Process development for biomass and lipid production from a local *Chlorella* isolate using a miniature parallel raceway pond reactor

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by

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Preface

The research contained in this dissertation was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences, of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was financially supported by the National Research Foundation.

The contents of this work have not been submitted in any form to another university and, except where the work of others was acknowledged in the text, the results reported are due to investigations by the candidate.

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Declaration 1: Plagiarism

I, Zanenhlanhla Gugulethu Gumbi, declare that:

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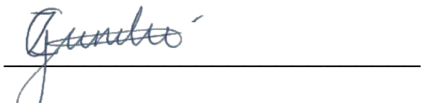

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Declaration 2: Publications

This thesis represents a compilation of manuscripts where each chapter was an individual entity prepared as per the journals' specifications therefore some repetition between chapters has been unavoidable. The first author (student) conducted all experimental work, data collection and manuscript preparation, under the guidance of the second and/or third (Supervisor) author.

1. Muthunarayanan, V., Chandran, T., Durawasamy, T., Muniraj, S., Sewsynker-Sukai, Y., Moodley, P., & Gumbi, Z. (2018). Biologically Renewable Resources of Energy: Potentials, Progress and Barriers. In *Microbial Fuel Cell Technology for Bioelectricity* (pp. 1-22). Springer, Cham. (Chapter 2)

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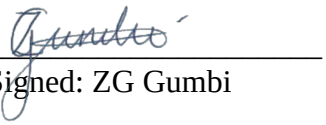
Conference proceedings and contributions

1. Gumbi, Z.G. and Gueguim Kana. Optimisation of biomass and lipid production from a local *Chlorella* isolate using response surface methodology and artificial neural network. 13th Biotechnology Congress, San Francisco, USA. 28 November 2016, Oral Presentation
2. Gumbi, Z.G. and Gueguim Kana. Biomass and lipid production from local *Chlorella* isolate: Process optimisation and kinetics. Annual College of Agriculture, Engineering and Science Research & Innovation Day. 26 October 2017, Poster Presentation



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Abstract

The consumption of fossil fuels such as coal and oil are unsustainable and has proved to be detrimental to the environment. There was a need to explore alternative sources of energy. Microalgal biomass and lipids have the potential to produce a variety of high value products; such as biofuels, bioactive medicinal products and food additives and these microorganisms have attracted worldwide interest. Microalgal biodiesel was an excellent substitute to liquid fossil fuels due to its cost effectiveness, renewability and environmental benefits. Although biodiesel production from microalgal feedstock was a feasible source of bioenergy, its commercialization was still curbed by a number of techno-economical challenges.

Bioprocess modelling and optimization at upstream and downstream stages was necessary to enhance product yields and improve viability of the technoeconomic output. In this study, the Response Surface Methodology (RSM) was used to model and optimize their biomass and lipid productivity of *Chlorella* sp. based on the input parameters; nitrogen concentration, iron concentration and phosphorus concentration within the ranges of 0.50 – 2.00 g L^{-1}, 3.00 – 9.00 mg L^{-1}, and 0.00 - 40.00 mg L^{-1}, respectively. The experiments were carried out in a novel miniature raceway pond photobioreactor. This reactor comprised of 15 units of 1.5L raceway ponds enclosed in a transparent plexiglass case with dimensions of 820 × 970 × 190 mm. This structure allowed for parallelization raceway pond experiments.

Experimental data were used to develop polynomial models. Analysis of variance (ANOVA) carried out on the developed models gave coefficient of determination values of 0.99 and 0.98 for biomass and lipid productivity, respectively. Nitrogen was found to be the most important input factor to biomass productivity whereas iron was most influential to lipid productivity. Optimized process yielded biomass and lipid productivities of 114.5 mg L^{-1} d^{-1} and 38.23 mg L^{-1} d^{-1}, respectively. The use of a parallel miniature raceway pond photobioreactor enabled high throughput experimentation for microalgae process development with the geometrical configuration of large-scale raceway ponds

The kinetic studies of *Chlorella* sp growth showed a maximum specific growth rate (μ_{max}) of 0.01 g L^{-1} h^{-1} and a cell concentration (X_{max}) of 1.78 g L^{-1}. The logistic model fitted well to the experimental data (R^2 value of 0.98). These kinetics data provide insights into *Chlorella* bioprocess scale up as well as the biological characteristics of a microorganism involved in a bioprocess. Knowledge of such characteristics will inevitably enhance the feasibility of a bioprocess.

An additional challenge in microalgae bioprocessing was the harvesting of microalgae biomass. In this study, the potential of using magnetic iron oxides for harvesting *Chlorella* sp. was investigated. The response surface methodology was used to optimize the recovery efficiency of crude (uncoated), tri-sodium citrate (TSC) and chitosan coated magnetic iron oxides. The operational parameters consisted of nanoparticles to microalgae culture exposure time, magnet retention time, pH and nanoparticles concentration. Experimental data were used to fit polynomial models using RSM. Analysis of variance gave coefficients of determination (R^2) values of >0.7 for crude, TSC and chitosan nanoparticles. Findings showed that tri-sodium citrate coated magnetic nanoparticles had the highest recovery efficiency of 95% compared to crude and chitosan (efficiencies of 85% and 87%, respectively). Additionally, the exposure time of the algae culture to the nanoparticle's solution was found to be a significant factor for the recovery efficiency of crude nanoparticles, whereas magnet retention time had a higher positive influence on the recovery efficiency of TSC nanoparticles. The concentration of nanoparticles positively affected the recovery efficiency of chitosan coated nanoparticles. This was evident from the polynomial model equations illustrating individual and interactive effects of the input parameters on the output.

Sensitivity studies on the recovery efficiency as a function of changes on process inputs revealed that for crude nanoparticles, the concentration of nanoparticles has a non-linear relationship with the recovery efficiency. Magnet retention time displayed a linear relationship for all nanoparticles types where an increase in this factor resulted in a proportional increase in the recovery efficiency. Increasing the exposure time of the algae culture to nanoparticles as well as pH increased recovery efficiency of chitosan coated nanoparticles whereas the opposite effect was observed for crude and TSC nanoparticles.

These findings demonstrated that *Chlorella* sp. was an attractive biodiesel feedstock. Modelling of biomass and lipid productivity revealed that optimal productivities of both biomass and lipid could be obtained using an appropriate mixture ratio of nitrogen and iron. The kinetic model provided crucial information on the growth of *Chlorella* sp for bioprocess development and scale up. The use of a novel miniature raceway pond photobioreactor provided a throughput experimentation using a geometrically similar environment for large scale microalgae production using raceway reactor. This ensured that reliable process data were generated for subsequent scale up. For downstream processing, tri-sodium citrate coated nanoparticles displaying the highest recovery efficiency. Sensitivity studies revealed shorter exposure time to algal culture and a lower pH resulted in a higher recovery efficiency from TSC coated nanoparticles. Findings from this study provided insight for upstream and downstream microalgae process development using the local isolate of *Chlorella sp*.

Keywords: *Chlorella* sp, bioprocess development, response surface methodology, kinetic models, artificial neural networks, miniature parallel photobioreactors

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Table of contents

Page

List of figures

Chapter 4

.

List of tables

Section **Page**

List of abbreviations

ANN - Artificial Neural Network

ANOVA - Analysis of Variance

ASTM – American Society for Testing and Materials

DoE - Department of Energy

EN - European Union

FA - Fatty Acid

FAMES - Fatty Acid Methyl Esters

GHG- Green House Gases

MION - Magnetic Iron Oxide Nanoparticles

NPs – Nanoparticles

PBR - Photobioreactor

PSI - Photosystem I

PSII - Photosystem II

PUFA - Poly-unsaturated Fatty Acid

ROS - Reactive Oxygen Species

RSM - Response Surface Methodology

SANS - South AfricouldNational Standards

SFE - Supercritical Fluid Extraction

TAG - Triacylglycerol

TSC - Tri-sodium phosphate

Chapter 1: General Introduction

1.1 Fossil fuel depletion and alternative energy source

Rapid increases in the human population and technological advancements have led to energy demands escalating to levels that cannot be sustained by fossil fuels alone. According to BP's 2019 World Energy Review, South Africa used 533 thousand barrels per day of fossil fuels, an increase from the 2008 data which were 511 thousand barrels per day (BP Statistical Review of World Energy 68th Ed; 2019) Rapidly depleting fossil fuel reserves and the release of greenhouse gases (GHG) from their combustion resulting in climate change, receding glaciers, increasing sea levels and biodiversity loss, necessitate the need for renewable, sustainable, efficient and cost-effective energy sources with less emissions (Gullison *et al.*, 2007).

Amongst the four most important sustainable fuel sources (biofuels, hydrogen, natural gas and syngas), biofuels have emerged as the most environmentally friendly energy source and have therefore become widely explored as a fossil fuel replacement due to their renewability, biodegradability and acceptable emissions (Bhatti *et al.,* 2008). A biofuel describes a liquid, solid and gas fuel derived from biomass such as bioethanol, biomethanol and biodiesel (Dermibas, 2008). In the year 2015, renewable energy fulfilled 19.3% of the global energy consumption and 0.8% of these renewable fuels was used for transportation, thus indicating the acceptance of renewable energy as a fossil fuel replacement. In 2016, government policies were put in place in 176 countries to increase renewable energy production (Renewables, 2017). In South Africa, the mandatory blending of biofuels are 5 and 2% for biodiesel and bioethanol, respectively (DoE., 2015). One of the major bioethanol producing countries, Brazil has increased biodiesel and bioethanol blend minimums from 7 to 8% and 25 to 27% , respectively (Biofuels Digest, 2016). The increasing worldwide interest in renewable biofuels requires the development viable bioprocess technologies to meet global targets.

1.2 Biodiesel production

Biodiesel was a diesel-fuel alternative produced by chemically reacting vegetable or animal fats with an alcohol such as methanol in a process known as transesterification (Figure 1.) A

strong acid or base catalyst was used to drive the reaction and the end product was methyl esters, which are biodiesel (van Gerpen, 2005).

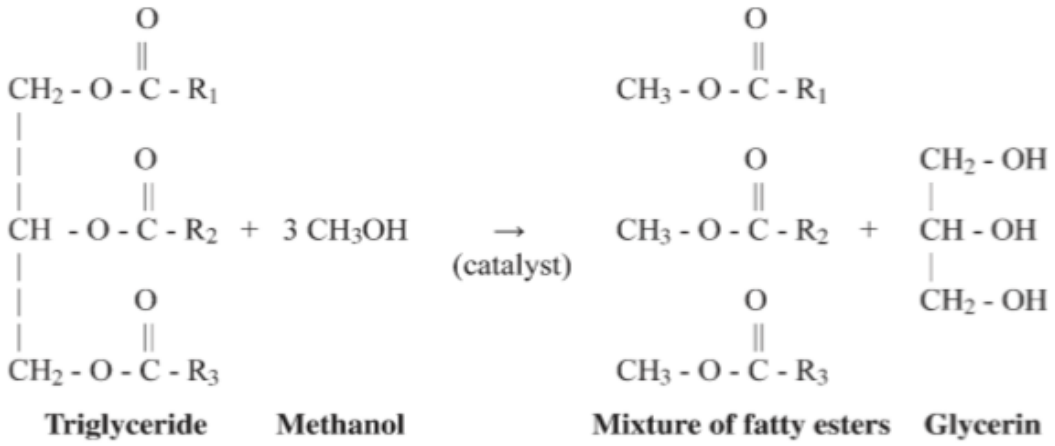

Figure 4: Transesterification mechanism (adapted from van Gerpen, 2005)

Biodiesel was an attractive energy resource due to its sustainable nature, high biodegradability, minimal toxicity, economic potential, closed carbon cycle, low emission profile and ability to be used in diesel engines with minimal or no modification (Ahmad *et al.,* 2011). Biodiesel can be produced from a variety of feedstock's (Table 1).

Table 1. Current feedstock for biodiesel worldwide (Adapted from Ahmad *et al.,* 2011)

Country/region	**Feedstock**
USA	Soybeans
Europe/EU	Rapeseed, sunflower
Western Canada	Canola oil
Africa	Jatropha
India	Jatropha
Malaysia/Indonesia	Palm
Philippines	Coconut
China	Waste cooking oil
Spain	Linseed oil
Greece	Cottonseed

First generation feedstock's are classified as edible oils such as rapeseed, soybeans, palm oil and sunflower oil. Such oils produce biodiesel but unfortunately succumb to global food security issues (Brennan and Owende, 2010). Most first generation plants form part of the human diet in various regions across the world, therefore their large scale use as biodiesel feedstock would inadvertently result in global food market imbalances (Gui *et al.,* 2008). The requirement of arable land and freshwater resources was another socio-economic challenge diminishing the use of these plants as a feedstock (Ahmad *et al.*, 2011).

Second generation feedstock's aim to reduce the dependency on edible oils by using energy crops such as, jatropha (Foidl *et al.*, 1996), jojoba (Canoira *et al.*, 2006), tobacco (Usta, 2005), waste cooking oils, restaurant grease and animal fats (Canacki, 2007). Second generation feedstock's eliminates competition for food and feed (Leung *et al.,* 2010), less farmland required for cultivation (Leung *et al.,* 2010), higher cetane number and non-corrosive qualities associated with animal fat methyl esters (Guru *et al.,* 2009). However the non-abundancy of these feedstock's challenges their sustainability and biodiesel derived from vegetable and animal oil perform poorly in colder temperatures (Singh and Singh, 2010; Janaun and Ellis, 2010).

Third generation feedstock's attempt to address the challenges associated with the first and second generation feedstock's.

1.3 Microalgae as biodiesel feedstock: major challenges

Microalgae are unicellular, photosynthetic, lipid producing microorganisms. They are considered as the third generation feedstock's. Interest has arisen in the use of microalgae as biodiesel feedstock's due to a number of advantages that these microorganisms present over first and second generation biodiesel feedstock's. Microalgae have higher biomass productivities than land plants, higher lipid accumulating capabilities (up to 20 -50% w/w_{dw}), their cultivation does not require arable land or fresh water resources and the resultant biomass after oil extraction can be used to produce other high value products such as bioethanol and pharmaceuticals (Mata *et al.*, 2010).

The production of biodiesel using microalgae as feedstock consists of 5 steps, namely strain selection, cultivation, harvesting, oil extraction and biodiesel production as seen in figure 2.

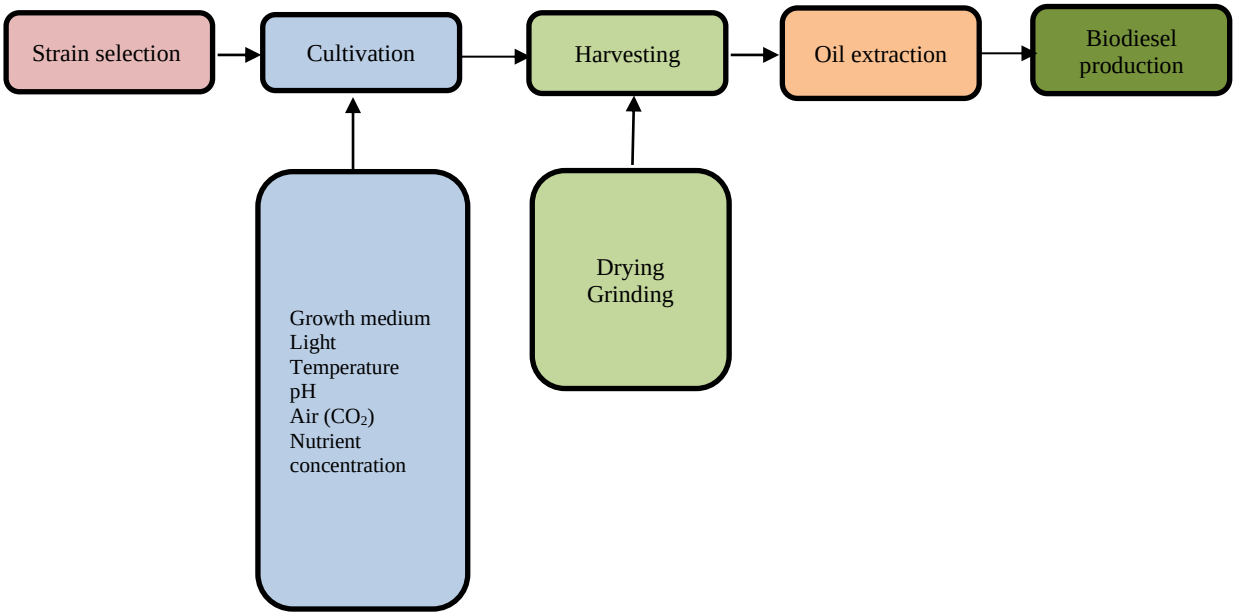

Figure 5: Biodiesel production process from microalgae

Fig. 2 shows a schematic of the production of biodiesel from microalgae. The selection of an appropriate species with a high lipid content and lipid quality suitable for transesterification to biodiesel was the first step. The cultivation conditions such as growth medium composition, cultivation temperature, pH, CO_2 and nutrient concentration have an impact on biomass and lipid productivity of microalgae. Following the cultivation step was harvesting.

Downstream operations focus on the separation of the biomass from the spent media. Commonly used methods are sedimentation, microscreens, centrifugation, flocculation or membrane filtration (Ahmad, 2011). The harvested biomass was then dried to release water and pulverized prior to oil extraction. Oil expeller/press, solvent extraction and supercritical fluid extraction are the most common methods used for oil extraction (Dermibas, 2009). The final stage was the transesterification process where oils are converted into biodiesel.

Screening of microalgae species for high oil producing species was a crucial step in the biodiesel production process. Selection of an appropriate species can result in a cell that was capable of producing up to 50% oil by weight (Ahmad *et al.*, 2011). *Nannochloropsis* sp. and *Chlorella* sp. have been reported to produce high lipid content (Widjaja *et al.*, 2009; Travieso *et al.*, 2006, Scragg *et al.*, 2003).

The cultivation conditions have an impact on biomass and lipid productivities. Converti et al. (2009) reported that nitrogen concentration and temperature influence the lipid content of *Chlorella vulgaris* and *Nannochloropsis oculata*. Yeesang and Cheirsilp (2010) increased lipid content of *Botryococcus* spp. by nitrogen deficiency, high light intensity (82.5 µE m^{-2} s^{-1}) and high iron levels (0.74mM). Another aspect of cultivation conditions affecting biomass and lipid productivities was cultivation reactors. These can be categorized into open ponds or closed photobioreactors. Open ponds have been the most commonly used large scale vessels since the 1950s. These can be described as shallow ponds with a paddle wheel to provide circulation of medium and nutrients. Open ponds are inexpensive to build and operate but present challenges in the form of contamination, poor mixing, dark zones and inefficient use of CO_2 (Chisti, 2007; Mata *et al.*, 2010). Tubular photobioreactors are currently the only types of closed photobioreactor systems used at large scale (Chisti, 2007). Such reactors allow for improved pH and temperature control, complete protection against contamination, improved mixing, less evaporative losses and higher cell densities (Mata *et al.*, 2010). Microalgal cultivation systems can have an impact on the biological characteristics of the microalgal cell therefore, ultimately affecting cell growth and product formation.

Separation of microalgae from growth medium (harvesting) remains a major hurdle at industrial scale due to small algal cell size (3-30µm) and the dilute nature of algal cultures (Grima *et al.*, 2003; Uduman *et al.*, 2010). Most microalgae processing industrial scale plants achieve harvesting by the use of chemical coagulation, followed by sedimentation or dissolved air flotation (Friedman *et al.*, 1977). This form of harvesting creates resultant chemical waste sludge which requires treatment before disposal, thereby increasing production costs (Hoffman, 1998). Centrifugation was a rapid and reliable method for harvesting microalgae but remains the most expensive method to carry out at large scale due to high energy requirements (Christenson and Sims, 2011). Lowering the costs of harvesting microalgae was a significant challenge hindering the commercialisation of biodiesel production from microalgae.

Microalgal feedstock cultivation was a multi-step process requiring the in-depth development and maximisation of each individual step to create a highly efficient and cost effective bioprocess. Bioprocess development was the sequential design of a process initiated at a laboratory scale and progressively scaled up to larger volumes, ultimately reaching production scale level (Clarke., 2013). Figure 3 shows the bioprocess development steps starting from shaken microtiter plates, successively increasing the scale until production scale bioreactor was reached.

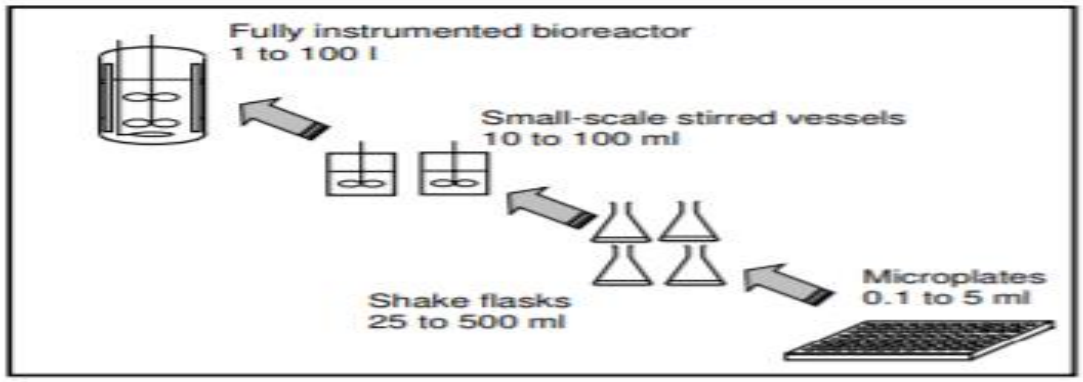

Figure 6. Schematic diagram of biopocess development (adapted from Betts and Baganz, 2006)

Parameters such as oxygen transfer, mixing and shear stress differ with changing scales, resulting in an alteration of microbial metabolism and ultimately compromising kinetic parameters such as yields and productivities (Clarke, 2013). It was essential that bioprocess development reactors used during scale up procedures allow for the optimum physiological parameters to be kept the same. Geometric similarity between small and large scales has been explored as a method to ensure symmetric scale up. Keeping important criteria constant between scales results in accurate and optimum bioprocess development (Betts and Baganz, 2006). Criteria such as oxygen mass transfer, mixing, shear stress and calculated power input are the most commonly kept constant between scales (Vallejos *et al.*, 2006, Clarke, 2013).

