Phycoremediation of industrial and municipal domestic wastewaters with concomitant biomass propagation for bioenergy production

By

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

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PREFACE

The experimental work contained in this thesis was conducted by the candidate while based in the Discipline of Microbiology, School of Life Science of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa, under the supervision of Prof A.O Olaniran and the co-supervision of Dr T Mutanda. The research was financially supported by the National Research Foundation (NRF).

These studies represent the original work by the author and the contents of this work have not otherwise been submitted in any form for any degree or diploma to another tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text. The result reported are due to investigation by the candidate.

DECLARATION I: PLAGIARISM

I, S'fiso Thuthukani Gumbi, declare that:

(i) The research reported in this thesis, except where otherwise indicated or acknowledged, is my original research;

(ii) This thesis has not been submitted in full or in part for any degree or examination to any other university;

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DECLARATION II – PUBLICATIONS

Details of contributions to publication 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 contribution of each author to the experimental work and writing of each publication)

Chapter 3

Gumbi, S. T*, Majeke, B.M., Olaniran, A. O and Mutanda, T. 2017. Isolation, Identification and High-Throughput screening of neutral lipid producing indigenous microalgae from South Africa aquatic habitats, *Applied Biochemistry and Biotechnology*, 182: 382 – 399. DOI 10.1007/s12010-016-2333-z.

Chapter 4

S'fiso T. Gumbi*, Ajit Kumar, and Ademola O Olaniran. 2021. Lipid productivity and biosynthesis genes response of indigenous *Chlorella* sp. T4 strain under different nitrogen and phosphorus load. Accepted to the journal, *BioEnergy*.

Chapter 5

S'fiso Thuthukani Gumbi*, Taurai Mutanda, and Ademola O Olaniran. 2021. Macronutrient removal from dairy and poultry wastewater with simultaneous biomass and biodiesel production by *Chlorella* sp. T4 isolated from a freshwater stream in South Africa. *Waste and Biomass Valorization*. http:// doi.org/10.1007/s12649-021-01492-0.

International Conference on Science, Technology, Engineering and Management (ICSTEM), Shanghai, China, 14th -15th December, 2019 (Oral presentation).

Chapter 6:

S'fiso T. Gumbi* and Ademola O Olaniran. 2021. Co-production of bioethanol and biodiesel using microalgae *Chlorella* sp. T4 cultivated in diary wastewater.

Signed 

Date: 18/01/2022

DEDICATION

This work is dedicated to my late grandfather, Mr. Sofeteza Ambrose Nsibande and Grandmother Nomatheku Nester Gumbi for their unconditional love and support

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ABSTRACT

The utilization of microalgae has been endorsed as a great source of biofuel generation and wastewater reclamation without any adverse effects. Microalgae have high growth rates, efficient photosynthesis process and biomass productivity which serve as an economic advantage. Microalgae can be used for the dual purpose of biodiesel production and wastewater treatment due to their ability to sequester organic pollutants such as nitrogen and phosphorus in wastewater. Thus, the aim of this study was to bioprospect for indigenous hyper lipid producing indigenous microalgal strain for biofuel production and wastewater treatment. Different water samples were collected from diverse aquatic habitats, including freshwater, brackish and marine water in KwaZulu-Natal, South Africa. Eight indigenous microalgal strains were isolated and screened for biomass accumulation and lipid yield using Nile red fluorescence microscopy screening and gravimetric analysis. The strains were identified based on their morphological characteristics and 18S rRNA gene sequence analysis to belong to the genera *Chlorella*, *Neochloris* and *Chlamydomonas*. They showed high lipid yield ranging from 14 ± 6.2 to 38 ± 8.8% dcw, proving to be a good feedstock for biodiesel production. Of the eight isolated microalgae, *Chlorella* sp. T4 was selected for further analysis based on the growth kinetic, lipid productivity and fatty acid profiles.

The strain was subjected to different cultivation conditions to enhance lipid productivity by varying nitrogen and phosphorus concentration. A significant decrease in biomass accumulation and low quantum efficiency of photosystem (Fv/Fm) value was observed under nitrogen and phosphorus limiting conditions. The lowest biomass yield of 0.58 ± 0.03 g L^{-1} was found in nitrogen limiting medium (0.75 g L^{-1}). High lipid productivity of 15.54 ± 0.7 mg L^{-1} d^{-1} was obtained under nitrogen limiting condition which was 1.37-fold higher than phosphorus limiting (0.02 g L^{-1}) condition after 21 days. Nutrient stress caused an increase in the expression of Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (*accD*), ketoacyl-ACP synthase-1 (*KAS*-1), omega-6 desaturase (*ω*-6 FAD) and omega-3 desaturase (*ω*-3 FAD) genes responsible for lipid biosynthesis. Whereas a decrease in Ribulose bisphosphate carboxylase large chain (*rbcL*) gene expression level was noted due to nutrient stress lowering the photosynthetic rate. Fatty acid methyl esters produced under nutrient limiting conditions were found to be suitable for the production of high-quality biodiesel with enhanced oxidative stability and cold flow properties.

The ability of *Chlorella* sp. T4 to utilize the different nutrient-rich environments and remove nutrients from poultry and dairy wastewater was investigated to ascertain its possible use for the sustainable and low-cost treatment of wastewater. *Chlorella* sp. T4 showed high nitrogen and phosphorus removal efficiency of 85 to 95% and 35 to 93%, respectively. This was followed by a high biomass yield of 1.28 g L^{-1} and 0.85 g L^{-1} obtained using raw poultry and dairy sludge wastewater, respectively. The biomass contained significant

amounts of lipids (16.2–25.7 % dry wt.), carbohydrates (20.7–33.1 % dry wt.), and proteins (24.5–34.6 % dry wt.), regardless of the wastewater type. Biodiesel properties of lipids extracted from the cell grown in poultry and dairy wastewater complied with most of the international standards by ASTM D6751 and EN 14,214.

Based on the lipid productivity and fatty acid profile, sludge dairy wastewater was used for biomass propagation for simultaneous bioethanol and biodiesel production. Optimization of cell disruption and extraction techniques resulted into high lipid and sugar recovery efficiency. Through acid hydrolysis using sulphuric acid, 2.14 g L^{-1} of sugar was recover from *Chlorella* sp. T4 biomass and fermented to ethanol (0.81 g L^{-1}) using *Saccharomyces cerevisiae*. High lipid content of 21.7 ± 0.5% was recovered from the residual biomass after hydrolysis and converted into biodiesel via transesterification. The biodiesel produced from the residual biomass meets most of the standards specified by ASTM D6751 and EN 14214. In conclusion, hyper lipid producing microalgal strain *Chlorella* sp. T4 isolated from KwaZulu-Natal, South Africa showed potential for biofuel production after proper optimization of growth conditions. The potential of *Chlorella* sp. T4 to utilize different wastewater high in nutrient concentration confirm potential application during large scale cultivation for biofuel production to address energy crisis and water shortage.

LIST OF TABLES

CHAPTER 6

LIST OF FIGURES

ABBREVIATIONS

ASTM – American Society for testing and materials

COD – Chemical oxygen demand

EN – European norms, European Committee for Standardization

FAMEs – Fatty acid methyl esters

Fv/Fm: Low maximum quantum efficiency

GC-MS – Gas chromatography- mass spectrometry

GPS – Global positioning system

HPLC – High pressure liquid chromatography

HRAPs – High rate algal points

MUFA – Monounsaturated fatty acid

PSB – Phosphate buffered saline

PS II – Photosystem II

PCR – Polymerase chain reaction

PUFA – Polyunsaturated fatty acid

PAM – Pulse amplitude modulation

SFA - Saturated fatty acid

SPV – Sulfo-phospho-vanilin

CHAPTER ONE

1.0 Introduction and scope of the study

The overuse of fossil fuel and the worldwide industrial revolution has resulted in ramped depletion of crude oil resources causing instability in its price and accretion of greenhouse gases. Generally, fossil fuels are non-renewable and expensive contributing to air/water pollution and global warming (Mathimani and Mallick, 2018). The reliance on fossil fuels contributes to the increase in the emission of greenhouse gases causing enormous damage to humanity and the environment (Brindhadevi et al., 2021). Besides energy shortage, domestic and industrial waste is the main cause of water pollution causing a critical shortage of freshwater (Daneshvar et al., 2019; Oliveira et al., 2018). This crisis has shifted the global focus to sourcing sustainable and feasible ways of biofuel production and wastewater treatment. At the same time, there is earnest attention for a renewable form of energy including biodiesel as the potential substitute for fossil fuel-based petroleum to overcome the energy crisis. Food crops such as starch and oil-based crops have been traditionally used for the generation of biofuels with low carbon emissions (Chng et al., 2017; Popa, 2018). However, these sources of liquid fuels are not sustainable due to food security, geopolitical and socio-economic concerns (Li et al., 2019).

Microalgae are the most attractive biological agent to address both energy and water treatment, compare to physical and chemical treatment methods (Razzak et al., 2017). Microalgae are classified as unicellular and photosynthetic organisms found in the diverse aquatic environment, producing various valuable compounds with application in the biofuel, pharmaceutical and cosmetic industries (Suparmaniam et al., 2019; Yin et al., 2020). They exhibit multiple advantages such as faster growth rate, high lipid accumulation that is suitable for biodiesel production and ability to utilize different wastewater makes them suitable feedstock for biodiesel production and wastewater treatment (Li et al., 2019). The triacylglycerol's (TAGs) in microalgae are converted to biodiesel via the transesterification process and monosaccharides are fermented for bioethanol production via fermentation (Odjadjare et al., 2017; Xu et al., 2019). At a large scale microalgae are cultivated in open cultivation systems (raceway pond etc.) and closed photobioreactor (tubular photobioreactor, flate photobioreactor etc.). A key constraint in cost-effective microalgal biodiesel production is the operation and capital investment owing to intricate expensive oleaginous materials and bioreactor design (Huang et al., 2017).

One possible way to improve the economics of microalgae-based technology involves bioprospecting for hyper lipid producing microalgal strains from the diverse aquatic environment that easily acclimatize to different growth conditions (Duong et al., 2012; Mutanda et al., 2011). Moreover, the utilization of wastewater as a cheap medium for microalgae cultivation can improve the economy of microalgae-based technology. Large quantities of wastewater from domestic and industrial activities are produced constantly that requires treatment before being discharged into the environment (Daneshvar et al., 2018; Ferreira et al., 2018). Wastewater contains various unwanted biochemical constituents and pathogens which have short- and long-term effects on the environment. Untreated

wastewater can cause eutrophication in the receiving water bodies, affect the aquatic ecosystem and constituent health risk to its users (Bhatia et al., 2020).

Conventional methods are faced with challenges to meet the stringent nutrients discharged standard with high efficiency and low cost (Kumar and Pal, 2015). Microalgae can remove a significant amount of nitrogen and phosphorus from different wastewater, which is more cost-effective in comparison to other methods used so far. Microalgae-based wastewater treatment does not require the addition of extremely hazardous chemicals, contribute to the reduction of carbon footprint and reduces energy demand and cost (Oliveira et al., 2018; Mennaa et al., 2015). The utilization of wastewater by microalgae as the growth medium for biomass propagation with concomitant wastewater treatment has also been reported by various researchers (Ferreira et al., 2018; Shahid et al., 2020). However, finding robust microalgae strain that can utilize different wastewater and produce high biomass is critical for the development of competitive microalgae-based fuel. Therefore, this research focused on bioprospecting for hyper lipid producing microalgal strain from aquatic environment around KwaZulu Natal, South Africa for biofuel production and wastewater treatment. The study was also undertaken to understand the role of nutrient stress on the accumulation of lipid and the expression of key lipid biosynthetic genes.

1.1 Hypothesis

Microalgae strains indigenous to KwaZulu-Natal province are hyper-lipid producer suitable for biodiesel production. Indigenous microalgae can efficiently remove nutrients from wastewater while simultaneously producing biomass for biodiesel production thus reducing the costs.

1.2 Aim

The aim of this study were:

1.2.1 To bioprospect for hyper lipid producing microalgal strain for biodiesel production.

1.2.2 To develop an integrated strategy for lipid enhancement strategy by varying the nutrient concentration on the growth medium and to study it effects on photosynthetic efficiency, expression of key function genes involved in photosynthesis and lipid biosynthesis.

1.2.3 To investigate the feasibility of utilizing industrial wastewater for simultaneous biomass propagation and wastewater treatment.

1.3 Objectives

The following objectives were set to achieve the general aims of the study;

1.3.1 Isolation and screening of unique indigenous microalgae strains from diverse aquatic environment. To identify and characterize the isolate obtained from the samples sites using light microscopy as well as PCR amplification, sequencing and analysis of 18S rRNA gene.

1.3.2 To analyze microalgae growth kinetic, biomass accumulation, lipid accumulation and suitability of fatty acid profile for biodiesel production.

1.3.3 To evaluate microalgae growth rates, photosynthetic efficiency and lipid productivity under nitrogen and phosphorus limiting and replete conditions.

1.3.4 To perform gene expression analysis of key function genes involved in carbon fixation (*rbcL*) and biosynthesis of fatty acid (*accD*, *KAS*-1, *ω-3* FAD and *ω-6* FAD).

1.3.5 To utilize poultry and dairy wastewater for microalgae growth with concomitant biomass propagation for biofuel production. Study the physiological response and lipid productivity of microalgae in wastewater and nutrient removal efficiency.

13.5 To optimize the cell disruption techniques and extraction solvent to enhance sufficient recovery of sugar and lipid from the microalgae biomass.

1.3.6 To use sludge dairy wastewater for biomass propagation for co-production of bioethanol and biodiesel by adoption of biorefinery.

1.4 Layout of the thesis

The dissertation comprises seven chapters and is based on publications referred to by their title in the text. Each chapter is self-contained, containing an abstract, an introduction for the study's motivation through literature, materials and methods, results and discussion, and conclusions.

Chapter 1 provided an overview that offers background information and the scope of the study, including the overarching aims and specific objectives of this study.

Chapter 2 consist of critically review the literature on the subject matter whilst highlighting areas of importance and future research on microalgal biotechnology.

Chapter 3 focuses on bioprospecting and screening for indigenous hyper lipid producing microalgae from the diverse aquatic environment around KwaZulu-Natal, South Africa for potential application in biodiesel production. This work has been published in a peer-reviewed journal, *Applied Biochemistry and Biotechnology*.

Chapter 4 focuses on enhancing lipid accumulation by the *Chlorella* sp. T4 by varying nutrient concentration on the growth medium. Also, it investigated the expression of key functional and fatty acid biosynthesis genes under different nutrient concentrations. The work generated here was prepared in form of a manuscript and has been submitted to the journal, *Fuel*.

Chapter 5 investigated the feasibility of utilizing of poultry and dairy wastewater for biomass propagation for biodiesel production and simultaneous nutrient removal using *Chlorella* sp. T4. This manuscripts has been published in a peer-reviewed journal, *Waste and Biomass Valorization*.

Chapter 6 demonstrated the feasibility of a biorefinery approach for bioethanol and biodiesel production from *Chlorella* sp. T4 biomass. The work generated here was prepared in form of a manuscript and has been submitted to the journal, *Molecules*.

Chapter 7 summarises the thesis, the various findings are discussed, conclusion are drawn, and recommendation for future work are made.

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CHAPTER 2

Literature review

Abstract

Currently, the world is faced with water and energy crises which are essential requirements for the growth and development of the global economy. The integrated approach of wastewater reclamation and microalgae biodiesel production based on phycoremediation is a promising process to mitigate some of the challenges created by these crises. Remediation of nutrients using microalgae presents technical and economic benefits compared to conventional techniques for wastewater treatment. However, the uptake of the technology to date has been limited mainly due to consideration of land requirement and volume of wastewater to be treated. This review provides an overview of the biology of microalgae, wastewater treatment using microalgae, benefits and challenges of microalgae-based wastewater treatment microalgae cultivation system and harvesting techniques, biodiesel production via transesterification and value-added products. Moreover, the issues and limitations of microalgae-based wastewater treatment are also discussed, and suggestions are proposed for further research and development.

Keywords: Microalgae, Wastewater, Biodiesel, Biorefinery, Value-added product

2.0 Introduction

The world is estimated to face 40% water shortage by 2030 causing a challenge in societal and economic development due to insufficient water reclamation technologies (Sun et al., 2016). The reuse of water is widely recommended for effective wastewater management since freshwater resources are being depleted through anthropogenic activities (Abinandan et al., 2018). Large quantities of wastewater from domestic and industrial activities are produced constantly that require treatment before being discharged to the environment. Wastewater contains various unwanted biochemical constituents and pathogen which have short- and long-term effects on the environment. Untreated wastewater can cause eutrophication in the receiving water bodies, affect the aquatic ecosystem and constitute health risk to its users (Bhatia et al., 2020). Generally, wastewater is treated using various physical and chemical methods in a three-step approach, involving, primary, secondary, and tertiary steps as summarized in Figure 1 (Salama et al., 2017). Conventional methods are faced with challenges to meet the stringent nutrients discharge standard with high efficiency and low cost (Li et al., 2019). Other drawbacks include, high energy consumption and use of expensive chemicals that often results in sludge formation with increased risk for secondary pollution (Sun et al., 2016; Guimarães et al., 2016).

Microalgae-based wastewater treatment has gained more attention as an environmental and economically friendly way to treat wastewater and produce biomass for biofuel production. Numerous studies have reported on the feasibility of applying microalgal technology for wastewater treatment, which could potentially reduce nutrient concentration up to 95% by converting it to biomass (Ferreira et al., 2019; Udaiyappan et al., 2017). Microalgal-based wastewater treatment has many advantages, does not require the addition of extremely hazardous chemicals, contributes to the reduction of carbon footprint, supplies the oxygen needed by bacteria as a by-product of photosynthesis, and reduces energy demand and costs (Mennaa et al., 2015). Microalgae utilize CO_2 through photosynthesis to generate O_2 which is used by heterotrophic bacteria to degrade organic matter and bioremediate inorganic compounds present in the wastewater (Munoz and Guieysse, 2006). The integration of wastewater treatment with CO_2 emission plants would result in more efficient carbon capture and sequestration. Algal technology of wastewater treatment provides significant benefits over traditional cultivation and treatment processes (Li et al., 2019). Some wastewater treatment plants use conventional stabilization ponds and high rate algal ponds as a cheap system for microalgae-based wastewater treatment and biomass generation (Rawat et al., 2013).

The biomass produced as by-products contains various valuable bioactive compounds such as lipids, protein, carbohydrates, and value-added products which can be widely used in a commercial application (Mutanda et al., 2011). Microalgae biomass that is rich in lipids can be used as an alternative source for biodiesel production through transesterification reaction using different acid, alkali, and enzymatic catalysts (Bhatia et al., 2018). There has been earnestly investigation directed toward the use of

microalgae as a feedstock for biodiesel production due to the growing demand and increase in the price for fossil fuel. Algal biofuel has less carbon emission with good potential to substitute fossil fuel petroleum. Integration of wastewater treatment and biofuel production will overcome the challenges that have impeded the feasibility of algal biotechnology on a commercial scale. Nevertheless, microalgae-based wastewater treatment is confronted by numerous challenges including contamination, low biomass yield, complex nutrients removal mechanisms, and impurities in the biomass after downstream processing. This review will focus on recent trends and developments in microalgae-based wastewater treatment technology biorefinery, advancement in microalgae cultivation system, harvesting technologies from wastewater, as well as utilization of microalga biomass for biofuel production and value-added products. Also, it provides an insight into the challenges of microalgae base wastewater treatment.

2.1 Biology of microalgae

Microalgae are some of the oldest microscopic autotrophic and/or heterotrophic microorganisms, found in freshwater and marine aquatic habitats. They are either unicellular or simple multicellular in structure which allows them to grow faster and survive under extreme conditions (Yin et al., 2020). When compared to other plants, microalgae lack complex structures and organs. Microalgae structure only contains a nucleus, chloroplast, endoplasm reticulum, cell wall, and pigments (Enamala et al., 2018). This photosynthetic organism can efficiently use CO_2 (as a carbon source), light (as an energy source), and water owing to the presence of photosynthesis pigment such as chlorophyll in their cells (Tan et al., 2020). Microalgae biomass can be used as a source for many products including oils (omega-3 fatty acids), protein, animal feed (larval bivalves) and chemicals (vitamins, pigments and antioxidant) and various biofuels such as bioethanol, biodiesel, biosyngas, bio-oil and bio-hydrogen (Suparmaniam et al., 2019).

There are four prominent groups of microalgae that are classified based on the pigmentation of the biological structure, namely *Baccilariophycea* (diatoms), *Chlorophyceae* (green algae), *Cyanophyceae* (blue algae) and *Chrysophyceae* (golden algae); (Vassilev and Vassileva, 2016). They can be separated based on the energy requirement as (i) autotrophic, which requires inorganic compounds to grow such as CO_2, nutrients (nitrates ion, phosphate) and lights, (ii) heterotrophic, obtain energy and carbon from organic compounds and (iii) mixotrophic, use different sources of energy and carbon for growth (Baicha et al., 2016). Microalgae can be cultivated in an open system (such as open raceway ponds) and closed aqueous systems (such as photobioreactor) under controlled growth conditions (Ullah et al., 2015). It is estimated that there are over 200,000 - 800,000 microalgae species, of which around 50, 000 species have been identified based on their morphological and molecular characteristics. However, few of these species have been comprehensively studied and characterised for their ability to produce important valuable compounds (Suparmaniam et al., 2019; Vassilev and Vassileva, 2016).

2.2 Microalgae based wastewater treatment

Wastewater contains substantial amounts of inorganic nitrogen and phosphorus which are essential for microalgae growth and biomass production. Nitrates, ammonia, phosphorus, urea and trace element are the major nutrients present in wastewater (Salama et al., 2017). The process of microalgae-based nutrient removal from wastewater is known as phycoremediation. The chemical energy that drives microalgae growth in wastewater is obtained through photosynthesis. Where, CO_2 is converted into organic compounds and provides O_2 for heterotrophic aerobic bacteria to oxidise organic matter using in turn CO_2 released from bacterial respiration (Munoz and Guieysse, 2006). Some of the advantages of microalgae-based wastewater treatment are summarized in Table 2.1 (Craggs et al., 2013). Potential microalgae for wastewater treatment should possess important traits, including the ability to grow under extreme conditions; fast growth rate; large cell size or colonial morphology; robust to environmental

conditions, and high lipid accumulation (Fazal et al., 2018). Several studies have been conducted in the last decade to investigate the feasibility of microalgae based-wastewater treatment to find cheap and simple process for wastewater reclamation (Ferreira et al., 2019; Udaiyappan et al., 2017). Figure 2.1 shows the simplified wastewater reclamation process using microalgae treatment system with multiple benefits.

Table 2.1 Some advantages and disadvantages of using microalgae in wastewater treatment.

Advantages	Disadvantages
• High growth rates, photosynthesis and oil content • Ability to grow on unproductive land and non-competition for farmland • Good capacity to capture flue gas from power plant via photosynthesis and tolerance of some microalgae to SOx and NOx • Ability to grow in different wastewater and remove pollutant • Algal biomass contains significant amounts of components that can be extracted to produce valuable products	• Risk of cultivating monoculture due to contamination by microorganism • Low biomass accumulation and lipid yield in wastewater • Physio-chemical parameter such as high-temperature major threat in the open pond cultivation system • Microalgae downstream process is expensive, especially biomass harvesting

Some other developed countries such as the USA, Australia, Mexico, Thailand and Taiwan have directed their research activities at a large scale toward efficient wastewater treatment with concomitant biomass accumulation for biofuel production at a large scale (Bux, 2013). Microalgae can grow in domestic wastewater (Ramsundar et al., 2017), dairy wastewater (Daneshvar et al., 2018), textile wastewater (Bhattacharya et al., 2017), agro-industrial wastewater (Ferreira et al., 2019), heavy metals rich wastewater (Bhattacharya et al., 2017), starch wastewater (Yang et al., 2015), pharmaceutical waste stream and palm oil and mill effluent (Xie et al., 2019). High-rate algal pond (HRAPs) have been used for the treatment of different types of wastewater on industrial scale. The concentration of total nitrogen (TN) and total phosphorus (TP) in wastewater varies significantly depending on the type of wastewater. For example, the concentration of ammonium nitrogen and phosphorus in secondary-treated wastewater falls into a range of 20-40 mg L^{-1} and 1-10 mg L^{-1}, respectively which is sufficient to support microalgae growth (Odjadjare et al., 2017). Commonly used microalgae species in wastewater treatment include *Chlorella* sp., *Scenedesmus* sp., and *Chlamydomonas* sp. These organisms have high adaptability and

tolerance rates to different concentrations and types of wastewater (Salama et al., 2017; Khan et al., 2019). Besides the above-mentioned species, various other microalgae species have been investigated for wastewater treatment (Li et al., 2019).

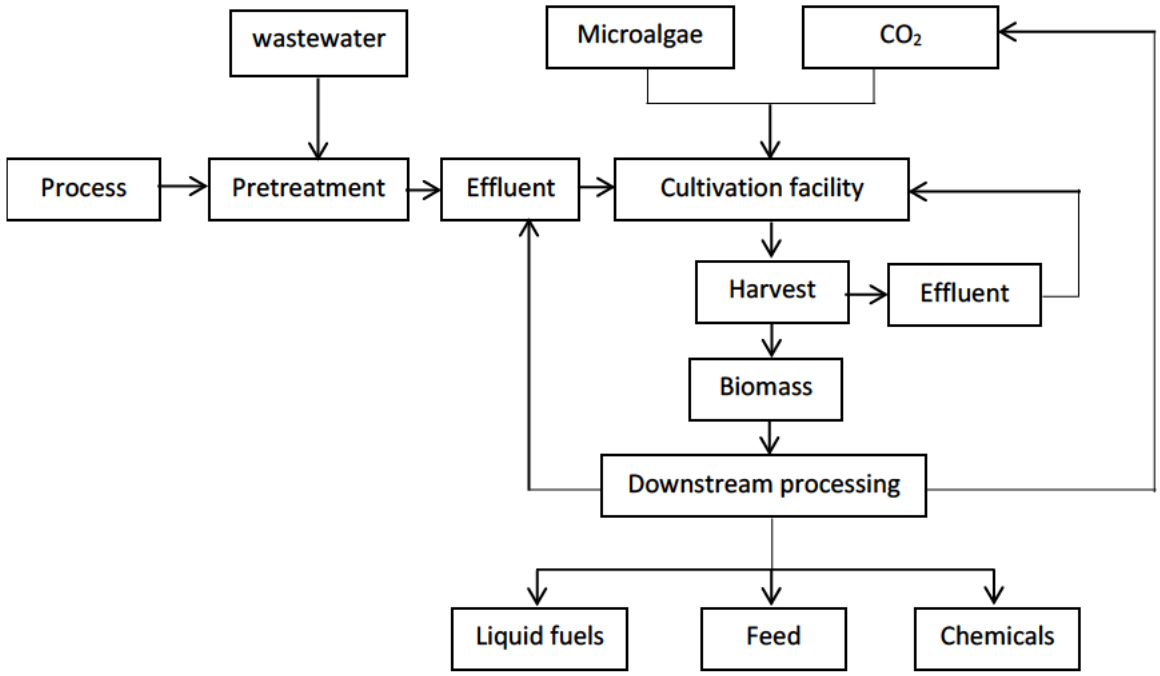

Fig. 2.1 Processes involved in wastewater treatment using microalgae (Tan et al., 2018).

A study by Guldhe et al., (2017) investigated nutrient removal by cultivated *C. sorokiniana* in aquaculture wastewater and found high removal of nitrate (84.51%), ammonium (75.56%), phosphate (73.35%) and COD (71.88%) along with the production of 129.9 mg $L^{-1}d^{-1}$ of carbohydrates, 150.2 mg $L^{-1}d^{-1}$ of lipids and 141.5 mg $L^{-1}d^{-1}$ of proteins. In another study, *Scenedesmus obliquus* showed a total nitrogen removal of 4.4 mg $L^{-1}d^{-1}$ with 1.4 g L^{-1} of biomass produced and 29.8 mg $L^{-1}d^{-1}$ of lipid productivity in urban wastewater (Álvarez-Díaz et al., 2017). Chinnasamy et al., (2010), cultivated native microalgae (*Botryococcus braunii*, *Chlorella protothecoides*, *Chlorella saccharophila*, *Crocosphaera carterae*, *Dunaliella tertiolecta*, *Nannochloris oculate*, *Spirulina platensis*, *Tetraselmis suecica*, *Tetraselmis chuii*, *Phaeodactylum tricornutum* and *Pleurochrysis carterae*) in wastewater that contained 85%-90% of carpet industry effluents and 10-15% of municipal sewage. The biomass productivity obtained was estimated to be 9.2 to 17.8 t ha^{-1} a^{-1} with a lipid content of 6.82%. Also, nutrient-removing microalgae have shown great potential at removing heavy metals, including Cu, Co, Fe, Hg, Ni, Zn, Cr, and U from industrial wastewater without secondary pollution (Chinnasamy et al., 2010).

Several microalgae species such as *Chlorella vulgaris*, *Chlamydomonas reinhardtti*, *Chlorococcum* spp., *Phaeodactylum tricornutum*, *Scenedesmus quadricauda*, *Spirogyr*a spp. have shown potential to remove heavy metals from wastewater. The removal of heavy metals through the microalgal process is cheap, ecologically safe, and efficient compared to the conventional method (Ummalyma et al., 2018). A study by Bhattacharya et al., (2017) cultivated *Chlorella variabilis* in textile effluent and reported 100% removal efficiency of nickel, aluminium and iron with 74 $gm^{-2}d^{-1}$ and a lipid yield of 20%. However, it should be noted that some wastewater contains high concentration of trace metals such as copper that can inhibit the growth of microalgae. Inconsistence in the composition of wastewater has been the main factor affecting the growth of microalgae in wastewater. Hence, it important to properly analyse the physical and chemical properties of wastewater before being used for microalgal cultivation (Tan et al., 2018).

2.3 Potential and challenges of using microalgae in wastewater treatment

The integrated approach of wastewater treatment with microalgae biomass accumulation offers a cost-effective wastewater treatment and algal cultivation for biotechnological applications. Domestic and agro-industrial wastewater contains significant amounts of important macro- and micro-nutrient that can provide sufficient support for microalgae growth without the need for supplementation with an exogenous sources of the nutrient. Microalgae-based wastewater treatment reduces the need for aeration as the oxygen produced through photosynthesis by algae provides a suitable environment for other microorganisms during wastewater treatment. Through biosorption, some microalgal species can to take up heavy metals from wastewater in a cheaper way compare to conventional method for wastewater treatment (Udaiyappan et al., 2017). Microalgae-based wastewater treatment does not require the use of chemicals and the sludge produced during treatment is mostly composed of algal biomass that can be used for biofuel production (Amenorfenyo et al., 2019). They have a high CO_2 fixing ability compare to terrestrial plants contributing to the mass reduction of greenhouse gases.

Nevertheless, the use of microalgae in wastewater treatment is faced with many challenges which need to be resolved before its application on a commercial scale. The microscopic size of microalgae makes harvesting difficult and expensive which is one of the major drawbacks in large-scale application of microalgae (Tan et al., 2020). Microalgae is negatively charged which prevents them from forming floc that can easily be recovered from wastewater at lower a cost. This impact cheap harvesting techniques such as filtration, sedimentation, and microstraining thus increasing the cost of the downstreaming process. The presence of large amounts of suspended solids in agro-industrial wastewater reduces the transmittance of light in the depth culture medium affecting the growth of microalgae and nutrient removal from wastewater (Udaiyappan et al., 2017). Commonly, agro-industrial wastewater is pretreated by filtration to remove large suspended solids and autoclaving which is not feasible at a commercial scale as it increases the overall cost for microalgae application.

Light availability in growth medium is regarded as one of the major controllers for the growth of microalgae in wastewater. The wastewater with high macronutrients can results in the multiply rapid growth of microalgae, reducing the log phase period (Udaiyappan et al., 2017). The rapid increase in microalgae concentration in the growth medium may decrease the amount of light access by a portion of the effluent due to the dense culture in the upper part of the medium, limiting the strength of light penetrating the water (Amaro et al., 2011). This will cause one-third of the water column to receive insufficient light required for photosynthesis by microalgae. In addition, the particulate matter in wastewater can further increase light attenuation in the cultivation medium.

The composition of wastewater varies daily and seasonal which can inhibit the growth of microalgae that are less adaptive to environmental changes. Hence, there is a need for bioprospecting for the robust microalgal strain that can grow and remove nutrients in different types of wastewater. Furthermore, wastewater with low carbon and nutrient ratios will hinder the growth of microalgae and biomass composition as high carbon/nutrient ratio stimulate high microalgae growth and lipid accumulation. Given all the challenges, microalgae based wastewater treatment still offer many benefits. The biomass recovered from wastewater treatment plants is rich in nutrient such as protein, lipids and carbohydrates can be used as a suitable feed for aquaculture. Furthermore, the biomass can be transformed into biofertilizer to supplement nutrient and improve the water holding capacity of the soil.

2.4 Utilization of flue gas for microalgae cultivation

Almost, over 33.4 Gt of carbon dioxide (CO_2) is emitted into the atmosphere yearly, 40% generated from fossil fuel power plants (Singh et al., 2019) and human activity accounts for about 68% of greenhouse gas emissions (Cheng et al., 2019; Zhou et al., 2017). Generally, three techniques are used for CO_2 mitigation from the atmosphere which includes chemical treatment, CO_2 storage and biological treatment (Molazadeh et al., 2019,). Chemical are harmful to the environment and requires large amounts of reagents for neutralization making it expensive. The process for CO_2 storage requires expensive tools for separation, collection and concentration CO_2. It has uncertainties such as a risk of CO_2 leakage over the years (Zhou et al., 2017). Biological treatment occurs through photosynthesis by terrestrial plants and trees removing up to 3-6% of CO_2. Interestingly, microalgae and cyanobacteria can fix CO_2 10-50 times faster than terrestrial plants due to faster growth rate (Molazadeh et al., 2019; Iasimone et al., 2017). Microalgae are known photosynthetic organism that requires light and CO_2 to grow while reducing greenhouse gas. Carbon forms one of the main components of microalgae cells accounting for about 50% of it biomass. It is estimated that the production of 1 kg of microalgal biomass results in about 1.83 kg of CO_2 fixed (Pavlik et al., 2017).

Microalgae can utilize low CO_2 concentration (0.04% v/v) that is readily available in the atmosphere as a cheap carbon source for growth. This concentration is insufficient to support high algal growth in a large-scale operation (Molazadeh et al., 2019). Flue gas produced from industrial plants represents a

good source of carbon that can be introduced directly to cultivation systems at a large production scale. A sufficient supply of CO_2 will positively enhance photosynthetic rates of microalgae resulting in high biomass accumulation and nutrient removal efficiency. The incorporation of flue gas with high-rate algal pond has proven too significantly enhance the removal of nutrient to the level similar to that achieved by mechanical treatment (Woertz et al., 2009). The integration of algal wastewater treatment with CO_2 capture from flue gas provides an environmentally and economically friendly way to reduce greenhouse gases and wastewater reclamation.

Several microalgal species of *Chlorella* spp., *Arthropira* spp., *Scenedesmus dimorphus*, *Botryococcus braunii*, and *Nannochloropsis oculate* have shown a strong potential to tolerate high CO_2 concentration (Cheah et al., 2015). Hanifzadeh et al., (2017), studied *Chlorella vulgaris* and *Scenedesmus obliquus* for CO_2 tolerance by supplying the cultivation media with 5% and 15% (v/v) CO_2 concentrations. They observed that *Chlorella vulgaris* had high CO_2 fixation rates compared to *Scenedesmus obliquus*. Another study by Yue and Chen, (2005) isolated *Chlorella* sp. from freshwater that showed strong tolerance to high CO_2 concentration with maximum cell growth rate achieved at a CO_2 concentration of 10% and 20%. The cell growth rates and density remained relatively higher as the CO_2 concentration was increased up to 30 and 50%, and decrease in cell growth rate at higher CO_2 concentration of up 70%.

Previous studies have shown that *Chlorella* spp. have the potential to tolerate flue gas emitted from industrial plates (Ding et al., 2020, Hariz et al., 2019). Flue gas contains compounds of sulphur (SO_X) and nitrogen (NO_X) that can inhibit the growth of some microalgae species. Microalgae species including *Nannocloropsis salina*, *Desmodesmus* sp., *Chlorella fusca,* and *Spirulina* sp. have high CO_2 removal rates and some species are more specific toward the removal of SO_X, NO_X, and VOC'S (Singh et al., 2019; Aslam et al., 2018; Duarte et al., 2017). A study by Kao et al., (2014) showed that *Chlorella* sp. MFT-15 can utilize CO_2, NO_X, and SO_2 present in the different flue streams with high microalgal growth rates. However, the selection of microalgae strains that has tolerance for high CO_2 concentration and proper analysis of flue gas composition is important for the success of this technology (Singh et al., 2019). The presence of high SO_X and NO_X in flue gas acidifies the media affecting the CO_2 removal rate by microalgae and biomass accumulation (Cheah et al., 2015). The CO_2 and nutrient loss in the growth medium caused by flue gas can be minimized by using close system, Close system provides high CO_2 removal efficiency of approximately 85% which is double compare to the open system (Aslam and Mughal, 2016).

