



UNIVERSITY OF TM
KWAZULU-NATAL
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**The Utilization of Wastewater for Microalgal Biomass
Propagation and Saccharification of Carbohydrates into
Fermentable Sugars for Bioethanol Production**

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Submitted in fulfilment of the academic requirements for the degree of Master of Science
in the School of Life Sciences, University of KwaZulu-Natal, Westville Campus

February 2017

As the candidate's supervisors, we have approved this thesis/dissertation for submission.

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Preface

The experimental work described in this dissertation was carried out in the School of Life Sciences, Discipline of Microbiology, University of KwaZulu-Natal, Westville Campus, from March 2015 to February 2017, under the supervision of Dr Taurai Mutanda and Prof Ademola Olaniran.

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

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Declaration 2 – Publications and Conferences

The outputs of the research include a poster presented at an international conference, one journal article in press and one journal article under review.

Majeke, B.M., Olaniran, A.O. & Mutanda, T. (2016). Response surface methodology for the optimization of biomass and carbohydrate production by an indigenous microalgal strain cultivated in modified BG-11 medium. *The 6th International Conference on Algal Biomass, Biofuels and Bioproducts*. 26 - 29 June, 2016. San Diego, CA, USA.

Gumbi, S.T., **Majeke, B.M.**, Olaniran, A.O. & Mutanda, T. (2017). Isolation, identification and high Throughput screening of neutral lipid producing indigenous microalgae from South African aquatic habitats. *Applied Biochemistry and Biotechnology* **182**, 382-399.

Majeke, BM., Gumbi, ST., Olaniran, A.O. & Mutanda, T. (2016). Microalgal isolates indigenous to various aquatic environments in KwaZulu-Natal (South Africa): Growth kinetics, physiological function and biochemical characterization for bioethanol production. Manuscript submitted to *Journal of Biotechnology Reports*.

Majeke, BM., Gumbi, ST., Olaniran, A.O. & Mutanda, T. (2016). Optimization of an indigenous *Chlorella vulgaris* strain for biomass and carbohydrate production under photoautotrophic growth conditions by response surface methodology. Manuscript in Preparation.

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Finally yet importantly, I would like to take this opportunity to express my deepest appreciation to my family.

Dedication

This dissertation is dedicated to my beloved mother Louisa Wongiwe Nomsuka. Her unconditional love and support is every reason for what I am and where I am today.

Abstract

Unstable oil prices and freshwater scarcity are of prime topical concerns. As part of pioneering efforts to assess the potential of indigenous microalgae as biofuel feedstock in South Africa, thirty one microalgal cultures were isolated from various habitats in KwaZulu-Natal and purified to monoculture. Six strains were profiled for their carbohydrate content, photosynthetic activity, growth rate and biomass production. Based on molecular and morphological characteristics, these strains were identified as belonging to the genera *Chlorella*, *Neochloris*, *Chlamydomonas* and *Chlorococcum*. Analysis of the biomass at the exponential and stationary phase showed that the strain *Chlorella vulgaris* (Toti RS4) had the highest growth rate (0.201 day^{-1}), biomass productivity ($70.8 \text{ mg L}^{-1} \text{ day}^{-1}$), and carbohydrate content (51% of the dry cell weight [dcw]) under nitrogen deficient conditions. The strain demonstrated high photosynthetic activity based on its high relative electron transport rates (rETR) of 40.3 units and was therefore selected for further experimentation. The selected microalgal strain (*C. vulgaris* (Toti RS4)) was optimized for its potential for biomass and carbohydrate production using statistical optimization strategies. First, important parameters affecting biomass and carbohydrate production by *C. vulgaris* (Toti RS4) were studied using the Plackett-Burman screening design. The results showed that nitrogen, K_2HPO_4 , Na_2CO_3 , temperature, light intensity and cultivation period were the most significant parameters ($p < 0.05$). Response surface methodology coupled with the Box-Behnken design was then used to identify the optimum levels of the significant parameters. The optimum values were identified as urea, 0.203 g L^{-1} ; K_2HPO_4 , 0.026 g L^{-1} ; Na_2CO_3 , 0.012 g L^{-1} ; temperature, $20 \text{ }^\circ\text{C}$, light intensity, $180.18 \mu\text{mol m}^{-2} \text{ s}^{-1}$ and cultivation time, 14 days. Under the optimized conditions, the carbohydrates were elevated to 78% of the dcw and the biomass was 488 mg L^{-1} . The identified optimum conditions, particularly light intensity, temperature and cultivation conditions were successfully adopted for the propagation of the microalgal strain using municipal wastewater. In this study, two types of wastewater media, namely; raw and autoclaved primary effluent municipal wastewater (PEMW) were used as substrates for simultaneous biomass accumulation and macronutrient removal. The microalga *C. vulgaris* (Toti RS4) demonstrated good growth (1.5 g L^{-1}), nutrient removal efficiency (100% nitrogen and phosphorus removal) and carbohydrate production (58% of the dcw) on raw PEMW. However, the microalgal cells demonstrated a decline in the maximum quantum efficiency from 0.681 to 0.490 after 14 days of cultivation. The biomass from wastewater grown cultures was easily harvested by flocculation and used for further downstream processing. Acid pre-treatment of the biomass under optimum conditions of HCl, 0.5 % (v/v); temperature of $120 \text{ }^\circ\text{C}$ and reaction time of 67.5 min, resulted in the decline of microalgal cell wall carbohydrate content (Optical density of ~ 0.10 to 0.5). The

concentration of reducing sugar after acid pre-treatment was 14.95 g L⁻¹. The maximum bioethanol concentration produced was 4.88 g L⁻¹. Enzymatic hydrolysis of microalgal carbohydrates into fermentable sugars was also evaluated in this study. The microalgal biomass was initially solubilized using various pre-treatments. FT-IR results showed that the autoclave pre-treatment had the highest effect on the breakdown and solubilisation of microalgal cells, with a decrease in cell wall size. The concentration of bioethanol produced via the separate hydrolysis and fermentation process was 6.3 g L⁻¹. The bioethanol concentration produced via the simultaneous hydrolysis and fermentation process was 7.9 g L⁻¹. This study demonstrated the feasibility of using microalgal biomass for bioenergy production.

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List of Abbreviations

- ATS - Algae turf scrubber
- BBM - Bolds basal medium
- BG-11 - Blue green medium
- CBP - Consolidated bioprocessing
- chl a - Chlorophyll a
- COD - Chemical oxygen demand
- DNA - Deoxyribonucleic acid
- DNS - dinitrosalicylic
- EDTA - Ethylenediaminetetraacetic acid
- FT-IR - Flourier transform infrared spectroscopy
- F_v/F_m - Maximum quantum yield of PSII of a dark-adapted sample (dimensionless)
- GC - Gas chromatography
- NPQ - Non-photochemical quenching
- NSSF- Non-isothermal simultaneous saccharification and fermentation
- PAM - Pulse amplitude fluorometry
- PBD - Plackett-Burman design
- PEMW - Primary effluent municipal wastewater
- PSII - Photosystem II
- rETR - Relative electron transport rate (dimensionless)
- RLC - Rapid light curve.
- RSM - Response surface methodology
- SEM - Scanning electron microscopy
- SHF- Separate hydrolysis and fermentation
- SSF - Simultaneous saccharification and fermentation
- TLC -Thin layer chromatography
- TN - Total nitrogen
- TP - Total phosphorus
- WW - wastewater
- WWTP - Wastewater treatment plant

List of Units

degrees Celsius - °C

Dry cell weight - dcw

Gram per litre - g L⁻¹

Gram per litre per day - g L⁻¹ day⁻¹

Hour - h

M - Mole

Microgram - µg

Micromole - µmol

Milligram per litre - mgL⁻¹

Milligram per litre per day - mg L⁻¹ day⁻¹

Minutes - min

Per day - day⁻¹

Micromole per square meter per second - µmolm⁻² s⁻¹

Per millilitre – m L⁻¹

Percentage - %

Volume per volume - v/v

Weight per volume - w/v

Weight per weight - w/w

Chapter 1. Introduction

1.1. General introduction

The rampant depletion of fossil fuel reserves is a worldwide topical phenomenon which requires abatement through research for alternative renewable fuels in order to avert dire catastrophic consequences in the future (Gumbi *et al.*, 2017). In addition, the ever-increasing world prices of fossil-based fuels are impeding industrial growth as well as contributing to the surge in world economies. The hardest hit countries are the so-called developing countries in Africa. With this scenario in mind, it is, therefore, prudent to explore sustainable and cost-effective strategies for the formulation and generation of readily available renewable energies for the future (Dalla Chiara and Pellicelli, 2016).

Microalgae have great potential as a source of alternative energy resource and a wide range of applications due to their unique characteristics such as high productivity, fast growth rates and short doubling time, when compared to terrestrial and aquatic plants, to which they are closely related phylogenetically (Buschmann and Zachgo, 2016). Considerable amounts of fuels such as biodiesel, biohydrogen, and bioethanol can be realized from microalgal biomass and strain selection is key to successful biofuel production (El-Dalatony *et al.*, 2016; Salam *et al.*, 2016). Although biodiesel and biohydrogen are considered as the best alternatives to fossil fuels, bioethanol remains the most widely used biofuel in several countries around the globe. Bioethanol can be employed to supplement or even replace gasoline. One other crucial attribute of bioethanol is that it is compatible with the current infrastructure (Sanchez Rizza *et al.*, 2017). A broad range of microalgal species can accumulate up to 51 to 60% of carbohydrates per dry weight, and the resulting carbohydrate profile has been shown to be suitable for bioethanol production (Ho *et al.*, 2013b). However, commercial production of microalgal biomass for bioethanol production requires large amounts of water input. The use of commercial media for the cultivation of microalgae cells at large scale is economically not feasible due to the prohibitive costs involved. Therefore, microalgal strains that can effectively grow in wastewater are more attractive as a feedstocks for commercial production of bioenergy (Cheah *et al.*, 2016).

The use of wastewater as a substrate for microalgal growth is a cost-effective initiative for sustainable biofuel production (Feng *et al.*, 2011). There are stringent guidelines and regulations to be met by municipalities before the effluent is discharged into receiving water bodies. Large amounts of nutrient-rich wastewater are clandestinely discharged by some municipalities into the water bodies causing serious socio-economic ramifications to the environment (Yan *et al.*, 2016). Microalgal growth in wastewater streams has a dual role

since they can phycoremediate wastewater as well as propagating biomass for bioenergy production. Microalgae use macronutrients (particularly nitrogen and phosphorus) in the wastewater for their growth and the 'treated' water can be safely discharged into receiving water bodies without causing any environmental problems such as eutrophication (Menna *et al.*, 2015). The acquired microalgal biomass can be harvested and converted into bioenergy by enzymatic and acid hydrolysis of complex polysaccharides (cellulose and starch) and subsequent downstream processing of the fermentable simple sugars (i.e. glucose) into bioethanol. However, the fact that microalgal biomass exists in an aqueous suspension and the microscopic size of the microalgal cell makes biomass harvesting technically challenging. Consequently, microalgae harvesting is the main stumbling block in downstream processing of the bioenergy production value chain (Feng *et al.*, 2011). It is therefore crucial to explore suitable cost-effective microalgal biomass harvesting strategies for the whole bioenergy chain to be feasible. Sustainable production of bioethanol is feasible but the main challenge is carbohydrate saccharification operational parameters as well as optimal fermentation conditions.

1.2. Aim

The overall aim of this research was to bioprospect for indigenous hyper-carbohydrate producing microalgal strains from different aquatic environments and use wastewater for the propagation of the selected microalgal strain for biomass accumulation with concomitant wastewater remediation. Enzymatic and chemical biomass hydrolysis strategies will be evaluated for the conversion of complex carbohydrates into fermentable sugars. The simple fermentable sugars were subsequently fermented into bioethanol using *Saccharomyces cerevisiae*.

1.3. Objectives

The specific objectives are as follows:

- 1.3.1. To bioprospect and select hyper-carbohydrate producing microalgal strain and optimize growth conditions.
- 1.3.2. To set up laboratory upstream processing and cultivation conditions.
- 1.3.3. To evaluate microalgal harvesting by auto-flocculation.
- 1.3.4. To saccharify by enzymatic hydrolysis of complex carbohydrates.
- 1.3.5. To saccharify by acid hydrolysis of complex carbohydrates.
- 1.3.6. To optimize for bioethanol production processes.

Chapter 2. Literature review

2.1. Introduction

Development of sustainable and environmentally benign energy resources is a necessity due to the dwindling fossil fuel reserves and increasing energy demand as a result of the rapidly expanding transport industry (Park *et al.*, 2016). Microalgae have been earmarked to hold great potential as a source of alternative energy resource and a wide range of applications due to their unique characteristics such as high productivity, fast growth rates and short doubling time, when compared to terrestrial or aquatic plants, to which they are closely related phylogenetically (Zhu, 2017). Considerable amounts of fuels such as biodiesel, biohydrogen, and bioethanol can be realized from microalgal biomass (El-Dalatony *et al.*, 2016; Salam *et al.*, 2016). Although biodiesel and biohydrogen are currently considered the best alternatives to fossil fuels, bioethanol still remains the most widely used biofuel in several countries around the globe. Bioethanol can be employed to supplement or even replace gasoline. One of the most crucial attributes of bioethanol is that it is compatible with the current infrastructure (Schulze *et al.*, 2016). A broad range of microalgal species can accumulate up to 51 to 60% of carbohydrates per dry weight, and the resulting carbohydrate profile has been shown to be suitable for bioethanol production (Ho *et al.*, 2013b). However, commercial production of microalgal biomass for bioethanol production requires large amounts of water input. This raises serious cost concerns if artificial water is used to grow microalgae. Therefore, microalgal strains that can effectively grow in wastewater are more attractive as a feedstock for microalgal bioenergy production (Chinansamy *et al.*, 2010; Mutanda *et al.*, 2011b; Rawat *et al.*, 2011).

The use of wastewater as a substrate for microalgal growth is a cost effective initiative for sustainable biofuel production (Wi *et al.*, 2015; Wijaya *et al.*, 2014). There are stringent guidelines and regulations to be met by municipalities before the effluent is discharged into receiving waters. Large amounts of nutrient-rich wastewater are clandestinely discharged by some municipalities into the water bodies causing serious socio-economic ramifications to the environment (Schulze *et al.*, 2016). Wastewater, particularly municipal and agricultural wastewaters are rich in organic and inorganic macronutrients such as nitrogen and phosphorus which are major requirements for microalgal growth (Zhu, 2017). These nutrients can be economically harnessed for the cultivation and propagation of microalgae with the added advantage of beneficiation of the wastewater quality suitable for their discharge into the environment or recycling (Yen *et al.*, 2013). This review paper highlights the major nutrient components of different wastewater streams, compares nutrient removal efficiency, biomass and carbohydrate production by some microalgal strains cultivated in

different wastewater streams, discusses the current microalgae cultivation systems and discusses in detail subsequent downstream bioenergy production processes.

2.2. Characteristics of microalgae

The green microalgae, also called *chlorophytes* are eukaryotic microorganisms that originated from primary endosymbiosis, the engulfment and retention by a eukaryotic, heterotrophic cell of a cyanobacterium (Fig. 2.1). Green microalgae bear chloroplasts containing chlorophylls a and b giving them their characteristic green colour but lack phycobilins. Most green microalgae inhabit freshwater and wastewater, although some are marine, and others are found in moist soil or growing in snow, to which they impart a pink colour. The sizes of microalgae can range from micrometres to millimetres, depending on the microalgal species (Mutanda *et al.*, 2011b).

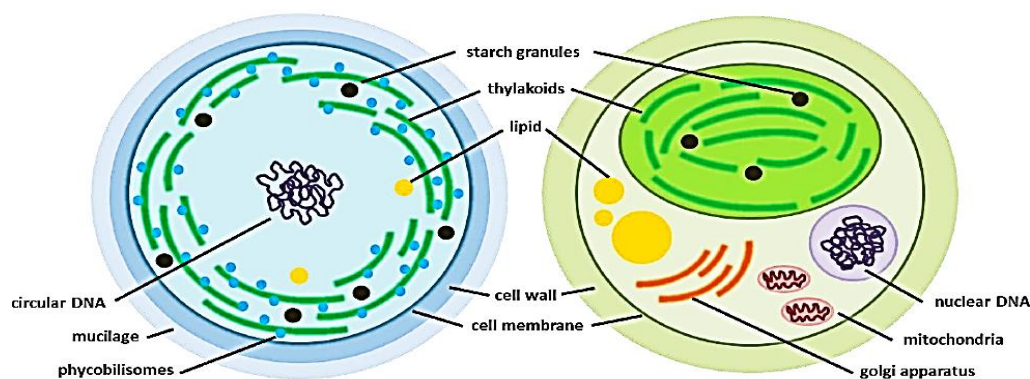


Fig. 2.1. The cell structure of a single-celled cyanobacterium (left) and eukaryotic green algal cell (right). Adapted from Wehr (2007).

Microalgae adopt various routes of metabolism for their growth and survival viz., autotrophic, heterotrophic and mixotrophic modes. They are also capable of shifting their metabolism in response to changes in the environmental conditions. Some microalgae can grow photoautotrophically by using light as a sole energy source for the generation of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) which provides the microalgae cells with chemical and reducing energy required for growth. Other microalgae can grow heterotrophically by utilizing only organic compounds such as carbohydrates and organic acids as both carbon and energy source and mixotrophically using both organic compounds and CO₂ as energy source. Among photoautotrophic and photoheterotrophic modes, heterotrophic cultivation of microalgae provides several advantages over the phototrophic cultivation strategy, such as minimization of light

requirement, higher growth efficiencies and easy biomass harvesting (Yen *et al.*, 2013). However, phototrophic cultivation is still the most widely used strategy for large-scale production systems because it is cost-effective and is less prone to contamination (Chokshi *et al.*, 2015; Mutanda *et al.*, 2014).

For the past decades, microalgae have been widely studied and exploited due to their advantages and special properties, which provide some solutions to environmental and energy shortages. Environmental problems, particularly wastewater management and greenhouse gas emissions, can be sequestered with the application of microalgae systems. A biological approach to treatment using microalgae provides a lower cost and added value to the remediation system through biomass production (Lizzul *et al.*, 2014). The biomass of microalgae is rich in lipids, carbohydrates and proteins (Fig. 2.2) which can be processed into energy sources, such as biodiesel, bioethanol, and biohydrogen. In addition to energy sources, microalgal biomass can be applied as animal feed and as fertiliser. Moreover, it has been found to be suitable for the production of bioactive compounds and other antioxidants that are in high demand in the health and pharmaceutical industries (Schulze *et al.*, 2016). However, due to the scope of this study, the focus is mainly on the utilization of microalgae as feedstocks for bioenergy production, particularly bioethanol.

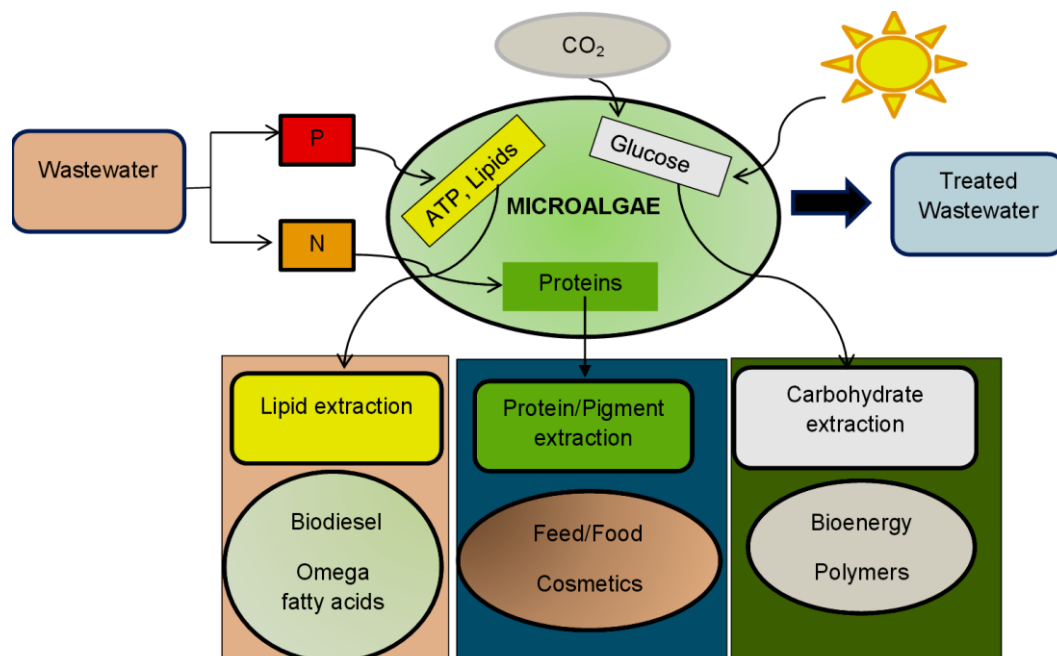


Fig. 2.2. The biotechnological applications of microalgae (Sanchez Rizza *et al.*, 2017; Zhu, 2017).

2.3. Microalgae as feedstocks for bioethanol production

Bioenergy production from renewable sources such as microalgae is gaining a lot of attention globally to avert fuel crises in the near future. The over reliance on fossil-based fuels is not sustainable since these fuels are projected to be depleted in less than half a century from now (Chisti, 2008). Considerable amounts of fuels such as biodiesel, biomethane, bioethanol, *inter alia* can be realized from microalgal biomass. A wide array of indigenous hyper-carbohydrate microalgal strains can be isolated from diverse aquatic habitats, cultured, purified to monoculture and propagated under lab conditions for biomass production. Unlike agricultural crops, the utilization of microalgae for biofuel production does not raise food security issues or increase demands on limited arable land and water supply. Similarly, the lignocellulosic material has been marked as a potential alternative feedstock for biofuel production since it is abundant in nature and a non-food source. However, application of this natural resource for biofuel production is hindered by low yields and the high costs involved in its hydrolysis stage. In addition, the hydrolysis of lignocellulosic biomass has been shown to produce by-products that are toxic to yeast cells, thus interfere with the downstream process of fermentation (Maitan-Alfenas *et al.*, 2015). Although detoxification strategies such as activated charcoal adsorption and lime treatments have been devised, an efficient strategy for the hydrolysis of lignocellulosic material into fermentable sugars is lacking (Wi *et al.*, 2015; Wijaya *et al.*, 2014). In this regard, microalgae are currently considered the most viable alternative.

Numerous microalgae potentially provide the benefit of accumulating considerable amounts of carbohydrates under optimal conditions, which are the major products formed by photosynthetic activity and CO₂ fixation. Microalgal carbohydrates mainly exist as cellulose in the cell walls and starch in the cytosol or chloroplast, depending on the prevailing environmental conditions (Chen *et al.*, 2013). The crystalline structure of cellulose comprises of linear glucan chains consisting of anhydroglucose units joined by β -(1, 4)-glycosidic bonds (Fig. 2.3(A)) (Doan *et al.*, 2012). On the other hand, the structure of starch consists of two main polysaccharides, amylose and amylopectin. Both polysaccharides consist of 1, 4 linked residues of α -D-glucose but amylose is linear while amylopectin highly branched (Fig. 2.3(B)) (Park *et al.*, 2016; Scholz *et al.*, 2013).

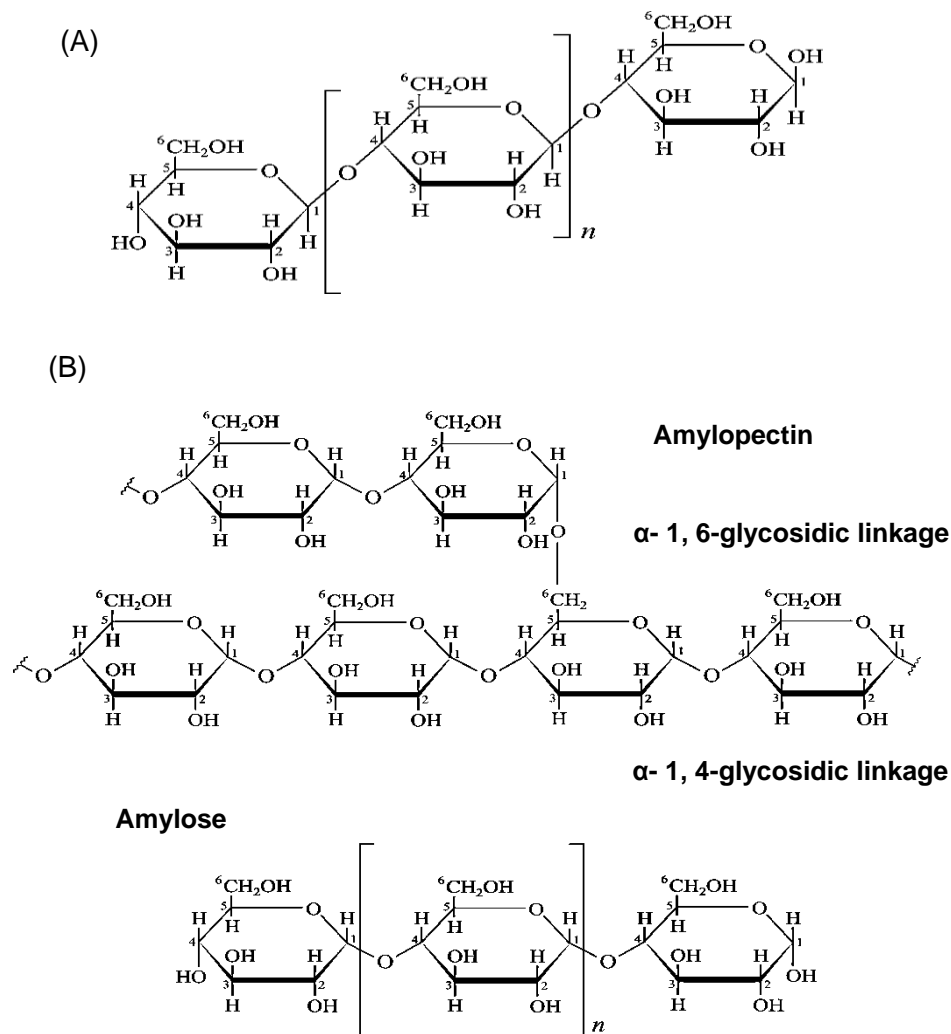


Fig. 2.3. Major microalgal carbohydrates. (A) Cellulose. (B) Starch (amylopectin and amylose). Adapted from [Doan et al. \(2012\)](#).

The desirable attribute of microalgal carbohydrates is that they are totally free of lignin which is a complex hydrophobic network of phenylpropanoid units that are synthesized by the oxidative polymerization of one or more types of hydroxycinnamoyl alcohol precursors ([Alvira et al., 2010](#); [Fernández-Rodríguez et al., 2017](#)). The lack of lignin in microalgae makes pre-treatment and hydrolysis of microalgal biomass easier and cheaper when compared to plant lignocellulosic biomass. According to some recent estimates, the yield of carbohydrates from microalgae has the potential to be several yields higher than the best performing crop plant/vegetable plant ([Sanchez Rizza et al., 2017](#); [Zhu, 2017](#)). Selection of microalgal strains capable of accumulating large quantities of carbohydrates (mainly existing as glucose) is crucial for bioethanol production. Some of the most promising species reported in the literature include *Chlorella*, *Chlamydomonas*, *Scenedesmus* as well as the cyanobacteria, *Spirulina* ([Do Nascimento et al., 2012](#); [Markou et al., 2013](#); [Sanchez Rizza et al., 2017](#)). There exist approximately 200 000 to 800 000 algal species, of which only 50 000

species have been described (Mutanda *et al.*, 2011b). Therefore, there is a great potential that more microalgal strains with unique characteristics still exist in nature and have not been described. Therefore, there is a great need for bioprospecting.

2.4. Wastewater resources for algal biofuel production

Microalgae can grow in various types of aquatic habitats, such as freshwater, marine water and wastewater streams (including municipal, agricultural or industrial wastewater), if there are adequate amounts of carbon (inorganic and organic), nitrogen (ammonium, nitrate or urea) and phosphorus, as well as other trace elements present (Chokshi *et al.*, 2015). Wastewater streams are unique in their chemical composition and physical properties as compared with fresh and marine waters. Recently, the integration of microalgae-based biofuel production with wastewater treatment has been considered a sustainable option for bioenergy production and also towards environmental sustainability (Rawat *et al.*, 2011). However, wastewater-based microalgae cultivation is still faced with many challenges, such as variation of wastewater composition due to the nature of anthropogenic activities occurring in the vicinity of the water body (Schenk *et al.*, 2008), improper nutrient ratios (e.g., N/P and C/N), high turbidity due to the presence of suspended solid particles or pigments which affect light penetration, as well as the presence of chemical and biological toxins which inhibit microalgal growth (Cheah *et al.*, 2016). In the rest of this section, a brief review of some of the studies of algal growth under three different wastewater conditions: municipal wastewater, agricultural wastewater and industrial wastewater, will be given.

2.4.1. Municipal wastewater

The increasing urbanization and growth in human population have resulted in an increase in the generation of municipal wastewater. Conventional municipal wastewater treatment plants consist of four different types of wastewater streams which are generated in different treatment stages, including wastewater before primary settling, wastewater after primary settling, wastewater after activated sludge tank and concentrated municipal wastewater generated during sludge centrifuge, also referred to as “centrate” wastewater. Table 2.1 shows the levels of the nitrogen and phosphorus in different wastewater streams, indicating variations in the nutrient content among the different wastewater streams. Microalgae species react differently to different wastewater streams due to the variations of the nutrient levels and profile as well as deficiency of some vital trace elements, and only a limited number of strains of a few species such as *Chlorella* sp. and *Scenedesmus* sp. have since been shown to adapt well in different wastewater environments (Mutanda *et al.*, 2014). For

example, (Li *et al.*, 2011) investigated the nutrient removal efficiency of *Chlorella* sp. on centrate wastewater and the results showed that the microalgae removed 93.9%, 89.1%, 80.9%, and 90.8% of ammonium total nitrogen, total phosphorus, and COD, respectively. *Chlorella* sp. had a daily biomass productivity of 0.92 g L⁻¹ day⁻¹ under the conditions used. In another study, Lizzul *et al.* (2014), reported that *Chlorella sorokiniana* could produce a maximum biomass concentration of 330 mg L⁻¹ when cultivated in final effluent enriched with 12% of CO₂. Han *et al.* (2015) reported 100 % removal of phosphorus and nitrogen from campus sewage wastewater using *Scenedemesus quadricauda*.

Overall, these reports indicate the potential use of municipal wastewater resources as a suitable and sustainable medium for microalgal biomass production. Although the main goal in the field of using wastewater for microalgae culture is to evaluate the viability of microalgae cultivated in wastewater for the inorganic nutrients removal, as the view of sustainable process, the value of the biomass produced after wastewater is undoubtedly an important point to be considered. Therefore, recent studies have focused on maximizing microalgal biomass and carbohydrate production (Zhu, 2017). A number of recent laboratory-based studies, where microalgae have been cultivated either in small batch cultures and continuous cultures or bioreactors, have reported reasonable carbohydrate accumulation in municipal wastewater-grown microalgae, ranging from low (~11 % dcw) to moderate (21.9- 42.6 % dcw) carbohydrate content (Table 2.2). These studies suggested that the utilization of municipal wastewater might have future promise to serve as a cheap nutrient source for microalgal biomass production. The wastewater-grown microalgal cells may serve the dual role of nutrient reduction and cost-effective feedstock for bioethanol production.

Table 2.1 Characteristics of wastewater used for the cultivation of microalgae.

Wastewater type	Wastewater source	COD (mg L ⁻¹)	TN (mg L ⁻¹)	NH ₄ ⁺ -N (mg L ⁻¹)	Nitrates (mg L ⁻¹)	PO ₄ ³⁻ -P (mg L ⁻¹)	TP (mg L ⁻¹)	pH	References
Municipal wastewater	Primary effluent	142	27.7	nd	nd	1.59	nd	nd	(Zhang <i>et al.</i> , 2013)
	Centrate	2304	116.1	82.5	nd	nd	212	nd	(Li <i>et al.</i> , 2011)
	Centrate autoclaved	2389.5	132.3	82.6	nd	nd	215.1	nd	(Li <i>et al.</i> , 2011)
Agricultural wastewater	Swine wastewater	20180	nd	1434.3	16.5	18.69	nd		(Wang <i>et al.</i> , 2015)
	Piggery wastewater	332	348	233	5.5	101.4	nd	7.7	(Marjakangas <i>et al.</i> , 2015)
Industrial wastewater	Brewery wastewater (filtered)	2000-3000	30-45	nd	nd	nd	12-16	5.5-6.0	(Farooq <i>et al.</i> , 2013)
	Brewery wastewater (centrifuged)	100-150	50-75	nd	nd	nd	15-20	6.5-7.5	(Farooq <i>et al.</i> , 2013)
	Dairy industry wastewater	6000	nd	18.45	78.31 5.03	5.58	nd		(Kothari <i>et al.</i> , 2013)
	Soybean processing wastewater	13215	267	52.1	nd	nd	56.3	nd	(Hongyang <i>et al.</i> , 2011)

nd: not determined

Table 2.2 Biomass and carbohydrate production by microalgae using wastewater from municipal, agricultural and industrial sources.

Wastewater type	Wastewater source	Microalgae	Biomass production	Carbohydrate content (% dcw)	References
Municipal					
	Primary effluent (Nitrogen sufficient)	<i>Scenedesmus obliquus</i>	nd	11.7	(Batista <i>et al.</i> , 2015)
		<i>C. vulgaris</i>	nd	27.7	
	Primary effluent (Nitrogen starvation)	<i>Scenedesmus obliquus</i>	nd	42.6	(Batista <i>et al.</i> , 2015)
		<i>Chlorella vulgaris</i>	nd	21.9	
Agricultural	Swine wastewater (5-fold dilution)	<i>Chlorella vulgaris</i> JSC-6	3.96 g L ⁻¹	58.3	(Wang <i>et al.</i> , 2015)
	Dairy wastewater	<i>Chlorella zofingiensis</i>	~0.35 g L ⁻¹	~25	(Huo <i>et al.</i> , 2012)
Industrial					
	Soy whey	<i>Chlorella vulgaris</i>	6.3 g L ⁻¹	6.64	(Mitra <i>et al.</i> , 2012)
	Thin stillage	<i>Chlorella vulgaris</i>	9.3 g L ⁻¹	15.48	(Mitra <i>et al.</i> , 2012)
	Brewery wastewater	<i>Chlorella vulgaris</i> UTEX and <i>Chlorella</i> sp.	2.28 g L ⁻¹	nd	(Farooq <i>et al.</i> , 2013)
	Soybean processing wastewater	<i>Chlorella pyrenoidosa</i>	0.64 g L ⁻¹ day ⁻¹	nd	(Hongyang <i>et al.</i> , 2011)

nd: not determined

2.4.2. Agricultural wastewater

The agricultural sector is considered the largest user of water in several parts of the world, including South Africa. This sector, therefore, generates copious amounts of wastewater, which poses serious environmental challenges globally. Compared to municipal and industrial wastewater, agricultural wastewater can be significantly high in nutrients such as nitrogen and phosphorus as well as organic matter in both soluble and insoluble forms, that are derived from manure and animal waste (Table 2.1). The main source of nitrogen in most agricultural wastewater is ammonium, which has been shown to constitute almost half of the total nitrogen content. The high level of ammonium present in agricultural wastewater e.g., piggery wastewater can be due to agricultural activities such as animal diet, age, productivity, and management (Cai *et al.*, 2013). In spite of the high nutrient concentration, studies have shown that microalgae would be potential candidates with efficient growth in agricultural wastewater, particularly benthic microalgal species due to their high nutrient tolerance and rapid nutrient removal rates (Kwon *et al.*, 2013).

Table 2.2 summarizes recent literature on microalgal growth and carbohydrate production on different types of wastewaters. The properties of agricultural wastewater show a great potential for their use for microalgal biomass and carbohydrate production. The utilization of this wastewater resource could be beneficial for countries with intensive agricultural activities. The growth mode (phototrophic, heterotrophic or mixotrophic) is another key factor affecting algae biomass production when grown on high turbid and high nutrient strength wastewater such as agricultural wastewater. Phototrophic growth in agricultural wastewater is often limited due to insufficient light supply. According to recent reports (Kwon *et al.*, 2013; Wang *et al.* 2015), agricultural wastewater, particularly dairy and swine effluents are rich in carbohydrates and organic acids (acetic acid, propionic acid and butyric acid) which are ideal carbon sources to be utilized for growth by some heterotrophic or mixotrophic microalgal strains. Wang *et al.* (2015) have demonstrated that using microalga *Chlorella vulgaris* JSC-6 cultivated on swine wastewater under mixotrophic growth conditions showed the advantage of nutrient removal and highest productivities of biomass and carbohydrates. The microalgal biomass and carbohydrate could achieve 3.96 g L⁻¹ and 58 % (per dry weight), respectively. It is worth mentioning that the diluted (5-fold dilution) swine wastewater provided an optimal nutrient concentration for *C. vulgaris* JSC-6.

Research conducted to date suggests that growing algae in agricultural wastewater is feasible. Therefore, research work needs to be continued for further development of microalgae biofuel production using agricultural wastewater.

2.4.3. Industrial wastewater

The composition of industrial wastewater is complex. Organic carbon is deficient in industrial wastewater but nitrogen and phosphorus, which are capable of supporting microalgal growth are present but at lower concentrations when compared to municipal and agricultural wastewater (Table 2.1). Industrial wastewaters are commonly considered unsuitable for microalgae cultivation due to their harsh intrinsic properties such as, relatively non-uniform nutrient profile, low pH and high content of heavy metal pollutants, phenols, and organic chemical toxins. In this regard, there are few studies involving microalgae cultivation in industrial wastewater for biomass and biofuel production (Mitra *et al.*, 2012). However, Chinnasamy *et al.* (2010) used carpet mill wastewater supplemented with municipal wastewater (5% (v/v)) and reported considerable quantities of microalgal biomass production. Food industry processing wastewaters such as brewery, cheese processing, soybean processing and chemical fermentation wastewaters are also proving to be promising as shown in Table 2.2.

Recently, Hodaifa *et al.* (2008) cultivated *S. obliquus* in olive oil processing wastewater. They reported a growth rate of 0.044 h⁻¹. In a further study by the same authors (Hodaifa *et al.*, 2009), *S. obliquus* was cultivated in three dilutions of oil mill wastewater and a carbohydrate yield of 65.8 % of the dry cell weight was achieved. In another study by Markou *et al.* (2012b), *Arthrospira (Spirulina) plantensis* was cultivated in olive oil mill wastewater (OMWW) treated with sodium hypochlorite (NaOCl). Maximum biomass production of 1696 mg L⁻¹ was obtained when the concentration of OMWW in the cultivation media was 10% with the supplementation of 1 g L⁻¹ NaNO₃ and 5 g L⁻¹ NaHCO₃. The maximum removal of COD and carbohydrates were 73.18% and 91.19%, respectively, while nitrates and phosphorus were completely removed. Overall, screening for robust microalgal strains which easily adapt to various industrial wastewater streams, seems to be the most promising approach for maximal algal biomass production as a biofuel feedstock.

2.5. Factors affecting microalgae growth in wastewater

Commercial production of microalgae biomass utilizing wastewater is possible only if the main parameters are maintained such as high biomass productivity, high carbohydrate content, high carbohydrate productivity, and high nutrient removal efficiency. These important factors can be optimised by response surface methodology (RSM) as opposed to one-factor-at-a-time-approach that is tedious and time-consuming (Ramirez-Lopez *et al.*, 2016). Important factors that are of special concern for the application of wastewater effluents as microalgal growth substrates are: nitrogen, phosphorous, carbon (CO₂),

temperature, light intensity, pH, dissolved oxygen (DO), oxygen demand (chemical and biological), oxidative reduction potential (ORP), conductivity, salinity and metals (Cabanelas *et al.*, 2013; Hena *et al.*, 2015; Li *et al.*, 2011). In the rest of this section, three-dimensional response surface plots and contour plots are used to illustrate the combined effect of the first five listed factors on biomass and carbohydrate production.

2.5.1. Nitrogen and phosphorus

Nitrogen and phosphorus are two fundamental macronutrients required for growth and metabolism by microalgal cells. Nitrogen plays a key role in the synthesis of essential biochemical and biological materials such as proteins and nucleic acids. Nitrogen is highly abundant in wastewater and most of which exist as ammonium (NH_4^+) or nitrate (NO_3^-), depending on the wastewater stream. Phosphorus is also present in wastewater but often at lower concentrations when compared with nitrogen. Being an integral part of essential molecules such as ATP, $\text{NADP}^+/\text{NADPH}$, DNA, RNA, and phospholipids, phosphorus is also a pivotal and important macronutrient. The individual effect and interaction effect of total nitrogen (TN) and total phosphorus (TP) on the microalgal biomass and carbohydrate production are presented in Figs. 2.4(A) and (B), respectively. From Fig. 2.4(A), it can be observed that varying TN and TP concentration mutual interactions has a significant effect on the biomass production value. The increase in TN and TP concentrations is directly related to the increase in the biomass production until a threshold value is reached, wherein the substrate concentration (especially ammonium) becomes growth limiting. This phenomenon has been reported in some studies in the literature. For example, in a study by Cabanelas *et al.* (2013), *Chlorella* sp. gave the highest biomass production (1.52 g L^{-1}) when cultivated in centrate wastewater (centrate III) with ammonium concentration of 466 mg L^{-1} .

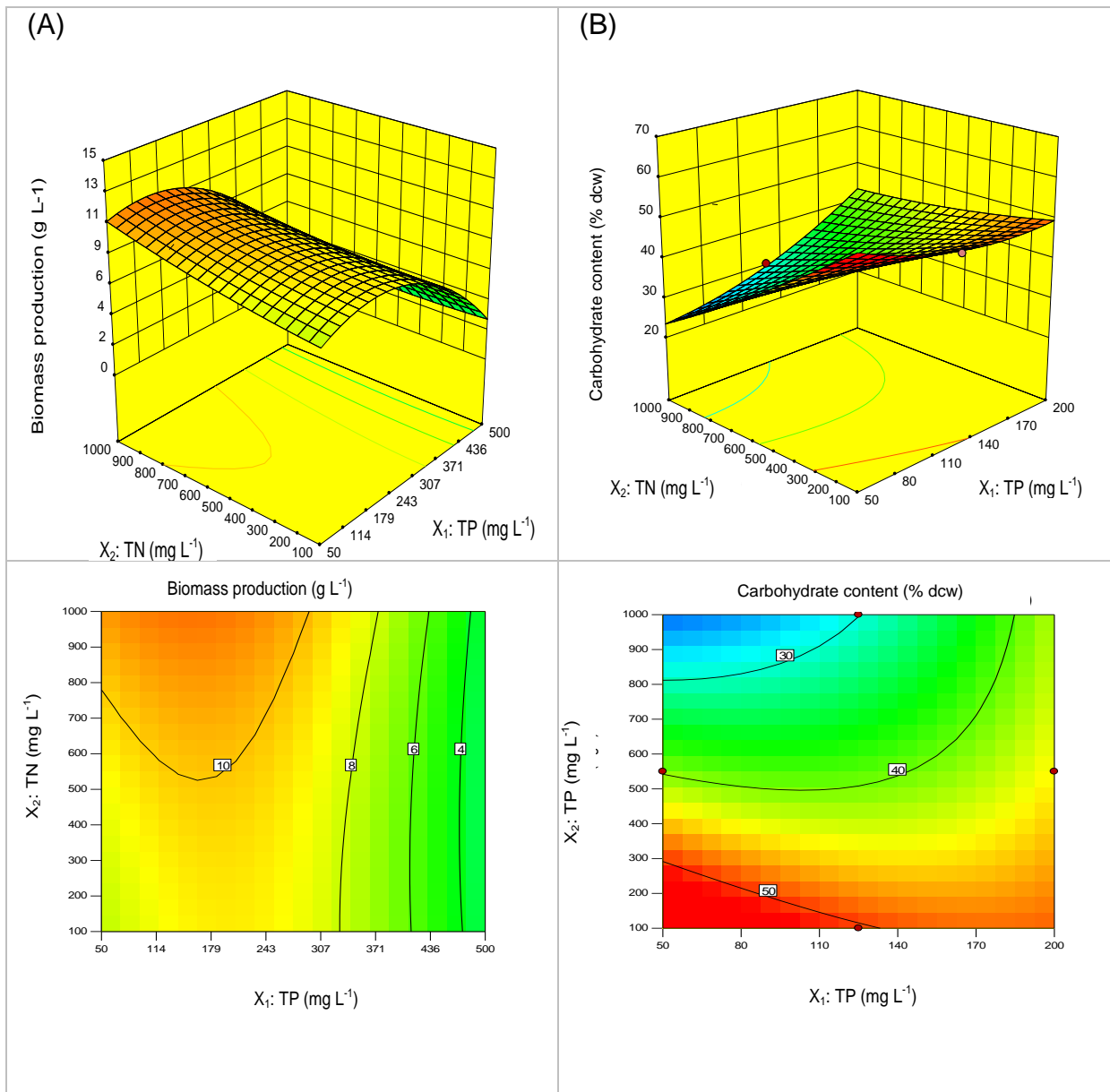


Fig. 2.4. The interactive effect of nitrogen and phosphorus on (A) microalgal biomass production and (B) carbohydrate content (Zhu *et al.*, 2013; Cabanelas *et al.* 2013).

However, they reported a decline of biomass when the cells were cultivated in centrate wastewater (centrate IV) containing higher concentrations of ammonium (905 mg L⁻¹). The concentration of TN of centrate (III) was 471 mg L⁻¹ while it was 909 mg L⁻¹ for centrate (IV). Lincoln *et al.* (1996) cultivated *Spirulina* in medium with diluted (1:1) anaerobically digested cattle manure. The growth of *Spirulina* was rapid and no inhibition was observed in the presence of N-NH₃ at a concentration of less than 75 mg N-NH₃ L⁻¹, but growth was inhibited at concentrations above 100 mg N-NH₃ L⁻¹. In a separate study, Zhu *et al.*, (2013) reported higher yields of *Chlorella zofingiensis* biomass when cultivated in 50% of piggery wastewaters containing higher concentrations of TN and TP.

Fig. 2.4(B) represents the effects of TN and TP levels individually and their mutual interaction on the carbohydrate content of microalgal cells. In contrast to biomass production, the carbohydrate content of microalgal cells is enhanced at low concentrations of both nitrogen and phosphorus (Fig. 2.4(A)). This phenomenon is known as nutrient starvation and is a technique widely used by many researchers to enhance the production of high energy compounds such as carbohydrates and lipids from microalgae (Ho *et al.*, 2013b; Pancha *et al.*, 2014). Under nutrient starvation, the carbon metabolic flux is turned from protein synthesis metabolic pathway to the lipid or carbohydrate synthesis pathway, resulting in the accumulation of either carbohydrate or lipids depending on the strain (Zhu *et al.*, 2013). For example, *C. vulgaris* accumulated carbohydrates up to 38-41% while *Spirulina maxima* accumulated 60-70% of carbohydrates when cultivated under nitrogen starvation (Dragone *et al.*, 2011).

2.5.2. Light intensity and temperature

Numerous studies have reported on the individual effects of temperature and light intensity on the biomass production of microalgae (George *et al.*, 2014; He *et al.*, 2015; Sriram and Seenivasan, 2015). However, a few studies have reported on the combined effect of these factors on biomass and carbohydrate production. Light is the most crucial element for photosynthesis, and together with photoperiod, is a crucial factor for microalgal growth and biochemical composition of microalgae. On the other hand, the temperature is important because it plays a pivotal role in all enzymatic reactions and physiological functions of the microalgal cells. Therefore, it is of great importance to study these factors simultaneously in order to get a better insight on how their interaction affects microalgal cells. Numerous studies analyzing the effect of light and temperature on microalgal growth and carbohydrate production have observed that changes in production yields respond to increments or decrement in light intensity and temperature (Sriram and Seenivasan, 2015). As observed in Fig. 2.5(A), increasing light intensity is viable until a threshold level is reached, further increase of light intensity beyond the threshold level results in a decrease of biomass production, a phenomenon called photoinhibition (Sun *et al.*, 2014). From Fig. 2.5(A) it can be observed that high biomass production is directly related to high temperature, which was also reported by Yang *et al.* (2012) for the growth of *Microcystis aeruginosa*. Similar observations were also made by Ota *et al.* (2015) during photoautotrophic cultivation of *Chlorococcum littorale*.