1.4 Bioprocess modelling and optimization

Optimisation of biomass and lipid productivity of microalgae has been achieved using modelling tools such as Response Surface Methodology (RSM) and Artificial Neural Network (ANN) (Wang and Lan, 2011; Mohamed *et al.*, 2013; Singh *et al.*, 2015). RSM was

a modelling tool that evaluates factors and their interactive effects on process yields as well as select optimum conditions for a desirable response (Rorke and Kana, 2016; Haland, 1989). ANNs can be described as a mathematical understanding of the neurological functioning of the human brain (Sewsynker-Sukair *et al.*, 2016) and they detect patterns and relationships within data (Agatonovic-Kustrin and Beresford, 2000). Mathematical modelling allows for the generation of quantitative knowledge that can be used to describe and predict the behaviour of bioprocesses under the influence of control variables (Omar *et al.*, 2006). Kinetic modelling allows for increased product yield, reduced by-product formation and high product quality (Almquist *et al.*, 2014). Monod models describe biomass growth in terms of the limiting substrate and modified Gompertz models describes production lag times, maximum product concentration and maximum production rate on a given substrate (Imamoglu and Sukan, 2013; Dodic *et al.*, 2012; Putra *et al.*, 2015). Bioprocess modelling allows for virtual experimentation as well as increasing the efficiency and success of process design, control and optimisation, thereby reducing scale up challenges (Linville *et al.*, 2013).

1.5 Problem statement and justification of study

The diminishing fossil fuels reserves and their negative environmental impacts make this energy source unsustainable for long term energy supply (Shafiee and Topal, 2009). Microalgal biodiesel was being explored as an alternative due to its sustainable nature, biodegradability, minimal toxicity, closed carbon cycle, low emission profile, ability to be used in diesel engines with minimal or no modification, and economic potential (Ahmad *et al.*, 2011).
However, the biodiesel production process was subject to a few challenges such as a low biomass and lipid productivities, inaccurate scale up methods and high harvesting costs. These combined challenges hinder the feasibility of microalgal biodiesel as a commercial fuel.

Challenges associated with microalgae process development could be addressed through efficient process modelling and optimization. Optimisation of biomass and lipid productivities can be carried out through the determination of optimal physico-chemical cultivation parameters. The Response Surface Methodology has been employed as a modelling tool to effectively optimise biomass and lipid productivities. Similarly, challenges associated with microalgae harvesting could be optimized using Response Surface Methodology. Kinetic

models such as the logistic model could provide insights into microalgae growth and lipids accumulation.

Previous optimisation studies on microalgae cultivation are based the flask culture data. These laboratory reactors lack the geometry configuration of raceway ponds system which are traditionally used for large scale microalgae cultivation. Therefore, the use of miniature reactors that are geometrically similar to large scale production systems could enhance large scale process yields and subsequently have an impact on biodiesel economics. Such findings therefore contribute to the implementation of microalgal biodiesel production at a large and commercial scale.

1.6 Aims and objectives

The aim of this study was to model and optimize the upstream and downstream stages of microalgae bioprocess development for biodiesel production using *Chlorella* sp. in a miniature parallel raceway pond photobioreactor.

To achieve the aim above, the following specific objectives were undertaken:

i. Modelling and optimisation of biomass and lipid productivity of *Chlorella* sp. for biodiesel production using miniature parallel raceway pond photobioreactor
ii. Kinetic studies of *Chlorella* sp. growth and lipid accumulation.
iii. Modelling and optimisation of microalgae biomass harvesting using various MION and the development of ANN based soft sensor for prediction of recovery efficiency of said MION.

1.7 Outline of dissertation/thesis

This thesis contains six chapters presented in research paper format as outlined in the dissertation/thesis template by the College of Agriculture, Engineering and Science (AES) of the University of KwaZulu-Natal. Each chapter contains a literature review, materials and methods, results, discussion and conclusion. The use of microalgae as a feedstock for the production of biodiesel was central to all chapters.

Chapter 2 was a literature review that describes the use of microalgae as a feedstock for biodiesel production. The challenges associated with biodiesel production, modelling and optimisation of biodiesel production as well as miniature microalgae cultivation systems

Chapter 3 investigates the modelling and optimisation of biomass and lipid productivity in *Chlorella* sp on input parameters of nitrogen, iron and phosphorus using BG11 medium. Kinetic studies of *Chlorella* sp. growth and lipid accumulation are presented.

In Chapter 4, microalgae biomass recovery was modelled and optimized on three type of magnetic iron oxide nanoparticles (MION). The process parameters of pH, algae-nanoparticles exposure time and magnet exposure time were considered. Additionally, three Artificial Neural Network models were developed for prediction of recovery efficiencies and knowledge extraction was implemented to reveal functional relationships between inputs and recovery efficiency.

Chapter 6 integrates the work, states major conclusions obtained from the study and provides recommendations for future research.

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Chapter 2: Process development for microalgal biofuel production: A mini review

* This mini review forms part of a book chapter titled:
Biologically Renewable Resources of Energy: Potentials, Progress and Barriers.
In *Microbial Fuel Cell Technology for Bioelectricity* (pp. 1-22). Springer, Cham.
(Chapter 2)

2.1 Abstract

This review discussed the effects of various culture conditions on biomass and lipid productivity, the kinetics of microalgal growth and potential harvesting efficiency of magnetic iron oxide nanoparticles. Various cultivation methods were discussed as well as optimisation methods and the economic feasibility of the process. Rapid fossil fuel depletion and the negative impact on the environment has necessitated the need for a sustainable, renewable, and environmentally friendly energy resource. Biodiesel was a potential energy source which has received a great amount of interest in the research sphere. Biodiesel was traditionally produced from crop plants albeit with a large amount of disadvantages. Microalgae offer a more feasible alternative to crop plants due to their higher growth rates and their minimal impact on food security; arable land and freshwater resources. Microalgae offer a range of commercially important products such as astaxanthin, poly unsaturated fatty acids such as omega-3, pigments used in the food and cosmetic industry, animal/aquaculture feed and biodiesel. The commercial production of biodiesel has yet to be commercialized due to challenges such as slow culture process and high production costs in comparison to conventional diesel. Separation of microalgal biomass from culture medium; harvesting, was a critical step in the production of algal biofuels. Centrifugation, flocculation, and filtration are currently being employed but are either energy intensive, costly, time consuming or generate large amounts of chemical waste. Magnetic iron oxide nanoparticles are a potential solution to this harvesting bottleneck by reducing the energy intensiveness of the process and costs. Bioprocess development and optimisation are necessary for the determination of large scale microalgal product production feasibility. Design of experiment (DoE) methods such as Response Surface Methodology are commonly used in the optimisation of bioprocesses as they could evaluate more than one factor

at a time and identify patterns often missed by the human eye. Artificial Neural Networks are less commonly used but offer the ability to model non-linear bioprocesses with greater accuracy. The study of the kinetics of microalgal growth and lipid production was also a useful tool in the commercialization of biodiesel production, once models are developed they allow for the understanding, design, and control of fermentation processes. Differences in reactors used at experimental scale and large scale reduce the accuracy of quantitative scale up after optimisation studies. Miniature parallel raceway reactors are a popular tool for process development as they allow for parallelization, thereby reducing labour and allowing for accurate quantitative scale up.

Keywords: Microalgae, Magnetic iron oxides, Process development, Miniature parallel raceway reactors, Biodiesel

2.2 Introduction

The rise in world-wide populations has resulted in increased energy demand as could be seen by the trend shown in Fig. 1 (Global energy statistical yearbook; 2019). Currently, conventional energies such as petroleum, coal and natural gas are being used to meet the world's energy demands with crude oil being the most utilized resource worldwide (Fig 2). Unfortunately, these conventional fossil fuels have limited reserves, impact the environment negatively as well as contribute to the global warming crisis (Abou-Shanab *et al.*, 2011). According to BP's 2019 World Energy Review, South Africa used 533 thousand barrels of fossil fuels per day (BP Statistical Review of World Energy 68th Ed, 2019), highlighting the country's dependency on such fuels. Due to the disadvantages associated with fossil fuel usage, an alternative energy source that was sustainable, renewable, and environmental friendly was required.

Biodiesel was a biofuel commonly produced from plant oils such as soybean oil, rapeseed oil, palm oil, corn oil, animal fat and waste cooking oil and was defined as fatty methyl esters derived from the transesterification of the oils mentioned above using an alcohol or acid as a catalyst (Satyanarayana *et al.,* 2011; Abomohra *et al.,* 2014). Feasibility of a biodiesel feedstock was dependent on many factors such as its impact on net energy supply, greenhouse gas (GHG) emissions, water and air quality and global food impact (Ahmad *et al.,* 2011). The use of plant oils, animal fat and waste cooking oils as feedstock's for the worldwide production

of biodiesel was therefore unsustainable as these feedstock's do not meet the criteria stated above, due to the fact that these feedstock's require large amounts of arable land, freshwater resources and would therefore have a severe negative impact on global food security (Liu *et al.*, 2007).

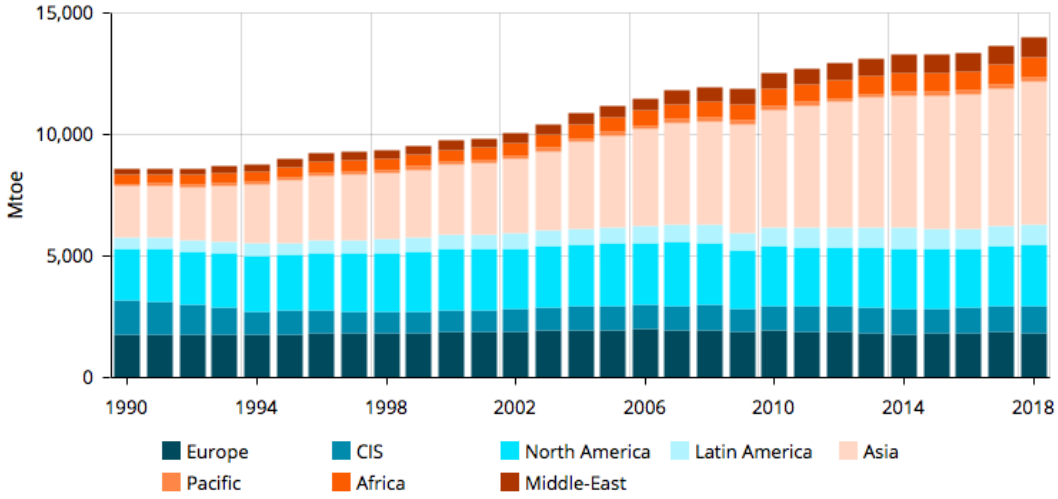

Figure 1. Total global energy consumption in 2018 (Global Energy Statistical Yearbook, 2019)

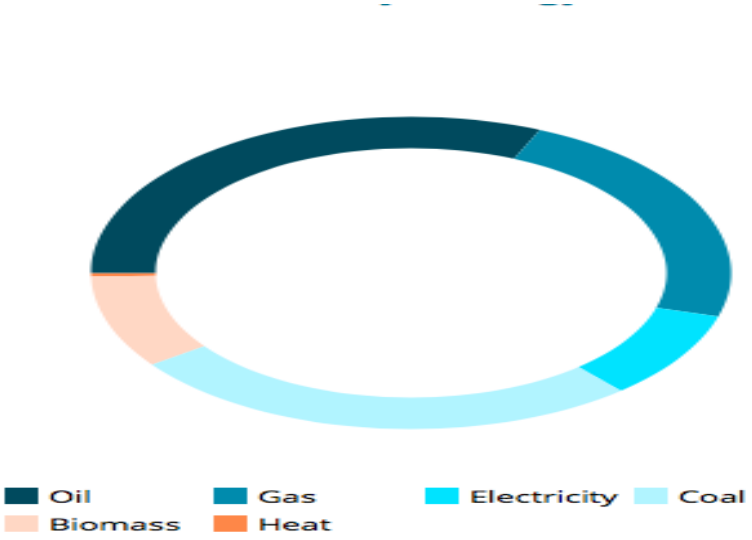

Figure 2. Breakdown of the types and amounts of energy consumed globally in 2018 (Global Energy Statistical yearbook, 2019)

Microalgae are a large group of unicellular, autotrophic, photosynthetic microorganisms. These microorganisms convert solar energy into chemical energy with a productivity of 10 – 20 times more than any other biofuel crop (Harun *et al.,* 2010; Mata *et al.,* 2010). This group of microorganisms have received a lot of attention as a promising biodiesel feedstock as they have a widespread availability, rapid growth rates in comparison to terrestrial food crops, higher oil yields, do not require arable land or freshwater resources used for food crops thereby minimising damage to food chain systems and food security (Chisti, 2007).

Microalgal biomass was a rich source of an immense variety of chemical products with applications in many different sectors such as food and feed (biomass), cosmetics (chlorophyll, β- carotene), pharmaceuticals (antioxidants, antibiotics, toxins and vitamins) and fuel industries (Olaizola, 2003; Borowitzkwa, 2013).

Key steps in microalgal biodiesel production process are; cultivation, biomass harvesting, and downstream processing of dry biomass (lipid extraction and transesterefication) (Wang *et al.,* 2015). As previously stated, microalgae are photosynthetic organisms therefore photoautotrophic cultivation was the most commonly used culture method where microalgae utilize carbon dioxide (CO_2) and sunlight as carbon and energy source , respectively (Zhu *et al.,* 2017). A large amount of algal species have been reported in studies employing the photoautotrophic cultivation method, including *Botyrococcus, Chlorella, Chlamydomonas, Desmodesmus, Dunaliella,Monallanthus and Neochloris* (Kiran *et al.,* 2014; Nascimento *et al.,* 2013; Leesing *et al.,* 2013). Heterotrophic cultivation differs from photoautotrophic cultivation in that microalgae utilize organic carbon materials as a carbon and energy source instead of CO_2 and sunlight for biomass accumulation. This method results in a decrease in formation of light induced products such as chlorophyll and carotenoids (Zhu *et al.,* 2017). Mixotrophic cultivation was a combination of both photoautrophic and heterotrophic cultivation method.

A variety of cultivation vessels could also be used for microalgal bioprocessing, including open ponds, photobioreactors (PBRs) and fermenters (Zhu *et al.,* 2017). Open raceway ponds are advantageous in terms of cost as they are cheaper to install and operate as compared to photobioreactors but present a disadvantage in their susceptibility to contamination (Chisti, 2007). Photobioreactors have a much higher initial cost and must be built specifically to the physiology of the cultivated strain but offer advantages in that they are less susceptible to

contaminants and allow control over nutrients and cultivation parameters required for growth such as temperature, dissolved carbon dioxide and pH (Harun *et al.,* 2010).

Harvesting was aimed at achieving a highly concentrated biomass slurry (Sustainable Energy Ireland Report; 2009). This step was considered to be the main bottleneck in microalgal bioprocessing as it was costly and energy intensive. This stage was reported to contribute to 20 -30% of the total production costs (Xu *et al.,* 2010; Sostanc *et al.,* 2012; Kim *et al.,* 2013). Traditional harvesting methods include centrifugation, sedimentation, flocculation, filtration, flotation and combined flocculation-filtration (Chen *et al.,* 2011; Rawat *et al.,* 2013; Zhou *et al.,* 2014, Bharathraja *et al.,* 2015). Such methods are disadvantageous due to high cost, high energy requirements, time-consuming nature and accumulation of chemical waste (Uduman *et al.,* 2010; Chen *et al.,* 2011). Low cost harvesting methods are a significant factor in microalgal bioprocess development, recently magnophoretic harvesting using naked or functionalized magnetite particles has been reported to be energy efficient and time saving (Prochazkova *et al.,* 2013).

Downstream processing of microalgal biomass includes biomass drying, extraction of lipids and transesterification of extracted lipids. Solvent extraction was the most widely reported method for extraction of lipids, it involves the use of polar organic solvents to disrupt the hydrogen bonds between polar lipids and non-polar organic solvents to disrupt hydrophobic interactions between non-polar/neutral lipids (Pragya *et al.,* 2013). Transesterification was the chemical process responsible for converting algal lipids into fatty acid methyl esters. It involves the reaction of alcohols such as ethanol, methanol. butanol, propanol and amyl alcohol with lipids (triglycerides) to produce glycerol and fatty acid methyl esters (Zhu *et al.,* 2017). The process requires a alkaline, acid or enzyme catalyst. Commonly reported alkaline catalysts are potassium hydroxide, sodium methoxide and sodium hydroxide (Aliya *et al.,* 2012). Reported acid catalyst examples include hydrochloric acid, sulfonic acid, phosphoric acid and sulfuric acid (Viegas *et al.,* 2015) and enzymatic catalysts include calcium oxide, magnesium oxide and lipases (Bharathiraja *et al.,* 2016).

The production of good quality bioproducts depends on the interactions of the culture medium components. Therefore, it was important to investigate and fully understand the effect of

various cultural conditions on the productivity of these microorganisms (Sforoza *et al.,* 2012; Garcia-camacho *et al.,* 2011). Optimisation of bioprocesses was necessary for the enhancement of the commercial feasibility of microalgae biotechnology (Ho *et al.,* 2015). Statistical methods such as Response Surface Methodology (RSM) and Artificial Neural Networks (ANNs) are commonly used for these purposes. Response Surface Methodology describes the interactive effects of process variables and develops a quadratic model illustrating these interactive effects, which was then used to optimise the desired process (Bezera *et al.,* 2008; Wang and Wan, 2009a). Artificial Neural Networks mimic the neurological functions of the brain by deciphering the patterns and relationships found in data and transforming this information into mathematical models (Vani *et al.,* 2015; Wang and Wan, 2009a). The use of such modelling tools allows for bioprocess development and reduction of production costs. Microalgae are not an extensively well-studied group in terms of an industrial biotechnological approach. Due to this, these microorganisms present an opportunity for discovery of a large range of highly valuable metabolites (Olaizola, 2003).

This review describes the importance of growth conditions on microalgal biomass and lipid productivities and how these factors could be manipulated to increase or decrease such outputs. The importance of mathematical and kinetics modelling was also discussed, focusing on how it could be used to gain relevant information on microalgal bioprocessing. The benefits of novel, modern microalgal harvesting techniques over conventional methods was explained. Large scale production of microalgae was focuses on optimizing culture conditions affecting the productivity of microalgal biomass and lipids, mathematical kinetic modelling for bioprocess development, novel biomass harvesting methods, the benefits of using miniature raceway pond photobioreactors for bioprocess development and the economic feasibility of a microalgal biorefinery at large commercial scale.

2.2 Microalgae cultivation methods

Microalgal cultivation requires light, carbon dioxide, water and inorganic salts and was commonly carried out in raceway ponds or photobioreactors (Chisti, 2007). Microalgae may assume three types of metabolism\ based on nutrients provided; autotrophic (light as sole energy source), heterotrophic (organic compounds as carbon and energy source) and

mixotrophic (light as sole energy source for photosynthetic activities but organic compounds or CO_2 play an essential role). Microalgae could shift from one type of metabolism\ to another based on changes in environmental conditions (Mata *et al.,* 2010)

2.2.1. Raceway ponds

Raceway ponds are open circular ponds in the form of natural waters such as lakes and lagoons or artificial ponds and containers. As seen in Figure 3, the configuration of raceway ponds was a closed loop oval recirculation channel typically 0.2-0.5m deep, depth was limited due to the penetration limit of light as an increase in depth would result in a decrease in the efficiency light penetration (Brennan and Owende, 2010). A paddlewheel provides mixing and circulation in the pond, evaporation achieves temperature regulation therefore temperatures in ponds fluctuate seasonally (Chisti, 2007). As the atmosphere only contains 0,03 – 0,06% of CO_2, most raceway pond structures sparge CO_2 in at the bottom of the bond to avoid mass transfer limitation (Mata *et al.,* 2010). Raceway ponds have a lower capital costs but present limitations in the form of maintaining monoculture conditions, poor mixing and media loss due to evaporation (Chisti, 2007). Monoculture conditions can be maintained by adopting high salinity, high nutrition or high alkalinity environments but this limits microalgal strains that could be cultivated in raceway ponds to strains such as *Dunaliella* sp., *Spirulina* sp. and *Chlorella* sp. are most commonly used (Lee, 2001). Raceway ponds are currently used in research and in industry in the form of shallow big ponds, circular pond tanks and closed ponds, which are usually operated in continuous mode to prevent sedimentation (Harun *et al.,* 2010). The algal culture is introduced into the pond directly after the position of the paddlewheels, the flow of the culture follows the shape of the pond and mechanical aeration is provide by CO_2 spargers. Culture is harvested before the paddlewheel point.