2.5 Cultivation mode and symbiosis

2.5.1 Autotrophic

Microalgae reproduce by capturing natural available light as a source of energy to generate chemical energy through photosynthesis. This is the most used mode for microalgae cultivation since all microalgae are photosynthetic organisms. Under autotrophic conditions microalgae can absorb industrial waste gas and CO_2 from the atmosphere, thereby reducing greenhouse gas (Yin et al., 2020). Light intensity plays a critical role in photoautotrophic mode as some microalgae species have tolerated high light intensity which increases their reproductive rate. Microalgal species such as *Chlorella* and *Scenedesmus* have shown the ability to grow under different levels of light intensity up to 200 µmol m^2s^{-1} (Cheah et al., 2015). However, low biomass productivity and insufficient light penetration in the cultivation medium due to high algal cell density have been major drawbacks under the autotrophic cultivation system (Gim et al., 2016; Mohan et al., 2015). The cost for biomass recovery may increase due to low algal cell density in photoautotrophic conditions.

2.5.2 Heterotrophic

Heterotrophic cultivation mode requires organic carbon as the main source of energy. Under heterotrophic cultivation systems microalgae converts sugars such as glucose into lipids in the absence of light making the process simple and easy to scale up (Mohan et al., 2015). This cultivation mode has higher growth rates and cell densities making harvesting simple compare to autotrophic cultivation mode (Gim et al., 2016). The growth of microalgae under heterotrophic conditions is four times greater than the autotrophic conditions in similar conditions (Miao and Wu, 2006). However, not all microalgal species can use organic carbon as the main energy source for growth. For instance, *Nostoc* sp. PCC7118 is unable to assimilate glucose as the energy source due to a lack of transmembrane transporters (Zhang et al., 1998). Microalgae species *Chlorella vulgaris*, *Haematococcus pluvialis*, *Nannochloropsis* sp., and *Spirulina* can grow under heterotrophic conditions (Li et al., 2019). However, the cost of organic carbon sources makes this cultivation mode expensive. Hence, utilization of wastewater can provide organic dye to the growth medium that can be up taken by microalgae as a carbon source.

2.5.3 Mixotrophic

Mixotrophic mode involves the combination of both autotrophic and heterotrophic metabolism in which inorganic and organic carbon are used by microalgae as the source of energy in the presence of light. The main energy source in this cultivation mode comes from photosynthesis by consuming atmospheric CO_2, although organic compounds are also important. The growth of microalgae under the mixotrophic mode is influnced by the dark and light cycle causes biomass loss, especially during the ligth cycle period (Andrade and Costa, 2007). Bouarab et al., (2004) reported a high specific growth rate of *Micractinium pusillum* under mixotrophic mode in the presence of glucose and light as energy source compares to heterotrophic conditions. In another study, *Chlorella vulgaris* showed low biomass productivity (0.35 ± 0.01 g $L^{-1}d^{-1}$) under autotrophic mode which was increased to 6.0 - 6.15 g L^{-1} under mixotrophic conditions (Li et al., 2014). Nevertheless, the cost of organic compounds and

contamination of the media by other organisms due to the presence of organic compounds is a major drawback.

2.6 Microalgae cultivation systems

There are mainly two types of microalgae cultivation systems classified based on the perspective design and configuration, which are open and closed systems. The selection of a microalgae cultivation system should be based on the operation cost, strain adaptability, application of the final products, source of carbon and the nutrient required. Open systems are mostly used for many commercial practices as they are cheaper and easy to construct, coupled with high production capacities. Photobioreactor (PBR) support the growth of most microalgae species, whereas the open pond is suitable for a few strains (Baicha et al., 2016; Tan et al., 2018). The advantages and disadvantages of each cultivation system are summarized in Table 2.2.

2.6.1 Open pond system

Open ponds are the most commercialized system for microalgae cultivation at the industrial scale level due to low operation and maintenance costs. The depth of the race pond is usually between 0.2 to 0.5 meters to ensure sufficient light penetration. Atmospheric CO_2 is continuously in contact with the pond surface to be used by microalgae as a carbon source (Tan et al., 2018). There are different types of open pond design available which include inclined systems, circular ponds, and raceway ponds (Fig. 2.2). The inclined system is designed to induce the flow of microalgal culture from the top to the bottom of the sloping surface. It uses gravity force as the turbulence to ensure proper mixing of culture to increase cell density. Microalgae species *Chlorella*, *Phaeodactylum,* and *Scenedesmus* are suitable for cultivation in the inclined systems as they can survive repetitive mixing environments (Fazal et al., 2018). The circular pond design is similar to the raceway pond with a diameter of 45 m and depth range of 30 to 70 cm and has a central pivoted agitator for mixing the culture (Chen et al., 2011). This system can be used for purpose of wastewater treatment and biomass accumulation for biofuel production. Rotating agitator becomes ineffective in large pond size (≥1 ha) and requires extensive energy for mixing (Fazal et al., 2018).

Raceway ponds are known as a high-rate algal ponds (HRAPs) with shallow design to be shallow and depth of approximately 0.3 m. HRAPs are used for wastewater treatment, where microalgae consume nutrients from wastewater for growth. The system is installed with a paddle wheel for continuous circulation of the culture to avoid possible sedimentation throughout the cultivation cycle (Suparmaniam et al., 2019). HRAPs has low operations costs and can work with high of CO_2 concentration released from industrial power plants (Baicha et al., 2016). However, the system is easily susceptible to contamination and requires large areas to scale up. It is difficult to control important growth parameters such as light, temperature, pH, and CO_2 in HRAPs (Chisti, 2007).

Table 2.2 Characterization of four promising photobioreactor**.**

Type of bioreactor	Conceptual Design	Advantages	Disadvantages
Open pond	• The depth of the pond is usually 0.2-0.5m to ensure adequate exposure to sunlight	• Easy to construct and operate • Low energy input and operation cost	• Water loss due to high evaporation • Difficulty to control temperature and pH • Susceptible to contamination
Tubular photobioreactor	• Tube diameter is limited (0.1 cm) to increase the surface/volume ration	• Large illumination surface area • Relatively higher biomass productivity • Minimize potential cell damage if an airlift system is used	• Require large land area due to use of long tubes • Potential of accumulating high O_2 in culture medium if tubes are too long • A decrease in CO_2 concentration along the tube may deprive microalgae of oxygen • Mixing is a problem
Vertical Column	• Optimum column diameter s 0.2 m with 4 m height	• High mass transfer rate with good mixing • Compact, easy to operate, and relatively low-cost • Lower power consumption	• Small illumination surface area • Cell sedimentation may occur if an airlift system is not used
Flat-panel airlift	• The light path (depth) is dependent on microalgae strain; it ranges between 1.3 and 10 cm	• Large illumination surface area gives maximum utilization of solar energy • Low concentration of dissolved oxygen • Can be position vertically or inclined at an optimum angle facing the sun • Lower power consumption	• Scale-up require many compartments and support materials • Difficulty to control culture temperature

Adapted from: (Lam et al., 2019; Mohan et al., 2019; Yen et al., 2019)

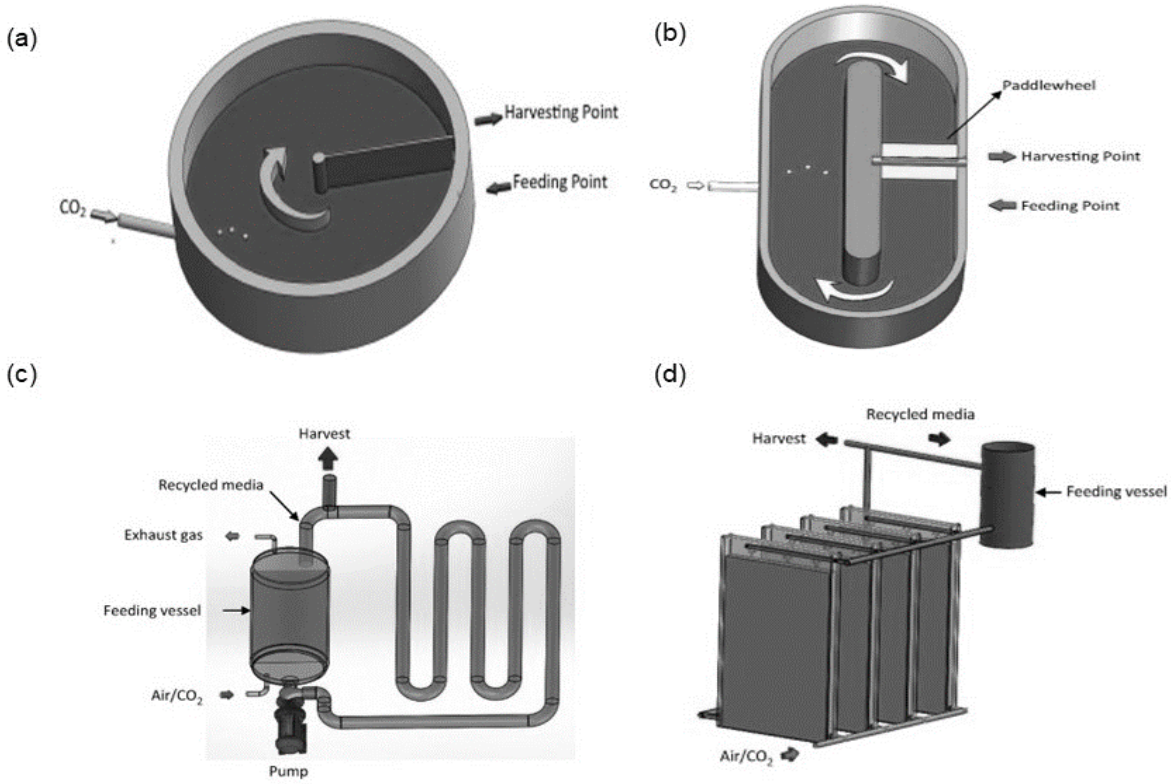

Fig 2.2 Type of cultivation system: (a) circular pond, (b) open raceway pond, (c) Tubular photobioreactor with parallel run vertical tubes (d) Flat plate photobioreactor.

Some microalgae such as *Chlorella* spp, *Spirulina*, and *Dunaliella* have shown resistance to severe environmental conditions and contamination by foreign predators suitable for use in HRAPs (Suparmaniam et al., 2019). Despite, low environmental impact of open ponds, there is still a need for further optimization of the system before it is implemented on a large scale for efficient biomass accumulation, nutrient removal, and CO_2 sequestration.

2.6.2 Photobioreactor

Photobioreactor (PBR) are designed to overcome several constraints associated with an open pond system. This system provides a better culturing system as it allows the control of important growth parameters such as temperature, CO_2 input, and O_2 output. The contamination level in PBR is reduced promoting the growth of monoculture for a longer period (Baicha et al., 2016). Furthermore, bioreactors are smaller in size, providing efficient land usage than an open pond system. It has a higher biomass productivity yield compare to the open system due to low volume-to-surface ratios (Acién et al., 2017). However, photobioreactor are not suitable for wastewater treatment since large volumes of wastewater are released daily for treatment. This could be solved by concentrating the wastewater before being diverted to the PBR for extra treatment (Tan et al., 2018). PBR powered with solar energy has been

successfully used for the removal of piggery wastewater at a low cost (Rawat et al., 2013). Types of PBR systems include tubular reactors, flat plate reactors, bubble column reactors, soft-frame reactors, and hybrid PBR. The selection of PBR depends on the type of microalgal strain, the space available, and the nature of the final products required (Baicha et al., 2016).

Tubular PBRs are designed using transparent long tubes made from glass or transparent plastic which are arranged in horizontal, vertical, helix, or slanted orientation to maximize capture of light (Tan et al., 2020). It is the most appropriate and feasible cultivation system preferred for outdoor cultivation of microalgae at an industrial scale because of large available surface area for illumination (Huang et al., 2017). A mechanical or airlift pump is used to circulate the culture with a loop of the tubular PBR. The length of the tube should range from 100 to 150 cm to maintain favourable pH, CO_2, and O_2 concentration in the culture medium (Acién et al., 2017). Flat-plate PBRs are constructed using rectangular transparent materials containers to keep a high surface area to volume ratio. The system is more popular for the cultivation of monoculture and wastewater treatment (Faried et al., 2017). The culture inside the reactor is mixed by the means of recirculating airlifts system (Tamburic et al., 2011). Its large illumination surface area contributes toward increase biomass productivity and allows better management of CO_2 accumulation (Smachetti et al., 2018).

Another configuration of PBR is known as airlift PBRs which contains transparent columns, internal columns, and air spargers. Airlift PBR provides high mass transfer rates, regulates light/ dark cycles allowing less shear stress. The culture is mixed using gas bubbling through moving the culture between the riser and bottom section of the reactor (Fazal et al., 2018). Column PBRs provide better mixing, gas-liquid mass transfer rate, and excellent control of culture conditions. It easy to control with maximum exposure to light for high biomass accumulation (Tan et al., 2018). Normally, photobioreactor is used to produce high value-added products that could afford the high investment costs and energy consumption by this system. The airlift system is susceptible to biofouling and cell damage by shear stress due to the accumulation of oxygen in the system.

2.7 Harvesting of microalgae

Harvesting is the process where algal cells are separated from the broth medium using various downstream techniques. Technically, harvesting microalgal cells from the suspension is challenging due to its small cell size (2-20 μm), low density, and colloidal stability of microalgae. Thus, making the process energy-intensive and expensive constituting about one-third of the total biomass production cost during biodiesel production (Liu et al., 2013; Tan et al., 2020). Harvesting methods should aim at obtaining high harvesting efficiency at moderate operating costs including low energy input and maintenance (Enamala et al., 2018). Harvesting methods mainly include centrifugation, filtration, flocculation, gravitational sedimentation, and floatation (Barros et al., 2015). In some cases, these techniques are applied individually or in combination to increase the harvesting efficiency. The

promising harvesting techniques are discussed hereafter in Fig. 2.3 with advantages and disadvantages are summarized in Table 2.3.

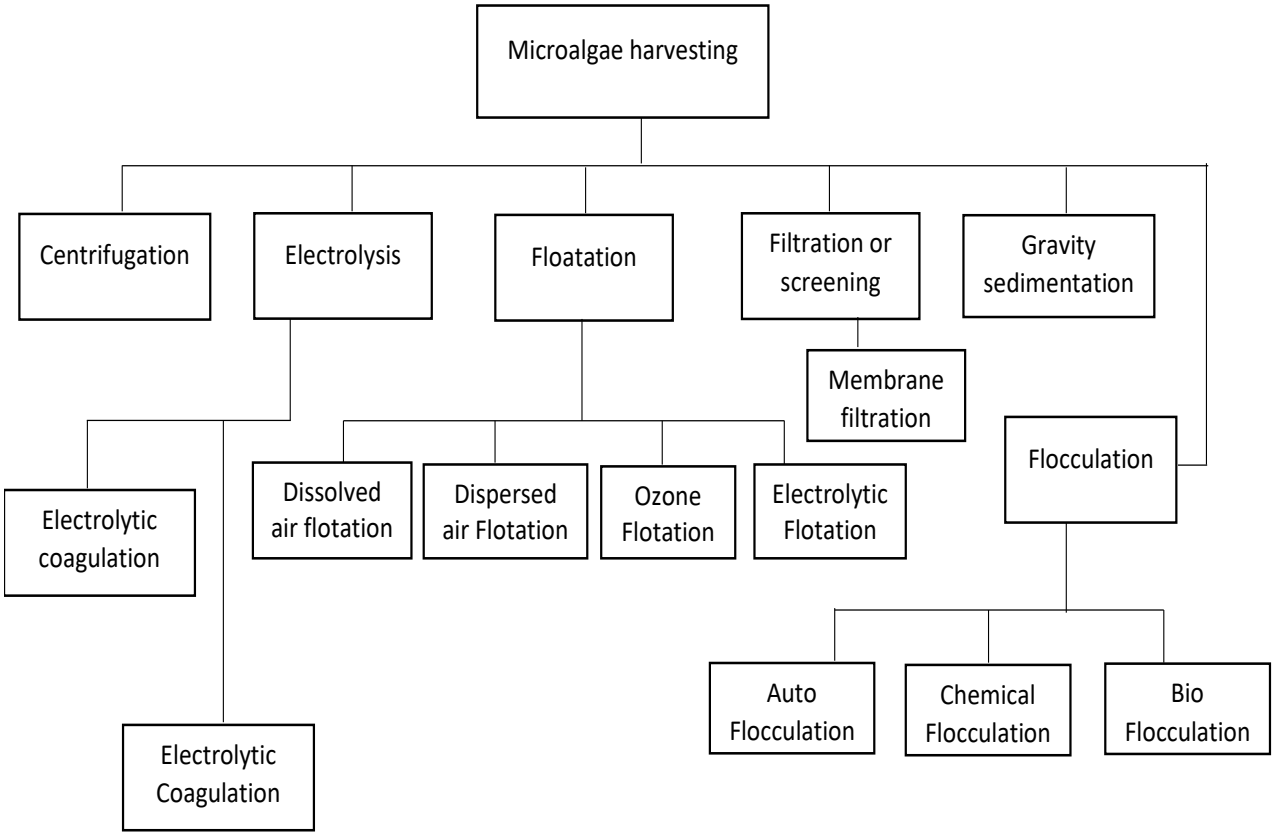

Fig. 2.3 Microalgae harvesting techniques

2.7.1 Centrifugation

The centrifugation method separates two immiscible matter by applying centrifugal forces based on each components density and particle size. Generally, centrifugation is used to harvest secondary metabolites, due to its high operation cost and energy input (Barros et al., 2015). There are various types of centrifugation techniques or systems available which include hydro-cyclone, solid bowl decanter, nozzle type, solid ejecting disk (Enamala et al., 2018). Nozzle type is regarded as the most promising type of centrifugation as it gives high harvesting efficiency and biomass with good quality, although it is expensive. Contrarily, the solid ejecting disk is the most commonly used technique to harvest microalgae cells on a commercial scale for biofuel production. It has the ability to recover microalgae cells with small size (3-30μ) and low density (0.2-0.05%) in medium (Milledge and Heaven, 2013). Centrifugation requires about 1 MJ/kg energy to recover 1 gram of microalgal biomass. To harvest microalgae culture from 0.04% to 4% dry weight on average costs 1.3 kW h/m^3 of pond water. However, gravitation force and shear stress may damage the cell during the centrifugation process (Suparmaniam et al., 2019).

Table 2.3 The advantages and disadvantages of harvesting techniques

Harvesting Methods	Advantages	Disadvantages
Centrifugation	• Fast and effective technique	• Expensive with a high energy requirement
	• High recovery efficiency (>90)	• High operation and maintenance costs
	• Preferable for small scale and laboratory	• Suitable for recovery of high-value products
	• Applicable to all microalgae	• Risk of cell destruction
Filtration	• High recovery efficiency and cheap	• Slow require pressure or vacuum
	• Low energy consumption	• Not suitable for small algae
	• Low shear stress	• Membrane fouling/clogging and replacement increase operation and maintenance costs
	• water recycle	• High energy consumption
Floatation	• Suitable for large scale	• Possibility of mineral or microbial contamination
	• Low cost and space requirement	• Needs surfactants
	• Short operation time	• Ozoflotation is expensive
Flocculation	• Fast and easy technique	• Use expensive chemicals
	• Suitable for large scale application	• High pH dependent
	• Applied to a vast range of species	• Difficult to separate the coagulant from the biomass
	• Low energy requirement	• Efficiency depends upon the coagulant used
	• Auto and Bioflocculation may be expensive methods	• Culture medium recycling is limited
Electrical based process	• Applicable to all microalgal species	• Metal electrodes required
	• No chemicals required	• High energy and equipment costs
		• Metal contamination

2.7.2 Filtration

Filtration is a mechanical or physical separation technique used to separate solids from liquids or gases by interposing the medium through the membrane that allows only fluids to pass (Enamala et al., 2018). Microalgae culture is passed through a filter or porous membrane, which retains algae slurry, while growth medium is passed through a filter via a driving force (Yin et al., 2020). This method is suitable for harvesting microalgae cells with large cell sizes and not suitable for microalgae with small cell sizes such as *Chlorella*, *Dunaliella,* and *Scenedesmus* (Suparmaniam et al., 2019). Filtration requires force drop in a form of either pressure, temperature, or concentration across the system to accelerate the suspension through the membrane. There are various types of filtration processes which include micro-filtration, macro-filtration, ultra-filtration, vacuum filtration, dead-end filtration, pressure filtration, and tangential flow filtration (TFF) (Enamala et al., 2018).

Micro-filtration is used to harvest microalgae that are fragile and smaller cells with a pore size of 0.1 – 10 mm such as *Chlorella* and *Cycotella* spp. Macro-filtration is suitable for harvesting microalgae that have larger cell sizes, especially the biomass obtained through flocculation (Mathimani and Mallick, 2018). In terms of operation cost, TFF and pressure filtration is cost-effective due to low energy consumption. However, filtration requires frequent changes of the filter membrane to avoid clogging or fouling which makes the filtration process expensive (Pragya et al., 2013). Hence, the use of cheap and easily accessible material to produce the filter has been proposed to make the technique cost-effective. Bejor et al., (2013) developed a filter membrane made out of cheap and easily accessible stretch cotton material and achieved the harvesting efficiency of 66-93%.

2.7.3 Flocculation

Flocculation is the process whereby free-floating unicellular microalgae aggregate together to form a floc, which is induced by the addition of flocculent or coagulants (Tan et al., 2020). This method is frequently used in various activities ranging from brewing to wastewater treatment and mining etc. (Molina Grima et al., 2003). Flocculation occurs by following any of the four modes: (1) charge neutralization, (2) patching, (3) sweeping, and (4) adsorption bridging (Mathimani and Mallick, 2018)**.** Chemical flocculation is induced by adding a flocculent either inorganic flocculants, inorganic polymer, and organic polymers (Yin et al., 2020).

2.7.3.1 Inorganic flocculation

Recently, inorganic flocculants have been extensively used to harvest microalgae during wastewater treatment as it is fast and high efficient. The ions in the chemicals interact with the negatively charged microalgal cells resulting in the efficient recovery of microalgal biomass (Ummalyma et al., 2018). The main types of inorganic flocculants includes $Fe(SO_4)_3$, $FeCl_3$, $Al_2(SO_4)_3$, $AlCl_3$, $ZnSO_4$, $ZnCl_2$, $CaSO_4$,

$CaCl_2$, $MgSO_4$, $MgCl_2$, $(NH_4)_2SO_4$, and NH_4Cl (Mathimani and Mallick, 2018). Among these flocculants, alum is considered as best flocculant because of it high density. Chatsungnoen and Chisti, (2016) reported that metals ion such as aluminium sulphate and iron chloride can recover up to 95% of microalgal biomass under standard conditions. Nonetheless, inorganic flocculants are toxic to the environment and generate enormous volumes of sludge that require further dewatering steps (Singh and Patidar, 2018). Metal that remains in the residue after lipid extraction require treatment if the residue is to be used as animal feed and this adds to the production cost (Goh et al., 2019).

2.7.3.2 Organic flocculants

Organic flocculants are natural, synthetic, or polyelectrolyte flocculants, either anionic, cationic, or non-ionic. Cationic coagulants are regarded as the best flocculent to recover microalgae cells from water as they attract negatively charged algal cells (Singh and Patidar, 2018). Cationic polymers flocculate by physically linking the cells together, whereas the anionic or non-ionic fails to form microalgal floc due to electro-repulsion. Polyelectrolyte flocculating power depends on the charge and functional groups of the surface of microalgae, pH, and density of microalgal cultivation medium (Chen et al., 2011). Chitosan is considered the best biodegradable flocculant used for microalgae harvesting. However, chitosan as the ideal flocculant is challenged by high cost, making it inappropriate for large-scale processing. Hence, the economic feasibility of polymeric flocculants is generally used to flocculate microalgae. The polymeric flocculants are found to be unsuitable for flocculating seawater/brackish water-grown microalgae. Due to that algal cell bridging fails if the ionic strength of the culture suspension is too high (Enamala et al., 2018).

2.7.3.3 Bioflocculation

Bioflocculation is the substitute concept for chemical-based flocculation, considered safe and economical compare to chemical-based flocculation. It occurs naturally and spontaneously in the lakes and ponds during microalgae blooms, because of extracellular polymer substances found in the water (Suparmaniam et al., 2019). Harvesting of microalgae by bio-flocculation allows the reusability of the broth culture for subsequent microalgae cultivation with no need for further treatment (Barros et al., 2015). The reusability of broth culture after bioflocculation has attracted more interest as it reduce the cost of upstream process making biomass recovery less expensive. This process can be induce using microorganisms such as bacteria, fungi, and other naturally flocculating microalgae (Goh et al., 2019). Nonetheless, co-cultivation of microalgae with bacteria, fungi, or flocculating microalgae can results in microbiological contamination, interfering with food or feed application of microalgae biomass (Barros et al., 2015). Bioflocculation technique has been effectively implemented in wastewater treatment plants because of it low cost and energy demand. However, the underlying reaction mechanism of this process is still not well understood.

2.7.3.4 Auto-flocculation

Auto-flocculation occurs naturally for certain microalgae species, induced by environmental stress such as pH variation, nutrient depletion, quantities of calcium and magnesium ions, and dissolved oxygen content in the media (Uduman et al., 2010, Ummalyma et al., 2018). The increase in the pH of a solution causes supersaturation of calcium and phosphate ions. The presence of many positively charged calcium ions in the media will cause calcium phosphate precipitate to be positively changed. As a result, microalgae cells act as a fixed carrier for the precipitate and charge neutralization is completed. Microalgae can form a stable suspension because of the negatively charged surface metals which make flocculation by pH adjustment feasible. The H^+ /OH^- ratio and Mg^{2+} charges in the medium destroy the electrostatic interactions between anionic algae (Wan et al., 2015).

Vandamme et al., (2012) investigated different techniques to promotes auto-flocculation by adding KOH, $Ca(OH)_2$, and $Mg(OH)_2$ resulting in the biomass recovery efficiency above 98%. Auto-flocculation can also be induced by adding alkaline compounds such as NaOH, KOH, $Ca(OH)_2$, $Mg(OH)_2$ (Enamala et al., 2018). Horiuch et al., (2003), reported a recovery efficiency of above 90% on *Dunaliella tertiolecta* by adding NaOH solution with the settling rate of few minutes with a pH ranging from 8.6 to 10.5. Auto-flocculation is considered a cheap, energy-efficient, and technically easy technique to recover microalgae biomass. However, the use of the base to induce flocculation and acid to neutralize the pH needs to be investigated in detail in terms of economic feasibility and environmental impact (Kim et al., 2013).

2.7.4 Gravity sedimentation

Gravity sedimentation is the simplest and highly energy-efficient method to harvest microalgae biomass. This method is the commonly used method for harvesting microalgae biomass from water and wastewater facilities (Pragya et al., 2013). Technically is the process of solid-liquid separation that separates a feed suspension into a slurry of higher concentration and effluent of substantially clear liquid (Tan et al., 2020). The competence of this method depends on sedimentation rate and time, which is related to the cytoplasmic density of microalgal cells (Milledge and Heaven, 2013). The biomass sedimentation rates can be enhanced by using lamella separators and sedimentation tanks. This application is usually designed for the auto-flocculation process (Uduman et al., 2010). However, the reliability of this method is uncertain, due to a low slurry output and the demand for further thickening. Low mass microalgae units cannot be efficiently harvested by the sedimentation process as some biomass might deteriorate during settling (Enamala et al., 2018; Yin et al., 2020).

2.7.5 Floatation

Flotation is the process whereby gas bubbles fed to the broth are used to carry suspended matter to the top of the liquid surface to form a floc that is lately collected through the skimming process (Singh and Patidar, 2018). This technique is frequently used in the wastewater treatment process which is followed by coagulation or flocculation to reduce the surface charge. The floatation technique has a relatively high harvesting efficiency, short operation time, and high flexibility with lower initial equipment cost (Barros et al., 2015). The success of this method depends on many factors such as the size of the suspended particle, likelihood of collision, and adhesion (Singh and Patidar, 2018). Presently, there are 3 main types of floatation techniques which include dissolved air flotation, dispersed air flotation, and electro-flotation (Tan et al., 2020).

Dissolved air flotation is a commonly used method in the wastewater treatment plants to separate microalgae from the liquid medium. The mechanism for dissolved air flotation is through saturating the culture with compressed air and discharging the culture at atmospheric conditions. Microalgal suspension is forced to float to the surface of the medium and recovered by skimming making the process energy-intensive. The created size of the bubble directly affects the microalgae harvesting efficiency which is induced by flotation (Suparmaniam et al., 2019). Nevertheless, dissolved air flotation is associated with high operation costs due to high energy consumption and usage of chemicals. Dispersed air flotation generates an air bubble by continuously passing the air through a porous material known as diffusers or spargers (Singh and Patidar, 2018). Contrary, dispersed air floatation is energy efficient compared to dissolve air flotation. However, it requires expensive equipment and high pressure drops for generating bubbles (Chen et al., 2011; Tan et al., 2018). Electro-flotation depends on the generation of microbubbles from its electrodes trap free floating microalgae by electrolysis operation. Electro-flotation has many advantages, as it allows simultaneous cell disruption operation when an alternating current is being used and is applicable to harvest a wide variety of microalgae species with no need for additional chemical (de Carvalho Neto et al., 2014). Nonetheless, cathode fouling and high power demand are the main drawbacks of this method (Chen et al., 2011).

2.8 Pre-treatment of microalgae biomass for biodiesel production

After obtaining high biomass concentration using different cultivation systems, the next is the extraction of lipids. Microalgae biomass is pre-treated and dried to degrade the cell wall liberating the lipid and removing high moisture content. The presence of high water content in the biomass decrease biodiesel yield during transesterification reaction (Odjadjare et al., 2017). Drying of microalgae biomass is essential to increase the viability of biomass for lipid extraction. Method for dewatering microalgae biomass includes oven drying, drum drying, fluidized bed drying, spray drying and freeze drying, which is selected based on the requirements of the final products (Guldhe et al., 2014). The different method

used for microalgal cell wall disrupting includes: microwave, ultrasonication, autoclaving, bead milling, homogenization, and chemical lysis.

Florentino de Souza Silva et al. (2014) compared four different cell disruption techniques namely microwave, ultrasonication, autoclaving, and electroflotation on 12 different microalgal species. Microwave resulted in high extraction efficiency from all 12 microalgal species (*Micractinium, Euclorine, Chlorella, Pandorina, Curteria, Aphanocapsa, Microcystis, Oscillotoria, Planktothrix, Hyalophacos, Phacus* and *Cyclotella*). Contrary, Prabakaran and Ravindran (2011), compared five cell disruption techniques (microwave, autoclaving, sonication, osmotic shock, and bead beating) and ultrasonication resulted in high lipid extraction efficiency from the four microalgal species *Chlorella, Nostoc* sp. and *Tolypothrix*. Cell disruption efficiency of microalgae cells varies from species to species due to the thickness of the cell wall different from each species (Hounslow et al., 2017). Microwave has been reported as the most efficient cell disruption technique since it is reliable, effective, quick, easily scalable, and applicable to a variety of microalgal species (Günerken et al., 2015). However, mechanical disruption methods are energy-intensive, when conducted under laboratory conditions need a specific energy consumption of at least 33 MJ/kg of dry biomass (Odjadjare et al., 2017).

2.9 Lipid extraction from microalgae biomass

Microalgae lipids are classified based on the polarity of the molecular head groups such as neutral lipids, hydrophobic and lacking charged groups, and polar lipids which are phospholipid and glycolipids. Efficient lipid extraction is critical for the overall production of carbon neutral biodiesel. Microalgae consist of a thick cell wall which makes lipid extraction challenging and energy-intensive. Conventionally, lipids are extracted from terrestrial plants by a mechanical pressing method that does not apply to microalgae (Lam and Lee, 2012). Currently, solvent extraction and supercritical fluid extraction are the two popular methods for extracting lipids from microalgae biomass.

2.9.1 Chemical method

Chemical extraction is a mostly practice technique for extracting lipid from dried microalgae biomass to reduce energy consumption compared to mechanical extraction. This process involves the use of polar and non-polar solvents which are paired together to ensure total extraction of all neutral lipids which includes free-standing globules and membrane associated complexes (Abomohra et al., 2016). The most used solvents in chemical extraction are alcohols (isopropanol, ethanol, or methanol), hydrocarbons (hexane or chloroform) (Ferreira et al., 2019). Microalgae lipids are usually extracted using Bligh and Dyer method (Bligh and Dyer, 1959, Ramluckan et al., 2013). In this method, chloroform and methanol create a two-phase system in which water is added to induce the biphasic partitioning and lipid will be fractioned in the chloroform phase (Odjadjare et al., 2017). Regardless of

the high lipid extraction efficiency by Bligh and Dyer method, chloroform is highly toxic and has adverse effects on the environment which is a disadvantage (Goh et al., 2019).

The mixture of hexane and isopropanol works in similar manner to chloroform but has high specificity toward neutral lipid compare to Bligh and Dyer method. The use of hexane has added advantages as it less toxic and readily separating the phases once in contact with water, thus improving the process (Arenas et al., 2017). The mixture of hexane with non-polar solvents breaks the hydrophobic interaction between neutral and non-polar lipids. Furthermore, polar solvents are involved in the disruption of hydrogen bonding between polar lipids (Rawat et al., 2013). Ramluckan et al. (2014) evaluated 13 different solvents for extracting lipid from the mixture of microalgae biomass containing mostly *Chlorella* sp. They found the highest lipid extraction efficiency was obtained using the mixture of ethanol and chloroform (1:1 v/v). D'Oca et al. (2011) evaluated 6 different solvents to extract lipids from *Chlorella pyrenoidosa* biomass assisted by soxhlet extraction, magnetic stirring, and ultrasonic bath. They found that highest lipid extraction efficiency using the mixture of chloroform and methanol (2/1 v/v). These results are consistent with many microalgae cultivation studies that use the Bligh and Dyer method for lipid extraction, which comprises of alcohol and chloroform. Considering the health and environmental issues associated with the used of conventional solvents, an attempt to look at new alternative methods for extraction of lipids and environmentally friendly is ongoing.

2.9.2 Supercritical fluid extraction of lipids

Supercritical fluid extraction has attracted too much attention as a green alternative approach for extracting lipids to replace the chemical-based methods. Supercritical fluid extraction functions by forcing the pressure and temperature above the critical point resulting in the fluid possessing properties of both liquid and gas (Odjadjare et al., 2017). The density of the supercritical fluid is similar to that of liquid with viscosity is similar to gas and its diffusivity is intermediate between the two states. The advantages of supercritical fluid include low viscosity, high affinity and dissolving power, easy separation, and low surface tension (Patil et al., 2017). Moreover, CO_2 waste from industries can be used under supercritical conditions adding to environmental advantage and reducing the cost for lipid extraction. Supercritical CO_2 is the most widely used supercritical fluid as it is non-flammable, non-toxic, cheap relatively inert, and moderate critical temperature (31.3 ^oC) and pressure (71 atm) (Taher et al., 2014, Odjadjare et al., 2017).

There is no requirement for further separation steps since CO_2 is gaseous at ambient pressure and permits easy recovery of lipids. The CO_2 utilized for extraction can be recycled to prevent the greenhouse gas effect (Mouahid et al., 2013). Taher et al., (2014) compared SC-CO_2 and conventional solvent for lipid extraction using biomass from *Scenedesmus* sp. and found that SC-CO_2 extraction showed high lipid extraction efficiency compare to solvent extraction. Millao and Uquiche, (2016) studied the effects of extracting lipids using supercritical CO_2 on *Nannochloropsis gaditana* and found

that the increase in temperature and CO_2 density resulted in higher lipid and carotenoid extraction yields. Due to the advantages are shown by supercritical fluid extraction more research has been reorienting to make supercritical fluid extraction of lipids more efficient and economical for large-scale production.

2.9.3 Pyrolysis

This technology has been studied for over the past 50 years as the biomass conversion method to bio-oil. Pyrolysis is defined as the decomposition of biomass process in the absence of oxygen under high temperatures (450 - 550 °C) (Tan et al., 2018). The main products of pyrolysis are a solid (char), an organic liquid (crude oil), and low calorific value gas (pyrogas), in different ratios depending on process conditions, reactor type, and feedstock characteristic (Chiaramonti et al., 2017). Microalgae biomass as the feedstock presents very different and peculiar characteristics compared to lignocellulose biomass as it is rich in cellular lipid and dissolvable polysaccharides and protein that are easily converted to bio-oils (Tan et al., 2018). Furthermore, bio-oil produced from microalgae has higher quality and stability compared to bio-oil produced from lignocellulostic material (Harman-Ware et al., 2013). The products of microalgae pyrolysis usually consist of a mixture of charcoal, organic liquid, acetic acid, acetone, methanol, and gases. The amount of liquid fuel produced increases as the temperature increases and charcoal decreases (Raheem et al., 2015).

