ISOLATION AND EVALUATION OF AUTOCHTHONOUS MICROALGAE STRAINS FOR BIODIESEL PRODUCTION AND WASTEWATER TREATMENT

By

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Submitted in fulfillment of the academic requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Microbiology; School of Life Sciences; College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Westville Campus, Durban.

As the candidate's supervisor, I approve this thesis for submission.

Signed: _____ Name: _____ Date: 17-August-2018

PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa from January 2015 to January 2018, under the supervision of Professor A. O. Olaniran, Dr. T. Mutanda and Dr. Y.F Chen.

These studies represent the original work by the authors and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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DECLARATION 2- PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis (include publications in preparations, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

Title: Potential Biotechnological Application of Microalgae: A critical Review.

Journal: Critical Review in Biotechnology

Authors:

E.C Odjadjare – Conceptualization, drafting and editing of manuscript

A.O. Olaniran – Conceptualization and editing of manuscript

T. Mutanda – Conceptualization and editing of manuscript

Publications 2:

Title: Microalgal diversity and community structure profiling of different aquatic ecosystems in Durban, South Africa using DGGE and T-RFLP.

Journal: **Freshwater Biology**

Authors:

E.C Odjadjare – Conceptualization, Laboratory work, drafting and editing of manuscript

A.O. Olaniran – Conceptualization, experimental design and editing of manuscript

T. Mutanda – Conceptualization and editing of manuscript

Y.F. Chen – Conceptualization and editing of manuscript

Publications 3:

Title: Characterization of autochthonous microalgae in South Africa with high biodiesel production potential

Journal: Applied Biochemistry and Biotechnology

Authors:

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A.O. Olaniran – Conceptualization, experimental design and editing of manuscript

T. Mutanda – Conceptualization and editing of manuscript

Y.F. Chen – Conceptualization and editing of manuscript

Publications 4:

Title: Evaluation of pre-chlorinated wastewater effluent for microalgal cultivation and biodiesel production.

Journal: Water

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Y.F. Chen – Conceptualization and editing of manuscript

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ACKNOWLEDGMENTS

I wish to express my profound gratitude to God Almighty for his grace and giving me the strength to complete this study. My sincere thanks go to my supervisor, Prof. A. O. Olaniran for his mentorship, assistance and guidance throughout the course of this study. I am immensely grateful for everything you did for me. Sincere thanks to my Co-Supervisors Dr. Taurai Mutanda and Dr. Yi-Feng Chen for their assistance and valuable input in this study.

I am immensely grateful to my parents Mr. and Mrs. E.U Odjadjare for their love and support all my life especially during this research. To my siblings Avwarosuoghene, Okieoghene, Oghenekowhiroro and Emuobosan for their prayers and love during this study.

Special thanks to the National Research Foundation for the PhD scholarship awarded me to conduct this research, the College of Agriculture, Engineering and Science, University of KwaZulu-Natal for giving me financial aid in the form of bursary and travel grant for conferences during the study. Thanks to the staff of Northern, Phoenix, Kingsburgh and Umbilo wastewater treatment works for their assistance in the collection of wastewater samples used for this study.

I also wish to thank my laboratory colleagues: Noxolo Nokubahle Mkhwanazi, Kerisha Ramessar, Rajnitha Magraj, Oladipupo Aregbesola, Oluwafemi Egbewale, Dr. Ajit Kumar Kanwal and the staff and postgraduate students of the Discipline of Microbiology (Westville campus) for their kind support. Special acknowledgement to my next of kin away from home Leanne Pillay. Six years of being a pal, next of kin and sister. Love you lots.

This Thesis is dedicated to my mother Mrs. Ese Lafua Odjadjare

Abstract

The current depletion of global fossil fuel reserves and increasing CO_2 emission are generating global climate change concerns hence, the increasing calls for the development of renewable biofuels with low CO_2 emission. Microalgae has been touted as the most suitable feedstock of renewable fuels due to a number of reasons including high biomass productivity, fast growth rates, cost-effective cultivation systems and alleviation of food security concerns associated with use of other feedstock for biofuel production. Bioprospecting could yet reveal microalgae with high lipid contents for biodiesel production and novel characteristics such as production of unique bioactive compounds. With an attractive climate and plenty of sunlight all year round, South Africa is in a unique position to take advantage of these organisms to guarantee her future energy needs. This research thus aims to bio-prospect for indigenous strains of microalgae in aquatic habitats and wastewater treatment plants in KwaZulu-Natal province of South Africa and evaluate their potential for biofuel production and wastewater remediation. DGGE and T-RFLP fingerprinting techniques were adapted to give an overview of the diversity of microalgae in various aquatic habitats including wastewater treatment plants (WWTP), rivers, estuaries and marine ecosystems in order to identify areas for potential bioprospecting. Ten (10) microalgae species from these aquatic environments were isolated, identified and evaluated for lipid, total carbohydrate and protein accumulation using well defined procedures. The lipid profile, biodiesel characteristics as well as effect of starvation on lipid accumulation were also determined. The biomass production and simultaneous phycoremediation potential of two autochthonous isolates *Asterarcys quadricellulare* and *Neochloris aquatica* were also investigated. Microalgae classes; Trebouxiophyceae and Chlorophyceae were observed to be dominant in fresh water habitat, while those belonging to the class Ulvophycea (*Oltmannseillopsis* spp.) dominated the salt water environment. At the brackish water habitat, microalgae belonging to the classes Trebouxiophyceae (*Chlorella minutissima*) and Chlorophyceae (*M. sturmi*) were dominant. A shift in community structure was observed at the maturation ponds of WWTPs and in rivers over time. The growth rates of 10 isolated microalgae ranged from 0.219 ± 0.003 to 0.175 ± 0.023 $gL^{-1}day^{-1}$ while dry weight ranged from 0.433 ± 0.208 to 1.167 ± 0.153 gL^{-1}. *Chlorococcum* LM1 showed high accumulation of lipid (11.93 ± 0.76 mg/L). The microalgae isolated in this study accumulated high carbohydrate content ranging from 25 to 61 % of their dry weight while protein content ranged from 1.06 ± 0.1 mg/L to 1.39 ± 0.1 mg/L accounting for 21 to 28% dry weight. Lipid accumulation also varied under nutrient limitation condition. Lipid accumulation was enhanced in some of the isolates such as *Chlorococcum* sp. LM1 (17.2%) and *C. sorokiniana* NWS5 (24%) while a decrease was observed in others such as *C. minutissima* TS9 (16%), *N. aquatica* Toti4 (3.5%) and *Chlorococcum* sp. LM2 (6.5%). Response to enhanced lipid accumulation via starvation seem to be unique to each algal strain irrespective of species. The lipid profile consisted mainly of saturated fatty acid such as oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0) with low amounts of polyunsaturated fatty acids

such as linoleic acid (C18:2 n-6). Characteristic of the biodiesel based on the lipid profile reveal a low viscosity and density. The biodiesel was determined to be of good quality with high oxidation stability, low viscosity and conformed to the ASTM guidelines. Low total phosphorus concentration in the wastewater resulted in an unbalanced N:P ratio of 44 at the Northern wastewater treatment works (NWWTW) and 4 at the Umbilo wastewater treatment works (UWWTW). *Asterarcys quadricellulare* utilized the wastewater for growth and reduced the COD of the wastewater effluent from the UWWTW by 12.4% in contrast to *Neochloris aquatica* which did not show any growth. *Asterarcys quadricellulare* was able to utilise the wastewater achieving a growth rate up to 0.18 day^{-1} in sterilized wastewater from the NWWTW and 0.17 day^{-1} in the unsterilized wastewater from UWWTW. *Asterarcys quadricellulare* accumulated high biomass of 460 mg/L compared to 180 mg/L in *Neochloris aquatica*. Total nitrogen (TN) and Phosphorus (TP) were reduced by 48% and 50% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced the TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% reduction were recorded in *Neochloris aquatica*. The study showed the diversity and community structure of microalgae in aquatic ecosystems in the study area. Autochthonous microalgae were rich in lipid, carbohydrate and protein and could be applied for biofuel production. Wastewater effluent can be used to generate biomass for biodiesel production while treating wastewater. However, optimization of the N:P ratio and carbon source are necessary to improve remediation and biomass productivity for future commercial scale production.

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List of Abbreviations

ASTM	American society of testing and materials
APE	Allylic position equivalent
BAPE	Bis-Allylic equivalent
B.C	Before Chlorination
CFPP	Cold filter plug point
CN	Cetane number
CP	Cloud point
DU	Degree of unsaturation
D.S	Downstream
DGGE	Denaturing gradient gel electrophoresis
HHV	Higher heating value
IV	Iodine value
LC-PUFA	Long chain polyunsaturated fatty acid
LCSF	Long chain saturated factor
MUFA	Monounsaturated fatty acid
MWWTP	Magabeni wastewater treatment plant
NWWTW	Northern wastewater treatment works
OS	Oxidative stability
OTU	Operational taxonomic unit
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
SV	Saponification value
TN	Total nitrogen
TP	Total phosphorus
TRFLP	Terminal restriction fragment length polymorphism
T-RFs	Terminal restriction fragments
U.S	Upstream
UWWTW	Umbilo wastewater treatment works
WWTP	Wastewater treatment plants

Abstract

The current depletion of global fossil fuel reserves and increasing CO_2 emission are generating global climate change concerns hence, the increasing calls for the development of renewable biofuels with low CO_2 emission. Microalgae has been touted as the most suitable feedstock of renewable fuels due to a number of reasons including high biomass productivity, fast growth rates, cost-effective cultivation systems and alleviation of food security concerns associated with use of other feedstock for biofuel production. Bioprospecting could yet reveal microalgae with high lipid contents for biodiesel production and novel characteristics such as production of unique bioactive compounds. With an attractive climate and plenty of sunlight all year round, South Africa is in a unique position to take advantage of these organisms to guarantee her future energy needs. This research thus aims to bio-prospect for indigenous strains of microalgae in aquatic habitats and wastewater treatment plants in KwaZulu-Natal province of South Africa and evaluate their potential for biofuel production and wastewater remediation. DGGE and T-RFLP fingerprinting techniques were adapted to give an overview of the diversity of microalgae in various aquatic habitats including wastewater treatment plants (WWTP), rivers, estuaries and marine ecosystems in order to identify areas for potential bioprospecting. Ten (10) microalgae species from these aquatic environments were isolated, identified and evaluated for lipid, total carbohydrate and protein accumulation using well defined procedures. The lipid profile, biodiesel characteristics as well as effect of starvation on lipid accumulation were also determined. The biomass production and simultaneous phycoremediation potential of two autochthonous isolates *Asterarcys quadricellulare* and *Neochloris aquatica* were also investigated. Microalgae classes; Trebouxiophyceae and Chlorophyceae were observed to be dominant in fresh water habitat, while those belonging to the class Ulvophycea (*Oltmannseillopsis* spp.) dominated the salt water environment. At the brackish water habitat, microalgae belonging to the classes Trebouxiophyceae (*Chlorella minutissima*) and Chlorophyceae (*M. sturmi*) were dominant. A shift in community structure was observed at the maturation ponds of WWTPs and in rivers over time. The growth rates of 10 isolated microalgae ranged from 0.219 ± 0.003 to 0.175 ± 0.023 $gL^{-1}day^{-1}$ while dry weight ranged from 0.433 ± 0.208 to 1.167 ± 0.153 gL^{-1}. *Chlorococcum* LM1 showed high accumulation of lipid (11.93 ± 0.76 mg/L). The microalgae isolated in this study accumulated high carbohydrate content ranging from 25 to 61 % of their dry weight while protein content ranged from 1.06 ± 0.1 mg/L to 1.39 ± 0.1 mg/L accounting for 21 to 28% dry weight. Lipid accumulation also varied under nutrient limitation condition. Lipid accumulation was enhanced in some of the isolates such as *Chlorococcum* sp. LM1 (17.2%) and *C. sorokiniana* NWS5 (24%) while a decrease was observed in others such as *C. minutissima* TS9 (16%), *N. aquatica* Toti4 (3.5%) and *Chlorococcum* sp. LM2 (6.5%). Response to enhanced lipid accumulation via starvation seem to be unique to each algal strain irrespective of species. The lipid profile consisted mainly of saturated fatty acid such as oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0) with low amounts of polyunsaturated fatty acids

such as linoleic acid (C18:2 n-6). Characteristic of the biodiesel based on the lipid profile reveal a low viscosity and density. The biodiesel was determined to be of good quality with high oxidation stability, low viscosity and conformed to the ASTM guidelines. Low total phosphorus concentration in the wastewater resulted in an unbalanced N:P ratio of 44 at the Northern wastewater treatment works (NWWTW) and 4 at the Umbilo wastewater treatment works (UWWTW). *Asterarcys quadricellulare* utilized the wastewater for growth and reduced the COD of the wastewater effluent from the UWWTW by 12.4% in contrast to *Neochloris aquatica* which did not show any growth. *Asterarcys quadricellulare* was able to utilise the wastewater achieving a growth rate up to 0.18 day^{-1} in sterilized wastewater from the NWWTW and 0.17 day^{-1} in the unsterilized wastewater from UWWTW. *Asterarcys quadricellulare* accumulated high biomass of 460 mg/L compared to 180 mg/L in *Neochloris aquatica*. Total nitrogen (TN) and Phosphorus (TP) were reduced by 48% and 50% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced the TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% reduction were recorded in *Neochloris aquatica*. The study showed the diversity and community structure of microalgae in aquatic ecosystems in the study area. Autochthonous microalgae were rich in lipid, carbohydrate and protein and could be applied for biofuel production. Wastewater effluent can be used to generate biomass for biodiesel production while treating wastewater. However, optimization of the N:P ratio and carbon source are necessary to improve remediation and biomass productivity for future commercial scale production.

CHAPTER 1

1.0 GENERAL INTRODUCTION AND SCOPE OF STUDY

Increased global industrialization and improved standard of living have led to a dramatic increase in fossil fuel consumption (Gouveia et al., 2017). Fossil fuel, the dominant global form of energy is a finite resource with a proven global reserve estimated at 1376 billion barrels and is projected to be exhausted as early as 2045 (Faried et al., 2017). Over a period of 60 years (195 -2011), more than 6 billion tonnes of CO_2 has been released annually into the atmosphere and this rate has accelerated in recent years speeding up global warming (Mondal et al., 2017). The current depletion of global fossil fuel reserves and increasing CO_2 emission are generating global climate change concerns hence, the increasing calls for the development of renewable biofuels with low CO_2 emission to compensate for the decreasing fossil fuel reserve (Dutta et al., 2016). Food crops (Starch and oil based crops) have been traditionally used for the generation of biofuels with low carbon emission (Popa, 2018). However, these sources of liquid fuels are not sustainable due to food security, geo-political and socio-economic concerns.

Microalgae are versatile photoautotrophic organisms producing valuable bioactive compounds with applications in the pharmaceutical, cosmetic and biofuel industries. They are very diverse with only a small fraction described in literature (Cheng & Ogden, 2011). These organisms have been touted as the most suitable source of renewable fuels due to a number of reasons including high biomass productivity, fast growth rates, cost-effective cultivation systems and alleviation of food security concerns associated with use of other feedstock for biofuel production. The triacylglycerol (TAG) in microalgal lipid can be converted into biodiesel via transesterification process by the reaction of TAG with methanol in the presence of an acid or alkali catalyst to form fatty acid methyl esters (biodiesel) and glycerol (Mata et al., 2010). The monosaccharides in microalgal biomass can be used to produce bioethanol or biogas via fermentation or anaerobic digestion, while bio-hydrogen can also be produced via biodegradation of starch extracted from microalgae. Besides the production of biofuels, microalgae have many other potential biotechnological applications. The microalgal biomass is a good and cheap source of protein that can be used as additives for animal and fish feeds. Some microalgae species produce several valuable bioactive compounds with nutritional and pharmaceutical benefits such as polyunsaturated fatty acids (DHA, EPA), astaxanthin and carotenoids (Chojnacka et al., 2012; Yaakob et al., 2014).

Bioprospecting in various ecosystems including water could yet reveal microalgae with high lipid content and novel or unique bioactive compounds, however, the process is slow, tedious and expensive. Molecular fingerprinting techniques such as DGGE and T-RFLP have been successfully used in microbial ecological systems to identify areas with high diversity and abundance (Diez et al., 2001; Su et al., 2012). The application of molecular biology approaches holds the advantage of including uncultivable and previously undescribed phylotypes in the profiles, thus providing a more comprehensive picture of the microbial community (Siqueira et al., 2010). These techniques could be adapted to microalgae and combined with traditional methods for more efficient and improved bio-prospecting (Ebenezer et al., 2012).

One major hurdle facing the commercialization of microalgal biodiesel is the enormous quantity of fresh water and nutrients such as phosphates and nitrates necessary to cultivate these organisms at commercial scale (Dutta et al., 2016; Park & Lee, 2016). In arid and semi-arid countries such as South Africa with limited fresh water resources, diversion of water for drinking to energy production would be unfavourable and meeting the nutrient requirements at large scale would be prohibitively expensive. Wastewater is rich in macronutrients such as nitrates and phosphates required for microalgal growth and can meet this water requirements. Unfortunately, wastewater may also contain microorganisms, organic and inorganic compounds depending on the source and require treatment before discharge into receiving waterbodies. High cost of treatment, inadequately trained staff amongst other factors has led to the discharge of inadequately treated wastewater into the environment with potential socio-economic repercussions especially in developing countries (Naidoo & Olaniran, 2014).

Microalgae can be utilized as a cheap source of phycoremediation of wastewater saving fresh water resource cost while, the organic compounds in the wastewater can be economically harnessed for the cultivation of microalgae. Microalgae has also been reported to be able to utilize wastewater as a growth medium for biomass propagation and biodiesel production by various researchers simultaneously remediating the wastewater (Farooq et al., 2013; Gentili, 2014; Sun et al., 2013). With an attractive climate and plenty of sunlight all year round, South Africa is in a unique position to take advantage of

these organisms to guarantee her future energy needs (Mutanda et al., 2011a). This research thus aim to bio-prospect for indigenous strains of microalgae in aquatic habitats and wastewater treatment plants in KwaZulu-Natal province of South Africa and evaluate their potential for fuel production and wastewater remediation.

1.1 Hypotheses

It is hypothesized that various aquatic ecosystems including wastewater, rivers and estuaries in Durban, KwaZulu-Natal Province of South Africa contain microalgae with biotechnological relevance in biodiesel production. It is also hypothesized that these microalgae can utilize municipal pre-chlorinated wastewater for growth and biodiesel production while simultaneously remediating the wastewater.

1.2 Aims

The aims of this study were:

- To profile microalgal diversity in selected aquatic ecosystems including domestic wastewater treatment plants, fresh water, brackish water and marine environments using denaturing gel electrophoresis (DGGE) and Terminal restriction fragment length polymorphism (T-RFLP).
- To evaluate the potential of autochthonous microalgal strains to produce high amount of lipids rich in long chain polyunsaturated fatty acid (LC-PUFAs), monosaccharides and protein.
- To evaluate the potential of domestic wastewater effluent to support biomass production of selected autochthonous microalgal strains while simultaneously remediating the wastewater.

1.3 Objectives

The objectives of the study were:

- To bio-prospect for, and isolate novel microalgae strains with potential characteristics for biodiesel production.
- To evaluate and analyze microalgal diversity and phylogenetic relatedness of microalgae in the water samples using denaturing gradient gel electrophoresis and phylogenetic analysis.
- To identify and characterize isolates obtained from sampled sites using light microscopy as well as PCR amplification, sequencing and analysis of 18S rRNA gene.
- To qualitatively and quantitatively analyze long chain polyunsaturated fatty acids and biodiesel in lipids from autochthonous microalgal isolates.
- To carry out quantitative analysis of carbohydrate accumulation in microalgae for potential bioethanol production.
- To evaluate the total protein in microalgal biomass using Lowry assay.
- To evaluate the ability of autochthonous isolates to maintain stable biomass and lipid production in domestic wastewater effluent while, removing nutrients in the form of phosphates and Nitrogen.

The study is divided into six chapters with chapter one dealing with a general introduction into the subject matter and scope of research. It also highlights the hypotheses, objectives and aims carried out in the study. Chapter two critically reviews the literature on the subject matter whilst highlighting areas of future research. Chapter three deals with the evaluation of denaturing gradient gel electrophoresis (DGGE) and terminal restriction length fragment polymorphism (T-RFLP) as molecular tools to identifying aquatic ecosystems with high microalgal biodiversity for efficient bioprospecting. Chapter four deals with the bioprospecting for and isolation of microalgal strains as well as evaluation of microalgal biomass for potential biodiesel production. Investigation into the evaluation of wastewater as a growth medium for microalgal biomass propagation for biodiesel production and simultaneous nutrient removal are reported in Chapter five, while Chapter six summarises the main findings of the study and highlights the future development of the study.

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CHAPTER 2

Literature Review

The chapter has been ***published in Critical Reviews in Biotechnology***

(2016 Impact Factor 6.542)

Critical Reviews in Biotechnology

ISSN: 0738-8551 (Print) 1549-7801 (Online) Journal homepage: http://www.tandfonline.com/loi/ibty20

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To cite this article: Ejovwokoghene C. Odjadjare, Taurai Mutanda & Ademola O. Olaniran (2015): Potential biotechnological application of microalgae: a critical review, Critical Reviews in Biotechnology, DOI: 10.3109/07388551.2015.1108956

To link to this article: http://dx.doi.org/10.3109/07388551.2015.1108956

Published online: 23 Nov 2015.

Critical Reviews in Biotechnology

http://informahealthcare.com/bty
ISSN: 0738-8551 (print), 1549-7801 (electronic)

Crit Rev Biotechnol, Early Online: 1–16
 DOI: 10.3109/07388551.2015.1108956

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Potential biotechnological application of microalgae: a critical review

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Abstract

Microalgae are diverse microorganisms inhabiting a wide range of habitats with only a small fraction being cultivated for human use. Recently, interest in microalgal research has increased in the quest for alternative renewable fuels due to possible depletion of fossil fuels in the near future. However, costly downstream processing has hampered the commercialization of biofuels derived from microalgae. Several value added products of industrial, pharmaceutical and agricultural relevance could be simultaneously derived from microalgae during bioenergy production. Despite these value-added products having the potential to offset the high cost of downstream processing of renewable fuels, their production has not been explored in-depth. This review presents a critical overview of the current state of biotechnological applications of microalgae for human benefit and highlights possible areas for further research and development.

Keywords

Bioethanol, biofuel, nutraceuticals, transesterification, wastewater remediation

History

Received 22 March 2015
Revised 24 July 2015
Accepted 17 August 2015
Published online 19 November 2015

2.0 Introduction

Microalgae are unicellular, microscopic (2–200 mm), polyphyletic, non-cohesive, artificial assemblage of carbon dioxide (CO_2) evolving, photosynthetic organisms. They are mostly eukaryotic although the prokaryotic cyanobacteria (blue-green algae) are frequently classified as algae (Greenwell et al., 2010; Mutanda et al., 2011). Microalgae are capable of living in harsh environmental conditions as well as proliferate rapidly due to their unicellular and simple multicellular structure (Mata et al., 2010). They first appeared in the ocean about 3 billion years ago utilizing CO_2, the main component of the atmosphere through photosynthesis and replacing it with oxygen resulting in creation of the current composition of the atmosphere (Sumi, 2009). The oxygen produced by photosynthesis not only constituted the atmospheric oxygen but also raised the level of dissolved oxygen in seawater. Microalgae also form the bottom of the food chain and serve as food for zooplankton, which in turn serve as food for fishes with humans at the top of the food chain. Microalgae are not only found in the aquatic ecosystem but in all earth ecosystems including terrestrial, and have a large variety of species living in a wide range of environmental conditions (Mata et al., 2010). It has been estimated that there are about 200 000–800 000 species in existence, of which only about 35 000 have been described in the literature underscoring their incredible diversity (Cheng & Ogden, 2011; Ebenezer et al., 2012). Classification of microalgae has traditionally been conducted by pigmentation, life cycle, and basic cellular structures, however, molecular methods such as denaturing gel electrophoresis and next-generation sequencing are emerging techniques in detecting and classifying new species of microalgae (Brennan & Owende, 2010; Ebenezer et al., 2012; Mutanda et al., 2011).

Recently, there has been steady increasing interest in microalgae due to the world energy crisis, depleting world fossil-fuel reserves, increasing demands for alternative energy with low greenhouse gases, and discovery of a wide range of bioactive compounds with nutritional, cosmetic and pharmaceutical applications from microalgae. Most research is focused on the production of biodiesel, bioactive compounds for cosmetic and nutraceuticals, remediation of wastewater and biomass feeds for livestock (Huang et al., 2010; Tabernero et al., 2012; Song et al., 2013; Singh et al., 2014). The triacylglycerol (TAG) in microalgal lipid can be converted into biodiesel via a transesterification process by the reaction of TAG with methanol in the presence of an acid or alkali catalyst to form fatty acid methyl esters (biodiesel) and glycerol (Figure 1).

The saccharides in microalgal biomass can be used to produce bioethanol or biogas via fermentation or anaerobic digestion. Bio-hydrogen can also be produced via biodegradation of starch extracted from microalgae. The microalgal biomass is a good and inexpensive source of protein that can be used as additives for animal and fish feeds. Some microalgae species produce several valuable bioactive compounds such as polyunsaturated fatty acids, astaxanthin, carotenoids etc. The potential applications of microalgae

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biomass are presented in Figure 2. Since microalgae are widely distributed in the environment with a large number still unknown and uncharacterized, there are many potentially beneficial species with novel characteristics and bioactive compounds yet to be discovered. This review presents an overview of the current state of potential biotechnological application of microalgae while highlighting areas that require further research and innovation.

2.1 Microalgae as potential sources of bioenergy

The current depletion of global fossil-fuel reserves and increasing emission of greenhouse gases leading to global warming, melting of the polar icecaps and a rise in sea levels are of major global concern (Olguin, 2012; Sawaengsak et al., 2014). Hence, the increasing calls for alternative sources of fuel with low emission of greenhouse gases makes biofuels a very attractive option for energy consumers. Microalgal biomass can reach high productivity rates ($\sim 10\ g\ l^{-1}$ per day) and accumulate a high lipid content (over 50% of their dry weight) that can be converted into biodiesel (Mata et al., 2010; Yen et al., 2013). They can grow in fresh and marine waters, which make up two-thirds of the earth's surface. Thus, microalgal biomass can serve as an excellent alternative source to meet present and future fuel demands (John et al., 2011). Biofuels, fuels generated from biomass, offer new opportunities to: (i) diversify income and fuel supply sources; (ii) develop long-term replacement for fossil fuels; reduce the emission of greenhouse gases; (iii) boost the decarbonisation of transportation fuels; and (iv) increase the security of energy supply (Mata et al., 2010). The two most common and successful biofuels are biodiesel and bioethanol which are aimed at replacing mainly the conventional liquid fuels such as diesel and petrol. Biodiesel is produced from the transesterification of oil usually obtained from oil rich crops such as rapeseeds, palm, and sunflower and emit very low greenhouse gases. Bioethanol, an environmentally friendly renewable liquid biofuel, can be produced from various biomass feedstocks such as sugar or starch crops (sugar cane, sugar beet, corn and wheat) and from lignocellulosic biomass. Sugar cane is the main feedstock for bioethanol production in Brazil, while corn and sugar beet are the major sources in the United States and Europe, respectively (John et al., 2011). However, the use of crop plants and arable lands for energy production has generated concerns over the risk of food scarcity and price increase, which is already beyond the reach of less developed countries. This is why there has been increasing research on microalgae as an alternative renewable feedstock for biofuel production in order to avoid the food versus fuel conflict since they do not compete with food crop production for arable land (Sun et al., 2013; Talebi et al., 2013; Zheng et al., 2012). They can be grown on non-arable land including saline soil land or even desert land not being employed for crop cultivation (Sawaengsak et al., 2014). They can also be grown in an open pond or in a closed photobioreactor system utilizing wastewater as a growth medium (Sun et al., 2013). Other advantages of exploitation of microalgae over other feedstocks for biodiesel production include: high oil yields; production from carbon dioxide and sunlight only; high growth rate with a generation time of 24 h (Chen et al., 2009; Christopher et al., 2014; Rittmann, 2008).

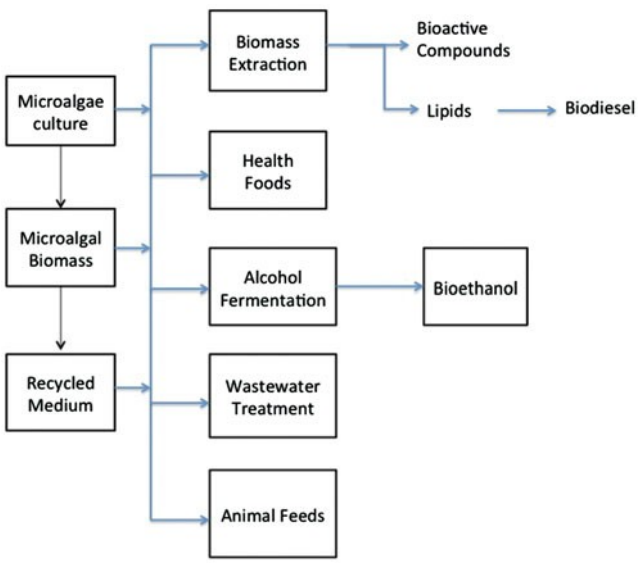
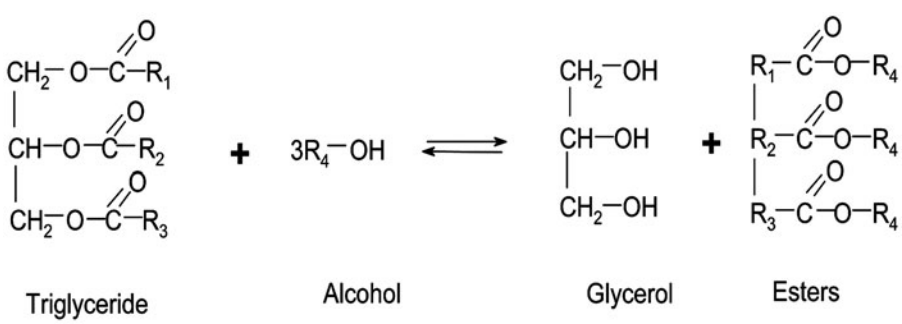


Figure 1. Transesterification reaction between triglyceride and alcohol in the biodiesel production.