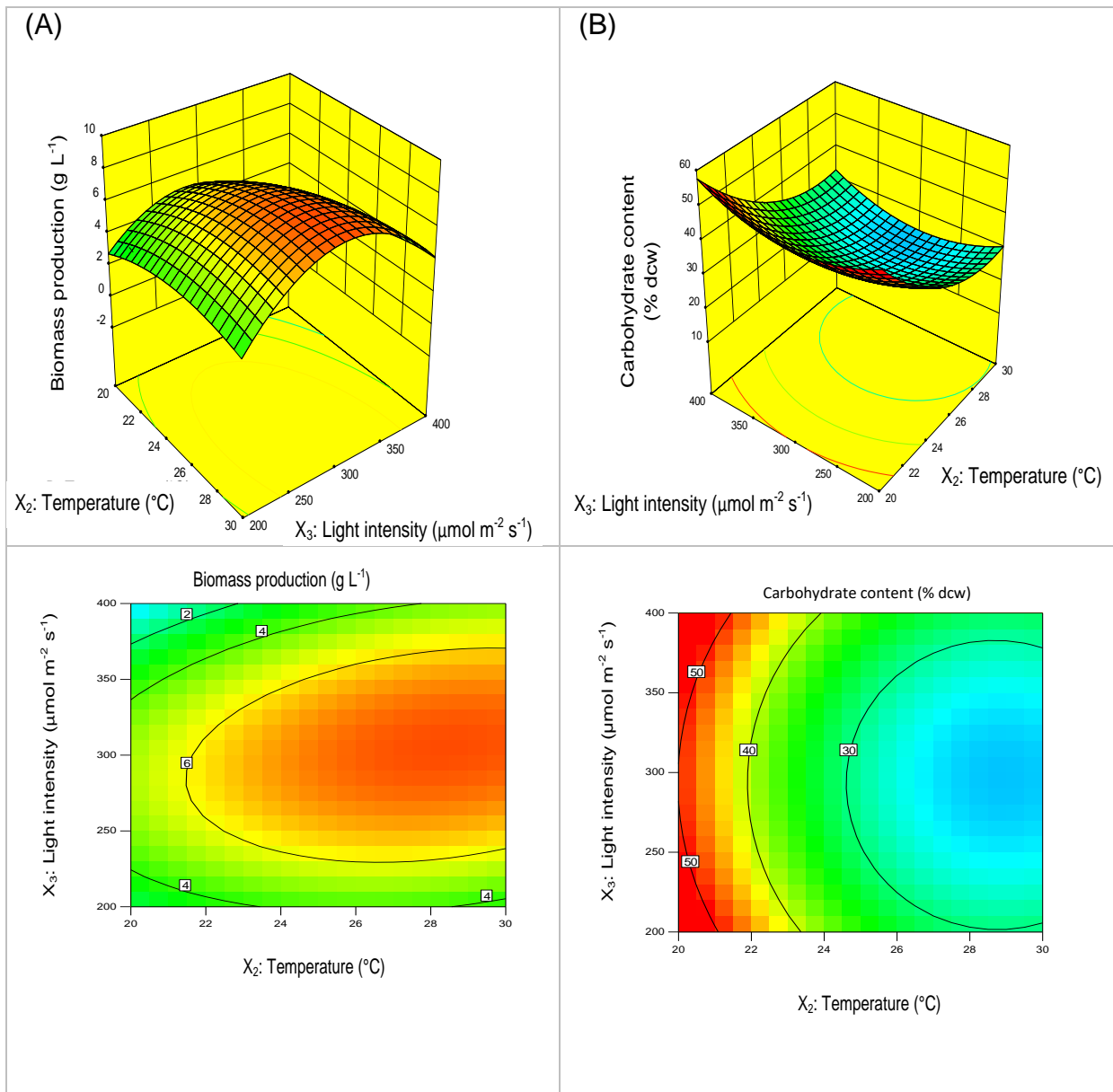


Fig. 2.5. The interactive effect of temperature and light intensity on (A) biomass production and (B) carbohydrate content (Mezhoud *et al.*, 2014; Wang *et al.*, 2016b).

In Fig. 2.5(B), it can be observed that high carbohydrate content is directly related to high levels of temperature and light intensity, although contradicting findings are reported in the literature (Cabello *et al.*, 2015; Ota *et al.*, 2015; Wang *et al.*, 2016b). It has been suggested that temperature affects the level at which photoinhibition occurs. For instance, Jensen and Knutsen (1993) reported that low temperatures enhance the light inhibition. However, Nakamura and Miyachi (1982) reported that increased temperatures lead to the degradation of starch. Enzymes that have been reported to play a role in the degradation of starch are α -amylase and α -glucan phosphorylase. Both these enzymes are temperature dependent (Nakamura and Miyachi, 1982). The interactive effect of light intensity and the temperature

was found to be highly influential in the production of the extracellular polymeric substances by *Graesilla* sp. (Mezhoud *et al.*, 2014).

2.5.3. Light intensity and carbon dioxide

During photosynthesis, CO₂ serves as the sole carbon source. Light provides the energy required for the reduction of CO₂ to organic compounds needed for cell growth. An increase in either one of these factors will result in an increase in the photosynthetic rate of microalgal cells, consequently, biomass and carbohydrate content will also increase (Fig. 2.6).

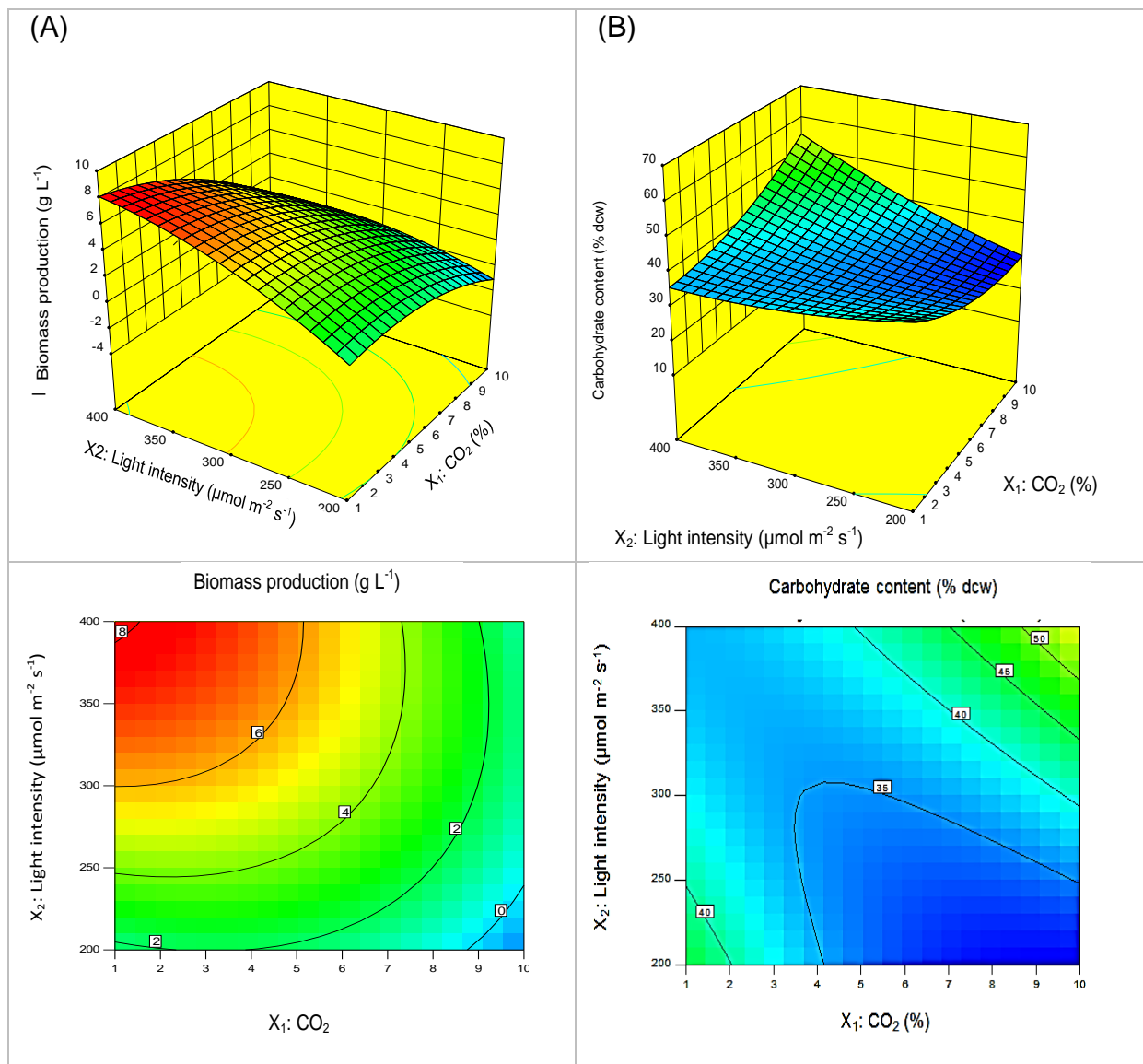


Fig. 2.6. The interactive effect of CO₂ and light intensity on (A) microalgal biomass production and (B) carbohydrate content (Ramanan *et al.*, 2010).

This phenomenon is most likely attributed to the increase in carboxylase activity of RuBisCo triggered by the supply of CO₂ and light (Ramanan *et al.*, 2010). In addition to total light intensity, the light: dark regime and spectral composition of incident light affect strongly the photosynthetic efficiency of microalgal cells. In an early study, Champigny (1985) showed that during irradiance periods, photosynthesis produces sucrose and starch that are consumed for respiration during the dark periods. Takeda and Hirokawa (1979) reported that in *Chlorella*, while the intracellular carbohydrate e.g., starch is consumed during the dark period, hemicellulose and cellulose (extracellular carbohydrates) increase. Miyachi and Kamiya (1978) studied the effect of red (660 nm) and blue (456 nm) light on the carbohydrate production by *Chlorella vulgaris*. The red light of high intensity was observed to incorporate carbon from CO₂ into sucrose and starch synthesis pathways. On the contrary, the application of blue light even at low intensities resulted in a significant decrease in sucrose and starch formation along with increasing levels of amino acids and lipids. In a study by You and Barnett (2004), the red and blue light were reported to increase the growth and polysaccharide production in *Porphyridium cruentum*.

2.6. Microalgae cultivation systems

Microalgae can be cultivated in several different ways. However, adoption of an efficient and cost-effective cultivation strategy on a large-scale basis is mandatory for the success of microalgal biomass propagation as a candidate in the renewable energy sector. Numerous designs for mass algal cultivation have been devised and can be separated into 1) suspended cultures, including raceway ponds and closed photobioreactors, and 2) attached culture systems, including algae turf scrubbers. These systems are discussed in detail in the following sections.

2.6.1. Raceway ponds

Raceway ponds have been the most widely used large-scale algae production systems in practice since their development in the 1950s. Raceway ponds are shallow (no more than 30 cm deep) and open to the environment. Most developed raceway ponds are a rectangular shape with algal culture current flowing from a supply end to an exit or harvesting end (Fig. 2.7(A)). The length to width ratio is an important parameter in designing a raceway pond. Technically, a larger width may result in weak current speed, which is not desirable for microalgal mixing and mass transfer (Richardson *et al.*, 2012). The length and depth of the raceway pond are determined by light penetration and the capacity of culture volume a unit can harbour (Mata *et al.*, 2010). Raceway ponds are also fitted with a paddle wheel that

provides mixing of nutrients and maximizes gas exchange (Fig. 2.7) (Cheng *et al.*, 2015). Raceway ponds have relatively low cost of installation, maintenance and operation. However, the major constraint is the relatively low biomass productivity when compared to closed photo-bioreactors. This can be attributed to a number of factors, including, poor mixing, dark zones and inefficient use of CO₂, evaporation losses and contamination with bacteria and other algal species and predators (Mata *et al.*, 2010). However, achieving high algae biomass production rates is possible with open pond systems, but there are still inconsistencies in the production rates reported in literature (Table 2.3).

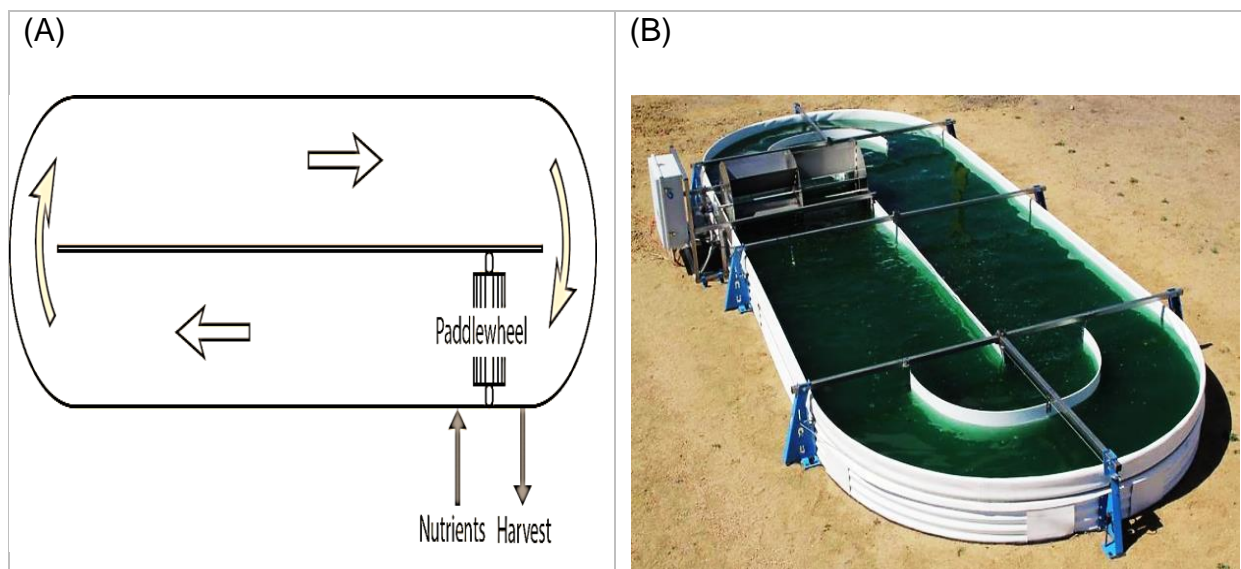


Fig. 2.7. Schematic diagram (A) and real photo (B) of the raceway pond cultivation system (Cheng *et al.*, 2015; Richardson *et al.*, 2012).

2.6.2. Closed photobioreactors

The design of closed photo-bioreactors helps avoid water evaporation, contamination, and increases photosynthesis efficiency, which are the limiting factors associated with open pond systems. Tubular photo-bioreactors are currently the only types of closed systems used for large-scale operations (Fig. 2.8) (Zhu *et al.*, 2013). Vertical, horizontal and helical designs are also common, but helical designs are considered the easiest to scale up (Min *et al.*, 2011). Compared with open ponds, tubular photobioreactors offer more control over culture conditions such as temperature and pH. In addition, closed photo-bioreactors give better mixing and higher cell densities, therefore lowering the costs associated with harvesting (Mata *et al.*, 2010). Reported productivities generally range from 20 to 40 g m⁻² day⁻¹ (Shen *et al.*, 2009). However, despite these benefits, photo-bioreactors have not achieved significant use due to installation and maintenance cost involved.

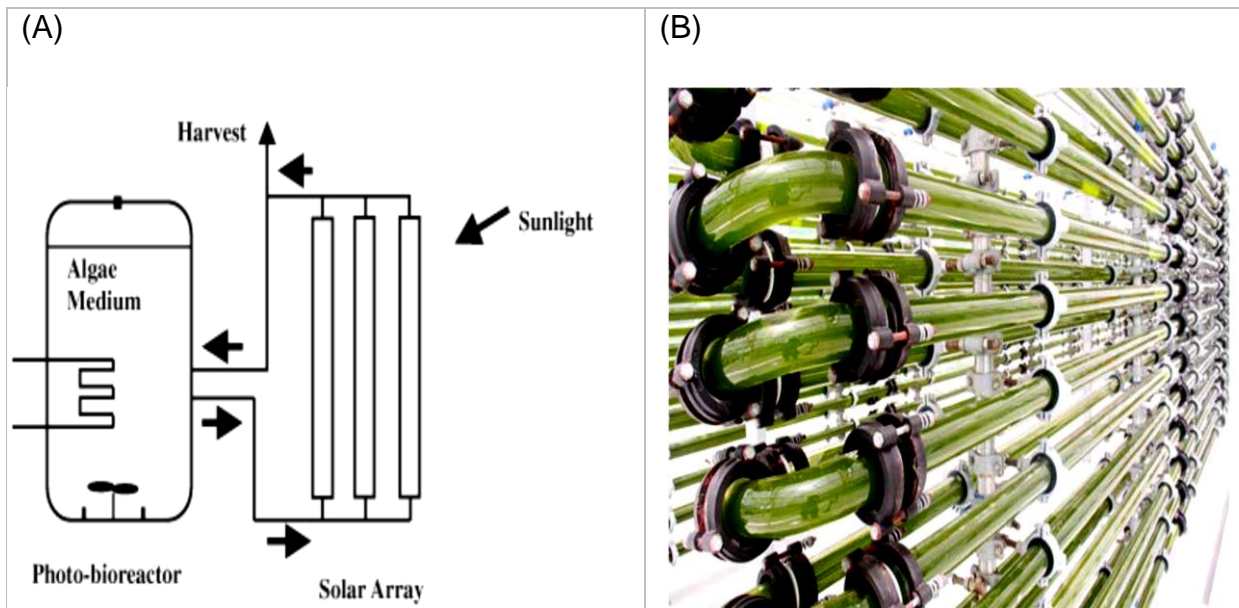


Fig. 2.8. Schematic drawing (A) and real photo (B) of a tubular photobioreactor cultivation system (Zhu *et al.*, 2013).

2.6.3. Attached microalgae cultivation system

Lack of an efficient and cost effective microalgal biomass harvesting method is regarded as one of the key limiting factors impeding commercial microalgal biofuel production systems (Liu *et al.*, 2016; Ray *et al.*, 2015). According to a recent life cycle analysis, harvesting can account for up to 20-30% of the total biofuel production costs (Reyes and Labra, 2016). Current harvesting methods include chemical, mechanical, electrical, and biological techniques. However, the large consumption of chemicals and energy are the major challenges associated with chemical, mechanical and electrical methods (Chatsungnoen and Chisti, 2016; Guldhe *et al.*, 2016).

Algal turf scrubber (ATS) is a controlled ecosystem that relies on keeping the algae in place and bringing the nutrients to it, rather than suspending the microalgae in a culture system (Fig. 2.9). Grown microalgae is harvested by scraping, and thus avoiding the use of settling tanks and subsequent centrifugation, which are used in traditional microalgal-suspended culture systems (Fig. 2.9).

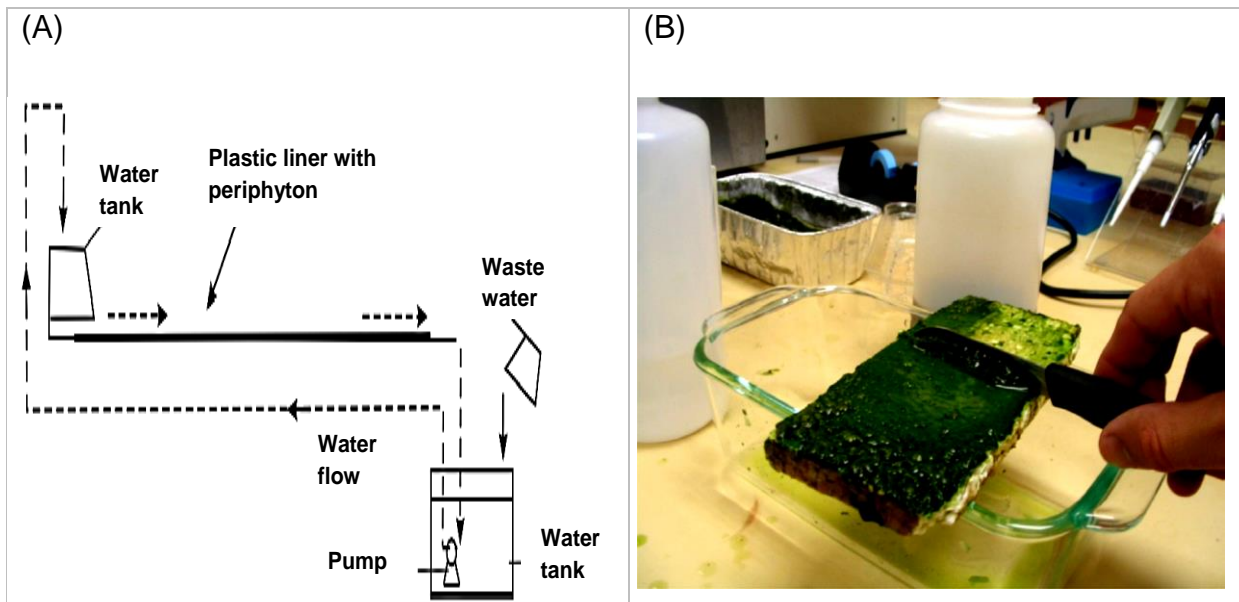


Fig. 2.9. Schematic drawing (A) and real photo (B) of the algal turf scrubber cultivation system (Liu *et al.*, 2016).

ATS systems have already been applied in treating polluted river water, agricultural and dairy manure wastewater with a growing area of 1-1000 m² (Adey *et al.*, 2011; Sandefur *et al.*, 2011). Different materials have been tested as supporting materials for algae attachment. For example, Sekar *et al.* (2004) investigated a great variety of substrates, such as perspex, titanium, stainless steel 316-L, glass, copper, aluminium brass, and admiralty brass. The results demonstrated that the highest attachment occurred on stainless steel and titanium. The remaining material exhibited weaker promotion of microalgal attachment.

Non-suspended cultivation methods could potentially be more commercially feasible than the traditional suspended microalgae cultivation since costs associated with harvesting and water consumption are lower in non-suspended cultivation. Currently no attached system operating at large scale for biofuel production is available; however, promising results have been obtained from laboratory scale experiments (D'Aiuto *et al.*, 2015). For instance, Johnson and Wen (2010) compared the performance of an attached culture system to a suspended culture cultivated under the same conditions. The total biomass produced using the attached cultivation system was 0.34 g while for the suspended cultivation system was 0.25 g. The biomass harvested from the attached growth system (through scraping) had a water content of 93.75%, similar to that harvested from suspended culture system (through centrifugation). Table 2.3 gives a comparison of the performance of attached culture systems and suspended culture systems with respect to biomass productivity, nutrient loading, and nutrient removal.

Table 2.3 Comparative analysis of algae biomass production and wastewater nutrient removal between the suspended cell and attached algae cultivation systems.

Design	Wastewater	Algal strain	Nutrient removal efficiency (%)	Biomass production (g m ⁻² d ⁻¹) ^a , (g L ⁻¹) ^c	References
Raceway pond	Dairy wastewater	<i>C. zofingiensis</i>	TN: 51.7; PO ₄ ³⁻ : 97.5	0.35 ^c	(Huo et al., 2012)
Open pond	Municipal Wastewaters	<i>C. minutissima</i>	NH ₄ ⁺ -N: >45; TP: 30	>0.5 ^c	(Bhatnagar et al., 2010)
Photobioreactor	Municipal wastewater	<i>Chlorella</i> sp.	TN: 61; TP: 61	34.6 TSS; 17.7 VSS	(Min et al., 2011)
Photobioreactor	Urine	<i>Spirulina platensis</i>	NH ₄ ⁺ -N: 99; TP: 99	1.05 ^c	(Yang et al., 2008)
Algal turf scrubber	Dairy manure	<i>Chlorella</i> sp.	TN: 100; TP: 70	2.59 ^a	(Johnson and Wen, 2010)
Algal turf scrubber		Periphyton	NO ₃ : 49; NO ₂ : 19; NH ₄ : 41 PO ₄ : 16	5.5 ^a	(D'Aiuto et al., 2015)

2.7. Microalgal biomass hydrolysis and solubilization

Obtaining bioethanol from microalgal biomass is a multiple step process that mainly involve cultivation, harvesting, pre-treatment, hydrolysis and fermentation, as shown in Fig. 2.10.

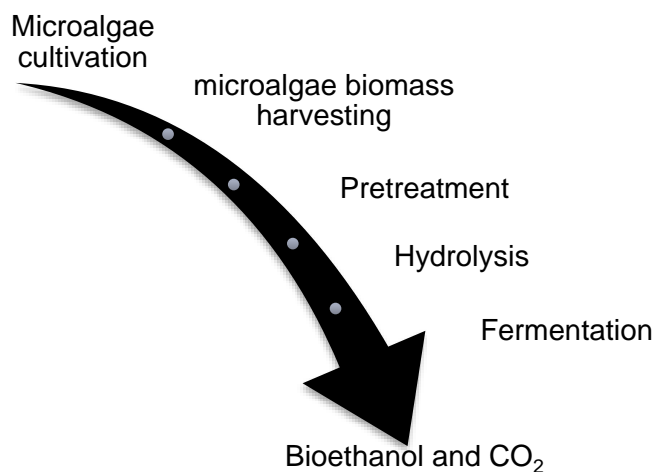


Fig. 2.10. Overall process diagram of bioethanol production by microalgae (Harun *et al.* 2011).

One of the main challenges in bioethanol production from microalgal biomass is to efficiently release fermentable sugars from microalgal cells (Eldalatony *et al.*, 2016). The conversion of native cellulose from the microalgal biomass to fermentable sugar is extremely slow since the cellulosic material is well protected by the cell matrix. Pre-treatment of the biomass enhances the rate of hydrolysis to fermentable sugar as it increases the surface area, enhances the sugar solubility, improves the substrate digestibility and weakens the cell wall for enzymes to be accessible (Miranda *et al.*, 2012). Numerous pre-treatment methods are available for cell disruption. These techniques can be divided into four categories: mechanical, thermal, chemical and biological processes (Fig. 2.11). A feasible cell disruption method should be established to ensure a low operating cost, high product recovery, and high quality of the recovered carbohydrates. The following sections summarise the state of the art on pre-treatment techniques for improving microalgal cell disruption for carbohydrate extraction.

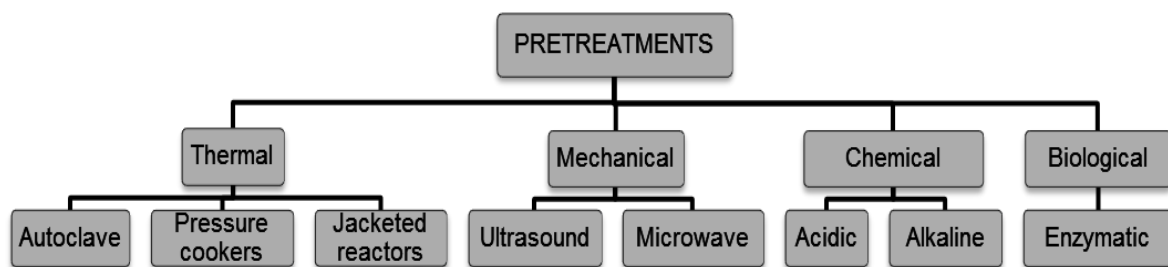


Fig. 2.11. Pre-treatment methods for improving microalgae carbohydrate extraction for the subsequent production of bioethanol (Choi *et al.*, 2011; Hernández *et al.* 2015).

2.7.1. Mechanical pre-treatment methods

2.7.1.1. Ultrasonication pre-treatment

During ultrasonication pre-treatment, high frequencies of ultrasonic waves are emitted and result in the formation of cavitation inside the cells and regions with liquid vapour, so-called microbubbles are formed. Depending mainly on the ultrasound frequency these microbubbles burst and emit high hydrodynamic shear forces which contain enough energy to shatter the microalgal cell walls. Ultrasound can be applied at both high and low frequencies. High frequencies promote the synthesis of free radicals while low frequencies favour mechanical effects. In addition to the ultrasound frequency, other factors that influence ultrasonication pre-treatment of microalgal cells include biomass concentration, output power, exposure time and temperature (Choi *et al.*, 2011). In particular temperature is vital since it affects vapour pressure inside the cell, thus, the lower the temperature, the lower the pressure and efficiency of pre-treatment (Choi *et al.*, 2011).

2.7.1.2. Microwave pre-treatment

Microwaves are short waves of electromagnetic energy varying in a frequency from 0.3 to 300 GHz, industrial and domestic microwave ovens can operate at up to 2.45 GHz. During microwave pre-treatment, the irradiation of the waves increases the kinetic energy of the water leading to a boiling state, the process causes rapid heat and pressure generation in the microalgal cell that produces cell burst. Similarly to ultrasound, the main controllable parameters influencing microwave pre-treatment efficiency are biomass concentration, irradiation frequency and exposure time.

Hernández *et al.* (2015) tested the carbohydrate extraction efficiency of microwave pre-treatment on three different microalgal strains. The recorded carbohydrate yield values were 21 mg g⁻¹ DW for *Chlorella sorokiniana*, 8 mg g⁻¹ DW for *Nannochloropsis gaditana*, and less than 2 mg g⁻¹ DW for *Scenedsmus almeriensis*. In another study, microwave pre-treatment was shown to enhance the extraction of major structural components such as amylose and cellulose from microalgae biomass (Budarin *et al.*, 2012). Whenever microwaves are chosen for pre-treatment, the sensitivity of the targeted cell compartment to irradiation must be first consideration since this method can damage cell organelles and other biological molecules (Miranda *et al.*, 2012). Other important considerations are scale-up and maintenance costs.

2.7.2. Thermal pre-treatment

2.7.2.1. Autoclave treatment

Thermal pre-treatment is often carried out in autoclaves, pressure cookers or jacketed reactors at temperatures higher than 100 °C, with gradual pressure release after pre-treatment. At this temperature range, generally, shorter pre-treatment times (15-30 min) are used in comparison with low temperature pre-treatment (3-24 h). Thermal pre-treatment has been proved successful in the degradation of microalgal cells; the heat generated breaks up the hydrogen bonds in the crystalline complexes of cellulose, causing the cells to open, thus causing solubilization of internal constituents such as starch (Hernández *et al.*, 2015). The effectiveness of thermal pre-treatment depends on the characteristics of microalgae biomass, such as toughness and structure of the cell wall. In a study by (Hernández *et al.*, 2015), the autoclave was used to pre-treat *C. sorokiniana*, *N. gaditana*, and *Scenedsmus almeriensis*. They reported yields of lower than 7 mg g⁻¹ DW regardless of the microalgal biomass and autoclave time used. These results were in agreement with those reported by Miranda *et al.* (2012), who obtained sugar release yields below 5 mg equivalent glucose g⁻¹ DW at 120°C for 30 min using the microalgae *S. obliquus*. Recent studies have opted for the application of the combined thermal and chemical pre-treatment for higher carbohydrate extraction from microalgae instead of thermal pre-treatment alone (Choi *et al.*, 2016).

2.7.3. Chemical pre-treatment methods

2.7.3.1. Acid pre-treatment

Acid pre-treatment is one of the most widely used methods among the chemical pre-treatment methods. It involves the use of organic and inorganic acids; however, inorganic acids are generally preferred due to their rapidness and effectiveness. The two most widely used inorganic acids for carbohydrate hydrolysis are HCl and H₂SO₄. They can be utilized in

the concentrated form (Wijaya *et al.*, 2014) or they can be diluted to desirable concentrations (Ho *et al.*, 2013a). The major drawback of using concentrated acids is that they are highly corrosive and can contribute heavily to the total cost of production. Diluted acids, on the other hand, have been suggested to have greater potential for wide scale applications. In addition, it has been shown to be effective in the pre-treatment of various microalgal biomasses. Recently, acid catalyzed pre-treatment was used for the treatment of lipid extracted biomass of *Scenedesmus* sp. (Pancha *et al.*, 2016). Using 0.5 M of HCl for 45 min at 121°C resulted in the highest saccharification yield of 37.87% (w/w) (Pancha *et al.*, 2016). In another study, Nguyen *et al.* (2009) reported a maximum glucose release of 58% from *Chlamydomonas reinhardtii* UTEX 90 after pre-treatment with 3% sulphuric acid at 110°C for 30 min. Harun *et al.* (2011) reported that using 1% sulphuric acid solution at 140 °C for 30 min as a pre-treatment strategy was effective in the decomposition of the cell walls of *Chlorococcum humicola*. Bioethanol yields were increased from 16% to 52 % (g bioethanol/ g microalgae). Even though dilute acid pre-treatment can significantly enhance microalgal cell digestibility, the use of chemicals may contaminate downstream products and processes.

2.7.3.2. Alkaline pre-treatment

Alkaline pre-treatment uses bases, such as ammonium, calcium, potassium and sodium hydroxide (Harun *et al.*, 2011; Martin Juarez *et al.*, 2016). It is hypothesized that the alkaline pre-treatment mechanism involves saponification of intracellular ester bonds crosslinking xylan, hemicellulose, and lignin. Furthermore, it has been hypothesized that alkaline pre-treatment eliminate acetyl and numerous other uronic acid substitutions on hemicellulose that prevents the hydrolytic enzymes to the hemicellulose and cellulose surface (Wi *et al.*, 2015). In a study by Harun *et al.* (2011), *Chlorococcum infusionum* was pre-treated with NaOH for bioethanol production. They reported a yield of *C. infusionum* glucose of 350 mg/g, and the maximum bioethanol yield of 0.26 g ethanol/ g algae. The concentration of NaOH used was 0.75% (w/v) and the reaction was performed using an autoclave system operated at 120 °C for 30 min. In a study of Kassim and Bhattacharya (2016) the effect of different bases viz. NaOH, Na₂CO₃, and NH₄OH on the pre-treatment efficiency of *Tetraselmis suecica* and *Chlorella* sp. was evaluated. They reported a maximum reducing sugar concentration of 81 mg g⁻¹ DW for *T. suecica* biomass after pretreatment with 2% (w/v) of KOH at 120 °C for 120 min. Meanwhile, the reported maximum reducing sugar concentration from *Chlorella* sp. biomass was 88mg g⁻¹ DW after the cells were pre-treated with 2% (w/v) of NaOH at 120 °C for 30 min. In summary, results on alkaline pre-treatment

of microalgae are scarce and sometimes contradictory. Even so, the combination of thermal and alkaline pre-treatments seems promising.

2.7.4. Enzymatic hydrolysis of microalgal polysaccharides

The advantage of using microalgae as a source of carbohydrates for fermentation is mainly the absence of lignin. Therefore, the lignin degrading enzymes such as laccase, lignin peroxidase and xylanase are avoided ([Fernández-Rodríguez *et al.*, 2017](#)). As mentioned earlier, microalgal carbohydrates are mainly composed of cellulose and starch. Cellulose is hydrolysed into D-glucose monomers by three main enzymes namely, endo- β -1,4-D-glucanase (EC 3.2.1.4), exo- β -1,4-D-glucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.2). Endo- β -1,4-D-glucanase first attacks the amorphous cellulose and cleaves cellulose into small fragments. Exo- β -1,4-D-glucanase hydrolyses the small fragments liberated by end- β -1, 4-D-glucanase into simple sugars such as cellobiose and cellodextrin. Finally, β -glucosidase hydrolyses cello-oligosaccharides into glucose ([Soni *et al.*, 2010](#)). Starch, on the other hand, is hydrolyzed mainly by amylase and glucoamylase. Amylase first attacks the internal α -1,4-glycoside bond of starch to produce dextrin. The liberated dextrin is then further hydrolyzed into D-glucose by glucoamylase ([Alvira *et al.*, 2010](#)).

Enzyme hydrolysis often results in higher yields of glucose without sugar degradation products or toxic by-products that may affect fermentation. Consequently, bioethanol produced from enzymatically hydrolyzed microalgal biomass is often higher when compared with the chemically hydrolysed microalgal biomass ([Table 2.4](#)).

Table 2.4 Reducing sugar and bioethanol yields via different hydrolysis methods.

Algal species (Substrate)	Pre-treatment	Sugar released	Hydrolysis type	Fermenting microorganism	Bioethanol yield	Reference
<i>Ulva prolifera</i>	H ₂ O ₂ and Cellulase	Glucose 0.42 g g ⁻¹	SHF	<i>S. cerevisiae</i>	13.2 g 100 g ⁻¹ biomass	(Li et al., 2016)
	Cellulase	Glucose 0.18 g g ⁻¹	SHF	<i>S. cerevisiae</i>	5.8 g 100 g ⁻¹ biomass	(Li et al., 2016)
<i>Chlorella vulgaris</i>	Cellulase and Amylase	Glucose 7.78 g L ⁻¹	SHF	<i>Z. mobilis</i>	0.178 g g ⁻¹ biomass	(Ho et al., 2013a)
	Cellulase and Amylase		SSF	<i>Z. mobilis</i>	0.214 g g ⁻¹ biomass	
<i>Ulva fasciata Delile</i>	Hot buffer and Viscozyme ® L	Reducing sugar ~206 mg g ⁻¹ biomass	SHF	<i>S. cerevisiae</i>	0.45 g g ⁻¹ reducing sugar	(Trivedi et al., 2013)
<i>Desmodesmus</i> sp.	H ₂ SO ₄	Total sugars 55.3 g L ⁻¹	SHF	<i>S. cerevisiae</i>	0.23 g g ⁻¹ biomass	(Sanchez Rizza et al., 2017)
SP2-3	H ₂ SO ₄	Total sugar 72.9 g L ⁻¹	SHF	<i>S. cerevisiae</i>	0.24 g g ⁻¹ biomass	(Sanchez Rizza et al., 2017)

SHF-Separate hydrolysis and fermentation.

SSF-Simultaneous hydrolysis and fermentation.

2.8. Fermentation of sugars to produce bioethanol

A wide variety of organic compounds can be fermented, and sugars, especially hexoses such as glucose, are excellent examples. A common pathway for the fermentation of glucose, glycolysis, also called the *Embden-Meyerhof* pathway, is widely distributed among the yeast species such as *Saccharomyces cerevisiae*. A variant of the glycolytic pathway called the *Entner-Doudoroff* pathway is one other pathway for sugar catabolism, which exists mainly in bacteria such as *Zymomonas mobilis*. In both pathways, glucose is converted into bioethanol and CO₂ but the *Entner-Doudoroff* pathway has a higher thermodynamic driving force than the *Embden-Meyerhof* pathway due to its lower ATP yield, and it also requires approximately 3.5-fold less enzymatic protein to achieve the same glycolytic flux as the *Embden-Meyerhof* pathway (Flamholz *et al.*, 2013). As shown in Table 2.4, several algal species are proficient candidates for bioethanol production due to their high glucose yields. The following sections cover the different fermentation processes used for the conversion of microalgal carbohydrates into bioethanol.

2.8.1. Simultaneous saccharification and fermentation (SSF)

In the traditional separate hydrolysis and fermentation (SHF) approach, enzymatic hydrolysis is allowed to proceed in its optimum conditions in a separate unit and to produce a high glucose yield prior to fermentation (Fig. 2.12) (Costa *et al.*, 2015). However, the main drawback of this approach is the inhibition of cellulase and α -amylase activity caused by the sugar released in the hydrolysis stage. This challenge can be resolved in SSF, which couples saccharification and fermentation into a single step. The advantage of the SSF is that the hydrolysis products such as glucose do not inhibit enzyme activity due to immediate and simultaneous fermentation (Fig. 2.12). SSF also offers a shorter reaction time and is less prone to contamination due to the elimination of some steps. Furthermore, some studies have shown that the overall bioethanol yield in SSF can be considerably higher when compared with SHF (Wingren *et al.*, 2003).

The major drawback of the SSF process is that fermentation and hydrolysis are not performed under optimum conditions. However, strategies to remedy this challenge have already been developed. For example, Goshadrou *et al.* (2013) reported an approach called non-isothermal simultaneous saccharification and fermentation (NSSF), in which enzymatic hydrolysis is briefly performed under optimum temperature and after inoculating the media, the temperature is adjusted to a suitable value for the fermenting organism. This process addresses the difference between the optimum temperatures of the hydrolyzing enzymes and fermenting microorganisms (Goshadrou *et al.*, 2013).

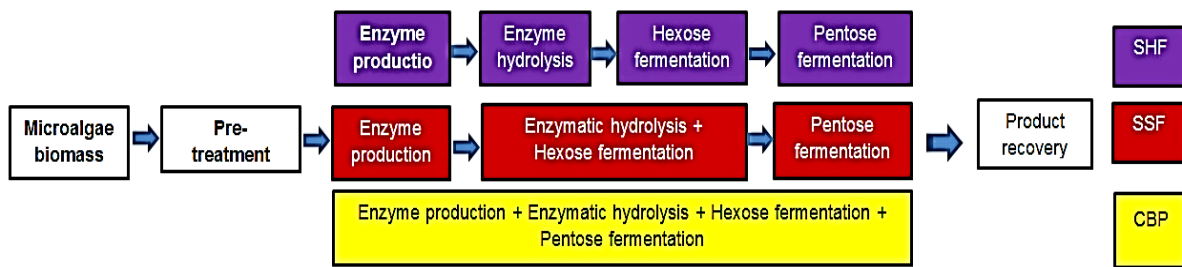


Fig. 2.12. Various bioprocessing strategies for the conversion of microalgal biomass. Abbreviations: SHF, separate hydrolysis and fermentation; SSF, simultaneous saccharification and fermentation; CBP, consolidated bioprocessing (Costa *et al.*, 2015; Kumagai *et al.*, 2014).

2.8.2. Consolidated bioprocessing (CBP)

The conversion of microalgal biomass into fermentable sugars suitable for bioethanol production could account for about two-third of the total production cost, of which the enzyme contribute the largest cost (Parisutham *et al.*, 2014). This cost constraint could be overcome by employing genetically engineered robust biocatalysts that combine pre-treatment, saccharification, and fermentation, within a single reactor, so-called consolidated bioprocessing (CBP) (Kumagai *et al.*, 2014). CBP could potentially also resolve the problem of sugar inhibition experienced in the conventional systems (SHF) since sugar is readily fermented into bioethanol before its concentration becomes inhibitive to hydrolysis (Schuster and Chinn, 2012). However, CBP is still in its infancy stages, the limiting factor for its application is finding robust microorganisms capable of CBP with high efficiency. Up to now no wild-type microorganism capable of efficient CBP has been identified, however, one bacterium *Chlostridium thermocellum*, is a strong candidate for CBP, due to its natural ability to rapidly hydrolyse cellulose using its cellulosome and produce bioethanol (Akinosho *et al.*, 2014).

2.9. Conclusion

Microalgae can be very effective in treating various wastewater streams, reducing considerable amounts of nitrogen and phosphorus. The produced biomass which may be rich in carbohydrates can be effectively harvested, pre-treated and hydrolyzed into fermentable sugars for bioethanol production. The cultivation conditions can be manipulated in order to increase the carbohydrate productivity of microalgae and hence the bioethanol yields. In this regard, it can be concluded that microalgae hold great future as the alternative source of renewable energy such as bioethanol.

Chapter 3. Microalgal isolates indigenous to various aquatic environments in KwaZulu-Natal (South Africa): Growth kinetics, physiological function and biochemical characterization for bioethanol production

3.1. Abstract

Microalgae are a major natural source for a vast array of valuable compounds as lipids, proteins, carbohydrates, pigments among others. Despite many applications, only a few species of microalgae are cultured commercially because of poorly developed of cultivation process. Nowadays some strategies of culture have been used for enhancing biomass and value compounds yield. In this study, a two-stage cultivation strategy was adopted to improve carbohydrate content and productivity of six indigenous microalgal strains belonging to the genera: *Chlorella*, *Chlamydomonas*, *Chlorococcum* and *Neochloris*. In the first stage, the microalgae were cultivated in nitrogen replete BG-11 medium to improve biomass yields. The highest biomass was recorded for the strain *Neochloris aquatica* (Toti RS7) at $816 \pm 0.77 \text{ mg L}^{-1}$. This strain also demonstrated the highest photosynthetic activity based on its high relative electron transport rates (rETR) of 40.3 units. In the second stage, nitrogen starvation resulted in stagnated cell growth and physiological stress, as demonstrated by the decrease in the maximum quantum efficiency (F_v/F_m) (18% to 86%) and an increase in non-photochemical quenching (NPQ) (58% to 81%) by the microalgal strains. *Chlorella vulgaris* (Toti RS4) demonstrated the highest peak intensity (OD~0.3 units) in the 1200-940 cm^{-1} band region of the Fourier transform infrared spectroscopy (FT-IR) and was found to be the highest carbohydrate producer (51% dcw). Up to 90% of *C. vulgaris* (Toti RS4) carbohydrates were attributed to glucose, making it an ideal strain for bioethanol production.

Keywords: Bioethanol; *Chlorella vulgaris*; Carbohydrates; rETR, F_v/F_m ; NPQ; FT-IR.

3.2. Introduction

Microalgae-based biofuels have been touted as the global panacea to the overreliance on fossil-based fuels due to attractive environmental benefits and high productivity associated with microalgae (Borines *et al.*, 2013; Chen *et al.*, 2014; Chisti, 2008). However, the development of products such as bioethanol from microalgae is still in its infancy since the production cost is currently higher than fossil based fuels (Do Nascimento *et al.*, 2012). Overcoming challenges associated with upstream processing such as strain selection,

cultivation systems and maximising carbohydrate yields as well as downstream processes such as biomass harvesting and carbohydrate extraction may potentially increase bioethanol production in the future (Srinophakun *et al.*, 2017). Maximising microalgal carbohydrate content and biomass productivity has been identified as one of the greatest potential targets for cost reduction and research in this direction is currently receiving global attention (Pancha *et al.*, 2015; Sun *et al.*, 2014). Under normal growth conditions (without nutritional or physical stress), microalgal biomass mainly consists of proteins, carbohydrates and lipids. Accumulation of energy-rich storage reserves such as starch and neutral lipids, mainly in the form of triacylglycerols (TAGs), occurs in numerous microalgae under stressful growth conditions such as nitrogen starvation (Chokshi *et al.*, 2015; Pancha *et al.*, 2014; Takeshita *et al.*, 2014). Thus, the greater understanding of the microalgal cell's physiological response with respect to abiotic influences is fundamental for the selection of appropriate microalgal strains.

Conventional methods (e.g., light microscopy and spectrophotometry) can identify the shifts in the growth and assemblage composition of the microalgal cells caused by external cues. However, these methods elucidate the final outcomes but do not reveal the underlying mechanisms such as cellular physiological response that trigger metabolite production via several pathways. Pulse amplitude modulation (PAM) fluorometry can serve as a rapid and sensitive technique for analysis of the physiology of cells in response to changes in the environmental conditions (Ramanna *et al.*, 2014). The key parameters measured by PAM fluorimeters include the relative electron transport rate (rETR), the maximum quantum efficiency (F_v/F_m), and non-photochemical quenching (NPQ). The rETR gives an estimate of the number of electrons passing through photosystem II (PS II) and so can be used to accurately estimate the photosynthetic capacity of the microalgal strains (Ritchie and Mekjinda, 2016). The F_v/F_m ratio estimates how the light absorbed is utilized by PS II (Jiang *et al.*, 2012). There have been some mixed views as to what the non-photochemical quenching parameter actually measures (Ramanna *et al.*, 2014; White *et al.*, 2011). However, in studies with proton ionophores and inhibitors of electron transport, it has been shown that this parameter is related to the size of the proton motive force present across the thylakoids in the chloroplast and has also been correlated with the production of storage compounds in microalgal cells (White *et al.*, 2011).

The microalgal biomass composition is also crucial in understanding the biotechnological application of the microalgal species. With the knowledge that bioethanol is derived from carbohydrates, microalgae with high photosynthetic rates and high carbohydrate content are desirable in the rapidly expanding field of microalgal biotechnology. Several conventional methods have been used to analyse the biochemical composition of microalgal cells (Chen

and Vaidyanathan, 2013; Martin Juarez *et al.*, 2016). However, most of the methods have various drawbacks such as being time-consuming and requiring complicated sample preparation. Fourier transform infrared spectroscopy (FT-IR) is an attractive technique because it offers the potential to analyse the key functional groups present in the biochemical compounds of the microalgal biomass within a short space of time and without the need for cell disruption (Dean *et al.*, 2010; Kose and Oncel, 2015).

The aim of this study was to bioprospect for indigenous hyper-carbohydrate producing microalgae and establish their potential application as feedstock for bioethanol production. The strains were characterized by their growth kinetics, physiological response and biochemical composition under nitrogen replete and deplete conditions. The physiological responses of the isolated microalgal strains were studied using a PAM fluorometer, while FT-IR was used to determine the changes in the biochemical composition of the cell in response to nitrogen availability. Qualitative thin layer chromatography (TLC) was used to study the composition and profiles of the microalgal carbohydrates.

3.3. Materials and Methods

3.3.1. *Microalgal strains and sampling sites*

Wild-type autochthonous microalgae were isolated from various aquatic environments such as freshwater, brackish water and wastewater in KwaZulu-Natal, South Africa during the winter months from June to August 2015 (Table 3.1). The major criterion for sample collection was based on the presence of abundant algal blooms in the water, indicating the existence of viable microalgal cells (Selvarajan *et al.*, 2015). The spatial and temporal sampling strategy was adopted and samples were collected from two depth levels to allow the collection of species which prefer different light intensities. The sampling sites were characterised by measuring the key physicochemical parameters such as temperature, pH, and salinity using an inoLab IDS meter, WTW (Model Multi 9310, Italy). The light intensity was measured using an HD 2102.2 photometer (Delta OHM, Italy). A fine mesh net of pore size of 100 μm was used to screen any potential grazers i.e. protozoa from the sampling site. The samples were transported to the laboratory in plastic bottles in dark and refrigerated conditions.

3.3.2. *Enrichment, isolation, purification and maintenance of microalgal cultures*

Microalgae samples were processed within 2-5 h after sampling. The samples were filtered through a Whatman filter paper with a pore size of 0.45 μm to remove any solid debris. Ten

millilitres of the filtered samples were transferred to 250 mL conical flasks and were enriched with 100 mL of sterile BG-11 medium, Bold's Basal medium (BBM) or f/2 medium. The microalgal samples were incubated under ambient CO₂ at 25 ± 2 °C with 12h:12h light-dark photoperiod at a light intensity of approximately 37 μmol m⁻² s⁻¹ (illuminated using cool white fluorescent light) for 24 days. The flasks were manually shaken twice daily to prevent sticking and biomass settling.