The process is divided into slow and fast pyrolysis including vapor to go through this catalyst bed immediately after pyrolysis (Wang et al., 2016). In the slow pyrolysis process, biomass is heated slowly to the desired temperature, and residence time varies from minutes to hours depending on the requirements products. The fast pyrolysis process uses the high heating rates (103–104 $°Cs^{-1}$) under low temperature (450-550 °C) and short gas residence to break the biomass into short chains (Odjadjare et al., 2017). Fast pyrolysis of microalgal biomass produces about 19-57% of bio-oil as the final product and char (Raheem et al., 2015). One of the recent studies showed a high bio-oil yield of about 78% using a temperature range of between 300-600 °C (Peng et al., 2020). The feasibility of producing bio-oil from microalgae biomass has been demonstrated in a wide range of microalgae species (Harman-Ware et al., 2013). However, microalgae biomass has high water content which requires dewatering steps making the process energy demanding (Razzak et al., 2013).

2.10 Composition of microalgae lipids

Lipids are divided into three classes saturated, monounsaturated, and polyunsaturated fatty acid. It is important to characterized lipids since not all microalgae species accumulate lipids that are suitable for biodiesel production (Chisti, 2007; Ferreira et al., 2019). The quality of biodiesel is determined by fatty acid chain length, degree of unsaturation, oxidative stability, viscosity, calorific value, acid value, and cetane number (Mathimani and Mallick, 2018). Fatty acid with a carbon chain length between 14 and

22 gives the finest quality biodiesel that meet the American Society for Testing and Materials (ASTM D6751) and European norms, European Committee for Standardization (EN 14214) vehicular fuel standard. Saturated fatty acid gives biodiesel high oxidation stability, making oxidation stability parameters easier to handle compared to unsaturated fatty acid (Menegazzo and Fonseca, 2019). Monounsaturated provides a fine compromise between oxidative stability and cold flow properties.

The presence of a high degree of polyunsaturated fatty acid in biodiesel indicates poor oxidative stability with good cold flow properties. Hence, virtuous biodiesel should have a low level of polyunsaturated and saturated fatty acid to avoid the issue of poor oxidative stability and cold clogging point of the filer in cold climates (Hoekman et al., 2012). Furthermore, unsaturated molecules have an allylic and bis-allic site, making them susceptible to degradation by reacting with atmospheric oxygen which in results in the production of unwanted products (Lanjekar and Deshmukh, 2016). The most present form of saturated fatty acid is palmitic acid (C16:0) and in other species, the percentage of palmitic acid reaches up to 50%. Whereas unsaturated fatty acid mainly presents in a form of oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) (Ferreira et al., 2019, Hoekman et al., 2012).

The quality of microalgae biodiesel is checked by measuring the important thermophysical properties of biodiesel and comparing those with international standards such as ASTM D675 or EN14214. Most of the microalgae species contain Linolenic content that is above 12% imposed by the EN14214 standard, which has been barricaded microalgal based fuel as an alternative for diesel engine fuel. Shanmugam et al., (2020), studied three microalgae species *Chlorella vulgaris, Scenedesmus,* and *Synechococcus* for their fatty acid profile. They found that lignoceric acid was predominant fatty acid in *Chlorella vulgaris* and linoleic acid and arachidic acid was prevalent in *Scenedesmus* and *Synechococcus*. The biodiesel produced from *Chlorella vulgaris* and *Scenedesmus* sp. had good oxidative stability and *Synechococcus* sp. produced biodiesel with a good cold filter plugging point. Nascimento et al., (2013), studied 12 microalgae species isolated from the environment and found that palmitic acid was the most abundant fatty acid. Furthermore, *Chlorella* sp., *Botryococcus braunii,* and *Botryococcus terribilis* had high lipid content, while *Chlamydomonas* sp. and *Scenedesmus obliquus* presented fatty acid with greater oxidative stability and high cetane number.

Similarly, Talebi et al., (2013) compared 11 microalgae species which 6 were Chlorophyceae, 4 were Trebouxiophyceae, and Bacillariophyceae. *Chlorella vulgar*is showed high lipid productivity and *Amphora* sp. had a superior lipid profile that is desirable for quality biodiesel production. They proposed mixing of oils from distinct cell culture to produce quality biodiesel that meets the vehicular fuel standards. Soares et al., (2014), investigated three different microalgae species, and found that *Chaetoceros muelleri* had a lipid profile that meets specified fuel standards by EN 14214. The composition of microalgae fatty acid is also affected by growth condition such as nutrient deficiency, temperature, and light stress. The cultivation of *Dunaliella tertiolecta* under nutrient limiting conditions

showed to enhance accumulation of saturated and monounsaturated (Chen et al., 2011). Moreover, light intensity has shown to enhance the accumulation of polyunsaturated fatty acids (C16, C18), mono and di-galactosyl-diglycerides, sphingolipids and phosphoglyceride in *Euglena gracilis*, and *Chlorella vulgaris*. The exposure of microalgae to low temperature has been shown to change lipid profile in *Monochrysis lutheri* and *Dunaliella salina* (Odjadjare et al., 2017).

2.11 Biodiesel production from microalgal lipids

Lipids extracted are converted into biodiesel via transesterification catalysed by either alkaline or acid catalysts. Biodiesel can also be produced directly from wet biomass through direct transesterification. Direct transesterification provides a simple way of producing biodiesel directly from wet algal biomass, eliminating the energy-intensive step for drying biomass. Microalgae biodiesel is reported to have similar fuel properties and performs in accordance with petroleum-derived fuel.

2.11.1 Transesterification

The transesterification process involves the reactions of triglyceride with alcohol in the presence of a suitable catalyst either acidic, alkaline, or enzyme to produce fatty acid methyl esters (FAMEs) and glycerol as the end product (Adeniyi et al., 2018). This reaction step is essential to make bio-oil less viscous by reducing it to low molecular weight fatty acid alkali ester. High viscosity in biodiesel creates problems in the atomization of the fuel and other issues such as engine deposits and piston rings sticking etc. During transesterification reaction, one mole of triacylglycerol molecules reacts with three moles of alcohol (generally methanol) to give three fatty acid alkyl ester molecule and glycerol as by-product of the reaction. The by-products can be used for pharmaceutical and cosmetics purposes (Suganya et al., 2016). This reaction is reversible, thus requires a minimum molar ratio of 1:6 (TAG: alcohol) to be maintained in order to have the forward reaction. Transesterification reaction rate is enhanced by the presence of the catalyst which includes alkaline catalysts (sodium hydroxide, potassium hydroxide, and sodium methoxide) or acid catalysts (hydrochloric acid, sulfuric acid, sulfonic acid, and phosphoric acid); enzymatic catalyst such as lipase; and inorganic heterogeneous catalysts (solid-phase catalyst) (Pragya et al., 2013).

Alkaline is the most used catalyst for biodiesel production because of higher reaction rates and greater yield compare to acid-catalysed process. Millao and Uquiche, (2016), reported a possibility of a great yield up to 90% FAME conversion from *Spirogyras* and *Oedogonium* using alkaline catalysed reaction. Unfortunately, free fatty acid reacts with alkaline to form a soap which can form a gel under ambient temperature and substantially reduce the production yield. Saponification can be avoided by including an esterification step, where free fatty acid is esterified to FAMEs in the presence of alcohol such as methanol (Odjadjare et al., 2017). In contrast, acid-based reactions can simultaneously catalyse esterification and transesterification without the formation of soap. However, compare to alkaline

catalysed reaction it has lower reaction rates, high energy consumption, and a high amount of methanol due to the high molar ratio of methanol to oil. It also requires additional steps for catalyst separation and can cause corrosion. The effects of other parameters such as alcohol to oil molar ratio, temperature, reaction time, and water content results on transesterification results have also been well investigated (Suganya et al., 2016).

In contrast, enzymatic catalysed reaction offers a potential alternative to replace chemical catalysed transesterification. Enzymatic catalysed transesterification requires mild reaction conditions and provides high selectivity of transesterification with regards to the feedstock. Furthermore, it offers an immense range of substrate due to its capacity to esterify both glyceride-linked and non-esterified fatty acid in one step without any side reactions and produce high-grade glycerol as by-products (Moazeni et al., 2019). Enzymatic transesterification offers a simple biodiesel purification procedure with reduced energy requirement and without the need for any difficult steps (Fukuda et al., 2008). Usually, intracellular lipase as a whole-cell biocatalyst is the ideal form of catalyst for biodiesel production. Intracellular lipase is cheap compare to extracellular lipase which requires further separation and purification procedure (Moazeni et al., 2019). Lipase is known as triacylglycerol acylhydrolases catalyse the hydrolysis of hydrogen of triglycerides to fatty acid and glycerol over the oil-water interface (Amini et al., 2017). Lipase can be isolated from several natural biological resources such as animals, plants, and microorganisms. The use of microbial lipase is advantageous since most of them produce enzymes at a faster rate and relatively low cost. Novozyme 453, is a commonly used lipase to catalyse biodiesel production reaction with a high conversion efficiency of above 90%. It is isolated and purified from the fungal resources of *Candida antarctica* and *Rhizopus oryzae* (Amini et al., 2017).

Despite all the advantages, enzymatic catalysed reaction is associated with the high cost and low reaction rate, which are the major disadvantages for its application at an industrial scale that needs to be further investigated. Immobilization of enzymes into the soluble surface through covalent bonding, encapsulation, adsorption, and entrapment has been studied as a solution to lower the cost associated with the application of enzyme-based catalyst. This approach permits the reusable of the enzyme derivatives and eases the recovery of the enzyme from the products and purer products. Immobilized enzymes are more stable towards temperature, chemical as well as shear denaturation (Moazeni et al., 2019). Bayramoglu et al. (2015) reported a conversion efficiency of 96.4% of the methyl ester with immobilised lipase as a catalyst, which was respectively higher than free lipase which had a conversion efficiency of 85.7%. Immobilised lipase demonstrated high stability of over 6 reaction cycles of repeated use and only 17% of its activity was lost. However, lipase has a low tolerance to acyl receptor (methanol) which could be overcome by stepwise addition of methanol (Ghaly et al., 2010). Shimada et al. (2002) reported a conversion efficiency of 95% of the methyl ester with stepwise methanol addition and the lipase maintained over 50 reaction cycles. Nevertheless, the low reaction rate and high

cost of the enzymes are currently major disadvantages for its application at the industrial scale (Ghaly et al., 2010; Odjadjare et al., 2017).

2.11.2 *In situ* transesterification

In this method, instead of extracting the lipids first and converting them into biodiesel, wet algal biomass is subjected to a solvent (alcohol) and catalyst (acid or alkaline) under high temperature to produce biodiesel in a single-step reaction (Menegazzo and Fonseca, 2019). Usually, acid is a preferred catalyst over alkaline for this reaction as it can result in saponification in the presence of free fatty acid (Park et al., 2015). Other researchers have suggested the addition of polar solvents such as chloroform or methanol to increase the extraction efficiency of algal oil (Pragya et al., 2013; Adeniyi et al., 2018). Furthermore, cell disruption using microwave and/ or ultrasonication can also be used to increase the cellular degradation which will increase mass transfer for higher biodiesel yield (Park et al., 2015; Rawat et al., 2013). The reacted biomass sample can be separated for biodiesel from cell debris, glycerol, and excess methanol using the centrifugation method.

Direct transesterification reduces the number of steps involved in biodiesel production, thus reducing the production and energy consumption (Menegazzo and Fonseca, 2019). Technically, the use of wet biomass is not easy as the presence of water molecules around the cell can prevent the interaction of the catalyst and reactant with the lipid molecules as it does not allow them to easily penetrate through the tick microalgae cell (Deshmukh et al., 2019; Liu et al., 2013). The comparative biodiesel produced through direct transesterification is more suitable for quality biodiesel compares to the conventional route of biodiesel production (Harrington and D'Arcy-Evans, 1985). Cheng et al. (2014) produced biodiesel from a wet *Nannochloropsis oceanica* (80%) biomass in the presence of 16 mL chloroform, 16 mL methanol, and 0.8 ml of sulphuric acid at 60 °C for 30 min with microwave-assisted extraction and reported biodiesel conversion efficiency of 100%. Sivaramakrishnan and Muthukumar (2014) obtained 82% biodiesel yield from *Oedogonium* sp. wet biomass in the presence of 7.5 mL dimethyl carbonate, 8% *Bacillus* sp. and 50 µL distilled water at 55 °C for 36 h**.** However, proper optimization of factors such as temperature control and stirring speed in the reaction vessel for biodiesel formation. The increase in the moisture content of the biomass resulting in significant reductions of the equilibrium FAME conversion yield which still needs to be investigated to improve the efficiency of the method.

2.12 Microalgae value-added products

The high capital cost and low biomass yield are some of the challenges preventing the commercialization of microalgae based fuel due which has geared focus toward exploring the biomass for value-added products. Recently, microalgae have gained more attention as the feedstock for the production of value-added products with wide application in the pharmaceutical, cosmetic, and nutritional industries. The valuable bioactive compound from algal biomass includes phycobiliproteins,

polyunsaturated fatty acids (PUFAs), and carotenoids. The production of value-added products by microalgae is affected by temperature, pH, light, salinity, and nitrogen concentration (Gatamaneni Loganathan et al., 2018).

2.12.1 Lutein

Lutein is a yellow pigment synthesized mainly by plants in large amounts, especially by all green leafy vegetables. It is used as feed additives to brighten the colours of poultry feathers and deepen the yellow of egg yolk. Humans obtain lutein from eating maize mill and egg yolk for health benefits. Lutein is proposed to assist in the prevention of cancer, cardiovascular disease, age related macular degeneration, and other disease related to retinal degeneration (Moreno-Garcia et al., 2017). Most of the current commercial production of lutein is from a marigold flower, with its production expected to grow rapidly by 3.6% annually. Unfortunately, high production costs, low lutein content (0.03%) from marigold flower, and difficult downstream process pose serious challenges. This has motivated the use of microalgae as the substitute for lutein production. Microalgae have higher lutein productivity, and small land area and labour involved in cultivating microalgae compare to marigolds (Gatamaneni Loganathan et al., 2018). Microalgae species that are rich in lutein include *Scenedesmus almeriensis*, *Muriellopsis* sp., *Chlorella prothecoides*, *Chlorella zofingiensis*, *Chlorococcum ciriforme*, *Neospongiococcus gelatinosum* and *Desmodesmus* sp. (Moreno-Garcia et al., 2017). Nevertheless, the production of lutein from microalgae is still in its early stage, more study needs to be done to improve the feasibility for commercial scale production of lutein.

2.12.2 Astaxanthin

Astaxanthin is another carotenoid produced from algae, it has two asymmetric carbon located at the 3 and 3' position of the benzenoids rings on either end of the molecules (Singh and Gu, 2010). Astaxanthin is reported to have a potent antioxidant activity which is 10 times higher than β-carotene, lutein, zeaxanthin and 500 times higher than vitamin E (Shah et al., 2016). A single cell microalgal strain, *Hematococcus pluvialis*, is a large producer of astaxanthin that has bioactive ingredients including dietary fibre. Astaxanthin accumulation by *Hematococcus pluvialis* is influenced by environmental stress (salt, light intensity, and temperature) and nutritional stress conditions (nitrates and phosphorus) (Markou and Nerantzis, 2013). Other certain microalgae like *Chlorella zofingiensis*, *Chlorococcum* sp., and *Scenedesmus* have been successfully used to produce astaxanthin commercially (Odjadjare et al., 2017). Consumption of astaxanthin is reported to help reduce the incidence of coronary heart disease, certain cancer types, macular degeneration, and increased resistance to several infections (Odjadjare et al., 2017). Astaxanthin produced from *Haematococcus pluvialis* has a market value of approximately 1.8 US$ for 1,000 mg of astaxanthin (Shah et al., 2016).

2.12.3 β-carotene

β-carotene is one of the important carotenoids due to the presence of provitamin-A and it is used as colouring agent on cheese, butter, and margarine. In addition, it has antiaging and anticancer properties (Odjadjare et al., 2017). Unicellular microalgae of genus *Dunaliella saline* found in halotolerant habitants is reported to contain about 10 - 14% of its dry matter as β-carotene (Shete and Quadro, 2013). Other microalgae species found to produce β-carotene includes *Scenedesmus almeriensis*, *Nannochloropsis oculata* and *Phordium autumnale*. Rodrigues et al., (2015), identified 24 different carotenoids classes from *Phordium autumnale* which had all-*trans*- β-carotene (225.44 μg/g), all-*trans*-lutein (117.56 μg/g, and all-*trans*-zeaxanthin (88.46 μg/g) as major contributors. *Dunaliella* sp. have been shown to accumulate high amounts of β-carotene consisting of the all-trans and 9-cis isomers. Thus, differs from the synthetic β-carotene which only has one form of all-trans isomer (Singh and Gu, 2010). The 9-cis isomer of β-carotene has shown positive results on plasma lipids and the potential of preventing the advancement of atherosclerosis in humans (Koyande et al., 2019). The use of *Dunaliella* as feedstock for β-carotene production is a substantial growing industry and its commercial production is economically viable. The amount of β-carotene produced from *Dunaliella bordawil* is about 1.65 pg/cell with an approximate market value of approximately 0.6 US$ per 1,000 mg of β-carotene (Gatamaneni Loganathan et al., 2018)**.**

2.12.4 Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA)

Polyunsaturated fatty acids (PUFAs) such as DHA and EPA are important for the treatment and prevention of a range of diseases and human nutrition. Fish oil is one major source of PUFAs that has been harnessed for the past decade (Suganya et al., 2016). This has resulted in the depletion of wild fish stocks and potential pollution of the marine environment, due to over increasing global demand for PUFAs. There is a growing concern over the accumulation of the toxic compound in fishes and the unpleasant “fishy” odor (Odjadjare et al., 2017). The increasing demand for PUFAs has motivated the discovery of microalgae as the alternative sources of DHA and EPA. Microalgae can produce long chain-PUFAs that have important properties for human health and nutrition (Chew et al., 2017). Studies have shown that certain long chain-PUFAs may be associated with physical, mental, and visual development in infants. Omega-3 fatty acids are part of the healthy diet that assist to lower the risks of disease such as cardiovascular disease, various cancers, arthritis, and dementia (Suganya et al., 2016).

Microalgae species such as *Schizochytrium*, *Ulkenia, Isochrysis galbana, Chlorella pyrenoidosa, Chlorella ellipsoidea,* and *Crypthecodinium* have been extensively studied for the production of PUFAs production (Borowitzka, 2013, Gatamaneni Loganathan et al., 2018). *Schizochytrium* have been used to produce DHA oil on a commercial scale for adult dietary supplements including cheese, yogurt, spread, dressing, cereals, and food for the pregnant and nursing woman (Hadley et al., 2016). Docosahexaenoic Acid is an essential compound in the cellular membrane of nervous tissues such as the grey matter of the brain and retina. It is important for brain and eye development in an infant and is

found in breast milk. Microalgae *Phaeodactylum tricornutum* and *Nitzschia laevis* have shown great potential as a source of EPA (Hadley et al., 2016).

Polyunsaturated fatty acids obtained from microalgae have a global market value of over 700 million US$/annum (Markou and Nerantzis, 2013). Another important PUFAs is gamma-linolenic acid (GLA) which is regarded as nutritionally essential fatty acids with a potential to lower low-density lipoproteins in hypercholesteraemic patients, which improves premenstrual syndrome and treat atopic eczema. GLA may reduce the signs and symptoms of inflammatory diseases such as rheumatoid arthritis and atopic dermatitis. In animal and human trials, dietary supplementation with GLA has been shown to modulate inflammation (Borowitzka, 2013).

2.12.4 Phycobiliproteins

Phycobiliproteins are coloured pigments found in cyanobacteria and red algae. Phycobiliproteins are commercially used as natural dyes in the food industry and pharmaceuticals sector for antioxidant, antiviral, anticancer, anti-inflammatory, and neuroprotective properties (Chew et al., 2017; Markou and Nerantzis, 2013). Based on their maximum UV-visible absorption spectra phycobiliprotein have four main classes namely: phycocyanin, allophycocyanin, phycoerythrin, and phycoerythrocyanin. Phycocyanin alone has reached an estimated market value between 5-10 million US$. The major sources of phycobiliprotein producers are *Spirulina* sp., *Arthrospira platensis*, and *Amphanizomenon floaaquae* (Odjadjare et al., 2017).

2.13 Current trend and challenges of microalgae

Microalgae are considered promising as future raw feedstock for bioenergy production because of their ability to produce derivative products during the biorefinery process. Microalgal biotechnology is faced with several practical and financial barriers that stand in the way of utilizing microalgae for wastewater treatment and producing biofuel on an industrial scale (Mohsenpour et al., 2021; Maryjoseph and Ketheesan, 2020). Increasing device reliability to maintain effluent efficiency and lowering the technology cost by minimizing the land needed for the process is the main challenge in microalgae-based wastewater treatment (Capodaglio et al., 2020). Ongoing studies are exploring the extensive use of microalgae for wastewater treatment to attain a higher cost efficiency than conventional wastewater treatments plants. Furthermore, a high level of microbial contamination is problematic in the microalgae cultivation system especially in open raceway ponds (Mutanda et al., 2020). Recently, researchers have focused on micro-algal-bacteria treatment using modern treatment technologies such as HRAP, hybrid microalgae cultivation techniques, and algae photobioreactor to eliminate contamination problems. This approach may theoretically increase N and P elimination and give an insight into the microalgal-bacteria consortium's morphology, development, and metabolism.

The cost of producing biofuel from microalgae biomass compared to conventional fuel remains the key techno-economic challenge due to low biomass accumulation and lipid productivity. Hence, the need to isolate and select robust microalgal strains with high photosynthetic efficiency and lipid yield. The future direction in the algal biotechnology industry is looking at metabolic engineering to enhance microalgal photosynthetic efficiency and productivity, and to create a more sustainable and cost-effective production platform (Sproles et al., 2021). This will contribute to the knowledge on the physiological characteristics of algae, to improve biomass yield, lipid accumulation, and wastewater treatment further. Quantitative analysis could be made regularly to map the expression of known genes in algal cells to allow for reasonably fast improvements in the operational parameters.

The recovery of biomass from the broth culture is an expensive and challenging issue in algal biotechnology. Recently, there have been some innovative approaches developed to improve the process of microalgae harvesting such as fungi-mediated harvesting, and the use of engineered nanoparticles. There is a need for in-depth research inventions including, further optimization and field-scale trials of such methods, which are crucial to developing models acceptable for growing biofuel requirements at an industrial scale. Pretreatment modules used for harvested biomass have a direct impact on biofuel recovery. Coupling of more than one type of pretreatment module has also emerged as an attractive approach, which needs further research intervention for acceptability at industrial scales. The conversion of extracted biofuel precursors into energy molecules is well developed. Moreover, the bio-refinery approach seems more sustainable in terms of the economic viability of algal biofuel (Bhushan et al., 2020).

2. 14 References

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CHAPTER 3

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Isolation, Identification and High-Throughput Screening of Neutral Lipid Producing Indigenous Microalgae from South African Aquatic Habitats

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Abstract Exploring indigenous microalgae capable of producing significant amounts of neutral lipids through high throughput screening is crucial for sustainable biodiesel production. In this study, 31 indigenous microalgal strains were isolated from diverse aquatic habitats in KwaZulu Natal, South Africa. Eight superior lipid producing strains were selected for further analysis, based on Nile red fluorescence microscopy screening. The microalgal isolates were identified to belong to the genera *Chlorella*, *Neochloris* and *Chlamydomonas* via morpho taxonomic and molecular approach by 18S rRNA gene sequencing. *Chlorella vulgaris* PH2 had the highest specific growth rate (μ) and lowest doubling time of 0.24 day ^{1} and 2.89 ± 0.05 day ^{1}, respectively. *Chlorella vulgaris* T4 had the highest biomass productivity of 35.71 ± 0.03 mg L ^{1}day ^{1}. *Chlorella vulgaris* PH2 had the highest lipid content of 34.28 ± 0.47 and 38 ± 9.2% (dcw) as determined by gravimetric analysis and the sulfo phospho vanillin (SPV) method, respectively. *Chlorella vulgaris* PH2 exhibited a high content of saturated fatty acids, while *Chlorella* sp. T4 exhibited a high total content of saturated and monounsaturated fatty acids with a low content of polyunsaturated fatty acids. The preponderance of neutral lipids suggests that *Chlorella* sp. T4 is a suitable candidate for biomass feedstock for biodiesel production.

Keywords Biodiesel · Bioprospect · *Chlorella* sp. · Indigenous · Fatty acid methyl esters · GC MS · Lipid profile · Microalgae

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Introduction

Microalgae are considered potential alternative sources of neutral lipids for the production of biofuels. The exhaustion of fossil fuels due to transportation, rapid industrialization and growing global population exacerbates the emission of toxic gases such as carbon dioxide which contributes to global warming [1]. The currently used major fuel resources around the world are the fossil fuels, renewable energy and nuclear energy sources. Recently, focus has shifted to renewable energy such as biodiesel as a potential alternative to fossil fuels to avert the prevailing global energy crises [2, 3]. Biodiesel has been produced from various feedstocks such as edible oil and non edible lignocellulosic biomass [4]. However, production of biodie sel from these feedstocks has significant socio economic and environmental impacts raising crucial sustainability and food security issues [5, 6]. Recently, microalgae have received immense attention as natural renewable feedstocks for biodiesel production. Microalgae as third generation feedstocks for biodiesel production have been reported to have several advantages compared to first and second generation feedstocks [5, 7, 8]. Microalgae are highly advantageous as a source of third generation biofuel production as microalgae have elevated growth rates, little land requirements for cultivation, reduced generation times, no pesticides or herbicides requirements, non food priority and high in lipid content [3, 8, 9].

The majority of microalgal species produce high amounts of neutral lipids, especially triacylglycerols (TAGs), which are highly desirable and suitable for biodiesel production [1, 10, 11]. Traditionally, the biodegradable and nontoxic biodiesel is produced through conver sion of TAGs into fatty acid methyl esters (FAMEs) via heterogenous, homogenous and enzymatic catalysis for the transesterification reaction. To date, biodiesel production from microalgal biomass feedstocks is considered not economically viable [12]; therefore, there is need to bioprospect and screen for indigenous hyper neutral lipid producing microalgal strains [13, 14]. Moreover, the suitable strain must have high growth rate, rapid biomass accumulation and specific composition of the produced lipid desirable for quality biodiesel production. It is important to select a strain that produces high quality biodiesel that meets biodiesel standards by EN 14214:2003 or ASTM D6751. Furthermore, selection of screening techniques that can accurately and reliably measure the neutral lipid content is vital for assessing and comparing the suitability of microalgal strains for biodiesel production.

The Nile red staining technique is a rapid and cost effective screening tool frequently used to detect the presence of intracellular neutral lipid globules in microalgal cells [15 17]. The Nile red dye interacts and binds with neutral lipids, which fluoresce yellow, with polar lipids stained red [18, 19]. Screening microalgae strains using the Nile red technique is inexpensive and quick but it is semi quantitative and cannot measure the composition and suitability of the lipids [20, 21]. Furthermore, the thick cell wall of some microalgae impedes the dye from penetrating the cells, hence a new high throughput and highly sensitive technique using a vital dye BODIPY 505/515 has been reported [22, 23]. However, highly sensitive techniques with great resolving power such as gas chromatography and mass spectrometry (GC MS) provide better information for effective screening and selection of potential microalgal strains. GC MS identifies and measures fatty acid content based on their mass to charge ratio which allows for individual quantification and identification of the fatty acids [24]. This advanced technique gives more information on the quality of biodiesel obtained from the selected microalgal strain after thorough screening. However, analysis of fatty acid with GC MS is expensive and requires specialised equipment and highly skilled and trained personnel [23]. Screening with both Nile red and GC MS will give more information on the candidate strain and allow for

proper selection of suitable neutral lipid producing microalgal strains for the production of quality biodiesel.

The aim of the current study was to isolate and screen indigenous microalgal strains with high specific growth rates and high neutral lipid content suitable for biodiesel production. Microalgal strains were isolated from diverse aquatic environments such as freshwater, brackish and marine habitats. The isolated strains were evaluated for growth rates and biomass productivity and screened for neutral lipid accumulation. Eight strains were selected based on the Nile red analysis of the lipids and identified using morphological taxonomic keys, PCR amplification and analysis of 18S rDNA sequences.

Materials and Methods

Collection of Water Samples

Water samples for the isolation of neutral lipid producing microalgal strains were collected during the summer period from various aquatic habitats. Forty water samples (freshwater, maturation ponds, brackish and marine water) were collected around KwaZulu Natal, South Africa. The main reason for sample collection at the various sites was the preponderance of microalgae in the water. The grab sampling technique was used for water sample collection where the sampling bottle was dipped directly into the water. The samples were transported to the laboratory in a dark thermo box for enrichment within 24 h of collection.

Isolation and Purification of Microalgal Strains

Freshwater samples were enriched with BG 11 [25] and BBM media [26], and brackish and marine samples were enriched with F/2 medium [27]. The medium pH was adjusted to 7.5 with 1 M NaOH before autoclaving at 121 °C for 15 min. A volume (10 mL) of the environmental water sample (10% *v*/*v*) was transferred to 250 ml conical Erlenmeyer flask containing 90 mL of the sterile enrichment medium. The flasks were incubated for 28 days at 25 °C under cool fluorescent illumination of 37 μmol photon m^{-2} s^{-1} (HD 2102.2 photo/radiometer, DELTA OHM, Italy) with 12 h:12 h light to dark photoperiod cycle. The cultures were hand shaken two to three times daily to avoid settling and sticking of the culture onto the bottom of the flask. The presence of viable microalgal cells was confirmed by microscopic examination of the culture. The cultures showing growth were subjected to purification by conventional serial dilution and spread plating on BG 11 and BBM plates. The single colonies that appeared were aseptically picked and transferred to the same medium for purification followed by sequential subculturing on broth and solid agar media (BG 11 and BBM). The process was repeated until axenic cultures of different microalgae strains, confirmed by microscopic examination, were obtained. The isolates were maintained at 4 °C in BG 11 liquid media, since it supported the growth of most microalgal strains.

DNA Isolation, PCR Amplification of 18S rDNA Sequence and Phylogenetic Analysis

Genomic DNA was extracted from each microalgal strain using the Xpedition™ Fungal/Bacterial DNA MiniPrep (Zymo Research) according to the manufacturer's instruction.

Genomic DNA concentration and quality was determined by the NanoDrop 200 (UV Vis Spectrophotometer). The genomic DNA was stored at 20 °C until used for PCR amplification. The universal eukaryotic 18S rDNA primers CHLORO F (5′ TGG CCT ATC TTG TTG GTC TGC 3′) and CHLORO R (5′ GAA TCA ACC TGA CAA GGC AAC 3′) were used for the amplification of the 18S rRNA gene. The PCR amplification was performed using 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 mM of $MgCl_2$, 0.4 mμ of each primer and 2 U of *Taq* DNA polymerase. The PCR reaction was performed in an Eppendorf Mastercycler ep gradient S thermocycler (ABI 2720, Applied Biosystems, USA) with the following conditions: Initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min, with an additional extension at 72 °C for 10 min [28]. The PCR product was separated by electrophoresis on 1.5% (*w*/*v*) agarose gels, stained with 1 mg/ml ethidium bromide (Bio Rad, USA) and visualized under UV light in G:BOX F3 system (Cambridge, UK) to determine amplification of the correct product size and sent for sequencing (Inqaba Biotechnical Industries (Pty) Ltd., South Africa). The overlapping fragments were assembled using Chromas Lite (version 2.1). The 18S rDNA sequences were compared to the nucleotide sequences of some known microalgae in GenBank database of the National Centre for Biotechnology Information (NCBI) by using Local Basic Aligned Search Tool (BLAST).

Preliminary Screening of the Microalgal Strains for Lipid Production

Cultivation of microalgae for biomass and lipid accumulation was carried out in triplicate in 250 mL conical Erlenmeyer flasks with a working volume of 90 mL BG 11 medium. Ten millilitres of each seed culture was used as the inoculum and cultivated under conditions as previously described. Microalgal growth was monitored by measuring optical density of each culture at 680 nm at regular time intervals of 3 days during the growth period until stationary phase. Equal amounts of culture suspension were retrieved at intervals from the bioreactors and harvested by centrifugation at 5000 rpm for 5 min. The accumulation of neutral lipids was observed with fluorescence microscopy after Nile red staining [15].

Nile Red Staining and Fluorescence Microscopy

Nile red (NR) was conducted to detect the presence of intracellular neutral lipid droplets [12]. One millilitre of the microalgal cell suspensions standardized to an absorbance of 0.05 at 680 nm was used for lipid screening. Microalgal cells were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 25% DMSO. The Nile red (9 (diethyl amino) benzo[a]phenoxazin 5(5H) one, Sigma Aldrich) solution aliquot (1 μL, 10 mg/L in ice cold acetone) was added to the microalgal cell suspension. The resulting suspension of the dye and microalgal cells was agitated for 1 min on a vortex mixer and stored in the dark for 10 min. After incubation, the suspension was mounted on a glass slide covered by a glass cover slip and observed at ×400 magnification with a Nikon 80E epi fluorescent microscope equipped with an imaging system (Zeiss, South Africa). The excitation and emission filters used were 540 and 605 nm, respectively. A Nikon DS Fl1 camera was used to capture images and data processing was done using the NIS Element D3.0 software.

Cultivation of Selected Microalgae and Biomass Determination

Microalgal suspension in BG 11 was adjusted and subsequently standardised to an absorbance of 0.05 at an OD of 680 nm and 10% (*v*/*v*) of the culture was used as the inoculum. Microalgae were cultivated in 500 mL conical Erlenmeyer flasks, containing a working volume of 200 mL BG 11 medium and incubated at 30 °C for 22 days under cool white fluorescent illumination of 37 μmol photon m ^{2} s ^{1}. The culture growth was monitored by measuring optical density at 680 nm using the UV 1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) at 2 day interval for 22 days. A 5 mL volume of the microalgal suspension was harvested by centrifugation, dried at 105 °C overnight and weighed for the determination of dry weights. The relationship between OD and DW was determined through linear regression and convert ed into gram per litre. Each experiment was performed in triplicate and the data obtained were expressed as means ± standard deviation.

Determination of Growth Kinetic Parameters and Lipid Accumulation

The uninterrupted growth was monitored for the entire 22 days. The growth kinetics of the selected microalgal isolates was evaluated based on two key parameters: the growth rates and lipid accumulation. Determination of the specific growth rate (μ) and the doubling time of the individual isolates were calculated using Eqs. 1 and 2, respectively [29]:

$$\text{Specificgrowth rate } \mu\left(\text{day}^{\ 1}\right) = \text{In}(N_2\ \ N_1)/(t_2\ \ t_1) \tag{1}$$

where N_1 is the optical density on the initial day t_1 and N_2 is the optical density measured on day t_2.

$$\text{Doubling time (DT) is defined as DT} = \left(\mu^{\ 1}\right)/\ln 2 \tag{2}$$

The accumulation of lipids with time was measured at regular time interval of 2 days during the growth period, spectrophotometrically at 530 nm using the sulfo phospho vanillin (SPV) method, which was correlated to lipid quantity obtained by a previously generated standard curve [30]. The microalgal biomass was harvested after reaching the stationary phase by centrifugation at 8000 rpm for 10 min and the cells were washed twice with distilled water. The cell pellets were dried in an oven at 60 °C until constant dry weight (DW) of the biomass was obtained using an analytical balance. Each experiment was carried out in triplicate.

Gravimetric Determination of Total Lipid Content and Productivity

Cell disruption and lipid extraction were carried out by a slightly modified method of Lee et al. [31]. The dried biomass (100 mg) was pulverized using a mortar and pestle and added to a co solvent mixture (20 mL) of chloroform and methanol (1:1 *v*/*v*) and subjected to cell disruption using a domestic microwave oven (Samsung, CE2877N, S. Korea) at high temperature (100 °C) for 5 min. The mixture was centrifuged at 2000 rpm for 10 min to collect the supernatant and the process was repeated three times to ensure complete extraction of lipids. The solvent was vacuum filtered and evaporated in a Dragon Lab RE 100 pro rotary evaporator (Polychem Supplies, South Africa) at 60 °C under vacuum to remove the remaining solvents. The total lipids obtained were measured gravimetrically and the percentage of the

lipid content (%) was determined based on the lipid recovered from the weight of a known biomass. The lipid content and lipid productivity were calculated according to Eqs. 3 and 4, respectively:

$$\text{Lipid content}(\%\text{dcw}) = W_{\text{L}}/W_{\text{dcw}} \times 100 \tag{3}$$

$$\text{Lipid productivity}\left(\text{mgL}^{-1}\text{day}^{-1}\right) = \text{PBX lipid content in}\% \tag{4}$$

Lipid content with W_{L} weight of the lipid and W_{dcw} weight of dry cell weight in milligram per litre, and PB is the biomass productivity expressed in mg $\text{L}^{-1}\text{day}^{-1}$.