2.1.1 Biomass production from microalgae

Large-scale microalgae biomass is usually cultivated using the open raceway pond or photo-bioreactor (Halim et al., 2012). Most commercial microalgae cultivation systems are carried out in open pond systems utilizing wastewater as a growth medium whilst using natural solar energy as a light source (Dassey et al., 2014). High rate ponds used in commercial algae production are typically operated at 20–40 cm in depth and mixed with paddle wheels. This depth allows for maximum penetration of sunlight. The total area of the pond could be up to 0.5 ha in size (Dassey et al., 2014; Malcata, 2011). The paddle wheel prevents the settling

Figure 2. Simplified flow diagram of microalgal biotechnology applications.



of the culture and has a shading effect (Rawat et al., 2013). Open ponds are generally regarded as more cost effective for microalgal biomass production compared to photobioreactors. The major drawbacks of open raceway ponds are the low biomass productivity compared to photobioreactors, ease of contamination and inability to control certain environmental factors such as rainfall, temperature humidity and evaporation (Ashokkumar & Rengasamy, 2012; Rawat et al., 2013; Zeng et al., 2011). Photobioreactors are closed systems generally used for culturing microalgae for high-value products such as pharmaceuticals that are difficult to grow as a monoculture in an open raceway pond. Photobioreactors may consist of a tubular column, flat plate or annular reactor configuration where culture conditions are tightly controlled. The photobioreactors can use sunlight or an artificial light source. This allows for a wider range of species cultivation and provides superior protection against contamination. The photobioreactor system requires constant carbon dioxide aeration and mixing of the culture, which is achieved by airlift, mechanical stirring or pumping (Rawat et al., 2013). Despite its advantage and obvious superiority, photobioreactors are not preferred due to the high capital expenditure and cost of operation. In large-scale biomass production, the selection of a cultivation system must take into account the intrinsic properties of the microalgae species to be cultivated. For example, as the physico-chemical properties of biodiesel are determined by the molecular structure of the constituent fatty-acid methyl ester (FAME), Do Nascimento et al. (2012) suggested that adequate fatty-acid composition of microalgae oil and the volumetric lipid productivity must be priority criteria for strains selection, to make viable the algae-based biodiesel industry. Table 1 shows commonly utilized microalgae feedstock for biomass production. Under laboratory conditions, microalgae are cultivated in suitably enriched media depending on their habitat and nutrient requirement (Mutanda et al., 2011). Antibiotics can be added to the media to prevent bacterial contamination however, prolonged exposure to antibiotics can have a negative impact on the physiology of microalgae (Abu et al., 2007; Hughes et al., 2013). Microalgae are usually harvested during the stationary phase of growth for maximal recovery of oil (Rawat et al., 2013). Harvesting of microalgal biomass is technically difficult due to their small size and association with epiphytes in dilute aqueous suspension. However, various microalgae harvesting techniques including flocculation, centrifugation, filtration, agglomeration and pressure dewatering have been developed (Halim et al., 2012). Of these processes, flocculation is considered the most promising for large-scale production of microalgae due to this process's low-energy requirements (Wijffels & Barbosa, 2010). During flocculation, microalgal cells adhere to one another to form heavy aggregates, which then settle out of suspension to become a concentrate (Uduman et al., 2010). The advantages and disadvantages of the different harvesting methods are shown in Table 2. Harvesting contributes 20–40% of the total cost of biomass production. Hence, development of a cost-effective and energy-efficient harvesting and dewatering technology is an active field of research that needs further development for an economically viable and sustainable microalgae based biofuel (Christopher et al., 2014; Halim et al., 2012; Liu et al., 2009).

2.1.2 Pretreatment of microalgal biomass for biodiesel production

Prior to lipid extraction, the microalgal concentrate is pretreated and dried to degrade the cell wall liberating the lipid and removing high moisture content. Water decreases the biodiesel yield during the transesterification reaction process thus, it is important to minimize the water content in the feedstock prior to transesterification (Atadashi et al., 2012). Various methods to remove the moisture content in microalgae concentrate include: freeze drying and heating at high temperature while, commonly used methods for disrupting the microalgal cell wall include: ultrasonication,

Table 1. Microalgae species with potential for use as feedstock in biomass production.

Microalgae Species	Biomass Productivity dry wt. ($g\,l^{-1}day^{-1}$)	References
Amphora sp. (Persian gulf)	0.16	Talebi et al. (2013)
Ankistrodesmus sp.	0.09	Talebi et al. (2013)
Chlamydomonas reinhardtii	0.05	Talebi et al. (2013)
Chlamydomonas reinhardtii	2.00	Kong et al. (2010)
Chlorella emersonii	0.29	Talebi et al. (2013)
Chlorella salina	0.17	Talebi et al. (2013)
Chlorella vulgaris	0.46	Talebi et al. (2013)
Chlorella vulgaris	0.015	Song et al. (2013)
Chlorella pyrenoidosa	0.25	Sun et al. (2013)
Chlorella protothecoides	2–7.70	Mata et al. (2010)
Chlorella pyrenedoisa	2.90–3.64	Mata et al. (2010)
Chlorella pyrenoidosa	0.05	Nautiyal et al. (2014)
Dunaliella sp. (Persian gulf)	0.12	Talebi et al. (2013)
Dunaliella salina (shariati)	0.05	Talebi et al. (2013)
Dunaliella salina (UTEX)	0.15	Talebi et al. 2013)
Scenedesmus sp.	0.10	Talebi et al. (2013)
Scenedesmus sp.	0.06	Guldhe et al. (2014)
Scenedesmus obliquus	0.004–0.74	Mata et al. (2010)
Scenedesmus obliquus	0.014	Song et al. (2013)
S. abundans PKUAC 12	0.11	Guo et al. (2013)
S. obliquus YSR01	0.08	Abou-Shanab et al. (2011)
S. obliquus YSR04	0.094	Abou-Shanab et al. (2011)
S. obliquus YSR05	0.083	Abou-Shanab et al. (2011)
S. obliquus YSW06	0.086	Abou-Shanab et al. (2011)
Spirulina platensis	0.04	Nautiyal et al. (2014)
Kirchneriella lunaris	0.02	Song et al. (2013)
P. tricornutum	0.017	Song et al. (2013)
Lyngbya kuetzingii	0.016	Song et al. (2013)
Isochrysis sphacrica	0.017	Song et al. (2013)
M. afer PKUAC 9	0.09	Guo et al. (2013)
Haematococcus pluvialis	0.06	Mata et al. (2010)
Monodus subterraneus	0.19	Mata et al. (2010)
Pavlova salina	0.16	Mata et al. (2010)
Skeletonema sp.	0.09	Mata et al. (2010)
Thalassiosira pseudonana	0.08	Mata et al. (2010)
Tetraselmis suecica	0.32–0.12	Mata et al. (2010)
Porphyridium cruentum	0.36–1.50	Mata et al. (2010)
Cryptothecodinium cohnii	10	Mata et al. (2010)
M. pussilum YSW08	0.11	Abou-Shanab et al. (2011)

Table 2. Advantages and disadvantages of techniques used for harvesting microalgal biomass.

Techniques	Advantages	Disadvantages	References
Filtration	Low cost, water reuse, high filtration rates attainable using tangential flow filtration; can be used for shear sensitive suspension.	Slow, membrane fouling and clogging, limited volume, cell damage; decreased concentration due to regular backwash of filter and energy intensive	Christenson & Sims, (2011); Danquah et al. (2009); Uduman et al. (2010)
Centrifugation	Rapid, easy, and efficient at laboratory scale	Energy intensive; exposure to high gravitational and shear forces can damage cell structure; expensive and time consuming when applied to large quantity; cell viability depends on specie type and method of centrifugation.	Uduman et al. (2010) Chen et al. (2011)
Gravitational sedimentation	Low cost, potential for water recycling; increased efficiency when combined with flocculation	Slow process; product deterioration; unreliable; Separation depends on cell density	Christenson & Sims, (2011); Uduman et al. (2010)
Flocculation	Can be applied to large volume; less expensive, low cell damage, can be applied to a wide range of species	Not suitable for harvesting marine microalgae; no water reuse, inefficient, potential to remove lipids, produces large quantity of sludge that increases difficulty in biomass dehydration. Highly dependent on the pH of the medium; size, and functional group of on microalgal cell wall; Chemicals such as aluminum and sulfates used for coagulation may lead to environmental pollution and biotoxicity	Chen et al. (2011); Christenson & Sims, (2011); Uduman et al. (2010)
Dissolved air floatation (DAF)	Low cost, proven at large scale	Requires flocculation pretreatment, water reuse and product value may be negatively affected due to chemical contamination	Greenwell et al. (2010); Uduman et al. (2010)
Foam fractionation	Small footprint, no addition of chemicals, can be used on large volume, relatively inexpensive, easy to maintain	Low yield due to inefficient flotation,	Csordas & Wang, (2004); Rawat et al. (2011)
Ozone fractionation	Small footprint, cell disruption because extraction occur simultaneously	Ozone generation is expensive, Loss of product	Rawat et al. (2011)
Micro-strainers	Easy operation, low construction cost, high filtration ratios	Highly dependent on cell concentration, smaller cells may undergo incomplete removal, difficulty in handling solids fluctuations	Rawat et al. (2011); Uduman et al. (2010)
Electrolytic flocculation	High efficiency (80–95%), inexpensive, safe, selective, environmentally friendly, versatile.	High energy input, increased temperature may damage systems, fouling of cathodes	Chen et al. (2011); Rawat et al. (2011)

autoclaving, bead milling, chemical lysis and microwaving. Lee et al. (2010) observed that the microwave oven method was the simplest, easiest and most effective method for lipid extraction from *Chlorella vulgaris*, *Scenedesmus* and *Botryococcus* sp. producing 10.0–28.6 g L^{-1} total lipid from all three species. Contrarily, Serive et al. (2012) evaluated several disruption techniques including: cryogrinding, soaking, homogenizing, bead beating, sonication, micro and mixer milling at laboratory scale on two biological microalgal models: *Phaedoactylum tricornutum* and *Porphyridium purpuruem* and observed that mixer milling technique gave the best results, offering access to a broad diversity of microalgal metabolites for a high throughput screening. Lee et al. (2012) suggested that mechanical disruption methods are highly energy inefficient when conducted under laboratory conditions and required a specific energy consumption of at least 33 MJ/kg of dry biomass. The specific energy consumption was found to be greater than the energy recoverable from microalgae and is also greater than the estimated minimum theoretical energy consumption required for feasibility by a factor of 10^5. The results of these researchers clearly show that no one extraction technique is suitable for all microalgae and suggests that further research and innovation is required for sustainable cell disruption and lipid extraction from microalgae.

Enzymatic conversion of microalgal biomass to methyl esters has the potential to exclude the dewatering step in the pretreatment process thus, saving cost. Two methods have been examined by Tran et al. (2012) for the conversion of microalgae to biodiesel: transesterification of extracted microalgal oil (M-I) and direct transesterification using disrupted wet microalgal biomass (M-II). In the M-II method, immobilized *Bukholderia* lipase attained a high methyl ester yield of 97% even though the water content was high. Nonetheless, the high cost of production will inhibit the use of lipases for commercial biodiesel production hence more research into cost effective lipases production are required. Olmstead et al., (2013) were able to extract 86% (w/w) of neutral lipids using a novel two-step process for disrupting concentrated paste (20–25% solids) of *Nannochlropsis* sp. cells. The highly resistant *Nannochloropsis* sp. cells were disrupted by incubation for 15 h at 37 ^{0}C followed by high-pressure homogenization at 1200 ± 100 bar. The cell rupture enabled recovery of up to 70% (w/w) of the total lipids and 86% (w/w) of neutral lipids using hexane, an extraction process requiring very low solvent to biomass ratios and performed at low temperature (35 ^{0}C). The process was shown to be effective on wet concentrated paste, required minimal solvent, moderate temperatures, and required no polar solvents.

2.2 Extraction of lipid from microalgae

2.2.1 Organic solvent extraction

Traditionally, lipid extraction from microalgal biomass is carried out using an organic solvent, thermal liquefaction or pyrolysis (Santana et al., 2012). However, supercritical fluid extraction (SFE) is an emerging green technology that has the potential to replace organic solvent extraction. During organic solvent extraction of lipids, the microalgal biomass is exposed to an eluting extraction solvent that extracts the lipid out of the cellular matrices. The mass of the extracted lipid can then be measured gravimetrically once the crude lipid has been separated from the cell debris, extraction solvent and water. Organic solvents used in microalgae lipid extraction include n-hexane, chloroform, diethyl ether, benzene and pure ethanol. Of these, chloroform and n-hexane are the most frequently used organic solvents (Halim et al., 2012; Rawat et al., 2013). The total lipids are extracted by mixing chloroform/methanol in different ratios (usually 1:2 [v/v]) with the disrupted microalgal biomass, transferred into a separation funnel and shaken for 5 min (Lee et al., 2010). Once the cell debris has been removed, more chloroform and water are added to induce biphasic partitioning. The lower organic phase (chloroform with some methanol) contains most of the lipids (both neutral and polar) while the upper aqueous phase (water and some methanol) constitutes most of the non-lipids such as proteins and carbohydrates (Halim et al., 2012; Robles Medina et al., 1998). Chloroform is highly toxic and thus not suitable for potential scale up of bioprocessing. However, hexane:isopropanol (3:2 v/v) has low toxicity and the potential to replace the chloroform/methanol system (Halim et al., 2011). The mixture works in a similar manner to chloroform but is more selective towards neutral lipids compared to chloroform/methanol (Halim et al., 2012; Lee et al., 1998). During biphasic separation, the upper organic phase (hexane and some propanol) contains most of the lipids (both neutral and polar) while the lower aqueous phase (water and some propanol) contains most of the non-lipids. Pure alcohol such as ethanol, butanol and isopropanol are mixed with non-polar organic solvents to ensure total extraction of lipids. This is because pure alcohol though inexpensive is volatile and has a strong affinity for phospholipids in membranes due to its ability to form hydrogen bonds thus, limiting interaction with neutral lipids globules (Halim et al., 2011).

2.2.2 Supercritical fluid extraction

Supercritical fluid extraction is a process whereby fluid is forced above its critical points (temperature and pressure) and is used for the extraction of compounds such as lipids. Above its critical point, the fluid possesses the properties of both liquid and gas (Herrero et al., 2006). Though the density of a supercritical fluid is similar to that of a liquid and its viscosity similar to a gas, its diffusivity is intermediate between the two states. Due to their low viscosity and high diffusivity, supercritical fluids have better transport properties than liquids, can diffuse easily through solid materials and can therefore give faster extraction yields (Herrero et al., 2006). Supercritical CO_2 is the most commonly used supercritical fluid, as it is non-flammable, non-toxic, inexpensive, relatively inert, and has moderate critical temperature (31.3 ^{0}C) and pressure (71 atm) (Herrero et al., 2006; Mendes et al., 2003). It can also be used for the extraction of thermolabile compounds without thermal degradation due to its moderate critical temperature. However, due to its low polarity, it is less effective in extracting polar lipids from microalgae and other natural matrices. To overcome this, polar solvents such as methanol or ethanol are added in various ratios as co-solvent.

Santana et al. (2012) examined the performance of supercritical CO_2 extraction of lipids from *Botryococcus braunii* for the production of biodiesel and concluded that decreasing temperature while increasing pressure resulted in higher lipid yield. Similar findings were previously reported by Halim et al. (2011). Current efforts in this area are geared towards simultaneous extraction and transesterification with supercritical CO_2 and methanol and the initial results showed that it has great potential (Paudel et al., 2015). Supercritical CO_2 extraction has several advantages over traditional methods for lipid extraction from microalgae including higher selectivity, shorter extraction times, and do not use toxic organic solvents (Sivasamy et al., 2009).

2.2.3 Pyrolysis

Pyrolysis is a phenomenon that involves decomposition of biomass under high temperatures (450– 550 ^{0}C) and oxygen deficient conditions. Pyrolysis is traditionally used for the production of bio-oil; biogas and bio-char from lignocellulose (Huang et al., 2010). Its application for lipid extraction from microalgae was first proposed in 1986, since microalgae contains higher amounts of cellular lipids, resolvable polysaccharides and proteins which are easier to pyrolize compared to lignocellulose (Huang et al., 2010). The production of liquid hydrocarbon mixtures from microalgae has been shown to have potential for large-scale application (Babich et al., 2011; Huang et al., 2010). Interestingly, Peng et al. (2001) have shown that the complete pyrolysis of the algae *Chlorella* resulted in high oil yields, above 40% on a dry biomass basis within a wide temperature range of 300 and 500 ^{0}C. The products of pyrolysis are usually mixtures of oxygenated components such as alcohols, ethers, aldehydes, ketones, phenols, esters, and acids (Babich et al., 2011). Fast pyrolysis has emerged as a new technology utilizing high heating rate (103–104 ^{0}C s^{-1}), short gas residence time and high temperature (450–550 ^{0}C) to degrade large organic compounds into short-chain molecules and be rapidly cooled to liquid (Huang et al., 2010; Pan et al., 2010). Pretreatment of the microalgae biomass with a catalyst such as Na_2CO_3 influences the process by shifting the decomposition temperature to a lower temperature but decreases the liquid yield while increasing the gas yield (Babich et al., 2011).

2.3 Microalgal lipid composition for biodiesel production

Microalgae lipids are mainly composed of free fatty acids (saturated and unsaturated), triacylglycerol (TAG) and hydrocarbons (Hama & Kondo, 2013). TAGs are the primary storage components energy reserves and are mainly produced as the metabolic rate of the microalgae slows down (Mairet et al., 2011; Singh & Gu, 2010). Characterization of lipids is important as different microalgae species produce different types of fatty acids. Some microalgae species are richer in neutral lipids than others (Lv et al., 2010), while some fatty acids are more suitable for transesterification to biodiesel than others. Most microalgal oils are rich in polyunsaturated fatty acids with four or more double bonds which are susceptible to oxidation during storage, thus reducing their desirability for use in a biodiesel (Chisti, 2008). Fatty acids with carbon chain lengths between 14 and 22 are recognized as the most common fatty acids contained in biodiesel and thus, considered desirable for production of good quality biodiesel (Demirbas, 2010; Zheng et al., 2012). The consensus view is that the most favorable biodiesel would have low levels of polyunsaturated and saturated fatty acids to decrease issues with oxidative stability and cold flow (Hoekman et al., 2012; Knothe, 2009; Song et al., 2013).

Thus, monounsaturated fatty acids of palmitoleic acid (16:1) and oleic acid (18:1) capable of giving the finest compromise between oxidative stability and cold flow are the most desirable lipid constituents for biodiesel production (Song et al., 2013). Abou-Shanab et al. (2011) reported that the major fatty acid composition of eight strains of microalgae studied were, linoleic acid (C18:3n3), palmitic acid (C16:0), linolenic acid (C18:3n3), oleic acid C18:1n9c), and g-linolenic acid while palmitoleic acid (C16:1) and eicosapentaenoic acid (C20:4) existed as minor fatty acids. Similarly, a study of 21 different species of algae isolated from the environment also reported that palmitic acid, oleic acid, linoleic acid and linolenic acid are the most abundant fatty acids (Do Nascimento et al., 2012). Certain factors such as specie growth cycle and nutrient limitation affect the composition of lipids accumulated in microalgae. For example, during the early growth phase of certain diatoms, large amounts of polar lipids and unsaturated fatty acids C16 and C18 are produced while, neutral saturated lipids 18:1 and 16:0 long chain fatty acids are the dominant lipids produced at the stationary phase (Rawat et al., 2013). Temperature and light also play a role in the type of lipid produced. For instance, the production of polyunsaturated C16 and C18 fatty acids, mono and di-galactosyl-diglycerides, sphingolipids and phosphoglycerides in *Euglena gracilis* and *Chlorella vulgaris* are enhanced by light, while synthesis of polyunsaturated C18 fatty acids in *Monochrysis lutheri*, and changes in the fatty acids composition in *Dunaliela salina* can be influenced by growth under low temperature conditions (Rawat et al., 2013).

2.3.1 Transesterification of microalgal oil to biodiesel

2.3.1.1 Chemical transesterification approach

Microalgal lipid can be transesterified to biodiesel using alkali or acid catalysts. Figure 3 shows a summary of the downstream processes required for biodiesel production. The alkali-catalyzed transesterification of oils and fats to biodiesel proceeds at faster rates and produces greater yields than the acid-catalyzed process (Christopher et al., 2014). To avoid yield losses from basic transesterification during the biodiesel production process, low acid value of the oil should be maintained. This is because the free fatty acid can produce soap in a parallel and undesirable reaction resulting in the consumption of the catalyst as well as a reduction in the efficiency of the catalyst. In addition, saponification leads to an increase in viscosity, forming gels, which make the subsequent separation process of biodiesel very difficult (Yan et al., 2014). Unfortunately, the acid value of microalgal oil is high, and it is recommended that an esterification step be performed to remove these free fatty acids (Tabernero et al., 2012). Esterification is an acid-catalyzed reaction in which the addition of an alcohol transforms the free fatty acids into alkyl-esters, producing biodiesel and water as reaction

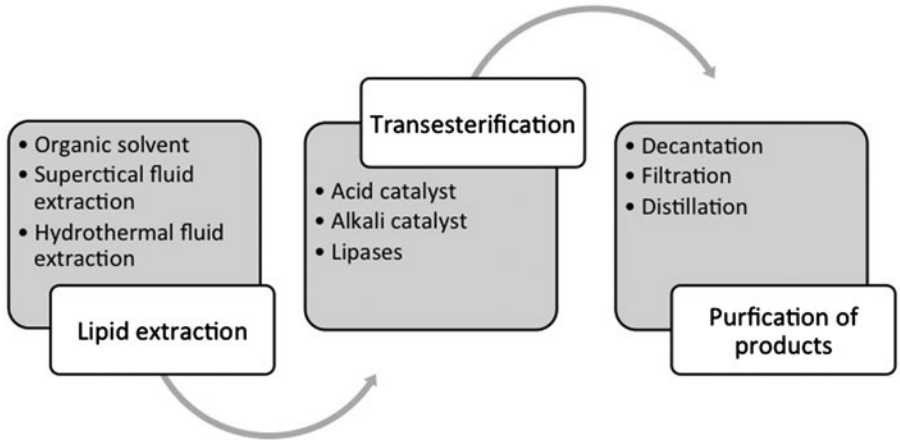


Figure 3. Downstream processing of microalgal lipid to biodiesel.

products. The oil is separated from the catalyst via centrifugation and transesterified using alkali catalysts such as potassium hydroxide (KOH). During transesterification of acylglycerols, the catalyst cleaves the ester bonds binding the fatty acids to the glycerol backbone (Chisti, 2008). The liberated fatty acids are

then reacted with methanol to form FAME. In laboratory scale experiments where only small amounts of crude microalgal lipids are available, a large amount of methanol in excess of stoichiometric requirement (6:1 molar) is used in order to drive the forward reaction and ensure quantitative transesterification (Stojkovic´ et al., 2014). The transesterification reaction kinetics in biodiesel production has been well studied by several authors (Darnoko & Cheryan, 2000; Komers et al., 2002; Vicente et al., 2005).

2.3.1.2 Enzymatic transesterification approach

Enzyme catalysts can be effectively used for low quality feedstocks with high free fatty acid content because they can catalyze both esterification and transesterification with resulting high yield and quality biodiesel (Atadashi et al., 2012). Several studies have shown that enzymes are highly efficient catalysts in the conversion of oil to biodiesel with purity ranging from 90 to 98% (Lai et al., 2012; Li et al., 2007; Tran et al., 2012). A comparison of different methods of transesterification is shown in Table 3. The enzymatic transesterification reaction is usually performed at a lower temperature compared to acid and alkali based reactions to prevent loss of enzyme activity (Gog et al., 2012). Optimum temperatures, determined for various lipases used in biodiesel synthesis, range between 30 ^{0}C and 55 ^{0}C (Iso et al., 2001; Thomas et al., 2002). Tran et al. (2012) reported an optimum temperature range of 25–40 ^{0}C when immobilized lipase originating from *Burkholderia* sp. was used to convert microalgal oil extracted from *Chlorella vulgaris* ESP-31. In enzymatic-catalyzed transesterification, the presence of water has insignificant effects on the conversion of triglycerides to biodiesel. However, water influences the activity of the enzymes in the surrounding region (Atadashi et al., 2012; Li et al., 2010). In an essentially non-aqueous medium, water plays several roles on the lipase-catalyzed transesterification reaction and has strong influence on the stability and catalytic activity of the lipase (Gog et al., 2012; Lu et al., 2009). Usually, some amount of water is required to sustain the activity of the enzyme in an organic solvent. Adding water to the reaction mixture could attenuate the resistance of mass transfer arising from the formation of glycerol, the main byproduct of transesterification (Tran et al., 2012). Accumulation of glycerol in the reaction mixture decreases the activity of immobilized lipase primarily due to an increase in mass transfer resistance. The effect of water on enzymatic transesterification has been extensively reviewed elsewhere (Atadashi et al., 2012). The high cost of lipase production due to the complex separation, purification and immobilization procedures is a major limiting factor to its application for commercial scale processes. The use of whole cell biocatalysts immobilized on a porous solid support using biomass solid particle (BSP) techniques developed by Atkinson et al. (1979) has been promoted as a potential low cost alternative (Gog et al., 2012). Immobilization of whole-cell lipase from filamentous fungi such as *R. oryzae* on reticulated polyurethane foam BSPs was found to yield a high conversion rate (90%) of soybean oil to biodiesel (Ban et al., 2002; Gog et al., 2012), however, whole cell immobilized lipases have not yet been tried on oil from microalgae. The major drawback of using immobilized whole cell lipase is a slow reaction rate attributed to inefficient mass transfer. This limitation can be mitigated by the use of packed bed reactors containing whole cell biocatalysts and can be operated under continuous operation conditions (Fukuda et al., 2009).

2.4 Microalgae as a feedstock for bioethanol production

Bioethanol currently accounts for more than 94% of global biofuel production, the majority (about 60%) coming from sugar cane (Balat et al., 2008) and 40% from other crops, with the US and Brazil accounting for the majority of bioethanol production worldwide (Dufey & Grieg-Gran, 2010). Bioethanol from biomass is commercially produced through fermentation or a thermochemical process known as gasification (Harun et al., 2010). The availability of feedstocks for bioethanol can vary considerably from season to season and depends on geographic locations. Microalgal biomass can be used as a potential alternative feedstock for bioethanol production and are gaining widespread research attention. This is because microalgae are highly efficient photosynthetic organisms; can accumulate high amount of starch and

Table 3. Comparison of enzymatic, chemical catalysis and non-catalyzed technology in biodiesel production modified from Fukuda, Hama Fukuda et al. (2008) and Taher, Al-Zuhair Taher et al. (2011).

Factors	Enzymatic process	Alkaline process	Acid process	Supercritical alcohol
Effect of free fatty acids	Not affected	Soap formation, hence requires pretreatment	Not affected	Not affected
Effect of water	Insignificant on lipid conversion, affect stability and catalytic activity of lipases	Soap formations, oil hydrolysis resulting in more soap formation	Catalyst deactivation	Increased FAME yield, decreased alkyl ester decomposition
Alcohol to oil ratio	Low (3:1)	Low (6:1)	High (30:1)	High (42:1)
Biodiesel yield	High, usually around 90%	High, usually 4 96%	High (490%) only for high alcohol to oil molar ratio, high catalyst concentration and high temperature	High (497 %)
Reaction rate	Low but can be sped up with the addition of organic solvent such as 1,4 dioxane, use of 1-propanol or 1-butanol as short chain alcohol.	High	Slower than alkaline processes	Very High
Glycerol recovery	Easy, high grade glycerol	Complex, low grade glycerol	Complex, low grade glycerol	Glycerol may undergo etherification which might affect fuel quality
Catalyst recovery and recycling	Easy, proven reusability but requires more study	Difficult, Partially lost in post processing, neutralized by acid	Difficult, the catalyst end up in the by-products, Not reusable	Not necessary
Energy cost	Low, Temperature: 20–50 ^{0}C	Medium, Temperature: 60–80 ^{0}C.	High, Temperature: 4100 ^{0}C	Very high Temperatures 350 ^{0}C
Catalyst cost	High	Low	Low but corrosion of equipment increases cost	Not necessary
Environmental impact	Low; does not require effluent treatment	High, require effluent treatment	High, require effluent treatment	Very low, does not require effluent treatment

cellulose that can be used for bioethanol production; does not compete with food crops for precious arable land and water, and contains no lignin, making saccharification an easier process (Yen et al., 2013).

Microalgae like *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Spirulina* are known to contain a large amount (4–50% of the dry weight) of starch and glycogen, useful as raw materials for ethanol production (Ho et al., 2013; Rodjaroen et al., 2007). Microalgal strains, *M. afer* and *S. abundans* isolated from coastal waters of Pearl River Delta, China by Guo et al. (2013) were successfully used for saccharification and subsequent fermentative bioethanol production. Saccharification of algal biomass resulted in the release of 10.752 g/l total sugars hydrolysate and 5.730 g/l of glucose hydrolysate from *S. abundans* biomass harvested in early stationary phase. Dilute acid and cellulase treated *S. abundans* biomass was identified to produce good feedstock for fermentative bioethanol production yielding 0.103 g of ethanol per gram of dry weight algae. Results showed that the highest bioethanol concentration yield of 7.20 g/L when the pretreatment step was performed with 15 g/L of microalgae at 140 ^{0}C using 1% (v/v) of sulfuric acid for 30 min. In terms of ethanol yield, 52% (g ethanol/g microalgae) maximum was obtained using 10 g/L of microalgae and 3% (v/v) of sulfuric acid under pretreatment at 160 ^{0}C for 15 min. The statistical analysis amongst the parameters investigated revealed that temperature is the most critical factor during acid pretreatment of microalgae for bioethanol production.