The f/2 medium was prepared according to the recipe by [Guillard et al. \(1962\)](#), with the following composition (g L⁻¹): NaNO₃, 0.75; NaH₂PO₄·H₂O, 0.005; Na₂SiO₃·9H₂O, 0.3; FeCl₃·6H₂O, 0.00315; Na₂EDTA·2H₂O, 0.00436; CuSO₄·5H₂O, 0.0098; Na₂MoO₄·2H₂O, 0.0063; ZnSO₄·7H₂O, 0.022; CoCl₂·6H₂O, 0.01; MnCl₂·4H₂O, 0.18 and vitamins; thiamine hydrochloride (vitamin B1), 0.2; biotin (vitamin H), 0.0001 and cyanocobalamin (vitamin B12), 0.0001. The composition of BG-11 medium was (g L⁻¹): NaNO₃, 1.5; K₂HPO₄, 0.03; MgSO₄·7H₂O, 0.075; citric acid anhydrous, 0.006; Na₂CO₃, 0.02, CaCl₂·2H₂O, 0.036; ammonium iron (III) citrate, 0.006; EDTA·2Na, 0.001 and 1 mL of trace metal solution. The trace metal solution was composed of (g L⁻¹): H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; Na₂MoO₄·2H₂O, 0.39; CuSO₄·5H₂O, 0.079; Co (NO₃)₂·6H₂O, 0.049 ([Rappika et al., 1979](#)). The composition of BBM was (g L⁻¹): K₂HPO₄, 0.075; KH₂PO₄, 0.175; NaNO₃, 0.25; NaCl, 0.025; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.025; EDTA, 0.05; KOH, 0.031; FeSO₄·7H₂O, 0.00498; H₂SO₄, 0.001; H₃BO₃, 0.01142; ZnSO₄·7H₂O, 0.001412; MnCl₂·4H₂O, 0.000232; CuSO₄·5H₂O, 0.000252; Co(NO₃)₂·6H₂O, 0.00008; Na₂MoO₄·2H₂O, 0.000192 ([Tan et al., 2016a](#)).

The collected microalgal samples adapted quickly and best in the BG-11 medium as compared to BBM and f/2 medium. Therefore, BG-11 was used for further experimentation. The microalgal samples were purified into monocultures by serial dilution and plating on BG-11 medium supplemented with 1.5 % (w/v) of bacteriological agar. Axenic cultures were obtained by repeated plating in medium pretreated with 25 μg L⁻¹ of tetracycline to discourage the growth of bacterial contaminants. The individual colonies were isolated and inoculated in sterile BG-11 medium and incubated under ambient CO₂ at 25 ± 2 °C and light intensity of 37 μmol m⁻² s⁻¹ (illuminated using cool white fluorescent light) with 12h: 12h light-dark period for 24 days. Repeated subculturing and microscopic investigation ensured the purity of the culture. Preliminary identification of the microalgal strains was based on morphological characterization by light microscopy using conventional botanical approaches ([Selvarajan et al., 2015](#)) and further confirmed by 18S rRNA gene sequence analysis.

3.3.3. DNA extraction, amplification, sequencing and analysis of 18S rDNA

The microalgal cells were recovered from the culture broth by centrifugation (4 000 x g, for 15 min at 4°C) during the late logarithmic phase using sterile microcentrifuge tubes. The microalgal whole genomic DNA was extracted using Xpedition™ Fungal/Bacterial DNA MiniPrep (Zymo Research, USA), according to the manufacturer's instructions and protocols. The concentration of DNA was measured at 260 nm using a UV-Vis spectrophotometer (Varian 50 bio UV-visible spectrophotometer, Aligent, USA). The eukaryotic primers ChloroF (5' TGG CCT ATC TTG TTG GTC TGT 3' as the forward primer) and ChloroR (5' GAA TCA ACC TGA CAA GGC ACC 3' as the reverse primer), previously described by [Moro et al., \(2009\)](#), were used for the amplification of the 18S rRNA gene. The PCR reactions were carried out in a final volume of 25 µl containing 2 µl genomic DNA, 0.2 mM of each deoxynucleotide (dNTP), 2 U *Taq* DNA polymerase (Inqaba Biotechnical Industries, (Pty) Ltd., South Africa), 1 x PCR buffer, 1 mM MgCl₂ and 0.4 mM of each primer. The following thermal profile was used: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min; and a further 10 min elongation at 72 °C. Amplicons were checked by electrophoresis on 1% agarose gel in 1 x TAE buffer with a positive and negative control, and a DNA marker (Thermo Scientific GeneRuler 1kd DNA Ladder) to determine if DNA of the correct fragment size had been amplified. The successful amplicons were sequenced by Inqaba Biotechnical Industries, (Pty) Ltd., South Africa. The DNA sequence fragments were analysed using Chromas Lite (version 2.1). The 18S rDNA sequences were compared to the nucleotide of the known microalgae in Genbank database of the National Centre for Biotechnology Information (NCBI) using Basic Local Aligned Search Tool (BLAST).

3.3.4. Two-stage cultivation of microalgae

In this study, the two-stage cultivation strategy was adopted for enhancing microalgal carbohydrates for meticulous selection of strains with high carbohydrate content and productivity ([Pancha et al., 2015](#); [Sun et al., 2014](#)). In the first stage, a standardised 10% (v/v) initial cell concentration of 0.03-0.05 (OD_{680nm}) was used as inoculum and cultivation was performed in 250 mL Erlenmeyer flasks containing 100 mL of nitrogen replete BG-11 medium (N= 1.5 g L⁻¹). The cells were incubated under ambient CO₂ at 25 °C and light intensity of 37 µmol m⁻² s⁻¹ in 12: 12 h light: dark photoperiod for 24 days. Following this stage, cells were recovered by centrifugation (4 000 x g for 15 min, 4 °C), aseptically washed thrice with sterile distilled water and directly transferred into nitrogen deficient BG-11 (N= 0 g L⁻¹) medium and incubated under similar conditions for additional 8 days.

3.3.5. Determination of microalgal growth kinetics

The microalgal biomass concentration was determined every second day by measuring the optical density of the samples at 680 nm using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA). The dry cell weight of the microalgae biomass was obtained by harvesting 5 mL aliquots of culture by centrifugation (4 000 x g for 15 min). The cell pellet was washed with distilled water, placed on a pre-weighed watch glass, and dried in an oven at 60 °C for 12 h until constant weight to determine the net mass of the microalgal cells. The OD₆₈₀ values were converted to biomass concentration via calibration between OD₆₈₀ and dry weight.

The specific growth rate (μ) of the microalgal cells was calculated during the logarithmic phase of growth using Eq. (3.1) (Gumbi *et al.*, 2016):

$$\mu = \frac{\ln(X_2 - X_1)}{t_2 - t_1} \quad (3.1)$$

where X_1 and X_2 are the dry cell weight concentration at the start (t_1) and the end (t_2) of the logarithmic growth phase, respectively.

The biomass productivity was calculated during the logarithmic growth phase using Eq. (3.2) (Gumbi *et al.*, 2016):

$$P = \frac{(X_2 - X_1)}{(t_2 - t_1)} \quad (3.2)$$

where X_1 and X_2 are the dry cell weight concentration at the start (t_1) and the end (t_2) of the logarithmic growth phase, respectively.

3.3.6. Measurement of chlorophyll a (chl a) concentration

The chl a content of the microalgal cells was determined using the method described by (Pancha *et al.*, 2014). Two millilitres of the cell culture was harvested by centrifugation (13 000 x g for 5 min) in a sterile microcentrifuge tube and the supernatant was decanted. The cell pellet was resuspended in 2 mL methanol (HPLC grade, $\geq 99.9\%$, Sigma-Aldrich, South Africa) and mixed by vortexing for 5 min and incubated at 45°C for 24 h in dark. After 24 h of incubation, the cell suspension was centrifuged (13 000 x g for 5 min) and the absorbance of the supernatant was read at OD_{665.2} and OD_{652.4} using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA). The concentration of chl a was then determined according to the equation previously reported by Lichtenthaler *et al.* (1987), as shown in Eq. (3.3):

$$\text{chl a } (\mu\text{g mL}^{-1}) = 16.72 A_{665.2} - 9.16 A_{652.4} \quad (3.3)$$

3.3.7. Assessment of microalgal physiological functions using PAM fluorometry

The physiology and photosynthetic efficiency of the microalgal cells were studied using a DUAL-PAM 100 Chlorophyll fluorescence and P700 photosynthetic analyser (Heinz Walz GmbH, 91090 Effeltrich, Germany) equipped with a Dual PAM software (v 1.9). The microalgal cells were dark-adapted for 15 min prior to analysis. The measurements were performed using a 10 mm quartz glass cuvette (10 x 10 x 40) containing a micromagnetic stirrer.

The maximum quantum efficiency of PS II was calculated using Eq. (3.4) as previously described in [Genty *et al.* \(1989\)](#):

$$F_v/F_m = \frac{(F_m - F_o)}{F_m} \quad (3.4)$$

Where F_m , F_o and F_v represents the maximum, minimum and variable fluorescence, respectively. All the measurements were taken from the dark-adapted samples as all PS II reaction centres are open ([Genty *et al.*, 1989](#)).

Non-photochemical quenching (NPQ) was calculated from the maximum fluorescence measured in the dark-adapted samples (F_m) and the maximum fluorescence measured in the light adapted samples (F'_m) as shown in Eq. (3.5) ([Cruz and Serôdio, 2008](#)):

$$\text{NPQ} = \frac{(F_m - F'_m)}{F'_m} \quad (3.5)$$

The dual PAM software calculated the relative electron transport rate (rETR) according to Eq. (3.6) ([White *et al.*, 2011](#)):

$$\text{rETR} = \frac{F'_q}{F'_m * \text{PPFD}} \quad (3.6)$$

Where $F'_q = (F'_m - F')$. $\frac{F'_q}{F'_m}$ is known as the PS II operating efficiency and is used to measure the utility of the light absorbed by PS II ([White *et al.*, 2011](#)). F'_m is the maximum fluorescence in the light adapted samples, F' is the dark fluorescence yield and PPFD refers to the photosynthetically active photon flux density which measures the quantum yield of linear electron flux through PS II.

3.3.8. FT-IR analysis of biochemical constituents of the microalgal strains

The biochemical composition of the finely powdered microalgal biomass was analyzed using an attenuated total reflection- Fourier transform infrared spectroscopy (ATR-FTIR) using an FTIR spectrometer (Model Alpha, Brucker) equipped with a diamond crystal cell (45 ° ZnSe; 80 mm long, 10 mm wide and 4 mm thick). The single beam spectra samples were collected by 32 scans and resolution of 4 cm⁻¹ in the spectral range of 500 cm⁻¹ to 4 000 cm⁻¹ for each sample. The absorbance spectrum was obtained by the equation $Abs = \log(I^{-1})$, where I is the single beam spectrum of interest. The FT-IR was operated using a Varian Resolution Software (Pro 4.0). The background measurement of the clean diamond was used as the initial spectrum and was auto-subtracted from the spectra of the sample. The FTIR band assignments of the major functional groups present in the microalgal biomass samples were based on previous studies (Jiang *et al.*, 2012; Pelusi *et al.*, 2016). The contribution of specific biological molecules to the bands was validated with authentic standards.

3.3.9. Colorimetric measurement of the biochemical component of the microalgal cells

Ten milligrammes of dry microalgal biomass was reconstituted in 10 mL of distilled water to prepare a known concentration of each sample (1 mg mL⁻¹). This was homogenised by vortexing for 10 min and used for lipid, protein and carbohydrate analysis. The analysis was performed in triplicate and the results were expressed as the average of three values ± standard deviation.

3.3.9.1. Quantification of total lipids

Total lipids were quantified using the colorimetric sulfo-phospho-vanillin (SPV) assay described by Mishra *et al.* (2014). In summary, the microalgal cells were pretreated by adding ice-cold concentrated sulphuric acid (96%) and boiling for 10 min at 100 °C. The resulting cell lysate was used to determine lipid content. Five millilitres of SPV reagent were added to 2 mL of the pre-treated microalgal sample and incubated for 15 min at 37 °C. The absorbance was measured at 530 nm using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA) (Mishra *et al.*, 2014). A calibration curve was prepared using canola oil as an authentic lipid standard for the determination of lipid concentration. The standard calibration curve was in the range of 0.05 to 1 mg mL⁻¹. The lipid content of biomass was determined using Eq. (3.7):

$$\text{Lipid content (\% dcw)} = \frac{\text{Lipid concentration } \left(\frac{\text{mg}}{\text{mL}}\right) * \text{dilution factor}}{\text{Biomass concentration } \left(\frac{\text{mg}}{\text{mL}}\right)} * 100 \quad (3.7)$$

3.3.9.2. Quantification of total proteins

The microalgal biomass was lyophilized with a freeze drier and disrupted with a pestle and mortar for 10 min prior to determination of protein concentration. The protein content was estimated using Bradford method using bovine serum albumin (BSA) as the authentic standard (Bradford, 1976). The protein content of microalgal biomass was determined using Eq (3.8) (Kassim and Bhattacharya, 2016):

$$\text{Protein content (\% dcw)} = \frac{\text{Protein concentration } \left(\frac{\text{mg}}{\text{mL}}\right) * \text{dilution factor}}{\text{Biomass concentration } \left(\frac{\text{mg}}{\text{mL}}\right)} * 100 \quad (3.8)$$

3.3.9.3. Quantification of total carbohydrates

The microalgal cells were pre-treated by autoclaving at 121°C for 15 min prior to carbohydrate analysis using Anthrone reagent method (Yemm and Willis, 1954). One millilitre aliquot of the cell lysate was reacted with 4 mL of Anthrone reagent in a boiling water bath for 10 min. The concentration of carbohydrates was determined spectrophotometrically (Varian 50 bio UV-visible spectrophotometer, Aligent, USA) at 620 nm against a calibration curve constructed from known concentrations of glucose. The carbohydrate content from microalgal biomass was determined using Eq (3.9) (Kassim and Bhattacharya, 2016):

$$\text{Carbohydrate or glucose content (\% dcw)} = \frac{\text{Carbohydrate or glucose concentration } \left(\frac{\text{mg}}{\text{mL}}\right) * \text{dilution factor}}{\text{Biomass concentration } \left(\frac{\text{mg}}{\text{mL}}\right)} * 100 \quad (9)$$

3.3.9.4. Quantification of total glucose content

The glucose content was determined using the glucose oxidase assay kit (GAGO-20, Sigma-Aldrich), following the manufacturer's guidelines. Sample preparation was done as reported by the National Renewable Energy Laboratory (NREL), USA (Ho *et al.*, 2013b). Briefly, 10 mg of the lyophilized microalgal biomass was added to 10 ml of 72% (v/v) of sulfuric acid and left for 30 min in a water bath operated at 30 °C for primary hydrolysis. The hydrolysate was then treated with 4% (v/v) sulfuric acid and autoclaved at 121 °C for 20 min for the secondary hydrolysis step. The sample was centrifuged at 4000 x g for 5 min and the supernatant was neutralized using 1 M of NaOH. An aliquot (1 mL) of the hydrolysate was added to 2 mL of assay reagent containing 500 U of glucose oxidase and 100 U of

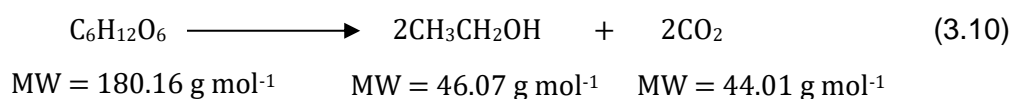
peroxidase (Product code D 3660, Sigma-Aldrich) and incubated at 37 °C for 30 min. The reaction was stopped by adding 2 mL of 12 N of sulphuric acid. Absorbance was measured spectrophotometrically at 540 nm using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA) and glucose concentration was determined from a calibration curve constructed from known concentrations of glucose (0.05 to 1 mg mL⁻¹). The glucose content of the biomass was determined using Eq (3.9).

3.3.10. Analysis of carbohydrate profiles using TLC

The carbohydrate profile of the microalgal strains was determined using TLC, according to the method described by Spollen and Nelson (1988). The microalgal samples were first hydrolyzed according to the NREL protocol (Ho *et al.*, 2013b). The microalgal hydrolysate was subjected to centrifugation (4 000 x *g* for 15 min) and neutralized using 100 µL of 1 M NaOH. Aliquots (5 µL) of the hydrolyzed samples were applied to a TLC plate of silica gel 60 F254 20x20 cm (Merck, South Africa). On the same plates, standards of xylose, glucose, and maltose (Merck, South Africa) were also loaded (5 µL) at a concentration of 1 mg mL⁻¹. The mobile phase consisted of 1-butanol: acetic acid: water (55:30:15) (v/v/v). The spraying reagent consisted of urea-Orthophosphoric acid, which was prepared as described elsewhere (Wise *et al.*, 1955). The plates were heated at 120 °C for 10 min and viewed under white and UV light (366 nm).

3.3.11. Theoretical ethanol yield

Theoretical ethanol yield was calculated using the balanced chemical equation shown in Eq (3.10)



Eq (3.10) depicts that one molecule of glucose can be fermented to 2 molecules of ethanol and 2 molecules of CO₂. Therefore, assuming 100% conversion, the conversion factor of glucose to ethanol is considered to be 51% based on the molecular mass of each compound (Vogel *et al.*, 2010).

3.3.12. Statistical analyses

Empirical data were analyzed using one-way analysis of variance (ANOVA) using Microsoft Office 2010 (Microsoft, USA). The correlation was analyzed using bivariate correlations, and

the Pearson's correlation coefficients are given with their significance levels. A significant difference was considered at the level of $p < 0.05$. All results were expressed as mean value \pm standard deviation of triplicate values.

3.4. Results and Discussion

3.4.1. Characterization of the sampling sites

Microalgae comprise of physiologically diverse groups of species that inhabit various niches of the aquatic environments. Previous reports on microalgal bioprospecting have shown that environmental conditions of the sampling sites can strongly dictate the diversity, performance and abundance of the microalgae present (Chen *et al.*, 2013; Li *et al.*, 2015a). Consequently, it is crucial to analyse and understand the key environmental conditions of the sampling sites in order to obtain the best performing microalgal strains from their natural aquatic habitats (Mutanda *et al.*, 2011b). Among the factors affecting the growth of microalgae, temperature, light intensity, pH and salinity are some of the most important contributors (Kumaran *et al.*, 2016; Ördög *et al.*, 2016). Most microalgae are photoautotrophic, obtaining their energy from light and use water as an electron donor to reduce CO₂ to organic matter during photosynthesis. These microorganisms can exist throughout the water column, sometimes accumulating in large numbers at a particular depth due to nutrient availability and environmental conditions such as the availability and intensity of light since they comprise of primary and accessory photosynthetic pigments which absorb light with specific spectral qualities and wavelengths (Mezhoud *et al.*, 2014; Wong *et al.*, 2015). The measured light intensities of the sampling sites ranged between 23.7 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and 43.3 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (Table 3.1), which is considered adequate for the growth of microalgae (Li *et al.*, 2015a; Zhou *et al.*, 2011).

Since sampling in this study was performed in winter, the temperature of the sampling sites ranged from 16 °C to 24 °C, (Table 3.1). Cold temperatures have been shown to promote the growth of benthic microalgal strains such as *Scenedesmus* sp. while some Chlorophytes such as *Chlorella* become dormant until temperatures are favourable (Mutanda *et al.*, 2011b). Therefore, in this study, the spatial and temporal sampling strategy was adopted and water samples were collected from different depth levels to cater for successional changes occurring in the sampling sites in order to ensure the success of isolating microalgal strains with unique properties.

Table 3.1 Characteristics of the sampling sites and identity of the indigenous microalgal isolates.

Sampling site (GPS coordinates)	Water type	Light intensity $\mu\text{mol m}^{-2}\text{s}^{-1}$	Temperature (° C)	pH	Salinity (g L ⁻¹)	Strain code	Microalgae identity	Similarity (%)	Accession number
Amanzimtoti river (-30.077537, 30.871276)	Freshwater	37.1	16	7.21	3.67	Toti RS4	<i>Chlorella vulgaris</i> AT-PSD-22	100	KP662699.1
Amanzimtoti river (-30.077537, 30.871276)	Freshwater	37.1	16	7.21	3.67	Toti RS7	<i>Neochloris aquatica</i> NIOT-223	100	AB983623.1
Umdloti river (-29.649058, 31.124596)	Freshwater	43.3	16	7.43	3.52	U-2	<i>Chlamydomonas sp.</i> BM3	100	KT720479.1
Tongaat pond (-29.590676, 31.038620)	Freshwater	23.7	20	7.43	2.67	TS5	<i>Chlorella sp.</i> HS-2	99	KU674363.1
Northern WWTP (-29.796361, 30.998788)	Wastewater	35.7	24	8.23	3.36	NWWTPS5	<i>Chlorella</i> QUCCM7	<i>sp.</i> 100	KM985379.1
La Mercy estuary (-29.812041, 31.041620)	Brackish water	29.7	16	7.34	19.7	L-3	<i>Chlorococccm sp.</i>	100	AB983624.1

The pH of the sampling sites was neutral to slightly alkaline, with the wastewater collected from Northern wastewater treatment plant (GPS coordinates: -29.796361, 30.998788) having the highest pH of 8.23 (Table 3.1). Microalgae typically require a pH between 7 and 9 for optimal growth (Chen *et al.*, 2013; Li *et al.*, 2015a). This has been observed in studies with *Chlamydomonas applanata* and *Ceratium lineatum* (Hansen, 2002; Visviki and Santikul, 2000). Only a few species can grow at pH values lower than 3 or greater than 9 and such microalgae can be isolated from extreme environments such as acid springs and soda lakes, respectively. Such microalgal species are of basic science interest particularly because they have unique properties such as the ability to accumulate high carbohydrates and lipids under these extreme and stressful environments. For instance, *Dunaliella acidophila* has been shown to grow in acidic environments (pH = 0.2 - 2.5) and accumulate high glycerol content to prevent the osmotic imbalance caused by acid build-up (Fuggi *et al.*, 1988).

The salt concentration of the sampling sites ranged from 2.67 to 19.7 g L⁻¹. The salt concentration of the water collected from an estuary in La Mercy (GPS coordinates: -29.812041, 31.041620) was significantly higher than other sites sampled in this study at a maximum value of 19.7 g L⁻¹ (Table 3.1). Microalgae residing in environments with high salt concentrations such as salt lakes and other marine environments have been shown to exhibit unique properties. A marine microalgae *Dunaliella* sp. exhibited a corresponding increase in lipid content after being subjected to a gradual increase in NaCl concentration from 23.4 to 233.8 g L⁻¹ (Xu and Beardall, 1997). Rao *et al.* (2007) demonstrated that carbohydrate synthesis by *Botryococcus braunii*, a freshwater microalgal strain, could be enhanced by increasing the salt concentration of the culture medium from 0.99 to 1.9 g L⁻¹. Thus, for the purpose of bioprospecting, sampling from local aquatic environments such as those listed in Table 3.1 is ideal for isolating robust microalgal strains with unique properties.

3.4.2. Morphological and molecular characterization of the microalgal isolates

In this study, up to thirteen microalgal strains were isolated into axenic monocultures. Of the thirteen, only six demonstrated good growths under *in vitro* conditions and were thus selected for further investigation. The six microalgal isolates showed variation in terms of cell size and structure (Fig. 3.1). Morphological characterization of the microalgal cultures revealed that the isolates Toti RS4, Toti RS7, U-2, TS5, NWWTPS5 and L-3 were affiliated to the genera *Chlorella*, *Neochloris*, *Chlamydomonas*, *Chlorella*, *Chlorella* and *Chlorococcum*, respectively. Based on the fact that different microalgal species may demonstrate similar morphologies and the fact that the morphological structure of the microalgal cells may vary depending on the environmental and growth conditions (George *et*

al., 2014), further molecular identification was performed for each microalgal isolate. The analysis of the 18S rDNA sequence of the six new isolates revealed the presence of four different genera, with *Chlorella* being the most abundant genus having 50% of the isolates. Table 3.1 presents an overview of the identified strains with their corresponding sampling locations, sampling site characteristics and GenBank accession numbers.

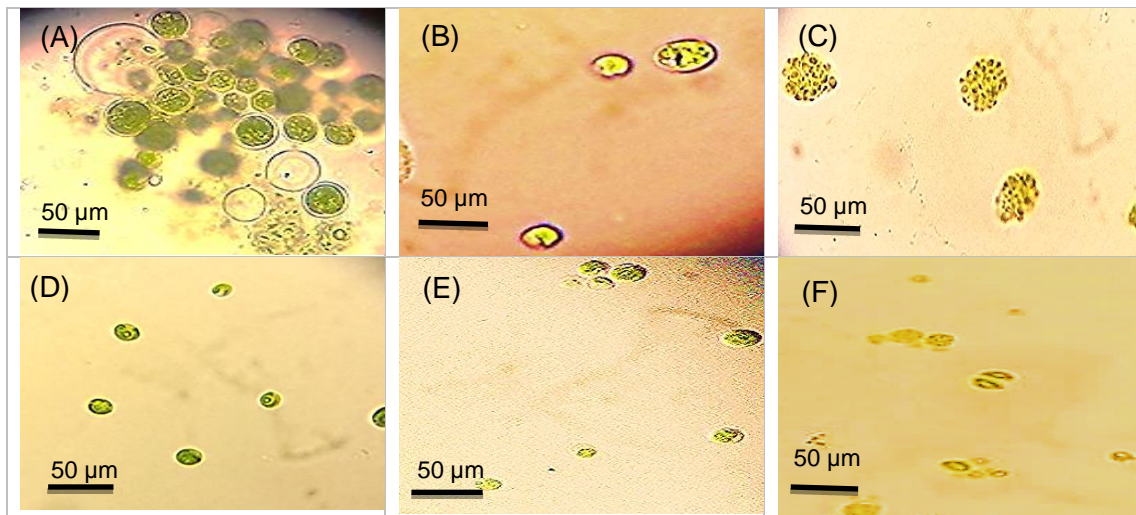


Fig. 3.1. Light microscopic images of the six indigenous microalgal isolates. (A): *Chlorococcum* sp. (L-3), (B): *Chlamydomonas* sp. (U-2), (C): *N. aquatica* (Toti RS7), (D): *C. vulgaris* (Toti RS4), (E): *Chlorella* sp. (NWWTPS5), and (F): *Chlorella* sp. (TS5).

3.4.3. Microalgal growth profile and biomass production

Rapid growth rates and high biomass productivity are important prerequisites for selection of suitable microalgal strains for large scale applications since the two parameters strongly influence the operational costs involved in the production chain (i.e., cultivation and harvesting) (Do Nascimento *et al.*, 2012; Gonzalez-Garcinuno *et al.*, 2014). It is widely acknowledged that microalgae require adequate nutrients for growth, however, the production of valuable products such as carbohydrates in microalgae occurs under nutrient deficient conditions (Arora *et al.*, 2016; Ho *et al.*, 2013b; Pancha *et al.*, 2014; Sun *et al.*, 2014). Hence, the adoption of the two-stage cultivation strategy in this study. The growth of the microalgal cells was monitored by measuring dry cell weight and chl a (Fig. 3.2). A positive correlation was observed between biomass and chl a concentration during the first growth phase for all the isolates (R^2 (average) =0.856; $p < 0.05$) (Fig. 3.2), in agreement with the findings reported by Vörös and Padisák (1991), demonstrating a significant correlation between the two growth indices ($R^2 = 0.68-0.92$).

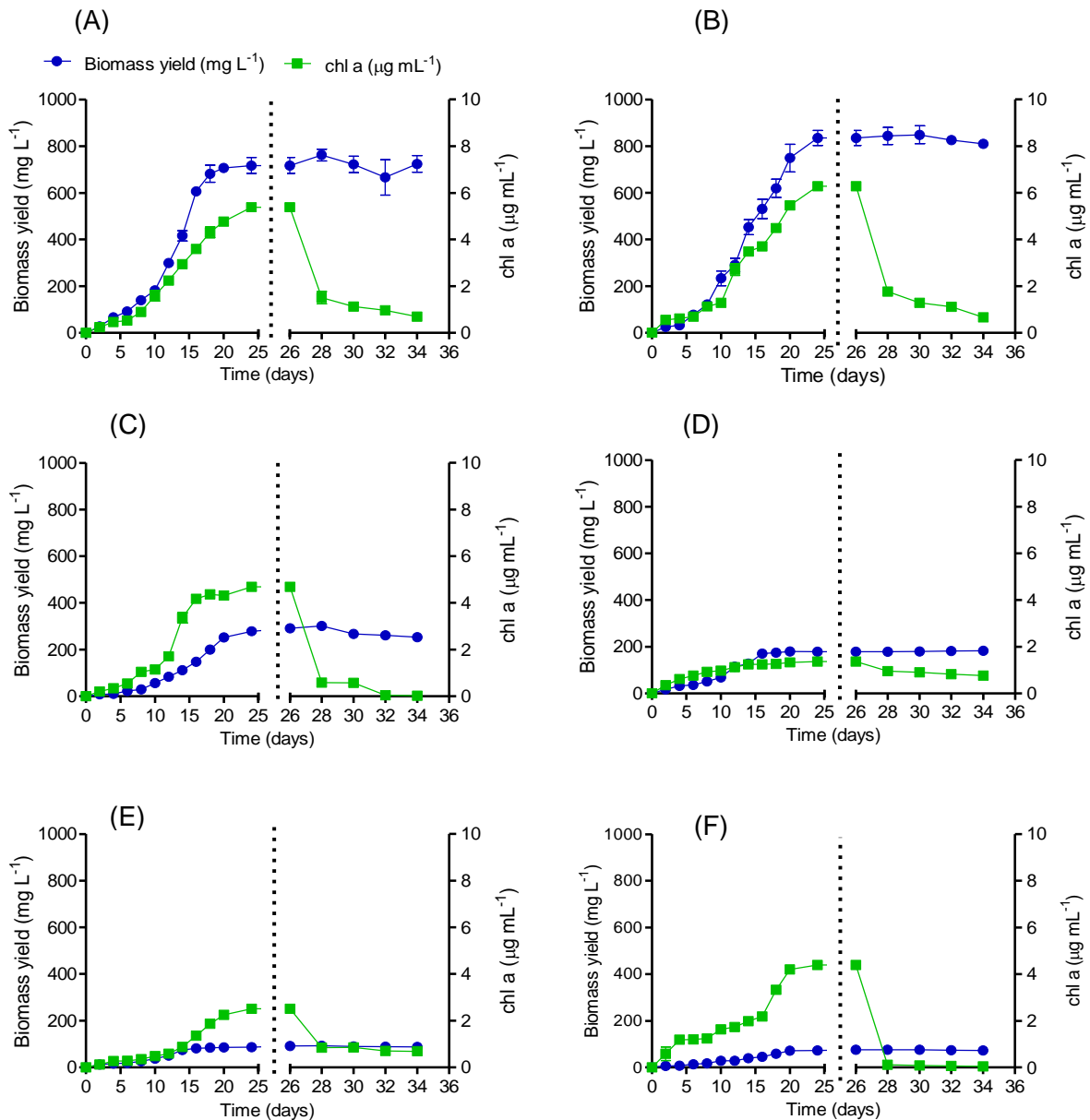


Fig. 3.2. Biomass and chl a concentration of the indigenous microalgal isolates cultivated under nitrogen replete conditions. (A); *C. vulgaris* (Toti RS4), (B): *N. aquatica* (Toti RS7), (C): *Chlamydomonas* sp. (U-2), (D): *Chlorella* sp. (NWWTPS5), (E): *C. nivale* (L-3), (F): *Chlorella* sp. (TS5). (Temperature= 25 °C, light intensity= 37 $\mu\text{mol m}^{-2} \text{s}^{-1}$, CO₂ concentration = ambient). The vertical line indicates the switch from nitrogen replete to nitrogen-deficient medium. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

The maximum biomass yields of the microalgal cultures under nitrogen replete conditions ranged between $178.9 \pm 5.6 \text{ mg L}^{-1}$ and $816 \pm 0.77 \text{ mg L}^{-1}$, with the highest biomass yield ($816 \pm 0.77 \text{ mg L}^{-1}$) produced by *N. aquatica* (Toti RS7). This value was found to be 11.2-

9.4-, 4.6-, 2.9-, and 1.2- fold higher than that of *Chlorella* sp. (TS5), *C. nivale* (L-3), *Chlorella* sp. (NWWTPS5), *Chlamydomonas* sp. (U-2), and *C. vulgaris* (Toti RS4), respectively. *N. aquatica* (Toti RS7) also showed the highest chl a concentration at $6.27 \mu\text{g mL}^{-1}$, which was consistent with its high biomass, while *Chlorella* sp. (NWWTPS5) showed the lowest chl a at $1.361 \mu\text{g mL}^{-1}$.

The average growth rate of the microalgal strains, estimated at the exponential growth phase, varied among the strains investigated (Table 3.2). Of all the isolates studied, *C. vulgaris* (Toti RS4) showed the highest growth rate ($0.201 \pm 0.012 \text{ day}^{-1}$) which is consistent with that reported elsewhere, for *Chlorella kessleri* (de Morais and Costa, 2007) and *Chlorella* sp. (Li et al., 2015a). The biomass productivity of the isolates was also variable, ranging from $4.30 \pm 0.3 \text{ mg L}^{-1} \text{ day}^{-1}$ to $70.8 \pm 3.4 \text{ mg L}^{-1} \text{ day}^{-1}$. Again, *C. vulgaris* (Toti RS7) showed the highest biomass productivity at $70.8 \pm 3.4 \text{ mg L}^{-1} \text{ day}^{-1}$, while *Chlorella* sp. (TS5) showed the lowest biomass productivity at $4.30 \pm 0.3 \text{ mg L}^{-1} \text{ day}^{-1}$ when compared to the other microalgal strains investigated. This is presumably attributed to physicochemical differences in the natural aquatic habitats of the sampling sites (e.g., pH, temperature, light intensity, salinity and altitude) which might have triggered different intrinsic growth potential of the microalgal strains (Zhou et al., 2011).

Cultivation of the microalgal strains under nitrogen-deficient conditions resulted in drastic changes to their growth efficiency, as previously reported by Jiang et al. (2012). A rapid drop in the chl a concentration was observed after two days of nitrogen starvation, indicating the degradation of chl a pigment protein. A slight but insignificant increase in the biomass production was observed for *C. vulgaris* (Toti RS4), *N. aquatica* (Toti RS7), *Chlamydomonas* sp. (U-2) and *Chlorococcum* sp. (L-3) ($p > 0.05$) (Fig. 3.2), probably due to chlorophyll in algal cells degrading for nutrient recycling to support algal growth under nitrogen starvation conditions. Zhu et al. (2014) also reported this phenomenon. The microalgal cells reached the stationary phase after 8 days of nitrogen starvation, with a concomitant change in the colour of the culture broth from green to yellow, indicating complete degradation of chl a pigment protein into storage reserves such as starch/lipids (Sun et al., 2014). The growth rates and biomass productivity of the indigenous microalgal strains also dropped in response to nitrogen deprivation (Table 3.2).

Table 3.2 The specific growth rates, biomass concentration and biomass productivity of the indigenous microalgal isolates cultivated under nitrogen replete conditions. (Temperature = 25 °C, light intensity= 37 $\mu\text{mol m}^{-2} \text{s}^{-1}$, CO_2 concentration= ambient).

	Toti RS4	NWWTPS5	Toti RS7	U-2	L-3	TS5
Nitrogen replete						
Growth rates (day^{-1})	0.201 ± 0.0	0.153 ± 0.01	0.137 ± 0.02	0.161 ± 0.0	0.130 ± 0.0	0.103 ± 0.0
Maximum biomass yield (mg L^{-1})	717 ± 34	179 ± 5.6	835 ± 32	278 ± 3.1	86.6 ± 0.3	73.1 ± 1.8
Biomass productivity ($\text{mg L}^{-1} \text{day}^{-1}$)	70.8 ± 3.4	17.1 ± 1.3	64.3 ± 7.6	19.5 ± 0.6	7.40 ± 0.3	4.30 ± 0.3
Nitrogen deficient						
Growth rates (day^{-1})	0.0305 ± 0.0	0.00213 ± 0.00	0.00395 ± 0.0	0.0179 ± 0.0	0.0162 ± 0.0	0.0102 ± 0.0
Maximum biomass yield (mg L^{-1})	762 ± 25	180 ± 6.6	848 ± 38	302 ± 4.1	92.4 ± 1.0	76.2 ± 2.0
Biomass productivity ($\text{mg L}^{-1} \text{day}^{-1}$)	24.4 ± 6.0	0.389 ± 0.12	3.36 ± 1.7	5.29 ± 3.3	1.08 ± 0.0	0.762 ± 0.7

The mechanism behind the disintegration of the photosynthetic apparatus (chl a) of the microalgal cells, leading to the downturn of growth was further analyzed using PAM fluorometry. The measured PAM parameters were rETR, F_v/F_m and NPQ as shown in Fig. 3.3 and Fig. 3.4(A) and (B), respectively. The rETR values were calculated and plotted as a function of PAR, as demonstrated in the rapid light curves in Fig. 3.3. The rapid light curves give insight on the photosynthetic activity of the microalgal cells by integrating the cells' ability to tolerate light fluctuations (Malapascua *et al.*, 2014). The rETR also measures the rate of linear transport through PSII, which is thus correlated with the overall photosynthetic performance of the microalgal cells (White *et al.*, 2011). As observed in Fig. 3.3, under nitrogen replete conditions, the microalgal strains demonstrated good resistance to light fluctuations, indicating good health of the physiology and photosystems of the microalgal cells acclimatized under nitrogen replete conditions. The highest rETR values were presented by *N. aquatica* (Toti RS7) and *C. vulgaris* (Toti RS4), suggesting that these strains had the highest photosynthetic rates, which correlates with their high growth rates and biomass production potential (Fig. 3.2). The F_v/F_m ratio of the microalgal strains acclimatized under nitrogen replete medium was greater than 0.5 (Fig. 3.4(A)), which is the accepted physiological acclimatization range of microalgae to environmental conditions (Ramanna *et al.*, 2014; White *et al.*, 2011).

When the microalgal strains were subjected to nitrogen-deficient conditions, there were drastic changes in the PAM parameters measured. The rETR values remained at very low magnitudes (< 0.1), signalling the impairment of PS II reaction centres and shutting down of the photosynthetic system of the microalgal cells (Fig. 3.3). This finding explains the sudden drop in the chl a content of the microalgal cells (Fig. 3.2) and the change in colour of the culture broth from green to yellowish cream upon nitrogen starvation. The F_v/F_m ratio of the cells decreased significantly under nitrogen-deficient conditions ($p < 0.05$) confirming that the greater majority of the PS II apparatus were damaged. The highest drop in the F_v/F_m ratio (86%) was presented by the brackish water strain *Chlorococcum* sp. (L-3), suggesting that this strain was the most sensitive strain to nitrogen starvation. A varying increase in the NPQ values of the microalgal cells in the nitrogen-deficient medium was observed among the strains (Fig. 3.4(B)). In a study by Ye *et al.* (2015), an increase in NPQ was correlated with the production of storage compounds, particularly lipids and starch. Evidence of this phenomenon was also observed in this study as shown in Fig. 3.2(B).

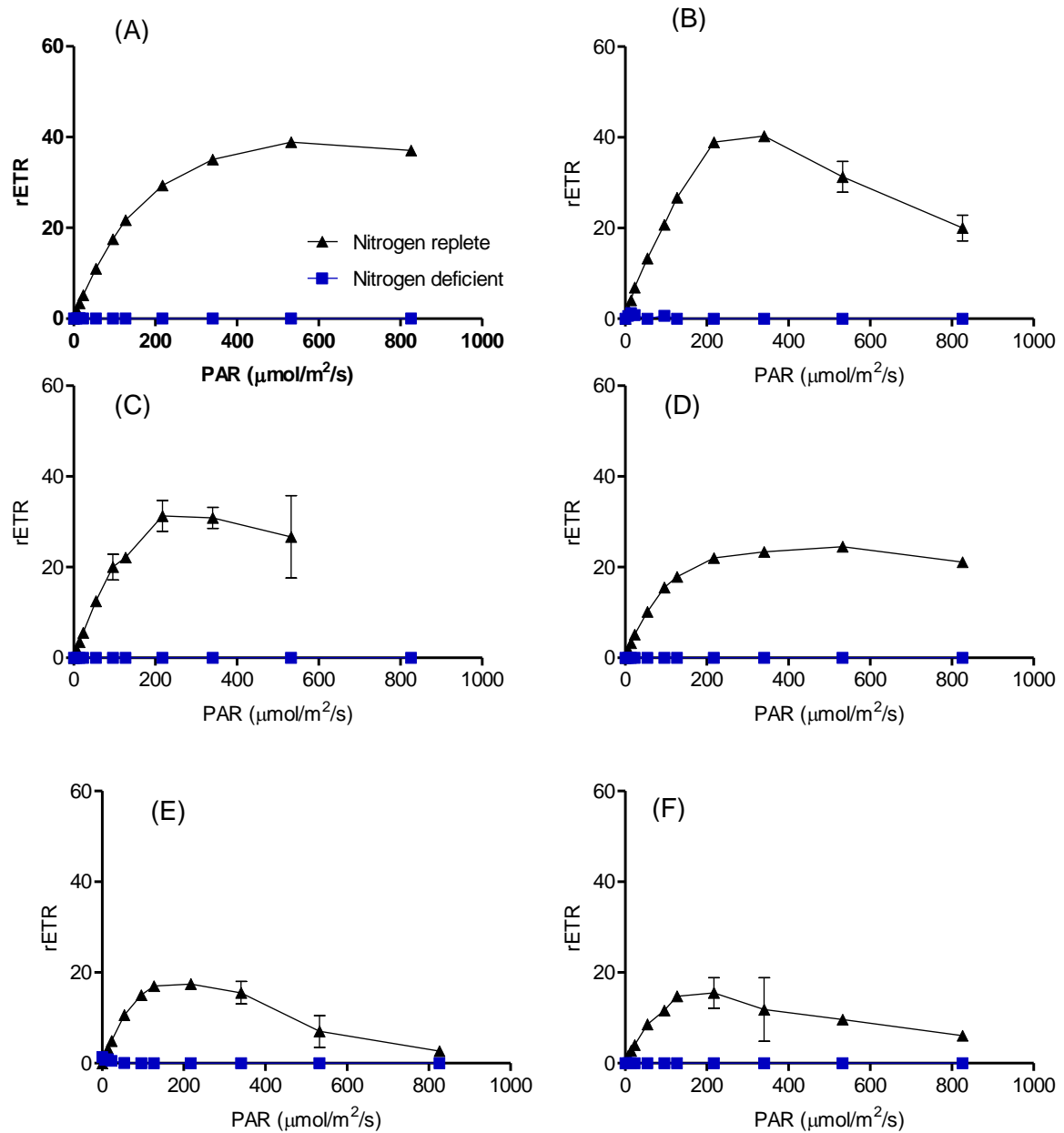


Fig. 3.3. Rapid light curves of the six indigenous microalgal strains cultivated in nitrogen replete BG-11 medium (on day 24) and nitrogen deficient BG-11 medium (on day 8). (A); *C. vulgaris* (Toti RS4), (B): *N. aquatica* (Toti RS7), (C): *Chlamydomonas* sp. (U-2), (D): *Chlorella* sp. (NWWTPS5), (E): *C. nivale* (L-3), (F): *Chlorella* sp. (TS5). The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

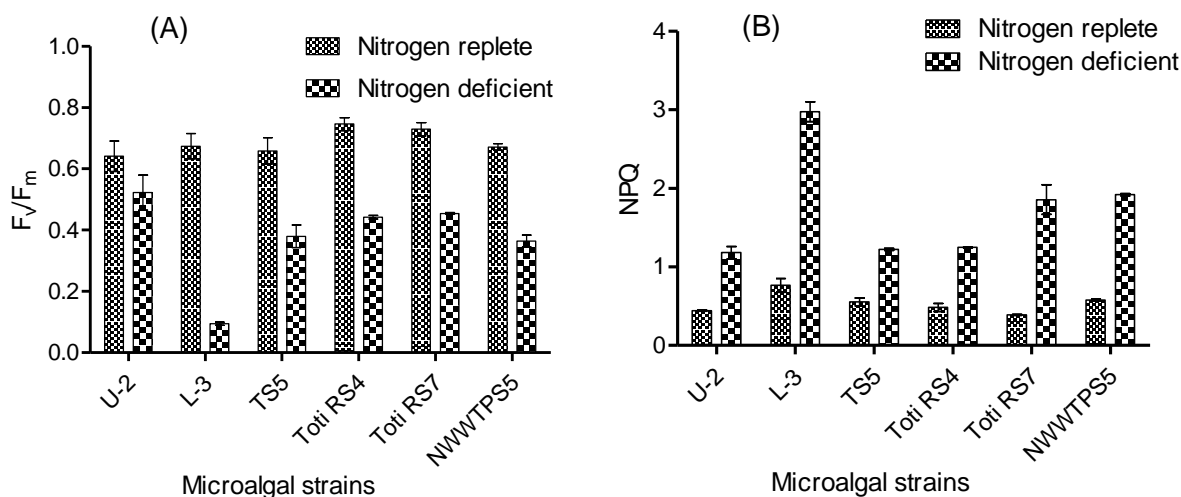


Fig 3.4. The physiological parameters F_v/F_m (A) and NPQ (B) recorded for microalgal strains cultivated in nitrogen replete BG-11 medium (on day 24) and nitrogen deficient BG-11 medium (on day 8). The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

3.4.4. Gross biochemical composition analysis of the indigenous microalgal isolates

To select the most suitable hyper-carbohydrate producing microalgae as the feedstock for bioethanol production, nitrogen starvation was employed to trigger the accumulation of carbohydrates (mainly starch) in the isolated microalgal strains. This is based on the phenomenon that when microalgal cells are subjected to nitrogen deficient conditions, carbon allocation diverts from protein synthesis to lipid or carbohydrate, which are two major carbon storage compounds. Whether the microalgae will accumulate only one or both of these compounds in considerable amounts is highly dependent upon the nature of the strain and the intensity of the administered stress (Do Nascimento *et al.*, 2012). Traditional methods (e.g., light microscopy) have been used to identify the shift in the biochemical composition of the microalgal cells and abundance of the biochemical compounds of interest due to external cues. However, these methods are time-consuming and require manipulation of the sample (Kose and Oncel, 2015). FT-IR is a reliable way to accurately study the dynamics of the biochemical components of the microalgal cells without sectioning or application of complicated sample preparation methodologies. This is largely attributed to the fact that microalgae typically have adequate cell wall thickness and contain enough cellular constituents to liberate a good IR signal (Pelusi *et al.*, 2016).

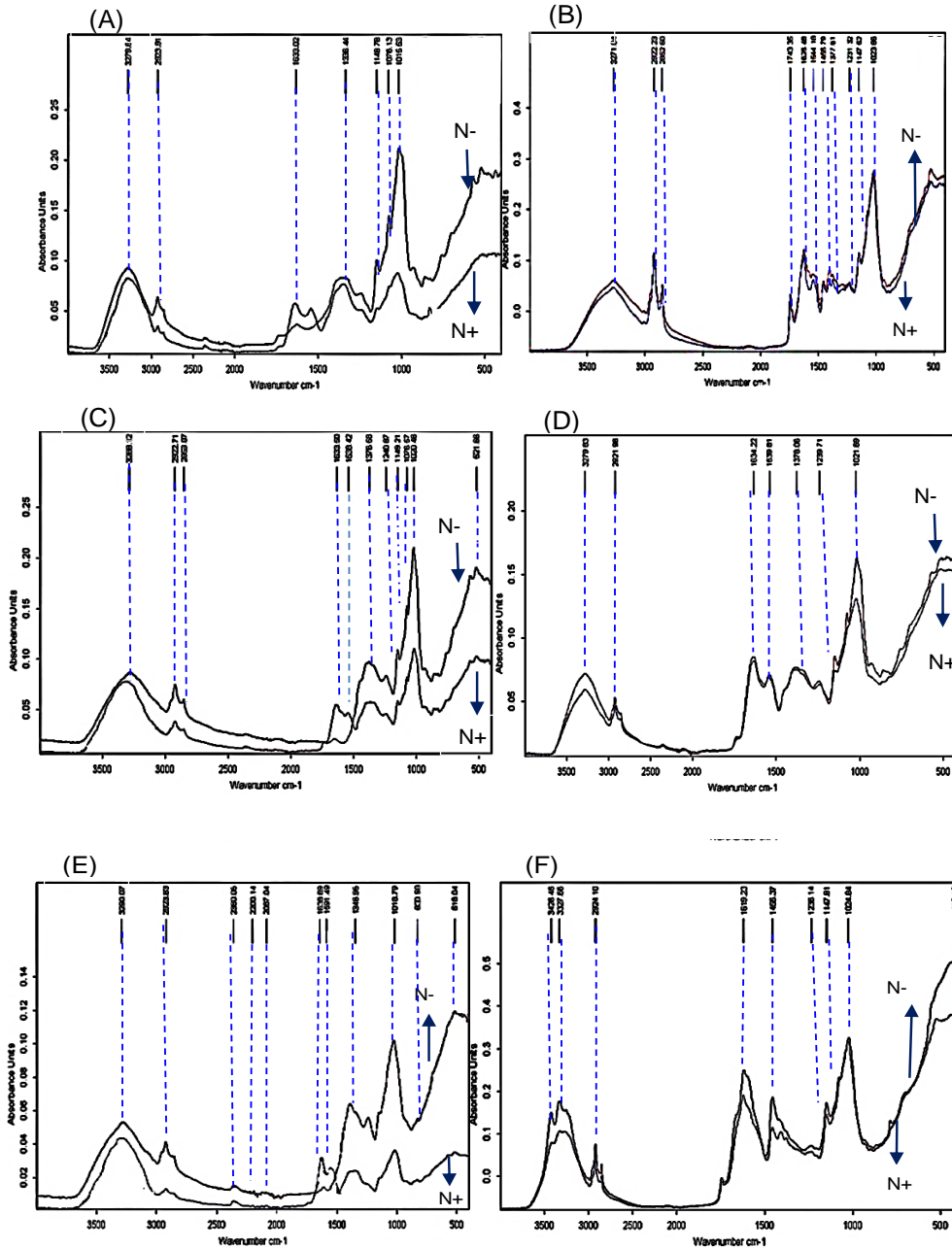


Fig. 3.5. FT-IR spectra of the six indigenous microalgal strains cultivated in nitrogen replete (N⁺) BG-11 medium (on day 24) and nitrogen-deficient (N⁻) BG-11 medium (on day 8). The dashed lines indicate regions of the spectra that have been assigned to band numbers of the functional groups. These assignments are detailed in [Pelusi et al. \(2016\)](#). (A): *N. aquatica* (Toti RS7), (B): *Chlamydomonas* sp. (U-2), (C): *Chlorococcum* sp. (L-3), (D): *Chlorella* sp. (NWWTPS5), (E): *Chlorella* sp. (TS5) and (F): *C. vulgaris* (Toti RS4).