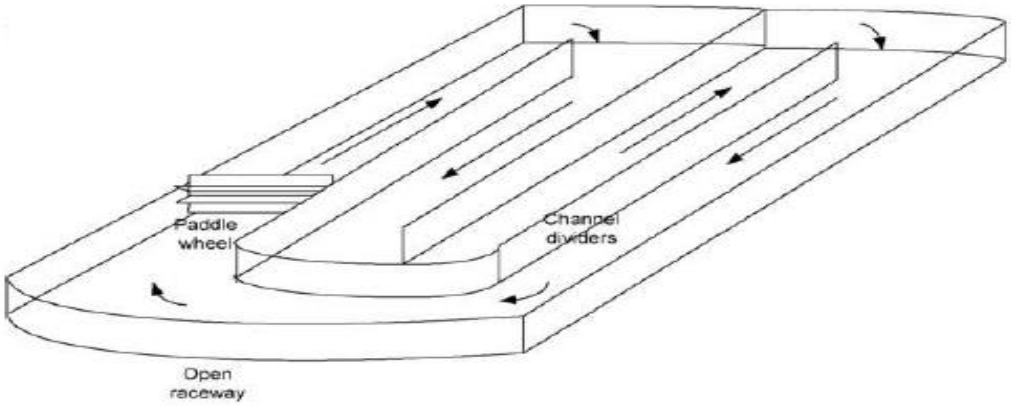

Figure 3. Schematic drawing of an open raceway pond (Molina-Grima, 1999)

2.2.2. Photobioreactors

Photobioreactors can come in several configurations such as airlift, tubular, flat plate, and vertical column photobioreactors. Photobioreactors are advantageous in that they show higher productivities than open ponds, have a greater ability to capture light energy, and efficient mixing and gas/liquid mass transfer (Jorquera *et al.*, 2010). Sunlight or artificial light was captured in an array of transparent tubes that are made of plastic or glass, less than 0.1m in diameter (Chisti, 2007) These tubular arrays can be aligned horizontally, vertically, inclined or as a helix (Brennan and Owende., 2010). The tubing configurations can have effect on a number of parameters in energy usage, horizontal tubing was more scaleable but requires large areas of land (Halim *et al.*, 2010). A degassing column functions in circulating the culture medium to the tubes and back (Chisti, 2007). Zhu *et al.*, 2013 cultivated *Chlorella zofingiensis* on piggery wastewater in tubular bubble column photobioreactor resulting in a net biomass productivity of 1.314 g l^{-1} day^{-1}. *Scenedesmus actus* was cultivated in a tubular photobioreactor with six vertical cylinders housed in a greenhouse illuminated by solar light, a biomass of 113.7g dry weight was obtained from 123.1l of wastewater (de Alva *et al.*, 2013). Feng *et al.*, 2011 used a 4 2.2L column aeration photobioreactors for the cultivation of *Chlorella vulgaris* in artificial wastewater, resulting in a cell concentration of 0.28g/l. Figures 4a and 4b showed schematic drawings of horizontal tubular and flat-plate photobioreactors, respectively.

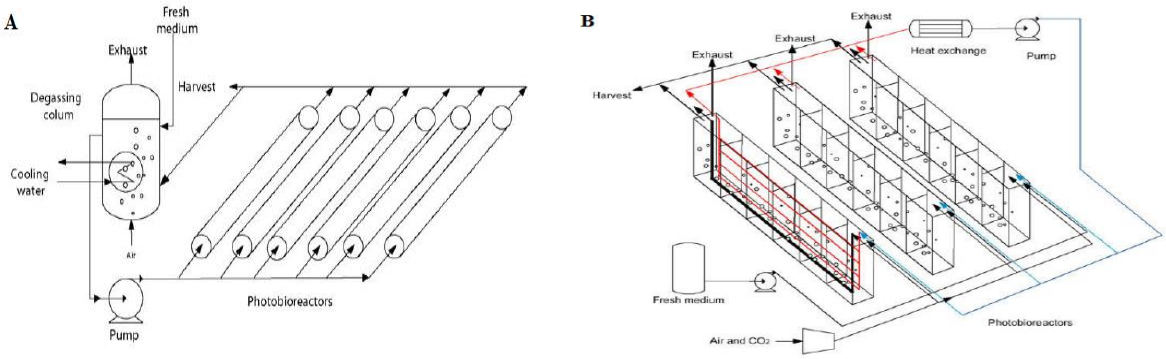

Figure 4. Schematic drawings of (a) horizontal tubular solar array (Chisti, 2007) and (b) flat-plate photobioreactors (Cheng-Wu *et al.*, 2001, Tredici and Rodolfi., 2004 and Sierra *et al.*, 2008)

2.3 Effect of nutrient concentration on biomass and lipid productivity in microalgae

Lipid productivity was dependent on both lipid content and biomass productivity in microalgal species. Therefore a suitable medium was paramount in achieving optimal lipid production in a microalgal species. The concentration of macro and micronutrients in a growth medium ultimately have impacts on the profile of the cellular macromolecular composition (Richardson *et al.*, 1969; Hu *et al.*, 2008; Khozin-Goldberg and Cohen, 2006; Li *et al.*, 2010), appropriate knowledge on the effects and interactions of each nutrient in a growth medium on product formation could therefore allow for increased bioprocess efficiency. Studies have shown that nutrient stress increases lipid production and this stress was most commonly exerted by nitrogen starvation (Singh *et al.*, 2015; Lv *et al.*, 2010; Fu *et al.*, 2017; Chu *et al.*, 2013).

Nitrogen was required for protein synthesis which was essential to the cell division and growth of microalgae. In conditions where nitrogen was at a sufficient concentration, a metabolic balance between carbon fixation rate and nitrogen assimilation rate could be observed, which is necessary for the cellular metabolism (Adams *et al.*, 2013). Nitrogen limitation has a negative effect on protein synthesis and reduces the photosynthetic rate of the cell, resulting in a metabolic flux towards lipid biosynthesis (Chu *et al.*, 2014; Ho *et al.*, 2014). Nitrogen limitation was the most efficient method of increasing the content of neutral lipids in

microalgae, but results in decreased biomass productivity. An increase in lipid accumulation due to nitrogen limitation has been reported in *Chlorella vulgaris, Chlorella zofingiensis, Neochloris oleoabundans, Scenedesmus obliquus, Ankistrodesmus falcatus* KJ671624 and *Scenedesmus dimorphus* KMITL(Breuer *et al.*, 2012, Singh *et al.*, 2015; Ruangsomboon *et al.*, 2012). A drawback to nitrogen limitation was the decrease in biomass productivity of the microalgal species which ultimately translates to a low lipid productivity.

Phosphorus was an essential nutrient to microalgal growth which plays an essential role in cellular metabolic processes related to energy transfer, signal transduction, photosynthesis, and respiration (Chu *et al.*, 2011; Sharma *et al.*, 2012; Xin *et al.*, 2010). Similar to nitrogen, phosphorus limitation has been shown to increase the lipid content in *Chlorella* sp, *Phaeodactylum trocornutum, Chaetoceros* sp, *Isochrysis galbana* and *Pavlova lutheri* (Liang *et al.*, 2012; Sharma *et al.*, 2012) In a study done by Goldberg and Cohen, under phosphorus limited conditions, the triacylglycerol content of the starved cells increased from 6.5% to 39.3% (Goldberg & Cohen, 2006).

Iron, a trace metal component in most microalgal growth mediums, was involved in the photosynthetic enzymatic reactions occurring in photosystem I (PSI) and photosystem II (PSII) which are linked to biomass accumulation (Cao *et al.*, 2014). Studies by Liu *et al.* concluded that a high iron concentration in combination with low nitrogen concentration results in an increase in lipid accumulation in *Chlorella vulgaris* (Liu *et al.*, 2008). Singh et al achieved the highest lipid content and lipid productivity of 59.6% and 74.07mg L^{-1} d^{-1}, respectively under high iron supplementation of 9 mg L^{-1} d^{-1} (Singh *et al.*, 2015). A combination of stresses and gains aids in relating the lipid productivity yields to biomass productivity yields and was required in order to achieve productivities that will ensure the feasibility of a commercial bioprocess. Other strategies used to enhance biomass or lipid productivities include CO_2, temperature influence, salinity stress, metal influence and oxidative stress. By altering these medium or environmental components a relative increase or decrease in biomass or lipid productivity can be observed (Sibi *et al.*, 2016).

2.4 Valuable products obtained from microalgae

2.4.1 Lipids

Microalgae are capable of accumulating a higher percentage of lipids than their terrestrial plant counterparts. Most oleaginous microalgal strains accumulate between 20-50% of lipids based on culture conditions and on the microalgal species making these organisms an attractive biodiesel feedstock (Chew *et al.,* 2017). Nitrogen starvation, high temperature, pH shift and high salt concentrations are all stress conditions that have been used to manipulate lipid accumulation in microalgae and enhance lipid productivity (Kwak *et al.,* 2016). The main lipid fraction of microalgae consists of fatty acids (FA), waxes, sterols, hydrocarbons, pigments and ketones (Halim *et al.,* 2011). Lipids are produced intra-cellularly and therefore need to be extracted during downstream processing. The following extraction methods have been used in various scientific literature; solvent extraction, ultrasonic extraction, microwave assisted extraction and electroporation (Biller *et al.,* 2013; Hernández *et al.,* 2014). Such methods are energy intensive, operate at high temperatures and generate organic solvent wastes, therefore hindering the commercial success of microalgal lipid production. Other than neutral lipids, microalgae produce poly unsaturated fatty acids (PUFAs) which are significant to human health and nutrition (Wang *et al.,* 2015b). Marine microalgal species are the most commonly used and the PUFAs are extracted using a variety of methods such as Bligh and Dyer extraction, solvent extraction and sonication, direct saponification and supercritical fluid extraction (SFE) (Li *et al.,* 2014)

2.4.2 Biodiesel

Fatty acid methyl esters originating from vegetable and animal fats are known as biodiesel (Widjaja *et al.,* 2009). The neutral lipid portion in microalgae can be converted into biodiesel by transesterification. The process of transesterification replaces the glycerol molecule with methanol to form fatty acid methyl esters (FAME) which are otherwise known as biodiesel. Neutral microalgal lipids with a low degree of saturation are suitable for conversion to biodiesel (Harun *et al.,* 2010). The conversion of triglycerides to fatty acid methyl esters was catalysed by an acid or base, using a homogenous or heterogenous catalytic process (Suganya *et al.,*

2016). Methanol and ethanol are most commonly utilized alcohols in literature, methanol was preferable due to its cost-effectiveness and physical and chemical advantages. Figure 5 illustrates the overall biodiesel production reaction where an alcohol was used to react with a triglyceride to produce glycerol and a fatty acid methyl ester using a catalyst to drive the reaction forward (van Gerpen, 2005; Zhu *et al.,* 2017). In-situ or direct transesterification was a single step method where lipid extraction and transesterification occur in one reactor (Maceiras *et al.,* 2011). It has emerged as a technique with the potential to reduce the fuel conversion process units therefore production costs (Zhu *et al.,* 2017). Research has shown that in-situ transesterification has the potential to produce more biodiesel than the conventional two-step method (Pragya *et al.,* 2013). Haas and Wagner (2011) obtained a biodiesel yield of 83% from microalgal biomass using the in-situ transesterification method. In-situ transesterification can also be applied to wet algal biomass as illustrated by Lopez *et al.,* (2016) where a 99.5% biodiesel conversion was achieved.

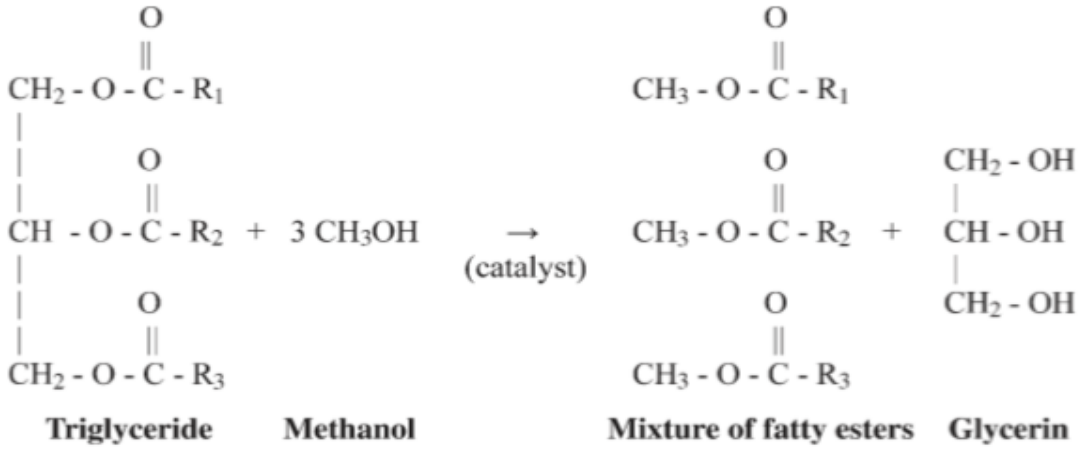

Figure 5: Transesterification mechanism (adapted from van Gerpen, 2005)

2.4.3 Pigments

There are three important classes of pigments found in microalgae; chlorophylls, carotenoids and phycobiliproteins (Chew *et al.,* 2017). These pigments play a role in the photosynthetic and pigmentation metabolism of microalgae but have been found to possess various beneficial biological activities such as being antioxidant, anti-carcinogenic, anti-inflammatory, anti-obesity and neuroprotective (Guedes *et al.,* 2011; Pangestuti and Kim, 2011). Chlorophyll was a greenwash, lipid soluble pigment with a porphyrin ring in its structure (Cuellar-Bermudez *et*

al., 2015). Under optimal conditions, most microalgal species can produce up to 4% dry weight of chlorophyll (Harun *et al.,* 2010). In general, cyanobacteria contain chlorophyll a and green algae contain chlorophyll b (Deng *et al.,* 2008; Dring, 2001). The chelating agents in chlorophyll allow for its addition into ointments, treatments for pharmaceutical benefits such as in liver recovery, ulcer treatment and its use as cosmetic and in food pigments due to increasing consumer demands for natural cosmetic and food additives (Puotinen, 1999). Carotenoids are fat-soluble pigments that are responsible for giving colour to certain parts of plants and are considered as "accessory pigments" (Chen *et al.,* 2016). Phycobiliproteins can be considered to be the major accessory pigment in microalgae (Chew *et al.,* 2017). Applications of these pigment classes include vitamin precursors in food and animal feed, additives, colourants, pharmaceuticals and biomaterials (Krupa *et al.,* 2010; Nobre *et al.,* 2013; Tamiaki *et al.,* 2014; Zhou *et al.,* 2015). Extraction of these pigments include organic solvent extraction and super critical CO_2 extraction, however the extraction pigments was tedious, time consuming and produces low yields (Chew *et al.,* 2017).

2.4.4 Microalgal biomass

Residual (spent) biomass refers to biomass left over after the extraction of lipids for biofuel production, consisting mainly of carbohydrates and proteins (Rizwan *et al.,* 2015). The application of residual (spent) algal biomass can have two objectives; the use of the residual biomass as a substrate for energy production (bioethanol, biomethane) or for the extraction of valuable metabolites for nutritional and economic value (Ansari *et al.,* 2015). Algal proteins contribute to 50-70% of the cells composition (Chew *et al.,* 2017). Proteins have become an important microalgal biorefinery product due to their nutritional value and amino acid profile and can be used for human and animal nutrition (Becker, 2007). Proteins can be extracted by solvent extraction and extraction was affected by pH, ionic strength and salt type (Vanthoor-Koopmans *et al.,* 2013). Microalgal carbohydrates are a favourable source of biologically active molecules due to the high carbohydrate content (<50% dcw) of microalgal cells. Algal carbohydrates consist of glucose, starch, cellulose and various kinds of polysaccharides (Chew *et al.,* 2017). Glucose or starch was commonly used for bioethanol or biohydrogen production (Fu *et al.,* 2010; Sun and Cheng, 2002) and polysaccharides are used as pharmaceutical agents, cosmetic additives and food ingredients due to their ability to regulate the immune system and inflammatory reactions (Aikwa *et al.,* 2012; John *et al.,* 2011).

2.5 Microalgal lipid profiles and fatty acid quality for biodiesel

2.5.1 Lipid profiles

There are many different classes of lipids produced in microalgal cells, their characterization was based on chemical structure and polarity and are divided into polar and neutral lipids. Polar lipids consist of phospholipids and glycolipids, and function as membrane structure components, neutral lipids consist of tri-di- and mono-acylglycerols, waxes and isoprenoid-type lipids e.g. carotenoids (Gong and Jiang *et al.,* 2011; Cuellar-Bermudez *et al.,* 2015). Triacylglycerols (TAGs) contain fatty acid esters that have been bonded onto a glycerol backbone and according to the number of fatty acid chains could be classified as triacylglycerols, diacylglycerols or monoacylglycerols (Halim *et al.,* 2011). Microalgal lipid accumulation, lipid content, lipid class and fatty acid composition is species dependant and can also be affected by certain changes in culture conditions such as light intensity periods, nitrogen depletion, salinity stress, temperature change and pH (Richmond, 2004; Guschina & Harwood, 2006). A study by Breur *et al*. (2012) reported an increase in the accumulation of triacylglycerols in *Chlorella vulgaris*, *Chlorella zofingiensis*, *Neochloris oleoabundans* and *Scenedesmus obliquus* under nitrogen stress conditions. Depending on strain or strains, microalgae produced fatty acids with chain lengths varying from C10 to C28 (Hu *et al.,* 2008). The filamentous cyanobacterium *Trichodesmium erythraeum* synthesizes C10 fatty acids (Parker *et al.,* 1967); *Crypthecodinium cohnii* produces docosahexaenoic acid (De Swaaf *et al.,* 1999). Table 1 showed lipid content and lipid productivities of various microalgal strains that have been reported in literature as suitable strains for commercial biodiesel production due to their high lipid contents and lipid productivities.

Table 1. Various microalgal species lipid content and productivities (Gouveia *et al.*, 2009; Li *et al.*, 2007; Mata *et al.*, 2010)

Microalgal species	Lipid content (% w/w)	Lipid productivity (mg L^{-1} d^{-1})
Chlorella protothecoides	15-58	1214
Chlorococcum sp.	19	54
Chlorella sorokiniana	19-22	45
Dunaniella salina	6-25	116
Ellipsoidion sp.	27	47
Nannochlropsis sp.	21-36	38-61
Nannochloropsis oculata	22-30	84-142
Neochloris oleoabundans	29-65	90-134
Pavlova salina	31	49
Pavlova lutheri	36	50
Phaeodactylum tricornutum	18-57	45
Scenedesmus sp	20-21	41-54

2.5.2 Fatty acid quality and biodiesel standards

Microalgae fatty acids are carboxylic acids with hydrocarbon chains between 4 and 36 carbons (DÁlessandro and Filho, 2016). These fatty acids are divided into 3 groups; monosaturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids (DÁlessandro and Filho, 2016). Commonly, microalgae fatty acids range from butanoic (C4:0) to octanoic (c28:0) with palmitic (c16:0) being the most commonly reported fatty acid (DÁlessandro and Filho, 2016). Polyunsaturated fatty acids (PUFA's) with 4 or more double bonds such as eicosapentaenoic acid containing 5 double bonds and docosahexaenoic acid with 6 double bonds are also very common in microalgae. Unfortunately, biodiesel produced from such fatty acids was extremely susceptible to oxidation during storage which reduces its acceptability as a fuel replacement (Chisti, 2007). Triglycerides are made up of 3 chains of fatty acids joined to a glycerol backbone (Halim *et al.,* 2011). The process of transesterification replaces the glycerol molecule with methanol to form fatty acid methyl esters (FAMEs) (Harun *et al.,* 2010).

Fatty acid profiles in microalgae are influenced by factors such as microalgal strain as well as growth conditions (nutrient levels, temperature, light intensities); this makes it difficult to determine a single compositional profile for all algal biodiesel (Hu *et al.,* 2008, Hoekman *et al.,* 2012). Ashokkumar *et al.,* (2014) showed that the major fatty acids found in *Botyrococcus braunii* were methyl palmitate and methyl oleate, biodiesel produced from these fatty acids yielded an acid number of 0.49 mg KOH/g and a cetane number of 55.4, which were both within the ASTM standards. Whereas, a study done by De Alva *et al.,* (2013) using *Scenedesmus actus* showed that the biodiesel produced from this microalgal species did not meet ASTM standards. In addition, the dominant fatty acids found; palmitic acid, hexadecadienoic acid and linoleic acid over the ASTM limit, and a 1.08 mg KOH/g acid value which does not comply with both ASTM D6751 and EN14214 standards. This highlights the difference in compositions based on microalgal strains and culture conditions. These differences can be manipulated in order to ensure fatty acid quality of a high standard when producing biodiesel at a commercial scale. The American Society for Testing and Materials (ASTM) definition of biodiesel was a fuel comprised of mono alkyl esters of long chain fatty acids derived from vegetable oils and animal fats (Hoekman *et al.,* 2012). It has the ability to serve as alternative to diesel fuel that could be used in diesel engines, only if its physical and chemical properties conform to the international standard specification. The relevant standard in the USA was the ASTM Biodiesel Standard D 6571 (Knothe, 2006). The European union uses separate standards for biodiesel used in vehicles (standard EN 14214) and biodiesel used as heating oil (standard EN 14213) (Knothe, 2006). South Africa's relevant standard was the SANS 342:2016

2.6 Modelling and optimisation of culture conditions for enhanced biomass and lipid productivities.

2.6.1. Response Surface Methodology

Response surface methodology (RSM) was a statistical tool that allows for the optimisation of multiple variables simultaneously at a reduced number of experimental runs (Singh *et al.,* 2015). RSM effectively depicts the synergistic interactions between various inputs, shows which inputs are most important and generates a polynomial equation which was used to determine the optimum process parameter set points (Mohamed *et al.,* 2013, Mandenuis and Brundin, 2008). RSM has been reported in the modelling and optimisation of microalgal

biomass and lipid productivity (Kirrolia *et al.,* 2014, Yang *et al.,* 2014, Binnal and Babu, 2017, Karpagam *et al.,* 2015).