Microalgae store starch mainly in the cells and biomass can be harvested at regular intervals from photobioreactors or shallow raceway ponds for the extraction of starch. The starch can be extracted by disruption of the cells using mechanical disruption techniques (e.g. ultrasonic, explosive disintegration, mechanical shear, etc.) or by dissolution of cell walls using enzymes (Costa & de Morais, 2011). The starch is then separated by extraction with water or an organic solvent and fermented to ethanol using technology similar to other starch-based feedstock, involving two processes, saccharification and fermentation (Matsumoto et al., 2003; Rubin, 2008). Prior to fermentation, the starch is hydrolyzed to simple sugars in a process known as saccharification using enzymes (alpha and glucoamylase) or acid. In the next step, the sugars are fermented to ethanol by a suitable yeast strain. Both processes can be simultaneously carried out in a single step if an amylase producing strain can be used for ethanol fermentation. Utilization of starch degrading ethanol producers can preclude the cost incurred for acid or enzymatic saccharification of starch. Finally, the ethanol is purified by distillation to remove water and other impurities in the diluted alcohol product (10–15% ethanol). The concentrated ethanol (95% ethanol) is drawn off and condensed into liquid form, which can be blended with fossil fuels or directly used as fuel (John et al., 2011; Nigam & Singh, 2011). However, the commercial production of bioethanol from microalgae is not yet feasible and requires further investigation and development. This is mainly due to the inherent difficulty in upstream processing of microalgae from cultivation to carbohydrate extraction, which makes it uncompetitive with plant based bioethanol feedstock such as corn and sugar cane.

2.5 Microalgal remediation of wastewater and simultaneous biomass production

Wastewater contains high amounts of N and P, two of the most essential nutrients required for microalgal growth. Considering the huge demand for fresh water and the high cost associated with large-scale microalgal biomass production, the production of microalgal biomass as a by-product of wastewater treatment in high rate algal ponds (HRAPs) provides a promising alternative (Park et al., 2011). High rate algal ponds are shallow, open raceway ponds and have been used for treatment of municipal, industrial and agricultural wastewaters (Gendy & El-Temtamy, 2013). Extensive research has been conducted to explore the feasibility of using microalgae for the treatment of wastewater, especially for the removal of nitrogen, phosphorus, and chemical oxygen demand from effluents (Hameed, 2007; Hernandez et al., 2006). Munoz & Guieysse (2006) reported the reduction of several heavy metal contaminants including copper in wastewater by microalgae. Microalgal cultivation for wastewater treatment coupled with biofuel generation is an attractive option in terms of reducing the energy, nutrient and fresh water resource costs and CO_2 emissions (Cho et al., 2013; Farooq et al., 2013; Kong et al., 2010; Sun et al., 2013). Hence, it has the potential to overcome the current limitation of microalgal biodiesel production while simultaneously treating wastewater (Olguin, 2012). Different wastewaters with varying characteristics have been studied for microalgal cultivation. The total nitrogen (TN) and total phosphorus (TP) concentrations differ significantly depending on the wastewater source and the different strata of the wastewater treatment process (Wang et al., 2010). Typical concentrations of ammonia nitrogen and phosphates in secondary-treated wastewater fall into the ranges of 20–40 mg/L and 1–10 mg/L, respectively, which are adequate to support high productivities from most fresh water microalgae strains (McGinn et al., 2011; Olguin, 2012). The highest concentrations of TN and TP are found in centrate from domestic and agricultural wastewater such as poultry litter effluent, dairy manure and olive oil mill wastewater (Wu et al., 2014).

In recent years, there has been considerable interest in the combination of wastewater treatment and microalgal biomass production. Wang et al. (2010) recommended the centrate as the best source of municipal wastewater for simultaneous biomass production and nutrient removal. Li et al. (2011) revealed that centrate is rich in nutrients including phosphorus and ammonium, and organic matter reflected in a high chemical oxygen demand (COD) of 1300 mg/L. They showed that a *Chlorella* strain isolated from wastewater was able to reach a productivity of 0.92 g/L/d in bench scale continuous cultures. In batch culture, the *Chlorella* strain reached a biomass yield of approximately 1.1 g/L and biodiesel yield of 0.12 g/L of an algae culture. The FAME content was 11% of the dry biomass. These productivities are comparable or higher than previously reported for other fractions of municipal sewage (Olguin, 2012). Sun et al. (2013) evaluated wastewater from the fermentation effluent of a riboflavin (B2) manufacturing plant for simultaneous biomass production, and reduction of COD, ammonia-N and phosphorus using *Chlorella pyrenoidosa*. Their

results indicated that at optimal conditions of 250 photons m^{-2} s^{-1}, 28 ^{0}C and 5%, the removal efficiency of COD reached 89.2%, while TN and TP decreased by 64.52% and 82.20%, respectively. After a second round of treatment, the COD of the effluent was reduced to less than 100 mg/L. They also reported 1.25 g/L of harvested microalgal biomass and 38.27% (dry basis, wt.%) of lipid content.

Carbon dioxide is known to have a positive effect on the growth and lipid production of microalgae, increasing photosynthesis and biomass by up to 256% and 260%, respectively (Sutherland et al., 2015). Flue gas contains up to 15% CO_2 and can be available as an inexpensive source of CO_2. This presents an additional economic incentive to incorporate flue gas into wastewater as a growth medium to reduce the cost of biomass production while simultaneously reducing the emission of greenhouse gases. Numerous reports have shown this idea to be feasible however, levels of SOx and NOx in the flue gases should be low to moderate [~150 ppm] (Cheah et al., 2015; Ji et al., 2015; Wang et al., 2008). Gentili (2014) used a consortia of municipal, dairy, paper and pulp wastewater in different proportions incorporated with flue gas from a local power plant containing 10% CO_2 to simultaneously treat wastewater and produce biomass and lipids. Results showed that all microalgae strains used grew in all wastewater mixtures with *Selenastrum minutum* exhibiting the highest biomass and lipid yields of up to 37% of the dry weight. One major hurdle to the incorporation of flue gas with wastewater treatment is the high temperature and noxious components of flue gases. The tolerance of microalgae to relatively high temperatures is essential in reducing the cost of cooling the flue gas. This can serve as a selective pressure in achieving species control in the wastewater treatment pond. A detailed review of flue gas incorporation with wastewater for algal production can be found elsewhere (Wang et al., 2008).

Despite the high promise of cultivating microalgal biomass cheaply via HRAPs, more research is still needed to make this technique feasible for large-scale processing. Algal production in HRAPs is still limited by many inhibitory factors including environmental (light and temperature), operational (pH, CO_2, dissolved oxygen and nutrients) and biological (grazers and pathogens such fungi and viruses). Maintaining the desirable algal species in the HRAP is important to achieve predictable and reliable algal biomass production. However, it is difficult to maintain a unialgal culture due to the contamination in wastewater treatment HRAPs. Algal dominance and species interactions in HRAPs are still poorly understood and require further research (Park et al., 2011).

2.6 Role of microalgae in agriculture

Microalgae can be employed in agriculture as biofertilizers, soil conditioners and as feed supplements. Biofertilizers consisting of cyanobacteria, which have the capability of fixing nitrogen, are used in tropical lowlands for rice cultivation. Eukaryotic unicellular green microalgae are applied as soil conditioning agents to sprinkler-irrigated farmlands to control erosion in temperate climate zones (Shaaban, 2001). Use of microalgae as a soil additive has been shown to significantly improve root volumes, chlorophyll formation, dry weight of shoots and plant height (Shaaban, 2001). Improved germination, nitrate reduction potential and increased carotenoid accumulation in carrots cultivated after conditioning in 0.5% Algaminoplant®, a biostimulant from algae has been previously reported (Chojnacka et al., 2012). Use of microalgae as a soil additive is environmentally safe, can improve soil fertility, the plant nutrient status and reduce the cost of secondary and micronutrients required to obtain good yields.

The use of microalgae in aquaculture is not new and has been extensively reviewed in the literature (Guedes & Malcata, 2012). However, the use of microalgae as an animal feed is relatively recent and targeted mainly at the poultry industry because it has been discovered to improve skin color, shanks and egg yolk. Microalgal biomass rich in proteins and bioactive compounds such as polyunsaturated fatty acids (PUFAs) can be used as animal feeds or supplements to improve the nutritional value of animals. Use of microalgae rich in PUFAs such as eicosapentaenoic acid (EPA) and arachidonic acid (AA) as feed supplement in poultry lead to the accumulation of PUFAs in the eggs while addition of algal supplements to the diet of cows resulted in a higher concentrations of PUFAs in meat and milk (Sjors & Alessandro, 2010). In light of all these benefits, certain countries such as Poland and the EU in general have legalized or recommended the addition of algae as feed material in animal farming (Chojnacka et al., 2012). The phycoremediation potential of microalgae, CO_2 utilization, coupled with their application as animal feed supplements could result in a self-sustained farming where wastewater rich in nitrogen and phosphorus generated by animal farming could be used to simultaneously cultivate microalgae as feed for aquaculture or supplement for livestocks while remediating wastewater and reducing greenhouse gas emission (Chojnacka et al., 2012).

2.7 Microalgae as potential sources of bioactive compounds and value added products

Most microalgal research is geared towards the production of bioenergy compounds such as biodiesel but the expensive cost of biomass production and downstream processing remains a bottleneck to commercialization (Misra et al., 2014). However, microalgae are also potential sources of valuable bioactive compounds with a wide application in the pharmaceutical, cosmetic and nutritional industries. Bioactive compounds from microalgae include phycobiliproteins, polyunsaturated fatty acids (PUFAs), sterols, pigments, enzymes and toxins. In this section, an overview of valuable bioactive compounds that could potentially be obtained commercially from microalgae is discussed.

2.7.1 Phycobiliproteins

Phycobiliproteins are hydrophilic proteins bonded to phycobilins, a photosynthetic pigment and are mainly found in cyanobacteria and some red algae (Markou & Nerantzis, 2013). Based on their maximum UV-visible absorption spectra, phycobiliproteins are classified as phycocyanin (blue pigment), phycoerythrin (red pigment), and allophycocyanin (pale-blue pigment). The annual market for phycocyanin is estimated at US$5–10 million (Sekar & Chandramohan, 2008; Yaakob et al., 2014). The major

sources of phycobiliproteins include *Spirulina* sp., *Arthrospira platensis* and *Amphanizomenon floa-aquae. Spirulina* sp. is a source of c-phycocyanins and allophycocyanins and contains relatively low nucleic acid contents. It is also composed of 55–70% protein, 6–9% fat and 15–20% carbohydrate and is rich in minerals, fibres and pigments (Yaakob et al., 2014). *Arthrospira platensis* is mainly used for the production of phycocyanin, a pigment extensively used as a food colorant while *Porphyridium* is used for the production of erythrin, a fluorescent agent used in flow cytometers and immunoassay assays, in microscopy and DNA assays as non-radioactive markers (Sekar & Chandramohan, 2008). Phycobiliproteins are utilized commercially as natural dyes and fluorescent agents, and in the pharmaceutical sectors as antioxidants, anti-inflammatory, neuroprotective and hepato-protective agents (Eriksen, 2008). Due to its stability, phycocyanin is also used in cosmetic formulations (de Jesus Raposo et al., 2013).

2.7.2 Long-chain polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) consists of three or more double bonds on a fatty acid skeletal chain containing 18 or more carbon atoms, and they are classified into !-3 or !-6 forms depending on the position of the last double bond proximal to the methyl end of the fatty acid (Yen et al., 2013). Long-chain PUFAs includes fatty acids such as EPA (20:5 !-3), docosahexaenoic acid (DHA, 22:6 !-3), a-linolenic acid (ALA, 18:3 !-3), AA (20:4 !-6) and linolenic acid (18:2 !-6). Polyunsaturated fatty acids such as !-3 fatty acids are mainly derived from fish oil but due to the increasing concern of accumulation of toxic compounds in fishes, coupled with the peculiar smell, unpleasant taste and oxidative instability of fish oil, there has been increasing interest in sourcing these PUFAs directly from microalgae.

The annual global demand for EPA is~300 tons (Wen & Chen, 2003) while the naturally produced PUFAs was valued at US$7.2 billion in 2011 and expected to increase to US$13 billion by 2017 (Yaakob et al., 2014). Higher animals and plants lack the enzymes to synthesize some of the !-3 and !-6 fatty acids hence, they are referred to as essential fatty acids. Microalgae are the natural sources of these essential fatty acids, which can be purified to provide a high value food supplement. Some examples of PUFAs producing microalgae include: *A. platensis*, *Isochrysis galbana*, *Odontella*, *Porphyridium cruentum*, *Cryptothecodinium cohni* etc. Microalgae of the genus *Phaeodactylum* and diatoms are rich in EPA while *P. lutheri* has been shown to contain large amounts of DHA (Guedes et al., 2011). *Cryptothecodinium cohnii* is a non-photosynthetic, marine dinoflagellate producing DHA predominantly (da Silva et al., 2006). Nearly 30–50% of its constituent fatty acids are C22: 6 (n-3) fatty acid and no other PUFAs are present in excess of 1%. It is, thus easy to separate DHA from the fatty acid mixtures. For this reason, *Cryptothecodinium cohnii* is used for the commercial production of DHA (Yaakob et al., 2014).

Although microalgae show promise of large-scale production of PUFAs, only DHA is commercially produced from the microalgae. Docosahexaenoic acid is a major structural fatty acid in the grey matter of the brain, required for brain and eye development in infant and abundant in breast milk (Thiombiano-Coulibaly et al., 2003; Yaakob et al., 2014). Therefore, it is recommended that infant formula should be fortified with DHA (Martins et al., 2013). The global wholesale market for infant formula is estimated at US$10 billion while other agricultural products such as eggs and dairy products fortified with PUFAs such as DHA are emerging on market shelves (de Jesus Raposo et al., 2013). Docosahexaenoic acid helps the body to fight against diseases especially cardiovascular diseases while a recent study revealed the significant role of PUFAs in the immune system through peroxisome proliferator-activated receptors (PPAR-a and PPAR-g), and favorable effects of n-3 PUFA supplementation on allergic reactions and novel therapeutic strategies to treat eosinophilic disorders (Tanigai et al., 2012). A balanced uptake of EPA/AA, precursors to two structurally and functionally different and sometimes antagonistic eicosanoid compounds such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), can prevent eicosanoid dysfunctions and may be effective in treating a number of illnesses and metabolic disorder (Wen & Chen, 2003). Gamma linolenic acid (GLA) has the potential to lower low-density lipoproteins in hypercholesterolemic patients, alleviate the premenstrual syndrome and treat atopical eczema. GLA may attenuate the signs and symptoms of inflammatory diseases such as rheumatoid arthritis and atopic dermatitis (Yaakob et al., 2014). From both animal and human trials, dietary supplementation with GLA has been shown to modulate inflammation (Johnson et al., 1997; Yaakob et al., 2014).

2.7.3 Carotenoids

Carotenoids are organic pigments that are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, consisting of a class of more than 600 naturally occurring pigments synthesized by plants, algae, and photosynthetic bacteria. These richly colored molecules are the source of the yellow, orange, and red colors of many plants. Carotenoids play a key role in oxygenic photosynthesis, as accessory pigments for harvesting light or as structural molecules that stabilize protein folding in the photosynthetic apparatus (Herrero et al., 2013). They have also been reported to have immense pharmaceutical values (Del Campo et al., 2007).

2.7.4 Asthaxanthin

Astaxanthin is a keto-carotenoid with two asymmetric carbon atoms located at the 3 and 3′ position of the benzenoid rings on either end of the molecule (Danxiang et al., 2013; Naguib, 2000). It is a natural pigment used in cosmetics, nutrition and aquaculture of salmons. It is commercially derived from the microalgae *Hematococcus pluvialis* under environmental (salt, light intensity and temperature) and nutritional (nitrate and phosphorus) stress conditions (Cysewski & Todd, 2007; Olaizola, 2000). A detailed review of the effect of environmental and nutritional stress conditions on the accumulation of astaxanthin in *H. pluvialis* can be found in Markou and Nerantzis (2013). Several other genera such as *Chlorella*, *Chlorococcum* and *Scenedesmus* are also potential producers

of astaxanthin (Del Campo et al., 2004; Ma & Chen 2001). The commercial value of astaxanthin is US$2500 per kilogram dry weight with an annual global market valued at US$200 million; though 95% of this are synthetically derived (Bishop & Zubeck, 2012). Astaxanthin is linked to several health benefits including protection against lipid peroxidases, age macular degeneration, reduction of atherosclerosis and increased immune response (Cysewski & Todd, 2007; de Jesus Raposo et al., 2013). Astaxanthin has a higher antioxidant activity than vitamin E, lutein and tocopherol (Focsan et al., 2014). It has also been reported that astaxanthin possesses antihypertensive and neuroprotective potential and confer protection from gastric ulcer by activating antioxidant enzymes such as catalases, superoxide dismutase and glutathione peroxidase (Hussein et al., 2006; Kamath et al., 2008).

2.7.5 Beta-Carotene

Beta-carotene is a carotene of increasing demand with a wide variety of commercial applications such as food colorant, provitamin A (retinol) in food and animal feed, additives to cosmetics and multivitamin preparations and as a powerful antioxidant in health food products. It has a market value that has been estimated at US$261 million and is expected to reach US$334 million by 2018 at an annual growth rate of 3% (Yaakob et al., 2014). The microalgae of the genus *Dunaliella*, are natural producers of b-carotenes. b-carotene, a component of the photosynthetic reaction center in *Dunaliella*, is accumulated in lipid globules in the interthylakoid spaces of the chloroplast (Del Campo et al., 2007). Under stress conditions, *Dunaliella* sp. is known to accumulate high amounts of b-carotene consisting of all *trans*- and 9-*cis* isomers, and is highly halotolerant (Ben-Amotz, 2007; Hosseini Tafreshi & Shariati, 2009). It has been documented that b-carotene rich in *Dunaliela* powder increases plasma high-density lipoprotein-cholesterol in fibrate-treated patients while lowering plasma triglyceride levels (Shaish et al., 2006). In their evaluation of carotenoid extract from *D. salina* against cadmium induced cytotoxicity and transformed growth induced expression of muscle a-actin with rat liver cell lines. Lin et al. (2010) reported that b-carotenes decreased lipid peroxidation and also inhibited activation of hepatic stellate cells (HSC).

2.7.6 Other bioactive compounds

Other important bioactive compounds that can be potentially derived from microalgae in large quantities include chlorophyll, lutein, toxins, vitamins and b-1,3-glucan. The microalgae *Chlorella* sp. is known to produce b-1,3-glucan, a bioactive compound important in human health. It is routinely used in tumor immunotherapy since it can stimulate recovery of the bone marrow following chemotherapy. It has also been reported to inhibit antiviral activity in HIV infected patients and accelerate hematopoietic recovery in both sub-lethally and lethally radiated mice (Amparyup et al., 2012; Vetvicka et al., 2002). The world annual sale of *Chlorella* sp. is estimated to be over US$38 billion (Yaakob et al., 2014). Microalgae also synthesize toxins, a wide variety of which have not been explored but could potentially be useful in health and industries. Known microalgal toxins include ciguatoxin, a heat resistant toxin produced by the dinoflagellate *Gambierdiscus toxicus* (de Jesus Raposo et al., 2013), okadaic acid, a cytotoxin produced by *Prorocentrum lima*, *Dinophysis acuminata* and *Dinophysis acuta* (Hu et al., 2010; MacKenzie et al., 2005; Rundberget et al., 2007) and domoic acid, a neurotoxin produced by diatoms of the genus *Pseudonitzchia*, which causes amnesic shellfish poisoning (Trainer et al., 2012). Recently, Washida et al. (2006) isolated two novel compounds Karatungiol A and B from cultivated symbiotic marine dinoflagellate *Amphidinium* sp. Karatungiol was revealed to possess antifungal property against *Aspergillies niger* at 12 mg/disc and antiprotozoan activity against *Trichomonas foetus* at 1 mg/ml. Chlorophyll is a photosynthetic pigment found in all photoautotrophic organisms and has been shown to stimulate liver function recovery and increase bile secretion (Bishop & Zubeck, 2012). It also repairs cells, increases hemoglobin in blood and promotes rapid cell growth. Being a natural pigment, chlorophyll is used in the food industry as a colorant due to the increasing consumer demands for natural foods (Harun et al., 2010). Although chlorophyll is extracted from inexpensive sources such as grass and lucerne (alfalfa), conventional extraction techniques yield only 5%. Microalgae such as the cyanobacterium *Aphanizomenon* contains significant amount of chlorophyll (~1–2% dry weight) and could be used as alternative source of chlorophyll (Bishop & Zubeck, 2012). However, more research is needed to develop an efficient downstream process for purification of chlorophyll a and b from algae (Harun et al., 2010).

2.8 Conclusion

Microalgae are versatile and diverse microorganisms inhabiting a wide range of ecological habitats with only a small fraction of the total number of species exploited for human benefit. There are probably more algal species that have not been identified and characterized than those known. Most microalgal research over the decades has focused on the use of microalgae for biodiesel production. However, microalgae also produce high value products most of which can be beneficial to the pharmaceutical, nutritional and cosmetics industries but remain largely unexplored. The simultaneous use of microalgae in phycoremediation of wastewater and biomass production still requires further research and innovation to make the production of microalgal biomass economical. The ability of algae, or their products, to treat and prevent numerous types of human diseases such as viral infections, heart diseases and cancer will undoubtedly continually raise interest and investigations into their application and value. However, novel and innovative methods of upstream processing such as harvesting, moisture removal and metabolite extraction as well as downstream processing are required to make the exploitation of microalgae economically viable, sustainable and competitive. Bioprospecting in habitats not yet explored will probably reveal microalgae with novel properties and usefulness.

2.8.1 Declaration of interest

The authors report no conflicts of interest.

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CHAPTER 3

This chapter has been submitted to the Journal: Freshwater Biology

Microalgal diversity and community structure profiling of different aquatic ecosystems in Durban, South Africa using DGGE and T-RFLP

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Abstract

Microalgae are very diverse photoautotrophic microorganisms and there are an estimated 800,000 species with only a small fraction (≈70,000) described so far. In this study, DGGE and T-RFLP fingerprinting techniques were adapted to give an insight into the diversity of microalgae in various aquatic habitats in Durban, South Africa and to identify potential areas for use in bioprospecting for novel green microalgae. Water samples were collected from various aquatic environments including estuarine, marine and freshwater habitats. Both fingerprinting techniques applied were robust enough to profile the microalgal diversity and community structure of the different aquatic ecosystems studied. Results from this study indicate the presence of some microalgae species in more than one aquatic habitat or ecosystem. Enrichment of water samples confirmed the presence of different types of microalgae with varied morphology and sizes. Nile red screening of microalgae in the enriched water samples confirmed the presence of lipids making them a potential feedstock for biodiesel production. Microalgae classes; Trebouxiophyceae and Chlorophyceae were observed to be dominant in fresh water habitat, while those belonging to the class Ulvophycea dominated the salt water environment. At the brackish water habitat, microalgae belonging to the classes Trebouxiophyceae (*Chlorella minutissima*) and Chlorophyceae (*M. sturmi*) were dominant.

Keywords: Molecular tools; Microalgae; Bioprospecting, Aquatic, Ecosystems.

3.0 Introduction

Microalgae are described as eukaryotic, oxygenic, microscopic, autotrophic, unicellular microorganisms that exist solitarily or in chains and are typically found in diverse aquatic systems (Mutanda et al., 2011). The microalgal taxon represents rich biodiversity of microorganisms, characterized by different biological, ecological and functional traits (Guiry, 2012). The number of species are estimated at approximately 200 000 – 800 000, of which only about 70000 are described in literature (Cheng & Ogden, 2011; Odjadjare et al., 2017). In recent years, there has been increasing research and development of third generation biofuels based on microalgal biomass as a renewable feedstock. Attributes of microalgae for potential application in biofuel production include; high oil yields per surface area and time, non-competition for arable land or food market with crops, better economy with water and nutrients through recycling, wastewater bioremediation and carbon dioxide sequestration, thus mitigating the effect of climate change (Do Nascimento et al., 2012). The first step in microalgal biotechnology is bioprospecting, isolation and characterization of microalgae with desirable traits for biotechnological applications (Pereira et al., 2016). Traditionally, monitoring of microalgal community and ecological studies are based on morphological characteristics using light and electron microscopy. These methods however, are cumbersome, time consuming and require expertise in phycological taxonomy and high-resolution microscopes to detect morphological differences and identify microalgae (Ghozzi et al., 2013; Godhe et al., 2007). Many algal species cannot be cultivated due to poor understanding of their cultivation and growth requirements. Other disadvantages of traditional methods include; loss of pigmentation, cell shrinkage and detachment of distinctive flagella. In addition, some species are known to undergo seasonal variation in morphology and at clonal level making ecological studies based on morphological specie concept more difficult (Kaggwa et al., 2013; Stoyneva et al., 2007).

Molecular approaches are rapid, easier and very effective for the identification and ecological studies of microalgae, especially for the pico-sized fractions that have very few morphological features that can be used for identification. Molecular techniques including denaturing gradient gel electrophoresis

(DGGE), terminal restriction fragment length polymorphism (TRFLP), random amplification of polymorphic (RAPD) and Next generation sequencing have been successfully used to analyze biodiversity of phytoplankton communities in marine environments as well as eukaryotic diversity in anoxic environments (Savin et al., 2004). These DNA-based methods have been developed for the taxonomic identification of species or strains of microalgae and are often used for phylogenetic analyses (Díez et al., 2001; Ebenezer et al., 2012) and has led to the discovery, identification and classification of many previously undescribed microalgae in the environment (Buchanan et al., 2013; Díez et al., 2001; Ebenezer et al., 2012; Joo et al., 2010).

Research into microalgal diversity not only provide insights into their community structure, biodiversity and abundance of phytoplankton species in various aquatic habitats including underexplored habitats, it could also help to streamline future bioprospecting to areas with high algal biodiversity. This would allow for bio-prospecting and characterization of previously unknown microalgae for potential biotechnological applications, including the production of biofuels, bioactive compounds and other valuable added products. This is the first study out of South Africa that provides a holistic view of the community structure and biodiversity of microalgae species in various aquatic habitat using DNA fingerprinting approach. Thus, in this study, DGGE and T-RFLP fingerprinting techniques were adapted to give overview of the diversity of microalgae in various aquatic habitats and identify areas in Durban, South Africa for potential bioprospecting for microalgae of biotechnological relevance.

3.1 Materials and Methods

3.1.1 Sample collection

Water samples (freshwater, brackish and seawater), irrespective of any visual evidence of the presence of microalgae, were collected from various aquatic sources in and around Durban, KwaZulu-Natal province of South Africa (Table 1) from May to July 2015. The grab sampling technique was used for sample collection into a clean 10 L plastic container. The plastic container was washed with a laboratory detergent, thoroughly rinsed with distilled water and disinfected with 70% ethanol before use. During sampling, the containers were rinsed twice with the water sample before collection. The samples were placed in icepacks, transported to the laboratory and processed within 24 h of collection.

Table 1: Sampling points, habitats and GPS coordinates of various sites sampled.

Sampled Point	Type of Habitat	Coordinate
NWWTW (B.C)	Freshwater	29.796011 S, 30.995920 E
NWWTW (D.P)	Freshwater	29.805613 S, 31.000624 E
PWWTW (B.C)	Freshwater	29.678156 S, 31.032978 E
Blue Lagoon	Brackish water	29.811604 S, 31.036894 E
Indian ocean	Salt water	29.813923 S, 31.041421 E
Umdloti River	Freshwater	29.647596 S, 31.118729 E
Tongaat River	Freshwater	29.645719 S, 31.122473 E
La Mercy Estuary	Brackish water	29.648042 S, 31.129976 E
MWWTP	Freshwater	30.165414 S, 30.781282 E
Amanzimtoti River	Freshwater	30.075529 S, 30.869149 E
Umgeni River (U.S)	Freshwater	29.808901 S, 31.000624 E
Umgeni River (D.S)	Freshwater	29.813923 S, 31.041421 E

NWWTW -Northern Wastewater treatment Works, PWWTW- Phoenix wastewater treatment works, MWWTP-Magabeni wastewater treatment plant, B.C- Before chlorination, D.P- Discharge Point, U.S- Upstream, D.S- Downstream

3.1.2 Metagenomic DNA extraction

Depending on water turbidity, 2 to 8 L of water samples were filtered through a 0.45 μm pore filter membrane prior to DNA extraction. In turbid water samples with large particles such as wastewater effluent, the water was pre-filtered through Whatman grade 1 filter paper with pore size of 11 μm (GE Healthcare, Buckinghamshire, UK). Metagenomic DNA was then extracted from the membrane filter using MoBio Power water kit (MoBio, Carlsbad, CA, USA) as per manufacturer's instruction and stored at -20 °C until further analysis. The quality of the DNA was checked using NanoDrop 2000c (ThermoFischer Scientific, Waltham, MA, USA).