The FT-IR spectra of the six indigenous microalgal strains tested in this study are shown in [Fig. 3.6](#). The selected microalgae gave the same wavenumbers with no significant

difference; however, the peak areas differed according to the microalgal strains and the cultivation conditions of the cells. Several different peaks were detected and were assigned to the major cellular constituents such as proteins (3400-3200 cm^{-1} , 1724-1590 cm^{-1} and 1585-1490 cm^{-1}) lipids (2960-2850 cm^{-1} and 1770-1720 cm^{-1}), carbohydrates (1200-900 cm^{-1}), and nucleic acids (1240 cm^{-1}) on the basis of reference standards (Fig. 3.5) and published FT-IR spectra in relationship to specific molecular groups (Pelusi *et al.*, 2016). Microalgae cultivated in nitrogen replete medium demonstrated a high presence of amide I (1724-1585 cm^{-1}) and amide II (1585-1490 cm^{-1}) bands representing proteins, while the “carbohydrate band” of 1200-950 cm^{-1} and “lipid band” were low (Fig. 3.6). This observation was in line with previously published data (Dean *et al.*, 2010).

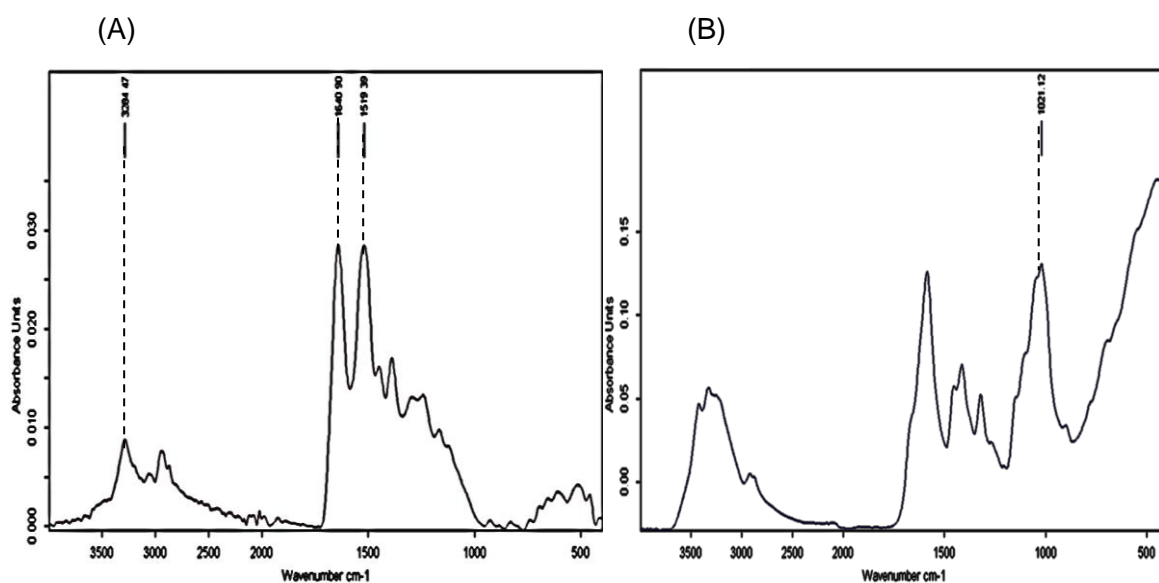


Fig. 3.5. FT-IR spectra of bovine serum albumin (A) and starch (B). The dashed lines indicate regions of the spectra that have been assigned to band numbers of the functional groups. These assignments are detailed in Pelusi *et al.* (2016).

Previously, PAM fluorometry demonstrated the mechanism behind the disintegration of the photosynthetic apparatus of the microalgal strains following nitrogen starvation (Figs. 3.3 and 3.4). Herein, FT-IR results show that during the course of nitrogen starvation, the synthesis of carbohydrates and lipids increased while that of proteins decreased (Fig. 3.6). This was in correspondence with the significant decrease of rETR (Fig. 3.4) and F_v/F_m ratio (Fig. 3.4A) of the microalgal strains, which was counteracted by an increase in NPQ (Fig. 3.4B). In acclimation experiments of nitrogen starved cultures of *Scenedesmus* sp. CCNM, various fundamental physiological processes were changed, which resulted in strongly reduced growth, reduced photosynthetic capacity and increased carbohydrate content (Panchar *et al.*, 2014). The microalgal cells showed the degradation of intracellular proteins

under nitrogen deprivation, in order to meet nitrogen supply requirements. Comparable results were also reported in the study of (Driver *et al.*, 2015).

To confirm the findings obtained from FT-IR in the monitoring of carbon allocation among proteins, lipids and carbohydrates, the dynamics of these biochemical compounds in response to nitrogen availability in the six indigenous microalgal strains were studied using the traditional biochemical methods (Table 3.3). Under nitrogen replete conditions, the protein content of the microalgal cells was variable, ranging from $17.8 \pm 0.1\%$ to $42.0 \pm 0.02\%$ of the dry cell weight, while the lipid and carbohydrate content ranged from 7.1 ± 1.2 to $30.5 \pm 7.6\%$, and $12.1 \pm 1.0\%$ to $38.2 \pm 1.2\%$ of the dry cell weight, respectively. Upon nitrogen starvation, the protein content of the microalgal cells decreased drastically to values as low as $3.6 \pm 0.04\%$ of the dry cell weight as presented by *Chlorella* sp. (NWWTPS5) (Table 3.3). There was a 29.5%, 24.5%, 23.2%, 15.4% and 12.9% reduction in the protein content of *Chlorella* sp. (TS5), *Chlorococcum* sp. (L-3), *Chlamydomonas* sp. (U-2), *N. aquatica* (Toti RS7) and *C. vulgaris* (Toti RS4), respectively following nitrogen starvation (Table 3.3).

In contrast to proteins, the lipid and carbohydrate content of the microalgal cells increased in response to nitrogen starvation (Table 3.3), which was in agreement with the FT-IR spectra data (Fig. 3.6) (Pelusi *et al.*, 2016). Under nitrogen starvation, the carbohydrate content of *C. vulgaris* (Toti RS4), *C. nivale* (L-3), *Chlamydomonas* sp. (U-2), and *N. aquatica* (Toti RS7) increased by 14%, 12%, 11%, and 10%, respectively. Ho *et al.* (2013) reported similar findings for two green microalgae, *Chlamydomonas* and *Chlorella*. When grown under nitrogen replete conditions the two strains possessed a low level of carbohydrates, but under nitrogen starvation, carbohydrate synthesis was transiently upregulated. No significant increase in carbohydrate production was observed for *Chlorella* sp. (NWWTPS5) and *Chlorella* sp. (TS5), however, these strains accumulated substantial amounts of lipids (Fig. 3.6 and Table 3.3), suggesting that they could be more suitable for biodiesel production rather than for bioethanol production. The microalgae *C. vulgaris* (Toti RS4) had the highest carbohydrate content (51.8% of the dry weight), similar to those reported for *C. vulgaris* FSP-E by Ho *et al.* (2013) and *Scenedesmus* sp. by Do Nascimento *et al.* (2012), suggesting its potential application as a bioethanol feedstock.

Table 3.3 Comparative investigation of the biochemical composition of microalgae cultivated in nitrogen replete BG-11 medium (on day 24) and nitrogen deficient BG-11 medium (on day 8).

	Toti RS4	NWWTPS5	Toti RS7	U-2	L-3	TS5
Nitrogen replete						
Total lipids (% dcw)	22.3 ± 2.6	20.3 ± 4.8	30.5 ± 7.6	15.4 ± 3.8	8.9 ± 2.9	7.1 ± 1.2
Total proteins (% dcw)	23.5 ± 0.0	17.8 ± 0.1	31.4 ± 0.1	42.0 ± 0.0	29.2 ± 0.1	40.3 ± 0.0
Total carbohydrates (% dcw)	38.0 ± 4.4	27.7 ± 0.2	32.1 ± 2.5	36.3 ± 1.9	15.5 ± 0.1	12.1 ± 1.0
Glucose (% dcw)	34.9 ± 0.1	23.4 ± 2.2	27.2 ± 0.6	32.8 ± 2.2	12.9 ± 0.5	9.2 ± 0.7
Theoretical ethanol (% dcw) ^a	17.8 ± 0.0	12 ± 1.1	13.9 ± 0.3	16.7 ± 1.1	6.6 ± 0.3	4.7 ± 0.4
Nitrogen deficient						
Total lipids (% dcw)	27.5 ± 1.6	35.4 ± 5.3	37.3 ± 3.5	32.2 ± 2.1	46.1 ± 14	34.6 ± 1.3
Total proteins (% dcw)	10.6 ± 0.0	3.6 ± 0.0	16.0 ± 0.1	18.8 ± 0.1	4.7 ± 0.0	10.8 ± 0.1
Total carbohydrates (% dcw)	51.8 ± 0.9	27.2 ± 2.6	42.0 ± 0.7	47.4 ± 1.3	27.5 ± 1.0	14.0 ± 0.7
Glucose (% dcw)	46.5 ± 1.2	23 ± 0.6	35.1 ± 0.2	44.8 ± 0.3	25.1 ± 0.5	10.7 ± 0.8
Theoretical ethanol (% dcw) ^a	23.7 ± 0.6	11.8 ± 0.3	17.9 ± 0.1	22.9 ± 0.1	12.8 ± 0.3	5.5 ± 0.4

^a 51 % glucose-to-ethanol conversion metabolic yield using *Saccharomyces cerevisiae* fermentation

3.4.5. Carbohydrate profile and microalga selection

The carbohydrate profile of the six indigenous microalgal strains obtained after acid hydrolysis was determined using TLC analysis (Fig. 3.7). Glucose was observed to be the main carbohydrate produced by the strains under the conditions used, indicating that any of these strains could potentially be used for bioethanol production. However, microalgal strains demonstrating high glucose content are regarded as the most feasible feedstock for industrial production of bioethanol and should, therefore, be selected (Guo *et al.*, 2013).

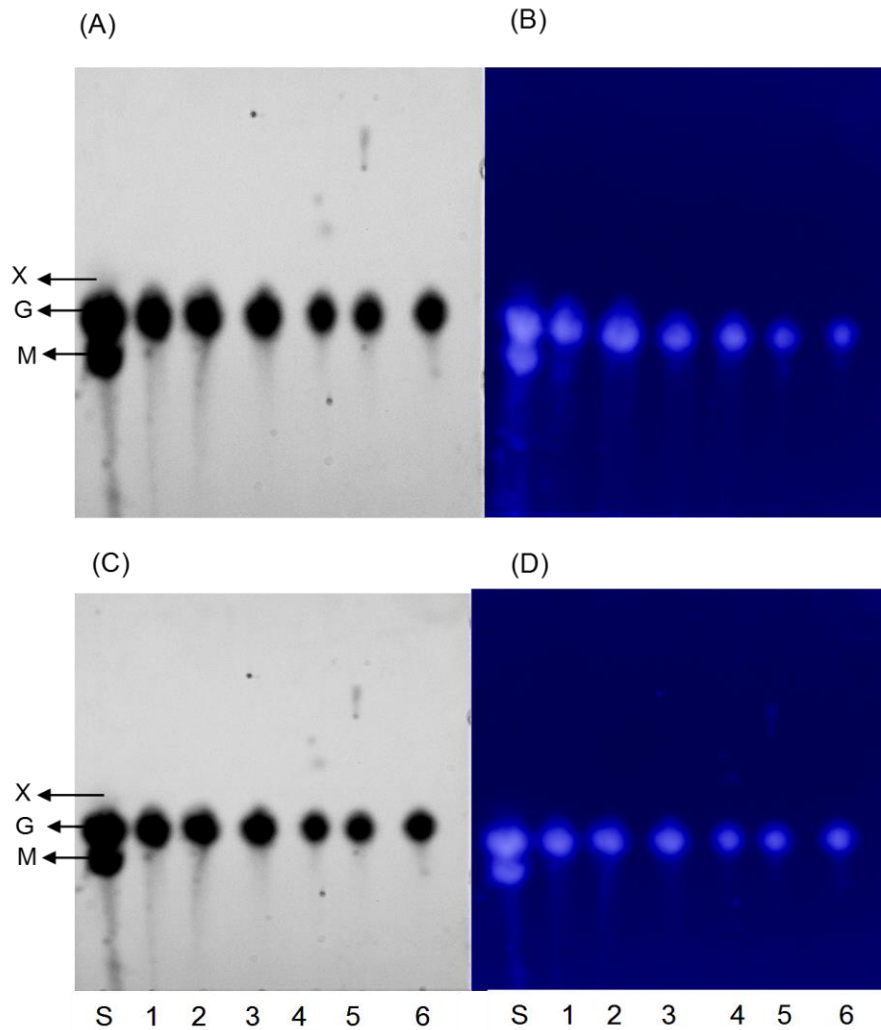


Fig. 3.7. TLC analysis of carbohydrates extracted from the six indigenous microalgal strains cultivated under nitrogen replete BG-11 medium (A and B) on day 24 and nitrogen-deficient BG-11 medium (C and D) on day 8. A and C are TLC silica gel plates stained using urea-phosphoric acid and observed under white light. B and D TLC plates viewed under UV light (366 nm). S: standards of xylose (X), glucose (G), and maltose (M). The numbers 1 to 6 represent *Chlamydomonas* sp. (U-2), *C. vulgaris* (Toti RS4), *N. aquatica* (Toti RS7), *Chlorella* sp. (NWWTPS5), *Chlorella* sp. (TS5) and *Chlorococcum* sp. (L-3), respectively.

The glucose content of the six indigenous microalgal cells under nitrogen replete and nitrogen deficient media is shown in [Table 3.3](#). Under the nitrogen replete conditions, the glucose content of the strains was low to moderate, ranging from 9.2 ± 0.7 % to 34.9 ± 0.1 % of the dry cell weight. However, once transferred to a nitrogen-deficient medium the glucose content increased by up to 1.3-fold, with *C. vulgaris* (Toti RS4) having the highest glucose content of 46.5 ± 1.2 % of the dry cell weight ([Table 3.3](#)).

The theoretical conversion of the glucose content of the six indigenous microalgal strains into bioethanol was calculated based on the conversion factor of 51 wt% (glucose to ethanol metabolic limit), as described in literature (Vogel *et al.*, 2010). The caveat of this dataset is that 100% extraction and conversion factors are assumed and no losses are built in the theoretical conversion projects. The data shown in Table 3.3 illustrate that low theoretical yields occurred during nitrogen replete conditions while the highest theoretical yields were obtained under nitrogen deficient conditions. The microalga *C. vulgaris* (Toti RS4) showed the highest theoretical ethanol yield (17.8% and 23.7% under nitrogen replete and nitrogen deficient condition, respectively) and can be therefore regarded as the most suitable strain for bioethanol production.

3.5. Conclusion

This study demonstrated that microalgae with unique properties such as high growth rates, high photosynthetic efficiency, high biomass production and high carbohydrate content could be isolated from various aquatic environments. The overall findings from this study confirm that some of the strains such as *C. vulgaris*, *Chlorella* sp., *Chlamydomonas* sp., and *N. aquatica* showed potential to produce significant quantities of carbohydrates. *C. vulgaris* gave the best carbohydrate content among the six strains tested in this study. In addition, *C. vulgaris* demonstrated the highest glucose content making it the most suitable candidate for carbohydrate production for bioethanol production.

Chapter 4. Optimization of biomass and carbohydrate production by an indigenous strain of *Chlorella vulgaris* under photoautotrophic growth conditions by response surface methodology

4.1. Abstract

Increasing energy prices demand a renewable, carbon neutral, transport fuel that is environmentally and commercially sustainable. The interest in the production of microalgae as biofuels is increasing due to their high carbohydrate content, rapid biomass production and small foot print. This study focused on the optimization of biomass and carbohydrate production by an indigenous microalgal strain *Chlorella vulgaris* (Toti RS4) using response surface methodology (RSM). The input variables; urea, K₂HPO₄, Na₂CO₃, temperature, light intensity and cultivation time were selected for optimization using RSM coupled with Box-Behnken design. Fifty-four batch experiments, including 6 replicates of the centre points were performed and the proximate data was fitted into the second order polynomial model. The predicted optimal levels were 0.203 g L⁻¹ of urea, 0.026 g L⁻¹ of K₂HPO₄, 0.012 g L⁻¹ of Na₂CO₃, 180.18 μmol m⁻²s⁻¹ of light intensity, 20 °C and 14 days of cultivation time. Under these conditions, the predicted values for biomass production and carbohydrate content were 488.4 mg L⁻¹ and 79.54% dcw, respectively. A mean biomass concentration and carbohydrate content of 486.30 mg L⁻¹ and 78.97% dcw with relative error % of 0.429 and 0.717, respectively was obtained from the experimental verification of the RSM model. The model for biomass production and carbohydrate content presented a coefficient of determination (R²) values of 0.999 and 0.983, respectively, suggesting the good quality of the generated models in predicting experimental data. The biomass yield and carbohydrate content suggest suitability of *C. vulgaris* (Toti RS4) as a candidate for potential biotechnological and bioenergy applications.

Keywords: Microalgae, Biomass, Carbohydrate, Response surface methodology.

4.2. Introduction

The rigorous search for renewable and sustainable biofuels as an alternative to fossil fuels is rapidly expanding and currently gaining global attention due to the imminent depletion of petroleum based fuels (Ang *et al.*, 2015). There are many available options in the bioenergy

sector, but unlike wind, solar and nuclear energy, biofuels have the capability of providing an energy source ideally suited to the existing infrastructure within the transport industry (Nejat *et al.*, 2015). Bioethanol is suggested as a suitable candidate biofuel to efficiently maintain or reduce levels of greenhouse gases. Microalgae are promising renewable bio-resources currently used as a feedstock for the commercial production of bioethanol, mainly because it does not compete with food supply, does not require the use of arable land and has higher growth rates when compared to terrestrial plants (Chen *et al.*, 2013).

Among several microalgal species existing in nature, *Chlorella vulgaris* is regarded as one of the leading candidates for bioethanol production due to its high growth rates and its ability to accumulate relatively high levels of carbohydrates over a short period of time (Takeshita *et al.*, 2014). How to trigger the synthesis of carbohydrates in microalgae is a hot topic in many industrial and academic research laboratories across the globe. Studies have shown that carbohydrate production in some microalgae could be increased by manipulating key nutritional and physical parameters such as nitrogen limitation (Pancha *et al.*, 2014), phosphorous limitation (Arora *et al.*, 2016; Markou *et al.*, 2012a; Singh *et al.*, 2015), high light intensity (Baer *et al.*, 2016; Niizawa *et al.*, 2014; Takeshita *et al.*, 2014), low temperature (Ota *et al.*, 2015; Wang *et al.*, 2016b) and high CO₂ supplementation (Chang *et al.*, 2016; He *et al.*, 2016; Sarat Chandra *et al.*, 2016). Under these stress conditions, microalgae respond by significantly increasing carbohydrate production in the range of 20-70% of dry cell weight (Tan *et al.*, 2016b; Zhang *et al.*, 2015). Of these factors, nitrogen limitation is the most widely used method since it is easy to manipulate in both open and closed cultivation systems and has been shown to be one of the factors with the strongest influence on the metabolism for carbohydrate production in various microalgal strains (Razaghi *et al.*, 2014). However, relatively low biomass associated with nitrogen limitation is the major obstacle limiting the application of this method for commercial bioethanol production since the low biomass concentration directly influences the operation cost for downstream processing, such as harvesting and carbohydrate extraction (Chatsungnoen and Chisti, 2016; Tan *et al.*, 2016b). Therefore, further research is required in order to achieve high carbohydrate content along with high biomass concentration to minimize operational costs.

Numerous studies have reported on the conventional parametric or one-factor-at-a time method to optimize microalgal biomass and carbohydrate production (George *et al.*, 2014; Wan *et al.*, 2013). However, this approach is laborious, expensive and time consuming. Moreover, it ignores the interaction between parameters and it does not ensure desirable conditions. Several reliable statistical experimental designs can be employed to reduce the

number of experiments under different conditions and can collectively eliminate the limitations associated with the single factor optimization strategy (Kumaran *et al.*, 2016; Quinlan and Lin, 2015). Moreover, statistical design methods are generally preferred because of various advantages they offer, such as rapid and reliable selection of key factors and understating their effects at varying concentrations or levels and significant reduction of the number of experiments, thus saving time and manpower. Numerous statistical methods such as full factorial, fractional factorial or Plackett-Burman design (PBD) and response surface methodology (RSM) have been described for process optimization. The PBD, a two-level factorial design, is used for identifying the most significant factors among a large number of factors and is, therefore, useful in preliminary studies to select factors that can be fixed or excluded in further optimization processes (Abdelhafez *et al.*, 2016; El-Sheekh *et al.*, 2016). The PBD has been favourably used by many researchers, for example, to identify which medium components give a maximum growth and production of metabolites (Naveena *et al.*, 2005). Srinophakun *et al.* (2017) used the PBD method to identify key nutritional medium components for the enhancement of biomass and biofuel production potential of *Arthrobacter* AK19.

Response surface methodology is a very useful method for identifying the best levels of the selected factors to obtain the best possible response. In contrast to the classical one-factor-at-a-time optimization strategy, RSM is a statistically designed method in which several factors are simultaneously varied (Baş and Boyacı, 2007). This method has already been successfully used in many fields, such as chemical industry, biology and engineering (Desai *et al.*, 2008). For instance, Ho *et al.* (2013b) examined variation in carbohydrate and biomass productivity by *C. vulgaris* FSP-E under different culture conditions. Process optimization using RSM was performed and interaction between operating variables was elucidated to establish the optimal conditions for maximum response.

The present study evaluates various optimization strategies to enhance biomass yield and carbohydrate production by an indigenous strain of *C. vulgaris* (Toti RS4). The significant factors were statistically identified using the PBD method. The most suitable nitrogen source for maximum biomass and carbohydrate production was established, having identified nitrogen as the most crucial factor affecting both biomass and carbohydrate production in *C. vulgaris* (Toti RS4). The optimum levels of factors for optimum biomass and carbohydrate yield was established using RSM. The predicted optimal growth conditions were empirically tested and statistically validated.

4.3. Materials and Methods

4.3.1. Microalgae and cultivation conditions

A strain of *Chlorella vulgaris* (Toti RS4), isolated from freshwater river at Amanzimtoti in the province of KwaZulu-Natal, South Africa, was used for this study. The standard BG-11 medium was used as the seed culture and growth medium as described in Section 3.3.2. The medium was initially sterilized by autoclaving at 121 °C for 20 min. Microalgal seeds (at an inoculum of 10% (v/v)) were cultivated in 250 mL Erlenmeyer flasks containing 100 mL of the total working volume. The flasks were exposed to an irradiance of 37 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a light:dark cycle of 12:12 h at a temperature of 25 ± 2 °C. The purity of the culture was maintained by sub-culturing on BG-11 agar and microscopic observation using a light microscope under 1000x magnification.

4.3.2. Analysis of cell growth

In all experiments, three replicates of batch cultures were set up for each treatment. Microalgal suspension in each treatment was adjusted to an OD of 0.05 at 680 nm using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA). Ten percent (v/v) of the microalgal culture was used as initial inoculum. Dry cell biomass was measured as the cell density (dcw, mg L^{-1}) at $\text{OD}_{680\text{nm}}$ of an actively growing culture at dilutions ranging from 0.2 to 1.0 (Appendix A). The dry biomass was calculated using the following regression equation:

$$X = 625.93 \times \text{OD}_{680} + 6.25; R^2 = 0.9953 \quad (4.1)$$

where, X is the cell density (in mg L^{-1}) and x is the optical density at $\text{OD}_{680\text{nm}}$

4.3.3. Carbohydrate analysis

Total carbohydrate and glucose content was determined using the Anthone reagent (Yemm and Willis, 1964) and glucose oxidase assay as previously described in sections 3.3.9.3 and 3.3.9.4, respectively.

4.3.4. Plackett-Burman design (PBD) for orthogonal growth conditions

In contrast to the RSM design methods such as Box-Behnken and central composite design (CCD), PBD does not describe the interaction between the factors and is based on the first order model as described by Eq (4.2) (Naveena et al., 2005):

$$Y = \beta_0 + \sum \beta_i x_i \quad (4.2)$$

Where Y is the response (biomass and carbohydrate production), β_0 is the model's intercept, β_i is the linear coefficient and x_i is the level of the independent variable.

In this study, PBD was used to evaluate medium components as well as environmental factors with regards to their influence on biomass and carbohydrate yield by the indigenous microalgal strain, *C. vulgaris* (Toti RS4). The chosen factors were (X_1 to X_{16}) representing nitrogen, K_2HPO_4 , Na_2CO_3 , $MgSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, EDTA, citric acid, ferric ammonium citrate, micronutrient solution, light intensity, temperature, cultivation time, salinity, pH, inoculum size and agitation, respectively. Each independent factor was investigated at two levels; -1 for a low level and +1 for a high level, according to the Plackett-Burman design matrix, as shown in [Table 4.1](#).

Table 4.1 Experimental variables and their respective units and levels determined in the Plackett-Burman design.

Factor	Unit	Code	Low level (-1)	High level (+1)
Nitrogen	g L ⁻¹	X ₁	0	1.5
K ₂ HPO ₄	g L ⁻¹	X ₂	0	0.04
Na ₂ CO ₃	g L ⁻¹	X ₃	0	0.02
MgSO ₄ ·7H ₂ O	g L ⁻¹	X ₄	0	0.075
CaCl ₂ ·2H ₂ O	g L ⁻¹	X ₅	0	0.036
EDTA	g L ⁻¹	X ₆	0	0.001
Micronutrient solution	mL L ⁻¹	X ₇	0	1
Citric acid	g L ⁻¹	X ₈	0	0.006
Ammonium ferric citrate	g L ⁻¹	X ₉	0	0.006
Light intensity	μmol m ⁻² s ⁻¹	X ₁₀	37	200
Temperature	°C	X ₁₁	20	30
Cultivation time	days	X ₁₂	14	24
Salinity	g L ⁻¹	X ₁₃	0	0.025
pH		X ₁₄	6	8
Inoculum size	% (v/v)	X ₁₅	5	15
Agitation speed	rpm	X ₁₆	100	200

The independent factor levels were decided based on the nutritional levels of BG-11 medium as well as from several reports in literature ([Bonfond et al., 2016](#); [Chang et al., 2016](#); [He et al., 2015](#); [Kumaran et al., 2016](#); [Ota et al., 2015](#)). Determination of biomass and carbohydrate yield (responses) was from the average of three replicate trials.

The main effect of each factor was calculated using Eq (4.3) (Naveena *et al.*, 2005):

$$E(x_i) = \frac{2(\sum M_i^+ - M_i^-)}{N} \quad (4.3)$$

Where $E(x_i)$ is the concentration effect of the tested variable M_i^+ and M_i^- are the responses from the trials where the factors (x_i) measured are present at high and low concentrations, respectively, and N is the number of trials. Experimental error was estimated by calculating the variance among the dummy variables as described in Eq (4.4) (Naveena *et al.*, 2005):

$$V_{\text{eff}} = \frac{\sum (E_d)^2}{n} \quad (4.4)$$

Where V_{eff} is the variance of the concentration effect, E_d is the concentration effect for dummy variable and n is the number of dummy variables. The standard error (SE) of the concentration effect was the square root of the variance of the effect. Factors significant at the 95% level ($p < 0.05$) were considered reliable.

4.3.5. Selection of nitrogen source

The microalgal strain was cultivated in BG-11 medium containing NaNO_3 (1.5 g L^{-1}), NH_4NO_3 (1.5 g L^{-1}) or urea (1.5 g L^{-1}) as nitrogen sources. BG-11 medium with no nitrogen source was used as a control. All the experiments were performed in triplicate in 1 L Erlenmeyer flasks with a working volume of 500 mL of respective medium and inoculated with 10% (v/v) of an actively growing culture of *C. vulgaris* (Toti RS4). All the experiments were performed in batch culture at $25 \pm 2 \text{ }^\circ\text{C}$ with 12h: 12h light: dark period under a light intensity of $37 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (illuminated using cool white fluorescent light). The light intensity was measured using an HD-2102.2 Meter with an LP-471PAR pyranometer sensor (Delta OHM, Italy). The flasks were shaken manually twice daily to prevent settling of microalgal cells. Urea resulted in the highest biomass and carbohydrate yield and was used as a nitrogen source in BG-11 for further experimentation.

4.3.6. Box-Behnken experimental design

The factors identified by the Plackett-Burman design to significantly influence the responses were further optimized using the RSM coupled with Box-Behnken design. The selected factors were: urea, K_2HPO_4 , Na_2CO_3 , temperature, light intensity and cultivation time. For this procedure, fifty-four batch experiments, including six replicates of the center points were

required. The factors were investigated at three levels, coded +1, 0, and -1 for high, intermediate and low value respectively, as shown in [Table 4.2](#).

Table 4.2 Generation of coded levels of the significant independent variables used in the Box-Behnken design.

Independent factors	Unit	Code	Level		
			-1	0	1
Urea	g L ⁻¹	X ₁	0	0.75	1.5
K ₂ HPO ₄	g L ⁻¹	X ₂	0	0.02	0.04
Na ₂ CO ₃	g L ⁻¹	X ₃	0	0.01	0.02
Temperature	°C	X ₄	20	25	30
Light intensity	μmol m ⁻² s ⁻¹	X ₅	37	118.5	200
Cultivation time	days	X ₆	14	19	24

These conditions were established and the variables were coded according to Eq (4.5) ([Desai et al., 2008](#)):

$$x_i = \frac{(X_i - X_{cp})}{\Delta X_i} \quad (4.5)$$

where x_i is the coded level of the variable, X_i is the real level of the variable, X_{cp} is the real level of the variable at the centre point and ΔX_i is the step change value at the real level.

The experimental data were fitted to the second-order polynomial model as described in Eq (4.6):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (4.6)$$

In this equation, Y is the response in coded units, β_0 is a constant; β_i is the linear coefficient, β_{ii} is the squared coefficient and β_{ij} is the interactive coefficient considered in the model. The experimental design and statistical analysis were performed using Design expert 10.0.0 (State-Ease, Inc, USA). The significance and adequacy of the model was evaluated according to the F -test and correlation coefficient (R^2). The statistical significance of the model equation and model term was evaluated using the p -value ($p < 0.05$).

4.4. Results and Discussion

4.4.1. Identification of significant growth factors using Plackett-Burman experimental design

Several investigations have been carried out to evaluate the effect of different media composition and environmental conditions on biomass yield and carbohydrate production by diverse microalgal strains (George *et al.*, 2014; Srinophakun *et al.*, 2017). In the present study, PBD was used as a decision making tool to identify key nutritional components in BG-11 medium as well as physico-chemical variables contributing to the enhancement of biomass and carbohydrate production in the indigenous microalgal strain, *C. vulgaris* (Toti RS4). The experimental design matrix and the corresponding experimental outputs are presented in [Table 4.3](#).

The maximum predicted and actual biomass yield values were 1049 mg L⁻¹ and 1045 mg L⁻¹, respectively, as shown in run 6 ([Table 4.3](#)). The maximum predicted and actual carbohydrate yield values were 95.45 mg L⁻¹ day⁻¹ and 94.41 mg L⁻¹ day⁻¹, respectively (in run 5) ([Table 4.3](#)). These findings demonstrate the strong influence of media composition and environmental conditions on biomass and carbohydrate production. Experimental run 20 gave the lowest biomass and carbohydrate yield values because no additional exogenous nutritional component was incorporated into the medium (BG-11) and the cultivation conditions were kept at their low levels (-1).

Table 4.3. The Plackett-Burman design matrix for screening of variables influencing biomass and carbohydrate production by *C. vulgaris* (Toti RS4), where X_1 to X_{16} are coded independent variables and D_1 to D_2 are dummy variables.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	D_1	D_2	Biomass production (mg L ⁻¹)		Carbohydrate production (mg L ⁻¹ day ⁻¹)	
																			Observed	Predicted	Observed	Predicted
1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	1	1	-1	692.5	698.2	63.74	63.77
2	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	601.2	602.8	63.21	63.55
3	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	1	1	1	1	478.4	479.2	72.81	73.82
4	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	1	-1	1	-1	238.4	238.2	66.12	66.55
5	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	-1	1	842.5	841	94.41	95.45
6	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	1045	1049	88.61	88.72
7	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	-1	1	-1	1	194.5	195.4	61.90	61.92
8	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	1	897.8	897.1	79.89	80.05
9	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	-1	902.4	903.7	88.12	88.08
10	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	1	1	465.9	465	91.36	92.31
11	1	1	1	1	-1	1	-1	1	-1	-1	-1	-1	1	1	1	1	-1	-1	1039	1041	55.12	55.15
12	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	1	-1	-1	-1	1	896.5	897.4	78.14	78.26
13	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	1	-1	-1	-1	683.1	682.2	79.45	79.85
14	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	-1	1	414.5	413.3	68.15	68.06
15	1	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	1	1	1	902.8	901.8	76.92	77.72
16	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	1	-1	-1	1	-1	-1	401	400	74.51	75.42
17	1	-1	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	-1	850	849.6	90.88	91.69
18	1	-1	-1	-1	-1	1	1	-1	1	1	-1	-1	1	1	1	-1	1	-1	669.1	669.7	49.24	49.11
19	1	1	-1	1	1	-1	-1	1	1	1	1	-1	1	-1	-1	-1	-1	-1	928.1	927.4	70.41	70.04
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	33.42	33.63	8.412	8.763

The nutritional and physico-chemical factors conditions have played an important role in the production of biomass and carbohydrates by the indigenous microalga *C. vulgaris* (Toti RS4) (Table 4.3). Thus, the relative significance of the sixteen factors, nitrogen (X_1), K_2HPO_4 (X_2) and Na_2CO_3 (X_3), $MgSO_4 \cdot 7H_2O$ (X_4), $CaCl_2 \cdot 2H_2O$ (X_5), EDTA (X_6), micronutrient solution (X_7), citric acid (X_8), ammonium ferric citrate (X_9), light intensity (X_{10}), temperature (X_{11}), cultivation time (X_{12}), Salinity (X_{13}), pH (X_{14}), inoculum size (X_{15}) and agitation speed (X_{16}) was investigated using by PBD (Table 4.4). These results suggest that biomass and carbohydrate yields were directly related to nitrogen, K_2HPO_4 and Na_2CO_3 under the conditions investigated. Factors such as $CaCl_2 \cdot 2H_2O$, EDTA, micronutrient solution, ammonium ferric citrate, light intensity, temperature, pH, inoculum size and agitation speed, had little effect on biomass production, while they had a notable effect on carbohydrate content. On the other hand, $MgSO_4 \cdot 7H_2O$, citric acid, and cultivation time suppressed biomass production, while promoting carbohydrate accumulation. Salinity was shown to suppress both biomass production and carbohydrate content under the experimental conditions investigated. This is in contrast to the findings of Kong *et al.* (2012), presumably due to salt concentration differences or the behaviour of the microalgal strains.

Table 4.4 Analysis of the effect of variance on biomass and carbohydrate production by *C. vulgaris* under different independent variable conditions.

Factor	Biomass production (mg L ⁻¹)				Carbohydrate production (mg L ⁻¹ day ⁻¹)			
	Effect	Coefficient	F-value	p-value	Effect	Coefficient	F-value	p-value
X_1 -Nitrogen	476.98	240.69	1016.74	0.0010	12.03	0.97	41.33	0.0234
X_2 - K_2HPO_4	222.02	120.57	255.14	0.0039	3.389	6.23	1689.12	0.0006
X_3 - Na_2CO_3	198.84	108.44	206.38	0.0048	10.46	4.50	880.33	0.0011
X_4 - $MgSO_4 \cdot 7H_2O$	-32.603	-48.90	41.97	0.0230	7.336	0.15	1.00	0.4226
X_5 - $CaCl_2 \cdot 2H_2O$	61.82	-45.67	36.61	0.0262	9.549	0.098	0.42	0.5833
X_6 -EDTA	21.02	6.67	0.78	0.4701	5.349	0.098	0.42	0.5833
X_7 -Micronutrient solution	24.10	30.50	16.33	0.0561	4.566	-0.098	0.42	0.5833
X_8 -Citric acid	-11.80	24.87	10.86	0.0811	4.294	0.098	0.42	0.5833
X_9 -Ammonium ferric citrate	50.02	-7.30	0.93	0.4357	2.667	0.15	1.00	0.4226
X_{10} -Light intensity	29.38	239.98	852.41	0.0012	7.706	4.81	0.54	0.5399
X_{11} -Temperature	32.12	121.50	218.49	0.0045	10.85	10.88	2.74	0.2396
X_{12} -Cultivation time	-0.203	107.58	171.30	0.0058	10.44	11.45	3.04	0.2235
X_{13} -Salinity	-17.66	-46.43	31.91	0.0399	-6.428	4.89	0.55	0.5341
X_{14} -pH	8.292	-46.50	32.00	0.0379	6.740	2.70	0.17	0.7205
X_{15} -Inoculum size	20.78	31.43	0.68	0.4958	9.314	6.47	1.93	0.2994
X_{16} -Agitation speed	26.07	7.10	9.60	0.0903	7.388	15.48	2.92	0.2298

The analysis of variance (ANOVA) of the data obtained was then performed with the aim of evaluating and ranking factors by their degree of impact on biomass and carbohydrate production (Table 4.4). Factors with a p -value lower than 0.05 were considered significant. Nitrogen was considered as the most significant factor for biomass accumulation ($p= 0.001$), followed by K_2HPO_4 ($p= 0.004$) and Na_2CO_3 ($p= 0.005$). On the other hand, K_2HPO_4 was considered the most significant factor for carbohydrate content ($p= 0.0006$), followed by Na_2CO_3 ($p= 0.001$) and nitrogen ($p= 0.023$). The PBD experiments on the production of biomass and carbohydrates from *C. vulgaris* under photoautotrophic conditions indicated that the most important factors were nitrogen, phosphorous (K_2HPO_4) and carbon (Na_2CO_3) which is in agreement with the previously published data (Singh *et al.*, 2015; Zhang *et al.*, 2015).

The physical factors such as light intensity and temperature have been reported to be some of the most crucial factors affecting growth and biochemical composition of microalgae cultivated under photoautotrophic conditions (Huesemann *et al.*, 2016; Ota *et al.*, 2015). Furthermore, previous studies have indicated that there could be a synergistic relationship between light intensity and temperature for carbohydrate production by microalgae. For example, at ambient temperatures (25 °C) and light intensity of $1720 \mu\text{mol m}^{-2} \text{s}^{-1}$, the photosynthetic rate of *Spirulina platensis* was reported to decrease by up to 50% in 1 h, but similar light intensities had no effect at high temperatures (35 °C) (Jensen and Knutsen, 1993). These observations indicate that investigating the combined effects of temperature and light intensity in the Box-Behnken design could be significant. Therefore, light intensity and temperature were considered for further investigation. In addition to light intensity and temperature, the cultivation period has recently been marked as one of the crucial factors affecting biomass and biochemical composition of microalgal cells (Hu *et al.*, 2015; Ördög *et al.*, 2016). For instance, Pancha *et al.* (2014) demonstrated that carbohydrate productivity of microalgae can be increased through nitrogen starvation. However, it is important to establish the cultivation period for optimum carbohydrate production. In another study, Chellamboli and Perumalsamy (2014) reported the significance of cultivation time on the growth of the microalgae, *Scenedesmus abundans*. The researchers found that lengthening the cultivation period resulted in an increase in growth rates and concluded that newly cultured cells required a certain period to overcome the lag/adaptation phase before optimum growth can be observed. Therefore, cultivation time was also considered for further optimization in this study.

The fitness of the model used was checked using the *F*-value, coefficient of variance (CV %) and R² values. The *F*-values of the model used to determine significant factors for biomass yield and carbohydrate production was 38.5 and 23.8, respectively. The large *F*-value for both models demonstrates that they were both statistically significant, as revealed by their corresponding *p*-values that were lower than 0.05. The R² values for biomass and carbohydrate production model were 0.9987 and 0.9992, respectively, indicating the goodness of fit of the data to the models. Lastly, the CV % for biomass and carbohydrate production models were 3.97 and 2.44 respectively, indicating a good reliability of the experiments performed, since the higher the value of CV %, the lower the reliability of that particular experiment.

4.4.2. Effect of nitrogen source on biomass and carbohydrate production

4.4.2.1. Effect of nitrogen source on biomass production by *C. vulgaris* (Toti RS4)

From the preliminary investigations, nitrogen was found to be the most influential factor on biomass and carbohydrate production by *C. vulgaris* (Toti RS4) under the conditions tested. Sources of nitrogen for algal growth include the inorganic ions such as ammonium, nitrate, and organic compounds such as urea, amino acids and purines. Nitrogen plays a crucial role in the regulation of cellular metabolites (Arora *et al.*, 2016). The preference of nitrogen source may vary substantially between the microalgal strains (Gonzalez-Garcinuno *et al.*, 2014). Therefore, it is important to identify the most suitable nitrogen source in order to increase the product yields. In this study, the nitrogen sources evaluated under *in vitro* conditions (urea, sodium nitrate and ammonium nitrate) were selected based on low cost and availability.

Among the different nitrogen sources investigated in this study, ammonium nitrate was the least effective since poor growth of *C. vulgaris* (Toti RS4) was observed under the growth conditions used, accompanied by slower growth rate and early entry into stationary growth phase by the organism (Fig. 4.1). This may be attributed to the drop in the pH of the culture medium (from 7.5 to 4.38) during the assimilation of ammonium ions in the cultivation process. Similarly, inhibition of growth of *Chlorella sorokiniana* has been attributed to decrease in pH value from 7.97 to 5.5 due to ammonium ion assimilation (Ramanna *et al.*, 2014). Studies have suggested that acidic culture conditions disrupt cellular respiration by depletion of tricarboxylic acid (TCA) cycle intermediates, resulting in slower growth rates. However, certain microalgal strains such as *Dunaliella acidophila* can grow in acidic environments by accumulating glycerol to prevent the osmotic imbalance caused by acid build-up while other species such as *Euglena mobilis* exhibit

a pH tolerance range of 3 to 9 by utilizing energy intensive proton pumps (Lane and Burris, 1981). In a review by Chiu *et al.* (2015), optimum concentration of ammonium for biomass production of the *Chlorella* cultures was found between 0.2-0.4 g L⁻¹, however, in the present study, 1.5 g L⁻¹ of ammonium concentration was used. Such high nutrient concentration used in this study could have also attributed to the inhibition of microalgal growth.

The maximum biomass yield obtained using urea as N source was 477.69 ± 9.3 mg L⁻¹, about 3-fold higher than 163.72 ± 2.3 mg L⁻¹ obtained with ammonium nitrate. The biomass yield obtained using sodium nitrate was approximately 434.06 ± 7.1 mg L⁻¹, value that is 6-fold higher than that obtained from the control experiment (Fig. 4.1). The effectiveness of urea as a nitrogen source could be attributed to its ability to maintain a constant pH due to the liberation of additional carbon source (CO₂ /2HCO₃⁻) during its decomposition, which plays a role in augmenting microalgal growth as well as providing excess carbon flux towards protein production (Ramanna *et al.*, 2014).

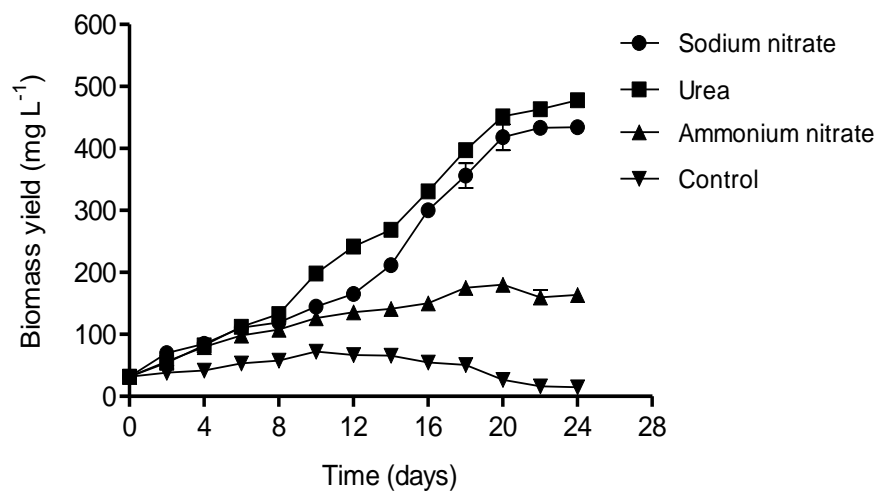


Fig. 4.1. Growth of *C. vulgaris* (Toti RS4) cultivated in BG-11 medium supplemented with different nitrogen sources. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

4.4.2.2. Effect of nitrogen source on carbohydrate production by *C. vulgaris* (Toti RS4)

The carbohydrate content obtained from the medium supplemented with urea as nitrogen source gave the total carbohydrate and reducing sugar content, of 50.1 ± 0.005 % dcw and 46.42 ± 0.001 % (dcw), respectively (Fig. 4.2). These values were consistent with those

reported by Ho *et al.* (2013b), where urea was the best nitrogen source for *C. vulgaris* FSP-E, with the highest carbohydrate and glucose content of 51.0 ± 0.7 % dcw and 46.6 ± 0.6 % obtained, respectively. In this study, ammonium nitrate gave the lowest total carbohydrate content (37.41 ± 0.0035 % (dcw) and lowest glucose yields (33.33 ± 0.009 % (dcw)), (Fig. 4.2). Unlike urea and sodium nitrate which require several energy intensive conversion steps prior to being utilized by microalgae, ammonium is readily utilized by microalgae, making the energy demand for ammonium utilization much lower, when compared with urea and sodium nitrate. Therefore, the energy stored in the form of lipids and carbohydrates in microalgae cultivated in ammonium rich medium should technically be higher than when cultivated in nitrate or urea rich medium (Li *et al.*, 2008; Razaghi *et al.*, 2014).

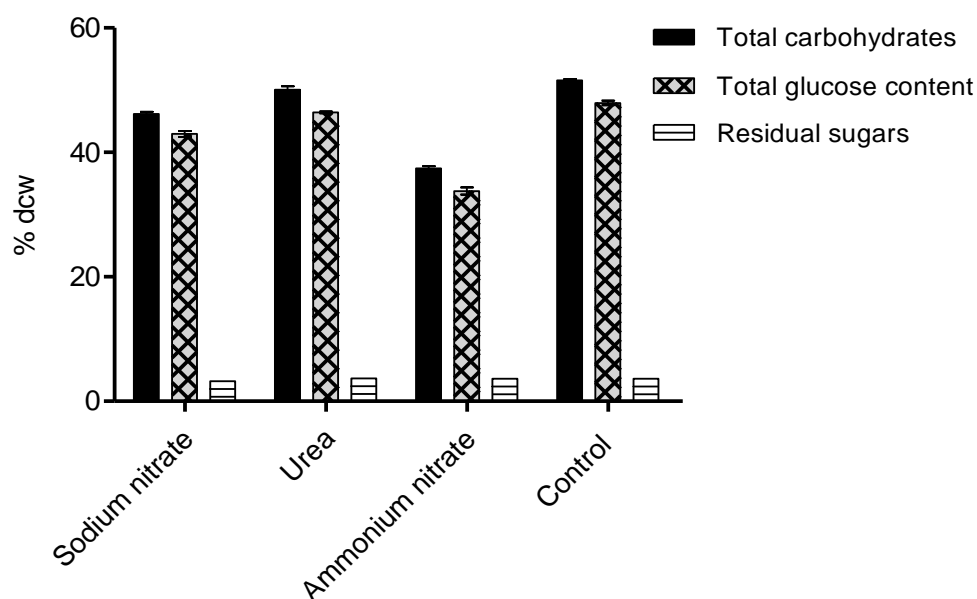


Fig. 4.2. Carbohydrate production by *C. vulgaris* (Toti RS4) cultivated using different nitrogen sources. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

The total carbohydrate content obtained from medium using sodium nitrate as the nitrogen source was 46.17 ± 0.004 % (dcw) and was higher than some of the values reported in the literature (Selvarajan *et al.*, 2015). Nevertheless, the highest carbohydrate (51.6% dcw) and glucose (47.94% dcw) content were obtained when *C. vulgaris* was cultivated under nitrogen starvation condition. The carbohydrate content obtained under this condition was 37.93%,

11.76% and 2.99% higher than that obtained for ammonium nitrate treatment, sodium nitrate treatment and urea treatment, respectively (Fig. 4.2). However, the high carbohydrate yields obtained under nitrogen starvation conditions was accompanied by low biomass yields that may greatly affect the cost involved during harvesting. For this reason, it is feasible and reasonable to utilize urea as a nitrogen source for the propagation of this *C. vulgaris* (Toti RS4) strain. Urea is very cheap compared to other nitrogen sources available for microalgae cultivation (≈US \$2.0/kg biomass), which makes it economically suitable for large-scale production of microalgae fuels. However, the major drawback to the use of urea for large-scale microalgal cultivation is that it could result in eutrophication and algal blooms if it leaches away from the raceway pond into receiving water bodies. Nevertheless, benthic microalgae with high nutrient removal rates can be used to circumvent this problem (Caporgno *et al.*, 2016).

