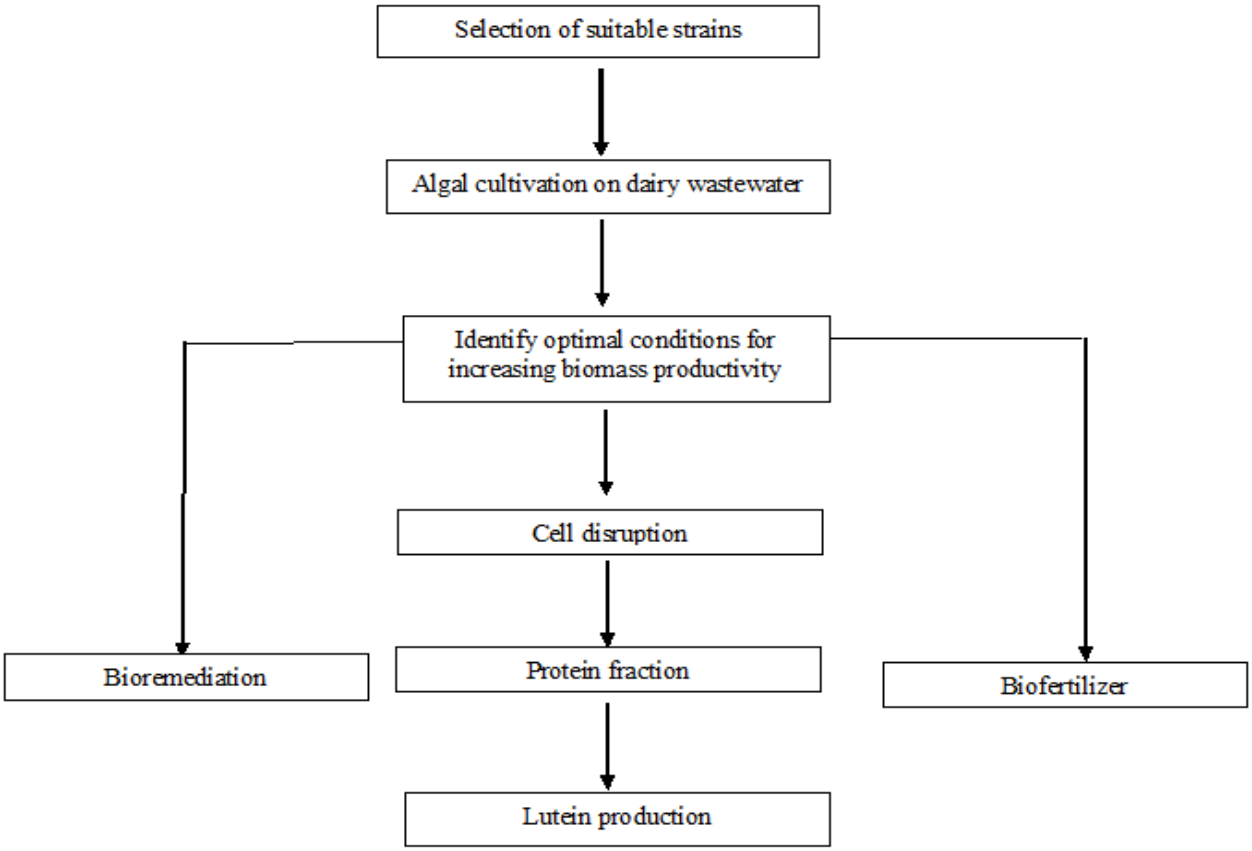


Figure 8.1 Proposed schema of a microalgal biorefinery according to this study

8.2 Contributions to Knowledge

The following are the findings that contribute to knowledge:

1. Though numerous studies have focused on employing dairy wastewater as a substrate for cultivation of various microalgal species, this study is the first of its kind to investigate the effect of various media including dairy wastewater on the growth performance, nutritional and lutein contents of the microalga *Chlorella variabilis*.
2. This thesis shows for the first time that the microalga *Chlorella variabilis* has the ability to produce significant amounts of lutein.
3. The introduction of amber light in this study for effectively growing the microalgal consortia *Chlorella variabilis* and *Scenedesmus obliquus* with significant increase in carbohydrate content opens up a new avenue for research which has been less explored by researchers.
4. This thesis shows for the first time that the algal concentration had a significant effect on pollutant reduction ability and biomass productivity with respect to time and light intensity. It showed for the first time that the microalgal consortia was able to produce higher amounts of lutein under optimal conditions when compared to other microalgal species.
5. The study revealed for the first time that the wet algal biomass of the microalgal consortia could be used as a biofertilizer for enhancing plant growth of both corn and soybean.
6. Finally, this study holds high significance from an industrial standpoint as this is the first study of its kind that has been carried out for developing a robust consortia to be incorporated into a biorefinery for performing multiple functions thus diversifying its development potential which would otherwise make commercialization of algal technology unfeasible.

8.3 Future work recommendations

The following are the recommendations for future research based on the current study:

a) The use of a microalgal consortia should be investigated as a feedstock for biofuel production.

b) Scale up studies should be carried out based on the conditions specified in the study utilizing a photobioreactor using real time dairy wastewater.

c) The possibility of utilizing a microalgal consortia for treating complex industrial wastewaters should also be assessed.

d) Optimization of extraction conditions for maximizing lutein content from the microalgal consortia should be investigated.

e) Field level studies employing the wet-algal biomass of the microalgal consortia as a biofertilizer for corn and soybean on different soil types should be studied.

f) Molecular level studies in understanding the mechanisms on how the consortia function when exposed to soil conditions should be investigated.

To conclude, there is no doubt that the microalgal consortia developed in this study will play a major role and could be the first step in the commercialization of microalgal technology in the near future with potential economic benefits.

CHAPTER IX

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