3.1.3 PCR amplification of 18S and 23Su rRNA genes

Polymerase chain reaction was carried out using three sets of microalgae specific primers (Table 1). The *Chloro* and *Baci* primers amplifies a region of the 18S rRNA subunit of the ribosome was previously described by Moro et al. (2009). The primer set *Chloro* was used to target green microalgae while the primer set *Baci* was used to amplify the Bacillariophyceae in the metagenome. The 23Su primers were used to target 23S rRNA region of the chloroplasts in Cyanobacteria (del Campo et al., 2010). A GC clamp was attached to the 5′ end of each forward primers (Muyzer et al., 1993). The PCR reaction mixture (50 μL) consisted of 1× buffer, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM of each dNTP and 2 U of SuperTherm *Taq* polymerase (SepSci, Johannesburg, South Africa). The reaction was carried out in an automated T100 thermocycler (Bio-Rad, Hercules, CA, USA) at the following temperature regime: first cycle at 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 60 s, annealing for 60 s at temperatures shown in Table 2, extension at 72 °C for 60 s; and a final extension at 72 °C for 10 min. Amplified DNA (5 μL) was then electrophoresed in a 1.5% agarose gel at 60V for 40 min. Thereafter, the gel was stained in 1% ethidium bromide for 10 min and visualized under UV light using the gel documentation system (Syngene, Cambridge, UK). The amplified DNA was then used for DGGE analysis.

Table 2: Nucleotide sequences and annealing conditions of the PCR primers used in this study.

Primer set	Primer Sequence (5' to 3')	Annealing Temp (°C)	Expected product size (bp)
ChloroF	TGG CCT ATC TTG TTG GTC TGT	60	473
ChloroR	GAA TCA ACC TGA CAA GGC AAC		
BaciF	AGA TTG CCC AGG CCT CTC G	66	516
BaciR	CCA TCG TAG TCT TAA CCA TAA AC		
23SuF	AGGGGTAAAGCACTGTTTCG	55	800
23SuR	CCTTCTCCCGAAGTTACG		

3.1.4 Denaturing gradient gel electrophoresis

DGGE analysis was performed on a DCode^{TM} Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California, USA) per manufacturer's instructions. In summary, samples containing approximately 800 ng of PCR amplicons were loaded onto 6% (w/v) polyacrylamide gels (37.5:1, acrylamide/bis-acrylamide) in 1× TAE buffer using a denaturing gradient ranging from 30 to 60% (100% denaturant solution contained 7 M urea and 40% (v/v) deionized formamide (Merck, Darmstadt, Germany)). Electrophoresis conditions were set at 60V for 16 h, after which the gel was stained in ethidium bromide for 30 min and de-stained in 1× TAE buffer for 15 min. The gel was then visualized under UV trans-illumination using the gel documentation system (Syngene, Cambridge, UK). The presence, intensity and patterns of the bands were analyzed using GelCompar II Bionumerics software Version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram was generated by cluster analysis of band matching pattern with band matching position tolerance set at 1% and optimization at 0.5%. Bright bands were excised and DNA eluted by immersing in elution buffer overnight (ThermoFisher Scientific, Waltham, MA, USA). The eluted DNA was re-amplified using primers previously described but without the GC clamp and sequenced at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The sequences were edited with Chromas Ver. 2.2.4 (Technelysium Pty Ltd, Brisbane, Australia) and compared against the NCBI non-redundant database using the basic local alignment search tool (BLAST) to reveal their identity. The phylogenetic relationship between

sequenced bands and sequences of microalgae obtained from GenBank database, some of which are known biodiesel producers was determined using Mega7 software (version 6.7).

3.1.5 Terminal restriction fragment length polymorphism

Both Chloro-F and Chloro-R primers were fluorescently labeled and used in the amplification step prior to T-RFLP analysis following the procedures previously described by Joo et al. (2010). The forward primer was labeled with phosphoramidite fluorophores 5-carboxy-fluorescein (FAM), while the reverse primer was labeled with phosphoramidite fluorochrome 6-carboxy-2′4′7′,7-hexachlorofluorescein (HEX). In each PCR reaction, 2 µL of extracted metagenomic DNA from the environmental samples was added to 23 µL of the amplification mixture, to final concentrations of 1× Buffer, 1 mM of $MgCl_2$, 0.2 mM of dNTPs mix, 0.4 µM of each primer and 2 U of Supertherm *Taq* polymerase (Sepsci, Johannesburg, South Africa). Thermal cycling conditions were as follows: an initial denaturation at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 60 s; annealing at 60 °C for 60s; elongation at 72 °C for 60 s; and a final extension step at 72 °C for 10 min. PCR products were purified using GeneJet PCR Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) and double-digested (10 µl) using restriction enzymes, *Hinf1* (ThermoFisher Scientific, Waltham, MA, USA) and *CviK-1* (New England BioLabs, Ipswich, MA, USA) in a 30 µL reaction with a final concentration of 1 U of each restriction endonuclease and 1× Tango Buffer (ThermoFisher Scientific, Waltham, MA, USA). The reaction was incubated at 30 °C for 60 min (Bio-Rad T100 Thermal cycler). The restriction enzymes were selected manually according to computer simulation that produced diverse T-RF lengths less than 500 bp with a minimum of 33 bp using SnapGene viewer version 3.1.2 (GSL Biotech LLC). Capillary gel electrophoresis and sequencing was done at Inqaba Biotechnical Industries Pty Ltd, Pretoria, South Africa with internal size standard ranging between 35 and 500 bp which covered most of the major peaks. T-RF profiles peaks were analyzed by Genemapper software (Van Mierlo Software Consultancy, Netherlands).

3.1.6 Enrichment of water samples, morphological confirmation and Nile Red staining of autochthonous microalgae

Water samples from the various aquatic ecosystems was enriched using appropriate enrichment medium. Commercial enrichment media used were: BG-11 for wastewater and freshwater samples, F2 medium for marine and brackish water samples and Bold basal medium (BBM) for wastewater and freshwater samples. All media were purchased from Sigma Aldrich (Germany). Trace metals for BG11 was prepared per standard protocols (Mutanda et al., 2011). The F2 medium was supplemented with 30% sodium chloride. The sample (15 ml) was inoculated into an Erlenmeyer flask containing 235 ml of sterile appropriate enrichment broth to which 50 mg/ml of Erythromycin was added. The flasks were sealed with cotton wool and placed under UV illumination (54.36 μmol/m^2s^{-1}) with shaking at 180 rpm for 14 to 21 days at 30 °C, with a 12:12 h light: dark cycles until visible cells were observed. After incubation, the mixed culture was serially diluted and viewed under light microscope. Nile red staining of enriched cells was done according to method previously described by Chen et al. (2011). A mixture of 100% DMSO (50 μl) and 5 μl of standardized algal cell suspension (OD_{760} 0.06) were mixed and heated in a microwave for 60 s (oven power 1350 W). Thereafter, 10 μl of Nile red stain (Sigma Aldrich, Germany) was added to the mixture and the volume brought up to 1 ml with distilled water. The mixture was heated in microwave oven for 60 s, incubated in the dark for 10 min. Air dried wet mount were then prepared and viewed under fluorescent microscope at 580 nm (Nikon, Tokyo, Japan).

3.2 Results and Discussion

The present study profiled microalgal diversity in various aquatic habitats including freshwater, brackish water, wastewater and saltwater in Durban, South Africa, using both DGGE and T-RFLP fingerprinting techniques, in order to gain an insight into the biodiversity, community structure and identity of dominant microalgae present in the region. The community profile was obtained directly from the total DNA extracted from the water sample using 18S primers capable of targeting microalgae while discriminating against other eukaryotic and prokaryotic DNA present in the metagenome (Moro et al., 2009). The classes Trebouxiophyceae, and *Chlorophyceae* were observed to be dominant in fresh water habitat, while microalgae belonging to the class *Ulvophycea* was observed to be dominant in the salt water environment. At the brackish water habitat, microalgae belonging to the classes Trebouxiophyceae (*Chlorella minutissima*) and Chlorophyceae (*M. sturmi*) were dominant (Table 3). Amplification of the microalgae in the class Bacillariophyceae were unsuccessful probably due to unfavorable conditions for growth as no bloom was observed at the sites sampled or the presence of robust silicate valves resulting in difficulty in DNA extraction (Bérard et al., 2005). In a similar study by Yu et al. (2015), Bacillariophyceae (diatoms) were also not detected using the PCR-DGGE method, highlighting the difficulty in studying the diversity of this class using molecular techniques.

3.2.1 Diversity of microalgae in aquatic ecosystems in Durban

The community structure and phylogenetic relationship of microalgae from 12 sampling sites in Durban, South Africa, based on DGGE analysis, is presented in Figure 1. The DGGE profile obtained vary across all sampling points, assuming that each band represents a different operational taxonomic unit (OTU). Certain bands appear to be common to some of the sampled points suggesting a common species at these points. The most microalgae diversity was observed in freshwater systems with 10 OTUs (each) recorded at the Umdloti river and Phoenix maturation pond (June 2015), while the lowest diversity was observed in brackish water system with 2 OTUs (each) observed at the Blue lagoon and La Mercy estuary in May 2015. This finding supports previous report of reduced algal diversity in brackish water systems compared to freshwater and seawater (Flöder & Burns, 2004). The reduction in

diversity may be attributed to frequent shift in salinity (from low to high) in brackish water (Buchanan et al., 2013).

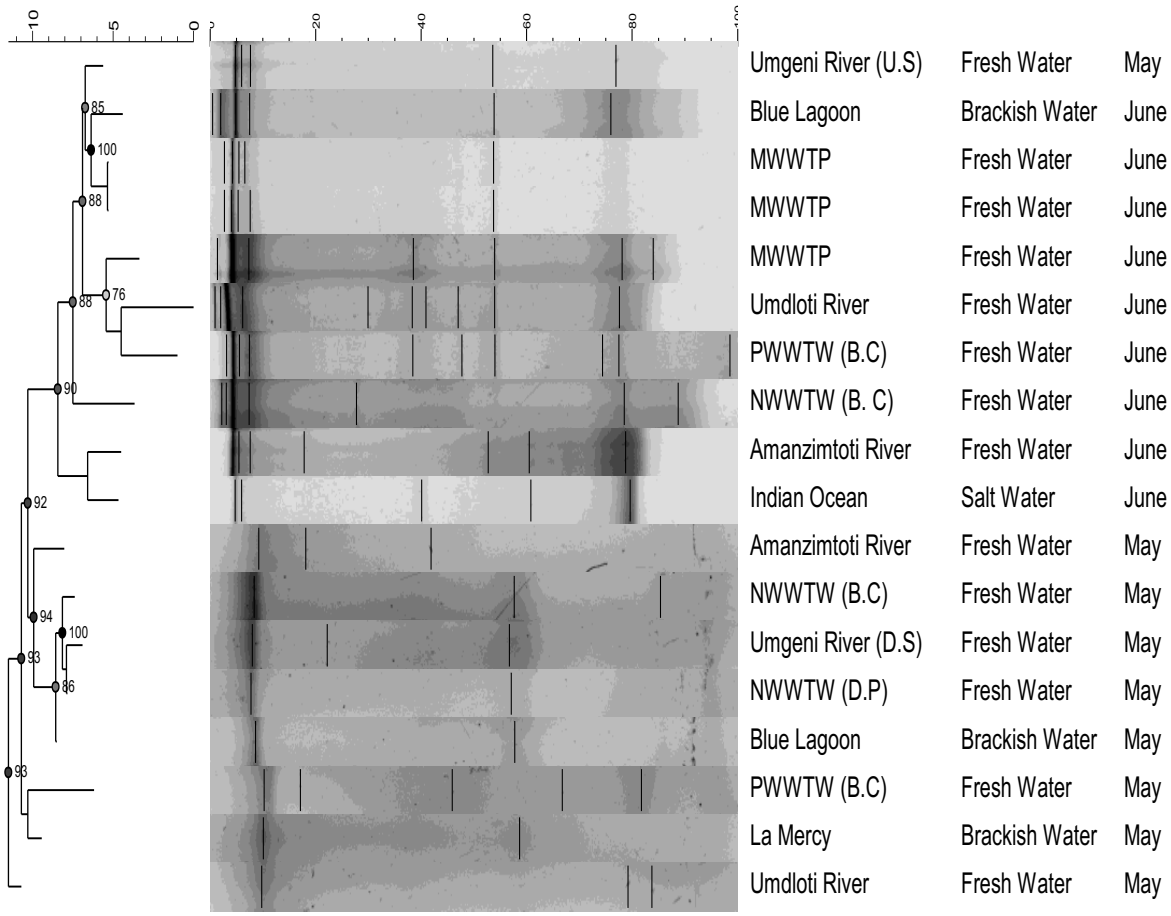


Fig. 1: Denaturing gradient gel electrophoresis showing the community profile of microalgae in various aquatic habitats sampled in KwaZulu-Natal province of South Africa. Community profiling of the various aquatic ecosystems and cluster analysis was performed using neighbor joining similarity coefficient. Each node represents a sample and nodes connected at the top of the figure are more closely related in terms of community structure than nodes connected at the bottom of the tree. NWWTW - Northern Wastewater Treatment Works, PWWTW- Phoenix Wastewater Treatment Works, MWWTW- Magabeni Wastewater Treatment pond, U.S- Upstream, B.C - Before Chlorination, D.S – Downstream

In general, maturation ponds of wastewater treatment plants (WWTPs) was observed to contain more OTUs compared to other aquatic ecosystems. A shift in community structure was observed at the maturation ponds of WWTPs and in rivers over time. For example, between the months of May and June 2015, the number of OTUs represented by visible bands obtained at the Blue lagoon increased from 2 to 6, while an increase from 3 to 10 OTUs was recorded at the Umdloti River. A similar trend was also observed at the Amanzimtoti River and the maturation pond of the Northern WWTW, with an increase in OTUs from 3 to 7. This suggests that season and other environmental factors may play a role in the community structure and diversity of microalgae at the sampling sites as previously reported (Yu et al., 2015). Interestingly, the microalgal community profile varied along the course of rivers, with more OTUs (7) observed upstream of Umgeni River in May 2015 compared to downstream (3), suggesting a higher microalgal diversity upstream. The Umgeni and Umdloti river has been previously reported to have varying levels of physicochemical parameters such as pH and turbidity as well as varying amount of nutrients (NO_3, NH_4 and PO_4) along the course of the rivers ranging from 0.58 mg/L to 1 mgL^{-1} for PO_4, 0.75 mgL^{-1} to 3.25 mgL^{-1} for NO_3 and < 0.5 mgL^{-1} to 1 mgL^{-1} for NH_4. These high levels of physicochemical and nutrient variation in the river have been attributed to increased and decreased flux in the river systems caused by discharge of treated effluents, season and storm runoffs (Olaniran et al., 2014) and could explain the variation in diversity observed.

Neighbor joining cluster analysis show a high similarity index amongst the various sites sampled suggesting a high similarity profile of microalgae community structure in the various aquatic ecosystems (Fig. 1). The Umdloti river and maturation pond of the phoenix wastewater showed 76% similarity index with the Magabeni wastewater treatment pond. In the month of May, the discharge point of the Northern wastewater treatment works (D.P) and downstream of the Umgeni River (D.S) showed a high similarity profile (100%) with the maturation pond of the NWWTW (B.C). Excision of dominant bands from the gel, PCR amplification of the 18S rRNA gene and sequencing revealed the identity of the dominant microalgal species at the

different sampling site. *Monactinus sturmi* was observed to be dominant in all types of aquatic ecosystem studied. This organism has reportedly been found in in-land water ways in India, Kenya and Germany suggesting its ubiquity in the aquatic ecosystems (Mrutyunjay et al., 2014). *Chlorella minutissima* was dominant in Brackish, freshwater and wastewater systems, *Neochloris aquatica* was dominant in Brackish and wastewater systems while *Picochlorum* and *Nanochloropsis* species were dominant in freshwater and wastewater systems. At the marine ecosystem, *Oltmannsiellopsis* sp*.,* a marine flagellate, was dominant (Table 3).

Table 3: Distribution of the dominant microalgal species in the studied aquatic habitats of KwaZulu-Natal province of South Africa.

Sampling point	*M. sturmi*	*C. minutissima*	*N. aquatica*	*Picochlorum sp.*	*Nannochloris sp.*	*C. cerasiformis*	*C. eugamestos*	*P. oviforme*	*H. pluvialis*	*S. similis*	*Oltmannsiellopsis sp.*
NWWTW (B.C)	-	-	-	+	-	+	+	+	+	-	-
NWWTW (D.P)	+	-	+	-	-	-	-	-	-	-	-
PWWTW (B.C)	-	+	+	-	-	-	-	-	-	-	-
Blue Lagoon	+	-	+	-	-	-	-	-	-	+	-
Indian ocean	+	-	-	-	-	-	-	-	-	-	+
Umdloti River	-	+	-	+	+	-	-	-	-	-	-
La Mercy Estuary	-	+	-	-	-	-	-	-	-	-	-
PWWTW (B.C)	-	-	+	-	+	-	-	-	-	-	-
MWWTP 1	-	-	-	-	+	-	-	-	-	-	-
MWWTP 2	+	-	-	-	+	-	-	-	-	-	-
MWWTP 3	+	-	-	-	-	-	-	-	-	-	-
Amanzimtoti River	-	-	-	-	+	-	-	-	-	-	-
Umgeni River (U.S)	+	-	-	-	-	-	-	-	-	-	-
Umgeni River (D.S)	-	+	-	-	-	-	-	-	-	-	-

+ indicates presence of microalgae, - indicates absence of microalgae

The phylogenetic relationships amongst the identified dominant microalgal species and some important microalgal species found in the NCBI GenBank database, based on their partial 18S rDNA sequence, are presented in Figure 2. Phylogenetic analysis revealed a high homology between the dominant microalgae species in aquatic environments of Durban, KwaZulu-Natal province of South Africa and other microalgae species known to produce biodiesel and other value added products (Bishop & Zubeck, 2012). For example, *Oltmannseillopsis* sp., a microalga dominant in the marine environment of KwaZulu-Natal (Table 3) is closely related to *Chlorella* sp. and *Neochloris aquatica*., species documented to be good sources of lipids for biodiesel production (Ji et al., 2015; Mutanda et al., 2011). Extracts from *Chlorella* have been used for face, skin and hair care products, as well as in sunscreen products in the cosmetics industry (Martins et al., 2014). This indicates that the sites sampled in this study could be suitable for prospecting hyper-lipid producing microalgae or microalgae with novel characteristics and capable of producing bioactive compounds. Information on microalgal diversity, community structure and dominant species can enhance the possibility of choosing the most appropriate species and enrichment conditions instead of the *ad hoc* cultivation system (Barra et al., 2014).

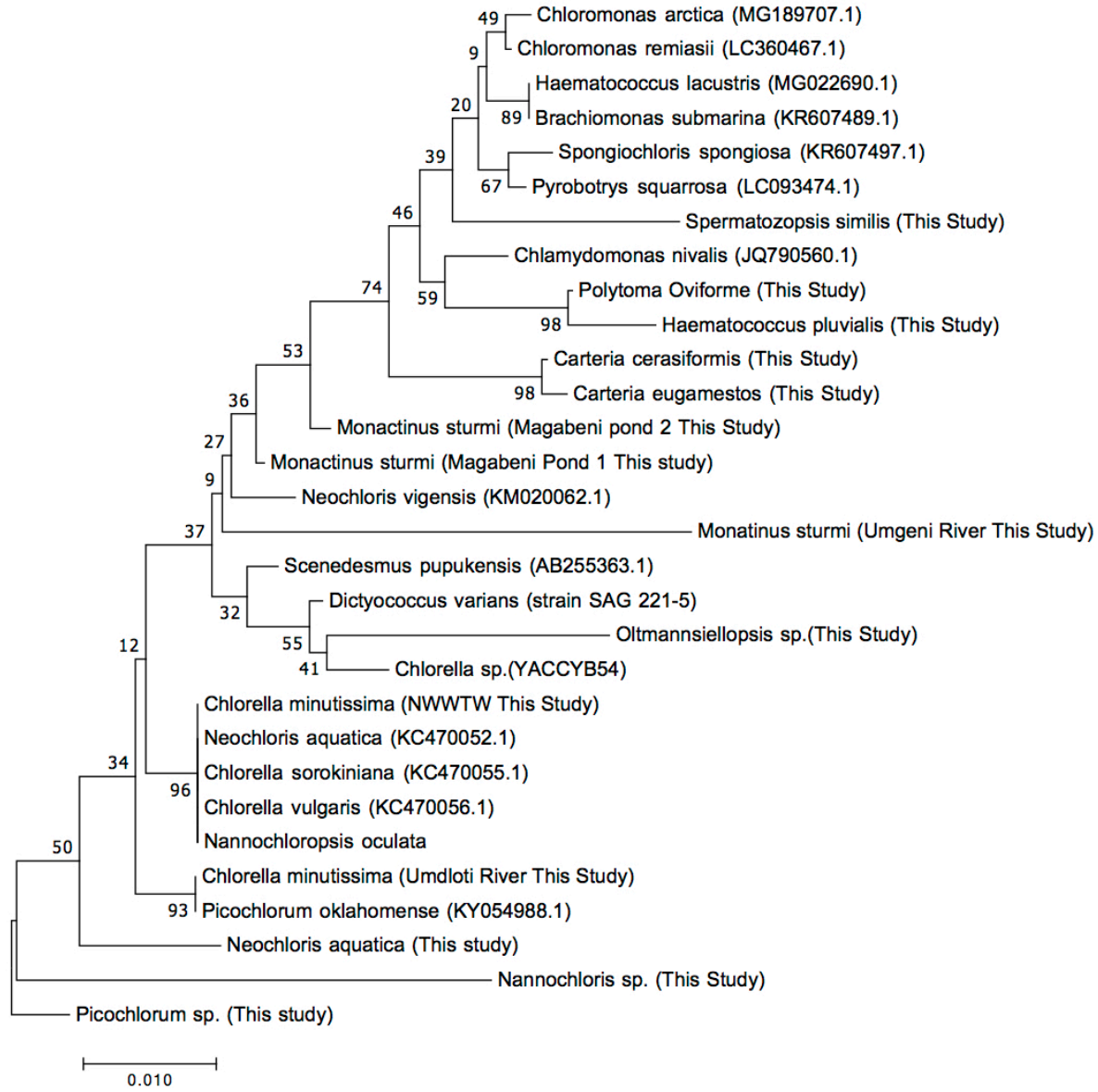
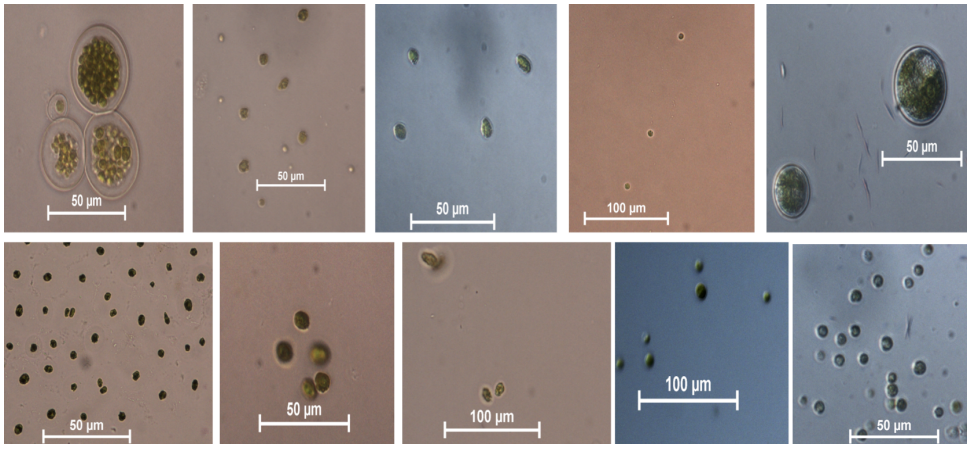
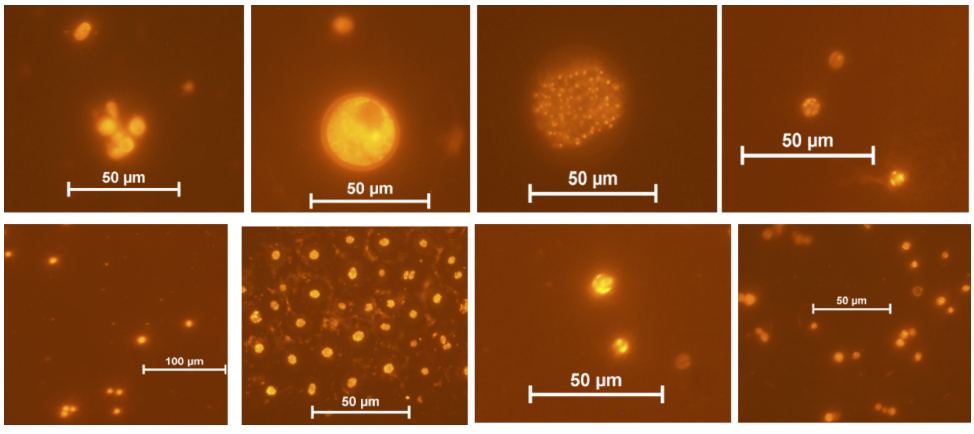


Fig. 2: Comparison of 18S rRNA gene sequences of dominant microalgae identified in this study with those of other important microalgae with known sequences in the NCBI GenBank database. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 34.84225147 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. A total of 442 positions were in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

To validate the feasibility of application of molecular tools in identifying areas of aquatic habitat with high microalgal community, enrichment of the water samples was done using appropriate enrichment culture media as described above. Figure 3 shows microalgae isolated from the different water samples which further supports molecular fingerprinting as a tool for enhanced and efficient bioprospecting. The microalgae observed were of different morphology and possessed green pigments which suggests they belong to the class Chlorophyceae in agreement with results observed from DGGE-PCR analysis. However, isolation, purification and molecular identification using PCR will be required to identify each organism to species level. They also possess lipids which can be beneficial for biodiesel production as evidenced by Nile red stain.



(a)



(b)

Fig. 3: Microscopic view (400× magnification) of enriched microalgae mixed culture from selected aquatic sampling sites showing different morphologies and sizes (a) as well as Nile red stain of lipid accumulated (b).

3.2.2 T-RFLP profile of microalgal sub-community

The fingerprint representative of the different phylogenetic groups in the microalgal community is shown in Figure 4. The species richness of natural communities was estimated by determining the number of unique T-RFs observed in digests of 18S rDNAs amplified by PCR from total community DNA. The community fingerprint represented by the T-RFLP pattern is a composite of the number of fragments with unique lengths and the relative abundance of each fragment as reflected by the size or area of each peak in the electropherogram (Liu et al., 1997). T-RFLP profile also suggest a varied community structure with few dominant taxa. T-TRFs 37, 39, 41 and 118 dominated the sampling points whilst exhibiting changes in abundance over time similar to observations in the DGGE profile (Fig. 3). For example, at the Blue Lagoon sampling site in May 2015, T-RF 38 was the dominant OTU with a relative abundance of 45.21% but reduced to 8.2% in June 2015, while at the same site in June 2015, T-RF 36 was dominant with a relative abundance of 81.81%. At the Umdloti river (May 2015), only two OTUs were recorded (TRFs 37 and 38) but increased to 5 OTUs in June. At the Umdloti river (May 2015), only two OTUs were recorded (TRFs 37 and 38) but increased to 5 OTUs in June. Buchanan et al. (2013) made similar observation in the dominance of few TRFs in water used in coal seam gas reactor systems while, Cutler et al. (2013) reported similar findings of few dominant taxa and varied specie abundance on the community structure of microalgae on sandstone building using T-RFLP and pyrosequencing. The changes in the dominant microalgae taxa and species richness in a particular aquatic environment with time suggests that time and or season of sampling is an important factor to consider when bio-prospecting for novel microalgae. Similarly, Joo et al. (2010) also concluded that seasonal changes affected the community structure of two reservoirs studied with TRF peaks identified *Cryptomonas* spp. showing high abundance in spring and winter.