Analysis of FAMEs by GC-MS

The fatty acid composition of the microalgal lipid was derivatised and determined as fatty acid methyl esters (FAMEs). The lipids were subjected to methanolysis with 5% H_2SO_4 and methanol in 30:1 methanol to lipid ratio at 60 °C with agitation on a benchtop orbital shaking incubator (MRC, London, UK) at 200 rpm for 4 h in the presence of 1 mL hexane as the reaction solvent. From the mixture, a 100 μL aliquot was retrieved and washed twice with distilled water. The fatty acid containing phase was recovered and used for GC MS analysis [32]. The composition of the FAMEs was analysed by a Gas Chromatography Mass Spec trometer QP2010 SE (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with FID using a slightly modified method of Zhou et al., 2011 [33]. The capillary column of the GC MS was a RXi® 5Sil MS capillary column (30 m × 0.25 mm id × 0.25 μm film thickness, RESTEK) and nitrogen was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was set at 80 °C and held for 5 min, while the injector and detector temperature were set at 250 and 230 °C, respectively. The peaks of fatty acids were identified from the NIST Mass Spectral library and the relative amount of fatty acid was calculated from the integrated area percentage from the total amount of fatty acid.

Statistical Analysis

The data were analysed by one way ANOVA at 95% confidence limit ($\alpha = 0.05$). All statistical tests were performed using GraphPad Prism Version 7 (trial version) for Windows (GraphPad software, San Diego, California, USA, www.graphpad.com). $p < 0.05$ denotes a statistically significant difference. The values were expressed as the means ± standard deviation.

Results and Discussion

Sampling and Isolation of Microalgae

Bioprospecting for indigenous microalgae from unexplored biodiverse aquatic habitats is critical for obtaining microalgal strains with novel properties. Therefore, water samples were collected from diverse aquatic habitats around Durban, KwaZulu Natal, South Africa including freshwater, brackish and marine aquatic environments. These sampling sites were selected based on the presence and dominance of microalgae in the water. In

addition, most microalgae are not fastidious and grow in diverse aquatic environments such as freshwater, brackish, marine, maturation ponds and hyper saline aquatic habitats [8, 21]. Sampling was suspended between late autumn and early winter where temper ature ranges were low and the growth of benthic microalgae was promoted [21]. The average temperature of the surface water during the sampling regime ranged from 16 to 25 °C, which was within optimal temperature range for microalgal growth (Table 1) [34]. Isolation of microalgae is a necessary prerequisite for obtaining pure cultures and presents the initial critical step towards selection of neutral lipid producing microalgal strains for biodiesel production [35, 36]. However, isolation of microalgae from natural water samples may require multiple media types containing various concentrations of essential nutrients promoting growth of numerous microalgal species to ensure complete recovery of all microalgal species. In this study, three different media (BG 11, BBM and F/2 media) were used to enrich environmental water samples. Thirty one microalgal strains which were able to grow on the agar solidified media were isolated and main tained on BG 11media since it can support the growth of most microalgal strains from diverse habitats [26]. Among the media used, BG 11 appeared to be more effective in supporting the growth of microalgae from various sampling points. Twenty eight of the microalgae were isolated from freshwater and three from brackish water. However, successional tendency in the habitat is one of the factors (nutrients variation, harsh weather and distribution of nutrients) affecting isolation of microalgae from the environ mental samples [21]. In this study, a spatial and temporal sampling strategy was not adapted instead a once off sampling was done. Isolates from freshwater environments were easy to adapt, propagate and maintain in artificial media because they are not fastidious. However, most of the freshwater isolates stuck at the bottom of the bioreactor and required intermittent gentle shaking to keep the cells in suspension. In contrast, microalgae strains isolated from brackish environments were buoyant forming a clump without adhering to the surface of the flask due to their larger cell size.

Table 1 In situ determination of physico chemical parameters of the habitats from which the eight microalgal strains were isolated

Strain name	Sampling area	T (°C)	pH	Salinity (%)	Habitat	GPS coordinates
PH1	Phoenix wastewater treatment works	19	7.32	3.22	Freshwater (maturation pond)	29.678156 S, 31.032978 E
PH2	Phoenix wastewater treatment works	19	7.32	3.22	Freshwater (maturation pond)	29.678156 S, 31.032978 E
T1	Klein Manzimtoti river, Amanzimtoti	16	7.21	3.67	Freshwater (stream water)	30.075529 S, 30.869149 E
T4	Klein Manzimtoti river, Amanzimtoti	16	7.21	3.67	Freshwater (stream water)	30.075529 S, 30.869149 E
T7	Klein Manzimtoti river, Amanzimtoti	16	7.21	3.67	Freshwater (stream water)	30.075529 S, 30.869149 E
TS7	Mdloti river point 1	20	7.43	2.63	Freshwater (stream water)	29.645719 S, 31.122473 E
TS10	Mdloti river point 1	20	7.43	2.63	Freshwater (stream water)	29.645719 S, 31.122473 E
U2	Mdloti river point 2	20	7.43	2.63	Freshwater (stream water)	29.647596 S, 31.118729 E

Screening of Indigenous Lipid Producing Microalgal Strains

Microalgal strain selection is critical for successful microalgal cultivation for biodiesel pro duction. Microalgae are composed of neutral lipids (including triacylglycerols and hydrocar bons) and polar lipids as the energy storage reserves. Triacylglycerols are easily and conveniently converted to carbon neutral biodiesel via transesterification [18, 37]. All the isolates were cultivated in normal unmodified BG 11 medium for the initial screening of indigenous neutral lipid producers. Of the purified microalgal isolates, approximately 70% of the isolates revealed the presence of neutral lipid including hydrocarbon and triacylglycerols by emitting yellow fluorescence after Nile red staining after 4 weeks of cultivation (Fig. 4). Microalgal strains PH1, PH2, T1 and TS7 emitted high fluorescence indicating the presence of neutral lipids. In some isolates, the quantities of lipid globules were relatively low (approxi mately 25% of the cell). Generally, microalgae under suitable growth conditions tend to synthesize polar lipids [38], as neutral lipid is mostly synthesized under nutrient limiting conditions [39 41]. Microalgal strains PH1, PH2, T1, T4, T7, TS7, TS10 and U2 were selected for further analysis as they showed faster growth rates and larger neutral lipid globules from Nile red analysis.

Identification of Selected Lipid Producing Microalgal Strains

Morpho taxonomic characterization of selected lipid producing microalgal strains showed the presence of polymorphism, demonstrated by a variation in terms of cell size, colour and structure (Fig. 1). However, morpho taxonomic identification of microalgal strains by light microscopy alone is insufficient, tedious and technically difficult due to the vast microalgal diversity and that most microalgae have analogous morphology [21, 42]. The application of morpho taxonomic keys for the identification of unknown microalgae is further compounded by the fact that generally most microalgal strains have similar cellular morphological charac teristics, dimensions and accessory pigmentation. Therefore, 18S rDNA sequencing was used as an accurate method to precisely and fully identify the selected lipid producing microalgal strains. The Chloro primers used to amplify the 18S rDNA successfully amplified the expected DNA fragments (500 bp) from all the microalgal strains. The BLAST analysis of the corresponding sequences indicate that microalgae strains PH1, PH2, T1, T4, T7, TS7, TS10 and U2 were closely related to *Chlorella* sp., *Chlorella vulgaris*, *Chlorella* sp., *Chlorella* sp.,

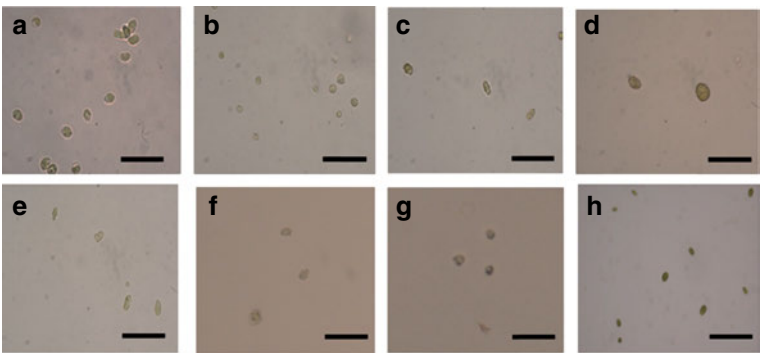

Fig. 1 Morphology of the selected isolates as revealed by light microscopy, *scale bar* **a** **h** represents 20 μm. **a** PH1. **b** PH2. **c** T1. **d** T4. **e** T7. **f** TS7. **g** TS10. **h** U2

Table 2 Molecular identification of isolated microalgal strains based on the partial amplification and analysis of the18S rRNA gene sequence

Strain code	Accession number	Sequence length (nt)	Microalgal identity	Similarity (%)
PH1	KP662698	477	*Chlorella* sp.	99
PH2	KP662699	459	*Chlorella vulgaris*	99
T1	KP662698	463	*Chlorella* sp.	99
T4	KP662697	466	*Chlorella* sp.	99
T7	KM677943	482	*Neochloris aquatica*	99
TS7	KM679943	457	*Neochloris aquatica*	99
TS10	KM677935	458	*Chlorella sorokiniana*	99
U2	KT720479	458	*Chlamydomonas* sp.	98

Neochloris aquatica, *Neochloris aquatica*, *Chlorella sorokiniana* and *Chlamydomonas* sp., respectively. The sequences of the selected isolates were deposited in NCBI database with specific accession numbers listed in Table 2.

Comparative Growth Kinetics and Lipid Accumulation

Rapid growth, high biomass yield and accumulation of intracellular neutral lipid are important prerequisites for selecting potential microalgal strains for sustainable biodiesel production [43, 44]. Eight microalgal strains were evaluated for their specific growth parameters and lipid accumulation. The average specific growth rates differed significantly among the strains in BG 11 media under the same culture conditions. The average specific growth rate was estimated at the exponential growth phase (Table 3). In our observation, there was no significant difference in the specific growth rates among the selected isolates (ANOVA, $p > 0.05$). The biomass yield of the eight microalgal strains cultivated in BG 11 medium over a 3 week period are presented (Fig. 2). The microalgal strain *Chlamydomonas* sp. U2 isolated from a freshwater stream demonstrated the highest specific growth rate of 0.2 ± 0.02; thus, it had the shortest doubling time of 0.29 ± 0.03 day ^{1}. *Chlorella* sp. T1 had a specific growth rate and a doubling time of 0.17 ± 0.01and 0.25 ± 0.01 day ^{1}, respectively. Microalgal strains C. sorokiniana TS 10 demonstrated a low specific growth rate of 0.09 ± 0.01 day ^{1}, suggesting

Table 3 Specific growth rate (μ) and doubling time (k) of the eight microalgal strains after 22 days of cultivation

Microalgal strain	μ, day ^{1}	k (doubling time per day)
Chlorella sp. PH1	0.1 ± 0.01	0.15 ± 0.01
C. vulgaris PH2	0.16 ± 0.03	0.23 ± 0.03
Chlorella sp. T1	0.17 ± 0.01	0.25 ± 0.01
Chlorella sp. T4	0.12 ± 0.01	0.17 ± 0.02
N. aquatica T7	0.16 ± 0.02	0.23 ± 0.03
N. aquatica TS7	0.1 ± 0.04	0.14 ± 0.05
C. sorokiniana TS10	0.09 ± 0.01	0.12 ± 0.01
Chlamydomonas sp. U2	0.2 ± 0.02	0.29 ± 0.03

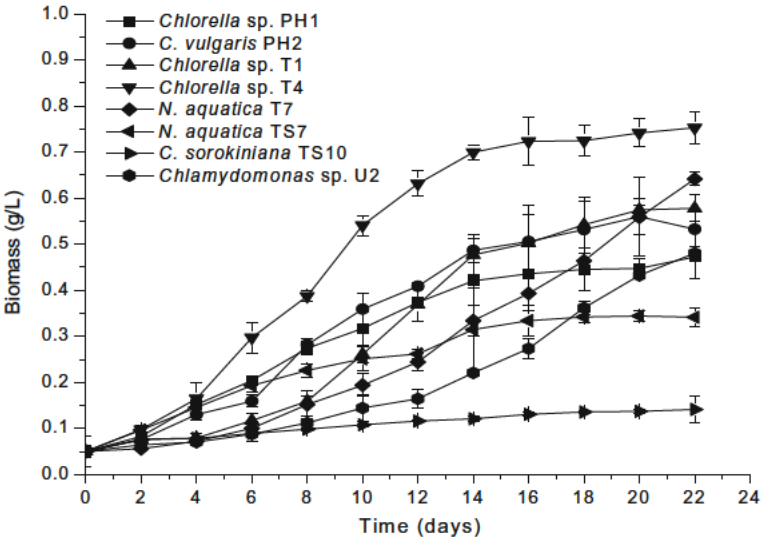

Fig. 2 Time course biomass yield of selected microalgal strains cultivated in BG 11 media

that these strains are not suitable for achieving high cell density which is one of the important parameters for selecting prospective microalgal strains [44].

The accumulation of lipids in the microalgal cells as monitored by the SPV colorimetric method revealed that rapid lipid accumulation among the eight microalgal strains occurred between 10 and 16 days of cultivation depending on the microalgal strain (Fig. 3).

Depletion of nutrients in the medium lowers the rate at which all cell components are produced resulting in low biomass [45, 46]. Most of the microalgal strains investigated in this study reached their stationary phase after 14 days of cultivation (Fig. 2). Several isolates

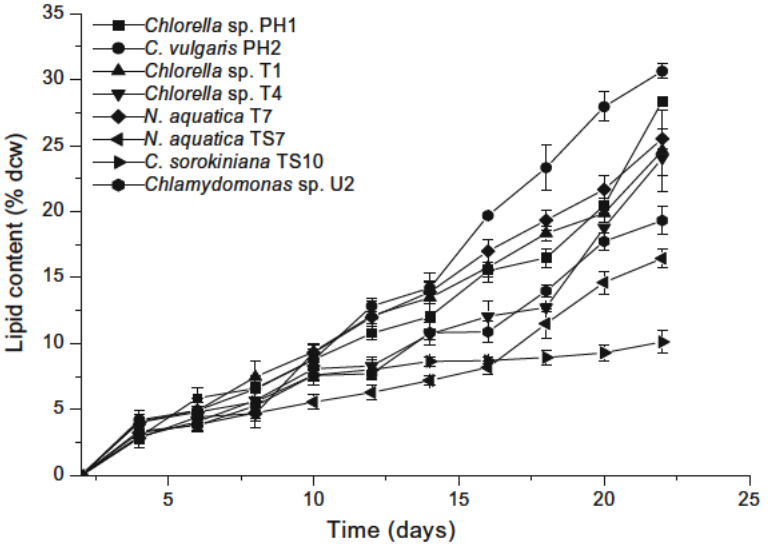

Fig. 3 Lipid content of eight microalgal strains cultivated in BG 11 media for 22 days. Lipid content was determined by the SPV method

investigated began to show a steady sharp increase in the accumulation of lipids after 16 days of cultivation indicating the onset of nutrient limitation as this corresponds to the time when stationary phase was attained by the isolates. Microalgal strain *C. vulgaris* PH2 showed the highest lipid content of 34.28 ± 0.47%, which is about 3.1 fold higher that the lipid content of *C. sorokiniana*, which produced the lowest lipid content of 11.04 ± 0.58% (ANOVA, $p > 0.05$).

Microalgae are known to accumulate substantial amounts of triacylglycerols (TAGs) during the stationary phase of growth [47, 48]. Nile red staining and fluorescence analysis was performed to confirm the presence of intracellular neutral lipid droplets by fluorescence microscopy after 22 days of cultivation. The NR fluorescence images of selected strains are presented in Fig. 4. The presence of large amounts of intracellular neutral lipid accumulation in microalgal cells as lipid globules was observed in all the eight microalgal strains (Fig. 4). There was inconsistency in the size of the lipid globules among the strains, as the accumulation of lipid is species specific [49]. Microalgal strains were further evaluated for their biomass and lipid content gravimetrically as they are equally important for attaining higher lipid productivity.

Analysis of Biomass, Lipid Content and Productivity

Microalgae are known to produce substantial amounts of intracellular neutral lipids that can be efficiently converted into biodiesel [8]. Measuring the microalgal lipid productivity is impor tant as it takes into account both lipid content and biomass production rate, which are essential parameters for sustainable biodiesel production. Therefore, cellular lipid productivity of the eight microalgal strains was estimated by evaluating biomass and lipid productivity (Table 4). The microalgal strain *Chlorella* sp. T4 had the highest biomass productivity of 35.85 ± 1.62 mg L^{-1} day^{-1} followed by *N. aquatica* T7 with biomass productivity of 30.55 ± 0.73 mg L^{-1} day^{-1} (ANOVA, $p < 0.05$).

The biomass productivity of *Chlorella* sp. T4 was close to the biomass productivity of 31.43 mg L^{-1} day^{-1} reported by Tale et al. [49]. The microalgal strain *C. sorokiniana* TS10 had the lowest biomass productivity of 6.72 ± 1.4 mg L^{-1} day^{-1}. The biomass productivity of this strain is in agreement with the findings of Piligaev et al. [3] showing a relatively low biomass productivity. The average lipid content of microalgae under normal cultivation

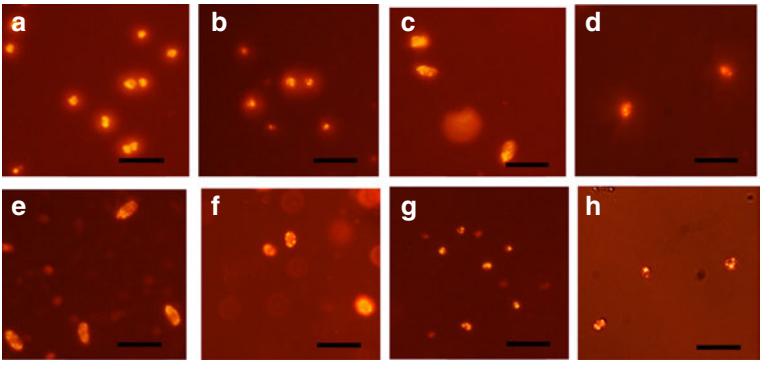

Fig. 4 Fluorescence microscopic images of selected microalgal strains stained with Nile red in the stationary phase of growth at the excitation and emission wavelengths of 540 and 605 nm, respectively. **a** *Chlorella* sp. PH1. **b** *Chlorella vulgaris* PH2. **c** *Chlorella* sp. T1. **d** *Chlorella* sp. T4. **e** *Neochloris aquatica* T7. **f** *Neochloris aquatica* TS7. **g** *Chlorella sorokiniana* TS10. **h** *Chlamydomonas* sp. U2. *Scale bar* **a** **h** represents 10 μm

Table 4 Biomass and lipid productivities of the eight selected microalgal strains. Lipid content was determined by the SPV method

Microalgal strain	Biomass productivity (mg L^{-1} day^{-1})	Lipid productivity (mg L^{-1} day^{-1})
Chlorella sp. PH1	22.49 ± 0.43	6.15 ± 0.79
C. vulgaris PH2	25.36 ± 1.76	9.46 ± 2.32
Chlorella sp. T1	27.51 ± 1.38	8.29 ± 1.95
Chlorella sp. T4	35.85 ± 1.62	8.03 ± 1.26
N. aquatica T7	30.55 ± 0.73	6.55 ± 0.65
C. aquatica TS7	16.25 ± 0.96	2.62 ± 0.39
C. sorokiniana TS 10	6.72 ± 1.40	0.90 ± 0.42
Chlamydomonas sp. U2	22.86 ± 2.63	5.72 ± 0.65

conditions has been reported to range between 6 and 42% [50, 51]. However, some microalgae can reach lipid content of up to 90% under optimized nutrient limited growth conditions that trigger rapid accumulation of lipids [36]. In this study, total lipid content after gravimetric analysis ranged from 14 ± 6.2 to 38 ± 8.8% of dry biomass weight (Fig. 3). The microalgal strain *C. vulgaris* PH2 exhibited the highest lipid content of 38 ± 8.8%, followed by *Chlorella* sp. T1 with a lipid content of 35 ± 7.1%.

Statistical analysis revealed no significant difference in the amount of lipid content between the two organisms (ANOVA, $p > 0.05$). In contrast, *Chlorella* sp. T4 was the highest biomass producer in the study with a lipid content of 25 ± 3.5% (dcw). Most microalgae exhibiting high biomass productivity are reported to have low lipid productivity [19]. However, irrespective of low lipid content by *Chlorella* sp. T4, this strain demonstrated a balance between biomass productivity and lipid productivity. The final lipid content (% biomass composition) of the eight strains after 3 weeks of growth is presented in Fig. 5**.** The results obtained are in agreement with a previous study in which the lipid content of 31 microalgal strains was compared and found to range between 6 ± 0.58 and 42 ± 0.08% of dry biomass weight [51]. The precise lipid content obtained after gravimetric analysis was closely equivalent to lipid content estimated spectrophotometrically by the SPV colorimetric method. Lipid productivity is one of the important parameters for selecting suitable candidate strains for biodiesel production, as lipid content alone is insufficient and unreliable for screening potential strains [12, 52]. The observed variation in the lipid productivity among strains confirmed the fact that there were no single optimized growth conditions to stimulate lipid accumulation. Interesting ly, the microalgal strains *C. vulgaris* PH2, *Chlorella* sp. T1 and *Chlorella* sp. T4 also demonstrated high lipid productivities of 9.46 ± 2.32, 8.29 ± 1.95 and 8.03 ± 1.26 mg L^{-1} day^{-1}, respectively (ANOVA, $p > 0.05$). The high lipid productivities of these oleaginous microalgal strains indicate their potential applications as feedstocks for biodiesel production [10]. However, potential microalgal strains are selected based on their lipid profile, saturation, carbon chain length and suitability for quality biodiesel production.

Fatty Acid Composition of the Microalgal Strains

Screening of indigenous neutral lipid producing microalgal strains should not only be based on the lipid content, but also the fatty acid content and composition, which determine the final

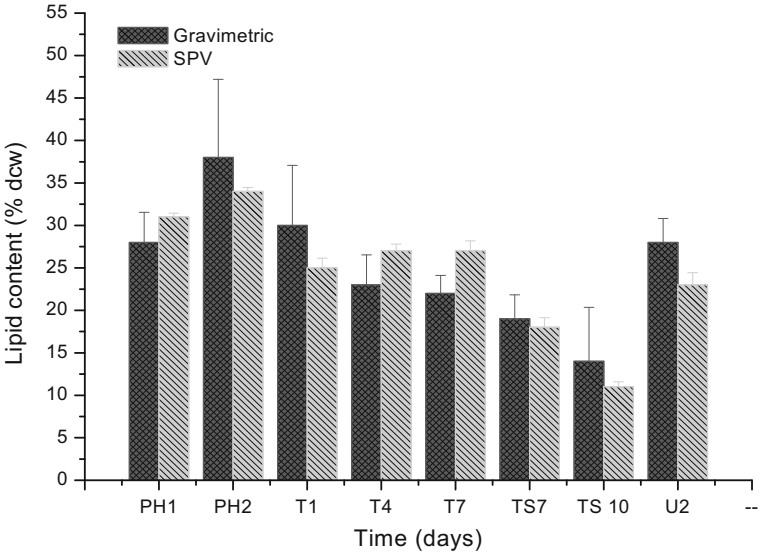

Fig. 5 Relative lipid content of microalgal isolates cultivated in BG 11 medium for 22 days as measured gravimetrically (gravimetric) and spectrophotometrically by the sulfo phospho vanillin colorimetric method (*SPV*). *PH1*: *Chlorella* sp. PH1, *PH2*: *C. vulgaris* PH2, *T1*: *Chlorella* sp. T1, *T4*: *Chlorella* sp. T4, *T7*: *N. aquatica* T7, *TS7*: *N. aquatica* TS7, *TS10*: *C. sorokiniana* TS10, *U2*: *Chlamydomonas* sp. U2

quality of biodiesel [53]. High quality biodiesel should meet EN and ASTM biodiesel quality specifications and must have good oxidative stability, cetane number, cold flow properties and iodine value, which are dependent on the degree of fatty acid saturation [54, 55]. Generally, the chain length of suitable fatty acids for biodiesel production ranges between 14 and 18 carbons [56, 57]. Most common FAMEs found in biodiesel are palmitate, oleic and linoleic acid [53, 58]. However, the degree of fatty acid saturation is influenced by cumulative effects of environmental growth conditions and also varies according to phylogenetic diversity of individual microalgal species [54, 55].

In this study, a distinct variation in the composition of fatty acids was observed among the eight microalgal strains. Analysis of fatty acid composition of the selected strains showed the presence of seven saturated fatty acids, tridecanoic, tetradecanoate, pentadecanoic, hexadecanoic, octadecanoic, nonadecanoic and eicosanoic. Seven monounsaturated fatty acids, methyl cis 6 octadecenoate, 6 octadecenoic, 7 octadecenoic, 8 octadeconic, 7 hexadecenoic, 18 methyllicosanoate and 16 methylheptadecanoic were identified. The pres ence of eight polyunsaturated fatty acids, 8,11,14 heptadecatrienoic, 9,12,15 octadecatrienoic, 9,12,5 octadecatrienoic, methyl 10 trans,12 cis octadecadienoate, methyl 9 cis,11 trans octadecadienoate, 9,11 trans octadecadienoate, 8,11 octadecadienoic and 11,14,17 eicosatetraenoate were also confirmed by GC MS. Most fatty acids were detected at the retention times ranging between 39.321 and 66.828 min among the different microalgal strains tested as depicted on the GC MS chromatogram obtained for *Chlorella* sp. T4 (Fig. 6). Methyl tetradecanoate (myristic acid), hexadecanoic acid methyl ester (palmitic acid) and pentadecanoic acid were the predominant fatty acids present in all the microalgal strains investigated (Table 5). These results are in agreement with the findings of Dermal et al. [58] in which hexadecanoic acid methyl ester and pentadecanoic acid methyl ester were the most abundant microalgal fatty acids detected. Palmitic acid is a saturated fatty acid and is known as

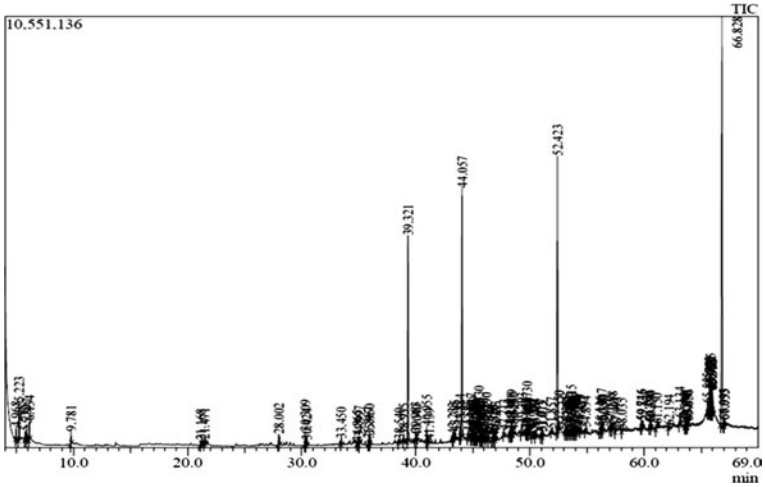

Fig. 6 Chromatogram obtained from GC MS equipped with FID for the identification and analysis of fatty acid methyl esters (FAMEs) in *Chlorella* sp. T4. The retention times of 39.321, 44.057 and 52.423 min correspond to saturated, monosaturated and polyunsaturated FAMEs, respectively

the most common fatty acid found in biodiesel [58]. High contents of only saturated fatty acids were detected in microalgal strains *Chlorella* sp. PH1, *C. vulgaris* PH2, *Chlorella* sp. T1, *N. aquatica* T7 and *Chlamydomonas* sp. U2. The fatty acid composition of *Chlorella vulgaris* PH1 depicts the preponderance of C16:0, therefore suggesting its suitability for biodiesel production. The fatty acid composition of *Chlorella vulgaris* PH2 determined by GC MS indicates the presence of C13:0 up to C16:0. However, the total fatty acid did not sum to 100% as microalgae produce different kinds of lipids, glycolipids, sterol esters, hydrocarbons and other complex oils which are not suitable for biodiesel production.

Saturated fatty acids have been reported to give a good cetane number and oxidative stability to biodiesel [56]. Microalgal strain *Chlorella* sp. T1 only showed the presence of high saturated and low polyunsaturated fatty acids. However, monosaturated fatty acids were not detected in *Chorella* sp. T1 which gives a compromise between the oxidative stability and cold flow properties of the final biodiesel. *Chlorella* sp. T4 showed the presence of high saturated and monounsaturated fatty acids with a low content of polyunsaturated fatty acids (Table 5). High content of oleic acid in microalgae oil confers balance to fuel properties such as better oxidative stability for longer storage, reduction in cold filter plugging point used in cold regions [53, 59]. However, microalgal strains *N. aquatica* TS7 and *C. sorokiniana* TS10 did not show the presence of fatty acids after GC MS analysis within the suitable range for biodiesel production, though Nile red analysis showed the presence of neutral lipids. None of the strains investigated showed the presence of oleic acid, which is the most important fatty acid for quality biodiesel. A reasonable balance of fatty acid composition with a high content of monounsaturated fatty acids such as oleic acid and a low content of saturated and polyunsaturated fatty acids is considered optimal from the biofuel quality standpoint [49, 53, 57]. Recently, research efforts have focused on improving the productivity of microalgae biomass and lipid, but improving the quality of biodiesel is still a technical challenge [55]. In this study, *Chlorella* sp. T4 was found to be a potential feedstock for biodiesel production due to its suitable fatty acid profile, composition and ready adaptability to the local regional climatic conditions.

Table 5 Identification and relative abundance (RA) of the fatty acids produced by the eight selected microalgal strains cultivated in BG 11 medium as analysed by GC MS equipped with FID. RA was calculated from the surface area of the peaks

FAMEs	Molecular formula	Relative abundance (RA) (%)					
		PH1	PH2	T1	T4	T7	U2
Tridecanoic acid methyl ester	$C_{14}H_{28}O_2$	25	20	7.48	12.28	23.37	0.0
Tetradecanoate methyl ester	$C_{15}H_{30}O_2$	0.0	20	7.48	14.58	3.26	0.0
Pentadecanoic acid methyl ester	$C_{16}H_{32}O_2$	25	20	7.48	12.28	23.37	20
Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	25	20	7.48	12.38	23.37	20
7 Hexadecenoic acid methyl ester	$C_{18}H_{36}0_2$	0.0	0.0	0.0	0.79	0.0	0.0
8, 11, 14 heptadecatrienoic acid methyl ester	$C_{18}H_{30}O_2$	0.0	0.0	5.25	0.0	0.0	0.0
Octadecanoic acid methyl ester	$C_{19}H_{38}O_2$	0.0	0.0	13.87	14.58	0.0	0.0
Methyl cis 6 octadecenoate	$C_{19}H_{36}O_2$	0.0	0.0	0.0	0.79	0.0	0.0
6 Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	0.0	0.0	0.0	0.79	0.0	0.0
7 Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	0.0	0.0	0.0	0.79	0.0	0.0
8 Octadecenoic acid methyl ester	$C_{19}H_{36}O_2$	0.0	0.0	0.0	0.79	0.0	0.0
16 Methylheptadecanoic acid methyl ester	$C_{19}H_{38}O_2$	0.0	0.0	0.0	14.58	0.0	0.0
Methyl 10 trans,12 cis octadecadienoate	$C_{19}H_{34}O_2$	0.0	0.0	0.0	0.29	0.0	0.0
Methyl 9 cis,11 trans octadecadienoate	$C_{19}H_{34}0_2$	0.0	0.0	0.0	0.29	0.0	0.0
8,11 Octadecadienoic acid methyl ester	$C_{19}H_{34}0_2$	0.0	0.0	0.0	0.29	0.0	0.0
9,12 Octadecadienoic acid methyl ester	$C_{19}H_{34}O_2$	0.0	0.0	0.0	0.29	0.0	0.0
9,12,15 Octadecatrienoic acid methyl ester	$C_{19}H_{30}O_2$	0.0	0.0	5.25	0.0	0.0	0.0
9, 12, 5 Octadecatrienoic acid methyl ester	$C_{19}H_{32}O_2$	0.0	0.0	5.25	0.0	0.0	0.0
Nonadecanoic acid methyl ester	$C_{20}H_{32}O_2$	0.0	0.0	0.0	12.38	23.37	0.0
5, 11, 14, 17 eicosatetraenoate acid methyl ester	$C_{20}H_{32}O_2$	0.0	0.0	5.25	0.0	0.0	0.0
Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	0.0	0.0	13.87	0.47	0.0	0.0
Cis 8 methylicosanoate methyl ester	$C_{22}H_{44}O_2$	0.0	0.0	0.0	0.47	0.0	0.0
Saturated fatty acids		75	80	57.66	78.95	96.74	40
Monounsaturated fatty acids (MUFA)		0.0	0.0	0.0	19	0.0	0.0
Polyunsaturated fatty acids (PUFA)		0.0	0.0	15.75	1.16	0.0	0.0

Conclusions

Bioprospecting of oleaginous indigenous microalgae from various aquatic habitats in KwaZulu Natal, South Africa, revealed microalgal strains with unique biochemical properties amenable for biotechnological exploitation. Of the 31 isolated strains, eight microalgal strains were selected based on their high growth rates and lipid accumulation through Nile red analysis in order screen and select microalgae with high biomass and neutral lipid productivity. Gravimetric analysis of the eight selected strains showed lipid content ranging from 14 ± 6.4 to 38 ± 9.2%. The strains presented high content of saturated fatty profiles with hexadecanoic acid methyl ester and pentadecanoic acid methyl ester as their main fatty acid. Among the strains, *Chlorella* sp. T4 showed a good combination of biomass productivity, lipid productivity and suitable lipid profile compared to other strains. The findings in this study suggest that naturally isolated indigenous strains of *Chlorella* sp. from unexplored diverse aquatic habitats in the region

are appropriate for biodiesel production based on biomass productivity, lipid productivity and fatty acid profiles.

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CHAPTER 4

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Lipid productivity and biosynthesis genes response of indigenous *Chlorella* sp. T4 strain under different nitrogen and phosphorus load

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Abstract

Microalgae can synthesize and accumulate high neutral lipids upon exposure to abiotic stress such as nutrient starvation or limitation. In this study, indigenous microalgae *Chlorella* sp. T4 was cultivated in nitrogen and phosphorus under both limiting and replete conditions. Growth, lipid yield, fatty acid profiles and biosynthetic gene expression levels were determined to ascertain cell's response under these conditions. An impaired cell growth was observed under nitrogen limiting condition, evident by the lowest biomass yield (0.58 ± 0.03 g L^{-1}) as revealed by low quantum efficiency of photosystem II (Fv/Fm) value and chlorophyll a content. An increase in lipid content yield was observed under nitrogen and phosphorus limiting conditions as compared to the control. Nutrient limiting conditions produced fatty acid methyl ester that is suitable for biodiesel production compared to the control (BG-11). Gene expression analysis using real time q-PCR for photosynthesis (*rbcL*) and lipid biosynthesis (*accD, KAS-1*, *ω-6* FAD, *ω-3* FAD) genes revealed different expression levels under both limiting and replete conditions. Under nutrient limiting conditions, increase in the expression of *accD*, *KAS-1*, *ω-6 FAD* and *ω-3 FAD* genes was observed, whereas a decrease in *rbcL* gene expression level was noted. A significant correlation could be drawn between the expression levels of the biosynthetic genes and growth rate, biomass yield, physiological response, lipid yield and fatty acid composition. These results provide an insight into the physiological response and gene expression level under different nutrient levels, which could be harnessed for future genetic engineering of *Chlorella* sp. T4 for improved lipid production.

Keywords: *Chlorella* sp. T4, Gene expression, Fatty acid, Nutrients, Lipid accumulation, Physiological response

4.0 Introduction

The demand for biodiesel, as a replacement for the conventional fossil fuel, is growing worldwide in recent years [1]. Microalgae have been a favourable feedstock for biodiesel production due to its fast growth rate, high lipid yield, suitable fatty acid composition and adaptability to a wide range of climatic environment [2, 3], but high production cost at commercial scale is still a major drawback [4]. Microalgae tend to accumulate energy storage material in form of lipids and starch under stress conditions, when the cell growth reaches the stationary phase [5]. It has been shown that optimization of microalgae culture condition can result into high lipid productivities in microalgal biomass. Manipulation of several key intrinsic and extrinsic factors such as nutrient stress, light intensity, temperature, and carbon source triggers lipid accumulation pathway [6-8]. Nutrient deprivation is often used by many researchers to improve overall microalgal lipid productivity, due to it's low cost and easy application during the cultivation process [9]. This approach causes decrease in photosynthetic rates, and compromises biomass accumulation, while resulting in enhanced overall lipid storage in form of triacylglycerol [10, 11]. Nitrogen (N) is regarded as important micro-nutrient for microalgal growth, as it is associated with protein synthesis and cell division [11], whereas phosphorus (P) contributes to various metabolic processes such as signaling pathways, energy generation and photosynthesis.