2.6.2. Artificial Neural Networks

Artificial Neural Networks (ANNs) are data-driven modelling tools capable of computing relationships between process parameters and process responses in order to describe the behaviour of the system (Sewsynker *et al.,* 2015). ANNs are extremely effective at modelling highly non-linear bioprocesses (Himmelblau *et al.,* 2008) such as most biological processes. Feed forward back propagation networks are most commonly used in bioprocess modelling due to their ability to effectively model these non-linear processes, having been applied in the modelling of microalgal growth in natural habitats, treatment of wastewaters with algal-bacterial mixed cultures in photobioreactors, bioremediation and in controlled photobioreactors (Garcia-Camacho *et al.,* 2016; Hu *et al.,* 2008; Das & Kundu, 2011). Models based on artificial neural networks do not require any in-depth understanding of the microalgal cell metabolism\ (Garcia-Camacho *et al.,* 2016) and thus can be easily applied to commercial scenarios where costs and labour play a huge factor. The downside of ANN was the requirement for a large set of data to train the network with a view to achieve accurate pattern recognition (Mohamed *et al.,* 2013).

2.6.3. Mathematical models

The use of mathematical models to understand, predict and optimise the behaviour of microorganisms in fermentation processes has increased significantly (Almquist *et al.,* 2014). Mathematical models can increase product yields and productivity of bioprocesses while minimising formation of unwanted by-products (Almquist *et al.,* 2014). Commonly used models are the Monod kinetic models and the modified Gompertz models. Monod kinetic models describe the formation of biomass with respect to limiting substrate (Imamglu and Sukan, 2013) and the modified Gompertz model determines lag time, maximum production rate and maximum product concentration for a given substrate (Dodić *et al.,* 2012, Putra *et al.,* 2015). Understanding the kinetics of microalgal biomass and lipid production will provide important insights into the development, scale up and commercialization of this bioprocess.

2.7 Mismatch between laboratory and industrial scale production reactor properties

A major problem with the industrial production of microalgal metabolites was presented in scale-up. Photobioreactors are the main cultivation method used in the photoautotrophic production of high value microalgal metabolites but the scale-up of research PBRs to commercial PBRs was a major obstacle as research scale reactors are unable to effectively mimic large commercial scale reactors. The process of scaling up needs to take into consideration the control of illumination source, gas transfer and temperature (Olaizola, 2003). Valid scale imitation can be achieved by maintaining geometric similarities which allows certain assumptions to remain valid (Betts and Baganz, 2006), if such similarities are maintained mechanisms such as oxygen mass transfer, mixing and power input can be based on the same principles as those at large scale (Vallejos *et al.,* 2006).

2.8: Downstream procedures in Microalgae cultivation

The separation of growth medium from microalgal cells was a critical step that accounts for 20 – 30% of total production costs due to its energy intensive nature (Gudin & Therpenier, 1986, Uduman *et al.,* 2010). Harvesting was dependent on the properties of the microalgal species; its size and density. Most commonly used harvesting methods include centrifugation, sedimentation, filtration, flocculation, flotation or a combination of these methods, with centrifugaion being the most popular (Milledge *et al.,* 2013). Centrifugation was a more reliable harvesting method but at a commercial scale, it is quite expensive and energy intensive (Olaizola, 2003). Magnetic separation was a separation technique that has been used in a variety of industries (Yavuz *et al.,* 2009). Iron oxide nanoparticless have been successfully used in the sepration of *Botryococcus braunii*, *Chlorella ellipsoidea* and *Nannochloropsis maritima* from growth medium (Hu *et al.,* 2013; Xu *et al.,* 2013). Iron oxide nanoparticles are beneficial in that they are low cost, biocompatible, strong paramagnetic behaviour, low toxicity and ease of synthesis (Kumar-Reddy and Lee, 2013).

The economic feasibility of biodiesel production at a commercial scale was highly dependent on high biomass productivity, high lipid yields and low production costs (Liu *et al.,* 2007). A microalgal species with a high biomass productivity will result in higher lipid yields which was essential to ensure the economic feasibility of commercial biodiesel production.

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Chapter 3. Biomass and lipid production from local *Chlorella* isolate: Process optimisation and kinetic studies

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Abstract

The economic viability of microalgal biodiesel production was dependent on high biomass and lipid productivities in microalgal species. This study investigated the effect of iron, nitrogen and phosphorus concentration on biomass and lipid productivity in a local microalgal isolate *Chlorella* sp. The isolate was cultivated in fifteen miniature parallel raceway pond reactors under varying concentrations of nitrogen, iron and phosphorus between the ranges of 0.5 – 2.0 g L^{-1}; 3.0 – 9.0mg L^{-1} and 0.0 – 40.0mg L^{-1} , respectively for a period of 20 days, after which biomass and lipids were extracted from the cultivated algae. The obtained experimental data on biomass and lipid accumulation was used to develop two response surface models with high coefficients of determination (R^2<0.80). Process optimisation yielded significant quantities of *Chlorella* sp. biomass and lipids (114.5 and 38.23mg L^{-1} d^{-1}), respectively. Kinetic studies using the Logistic model showed a maximum biomass concentration and specific growth rate of 1.78g $L^{-1}$and 0.01 g L^{-1} h^{-1}, respectively with a coefficient of determination (R^2) of 0.98. Biomass and lipid productivity were successfully optimized demonstrating the commercial potential of *Chlorella* sp. as a biodiesel feedstock.

Keywords: Response surface methodology**,** logistic model, miniature reactors, microalgae, process development

3.1. Introduction

Alleviating climate change issues and meeting the worlds' increasing energy demands have resulted in significant focus on sustainable, renewable and alternative fuels driving intense research and development efforts into biofuels (Hallenbeck *et al.,* 2015). Biofuels produced from microalgae have the unique ability of producing a variety of replacement fuels; such as biodiesel, biomethane and bioethanol while addressing sustainability issues faced by large scale fuel production from first and second generation feedstock's (Hu *et al.,* 2008; Abdelaziz *et al.,* 2013).

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms with the rapid growth and survival capabilities due to their simple and unique unicellular or multicellular structures (Mata *et al.,* 2010). Microalgae, as a third generation feedstock, have been widely reported to be advantageous over first and second generation feedstock's when considering biofuel production and this was due to reasons such as ease of cultivation; being able to grow with little or no attention, using water unsuitable for plant or human consumption and not requiring any arable land (Mata *et al.,* 2010).

Despite these advantages, high production costs are still a major bottleneck in the successful large scale commercialization of microalgal biofuels. Large amounts of microalgal lipids are required to fulfil the worlds' biodiesel demands. Most strains can accumulate 20 – 50% lipids based on their dry cell weight (Amaro *et al.,* 2011). The exploitation of microalgae species with high biomass and lipid productivities was essential for biodiesel production capable of meeting high world demands (Singh *et al.,* 2015).

Factors such as nutrients stress directly influence biomass and lipid productivities in microalgae (Chu *et al.,* 2014; Hao *et al.,* 2013; Converti *et al.,* 2009). Nitrogen deficiency affects protein synthesis and photosynthetic rates, resulting in a metabolic shift towards lipid synthesis as opposed to biomass accumulation (Chu *et al.,* 2014; Ho *et al.,* 2014). Phosphorus deprived conditions increase lipid contents in a variety of microalgal strains including *Chlorella* sp. (Liang *et al.,* 2012). Iron was an essential trace metal involved in the reactions of photosystem I and photosystem II which are directly linked to biomass accumulation (Cao *et al.,* 2014). It has been reported that increasing iron concentrations in growth media ($1.2x10^{-5}$ mol L^{-1}) resulted in an increase in both biomass and lipid content of *Chlorella vulgaris* (Liu *et*

al., 2008). A study by Niagam *et al.* (2011), showed that biomass concentration in *Chlorella pyrenoidosa* increased as the concentration of nitrogen source, KNO_3 increased from 0 – 0.4g L^{-1} over a period of 24 days whereas the opposite was observed for lipid content. Liang *et al.* (2012) investigated the effect of phosphorus on lipid accumulation in *Chlorella* sp. and observed and increase in lipid accumulation when phosphorus concentration was decreased from 240 to 32 µM. Biomass accumulation increased with phosphorus concentration increase from 16 to 80 µM but above 80µM, biomass concentration was negatively affected.

Understanding the synergistic effects of environmental growth parameters on cell growth and product formation provides knowledge for process design and enhances yields. It was therefore essential that the interactive effects of key parameters affecting microalgae biomass and lipid accumulation be investigated and optimized.

Bioprocess optimisation is a complex and necessary stage that results in the improvement of product yields and allows for consistency during scale-up (Cheng *et al.,* 2017). Response surface methodology (RSM) has been used to identify the individual and interactive effects of process variables and determine optimum operational conditions for investigated processes. Fermentation process development was an integral part into achieving commercialization of bioproducts formed from bioprocesses.

Currently, microalgal bioprocess operations are carried out in illuminated shake flask systems as pilot and lab-scale photobioreactors tend to limit the number of experimental variables examinable in parallel (Ojo *et al.,* 2015). The shake flask system of experimentation was challenged with differences in agitation, mixing efficiency as well as gas-liquid mass transfer resulting in a significant impact on reproducibility of cell cultivations in scale up bioreactors. Commercial scale raceway systems have reported performances that are considerably less than the theoretical values as productivities at large scale do not mimic results obtained at lab scale (Posten, 2009).

The use of miniature bioreactors has become increasingly necessary due to their ability to simulate the mechanisms of large scale processes. Their size allows for parallelization, thereby reducing the labour involved in performing a large number of cell cultivations necessary for bioprocess development. Miniature reactors are able to minimize differences in agitation methods, mixing and gas-liquid mass transfer that significantly challenge the reproducibility

of results at large scale (Ojo *et al.*, 2015). Betts and Baganz reported a variety of parallel reactor systems such as miniature stirred-tank and miniature bubble column reactor systems for microalgal growth (Betts and Baganz, 2006), however there was a dearth of knowledge on the potential of miniature raceway pond photobioreactors for laboratory scale bioprocess modelling and optimisation of biomass and lipid productivities in microalgae.

Bioprocess kinetic modelling represents the complex biochemistry of microbial cultures and can describe microbial growth, substrate utilization and product formation (Ordonez *et al.,* 2016). Kinetic modelling provide knowledge to improve the design, optimisation and control of biological systems (Linville *et al.,* 2013), thus increase product yield and productivity and reduces the formation of unwanted by-products (Alquimist *et al.,* 2014). The logistic kinetic model has been used to describe biomass growth and the modified Gompertz model describes product formation as functions of time (Dodic *et al.,* 2012; Phukoetphim *et al.,* 2017).

In this study, a miniature raceway pond photobioreactor was used to model and optimize the growth and lipid formation in *Chlorella* sp. using miniature raceway ponds. The considered response surface model inputs were nitrogen, iron and phosphorus concentrations ranging from 0.5 – 2.0 g L^{-1}; 3.0 – 9.0mg L^{-1} and 0.0 – 40.0 mg $L^{-1,}$ respectively. Furthermore, kinetic studies of *Chlorella* sp. growth were carried out using the Logistic model.

3.2. Materials and methods

3.2.1. Culture maintenance

Chlorella sp. was isolated from the Botanical Garden ponds at the University of Kwa-Zulu Natal, Pietermaritzburg campus (29° 33’ S, 30° 19’ E) and maintained on both solid and liquid BG11 medium (Allen and Stainer, 1968). Sub-culturing was performed every 4-6 weeks as means of culture maintenance (Feng *et al.,* 2011). The BG11 agar plates were para filmed and incubated in a MRC Conviron at 25°C for 12h:12h light:dark cycle with light intensity of 30umol m^{-2} s^{-1}. Liquid cultures were maintained in 50ml of BG11 medium grown in Erlenmeyer flasks at ambient temperature agitating at 200rpm on orbital shakers (DragonLab SK-O330-Pro-benchtop orbital shakers). A fluorescent lighting system was assembled above the orbital shakers set at a light:dark cycle of 16h:8h, light intensity of 100µmol m^{-2} s^{-1}.

3.2.2. Photobioreactor configuration

A miniaturized parallel raceway photobioreactor was designed with geometric similarities to pilot scale raceway ponds as (Figure 1). The transparent reactor components (paddles, central baffle and incubator box) were constructed using Perspex due to its favourable mechanical and optical properties (>62 MPa tensile strength, >92% light transmittance) (Ojo *et al.*, 2015). The reactor consisted of 15 high density polyethylene (HDPE) miniature raceway vessels configured at 90mm wide and 285mm long. A 19mm long central baffle was included in each miniature raceway vessel. The total surface area accessible for light absorption in each vessel was 0.026m^2 and a liquid height/light path through the media of maximum 55mm for each vessel. The total working volume of the photobioreactor was 15L. Mixing in each vessel was achieved by a 30 × 60mm paddle which was anchored on a stainless steel agitation shafts. The 15 raceway vessels and mixing system were encased in an 820 × 970 × 190 mm transparent incubator box. Illumination of the photobioreactor was achieved by four 36W fluorescent lamps mounted above the photobioreactor Perspex box using height adjustable clamps. Temperature thermocouple (0-150°C) and light dependant resistor (0-6000lux) sensors were mounted inside the incubator box. Lab Quest 2 light sensor (Vernier Software and Technology, Beaverton, USA) were used to calibrate the light dependant resistor. Three stepper motors with programmable speed between 40 – 150rpm were used to rotate the agitation shafts. The actuators and sensors were interfaced with programmed Arduino microcontroller.

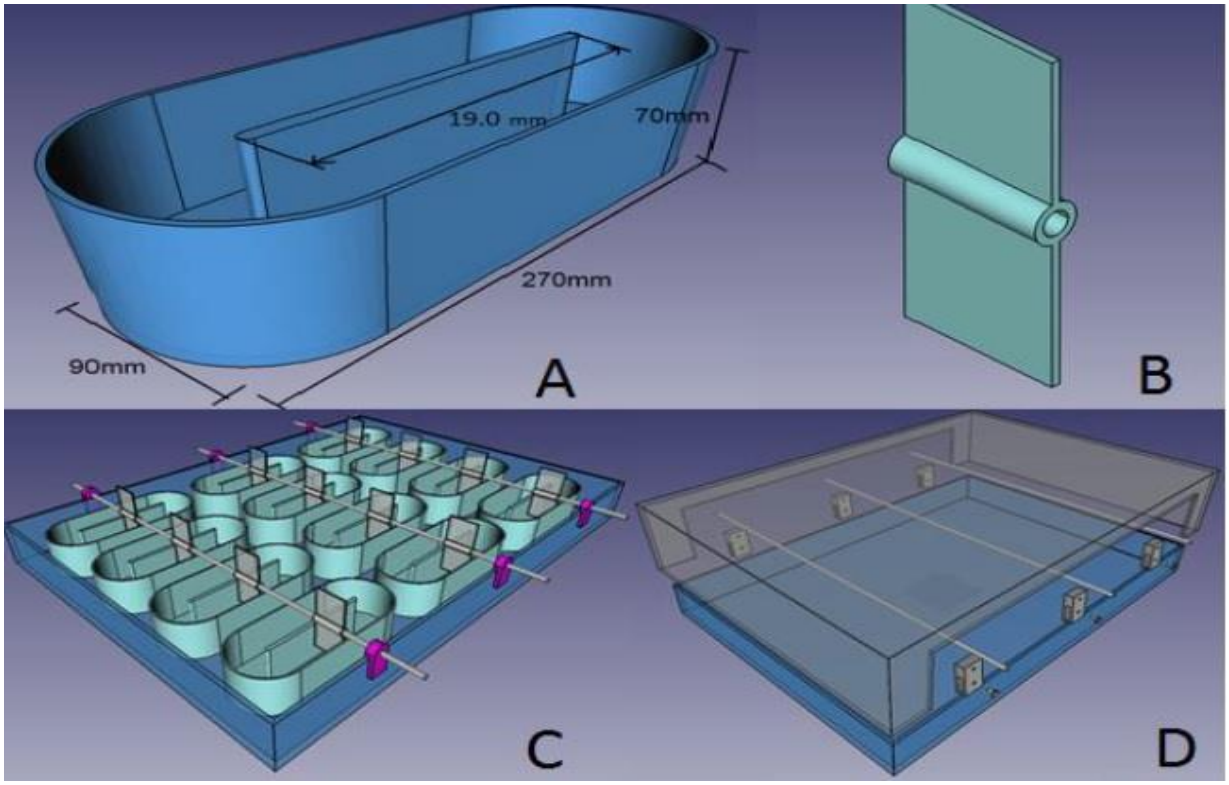

Figure 1. Implemented photobioreactor structures: (A) raceway vessel, (B) impeller, (C) reactor arrangement, (D) photo-incubator box

3.2.3. Experimental design

The selection of process parameter type and ranges was aimed at increasing both biomass and lipid productivity in *Chlorella* sp., and was guided by previous reports (Singh *et al.*, 2015). The input parameters consisted of nitrogen concentration (0.5 – 2.0g L^{-1}), iron concentration (3.0-9.0mg L^{-1}) and phosphorus concentration (0.0 – 40.0mg L^{-1}) with biomass and lipid productivity as the response outputs (Table 1). The Box-Behnken design was used generating seventeen experiments. Experiments were carried out in duplicate.

3.2.4. Cultivation process

Each raceway vessel was filled to 800ml with BG11 medium modified with various parameters as specified by the experimental design and inoculated with 10% (v/v) of *Chlorella* sp. culture (OD_{680nm} of 0.1). The runs were carried out for 20 days at 25°C ± 2°C at continuous light of 4600 lux and an agitation speed of 40rpm. The process was terminated on the 20^{th} day and the cultures were analysed for biomass and lipid productivity. A control experiment was also

carried out simultaneously under BG11 medium conditions (1.5g L^{-1} nitrogen concentration, 6.0mg L^{-1} iron concentration and 40mg L^{-1} phosphorus concentration).

Table 1. Experimental design input variables and their corresponding ranges

Coded factor	Variable	Unit	Input range/ coded values		
			-1	0	+1
A	Nitrogen concentration	g L^{-1}	0.50	1.25	2.00
B	Iron concentration	mg L^{-1}	3.00	6.00	9.00
C	Phosphorus concentration	mg L^{-1}	0.00	20.00	40.00

Table 2. Box-Behnken experimental design used for optimisation of biomass and lipid production.

		Investigated factors			**Response 1**	**Response 2**	
Std	Run	A: Nitrogen	B: Iron	C: Phosphorus	Biomass productivity	Lipid productivity	Lipid Content
		g L^{-1}	mg L^{-1}	mg L^{-1}	mg L^{-1} d^{-1}	mg L^{-1} d^{-1}	(%)
15	1	1.25	6	20	65.00	13.32	20.49
8	2	2	6	40	118.9	28	23.54

11	3	1.25	3	40	56.78	18.94	33.36
10	4	1.25	9	0	46.57	15.65	33.61
14	5	1.25	6	20	62.50	12.28	19.65
17	6	1.25	6	20	65.00	13.48	20.74
3	7	0.5	9	20	57.50	41.4	72.00
6	8	2	6	0	87.65	26.43	30.15
9	9	1.25	3	0	38.67	28.55	73.83
12	10	1.25	9	40	82.5	32.14	38.96
1	11	0.5	3	20	41.97	29.88	71.20
16	12	1.25	6	20	67.89	15.67	23.08
13	13	1.25	6	20	68.00	16.00	23.53
5	14	0.5	6	0	47.98	34.67	72.26

4	15	2	9	20	123.45	39	31.59
2	16	2	3	20	96.78	21	21.69
7	17	0.5	6	40	62.5	42.37	67.79

3.2.4. Analytical methods

3.2.4.1. Biomass quantification

Biomass concentration (mg/l) was determined by measuring optical density of the algal culture at 680nm by spectrophotometer (UV mini-1240 UVVWAS, Shimadzu). Optical density was related to biomass concentration (mg/L) using the equation $y=0.0679x - 0.0025$ (R^2=0.9626) for *Chlorella* sp. where y was the biomass concentration and x was the OD680nm. Biomass productivity was calculated as per Eq. (2) (Singh *et al.*, 2015)

$$\textit{Biomass productivity (mg } L^{-1} d^{-1}) = \textit{Biomass concentration (mg } L^{-1}) / \textit{days} \quad (2)$$

3.2.4.2. Total lipids determination

Microalgal cells were harvested by centrifugation at 8000rpm for 10 min using a Beckman Coulter centrifuge. Microalgal paste was washed twice with distilled water and dried in an oven overnight at 60°C. A modified method of extraction adapted from Lee *et al.* (2010) and Blight and Dyer. (1959) was used for solvent extraction of lipids. Ultrapure water (0.8ml) was added to 10mg of algal biomass, this mixture was homogenized and placed in a microwave (Samsung) for 5 minutes at 2450 MHz to achieve cell lysis. Chloroform, Methanol and Ultrapure water was added in a 2:2:1 ratio vortexing in between solvent addition. The mixture was filtered using Whatman No.1 filter paper to remove residual biomass. Layers were allowed to separate and

the solvent layer was evaporated in a fume hood for 24 hours after which lipids were measured gravimetrically. Lipid productivity was calculated as per Eq 3 (Singh *et al.*, 2015)

Lipid productivity (mg L^{-1} d^{-1}) = Biomass productivity x *Lipid content (%) / 100* (3)

3.2.4.3. Optimisation of biomass and lipid productivities using Response Surface Methodology (RSM)

The experimental biomass and lipid productivity data were used to fit two polynomial equations relating the input parameters to the biomass and lipid productivity using Design Expert software (Stat-Ease Inc., USA). The general form model equation was shown in Eq. (4) where Y represents the process response (biomass or lipid productivity), a_0 was the free term; a_1, a_2 and $a_3$are the linear coefficients; a_{11} , a_{22} and a_{33} are the squared term coefficients ; a_{12} , $a_{13,}$ and a_{23} are the interaction coefficients also X_1, X_2, X_3 are the nitrogen, iron and phosphorus , respectively. Process optimisation was carried out using the method of Myers and Montgomery (Myers and Montgomery, 1995).The optimized process conditions for biomass and lipid productivity were validated experimentally in duplicate.