4.4.3. Modelling and optimization of significant process variables

4.4.3.1. Model building and statistical significance test

The Box-Behnken design was used to examine the combined effects of six different factors on biomass and carbohydrate production (Appendix I). The second-order polynomial equations were used to determine the relationship between factors and the responses (biomass and carbohydrate production). The second-order polynomial coefficient for each term of the equation was determined using multiple regression analysis using the Design expert software. Analysis of variance was used in order to ensure a reliable model (Table 4.5). Factors with a p -value of <0.05 were considered significant.

Table 4.5 One-way ANOVA results of the fitted model for biomass production and carbohydrate content.

Factor	Biomass production (mg L ⁻¹)					Carbohydrate content (% dcw)				
	Sum of Squares	df	Mean Square	F-value	p-value Prob>F	Sum of Squares	df	Mean Square	F-value	p-value Prob>F
Model	1.879E+006	27	69580.12	19298.22	< 0.0001	9380.33	27	347.42	56.79	< 0.0001
X ₁ -Urea	1.319E+006	1	1.319E+006	3.658E+005	< 0.0001	811.43	1	811.43	132.64	< 0.0001
X ₂ -K ₂ HPO ₄	3.108E+005	1	3.108E+005	86204.07	< 0.0001	404.59	1	404.59	66.13	< 0.0001
X ₃ -Na ₂ CO ₃	1.680E+005	1	1.680E+005	46586.67	< 0.0001	50.81	1	50.81	8.31	0.0078
X ₄ -Temperature	22368.72	1	22368.72	6204.02	< 0.0001	143.52	1	143.52	23.46	< 0.0001
X ₅ - Light intensity	29736.96	1	29736.96	8247.62	< 0.0001	508.30	1	508.30	83.09	< 0.0001
X ₆ -Cultivation time	4887.76	1	4887.76	1355.63	< 0.0001	47.26	1	47.26	7.73	0.0100
X ₁ X ₂	16882.03	1	16882.03	4682.27	< 0.0001	716.69	1	716.69	117.15	< 0.0001
X ₁ X ₃	155.76	1	155.76	43.20	< 0.0001	154.00	1	154.00	25.17	< 0.0001
X ₁ X ₄	374.42	1	374.42	103.85	< 0.0001	621.88	1	621.88	101.65	< 0.0001
X ₁ X ₅	818.10	1	818.10	226.90	< 0.0001	711.59	1	711.59	116.32	< 0.0001
X ₁ X ₆	63.28	1	63.28	17.55	0.0003	13.99	1	13.99	2.29	0.1425
X ₂ X ₃	571.22	1	571.22	158.43	< 0.0001	108.34	1	108.34	17.71	0.0003
X ₂ X ₄	682.65	1	682.65	189.33	< 0.0001	252.90	1	252.90	41.34	< 0.0001
X ₂ X ₅	167.06	1	167.06	46.33	< 0.0001	243.67	1	243.67	39.83	< 0.0001
X ₂ X ₆	603.78	1	603.78	167.46	< 0.0001	935.71	1	935.71	152.95	< 0.0001
X ₃ X ₄	301.35	1	301.35	83.58	< 0.0001	88.31	1	88.31	14.44	0.0008
X ₃ X ₅	800.00	1	800.00	221.88	< 0.0001	68.09	1	68.09	11.13	0.0026
X ₃ X ₆	18.49	1	18.49	5.13	0.0321	12.96	1	12.96	2.12	0.1575
X ₄ X ₅	318.78	1	318.78	88.41	< 0.0001	1024.91	1	1024.91	167.53	< 0.0001
X ₄ X ₆	341.91	1	341.91	94.83	< 0.0001	362.34	1	362.34	59.23	< 0.0001
X ₅ X ₆	7.41	1	7.41	2.06	0.1636	4.38	1	4.38	0.72	0.4051
X ₁ ²	120.46	1	120.46	33.41	< 0.0001	131.23	1	131.23	21.45	< 0.0001
X ₂ ²	814.99	1	814.99	226.04	< 0.0001	868.11	1	868.11	141.90	< 0.0001
X ₃ ²	370.97	1	370.97	102.89	< 0.0001	408.01	1	408.01	66.69	< 0.0001
X ₄ ²	302.41	1	302.41	83.87	< 0.0001	454.78	1	454.78	74.34	< 0.0001
X ₅ ²	305.67	1	305.67	84.78	< 0.0001	373.15	1	373.15	61.00	< 0.0001
X ₆ ²	381.86	1	381.86	105.91	< 0.0001	325.53	1	325.53	53.21	< 0.0001
Residual	93.74	26	3.61			159.06	26	6.12		
Lack of Fit	62.82	21	2.99	0.48	0.8898	125.74	21	5.99	0.90	0.6165
Pure Error	30.93	5	6.19			33.32	5	6.66		
Cor Total	1.879E+006	53				9539.39	53			

All the linear (X₁ to X₆) and quadratic (X₁² to X₆²) terms were significant model terms for biomass production (Table 4.5). Of all the interactive terms in this model, the term (X₅X₆) was the only insignificant model term, suggesting that it had the least contribution towards biomass production. For carbohydrate production, all of the linear (X₁ to X₆) and quadratic (X₁² to X₆²) terms were significant model terms. The three interactive terms (X₁X₆, X₃X₆ and X₅X₆) were found to be insignificant models, suggesting that they contributed less to the production of carbohydrates by *C. vulgaris* (Toti RS4) under the described conditions.

The model *F*- values of 19298.22 and 56.79 for biomass and carbohydrate production, respectively and the probability values (Prob > *F*) of less than 0.05, indicated that the models

used were significant. The lack of fit of the model (p -value of > 0.05) for biomass and carbohydrate production demonstrated that the model used was insignificant (Table 4.5).

The final response equations for biomass production and carbohydrate content from *C. vulgaris* could be expressed in the equations below:

$$Y_{\text{Biomass production}} = 524.117 + 234.421X_1 + 113.8X_2 + 83.6583X_3 + 30.5292X_4 + 35.2X_5 + 14.2708X_6 - 45.9375X_1X_2 - 4.4125X_1X_3 - 4.8375X_1X_4 - 10.1125X_1X_5 - 2.8125X_1X_6 - 8.45X_2X_3 - 9.2375X_2X_4 - 3.23125X_2X_5 - 8.6875X_2X_6 - 6.1375X_3X_4 - 10X_3X_5 - 1.075X_3X_6 - 6.3125X_4X_5 + 6.5375X_4X_6 + 0.9625X_5X_6 + 3.42222X_1^2 + 8.90139X_2^2 + 6.00556X_3^2 + 5.42222X_4^2 + 5.45139X_5^2 + 6.09306X_6^2 \quad (4.7)$$

$$Y_{\text{Carbohydrate content}} = 36.7317 - 5.81458X_1 - 4.10583X_2 + 1.455X_3 - 2.44542X_4 + 4.60208X_5 - 1.40333X_6 - 9.465X_1X_2 - 4.3875X_1X_3 - 6.23437X_1X_4 - 9.43125X_1X_5 - 1.3225X_1X_6 - 3.68X_2X_3 - 5.6225X_2X_4 - 3.9025X_2X_5 - 10.815X_2X_6 - 3.3225X_3X_4 - 2.9175X_3X_5 - 0.9X_3X_6 - 11.3188X_4X_5 + 6.73X_4X_6 + 0.74X_5X_6 + 3.57194X_1^2 + 9.18694X_2^2 + 6.29819X_3^2 + 6.64944X_4^2 + 6.02319X_5^2 + 5.62569X_6^2 \quad (4.8)$$

Where Y is the predicted biomass/ carbohydrate production; X_1 , X_2 , X_3 , X_4 , X_5 and X_6 are the coded values of urea, K_2HPO_4 , Na_2CO_3 , temperature, light intensity and cultivation time, respectively.

The model for biomass production and carbohydrate content presented a coefficient of determination (R^2) values of 0.999 and 0.9833, respectively, which translated to response variability of 99.9% and 98.33%, respectively. From these values, a good correlation was achieved, indicating a good fit of the suggested models.

4.4.3.2. Mutual interaction between significant factors

Response surface methodology is a very convenient statistical tool aimed at identifying the best levels of process components in order to obtain the best possible response. The main advantage of the RSM is that the effect of the individual components (factors) on the system can be studied much easier when compared to other statistical tools such as artificial neural networks. Since the variables in the quadratic equation of the RSM are in the normalized form, the coefficients of the equations give a direct insight of the contribution of the various factors in the system. As shown in Eq. (4.7) and Eq. (4.8), (X_1) has the largest coefficient of 243.421 and 5.81458, respectively. This indicates that urea (X_1) was the most influential factor on biomass and carbohydrate production. For the interactive terms, the interaction (X_1X_2) had the highest coefficient (45.9375) for biomass production (Eq. 4.7). For carbohydrate production, the interaction (X_4X_5) had the highest coefficient (11.3188) which indicates that it was the most influential interaction (Eq. 4.8). These findings are presented in detail using ANOVA (Table 4.5). Three-dimensional graphs were also included in determining the sensitivity of the responses of two interacting factors by holding the other factors at the central value. Response surface plots, as shown in Fig. 4.3 represent the top four of the most influential interactive factors on biomass production.

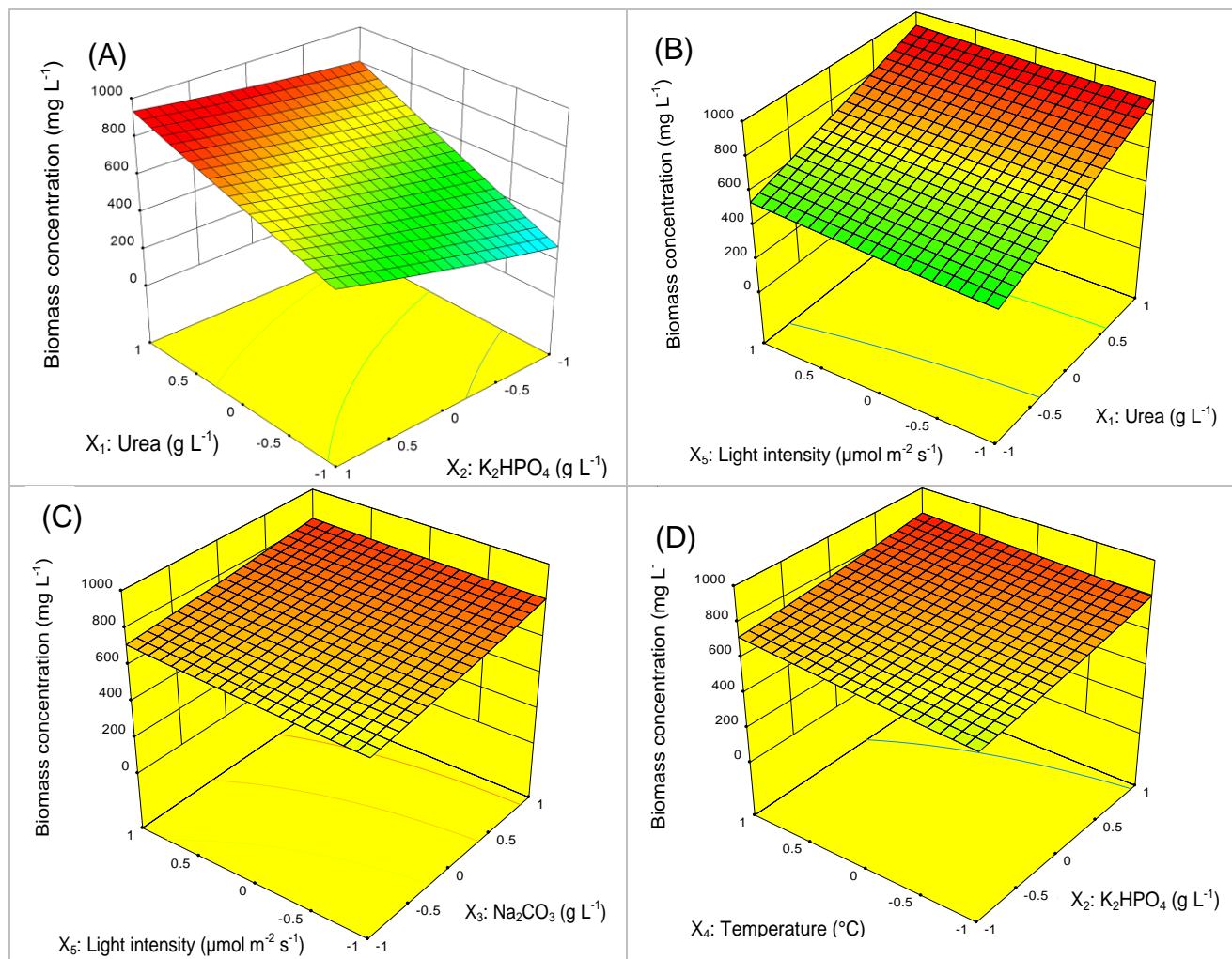


Fig. 4.3. Three – dimensional response surface plots for biomass production with varying urea vs. K_2HPO_4 (A), varying urea vs light intensity (B), varying light intensity vs. Na_2CO_3 (C), Temperature vs. K_2HPO_4 (D), and all other variables were kept at their central values.

Fig. 4.3 (A) represents the effect of urea and K_2HPO_4 individually and their combined interactive effect on biomass production. The mutual interaction between urea and K_2HPO_4 had a significant effect on biomass production ($p < 0.05$) (Table 4.5). An increase in the amount of urea and K_2HPO_4 led to higher yields of biomass. This result was in agreement with the work reported by *Xin et al. (2010)*, in which high concentrations of phosphorus and nitrogen enhanced biomass production in *Scenedesmus* sp. This is likely due to the requirements of these macromolecules for the synthesis of essential biological material such as nucleic acids, pigments and proteins. Fig. 4.3 (B) shows the interactive effect between urea and light intensity,

which also had a significant effect on biomass production ($p < 0.05$) (Table 4.5). Higher biomass yields were obtained under high levels of urea and light intensity. The light intensity affects the uptake of nutrients during photoautotrophic growth. In addition, light intensity has been shown to influence the activity of PSII (White *et al.*, 2011; Yang *et al.*, 2012). Microalgae can tolerate a broad range of light intensity, typically between 200-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$, depending on the microalgal species. Increasing the light intensity up to optimum level results in the improvement of microalgal growth (Fig. 4.3 (B)). However, above the optimum level of light intensity, the growth rate decreases due to photo-oxidation (Wahidin *et al.*, 2013).

Fig. 4.3 (C) shows the interactive effect between light intensity and Na_2CO_3 , where biomass production was shown to increase with an increase in light intensity and Na_2CO_3 . Therefore, Na_2CO_3 may serve as an excellent carbon source for microalgae in cases where CO_2 is limited such as in large-scale cultivation systems (e.g., open raceway ponds). In the presence of light, microalgae can drive the conversion of a carbon source (e.g., CO_2 , HCO_3^-) into organic and inorganic substances such as glucose and oxygen. The glucose can be stored as starch or used for the synthesis of adenosine triphosphate (ATP) which provides the cells with the chemical energy needed for growth. Finally, Fig. 4.3 (D) shows the interactive effect between temperature and K_2HPO_4 . From the plots, the biomass production was directly related to both temperature and K_2HPO_4 . High biomass yields were attained at high temperatures and high concentrations of K_2HPO_4 .

The combined effect between factors on carbohydrate yields was also studied using the three-dimensional response surface plots as shown in Fig. 4.4. Fig. 4.4 (A) represents the effect of urea and K_2HPO_4 individually and their combined interactive effect on carbohydrate yields. The mutual interaction between urea and K_2HPO_4 had a significant effect on the production of carbohydrates ($p < 0.05$) (Table 4.5). A decrease in the concentration of urea and K_2HPO_4 led to an increase in the carbohydrate content. This is likely due to the turn of the flow of the photosynthetically fixed carbon from the protein synthesis metabolic pathway to the carbohydrate synthesis pathway (Xin *et al.*, 2010).

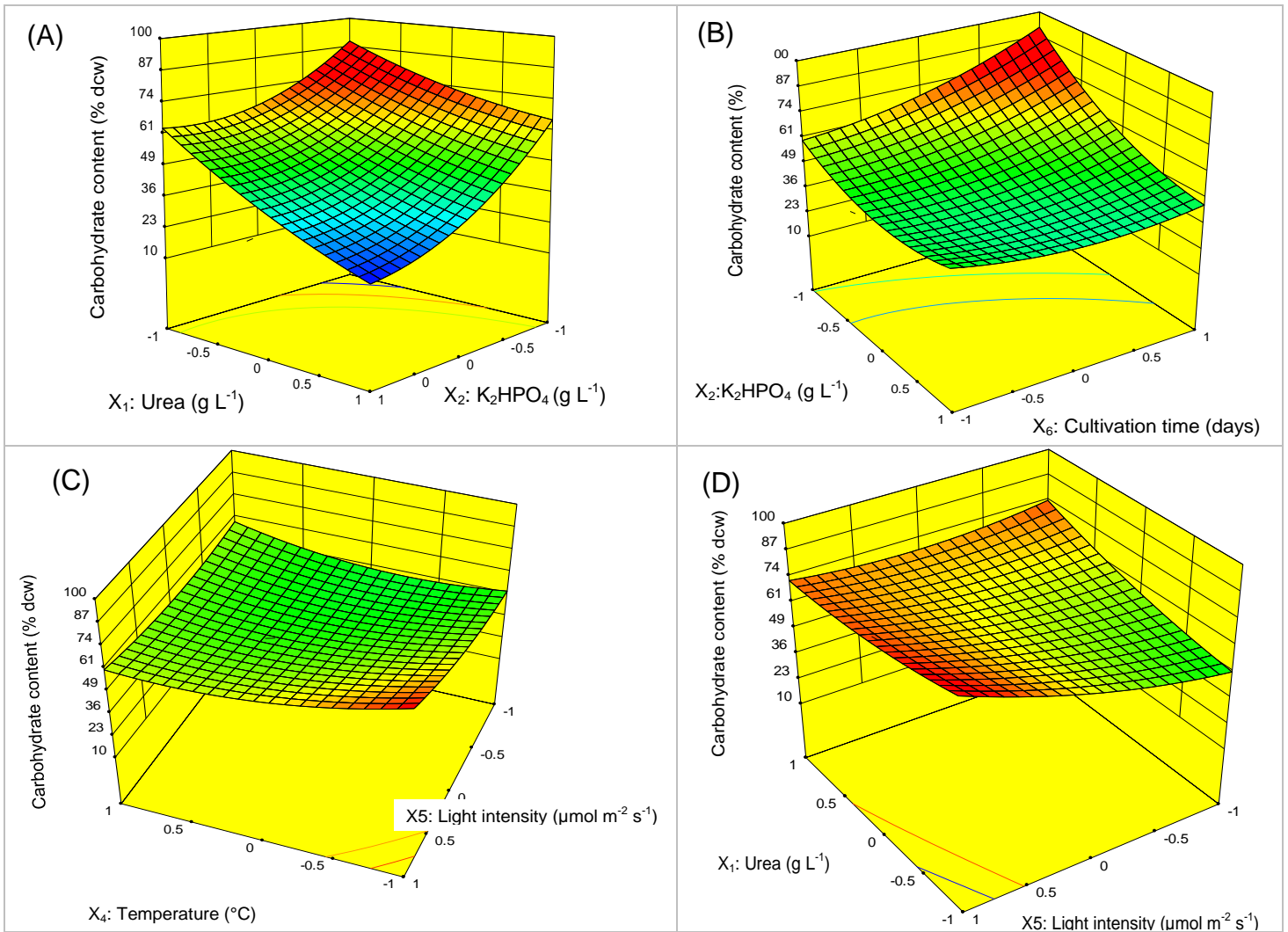


Fig. 4.4. Three-dimensional response surface plots for carbohydrate content with varying urea vs. K₂HPO₄ (A), varying cultivation time vs. K₂HPO₄ (B), varying temperature vs. light intensity, (C) and varying urea vs. light intensity (D), all other variables were kept at their central values.

A similar phenomenon was reported by [Pancha et al. \(2014\)](#), wherein nitrogen starvation led to an increase in the carbohydrate production by *Scenedesmus* sp CCNM 1077 to a value of 45.74 % dcw, while in *C. vulgaris* the carbohydrates were increased to a value of 55 % dcw under phosphorus starvation ([Branyikova et al., 2011](#)). [Fig. 4.4 \(B\)](#) show the interactive effect between the cultivation period and K₂HPO₄ which was also significant to carbohydrate production ($p < 0.05$) ([Table 4.5](#)). From this interaction, it can be observed that carbohydrate content increased as the concentration of K₂HPO₄ decreased and cultivation period increased. In a

study by [Hu et al. \(2015\)](#) the carbohydrate production by *Nannochloropsis gaditana* 1049 increased from 16.78 % dcw to 18.56 % dcw from 7 days to 9 days of cultivation, and then the content fluctuated around the value of 17 % dcw with the cultivation period extension and eventually the lowest carbohydrate content of 14.07 % dcw was observed after 21 days of cultivation. [Fig. 4.4 \(C\)](#) shows the interactive effect of light intensity and temperature. This interaction was also significant to carbohydrate production ($p < 0.05$) ([Table 4.5](#)). From this interaction, it was found that carbohydrate content was high at low levels of temperature and high levels of light intensity. As mentioned earlier, the interactive effect of light intensity and temperature seems cooperative. Studies have reported that low temperatures enhance photo-inhibition ([Nejat et al., 2015](#)). While the biomass production drops with decreasing temperatures, the carbohydrate content increases. This phenomenon has been observed in the cultures of *Chaetoceros cf. wighamii* ([de Castro Araújo and Garcia, 2005](#)).

[Hosono et al. \(1994\)](#) reported that adjusting the temperature to 20 °C during exponential growth, and then rapidly decreasing the temperatures down to ~14 °C resulted in increased carbohydrate production by up to 25% in *C. vulgaris*. Presumably, this phenomenon arises because the catalytic enzymes involved in the synthesis of carbohydrates have different optimum temperatures. For instance, in *Chlorella* sp., amylopectin synthesis is higher at 20 °C while amylase synthesis has been shown to be enhanced at temperatures above 30 °C ([Ördög et al., 2016](#)). Light intensity is generally believed to increase carbohydrate production. This phenomenon has been observed in the cultures of *Chlorella* and *Botryococcus brainii* ([Lupi et al., 1994](#); [Ogbonna and Tanaka, 1996](#)). Although it is certain that light intensity affects biomass and biochemical composition of many microalgal strains, its manipulation in cultures cultivated under natural irradiation such as raceway ponds presents a huge challenge because of the strong variation in the weather conditions.

Finally, [Fig. 4.4 \(D\)](#) shows the interactive effect of urea and light intensity on carbohydrate yields. From this plot, it can be observed that carbohydrates increased as the level of urea and light intensity increased. This was in disagreement with the findings of [Ho et al. \(2012\)](#), showing that increasing light intensity and decreasing nitrogen concentration resulted in increased carbohydrate production by *S. obliquus* CNW-N. This phenomenon could be strain dependent.

4.4.4. Validation of optimal conditions best predicted by RSM model

The proposed model will have no meaning until its prediction abilities are confirmed under *in vitro* conditions. Validation experiments were conducted using the optimum conditions derived from analysis of RSM. These experiments were conducted with the goal of achieving maximum carbohydrate content while keeping biomass production within the reference range. Biomass concentration and carbohydrate content are critical indicators of commercialization potential of a microalgal strain. Thus, the main objective of this study was to optimize both parameters simultaneously. However, the main challenge was that conditions required for maximum carbohydrate production were often the opposite of that required for maximum biomass production. Hence, the end product (carbohydrate content) was given more priority since biomass is mainly critical for the operation cost for downstream processing (e.g., harvesting).

The optimal levels of urea, K_2HPO_4 , Na_2CO_3 , light intensity, temperature and cultivation time obtained using RSM were 0.203 g L^{-1} , 0.0264 g L^{-1} , 0.0122 g L^{-1} , $180.18\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, $20\text{ }^\circ\text{C}$ and 14 days, respectively. For this combination of conditions, the predicted responses of biomass and carbohydrate production were 488.396 mg L^{-1} and 79.54% dcw, respectively, with a desirability of 1.0. The optimized conditions were used for triplicate runs in batch experiments (Fig. 4.5). From these cultivations, the RSM was accurate and adequate in predicting the final biomass and carbohydrate production by *C. vulgaris*. A mean biomass concentration and carbohydrate content of 486.30 mg L^{-1} and 78.97% dcw with relative error % of 0.429 and 0.717, respectively was obtained from the experimental verification of the RSM model (Fig. 4.5). The results obtained in our study were consistent with previously reported data in literature (Branyikova *et al.*, 2011; Lv *et al.*, 2010; Tan *et al.*, 2016b).

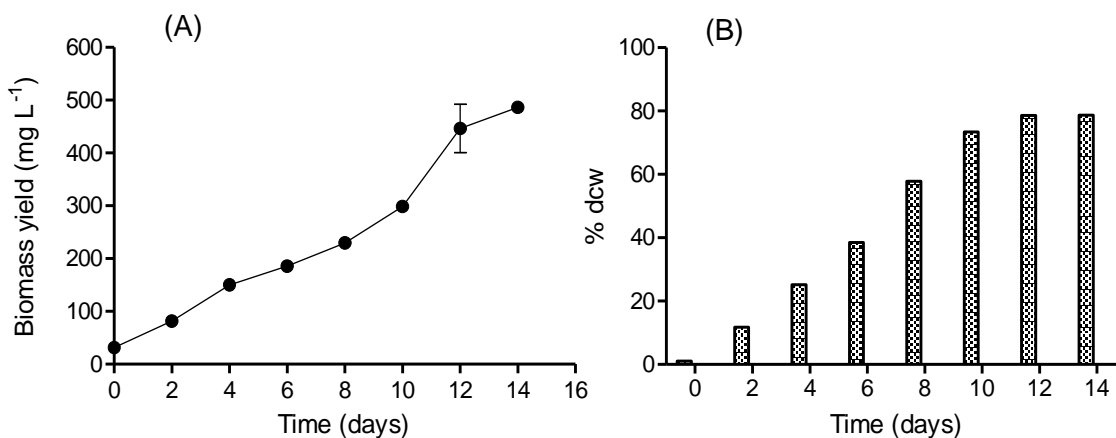


Fig. 4.5. Time-course profiles of biomass production (A) and carbohydrate content (B) of *C. vulgaris* (Toti RS4) cultivated under optimum culture conditions established using RSM. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation.

4.5. Conclusion

This study demonstrated effective use of statistically based methods to optimize the carbohydrate content and biomass yield of indigenous microalgae, *C. vulgaris* (Toti RS4) for possible use as feedstock for bioethanol production. The carbohydrate content was improved by selecting the most significant factors using the Plackett-Burman design. Nitrogen was found to be the most influential factor affecting biomass and carbohydrate production. Of the different nitrogen sources evaluated, urea was shown to be the most suitable nitrogen source for the cultivation of *C. vulgaris* (Toti RS4) tested in this study for biomass and carbohydrate production. The factors: urea, K₂HPO₄, Na₂CO₃, temperature, light intensity and cultivation time were then optimized using RSM coupled with Box-Behnken design. The optimal levels of urea, K₂HPO₄, Na₂CO₃, temperature, light intensity and cultivation time as per RSM were 0.203 g L⁻¹, 0.0264 g L⁻¹, 0.0122 g L⁻¹, 180.18 μmol m⁻² s⁻¹, 20 °C and 14 days, respectively. The maximum biomass production and carbohydrate content under the optimized conditions were 486.3 mg L⁻¹ and 78.97 % dcw, respectively. Results from this study indicate the feasibility of using *C. vulgaris* (Toti RS4) as a suitable candidate for the optimal production of biomass and carbohydrates as feedstock for downstream bioethanol production.

Chapter 5. Municipal wastewater for integrated nutrient removal, growth and carbohydrate production by *Chlorella vulgaris* (Toti RS4)

5.1. Abstract

Wastewater remediation by microalgae is an eco-friendly process with no secondary pollution as long as the biomass produced is reused and allows efficient nutrient cycling. The aim of this study was to investigate the feasibility of growing *Chlorella vulgaris* (Toti RS4) in the primary effluent municipal wastewater (PEMW) for simultaneous wastewater treatment and biomass production for bioenergy. The performance of *C. vulgaris* (Toti RS4) on raw and autoclaved (PEMW) was evaluated, in terms of biomass and carbohydrate production, nutrient removal efficiency and physiology of the photosynthetic apparatus. The results showed that by the end of a 14-day batch culture experiment, microalgae grown on raw PEMW could remove total nitrogen (TN) and total phosphorus (TP) at 100% efficiency, with maximum biomass yield and carbohydrate content of 1.53 g L⁻¹ and 58% of the dry cell weight, respectively. Pulse amplitude fluorometry results demonstrated a decline in the maximum quantum efficiency (F_v/F_m) of the microalgal cells grown in raw PEMW from 0.681 to 0.490 units during the 14-day incubation period. Furthermore, the microalgal cells in raw PEMW demonstrated promising auto-flocculation properties. Up to 68% of the biomass of *C. vulgaris* (Toti RS4) was recovered by auto-flocculation from raw PEMW after 240 min.

Keywords: Municipal wastewater, nutrient removal, biomass, carbohydrate, auto-flocculation.

5.2. Introduction

Presently, the development of environmentally friendly and sustainable energy resources is of prime concern because of increasing global energy demands, increasing greenhouse gas emissions, global warming issues and declining petroleum reserves. Biofuels are an excellent substitute for conventional energy fuels because they are carbon-neutral, non-toxic and biodegradable (Vieira Salla *et al.*, 2016). In addition, these fuels are particularly optimal options for sustainable energy use because they can replace petroleum-based fuels without extensive changes in energy infrastructure (Guo *et al.*, 2013). For sustainable biofuel and bioenergy production, research efforts are geared towards the utilization of biomass such as

lignocellulosic and animal waste, however, microalgae biomass is the most promising feedstock (Abomohra *et al.*, 2017). Microalgae draw high research interest due to its compelling potential for the production of a wide range of products because of the high concentrations of proteins, lipids, carbohydrates, vitamins, pigments and enzymes (Karapatsia *et al.*, 2016; Lv *et al.*, 2010; Postma *et al.*, 2015). The major advantages associated with the utilization of microalgae for biofuel production include reduced environmental impact and no competition for land with food crops in view of the ability to grow in marine as well as wastewater, without the additional demands for freshwater.

Biofuel production from microalgae is technically promising but not yet economically feasible at the commercial scale. One of the major technical glitches with the large-scale production of microalgal biomass is the cost of the culture medium (Cheah *et al.*, 2016). Therefore, it is prudent to carefully plan production systems and facilities for a feasible production strategy. The utilization of wastewater to cultivate microalgae is an alternative that can reduce production costs, consequently several studies have been undertaken to validate this concept (Batista *et al.*, 2015; Cheng *et al.*, 2017; Labbé *et al.*, 2017). Wastewater-based cultivation systems save enormous amounts of freshwater and required nitrogen and phosphorus nutrients for microalgae growth. Nitrogen and phosphorus alone can contribute 10-20 % to the total production cost (Cai *et al.*, 2013) and this share is expected to increase due to the recent inflation in fertiliser prices. There is a great potential for exploitation of various wastewater sources such as municipal, agricultural and industrial wastewater for the harnessing of microalgal growth for biofuel production. However, municipal wastewater holds the greatest potential because of its relatively stable nutrient profile and low presence of inhibitory compounds. Municipal wastewaters collected from various stages during treatment process have been tested for their ability to support algae growth in previous studies (Cheah *et al.*, 2016; Rawat *et al.*, 2011). These different types of wastewater include primary clarifier effluent (AlMamani and Örmeci, 2016), activated sewage filtrates (Lee *et al.*, 2016), effluent from secondary treatment tank (AlMamani and Örmeci, 2016) and post-chlorinated wastewater (Mutanda *et al.*, 2011a). The primary effluent wastewater has the characteristics of rich nutrients such as phosphorus, nitrogen and COD, serving as the potential source of carbon for microalgae cultivation (Huang and Goel, 2015). Up to now, few studies have been conducted to test the suitability of growing algae in primary effluent, thus research effort in this area is necessary and important. Carbohydrate content in microalgae cultivated in synthetic media solution has been reported to exceed 70% of dry cell weight (Sanchez Rizza *et al.*, 2017). However, few reports have explored the carbohydrates produced by microalgae grown in municipal wastewaters (Batista *et al.*, 2015).

The fact that microalgal biomass exists in an aqueous suspension makes biomass harvesting technically challenging. Consequently, microalgae harvesting is regarded as a major obstacle in downstream processing of the bioenergy production value chain. It is therefore crucial to explore suitable cost-effective microalgal biomass harvesting strategies for the whole bioenergy chain to be feasible. Previous studies have shown that some microalgal strains can be harvested by flocculation in a short time, without the addition of any flocculating agent (Markou *et al.*, 2012a).

Therefore, the objectives of this study were to; (1) determine the growth rate, biomass yield, nutrients removal efficiency, and carbohydrate production during growth of microalgae in primary effluent municipal wastewater (PEMW), and (2) study the flocculation kinetics of microalgae in wastewater.

5.3. Materials and Methods

5.3.1. Experimental set-up

The experiments in this study were performed in two successive phases. In the first phase, effect of autoclaved and raw primary effluent municipal wastewater (PEMW) was studied in terms of algal growth, photosynthetic efficiency, wastewater nutrients removal and carbohydrate production. Autoclaved PEMW was particularly used to provide the baseline information on the performance of microalgae alone in wastewater by eliminating bacterial contamination and excess heterotrophic growth. In this stage, BG-11 was used as a positive control to compare the performance of microalgae in wastewater. The experiments were performed for 14 days. The second phase was aimed at testing the flocculation kinetics of microalgae after cultivation. The flocculation experiment was performed for a period of 240 min (4 h) and no flocculation agent was added. A positive control was also performed by adding FeCl₃ (a flocculating agent) at a concentration of 0.3 g L⁻¹. Based on the results of Chatsungnoen and Chisti (2016), this concentration was optimum for the flocculation of *Chlorella vulgaris* biomass.

5.3.2. Pre-treatment and physicochemical characterization of wastewater

The wastewater used in this study was the primary effluent of municipal wastewater collected from Phoenix wastewater treatment works located in Phoenix, Durban, South Africa. The wastewater effluent was collected in 2 L plastic bottles and preserved at 4 °C. Prior to use, the wastewater was filtered using a 2.36 mm pore sieve to remove suspended solids and then divided into two equal proportions. One proportion was sterilised by

autoclaving (121 °C, 15 min), after which, the liquid was centrifuged (4000 x g for 15 min) and the supernatant was stored at 4 °C until use in experiments. The other untreated portion was stored at 4 °C until use in experiments.

The physico-chemical parameters of the wastewater effluent analysed include salinity, pH and temperature measured with an inoLab IDS meter, WTW (Model Multi 9310) and dissolved micronutrients (e.g., S, Cd, Cu, Fe, Pb, Mn, Hg, Se, Zn, Ca, Mg, K, SO₄, Na and Mo) as well as macronutrients (e.g., P and NO₃) using standard methods (APHA, 1995), with a gallery discreet water analyzer (Thermo Scientific, USA).

5.3.3. Microalgal strain and cultivation conditions

The microalgae used in this study was *Chorella vulgaris* (Toti RS4), which was isolated from freshwater in Amanzimtoti river, Durban, South Africa (GPS: -30.077537, 30.871276). This microalga was pre-cultured in wastewater (sterile and non-sterile) and in the BG-11 medium. The constituents of BG-11 were as follows (g L⁻¹): NaNO₃, 1.5; K₂HPO₄, 0.03; MgSO₄·7H₂O, 0.075; citric acid anhydrous, 0.006; Na₂CO₃, 0.02, CaCl₂·2H₂O, 0.036; ammonium iron (III) citrate, 0.006; EDTA.2Na, 0.001 and 1 mL of trace metal solution. The trace metal solution was composed of (g L⁻¹): H₃BO₃, 2.86; MnCl₂·4H₂O, 1.81; ZnSO₄·7H₂O, 0.222; Na₂MoO₄·2H₂O, 0.39; CuSO₄·5H₂O, 0.079; Co (NO₃)₂ ·6H₂O, 0.049 (Rappika *et al.*, 1979). The microalgal suspension at 10% (v/v) inoculum concentration at a cell load of 1.2 x 10⁷ cells mL⁻¹ was added to 100 mL working volume of BG-11 or two different wastewaters (sterile and non-sterile) medium in 250 mL capacity Erlenmeyer flask. The flasks were incubated under ambient CO₂ at 20 ± 2 °C with 12h: 12 h light: dark period under a light intensity of 180.2 μmol m⁻² s⁻¹ (illuminated using cool white fluorescent light) for 14 days. The light intensity was measured using an HD-2102.2 Meter with an LP-471PAR pyranometer sensor (Delta OHM, Italy). The experiments were conducted in triplicate.

5.3.4. Analytical methods

Dry cell concentrations were obtained based on the optical density (OD) values measured at 680 nm with a spectrophotometer (model U-8000, Shimadzu, Japan) via appropriate calibration (Appendix A). The biomass concentration (X; mg L⁻¹) of the microalgal cultures in wastewater and synthetic medium (BG-11) was measured using Eq. (5.1) and (5.2), respectively:

$$X \text{ (mg L}^{-1}\text{)} = 789.53 \times \text{OD}_{680} - 46.65; R^2 = 0.9879 \quad (5.1)$$

$$X \text{ (mg L}^{-1}\text{)} = 625.93 \times \text{OD}_{680} + 6.25; R^2 = 0.9953 \quad (5.2)$$

The time-course profile of the biomass concentration (X ; mg L^{-1}) was used to calculate the specific growth rate μ (d^{-1}) and biomass productivity, as previously shown in Eqs (3.1) and (3.2), respectively.

To determine nutrient removal, 5 mL samples were drawn from each flask starting from the day of inoculation. The samples were first centrifuged at 7000 rpm for 2 min. The collected supernatant was then filtered through 0.45 μm cellulose membrane. The filtrate was properly diluted and analyzed for total nitrogen and total phosphorus using Spectroquant test kits 1.14763.0001 (TN) and 1.00673.0001 ($\text{PO}_4\text{-P}$) (Merck Millipore, Germany), following manufacturer's instructions (Appendices G and H) and read via a Spectroquant Nova 60 spectrophotometer (Merck Millipore, Germany). The nutrient uptake rates were calculated using Eq (5.3) as previously reported by [Markou et al. \(2012a\)](#):

$$R_i = \frac{S_i - S_f}{t_i - t_f} \quad (5.3)$$

where, S_i is the initial substrate concentration as TN or TP at time t_i , and S_f is the corresponding substrate concentration at time t_f .

Chlorophyll a (chl a) was determined spectrophotometrically after extraction in absolute methanol (HPLC grade, $\geq 99.9\%$, Sigma-Aldrich, South Africa), as previously described in section 3.3.6.

The physiology and photosynthetic efficiency of the microalgal cells were studied using a DUAL-PAM 100 Chlorophyll fluorescence and P700 photosynthetic analyser (Heinz Walz GmbH, 91090 Effeltrich, Germany) equipped with a 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 x 10 x 40) containing a micromagnetic stirrer. The measured parameters were F_v/F_m , rETR and NPQ, as previously described in section 3.3.7.

Total carbohydrates were determined using the Anthrone method ([Yemm and Willis, 1964](#)), after lyophilization of the microalgal biomass in the oven at 60 $^{\circ}\text{C}$ for 48 h. The concentration of the carbohydrates was determined from a calibration curve constructed using a standard of D-glucose (Appendix B). The flocculation efficiency of *C. vulgaris* (Toti RS4) was tested in the cultures with optical density $\text{OD}_{680} = 1.8$ (approximately 1 g L^{-1}). Flocculation

experiments were performed in sterile glass test tubes with screw caps (size 25 x 200 mm, with a capacity of 70 mL) with 50 mL testing volume with two replications containing microalgae for a maximum period of 240 min. Samples were periodically taken (each 30 min) close to the interface zone in the clarified zone to determine the flocculation efficiency. The percentage of the microalgal biomass (B, %) remaining in the broth was calculated using Eq. (5.4)

$$B (\%) = \frac{C_f}{C_i} \times 100 \quad (5.4)$$

where C_f (g L^{-1}) was the biomass concentration in the supernatant after flocculation and C_i (g L^{-1}) was the initial biomass concentration in the broth.

5.3.5. Statistical analysis

Statistical analysis was done as previously described in section 3.3.12.

5.4. Results and Discussion

5.4.1. Physico-chemical characteristics of the wastewater

The various physical and chemical characteristics of the primary effluent wastewater (PEMW) used in this study is shown in Table 5.1. The wastewater has a broad range of inorganic microelements suggesting the presence of potential metallic inhibitors to the microalgal growth. High concentrations of Ca, S^{2-} , SO_4^{2-} , Mg and Na as well as the deficiency of Cu, Cd, Se and Zn were observed in this study. Compared to the standard BG-11 medium and other wastewater streams (e.g., centrate), the PEMW had low concentration of macronutrients (particularly nitrogen and phosphorus), which may have been due to assimilation of these nutrients by the intrinsic microorganisms in the wastewater. Nevertheless, PEMW demonstrated an inorganic N/P ratio of 7.2, which was within the recommended range (6.8 to 10) for microalgal growth (Zhang *et al.*, 2013). In a study by Wang *et al.* (2010), it had been demonstrated that only wastewaters before and after primary settling had optimal inorganic N/P ratios. The wastewater effluent from aeration tank (secondary effluent) had an inorganic N/P ratio of 53.2, much higher than the optimal ratio, indicating high phosphorus limitation. In contrast, the inorganic N/P of the centrate wastewater was 0.36, which was much lower than the optimal inorganic N/P ratio, indicating high nitrogen limitation. Thus, from these observations, it can be ruled that the wastewater

effluent used in this study can adequately serve as a growth medium for microalgae without nutrient supplementation or dilution.

Table 5.1. Physico-chemical profiles of the primary effluent of the municipal wastewater used in this study.

Substance/Parameter	Units	Quantity
Temperature	°C	19
pH	-	7.0
Nitrate as NO ₃	mg L ⁻¹	<5
Nitrate as N	mg L ⁻¹	<1,3
Total N	mg L ⁻¹	61
Sulphide as S ²⁻	mg L ⁻¹	13
Conductivity	mS m ⁻¹	10
Salinity	ppt	<2
Total P	mg L ⁻¹	8.48
Cadmium as Cd - Dissolved	mg L ⁻¹	<0,01
Copper as Cu - Dissolved	mg L ⁻¹	0.065
Iron as Fe - Dissolved	mg L ⁻¹	2.8
Lead as Pb - Dissolved	mg L ⁻¹	0.092
Manganese as Mn - Dissolved	mg L ⁻¹	0.159
Mercury as Hg	mg L ⁻¹	0.001
Selenium as Se - Dissolved	mg L ⁻¹	0.08
Zinc as Zn - Dissolved	mg L ⁻¹	0.151
Calcium as Ca	mg L ⁻¹	29
Magnesium as Mg	mg L ⁻¹	11
Potassium as K	mg L ⁻¹	3.0
Sulphate as SO ₄	mg L ⁻¹	60
Sodium as Na	mg L ⁻¹	83
Molybdenum as Mo	mg L ⁻¹	0.007

Note: P represents phosphorus and N represents nitrogen.

5.4.2. Microalgal growth

The growth potential of *C. vulgaris* (Toti RS4) on the three different culture media within 14 days was investigated (Fig. 5.1). The growth patterns of microalgae on wastewater exhibited all the characteristic growth phases expected in a batch culture of microalgae, except the lag and the death phase (also called lysis phase), which were not evident. In contrast, the microalgal cultures grown in the BG-11 medium demonstrated an extensive lag phase,

which lasted about 8 days although the cells were already in the logarithmic phase when they were introduced to the medium. The logarithmic growth phase in the autoclaved PEMW lasted for about 10 days (Fig. 5.1(A)), compared to the microalgal culture grown on the raw PEMW, which had an exponential phase of only 6 days (Fig. 5.1(B)). One possible explanation for the sooner onset of stationary phase for the raw PEMW is the early depletion of nutrient, particularly nitrogen and phosphorus caused by competition or substrate limitation owing to the presence of microbes in the raw PEMW (Fig. 5.3). The early decrease in the microalgal growth in raw PEMW may also be due to the high levels of toxins and competitive bacteria. Another possible reason could be low light availability arising from shading by particles in raw PEMW (Anbalagan *et al.*, 2017; Fowdar *et al.*, 2016).

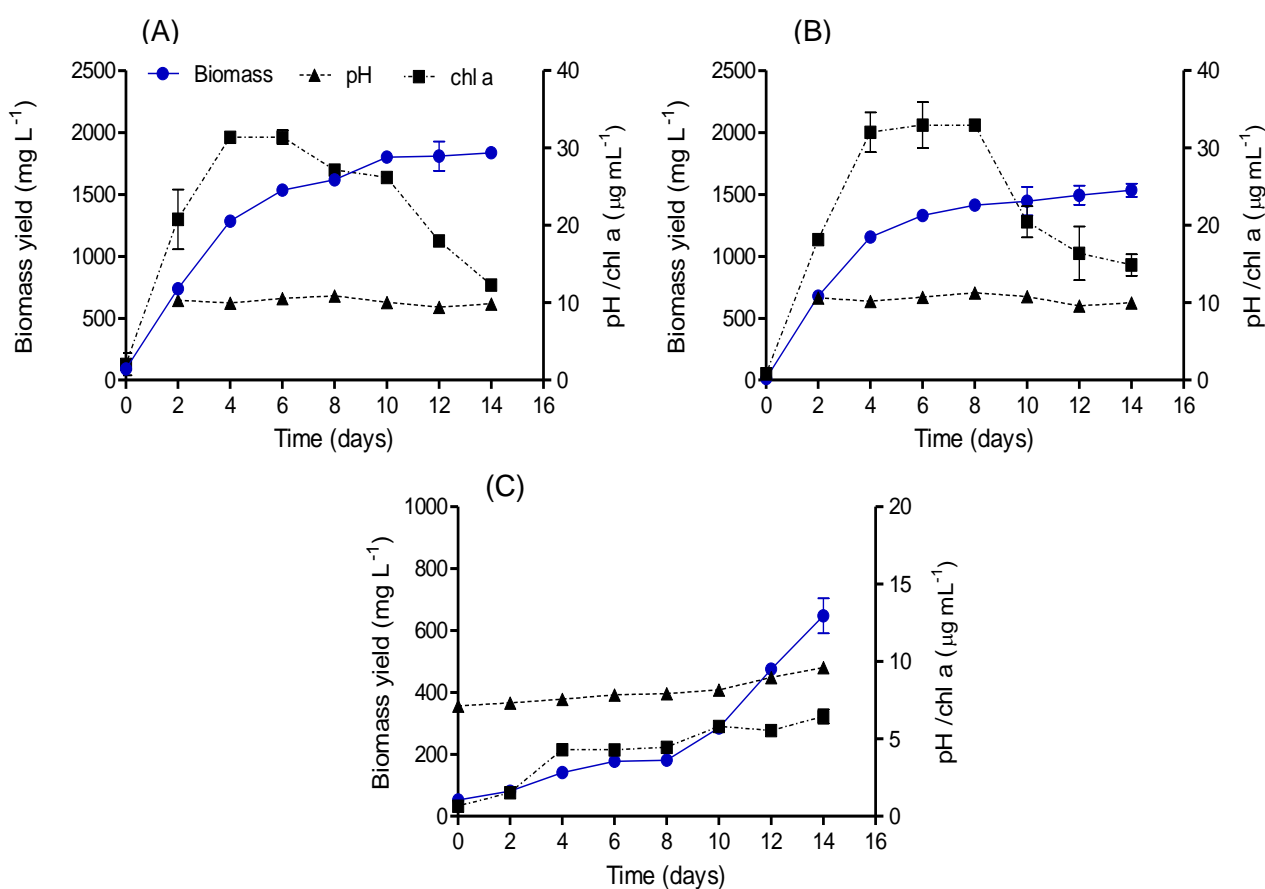


Fig. 5.1. Biomass, chl a and pH values obtained from the batch cultivation in (A) autoclaved PEMW, (B) raw PEMW, and (C) BG-11 medium for 14 days. The light intensity and temperature were kept constant at $180.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $20 \text{ }^\circ\text{C}$, respectively. Carbon dioxide was ambient. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

The maximum specific growth rate in all culture media ranged from 0.20 day⁻¹ to 0.28 day⁻¹ (Table 5.2), with the cultures grown in the autoclaved PEMW having the highest growth rate of 0.28 day⁻¹.

Table 5.2 Specific growth rates, biomass concentration and biomass productivity for *C. vulgaris* (Toti RS4) grown on the three culture media types.