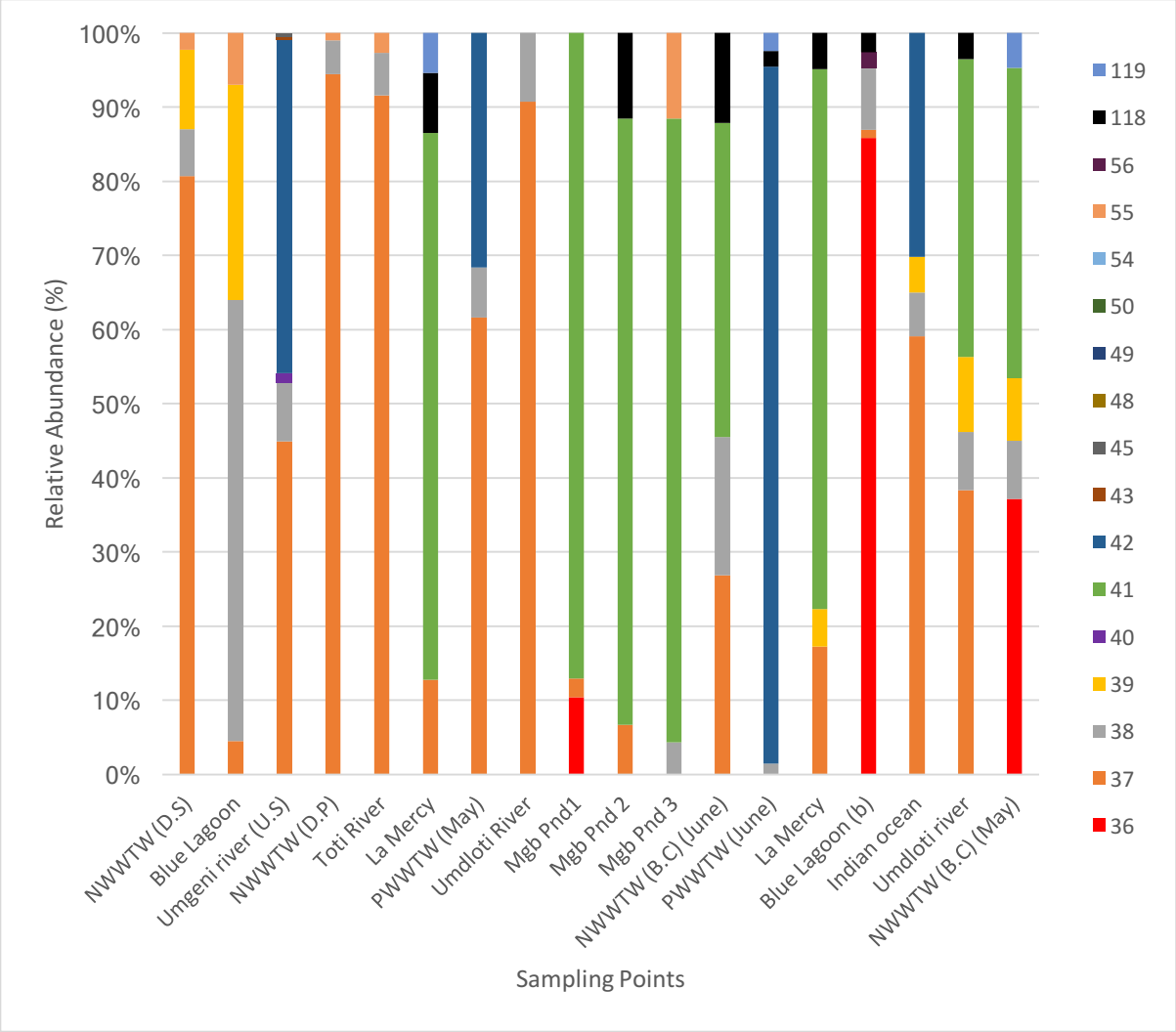


Fig. 4: Relative abundance of microalgal phylogenetic groups in various aquatic ecosystem and sampling sites in KwaZulu-Natal, South Africa. Each different colored T-RF represent a different phylogenetic group as determined by the T-RFLP analysis of the 18S rRNA genes. NWWTW - Northern Wastewater Treatment Works, PWWTW- Phoenix Wastewater Treatment Works, Mgb pnd - Magabeni Wastewater Treatment pond, U.S- Upstream, B.C - Before Chlorination, D.S - Downstream

A major limiting factor to molecular algal diversity study is the lack of suitable genetic markers for the identification, quantification, diversity analysis, and isolation of genomic DNA from environmental samples (Ebenezer et al., 2012). Though this has improved in recent years, these factors were still evident in this study, especially the lack of robust discriminating primers that can target all microalgal phyla in the metagenome while discriminating against other DNA from both prokaryotic and eukaryotic sources. The 18S primers utilized in this study, targeted mainly Chlorophyceae and Trebouxiophyceae but did not target the Cyanobacteria and other microalgal phylogenetic groups (Moro et al., 2009). This problem is further compounded by the fact that microalgae cut across both prokaryotes such as Cyanobacteria which contains 16S ribosome (Singh et al., 2015; Sudek et al., 2015) and eukaryotes such as Chlorophyceae and Bacillariophyceae which possesses the 18S ribosome (Bornet et al., 2005; Chen et al., 2015; Jung et al., 2010; Lin & Bishop, 2015). Primers based on the 23S rRNA gene described by del Campo et al. (2010) was used to target cyanobacteria and other microalgal DNA in the metagenome and used for DGGE analysis. However, sequencing of excised bands revealed that the primers was unable to discriminate against prokaryotic DNA especially *Acinetobacter* sp. (Data not shown). Thus, development of robust primers that can target all microalgae while discriminating against non-microalgal DNA is crucial to increase the accuracy of microalgal diversity and community study.

3.3 Conclusion

Using molecular approach, this study elucidated the diversity and phylogenetic profile of microalgae in various aquatic systems. The study show that molecular tools such as DGGE and T-RFLP can be beneficial in streamlining bioprospecting, a starting point in microalgal biotechnology by identifying the best sampling sites with high diversity and abundance thus saving time, cost and resources. Both fingerprinting techniques employed in this study showed that the community structure of microalgae varied amongst the sites sampled with certain OTUs common to several sampling sites. Temporal variation was observed at various points suggesting that time or season may play a role in the structure and diversity of the microalgal community. Enrichment of water samples from sampled site confirmed the presence of microalgae in the water even without visual evidence at the time of sampling. Nile red screening of isolated microalgae from sampled sites showed that they contain high amount of lipid making these indigenous microalgae a promising feedstock for biodiesel production. Adaptation of these fingerprinting techniques requires further development and refinement especially at the primer design stage to increase the accuracy of microalgal diversity study.

3.4 Acknowledgements

The authors are grateful to the National Research Foundation of South Africa (UID: 92803 to AOO; UID: 94043 to TM) and the National Science Foundation of China (31570367 to YFC) for funding this study.

3.5 Declaration of Interest

The authors declare no conflict of interests.

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CHAPTER 4

This chapter has been submitted to the Journal: Applied Biochemistry and Biotechnology

Characterization of autochthonous lipid-rich microalgae in South Africa with high biodiesel production potential

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Abstract

Increasing concern for world fossil fuel depletion, global climate change, a thirst for renewable and sustainable energy, production of bioactive compounds of pharmaceutical and nutritional benefits has led to an intense interest in microalgal research. Microalgae are found in diverse aquatic habitats and vary incredibly in nature. The current study aimed to bioprospect, screen and identify indigenous high lipid accumulating microalgae strains with potential use for biodiesel production. Ten microalgae species were isolated from terrestrial aquatic environments in Durban, and identified via the analysis of their 18S rRNA gene. The growth rates of the isolates were determined after 21 days of uninterrupted growth under batch photoautotrophic conditions in BG11 medium at 30 °C, 54.36 μmol/m^2s^{-1} and 12:12 Light/Dark period. Their lipid, carbohydrate and protein accumulation potential was evaluated using well-established methods. The characteristics of the produced biodiesel as well as the effect of nutrient limitation on their lipid, carbohydrate and protein accumulation potential was also evaluated. The indigenous isolates were identified as *Chlorella* spp., *Asterarcys quadricellulare*, *Chlorococcum* spp. and *Neochloris aquatica*. Their growth rates ranged from 0.219 ± 0.003 to 0.175 ± 0.023 $gL^{-1}day^{-1}$ while dry weight ranged from 0.433 ± 0.208 to 1.167 ± 0.153 gL^{-1}. *Chlorococcum* sp LM1 showed high accumulation of lipid (11.93 ± 0.76 mg/L), carbohydrate (15.22 ± 2.64 mg/L) and protein (1.32 ± 0.46 mg/L). The lipid profile consisted mainly of linoleic acid (C18:2 n-6), oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0). Characterization of the biodiesel based on the lipid profile reveal a low viscosity and density ranging from 1.66 to 4.264 mm^2/s and 0.327 to 0.867 g/cm^3 respectively. Cetane number ranged from 51.66 to 111.262, while oxidative stability ranged from 5.51 h to infinity. Under nutrient limitation, some of the isolates showed an increase in lipid accumulation such as *Chlorococcum sp*. LM1 (17.2%), *C. sorokinaina* NWS5 (24%), while it decreased in others such as *Chlorella minutissima* TS9 (16%), *Neochloris aquatica* Toti4 (3.5%) and *Chlorococcum sp.* LM2 (6.5%). The results suggest that these indigenous microalgae species can be used for biodiesel and bioethanol production due to their high lipid and carbohydrate accumulation. Biomass of these microalgae can also be applied in the agricultural sector as animal feed additives given their protein content.

Keywords: Microalgae; Biodiesel; Freshwater; Lipid; Bioprospecting.

4.0 Introduction

Modern life and socio-economic status is intimately linked with the availability of energy, a major source of which is derived from fossil fuels, though their use continues to drive climate change, exhausts finite reserves and contributes to global political strife (Dean 2017, Georgianna and Mayfield 2012). Increasing concerns for world fossil fuel depletion, global climate change, a thirst for renewable and sustainable energy, production of certain bioactive compounds of pharmaceutical and nutritional benefits have led to an intense interest in microalgal research (Odjadjare et al. 2017). Many microalgae can produce substantial amounts of triacylglycerol (TAG) and carbohydrates that can be converted to biofuel (Guo et al. 2013, Ho et al. 2013, Nautiyal et al. 2014, Song et al. 2013). Microalgae are versatile, exceptionally diverse and highly specialized group of microorganisms adapted to various ecological habitats. They are touted as biofuel feedstock that can potentially alleviate the food versus fuel concern associated with utilizing starch and oil rich crops for biofuel production, mitigate climate change via the sequestration of CO_2 and remediate wastewaters (Talebi et al. 2013).

Bioprospecting for the ideal microalgae strain with high lipid content is key to improving the feasibility of commercial scale microalgal biodiesel production. This is made easier by the immense diversity of the group, which has diverged over billions of years (Cheng and Ogden 2011). Thus, microalgae have a potential genetic pool that is orders of magnitude larger than that of animals or land plants. The potential is reflected in the diversity of algal species being explored for fuel and bioactive compound production, which include green algae, diatoms, and cyanobacteria (Georgianna and Mayfield 2012, Odjadjare et al. 2017). South Africa's climate generally range from Mediterranean to temperate in the interior plateau, to subtropical. With an attractive climate and plenty of sunlight all year round, South Africa is in a unique position to take advantage of these organisms to guarantee her future energy needs (Mutanda et al. 2011a). However, very few microalgae bioprospecting and characterization studies have been carried out in South Africa compared to other regions of the world such as the US and China (Mutanda et al. 2011b). The unique temperate and subtropical ecological environments in South Africa suggest the presence of microalgal strains with appropriate growth rates and high oil content. Under optimized culture conditions, these strains could express rapid growth and high lipid accumulation,

which could be harnessed for biodiesel production. Hence, this study aimed to bio-prospect, screen and identify indigenous high-lipid accumulating microalgae strains with potential use for biodiesel production.

4.1 Materials and Methods

4.1.1 Sample collection and Isolation

Water samples were collected from various aquatic sources (Table 1) ranging from freshwater to seawater in and around Durban, KwaZulu-Natal province of South Africa from May to July 2015. The grab sampling technique was used for sample collection from sites, irrespective of any visual evidence of the presence of microalgae, into a clean 10 L plastic container. The plastic container was washed with a laboratory detergent, thoroughly rinsed with distilled water and disinfected with 70% (v/v) ethanol before use. During sampling, the containers were rinsed twice with the water sample before collection. The samples were transported to the laboratory on ice and processed within 24 h of collection for enrichment, purification and isolation.

Table 1: Sampling points, habitats and GPS coordinates of various sites sampled in this study.

Sampled Point	Type of Habitat	Coordinate
NWWTW (B.C)	Freshwater	29.796011 S, 30.995920 E
NWWTW (D.P)	Freshwater	29.805613 S, 31.000624 E
PWWTW (B.C)	Freshwater	29.678156 S, 31.032978 E
Blue Lagoon	Brackish water	29.811604 S, 31.036894 E
Indian ocean	Salt water	29.813923 S, 31.041421 E
Umdloti River	Freshwater	29.647596 S, 31.118729 E
Tongaat Pond	Freshwater	29.645719 S, 31.122473 E
La Mercy Estuary	Brackish water	29.648042 S, 31.129976 E
PWWTW (B.C)	Freshwater	29.678156 S, 31.032978 E
MWWTP	Freshwater	30.165414 S, 30.781282 E
Amanzimtoti River	Freshwater	30.075529 S, 30.869149 E
Umgeni River (U.S)	Freshwater	29.808901 S, 31.000624 E
Umgeni River (D.S)	Freshwater	29.813923 S, 31.041421 E

4.1.2 Microalgae isolation and cultivation

Water samples from the various aquatic ecosystems was enriched using appropriate enrichment medium. Commercial enrichment media used were BG-11 for freshwater samples, F2 medium for marine and brackish water samples and Bold basal medium (BBM) for wastewater and freshwater samples. All media were purchased from Sigma Aldrich (Germany). Trace metals for BG11 was prepared as per standard protocols previously described by Mutanda et al. (2011a). The F2 medium was supplemented with 30% sodium chloride. The sample (15 ml) was inoculated into an Erlenmeyer flask containing 235 ml of sterile appropriate enrichment broth to which 50 mg/ml of Erythromycin was added. The flasks were sealed with cotton wool and placed under UV illumination (54.36 μmol/m^2s^{-1}) with shaking at 180 rpm for 14 to 21 days at 30 °C and 12:12 h light: dark cycles until visible cells were observed. After incubation, the mixed culture was serially diluted and used to inoculate appropriate growth media solidified with 1.5% (w/v) bacteriological agar using the spread plate technique. The plates were further incubated under the same conditions previously described. Plates showing growth of well isolated microalgal colonies were further inoculated into fresh broth media while plates showing colonies not well isolated were further purified using the triple streaking technique until well isolated colonies could be observed and inoculated into fresh media. Ten random samples from freshwater and brackish water systems were chosen for further studies and grown for 12 weeks under continuous batch photoautotrophic growth condition to accumulate enough biomass.

4.1.3 Growth rate determination of microalgal isolates

Algal growth was determined using the method described by Wang et al. (2010). Standardized (0.5 McFarland) culture (15 ml) was inoculated into 225 ml of sterile BG11 broth. Samples were taken every day for 21 days for spectrophotometric determination of optical density at 680 nm (OD_{680}) using a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) as the algal density indictor. The growth rate per day (GR, per day) was calculated by fitting the OD for the first three days of culture to an exponential function: $GR = (\ln OD_t - \ln OD_0)/t$. where OD_0 is the optical density at the initial day,

OD_t is the optical density measured on day t. After twenty one days, 5 ml of algal cells were filtered through a membrane filter (Millipore, USA), washed twice with sterile deionized water and dried in an oven at 40 °C overnight. Prior to filtration, the filter was weighed in a glass petri dish and reweighed after drying. The experiment was done in triplicates and results expressed as a mean of obtained data.

4.1.4 Molecular identification of microalgal isolates

Microalgal isolates were identified by the amplification of the 18S rRNA gene using primers and conditions previously designed and described by Moro et al. (2009). DNA was extracted using the Quick-DNA Fungal/Bacterial Miniprep kit as per manufacturer's instructions (Zymo Research Corp. USA). PCR was carried out in a 25 µl reaction consisting of 1× buffer, 1 mM $MgCl_2$, 200 µM of dNTPs, 0.4 µM of each primers and 2U of Supertherm *Taq* polymerase (Southern Cross Biotech., Cape Town, South Africa). The reaction mixture was subjected to a cycling condition of 94 ℃ for 3 min and 35 cycles of 94 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 1 min and a final temperature of 72 ℃ for 10 min. Amplified DNA (5 µl) was electrophoresed in a 1.5% agarose gel at 60 V for 60 min. Thereafter, the gel was stained in ethidium bromide for 10 min and visualized under a UV trans illuminator (Syngene, UK). The amplified products were sequenced (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa) and the sequences edited using Chromas Ver. 2.2.4 (Technelysium Pty Ltd, Brisbane, Australia). The sequences were then compared against the sequences in GenBank database using the basic local alignment search tool (BLAST) to identify the organism (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.1.5 Lipid accumulation and biodiesel production

4.1.5.1 Evaluation of lipid accumulation in microalgae isolates

Lipid content was quantified using the gravimetric method previously described by Halim et al. (2011). Lyophilized cells (50 mg) was finely ground in a mortar and pestle and suspended in 4 ml hexane: isopropanol (3:1 v/v) and vortexed for 5 min. The mixture was sonicated for 15 min at an interval of 5 min, 50 % power and 60 pulse to break the cells. The samples were then sealed to prevent evaporation of hexane and agitated (180 rpm) at ambient conditions overnight. The next day, the cells were vortexed for 5 min and 1.5 ml of 1% (w/v) sodium chloride solution was added to the mixture to induce biphasic phase separation in a separating funnel. The mixture was left to stand until phase separation was observed. After settling, a top dark-green hexane layer containing most of the extracted lipids was collected in a pre-weighed Eppendorf tube and heated to dryness in an oven at 60 °C overnight to enable gravimetric quantification of the lipid extract. All experiment was carried out in triplicates and results expressed as a mean of replicate data.

4.1.5.2 Production and characterization of biodiesel from microalgal lipid

Biodiesel production was carried out using methods previously described by Ramanna et al. (2014). Briefly, dried lipid samples (20 mg) were dissolved in hexane (1 ml) and reacted with methanol (1:30 v/v) containing 5% sulphuric acid as catalyst. The reaction proceeded at 60 °C for 4 h in an incubator with shaking at 200 rpm. Thereafter, the reaction mixture was transferred into a separating funnel and a mixture of distilled water and hexane (1:1 v/v) was added to induce biphasic separation. The organic layer was collected and analysed using GC-MS. The oven temperature was programmed to start at 60 °C and kept on hold for 2 min, then initially increased to 160 °C at a ramp rate of 10 °C min^{-1} and then to 240 °C at a ramp rate of 7 °C min^{-1} and again kept on hold for 1 min. The injector and detector temperature was 250 °C and nitrogen was used as a carrier gas. Identification of lipids was determined by comparing the mass spectra of the resolved components using electronic library search routines

(Shuping et al. 2010). Biodiesel properties were estimated using the web version of the BiodieselAnalyzer© ver 2.2 (http://www.brteam.ir/biodieselanalyzer)

4.1.6 Evaluation of carbohydrate accumulation in microalgae isolates

Carbohydrate extraction and quantitation was carried out using the laboratory analytical procedure developed by the national renewable energy laboratory (USA) as described by Lee et al. (2013). Briefly, 25 mg of freeze dried algal biomass was hydrolysed in a 250 µl of 72% (w/v) sulphuric acid at 30 °C for 60 min. Thereafter, 7 ml of sterile Millipore water was added to achieve a concentration of 4% (w/v). The setup was autoclaved at 121 °C for 60 min and cooled to room temperature. The hydrolysate was then filtered through a 0.2 µm nylon filter and the filtrate stored at 4 °C for further analysis. Spectrophotometric analysis of monomeric sugars was carried out using 500 µl of diluted filtrate (1:50) to which 500 µl each of MBTH and 0.5N NaOH was added. The setup was carefully vortexed and immediately placed in a preheated dry block at 80 °C for 15 ± 1 min. Thereafter, 1 ml of Ferric solution (made up of 0.5 % ammonium sulphate dodecahydrate and 0.5 % of sulfamic acid (w/v) in 0.25 M HCl) was added to the setup. The setup was carefully vortexed and allowed to cool to room temperature, there after 2.5 ml of Millipore water was added and vortexed. The optical density was measured at 620 nm using the Cary 60 UV-1800 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The concentration of glucose was calculated from standard curve generated using glucose as standard.

4.1.7 Protein extraction and quantitation

Protein extraction was carried out using the hot TCA method and quantified using the Lowry method as previously described by Slocombe et al. (2013). Calibration curve was prepared with bovine serum albumin (BSA) stock solution (200 mg/ml) using a polynomial line of best fit generated in Microsoft Excel 2016 (Microsoft Corp. Redmond, WA, USA).

4.1.8 Effect of nutrient limitation on biochemical accumulation potential of isolated microalgae

The effect of nutrient limitation on the accumulation of lipid, protein and carbohydrate in the isolates were evaluated. Fifteen millimetre of cultures standardized at an OD_{680} of 1 was inoculated onto sterile BG11 broth containing 3 mg/L Nitrogen and 3.14 mg/L Phosphates (Sigma Aldrich), incubated at 30 ℃ for 14 days with a 12:12 h light: dark cycles. Thereafter, the cells were centrifuged, washed twice with deionized water and inoculated into BG-11 medium containing 0.003 mg/L Nitrogen and 0.00314 mg/L Phosphate. The setup was incubated for a further 14 days at previously described conditions after which, cells were then harvested and lyophilized. A control (cultivated in nutrient rich medium) was setup in parallel for 28 days. Carbohydrate, protein and lipid accumulation potential was evaluated as described above. The fatty acid methyl esters of the generated biodiesel were also evaluated using methods described above.

4.2 Results and Discussion

4.2.1 Isolation and identification of microalgae

Microalgae are diverse and found in all earth ecosystems including aquatic and live in a wide range of environmental conditions. In this study, a total of 10 microalgae species were isolated from aquatic environments ranging from maturation ponds of wastewater treatment plants, estuaries, marine and rivers located in Durban, KwaZulu-Natal province of South Africa. The isolates were identified based on the amplification and analysis of 18S rRNA genes (Table 2). Most isolates (5/8) from the freshwater habitats were identified to belong to the genus *Chlorella*, followed by *Neochloris aquatica* (2/8), and *Asterarcys quadricellulare* (1/8), belonging to the family Scenedesmaceae, while *Chlorococcum* spp. was identified in the brackish water ecosystem. Phylogenetic analysis (Fig. 1) suggest close ancestry to microalgae of biotechnological relevance such as *Haematococcus* from which the antioxidant Astaxanthin is commercially produced (Shah et al. 2016), *Dunaliela* spp. reported to contain large amounts of carotenoids (Diprat et al. 2017), *Scenedesmus* spp., *Chlorella vulgaris* which have been reported as good feedstock for biodiesel production due to their high lipid content (Chiu et al. 2015).

Table 2: List of microalgae isolated from fresh and brackish water samples identified in this study

Organism ID	Type of habitat	Identity	% similarity	Max Score
TS4	Fresh water	*Asterarcys quadricellulare*	99	826
TS9	Fresh water	*Chlorella minutissima*	99	789
NWS5	Fresh water	*Chlorella sorokiniana*	99	832
Toti4	Fresh water	*Neochloris aquatica*	99	833
NWS1	Fresh water	*Neochloris aquatica*	99	832
PHX1	Fresh water	*Chlorella sorokinaina*	99	826
UMS2	Fresh water	*Chlorella sorokinana*	99	826
LM1	Estuary	*Chlorococcum spp.*	99	778
LM2	Estuary	*Chlorococcum spp.*	97	813
Toti7	Fresh water	*Chlorella sorokiniana*	98	826

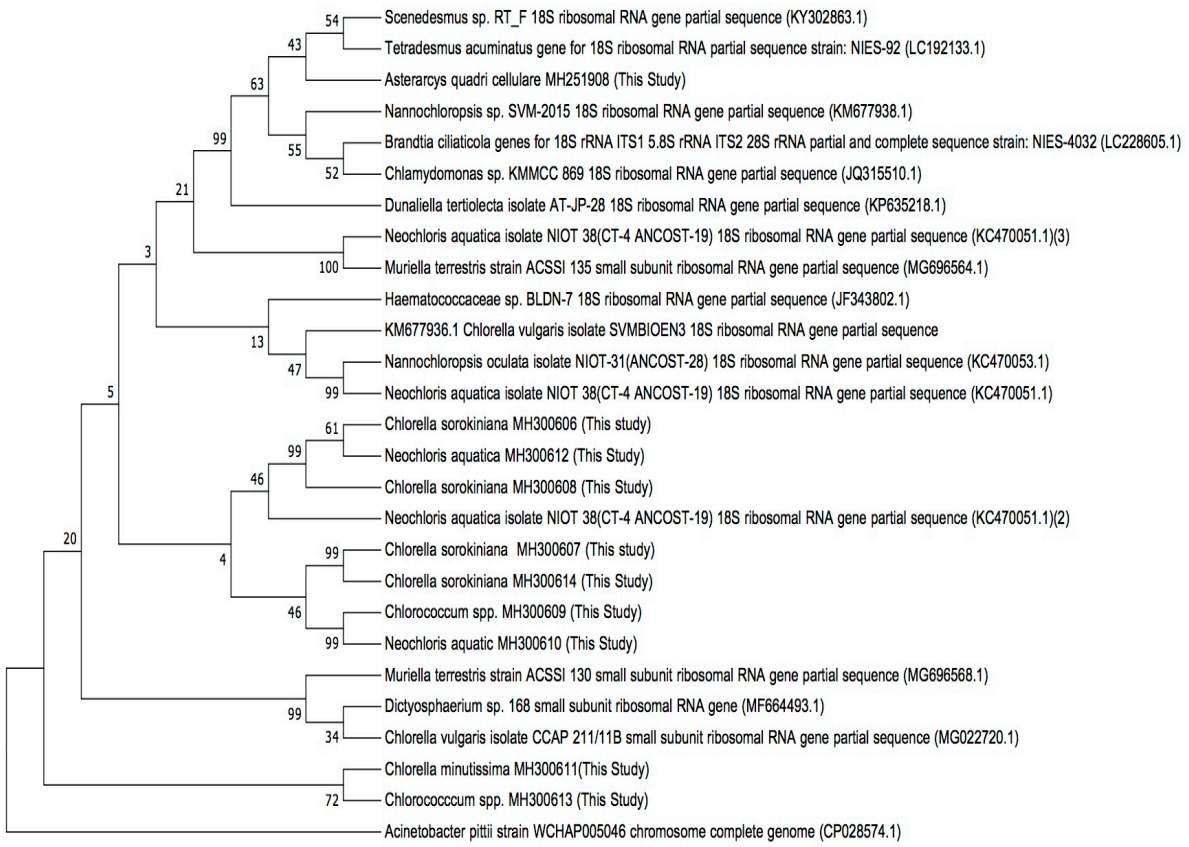


Fig. 1. Evolutionary relationships of isolated microalgae taxa. The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analysed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 429 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

4.2.2 Comparative growth rate, dry weight and productivity of microalgal isolates

The net growth rate differed among the studied microalgal species with *Chlorella sorokiniana* Phx1 showing the highest $O.D_{680}$ of 1.6 while *Chlorella minutissima* TS9 showed the lowest $O.D_{680}$ of 0.6 (Fig. 2). The average growth rate of most isolates after 21 days of incubation ranged from 0.175 ± 0.02 to 0.219 ± 0.003 g L^{-1} d^{-1}. The average biomass yield ranged from 0.433 ± 0.208 g L^{-1} to 1.167 ± 0.153 g L^{-1} with a dry weight productivity yield ranging from 0.021 to 0.056 g L^{-1} d^{-1} (Table 3). The *Chlorella* strain NWS5 exhibited the highest growth rate (0.219 ± 0.003 g L^{-1} d^{-1}), dry weight (1.167 ± 0.153 g/L) and biomass productivity (0.056 g L^{-1} d^{-1}). The lowest dry weight and biomass productivity were recorded for *Asterarcys quadricellulare* TS4 with values of 0.433 ± 0.208 g L^{-1} d^{-1} and 0.021 g L^{-1} d^{-1} respectively. However, the lowest growth rate of 0.175 ± 0.023 g L^{-1} d^{-1} was recorded for Toti7.

High growth rate, dry weight and biomass productivity were recorded in this study though variation were observed even amongst isolates of the same genus. Similar observations were made in a previous report (Abou-Shanab et al. 2011). Under similar growth conditions, *Chlorella sorokoniana* NWS5 exhibited the highest growth rate (0.219 ± 0.003 $gL^{-1}day^{-1}$), dry weight (1.167 ± 0.153 gL^{-1}) and highest productivity (0.056 $gL^{-1}day^{-1}$) indicating its suitability for high density culture. The biomass productivity recorded in this study was higher than those reported elsewhere (Song et al. 2013). High growth rate, biomass productivity and accumulation of lipids and carbohydrates are some of the major traits that makes microalgae attractive feedstock for the biofuel applications over plant-based feedstocks (Guo et al. 2013, Odjadjare et al. 2017, Yen et al. 2013, Zhang et al. 2014).

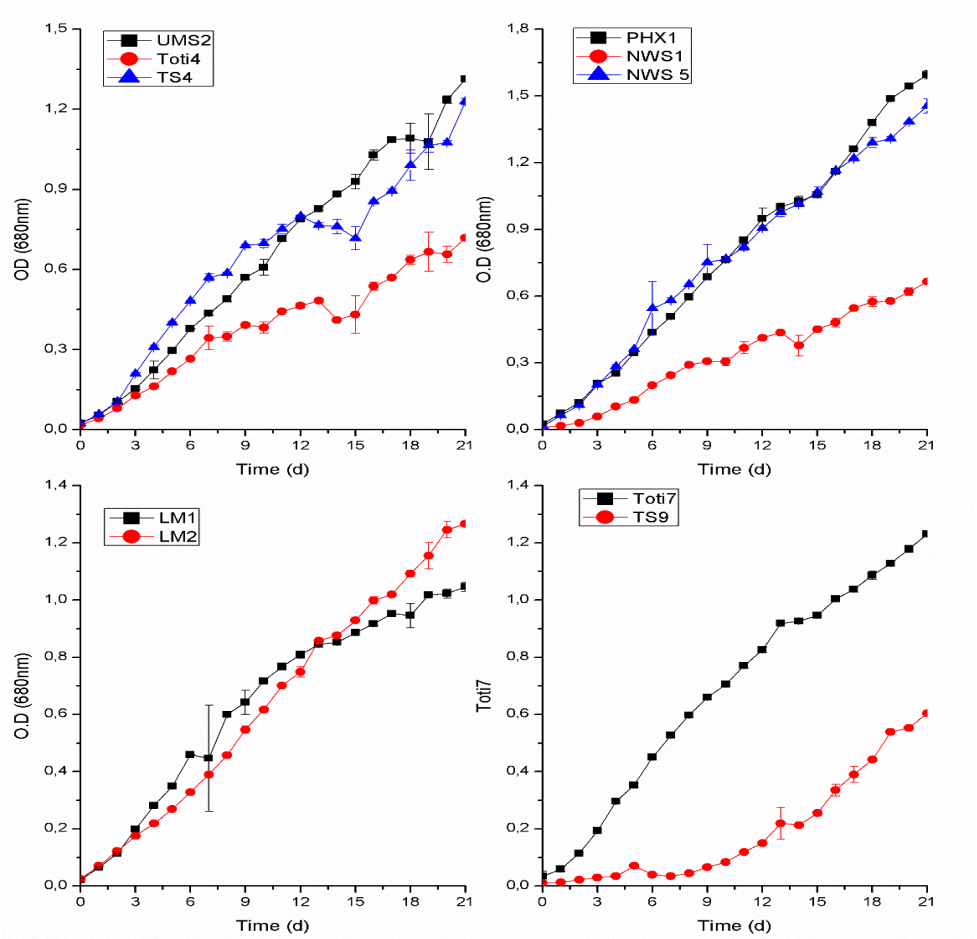


Fig. 2: Time-course growth profiles of the microalgae isolates.