Most studies have been focused on obtaining high lipid productivity yield under nutrient-stress conditions, without a proper understanding of the effect of these conditions on the microalgae photosynthesis activity, physiological response, and gene expression levels under these conditions [12, 13]. The understanding of microalgal response at the molecular level is limiting to few species, such as *Chlamydomonas reinhardtti, Thalassiosira psuedonana* and *Dunaliella salina* [14]. The microalgae under study, *Chlorella* sp. T4 strain, isolated in our laboratory, have demonstrated huge potential to accumulate large amount of fatty acid that can be used for biodiesel production [15]. In this study, different concentrations of N and P were applied to trigger hyper lipid accumulation in this indigenous microalgae. Furthermore, the physiological response of the microalgae under different nutrient conditions and expression levels of five key fatty acid biosynthetic genes (*rbcL*, *accD*, *KAS-1*, *ω*-6 *FAD* and *ω*-3 *FAD*) were investigated. The *rbcL* gene encodes the catalytic large subunit of the enzyme RuBisCO (ribulose 1.5-bisphosphate carboxylase/oxygenase) which is responsible for carbon fixation, catalyzing the first step in the Calvin cycle [14]. Previous study reported a decrease in *rbcL* gene expression under N and P deficient conditions in *Chlorella sorokiniana* [3]. *accD* encodes for acetyl-coenzyme A carboxylase carboxyl transferase subunit beta which is responsible for fatty acid biosynthesis, and catalyses the conversion of acetyl-CoA to malonyl-CoA during the lipid biosynthesis [6]. An increase in *accD* expression under nutrient deficient conditions has been reported in *Chlorella pyrenoidosa* [14]. The expression levels of three fatty acid biosynthetic genes; *KAS-1*, *ω-6 FAD* and *ω-3 FAD* in relation to fatty acid yield under different N and P concentrations have been investigated. The *KAS-1* gene encodes for ketoacyl-ACP synthase-1 responsible for the addition of malonyl-CoA to

elongate 4-carbon fatty acid to 6-, 12- and 16 carbon fatty acid chains, for the production of palmitic and stearic acid. Furthermore, *ω-6 FAD* gene which encodes for omega-6 desaturase responsible for catalyzing the conversion of oleic acid into linoleic acid, while *ω-3 FAD* gene code for omega-3 desaturase which is responsible for the conversion of *ω*-6 fatty acid into *ω*-3 fatty acid [16-18]. Therefore, the aim of the present study was to design suitable cultivation conditions for high lipid accumulation that can be used for biodiesel production. To understand the expression of key functional genes (*rbcL*, *accD*, *KAS-1*, *ω-6 FAD* and *ω-3 FAD*) by varying N and P concentration in the growth medium.

4.1 Experimental

4.1.1 Algal strain and seed preparation

The microalgal strain *Chlorella* sp. T4 used in this study was isolated from freshwater body in KwaZulu-Natal, South Africa [15]. The strain was preserved in BG-11 medium which is composed of (g L^{-1}): $NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.75; $CaCl_2*2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na_2CO_3, 0.02 and 1 mL of micronutrient or trace metal solution containing (g L^{-1}): H_3BO_3, 2.86; $MnCl_2*4H_2O$, 1.81; $ZnSO_4*7H_2O$, 0.22; $NaMoO_4.5H_2O$, 0.079; $Co(NO_3)_2*6H_2O$, 0.04. The medium was adjusted to a pH of 7 and sterilized in an autoclave at 121 °C for 15 min.

The culture (10% v/v) was inoculated in 500 mL conical Erlenmeyer flaks containing 200 mL of BG-11 medium. An aliquot of tetracycline (0.5 µl mL^{-1}) was added to the growth medium to prevent any bacterial contamination in the microalgal samples. The culture was incubated at 25 °C under cool white fluorescent illumination of 200 µmol $m^{-2}s^{-1}$ with a photoperiod of 12h: 12h, light: dark cycle under ambient CO_2. Similar cultivation conditions were maintained for all the subsequent experiments. The cultures were hand-shaken two to three times daily to avoid settling and sticking of the culture onto the bottom of the flask

4.1.2 Experimental design and physiological parameters analysis

To find the best nitrogen (N) and phosphorus (P) concentration for high lipid productivity yield, *Chlorella* sp. T4 was cultivated in BG-11 medium containing sodium nitrates (0.75 and 2.25 g L^{-1}) and di-potassium-ortho-phosphate (0.02 and 0.06 g L^{-1}). These nutrient concentrations were selected based on the lipid productivity yield obtained from the preliminary study conducted by growing the microalgae strain on BG-11 containing different concentrations of N (0, 0.35, 0.75, 2.25 g L^{-1}) and P (0, 0.02, 0.04, 0.06 g L^{-1}). Optimization was conducted with one factor at a time, and other individual media composition kept constant as in BG-11 to assess the individual effect of the culture treatment on *Chlorella* sp. T4. In addition, control experiment was conducted using BG-11 medium with normal concentration of sodium nitrates (1.5 g L^{-1}) and di-potassium-ortho-phosphate (0.04 g L^{-1}) that is known

to support microalgae growth. The algal cell was standardized to the optical density of 0.05 at 680 nm. The cells were harvested by centrifugation at 5000 rpm for 10 min, washed with distilled water, and resuspended into appropriate medium containing different concentrations of sodium nitrate and di-potassium-ortho-phosphate. The culture condition was maintained at 25 ºC under continuous fluorescent light with light intensity of approximately 100 µmol $m^{-2}s^{-1}$, and the flasks were hand shaken 2 to 3 times a day.

The Chlorophyll a content of *Chlorella* sp. T4 was measured as described previously [19]. The physiological and photosynthesis efficiency of the microalgal cells were studied as described previously [20]. The maximum quantum efficiency of Photosystem II (PS II) was calculated using the equation: $F_V/F_m = (F_m-F_0)/F_m$ as previously described [20], Where F_m, F_o, and Fv represents the maximum, minimum and variable fluorescence, respectively.

4.1.3 Measurement of cell growth, biomass concentration, lipid yield and fatty acid content

Cell growth determination, biomass concentration measurement, and algal lipids extraction and weight determination were carried out as described previously [15]. The harvesting was done by centrifugation at 5000 rpm for 10 min. The fatty acid content were quantified as described previously [15] while the biodiesel properties were estimated using the web version of the Biodiesel Analyzer 2.2 [21].

4.1.4 Gene expression analysis

The expression levels of five key fatty acid biosynthetic genes (*rbcL*, *accD*, *KAS-1*, *ω*-6 *FAD* and *ω*-3 *FAD*) were determined in samples collected at the early log phase (day 7), late log phase (day 14) and stationary phase (day 21) of growth in the different nutritional growth conditions. The total RNA was extracted from ≈100 mg of algal cells using GeneJet RNA purification kit (Thermo Fisher Scientific, MA, USA) followed by synthesis of first-strand cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific, MA, USA) according to the manufacturer's instruction. The level of gene expression was monitored by Real-time quantitative PCR performed with Universal SYBR Green Supermix (Bio-Rad, CA, USA) in Hard-Shell High-Profile 96-Well Semi-Skirted PCR Plates (Bio-Rad, CA, USA) using 50 ng cDNA as the template and primer pairs listed in Table 1.

All PCR reactions consists of 1 µL 50 ng cDNA template, 1.5 µL of 10 mM deoxyribonucleotide triphosphates, 0.4 µM final concentration of each forward (F) and reverse (R) primers (Table 1), 10 µL 2 × iQ SYBR Green Supermix and nuclease-free water to final volume of 20 µL. RT-qPRC amplification protocol for targeted genes consists of initial denaturation for 3 min at 95 °C, followed by 40 cycles of three steps consisting of 15 s at 95 °C, 20 s at appropriate annealing temperature and 30 s at 72 °C**.** The specificity of all PCR amplifications was verified with melting curve calculation at the completion of each run, set from 55°C to 95°C at 0.5°C increment. The gene expression levels were

normalized by the expression level of 18S rRNA gene and data presented as fold increase or decrease of the target gene expression levels in the treated samples relative to the control sample [3, 22, 23].

4.1.5 Statistical analyses of experimental results

The data were analysed by one-way ANOVA at 95% confidence limit ($\alpha = 0.05$). All statistical tests were performed using SPSS (v. 20, IBM). Unless otherwise stated, $p < 0.05$ denotes a statistically significant difference. The values were expressed as the mean ± standard deviation.

Table 1 Primers used in the real time RT-PCR for quantifying the biosynthetic genes.

Gene	Sequence (5′-3′)	Tm (°C)	Amplicon size (bp)	Reference
accD	(F) TTTGGTTTGTGCTTCTGGTG	51.9	149	[3]
	(R) CACCACCAGTTGTTGGAGAA			
rbcL	(F) CTTTCCAAGGTCCTCCTCAC	56.4	208	[3]
	(R) TCTCTCCAACGCATAAATGG			
KAS-1	(F) CCATGATTGGTCATTGCTTGGGAGC	58	151	[16]
	(R) GCTCTTGCTTCATGTTTGGGACCAC			
ω -6 FAD	(F) CTTCACCCACGAAGGCACAGGC	58.8	129	[16]
	(R) CCTGCACACTGCTGGGAACG			
ω -3 FAD	(F)CATGTTGAGAACGACGAGTCCTGGTAT	59	162	[16]
	(R) GTCAAAGTGGGAGCCAGTCTTGC			
18S rRNA	(F) CCTGCGGCTTAATTTGACTCAACACG	60	172	[16]
	(R) TAGCAGGCTGAGGTCACGTTCG			

F-forward, R-reverse

4.2 Results and discussion

4.2.1. Cell growth and biomass accumulation

The effects of varying nutrient concentrations on the growth of *Chlorella* sp. T4 were investigated to ascertain suitable condition for biomass yield and high lipid productivity. Cultivation of microalgae under nutrient limiting condition has been reported to decrease the overall algal biomass, while inducing synthesis of neutral lipid suitable for biodiesel production [12]. In this study, nutrient stress conditions produced adverse effects on the proliferation of *Chlorella* sp. T4 cells (Fig 1). As shown in Table 2, low specific growth rates 0.055 ± 0.004 h^{-1} was observed when *Chlorella* sp. T4 was cultivated under N-limiting medium, with short generation time of 0.079 ± 0.005 day^{-1} compared to the control. High specific growth rate of 0.079 ± 0.004 h^{-1} was observed under N-replete medium which was not significantly higher than that the growth rate in the control medium. This is also reflected by similar growth pattern of *Chlorella* sp. T4 obtained in N-replete medium and control medium (Fig 1a). It proves the importance of nitrogen as a macro nutrient required for protein synthesis and cell division in microalgae [11]. The observed growth patterns under N and P-limiting medium (Fig 1) are consistent with those reported for other *Chlorella* strains [14, 16, 24].

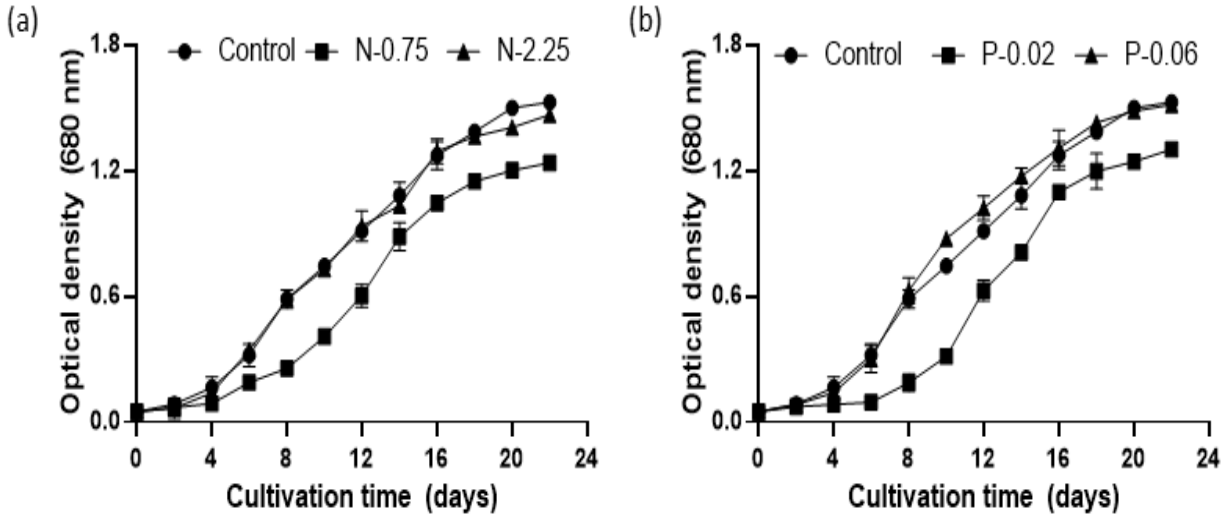


Fig. 1. Growth curve of *Chlorella* sp. T4 under different nutrient conditions (a) N-treatment (g L^{-1}), (b) P-treatment (g L^{-1}). N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Values show the average of three replicates ±SD.

The microalgae *Chlorella* sp. T4 showed tolerance to high N concentration as demonstrated by the growth curve similar to that obtained in the control medium. Similarly, higher specific growth rate was observed in medium with higher concentration of phosphorus. The specific growth rate (0.050 ± 0.011

h^{-1}) observed under P-limiting condition is about two-fold less compared to the value obtained in the P-replete medium (0.098 ± 0.014 h^{-1}). This was also corroborated by the growth patterns of the strain under P-limiting condition (Fig 1b). P-replete medium produced higher generation time of 0.147 ± 0.020 day^{-1}, which is 1.35- and 2.04-fold higher than the generation times obtained in the control and P-limiting medium. Phosphorus is a constituent element of ATP, essential for photophosphorylation which has significant relevance to the cell growth and metabolism of microalgae. Photosynthetic microalgae require large amounts of proteins (mainly RuBisCO) which is synthesized by phosphorus-rich ribosome [25].

Table 2 Specific growth rate and doubling time of *Chlorella* sp. T4 cultivated in BG-11 medium with different N and P concentration.

Nutrient treatment	Growth rate (h^{-1})	Generation time (day^{-1})
Control	0.076 ± 0.014^{b}	0.109 ± 0.020^{ab}
N-0.75	0.055 ± 0.004^{c}	0.079 ± 0.005^{ab}
N-2.25	0.079 ± 0.004^{b}	0.114 ± 0.006^{b}
P-0.02	0.050 ± 0.011^{c}	0.072 ± 0.016^{c}
P-0.06	0.098 ± 0.014^{a}	0.147 ± 0.020^{a}

For all the experiments, biomass yield and productivity together with lipid content and productivity were calculated after 21 days cultivation period. Nitrogen replete medium (N-2.25) produced the highest biomass yield of 0.82 ± 0.06 g L^{-1} which is 41.4% significantly ($p < 0.05$) higher than the biomass yield in the nitrogen deficiency (N-0.75) medium but not significantly different from that obtained in the control medium (Table 3). Similarly, 23.4% significantly ($p < 0.05$) higher biomass yield was obtained in P-replete (P-0.06) medium compared to the P-deficiency (P-0.02) medium.
This is further correlated by the high biomass productivity obtained under N-replete medium (38.95 ± 0.84 mg L^{-1} d^{-1}) and P-replete medium (37.52 ± 0.53 mg L^{-1} d^{-1}) due to high nutrient availability to utilize for cell division (Table 3).
Reduction in nutrients concentration in media has been shown to slow down the metabolic activity and cell division in most microalgae, while triggering lipid accumulation [26]. It is therefore not surprising that a significantly 1.6-fold and 1.2-fold increases in lipid productivity and lipid content were obtained in nitrogen limiting and phosphorus limiting medium, respectively, relative to the nutrient replete medium. High biomass productivity by this microalgae strain under both nutrient rich and nutrient stressed conditions is very promising, since biomass productivity is one of the major traits that makes microalgae attractive feedstock for biofuel applications over plant-based feedstocks [27]. Similarly, Paranjape et al. [28] found that an increase in N concentration resulted high biomass yield of 1.56 and

1.78 g L^{-1} for *Chlorella sorokiniana* (PCH02) and *Chlorella vulgaris* (PCH05), respectively. The observed overall increase in biomass yield and biomass productivity in N and P rich medium of this strain may be due to luxurious uptake of phosphorus which gets deposited in the cell as polyphosphate involved in metabolic pathway and storage for further use during phosphorus starvation/ limitation [29]. Similarly, Chakraborty et al. [24] reported an increase in biomass concentration of *Chlorella minutissima* MCC as the phosphate concentration increase from 0 to 3 mM.

Table 3 Cumulative lipid content (% dcw) of *Chlorella* sp. T4 under different culture conditions

Experiments	Biomass (g L^{-1})	Biomass productivity (mg L^{-1} d^{-1})	Lipid content (%dcw)	Lipid productivity (mg $L^{-1}d^{-1}$)
Control	0.77 ± 0.01^{ab}	36.48 ± 0.64^{a}	25.87 ± 1.03^{c}	12.93 ± 0.51^{b}
N-0.75	0.58 ± 0.03^{ab}	27.58 ± 1.23^{a}	31.07 ± 0.53^{a}	15.54 ± 0.27^{a}
N-2.25	0.82 ± 0.06^{ab}	38.99 ± 0.85^{b}	19.99 ± 0.01^{e}	10 ± 0.15^{b}
P-0.02	0.64 ± 0.01^{b}	30.28 ± 0.68^{a}	28.33 ± 1.35^{b}	14.17 ± 0.67^{b}
P-0.06	0.79 ± 0.50^{a}	37.52 ± 0.53^{a}	23.80 ± 0.26^{d}	11.90 ± 0.13^{b}

N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Different letters depict significance difference within the group according to one-way ANOVA at $p < 0.05$. Values show the average of three replicates ±SD.

4.2.2 Chlorophyll content and physiological response of *Chlorella* sp. T4 under different nutrient conditions

Chloroplast is an important unit component for most photosynthetic plants and algae. Hence, the chlorophyll content and the viability of the photosynthetic process are critical physiological indicator for monitoring microalgae adaptability to different culture conditions [14]. The chlorophyll a content was measured during the cultivation period as an indicator of the physiological response of *Chlorella* sp. T4 in different N and P conditions. An increase in chlorophyll a content was observed as the concentration of N and P increased in media. Highest chlorophyll a content (in mg/g dcw) of 27.11 ± 0.01 (Fig 2a) and 26.50 ± 0.67 (Fig 2b) was observed after 21 days in N and P-replete medium, respectively. A significantly ($p < 0.05$) decrease in chlorophyll a content by 1.2-fold and 1.4-fold under

N and P-deficient conditions compare to the control, respectively, were recorded. It suggests that the decrease of N and P concentration in the medium results in lower cell chlorophyll accumulation due to scarcity of intracellular nitrogen and phosphorus pool to synthesize chlorophyll for further cell reproduction. Nitrogen and phosphorus are most important elements contributing to the growth of microalgae cell, it limitation significantly changes the biosynthesis of cell pigment [30]. The results clearly show the influence of nutrient limitation on the growth physiology of *Chlorella* sp. T4 as the growth rate and biomass yield also decreased under these conditions.

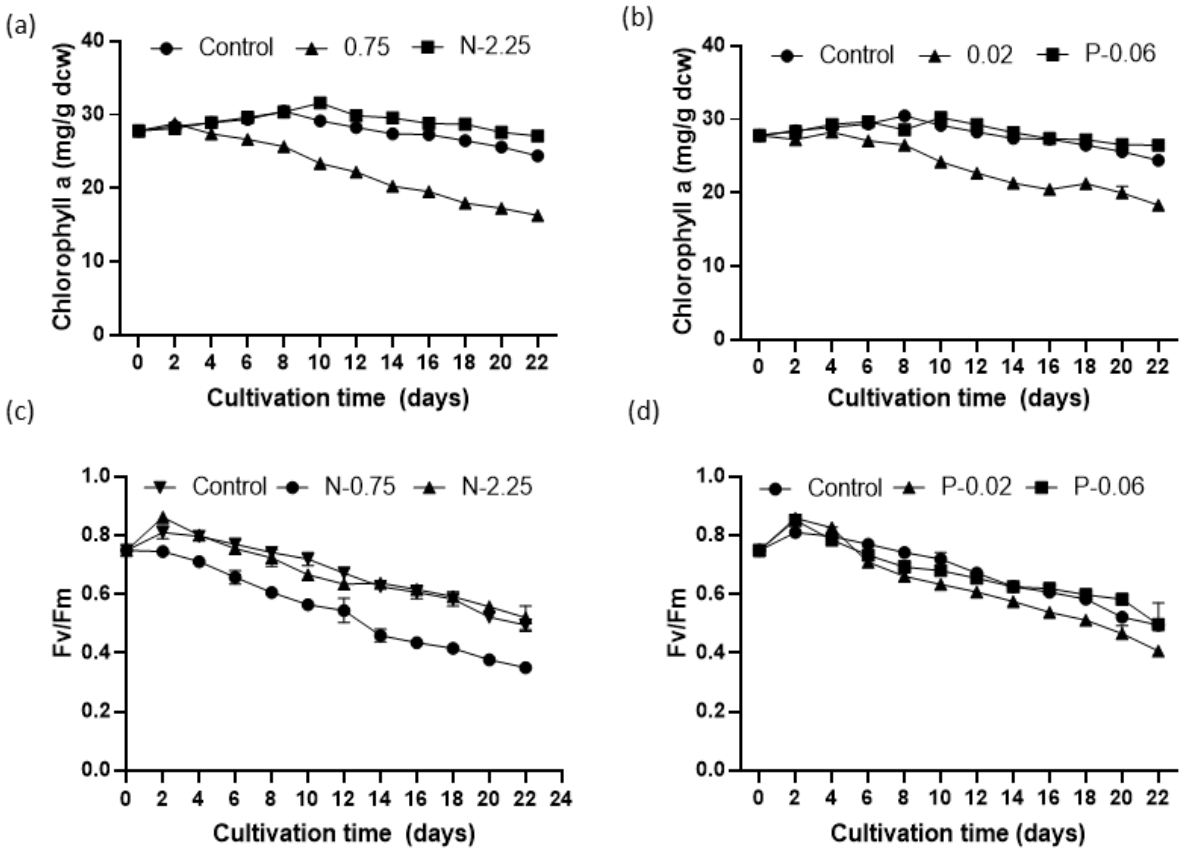


Fig. 2. Photosynthetic activity of *Chlorella* sp. T4 under different nutrient conditions (a) Chlorophyll a under N treatment, (b) Chlorophyll a under P treatment, (c) Fv/Fm value under N treatment and (d) Fv/Fm value under P treatment. N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Values show the average of three replicates ±SD.

The widely used fluorescence parameter Fv/Fm, an index reflecting irradiance acclimation status [31] was investigated under nutrient deficient and replete medium. It represents the measure of photosystem II (PSII) quantum yield and could be used to evaluate the photo induced damage to protein complex [32]. The positive influence of N and P treatment conditions was observed on the growth of *Chlorella* sp. T4 (Fig 2c, d). A significantly ($p < 0.05$) decrease in Fv/Fm by 1.21-fold and 1.12-fold was observed

under N and P-limiting condition compare to the control, respectively. The decrease in Fv/Fm is an indicative of the photoinhibition of PSII by *Chlorella* sp. T4. Microalgae tend to redirect their energy from photosynthetic process under nutrient starved conditions toward maximizing nutrient uptake upon nutrient addition, leading to a net decrease in the capacity of cells to dissipate energy photochemically [33]. *Chlorella* sp. T4 exhibited a steady decline in the photosynthetic parameter Fv/Fm under N and P-replete medium by 1.13-fold and 1.12-fold compare to the control, respectively. It has been shown that the Fv/Fm ratio in microalgae increase markedly when cultured under nutrient sufficient condition [3].

4.2.3 Analysis of lipid content and composition

Studies have shown that cultivation of microalgae under nutrients deficiency conditions stimulates lipid biosynthesis in many microalgae species [3, 8]. In this study, *Chlorella* sp. T4 showed a significant ($p < 0.05$) increase in total lipid yield under N and P-limiting conditions, accounting for 31.07 ± 0.53 % and 28.33 ± 1.35 % of dry cell weight, respectively (Table 3). Feng et al. [34], cultivated *C. zofingiensis* under N and P-deficient medium and reported higher lipid contents as compared to the nutrient sufficient medium which is similar to the present study. Singh et al. [3] also observed high lipid accumulation by *Chlorella sorokiniana* under N and P-deficient medium compare to the control medium. Contrary, Kiran et a., [12] cultivated *Chlorella* sp. in medium containing different N concentration and observed an increase in lipid accumulation under N-replete medium compare to N-deficient conditions.

Lipid productivity is one of particular importance microalgal lipid production process as it considers both lipid content and biomass production rate. High lipid productivity of 15.54 ± 0.7 mg L^{-1} d^{-1} was obtained under N-limiting condition which was 1.37-fold higher than P-limiting condition after 21 days (Table 3). Nonetheless, nutrient limiting conditions repressed the growth of *Chlorella* sp. T4 and the overall productivity caused by nutrient deficiency was not offset by biomass loss. Similarly, Fan et al., [14] reported high lipid productivity of 47.05 mg L^{-1} d^{-1} under N-deficiency in *Chlorella pyrenoidosa* after 5 days of cultivation. Also, Chu et al., [35] investigated the effects of phosphorus on lipid accumulation of *Chlorella vulgaris* and reported a lipid productivity of 19.40 mg $L^{-1}d^{-1}$ in phosphorus deficient medium. Low lipid productivity was observed under N-replete medium (10 ± 0.15 mg L^{-1} d^{-1}) which was 2.32-fold lower than the control medium. These findings clearly show that high lipid productivity yield can be obtained by cultivating microalgae under nutrient deficiency conditions that has just enough nutrients to support the growth.

Microalgae biomass contains significant quantities of lipids in the form of triacylglycerol, which can be converted to biodiesel via transesterification process. This has attracted huge commercial interest of

using microalgae as feedstock for biodiesel production [27]. The lipid composition of *Chlorella* sp. T4 varied according to the nutrient concentration of the growth medium (Table 4). Previous studies have also shown that the concentration of nitrogen and phosphorus in microalgae culture alter total fatty acid content and composition [16, 36].

Table 4 Fatty acids profile of biodiesel produced under different N and P condition after 21 days of cultivation.

Fatty acids	Control	N-0.75	N-2.25	P-0.02	P-0.06
Palmitic acid (C16:0)	29.7 ± 1.3^{a}	31.1 ± 0.1^{a}	30.8 ± 0.7^{c}	25.5 ± 0.5^{b}	30.4 ± 0.9^{a}
Stearic acid (C18:0)	2.8 ± 0.2^{d}	8.3 ± 0.2^{b}	6.8 ± 0.8^{c}	9.5 ± 0.1^{a}	12.9 ± 0.2^{e}
Oleic acid (18:1)	37.1 ± 0.1^{a}	35 ± 1.7^{b}	30.2 ± 0.3^{c}	27.4 ± 0.7^{d}	29.6 ± 1.7^{c}
Linoleic acid (C18:2)	27.6 ± 0.4^{b}	21.4 ± 0.0^{a}	28.8 ± 0.6^{a}	29.9 ± 1.5^{a}	24.5 ± 0.0^{a}
α-Linoleic acid (C18:3n3)	2.58 ± 0.8^{a}	4.2 ± 0.5^{a}	3.3 ± 0.5^{a}	7.4 ± 0.4^{a}	2.6 ± 0.1^{a}

N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Different letters depict significance difference among the group according to one-way ANOVA at $p < 0.05$. Mean value shown is the average of three replicates ±SD.

Palmitic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) constitute the major fatty acids in algal oil. Fatty acids of this chain length are reported to be suitable for high quality biodiesel production [1]. Further analysis reveals that saturated fatty acid (SFA) ranged from 32% to 43.3%, monounsaturated fatty acid (MUFA) ranged from 21.4% to 37.1%**,** whereas polyunsaturated fatty acid (PUFAs) ranged from 27.1% to 37.3%. Polyunsaturated fatty acids are important with nutritionally benefits for infant development and with estimated market value of over 11 billion US dollars [27]. There was high level of saturated fatty acid observed under P-replete (49.9 %) medium, which is 1.3-fold higher than the control medium (Table 4). High level oleic acid (37.1 ± 0.1 %) was obtained in the control medium, but not significantly ($p > 0.05$) different from the level obtained in both the N and P-limiting medium. High content of oleic acid is beneficiary for excellent oxidative stability, increases biodiesel's flow properties and reduces it solidification temperature [37, 38]. Furthermore, high content of PUFAs was found in P-limiting condition which could cause decline of cetane number and oxidation stability, making biodiesel prone to oxidation-dependent degradation [37, 39]. This finding was well with the results reported by Aziz et al., [16] who reported that high accumulation of PUFAs was obtained under P-limiting condition.

The quality of microalgae biodiesel is measured by the important thermophysical properties of biodiesel and comparing those with the international standards such as ASTM D675 or EN14214 (Table 5). Previous studies have demonstrated that fatty acid profile significantly affected the quality of biodiesel [1, 21]. The oxidative stability of the biodiesel produced in this study ranged between 5.75 to 7.20 h which is favourable for biodiesel production due to saturated fatty acid [40]. This microalgae strain showed low cold filter plugging properties (-2.75 ºC) under N-limiting condition which is preferable for biodiesel production. This was caused by a good balance between the saturated fatty acid and monounsaturated fatty acid observed under the N-limiting condition. High saturated fatty acid content may reduce the cold filter plugging point properties of biodiesel because saturated fatty acid has higher melting points than unsaturated fatty acid [41]. Furthermore, kinematic viscosity ($mm^2 s^{-1}$) produced by *Chlorella* sp. T4 under all N and P conditions was outside the range recommended by ASTM D675 and EN14214. This property may result into biodiesel produced with high viscosity affecting the fuel atomization and lead to deposits forming inside the engine, due to high PUFAs contain by microalgae compare to the other seed oils. Linoleic acid was above 12 recommended by EN14214 for all the conditions, an indication of poor oxidative stability with good cold flow properties [27]. In this study, the best biodiesel was produced under N-limiting condition, with high ignition quality, good oxidative stability, cetane number value and saponification value (Table 5).

Table 5 Properties of *Chlorella* sp. T4 biodiesel cultivated in BG-11 medium with different N and P concentration compared to ASTM D6751 and EN 14214 specification [1].

Biodiesel properties (Units)	Standard fuel parameters		Transesterification				
	ASTM D6751	EN 14214	Control	N-0.75	N-2.25	P-0.02	P-0.06
Iodine value (gl_2/100 g)	-	120 (max)	82	90.54	88.36	99.05	78.11
Saponification value (mg KOH)	-	-	205.42	211.12	204.67	210.51	204.62
Cetane number	47 (min)	51.0 (min)	54.45	52.61	50.9	49.94	55.40
Degree of unsaturation (% wt)	-	-	86.50	97.60	94.40	102.00	83.80
Long-chain saturation factor (% wt)	-	-	7.26	4.37	6.48	7.63	9.49
High heating value (MJ kg^{-1})	-	-	39.56	39.51	39.38	40.60	39.45
Cold filter plugging properties (ºC)	-	-	6.33	-2.75	3.88	7.49	13.34
Kinematic viscosity (mm^2 s^{-1})	1.9-6.0	3.5-5.0	1.35	1.33	1.33	1.36	1.35
Density (g cm^{-3})	-	0.86-0.90	0.87	0.87	0.87	0.90	0.87
Oxidative stability (h)	3 (min)	≥ 6	7.20	6.50	6.26	5.75	6.94
Linoleic acid (%)	-	12	27.6	21.4	28.8	29.9	24.5

ASTM D-6751-American Society for Testing and Materials, EN 14214-European standard for biodiesel, N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}.

4.2.4 Effect of culture conditions on the expression of *rbcL* and *accD* genes of *Chlorella* sp. T4

During photosynthesis, the RuBisCo enzyme is involved in carbon fixation process. A large subunit of this enzyme encoded by gene *rbcL* which harbour binding site [42, 43]. The present study evaluates the effect of nutrient conditions on the expression levels of some functional and fatty acids biosynthetic genes from *Chlorella* sp. T4. A significant (p<0.05) decrease of 2.09-fold in the *rbcL* gene expression was observed under N-limiting condition after 21 days compare to the control (Fig 3a). There was no significant difference in the expression level of *rbcL* under N-replete compared to the control medium (Fig 3a). Similarly, the *rbcL* gene was significantly ($p < 0.05$) decreased by 1.59-fold under P-limiting condition after 21 days compare to the control (Fig 3b). Under nutrient stress, the cell protein synthesis and photosynthetic rates is affected as chlorophyll a is utilized as an intracellular nitrogen to support the growth of microalgae [13].

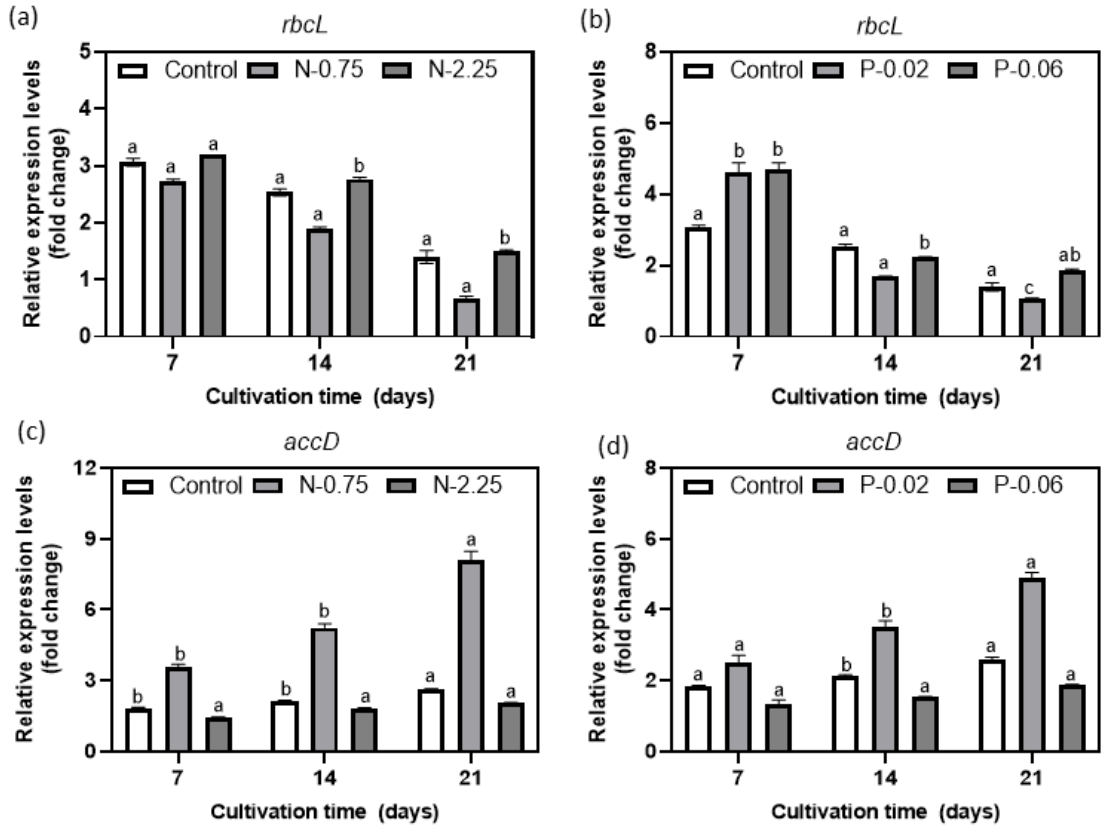


Fig. 3. Mean fold of relative gene expression of *rbcL* gene of *Chlorella* sp. T4 cultured in different N and P concentration. Fold change was relative to the control treatment. (a) *rbcL*- N-treatment, (b) *rbcL*-P-treatment, (c) *accD*- N-treatment and (d) *accD*-P treatment. N-0.75, N-limiting condition; N-2.25, N-replete medium; P-0.02, P-limiting condition and P-0.06, P-replete medium; Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Different letters depict significance difference among the gene expression according to one-way ANOVA at $p < 0.05$. Values show the average of three replicates ±SD.

Fan et al. [14] cultivated *Chlorella pyrenoidosa* under N and P-deficient conditions and reported high expression of *rbcL* gene in the nutrient condition which was two to five times higher than N-deficient condition. Singh et al., [3] also reported 78% and 56% down regulation of *rbcL* gene in N and P stress conditions on *Chlorella sorokiniana*, respectively. The decrease in the expression of *rbcL* gene was also translated by low specific growth rate (Table 2) and decrease of maximum quantum efficiency of PSII (Fig 2) under N-limiting condition in this study. The expression of *rbcL* gene was significantly ($p < 0.05$) increased by 1.12-fold under P-limiting condition compare to the control medium just after 7-day incubation periods. (Fig 3b). Microalgae utilizes phosphorus for the transfer and signal transduction during photosynthesis [44]. Microalgae under nutrient sufficient medium tend to require more fixed carbon cell construction, which then demand for more RuBisCO to sequester the CO_2 in the air.