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad (4)$$

3.2.4.3. Kinetic model and calculation of kinetic parameters

The logistic model equation in the differential form as seen in Eq. (5) representing the exponential and stationery phases of growth, was integrated to give Eq. (6) where biomass (X) was related to initial biomass concentration (X_0), maximum cell concentration (X_{max}) and maximum specific growth rate (μ_{max}) at specific times (t) during exponential and stationery phases of *Chlorella* sp. growth. This model does not predict the death phase of microorganisms (Zajsek and Gorsek, 2010). Model coefficients were determined using CurveExpert (Hyams Development).

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \quad (5)$$

$$X = \frac{X_0 exp(\mu_{max} t)}{1 - \left[\left(\frac{X_0}{X_{max}}\right)(1 - exp(\mu_{max} t))\right]} \quad (6)$$

3.3. Results and discussion

3.3.1. Modelling of biomass and lipid productivity

The experimental design generated 17 experimental conditions (Table 2). Process data was used to generate polynomial equations (Table 4) relating biomass and lipid productivities to the investigated parameters (nitrogen, iron and phosphorus concentration). The statistical suitability of the developed models was assessed using Analysis of Variance (ANOVA) (Table 3). F-values of 63.91 and 31.20 were observed for the biomass and lipid productivity models, respectively. In addition, biomass and lipid productivity models both yielded p-values of <0.0001. High F-values and low P-values are indicative of the statistical significance by relating the response and selected factors at a 95% confidence level (Singh *et al.,* 2015).

The coefficient of determination (R^2) value was a measure of variation where values above 0.70 translate the models ability to accurately predict the bioprocess. R^2 values of 0.9880 and 0.9757 were obtained for biomass and lipid productivity models respectively, highlighting the models ability to relate the input parameters to the responses. ANOVA coefficients such as p-values, indicate statistically the model's ability to fit the data. Model terms with p- value's less than 0.05 are termed to be significant to the corresponding model response (Qing *et al.,* 2016). Nitrogen, iron and phosphorus all obtained p-values less than 0.05 illustrating the significance

of these factors to biomass productivity whereas significant factors to lipid productivity were shown to be iron concentration (Table 3).

This was due to nitrogen being essential to microalgal cell structure as well as functional processes of microalgae. It was an integral component of proteins, amino acids, nucleic acids, enzymes and photosynthetic pigments (Sajjadi *et al.,* 2018). Iron plays a major role in photosynthesis due to its involvement in the enzymatic reactions of photosystem I (PSI) and photosystem II (PSII) (Sun *et al.,* 2014). Efficient photosynthetic activity results in efficiency and high biomass productivity. Phosphorus was also a significant medium composition to the biomass productivity of *Chlorella* as shown by the p-value obtained in Table 3. Mediums that are replete in phosphorus allow for the accumulation of large amounts of Poly-P in microalgal cells which cells then use to synthesize ATP which could be used for protein, DNA and RNA anabolism during unfavourable growth conditions (Harold., 1966).

A study by Singh *et al.,* 2015 showed that both nitrogen and iron were essential for high biomass productivity as depicted by the p-values obtained for both input factors (>0.05). An increase in nitrogen, iron and phosphorus levels resulted in an increase in both biomass productivity and chlorophyll a concentration as shown in a study by Ruangsomboon *et al.,* 2013.

Table 3. Analysis of Variance (ANOVA) for response surface quadratic models

Response 1 Biomass Productivity ($mg\ L^{-1}\ d^{-1}$)			Response 2 Lipid Productivity ($mg\ L^{-1}\ d^{-1}$)		
Source	**F-value**	**p-value**	**Source**	**F-value**	**p-value**
Model	63.91	<0.0001	Model	31.20	<0.0001
A	350.67	<0.0001	A	12.74	0.0076
B	43.03	0.0003	B	25.19	0.0015
C	75.15	<0.0001	C	0.064	0.7998
AB	1.86	0.2122	AB	2.61	0.1505
AC	4.39	0.0782	AC	7.20	0.0314
BC	4.75	0.0641	BC	46.14	0.0003
A^2	87.59	<0.0001	A^2	160.15	<0.0001
B^2	4.86	0.0596	B^2	4.70	0.0668
C^2	6.70	0.0363	C^2	11.35	0.0119
Lack of fit	5.98	0.0600	Lack of fit	5.02	0.0765

$R^{2:}$ biomass productivity = 0.9880 and lipid productivity = 0.9757 (F value – probability distribution; p value – probability)

Table 4. RSM polynomial model equations relating input parameters to the biomass and lipid productivities of *Chlorella* sp.

Model	Equation	Equation number
Biomass productivity	$+65.68 + 27.06A + 9.48B + 12.53C + 2.78AB + 4.28AC + 4.46BC + 18.64A^2 - 4.39B^2 - 5.16C^2$	7
Lipid productivity	$+14.15 - 3.48A + 4.72B - 0.2475C + 2.14AB - 3.75AC + 9.03BC + 16.39A^2 - 2.81B^2 - 4.36C^2$	8

A: nitrogen concentration, B: iron concentration, C: phosphorus concentration

3.3.2. Interactive effects of input process parameters;

The highest biomass productivity was obtained for run 15 (2.0g $L^{-1}$of nitrogen, 9.0mg L^{-1} of iron and 20.0mg L^{-1} of phosphorus), resulting in a biomass productivity of 123.45mg L^{-1} d^{-1} and a corresponding lipid productivity of 39mg L^{-1} d^{-1} and lipid content of 31.59%. The highest lipid productivity of 42.37 mg L^{-1} d^{-1} was obtained from experimental run 17 (0.5g L^{-1} of nitrogen, 6.0 mg $L^{-1}$of iron and 40.0 mg L^{-1} of phosphorus) with a corresponding biomass productivity of 62.5 mg L^{-1} d^{-1} and lipid content of 67.79% (Table 2).

When comparing the above results to the control (standard BG11 medium; 1.5g L^{-1} nitrogen, 6.0mg L^{-1} iron and 40.0mg L^{-1} phosphorus) a biomass productivity, lipid productivity and lipid content of 71.43mg L^{-1} d^{-1}, 18.43mg L^{-1} d^{-1} and 67.79% , respectively was obtained. By increasing the nitrogen concentration from 1.5 g L^{-1} to 2.0 g L^{-1} a 2-fold increase in biomass productivity was observed. When comparing the control to the run 17 it could be seen that decreasing nitrogen concentration from 1.5 g L^{-1} to 0.5 g L^{-1} resulted in a 2-fold increase in lipid productivity and a 3-fold increase in lipid content. Singh *et al.* (2015) reported a 2.55 fold increase in lipid content when nitrogen concentration was reduced to 750 mg L^{-1}. Ben-Atmoz

et al. (1985) reported an improvement in the lipid content of *Ankistrodesmus* sp. of up to 45% under nitrogen deficient conditions.

Nitrogen deficient medium shifts metabolic flux from protein synthesis, photosynthetic efficiency and growth to lipid or carbohydrate storage (Jiang *et al.*, 2012). This explains the high lipid productivity achieved under nitrogen deficient conditions. Nitrogen deficient conditions have been reported to be effective in increasing lipid productivities in *Chlorella* sp in various studies; Lv *et al.* (2010); Li *et al.* (2014); Fu *et al.* (2017) and Arora *et al.* (2016) used a nitrogen deficiency strategy to increase lipid productivity and lipid content in various strains of *Chlorella* sp.

A significant drawback in using nitrogen limited medium to increase lipid productivity was the slow growing nature of the culture due to the shift in metabolic flux. This decrease in cell growth rate and biomass generation ultimately affects the rate at which lipids are being produced (Tan and Lee, 2016). In order to overcome this phenomenon and achieve conditions suitable to increasing both biomass and lipid productivities, a nutrient stresses and gains combination medium must be used (Singh *et al.*, 2015). As illustrated in Table 2; runs 7, 14 and 17, the combination of a low nitrogen concentration (0.5g $L^{-1)}$ with a high iron concentration (6 - 9 mg L^{-1}) and high phosphorus concentration (20 mg L^{-1} - 40 mg L^{-1}) can be seen to supplement the growth of *Chlorella* sp.

Previous research by Li *et al.* (2014) showed a high lipid productivity (224.14 mg L^{-1} d^{-1}) was obtained in *Chlorella* sp using a nitrogen deficient medium supplemented with phosphorus. Li *et al.* (2015) achieved a lipid productivity of 820.17 mg L^{-1} d^{-1} in *Chlorella protothecoides* when using a heterotrophic iron induction strategy. Another study by Fu *et al.* (2017) used a nitrogen limited medium combined with surplus phosphorus to obtain a lipid productivity of 310 mg L^{-1} d^{-1} in *Chlorella regularis* .Chu *et al*, (2013) reported a 58.39 mg L^{-1} d^{-1} lipid productivity in *Chlorella vulgaris* when using a nitrogen deficient phosphorus sufficient medium. Lv *et al.* (2010) obtained a lipid productivity of 40mg L^{-1} d^{-1} when using nitrogen deficient medium supplemented with phosphorus in *Chlorella vulgaris*. A study by Arora *et al.* (2016) achieved similar results in which supplementing nitrogen deficient mediums with phosphorus yielded lipid productivities of 49.1 mg L^{-1} d^{-1} in *Chlorella minutissima* whereas using a medium deficient in nitrogen and phosphorus yielded low lipid productivities of 1mg L^{-1} d^{-1}. The data shown in table 5 successfully demonstrates the importance of supplementing nitrogen deficient mediums for high biomass and lipid productivities.

Table 5. Biomass and lipid productivities obtained from various *Chlorella* species under different nutrient stress conditions

Strain	BP $mgL^{-1}d^{-1}$	LP mgL^{-1} d^{-1}	Medium	Temp (°C)	Nutrient status	Reference
Chlorella sp.	57.50	41.40	BG11	25	$N^{-}Fe^{+}P^{+}$	Present study
C.protothecoides	-	224.14	Basal medium	28	$N^{-}P^{+}$	Li *et al.*, 2014
C.protothecoides	-	820.17	Modified Basal medium	28	Heterotrophic iron induction	Li *et al.*, 2015
C.minutissima	119±0.3	49.1±0.41	BBM	25	N+P+ (BP) N-P-(LP)	Arora *et al.*, 2016
C.vulgaris	-	40	-	-	$N^{-}P^{+}$	Lv *et al.*, 2010
C.regularis	720	310	BG11	25	$N^{lim}P^{++}$	Fu *et al.*, 2017
C.vulgaris	100.4	58.39	BG11	25	$N^{-}P^{sufficient}$	Chu *et al.*, 2013

The response surface graphs in Fig 2(A-F) showed the interactive effects of the process input parameters on biomass and lipid productivities. In Figure 2A it can be seen that a combination of high iron concentration (9mg/l) and 1.5g/l nitrogen resulted in high levels of biomass productivities (±118mg/l). This was in alignment with a study done by Singh *et al.* (2015) where 750mg/l nitrogen concentration combined with 9mg/l iron resulted in a biomass productivity of 124.6 mg/l/d in *Ankistrodesmus falcatus* KJ671624. Increasing phosphorus concentration from 35 to 40mg/l and nitrogen from 1.5 to 2g/l resulted in an increase in biomass productivity from 60mg/l/d to ±135mg/l/d. Studies reported by Chu *et al.* (2013) and Ruangsomboon *et al.* (2013) suggested that a combination of high concentrations of nitrogen, iron and phosphorus result in increased biomass productivities due to the importance of these components to the growth of all microalgal species. As shown in Fig 2D, ±1.7g/l of nitrogen combined with 8.5mg/l iron resulted in high lipid productivities of 40mg/l/d. Fig 2E combines 35mg/l of phosphorus and 1.5g/l nitrogen to give a lipid productivity of 35mg/l/d. A combination of lower level nitrogen concentrations with excess iron or phosphorus was known to enhance lipid productivity. Phosphorus limited media result in a large number of Poly-P accumulated in microalgal cells. This poly-p are utilized by microalgae to synthesize ATP which was used in the carbon concentration mechanism (CCM) pathways responsible for capturing CO2 and converting the captured CO2 into lipid and/or carbohydrate (Young and Beardall., 2005). Iron was an essential trace element for microalgal growth as it plays a role as a precursor for many enzymatic and photochemical reactions (Terauchi *et al.*, 2010).

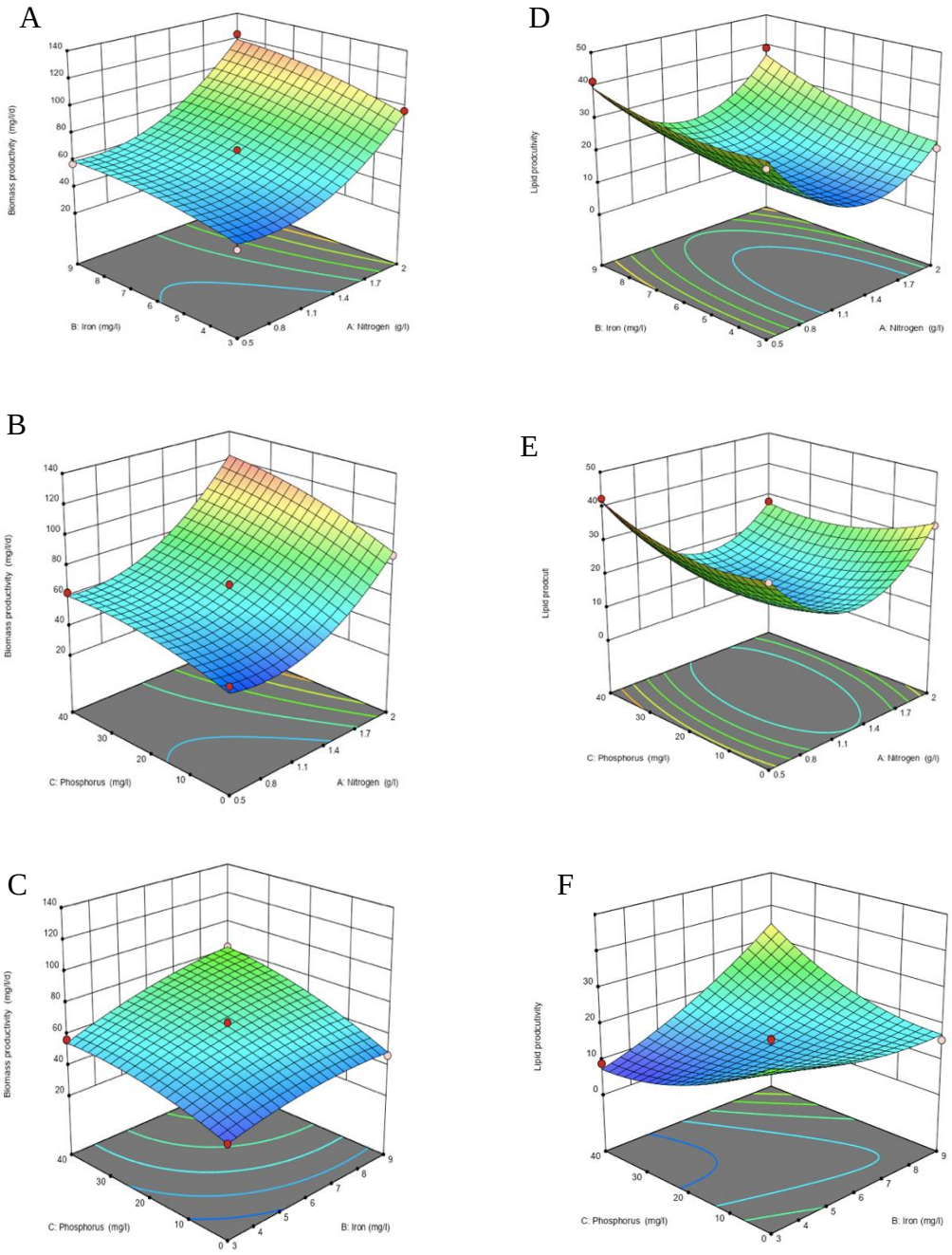

Figure 2. Response surface plots showing interactive effects of: (A) nitrogen and iron concentration on biomass productivity, (B) nitrogen and phosphorus on biomass productivity, (C) phosphorus and iron on biomass productivity, (D) nitrogen and iron on lipid productivity, (E) nitrogen and phosphorus on lipid productivity, (F) phosphorus and iron on lipid productivity.

The predicted optimal conditions for biomass productivity, lipid were validated in duplicate (Table 6). Validated experimental condition 1 demonstrated the importance of the micronutrients; iron and phosphorus for biomass accumulation. High concentrations of these micronutrients (7 mg L^{-1} and 40mg L^{-1}) in combination with a high nitrogen concentration (2.00g L^{-1}) resulted in an observed biomass productivity of 114.5mg L^{-1} d^{-1}. Validated experimental condition 2 shows a high lipid productivity (38.23mg L^{-1} d^{-1}) can be obtained with a combination of low nitrogen concentration and high iron concentration (0.5g L^{-1} nitrogen, 8mg L^{-1} iron). This was due to the presence of iron which supplements the growth of *Chlorella* sp. to ultimately yield a high lipid productivity in deficient nutrient conditions as described in section 3.3.2.

Table 6. Validation of the optimized conditions for biomass and lipid productivity

Run	**Nitrogen (g/L)**	**Iron (mg/L)**	**Phosphorus (mg/L)**	**Biomass productivity (mg/L/d)**		**Lipid productivity (mg/L/d)**	
				Predicted	Observed	Predicted	Observed
1	2.00	7	40	125.6	114.5	-	-
2	0.5	8	0	-	-	42.56	38.23

3.3.3. Kinetics of *Chlorella* sp. growth

The microbial biomass grown in BG11 medium was shown in figure 3. An exponential phase of 288 hours was observed and the stationery phase began at 384 hours. The experimental data for biomass concentration over time were used to fit the logistic model and a high coefficient of determination (R^2) of 0.98 was obtained showing the models ability in predicting the growth of *Chlorella* sp. Kinetic coefficients showed a maximum specific growth rate (μ_{max}) of 0.01 g $L^{-1,}$ an initial cell concentration (X_0) of 0.03g/l and a maximum cell concentration (X_{max}) of 1.78g/l (table 7). These were similar to the experimental values of X_0= 0.01g/L and X_{max} = 1.5g/L. The results obtained in this study agree satisfactorily with other similar studies. The μ_{max} of *Chlorella vulgaris* has been reported in literature as 0.01h^{-1}, 0.0125h^{-1} and 0,0309h^{-1} which was in line with the μ_{max} obtained in this study (Morais and Costa, 2007; Chiu *et al.*, 2008 and Mansouri, 2017). Chiu *et al.*, 2008 reported a maximum cell concentration (X_{max}) of 1.4g/l for *Chlorella vulgaris* which was comparable to the X_{max} of 1.78g/l obtained in this study.

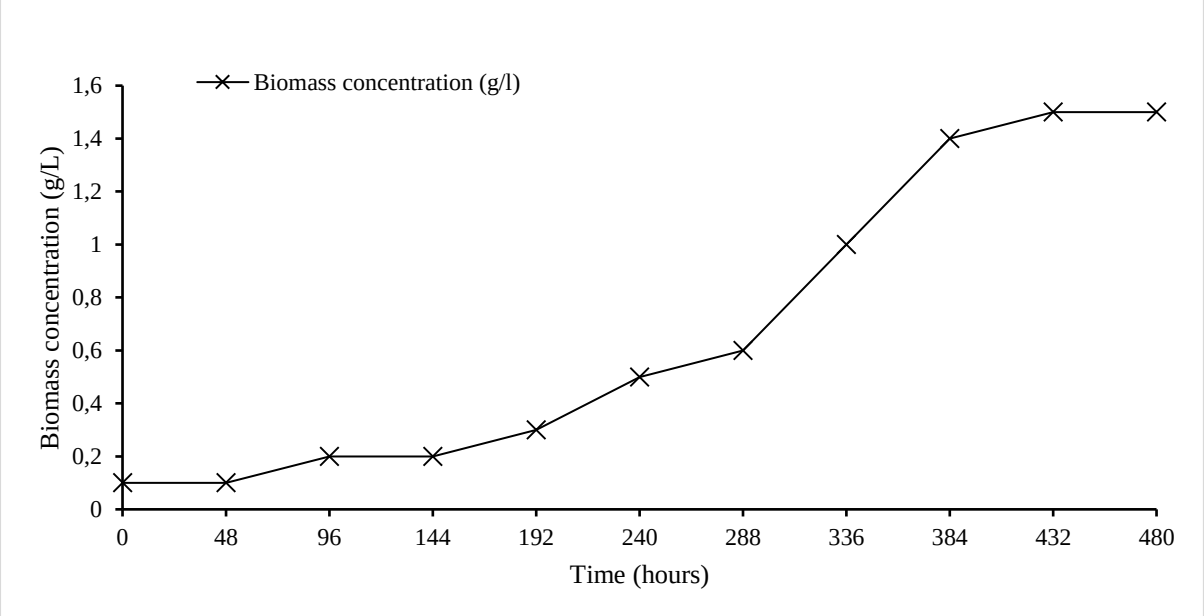

Figure 3. *Chlorella* sp. cell growth and lipid production in BG11 medium over a 20 day period

Table 7. Logistic model kinetic coefficients for cell biomass growth

Kinetic parameter	Biomass kinetic process
μ_{max} (h^{-1})	
	0.01
X_0 (g/L)	
	0.03
X_{max} (g/L)	
	1.78

3.4. Conclusions

In this study, optimisation of process conditions for biomass and lipid accumulation in *Chlorella* sp as well as the employment of the logistic model to model and predict the growth kinetics of Chlorella when grown in BG11 media was investigated. The highest biomass productivity of 123.45 mg L^{-1} d^{-1} was obtained under high nitrogen (2.0g/l), high iron (9mg/l) and moderate phosphorus (20.0mg/l) growth conditions. The process model revealed that biomass accumulation is highly dependent on all three input parameters; nitrogen, iron and phosphorus. The highest lipid productivity of 42.37mg L^{-1} d^{-1} was obtained under low nitrogen (0.5g/l), moderate iron (6mg/l) and high phosphorus (40mg/l). Decreasing the nitrogen concentration resulted in a 2 fold increase in lipid productivity in comparison with BG11 media. The RSM models gave R^2 values of >0.80, highlighting the models significance. Experimental validation gave biomass productivities of 114.50mg L^{-1} d^{-1} under conditions of high nitrogen (2.0g L^{-1}), high iron (7mg L^{-1}) and high phosphorus (40mg L^{-1}) . Lipid productivity of 38.23mg L^{-1} d^{-1} , respectively was obtained under conditions of low nitrogen (0.5g L^{-1}), average iron (3mg $L^{-}1$) and no phosphorus (0mg L^{-1}), highlighting the importance of micronutrients such as iron and phosphorus in growth medium as the presence of such nutrients supplements *Chlorella* sp. growth, thereby improving both biomass and lipid productivities. Kinetic modelling of microalgal growth using the Logistic model gave important insights into *Chlorella* sp growth characteristics. A maximum specific growth rate (μ_{max}) value of 0.01h^{-1} , an initial (X_0) and maximum (X_{max}) biomass concentrations of 0.03 g/L and 1.73g/L , respectively were obtained which strongly correlated to the experimental data. The miniature parallel raceway pond reactors used in this study mimicked the geometry configuration of large scale raceway ponds , unlike laboratory flask experiments thus

providing accurate data for process development. The findings demonstrate the importance of understanding interactions between nutrients in growth medium and how this affects the growth kinetics and product formation. The potential of replacing laboratory flasks with miniature parallel reactors was also demonstrated. *Chlorella* sp. was therefore a viable feedstock for biodiesel production.