Table 3: Growth rate and biomass productivity of microalgal cells isolated from various aquatic habitat in KwaZulu-Natal, South Africa

Microalgae strain	Growth rate (g/L/day)	Dry weight (g/L)	Productivity (g/L/day)
TS4	0.191 ± 0.013	0.433 ± 0.208	0.021
TS9	0.192 ± 0.005	0.600 ± 0.1	0.029
NWS5	0.219 ± 0.003	1.167 ± 0.153	0.056
Toti4	0.177 ± 0.006	0.967 ± 0.115	0.046
NWS1	0.198 ± 0.008	0.867 ± 0.230	0.041
PHX1	0.199 ± 0.002	0.600 ± 0.1	0.029
UMS2	0.193 ± 0.019	0.767 ±0.201	0.037
LM1	0.187 ± 0.013	0.733 ± 0.058	0.035
LM2	0.189 ± 0.012	0.900 ± 0.01	0.043
Toti7	0.175 ± 0.023	0.733 ±0.153	0.035

Table 4: Lipid, protein and carbohydrate yield of microalgae isolated from freshwater and aquatic ecosystems in KwaZulu-Natal South Africa.

Microalgal strain	Lipid dry wt. mg/L	% lipid dry wt.	Protein content (mg/L)	% protein dry wt.	Carbohydrate conc. (mg/L)
TS4	10.93 ± 1.53	22	1.21 ± 0.15	24	6.32 ± 0.17
TS9	12.17 ± 0.97	24	1.24 ± 0.09	25	8.25 ± 0.43
NWS5	8.20 ± 0.80	16	1.39 ± 0.10	28	8.80 ± 0.10
Toti4	9.87 ± 1.42	20	1.06 ± 0.10	21	10.42 ± 1.88
NWS1	10.13 ± 0.15	20	1.14 ± 0.11	23	13.70 ± 2.98
PHX1	9.77 ± 3.26	20	1.29 ± 0.09	26	10.60 ± 3.14
UMS2	10.67 ± 1.55	21	1.16 ± 0.23	23	15.08 ± 1.70
LM1	11.93 ± 0.76	24	1.32 ± 0.46	26	15.22 ± 2.64
LM2	6.67 ± 0.513	13	1.28 ± 0.02	26	10.64 ± 1.91
Toti7	9.90 ± 0.5	20	1.32 ± 0.12	26	11.57

4.2.3 Lipid content and Fatty acid methyl esters composition of the different microalgal isolates

Microalgal isolates in this study accumulated high lipid content with the isolates *Chlorella minutissima* TS9 and *Chlorococcum* spp. LM1 accumulating 24% dry weight (Table 4). This highlights the potential use of these organisms in large scale lipid production for biodiesel. Similar findings have been reported by Talebi et al. (2013) whose examination of 11 microalgal species revealed that *Chlorella* spp. had the highest lipid content and productivity. The lipid content was however, two fold lower than those recorded in previous study and could be due to the difference in species, type of media used for cultivation and solvent for lipid extraction (Abou-Shanab et al. 2011). The amount of lipid that can be accumulated appears to be strain specific as the accumulated lipid recorded in this study varied amongst all isolates including isolates of the same species (Table 3). This phenomenon is also observed in literature. For instance, in the work of Abou-Shanab et al. (2011), *S. obliquus* YSR01 had a 58 ± 1.5 % lipid content of dry biomass while, only 27 ± 1.9 % lipid content was recorded in *S. obliquus* YSR04. Similarly, the lipid content (% dry weight) of *D. salina* was also observed to be different among the

strains studied by Talebi et al. (2013). This highlights the importance of bioprospecting for the right strains that can accumulate high lipid content while also exhibiting other desirable characteristics for the biotechnology industry (Mutanda et al. 2011b).

The lipid profile of the 10 microalgal isolates is shown in Table 5 and consisted mostly of fatty acids with carbon chain between 14 and 22 making them suitable for biodiesel production (Zheng et al. 2012). The lipids consisted majorly of stearic acid (C18:0), palmitic acid (C16:0) linoleic acid (C18:2 n-6). Saturated fatty acid was dominant in most isolates accounting for up to 96.58% of the lipid in isolates such as *Chlorella sorokoniana* while polyunsaturated fatty acid accounted for 40.4%, 32.69% and 17.47% of the lipid recorded in *Neochloris aquatica*, *Chlorella sorokoniana*, and *Chlorella minutissima* respectively (Table 5). Polyunsaturated fatty acids (PUFAs) are nutritionally important fatty acids for infant development and its industry is estimated to be worth over 11 billion US dollars (Yaakob et al. 2014). The nutritionally essential fatty acid linoleic acid (C18:3 n-3) consisted up to 32.69% of the fatty acid in the isolate *Chlorella sorokoniana* making it a good candidate for the optimization and large scale production of PUFAs in the nutraceutical industries. Other PUFAs recorded include gamma-linoleic acid (C18:3 n-6) and linoleic acid (C18:2 n-6). However, docosahexaenoic acid and eicosapentaenoic acid, two major PUFAs used in baby formula were not detected in this study. The profile of lipid in the microalgae determines the characteristic of the biodiesel (FAMEs) that will be produced (Guldhe et al. 2014). Two important properties of a desirable biodiesel are its oxidative stability (OS) and cold flow properties which bear an inverse relationship to each other (Singh et al. 2014). Biodiesel with high saturated fatty acid content has a higher OS while the one with higher amount of unsaturated fatty acid is beneficial for cold filter (cloud point, cold filter plug point, pour point) properties of the biodiesel. A mixture of saturated and polyunsaturated fatty acid in the biodiesel is recommended to strike a balance between the cold flow and OS. However, the concentration of linolenic acid in the lipid should not exceed 12% (Guldhe et al. 2014, Singh et al. 2014). Linolenic acid was generally below 12% in most isolates in this study except *Neochloris aquatica* (20%) and had the lowest OS (5 h). In comparison with biodiesel standard ASTM D6751-07, the biodiesel produced from the isolated algae exceeded the recommended minimum OS of 3 h. The kinematic viscosity ranged from

1.669 to 4.624 mm^2/s, well within the recommended range of the ASTM D6751-07 biodiesel standard (ASTMD6751-07b 2007). The Cetane number recorded (51.66 to 111.262) was also higher than the recommended limit of 47. The properties of biodiesel in this study suggests that the microalgae isolates will be good feedstock for large-scale biodiesel production. The low cold filter plug point (-5 °C) and pour point (-12 °C) of *Chlorella minutissima* TS9 suggests it will be a good feedstock for biodiesel production in areas with cold climate.

Table 5: Percentage composition of FAME in microalgae isolated from fresh and brackish water systems in KwaZulu-Natal, South Africa

FAME	LM1	LM2	PHX1	Toti4	UMS2	Toti7	TS4	NWS1	NWS5	TS9
Linoleic acid	7.57	10.91	0.00	8.14	0.00	2.33	13.10	19.89	32.69	17.47
Eicosatrienoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.26	3.00
Palmitoleic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.05	1.05
Palmitic acid	42.74	32.46	46.72	45.91	48.78	43.20	37.42	54.62	24.99	21.35
Margaric acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.79
Linolenic acid	0.00	9.86	5.74	0.00	0.00	6.33	0.00	20.51	0.00	0.00
Petroselinate	0.00	0.00	0.00	0.00	3.42	0.00	12.46	2.49	22.08	26.92
Oleic acid	0.00	3.57	0.00	0.00	0.00	0.00	2.35	2.49	2.61	6.26
Vaccenic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.74	7.42
Stearic acid,	44.70	40.87	47.53	45.95	47.80	48.14	34.66	0.00	10.72	11.29
Gondoic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.95	3.64
Methyl Erucate	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40
Behenic acid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.91	0.43
gamma-linolenic acid	4.99	2.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Total (%)	100	100	100	100	100	100	100	100	100	100

4.2.4 Carbohydrate and Protein accumulation potential

Carbohydrate accumulation recorded was also high with *Chlorococcum* strain LM1 accumulating 15.22 ± 1.70 mg/L corresponding to 61% of its dry weight, while *Asterarcys quadricullare* TS4 accumulated the lowest (6.332±0.17 mg/L) corresponding to 25% of its dry weight. Microalgae are highly efficient photosynthetic organisms and can accumulate high amounts of carbohydrate that can be used as feedstock for bioethanol production (Guo et al. 2013, Harun and Danquah 2011, Harun et al. 2010). The microalgae isolated in this study accumulated high carbohydrate content ranging from 25 to 61 % of their dry weight (Table 6) and are therefore suitable feedstock for bioethanol production. *Chlorella sorokoniana* UMS2 accumulated higher carbohydrate content (61% dry weight) than *Chlorella vulgaris* FSP-E which was used to produce 11.7g/L of ethanol via separate hydrolysis fermentation (Ho et al. 2013). Though microalgae accumulate high carbohydrate content and have certain advantages over crop-based feedstocks, its utilization for commercial bioethanol production is still hampered by the expensive upstream and downstream processing of the algal biomass from cultivation to carbohydrate extraction, fermentation and purification processes and requires more research and innovation (Odjadjare et al. 2017, Zhao et al. 2013). However, identification of potential feedstock is still necessary for the development of viable commercial bioethanol industry.

Biofuel production is expensive and uncompetitive with fossil based fuel due to expensive downstream processing and require more innovation (Petrova and Ivanova 2010). However, agricultural use of microalgae require little additional downstream processing, can be used as feed additives in animal husbandry due to their high protein content and has been recommended in certain countries and the EU countries in general as feed additives (Chojnacka et al. 2012). Their use in aquaculture is already established and they are beginning to be considered for use in poultry (Guedes and Malcata 2012, Odjadjare et al. 2017). The microalgae isolates were observed to be able to accumulate protein at concentrations ranging from 1.06 ± 0.1 mg/L to 1.39 ± 0.1 mg/L accounting for 21 to 28% dry weight and are thus suitable as feed additives for animal feed. These microalgae also possess high amounts of lipids and if used in animal feeds could improve nutritional value of meat and eggs.

Table 6: Characterization of biodiesel produced from microalgal isolates from various aquatic habitat in KwaZulu-Natal, South Africa.

Parameters	LM1	LM2	PHX1	Toti4	UMS2	Toti7	TS4	NWS1	NWS5	TS9
SFA	87.44	73.33	94.25	91.86	96.58	91.34	72.08	54.62	36.62	12.51
MUFA	0	3.57	0	0	0	0	0	2.49	3.66	7.31
PUFA	7.57	20.77	5.74	8.14	0	8.66	13.1	40.4	32.69	17.47
DU	15.14	45.11	11.48	16.28	0	17.32	26.2	83.29	69.04	42.25
SV	196.448	200.037	207.14	206.97	200.626	206.493	176.108	205.19	149.936	74.173
IV	13.712	49.957	15.709	14.745	0	21.544	23.729	94.399	62.609	38.322
CN	70.998	62.345	69.115	69.353	73.505	67.884	71.953	51.66	68.615	111.262
LCSF	26.624	23.681	28.437	27.566	28.778	28.39	21.072	5.462	9.224	6.29
CFPP	67.168	57.922	72.864	70.127	73.935	72.716	49.725	0.683	12.502	3.284
CP	17.489	12.082	19.583	19.157	20.666	17.731	14.691	23.738	8.153	-4.992
PP	12.165	6.295	14.437	13.975	15.613	12.427	9.127	18.948	2.029	-12.24
APE	15.14	45.11	11.48	16.28	0	17.32	26.2	83.29	67.99	41.2
BAPE	7.57	30.63	11.48	8.14	0	14.99	13.1	60.91	32.69	17.47
OS	18.169	8.268	23.136	17.078	Infinity	16.208	11.593	5.51	6.198	9.341
HHV	37.56	38.59	39.513	39.525	38.183	39.525	33.648	38.248	28.757	14.759
Viscosity	3.978	3.943	4.246	4.264	4.137	4.232	3.372	3.375	2.637	1.669
Density	0.823	0.851	0.866	0.866	0.835	0.867	0.739	0.855	0.639	0.327

SFA: Saturated Fatty Acid (%); MUFA: Mono Unsaturated Fatty Acid (%); PUFA: Poly Unsaturated Fatty Acid (%); DU: Degree of Unsaturation; SV: Saponification Value (mg/g); IV: Iodine Value; CN: Cetane number; LCSF: Long Chain Saturated Factor; CFPP: Cold Filter Plugging Point (°C); CP: Cloud Point (°C); PP: Pour Point (°C); APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability (h); HHV: Higher Heating Value; υ: Kinematic Viscosity (mm2/s); ρ: Density (g/cm^3).

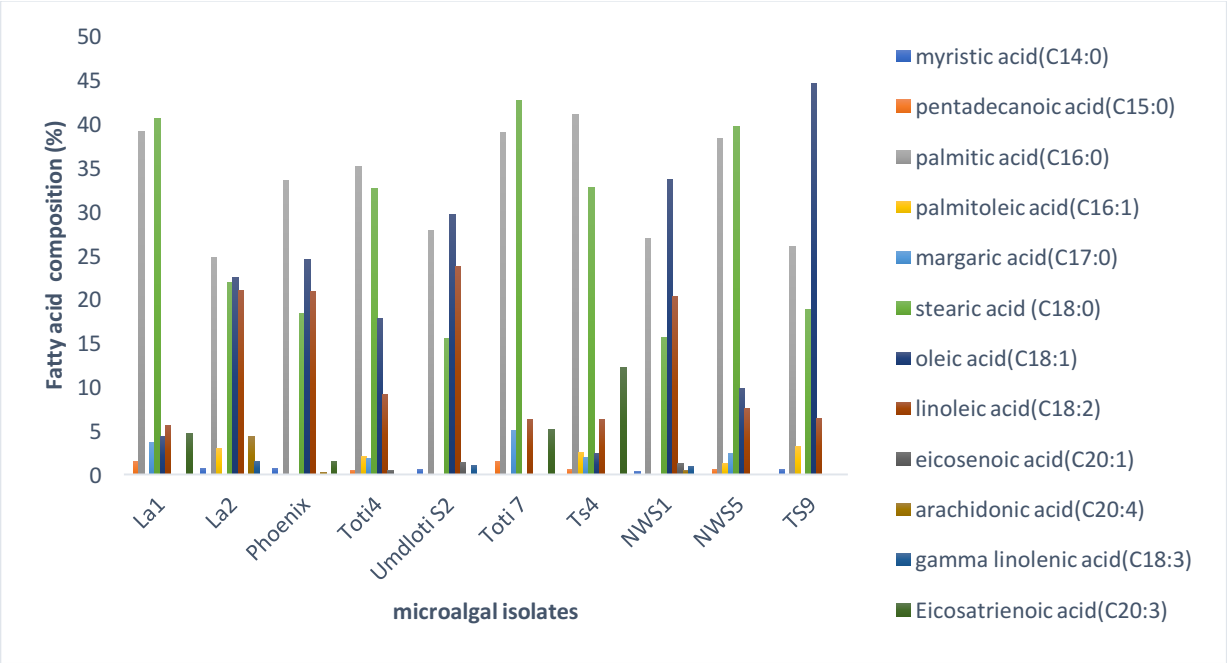
4.2.5 Effect of starvation on lipid, carbohydrate and protein accumulation potential

The effect of nitrogen and phosphorus starvation was examined in the microalgae isolates with the results showing that some isolates responded differently to nutrient starvation under the same growing conditions. The effect of nutrient limitation on protein accumulation by the microalgae in this study is depicted in Figure 4b. Fifty percent (5/10) of the isolates showed a decrease in protein accumulation with the highest decrease recorded in *Chlorella minutissima* TS9 (7% dry weight) and the lowest in *Chlorococcum spp*. LM2 (1% dry weight) while 4 of the isolates showed increased protein accumulation of up to 4% (*Neochloris aquatica* NWS1) due to nutrient starvation. However, *Neochloris aquatica* Toti4 was not affected by nutrient limitation.

Lipid accumulation also varied under nutrient limitation condition (Fig. 4c). Lipid accumulation was enhanced in some of the isolates such as *Chlorococcum* sp. LM1 (17.2%) and *C. sorokiniana* NWS5 (24%) while a decrease was observed in others such as *C. minutissima* TS9 (16%), *N. aquatica* Toti4 (3.5%) and *Chlorococcum* sp. LM2 (6.5%). The amount of carbohydrate decreased amongst most of the isolates except *C. minutissima* TS9 which was not affected by starvation (Fig. 3a). Decrease in carbohydrate ranged from 5% in *Neochloris aquatica* NWS5 and Toti4 to 20% recorded in *Chlorococcum spp*. LM2. Nutrient limitation enhances the lipid accumulation in microalgae species previously reported (Vitova et al. 2015, Zhu et al. 2016) but can also impact negatively on the growth rate and biomass accumulation (Fields et al. 2014). Based on the evidence of these results, response to enhanced lipid accumulation via starvation seem to be unique to each algal strain irrespective of species. Starvation of microalgae resulted in the decreased accumulation of carbohydrates in most of the isolates examined except in TS9 which did not show any appreciable change in carbohydrate while protein accumulation increased in some isolates and decreased in others (Fig. 4b and c). The general increase in lipid and protein content and corresponding decrease in carbohydrate may suggest that the organisms increased production of enzymes involved in the conversion of glucose, the end product of photosynthesis to high energy storage lipid molecules as an adaptation to the limited nutrient in the growth medium (Valenzuela et al. 2012, Zhu et al. 2016).

The lipid profile of microalgae grown under stressed condition is shown in Figure 3. The lipid profile under stressed condition was dominated by the saturated fatty acids: stearic acid (C18:0) and palmitic acid (C16:0) in contrast to Oleic acid (C18:1), stearic acid (C18:0) and palmitic acid (C16:0) which were dominant in the unstressed microalgae isolates. Linoleic acid reduced from percentage composition of 20 % in the microalgae grown under normal condition (Fig. 3a) to maximum composition of 12.14 % in microalgae grown in limited nutrient media (Fig. 3b). The amount of PUFAs decreased from 23. 72 % to 15.84 %) in stressed *Chlorella sorokiniana* UMS2 23, while an increase was recorded in Chlorella minutissima T9, Chlorococcum spp. LM1, Chlorella sorokiniana PHX1. In comparison, Yodsuwan et al. (2017) reported palmitic acid (C16:0) and palmitoleic acid (C16:1) as the dominant fatty acids in the lipid profile of nitrogen starved marine algae *Phaeodactylum tricornutum*. Similarly, palmitic acid and oleic acid (C18:1ω9) were reported as the dominant saturated fatty acid in nutrient starved (N and P*) Scenedesmus obliqu*s (Darki et al. 2017). The characteristics of biodiesel produced from unstressed and stressed microlage are presented in Table 7 and 8. The biodiesel produced from microalgae grown in nutrient limited condition was of good quality and had an OS ranging from 10.10 h to 37.28 h well above the recommended 3h ASTM limit (table 8). The kinematic viscosity of biodiesel derived from stressed microalgae ranged from 3.7 to 4.27 mm^2/s, well within the recommended range (1.9 to 6 mm^2/s) of the ASTM D6751-07 biodiesel standard (ASTMD6751-07b 2007). The Cetane number recorded (65.7 to 72.65) was also higher than the recommended limit of 47.

A



B

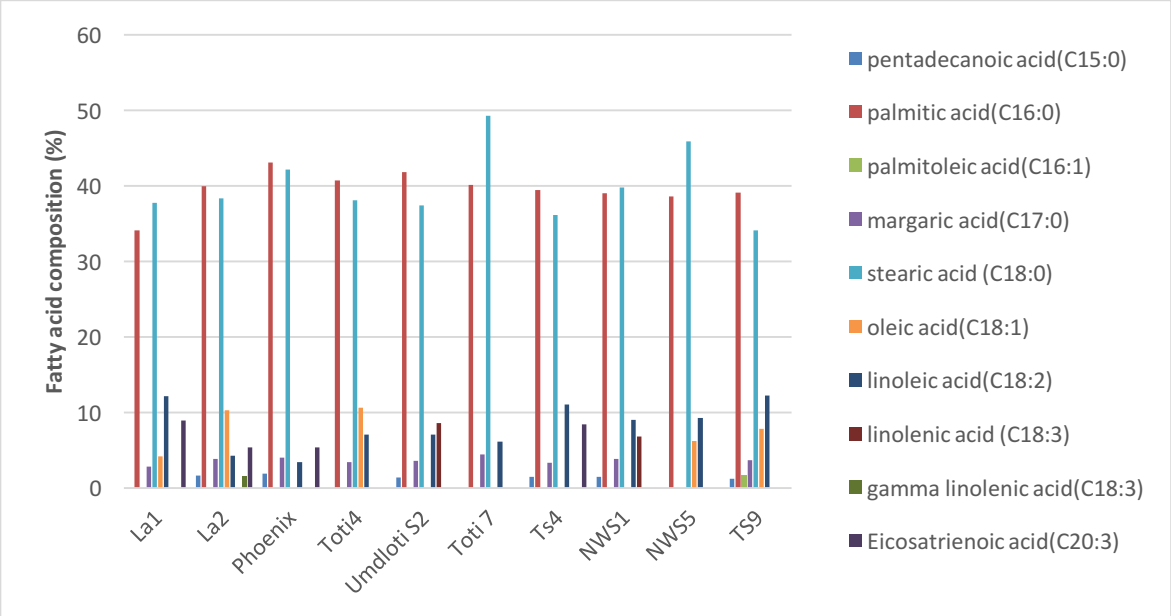
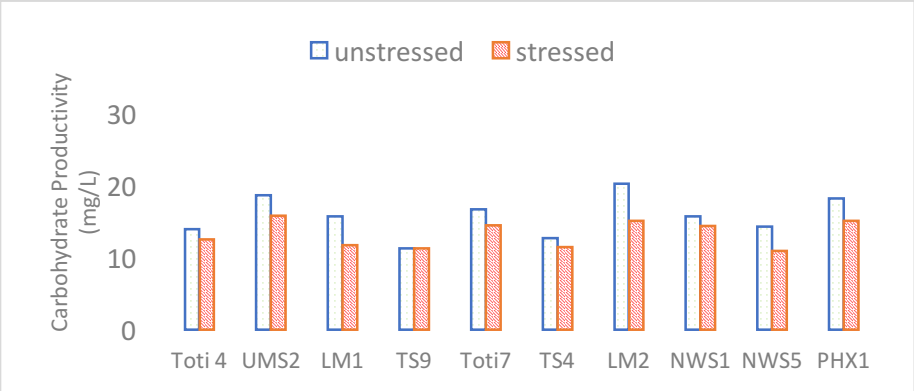


Fig. 3: Fatty acid profile of microalgae grown under normal nutrient condition (A) and nutrient limiting conditions (B).

A



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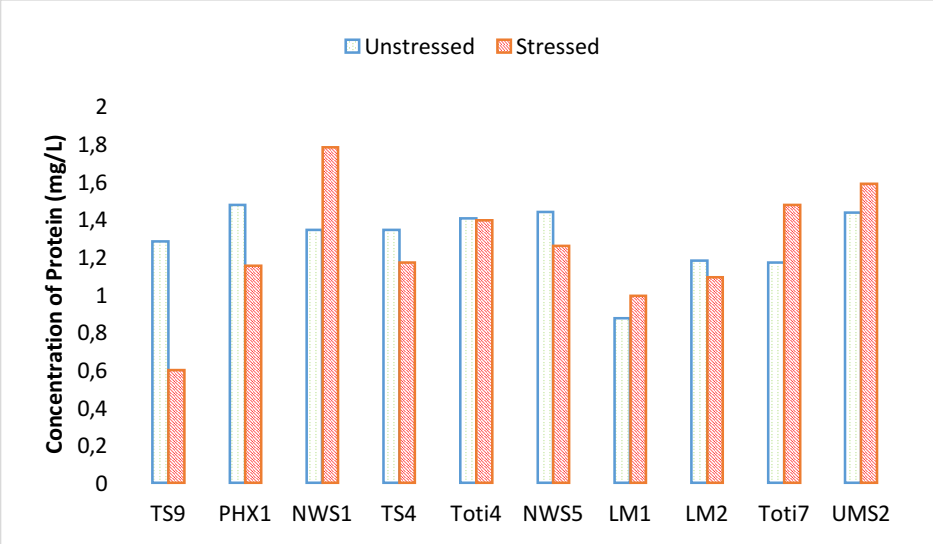

C

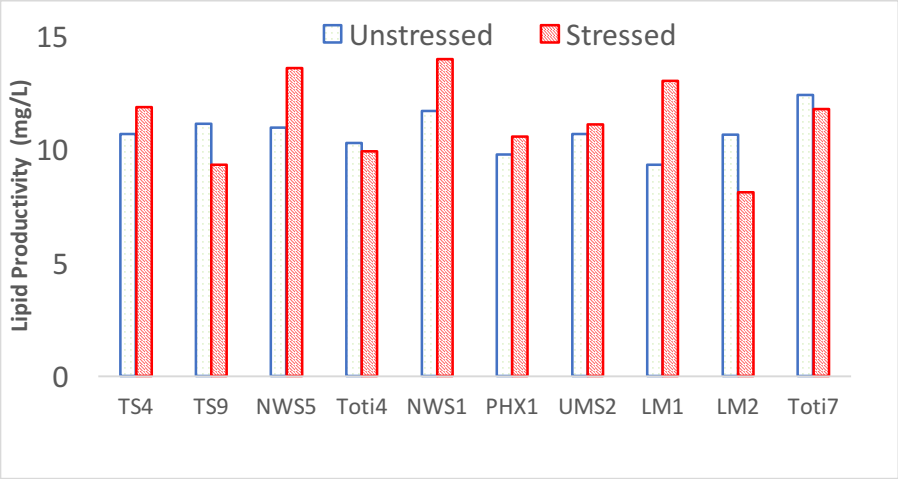

Fig. 4: Effect of nutrient limitation on (A) carbohydrate, (B) protein and (C) lipid accumulation potential of microalgae isolates recovered various aquatic habitats in Durban.

Table 7: Characterization of biodiesel produced from unstressed microalgal isolate from various aquatic habitat in KwaZulu-Natal, South Africa

Parameters	LM1	LM2	PHX1	Toti4	UMS2	Toti7	TS4	NWS1	NWS5	TS9
SFA	85.05	47.4	52.64	70.16	44.05	88.38	76.29	42.95	81.19	45.7
MUFA	4.47	25.47	24.47	20.55	31.07	0	5.09	35	11.25	47.85
PUFA	5.71	25.51	21.24	9.28	23.72	6.39	6.38	20.94	7.56	6.45
DU	15.89	76.49	66.95	39.11	78.51	12.78	17.85	76.88	26.37	60.75
SV	197.036	200.422	202.073	205.71	201.715	196.227	182.828	201.391	206.259	204.418
IV	14.363	76.154	61.043	35.456	70.783	11.575	16.397	70.205	23.944	55.043
CN	70.769	56.398	59.575	64.855	57.432	71.51	72.464	57.605	67.374	60.615
LCSF	24.21	13.404	12.54	19.783	10.565	25.219	20.452	10.519	23.664	12.032
CFPP	59.584	25.634	22.92	45.675	16.715	62.754	47.777	16.571	57.868	21.324
CP	15.548	8.021	12.629	13.46	9.657	15.517	16.532	9.152	15.149	8.695
PP	10.058	1.887	6.888	7.791	3.662	10.023	11.125	3.114	9.624	2.617
APE	15.89	77.89	67.32	36.37	77.01	12.78	15.19	76.08	25.02	57.41
BAPE	5.71	29.93	21.61	9.28	23.72	6.39	6.38	21.54	7.56	6.45
OS	23.244	8.182	8.241	15.298	7.562	21.046	21.075	8.388	18.19	20.874
HHV	37.641	38.842	38.812	39.513	39.027	37.461	34.642	39.062	39.527	39.514
Viscosity	3.979	3.828	3.865	4.155	3.883	3.967	3.497	3.909	4.224	4.062
Density	0.825	0.86	0.858	0.869	0.863	0.821	0.761	0.864	0.867	0.871

SFA: Saturated Fatty Acid (%); MUFA: Mono Unsaturated Fatty Acid (%); PUFA: Poly Unsaturated Fatty Acid (%); DU: Degree of Unsaturation; SV: Saponification Value (mg/g); IV: Iodine Value; CN: Cetane number; LCSF: Long Chain Saturated Factor; CFPP: Cold Filter Plugging Point (°C); CP: Cloud Point (°C); PP: Pour Point (°C); APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability (h); HHV: Higher Heating Value; υ: Kinematic Viscosity (mm2/s); ρ: Density (g/cm^3).