Acetyl-CoA carboxylase (ACCase) is regarded as rate-limiting enzyme for fatty acid synthesis and it has been overexpressed in different organism to enhance lipid production [6]. A study by Fan et al. [14] suggested a strong involvement of *accD* in triggering lipid accumulation by the cell under nutrient deficient conditions. The present study evaluated the expression of heteromeric ACCase unit (*accD* gene) as a function of different N and P concentrations on lipid synthesis. A significant increase in the expression of *accD* gene was observed under nutrient limiting conditions during the cultivation period as compared to the cells grown in standard BG-11 medium (Fig 3 c & d). In N-limiting condition, 3.11-fold increase of *accD* gene expression was observed after 21 days cultivation compare to the control (Fig 3c). Likewise, a significant ($p < 0.05$) 1.89-fold increase in the expression *accD* gene by was observed under P-limiting condition after 21 days of cultivation compare to the control (Fig 3d). Usually, lower photosynthetic rates cause NADH accumulation inhibiting enzyme citrate synthase so that the acetyl-CoA is blocked from entering TCA cycle. By increasing the acetyl-CoA concentration, ACCase is activated resulting in the enhancement of lipid content in microalgae [6]. This was evidently shown by an increase lipid yield by *Chlorella* sp. T4 under N and P-limiting conditions compare to the control medium (Table 3). During nutrient starvation, cell tends to synthesis lipids as a protective mechanism against stressful condition [45].

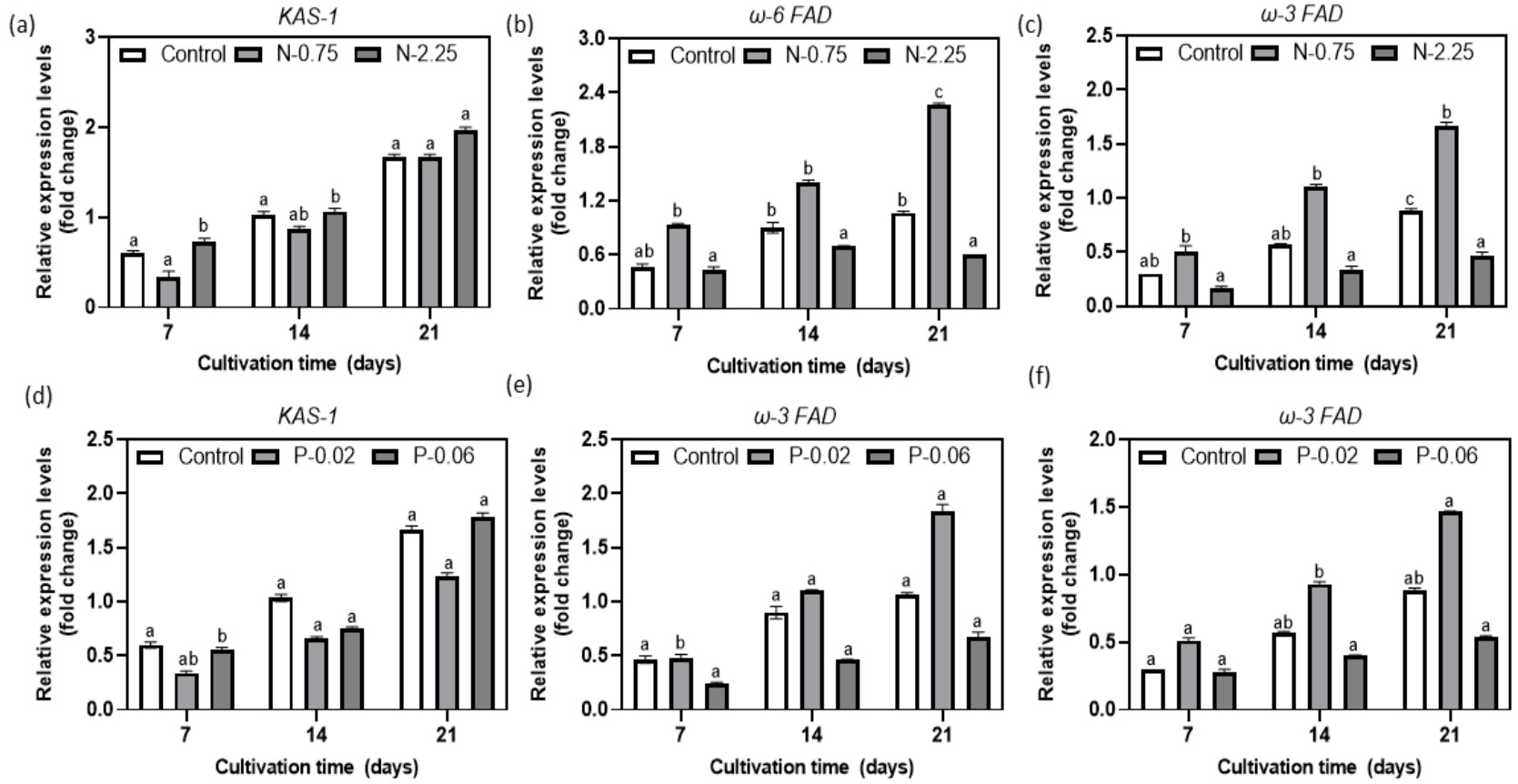
The expression of *accD* gene under N-replete medium was significantly lower compared to the control and N-deficient medium, with 1.3-fold and 2.6-fold increase obtained, respectively at day 21 (Fig 3c). Similarly, the expression of *accD* gene under P-replete medium was significantly lower by 2.95-fold compare to the control at day 21 (Fig 3d). Singh et al. [3], cultivated *Chlorella sorokiniana* under N and P-limiting conditions along with metal stress. They recorded a 3.24-fold and 2.93-fold increase in the expression of *accD* gene at the late log phase compared to the control medium (BG-11). Also, a significant correlation was found between the expression of *accD* gene, growth rate, photosynthetic efficiency, and lipid accumulation. Based on the results obtained in the present study, the expression of *accD* was observed to be higher under nutrient limiting medium. This was attributed by higher amount

of lipid content under nutrient limiting medium despite lower biomass yield compare to N-replete medium.

4.2.5 Effect of culture conditions on the expression of *KAS-1*, *ω*-6 and *ω*-3 desaturase gene of *Chlorella* sp. T4

Another key gene in the process for fatty acid biosynthesis is *KAS-1* which is required for the addition of malonyl-CoA to elongate a 4-carbon fatty acid to 6-, 12- and 16 carbon fatty acid chains [17]. There was no significant difference in the expression level of *KAS*-1 gene under N-deficiency and control medium after 21 days as observed in this study (Fig 4a). The expression of *KAS*-1 gene was significantly ($p < 0.05$) increased under N and P-replete medium by 1.12-fold (Fig 4a) and 1.19-fold (Fig 4d) after 21 days compare to the control, respectively. Usually, microalgae under normal growth condition consume ATP and NADPH produced by the cell though photosynthesis resulting in the formation of ADP and $NADP^+$ that are being available again as acceptor molecules in photosynthesis [46]. This was translated by high biomass (Table 3) and abundance of saturated fatty acid (Table 4) observed in N and P-replete medium which can be attributed to the high expression level of *KAS*-1 gene under these conditions. Aziz et al. [16] cultivated *Chlorella* strain KS-MAS under different P concentration and observed high expression of *KAS*-1 gene under P-replete condition, which is about 3.7 and 4.3-fold higher than the control. They found a strong correlation between the expression of *KAS*-1 and saturated fatty acid and biomass yield. The *KAS*-1 gene is known to catalyze the production of 18-carbon fatty acid from 16-carbon fatty acid in which palmitic and steric acid are the final product of the fatty acid synthesis [47].

The expression of *KAS*-1 gene significantly decreased under N and P-limiting conditions by 1.13- and 1.23-fold after 21 days compare to the control, respectively. This showed a strong correlation with the expression of *KAS*-1 gene with high biomass yield and saturated fatty acid accumulation by *Chlorella* sp. T4 under nutrient replete medium. Microalgae structural lipid is known to have a high content of polyunsaturated fatty acid. Under nutrient starvation, the carbon metabolism is affected and results in cell physiology regulation which increases the cellular demand for the synthesis of membrane phospholipids [46, 48]. Omega-6 desaturase encoded by gene *ω-6 FAD* catalyzes the conversion of oleic acid to linoleic acid. The expression level of *ω*-6 FAD by *Chlorella* sp. T4 was affected by nutrient conditions. The expression of *ω-6 FAD* was significantly ($p < 0.05$) increased under N-limiting condition by 2.09-fold at day 21 compare to the control (Fig 4b). The increase in the expression of *ω-6 FAD* under N-limiting condition was corroborated by high level of linoleic acid ascertain under N-limiting condition. The expression of *ω-6 FAD* gene by *Nannochloropsis oceanica* under N-starvation condition which led to an increase in linoleic acid content.



Control, BG-11 containing N-1.5 g L^{-1} + P-0.04 g L^{-1}. Different letters depict significance difference among the gene expression according to one-way ANOVA at $p < 0.05$. Values show the average of three replicates ±SD

Microalgae requires sufficient *ω-6 FAD* gene expression to convert oleic acid substrate to linoleic acid [44]. The expression of *ω-6 FAD* gene was significantly decreased in N-replete medium by 1.83-fold compare to the control medium (Fig 4b). Kaye et al., [45] reported high expression. The expression of *ω-6 FAD* was significantly increased under P-limiting condition by 1.97-fold after 21 days compare to the control (Fig 4e). The increase in the expression of *ω-6 FAD* under P-limiting condition was corroborated by high level of oleic acid ascertain under N and P-limiting condition (Table 4). Omega-6 desaturase is activated by the availability of oleic acid and α-linoleic acid. This suggests that the function of desaturase enzyme was satisfaction to the demand of membrane phospholipids for synthesis of PUFAs by. The present study demonstrated that nutrient limiting conditions had a significant impact on the expression of *ω-6 FAD* and monounsaturated fatty acids.

Omega-3 desaturase encoded by gene *ω-3 FAD* plays important role in the conversion of linoleic acid to form trienoic fatty acid known as α-linoleic acid [49]. The expression of *ω-3 FAD* gene was significantly ($p < 0.05$) increased by 1.93-fold and 1.65-fold under N and P-limiting condition after 21 days compare to the control, respectively (Fig 5c, f). The expression levels of *ω-3 FAD* gene is strongly associated with the increase in α-linoleic acid level [50], which was corroborated by high levels of α-linoleic acids content that was ascertain under N and P-limiting conditions by *Chlorella* sp. T4 (Table 4). Suga et al., [51], reported overexpression of *ω-3 FAD* gene by *Chlorella vulgaris* in transgenic tobacco plant which resulted to an increase in α-linoleic acids content. The expression of *ω-3 FAD* gene was significantly ($p < 0.05$) decreased under N and P-replete medium by 2-fold and 1.89-fold after 21 days compare to the control, respectively (Fig 5c, f). Nevertheless, the accumulation of α-linoleic acid by *Chlorella* sp. T4 was relatively low to compare to other fatty acid. Omega-3 desaturase gene have been successfully overexpressed to increase the α-linoleic acids content [50, 52]. Polyunsaturated fatty acid are major constituents of biological membrane which plays important role in maintaining the membrane fluidity and are essential for cell growth at low temperatures [49].

4.4. Conclusion

The cultivation of *Chlorella* so. T4 in nutrient replete medium has resulted in increase in the cell growth rate which was attributed by high chlorophyll content and quantum efficiency of photosystem II (Fv/Fm) value. The biomass was significantly decreased under nutrient-stress condition, as corroborated by significantly decrease in the expression of *rbcL* gene. The correlation between the upregulation of *accD* gene and enhanced lipid productivity by N and P limitation was observed indicating a clear impact of nutrient stress in *Chlorella* sp. T4. The level of *KAS-1* gene was upregulated under nutrient replete medium, translated by high level of saturated fatty acid under non-nutrient stress conditions. Furthermore, an increase in the expression level of *ω-6 FAD* and *ω-3 FAD* genes under N and P-limiting medium was observed which corresponded to high levels of monounsaturated and polyunsaturated fatty acid. This provides a clue for future prospective metabolic engineering to make

microalgal biodiesel economically viable. FAMEs produced under nutrient limiting condition were suitable for production of high-quality biodiesel with better oxidative stability and cold flow properties. Future research may focus on the overexpression of these key biosynthetic genes through metabolic engineering for higher yield of neutral lipid with good biodiesel properties.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Chapter 5

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ORIGINAL PAPER

Nutrient Removal from Dairy and Poultry Wastewater with Simultaneous Biomass and Biodiesel Production by *Chlorella* sp. T4 Isolated from a Freshwater Stream in South Africa

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Abstract

Introduction: Huge amounts of wastewater are generated during food process, and usually contain high nutrient concentration. The present study investigated the feasibility of dairy and poultry wastewaters as growth media for *Chlorella* sp. T4 cultivation for concomitant nutrient removal and biomass propagation for biofuel production.
Methods: Microalgae *Chlorella* sp. T4 was cultivated in poultry and dairy wastewater collected at different stage of wastewater treatment to study the growth, physiological respond, nutrient removal efficiency and biochemical composition.
Results: The strains showed phycoremediation potential resulting to high nitrogen and phosphorus removal efficiency in dairy and poultry wastewater ranging from 85–95% and 35–93%, respectively. High biomass yield of 1.28 ± 0.1 g L^{-1} was obtained in poultry wastewater compared to 0.85 ± 0.02 g L^{-1} obtained in dairy wastewater. The biomass contained significant amounts of lipids (16.2–25.7 % Dry wt.), carbohydrates (20.7–33.1 % Dry wt.), and proteins (24.5–34.6 % Dry wt.), regardless of the wastewater type. The fatty acid analysis revealed that palmitic (16:0), oleic (18:1), and linoleic (18:2) acids were the major fatty acids accumulated by *Chlorella* sp. T4 when cultivated in poultry and dairy wastewater. Biodiesel properties of lipids extracted from the cell grown in poultry and dairy wastewater complied with most of the international standards by ASTM D6751 and EN 14214.
Conclusion: The results of this study revealed that *Chlorella* sp. T4 is a potential candidate for dairy and poultry wastewater treatment, with a significant accumulation of lipid, protein and carbohydrates for use in biofuel production.

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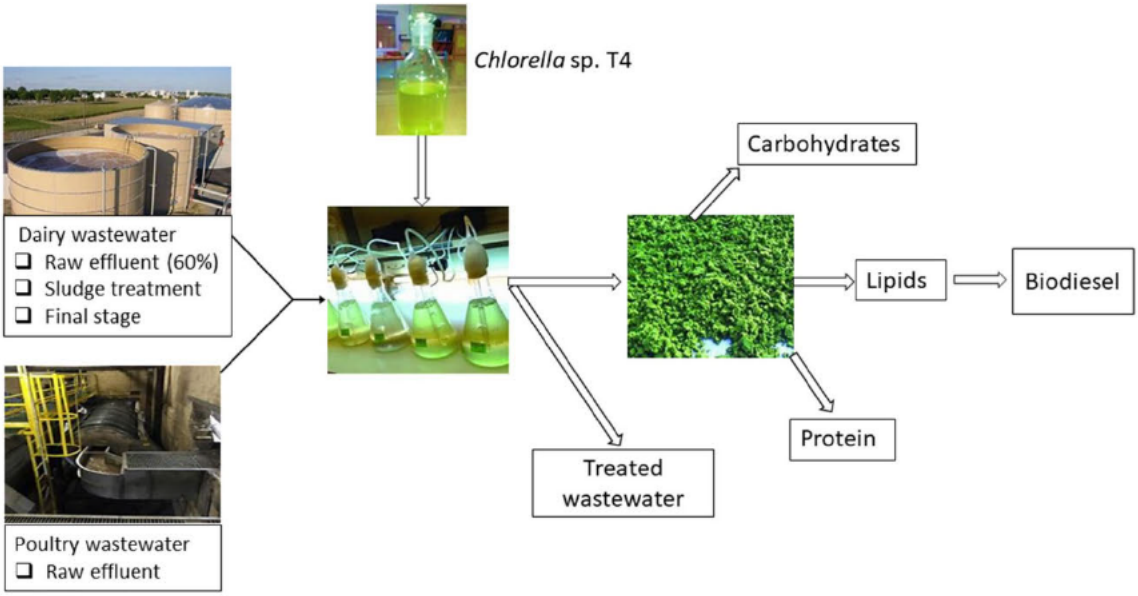
Graphical Abstract



Keywords Dairy wastewater · Poultry wastewater · *Chlorella* sp. T4 · Nutrient removal · Lipid profile

Statement of Novelty

The application of indigenous strain of microalgae in utilizing poultry raw and dairy wastewater for simultaneous removal of nutrient and biomass accumulation for biofuel is underreported. The novelty of the presented study's findings is mainly in the application of a unique and robust autochthonous microalgae strain isolated from local aquatic habitant for its ability to utilize wastewater from poultry and dairy industry containing high amounts of organic compounds such as nitrogen and phosphorus. Our finding shows high nutrient removal rates by the strain with concomitant biomass accumulation. The biomass contained significant amounts of lipids, carbohydrates and protein that can be used for biotechnological application. Further, we confirmed and validated the suitability of the lipids produced by GC-MS for biodiesel production.

Introduction

The growing global population and agricultural sector such as poultry and dairy industry have raised major concerns regarding waste management, as huge amounts of wastewater are produced daily. Wastewater generated from industrial and agricultural sources contain harmful pollutants such as metals, dyes, phenols, detergents, antibiotics, disinfectants, pesticides and nutrients [1]. The discharge of these untreated wastewater from poultry and dairy activities into the receiving water bodies poses harmful and ecotoxicological effects on aquatic life and can cause eutrophication [2]. Therefore, it is necessary to reduce the risk of pollution by treating the wastewater before discharge into the coastal water, rivers, and lakes. Apart from the water crises, depletion of fossil fuel reserves and a continuous increase in the emission of greenhouse gases continue to be of growing concern. Many researchers have devoted extraordinary efforts to search for cheap, renewable and environmentally friendly alternative energy sources to replace fossil fuel-based petroleum [3, 4].

Interestingly, microalgae have been considered as the best option to address the water and energy crisis in an environmental and eco-friendly way. They are unicellular and photosynthetic organisms that are found in diverse aquatic environments [5], and exhibit multiple advantages; viz., they do not compete with crops for arable land, can be cultivated in brackish water and on non-arable land, grow rapidly and accumulate high lipid content [6]. The robustness of microalgae allows them to grow in wastewater while removing nutrients and convert them into biomass. Wastewater is rich in nitrogen, phosphorus compounds and other important macro- and micronutrients which are important elements for microalgae growth [7, 8]. Consequently, the operational cost of microalgae cultivation in

wastewater is low and enhances the economic feasibility of large-scale biodiesel production [2, 9].

The biomass from microalgae has large amounts of lipids in the form of triacylglycerols (TAGs) that can be converted to biodiesel via transesterification. Biodiesel produced from microalgae is reported to be one of the biofuels with characteristics compatible with fossil fuel. Thus, biodiesel can be directly used in an existing diesel engine without any modification. The TAGs are the most suitable feedstock for biodiesel production should have a chain length between C14–C22 and a low level of unsaturation. In wastewater, microalgae have shown the ability to produce a variety of fatty acids that yield biodiesel of good quality which meets European and ASTM standards [10, 11]. Biodiesel produced from microalgae has many advantages as it is considered clean, and a suitable carbon-neutral fuel and a potential fossil fuel alternative.

Proper selection of the type of wastewater and microalgae strain is important for successful and cost-effective simultaneous wastewater treatment via nutrient removal and biomass generation for biodiesel production. It is worth noting that the composition of wastewater varies based on the wastewater type and this can have a detrimental effect on the nutrient removal rates, growth of microalgae and lipid compositions. Some microalgae species can tolerate wastewater with high nutrient concentration, that can be toxic to other microalgal strains. Effluents derived from the dairy and poultry agricultural sectors are rich in organic compounds, such as phosphate and nitrogen [12, 13]. The organic waste contained in these wastewaters needs to be reduced before being discharged to the environment to prevent rapid putrefaction [14]. The nutritional composition of poultry and dairy wastewater makes it suitable for dual microalgal wastewater treatment and biomass propagation [12, 14, 15].

In this study, the feasibility of dairy and poultry wastewater as a growth medium for *Chlorella* sp. T4 cultivation for concomitant nutrient removal and biomass propagation for biofuel production was investigated. The growth and physiological response of *Chlorella* sp. T4 cultivated in the wastewater was studied. The removal rate of nitrogen, phosphorus and chemical oxygen demand during the growth of the microalgae was determined. Lastly, the biochemical composition and fatty acid methyl ester (FAMEs) profiles of the microalgal biomass produced were analyzed. The finding from this study will help to establish the potential of the *Chlorella* sp. T4 strain for nutrient removal from wastewater and the possible application of the propagated biomass for biofuel production.

Materials and Methods

Microalgal Strain and Culture Conditions

The microalga *Chlorella* sp. T4 (Accesion number: KP662697) was isolated from a freshwater stream in KwaZulu-Natal, South Africa [5]. The strain was preserved in BG-11 medium [16], which is composed of (g L^{-1}): $NaNO_3$, 1.5; K_2HPO_4, 0.04; $MgSO_4.7H_2O$, 0.75; $CaCl_2{\cdot}2H_2O$, 0.036; citric acid, 0.006; ferric ammonium citrate, 0.006; EDTA, 0.001; Na_2CO_3, 0.02 and 1 mL of micronutrient or trace metal solution containing (g L^{-1}): H_3BO_3, 2.86; $MnCl_2{\cdot}4H_2O$, 1.81; $ZnSO_4{\cdot}7H_2O$, 0.22; $NaMoO_4.5H_2O$, 0.079; $Co(NO_3)_2{\cdot}6H_2O$, 0.04. The medium was adjusted to a pH of 7 and autoclaved at 121 °C for 15 min. The culture (10 % v/v) was inoculated in 500 mL conical Erlenmeyer flask containing 200 mL of BG-11 medium. An aliquot of tetracycline (0.5 µl mL^{-1}) was added to the growth medium to prevent any bacterial contamination in the microalgal samples. The culture was incubated at 25 °C under cool white fluorescent illumination of 200 µmol m^{-2} s^{-1} with a photoperiod of 12 h: 12 h, light: dark cycle under ambient CO_2. The cultures were hand-shaken two to three times daily to avoid settling and sticking of the culture onto the bottom of the flask.

Wastewater Collection and Pretreatment

The wastewater samples were collected from two different sources namely, dairy and poultry processing industries, all in the KwaZulu-Natal province of South Africa. Raw dairy effluent was collected from the milk processing industry before being sent to the effluent treatment plant. Furthermore, sludge effluent and final effluent was collected from the dairy effluent treatment plant using grab sampling technique. Raw poultry effluent was collected from poultry industry before being discharged using grab sampling technique. The wastewater was generated mainly from the production line, equipment, and floor cleaning operations. Due to variation in wastewater composition with collection time, the poultry and dairy effluent were collected in one batch of 25 L sterile plastic containers. Large solid particles were removed from the wastewater by sedimentation followed by filtration through two layers of wire gauze. The sample was then autoclaved for 15 min at 121 °C and stored at 4 °C until required for analysis.

Physicochemical Characterization of Wastewater

The wastewater samples were characterized for chemical oxygen demand (COD) via the $K_2C_{r2}O_7$ assay using the

spectroquant COD cell test kit (Merck, Darmstadt, Germany) following the manufacturer's instructions. Total nitrogen (TN) and total phosphorus (TP) were determined using the spectroquant total nitrogen and phosphate cell test kits, respectively (Merck, Darmstadt, Germany). Salinity and electrical conductivity (EC) were determined using the CDC 401 probe and HQ40d multimeter (HACH, Loveland, CO, USA) respectively. The pH and temperature of the wastewater were determined by direct measurement using a pH meter (HI2002-01, Hanna Instruments, Woonsocket, RI, USA) and a glass thermometer, respectively. Turbidity was measured by the nephelometric method using a turbidimeter (21000P, HACH, CO, USA).

Cultivation of *Chlorella* sp. T4 in Poultry and Dairy Wastewater

Dairy wastewater effluent was diluted with distilled water to achieve different concentrations, i.e. 20 % (WW-20), 40 % (WW-40), 60 % (WW-60) and 80 % (WW-80) as microalgal strain failed to grow in concentrated dairy wastewater. The ideal concentration for dairy wastewater effluent (60 % dairy effluent), sludge, final and raw poultry effluent were inoculated with 10 % (v/v) of the seed culture. The experiments were set up in 1 L conical Erlenmeyer flasks with a working volume of 500 mL. The cells were harvested by centrifugation at 3024 x g for 10 min at 4 °C and washed with once with sterile deionized water to remove BG-11 medium nutrient residues. The seed culture in sterile distilled water was adjusted to an absorbance of 0.1 at 680 nm using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was then inoculated into the different concentrations of dairy and poultry wastewater. In addition, the control experiment was conducted using BG-11 medium which was treated the same as wastewater samples. The flasks were incubate 25 °C under cool white fluorescent illumination of 200 µmol m^{-2} s^{-1} with a photoperiod of 12 h: 12 h, light: dark cycle supplied with ambient CO_2 for 22 days. The experiment was done in triplicate and results expressed as a mean of the data obtained. During the incubation period, a 10 mL volume of the culture was taken at 2-day interval from each flask in order to monitor the growth rates, physiological responses, TN, TP and COD.

Determination of the Growth Kinetics Parameters

The growth of *Chlorella* sp. T4 was monitored by measuring the optical density of the culture at 680 nm using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at a 2-day interval. Determination of specific growth rate (μ) and the doubling time in the different wastewater concentrations was calculated using Eqs. 1 and 2, respectively [5]:

$$\text{Specific growth rate } \mu(\text{day}^{-1}) = \frac{1}{t} \times \ln\left(\frac{Xm}{Xo}\right) \quad (1)$$

Where Xm and Xo is the optical density at the exponential phase and at the beginning of a batch run, respectively with t (in days) the time duration of the batch run.

$$\text{Doubling time (DT) is defined as DT} = \frac{\mu^{-1}}{\ln 2} \quad (2)$$

For chlorophyll a determination 5 mL volume of the culture was taken in 2-day interval in each flask. The Chlorophyll a was extracted using 90 % methanol at 50 °C in a water bath overnight and centrifuged again for 5 min at 12,096 x g. The absorbance of the solution was measured at 665 and 652 nm using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) and chlorophyll a concentration was calculated using Eq. 3 [17].

$$\text{Chlorophyll a } (\mu\text{g mL}^{-1}) = 16.29 \times A_{665} - 8.45 \times A_{652}. \quad (3)$$

Assessment of Microalgal Physiological Functions Using Pam Fluorometry

The physiological and photosynthetic efficiency of the microalgal cells were studied using a Dual-PAM 100 Chlorophyll fluorescence and P700 photosynthetic analyzer (Heinz Walz GmbH, 91,090 Effeltrich, Germany) analysed with the Dual PAM software (v 1.9). The microalgal cells were dark-adapted for 15 min prior to analysis. The measurements were performed using 10 mm quartz glass cuvette (10 × 10 × 40) containing a micromagnetic stirrer.

The maximum quantum efficiency of PS II was calculated using Eq. (4) as previously described by Gupta et al. [18].

$$F_V / F_m = \frac{Fm - Fo}{Fm} \quad (4)$$

Where F_m, F_o, and Fv represent the maximum, minimum and variable fluorescence, respectively. All the measurements were taken from the dark-adapted samples as all PS II reaction centers are open [19].

Wastewater Analysis and Determination of Pollutants Removal Efficiency

Samples of wastewater undergoing treatment by *Chlorella* sp. T4 were analysed at day zero and day 21 for total nitrogen, total phosphorus, and chemical oxygen demand (COD) as previously described following initial centrifugation for 10 min at 3024 x g. The percentage removal of nutrients was calculated using the following Eq. 5 :

$$\text{Removal efficiency (RE)} = \frac{Ci - Cn}{Ci} \times 100 \quad (5)$$

Where C_1 is the initial nutrient concentration and C_n is the nutrient concentration on day n.

Harvesting and Biochemical Analysis Microalgal Biomass

At the end of the cultivation period, algal biomass was collected by centrifugation at 15,008 x g for 10 min from the cultivation media (poultry, 60 % dairy, sludge dairy, and final dairy wastewater) and analysed for the biochemical contents (total protein, carbohydrates, and lipids). The total protein was extracted and analyzed using a modified method by Daneshvar et al. [1]. Twenty milligrams of freeze-dried microalgal powder was resuspended in 10 mL 0.5 M NaOH and vortexed for 1 min before cell disruption by sonication (Omni Sonic Ruptor 400, Omni International, Kennesaw, Georgia) at an interval of 5 min for 15 min at 50 % power and 60 pulse to break the cells. Thereafter, the solution was incubated in an oven at 100 °C for 2 h and then centrifuged at 4355 x g for 5 min. The total protein concentration of the supernatant was determined according to the Bradford method [20]. One milliliter of the Bradford reagent was added to 1 mL of the crude supernatant and incubated for 5 min before absorbance was measured at 595 nm. The absorbance was converted into protein concentration by using a calibration curve established using bovine serum albumin (BSA) as a standard for this analysis.

The total carbohydrate concentration was determined by the Anthrone reagent method [21]. Briefly, 20 mg of dried microalgal powder was added to 20 mL deionized water and vortexed for 1 min. The suspension was pre-treated by autoclaving at 121 °C for 15 min and 1 mL from the cell lysate was reacted with 4 mL of Anthrone reagent in boiling water for 10 min. The concentration of carbohydrates was determined spectrophotometrically at 620 nm and compared with the glucose standard curve. The carbohydrate content from microalgal biomass was determined using the equation previously explained by Kassim et al. [22].

To extract algal lipids, 5 ml of chloroform and methanol (1:1 v/v) was added to 100 mg of dried microalgal powder. After vortexing for 1 min, the suspension was microwaved (Samsung, CE2877N, S. Korea) at 100 °C for 5 min. The microalgal biomass at the bottom of the tube was separated by centrifugation at 12,096 x g for 10 min and the supernatant was collected into another sterile tube. The extraction procedure was repeated twice with half of the solvents. The solvent was vacuum filtered and evaporated in a Dragon Lab RE 100-pro rotary evaporator (Polychem Supplies, South Africa) at 60 °C under vacuum to remove the remaining solvents. The chloroform phase was collected and evaporated, and the weight of the lipid was calculated gravimetrically [5].

Fatty Acid Profile Analyses

The analysis of fatty acid was based on a modified method previously described by Gumbi et al. [5]. Briefly, the dried lipid sample was subjected to methanolysis with methanol containing 5 % sulphuric acid as a catalyst (30:1 v/v). The reaction was carried out at 60 °C for 4 h in a benchtop Orbital shaking incubator (MRC, London, UK) at 484 x g in the presence of 1 mL hexane as the reaction solvent. From the reaction mixture, a 100 µL aliquot was transferred to a separated test tube and washed twice with a mixture of distilled water and hexane (1:1 v/v) to induce biphasic separation. The organic layer containing fatty acid methyl esters was collected and subsequently analyzed on a gas chromatography-mass spectrometer (Shimadzu Scientific Instruments, Inc., Columbia, MD). The capillary column of the GC-MS was RXI®-5Sil MS Capillary column (30 m x 0.25 mm id x 0.25 µm film thickness, RESTEK) and nitrogen was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was programmed to start at 80 °C and kept on hold for 2 min, while the injector and detector temperatures

Table 1 Physico-chemical and nutritional profile of the poultry and dairy wastewater used in this study. All wastewater samples used undiluted except 60 % dairy

Parameter	Wastewater			
	60 % dairy	Sludge dairy	Final dairy	Poultry
Color	Whitish	Grey	Clear	Brownish
Odor	Unpleasant	Unpleasant	Unpleasant	Unpleasant
Temperature (°C)	29	24	22	25
pH	6.72	8.26	8.26	7.13
COD (mg L^{-1})	4346 ± 1.4	2571 ± 0.7	105 ± 0.7	1435 ± 0.1
Salinity (%)	5.88 ± 0.5	3.7 ± 0.3	0.21 ± 0.9	0.81 ± 1.2
Total Nitrogen (mg L^{-1})	150 ± 0.2	130 ± 0.1	64 ± 0.1	190 ± 0.1
Total Phosphorus (mg L^{-1})	20 ± 0.1	30 ± 0.1	12 ± 0.1	31 ± 0.1
Turbidity (NTU)	1962 ± 7	1210 ± 17	369 ± 13	2077 ± 83

were set at 250 and 230 °C, respectively. The peaks of fatty acids were identified from the NIST Mass Spectral Library and the related amount of fatty acid was calculated from the integrated area percentage from the total amount of fatty acid. The biodiesel properties were estimated using the web version of the BiodieselAnalyzer version 2.2 [23].

Statistical Analyses

The data were analyzed by one-way ANOVA at 95 % confidence limit ($\alpha = 0.05$). All statistical tests were performed using GraphPad Prism Version 7 (trial version) for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). $P < 0.05$ denotes a statistically significant difference. The values were expressed as the mean ± standard deviation.

Results and Discussion

Characterization of Wastewater

The detailed composition of the untreated poultry and dairy (effluent, sludge, and final treatment stage) wastewater is shown in Table 1. The physical parameters like temperature, odor and color of diary (effluent, sludge and final) and poultry wastewater samples were recorded on site during collection. The unpleasant odor of the wastewater may be attributed to the anaerobic decomposition of organic matter present in the water. The color of dairy effluent was whitish, while sludge dairy wastewater was gey in color with large flocs of suspended matter. The final dairy wastewater was clear in colour, while poultry wastewater was brownish in appearance. Wastewater color is one limiting factor for algal growth, as influences the transmittance of light into the culture for photoautrophic microalgae resulting to low biomass yield and intracellular composition by the strain [24]. Dairy effluent had significantly higher COD concentration (4344 ± 0.81 mg L^{-1}) followed by sludge dairy wastewater

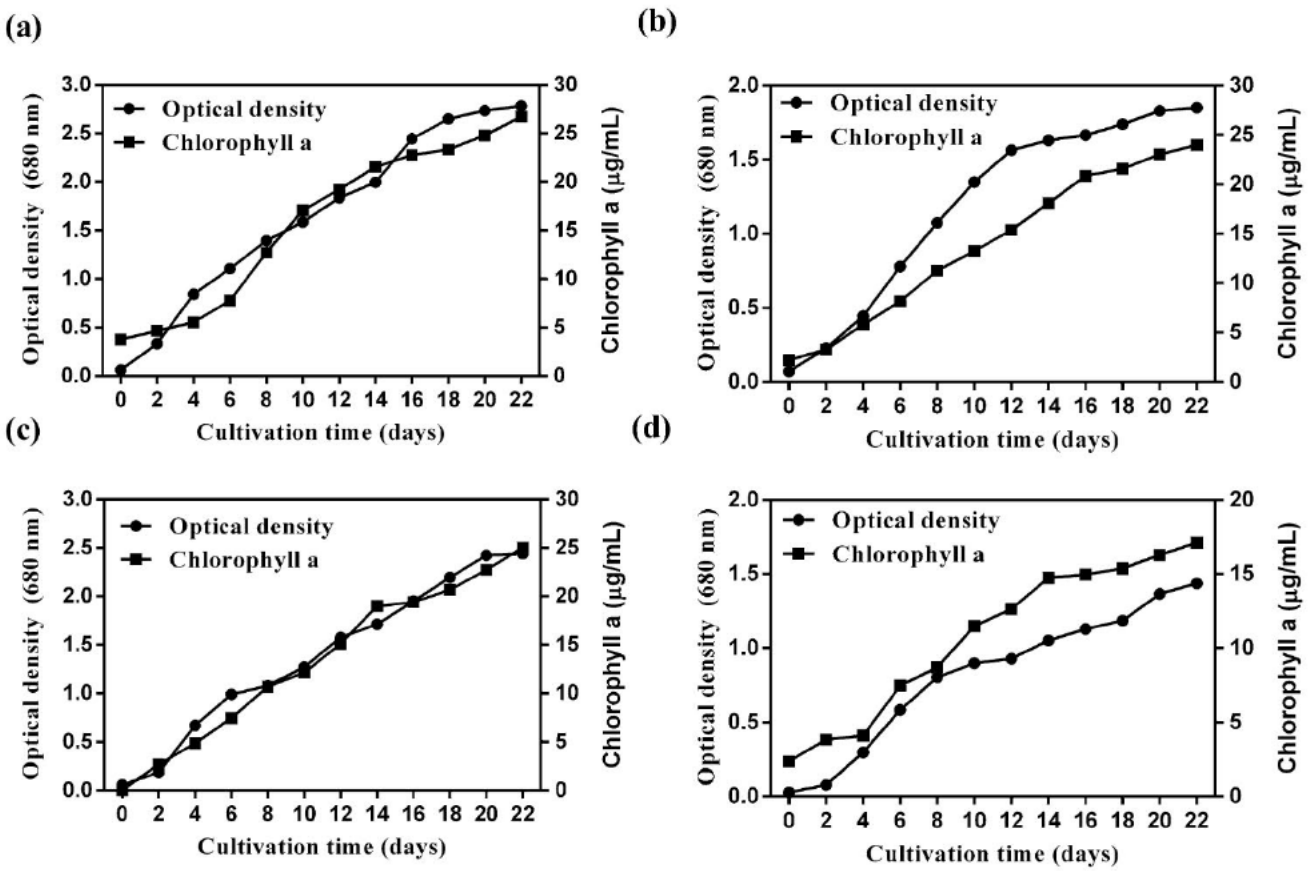


Fig. 1 The growth profile and chlorophyll a of *Chlorella* sp. T4 cultivated in dairy and poultry wastewater for 21 days. (**a**) poultry wastewater, (**b**) 60 % dairy wastewater, (**c**) sludge dairy wastewater and (**d**) final dairy wastewater as measured by the optical density values and chlorophyll a content. Values show the average of three replicates ± SD

Table 2 Specific growth rate, chlorophyll a concentration and biomass yield of *Chlorella* sp. T4 cultivated in poultry and dairy wastewater

Type of wastewater	Growth rate (h^{-1})	Chlorophyll a (μgmL^{-1})	Biomass ($g\ L^{-1}$)	Biomass productivity ($mg\ L^{-1}\ day^{-1}$)
Control	0.071 ± 0.1	23.75 ± 0.31	0.66 ± 0.03	6.30 ± 0.26
Poultry	0.099 ± 0.1	26.71 ± 0.05	1.28 ± 0.01	12.17 ± 0.45
Sludge dairy	0.12 ± 0.2	25 ± 0.66	0.85 ± 0.05	8.11 ± 0.44
Final dairy	0.079 ± 0.1	17.12 ± 0.5	0.37 ± 0.03	3.57 ± 0.26
60 % dairy	0.098 ± 0.1	23.98 ± 0.02	0.74 ± 0.02	7.02 ± 0.20

compared to poultry and final dairy wastewater. The high nutrient concentration in dairy and poultry wastewater, as indicated by total nitrogen and phosphorus values makes it low-cost source of nutrient for microalgae culture. The concentration of nutrients in dairy and poultry wastewater will provide sufficient support for the growth of *Chlorella* sp. T4 without the need for supplementation with exogenous source of nutrient, while represent a sustainable system for removing undesirable compounds from wastewater [25]. The levels of COD, nitrogen and phosphorus concentrations in poultry and dairy wastewater are much higher than the allowable effluent discharge standard set by the Department of Water and Sanitation, South Africa and World Health Organisation (WHO). These results are in agreement with other values previously reported in other studies for dairy and poultry wastewater [8, 12, 26].