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Chapter 4: Development and assessment of intelligent models to predict the recovery efficiency of *Chlorella* sp. using coated and non-coated iron oxide magnetic particles

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Abstract

This study described the modelling, optimisation and prediction of microalgae recovery using magnetic iron oxide nanoparticles (MION). The input parameters consisted of nanoparticles to algae exposure time, magnet retention time, pH and nanoparticles concentration for three MION types; crude, tri-sodium citrate and chitosan. Using three response surface models, recovery efficiencies of 85%, 95% and 87% were obtained for crude, tri-sodium citrate and chitosan MIONs, respectively. Three multilayer Artificial Neural Network models were developed to predict microalgae removal efficiencies under novel process conditions. R^2 values up to 0.82 were obtained for the three MION types. Knowledge discovery on ANN models revealed that the impact of MION operational inputs on microalgae recovery efficiency could be illustrated with sigmoidal and dose response type relationships. The prediction of microalgae removal efficiency under varied MION conditions provides a virtual analytical tool highly suitable for downstream process design in microalgae production and impact the technoeconomic output.

Keywords: Microalgae, Magnetic Iron Oxide Nanoparticles, Harvesting, Artificial Neural Networks

4.1. Introduction

Currently, 80% of the world's energy was obtained from fossil fuels (Brennan and Owende., 2010). Due to their unsustainable nature and negative impacts on the environment, the search for new fuels that are sustainable and eco-friendly has been on the rise worldwide (Wu *et al.,* 2012). Amongst such sustainable and eco-friendly biofuels, biodiesel has received great interest. Traditionally used plantation crops such as soybean and rapeseed will not be able to meet the worldwide biofuel demand because their sustainable production and availability require large amounts of arable land and freshwater (Clarens *et al.,* 2010; Feng *et al.,* 2011).

This has brought much attention to microalgae as a biodiesel feedstock. Microalgae offer several advantages over plant based feedstock's in that their cultivation does not require arable land, microalgae have higher lipid productivities than plants and their cultivation could be coupled to wastewater treatment which reduces the need for fertilizers and fresh water during cultivation, microalgae have the ability to bio-mitigate carbon dioxide from the atmosphere and flue gases (Lam & Lee, 2014).

In addition to biodiesel, microalgae also produce a variety of products such as poly unsaturated fatty acids, chlorophyll, antioxidants and pharmaceuticals (Wang *et al.,* 2015). Microalgae have also been reported in the treatment of industrial wastewaters such as those contaminated with heavy metals, organic chemical toxins, hydrocarbons etc. (de-Bashan and Bashan, 2010) and domestic wastewaters (Abdel-Raouf *et al.,* 2012). Unfortunately, the commercialization of microalgal based products especially biodiesel was quite limited due to economic and technological challenges such as the dilute nature of microalgal cultures and high harvesting costs (Xu *et al.,* 2009, Stephens *et al.,* 2010, Liu *et al.,* 2012). To improve the technoeconomic output of microalgal production, process modelling and optimisation are required at both upstream and downstream stages.

Microalgal cells typically fall into a size range of 5 – 50 µm with algal cells having a negative surface charge therefore forming stable suspensions with growth media (Wu *et al.,* 2012). The separation of these cells from the growth medium was a critical step that couldaccount for 20 to 30% of the total production costs due to its energy intensiveness, thereby emphasizing the need for an efficient, cost effective harvesting technique (Gudin & Therpenier, 1986; Uduman *et al.,* 2010).

Various harvesting methods have been assessed in the separation of microalgal cells from growth medium such as centrifugation, sedimentation, filtration, flocculation, flotation or a

combination of such methods. Unfortunately none of these strategies was superior over the other, or was best suited for a particular algal species and are all disadvantageous in that they are either too energy intensive, have a high cost or are time consuming (Uduman *et al.,* 2010, Milledge *et al.,* 2013). A cost-effective and efficient harvesting method was required for the commercialisation of microalgal based products.

Magnetic separation was a simple, easy, low energy consuming and low cost separation process that has been employed in a variety of industries (Yavuz *et al.,* 2009). This separation was based on the movement of the magnetically tagged particles in response to a magnetic field (Yavuz *et al.,* 2009, Borlido *et al.,* 2013). Naked magnetites are a useful tool in the separation of microalgae from growth medium. Fe_3O_4 particles have been successful in the separation of *Botryococcus braunii*, *Chlorella ellipsoidea*, *Nannochloropsis maritima* (Hu *et al.,* 2013; Xu *et al.,* 2013). Iron oxides are preferred due to their biocompatibility, strong paramagnetic behaviour, low toxicity and easy synthesis (Kumar-Reddy and Lee, 2013). Viable industrial application of flocculation using magnetic iron oxides for microalgal harvesting requires an in-depth understanding of the interactions between various input parameters. The process of magnetic separation was greatly influenced by a variety of factors such as the algal cell type and surface charge, the characteristics of the magnetic particles, the pH of the solution and the nanoparticles concentration (Wang *et al.,* 2015). To achieve an efficient separation procedure, optimisation of significant factors will be required. To the best of our knowledge there was a dearth of information in public domain on the modelling, optimisation and sensitivity of process inputs on *Chlorella* sp. harvesting using crude, chitosan and tri-sodium citrate MION.

Response Surface Methodology (RSM) was a suitable and efficient tool for the optimisation of process conditions for the maximisation of the desired output (Yang *et al.,* 2014). RSM works by evaluating the interactions of the input variables and the effect that these interactions have on the process output (Moodley & Kana, 2015). This provides knowledge on the process dynamics and efficiency which can be used to determine the optimum process operational setpoints.

Artificial Neural Network (ANN) is a data driven modelling technique that mimics the learning process of the human brain. ANN are highly efficient in modelling the non-linear relationship patterns between the process inputs and outputs based on experimental data used in training (Whiteman & Kana, 2013). ANN has been used to predict the growth dynamics of the microalga *Karlodinium veneficum* in a growth medium based on the concentration of key

nutrients (Garcia-Camacho *et al.,* 2016). Nasr *et al* (2013) used artificial neural network to predict the hydrogen production profile over time in batch studies (Nasr *et al.,* 2013).

This study investigated the interactive effects of process input parameters; nanoparticles concentration, pH, algae-nanoparticles exposure time and magnet exposure time, for optimal recovery of *Chlorella* sp. using crude, tri-sodium citrate (TSC) and chitosan nanoparticles. Additionally, three Artificial Neural Network models were developed and validated to predict the microalgal harvesting efficiency using crude, TSC and chitosan nanoparticles under novel operational conditions. Knowledge discovery on ANN models was implemented to reveal functional relationships between the various inputs and the recovery efficiency.

4.2. Methods and Materials

4.2.1. Culture Conditions

A local strain of *Chlorella sp*. was isolated from the Botanical garden ponds at the University of KwaZulu Natal, Pietermaritzburg. Cultivations and maintenance were conducted in a liquid BG11 medium comprising of the following constituents: $NaNO_3$ (15g/l), $MgSO_4.7H_2O$ (0.075g/l), $CaCl_2.H_2O$ (0.036g/l), citric acid (0.006g/l), ammonium ferric citrate green (0.006g/l). EDTA (0.04g/l), $EDTA.Na_2$ (0.001g/l), Na_2CO_3 (0.02g/l), K_2HPO_4 (0.02g/l) and 1g/l of a trace metal solution that comprised of H_3BO_3 (2.86g/l), $MnCl_2.4H_2O$ (1.81g/l), $ZnSO_4.7H_2O$ (0.22g/l), $Na_2MoO_4.2H_2O$ (0.39g/l), $CuSO_4.5H_2O$ (0.08g/l) and $Co(NO_3)2.6H_2O$ (0.05g/l). pH was adjusted to 7.1 using 1M NaOH or HCl. Cultures were grown on an orbital shaker at 250rpm at a 12h:2h light and dark cycle with a light intensity of $30\mu molm^{-2}s^{-1}$. Biomass concentrations of the cells were determined using a spectrophotometer at 680nm.

4.2.2 Synthesis of magnetic iron oxide nanoparticles (MION)

4.2.2.1 Crude MION

The synthesis of the magnetic iron oxide was carried out as described by Zheng et al. (2010). A known weight of $FeSO_4.7H_2O$ (0.556g) was dissolved in 100ml of deionized water in an 800ml beaker. NaOH was added to the mixture in a dropwise manner under constant stirring, until a pH of 11 was obtained. The solution gradually changed from clear to black, indicating the formation of $Fe(OH)_2$ nanoparticles. The beaker was heated in a regular microwave for 1

minute at 700W for the formation of Fe_3O_4 nanoparticles created by the oxidation of $Fe(OH)_2$. The solution was cooled to ambient temperature and washed several times with deionized water and ethanol. The magnetic nanoparticles were then dispersed in deionized water and stored at room temperature (~25°).

4.2.2.2. Tri-sodium citrate (TSC) coated MION

Crude MION were coated as described by Lakshmanan (2013). The TSC of 0.2g was dissolved in 20ml of deionized water to form a TSC solution. The MION was maintained at 90°C and continuously stirred for 30min, whilst the TSC solution was added dropwise. Citrate groups were charged on the surface of the TSC coated MION.

4.2.2.3. Chitosan coated MION

Chitosan coated MION were synthesized similar to Lakshman (2013). Chitosan of 1g was dissolved in 50ml of deionized water. Subsequently, 50ml of a 2% acetic acid solution (2ml water and 98ml acetic acid) was added to the mixture, and continuously stirred for 30 minutes to create a 1% chitosan solution. The chitosan solution was then added to the crude MION and stored at room temperature, undisturbed for 24 hours. The solution was then washed several times with deionized water and ethanol. The chitosan coated nanoparticles were dispersed in deionized water and stored at 4°C. Concentrations of crude, TSC and chitosan MION were determined gravimetrically.

4.2.3 Microscopy

The synthesized MION were observed using transmission electron microscopy and scanning electron microscopy

4.2.3.1. Transmission electron microscopy (TEM)

A TEM grid was dipped into the crude, chitosan and TSC coated MION which were stored in water. Each grid was placed on a filter paper under a light source to dry for 30minutes. Once dried, the grids were loaded into the JEOL 1400 TEM and viewed.

4.2.3.2. Scanning electron microscopy (SEM)

The MION samples were dried using a rotorvap for 15minutes. Subsequently, the dried powdered form of MION was added onto carbon paper attached to the SEM stubs. The SEM stubs were then viewed using the Zeiss EVO Ls15 under variable pressure.

4.2.4. Experimental design for process modeling and optimisation

A four factor Box-Behnken experimental design was used to generate 29 experiments with varied input conditions of algae-nanoparticles exposure time (sec), magnet retention time (min), pH and nanoparticles concentration (g/l). The input ranges and coded values are shown in Table 1 and the experimental design was presented in Table 2.

Table 1: Input variables and their corresponding ranges used un the experimental design

Independent variable	**Coded Factor**	**Input range**	**Coded values (-1, 0, +1)**
Algae – Nanoparticles Exposure time (sec)	A	30 – 90	30, 60, 90
Algae solution – Magnet exposure time (min)	B	5 -15	5, 10, 15
pH	C	6 – 10	6, 8, 10
Nanoparticles concentration (g/l)	D	0.10 – 1.00	0.10, 0.55, 1.00

Table 2: Box-Behnken design for crude, TSC and chitosan coated NPs to determine removal efficiency (%) by varying four parameters.

Run	Factor 1 Microalgae-NP exposure time (s)	Factor 2 Magnet exposure time (min)	Factor 3 pH	Factor 4 NP concentration (g/l)
1	60.00	10.00	8.00	0.55
2	90.00	10.00	8.00	0.10
3	60.00	10.00	8.00	0.55
4	60.00	15.00	8.00	1.00
5	60.00	10.00	8.00	0.55
6	60.00	10.00	8.00	0.55
7	60.00	5.00	8.00	0.10
8	60.00	15.00	8.00	0.10
9	60.00	5.00	8.00	1.00
10	30.00	5.00	8.00	0.55
11	60.00	5.00	10.00	0.55
12	60.00	10.00	10.00	0.10
13	60.00	15.00	6.00	0.55
14	90.00	5.00	8.00	0.55
15	60.00	10.00	6.00	0.10
16	30.00	10.00	6.00	0.55

17	60.00	5.00	6.00	0.55
18	60.00	10.00	6.00	1.00
19	90.00	10.00	8.00	1.00
20	30.00	15.00	8.00	0.55
21	60.00	10.00	10.00	1.00
22	30.00	10.00	10.00	0.55
23	90.00	15.00	8.00	0.55
24	60.00	10.00	8.00	0.55
25	60.00	15.00	10.00	0.55
26	30.00	10.00	8.00	0.10
27	90.00	10.00	6.00	0.55
28	90.00	10.00	10.00	0.55
29	30.00	10.00	8.00	1.00

4.2.5. Microalgal harvesting procedure

Crude, TSC and chitosan coated nanoparticles were added at varied concentrations according to the experimental design to 3 ml of a 5-day old algal culture. The exposure time to nanoparticles and magnet was varied as per the design. After harvesting, the optical density of the supernatant was measured at 680nm using a UV mini -1240 UV VWAS Spectrophotometer (Shimadzu) to determine the removal efficiency percentage as shown in Equation 1.

$$Removal\ efficiency = \frac{Optical\ density\ of\ supernatant}{Optical\ density\ of\ algal\ culture} \times 100 \quad (1)$$

4.2.6. Magnet Properties

The magnet used in the harvesting process was a 10mm x 5mm round super strong neodymium magnet, grade N50 with a Ni-Cu-Ni plating silver in colour. The magnetized direction was towards both North and South pole.

4.2.7 Optimisation of removal efficiency using Response Surface Methodology (RSM)

The experimental data were used to fit polynomial model equations for each type of nanoparticles using Design Expert software (Stat-Ease Inc., USA). The model equations relate input parameters to the removal efficiency. Optimum set-point values for removal efficiencies were obtained by solving the polynomial equations using the method of Myers and Montgomery (1995), followed by an experimental validation in duplicate for each nanoparticles type.

4.2.8. Artificial Intelligent Model Development for the prediction of Removal Efficiency (%)

Three Artificial Neural Network models were used to develop intelligent models for the prediction of microalgal removal efficiency under varied conditions. The input vector consisted of nanoparticles- algal solution exposure time (s), magnet exposure time (min), pH and nanoparticles concentration. The output was the microalgae removal efficiency. The networks topology for each model consisted of 1 input layer with 4 neurons, 1 hidden layer consisting of 3 neurons and 1 output layer of 1 neuron (Figure 1).

The hidden layer functioned in the simultaneous addition of weighted inputs and linked bias and in shifting input data to a non-liner form, as shown in equations 2 and 3. (Desai *et al.*, 2008)

$$sum = \sum_{i}^{n} = 1^{xiwi} + \theta \qquad (2)$$

where w_i (i = 1, n) are the connection weights, θ was the bias and x_i was the input variable

$$f(sum) = \frac{1}{(1 + \exp(-sum))} \quad (3)$$

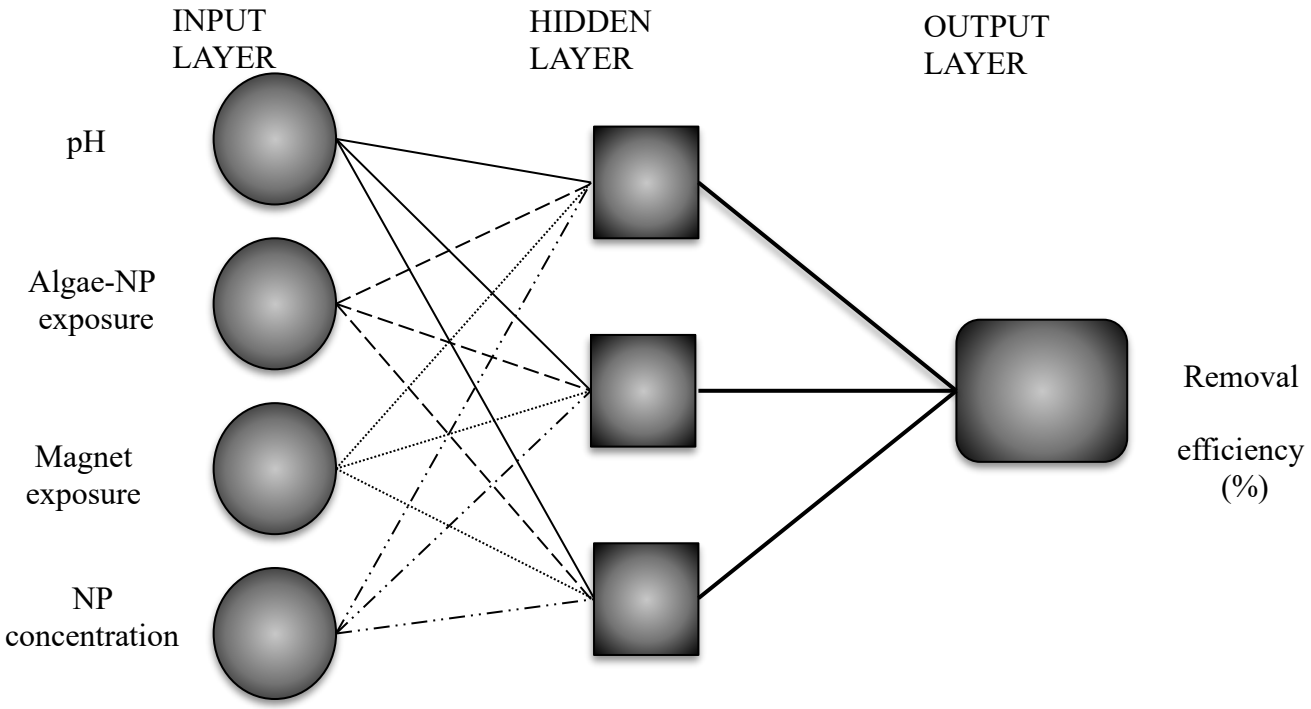


Figure 1: Neural network topology used for the development of three models (crude, TSC and chitosan). The network consists of input layer (4 neurons), 1 hidden layer (3 neurons) and an output layer (1 neuron).

4.2.9. Artificial Neural Network Training and Validation

Prior to implementing the models, each experimental dataset was normalized using equation 4.

$$\text{Normalized } (e_i) = \frac{e_{i-E_{min}}}{E_{max}-E_{min}} \quad (4)$$

where e_i was the normalized data and E_{min} and E_{max} denote the minimum and maximum values.

For each model, the normalized experimental data were divided into 75% set used for training and 25% set used for validation.

The network was trained using a back-propagation algorithm with the aim of achieving a minimal net error value on the validation set while preventing an overtraining or memorisation of the data. After training, regression analyses were performed with the predicted and observed outputs and the coefficients of determination (R^2) were obtained.

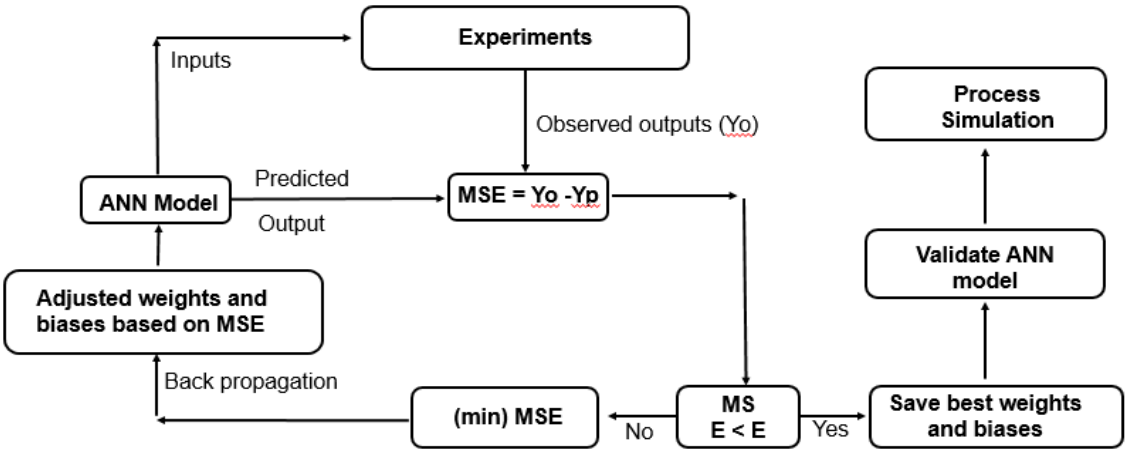


Figure 2: Back propagation training algorithm for artificial neural network

4.2.10. Sensitivity Analysis and Knowledge Discovery

In this study, sensitivity analyses were performed to give insight into the rate and direction of change in output (microalgae removal efficiency) when each of the inputs were varied within its operational range. The rest of the inputs were kept at their median values using the developed predictive models. Knowledge extraction was then carried out with the aim of discovering the functional relationships between the inputs and outputs. These relationships were derived using curve fitting and illustrated with mathematical expressions.