Table 8: Characterization of biodiesel produced from stressed microalgal isolates from various aquatic habitat in KwaZulu-Natal, South Africa

Parameters	LM1	LM2	PHX1	Toti4	UMS2	Toti7	TS4	NWS1	NWS5	TS9
SFA	74.67	83.85	91.26	82.28	84.30	93.89	80.46	84.16	84.50	78.13
MUFA	4.21	10.27	0.00	10.66	0.00	0.00	0.00	0.00	6.25	9.64
PUFA	12.14	4.29	3.40	7.06	15.70	6.11	11.08	15.84	9.25	12.23
DU	28.49	18.85	6.80	24.78	31.40	12.22	22.16	31.68	24.75	34.10
SV	187.21	203.55	196.79	206.32	207.29	206.13	189.87	206.70	205.52	206.94
IV	25.78	17.01	6.16	22.37	36.40	11.07	20.07	34.98	22.38	31.00
CN	69.66	69.29	72.65	67.72	64.44	70.29	70.53	64.84	67.82	65.70
LCSF	22.28	23.20	25.40	23.14	22.89	28.67	22.01	23.81	26.81	20.97
CFPP	53.52	56.40	63.33	56.22	55.43	73.60	52.66	58.31	67.74	49.42
CP	12.95	16.05	17.69	16.41	17.01	16.13	15.76	15.53	15.32	15.57
PP	7.23	10.60	12.39	10.99	11.64	10.69	10.29	10.04	9.81	10.08
APE	28.49	18.85	6.80	24.78	31.40	12.22	22.16	31.68	24.75	32.35
BAPE	12.14	4.29	3.40	7.06	24.30	6.11	11.08	22.63	9.25	12.23
OS	12.31	30.08	37.28	19.29	10.10	21.89	13.23	10.04	15.34	12.23
HHV	35.99	38.90	37.41	39.53	39.47	39.56	36.15	39.49	39.55	39.49
Viscosity	3.70	4.15	3.97	4.23	4.07	4.34	3.71	4.11	4.27	4.13
Density	0.79	0.85	0.82	0.87	0.87	0.87	0.79	0.87	0.87	0.87

SFA: Saturated Fatty Acid (%); MUFA: Mono Unsaturated Fatty Acid (%); PUFA: Poly Unsaturated Fatty Acid (%); DU: Degree of Unsaturation; SV: Saponification Value (mg/g); IV: Iodine Value; CN: Cetane number; LCSF: Long Chain Saturated Factor; CFPP: Cold Filter Plugging Point (°C); CP: Cloud Point (°C); PP: Pour Point (°C); APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability (h); HHV: Higher Heating Value; υ: Kinematic Viscosity (mm2/s); ρ: Density (g/cm^3).

4.3 Conclusion

Indigenous microalgae isolates studied exhibited high growth rate (0.219 $gL^{-1}day^{-1}$) and biomass accumulation (1.167 $gL^{-1}day^{-1}$). The microalgae proved to be suitable feedstock for biodiesel production accumulating high lipid content up to 12.17 mgL^{-1} (*Chlorella minutissima* TS9) and desirable saturated fatty acids including stearic acid (C18:0), Palmitic acid (C16:0). In-silico characterization of biodiesel suggests that the biodiesel of desirable characteristics within the ASTM guidelines with low viscosity, high OS and low polyunsaturated fatty acid. Microalgal response to nutrient deprivation suggests specie specificity. While, lipid accumulation was enhanced in some of the isolates, it decreased in others. Thus, the nature and specie of microalgae should be considered when applying this strategy for lipid optimization and enhancement. High total carbohydrate content (structural and storage) was also recorded in this study (Table 3) suggesting the isolates would be good feedstocks for bioethanol production. Though starvation of microalgae resulted in increased accumulation of lipids and protein, the negative impact on carbohydrate accumulation makes it undesirable as an enhancement strategy if the microalgae is to be used as a feedstock for bioethanol production.

4.4 Acknowledgements

The authors are grateful to the National Research Foundation of South Africa (UID: 92803 to AOO; UID: 94043 to TM) and the National Science Foundation of China (31570367 to YFC) for funding this study.

4.5 Declaration of Interest

The authors declare no conflict of interests.

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CHAPTER 5

This chapter has been submitted to the Journal: Water

Evaluation of Pre-Chlorinated Wastewater Effluent for Microalgal Cultivation and Biodiesel Production.

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Abstract

Microalgae are promising feedstock to produce biodiesel and other value added products. Their high growth rate, biomass and lipid accumulation potential, and ability to grow in even harsh environment gives them an advantage over traditional first generation biofuel feedstock such as oil rich crops. However, the water footprint for producing microalgal biodiesel is rather large requiring about 3,726 kg of water, 0.33 kg of Nitrogen and 0.71 kg of Phosphate to produce 1 kg of biodiesel if freshwater is used without recycling, putting a strain on the freshwater resources, especially in water stressed countries like South Africa. This study thus, evaluate the utilization of pre-chlorinated wastewater as a cheap growth media for microalgal biomass propagation with the aim of producing biodiesel whilst simultaneously remediating the wastewater. Wastewater was collected from two wastewater treatment plants (WWTPs) in Durban. Sterilized and unsterilized wastewater were inoculated with *Neochloris aquatic* and *Asterarcys quadricellulare* and growth kinetics was monitored for a period of 8 days. The physicochemical parameters; including chemical oxygen demand (COD), total nitrogen (TN), total Phosphorus (TP), salinity, pH, resistivity and conductivity was determined before microalgal cultivation and after harvesting and the nutrient removal efficiency calculated. Total lipids were quantified gravimetrically after extraction using hexane/isopropanol (3:2 v/v). Biodiesel was produced by transesterification of the lipids with methanol in the presence of sulphuric acid and the fatty acid methyl esters (FAMES) characterised by GC-MS. The total carbohydrate was also quantified using well-established methods. TP concentration was low resulting in an unbalanced N:P ratio of 44 at the Northern wastewater treatment works (NWWTW) and 4 at the Umbilo wastewater treatment plant (UWWTP). *Asterarcys quadricellulare* utilized the wastewater for growth and reduced the COD of the wastewater effluent from the UWWTP by 12.4% in contrast to *Neochloris aquatica* which did not show any growth. Total nitrogen (TN) and Phosphorus (TP) were reduced by 48% and 50% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced the TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% were recorded in *Neochloris aquatica*. Although the highest biomass yield (460 mg) was obtained for *Asterarcys* , the highest amount of lipid (14.85 mg) and carbohydrate (14.84 mg) content were recorded in *Neochloris aquatica* . The dominant fatty acids in the microalgae were palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). The biodiesel produced was determined to be of good quality with high oxidation stability and low viscosity, and conformed to the ASTM guidelines. This study thus showed the feasibility of wastewater remediation and microalgal biomass propagation and biodiesel production using treated wastewater effluent from WWTPs in Durban, South Africa. However, optimization of the N:P ratio and carbon source are necessary to improve biomass productivity for commercial scale production of biodiesel.

Keywords: Wastewater, Wastewater treatment plant, Microalgae, Biodiesel, Phycoremediation

5.0 Introduction

Microalgae are photoautotrophic organisms utilizing sunlight and carbon dioxide to generate energy which are stored as lipids. These lipids, usually stored as triglycerides, can be converted to biodiesel, thus the increasing interest in the propagation and cultivation of these organisms. Furthermore, increasing greenhouse gas emissions, concern towards global warming and climate change has spurred the need for the development of environmentally friendly forms of energy including biodiesel. Biodiesel can be derived from oleaginous feedstock such as oil rich crops but this however, creates a concern of diversion of scarce arable land resources towards energy production instead of food. Microalgae are versatile organisms that require less land space for cultivation and can accumulate a high amount of lipids amongst other advantages. However, it has a large water footprint that is unsustainable for a semi-arid and water stressed country like South Africa, with low average rainfall of 465 mm, which is below the global average of 860 mm (Pitman et al., 2011). Demand for this important scarce resource is expected to increase due to rapid industrial development, increasing human population, per capita consumption increase and the resulting impact of human activities on the environment (Okello et al. 2015). High water demand and consumption leads to increases in the volume of wastewater generated. Wastewater effluent from wastewater treatment plants (WWTPs) have been identified as a potential solution to the large water requirement for the cultivation of microalgae for biodiesel production.

Wastewater effluent characteristics is dynamic and dependent on several factors including wastewater type, treatment applied, location of treatment plant, climate, population etc… Agricultural wastewater such as diary, pig slurry and others are known to contain very high amount of nutrient, while the level of nutrient content varies within municipal wastewater treatment plants (de Godos et al. 2009). Untreated or partially treated municipal wastewater contain a large amount of nutrient including Ammonia (NH_4^+), Nitrates (NO_3^-) and phosphates (PO_4^{3-}) which if released into receiving water bodies results in eutrophication of these natural waterbodies (Li et al., 2017). Microalgae can utilize these nutrients in addition to sunlight and CO_2 for their growth while simultaneously treating the wastewater hence, it was first proposed and developed in the 1950s as a cheap method of treating wastewater

(Craggs et al., 2014). Since then, other researchers have reported the use of different types of wastewater and microalgae species for the simultaneous remediation and biomass propagation of microalgae for the biodiesel production (Cho et al. 2013, Gentili 2014, Li et al. 2011, McGinn et al. 2011). Mahapatra et al. (2014) reported the use of mixotrophic algal consortia for the bioremediation of municipal wastewater and simultaneous lipid accumulation in municipal wastewater in India with nutrient removal reaching as high as 90% and lipid accumulation reaching 28.5% of dry algal biomass.

South Africa possess an attractive climate for the cultivation of microalgae and huge microalgal biodiversity which can be applied as feedstock for biodiesel production and other biotechnological application. Utilizing wastewater for microalgal cultivation can reduce the amount of freshwater required for large scale biofuel production. While, nutrients in wastewater can reduce cost of biodiesel production with the added benefit of generating wastewater fit for discharge into receiving waterbodies. However, there is very little information regarding the suitability of wastewater effluent in South Africa for microalgae cultivation. Hence, this study investigated the potential use of treated pre-chlorinated wastewater effluent from two major WWTPs in Durban, KwaZulu-Natal for growth and biomass propagation of two microalgae isolates, *Asterarcys quadricellulare* and *Neochloris aquatica* for biodiesel production. The phycoremediation potential of the isolates during growth in the wastewater effluent was also established.

5.1 Materials and Methods

5.1.1 Sample collection and processing

Wastewater samples were collected from the Northern wastewater (29°48′45.62″ S; 30° 59′ 45.62″ E) and Umbilo wastewater treatment plants (29°50′41.431″ S; 30° 53′ 29.122″ E) in Durban, KwaZulu-Natal province of South Africa. The treatment plants are amongst the major wastewater treatment plants in the province and utilize the activated sludge treatment system to treat wastewater. Water samples were collected from the secondary clarifier tank of each wastewater treatment plant in a clean 10 L plastic container previously washed and thoroughly rinsed twice with the sample water before collection. The wastewater samples (5 L) were filtered through Whatmann 1 filter paper to remove solid particles and reduce turbidity before autoclaving at 121 °C for 15 minutes to sterilize it.

5.1.2 Determination of physico-chemical parameters of wastewater samples

The physico-chemical parameters of the wastewater samples were determined prior to inoculation and after 8 days of inoculation with the microalgae using standard methods. The pH was determined using Hanna Edge pH meter (Hannah Instruments, Rhode Island, USA), Chemical oxygen demand (COD) was determined via the $K_2Cr_2O_7$ assay using spectroqaunt COD cell test kit (Merck, Darmstadt, Germany) following the manufacturer's instructions, Total nitrogen and phosphates were determined using Spectroquant total Nitrogen and Phosphate cell test kit respectively (Merck, Darmstadt, Germany). Electrical conductivity (EC), salinity, resistivity and total dissolved solids (TDS) were determined using the CDC 401 probe and HQ40d multimeter (HACH, USA). Physicochemical parameters were determined in duplicates and results expressed as a mean of the obtained data.

5.1.3 Microalgal strain cultivation and growth kinetics

Monocultures of two microalgae strains *Asterarcys quadricellulare* and *Neochloris aquatic* previously isolated from the Toongati river and maturation pond of the Northern wastewater treatment plant, respectively between May and July 2015, were maintained on commercial BG11 medium (Sigma Aldrich, Germany) supplemented with trace metals prepared as previously descried by Mutanda et al.

(2011). The cells were standardized to an optical density of 1 at a wavelength of 680 nm, centrifuged at 8000 rpm (Beckman Coulter Avanti J-30i, California, USA) and washed three times with sterile distilled water and once with sterile wastewater sample to remove every trace of phosphates or nitrogen carried over from the commercial chemically defined media to the experimental setup. Thereafter, the cells suspended in sterile autoclaved wastewater sample (10%) were inoculated into 800 ml (total volume) of the filtered autoclaved wastewater and unsterilized wastewater samples. The flasks were sealed with cotton wool and placed under cool white light illumination (54.36 μmol/m^2s^{-1}) with shaking at 180 rpm for 8 days at 30 °C, under ambient air diffusion with a 12:12 h light: dark cycles. Samples were taken daily over the 8 days incubation period from the purified culture for growth determination at an optical density of 680 nm (OD_{680}) using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, California, USA) as the algal density indictor. The growth rate per day (GR, per day) was calculated using the equation $GR = (\ln OD_t - \ln OD_0)/t$. where OD_0 is the initial optical density, OD_t is the optical density measured on day t. Every four days, 5 ml of algal cells were filtered through a membrane filter with 0.45 μm pore size (Millipore, USA), washed twice with sterile deionized water and dried in an oven at 40 °C overnight. Prior to filtration, the filter was weighed in a glass dish and reweighed after drying. The experiment was done in triplicate and results expressed as a mean of the data obtained.

5.1.4 Dry Weight Determination, Lipid extraction and Quantitation

Dry weight was determined after 7 days by filtering 10 ml of algal broth through a pre-weighed Whatmann 1 filter paper and placed on a Petri dish. The Petri dish and filter paper were dried in an oven at 60 °C for 12 hours after which it was allowed to cool to room temperature. The net mass of the microalgal cells was determined by subtracting the weight of microalgae and filter paper from the pre-weighed filter paper. Lipid content was quantified using the gravimetric method previously described by Halim et al. (2011). Lyophilized biomass (50 mg) was finely ground in a mortar and pestle and suspended in 4 ml hexane: isopropanol (3:2, v/v) and vortexed for 5 min. The mixture was sonicated at an interval of 5 min for 15 min at 50% power and 60 pulse to break the cells. The samples were then

sealed to prevent evaporation of hexane and agitated at 180 rpm at ambient conditions overnight. The next day the cells were vortexed for 5 min and 1.5 ml of sterile 1% NaCl solution was added to the mixture to induce biphasic phase separation using a separating funnel. The mixture was left to stand until phase separation was observed. After settling, a top dark-green hexane layer containing most of the extracted lipid was collected in a pre-weighed Eppendorf tube and the tubes were heated to dryness in the oven (60 °C) overnight until constant weight to enable gravimetric quantification of the lipid extract. All experiments were carried out in triplicate and results expressed as means of replicate data.

5.1.5 Biodiesel production and characterization

Biodiesel production was carried out using methods previously described by Ramanna et al. (2014). Briefly, dried lipid samples (20 mg) gravimetrically extracted were dissolved in 1 ml of hexane and reacted with methanol containing 5% sulphuric acid as a catalyst in a 30:1 (v/v) ratio. The reaction proceeded at 60 °C for 4 h in an incubator with shaking at 200 rpm. Thereafter, the reaction setup was transferred to a separating funnel and washed with a mixture of distilled water and hexane (1:1 v/v) to induce biphasic separation. The organic layer was collected and analysed using GC-MS (Shimadzu Corp, Kyoto, Japan). The oven temperature was programmed to start at 60°C and kept at hold for 2 min, then initially increased to 160 °C at a ramp rate of 10 °C min^{-1} and then to 240 °C at a ramp rate of 7 °C min^{-1} and again kept at hold for 1 min. The injector and detector temperature was 250 °C and nitrogen was used as a carrier gas. Identification of lipids was done by comparing the mass spectra of the resolved components using electronic library search routines (Shuping et al. 2010). Biodiesel properties were estimated using the web version of the BiodieselAnalyzer© ver 2.2 (http://www.brteam.ir/biodieselanalyzer).

5.1.6 Evaluation of carbohydrate accumulation potential of microalgal isolates

Total carbohydrate extraction and quantitation was carried out using the laboratory analytical procedure developed by the national renewable energy laboratory (USA) and described by Lee et al. (2013a). Briefly, 25 mg of freeze dried algal biomass was hydrolysed in a 250 µl of 72% (v/v) sulfuric acid at 30 °C for 60 min. Thereafter, 7 ml of sterile Millipore water was added to achieve a concentration of 4% (w/v). The setup was autoclaved at 121 °C for 60 min and cooled to room temperature. The hydrolysate was then filtered through a 0.2 µm nylon filter and the filtrate stored at 4 °C for further analysis. Spectrophotometric analysis of monomeric sugars was carried out using 500 µl of diluted filtrate (1:50) to which 500 µl each of MBTH and 0.5N NaOH was added. The setup was carefully vortexed and immediately placed in a preheated dry block at 80 °C for 15 ± 1 min. Thereafter, 1 ml of Ferric solution (made up of 0.5% ammonium sulphate dodecahydrate and 0.5% of sulfamic acid (w/v) in 0.25 M HCl) was added to the mixture. The setup was carefully vortexed and allowed to cool to room temperature after which 2.5 ml of Millipore water was added and vortexed. The optical density was measured at 620 nm using the Cary 60 UV-1800 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The concentration of monosaccharide was extrapolated from a standard curve made with 0.25 mg/ml glucose after correcting for dilution. The total carbohydrate content was calculated by multiplying the obtained concentration with the total volume of extract (7.25 ml). The analysis was done in triplicate and the result expressed as a mean of the obtained data.

5.1.7 Statistical Analysis

In order to arrive at a validated conclusion, the growth rate and physicochemical parameters were subjected to Pearson's correlation to ascertain significant correlation between the obtain data. The statistical analysis was performed using SPSS software package ver 25 (IBM Corporation, New York, USA).

5.2 Results

5.2.1 Physico-chemical profiles of the treated wastewater effluent

The physico-chemical profiles of the treated pre-chlorinated wastewater used in this study is summarised in Table 1. The initial pH, TP, TN and COD of the wastewater sample from the NWWTW were found to be 7.66, 0.4 mg/L, 17.77 mg/L, and 23.33 mg/L, respectively while at the UWWTP, initial values of 7.37, 3.40 mg/L, 15.03 mg/L and 45.67 mg/L, respectively were recorded. The N/P ratio of the wastewater samples were 44.42 (NWWTW) and 4.42 (UWWTP). The highest TP and TN reduction at the NWWTW were achieved by *Asterarcys quadricellulare* inoculated in sterilized wastewater (50%) and unsterilized wastewater (48%). While at the UWWTP, the highest TP and TN reduction was achieved by *Asterarcys quadricellulare* inoculated in sterilized wastewater (32%) and unsterilized wastewater (45%). The salinity of the wastewater from the NWWTW increased after treatment from an initial value of 0.38 up to 0.43 in the unsterilized wastewater inoculated with *Chlorella* spp., while at the UWWTP, salinity was relatively stable and unchanged (Table 1). COD was reduced by up to 12.4% in the wastewater from the UWWTP after 8 days, however an increased COD of up to 30.7% was recorded in the wastewater from the NWWTW. A significant positive correlation was observed between COD and TP ($p < 0.01$; $r = 0.937$), whereas an inverse correlation between COD and salinity ($p<0.01$; $r = -0.911$).

Table 1: Physico-chemical profiles of treated pre-chlorinated wastewater effluent before and after cultivation with microalgae.

Wastewater Treatment Plant	Organism ID	pH	Conductivity μScm^{-1}	Salinity %	TDS mgL^{-1}	Resistivity Ω.cm	TP (mgL^{-1})	TN (mgL^{-1})	COD (mgL^{-1})
NWWTW	Before cultivation	7.66	775	0.38	ND	1290	0.40	17.77	23.33
	Asterarcys[a]	8.43	804	0.39	394	1242	0.25	9.20	30.00
	N. aquatica[b]	7.27	867	0.43	425	1151	0.30	12.50	30.50
	Asterarcys[a]	7.62	879	0.42	422	1162	0.20	10.35	28.50
	N. aquatica[b]	7.46	871	0.43	429	1132	0.25	13.55	24.50
UWWTP	Before cultivation	7.37	549	0.26	ND	1822	3.40	15.03	45.67
	Asterarcys[a]	8.33	527	0.25	255	1899	2.30	8.30	40.00
	Asterarcys[b]	7.71	537	0.26	260	1864	2.75	9.20	41.00
	N. aquatica[a]	7.25	547	0.26	265	1826	2.40	12.10	44.00
	N. aquatica[b]	7.45	555	0.27	269	1757	3.10	12.75	41.00

a- Sterilized wastewater; *b*- unsterilized wastewater; NWWTW- Northern Wastewater treatment works, UWWTP- Umbilo wastewater treatment plant.

5.2.2 Growth Kinetics, Biomass Yield and Total Carbohydrate Content of the Microalgae

Growth profiles of *Neochloris aquatica* and *Asterarcys quadricellulare* in the different wastewater samples are presented in Figure 1. The results suggest *Neochloris aquatica* did not grow in the wastewater (sterilized and unsterilized) from both wastewater treatment plants for biomass propagation. *Asterarcys quadricellulare* was able to utilise the wastewater achieving a growth rate up to 0.18 day^{-1} in sterilized wastewater from the NWWTW and 0.17 day^{-1} in the unsterilized wastewater from UWWTP. *Asterarcys quadricellulare* accumulated high biomass ranging from 250 mg to 460 mg compared to 130 mg/L to 180 mg/L in *Neochloris aquatica*. Highest biomass productivity of up to 65.71 mg/day was recorded for *Asterarcys quadricellulare*, values which is 2.56-fold higher than that recorded for *Neochloris aquatica* (Table 2). The highest total monosaccharide content (57.9% dry wt.) was recorded in *Neochloris aquatica* cultivated in sterilized wastewater and the lowest (24.29% dry weight) in unsterilized wastewater.

Despite having a low biomass productivity, *Neochloris aquatica* accumulated higher amounts of lipids than *Asterarcys quadricellulare*. *Neochloris aquatica* cultivated in sterilized wastewater from the UWWTP accumulated up to 14.85 ± 1.36 mg (29.7% dry wt.) and 11.35 ± 2.48 mg (22.7 % dry wt.) lipids in sterilized and unsterilized wastewater from UWWTP, respectively. In contrast, *Asterarcys quadricellulare* accumulated 10.15 mg (20.3% dry wt.) in the unsterilized wastewater and 8.25 mg (16.5% dry wt.) in the sterilized wastewater. In the wastewater from the NWWTW, *Neochloris aquatica* accumulated comparable amounts of lipids (up to 20.8% dry wt.) to *Asterarcys quadricellulare* (21.9% dry weight) despite having a lower biomass dry weight and productivity. Significant positive correlation ($p < 0.01$) was recorded between growth rate and dry weight while the dry weight inversely correlated with TN ($p < 0.01$, $r = -0.82$).

Total carbohydrate accumulated by microalgae are presented in Table 2. The total sugar content was highest in *Neochloris aquatica* cultivated in sterilized wastewater from the NWWTW (14.48 ± 0.1 mg) accounting for 57% of its dry weight while the lowest (6.07 ± 0.45 mg) was recorded in *Neochloris aquatica* cultivated in unsterilized wastewater from the NWWTW. Total carbohydrate accumulated by *Asterarcys quadricellulare* ranged from 10.77 ± 1.55 mg (UWWTP) to 12.61 ± 0.61 (NWWTW).

5.2.3 Fatty Acid Methyl Esters Profile

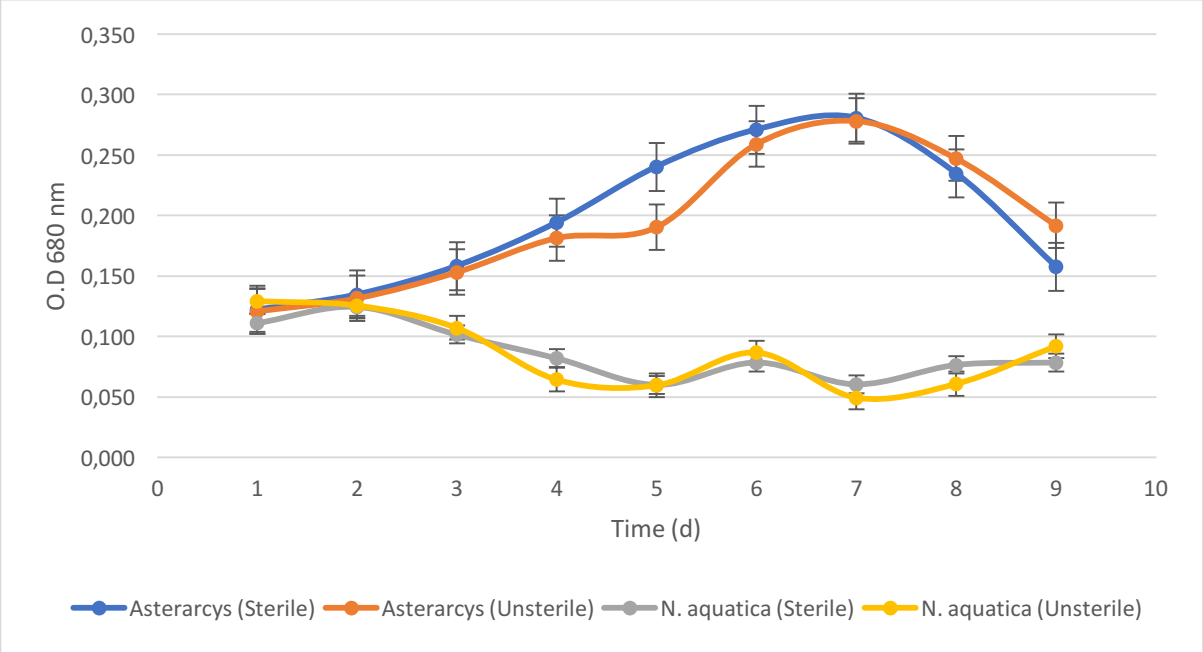
Fatty acid profile of the extracted lipid is presented in Figure 2. The fatty acid profile was generally the same irrespective of the wastewater source or sterility. Palmitic acid (C16:0), Stearic acid (C18:0) and Oleic acid (C18:1) constituted the dominant fatty acids the microalgae. Minor fatty acids recorded include myristic acid (C14:0), Arachidonic acid (C14:0) and Pentadecanoic acid (C15:0). The biodiesel property determined in-silico is presented in Table 3. Saturated fatty acids constituted the bulk of the types of fatty acid present ranging from 69.5% to 77.2%. Monounsaturated fatty acids (MUFAs) were the second most dominant type of fatty acid recorded ranging from 22.89% to 34.82%. Polyunsaturated fatty acids were present in small quantity ranging from 0 to 4.75%. Oxidative stability is dependent on the type fatty acid present in the oil varied and ranged from 36 h to infinity. Viscosity was low with a minimum of 3.96 mm^2/s and a maximum value of 4.13 mm^2/s. Cetane number was high ranging from 64.9 to 66.5.

Table 2: Growth kinetics and metabolite accumulation in microalgae cultivated in sterilized and unsterilized wastewater effluent

Treatment Plant	Organism ID	Lipid Dry Wt. (mgL^{-1})	Lipid % Dry Wt.	Total carbohydrate content (mg)	Total carbohydrate % dry wt.	Growth rate ($gL^{-1}day^{-1}$)	Biomass Dry wt. (mgL^{-1})	Biomass productivity ($mgday^{-1}$)
NWWTW	***Asterarcys***[b]	9.95 ± 0.50	19.90	11.05 ± 0.02	44.20	0.14	250 ± 1.20	35.71
	N. aquatica[a]	10.4 ± 0.71	20.80	14.48 ± 0.10	57.90	0.00	180 ± 0.70	25.71
	Asterarcys[a]	10.95 ±1.49	21.90	12.61 ± 0.61	50.44	0.18	460 ± 5.28	65.71
	N. aquatica[b]	8.75 ± 0.92	17.50	6.07 ± 0.45	24.29	0.00	163 ± 0.56	23.33
UWWTP	***Asterarcys***[a]	8.25 ± 1.63	16.50	10.77 ± 1.55	43.09	0.15	400 ± 2.77	57.14
	Asterarcys[b]	10.15 ± 1.20	20.30	11.42 ± 0.10	45.68	0.17	433 ± 0.61	61.91
	N. aquatica[a]	14.85 ± 1.63	29.70	9.83 ± 0.96	39.31	0.00	130 ± 0.36	18.57
	N. aquatica[b]	11.35 ± 2.48	22.70	12.82 ± 0.51	51.29	0.00	180 ± 0.26	25.71

a- Sterilized wastewater; *b*- unsterilized wastewater; NWWTW- Northern Wastewater treatment works, UWWTP- Umbilo wastewater treatment plant

(a)



(b)

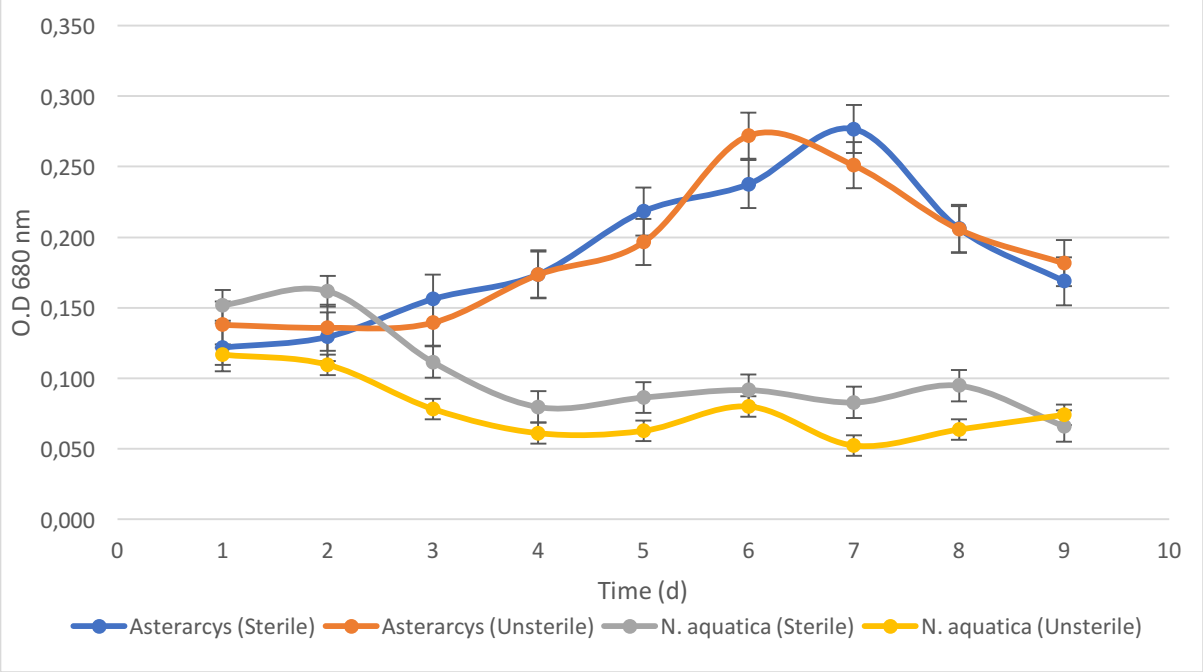


Fig. 1: Growth rate of microalgae cultivated in treated wastewater effluent from the NWWTW (a) and UWWTP (b).