Cultivation media	Specific growth rate (day ⁻¹)	Biomass at day 14 (g L ⁻¹)	Overall biomass productivity (mg L ⁻¹ day ⁻¹)
BG-11	0.20 ± 0.03	0.65 ± 0.08	42.5 ± 5.4
Autoclaved PEMW	0.28 ± 0.00	1.84 ± 0.02	124.7 ± 1.7
Raw PEMW	0.27 ± 0.02	1.53 ± 0.08	108.5 ± 6.3

Overall, these results indicate that the net biomass production in autoclaved and raw PEMW is higher than that of the BG-11 medium. However, the growth of contaminating microorganisms in raw PEMW induces bias to this study and thus complicates the interpretation of the results. In this regard, chl a concentration was determined parallel to microalgal cell growth to serve as an indicator of microalgae viability and subsequent biomass accumulation. From Fig. 5.1, a direct relationship between microalgal growth and chl a concentration can be observed, as previously described elsewhere (Vörös and Padisák, 1991). The microalgal cultures grown on autoclaved PEMW and raw PEMW demonstrated similar chl a concentration patterns and were both comparatively higher than that of microalgae grown on BG-11 medium ($p < 0.05$). This demonstrates the superiority of utilizing wastewater as growth medium for *C. vulgaris* (Toti RS4) under the tested conditions. In particular, autoclaved wastewater showed the highest growth potential of *C. vulgaris* (Toti RS4). However, autoclaving even a small portion of the wastewater in any size of the municipal wastewater treatment plant is impractical and could have catastrophic implications on process economy due to the intensive energy demand of the autoclave systems. Therefore, the focus should be on raw PEMW. The fact that the microalgal cells showcased high growth rates immediately after inoculation without any supplementation suggest that raw PEMW may serve as a cheap alternative nutrient source for the microalgae nourishment.

As a significant parameter affecting growth in wastewater, pH was also analysed with time (Fig. 5.1). The pH values for the three culture media were in the desirable range and hence medium supplementation with additional CO₂ was not necessary. The pH ranged from 7.1-9.6, 9.6-11.3 and 9.6-10.7 for BG-11, raw PEMW and autoclaved PEMW, respectively. CO₂

has been shown to have a strong influence on pH fluctuations on the cultivation medium. The data presented in this study suggests that the utilisation of CO₂ during phototrophic growth results in the formation of carbonates in the medium that elevates the pH into the alkaline range. High pH levels can be desirable because contaminants such as protozoans and rotifers are inhibited under these conditions (Mutanda *et al.*, 2014).

5.4.3. Analysis of the physiological state and photosynthetic efficiency

The photosynthetic efficiency of microalgae in wastewater is one rather under-researched topic, despite the fact that photosynthesis is the fundamental reaction behind the phototrophic growth of microalgal cells. Previously, researchers monitored the photosynthetic activity of microalgal cells using chambers, the ¹⁴C-technique, or oxygen microelectrodes (Li *et al.*, 2015b). However, these evaluations are subjective and do not address the invisible photosynthetic mechanism and physiological state of microalgal cells. On this basis, chlorophyll fluorescence devices such as the pulse amplitude modulated fluorometer (PAM) can serve as an important tool for the determination photosynthetic response of microalgae subjected to wastewaters and effluents generated from large-scale applications (Ritchie and Mekjinda, 2016).

At the initial stage of growth, all the microalgal cultures were within the accepted F_v/F_m range of >0.5 (Fig. 5.2 (A)), which suggests that the microalgal cells were adequately acclimatized under the cultivation conditions used (Ramanna *et al.*, 2014). As shown in Fig. 5.2 (A), microalgal cultures grown in autoclaved PEMW demonstrated increased F_v/F_m values during the first 2 days of cultivation, followed by a steady decrease to a value of 0.658 on day 14. In contrast, the microalgal cultures grown in BG-11 medium showed a rapid decline in the F_v/F_m value from 0.961 to 0.229 within the first 2 days of cultivation. The F_v/F_m values of the cultures were maintained at the local minimum for 2 additional days, while, after day 8, this trend was reversed. The microalgal cultures grown in raw PEMW exhibited a slow but a significant decrease in the F_v/F_m values from 0.681 to 0.490. The current investigations on the photosynthetic response of microalgae suggest that the drop in F_v/F_m value can be brought about by changes in the cultivation conditions such as nutrient or light availability. For example, Samori *et al.* (2013) observed a decline in maximum PSII photochemistry during growth of *Desmodesmus communis* in primary wastewater effluent, under nitrogen-depleting conditions. In another study, Solovchenko *et al.* (2014) postulated that the drop in the F_v/F_m value of microalgal cells can be attributed to the failure of microalgae to grow or a pronounced lag phase in highly diluted samples. Such a phenomenon was observed for microalgae grown on BG-11 medium in this study (Fig. 5.2 (A)). Nevertheless, after 14 days of cultivation, F_v/F_m values of microalgal cultures grown in BG-11 medium were higher compared to raw PEMW or autoclaved PEMW grown cultures. This was likely because the

microalgal cultures grown on BG-11 were subjected to lower toxicity when compared to the wastewater samples. Also, it could be due to the fact and on day 14, the microalgal cultures grown on BG-11 medium were in the logarithmic growth phase thus had abundant supply of nutrients, compared to the wastewater cultures that were in their stationary phase on day 14 and were subjected to nutrient stress and light shading due to high cell density.

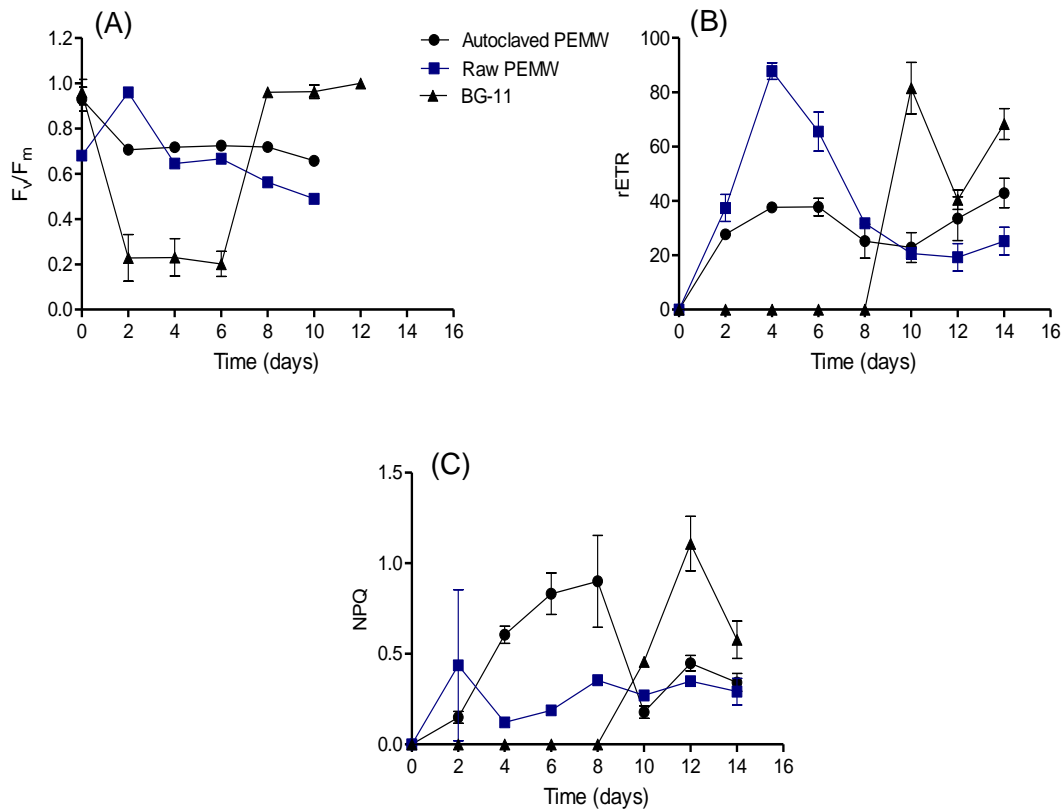


Fig.

5.2. (A): The maximum quantum efficiency (F_v/F_m), (B): relative electron transport rate (rETR) and (C): non-photochemical quenching (NPQ) of *C. vulgaris* (Toti RS4) grown in the three different culture media. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

The rETR of the microalgal cells grown in the three culture media types was also determined as shown in Fig. 5.2(B). The microalgal cultures grown in BG-11 displayed low rETR values from day zero to day 8 of the experiment, most probably since the microalgal cells were in the lag phase. However, on day 10 an increase in the rETR value from zero to 81.5 units was observed, correlating to the transitioning of the microalgal cells from the lag phase to the logarithmic phase as well as the increase in the biomass concentration. At the end of the experiment (day 14), the rETR value of the microalgal cultures in BG-11 was 68.4 units. In the raw PEMW, microalgae demonstrated an increase in the rETR values from zero to 87.8

units during the first 4 days of cultivation, thereafter a steady decline in the rETR value was observed until it finally stabilized at 19.3 units on day 14. The rETR value of the microalgal cultures grown in the autoclaved PEMW demonstrated a rapid increase from day 0 to day 4, followed relatively constant phase, which lasted for 2 days. Also from day 10 to 14, an increase in the rETR value was observed. For this, we have no explanation only that it seems to coincide with the changes in the composition of the cell. The correlation (R^2) between biomass and rETR of the microalgal cultures grown in autoclaved was 0.55 and this was found to be significant ($p < 0.05$). Thus, these results suggest that an increase in biomass yield is directly proportional to the increase in the operating efficiency of photosystem II. Indeed, the higher the photosynthetic efficiency or activity of the microalgal cell, the higher the growth and the biomass produced.

[Fig. 5.2 \(C\)](#) shows the NPQ values versus time for *C. vulgaris* (Toti RS4) grown in the three cultivation media types. The maximum values estimated for NPQ on day 14 were 0.58, 0.32 and 0.29 for microalgae grown in BG-11, autoclaved PEMW and raw PEMW, respectively. Generally, high NPQ values are associated with the elevated capacity for photoprotection developed as an adaptation against the damaging effect of prolonged light exposure and the rate at which the heat is dissipated from the PS (II) apparatus representing the physiological state of the microalgal cell. This study therefore demonstrated that increased cultivation results in the deterioration of the activity of the photosystems of microalgae, primarily due to nutrient limitation and toxin build up in the cultivation media ([Fig. 5.1](#)).

5.4.4. Wastewater nutrients removal by microalgae

In coupling the system of bioethanol production and wastewater treatment, nutrient removal should be studied in addition to microalgal growth and carbohydrate accumulation for practical application. The variation in TN and TP removal with time by *C. vulgaris* (Toti RS4) in the different wastewaters (raw and autoclaved) for 14-day batch culture is shown in [Fig. 5.3](#). Results indicate that the total nitrogen (TN) content was reduced from 60.5 ± 0.7 to 8.5 ± 2.1 mg L⁻¹ in the autoclaved PEMW during the first 6 days and followed by a stationary phase in which the TN content was constant until the end of the 14-day batch experiment, resulting in the TN removal efficiency of 91.2%. In raw PEMW, TN removal efficiency was higher than in the autoclaved PEMW, as the removal efficiency reached a maximum of 100% after 6 days of cultivation. This removal efficiency was comparable with various studies using other municipal wastewaters ([Ji et al., 2013](#); [Zhang et al., 2014](#)).

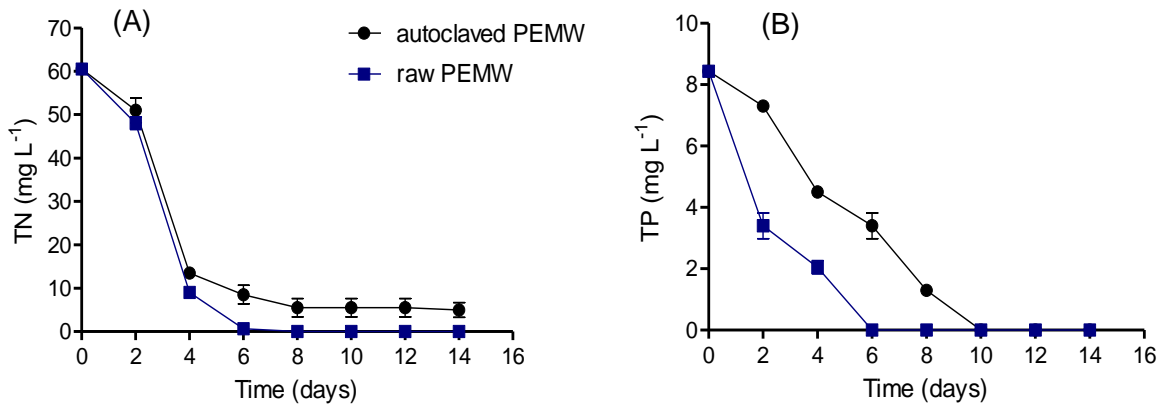


Fig. 5.3. (A) Total nitrogen (TN) and (B) total phosphorus (TP) removal profile of *C. vulgaris* (Toti RS4) grown on autoclaved and raw PEMW. The light intensity and temperature were held constant at $180.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $20 \text{ }^\circ\text{C}$, respectively. Carbon dioxide was ambient. The analysis was performed in duplicate and the results were expressed as the average with a standard deviation of two measurements.

There are numerous chemical species contributing to TN, such as nitrate, nitrite, organic nitrogen and ammonia. Microalgae can assimilate various species of inorganic nitrogen such as nitrates, nitrite and ammonium as well as organic nitrogenous sources such as urea. Since nitrates account for less than 5% of the TN (Table 5.1), most nitrogen present in the municipal wastewater used in this study was assigned to ammonium and other organic nitrogen sources such as urea. The fact that the removal efficiency in the autoclaved PEMW medium reached a maximum of 91.2%, and 100% in the raw PEMW suggests that the higher removal efficiency of TN on the raw PEMW was due to the participation of the water-borne bacterial consortia in the degradation of nitrogen sources that microalgae alone could not degrade or assimilate. Some studies have suggested that the nitrogen/phosphorus ratio was the critical factor that greatly influenced nutrient removal (Li *et al.*, 2011; Zhang *et al.*, 2014). Other studies have reported that a decrease in nitrogen removal efficiency may be due to phosphorus limitation, causing an imbalance in the nitrogen/phosphorus ratio (Cai *et al.*, 2013; Ge and Champagne, 2016; Zhang *et al.*, 2014).

In the present study, ~ 78% of TP was removed within 4 days in the raw PEMW, and 100% after 6 days (Fig. 5.3 (A)), compared to microalgal cultures grown in the autoclaved PEMW media, which required about 10 days to achieve comparable removal efficiency (Fig. 5.3 (B)). It had been reported that increased pH values above 8 in the solution due to microalgae in the system might cause coagulation and adsorption of inorganic phosphates (Cai *et al.*, 2013; Ge and Champagne, 2016). Since the pH values in the wastewater effluents tested in

this study exceeded 9, it is reasonable to conclude that the removal of phosphorous was due to both microalgal activity and phosphate precipitation.

Overall, this study indicated that *C. vulgaris* (Toti RS4) was successful in the removal of nitrogen and phosphorus from the wastewater. It is also worth mentioning that the nutrient levels after 14-day experimentation were below the permissible dischargeable limits for wastewater treatment facilities (Farooq *et al.*, 2013). Moreover, a cluster formation or coagulation of microalgal biomass was observed, which caused sedimentation of microalgae during growth, and which could help reduce the cost of microalgal biomass recovery from the culture broth. The nutrient removal efficiencies obtained in our study was in reasonable agreement with the previously reported data (Cabanelas *et al.*, 2013; Ji *et al.*, 2013).

5.4.5. Carbohydrate content and productivity

The carbohydrate content of the microalgae grown in the three different culture media types is shown in Fig. 5.4. A common trend can be observed, in which the carbohydrate content (Fig. 5.4) increased rapidly in response to a decrease in the nitrogen and phosphorus concentration (Figs. 5.3 (A) and (B)). This is in agreement with previous studies showing that carbohydrate accumulation in microalgae is often triggered by nutrient limitation or starvation (Driver *et al.*, 2015; Pancha *et al.*, 2015). After cultivation for 14 days on raw PEMW, autoclaved PEMW and BG-11, the carbohydrate content ranged from 32.9% to 57.6%, 34.1% to 55.7% and 8.7% to 47% of the dry cell weight, respectively (Fig. 5.4). Statistical analysis of the results induced no statistically significant differences in carbohydrate content values observed on day 14, between microalgal cells grown on autoclaved PEMW and raw PEMW ($p > 0.05$). Because most microalgal carbohydrates are mainly derived from cellulose and starch, glucose is often the most dominating monosaccharide obtained from microalgal biomass after hydrolysis (Park *et al.*, 2016), therefore making microalgal biomass attractive as a feedstock for bioethanol production via the fermentation process.

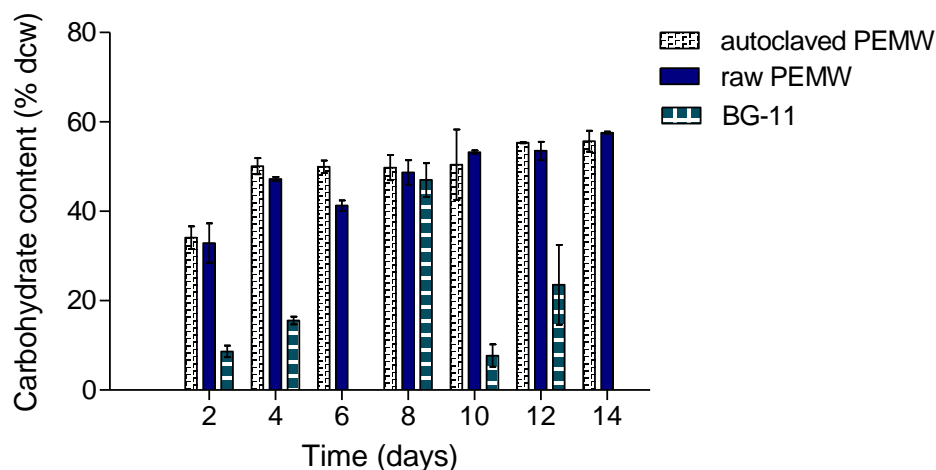


Fig. 5.4. Time-course profile of carbohydrate content during the batch growth of *C. vulgaris* (Toti RS4) on the three different culture media. (Light intensity= 180.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; temperature = 20 °C; CO_2 = ambient). The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

The carbohydrate productivities ranged between 10.3 ± 5.1 and $69.5 \pm 3.8 \text{ mg L}^{-1} \text{ d}^{-1}$ (Table 5.3). The microalgal cells grown in the autoclaved PEMW demonstrated the highest carbohydrate productivity while the cells cultivated in BG-11 media exhibited the lowest carbohydrate productivity. The carbohydrate productivity of *C. vulgaris* (Toti RS4) grown on the raw PEMW was $62.5 \pm 3.9 \text{ mg L}^{-1} \text{ d}^{-1}$, approximately 10% lower than that obtained for the autoclaved PEMW.

Table 5.3 Carbohydrate content and productivity of *C. vulgaris* (Toti RS4) under the three different culture media (Light intensity = 180.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; temperature = 20 °C; CO_2 = ambient).

Duration of cultivation	Cultivation media	Carbohydrate content at day 14 (% dcw)	Overall carbohydrate productivity ($\text{mg L}^{-1} \text{ day}^{-1}$)
14 days	BG-11	23.6 ± 8.9	10.3 ± 5.1
	Autoclaved PEMW	55.7 ± 2.3	69.5 ± 3.8
	Raw PEMW	57.6 ± 0.3	62.5 ± 3.9

Carbohydrate production had been demonstrated to cause a decline in the physiological function of the cell, which can be indicated by the decrease in the biomass production due to

the metabolic costs involved in carbohydrate synthesis. Therefore, correlation between carbohydrate content, biomass concentration and the PAM parameters (F_v/F_m , rETR and NPQ) for the strain *C. vulgaris* (Toti RS4) in raw and autoclaved PEMW were analyzed (Fig. 5.5).

Figs. 5.5 (A) and (B) showed that the carbohydrate content was directly proportional to the biomass production in both the autoclaved and raw PEMW and a significant correlation ($R^2 = 0.9463$ and 0.9406 , respectively) ($p < 0.05$) was obtained. Figs. 5.5(D), (F) and (H), showed that no significant correlation was found between the carbohydrate content and the PAM parameters (F_v/F_m , rETR and NPQ) on microalgae grown in the raw PEMW. The correlation coefficients (R^2) values were 0.148 , 0.106 and 0.349 , respectively ($p > 0.05$). Interestingly, for microalgae grown in the autoclaved PEMW, significantly higher correlation coefficients were found between carbohydrate content and the PAM parameter (F_v/F_m , rETR and NPQ). The correlation coefficients (R^2) were 0.676 ($p < 0.05$), 0.4702 ($p > 0.05$) and 0.6812 ($p < 0.05$), as shown in Figs. 5.5 (C), (E) and (G), respectively. This seems to coincide with the nutrient availability, biomass yields and lack of biological interference (bacterial, fungal or algal contaminants) in the autoclaved PEMW compared to the raw PEMW.

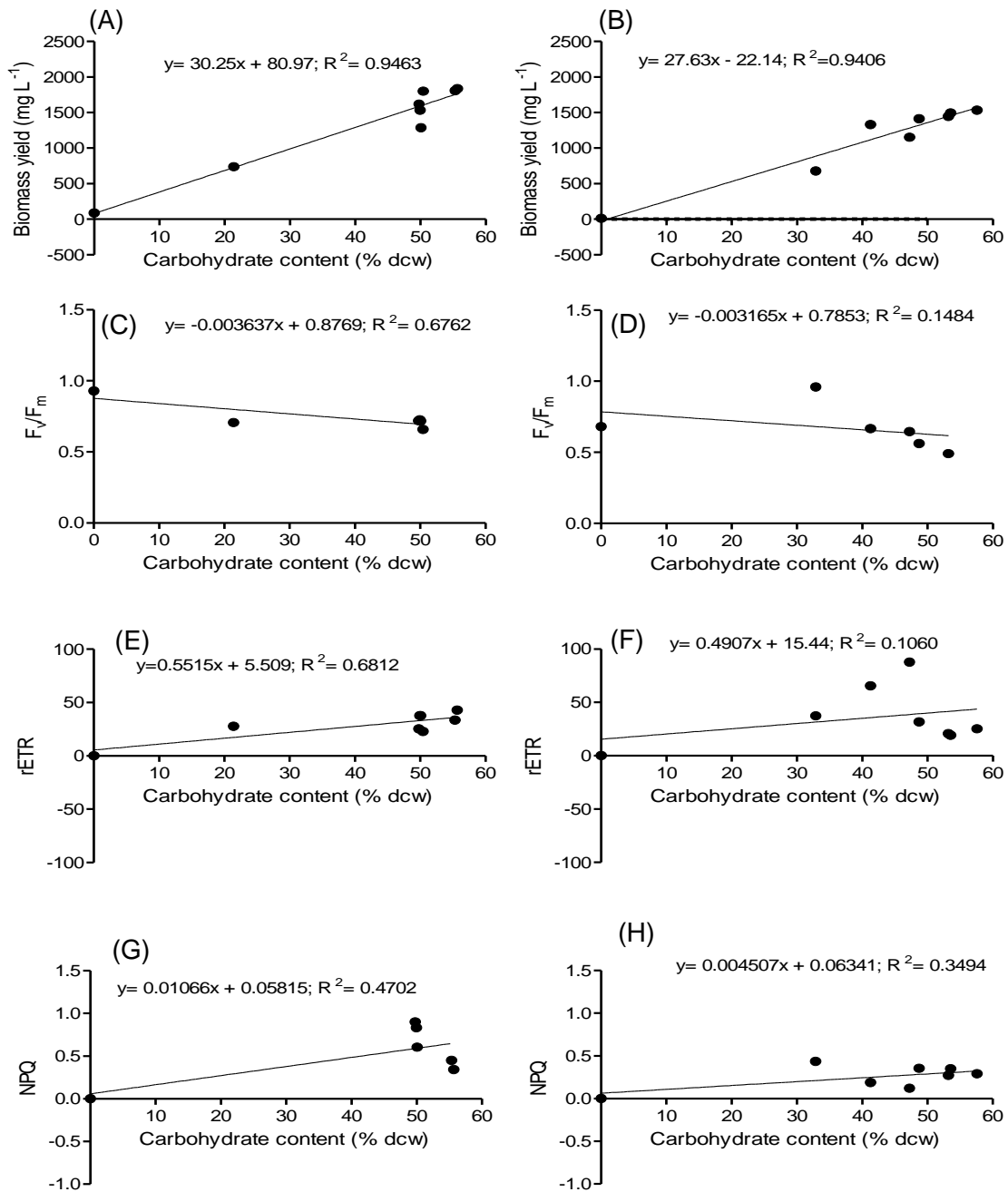


Fig. 5.5. Correlation between carbohydrate content and biomass yield (A) and (B) in autoclaved and raw PEMW, respectively. Correlation between carbohydrate content and F_v/F_m (C) and (D) in autoclaved and raw PEMW, respectively. Correlation between carbohydrate content and rETR (E) and (F) in autoclaved and raw PEMW, respectively. Correlation between carbohydrate content and NPQ (G) and (H) in autoclaved and raw PEMW.

5.4.6. Microalgal biomass flocculation

One of the most challenging steps in microalgae biotechnology, and especially for the large-scale production of biofuels, is the efficient and reliable separation of microalgae from the culture broth. It is worth pointing out that microalgae harvesting is one of the major bottlenecks of microalgal biotechnology. The cost of harvesting of microalgal biomass is estimated to account for up to 20-30 % of the total production cost depending on species, cell density and cultivation conditions. The most common harvesting strategies in the microalgal industry are mechanical methods such as filtration and centrifugation as well as chemical methods such as auto-flocculation or coagulation. Natural aggregation of suspended cells into larger particles and colony formation is promisingly a cost-effective means of microalgal biomass recovery. In this study, *C. vulgaris* (Toti RS4) demonstrated notable auto-flocculation characteristics in wastewater. The microalgal auto-flocculation mechanism in wastewater is not clearly understood because numerous factors are involved, such as high pH (Yang *et al.*, 2015), production of intracellular or extracellular carbohydrates (Markou *et al.*, 2012a) and the coexistence of other bacterial or fungal species (Cho *et al.*, 2016).

For flocculation experiment in this study, all algae were harvested in the stationary phase of growth. The biomass concentration was adjusted to $\sim 1 \text{ g L}^{-1}$ ($\text{OD}_{680} = 1.8$) via appropriate dilutions. Fig. 5.6 shows the kinetics of the auto-flocculation of *C. vulgaris* (Toti RS4) grown on the three different cultivation media.

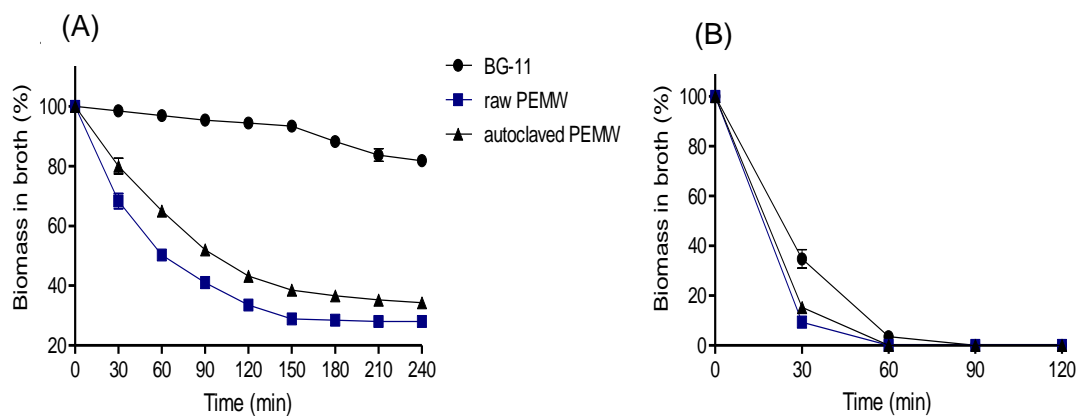


Fig. 5.6. (A): auto-flocculation of *C. vulgaris* (Toti RS4) cultivated in the three different culture media, (B): flocculation of microalgae using FeCl_3 at a concentration of 0.3 g L^{-1} . The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

During the 240 min experiment, 63.6 ± 0.3 % of the microalgal biomass had settled in the autoclaved PEMW and 69.6 ± 0.3 % of the total biomass in the raw PEMW had settled. It is worth mentioning that in the BG-11 medium the microalgal cells had a trend to ascend and float in the medium, thus making cells difficult to harvest by sedimentation. Only 18.2 ± 0.5 % of the microalgal biomass had settled by the end of the experimental (Fig. 5.6). Overall, the results illustrate the possibility of microalgal harvesting without the addition of any flocculating agent in wastewater. However, more research is required on this topic to establish the effect of various parameters such as cell density, cell surface charge, the presence of biological contaminants (fungi or bacteria), pH etc., on the auto-flocculation of microalgae in order to know the parameters to manipulate to achieve maximum flocculation efficiency. The use of chemical coagulants such as FeCl_3 for microalgae cell recovery is an attractive approach based on its rapidness (Fig. 5.6) as well as its relatively low cost when compared with mechanical methods such as centrifugation or filtration. Up to 96.45, 100 and 100% of the microalgae had settled within 60 min of experimentation in BG-11, autoclaved PEMW and raw PEMW, respectively. Despite this benefit, the use of chemical coagulants is a biohazard because they introduce chemicals to the system, which increases toxicity and carcinogenicity. In addition, the use of chemical coagulants also induce a color change of the growth medium which prevents its reuse and recycling. Recent reports stated that chemical coagulants are toxic and are not recommended for harvesting microalgae biomass that is being processed for food supplements and food additives (Chatsungnoen and Chisti, 2016; Guldhe *et al.*, 2016). Therefore, whenever chemical coagulants are selected for the harvesting of microalgal biomass, their effect on the downstream processing of microalgae should be of concern.

5.5. Conclusion

The study demonstrated that both autoclaved and raw PEMW could support the growth of the microalgal strain *C. vulgaris* (Toti RS4), and the growth potential, photosynthetic activity and nutrient removal capabilities were in agreement with the previously reported studies. Because the daily process rate of municipal wastewater is as high as one million gallons in wastewater treatment plants, pretreatment of the wastewater by autoclave is not feasible. In this view, raw PEMW is more desirable than autoclaved PEMW for cultivation despite the low growth rates observed. The carbohydrate content for microalgae grown in raw PEMW was 57.6% of the dry cell weight, and the carbohydrate productivity was 62.5 ± 3.9 mg L^{-1} day⁻¹, providing a net biomass recovery efficiency of 69.6 ± 0.3 % (after 240 min of experimentation). Therefore, it can be concluded that the raw PEMW can be used as a suitable medium for microalgal biomass and carbohydrate production.

Chapter 6. Optimization of concomitant chemical and thermal hydrolysis of complex microalgal polysaccharides for the production of fermentable sugar monomers for enhanced bioethanol production

6.1. Abstract

Microalga is one of the most compelling microbial biomass for bioethanol production. Carbohydrate hydrolysis is an integral part of bioethanol production, as it produces sugar monomers required for fermentation. Statistical modelling and optimization of dilute hydrochloric acid pre-treatment of a robust carbohydrate producing microalga strain *Chlorella vulgaris* (Toti RS4) were performed using response surface methodology (RSM). The Box-Behnken design was applied to study the effect of acid concentration, reaction time and temperature on the efficiency of microalgal biomass deconstruction into simple sugar monomers. The second-order polynomial model was fitted to experimental data to find the optimum reaction conditions by multiple regression analysis. The maximum reducing sugar concentration of 14.95 g L⁻¹ (vs. 16.37 g L⁻¹ predicted) was achieved under optimized hydrolysis conditions (0.5% (v/v) acid concentration, 120 °C and 67.5 min), confirming the high quality and accuracy of the developed model ($R^2 = 0.9407$; $p < 0.05$). The microalgal cell structure was analysed post- and pre-treatment using Fourier transform infrared (FT-IR) spectroscopy and scanning electron microscopy (SEM). These analyses revealed that functional groups, structure and surface of *C. vulgaris* (Toti RS4) were completely altered through dilute acid pre-treatment. Fermentation of the pre-treated microalgal biomass of *C. vulgaris* (Toti RS4) with *Saccharomyces cerevisiae* resulted in bioethanol concentration of 4.88 g L⁻¹ and ethanol yield of 0.16 g bioethanol/ g microalgal biomass.

Keywords: Microalgal biomass, dilute acid pre-treatment, optimization, fermentation.

6.2. Introduction

With the increasing shortages of fossil fuels as well as the continuous build-up of greenhouse gas emissions in the atmosphere, the focus has been drawn towards the development of renewable and sustainable energy resources (Yang *et al.*, 2016). Potentially valuable alternatives include those derived from microalgae. Microalgae represent a broad group of photosynthetic eukaryotic microorganisms that have received growing interest in

recent years as a platform for biofuel and biochemical production (He *et al.*, 2016). Some microalgal species such as *C. vulgaris* and *Scenedesmus* sp. have high growth and photosynthetic rates and are crucial for global carbon dioxide sequestration (Chang *et al.*, 2016; Ho *et al.*, 2012; Lizzul *et al.*, 2014). Other microalgal strains present numerous attractive properties for biofuel production. In particular, microalgal species such as *Chlorella vulgaris*, can accumulate over 50% carbohydrates in the biomass, and thus serve as an alternative fermentation substrate for the production of bioethanol (Ho *et al.*, 2013b).

Prior to use for fermentation, the microalgal biomass has to undergo pre-treatment and hydrolysis to generate fermentable sugars (Eldalatomy *et al.*, 2016). Chemical pre-treatment methods particularly using dilute acids or alkaline pre-treatment under moderate reaction conditions appear promising for effective depolymerisation of microalgae complex sugars into fermentable monomers (Eldalatomy *et al.*, 2016). In addition, they are environmentally friendly and cost effective since they can be administered at very low concentrations during the pre-treatment process. Optimization of the pre-treatment conditions must be performed to extract high concentration of reducing sugars from microalgal biomass with minimal inhibitors that can affect the fermentation process. The most important factors affecting carbohydrate depolymerisation through the chemical pre-treatment process include acid/alkaline concentration, temperature and reaction time (Borines *et al.*, 2013). The acid or alkaline concentration is a crucial factor that affects the sugar yield, while the temperature is mainly responsible for sugar degradation into monomers and various by-products such as furfural. Optimization of pre-treatment conditions often employs changing each variable at a time and keeping other variables constant. Thus, to test all combinations, this involves a large number of experiments and hard labour. Alternatively, response surface methodology (RSM) can be employed to reduce the number of experiments and optimization of experimental conditions with high efficiency. Few studies (Borines *et al.*, 2013; Pancha *et al.*, 2016; Scholz *et al.*, 2013) have reported the application of RSM on chemical (acid or alkali) pre-treatment of microalgal biomass, while most studies have focused on the pre-treatment of first and second generation feedstocks. However, there is currently limited information on chemical pre-treatment of microalgal biomass to produce bioethanol.

Therefore, the aim of this work was to study the chemical pre-treatment of *C. vulgaris* (Toti RS4) using response surface methodology (RSM) approach. The effect of different parameters of the chemical pre-treatment process such as the type of chemical agent, concentration, reaction temperature and reaction time on reducing sugar production from the microalgal biomass was investigated. The resulting reducing sugars achieved after hydrolysis were subsequently fermented into bioethanol by adopting the separate hydrolysis and fermentation (SHF) strategy.

6.3. Materials and Methods

6.3.1. Microalgal biomass

The microalgal species, *Chlorella vulgaris* (Toti RS4) was used in this study. The microalga was grown in raw primary effluent municipal wastewater (PEMW) as previously described in section 5.3.3. The nutrient profile and level of the wastewater is presented in [Table 5.1](#). The microalgal cells were grown under light with a photon intensity of $180.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature of 20 °C and ambient CO₂ for a maximum period of 14 days. The microalgae cultures were harvested during the stationary growth phase by autoflocculation for 240 min (4h), as previously described in section 5.4.6. The resulting cell pellet was washed twice with distilled water and subsequently dried at 60 °C for 24 h. The dried biomass obtained was used for further study.

6.3.2. Analysis of microalgal biomass composition

The lipid, protein and carbohydrate content of the microalgal biomass were analyzed using the sulfo-phospho-vanillin (SPV) ([Mishra et al., 2014](#)), Bradford method ([Bradford et al., 1976](#)) and Anthrone reagent method ([Yemm and Willis, 1964](#)), respectively as previously described in section 3.3.9. The analysis was performed in triplicate and the results were expressed as the average \pm standard deviation.

6.3.3. Chemical pre-treatment procedure

6.3.3.1. Preliminary screening

Hydrochloric acid (HCl), sulphuric acid (H₂SO₄), phosphoric acid (H₃PO₄), formic acid (CH₂O₂), acetic acid (CH₃CO₂H), sodium hydroxide (NaOH), ammonium hydroxide (NH₄OH) and disodium carbonate (Na₂CO₃) were investigated to establish the most effective hydrolytic agent for reducing sugar production from the dried biomass of *C. vulgaris* (Toti RS4). In this experiment, the microalgal biomass at a concentration of 30 g L⁻¹ (prepared by reconstituting 0.3 g of algal powder in 10 mL of distilled water) was reacted with 1% (v/v) of each acid reagent in a 250 mL Erlenmeyer flask and the reaction was performed by autoclaving at 121 °C for 15 min. The reaction was terminated by adding different volumes of 1 M of NaOH (for acids) or 1 M of HCl (for bases) until a pH of 7 was achieved.

6.3.3.2. Optimization of acid and thermal hydrolysis of microalgal carbohydrates using RSM

After selecting the best acid agent, the pre-treatment conditions were optimized using response surface methodology (RSM) coupled with the Box-Behnken design (BBD). Acid concentration (0.5-5 % (v/v)), heating temperature (100-121 °C) and reaction time (15-120 min) were chosen as input variables based on previous reports on microalgae hydrolysis (Borines *et al.*, 2013; Choi *et al.*, 2016; Kassim and Bhattacharya, 2015). The factor levels were coded as shown in Table 6.1. The relationship between the coded values and actual values is described as Eq. (6.1):

$$X_i = \frac{X_i - X_{cp}}{X_i} \quad (6.1)$$

where x_i is the coded level of the variable, X_i is the real level of the variable, X_{cp} is the real level of the variable at the centre point and X_i is the step change value at the real level.

Table 6.1 Range and levels of the independent variables based on BBD design.

Independent variable	Coded factor	Units	Levels and range		
			-1	0	1
Acid concentration	X_1	% (v/v)	0.5	2.75	5
Temperature	X_2	°C	100	110	121
Time	X_3	min	15	67.5	120

The polynomial model for predicting the response was expressed as described in Eq. (6.2):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (6.2)$$

where Y is the response in coded units, β_0 is a constant; β_i is the linear coefficient, β_{ii} is the squared coefficient and β_{ij} is the interactive coefficient considered in the model. The accuracy of the above polynomial model was evaluated using the coefficient of determination R^2 and adjusted R^2 .

For BBD with three input variables ($K= 3$) and five centre points ($C_0 = 5$), a total of 17 runs of experiments ($N= 17$) were required (Table 6.2) and experimental data analysis was performed using the Design-Expert statistical software program (10.0 trial version; State-Ease, Minneapolis, MN). The significance of the model coefficients was determined by the analysis of variance (ANOVA) combined with the application of Fischer's F test.

Table 6.2 Experimental design (conditions and responses) for glucose concentration.

Run	Experimental variables			Response variables
	X ₁ (Acid (%))	X ₂ (Temperature (°C))	X ₃ (Time (min))	Reducing sugar concentration (g L ⁻¹)
1	0.5	120	67.5	16.37
2	2.75	100	120	8.98
3	5	100	67.5	7.33
4	5	110	15	9.04
5	5	120	67.5	5.02
6	0.5	100	67.5	10.18
7	2.75	110	67.5	10.61
8	5	110	120	9.07
9	2.75	110	67.5	10.61
10	2.75	110	67.5	10.61
11	0.5	110	120	15.83
12	2.75	120	120	12.68
13	0.5	110	15	15.39
14	2.75	110	67.5	10.61
15	2.75	110	67.5	10.61
16	2.75	120	15	10.14
17	2.75	100	15	10.50

6.3.4. Determination of reducing sugar concentration

The total concentration of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Briefly, 1 mL of the acid hydrolyzed sample was filtered and added to 3 mL of DNS reagent and then boiled for 10 min in a water bath. The amount of reducing sugars was determined by measuring absorbance at a wavelength of 540 nm using a Varian 50 bio UV-visible spectrophotometer (Aligent, USA). The actual sugar concentration was determined from a calibration curve constructed from known concentrations of D-glucose.

6.3.5. Scanning electron microscopy

The detailed morphological analysis of untreated (control) and acid-hydrolysed microalgal cells were studied using a scanning electron microscope (ZEISS, Model EVO18). The microalgal samples were fixed using glutaraldehyde (3% v/v) for 24 h at 4 °C and washed twice with distilled water, followed by dehydration through a series of graded ethanol solutions (30%, 50%, 70% and 90% (v/v)). After the dehydration step, cells were mounted on aluminium stubs, sputter-coated with a gold layer, and used for SEM with an accelerating voltage of 15.0 kV.

6.3.6. Fourier transmission infrared (FT-IR) spectroscopy

The FT-IR procedure was done as previously described in section 3.3.8.

6.3.7. Fermentation of reducing sugars into bioethanol

6.3.7.1. Preparation of yeast inoculum

The commercial baker's yeast, *Saccharomyces cerevisiae* was used in this study. The yeast was first cultivated on YPD agar plates containing yeast extract (10 g L⁻¹), peptone (20 g L⁻¹), glucose (20 g L⁻¹) and agar (15 g L⁻¹) at 30 °C for 48 h. A single colony of *S. cerevisiae* was inoculated into 100 mL of pre-autoclaved YPD broth medium. The cells were cultivated at 30 °C for 24 h with a gentle agitation at a rotation of 120 rpm.

6.3.7.2. Fermentation using hydrolysate of *C. vulgaris*

The fermentation was performed in 250 mL Schott glass bottles which contained 100 mL of the microalgal hydrolysate, inoculated with 10% (v/v) of the inoculum prepared according to section 6.3.7.1. The yeast cells were cultured anaerobically at 30 °C for 24 h at 120 rpm. The concentration of reducing sugars and produced ethanol were analyzed using 3,5-DNS method (Miller, 1959) and gas chromatography, as described in sections 6.3.4 and 6.3.7.4, respectively.

6.3.7.3. Ethanol extraction procedure

Samples (600 µL) were drawn at regular intervals through the rubber septum by a syringe equipped with an 18G needle. The broth was transferred to a 2 mL Eppendorf tube and subjected to centrifugation at 7 000 x g to sediment the yeast cells. Afterwards, 500 µL of the cell-free supernatant was transferred to a new tube, and then 5 µL of butan-1-ol (as internal standard) was added and the tubes were vortexed for 1 min at maximum speed. One millilitre of ethyl acetate was added, followed by 5 min of vortexing at maximum speed. Finally, the tubes were subjected to centrifugation (5 000 x g for 2 min at room temperature) to facilitate phase separation. The organic phase was drawn and used for GC analysis.

6.3.7.4. Analysis of bioethanol using gas chromatography

Ethanol concentration was analyzed using gas chromatography (GC 14B, Shimadzu, Japan) equipped with a FID detector and a DB-5 column (30 m x 0.25 mm x 1 µm) (Agilent, USA) using helium as a carrier gas at a flow rate of 0.7 mL min⁻¹. The sample was filtered through a 0.22 µm PVDF syringe filter and then injected for analysis. The column temperature was

45 °C while the injector and detector temperatures were kept constant at 200 °C and 250 °C, respectively. The injection volume was 1 µL. The data was monitored and processed using an LCsolution software v 1.0 (Shimadzu, Japan). The concentration of ethanol was determined using a calibration curve obtained from standard ethanol solutions (Appendix F).

6.3.8. Statistical analysis

Statistical analysis was done as previously described in section 3.3.12.

6.4. Results and Discussion

6.4.1. Microalgal biomass composition

The biochemical composition of *C. vulgaris* (TotiRS4) grown on the raw PEMW is shown in Fig. 6.1. On a dry cell weight basis, the amount of carbohydrate in the sample was $57.6 \pm 0.3\%$, which was within the reported percent carbohydrate content of *Chlorella* species in wastewater (Wang *et al.*, 2015). The lipid content of the sample on dry basis was $13.8 \pm 0.3\%$ dcw and was found to be lower compared to *Chlorella sokoriana* at 58% dcw (Ramanna *et al.*, 2014) but higher than that of *Chlorella* sp. (11.04% dcw) (Li *et al.*, 2011). The protein content of the sample on a dry biomass basis was $22.9 \pm 1.9\%$ and agreed with values reported for *Chlorella vulgaris* by Ji *et al.* (2014). The high carbohydrate content of *C. vulgaris* (Toti RS4) demonstrates its suitability as a potential source of feedstock for bioethanol production.

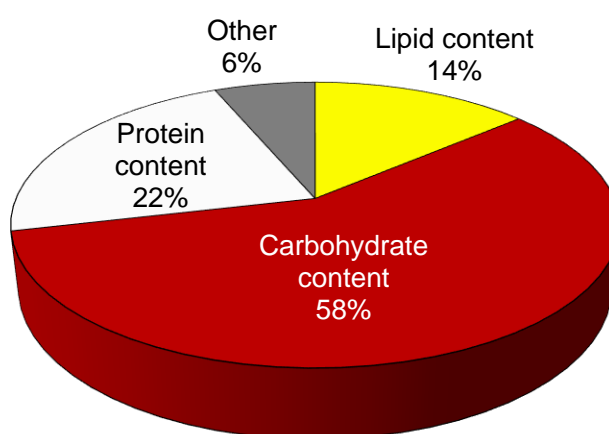


Fig. 6.1. Biochemical composition of raw PEMW grown *C. vulgaris* (Toti RS4) biomass.

6.4.2. Selection of chemical agent

In this study, chemical treatment using five different acids, HCl, H₂SO₄, H₃PO₄, CH₂O₂ and CH₃CO₂H as well as three different alkali, NaOH, NH₄OH and Na₂CO₃ was investigated to identify the most effective hydrolytic agent for maximum reducing sugar production from the carbohydrate-rich biomass of *C. vulgaris* (Toti RS4). Fig. 6.2 shows the reducing sugars produced from microalgal biomass after pretreatment. The highest concentration of reducing sugars was obtained using 1% (v/v) (or 0.33 M) of HCl. Approximately 7.4 g L⁻¹ (~24% dcw) of reducing sugar concentration was achieved from the total biomass of *C. vulgaris* (Toti RS4) during the pretreatment process. Meanwhile, pretreatment using the organic acids (CH₂O₂ and CH₃CO₂H) showed an insignificant effect on reducing sugar production during the acid pre-treatment process, which was in disagreement with the study of [Du et al. \(2016\)](#). This is presumably due to the strong cell wall structure of the microalgal cells used in this study. The reducing sugar concentrations achieved during alkaline pre-treatment using NaOH, NH₄OH and Na₂CO₃ were 2.4, 1.4 and 0.95 g L⁻¹, respectively. These values were comparatively lower than those obtained using inorganic acids such as HCl, H₂SO₄ and H₃PO₄ but were in a similar range to those reported by [Kassim and Bhattacharga \(2016\)](#).

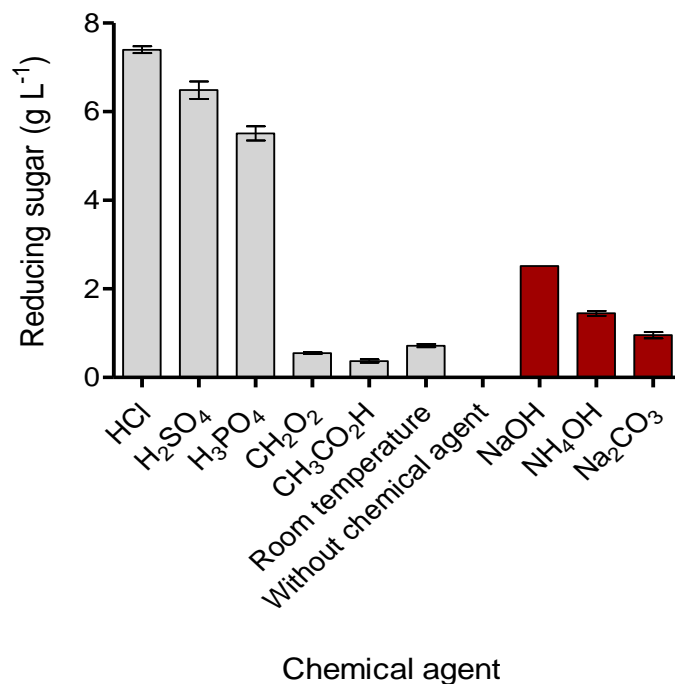


Fig. 6.2. Reducing sugar concentration from dried microalgal biomass pre-treated with different chemical agents. Control represents untreated biomass. The reaction was performed at 121°C for 15 min using the chemical agent concentration of 1% (v/v).

The comparison of pretreatment using controls with thermally pretreated (without chemical i.e using distilled water) and chemical pretreated (at room temperature using HCl) was also performed in order to determine the degree of chemical and thermal effect on pretreatment in this study. It was found that the reducing sugars produced in the thermally pretreated sample (without chemical) were below the detection range. Meanwhile, the maximum reducing sugar concentration produced in the chemically pretreated sample (at room temperature) was 0.718 g L⁻¹ and was found to be in a similar range to the microalgal biomass pretreated with CH₂O₂ (0.548 g L⁻¹) and CH₃CO₂H (0.373 g L⁻¹) under autoclave conditions (121 °C, 15 min) (Fig. 6.2).

The ineffectiveness of thermal treatment “alone” (or without chemical) is likely attributed to the inability of the used mechanical method to hydrolyse glycosidic bonds of the carbohydrates to produce sugar monomers. This phenomenon was also reported by Yuan *et al.* (2016). These results therefore indicate that the production of reducing sugars from *C. vulgaris* (Toti RS4) could be attributed to the effect of combined interaction of temperature and chemical agent rather than their individual effects (Choi *et al.*, 2016).