4.3. Results and Discussion

4.3.1 Microscopy and composition of crude, TSC and chitosan nanoparticles

The size and characteristics of the crude and coated MION show non-uniformity. The crude (Figure 3(a)) consisted of nanoparticles within the size range of 80-90nm, however smaller nanoparticles in the range of 55-65nm were also observed. Prochazkova *et al.* (2013) obtained crude nanoparticles within the range 150-200nm. The nanoparticles were either cubic or spherical. These structural observations coincide with previous reports, Zheng *et al.* (2010) microwave synthesized MION that were spherically shaped and approximately 80nm in size. The chitosan MION (Figure 3(b)) displayed a broad range in size of approximately 50-60nm and 100-105nm. Chitosan coated nanoparticles obtained in this study were smaller in size compared to those reported by Lakshmanan (2013), which were synthesized using a co-precipitation technique. The TSC coated NPs (Figure 3(c)) had a broad size range of 20-55nm and 80-90nm. MION size correlates to their magnetic properties where MION below 20nm are supermagnetic, between 20-100nm are stable and single-domain ferromagnetic and MION over 100nm are multi-domain ferromagnetic (Mirabello *et al.*, 2016). The synthesized MION in this study were stable and single-domain ferromagnetic. According to Lee *et al.* (2015), separation of smaller MION (>20nm) from microalgae was difficult and costly, therefore larger sized MION are preferred.

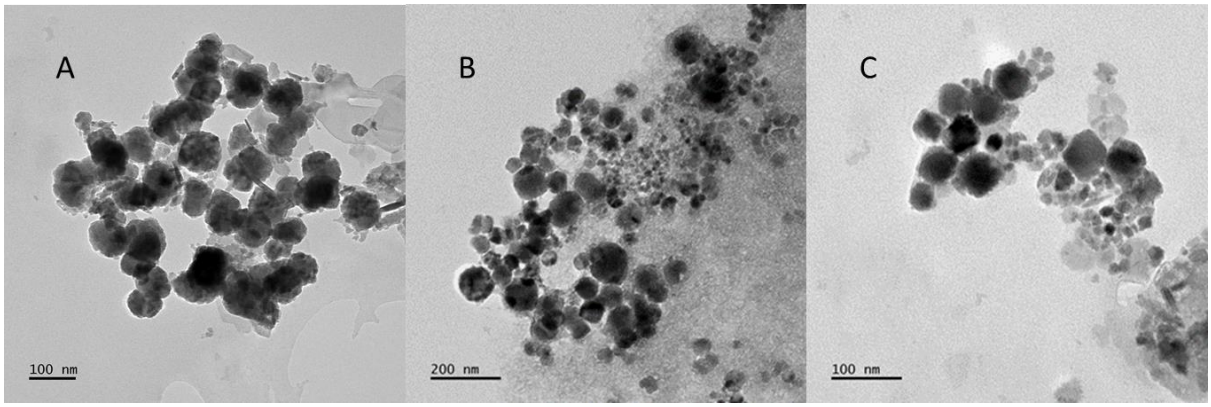


Figure 3: Crude (A), chitosan coated (B) and TSC coated (C) MION viewed under JEOL 1400 transmission electron microscope.

4.3.2 Optimisation of removal efficiency using the Response Surface Model

The fitness of the polynomial equation models was assessed by performing analysis of variance (ANOVA). Coefficients of determination (R^2) of 0.75, 0.87 and 0.70 were obtained for the crude, chitosan and TSC MION's , respectively (Table 3). This was a measure of variance that falls between a range of 0-1, 1 indicating the model's ability to accurately predict the bioprocess and 0 indicating complete inability (Rorke and Gueguim Kana, 2016). Each of the 3 polynomial models accounted for more than 70% of the variation observed in the experimental data. The developed polynomial equations 5,6 and 7 for crude, chitosan and TSC MIONs, respectively are as presented.

Crude MION:

$$\text{Removal efficiency} = 71.65 + 0.73A + 12.28B + 0.50C - 3.59D + 5.65AB - 0.88AC - 8.80AD + 4.86BC + 3.25BD + 2.35CD + 1.04A^2 - 6.54B^2 + 1.43C^2 - 1.68D^2 \quad (5)$$

Chitosan MION:

$$\text{Removal efficiency} = 77.47 + 0.20A + 3.46B - 3.79C + 9.06D - 0.20AB + 0.73AC - 4.09AD - 3.37BC + 2.04BD - 11.22CD + 1.07A^2 - 1.85B^2 - 5.54C^2 - 14.83D^2 \quad (6)$$

TSC MION:

$$\text{Removal efficiency} = 65.81 - 2.02A + 16.20B + 2.88C + 3.41D + 8.11AB - 9.68AC + 4.86AD - 0.95BC + 1.93BD - 2.68CD + 2.71A^2 - 2.58B^2 + 8.00C^2 - 2.24D^2 \quad (7)$$

Where A was the microalgae and nanoparticles exposure time, B was microalgae and magnet exposure time, C was the pH and D was nanoparticles concentration.

Table 3: Statistical analyses of variance (ANOVA) for removal efficiency using crude, chitosan coated and TSC coated MION.

MION	Sum of squares	Degrees of Freedom	Mean Squares	F-Value	P-Value	R^2

Crude	2930.47	14	209.32	3.04	0.0229	0.75
Chitosan	3545.83	14	253.27	6.47	0.0060	0.87
TSC	4849	14	346.40	2.35	0.0606	0.70

All RSM models were significant with an R^2 value above 0,7.

The predicted optimum setpoints for crude, chitosan and TSC MION are presented in Table 4. Crude MION gave the highest predicted and observed removal efficiency of 90% with optimal setpoints of 81 seconds, 15 minutes, 10 and 0.30g/l for algae-nanoparticles exposure time, algae-magnet exposure time, pH and nanoparticles concentration , respectively. For chitosan and TSC MION, the observed removal efficiencies were 83 and 75% with prediction errors of 6 and 27% , respectively. As seen in Table 4, the optimal pH set point for chitosan was 6, which was lower than the optimal pH set point of 10 for both crude and TSC MION. Microalgal recovery was dependent on pH as changes in pH affect the electrostatic forces of the MION (Xu *et al.,* 2011; Hu *et al.,* 2014). A pH of 6 was optimal for chitosan MION, increases in pH decreased the removal efficiency of chitosan MION due to increasing competition between algal cells and hydroxyl groups for an adsorption site on the MION (Hu *et al.,* 2014). The optimal pH of 10 for crude MION correlates to literature where Cerff *et al.,* (2012) and Xu *et al.,* (2011) used pH between 7 and 12 for harvesting of *Chlorella* sp. using naked magnetite. Nanoparticles concentration (0.30-0.38g/l) was favourable for microalgae removal when using crude or TSC coated nanoparticles, this correlates with results obtained by Xu et al. (2011). A higher nanoparticles concentration of 0.90g/l was suitable for microalgae removal when using chitosan MION. Liu *et al.* (2016) observed that an increase in graphene MION concentration enhanced the removal efficiency of the microalgae. A shorter nanoparticles-algae exposure time (30-31 sec) was optimal when using chitosan and TSC MION as compared to crude MION (81 sec).

Table 4: Optimized conditions obtained using the three RSM polynomial models

MION	Algae-Nanoparticles Exposure Time (sec)	Algae-Magnet Retention Time (Min)	pH	Nanoparticles Concentration (g/l)	Predicted Removal Efficiency (%)	Observed Removal Efficiency (%)
Crude	81	15	10	0.30	90	90
Chitosan	30	10	6	0.90	88	83
TSC	31	14	10	0.38	95	75

4.3.3 Prediction of algae removal efficiency using Artificial Neural Network models

To assess the accuracy of the developed ANN models, regression analyses were performed on model predicted and observed removal efficiency values. The obtained coefficients of determination (R^2) are shown in Figure 4 with values of 0.80, 0.76 and 0.76 for crude, chitosan and TSC MION, respectively. Regression analysis highlights the significant impact of outliers on the prediction accuracy of the model (Khamis *et al.*, 2005) and illustrates the predictive accuracy of the model (Desai *et al.*, 2008). All the models could account for more than 75% variation in observed data, thus indicating their suitability to predict the removal efficiency under novel MION process conditions. A higher R^2 value correlates to a higher prediction accuracy therefore indicating potential for using the model as a virtual analytical tool.

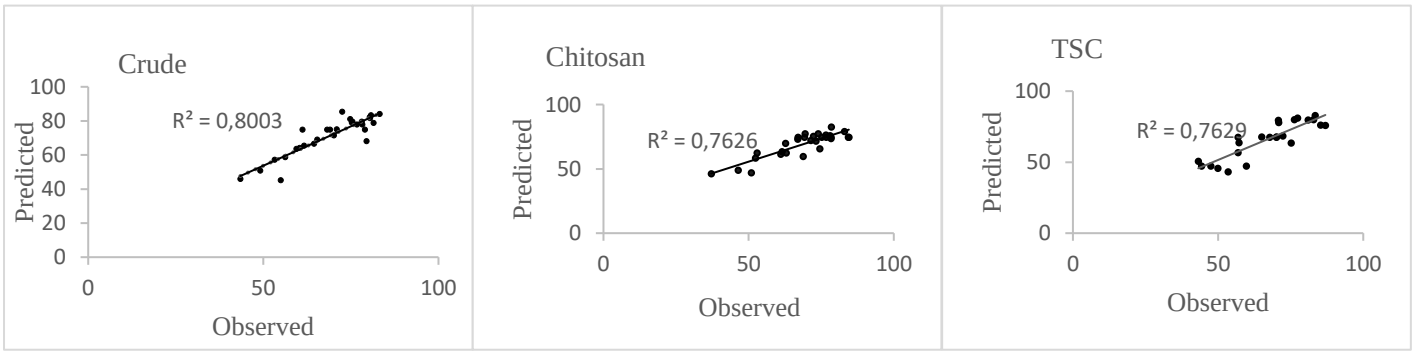
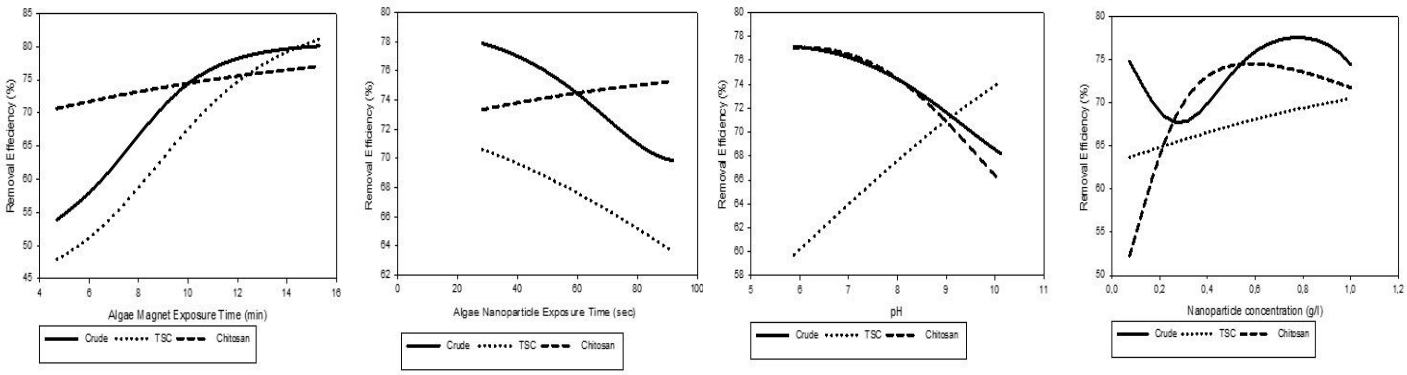


Figure 4: Regression plots for crude, chitosan and TSC MION showing R^2 values ranging from 0.76 to 0.8.

4.3.4 Knowledge discovery on ANN model

A high sensitivity to an input shows that the process output was significantly affected by minimal changes in the input and vice versa (Sewsynker *et al.,* 2015). Variation of the input parameter algae-magnet exposure time within its operational range for all 3 MION types showed an increase in the removal efficiency only up to a 10-minute time point after which no significant improvement was observed (Figure 5(a)). The mathematical relationship illustrating the algae-magnet exposure time and removal efficiency showed a sigmoidal type relationship for the crude, TSC and chitosan MION (Table 5(b), (f) and (j)). The magnet exposure time affects the rate at which the MION bind to the microalgae, binding was dependent on the size and force of the magnet, quantity of MION and size of the reactor used (Hu *et al.,* 2013). Figure 5(b) showed that an increase in algae-nanoparticles exposure time from 38 seconds to 92 seconds resulted in a decrease in the removal efficiency from 74 to 63% for both crude and TSC coated MION. Whereas for chitosan coated MION showed that an increase in the exposure time, from 28 to 92 seconds resulted in an increase removal efficiency up to 75%. Hu *et al.* (2014) reported an optimal duration of 120 seconds for the recovery of *Chlorella ellipsoidea* using MION coated with polyethylenimine. These functional relationships between the algae-nanoparticles exposure time and the removal efficiency were best described by sigmoidal class of relationships for all MION types (Table 5(a), (e), (i)). The microalgal solution together with the MION solution needs to be adequately mixed to promote the electrostatic adhesions. Figure 5(c) showed that an increase in pH from 5 to 10 resulted in a decrease in removal efficiency from 76 to 68% for both crude and chitosan MION whereas for TSC coated MION, an increase in pH from 5 to 10 enhanced the removal efficiency (74%). These relationships were well illustrated by Weibull type of sigmoidal models for all MION (Table 5(c), (g), (k)). Microalgal cell wall surfaces carry a negative charge, therefore nanoparticles carrying a positive surface charge will bind to such algae. At a pH above the isoelectric point of iron oxide nanoparticles (6,5), nanoparticles surface charges become negative therefore preventing binding of microalgal cells. This phenomenon can be seen in both the crude and chitosan MION (Lee *et al.,* 2013; Xu *et al.,* 2011). A non-linear relationship was observed between crude MION and nanoparticles concentration (Figure 5(d)). The highest removal efficiencies (77.32 – 77.54%) were observed when nanoparticles concentration was

between 0.71 to 0.76 g/l. A nanoparticles concentration of 0.61g/l corresponded to a removal efficiency of 76% highlighting the fact that minimal changes in nanoparticles concentration significantly affect the removal efficiency. A Dose Response Multistage type of equation could be used to describe this non-linearity (Table 5(d)). Increase in nanoparticles concentration (0.073 – 0.66g/l) increased the removal efficiency of chitosan MION from 52% to 74.4%. Further increase in nanoparticles beyond this threshold resulted in a steep decline in removal efficiency from 74.4% to 71.8%. The interaction between TSC MION removal efficiency and nanoparticles concentration follow a linear relationship where a continual increase in nanoparticles concentration from 0.073 – 1g/l resulted in an increase in removal efficiency from 63.6% to 70.5%.The rate and direction of change for both chitosan and TSC MION are both illustrated by a dose response model (Table 5(h) and (l)).



(a) **(b)** **(c)** **(d)**

Figure 5: Impact of input variations on the process output (algae removal efficiency (%))

Table 5: Model equations describing the direction and rate of change of removal efficiency due to variation of input parameters within their limits

MION	Equation number	Process Input/output	Model Equation Form	Equation Type	Fitted Model	R^2 value
Crude	(a)	Algae:NP exposure time	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$	Richards	$y = \frac{7{,}80}{(1 + e^{-5{,}69+1{,}52x})^{1/7{,}07}}$	0.99
	(b)	Algae-Magnet exposure time	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$	Richards	$y = \frac{7{,}99}{(1 + e^{9{,}30-8{,}86x})^{1/1{,}27}}$	0.99
	(c)	pH	$y = a - be^{-cx^d}$	Weibull	$y = 7{,}72 - 3{,}31e^{-7{,}27x^{-2{,}74}}$	0.99
	(d)	Nanoparticles concentration	$y = \gamma + (1-\gamma)^{[1-e^{-\beta 1x-\beta 2x^2-\beta 3x^3-\beta 4x^4}]}$	DR-Multistage 4	$y = 8{,}51 + (1 - 8{,}51)[1 - e^{-2{,}11x+6{,}30x^2-6{,}49x^3+2{,}17x^4}]$	0.98
TSC	(e)	Algae:NP exposure time	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$	Richards	$y = \frac{7{,}44}{(1 + e^{-1{,}21+2{,}61x})^{1/9{,}24}}$	0.99
	(f)	Algae-Magnet exposure time	$y = a - be^{-cx^d}$	Weibull	$y = 8{,}21 - 3{,}78e^{-9{,}27x^{3{,}01}}$	0.99
	(g)	pH	$y = a - be^{-cx^d}$	Weibull	$y = 8{,}25 - 3{,}54e^{9{,}25x^{3{,}01}}$	0.99
	(h)	Nanoparticles concentration	$y = \gamma + (1-\gamma)^{[1-e^{-\beta 1x-\beta 2x^2-\beta 3x^3-\beta 4x^4}]}$	DR-Multistage 4	$y = 6{,}30 + (1 - 6{,}30)[1 - e^{-1{,}53x-2{,}94x^2-1{,}92x^3+7{,}14x^4}]$	0.99
Chitosan	(i)	Algae:NP exposure time	$y = \frac{a}{(1 + e^{b-cx})^{1/d}}$	Richards	$y = \frac{7{,}67}{(1 + e^{-5{,}27-1{,}51x})^{1/7{,}24}}$	0.99
	(j)	Algae-Magnet exposure time	$y = a - be^{-cx^d}$	Weibull	$y = 8{,}15 - 1{,}52e^{-6{,}26x^{1{,}09}}$	0.99
	(k)	pH	$y = a - be^{-cx^d}$	Weibull	$y = 7{,}71 - 4{,}48e^{-1{,}66x^{-3{,}07}}$	0.99
	(l)	Nanoparticles concentration	$y = \gamma + (1-\gamma)^{[1-e^{-\beta 1x-\beta 2x^2-\beta 3x^3-\beta 4x^4}]}$	DR-Multistage 4	$y = 4{,}33 + (1 - 4{,}33)[1 - e^{-2{,}96x-5{,}79x^2+4{,}95x^3-1{,}61x^4}]$	0.99

DR: Dose -Response, NP: Nanoparticles

4.4. Conclusion

Process optimisation enhanced the removal efficiency of all MION types, resulting in 85%, 87% and 95% removal efficiencies for crude, chitosan and TSC MION , respectively. Three predictive tools built on Artificial Neural Network were implemented and assessed with great accuracy as illustrated by their coefficient of determination values up to 0.82. The impact of MION operational input changes on microalgae recovery efficiency showed sigmoidal and dose response type relationships. The elucidated functional relationships between microalgae recovery efficiency and MION operational parameters provide knowledge for an efficient design of harvesting regimes for microalgae bioprocessing, thus enhancing technoeconomic output.

Declaration of interests

The authors declare no conflict of interest.

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Chapter 5: Conclusions and recommendations for further research

This study investigated microalgae process development for biomass and lipid production using a local *Chlorella* isolate cultivated in a novel miniature parallel raceway pond photobioreactor. Biomass and lipid productivity was optimized in *Chlorella* sp using Response Surface Methodology (RSM), revealing optimal nutrient concentrations for high biomass and lipid productivity. Early stage microalgae bioprocess development using parallel miniaturised raceway ponds reactor has been demonstrated. Kinetic studies of cell growth using the logistics was undertaken. Three types of magnetic nanoparticles (crude, chitosan and TSC) were assessed for downstream harvesting of *Chlorella* biomass from growth medium. This process was optimized using RSM and artificial neural networks were used to further explain the impact of input changes on the recovery efficiency of all three types of nanoparticles.

5.1. Biomass and lipid production from local *Chlorella* isolate: Process optimisation and kinetics

The generated response surface quadratic models were statistically analysed using Analysis of Variance (ANOVA). Model coefficients of determination (R^2) of above 0.80 were obtained which was an indication of the model's accuracy in describing the relationship between the process inputs and outputs (biomass and lipid productivity). Nitrogen was revealed to be an important nutrient component to biomass productivity as illustrated by the p value (<0.0001), whereas iron was shown to be important to lipid productivity. The developed process model equations described the individual and interactive effects of nitrogen, iron and phosphorus concentrations on both biomass and lipid productivity of *Chlorella* sp. As per the model equation, nitrogen was most influential to biomass productivity in a positive manner. Nitrogen was most influential to lipid productivity albeit in a negative manner. Iron had the highest positive effect on lipid productivity. The interactive effects as determined by the model equations showed how supplementation using micronutrients was important in obtaining high lipid productivity. The lipid productivity model equation showed a positive interaction between micronutrient iron and macronutrient. Therefore, iron can act as a growth supplement in nitrogen deficient medium to ensure high lipid productivity. Individually, the importance of such micronutrients can be overlooked highlighting the importance of determining interactive effects and not only individual effects. The model predicted a biomass productivity of 125.60

mg L^{-1} d^{-1} under the conditions 2.0g L^{-1}nitrogen, 7.0mg L^{-1} iron and 40.0mg L^{-1}phosphorus concentrations. A lipid productivity of 42.56mg L^{-1} d^{-1} was predicted under the conditions 0.5g L^{-1}nitrogen, 3.0mg L^{-1} iron and 0.00mg L^{-1}phosphorus concentrations. Experimental validations gave 114.50mg L^{-1} d^{-1} and 38.23mg L^{-1} d^{-1} biomass and lipid productivities, respectively. The optimized culture conditions underscored the models predictions.