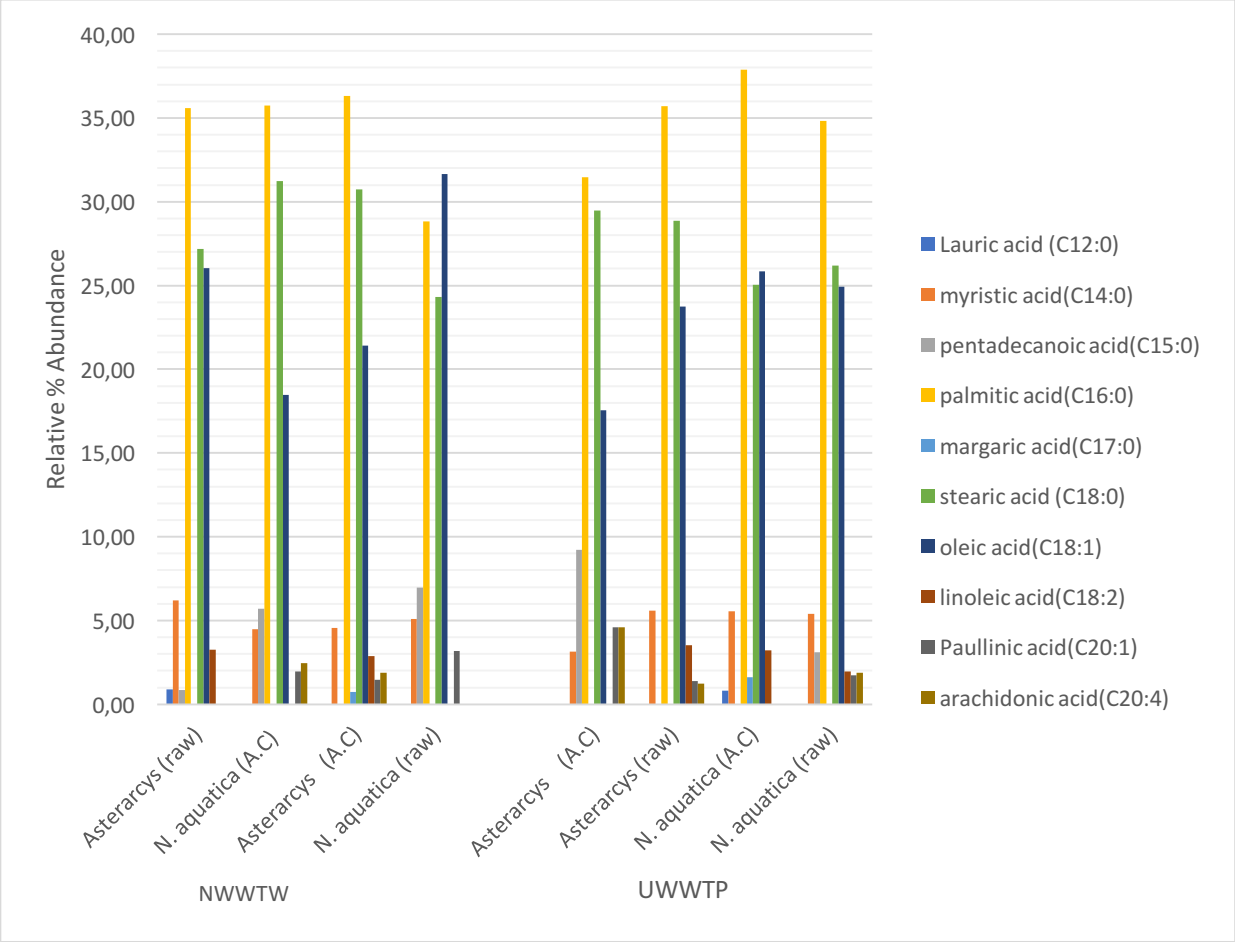


Fig. 2: FAMEs composition of indigenous microalgae grown in sterilized and raw wastewater effluent.

Table 3: Biodiesel characteristics of microalgae grown in sterilized and unsterilized wastewater effluent.

	Asterarcys (raw)	*N. aquatica* (A.C)	*Asterarcys* (A.C)	*N. aquatica* (raw)	*Asterarcys* (A.C)	*Asterarcys* (raw)	*N. aquatica* (A.C)	*N. aquatica* (raw)
SFA	70.7	77.16	72.37	65.18	73.26	70.13	70.92	69.5
MUFA	26.05	20.41	22.89	34.82	22.13	25.12	25.86	26.67
PUFA	3.25	2.44	4.75	0	4.6	4.74	3.23	3.83
DU	32.55	25.29	32.39	34.82	31.33	34.6	32.32	34.33
SV	209	208.312	206.878	207.821	209.975	207.263	209.018	207.89
IV	29.312	26.336	31.952	31.054	34.878	32.925	29.105	33.648
CN	65.82	66.575	65.493	65.576	64.446	65.226	65.864	64.983
LCSF	17.145	19.187	19.003	15.034	15.651	18.004	16.314	16.582
CFPP	37.387	43.803	43.225	30.755	32.694	40.086	34.777	35.619
CP	13.734	13.797	14.118	10.178	-0.148	13.781	14.938	13.323
PP	8.088	8.156	8.504	4.228	-6.981	8.139	9.395	7.642
APE	32.55	25.77	32.82	31.65	31.35	34.45	32.32	34.48
BAPE	3.25	4.88	6.64	0	9.2	5.96	3.23	5.71
OS	38.877	Infinity	43.825	Infinity	Infinity	36.093	39.101	63.067
HHV	39.426	39.458	39.491	39.459	39.374	39.468	39.431	39.45
υ	4.055	4.107	4.13	4.096	3.962	4.102	4.055	4.067
ρ	0.868	0.868	0.869	0.869	0.869	0.869	0.868	0.869

SFA: Saturated Fatty Acid (%); MUFA: Mono Unsaturated Fatty Acid (%); PUFA: Poly Unsaturated Fatty Acid (%); DU: Degree of Unsaturation; SV: Saponification Value (mg/g); IV: Iodine Value; CN: Cetane number; LCSF: Long Chain Saturated Factor; CFPP: Cold Filter Plugging Point (°C); CP: Cloud Point (°C); PP: Pour Point (°C); APE: Allylic Position Equivalent; BAPE: Bis-Allylic Position Equivalent; OS: Oxidation Stability (h); HHV: Higher Heating Value; υ: Kinematic Viscosity (mm2/s); ρ: Density (g/cm^3)

5.3 Discussion

Compared to available fossil fuel, biofuels from microalgae are more water intensive (Batan et al. 2013) with estimated 3,726 kg of water, 0.33 kg of Nitrogen and 0.71 kg of Phosphate required for the production of 1 kg of microalgae based biodiesel, if freshwater is used without recycling (Lee et al. 2013b, Yang et al. 2011). This is unsustainable for a water stressed country like South Africa. Thus, wastewater present a solution to this dilemma as it contains nitrogen in the form of nitrates and nitrites, phosphates as well as trace metals necessary for algal growth. Treated wastewater effluent is dynamic in its composition of these nutrients and compounds depending on the location of treatment plant, season and type of treatment applied.

In this study, treated municipal wastewater effluent was accessed for microalgal biomass propagation and subsequent biodiesel production. The treated effluent from the Northern wastewater treatment works (NWWTW) contained low amounts of nitrogen and phosphorus compared to the samples from the Umbilo wastewater treatment plant (UWWTP) while, initial pH values of 7.66 (NWWTW) and 7.37 (UWWTP) as well as low salinity suggests its suitability for the cultivation of freshwater microalgae. COD was reduced by up to 12.4% in the wastewater from the UWWTP after 8 days, however an increased COD of up to 30.7% was recorded in the wastewater from the NWWTW suggesting the excretion of organic compounds such as glycosylic acid into the medium during photosynthesis by the microalgae (Wang et al. 2010). Total nitrogen (TN) and Phosphorus (TP) were reduced by 48% and 50% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced the TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% were recorded in *Neochloris aquatica*. The difference in phycoremediation of wastewater by microalgae grown under the same conditions suggests that selecting the right microalgal specie is an important factor to consider when remediating wastewater with microalgae. This is also in agreement with observations by Kim and Kim (2017) in their study of phycoremediation potential of tertiary livestock stock wastewater and bioresource recovery.

Sterilization of wastewater did not seem to have any significant effect on the amount of biomass produced or the growth rate of the microalgae, thus unsterilized treated effluent can be used for microalgal cultivation without any pre-treatment step. However, a significant inverse correlation ($p < 0.01$) exist between the biomass produced and total nitrogen concentration in the water. when using wastewater as growth medium for microalgae for increased growth and nutrient removal, the N: P ratio should be balanced (Lee et al. 2013b, Whitton et al. 2016). In this study, the total nitrogen concentration far exceeded the total phosphorus concentration at the NWWTW with its optimal N: P of 44.2 exceeding the optimal range of 6.8 to 10 (Wang et al. 2010). The inability of the microalgae *Chlorella* spp. to utilize treated wastewater for growth was previously reported by Mutanda et al. (2011) and it was attributed to the presence of predators such as rotifers and protozoans in the wastewater. However, the inhibited growth of *Neochloris aquatica* and growth of *Asterarcys quadricellulare* observed in both autoclaved and unsterilized wastewater coupled with in both unsterilized and sterilized wastewater suggests that the ability of microalgae to utilise wastewater for growth may also be specie dependent amongst other factors. Another factor that affects the growth of microalgae in municipal wastewater is the optimal N:P ratio, said to be between 6.8 and 10 (Wang et al. 2010). While the UWWTP the N:P ratio value of 4.2 is close to the optimum value of 6.8, the N:P of the NWWTW far exceeds the optimum value suggesting a high Phosphorus limitation in the wastewater. The growth rate and biomass productivity of microalgae can be improved by supplementing the wastewater with cheap source of Nitrogen and Phosphorus such as food waste (Ji et al. 2015) and centrate (Udom et al. 2013) . The biomass can further be enhanced by the addition of CO_2 from flue gas (Kuo et al. 2016, Lage et al. 2018). Though *Asterarcys quadricellulare* had the highest biomass and growth rate when cultivated in wastewater compared to *Neochloris aquatica,* the highest lipid accumulation was recorded in *Neochloris aquatica* (14.85 ± 1.63 mg). This also indicates that a high growth rate or biomass productivity does not necessarily translate to high lipid accumulation and that lipid accumulation potential may vary from specie to specie. This may be a necessary factor to consider when cultivating microalgae in wastewater for large-scale biodiesel production.

Saturated fatty acid; Palmitic acid (C16:0), Stearic acid (C18:0) and the monounsaturated fatty acid Oleic acid (C18:1) were the dominant fatty acid present in the microalgal lipid. Linoleic acid (C18:2) and Arachidonic acid (C20:4) are important (omega 6) polyunsaturated fatty acid (PUFAs) that make up the minor components of the microalgal lipid. Polyunsaturated fatty acid are nutritionally important fatty acid essential for infant development with an estimated industrial worth of 11 billion dollars (Yaakob et al. 2014). The microalgae are therefore, suitable for commercial production of these fatty acids. However, the long chain PUFAs; Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), two major essential fatty acids used in baby formula production were not detected in the lipid profile. The biodiesel was deemed to be of high quality as it contained a mix of saturated, monounsaturated and polyunsaturated fatty acids recommended in the ASTM standard D6751-07 (ASTMD6751-07b 2007). Lipids containing high amounts of Oleic acid have been reported to have a good balance of fuel in terms of its ignition quality, combustion heat, cold filter plug point, oxidative stability and viscosity, all of which are determined by the fatty acid composition of the biodiesel (Abou-Shanab et al. 2011). Insilco characterization of the biodiesel revealed a quality biodiesel with good oxidation stability ranging from 36 hours to infinity well above 3 mins limit set by the ASTM standard. Biodiesel viscosity recorded ranged from 3.96 mm^2/s to 4.13 mm^2/s, well within the specified limits of 1.6 and 6 mm^2/s prescribed by the ASTM biodiesel standard for second generation biodiesel (ASTMD6751-07b 2007).

Carbohydrates in microalgae consists mainly of starch found as storage molecule in chloroplasts and cellulose/polysaccharides found as structural components within the cell walls. The potential of microalgae as a promising feedstock for bioethanol production has been reported by several researchers, while the utilization of wastewater for growth by these organisms can drastically reduce the cost of bioethanol production making it competitive with starch rich crop-based feedstock such as sugarcane currently used in bioethanol production (Guo et al. 2013). *Chlorella* spp. grown in sterilized wastewater from the NWWTW accumulated the highest amount of total carbohydrate (14.48 ± 0.10 mg) accounting for up to 57.9% of its dry weight and would be excellent candidate as a feedstock for bioethanol production.

5.4 Conclusion

Pre-chlorinated treated wastewater effluent in Durban were shown to be feasible growth media for cultivation of microalgae for biofuel production as it possess adequate physicochemical characteristics for the cultivation of freshwater microalgae. Though microalgae utilized the wastewater for growth, unbalanced N:P ratio, incomplete nutrient removal and low biomass yield are some of the challenges that needs to be overcome to increase biomass propagation. The growth rate and biomass productivity may be enhanced by supplementation of the wastewater with phosphates to balance the N:P ratio and addition of carbon dioxide (Lage et al. 2018). Bacterial contamination and predation were less of an issue in this study as sterilization of the wastewater did not result into any significant increase in the growth rate of the microalgae. Thus, the microalgae can be cultivated without pre-treatment of the wastewater saving costs. The microalgae also accumulated good amounts of lipids which are desirable for biodiesel production, while the high carbohydrate content also make them good candidates for bioethanol production. . Some studies have reported increase in carbohydrate accumulation after addition of CO_2 (Ji et al. 2015). Thus, future research could incorporate this to achieve high carbohydrate content if bioethanol production is the goal.

5.5 Acknowledgements

The authors are grateful to the National Research Foundation of South Africa (UID: 92803 to AOO; UID: 94043 to TM) and the National Science Foundation of China (31570367 to YFC) for funding this study.

5.6 Declaration of Interest

The authors declare no conflict of interests.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSION

6.1 The Research in Perspectives

Microalgae are ubiquitous, unicellular and simple organism with rapid growth rate, high biomass, lipid accumulation, and ability to grow in harsh environment (Mutanda et al., 2011; Rawat et al., 2013). The triglycerides in their lipids can be converted to biodiesel, a renewable energy that can mitigate the growing concerns over the finite nature of fossil-based fuel, increasing carbon emission and climate change. Second generation biofuel feedstock such as oleaginous and starch rich crops are regarded as unsuitable for commercial production of biofuels due to the concern over increasing food cost and its impact on the poor (Shurson, 2017). Microalgae are found in most of earth's ecosystems and there exists an estimated 200 000 – 800 000 species with only about 35 000 has been characterized and described (Barra et al., 2014). The vast majority remains unknown in the environment and may possess novel characteristics with potential biotechnological applications, such as biodiesel production, polyunsaturated fatty acid generation, carbohydrate accumulation for bioethanol production, carotenoids etc. Bioprospecting for microalgae with biotechnological relevance including high lipid accumulation is necessary for the realization of a viable commercial microalgae derived biofuel industry (Georgianna & Mayfield, 2012; Mutanda et al., 2011). However, traditional bioprospecting for novel microalgae is tedious, time consuming and expensive. Molecular fingerprinting tools such as denaturing gradient gel electrophoresis (DGGE) and Terminal restriction length fragment polymorphism (T-RFLP) have been used extensively to profile the community structure of microbial ecosystems including bacteria and phytoplankton (Buchanan et al., 2013; Liu et al., 1997; Muyzer et al., 1993; Yu et al., 2015). However, there is little information about the community structure of microalgae in various aquatic ecosystems in South Africa. Thus, this study used two DNA fingerprinting techniques (DGGE and T-RFLP), to provide an overview of the microalgal diversity in various aquatic ecosystem in Durban, KwaZulu-Natal province of South Africa in order to identify areas with high diversity as well as identify the dominant species. The sampled sites include; rivers, estuaries, and maturation ponds of wastewater treatment plants and marine environment of the Indian Ocean. The classes Trebouxiophyceae and Chlorophyceae were observed to be dominant in fresh water habitat, while

microalgae belonging to the class Ulvophyceae was observed to be dominant in the salt water environment. At the brackish water habitat, microalgae belonging to the classes Trebouxiophyceae (*Chlorella minutissima*) and Chlorophyceae (*M. sturmi*) were dominant. The presence of three or more OTUs from the DGGE and T-RFLP results suggests high microalgal diversity however, specie richness varied in agreement with other studies (Buchanan et al., 2013; Cutler et al., 2013). Enrichment of water samples confirmed the presence of different types of microalgae with varied morphology and sizes with green pigmentation in agreement with results obtained from DGGE while, Nile red screening confirmed the presence of lipids making them a potential feedstock for biodiesel production. Thus, DGGE and T-RFLP methods can complement traditional method of bioprospecting in enhancing the chance of rapid discovery and isolation of novel microalgae for biotechnological applications. However, further research on the development of more robust primers capable of amplifying all microalgae, while discriminating against other sources of DNA, is required to improve the accuracy of the method.

The enriched microalgae from the different terrestrial aquatic ecosystems sampled in Durban were isolated, purified and monocultures were maintained in commercial BG-11 media (Sigma Aldrich, Germany) while 10 randomly selected pure isolates were identified using PCR amplification, sequencing and analysis of 18S rRNA gene and used for further studies. The growth rates of the isolates were determined after 21 days of uninterrupted growth under batch photoautotrophic conditions in BG11 medium at 30 °C, 54.36 μmol/m^2s^{-1} and 12:12 Light/Dark period. Their lipid, carbohydrate and protein accumulation potential was evaluated using well-established procedures. The characteristics of biodiesel was estimated using the biodiesel analyzer software. The effect of nutrient limitation on their lipid, carbohydrate and protein accumulation potential was also evaluated.

The indigenous isolates were identified to consist of *Chlorella* spp., *Asterarcys. quadricellulare*, *Chlorococcum* spp. and *Neochloris aquatica*. Phylogenetic analysis suggest close relatedness to microalgae of biotechnological relevance, such as *Haematococcus* from which the antioxidant Astaxanthin was commercially produced (Shah et al., 2016), *Dunaliela* spp. reported to contain large amounts of carotenoids (Diprat et al., 2017), *Scenedesmus* spp. and *Chlorella vulgaris* which have been

reported as good feedstock for biodiesel production due to their high lipid content (Chiu et al., 2015). The growth rates ranged from 0.219 ± 0.003 to 0.175 ± 0.023 $gL^{-1}day^{-1}$ while dry weight ranged from 0.433 ± 0.208 to 1.167 ± 0.153 gL^{-1}. *Chlorococcum* LM1 showed high accumulation of lipid (11.93 ± 0.76 mg/L), carbohydrate (15.22 ± 2.64 mg/L) and protein (1.32 ± 0.46 mg/ml). Although the growth rate, dry weight and biomass productivity recorded in this study are high, the values varied amongst isolates even for those of the same genus as previously reported by Abou-Shanab et al. (2011). Under similar growth conditions, *Chlorella sorokoniana* NWS5 exhibited the highest growth rate (0.219 ± 0.003 $gL^{-1}day^{-1}$), dry weight (1.167 ± 0.153 gL^{-1}) and highest productivity (0.056 $gL^{-1}day^{-1}$) indicating its suitability for high density culture. The biomass productivity recorded in this study was higher than those reported elsewhere (Song et al., 2013). *Chlorella sorokiniana* accumulated the highest amount of lipid up to 24% of its dry weight similar to previous report by Talebi et al. (2013), demonstrating its suitability for possible commercial scale applications.

Biodiesel was produced by transesterification of lipid and the lipid profile was analyzed using GC-MS (Shimdzu Corp., Kyoto, Japan). The lipid profile consisted mainly of saturated fatty acids (80%) including oleic acid (C18:1), palmitic acid (C16:0) and stearic acid (C18:0) and low amounts of polyunsaturated fatty acids such as linoleic acid (C18:2 n-6), making it desirable for biodiesel production (Zheng et al., 2012). Characteristics of the biodiesel based on the lipid profile reveal a low viscosity and density ranging from 1.66 to 4.264 mm^2/s and 0.327 to 0.867 g/cm^3, respectively. Cetane number ranged from 51.66 to 111.262 and oxidative stability ranged from 5.51 h to infinity, values well within the recommended ASTM D6751-07 biodiesel standard (ASTMD6751-07b, 2007). Under nutrient limitation, some isolates showed an increase in lipid accumulation, e.g. *Chlorococcum sp*. LM1 (17.2%) and *C. sorokiniana* NWS5 (24%), while it decreased in others such as *Chlorella minutissima* TS9 (16%), *Neochloris aquatica* Toti4 (3.5%) and *Chlorococcum sp.* LM2 (6.5 %). Nutrient limitation has been previously reported to enhance lipid accumulation in microalgae species (Vitova et al., 2015; Zhu et al., 2016), however, this can also impact negatively on the growth rate and biomass accumulation (Fields et al., 2014). As evident in this study, enhanced lipid accumulation via starvation seemed to be unique to each algal strain irrespective of species. The results suggest that these indigenous microalgae

species can be applied for biodiesel and bioethanol production due to their high lipid and carbohydrate accumulation. Biomass of these microalgae can also be applied in the agricultural sector as animal feed additives given their high protein content.

The ability of microalgae to attain high growth rate, and accumulate high biomass and lipid even in harsh environment gives them an advantage over traditional first generation biofuel feedstocks such as oil rich crops but huge amount of water is required for producing microalgal biodiesel (Batan et al., 2013; Farooq et al., 2015) , with an estimate of 3.726 kg of water, 0.33 kg of Nitrogen and 0.71 kg of Phosphate required to produce 1 kg of biodiesel, if freshwater is used without recycling (Lee et al., 2013; Yang et al., 2011). Thus, putting a strain on the freshwater resources of water stressed countries like South Africa. Wastewater usually contains nitrogen in the form of nitrates and nitrites, and phosphorus in the form of phosphates as well as other trace metals necessary for the growth of microalgae. Discharge of wastewater containing these nutrients can lead to eutrophication of the receiving water body. Microalgae can utilise these macronutrients in the wastewater for their growth while simultaneously remediating the wastewater. The potential use of pre-chlorinated wastewater as a cheap growth media for microalgal biomass propagation was therefore evaluated with the aim of concomitant biodiesel production and wastewater remediation. Pre-chlorinated wastewater samples were collected from the secondary clarifier tank of two wastewater treatment plants in Durban, South Africa. Sterilized (by autoclaving) and unsterilized wastewater were inoculated separately with *Neochloris aquatica* and *Asterarcys quadricellulare* and growth profiles of the isolates were monitored for a period of 8 days at 680 nm. The physicochemical parameters of the wastewater samples, including chemical oxygen demand (COD), total nitrogen (TN), total Phosphorus (TP), salinity, pH, resistivity and conductivity were determined before microalgal cultivation and after harvesting. The total lipids were quantified gravimetrically after extraction in hexane/methanol (3:2 v/v) solution mixture. Biodiesel was produced by transesterification of the lipids with methanol in the presence of sulfuric acid and the fatty acid methyl esters (FAMES) characterised by GC-MS (Shimadzu Corp., Kyoto, Japan). The total carbohydrate was also quantified using well-established procedures. TP concentration

was low resulting in an unbalanced N:P ratio of 44 at the NWWTW and 4 at the UWWTP. However, statistical analysis revealed a significant inverse correlation ($p < 0.01$) between the biomass produced and total nitrogen concentration in the water. Several authors have reported the need for a balanced N:P ratio when using wastewater as growth medium for increased growth and nutrient removal (Lee et al., 2013; Whitton et al., 2016).

Asterarcys quadricellulare utilized the wastewater for growth, with 12.4% reduction in the COD of sample from the UWWTP recorded. Total nitrogen (TN) and Phosphorus (TP) were reduced by 48% and 50%, respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater from NWWTW while, *Neochloris* reduced TP by 37% and TN by 29%. At the UWWTP, TP and TN were reduced by 32% and 44% respectively by *Asterarcys quadricellulare* cultivated in sterile wastewater while 29% and 19% TP and TN reduction, respectively were recorded in *Neochloris aquatica*. The difference in phycoremediation of wastewater by microalgae grown under the same conditions suggests that selecting the right microalgal specie is an important factor to consider when remediating wastewater with microalgae. This is also in agreement with observations by Kim and Kim (2017) in their study of phycoremediation potential of tertiary livestock stock wastewater and bioresource recovery. Sterilization of wastewater did not seem to have any significant effect on the amount of biomass produced nor the growth rate of the microalgae, thus raw treated effluent can be used for microalgal cultivation without any pre-treatment step.

Though *Asterarcys quadricellulare* accumulated the highest biomass (460 mg), the highest amount of lipid and carbohydrate content were recorded in *Neochloris aquatica* (14.85 and 14.84 mg, respectively). The dominant fatty acids in the microalgae were palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1). The omega-6 polyunsaturated fatty acids; arachidonic acid (C14:0) and linoleic acid (C18:2) constituted the minor fatty acids. The biodiesel was determined to be of good quality with high oxidation stability and low viscosity, and conformed to the ASTM guidelines. This study thus, showed the feasibility of microalgal biomass propagation and biodiesel production using treated wastewater effluent from wastewater treatment plants in Durban, KwaZulu-Natal, South Africa.

However, optimization of the N:P ratio and carbon source are necessary to improve biomass productivity for commercial scale production.

6.2 Potential for Future Development of the Study

Very little research (if any) has been done on the diversity and community structure of microalgae in aquatic ecosystems in Durban, KwaZulu-Natal, South Africa. Molecular fingerprinting approach including 18S rDNA analysis, pyrosequencing, whole genome sequencing and phylogenetic profiling could enhance the discovery of new microalgae capable of biodiesel production and their phylogenetic analysis may reveal many more potential species (Duong et al., 2012). This research provided an overview of the community structure and diversity using molecular approach (DGGE and T-RFLP). However, these methods could be improved upon by the development of more robust primers that can target both eukaryotic and prokaryotic microalgae. Furthermore, the primers used in this study were only able to amplify microalgae of the division Chlorophyta, excluding other divisions and classes such as the Bacillariophyceae, Eustigmatophyceae etc., thus skewing the data on the diversity, identity and community structure of microalgae present in the study area. Microalgae are found in other ecosystems besides aquatic environment, hence, future research could extend molecular evaluation to other ecosystems such as soil.

The indigenous microalgae isolates evaluated in this study proved to be potential feedstock for biodiesel and bioethanol production due to their high lipid content (e.g. *Chlorella sorokinaina* NWS5, 12.9 mg/L) and total carbohydrate content (e.g. *Chlorococcum* spp. 60% dry weight) when grown in commercial BG-11. However, it would be interesting to compare and evaluate the lipid accumulation in other types of growth media such as Bold's Basal medium (BBM) and Tris-acetate phosphate medium (TAP). Future optimization of lipid accumulation could also include the addition of carbon dioxide which has been proven to accelerate growth of microalgae. In this study, ambient carbon dioxide from the air passively diffused into the growth medium due to agitation (150 rpm). However, future research could incorporate carbon dioxide in the form of bubbled air, sodium carbonate or bubbled CO_2 gas.

Microalgae grown in pre-chlorinated wastewater could also be optimized in this way as well. Future studies can also incorporate the addition of small metabolic compounds such as acetyl CoA to the growth medium for improved lipid synthesis and accumulation. Other growth factors such as the amount of light, temperature and salinity conditions could be further optimized using optimization tools such as response surface methodology.

Upstream processing of microalgae such as harvesting, dewatering and lipid extraction are cumbersome and require lots of energy. In this research, harvesting was done by centrifugation and dewatering was achieved by lyophilization, both energy intensive process, and could increase the cost of microalgal biodiesel production on a commercial scale. Future research into other less energy intensive methods such as flocculation and sedimentation is recommended while, the feasibility of using wet biomass for transesterification should also be explored as this would eliminate the dewatering step and reduce cost. Solvent used for lipid extraction can also affect the amount of lipid recovered from microalgae. In this study, hexane: isopropanol mixture (3:2 v/v) was used to extract lipid using gravimetric approach. However, the extraction yield of other extraction solvents such as chloroform, absolute ethanol and supercritical carbon dioxide can be evaluated and compared to the current study in future research. It would also be interesting to compare the use of enzymes for transesterification of microalgae lipids during biodiesel production against the chemical catalysts (H_2SO_4) used in this study.

Future research can also be geared towards the identification and detection of genes necessary for lipid and other valuable metabolites accumulation such as genes coding for acetyl-CoA carboxylase and nitrate reductase in the microalgae isolates. These genes can be manipulated via random mutation to improve microalgae strain, cloned and expressed in suitable expression host such as *Chlymodomonas* spp.

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