Microalgae Growth Kinetics and Biomass Yield

In this study, the feasibility of wastewater from poultry and dairy factories as a cheap and readily available medium for microalgal growth was investigated. The growth of *Chlorella* sp. T4 was monitored by measuring the optical density at 680 nm and the chlorophyll a content. In addition, the biomass yield was determined after 21 days of cultivation. There was no growth observed in undiluted dairy effluent which can be attributed to the presence of suspended solids in wastewater. High turbidity decreases the penetration power of light in the cultivation medium which leads to inhibition of microalgae growth [27, 28]. Excess nutrients in wastewater can have repressive effect on microalgae growth in wastewater [29]. Therefore, the wastewater was diluted to different concentrations (WW-20, WW-40, WW-60, and WW-80 %) to find an ideal concentration for maximum microalgal growth and biomass yield. This approach has been previously done by other researchers to optimise microalgal growth in wastewater [1, 18]. The strain *Chlorella* sp. T4 demonstrated high growth and biomass accumulation in WW-60 % which was then used for further investigation (Fig. 1a). The low specific growth rates in other wastewater concentrations lower and higher than 60 % could be due to insufficient concentration of nutrients and toxicity of ammonium, respectively. Poultry (Fig. 1b), sludge dairy (Fig. 1c) and final dairy (Fig. 1d) and poultry wastewater provided well favourable growth conditions since the dilution of the effluent was not a prerequisite.

The strain *Chlorella* sp. T4 was able to utilize poultry and dairy wastewater achieving highest specific growth rates of 0.12 h^{-1} in sludge dairy wastewater which was 1.71-folds higher than the control medium (Table 2). However, there was a significant difference shown by the strain in poultry and 60% dairy effluent. The lowest specific growth rate of 0.08 h^{-1} observed in final dairy wastewater may be due to the low nutrient level compared to other conditions. The total nitrogen and total phosphorus level in the final dairy wastewater was approximately 3-fold and 2.6-fold less respectively, compared to the values recorded for the poultry wastewater (Table 1). Also, the final dairy wastewater was more alkaline compared to the poultry wastewater. Daneshavar et al. [1] reported high specific growth rates of 1.08 d^{-1} by *Chlorella* sp. cultivated in dairy wastewater that had nitrogen and phosphorus concentration of 86 ± 2.2 and 8.75 ± 0.1 %, respectively. Another study by Oliveria et al., [26] demonstrated the feasibility of *Scenedesmus obliquus* to utilize raw poultry and poultry flocculated wastewater with nitrogen concentration of 122.7 and 259.3 mg L^{-1} and phosphorus concentration of 27.9 and 23.4 mg L^{-1}, respectively. Also, other studies were reported on the feasibility of

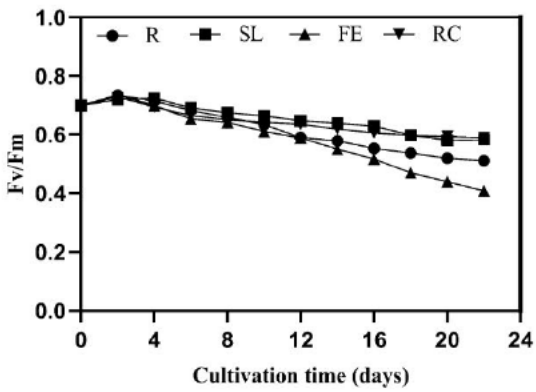


Fig. 2 The maximum quantum yield of PSII (Fv/Fm) of *Chlorella* sp. T4 cultivated in dairy and poultry wastewater for 21 days. Curves indicate, control medium (BG-11), 60 % dairy wastewater (R), sludge dairy wastewater (SL), final dairy wastewater (FE), poultry effluent (PW). Values show the average of three replicates ± SD

using wastewater as a cheap medium for microalgae growth [8, 26]. *Chlorella* sp. T4 accumulated high biomass in raw poultry (1.28 ± 0.1 g L^{-1}) and sludge dairy (0.85 ± 0.02 g L^{-1}) wastewater which was 1.94-folds and 1.29-folds higher than the control medium, respectively. Lowest biomass yield (0.37 ± 0.03 g L^{-1}) obtained in final diary wastewater was 3.5-fold less compared to the yield in poultry wastewater (Table 2).

Similarly, high biomass productivity of 12.2 ± 0.05 mg L^{-1} day^{-1} was recorded in raw poultry effluent, followed by sludge dairy wastewater (8.11 ± 0.44 mg L^{-1} day^{-1}) with the least value (3.57 ± 0.26 mg L^{-1} day^{-1}) obtained in final diary wastewater. High biomass accumulation in this wastewater can be related to high specific growth and concentration of nutrients in the wastewater [30]. Furthermore, 60% dairy wastewater had high biomass productivity of 7.02 ± 0.20 mg L^{-1} day^{-1} which is 1.73-folds lower than poultry effluent. Chlorophyll a concentration was also measured to identify the direct relationship between microalgal cell growth and chlorophyll a concentration. As shown in Fig. 1, the increase in chlorophyll a concentration follow a similar pattern as the microalgae growth curve for all culture conditions tested. High concentration of chlorophyll a content was obtained in raw poultry (26.71 ± 0.05 µg mL^{-1}) and sludge dairy wastewater (25 ± 0.66 µg mL^{-1}). The least value of 17.12 ± 0.5 µg mL^{-1} was obtained in final diary wastewater which was 1.39-folds lower than the control medium. Damm et al. [31], reported that high availability of initial nitrate in the growth media positively contribute to high chlorophyll a accumulation and growth rates, as observed in this study. The low concentration of chlorophyll a obtained in final dairy wastewater can be related to the low concentration of microalgal cells in the culture medium (Table 2). Based on the findings of this study, suitably diluted raw poultry and sludge dairy wastewater can be used as suitable media for the growth of *Chlorella* sp. T4 to achieve maximum biomass yield.

Photosynthetic Quantum Yield (Fv/Fm) of Microagal Cells in Wastewater

The composition of wastewater varies depending on the wastewater type, thus, it is important to investigate the stress imposed on the microalgal culture physiology in order to determine their sustainable application [18]. The suitability of wastewater as a substrate for *Chlorella* sp. T4 cultivation was observed by calculating the quantum efficiency (Fv/Fm) of reaction centres in PS-II of chlorophyll by non-invasive fluorescence measurement. Normally, the Fv/Fm value of < 0.5 indicates that the culture is undergoing physiological stress [19]. The physiological changes of *Chlorella* sp. T4 during growth in poultry and dairy wastewater is represented in Fig. 2. A low Fv/Fm value of 0.41 ± 0.02 recorded in the final dairy wastewater compare to 0.472 ± 0.26 in the control medium, might have been caused by nutrient limitation in the cultivation media since the initial nutrient level in the wastewater was generally lower compared to the other wastewater types. However, the final dairy wastewater is suitable for biomass production and lipids accumulation. Previous studies have also confirmed that *Chlorella* sp. have great potential for maintaining high lipid accumulation [8, 18, 32].

For poultry wastewater, 60% dairy effluent and sludge dairy effluent the Fv/Fm value above 0.5 was observed after 21 days of cultivation, indicative of healthy culture in these growth media. The decrease in Fv/Fm value observed for all the cultures just after 4 days of cultivation can be explained by the need for the *Chlorella* sp. T4 to acclimatize in the wastewater. These findings provide insight into the suitability of poultry and dairy wastewater as a cheap medium for the cultivation of microalgae for biomass accumulation. The potential of poultry and sludge dairy wastewater as a suitable cultivation medium for *Chlorella* sp. T4 was evident by high growth rates and biomass accumulation (Table 2). Thus, this could eliminate the need for the use of expensive clean water and synthetic medium for the cultivation of microalgae.

Table 3 Nutrient and COD level in poultry and dairy wastewater before and after 21-day cultivation of *Chlorella* sp. T4

Wastewater sample	COD (mgL^{-1})			TN (mgL^{-1})			TP (mgL^{-1})		
	Initial	Final	Removal efficiency	Initial	Final	Removal efficiency	Initial	Final	Removal efficiency
Poultry	1435 ± 1.41	458 ± 0.53	66.2 ± 0.34	190 ± 0.78	11 ± 0.28	94.5 ± 0.15	30 ± 0.17	15 ± 0.21	50.9 ± 0.69
Sludge dairy	2575 ± 0.71	580 ± 0.34	77.4 ± 0.66	130 ± 0.14	22 ± 0.71	91.5 ± 0.27	30 ± 0.71	2 ± 0.49	92 ± 1.63
Final dairy	105 ± 0.57	19 ± 0.80	81.9 ± 0.21	64 ± 0.71	11 ± 0.35	88.8 ± 0.45	12 ± 0.41	3 ± 0.14	79.1 ± 1.10
60% dairy	4344 ± 0.81	1752 ± 0.22	59.7 ± 0.31	150 ± 0.40	7 ± 0.28	85.4 ± 0.35	20 ± 1.20	13 ± 0.35	34.7 0.73

Table 4 Comparative biochemical composition of *Chlorella* sp. T4 biomass recovered after cultivation in poultry and dairy (raw, sludge and final) wastewater

Growth medium	Total lipid (% Dry wt.)	Total carbohydrates (% Dry wt.)	Total protein (% Dry wt.)
Control	22.72 ± 0.4	31.41 ± 0.3	26.1 ± 0.2
Poultry	21.7 ± 0.5	22.4 ± 0.1	34.6 ± 0.6
Final dairy	25.7 ± 0.3	33.3 ± 0.7	24.5 ± 0.3
Sludge dairy	23.6 ± 0.2	25.3 ± 0.6	27 ± 0.8
60% dairy	16.2 ± 0.1	20.7 ± 0.7	32.7 ± 0.4

Nutrient Removal from Wastewater

The *Chlorella* sp. T4 strain investigated in this study showed exceptionally good nitrogen removal efficiency in all the wastewater used for the phycoremediation experiment (Table 3). The high nitrogen removal efficiency was achieved in poultry wastewater (94%), and was closely followed by sludge dairy wastewater (92%). In all four wastewater types, an increase in the total nitrogen removal efficiency with time was observed. The strain showed high removal of nitrogen final dairy wastewater which was 1.04 folds than 60% dairy wastewater. Removal of nutrients by the microalgae has been reported to take place between 3 and 26 days of cultivation [33]. In this study, the total phosphorus reduction efficiency in poultry and dairy wastewater ranged between 35 and 92% (Table 3). Total phosphorus removal rates were highest in sludge dairy wastewater followed by final dairy wastewater. The observed high phosphorus removal by *Chlorella* sp. T4 is promising for its possible application in wastewater treatment since most treatment plants show less efficiency in removing phosphorus [34].

Kothari et al. [35] reported the removal efficiency of about 60–80% of nitrogen and 80–85% of phosphorus from wastewater by *Chlorella pyrenoidosa*. Moreover, Ferriera et al. [12] investigated the removal of nitrogen and phosphorus in poultry, swine, cattle, brewing, dairy and urban wastewater by *Scenedesmus oblique*. They achieved a removal range of 95–100% for nitrogen and 63–99% for phosphorus and 48–70% for COD. Compared to nitrogen, the process for phosphorus removal is more complex in that it is utilized in a form of orthophosphate for assimilation by microorganisms. Besides cell growth and nucleic acid synthesis, microalgae utilise phosphorus also for the synthesis of important value-added products such as astaxanthin [36]. The current study demonstrates the potential of using poultry and dairy wastewater for algal cultivation with efficient nutrient removal and biomass accumulation that can be used for biodiesel production.

Another important parameter in wastewater treatment is COD which is the indicator of the overall concentration of dissolved and suspended organic matter in the wastewater. The COD removal by the strain *Chlorella* sp. T4 ranges from 59.67 to 81.90% in the dairy (60% dairy, sludge, and final) and poultry wastewater (Table 3). In wastewater, COD is removed through the oxidation of organic matter by bacteria, utilizing the oxygen supply provided by microalgae through photosynthesis [12]. Other researchers have observed comparable COD removal in different wastewaters by microalgae [18, 37]. The lowest COD removal efficiency of 59.67% observed in 60% dairy wastewater can be due to a high initial concentration in this culture medium.

Biochemical Profiles of the Algal Biomass

Microalgae are highly efficient at collecting solar energy and transforming it into cellular biomass composed of protein, carbohydrates, and lipids, estimated to be about 60–85% of microalgal dry weight [38]. Biochemical composition of *Chlorella* sp. T4 derived from four different culture media

Table 5 Fatty acid profile (%) of oil extracted from *Chlorella* sp. T4 following cultivation in the different wastewater

FAMEs	Control	Final diary	60% dairy	Sludge dairy	Poultry
Myristic acid (C14:0)	5.15	9.5	3.27	6.13	4.27
Palmitic acid (C16:0)	30.06	35.8	33.8	36.8	37.6
Palmitoleic acid (C16:1)	3.07	1.13	2.21	1.22	1.99
Stearic acid (C18:0)	2.87	1.61	1.41	1.39	11.92
Oleic acid (18:1)	33.46	30.4	36.5	36.6	24.1
Linoleic acid (C18:2)	20.16	19.1	20.1	14.90	17.21
Arachidic acid (20:0)	2.31	1.5	1.55	2.0	0.56
Behenic acid (C22:0)	1.05	1.0	0.63	0.57	2.34
Lignoceric acid (C24:0)	1.82	nd	0.48	0.36	nd
Saturated	46.6	49.4	40.6	46.9	56.7
Monounsaturated	37.14	31.5	38.7	37.8	26.1
Polyunsaturated	19.1	19.7	20.1	14.9	17.2

nd non-detectable level

were found to vary depending on the composition of the culture medium (Table 4). The total lipid in poultry and dairy wastewater after a cultivation period of 21 days varied from 16.2 to 25.7 %, which is within the range obtained in previous studies [1, 39]. There was variation in the lipid content yield by *Chlorella* sp. T4 due to the nutrient composition of each wastewater used as culture media. As compared to the BG-11 medium, the concentration of lipid increased in final dairy wastewater and sludge diary wastewater to 25.7 and 23.6 %, respectively. Microalgae have been reported to accumulate lipids in wastewater with low nutrient concentration as they undergo nutrient starvation [18, 40]. This was evident by low Fv/Fm value by the strain in the final and sludge dairy wastewater causing cell physiological stress due to nutrient starvation (Fig. 2). These findings confirmed that poultry wastewater and dairy wastewater can be used as a growth medium for sustainable propagation of microalgae for biodiesel production.

Carbohydrates act as a structural component in the cell wall and as storage components inside the microalgal cell [41]. In this study, the carbohydrate content ranged from 22.4 to 33.3 % under poultry and dairy wastewater cultivation (Table 4). This range was found to be in agreement with other reports [4, 12]. The carbohydrates yield was increased to 33.3 % in final daily wastewater compare to the control medium (31.4 %). This was followed by 25.3 % obtained in sludge dairy wastewater which was 1.24-folds lower than the control. The exhaustion of nitrogen concentration in the growth medium triggered the accumulation of carbohydrates in the microalgal cells [42]. Ferreira et al. [12], reported a carbohydrate content of 14 % in dairy wastewater which was lower compared to our findings. For poultry wastewater, they reported higher carbohydrate yield of 36.2 % which was higher than the values obtained in the present study. The high carbohydrate yield obtained in this study suggest that the microalgal biomass propagated in poultry and dairy wastewater may have higher potential as a substrate for bioethanol production.

For the four studied wastewater types, there was a predominance of protein over lipids and carbohydrates in the biomass composition. A significant increase in the protein concentration by *Chlorella* sp. T4 compare to the control medium was observed in this study. The high protein content of 34.6 and 32.7 % were found in 60 % dairy and poultry wastewater, respectively (Table 4). This may be due to the high nitrogen content present in this wastewater which resulted in high biomass yield. The presence of high available nitrogen in the culture medium contributes to high protein accumulation in microalgae [12, 39]. Low protein content yield (24.5 %) was found in final dairy wastewater. The algal strain *Chlorella* sp. T4 produced high protein content which was found to be higher than the amount in common several vegetable feeds [4, 39].

Fatty Acid Methyl Ester Profile

The fatty acid composition of *Chlorella* sp. T4 grown in poultry and dairy wastewater was analysed by GC-MS. The results showed that *Chlorella* sp. T4 mainly produced high amounts of palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) for all the wastewater types (Table 5). Previous studies reported a similar prevalence of the above-mentioned fatty acid [1, 40]. The variation in the composition of wastewater was shown to influence the fatty acid composition in microalgae [6]. The fatty acid chain length ranging from C16 to C18 are considered suitable for biodiesel production. In view of biodiesel properties, high abundance of palmitic acid and oleic acid gives good oxidative

Table 6 Biodiesel properties of *Chlorella* sp. T4 cultivated in dairy and poultry wastewater compared to ASTM D6751 and EN 14214 specification

Biodiesel property (Units)	Standard fuel parameters		After transesterification				
	ASTM D6751	EN 14214	Control	60 % dairy	Sludge dairy	Final dairy	Poultry
Iodine value ($gI_2100\ g^{-1}$)	–	120 (max)	59.34	63.19	76.61	57.13	49.38
Saponification value (mg KOH)	–	–	186.19	207.19	210.96	208.30	206.43
Cetane number	47 (min)	51.0 (min)	62.26	58.43	54.93	59.65	61.63
Degree of unsaturation (% wt)	–	–	61.55	69.81	82.01	63.25	54.63
Long-chain saturation factor (% wt)	–	–	9.66	8.09	6.19	7.95	12.45
High heating value ($MJ\ kg^{-1}$)	–	–	35.48	39.40	40.15	39.38	39.46
Cold filter plugging properties (°C)	–	≤5/≤-20	13.87	8.94	2.97	8.50	22.64
Kinematic viscosity ($mm^2\ s^{-1}$)	1.9-6.0	3.5-5.0	1.22	1.35	1.36	1.35	1.39
Density ($g\ cm^{-3}$)	–	0.86–0.90	0.78	0.87	0.88	0.87	0.87
Oxidative stability (h)	3 (min)	≥6	0	0	0	0	0
Linolenic acid (%)	–	12	20.16	14.09	10.51	9.07	5.86

ASTM D6751, American biodiesel standards; EN 14214, European biodiesel standard

stability and better low temperature performance [6]. A significant amount of palmitic acid (C18:1), ranging from 33.8 to 37.6 % was obtained for the four-wastewater used in this study. The monounsaturated fatty acid proportion was slightly higher in 60 % dairy, sludge dairy and final dairy effluent with relative index ranging between 31.5 and 38.7 %. Among the monounsaturated fatty acids, oleic acid (C18:1) was the most dominant with sludge dairy having the highest relative index of 36.6 % which was 1.2-folds higher than control. The biodiesel with a higher content of monounsaturated fatty acid such as oleic acid (C8:1) has higher distillation temperature [10]. Dominant polyunsaturated fatty acid was linoleic acid (C18:2) with the relative index ranging from 14.90 to 20.1 %. Minor component of arachidic acid (C20:0) and lignoceric acid (C24:0) was also detected. Linoleic acid is an important (Omega 6) polyunsaturated fatty acids, and are of therapeutic and nutritional importance for infant development with an estimated industrial worth of 11 billion [7]. The insignificant variations in the fatty acid profile of *Chlorella* sp. T4 in control medium and wastewater revealed that dairy and poultry wastewater can be used as growth medium and preserve the diversity of microalgae fatty acids.

Furthermore, the biodiesel properties of *Chlorella* sp. T4 is presented in Table 6, and was compared with ASTM D6751 (American Society for Testing and Materials) and EN 14214 (European norms, European Committee for Standardization) vehicular fuel standard. The parameters mainly considered for determining the biodiesel quality are cetane number (CN), saponification value (SV), iodine value (IV), degree of unsaturation (DU), oxidative stability (OS), and kinetic viscosity (Kv) [43]. The specified minimum value of CN by ASTM D6751 is 47 min, while EN 14214 is 51 min. The CN value in this study for all four wastewaters was found to be higher than the specified limits of fuel standard. Higher CN value in biodiesel is an indication of high combustion efficiency and ignition delay time with low nitrous oxide emission [11, 44]. The low level of polyunsaturated fatty acid compared to saturated fatty acid in the present study resulted in superior CN value. The CN value depends on the content of saturated and monounsaturated fatty acid content [11].

The predicted Kv value determined in this study is not in compliance with fuel standard, as it was lower than the specified range for all the tested conditions (Table 6). Low Kv value causes inadequate fuel supply to the injector at different operating temperatures. This parameter is important for defining cold flow properties (CFPP) which determine the suitability of biodiesel operations in low temperatures. From the cultivation conditions, only sludge dairy wastewater had a CFPP value of 2.97 which was within the EN 14214 specified standard. Another important parameter in fuel is oxidative stability which is the indication of high combustion efficiency for fuel. The fuel produced under these conditions displayed poor oxidative stability due to an imbalance in degree of unsaturation of FAMEs produced by *Chlorella* sp. T4. Biodiesel produced under this condition will not be stable for long-term storage [44]. In the present study, the IV of all the culture conditions was in accordance with the biodiesel standard due to the moderate amount of unsaturated fatty acid. Usually, oil with higher IV value tends to polymerize and results in engine deposition [11].

Conclusions

The present study investigated the feasibility of *Chlorella* sp. T4 in removing nutrients and to accumulate biomass for biofuel production when cultivated in poultry and dairy (60 % raw, sludge and final) wastewater. The increased specific growth, photosynthetic parameters and biomass yield show the adaptability of the strain to utilize poultry and dairy wastewater as the cheap growth medium. *Chlorella* sp. T4 demonstrated high nutrient removal efficiency, with the highest nitrogen (94.5 %) and phosphorus (92 %) removal efficiency obtained in poultry and sludge diary wastewater, respectively. This was attributed to high biomass accumulation ascertain in poultry and sludge dairy wastewater. Furthermore, high COD removal efficiency in final dairy wastewater (81.9 %) showed the potential use of *Chlorella* sp. T4 in phycoremediation process to reduce pollutants levels in dairy and poultry wastewater, and prevent eutrophication. The biomass produced in wastewater was rich in lipids, carbohydrates and proteins as good feedstock for value-added product production. The fatty acid profile produced by *Chlorella* sp. T4 was completely dependent on the wastewater type used for cultivation. The FAMEs profile of the strain, especially when cultivated in the final dairy wastewater complied with most of the guidelines specified in the ASTM D6751 and EN 14214 standards. Findings of this study showed that *Chlorella* sp. T4 could be used to reduce the pollutant load in poultry and dairy wastewater with concomitant biomass propagation for biofuel production. However, dairy wastewater effluent needed to be diluted to 60 % due to the extremely high nitrogen and phosphorus concentration in the raw wastewater, which are toxic to microalgae. Future studies could investigate the suitability of the sugars extracted from the biomass of *Chlorella* sp. T4 for bioethanol production.

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Author contributions S.G., T.M. and A.O. conceived and designed the project; S.G. and A.O. designed the experiments; S.G. performed

the experiments; A.O. contributed reagents and materials; S.G., T.M. and A.O. wrote the manuscript; all the authors have read and approved the manuscript.

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Declarations

Conflict of interest All the authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Chapter 6

Co-production of bioethanol and biodiesel using microalgae *Chlorella* sp. T4 cultivated in diary wastewater

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Abstract: Biomass from microalgae consists of high neutral lipids and carbohydrates that can be converted to biodiesel and bioethanol, thus making them promising feedstock for bioenergy production. This study investigated concomitant production of bioethanol and biodiesel by an indigenous microalgal strain following a biorefinery approach using different pretreatment and extraction techniques. Lipids were efficiently recovered using microwave pretreatment and a mixture of chloroform-methanol (2:1, v/v) as the extraction solvents. The maximum sugar recovery of 2.14 g L^{-1} was obtained using 1% (v/v) of sulfuric acid at 120 °C for 30 min. The hydrolysis produced ethanol yield of 0.81 g L^{-1} following fermentation with *Saccharomyces cerevisiae*. The lipid recovery from the residual biomass was efficient and converted into fatty acid methyl esters as biodiesel products using acid catalyzed transesterification reaction. The biodiesel produced from the residual biomass meet most of the standards specified by ASTM D6751 and EN 14214. This study demonstrated the potential of *Chlorella* sp. T4 as suitable feedstock for simultaneous production of bioethanol and biodiesel. This is very promising for future improvement of the economics of microalgae biorefinery approach.

Keywords: Biorefinery, Biodiesel, Bioethanol, *Chlorella* sp. T4, Pretreatment

6.1. Introduction

The rapid increase in the global population has resulted in high energy demand. With a projected population in excess of 9 billion by 2050, demand for energy is expected to be on the increase. Excessive consumption of fossil fuel has damaging impact on the environment due to emission of greenhouse gasses (Xu et al., 2019), thus search for alternate and cleaner energy source is on the rise. Traditionally, food crops such as sugarcane and corn have been used as alternative feedstocks for fuel production. However, ethical issue involving food securing, geopolitical and socio-economic concerns makes it unsustainable alternative to replace fossil fuel-based petroleum (Chng et al., 2017, Popa et al., 2018). Microalgae have been proposed as potential feedstock for biofuels production as they do not compete with human food and can grow under harsh conditions in arid land (Ashokkumar et al., 2015). Microalgae have superior photosynthetic efficiency with short generation cycle and high CO_2 fixing efficiency which is 10-fold higher than that of crop plants (Mutanda et al., 2011, Odjadjare et al., 2017). Researchers are still looking for solution to fix the hurdles in the upstream and downstream processing of the biofuel production. *Chlorella* sp. is a well-known microalgae for its ability to utilize different types of wastewater and accumulate both lipid and starch (Daneshvar et al., 2019, Ferriera et al., 2018).

In recent years, biorefinery concept has attracted a lot of attention as it involves the use of the whole cell components to produce various products to enhance the economically competitiveness of microalgae-based technology Xu et al. 2019, Karemore et al., 2016). Microalgae-derived products are very satisfactory for biorefinery concepts. Furthermore, microalgae stores significant amounts of neutral lipids and carbohydrates composed mainly of starch and cellulose polysaccharides suitable for biofuel production (Xu et al., 2019, Wang et al., 2014). Bioethanol production from microalgae has many advantages over first and second-generation biofuel as algal cell lack lignin content which makes it easy to disrupt compare to lignocellulosic material (Phwan et al., 2019). The residual biomass after lipid and sugar extraction contains significant amounts of lipids and carbohydrates that can be extracted for biofuel production (Chng et al., 2017). Adopting a biorefinery approach, Karemore et al. (2016) recovered fermentable sugar yield of 86.5 ± 2.6% from the supernatant and 74.1 ± 1.8% lipid from *Chlorococcum* pellet after sugar extraction. After transesterification, they obtained FAMEs conversion of 94.7 ± 2.5% and ethanol concentration of 4.1 ± 0.2 g L^{-1}. Apart from biofuel production, microalgae have been used in CO_2 sequestration, wastewater reclamation and production of various commercially important bioproducts (Seo et al., 2015).

Although for the biorefinery process to be economical, attention needs to be given to downstream processes to improve their complexity and the cost for biofuel production from microalgae (Bwapwa et al., 2019). Harvesting of algal cells is energy-intensive and expensive due to lack of a cost-effective harvesting technology for easy separation of the biomass from broth culture. Before extraction, pretreatment of algal biomass is required to enhance lipid and carbohydrates extraction efficiency, but

whether the process is energy- and cost-efficient needs to be considered for efficient biofuel production. Pretreatment of algal biomass by physical, chemical and enzymatic disruption techniques enhances the lipid and carbohydrates extraction efficiency from the biomass (Dave et al., 2019). After cell disruption, carbohydrates are extracted from the biomass either by chemical, acid or enzymatic hydrolysis. Acid hydrolysis is a frequently used method as it allows for depolymerization of the intracellular starch and structural polysaccharide into their monomers, which are further quantified (Souza et al. 2017). The hydrolysis conditions (acid, temperature, and time) must be kept at optimum to ensure complete hydrolysis of the polysaccharides and avoid excessive degradation of monosaccharides (Schulze et al. 2017).

The key practical barrier for the algal biofuel industry depends on achieving a net positive cost and energy balance for utilizing microalgae as the feedstock. Hence, it is necessary to improve the economics and sustainability of the biofuel production process by adopting a biorefinery approach, optimization of downstream processes, and utilizing wastewater as a cheap growth medium for biomass propagation. Numerous studies have reported on biodiesel and bioethanol production from microalgae. Yet, there are gaps in literature about sequential biodiesel and bioethanol production from microalgae using nutrient rich wastewater as a cheap medium for microalgae cultivation. Therefore, the aim of this study was to evaluate the systematic downstream process for efficient pretreatment and extraction of lipids and sugars from *Chlorella* sp. T4 cultivated in diary wastewater. Furthermore, integrated approach for bioethanol and biodiesel production was adopted to develop a competitive microalgal based biorefinery model.

6.2. Materials and method

6.2.1 Organism and culture conditions

Chlorella sp. T4 obtained from the previous study (Gumbi et al., 2017) was maintained and grown in BG-11 medium under continuous illumination of 200 μmol $m^{-2}s^{-1}$ with a photoperiod of 12h: 12h, light: dark cycle under ambient CO_2. The organism was isolated from freshwater stream, identified and the 16S rRNA gene sequences deposited in the GenBank with the accession number: KP662697. To ensure the purity of the culture, regular subculturing and microscopic analysis was carried out. Dairy wastewater was used for biomass propagation. Large solid particles were first removed from the wastewater by sedimentation followed by filtration through a two layer of wire gauze. The wastewater samples were then autoclaved for 15 min at 121 °C, and stored at 4 °C until required for analysis. The wastewater sample was characterized for physiochemical parameters listed in Table 1.

Table 1. Physico-chemical and nutritional profile of sludge dairy wastewater used in this study.

Parameter	Sludge dairy
Colour	Grey
Odour	Unpleasant
Temperature (°C)	24
pH	8.26
COD (mg L^{-1})	2571 ± 0.7
Salinity (%)	3.7 ± 0.3
Total Nitrogen (mg L^{-1})	130 ± 0.1
Total Phosphorus (mg L^{-1})	30 ± 0.1
Turbidity (NTU)	1210 ± 17

The experiments were set up in 5 litre conical Erlenmeyer flasks with 2 L working volume of the wastewater. The seed culture in sterile distilled water was adjusted to an absorbance of 0.1 at 680 nm using a Cary 60 UV-Vis Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA), and 10% of the seed culture was then inoculated into the wastewater. The experiment was done in triplicate and results expressed as a mean of the data obtained. The culture was incubated at 25 °C under cool white, fluorescent illumination of 200 μmol $m^{-2}s^{-1}$ with a photoperiod of 12h: 12h, light: dark cycle under ambient CO_2, with shaking at 120 rpm. The 21 days grown cell suspension was harvested by sedimentation for 12 h and the harvested biomass was centrifuged (Beckman Coulter Avanti J-25i) at 5000 rpm for 10 min to removed remaining solvent and freeze-dried overnight.

6.2.2 Optimization of different pretreatment and extraction techniques for high lipids and sugar yield

One gram of freeze-dried biomass was powdered using mortar and pestle and mixed with 20 mL extraction solvent (chloroform-methanol (1:1 v/v)) in 125 mL flaks. The mixture was pretreated using different techniques including bead-beating, sonication, microwaving, and autoclaving. Thereafter, different extraction solvents mixture including chloroform-methanol (1:1, v/v), chloroform-methanol (2:1. v/v), hexane-methanol (1:1, v/v), and hexane-methanol (2:1, v/v) were used in order to find a suitable solvent combination for maximum lipid extraction. Subsequently, the samples were filtered using Whatman 40 filter paper to remove suspend particle. The solvent and crude lipid were separated using Dragon Lab RE 100-pro rotary evaporator (Polychem supplies, South Africa) at 60 °C under vacuum. Lipid content were measured gravimetrically and expressed as percentage per dry cell weight (Gumbi et al., 2017).

For maximum sugar recovery, efficiency of different acids (sulfuric acid (H_2SO_4), hydrochloric acid (HCl)) and alkali (sodium hydroxide (NaOH), ammonium) were evaluated. The effects of the chemical concentration (1 to 4 % (v/v)) and temperature (100 °C, 110 °C and 120 °C in autoclave (15 psi)) were determined, while reaction time was varied in the range of 15-45 min. After hydrolysis, the samples were cooled to room temperature and the supernatant containing sugar fractions was collected by centrifugation at 5000 rpm for 10 min and analyzed using dinitrosalicylic acid (DNS) assay. The carbohydrates content was determined using previously explained equation (Kiran et al., 2016).

6.2.3 Simultaneous pretreatment and extraction of carbohydrates and lipids

The pre-determined conditions were used for simultaneous pretreatment and extraction of carbohydrates and lipids. The biomass (1 g) was mixed with 1% of H_2SO_4 and microwaved (Samsung, CE2877N, S. Korea) at 100 °C for 5 min. Then, the mixture was autoclaved at 120 °C at 15 psi for 30 min and centrifuged at 5000 rpm for 10 min. The supernatant was collected as the hydrolysate for ethanol fermentation and neutralized to pH 5 using pH meter (HI2002-01, Hanna Instruments, Woonsocket, RI, USA). The hydrolysate was filtered using 0.2 µm cellulose membrane filter under vacuum and sterilized by autoclaving at 121 °C for 15 min. The residual biomass was dried to a constant weight using a vacuum desiccator to remove residual organic solvents. Lipids were extracted from the residual biomass using 20 mL solvent mixture of chloroform-methanol (2:1, v/v) and centrifuged (Beckman Coulter Avanti J-25i) at 5000 rpm for 10 min to collect the supernatant. The process was repeated three times to ensure complete extraction of lipids. The solvent was vacuum filtered and evaporated in a Dragon Lab RE 100-pro rotary evaporator (Polychem supplies, South Africa) at 60 °C under vacuum to remove the remaining solvents.