The mechanism of reducing sugar production using different acids and alkali on the microalgal biomass is not fully understood and this has led to some data that is difficult to reproduce and confidently used for industrial applications (Miranda *et al.*, 2012). For example, in a study of Lee *et al.* (2013b), a comparison between the use of HCl and H₂SO₄ for pre-treatment of *Chlorella* indicated that the use of HCl resulted in a greater saccharification efficiency compared to H₂SO₄, which concurred with this study. In contrast, Markou *et al.* (2013) found H₂SO₄ to be the most effective acid for the hydrolysis of *Anthrospira plantensis* when compared to HCl, HNO₃ and H₃PO₄. Thus, it can be concluded from these studies that the selectivity of the hydrolytic agent is mostly influenced by the nature of the biomass feedstock. Wang *et al.* (2016a) also indicated that the state of the biomass feedstock, such as cell age, cell size and cell wall architecture and thickness play a pivotal role in the pre-treatment process. Thus, the insignificant effects presented by the organic acids (CH₂O₂ and CH₃CO₂H) on reducing sugar production during the pre-treatment process may have been attributed to the pre-treatment conditions applied. Technically, organic acid pre-treatment is milder when compared to the mineral acid pre-treatment. In this respect, organic acids require catalysts and longer reaction time (over 10 h) and higher temperature (over 100 °C) to obtain better performance (Du *et al.*, 2016).

6.4.3. Model fitting and statistical analysis

Based on the preliminary investigations, HCl demonstrated the highest yield of reducing sugars from the biomass of *C. vulgaris* (Toti RS4) (Fig. 6.2). Therefore, this acid was selected and used for pre-treatment optimization studies. Identification of optimum conditions is vital for obtaining higher yields of reducing sugar and lower yields of degradation products and inhibitors. In this study, reducing sugar production from the microalgal biomass was evaluated as a function of acid concentration (X₁), time (X₂), and temperature (X₃) using the Box-Behnken experimental design to identify the optimum conditions. The statistical combination of the test variables along with the measured response values expressed as reducing sugar corresponding to each combination are summarized in Table 6.2. The second order polynomial equation, which was an empirical relationship between the amount of reducing sugar and test variables in coded values, is shown in Eq (6.3):

$$Y = + 14.242 - 3.283X_1 + 0.53025X_2 + 0.642X_3 + 0.933X_1X_2 - 0.6585X_1X_3 + 0.6175X_2X_3 - 3.30025X_1^2 - 1.35525X_2^2 + 0.53575X_3^2 \quad (6.3)$$

Where Y is the reducing sugar concentration (g L^{-1}), X_1 is the acid concentration (% (v/v)), X_2 is temperature ($^{\circ}\text{C}$) and X_3 is time (min).

A one-way analysis of variance (ANOVA) for the response surface quadratic model was used to evaluate the impact and significance of test variables on the reducing sugar production from *C. vulgaris* (Toti RS4) (Table 6.3). The model F -value of 12.32 and p -value of 0.0016 implies that the model was significant. In addition, the model lack of fit was observed to be insignificant ($p= 0.9605$), which suggests the goodness of fit of the experimental data to the model.

Table 6.3 Analysis of variance (ANOVA) for the generated quadratic model*.

Source	Sum of square	df	Means square	F -value	p -value
Model	136.86	9	15.21	12.32	0.0016
Residual	8.64	7	1.23		
Lack of fit	0.56	3	0.19	0.092	0.9605
Pure Error	8.08	4	2.02		
Cor Total	145.50	16			

*Coefficient of determination (R^2) = 0.9407; adjusted R^2 = 0.8643; predicted R^2 = 0.8519.

The fitness of the model was also expressed by the coefficient of determination (R^2). The R^2 value was 0.9407 (Table 6.3), implying that the model generated could explain 94.07 % of the variability in the response and only 5.94 of the total variation was not attributed to the model. The closer the R^2 is to 1.0., the stronger the model and the better it predicts the response. Generally, a model with R^2 higher than 0.90 is considered to have a good correlation (Yun *et al.*, 2012). The experimental value versus the predicted value of reducing sugar production shown in Fig. 6.3 reveals a satisfactory correlation between these values ($R^2= 0.9407$). In addition, it demonstrated that a Box-Behnken design could be applied for the optimization of pre-treatment conditions.

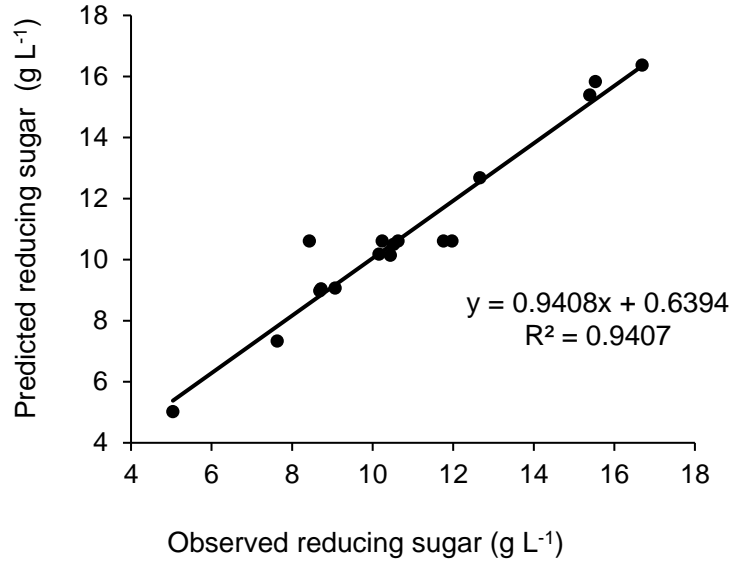


Fig. 6.3. The correlation of experimental and predicted glucose concentration.

The adjusted R^2 value of the model was 0.8643 which suggests a good adjustment of the predicted response values to the experimental data by the developed model. Furthermore, the vicinity of the predicted R^2 value of 0.8519 was found to be in reasonable agreement to the adjusted R^2 value. These results suggest a good fit of the generated model.

Table 6.4 shows the F -test and the corresponding p -values of the test variables and the interaction. Generally, p -value lower than 0.05 indicates that the terms are statistically significant at 95% confidence while the p -value greater than 0.05 suggest that the terms are insignificant. Therefore, based on the results obtained, the linear (X_1), interactive (X_1X_2), and quadratic (X_2^2 , and X_3^2) terms were significant model terms for reducing sugar concentration. In contrast, the linear (X_2 and X_3), interactive (X_2X_3), and quadratic (X_1^2) terms were insignificant model terms for reducing sugar concentration. Therefore, a statistically significant model term only with significant terms can be written as shown in Eq (6.4)

$$Y = +14.242 - 3.283X_1 + 0.933X_1X_2 - 1.35525X_2^2 + 0.53575X_3^2 \quad (6.4)$$

Table 6.4 Analysis of variance (ANOVA) for the model terms.

Model term	Sum of squares	df	Mean square	F-value	p-value
X ₁ -Acid concentration	93.21	1	93.21	75.54	< 0.0001
X ₂ -Temperature	5.56	1	5.56	4.51	0.0714
X ₃ -Time	0.52	1	0.52	0.42	0.5372
X ₁ X ₂	15.86	1	15.86	12.85	0.0089
X ₁ X ₃	0.22	1	0.22	0.18	0.6827
X ₂ X ₃	4.11	1	4.11	3.33	0.1107
X ₁ ²	0.81	1	0.81	0.66	0.4447
X ₂ ²	8.90	1	8.90	7.21	0.0313
X ₃ ²	8.55	1	8.55	6.93	0.0338

6.4.4. Effect of processing variables on reducing sugar production

In the classical 'one-factor-at-a-time' optimization strategy, the individual effects of bioprocess factors can be graphically illustrated without the need for statistical analysis. Unfortunately, this method often fails to seek the region of optimum response since the combined effects of factors on the response are not considered in the technique. In this respect, RSM is more attractive because it gives a strong detection of the optimum response. In Fig. 6.4, two-dimensional contour plots and three-dimensional response surface plots are shown. In the design boundary, the response surface of the reducing sugar concentration had a clear peak, which suggests that the key variables could be optimized inside the design boundary. The interaction effect between temperature and acid concentration on the reducing sugar production is shown in Fig. 6.4(A). When the reaction time is set at 67.5 min as a center point of statistical experimental design, the maximum reducing sugar production can be obtained by conducting the hydrolysis experiment at temperatures between 110 °C and 120 °C using 0.5 - 2.75 % (v/v) of HCl. ANOVA analysis for the interaction of acid concentration and temperature was lower than 0.05 ($p= 0.0089$) suggesting that the combined effect of acid concentration and temperature plays a pivotal role in the production of reducing sugar from microalgal biomass. However, the acid concentration was the most significant factor ($p< 0.0001$) which suggests that changes in this factor could significantly affect the reducing sugar production from microalgal biomass. Based on Fig. 6(A), pre-treatment of *C. vulgaris* (Toti RS4) with HCl concentration higher than 2.75 % (v/v) would result in the drop of reducing sugar production.

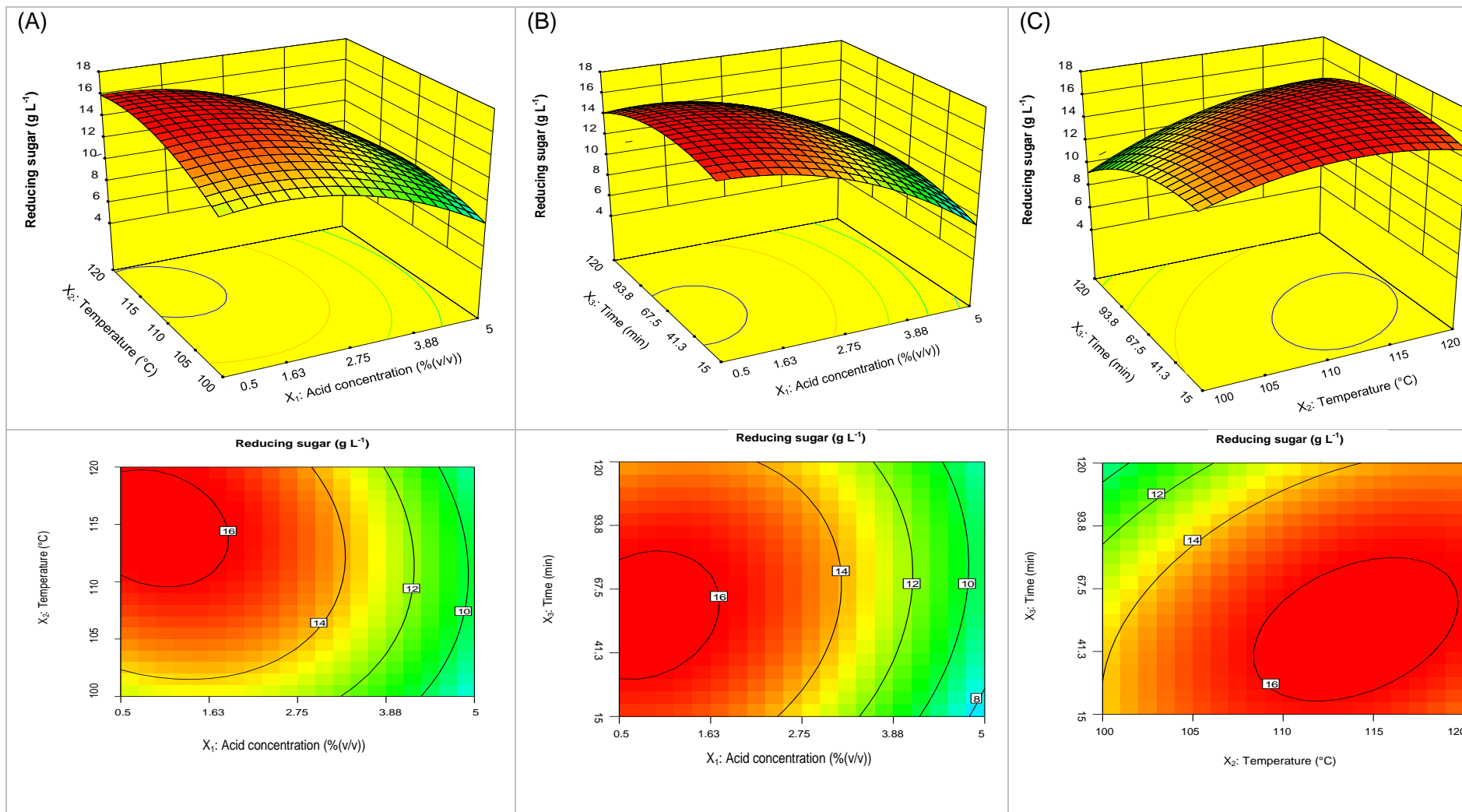


Fig. 6.4. Three-dimensional surface plots and two-dimensional contour plots for reducing sugar yield.

Studies have shown that an increase in medium acidity causes monosaccharide degradation and respective drop in reducing sugar recovery (Pancha *et al.*, 2016). Fig. 6.4(B) shows the interaction effect between acid concentration and reaction time at a constant temperature of 110 °C set as a center point. It can be observed that the acid concentration range of 0.5 - 2.75 % (v/v) and reaction time of 67.5 min are desirable to maximize the reducing sugar production from the biomass of *C. vulgaris* (Toti RS4). The interaction effect between time and temperature on reducing sugar concentration is shown in Fig. 6.4(C). The desirable ranges of reaction time and temperature (67.5 min and 110-120 °C) are particularly notable where the acid concentration was kept constant at 2.75% (v/v). Although the interactive effect between temperature and reaction time was found to be insignificant from the ANOVA analysis (Table 6.4), the response surface and contour plots demonstrated that the effect of different temperatures at different reaction time provoke different effects on the production of reducing sugar. For instance, a combination of high temperature for long reaction time was disadvantageous for the pre-treatment of *C. vulgaris* (Toti RS4). From the financial point of view, lower operational temperatures or shorter reaction time would greatly reduce the cost of this process since the autoclave system consumes immense amounts of energy in their operation.

Overall, this study indicated that the pre-treatment test factors such as acid concentration, reaction temperature and reaction time have a significant effect on reducing sugar released from *C. vulgaris* (Toti RS4) biomass. Pre-treatment at below or above the identified optimum conditions could result in low reducing sugar concentration. The results indicate that the combination of high acid concentration, temperature and reaction time produced low reducing sugar concentration mainly due to the breakdown of reducing sugar under those conditions (Fig. 6.4). It has been shown that degradation of reducing sugar at high acid concentration and temperature leads to the formation of derivatives such as organic acids and phenols, which inhibit the growth of yeast during the fermentation process. Gas liquid chromatograph analysis has demonstrated these by products (Rahman *et al.*, 2007).

6.4.5. Confirmation test of reducing sugar production at optimal conditions

In order to confirm the validity of the statistical experimental strategies and to gain a better understanding of reducing sugar production, a confirmation test was performed under the following optimized conditions: acid concentration = 0.5 % (v/v), temperature = 120 °C, time = 67.5 min. Fig. 6.5 shows the time course profile reaction of this experiment. The maximum reducing sugar concentration of 14.95 g L⁻¹ was achieved (Fig. 6.5), which was close to the

predicted reducing sugar concentration of 16.37 g L⁻¹. These results indicate that optimization of key factors was successfully achieved by RSM with Box-Behnken enhancing reducing sugar release from the biomass of *C. vulgaris* (Toti RS4).

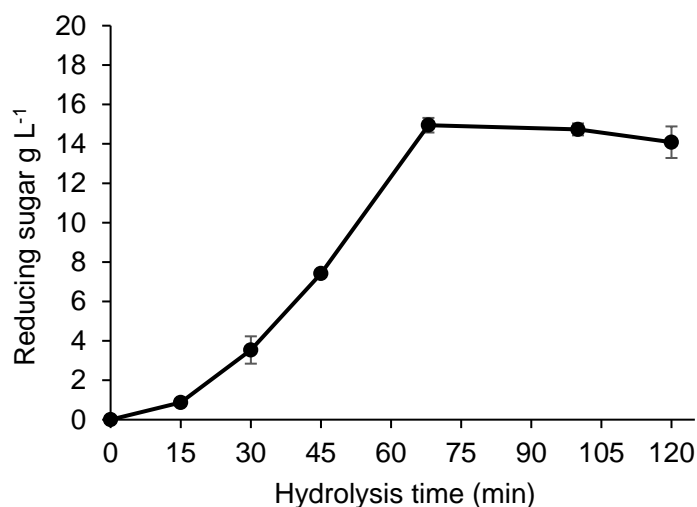


Fig. 6.5. Time-course analysis of glucose concentration produced from *C. vulgaris* (Toti RS4) biomass pre-treated under optimum conditions. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation.

The concentration of reducing sugar produced was comparable to the concentrations obtained from other microalgae reported in the previous studies. Approximately, 14.8 mg mL⁻¹ of glucose was produced from acid pre-treatment of *Dunaliella tertiolecta* performed using 0.5 M of HCl at 121°C for 15 min (Lee *et al.*, 2013b). In another study, Jang *et al.*, (2012) reported glucose concentration of 13.5 g L⁻¹ after pre-treatment with 40 mM H₂SO₄ with autoclaving at 121°C for 60 min. According to Park *et al.*, 2016, approximately 5.74 g L⁻¹ of reducing sugar were produced from acid pre-treatment of *C. vulgaris* using 2% (w/w) HCl at 121°C for 20 min.

6.4.6. FT-IR spectroscopy and SEM

FT-IR spectra showed the changes in the functional groups of the microalgal biomass during acid pre-treatment. In the FT-IR spectra, the major components of microalgal biomass such as carbohydrates showed a peak at 1200-900 cm⁻¹, lipids showed a peak near 2960-2850 cm⁻¹ while proteins showed a peak near 3400-3200 cm⁻¹ as well as near 1545-1655 cm⁻¹ (Pelusi *et al.*, 2016). The comparison of FT-IR spectra for untreated and pre-treated microalgal biomass is shown in Fig. 6.6. Typically, during the acid-thermal pre-treatment, the α - and β -aryl ether glycosidic bonds of carbohydrates polymers are broken down and an

acetyl group is removed (Miranda *et al.*, 2012). This mechanism was instantly confirmed in this study, evidenced by the large decline in the carbohydrate bands (1200-900 cm^{-1}). FT-IR also indicates that acid pre-treatment had a reduction on band 1655 cm^{-1} and 1545 cm^{-1} , which are associated with amide I and amide II, respectively. A reduction was also observed in the 1240 cm^{-1} band illustrating the degradation of phosphodiester bonds of nucleic acids and phospholipids.

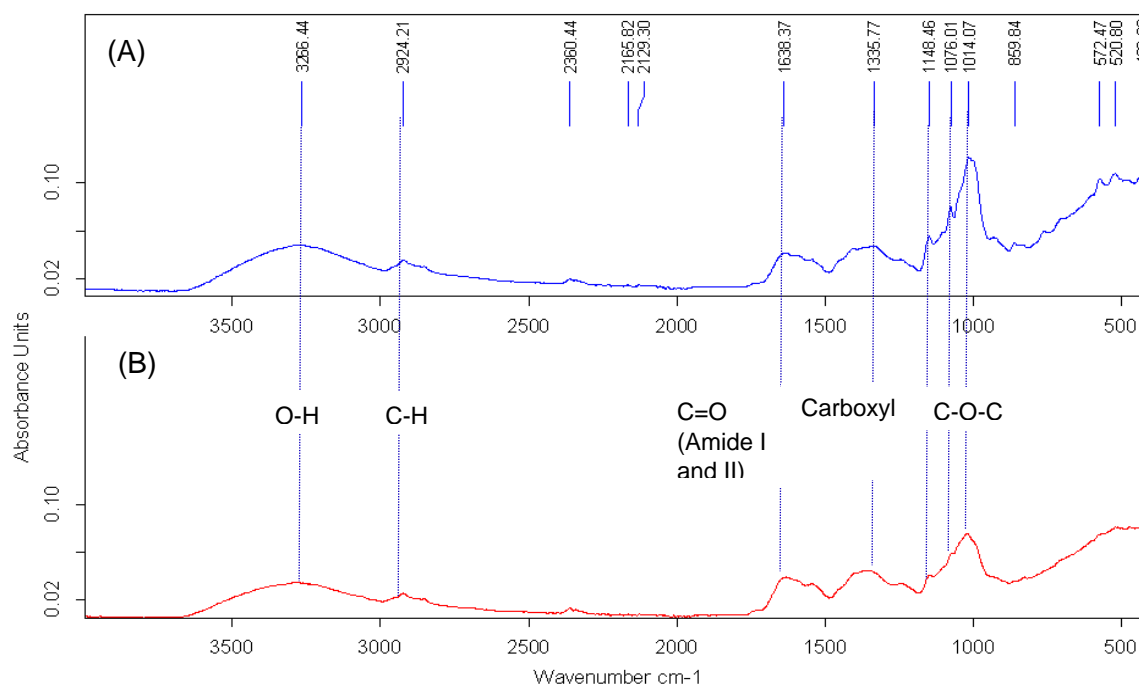


Fig. 6.6. FT-IR spectra of untreated (A) and combined acid and thermal pre-treated (B) cells of *C. vulgaris* (Toti RS4).

SEM analysis was performed to study the ultrastructural changes and surface characteristics of the microalgal cells under the optimum acid-thermal pre-treatment conditions. The SEM images of microalgae before pre-treatment (Fig. 6.7(A)) shows that the cells were clumped together, intact, and mostly cocci in shape. On the other hand, the SEM images of the acid treated microalgal cells instantly shows that the cells were lysed, evident by the uneven cell structure and large cell debris (Fig. 6.7(B)). The change from intact cells to broken cells after the combined acid and thermal pre-treatment process confirms the efficiency of this method in the disruption of microalgal cell walls to release entrapped carbohydrates.

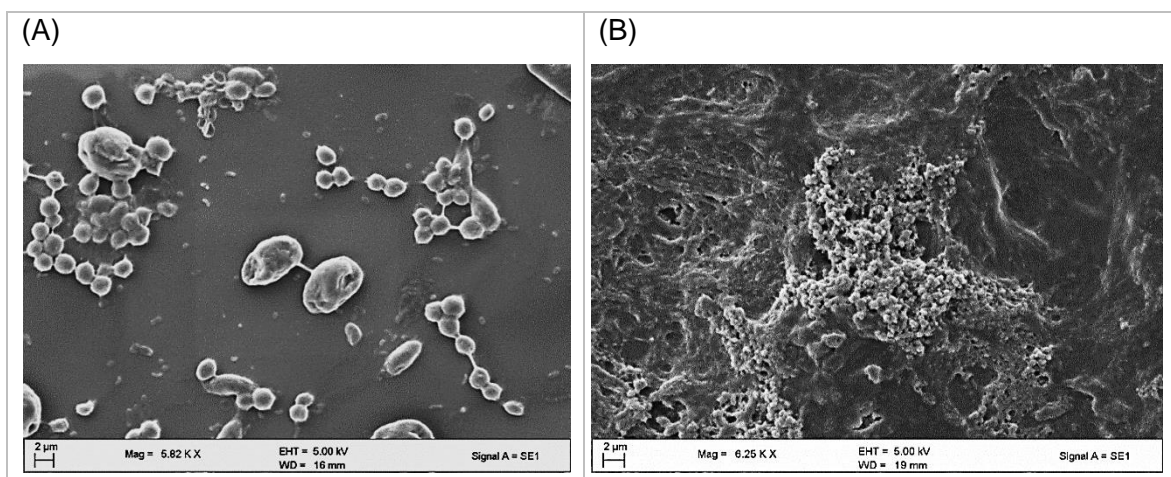


Fig. 6.7. SEM images of *C. vulgaris* (Toti RS4) without pre-treatment (A) and with pre-treatment under optimum conditions (B).

6.4.7. Fermentation of *C. vulgaris* reducing sugars produced from biomass hydrolysis under optimal pre-treatment conditions

Fermentative conversion of hydrolysed microalgae biomass to bioethanol was investigated using *S. cerevisiae* with separate hydrolysis and fermentation (SHF) process. The cell growth, reducing sugar consumption and ethanol production profiles are presented in Fig. 6.8. The results indicate that the acid pre-treated biomass of *C. vulgaris* (Toti RS4) containing 14.95 g L⁻¹ reducing sugars was fermented with *S. cerevisiae*. The fermentation process produced 4.88 g L⁻¹ of bioethanol with a yield of 0.16 g bioethanol/ g microalga biomass after 24 h.

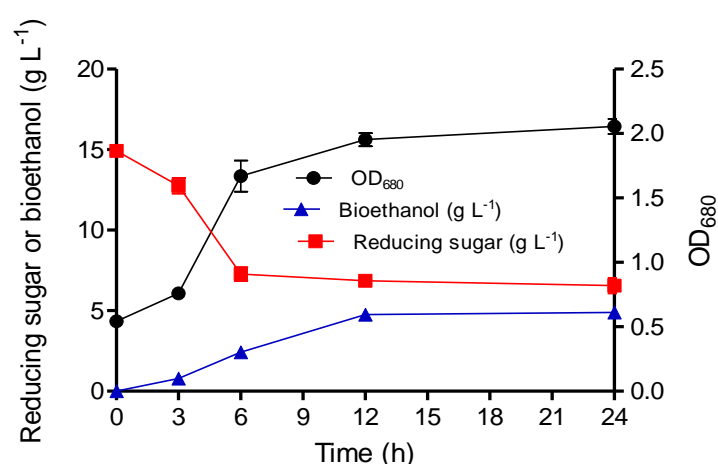


Fig. 6.8. The time-course profile of cell growth, reducing sugar utilization and bioethanol production by *S. cerevisiae*.

The ethanol yield obtained in this study was comparatively similar to that reported by [Ellis et al. \(2012\)](#) and [Lee et al. \(2013b\)](#) but was lower than that reported by [Sanchez Rizza et al. \(2017\)](#). The reducing sugar concentration decreased gradually with incubation time and finally stabilized at 6.55 g L⁻¹. This seems to coincide with the death of *S. cerevisiae* cells after 12 h of incubation ([Fig. 6.8](#)). The probable explanation for the early onset of the stationary phase by *S. cerevisiae* was due to the presence of inhibitory compounds such as furfurals and anhydrides generated during acid hydrolysis. For future studies, it could be interesting to validate this assumption in order to administer a suitable detoxification strategy prior to inoculation of the yeast for fermentation.

6.5. Conclusion

This study demonstrated the successful utilization of the green microalgae *C. vulgaris* (Toti RS4) as a potential feedstock for bioethanol production. Acid hydrolysis of the microalgal biomass produced suitable carbohydrates that ensured its fermentability. The total concentration of 14.95 g L⁻¹ of reducing sugars was obtained after hydrolysis and 4.88 g L⁻¹ of bioethanol concentration was achieved.

Chapter 7. Enzymatic hydrolysis of pretreated microalgal (*Chlorella vulgaris* Toti RS4) biomass for bioethanol production

7.1. Abstract

Enzymatic hydrolysis of microalgal biomass for reducing sugar production is one of the crucial steps for biofuel production through biochemical conversion. Initially, the microalgal biomass was subjected to pretreatment using sonication, microwave and autoclave. Subsequently, the biomass was characterized using fourier transform infrared spectroscopy (FT-IR) and scanning electron microscope (SEM). The analyses revealed the functional groups and surface structure of *Chlorella vulgaris* (Toti RS4) changes during pretreatment, which is favourable for enzymatic accessibility to microalgal carbohydrates. Comparison of carbohydrate solubilization after pretreatment revealed that autoclave pretreatment was the most effective method, with a maximum sugar solubilization of 66%. Enzymatic hydrolysis of the autoclave pretreated *C. vulgaris* (Toti RS4) was performed using α -amylase (E.C 3.2.1.1) obtained from *Aspergillus oryzae*. Enzymatic hydrolysis was optimized by varying temperature, pH and enzyme concentration to substrate ratio ([E]/[S]). The highest concentration of reducing sugar of 16.9 g L⁻¹ and conversion yield of 98% out of the total carbohydrates (57.6% dcw) from the pretreated biomass was obtained from hydrolysis at temperature: 25 °C, pH: 6.0 and [E]/[S] of 0.08 after 72 h incubation. Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes were used to ferment the enzymatic microalgal hydrolysate into ethanol with a yield of 0.21 and 0.26 g bioethanol/ g microalgal biomass, respectively. These findings demonstrate the feasibility of using microalgae as feedstock for bioethanol production.

Keywords: Microalgal biomass, pretreatment, enzymatic hydrolysis, bioethanol

7.2. Introduction

The environmental and economic pitfalls associated with the utilization of fossil-based fuels have led to the search for alternative energy resources which are renewable, sustainable and environmentally friendly (Ang *et al.*, 2015). Among the various forms of renewable energy resources present, bioethanol is realized as one of the most promising alternative biofuels and has been developed extensively for two decades (Yang *et al.*, 2016). Bioethanol has been produced largely from sugar cane and starch crops as well as from lignocellulosic biomass (Wi *et al.*, 2015). However, recently microalgae have gained

heightened interest as third generation feedstock for biofuel production due to their high polysaccharide production efficiency and their lack of lignin content. The absence of lignin in microalgal cell walls suggests that microalgal cells have relatively loose structure when compared to terrestrial plants, to which they are closely related phylogenetically. However, despite this advantage, the hydrolysis of microalgal biomass into fermentable sugars aside from high-value products is still regarded as the major technological bottleneck hindering the utilization of microalgal biomass. The cellulose-rich cell wall and firm cell membrane of microalgae delay the subsequent hydrolysis of microalgae in the fermentation process. Thus, pre-treatment is often required in the bioconversion of microalgal biomass into fermentable monomers (Lee *et al.*, 2013a).

Current pretreatment technologies can be grouped into four categories: mechanical (ultrasonication, bead-beating and milling), thermal (microwave, autoclaving and freezing), chemical (organic solvents and acid-alkaline reactions) and biological processes (microbial degradation, enzymatic reactions) (Borines *et al.*, 2013). There are already a few studies evaluating the potential application of these techniques for microalgae cell pretreatment in biorefinery. For instance, the review by Gunerken *et al.* (2015) gives a comparison of conventional and non-conventional emerging techniques for microalgae pretreatment in terms of disruption efficiency, product quality, scalability and specific energy consumption. The authors suggested that the process efficiency and specific energy consumption are highly attributed to the microalgal species, growth conditions of biomass and cell dry weight loading. Previously, chemical pretreatment using dilute acids or alkaline reactions have been deemed to be the most effective approaches due to their rapidness and ability to effectively hydrolyze the microalgal carbohydrates with high sugar yields (Lv *et al.*, 2010). Nevertheless, microalgal hydrolysis via the chemical approach is accompanied by the production of undesirable compounds such as organic acids, furfurals and phenolic compounds, which interfere with the downstream processing of microalgae into bioethanol. Although detoxification methods can be applied, they are however expensive and can contribute significantly to the total cost of production (Fernández-Rodríguez *et al.*, 2017).

Enzymatic saccharification of microalgal biomass provides superior yields without the generation of undesirable products (Szabo and Csiszar, 2017). Individual enzymes and enzyme mixtures can be used for the hydrolysis of microalgal biomass. The most effective enzymes reported in literature include cellulases (endoglucanase (E.C 3.2.1.4), exoglucanase (E.C 3.2.1.19) and β -glucosidase (E.C 3.2.1.21), pectinase (E.C 3.2.1.15), chitinase (E.C 3.2.1.14), and amylase (E.C 3.2.1.1) (Ho *et al.*, 2013a; Kumar *et al.*, 2013; Lee *et al.*, 2013b; Pancha *et al.*, 2016). The released microalgal carbohydrates after enzymatic hydrolysis are mainly in the form of glucose, xylose or arabinose, depending on

the microalgal strain (Ho *et al.*, 2013a; Kermanshahi-pour *et al.*, 2014). *S. cerevisiae* can ferment hexoses with great efficiency, making it an ideal organism for industrial production of bioethanol from microalgal biomass. In a study by Choi *et al.* (2010), bioethanol yield of 0.235 g bioethanol/ g microalgae biomass was achieved. Considering the need for alternative sustainable energy resources that do not compete with the food supply, microalgal biomass is viewed as a promising alternative. However, a limited number of studies conducted in this research area are present in the international literature, thus current research effort is necessary.

The objectives of this study were to; (1) determine the most efficient pretreatment strategy for *C. vulgaris* (Toti RS4), (2) determine the most suitable hydrolytic enzyme and optimize conditions such as pH, temperature and enzyme concentration for enhanced enzymatic hydrolysis of pretreated *C. vulgaris* (Toti RS4) biomass, and (3) investigate the potential of bioethanol production from microalgal hydrolysate via separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes.

7.3. Materials and Methods

7.3.1. Microalgae cultivation conditions

Microalgal species, *Chlorella vulgaris* (Toti RS4) was used in this study. The microalga was grown on raw primary effluent of municipal wastewater (PEMW), as previously described in section 5.3.3. The nutrient profile and level of the wastewater is presented in Table 5.1. The microalgal cells were grown under light with a photon intensity of 180.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$, temperature of 20 °C and ambient CO₂ for a maximum period of 14 days (section 5.3.3).

7.3.2. Preparation of substrate (microalgal biomass)

The microalgae biomass was recovered from the cultivation broth by autoflocculation for 240 min as previously described in section 5.4.6. The recovered cell pellet was washed twice with distilled water and dried at 60 °C in an oven until constant mass was achieved. For preparing a substrate with concentration of 30 g L⁻¹, 0.3 g dry microalgae were weighed and then re-suspended in 10 mL of 10 mM sodium acetate buffer solution (pH 4.5-6.0, depending on the enzyme) for experimentation.

7.3.3. Pretreatment of microalgal biomass

Pretreatments were performed using 30 g L⁻¹ of microalgal biomass prepared as previously described in section 7.3.3. Three different cell disruption methods; autoclaving, sonication, and microwave treatment were used in this study.

The autoclave pretreatment experiment was performed at 121 °C for 15 min (MLS-3020, Sanyo, Japan), followed by cooling in an ice bath for 10 min. The sonication pretreatment experiment was performed using an OMNI sonic rupture 400 apparatus operated at a frequency of 40 kHz and 40% amplitude for 3 min, followed by cooling in an ice bath for 10 min. This procedure was repeated five times. In the microwave pre-treatment experiment, the microalgal biomass was processed for 40 s at 600 W, followed by cooling in an ice bath for 10 min. This procedure was repeated three times for each sample.

7.3.4. Enzymatic hydrolysis

Enzymatic hydrolysis was performed using two coupled experiments. In the first experiment, the effect of the type of enzyme on reducing sugar production from microalgal biomass was investigated. In this experiment, hydrolysis was performed using four different commercial hydrolytic enzymes; α -amylase (BCB7609V), pectinase (BCBP9450V), cellulase (SLBM6034V), and β -glucosidase (BCD9743V) purchased from Sigma-Aldrich, South Africa. The enzymes α -amylase, pectinase, cellulase and β -glucosidase were extracted from *Aspergillus oryzae*, *Aspergillus niger*, *Trichoderma reesei* ATCC 29621 and almonds, respectively. The specific activities of the enzymes were 36 U mg⁻¹ (α -amylase), > 1U mg⁻¹ (pectinase), \geq 1U mg⁻¹ (cellulase), and \geq 6 U mg⁻¹ (β -glucosidase). One enzyme unit (U) was defined as the amount of enzyme that catalyzed the liberation of 1 μ mol of glucose per minute under the experimental conditions used (Sigma-Aldrich). The amount of protein content of the enzymes was determined using the Bradford method with bovine serum albumin (BSA) as standard (Appendix E). The enzyme addition was conducted at the optimal loads and conditions recommended by the supplier (Sigma-Aldrich, South Africa). The enzyme mixture consisting of different combinations (ratios) of cellulase and β -glucosidase was also evaluated in this study. The studied ratios were 1:1 (cellulase: β -glucosidase), 2:1 (cellulase: β -glycosidase) and 1:2 (cellulase: β -glucosidase). The pH and temperature were kept constant at 4.5 and 50 °C, respectively (supplier recommendation) (Sigma-Aldrich).

In the second set of experiments, optimisation of hydrolysis conditions was carried out using the selected hydrolytic enzyme. The enzyme used for this study was selected based on its ability to produce a high concentration of reducing sugars in the preliminary study.

Optimization of the enzymatic hydrolysis was carried out using one-variable-at-a-time (OVAT) method. Enzymatic hydrolysis was carried out by incubating the sample at a temperature ranging between 20-35 °C, pH ranging between 4.5-7.5 and an enzyme concentration to substrate ratio ($[E]/[S]$) of 0.02 to 0.1. These experiments were conducted for 72 h. Five different enzyme concentrations varying from 6 mg to 30 mg with an initial amount of 0.3 g microalgal biomass was used. The samples were withdrawn every 3 to 24 h for analysis and heated at 100 °C to deactivate the enzymes. The samples were then centrifuged at 4 000 x *g* for 5 min and the supernatant was used for reducing sugar analysis using 3,5-DNS method (Miller, 1959), as previously described (section 6.3.4).

7.3.5. FT-IR and SEM analysis

The procedure for FT-IR analysis was done as previously described in section 3.3.8. SEM procedure was done as previously described in section 6.3.5.

7.3.6. Fermentation studies

7.3.6.1. Preparation of yeast inoculum

The yeast inoculum was prepared as previously described in section 6.3.7.1.

7.3.6.2. Separate hydrolysis and fermentation (SHF)

The autoclave pretreated *C. vulgaris* (Toti RS4) biomass (at a concentration of 30 g L⁻¹) was hydrolyzed by α -amylase (at a concentration of 0.08 mg enzyme/ g microalgal biomass) at 25 °C and pH of 6.0 until a constant concentration of reducing sugars was achieved (typically 72 h). After enzymatic hydrolysis, the pre-cultured cells of *S. cerevisiae* were inoculated (at an inoculum size of 10 % (v/v)) to carry out fermentation at 30 °C for 48 h under anaerobic conditions.

7.3.6.3. Simultaneous saccharification and fermentation (SSF)

The autoclave pre-treated biomass (at a concentration of 30 g L⁻¹) was used as SSF medium. *S. cerevisiae* (at an inoculum size of 10 % (v/v)) and α -amylase (at a concentration of 0.08 mg enzyme/ g microalgal biomass) were simultaneously added into the microalgal medium. The fermentation was performed in a single reaction vessel at 30 °C for 72 h under anaerobic conditions.

7.3.6.4. Ethanol extraction and analysis

Ethanol was extracted as previously described in section 6.3.7.3. The extracted ethanol was analyzed by gas chromatography, following the procedure described in section 6.3.7.4

7.3.7. Statistical analysis

Statistical analysis was done as previously described in section 3.3.12.

7.4. Results and Discussion

7.4.1. Identification of a suitable pre-treatment method

The carbohydrates contained in microalgae are in the cell wall and storage polysaccharides. Therefore, to hydrolyze polysaccharides, the enzyme should have access to hydrolyse sugars in the cell wall and inside the microalgal cell to release fermentable sugars. One way of improving enzyme accessibility to microalgal carbohydrates is through pre-treatment of the biomass. In this study, pre-treatment using three different cell disruption techniques, autoclave, microwave and sonication on *C. vulgaris* (Toti RS4) biomass was investigated.

The biochemical determination of *C. vulgaris* (Toti RS4) revealed that the carbohydrate fraction accounts for 57.6 % out of the total dry weight. Out of this percentage, approximately 3.0 ± 0.1 % carbohydrates were in the soluble fraction at the beginning of the experiment (non-pre-treated sample). However, after pre-treatment, an increase in carbohydrate solubilization was observed (Fig. 7.1).

Autoclave pre-treatment resulted in the highest soluble carbohydrate release of 66.4 ± 0.8 %, which was 1.7 and 1.2 times higher than that obtained for sonication and microwave pre-treated microalgal biomass, respectively. In a study by Mendez *et al.* (2013), thermal pre-treatment of *C. vulgaris* at 120 °C for 20 min resulted in 35.2% carbohydrate solubilisation, which was comparatively lower than *Chlorella* reported in this study. This difference may be attributed to the differences in cell wall structure presumably caused by different cultivation conditions or growth phase.

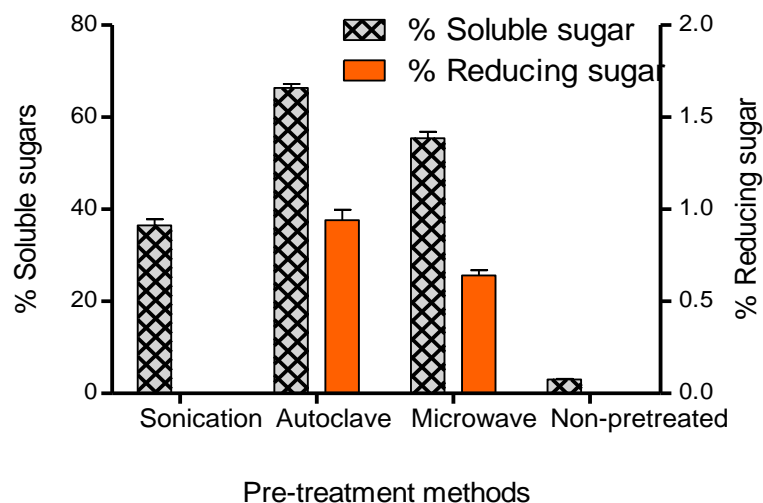


Fig. 7.1. Sugar solubilisation and reducing sugar production under different pre-treatment methods. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation.

In order to study the extent of carbohydrate solubilisation, the supernatant obtained was analysed for reducing sugars. As depicted in Fig. 7.1, the presence of reducing sugars in the soluble fraction was only observed when microalgae biomass was subjected to autoclave and microwave pre-treatment but at very low concentrations (below 1 %). No reducing sugars were detected in the soluble fraction after sonication pre-treatment, which was in agreement with the results of Asada *et al.* (2012). These researchers observed that regardless of treatment time, glucose extraction ratio was maintained at a low constant value (i.e., below 1%), claiming that the ultrasonic treatment does not degrade starch to glucose directly because it cannot break down α -1,4- and/or β -1,6-glucoside linkages. Therefore, results obtained in this study indicated that the pre-treatment methods and conditions used were effective not for the degradation but for the extraction of polysaccharides (i.e., cellulose and starch) from microalgae. Higher disruption and hydrolysis efficiency can be obtained from the combination of mechanical pre-treatment with a hydrolytic agent such as acids (HCl and H₂SO₄) as opposed to mechanical pre-treatment alone (Miranda *et al.*, 2012).

Based on results of the present study, autoclave pre-treatment is the most effective pre-treatment method since its utilisation resulted in the greater solubilization of the microalgal carbohydrates compared to the use of microwave and ultrasonication pre-treatment. However, further investigation of the biomass physical structure needs to be performed in order to effectively select the most suitable pre-treatment method. Thus, further analyses of

cell structure were carried out using FT-IR and SEM to determine the effect of pre-treatment on the microalgal cell structure.

7.4.2. The effect of biomass pre-treatment on ultrastructure and chemical composition

The fingerprint regions of the FT-IR spectra of non-pretreated and pre-treated microalgal biomass are presented in Fig. 7.2.

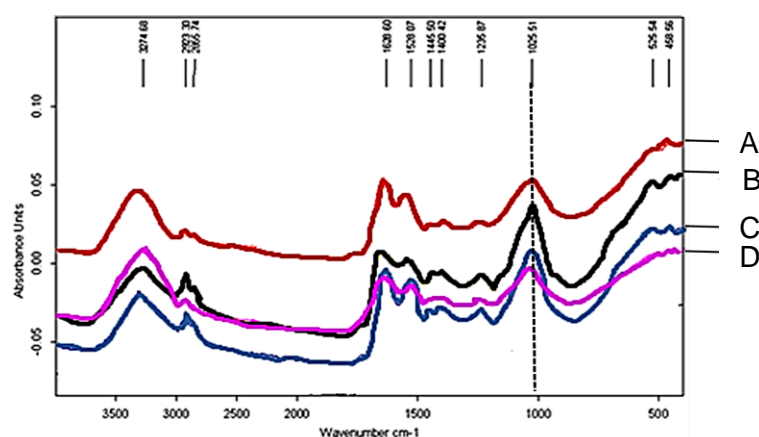


Fig. 7.2. FT-IR spectra of microwave pre-treated (A), non-pretreated (B), sonication pre-treated (C) and autoclave pre-treated (D) cells of *C. vulgaris* (Toti RS4).

This analysis has been widely used for understanding the functional groups on cell surface structure after pre-treatment. The microalgal proteins are registered by the band wave numbers 3400 cm⁻¹ to 3200 cm⁻¹ (Pelusi *et al.*, 2016). Comparing spectra obtained upon pre-treatments, an up-shift of the protein peaks was observed due to protein extraction. However, rearrangement of the protein structure upon pre-treatment was not the main focus of the FT-IR spectra. Particularly in this study, FT-IR spectra were used to elucidate whether or not the cell wall polysaccharides have been affected by pre-treatment, particularly cellulose since this component has been noted to be the main fraction hampering microalgae cell hydrolysis and the subsequent production of bioethanol. In the FT-IR spectra, the bond C-O-C of polysaccharides was assigned to wavenumber range of 1200-940 cm⁻¹. As shown in Fig. 7.2, untreated sample had high-intensity peaks within polysaccharide range (1200-940 cm⁻¹). However, the intensity decreased after the cells were subjected to pre-treatment, suggesting that pre-treatment affected the carbohydrate structure. The lowest peaks were observed for the autoclave pre-treated samples which suggests that this pre-treatment method had the highest effect on the carbohydrate

structure. In contrast, the sonication pre-treated samples demonstrated the highest polysaccharide peaks, indicating that sonication pre-treatment had the least effect on the carbohydrate structure under the experimental conditions.

Scanning electron microscopy (SEM) was employed to determine the structural changes of the microalgal cells following pre-treatment. It must be noted that no counting of cells was performed since this analysis was only employed to qualitatively elucidate cell disruption. SEM image of both microalgal biomass before (control) and after pretreatment are shown in [Fig. 7.3](#). Comparison of the SEM images shows a change to the microalgal cell structure after pre-treatment. As shown in [Fig. 7.3\(A\)](#), the untreated sample showed intact cell structure with a smooth surface. The opposite trend was observed with the autoclave pre-treated cells. The high temperature (121 °C) and pressure (120 kPa) of this method resulted in cell breakage and release of intracellular constituents as shown in [Fig. 7.3\(B\)](#). Microwave pre-treatment also resulted in cell breakage ([Fig. 7.3\(C\)](#)). However, the microalgal cells remained intact after sonication pre-treatment ([Fig. 7.3\(D\)](#)). Therefore, carbohydrates solubilized during this pre-treatment did not correspond to intracellular carbohydrates but likely to exopolymers released from cell walls. In fact, some *Chlorella* species have been reported to consist of cellulose and some are coated by chitin and pectin-like polysaccharides ([Kassim and Bhattacharya, 2016](#)).

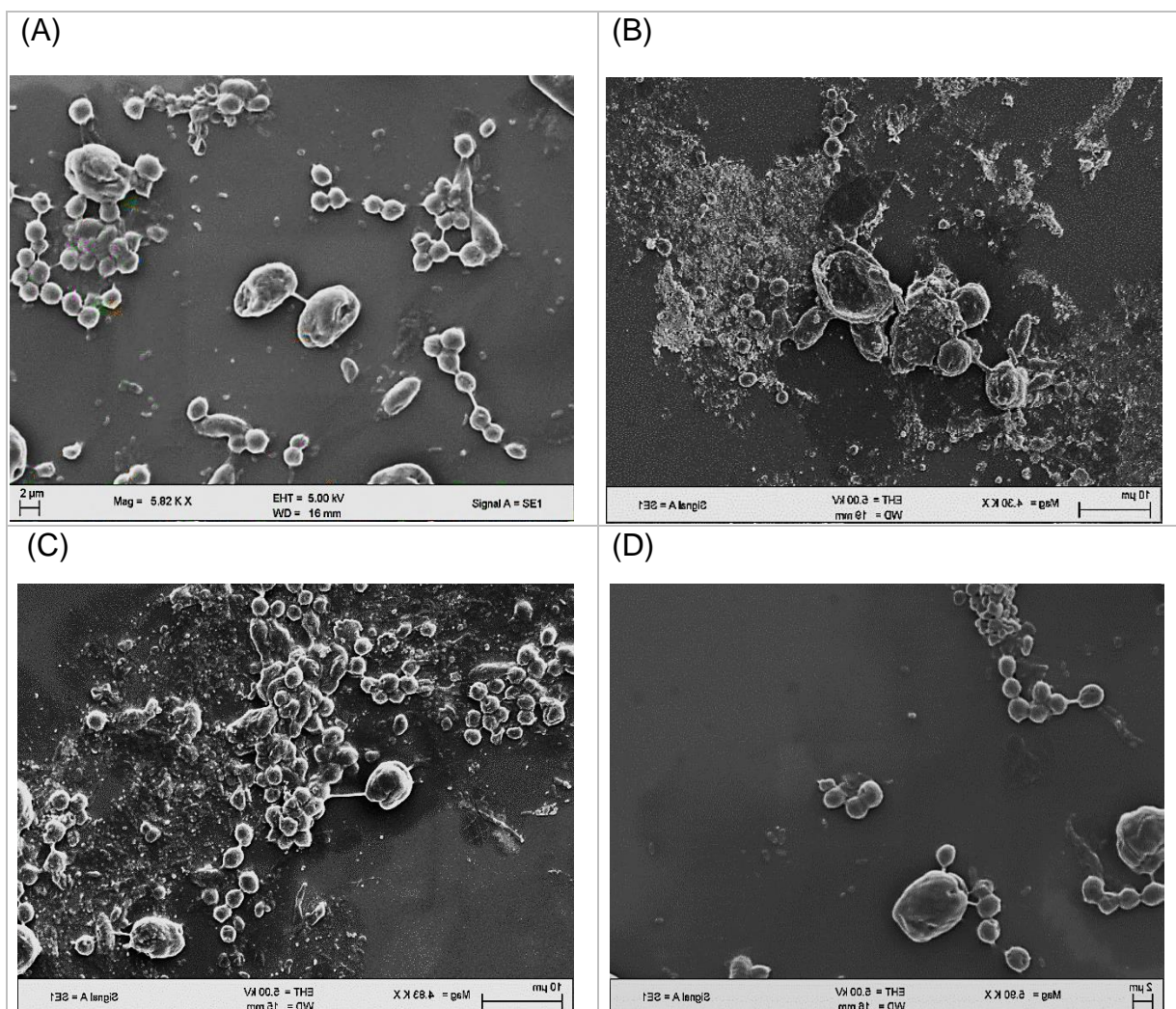


Fig. 7.3. SEM micrographs of non-pretreated (A), autoclave pre-treated (B), microwave pre-treated (C) and sonication pre-treated (D) cells of *C. vulgaris* (Toti RS4).