The logistic model gave a R^2 value of 0.98, a maximum biomass concentration and maximum specific growth rate of 1.78g L^{-1} and 0.01g L^{-1} h^{-1} , respectively. These results highlight the potential of *Chlorella* sp. to be used as a feedstock for biodiesel production under optimized process conditions.

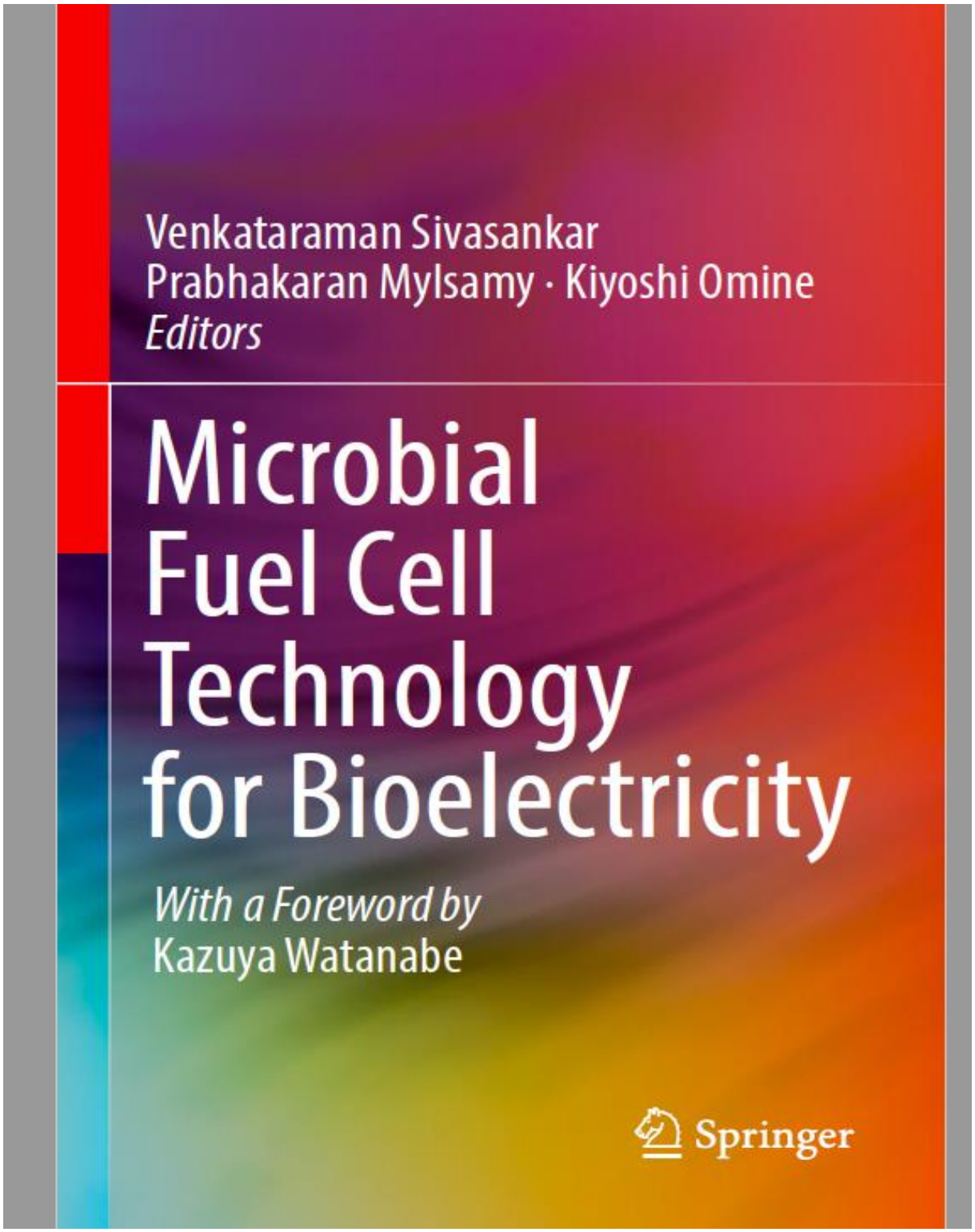
5.2. Development and assessment of intelligent models to predict the recovery efficiency of *Chlorella* sp. using coated and non-coated iron oxide magnetic particles

Using ANOVA, R^2 values of above 0.70, was obtained for all nanoparticles types (crude, chitosan and TSC), indicating the model's accuracy in describing relationships between inputs and outputs. Microalgae-magnet exposure time, nanoparticles concentration and microalgae – MION exposure time had the highest positive individual effects on crude, chitosan and TSC nanoparticles types, respectively. It was observed that interactions between microalgae and magnet exposure time and nanoparticles type had a positive impact on recovery efficiency of crude nanoparticles. The interactions between microalgae to nanoparticles exposure time and nanoparticles concentration positively impacted the recovery efficiency of chitosan coated nanoparticles. TSC coated nanoparticles were positively influence by the interactions between microalgae-magnet exposure time and nanoparticles concentration. The model predicted 90%, 88% and 95% removal efficiency of crude, chitosan and TSC coated nanoparticles, respectively. Experimental validation resulted in 90%, 83% and 75% for crude, chitosan and TSC nanoparticles, respectively. Regression analysis using artificial neural network gave R^2 values of above 0.70 for all nanoparticles types indicating the ability of ANN to be used as a virtual analytical tool. Sensitivity analyses revealed that minor changes in nanoparticles concentration has a significant impact on the recovery efficiency. This was indicated by the dose response multistage equation developed by the model.

5.3. Recommendations for future research

The following recommendations can be implemented to future research based on the findings obtained in this study:

- The use of miniature parallel raceway pond photobioreactors was a useful tool for bioprocess development as it was able to mimic the geometric configuration of large scale raceway ponds, resulting in laboratory scale work that was able to be correlated accurately to larger scales. The use of miniature reactors also decreases the tedious nature of the multiple experimentation required in research and development, increasing experiment efficacy.
- Assessment of various industrial wastes for microalgae cultivation using mathematical models could enhance knowledge of microalgal biomass and lipid production, and reveal a cost effective medium to use for commercial biodiesel production
- The effect of growth media, macro and micronutrients on fatty acid methyl ester (FAME) profiles could be investigated allowing for a full assessment of different media and their suitability for microalgal biodiesel production.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Contents

1.2.4 Production of Biodiesel from Biomass

The biodiesel is scientifically defined as alkyl esters of especially long-chain fatty acids. Such acids are derived from either animal fats or vegetable oils. This biodiesel has higher energy density, facilitates favourable combustion and has an enhanced lubricating property. Biomass is an energy resource which could facilitate the production of such biodiesel. Various reports explain the possibility of utilizing agricultural residues such as tea waste, bagasse of sugarcane and hazelnut, cotton waste, corn cobs and residues of olive seeds for the production of different valuable products including biofuels (Conesa et al. 2009; Blanco et al. 2002; Demiral and Sensoz 2008).

1.2.4.1 Production of Biodiesel from Microalgae

Microalgae are a large group of unicellular autotrophic microorganisms that carry out photosynthesis. These microorganisms can convert solar energy into chemical energy with a greater efficiency than plants due to their unicellular structure (Harun et al. 2010). This group of microorganisms has received significant attention as a promising biodiesel feedstock due to their widespread availability, ability to grow on nonarable land and being cultivable on wastewater or saltwater (Brennan and Owende 2010; Mata et al. 2010). Microalgae have higher growth rates and oil yields compared to crop plants with a productivity unit per area of 13,69 l/m² oil yield which is higher than the 0,6 l/m2 oil yield obtained from feedstocks such as soybean, coconut and palm oil (Chisti 2007). Microalgae are also a source of different value-added products such as carotenoids, β-carotene and chlorophyll which all have uses in food and pharmaceutical industries thereby increasing the economic potential of microalgal bio-refineries (Mata et al. 2010; Harun et al. 2010).

As seen in Table 1.4, microalgae can produce much higher amounts of biodiesel as compared to plant crops. Under optimal growth conditions, microalgae are capable of producing and accumulating hydrocarbons of between 30% and 70% of their dry weight (Kong et al. 2007); most commonly reported oil yields are between 20% and 50% of the biomass dry weight (Spolaore et al. 2006). Parameters that are important for microalgal cultivation include light, CO_2, temperature and pH (Faried et al. 2017).

Microalgal fatty acids can be divided in two groups based on the polarity of the molecular headgroup: polar and neutral lipids. Polar fatty acids consist of phospholipids and glycolipids. The neutral fatty acids consist of free fatty acids and acylglycerols, which are of interest in terms of biodiesel production (Cuellar-Bermudez et al. 2015). Acylglycerols are fatty acid esters that have been bonded onto a glycerol backbone and according to the number of fatty acid chains can be classified as triacylglycerols, diacylglycerols or monoacylglycerols (Halim et al. 2011). The content and lipid profile of microalgae are species dependent but can also be affected by culture conditions such as light intensity periods, nitrogen depletion, salinity

Table 1.4 Comparison of some sources of biodiesel

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]	Percent of existing US cropping area
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae[b]	136,900	2	1,1
Microalgae[c]	58,700	4,5	2,5

Chisti (2007)
[a]For meeting 50% of all transport fuel needs of the USA
[b]70% oil (by wt) in biomass
[c]30% oil (by wt) in biomass

stress, temperature change and pH (Richmond. 2008; Guschina & Harwood. 2006). Nitrogen limitation is a commonly used strategy in the increase of lipid and triacylglycerols in microalgae to produce biodiesel (Cuellar-Bermudez et al. 2015; Xin et al. 2010).

The ASTM definition of biodiesel is a fuel comprised of mono-alkyl esters of long-chain fatty acids derived from vegetable oils and animal fats (Hoekman et al. 2012). It can serve as alternative to diesel fuel that could be used in diesel engines, only if its physical and chemical properties conform to the international standard specification. The relevant standard in the USA is the ASTM Biodiesel Standard D 6571 (Knothe 2006). The European Union uses separate standards for biodiesel used in vehicles (standard EN 14214) and biodiesel used as heating oil (standard EN 14213) (Knothe 2006). In South Africa, the SANS 342:2016 is used to specify automotive diesel fuel blended with 5% of biodiesel.

Microalgae are rich in polyunsaturated fatty acids with four or more double bonds; for example, eicosapentaenoic acid which has five double bonds and docosahexaenoic acid which has six double bonds occur very commonly in microalgal oils; unfortunately such fatty acids and fatty acid methyl esters (FAMEs) are susceptible to oxidation during storage which reduces their acceptability for use as biodiesel (Chisti 2007). Triglycerides consist of three chains of fatty acids joined to a glycerol backbone (Halim et al. 2011); the process of transesterification replaces the glycerol molecule with methanol forming fatty acid methyl esters (Harun et al. 2010). The fatty acid profiles of microalgae are influenced by specific growth conditions such as nutrient levels, temperatures and light intensities; this can make it difficult to define a compositional profile for algal biodiesel (Hoekman et al. 2012). Ashokkumar et al. (2014) showed that the major fatty acids found in *Botryococcus braunii* were methyl palmitate and methyl oleate and the acid number of 0.49 mg KOH/g and a cetane number of 55.4 were both within the ASTM standards, whereas a study done by De Alva et al. (2013) using *Scenedesmus acutus* showed that the biodiesel produced from this microalgal species did not meet ASTM standards and

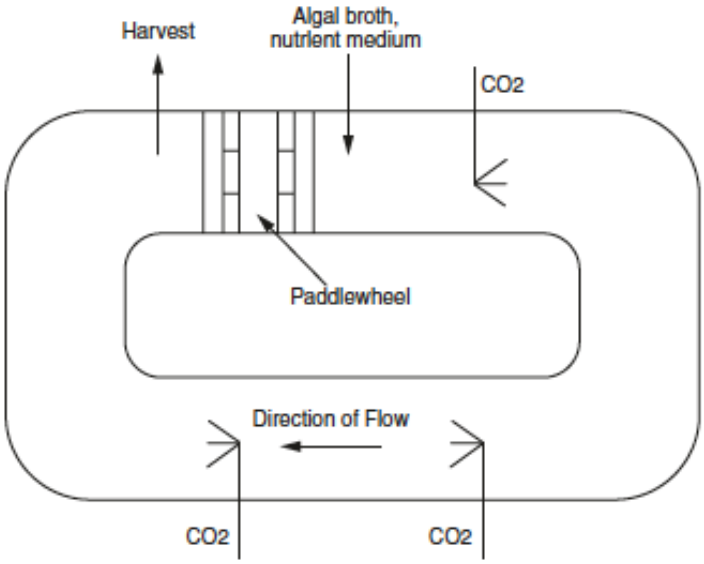

Fig. 1.4 Aerial view of an open raceway pond. (Adapted from: Brennan and Owende 2010)

the dominant fatty acids being palmitic acid, hexadecadienoic acid and linoleic acid were over the ASTM limit and the 1.08 mg KOH/g acid value did not comply with both ASTM D6751 and EN14214 standards (de Alva et al. 2013), highlighting the different fatty acid compositions found in different microalgal species.

The production of microalgal biomass is generally more expensive than crop cultivation since microalgal cultivation requires light, carbon dioxide, water and inorganic salts. Therefore, to minimize costs, cultivation should rely solely on freely available sunlight (Chisti 2007). Cultivation of algae is most commonly carried out in raceway ponds or photo bioreactors.

Raceway ponds are open circular ponds and can be in the form of natural waters such as lakes and lagoons or artificial ponds and containers. The configuration of raceway ponds is a closed loop oval recirculation channel that is typically 0.2–0.5 m deep, the depth is limited due to the penetration limit of light and an increase in depth would result in a decrease in the efficiency of the pond (Brennan and Owende 2010). A paddlewheel provides mixing and circulation in the pond, and cooling is achieved only by evaporation which often results in significant water losses; also there is no temperature control as temperature fluctuates seasonally (Chisti 2007) (Fig. 1.4). Carbon dioxide can be sparged at the bottom of the pond as a carbon source for autotrophic cultivation as the atmosphere only contains about 0.03–0.06% CO_2; therefore, it is expected that the mass transfer limitation could slow down the growth of the microalgae (Mata et al. 2010). Ashokkumar et al. (2014) used a 25m² open raceway pond in semi-continuous mode for the cultivation of *B. braunii*-TN101 resulting in a biomass productivity of 33.8 g m^{-3} day^{-1} (Ashokkumar et al. 2014). Raceway ponds are less expensive cultivation method but

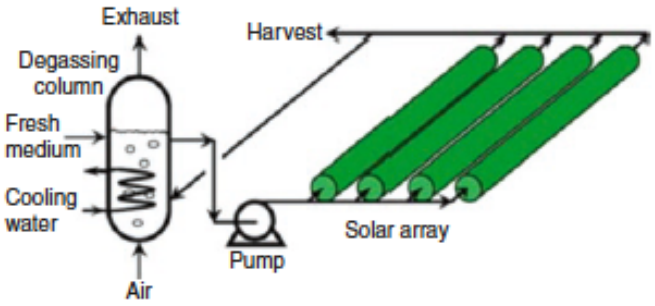

Fig. 1.5 Photo bioreactor schematic with horizontal tubular solar array. (Adapted from: Chisti 2007)

have several limitations such as high risks of contamination that cannot be completely prevented especially in open outdoor raceway ponds, and this can greatly affect the productivity of the algae. Poor mixing in the pond can also affect biomass concentration (Chisti 2007). Raceway ponds are currently used in research and in industry in the form of shallow big ponds, circular pond tanks and closed ponds; these are usually operated in continuous mode to prevent sedimentation (Harun et al. 2010).

The algal culture is introduced into the pond at a point after the paddlewheels and follows the shape of the pond with mechanical aeration from CO_2 spargers; culture is harvested before the paddlewheel point.

There are several types of photo bioreactors such as airlift, tubular, flat plate and vertical column photo bioreactors. The main advantage of photo bioreactors is that it allows for the maintenance of optimum growth conditions allowing for consistency in biomass and lipid productivity (Lam and Lee 2012). Sunlight or artificial light is captured in an array of transparent tubes that are made of plastic or glass, less than 0.1 m in diameter (Chisti 2007). These tubular arrays can be aligned horizontally, vertically, inclined or as a helix (Brennan and Owende 2010) (Fig. 1.5). The tubing configurations can influence a number of parameters in energy usage; horizontal tubing is more scalable but requires large areas of land (Halim et al. 2011). A degassing column functions in circulating the culture medium to the tubes and back (Chisti 2007). Zhu et al. (2010) cultivated *Chlorella zofingiensis* on piggery wastewater in tubular bubble column photo bioreactor resulting in a net biomass productivity of 1.314 g l^{-1} day^{-1}. *Scenedesmus acutus* was cultivated in a tubular photo bioreactor with six vertical cylinders housed in a greenhouse illuminated by solar light. A biomass of 113.7 g dry weight was obtained from 123.1 l of wastewater (de Alva et al. 2013). Feng et al. (2011) used four 2.2 L column aeration photo bioreactors for the cultivation of *Chlorella vulgaris* in artificial wastewater resulting in a cell concentration of 0.28 g/l (Feng et al. 2011).

Microalgal broth is circulated from the degassing column into the solar array of horizontal tubes and back to the degassing column.

1.2.4.2 Current Progress in Biodiesel Production

The most pressing and urgent need for biodiesel production is high lipid accumulation in microalgal strains. Naturally, microalgae produce different amounts of proteins and lipids, and these amounts vary strain to strain, but physiological stress has been used as a technique for driving metabolic fluxes towards biosynthesis of the target products.

Nitrogen limitation/deficiency has been found to be the most efficient stimulant for high lipid production in several different microalgal species (Mallick et al. 2016). A lipid content increase of 33% dry cell weight (dcw) in *Choricystis minor* was achieved under simultaneous nitrate and phosphate deficiencies (Sobczuk and Chisti 2010). Mallick et al. (2016) observed an increase in lipid content from 8% to 57% (dcw) under simultaneous nitrate, phosphate and iron limitations in the microalgae *Chlorella vulgaris*. A high light intensity together with nitrogen-depleted conditions has also been found to increase lipid contents to 54% (dcw) in *Nannochloropsis oceanica* IMET1 (Xiao et al. 2015). Nitrogen limitations can also be applied to municipal wastewater mediums; Robles-Heredia et al. (2015) achieved a 63% (dcw) lipid content in *Chlorella vulgaris* (Robles-Heredia et al. 2015).

Other methods that may be used to increase the accumulation of lipids in algae include metabolic engineering. In order to achieve optimal metabolic engineering for optimal lipid production, an in-depth knowledge and understanding of the microalgal lipid biosynthesis is required. This has not been extensively examined till this date (Mallick et al. 2016). Mass cultivation of microalgae in raceway ponds or enclosed photo bioreactors is another technique to increase the feasibility of microalgal biodiesel by producing algae biomass at a large scale for various other products including biodiesel (Mallick et al. 2016).

The cultivation of microalgae at a laboratory scale differs when compared to common cultivation methods at large scale. This poses a problem with scale-up studies concerning microalgae production. A miniature parallel raceway pond was developed in our laboratory to bridge the gap between laboratory-scale and commercial-scale production. The similar cultivation methods will be similar in the important configurational structures and therefore allow for easy translation of laboratory results to commercial results.

1.2.4.3 Challenges with the Commercialization of Biodiesel

The cost of microalgal biodiesel production at a commercial scale is one of the major drawbacks to the commercialization of this product. Factors that contribute to these costs are harvesting, drying and oil extraction and transesterification.

Harvesting

The solid-liquid separation is a critical step in the production of microalgal biodiesel. Though, it is most commonly achieved by centrifugation, this is an extremely cost-intensive step. Research has shifted to finding less energy and cost-intensive methods to separate microalgae from their growth liquid. The use of chemical flocculants such as $FeCl_3$ and $AlCl_3$ increases the speed of the sedimentation process but is not environmentally friendly; therefore, less toxic and cheaper flocculants need to be investigated (Mallick et al. 2016). In a study carried out by Knuckey et al. (2006), chitosan was found to be an excellent flocculant for freshwater microalgae such as *Chlorella* sp. and the marine alga *Isochrysis galbana*. Electro-coagulation-flocculation (ECF) is an effective method for microalgal flocculation resulting in faster flocculation at higher current densities; when using an aluminium node, the ECF method is effective at laboratory scale, but at large scale the use of much more power due to the higher current densities required is not feasible (Vandamme et al. 2011). Magnetic separation is a simple, easy, low energy consuming and low-cost separation technique that can be applied to separation of microalgae from growth medium (Yavuz et al. 2009). The separation is based on the movement of magnetically tagged particles in response to a magnetic field (Yavuz et al. 2009; Borlido et al. 2013). Fe_3O_4 particles have been successful in the separation of *Botryococcus braunii*, *Chlorella ellipsoidea* and *Nannochloropsis maritima* (Hu et al. 2013; Xu et al. 2011). Iron oxides are preferred due to their biocompatibility, strong paramagnetic behaviour, low toxicity and easy synthesis (Reddy and Lee 2013).

Drying

The drying of algal cells after harvesting is necessary for the storage of the feedstock as well as for downstream processing. The drying step can account for up to 30% of the total production costs of algal fuel therefore making this a big hurdle in the commercialization of algal biofuel (Grima et al. 2003). Generally, heat is used to dry algal biomass, but due to the high moisture content of microalgae, more heat is required to dry larger quantities of biomass relating to higher energy costs, and the high moisture content of microalgae makes sun drying very low in efficiency. Several artificial drying methods have been used in food industries such as drum, freeze, spray, oven and vacuum drying to name a few (Mallick et al. 2016). Solar drying is the most feasible drying method that can be employed at large scale but poses problems relating to large areas of land required for the drying of large amounts of biomass. There is a pressing need for the development of solar dryers that can overcome the issues of feasibility at a commercial scale.