6.2.4 Fermentation

The hydrolysate was fermented using *Saccharomyces cerevisiae*, pre-cultured in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 15 g L^{-1} of agar) at 30 °C for 48 h. The growth of yeast was monitored by measuring optical density at 600 nm using a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). The fermentation was initiated by adding *S. cerevisiae* solution (5% v/v) to sterile hydrolyzate to make a total volume of 50 mL and grown for 48 h at 30 °C in a shaking incubator at 150 rpm. Sample (2 mL) was taken from the fermentation broth at certain time intervals (0, 12, 24, 36 and 48h). The samples were centrifuged at 10 000 rpm for 5 min and the supernatant was used for ethanol and sugar analysis. HPLC (PerkinElmer) consisting of PerkinElmer binary LC pump, a Rezex ROA-Organic acid column, and refractive index detector was used to quantify sugar concentration and ethanol yield using 1 µL of filtered sample. The mobile phase consisted of aqueous sulphuric acid (2.5 mM) set at a flow rate of 0.5 mL/min. The column was operated at 55 °C, the pH of the reaction mixture ranges from 4 to 7. The theoretical bioethanol yield was calculated based on Eq. (1) previously described by Chng et al. (2017).

$$\text{Bioethanol Yield, \%} = \frac{\text{Ethanol (g)}}{\text{Total sugar (g)} \times 0.511} \times 100 \quad (1)$$

$$\text{Sugar conversion (\%)} = \frac{S0 - S1}{S0} \times 100 \quad (2)$$

Where S0 is the initial sugar concentration (g L^{-1}), and S1 is the final sugar concentration (g L^{-1})

6.2.5 Transesterification

Lipids extracted from the microalgae biomass were subjected to methanolysis with methanol containing 5% sulphuric acid as the catalyst (30:1 v/v). The reaction was carried out at 60 °C for 4 h in a benchtop Orbital shaking incubator (MRC, London, UK) at 200 rpm in the presence of 1 mL hexane as the reaction solvent. From the reaction mixture, 100 µL was transferred to a separate test tube and washed twice with a mixture of distilled water and hexane (1:1 v/v) to induce biphasic separation. The organic layer containing fatty acid methyl esters was collected for fatty acid profile analyses using gas chromatography-mass spectrometer (GC-MS) (Shimadzu, Corp., Kyoto, Japan). The oven temperature was programmed to start at 80 °C and kept on hold for 2 min, while the injector and detector temperature were set at 250 and 230 °C, respectively. The peaks of fatty acid were identified from the NIST Mass Spectral Library and the relative amount of fatty acid was calculated from the integrated area percentage from the total amount of fatty acid. The biodiesel properties were estimated using the web version of the BiodieselAnalyzer© version 2.2 (Talebi et al., 2013).

6.2.6 Statistical Analyses

The data were analysed by one-way ANOVA at 95% confidence limit ($\alpha = 0.05$). All statistical tests were performed using SPSS (v. 20, IBM). $p < 0.05$ denotes a statistically significant difference unless otherwise stated. The values were expressed as the mean ± standard deviation.

6.3 Results and discussion

6.3.1 Biomass pretreatment for lipid and carbohydrates extraction

Microalgae have a thick cell wall and membrane made single layer of phospholipid making extraction of intracellular biochemical products difficult. It has been reported that cell pretreatment enhance not only the release of stored lipids but also protein and carbohydrates (Bai et al., 2015). The study, investigated different pretreatment and extraction methods in order to optimize the conditions for optimum recovery of both lipid and carbohydrates from the indigenous *Chlorella* sp. T4 isolate used in this study. The parameters for pretreatment and extraction of lipid and carbohydrates from *Chlorella* sp. T4 were selected from literature that reported on the production of biofuel from *Chlorella* spp.

6.3.1.1 Optimization of lipid extraction from *Chlorella* sp. T4

In this study, different cell disruption techniques were investigated including sonication, autoclaving, bead-beating and microwaving. A significantly high lipid yield of 21.03 ± 1.17 % was obtained using microwave assisted pretreatment technique compared to the other cell disruption techniques investigated (Figure 1a). Lee et al. (2010) investigated the effect of different pretreatment techniques on the lipid yield from *Botryococcus* sp., *Chlorella vulgaris* and *Scenedesmus* sp. and found that microwave pretreatment resulted in the maximum lipid extraction. In another study by Ansari et al. (2018), high lipid extraction was reported using microwave-assisted pretreatment technique on *Scenedesmus obliquus* compared to autoclaving and osmotic shock pretreatment. Microwave provides a larger amount of thermal energy from electromagnetic radiation with a certain frequency to cells which enhances the kinetics of lipid extraction process by disrupting the cellular wall. Furthermore, sonication showed high pretreatment efficiency resulting in a lipid yield of 17.95 ± 0.97 % which was 1.17-fold lower compared to microwave assisted cell disruption. Another study by Guldhe et al. (2014), reported high lipid yield (19.85 ± 0.35%) using sonication assisted cell disruption on freeze dried biomass. Also, found that lipid yield using microwave assisted cell disruption was 1.49-fold higher compare to sonication assisted cell disruption.

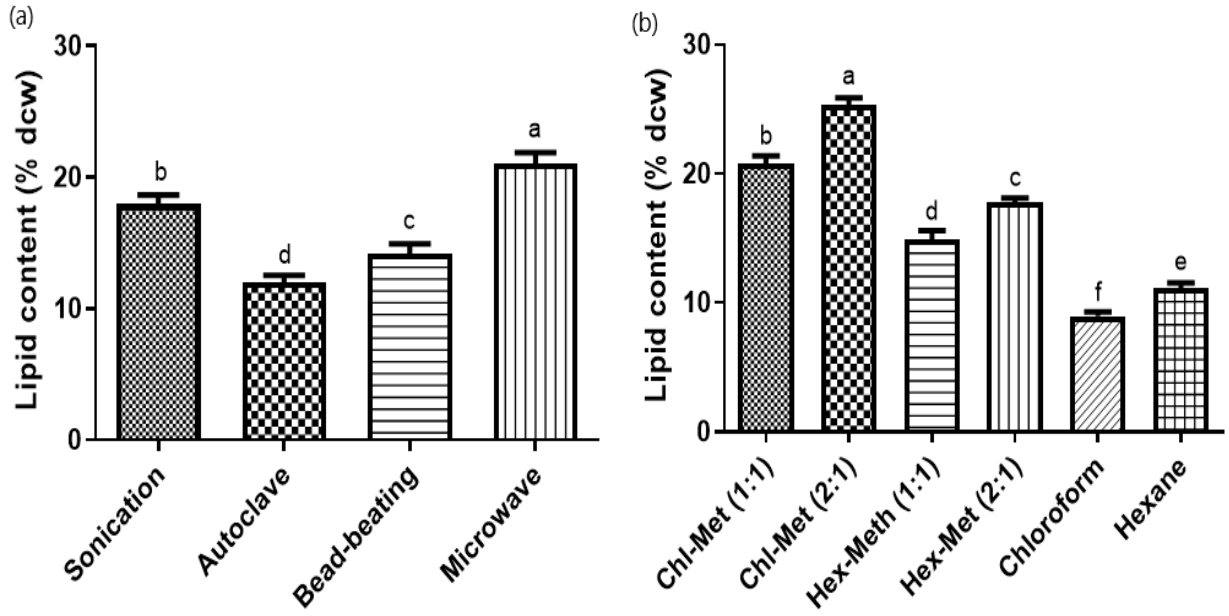

Figure 1. Lipid yield of *Chlorella* sp. T4 under (a) different cell disruption methods, and (b) using different extraction solvents. Chl: Chloroform, Met: Methanol, Hex: Hexane. Different letters depict significant difference amongst the group according to one-way ANOVA at $p < 0.05$.

Bead-beating and autoclaving pretreatment methods resulted in lower lipid yield of 14.19 ± 1.04 and 12 ± 0.73 %, respectively (Figure 1a). Microwave cell disruption was therefore used for further analysis

as it resulted in the highest cell disruption efficiency which was translated into significant high lipid yield.

The extraction solvent plays a critical role in ensuring maximum recovery of lipid and prevents the loss of other important metabolites found in microalgae biomass. In this study, lipids were extracted using different polar and non-polar solvents to find suitable solvent for efficient lipid recovery. The mixture of chloroform-methanol (2:1 v/v) was most efficient solvent, resulting in the highest lipid extraction of 25.30 ± 0.85% compared to the other solvents tested (Figure 1b). The mixture of chloroform and methanol have been found by other researchers to be efficient for high lipid recovery from different microalgal species (Odjadjare et al., 2017, Goh et al., 2019). Moreover, the mixture of hexane-methanol (2:1 v/v) showed high lipid extraction efficiency of 17.80 ± 0.42%, which was 1.42-fold lower compared to mixture of chloroform-methanol (2:1 v/v). However, hexane is known to be less toxic with a minimal affinity toward non-lipid contaminant compared to chloroform. It was observed that using a single extraction solvent resulted in low lipid extraction efficiency compared to a binary mixture of polar and non-polar solvents. This has been attributed to the fact that a combination of polar and nonpolar solvent ensures sufficient recovery of all neutral lipids including free-standing globules and membrane-associated complexes (Abomohra et al., 2013). Overall, the results from this study showed that the binary mixture of chloroform-methanol (2:1 v/v) with microwave-assisted cell disruption is suitable for high lipid recovery efficiency.

6.3.1.2 Optimization of sugar extraction from *Chlorella* sp. T4

Microalgae sugars are covered by thick cell wall, thus making the recovery of fermentable sugars difficult. The sugar recovery efficiency from *Chlorella* sp. T4 was optimized by investigating the effects of different hydrolysis methods, solvent concentrations, reaction temperatures and reaction times on sugar recovery. The maximum sugar yield of 23.53 ± 0.64% was obtained using 2% of H_2SO_4, followed by 20.01 ± 0.82% when 2% of HCl was used (Figure 2a). Sivaramakrishnan and Incharoensakdi, (2018) showed high sugar recovery efficiency of 80% from *Scenedesmus* using 0.3 N of H_2SO_4. Sulphuric acid (H_2SO_4) is well known to stimulate the degradation of cellulose matrix found in microalgae cell wall, and assist to depolymerize hemicellulose and hydrolyse starch into simple molecules (Velazquez-Lucio et al., 2018). Low sugar recovery efficiency was obtained using NaOH and ammonium compared to acid hydrolysis as shown in Figure 2a. Acid hydrolysis is considered the most efficient and economically friendly method to recover fermentable sugars from microalgae biomass (Karemore et al., 2016).

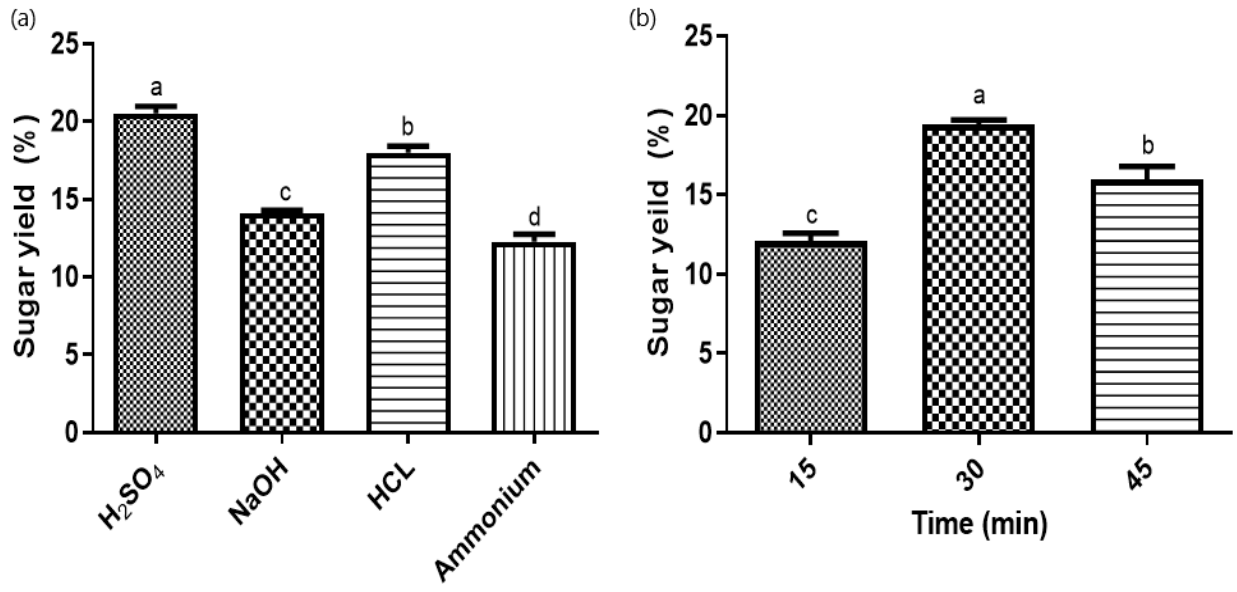

Figure 2. Sugar yield from *Chlorella* sp. T4 when subjected to (a) different chemicals for hydrolysis, and (b) different hydrolysis times. Different letters depict significant difference amongst the group according to one-way ANOVA at $p < 0.05$.

The hydrolysis time is a critical parameter for the release of sugar from microalgae biomass. As shown in Figure 2b, the maximum sugar recovery efficiency of 23.11 ± 0.36% was obtained when the *Chlorella* sp. T4 biomass was hydrolysed with 2% H_2SO_4 for 30 min, with 1.59-fold and 1.3-fold reduction in the amount of sugar recovered obtained when the hydrolysis was carried out for 15 min and 45 min, respectively. The incubation time significantly affects the availability of free fermentable sugar during acid hydrolysis. Abdulla et al. (2020) observed a significant decrease in the sugar recovery efficiency by 2.84-fold as the pretreatment time increase from 30 to 50 min using dilute acid hydrolysis of 2% H_2SO_4. In another study, Sivaramakrishnan and Incharoensakdi, (2018) reported a decrease in sugar yield as the pretreatment time increase above 20 min using 0.3 N H_2SO_4 The optimum pretreatment time is required to break down the hierarchical structure of the fibre into a nanocrystalline structure to increase the sugar recovery efficiency (Razali et al., 2017).

The use of strong acid can cause equipment corrosion, contaminate residual biomass, and could result in environmental damage, which can be avoided by with the use of weaker acid. Different concentrations of H_2SO_4 and reaction temperatures ranges were optimized for efficient sugar recovery at a constant hydrolysis time of 30 min. The maximum sugar recovery of 33.31 ± 0.46% was obtained using 1% of H_2SO_4 at 120 °C in an autoclave (Table 2). Previous study showed high sugar recovery from *Chlorococcum humicola* using 1% (v/v) H_2SO_4 compared to higher acid concentration, after varying the acid concentration from 1 to 10% (v/v) (Harun et al., 2011). Wang et al. (2014), obtained high sugar recovery efficiency of 18.8 g L^{-1} using 2.5% (v/v) of H_2SO_4, with a significant 1.34-fold decrease in sugar yield when 5% (v/v) of H_2SO_4 was used. In this study, hydrolysis with 2% (v/v) of

H_2SO_4 at 120 °C resulted in high sugar recovery efficiency of 31.75 ± 0.94%, which is 95.32% of the sugar yield obtained using 1% of H_2SO_4 at the same temperature. The decrease in sugar yield can be explained by the inhibitory effects of high concentration of sulphuric acid concentration. Usually hydrolysis conducted at high acid concentration and relatively high temperature might results in the lower amount of glucose concentration in the hydrolyte as the extracted glucose can be converted to organic acid (Ajani et al., 2011). Hydrolysis using dilute acid is regarded as an inexpensive pretreatment method, and is widely used to lower the cost of bioethanol production (Phwan et al., 2019).

Table 2. Optimization for sugar extraction from *Chlorella* sp. T4 at different concentrations of H_2SO_4 and reaction temperatures.

No. of experiments	Acid % (v/v)	Temperature (ºC)	Time (min)	Sugar yield (% dcw)
1	1	100	30	27.14 ± 0.15^{d}
2	1	110	30	28.60 ± 0.26^{c}
3	1	120	30	33.31 ± 0.46^{a}
4	2	100	30	25.69 ± 0.79^{e}
5	2	110	30	26.74 ± 0.33^{d}
6	2	120	30	31.75 ± 0.94^{b}
7	3	100	30	22.67 ± 0.67^{f}
8	3	110	30	22.06 ± 0.43^{fg}
9	3	120	30	24.66 ± 0.11^{e}
10	4	100	30	20.69 ± 0.66^{h}
11	4	110	30	21.50 ± 0.17^{gh}
12	4	120	30	21.47 ± 0.63^{gh}

Different letters depict significant difference amongst the group according to one-way ANOVA analysis at $p < 0.05$.

Furthermore, a significant decrease in sugar recovery, ranging from 20.69 to 21.47 % dcw was observed using 4% (v/v) of H_2SO_4 at high and low temperatures. High acid concentration can cause side reaction during hydrolysis leading to the degradation of glucose and xylose to furfural and hydroxymethylfurfural, thus reducing the total sugar yield (Talukder et al., 2012). Considering the need to minimize acid dosage, 1% (v/v) of H_2SO_4 is the most desirable acid concentration for maximum sugar recovery. Sugar recovery efficiency was enhanced by 22.73% at the reaction temperature of 120 °C as compared to the yield obtained at 100 °C using 1% of H_2SO_4 for hydrolysis (Table 2). Chng et al. (2017) reported that a low concentration of acid at high temperature is desirable compared to acid

concentration, as it does not cause the degradation of fermentable sugars to other unfavourable compounds which could affect the hydrolysis yield. However, high temperature of up to 180 °C during acid hydrolysis can significantly reduce the release of fermentable sugars (Saha et al., 2005). This is due to the direct solubilisation of the complex sugars at high temperature, changing the equilibria formation of simple sugar moieties. The results from this study suggest that acid pre-treatment with 1% (v/v) of H_2SO_4 at 120 °C for 30 minutes is most suitable.

6.3.2 Simultaneous sugar and lipid extraction

The optimized conditions for lipids and sugar extraction were used for simultaneous extraction of carbohydrates and lipids from microalgae biomass. The biomass of *Chlorella* sp. T4 was initially pretreated using microwave, followed by autoclaving under acid conditions. The liquid fraction was used for carbohydrates analysis while lipid content were extracted from the residual biomass. The maximum lipid and carbohydrates recovered during the simultaneous extraction process was 21.7 ± 0.5 % and 31.6 ± 0.1%, respectively. However, compared to the step-wise extraction process, a significant 1.07-fold decrease in lipid extraction efficiency was observed in residual biomass. Similarly, Karemore and Sen, (2016) reported a significant decrease in the total lipid yield from *Chlorococcum* sp. after hydrolysis, which was caused by the decrease in phospholipid content and high free fatty acid content in the lipids that could be totally converted to FAME. Optimization of cell disruption and extraction technique increased mass transfer to support the concomitant extraction of both sugars and lipids from *Chlorella* sp. T4 with good potential for bioethanol and biodiesel production. The remaining biomass after sugar and lipid extraction can be used to generate biogas or animal feed.

6.3.2.1 Bioethanol production from acid hydrolysate of *Chlorella* sp. T4

The hydrolysate derived from *Chlorella* sp. T4 was fermented to produce bioethanol using *S. cerevisiae* and results shown in Figure 3. Through acid hydrolysis, 2.14 g L^{-1} of sugar was obtained from the biomass using 1% (v/v) of H_2SO_4 at 120 °C for 30 minutes. Hence, these conditions were found to be optimum for high sugar recovery efficiency after proper optimization of extraction conditions. Similarly, Abdulla et al. (2020) reported high sugar recovery efficiency of 5.382 ± 0.063 g L^{-1} on *Chlorella* sp. with 2.0 M of H_2SO_4 at 130 °C for 30 min after proper optimization of hydrolysis conditions. Other studies, have shown different microalgae species as the excellent feedstock for bioethanol production (Xu et al., 2019, Abdulla et al., 2020, Lee et al., 2015). Figure 3 shows the time course profile of ethanol production and residual sugar consumption rate during the fermentation process. The sugar levels dropped rapidly after 24 h of fermentation process, which was accompanied by a sharp increase in the accumulation of ethanol. The rapid consumption of fermentable sugar resulted in ethanol concentration and yield of 0.81 g L^{-1} and 74.1%, respectively after 48 hours. Similarly, Lee et al. (2017), reported high sugar conversion efficiency to ethanol using acid-pretreated hydrolysate from *Chlorella* sp. KR-1 catalyzed by *S. cerevisiae*. They reported ethanol concentration and yield of

5.9 g L^{-1} and 79.3% from glucose concentration of 14.75 g L^{-1}, respectively. Another study by Harun and Danquah, (2011) reported high bioethanol concentration and yield of 7.2 g L^{-1} and 52% from *Chlorococcum humicola* biomass catalyzed by *S. cerevisiae*. This study showed that indigenous *Chlorella* sp. T4 contains significant amounts of carbohydrates that can be recovered and fermented to produce bioethanol.

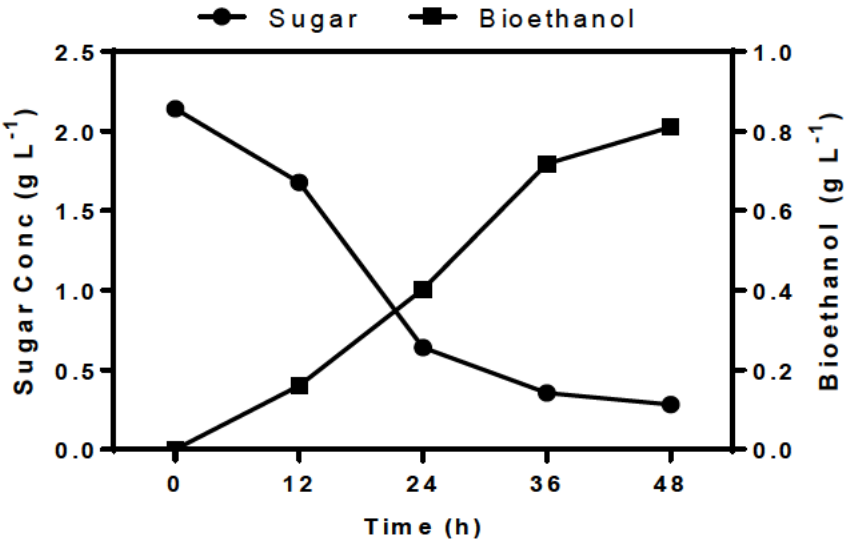

Figure 3. Time-course bioethanol production from acid hydrolysate of *Chlorella* sp. T4

6.3.2.2 Transesterification for biodiesel production

The GC-MS profile of the biodiesel obtained from the residual biomass is shown in Table 3. The fatty acids analysis revealed palmitic acid (C16:0), oleic acid (C18:1) and linoleic acid (C18:2) as the major constituents in *Chlorella* sp. T4 biomass. There was 1.19-fold increase in the amount of saturated fatty acid in residual biomass compared to the biomass before hydrolysis, especially palmitic acid. High content of palmitic acid gives biodiesel with high cetane number and good oxidative stability with lower nitrogen oxide emissions into the environment (Ha et al., 2020). Wang et al. (2014) found that the conversion rates of triacylglycerol to lipids was higher before hydrolysis than after hydrolysis. However, there was no significant decrease in total monounsaturated fatty acid in the residual biomass compared to that before hydrolysis. Oleic acid was the most abundant monounsaturated fatty acid which gives biodiesel higher oxidative stability, quality ignition and good lubricity (Odjadjare et al., 2018). A 1.17-fold decrease in polyunsaturated fatty acid content, was observed in the residual biomass after hydrolysis compared to the content before hydrolysis. This is especially notable in linoleic acid with 1.25-fold decrease. High content of linoleic acid is not desirable for quality biodiesel as is associated with poor oxidative stability and tend to results in biodiesel degradation during long-term storage and high temperatures (Nzayisenga et al., 2018).

Table 3. Fatty acid methyl ester profiles extracted from residual biomass of *Chlorella* sp. T4.

Fatty acid compositions (%)	Before hydrolysis	After hydrolysis
Myristic acid (C14:0)	5.33 ± 0.02	7.63 ± 0.27
Palmitic acid (C16:0)	26.92 ± 0.75	29.59 ± 0.92
Palmitoleic acid (C16:1)	2.88 ± 0.09	4.65 ± 0.06
Stearic acid (C18:0)	3.09 ± 0.11	5.19 ± 0.05
Oleic acid (18:1)	34.81 ± 0.91	31.94 ± 0.71
Linoleic acid (C18:2)	21.74 ± 1.69	17.37 ± 0.16
Arachidonic acid (C20:4)	2.09 ± 0.03	2.94 ± 0.09
Behenic acid (C22:0)	0.60 ± 0.02	0.93 ± 0.19
SFA (%)	35.94	42.6
MUFA (%)	37.69	36.59
PUFA (%)	23.83	20.31

SFA: Saturated fatty acid, MUFA: Monounsaturated fatty acid, PUFA: Polyunsaturated fatty acid. Data are mean of triplicate value ± standard deviation.

Biodiesel as a sustainable alternative for petroleum fuel can only be used directly in diesel engine if the fuel properties meet the ASTM D6751 and EN 14214 standards (Table 4). Therefore, it was important to evaluate the potential of biodiesel produced from the residual biomass to ascertain its potential use. The biodiesel obtained from residual biomass had a cetane number of 55.81, which was within the recommended value by ASTM and EN standard. Cetane number is determined by carbon chain length and higher number causes a shorter delay between injection and combustion. In addition, ideal cetane number reduce engine knock and improve exhaust emissions (Odjadjare et al., 2017, Goh et al., 2019). The viscosity of the biodiesel obtained was within the recommended value set by ASTM and EN specification. Density is one key properties for biodiesel combustion systems and influences the efficiency of atomization. Higher density value and viscosity will lead to poor atomization and air fuel mixing which ultimately result in poor combustion and reduced engine efficiency (Ashokkumar et al., 2015). The properties of biodiesel from residual biomass meet most the ASTM D6751 and EN 142114 specification showing that the *Chlorella* sp. T4 biomass can be used for integrated biorefinery approach to produce biodiesel and other fuels.

Table 4. Properties of biodiesel obtained from *Chlorella* sp. T4.

Methyl ester properties	ASTM D6751	EN 14214	Before hydrolysis	After hydrolysis
Iodine value (gl_2/100 g)	-	120 (max)	80.53	74.64
Saponification value (mg KOH)	-	-	201.54	207.51
Cetane number	47 (min)	51.0 (min)	55.26	55.81
High heating value (MJ kg^{-1})	>35	-	38.37	39.14
Cold filter plugging properties (°C)	-	≤5/≤-20	-0.33	1.87
Kinematic viscosity (mm^2 s^{-1})	1.9-6.0	3.5-5.0	1.29	1.31
Density (g cm^{-3})	0.875-0.90	0.86-0.90	0.85	0.86
Oxidative stability (h)	3 (min)	≥ 6	0	0
Cloud point (°C)	-	-	9.17	10.57

ASTM D-6751-American Society for Testing and Materials, EN 14214-European standard for biodiesel.

6.4 Conclusion

An integrated microalgal biorefinery process for biodiesel and bioethanol production from *Chlorella* sp. T4 was demonstrated in this study. The successful concomitant extraction of fermentable sugars and lipids confirmed the feasibility of using *Chlorella* sp. T4 for biorefinery process. Biomass pretreatment resulted in high lipid and sugar recovery efficiency, resolving the limitation of energetic and economic issues. Maximum sugar yield of 2.14 g L^{-1} and bioethanol yield of 0.81 g L^{-1} was obtained after proper optimization of the downstream process using low acid hydrolysis. The lipid yield in the residual biomass was not adversely affected by hydrolysis of sugar prior to lipid extraction as the biodiesel meet most of the standards specified by ASTM D6751 and EN 14214. High-value co-production opportunities possess great potential to significantly reduce the high cost of microalgae biofuel production. The indigenous microalgal strain *Chlorella* sp. T4 used in this study showed immense potential to offer a suitable alternative to the current biofuel production process, for the sustainable development of a bio-based economy in the near future.

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Chapter Seven

7.0 Concluding remarks

7.1 The research in perspective

The increase in urbanization, industrialization and commercialization has caused depletion of energy and water resources while increasing the emission of carbon and contaminating water bodies with toxic pollutants (Ferreira et al. 2018, Shahid et al. 2020). Microalgae are considered as the potential alternative to address both energy and water shortage to a significant extent in an eco-friendly and inexpensive way. They have remarkable advantages compare to terrestrial plants with high adaptability toward a wide range of climate and environmental conditions (Rawat et al. 2013, Xie et al. 2019). Microalgae can utilize nitrogen, phosphorus and other macronutrients in wastewater as the main enricher for growth (Daneshvar et al., 2019, Odjadjare et al., 2018). This provides cheap was for wastewater treatment and the biomass can be recovered for bioenergy production (Mishra and Mohanty, 2019). However, large scale of microalgae biofuel is hindered by the lack of economic competitiveness due to high cultivation cost, harvesting of biomass and extraction valuable products. Bioprospecting for robust native microalgae that can growth in different type wastewater and accumulated high biomass and lipids that are suitable for biodiesel can improve the economic microalgal-based technology (Duong et al. 2012).

In this study, thirty-one indigenous microalgal strains were isolated from different water sites in Kwazulu Natal, South Africa. Eight of the isolated strains demonstrated potential showed high biomass and lipid accumulation. They were identified belong to genera *Chlorella*, *Neochloris* and *Chlamydomonas*. The biomass productivity produced by the strains ranged between 6.72 ± 1.4 mg $L^{-1}d^{-1}$ to 30.44 ± 1.62 mg $L^{-1}d^{-1}$. Among the isolates, *Chlorella* sp. T4 showed highest biomass productivity, which was in a similar range to biomass productivity reported on *Chlorella* sp. by Tale et al. (2014). Using Nile red stain, lipid globules were visualized inside which was ascertain by gravimetric analysis revealing lipid yield ranging from 14 ± 6.2 to 38 ± 8.8% dcw. Microalgae are reported to have lipid range between 6 to 42% under normal growth conditions (Lin et al. 2015). *Chlorella* sp. T4 showed a good balance of biomass accumulation and lipid yield resulting into high lipid productivity of 8.03 ± 1.26 mg $L^{-1}d^{-1}$ compare to other isolates. Lipid productivity

is regarded as one importance parameters for selecting microalgae strain for biodiesel production as it considers both biomass and lipid content (Mohan et al. 2019). The precise lipid content obtained after gravimetric analysis was closely equivalent to lipid content estimated spectrophotometrically by the SPV colorimetric method. Fatty acids analyses showed that myristic acid, palmitic acid and pentadecanoic acid were the dominant fatty acids present in all the microalgal strains investigated. Fatty acid with a carbon chain length between 14 and 22 gives the finest quality biodiesel that meets the ASTM D6751 and EN 14214 vehicular fuel standards. The findings suggest that naturally isolated indigenous strains of *Chlorella* sp. from unexplored diverse aquatic habitats in the region are appropriate for biodiesel production based on lipid productivity and fatty acid profile.

Algal fuel production has not been commercialized at a large scale due to biomass and low lipid yield, and the cost associated with biodiesel production. In order to enhance lipid productivity and fatty acid suitability, *Chlorella* sp. T4, was cultivated in medium containing different nitrogen and phosphorus concentrations. Alteration of nutrient concentration in the growth is regarded as the viable approach to stimulate high lipid accumulation in microalgae (Mandotra et al. 2016, Sukačová et al. 2019). The cultivation of *Chlorella* sp. T4 under nitrogen and phosphorus limiting conditions alleviated a significant increase in lipid yield with decrease in the specific growth rate and biomass accumulation as compared to nutrient replete conditions. This was explained by a sharp decrease in chlorophyll a content and maximum quantum efficiency (Fv/Fm) yield under nitrogen and phosphorus limiting conditions. It was essential to systematically understand the genetic response of *Chlorella* sp. T4 under different nitrogen and phosphorus conditions to provide theoretical and experimental bases for improvement in biomass accumulation and lipid yield.

I was observed that nutrient limitation resulted to a significant decrease in the expression of *rbcL* gene involved in photosynthesis. The rate limiting enzyme responsible for fatty acid biosynthesis (*accD*) was upregulated by 3.11 and 1.89-fold in nitrogen and phosphorus limiting conditions compare to the control, respectively. The increase in acetyl-CoA concentration activates the ACCase gene triggering an increase in the accumulation of lipids microalgae (Fan et al. 2014, Singh et al. 2017) . The expression of *KAS-1* FAD gene showed a strong correlation with high biomass accumulation and abundance of saturated fatty acid in nitrogen and phosphorus replete medium. While, *ω-6 FAD* and *ω-3 FAD* was significantly increased in nitrogen and phosphorus

limiting conditions with a strong correlation to the abundance of monounsaturated and polyunsaturated fatty acid, respectively. This finding provided a better understanding of the response of key genes involved in the basic metabolism of *Chlorella* sp. T 4 during photosynthesis and biosynthesis of fatty acid (Shin et al. 2015). This results form the basis for further studies targeting genetic modifications of the strain to enhance biomass and lipid accumulation for biodiesel production. Important fatty acid such palmitic acid, stearic acid, oleic acid were synthesized by *Chlorella* sp. T4 under nutrient limiting conditions resulting into biodiesel with better oxidative stability and cold flow properties.

Utilization of wastewater is important in microalga technology in order to develop a cheap cultivation for high biomass production and wastewater treatment. To produce 1 kg of biodiesel from microalgae, requires about 3.726 kg of water, 0.33 kg of nitrogen and 0.71 kg of phosphorus. The continuous used of freshwater for microalgae cultivation put more tension on the available freshwater resource with South Africa regarded as a water-scarce country due to climatic conditions and escalating water demand (Odjadjare et al. 2018). In study, wastewater from poultry and dairy industry was used the cheap medium for cultivation *Chlorella* sp. T4. Poultry and dairy wastewater are good source of ready available macronutrients required for microalgae growth (Li et al., 2019, Ummalyma et al., 2018, Daneshvar et al., 2019). The strain showed strong adaptability to poultry and dairy wastewater expected raw dairy effluent which was diluted to 60% using distilled water as previously done study. Raw dairy effluent had high presence of suspended solids and nutrient concentration in raw dairy wastewater that had repressive effects on the growth of *Chlorella* sp. T4 (Cai et al. 2013, Udaiyappan et al. 2017).

The strain showed high specific growth rate in wastewater with the Fv/FM value above 0.5 in poultry, 60% dairy and sludge dairy, showing the algal strain can use wastewater as cheap growth medium (White et al. 2011). The dual purpose of microalgae wastewater treatment and biomass accumulation for biofuel production was achieved in this study. This was shown by high nitrogen (85-94%) and phosphorus (35-93%) removal efficiency by *Chlorella* sp. T4 which similar to nutrient removal efficiency reported by other researcher (Ferreira et al. 2018, Jais et al. 2017)**.** A high biomass yield of 1.28 ± 0.1 g L^{-1} and 0.85 ± 0.02 g L^{-1} was ascertain in poultry and dairy sludge wastewater. The biomass contained significant amounts of protein, carbohydrates, and lipids that can be used for various biotechnological application. The FAMEs profile of the strain,

especially when cultivated in the final dairy wastewater complied with most of the guidelines specified in the ASTM D6751 and EN 14,214 standards.

The biomass composition of *Chlorella* sp. T4 obtained in sludge dairy wastewater was revealed to contain high amounts of fermentable sugar and lipids. The feasibility of utilizing the biomass for co-production bioethanol and biodiesel was also investigated. The biomass was harvested by sedimentation on order to minimize harvesting cost which contribute about 20-30% of total production cost (Mohd Udaiyappan et al. 2017). Microwave-assisted cell disruption and chloroform: methanol (2:1 v/v) resulted in a significantly increase in the extraction of lipids compare to other cell disruption techniques and extraction solvent mixtures. The mixture of chloroform and methanol is known to be efficient for maximum lipid extraction from different microalgal species (Goh et al. 2019). High recovery efficiency of sugar was achieved using 1% H_2SO_4 at 120 °C for 30 min in autoclave supported hydrolysis, which was similar to a previous study done by Harun and Danquah, (2011). HPLC analyzed revealed that the biomass contains 2.14 g L^{-1} of sugar, after fermentation using *Saccharomyces cerevisiae* to produce 0.81 g L^{-1} of ethanol. The fatty acid methyl ester extracted from the residual biomass contained palmitic acid, oleic acid, and linoleic acid with a slight decrease than before hydrolysis. The study shows that the indigenous strains can be used for the biorefinery approach for biodiesel and bioethanol production.

7.2 Limitation of the current study

The process of isolation, and purification of native microalgae strain using the conventional plating and dilution technique is generally time consuming and may result in the loss of novel microalgae strain that has potential application in biofuel industry. The suitability and adaptability of the strain for outdoor cultivation in a large scale such raceway pond using wastewater as the growth medium was not investigated in study. This can provide valuable information on feasibility of *Chlorella* sp. T4 to naturally acclimate to the prevailing environmental conditions and produce biomass that is rich in lipids and carbohydrates. Designing, a high efficiency and cost effective strategy for biomass harvesting is still major challenge on the economics of microalgae based fuel to compete with petroleum based fuel.

7.3 Potential for future development of the study

Based on finding obtained on the current study, it evident that *Chlorella* sp. T4 is a robust strain with great potential to be used for biodiesel and bioethanol production. The strain showed strong ability to utilize different type for wastewater and remove high amounts of nitrogen and phosphorus. It may be worthwhile to perform the whole genome sequencing on the strain which will assist in overcoming the inherent limitation of metabolic capacity for higher accumulation of desired product, thus eventually improve the economic feasibility of the production process. Harvesting of microalgae biomass from a large culture volume of culture is still a challenging and costly for their biorefinery. The adoption of cheap and less energy-intensive harvesting techniques such as the flocculation and sedimentation is recommended. Lastly, it important to look at cultivating the strain in an open raceway or bioreactor using wastewater to monitor the physiological response of the strain outside the laboratory conditions. Also, do techno-economic analysis to assess the economic feasibility of utilizing macroalgae for biofuel production and wastewater treatment on a pilot scale.

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