7.4.3. Enzyme selection for hydrolysis of *C. vulgaris* (Toti RS4) biomass

In this study, enzymatic hydrolysis with four different enzymes, α -amylase, pectinase, cellulase and β -glucosidase was investigated to identify the most efficient enzyme for the hydrolysis of *C. vulgaris* (Toti RS4) biomass. Fig. 7.4 shows the reducing sugar production from the untreated and pre-treated *C. vulgaris* (Toti RS4) biomass during enzymatic hydrolysis.

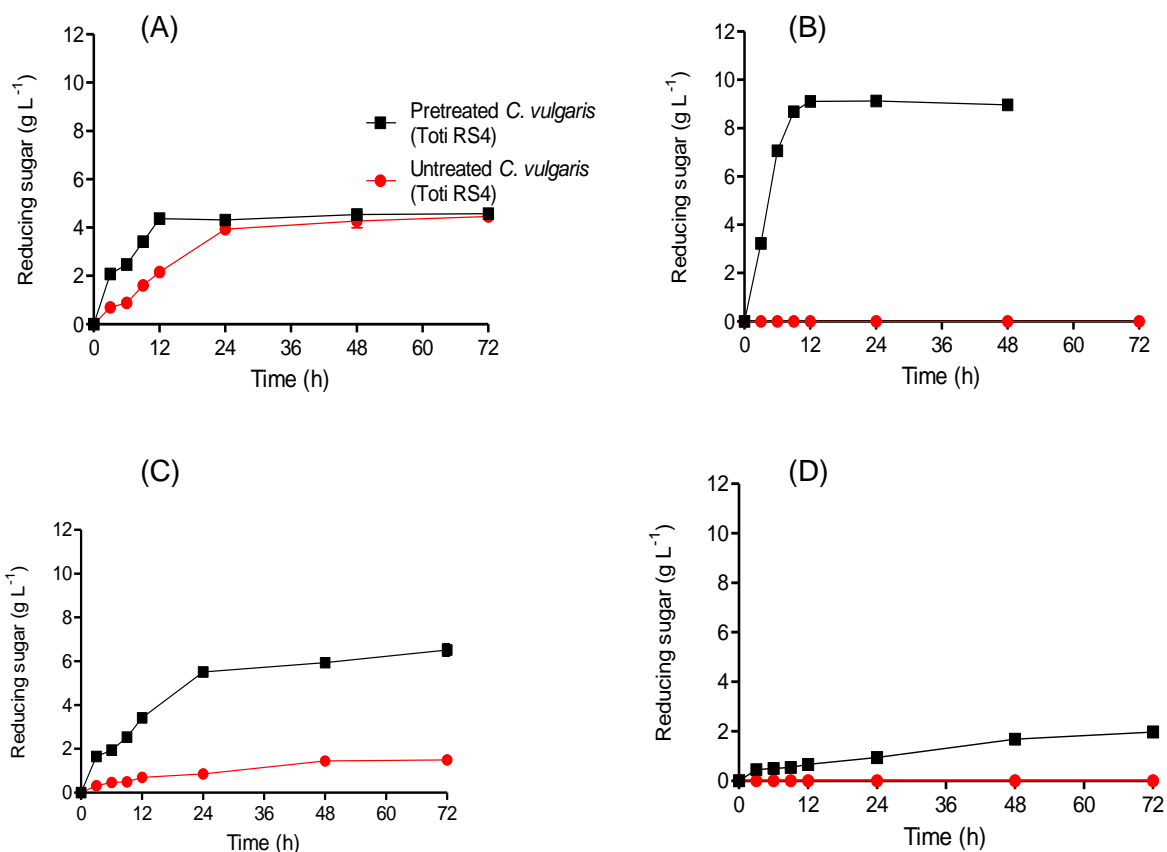


Fig. 7.4. Effect of enzymes on reducing sugar production from untreated and autoclave pre-treated biomass of *C. vulgaris* (Toti RS4). (A) cellulase, (B) α -amylase, (C) pectinase and (D) β -glucosidase. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation.

As expected, the pre-treated microalgal biomass rendered higher reducing sugar concentration due to improved enzyme accessibility. Interestingly, it was found that hydrolysis using cellulase led to a similar reducing sugar production under the conditions used (i.e., pretreated and non-pretreated) indicating that pre-treatment was not necessary to enhance the process of reducing sugar production. This is due to the fact that substrate of cellulase (i.e., cellulose) resides in the cell walls of the microalgae cells. Thus, cellulase was readily accessible to its substrate. In general, renewable feedstocks that do not require any intensive pre-treatment procedures prior to enzymatic hydrolysis are preferable because they require a lower input cost. Nevertheless, hydrolysis was found to be faster when pre-treatment was used in this study (Fig. 7.4(A)).

α -Amylase, pectinase, cellulase and β -glucosidase resulted in reducing sugar yields of 8.97 ± 0.10 , 6.51 ± 0.27 , 4.58 ± 0.19 and 1.97 ± 0.15 g L⁻¹ from the autoclave pre-treated microalgal biomass, respectively (Fig 7.4). As the major component of the total

carbohydrates of the microalgal biomass existed as starch, it was not surprising that α -amylase resulted in the highest concentration of reducing sugar since this enzyme has a high activity towards starch, in contrast to cellulases and β -glucosidases, which are mainly specific for the cellulose fraction of the biomass.

The combined use of cellulase and β -glucosidase was also evaluated (Fig. 7.5) as these enzymes can work synergistically to degrade polysaccharides. The use of a mixture of cellulase and β -glucosidase (2:1) displayed good results but did not substantially enhance reducing sugar production from the pre-treated biomass of *C. vulgaris* (Toti RS4).

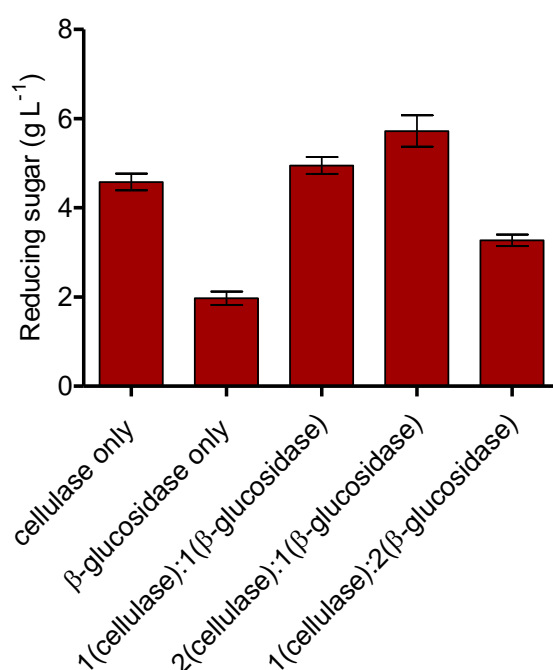


Fig. 7.5. Effect of the combination use of different enzymes on the hydrolysis of the pre-treated *C. vulgaris* (Toti RS4) biomass. The analysis was performed in triplicate and the results were expressed as the average with a standard deviation of three measurements.

Overall, it can be concluded that the selectivity of the enzyme is largely influenced by the nature of the biomass composition. According to a study by Kim *et al.* (2014), cellulase and amylase had no major influence on sugar conversion yield from the biomass of *C. vulgaris*, which is in contrast to the present study. The same workers found that only pectinase enzyme had a significant effect on polysaccharide hydrolysis. According to the study of Kermanshahi-pour *et al.* (2014), fungal pectinase from *Aspergillus niger* was accountable for the complete degradation of starch from *Tetraselmis suecica*. In the present study,

pectinase also demonstrated good reducing sugar yields. However, based on the results obtained, α -amylase produced the highest concentration of reducing sugars and was therefore considered for further experimentation.

7.4.4. Effect of operation conditions on enzymatic hydrolysis using α -amylase

7.4.4.1. Effect of temperature on enzymatic hydrolysis of the autoclave pre-treated biomass of *C. vulgaris*

The effect of temperature on reducing sugar production from pre-treated *C. vulgaris* (Toti RS4) was investigated and the results are presented in Fig. 7.6.

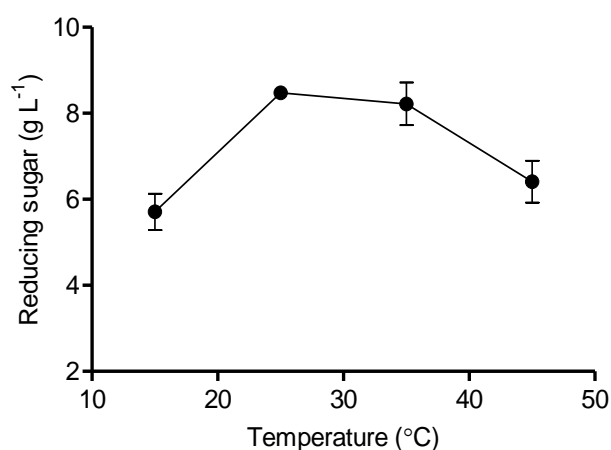


Fig. 7.6. The effect of temperature on enzymatic hydrolysis of autoclave pre-treated *C. vulgaris* (Toti RS4) biomass. Results represent the average value of three replicates.

The optimal production of reducing sugar with the highest reducing sugar concentration obtained at 25 °C, which was consistent with the manufacturer's recommendations (Sigma-Aldrich). Temperature below and above the optimal level (25 °C) resulted in low reducing sugar concentrations. There was a significant difference ($p < 0.05$) between experimental temperatures, suggesting that temperature plays a pivotal role in reducing sugar production from *C. vulgaris* (Toti RS4) biomass. Similar observation has been reported on the saccharification of microalgae *Dunaliella tertiolecta* (Lee et al., 2015). Generally, temperature affects the molar kinetic energy of the hydrolysis process by influencing the interactions between substrate and enzyme. Increasing temperature results in an increase of substrate-enzyme interaction. However, temperature above the optimum range results in denaturation and inactivation of the enzyme.

7.4.4.2. Effect of pH on enzymatic hydrolysis of the autoclave pre-treated biomass of *C. vulgaris*

The effect of pH on enzymatic hydrolysis of pre-treated *C. vulgaris* (Toti RS4) was also investigated in this study. According to Fig. 7.7, the optimum hydrolysis pH was 6.0 reflecting the highest reducing concentration of $8.9 \pm 0.3 \text{ g L}^{-1}$. A suitable pH is necessary to maintain the three-dimensional conformation of the active site of the enzyme to assist the electrostatic binding between the substrate and enzyme during hydrolysis (Harun *et al.*, 2011). In this study, hydrolysis in strongly acidic or slightly alkaline conditions resulted in the decreased production of reducing sugars due to protein unfolding or denaturation under those conditions. According to Lee *et al.* (2015), the optimum pH for Pectinex Ultra SP-L, an enzyme cocktail consisting of α -amylase, cellobiases and xylanase was 5.5, which was comparable to this study.

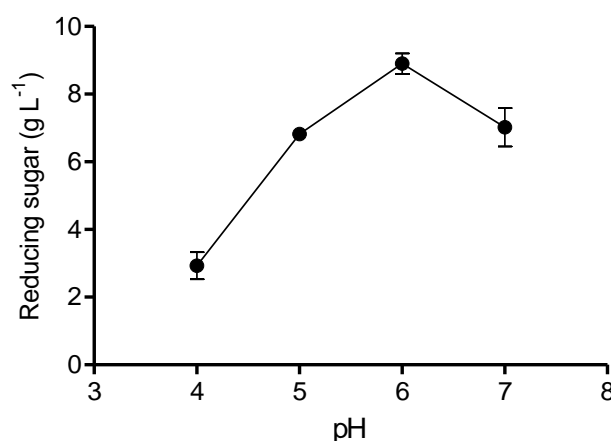


Fig. 7.7. The effect of pH on enzymatic hydrolysis of pre-treated *C. vulgaris* (Toti RS4) biomass. Results represent the average and \pm standard deviation of three replicates. If the error bars are not visible, they are smaller than the symbol.

7.4.4.3. Effect of enzyme concentration on enzymatic hydrolysis of the autoclave pre-treated biomass of *C. vulgaris*

The effect of enzyme concentration to substrate ratio in the range of 0.02 to 0.1 was investigated and the results are shown in Fig. 7.8. Results indicated that the rate of hydrolysis increased with an increase in enzyme concentration. The highest reducing sugar concentration of $16.9 \pm 0.4 \text{ g L}^{-1}$ was obtained when the hydrolysis was carried out with [E]/[S] of 0.08 and the lowest reducing sugar concentration of $7.2 \pm 0.3 \text{ g L}^{-1}$ was displayed when the hydrolysis was carried out with [E]/[S] of 0.02. Adding a small concentration of enzyme (i.e., [E]/[S] = 0.02), the substrate and product concentrations remains constant and

requires longer operation time scale for optimum release of reducing sugars. The long duration for optimum release of reducing sugars increases the chance of contamination and production of inhibitory effects. As shown in Fig. 7.8, there was an effect but no improvement on reducing sugar production due to the fact that high enzyme dosage results in high concentration of reducing sugars (glucose) in the medium, which can reversibly inhibit the enzyme action and slow down the overall hydrolysis process (Pancha *et al.*, 2016). Alternatively, high substrate concentration results in increased viscosity, which hinders the efficiency of the enzyme to hydrolyze substrate, and cause mass transfer limitation within the reaction mixture, leading to low reducing sugar yields. Therefore, for effective hydrolysis of pre-treated *C. vulgaris* (Toti RS4) biomass, enzyme to biomass loading of 0.08 was used for subsequent hydrolysis experiments.

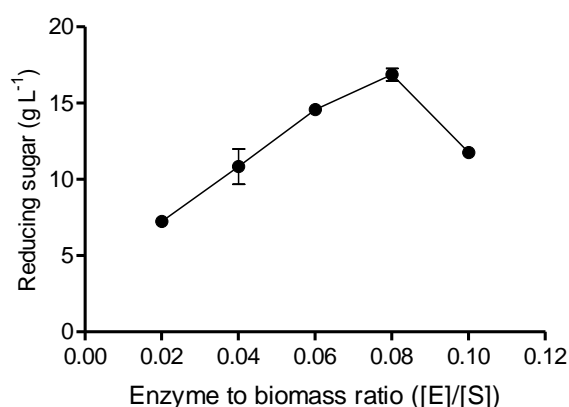


Fig. 7.8. The effect of the enzyme to biomass ratio ([E]/[S]) of pre-treated *C. vulgaris* (Toti RS4) biomass. The results represent the average value \pm standard deviation of three replicates.

7.4.5. Bioethanol production using enzymatic hydrolysates of the microalgal residual biomass via SHF and SSF process

The potential to produce bioethanol from enzymatic hydrolysis of pre-treated *C. vulgaris* (Toti RS4) was evaluated with SHF and SSF processes. The time course profile of residual sugar and bioethanol concentrations during the SHF process is shown in Fig. 7.9 (A). After 72 h of enzymatic hydrolysis at 25 °C, pH 6 and 0.08 of [E]/[S], the reducing sugar concentration was 16.9 ± 0.4 g L⁻¹. Shortly after *S. cerevisiae* inoculation, the reducing sugars of pre-treated *C. vulgaris* (Toti RS4) were consumed significantly, accompanied by a rapid increase in bioethanol concentration, reaching a maximum bioethanol concentration of 6.3 g L⁻¹ and ethanol yield of 0.21 g bioethanol/ g biomass.

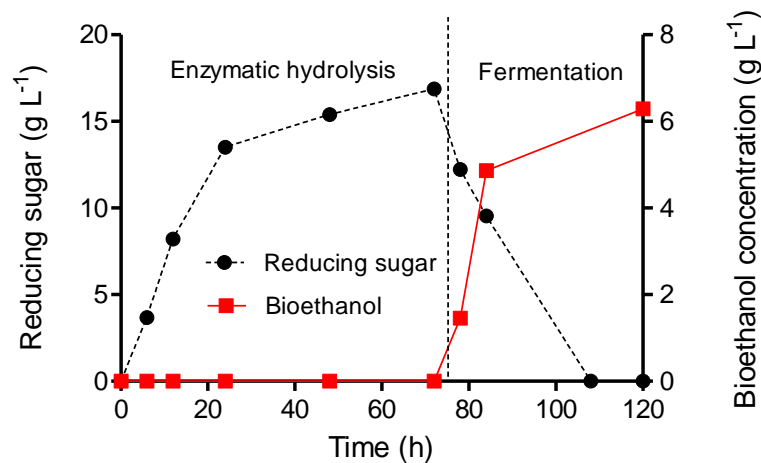


Fig. 7.9. Time-course profile of fermentation of the enzymatic hydrolysates of the residual biomass by *S. cerevisiae* via the SHF process. The vertical line indicates the switch from hydrolysis to fermentation.

In the control fermentation experiment using YPD medium, a similar trend was observed for reducing sugar assimilation and bioethanol production (Table 7.1). Therefore, it may be concluded that the enzymatically hydrolyzed biomass of *C. vulgaris* (Toti RS4) did not produce any significant inhibitory effects on yeast performance and contained adequate nutrients to support growth and ethanol production without requiring further supplementation. The bioethanol concentration observed in this study was higher than the value of 5.9 g L⁻¹ reported by Lee *et al.* (2015) from the enzymatic (amylase) hydrolysis of the lipid extracted biomass of *Chlorella* sp. KR-1.

Table 7.1 Comparison of the bioethanol concentration and yield of *C. vulgaris* (Toti RS4) using different hydrolysis methods with the performance given in YPD medium.

Substrate	Hydrolysis type	Initial algal biomass conc. (g L ⁻¹)	Maximum reducing sugar conc. (g L ⁻¹)	Maximum bioethanol conc. (g L ⁻¹)	Bioethanol yield (g bioethanol. (g algae) ⁻¹)
YPD medium	-	-	16.9 (glucose)	6.3	0.21
<i>C. vulgaris</i> (Toti RS4)	SHF	30	16.9	6.3	0.21
<i>C. vulgaris</i> (Toti RS4)	SSF	30	ND	7.9	0.26

Bioethanol production from *C. vulgaris* (Toti RS4) was also conducted via the SSF process and the results of this experiment are presented in Fig. 7.10. The highest bioethanol concentration obtained in the SSF process was 7.9 g L⁻¹ and the bioethanol yield was 0.26 g ethanol/ g biomass after 72 h of incubation and thereafter it remained constant, showing the reciprocal relationship similar to earlier reports (Lee *et al.*, 2015). Overall, these results indicate that the SSF process is a more superior approach for enzymatic hydrolysis based bioethanol production, since it not only shortens the reaction time required to reach maximum bioethanol production, but also achieves a higher bioethanol yield than the SHF process (Table 7.1).

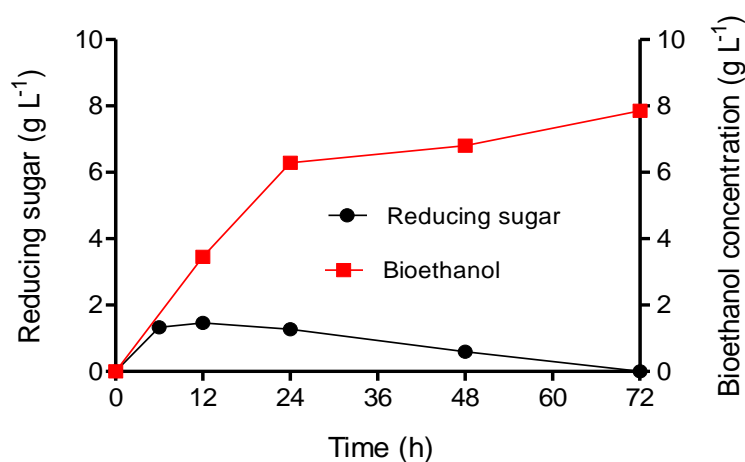


Fig. 7.10. Time-course profile of fermentation of the enzymatic hydrolysates of the residual biomass by *S. cerevisiae* via the SSF process.

7.5. Conclusion

This study demonstrated the feasibility of producing bioethanol from microalgal biomass. The combination of autoclave pre-treatment and enzymatic hydrolysis using α -amylase from *A. oryzae* successfully converted the total carbohydrate content in microalgal biomass into reducing sugar. Based on this study, enzymatic hydrolysis parameters such as temperature, pH and enzyme concentration demonstrated a significant effect on reducing sugar release from *C. vulgaris* (Toti RS4) biomass. The reducing sugars produced were effectively converted into bioethanol via the SHF and SSF processes. However, bioethanol production by SSF was found to be more effective, producing ethanol concentration of 7.9 g L⁻¹ with a yield of 0.26 g ethanol/ g microalgal biomass. The SSF process shows more potential for large-scale production of bioethanol using *C. vulgaris* (Toti RS4) as feedstock.

Chapter 8. General Discussion and Recommendations

In the quest for sustainable development, microalgal bioethanol as a renewable and sustainable energy source, has enjoyed a surge in popularity. The objectives of this study were to bioprospect for indigenous hyper-carbohydrate producing microalgal strains from various aquatic habitats in KwaZulu-Natal, develop an operational strategy optimal for biomass and carbohydrate production, and establish the feasibility of utilizing wastewater as a cheap nutrient source for microalgal biomass propagation for subsequent production of bioethanol via separate hydrolysis and fermentation as well as simultaneous hydrolysis and fermentation processes.

The first phase of this study was targeted at sample collection, isolation, identification and characterization of indigenous microalgal isolates for their potential as feedstock for bioethanol production. The two-stage cultivation strategy was used in which the microalgae were exposed to nutrient rich medium and subsequently transferred to nutrient-starved medium to trigger carbohydrate synthesis in order to select for strain with the highest carbohydrate producing capacity. After preliminary analysis of six indigenous microalgal strains, *C. vulgaris* (Toti RS4) was selected because its biomass could be enriched in carbohydrates over 50% on a dry biomass basis, which was the highest in this study. In addition, over 90% of its carbohydrates were attributed to glucose and it also had the highest theoretical ethanol potential (23.7% of the dry cell weight) which suggested that it had a better prospect for bioethanol generation.

The second phase involved optimization of conditions for the enhancement of biomass and carbohydrate production by *C. vulgaris* (Toti RS4). The experiments in this study were carried out in two consecutive stages. In the first stage, the Plackett-Burman screening design was used as a decision making tool to identify the most significant factors on the response. Six parameters namely, nitrogen, K_2HPO_4 , Na_2CO_3 , light intensity, temperature and cultivation time were found to be significant factors affecting biomass and carbohydrate production. Therefore, they were selected for further investigation in the Box-Behnken design. The optimum conditions obtained were as follows: urea, 0.203 g L^{-1} ; K_2HPO_4 , 0.026 g L^{-1} ; Na_2CO_3 , 0.012 g L^{-1} ; light intensity, $180.18\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$; temperature, $20\text{ }^\circ\text{C}$; cultivation time 14 days. Under these optimized conditions the biomass concentration of 488.4 mg L^{-1} and carbohydrate content of 79.54 % (w/w) of dry biomass basis was obtained. The optimum conditions were subsequently used for further experimentation.

In the third phase, the feasibility of growing *C. vulgaris* (Toti RS4) on the primary effluent of municipal wastewater (PEMW) for simultaneous wastewater treatment and energy production was evaluated. The characteristics of microalgae growth, photosynthetic

performance, nutrient removal efficiency, carbohydrate production and biomass recovery were examined. Two culture media, namely autoclaved and raw PEMW were compared in this study. The results showed that by the end of 14-day batch culture, *C. vulgaris* (Toti RS4) could remove 100% of total nitrogen and total phosphorus from raw PEMW and the carbohydrate content was 57.6% of dry cell weight. The biomass productivity reached a maximum of $108.5 \pm 6.3 \text{ mg L}^{-1} \text{ day}^{-1}$. Auto-flocculation resulted in 68% of biomass recovery from broth after 4 h.

In the fourth phase, response surface methodology coupled with the Box-Behnken design was used to study the combined effect of acid and thermal pre-treatment on reducing sugar production from the biomass of *C. vulgaris* (Toti RS4). The studied parameters were acid concentration, temperature and reaction time. The maximum reducing sugar concentration of 14.96 g L^{-1} was obtained under the following optimum conditions: HCl concentration of 0.5 % (w/v), the temperature of $120 \text{ }^\circ\text{C}$ and reaction time of 67.5 min. The maximum bioethanol concentration was 4.88 g L^{-1} and bioethanol yield of $0.16 \text{ g bioethanol/ g biomass}$.

Finally, hydrolysis of the microalgal biomass was performed via the enzymatic approach. Various pre-treatment strategies were evaluated including autoclaving, sonication and microwave treatment. The autoclave pre-treatment strategy was deemed to be the most effective pre-treatment method because it had the highest effect on the biomass structure and solubilisation of microalgal carbohydrates. Hydrolysis of the autoclave pre-treated microalgal biomass was performed using a commercial α -amylase enzyme (specific activity = 36 U mg^{-1}) isolated from *Aspergillus oryzae*, under the following optimised condition: [E/S] of 0.08, the temperature of $25 \text{ }^\circ\text{C}$ and pH of 6.5. The maximum reducing sugar concentration obtained was 16.9 g L^{-1} . The concentration of bioethanol produced via SHF was 6.3 g L^{-1} , and 7.9 g L^{-1} via the SSF process.

It can be concluded that this work has demonstrated that the cultivation of freshwater microalgae *C. vulgaris* (Toti RS4) in wastewater (particularly municipal wastewater primary effluent) for bioethanol production and pollutant removal is technologically feasible. Therefore, this work needs to be continued for further development of bioethanol from this biomass feedstock. The following suggestions could be of interest for future studies:

- 1) Validate microalgal biomass and carbohydrate productivity at large scale

Future work is needed in this research area to investigate the feasibility of scale-up and stability of continuous operation since the daily process rate of municipal wastewater is as high as one million gallons in wastewater treatment plants. Most of the studies to date have analysed microalgal growth and carbohydrate production in laboratory conditions and

following microalgae in batch culture flasks rather than in large culture systems such as raceway ponds.

2) Contamination control and species protection

Without sterilization of wastewater, *C. vulgaris* (Toti RS4) was able to grow, but the culture was contaminated by intrinsic bacteria and fungi. Sterilization of wastewater by autoclaving is energy intensive and not feasible at large scale. In this regard, it is prudent to search for easier and cheaper methods (e.g., disinfection by UV, NaClO etc.) for wastewater sterilization before the introduction of microalgae.

Although wastewater can be sterilized beforehand using UV or NaClO, there is no guarantee that during long-term operation, the system (especially the open system) will not be contaminated by other intrinsic microorganisms. Control of contamination will be demanding and thus costly, and this should therefore be a further focus for future research.

3) Biorefinery-based production system

In addition to carbohydrates, microalgae can accumulate notable amounts of proteins and lipids. Depending on the conversion process, a wide range of products can be obtained from microalgal biomass feedstock. Biorefining is an approach in which all the components of the biomass raw material can be used to produce valuable products. This system allows for the production of multiple product streams and thus multiple income streams from a single biomass feedstock and, therefore, more economically viable than single product-based production schemes. Microalgal based biorefinery systems can simultaneously produce bioethanol (from the carbohydrate fraction), biodiesel (from the lipid fraction) and animal feed (from the protein fraction). Development of new processes, the design of the system, and life assessments are necessary for the development and implementation of algae-based biorefineries at a commercial level.

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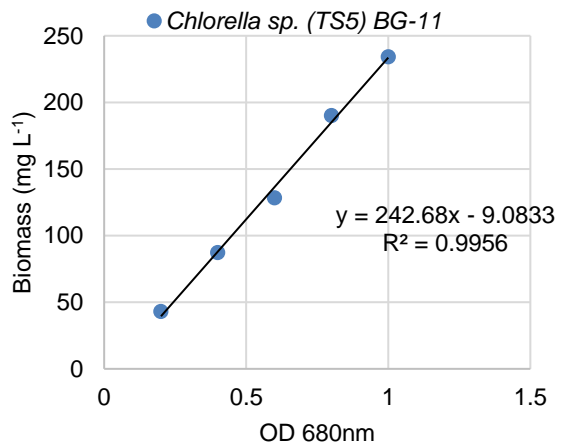
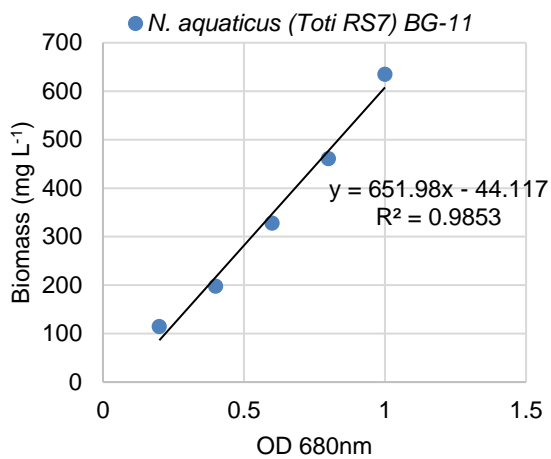
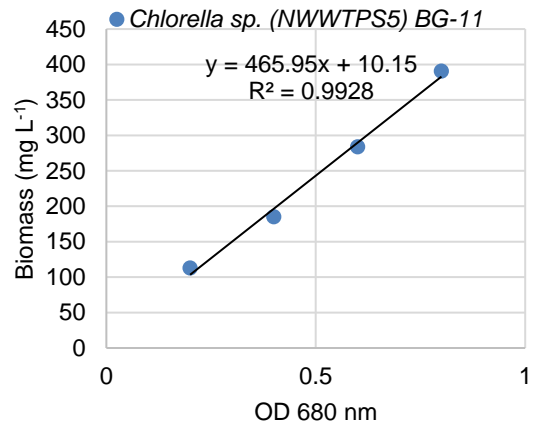
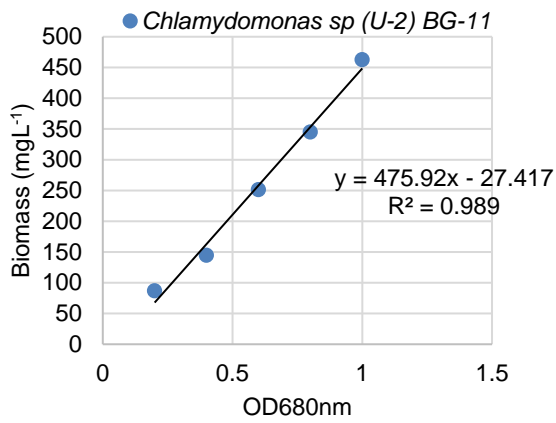
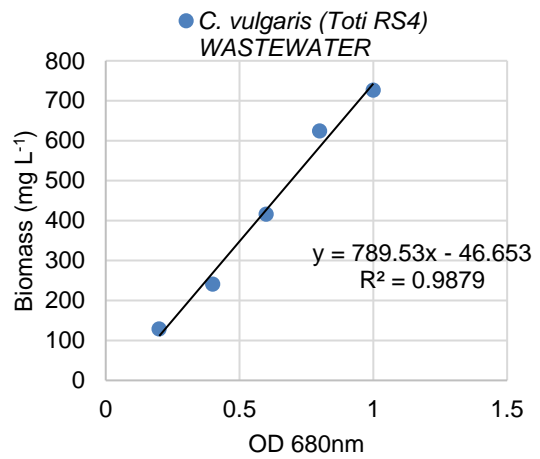
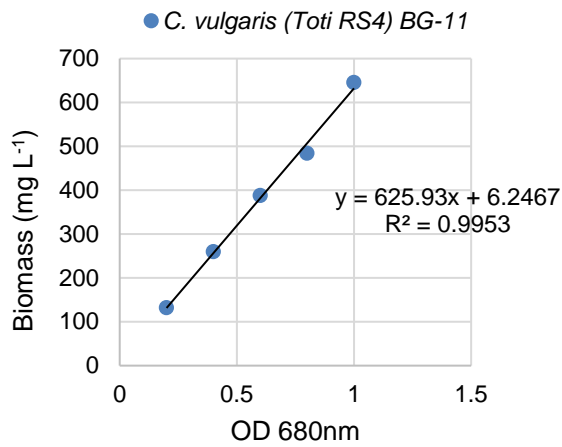
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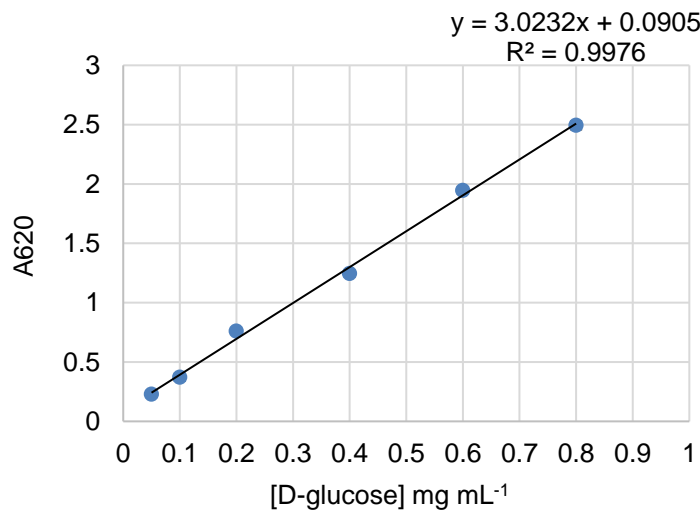
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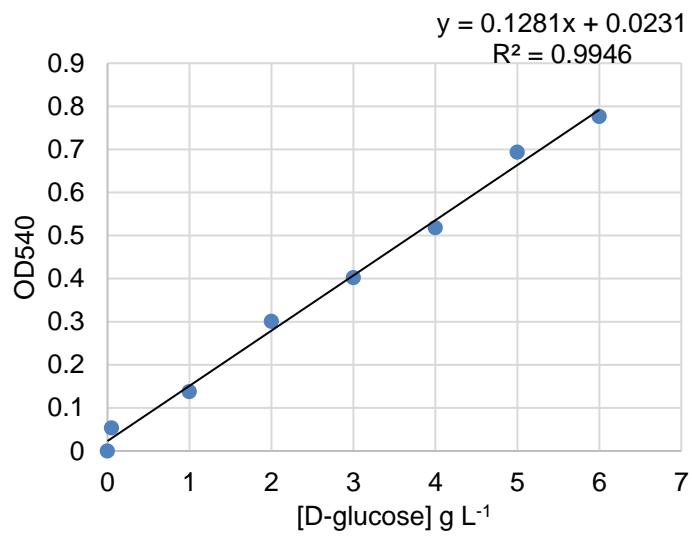
Appendix A: Standard curves: Biomass yield vs. OD680_{nm}



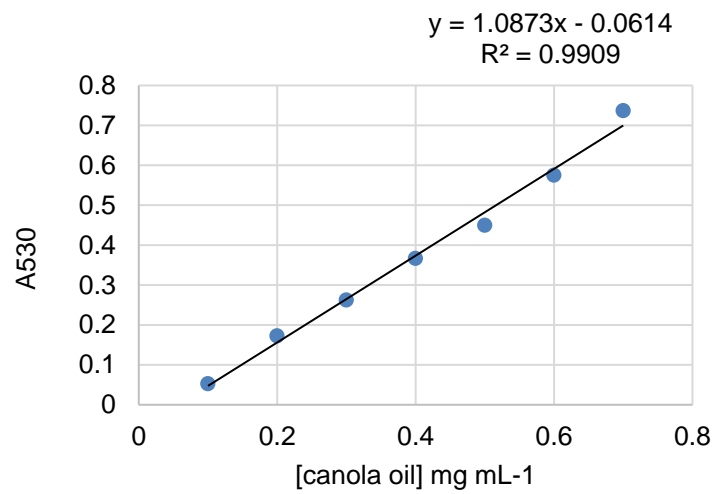
Appendix B: D-glucose standard using Anthrone reagent method



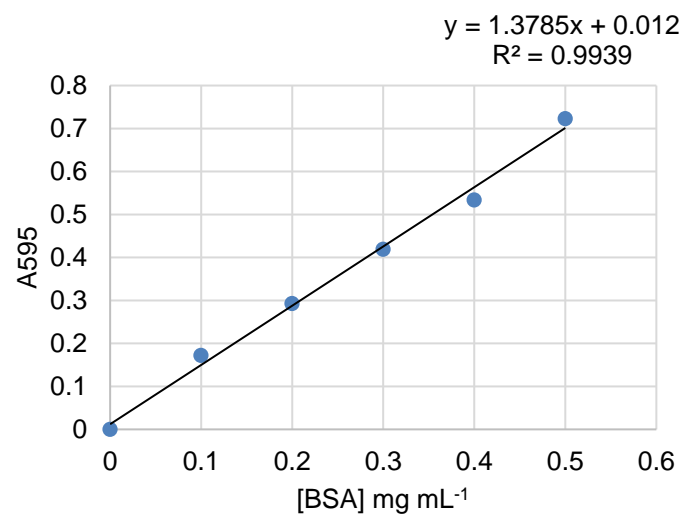
Appendix C: D-glucose standard using 3,5-DNS method



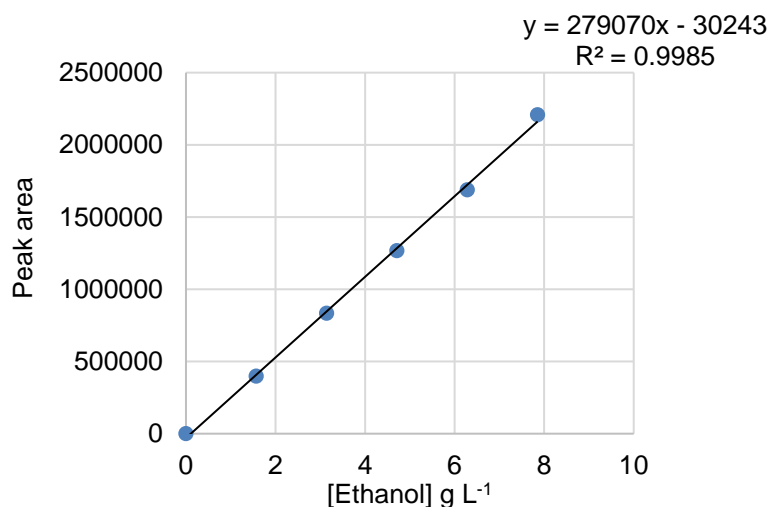
Appendix D: Canola oil standard using SPV method



Appendix E: Bovine serum albumin standard using the Bradford method



Appendix F: Ethanol standard



Appendix G: Determination of Total nitrogen

Principle

The organic and inorganic nitrogen compounds are converted into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor. In a solution acidified with H_2SO_4 and H_3PO_4 , this nitrate reacts with 2,6-dimethylphenol (DMP) to form 4-nitro-2,6-dimethylphenol that is determined photometrically.

Procedure

Water samples were drawn and filtered using a Whatman filter paper with the pore size of $0.45 \mu\text{m}$. One milliliter of the filtrate was added to 9 ml of distilled water and was mix until a homogenous solution was obtained. One milliliter of reagent N-1K was added, followed by 6 drops of reagent N-2K. The solution was mixed and heated at $120 \text{ }^\circ\text{C}$ for 1 h. After the incubation period was elapsed the samples were vigorously mixed and left to cool at room temperature. One milliliter of this sample was drawn and pipetted into a reaction cell and 1 mL of reagent N-3K was added. The sample was left for 10 min at room temperature and was then measured for total nitrogen concentration using Spectroquant Nova 60.

Appendix H: Determination of Total phosphorus

Principle

In H_2SO_4 solution orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Asorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically.

Procedure

Water samples were drawn and filtered using a Whatman filter paper with the pore size of $0.45\ \mu\text{m}$. The filtrate (0.20 mL) was pipetted into a reaction cell and 1 dose of reagent P-1K was added and mixed. The sample was heated at $120\ ^\circ\text{C}$ for 30 min and allowed to cool at room temperature. Five drops of reagent P-2K were added and mixed by vortexing. One dose of reagent P-3K was then added and mixed by vortexing. The reaction was left for 5 min at room temperature and read using Spectroquant nova 60.

Appendix I: The Box-Behnken design with experimental and predicted values of biomass production and carbohydrate content.

Run	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	Biomass production (mg L ⁻¹)		Carbohydrate content (% dcw)	
							Experimental value	RSM prediction	Experimental value	RSM prediction
1	-1	-1	0	1	0	0	191.6	195.1	61.85	62.41
2	0	0	1	1	0	-1	628.9	627.3	45.85	44.40
3	0	1	0	0	1	1	698.2	694.5	50.86	50.87
4	1	1	0	1	0	0	862.0	859.9	41.33	38.24
5	0	-1	-1	0	1	0	388.4	386.3	63.94	63.61
6	0	0	0	0	0	0	522.6	524.1	34.47	35.82
7	0	1	-1	0	1	0	624.9	623.5	78.09	79.96
8	0	0	-1	1	0	1	513.6	521.8	70.58	68.93
9	0	-1	1	0	-1	0	493.5	494.6	59.85	68.71
10	0	1	0	0	-1	-1	621.6	620.5	67.32	66.25
11	0	-1	0	0	-1	1	414.5	412.3	54.99	53.80
12	-1	-1	0	-1	0	0	102.1	101.4	32.8	29.56
13	1	-1	0	-1	0	0	674.3	676.7	52.07	55.71
14	0	-1	-1	0	-1	0	291.7	291.2	29.4	27.29
15	0	-1	0	0	1	-1	443.4	445.1	49.73	50.36
16	0	0	0	0	0	0	521.8	524.1	37.42	35.82
17	0	-1	0	0	1	1	492.3	490.4	69.72	70.72
18	1	1	0	-1	0	0	828.0	829.9	66.26	68.73
19	-1	0	1	0	0	-1	377.8	378.2	58.13	59.82
20	-1	0	-1	0	0	1	235.5	232.7	49.42	48.11
21	1	0	0	1	1	0	816.8	814.6	34.98	37.55
22	0	0	-1	-1	0	-1	412.5	414.1	55.65	50.58
23	0	0	1	-1	0	1	606.9	608.7	56.64	54.92
24	1	0	0	-1	-1	0	715.2	716.6	56.38	56.97
25	0	0	0	0	0	0	521.4	524.1	38.74	35.82
26	0	0	0	0	0	0	525.6	524.1	36.14	35.82
27	1	0	1	0	0	-1	844.3	840.0	56.27	52.20
28	1	-1	0	1	0	0	744.0	744.7	60.25	65.88
29	0	0	-1	1	0	-1	475.0	474.9	54.67	51.40
30	1	0	1	0	0	1	863.4	863.9	48.86	51.44
31	0	0	0	0	0	0	527.3	524.1	32.77	35.82
32	1	0	0	1	-1	0	778.0	781.2	58.14	58.79
33	-1	0	0	-1	-1	0	217.1	215.0	21.48	25.59
34	0	1	1	0	1	0	752.9	753.8	43.74	45.57
35	-1	1	0	1	0	0	492.1	496.6	64.58	60.86
36	-1	0	0	-1	1	0	321.6	318.4	62.26	64.88
37	0	1	-1	0	-1	0	541.1	544.9	59.68	57.94
38	0	0	1	-1	0	-1	595.6	598.0	70.63	70.08
39	-1	0	0	1	1	0	379.3	377.5	64.13	65.41
40	1	0	-1	0	0	1	708.4	709.5	56.17	59.25
41	1	0	-1	0	0	-1	680.7	683.0	55.69	54.55
42	0	-1	1	0	1	0	552.2	552.8	62.51	64.74
43	0	0	0	0	0	0	526	524.1	36.77	35.82
44	0	0	-1	-1	0	1	429.6	427.4	40.97	40.89
45	0	0	1	1	0	1	671	671.6	57.05	56.45
46	-1	0	0	1	-1	0	301.7	305.4	49.6	50.08
47	0	1	0	0	1	-1	684.6	679.2	66.28	67.38
48	-1	1	0	-1	0	0	440.8	440.9	64.70	68.67
49	0	-1	0	0	-1	-1	370	369.9	37.34	34.93
50	0	1	0	0	-1	1	631.9	632.0	47.21	48.25
51	-1	0	1	0	0	1	409.3	409.3	53.17	57.48
52	-1	0	-1	0	0	-1	197.7	198.9	43.7	44.98
53	0	1	1	0	-1	0	711.1	712.2	61.31	63.84
54	1	0	0	-1	1	0	777.6	781.2	59.94	59.69



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 $2.89 \pm 0.05 \text{ day}^{-1}$, respectively. *Chlorella vulgaris* T4 had the highest biomass productivity of $35.71 \pm 0.03 \text{ mg L}^{-1} \text{ day}^{-1}$. *Chlorella vulgaris* PH2 had the highest lipid content of 34.28 ± 0.47 and $38 \pm 9.2\%$ (dew) 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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RESPONSE SURFACE METHODOLOGY FOR THE OPTIMIZATION OF BIOMASS AND CARBOHYDRATE PRODUCTION BY AN INDIGENOUS MICROALGAL STRAIN CULTIVATED IN MODIFIED BG-11 MEDIUM



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1.0 INTRODUCTION

The rigorous search for renewable and sustainable biofuels for the future is rapidly expanding and currently gaining global attention due to the imminent depletion of petroleum-based fuels. Microalgae are the current promising renewable bioresource used as a substrate for the commercial production of biofuels. However, the development of microalgae biofuels faces a great challenge since production costs of microalgae as biofuel feedstock is higher compared to fossil fuels. Numerous attempts have been made to enhance the economic feasibility of microalgal biofuel production such as: improving strain performance, development of culture systems, and harvesting/ extraction technologies.

In recent years, there were increasing reports demonstrating the potential of *Chlorella* for biofuel production. Moreover, *Chlorella* has been accepted for nutraceutical and for wastewater treatment. In addition, *Chlorella* species have high flexibility to adapt to diverse culture conditions and are very promising for the commercial applications. *Chlorella* cells have also been shown to accumulate abundant carbohydrates mainly as starch under various conditions.

In this scenario, this study was, therefore, conducted to improve carbohydrate and biomass production from an indigenous freshwater isolate *C. vulgaris* Td1 R84 by combined optimization of the cultivation conditions using response surface methodology (RSM).

3.0. RESULTS AND DISCUSSION

3.1. Effect of factors identified using the Plackett-Burman screening design

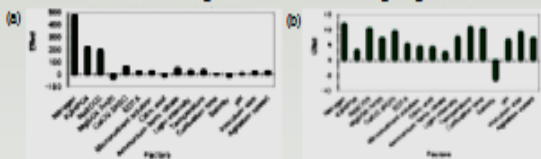


Fig. 3. The effect of factors on biomass accumulation (a), the effect of factors on carbohydrate content and profile (b). The experiments were performed in triplicate in the Plackett-Burman design.

3.2. Identification of suitable nitrogen source based on biomass and carbohydrate yield

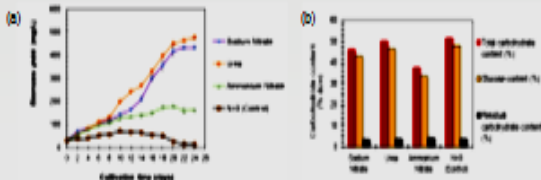


Fig. 3. The effect of nitrogen source on biomass accumulation (a), the effect of nitrogen source on carbohydrate content and profile (b). All the experiments were performed in triplicate.

3.3. Optimization and validation of model generated using RSM

Table 2. Brief description of the ANOVA table for the proposed models

Model	Biomass yield (mg/L)	Carbohydrate content (% dw)
Regression coefficient (R ²)	0.999	0.9833
F-value	19236.22	56.79
Prob >F	<0.0001	<0.0001
Lack of fit	F= 0.48; P= 0.8898	F= 0.90; P= 0.6165

2.0 MATERIALS AND METHODS

2.1. Identification of significant factors with Plackett-Burman design

The Plackett-Burman screening design was used to identify and select the most significant factors influencing biomass and carbohydrate production by *C. vulgaris* Td1 R84. The chosen factors were Nitrogen, KH₂PO₄, Na₂CO₃, MgSO₄·7H₂O, CaCl₂·2H₂O, EDTA, citric acid, ferric ammonium citrate, micronutrient solution, light intensity, temperature, culture time, salinity, pH, inoculum size and agitation. All factors were investigated at two levels; -1 for a low level and +1 for a high level, according to the Plackett-Burman design matrix.

2.2. Selection of nitrogen source

The microalgae was cultivated in BG-11 medium containing either NaNO₃ (1.5 g/L), NH₄NO₃ (1.5 g/L) or urea (1.5g/L) serving as nitrogen source. BG-11 with N=0g/L was used as a control. All the experiments were performed in batch culture at 25 ± 2 °C with 12h: 12h light:dark period under a light