Optimization of Biomass Production and Lipid Formation from *Chlorococcum* sp. Cultivation on Dairy and Paper-Pulp Wastewater

By

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PREFACE

The research contained in this thesis 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 campus, South Africa.

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

……..... …….....

Signed: Prof. Gueguim E. B. Kana Signed: Prof. Ademola Olaniran

Date: 27 July 2021 Date: 27 July 2021

DECLARATION BY SUPERVISOR

I hereby declare that I supervised this MSc student:

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Thesis Title: Optimization of Biomass Production and Lipid Formation from *Chlorococcum* sp. Cultivation on Dairy and Paper-Pulp Wastewater

Consultation took place between the student and me throughout the duration of this research. I advised the student to the best of my ability and approved the final document for submission to the College of Agriculture, Engineering and Science Higher Degrees Office for examination by the University appointed Examiners.

Signed:

……...	……...
Prof E.B. Gueguim Kana (Supervisor)	Prof Ademola Olaniran (Co-supervisor)
Date: 27 July 2021	Date: 27 July 2021

Declaration 1: Plagiarism

I, Emmanuel C. Ngerem, declare that:

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

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

(iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons;
(iv) This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

> a) Their words have been re-written but the general information attributed to them has been referenced;
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(v) Where I have used material for which publications followed, I have indicated in detail my role in the work;

(vi) This dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;

(vii) This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Emmanuel C. Ngerem (Student)

Date: 27 July 2021

Declaration 2: Publications

This dissertation involves a compilation of manuscripts. Each chapter is an individual entity prepared as per the journals' specifications hence some repetition between chapters has been inevitable. The first author (student) conducted all experimental work, data collection and manuscript preparation, guided by the second and/or third (supervisor) author. The * indicates corresponding author.

Chapter 3

Emmanuel. C. Ngerem[1], Gueguim. E.B. Kana[1], Ademola O. Olaniran[2]*. Optimization of mixed wastewater *Chlorococcum* cultivation for biomass production and lipid accumulation.

Chapter 4

Emmanuel. C. Ngerem[1], Gueguim. E.B. Kana[1], Ademola O. Olaniran[2]*. Microwave-assisted pre-treatment of microalgal biomass for the release of fermentable sugar: An optimization and enzymatic studies.

Signed: Emmanuel C. Ngerem (Student)

Date: 27 July 2021

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Abstract

The ever-increasing depletion of the dominant global form of energy (fossil fuels), calls for the development of sustainable and green alternative energy sources such as bioethanol, biohydrogen, and biodiesel. The production of the major biofuels relies on biomass feedstocks that are mainly derived from edible food crops and some inedible plants. One suitable feedstock with great potential as raw material for biofuel production is the microalgal biomass. Despite the tremendous attributes of microalgae as a source of biofuel, their cultivation requires huge volumes of freshwater, thus posing a serious threat to commercial-scale production and utilization of algal biomass. In this study, a multi-media wastewater mixture for microalgae growth was formulated and optimized. Moreover, the obtained microalgae biomass was pre-treated for reducing sugar recovery and was compared with previous studies on microalgae biomass pre-treatment.

The formulated and optimized mixed wastewater media for biomass and lipid accumulation was established using the simplex lattice mixture design. Based on the superposition approach of the potential results, numerical optimization was conducted, followed by the analysis of biomass concentration and lipid accumulation. The coefficients of regression (R^2) of 0.91 and 0.98 were obtained for biomass concentration and lipid accumulation models, respectively. The developed optimization model predicted optimal biomass concentration and lipid accumulation of 1.17 g/L and 0.39 g/g, respectively. It suggested 64.69% dairy wastewater (DWW) and 35.31% paper and pulp wastewater (PWW) mixture for biomass concentration, 34.21% DWW and 65.79% PWW for lipid accumulation. Experimental validation generated 0.94 g/L and 0.39 g/g of biomass concentration and lipid accumulation, respectively. The obtained microalgae biomass was pre-treated, enzymatically hydrolysed and subsequently assessed for reducing sugars. The optimization of microwave pre-treatment of *Chlorococcum* sp. was achieved using response surface methodology (RSM). Microwave power (100 – 700 W), pre-treatment time (1 – 7 min) and acid-liquid ratio (1 – 5%) were selected as independent variables for RSM optimization. The

optimum conditions were achieved at microwave power, pre-treatment time, acid-liquid ratio of 700 W, 7 min, and 32.33:1, respectively. These conditions provided the highest amount of reducing sugars of 10.73 g/L. Process optimization predicted reducing sugar yields of 11.14 g/L on microwave-assisted pre-treatment of 2.52% HCl for 4.06 min at 700 Watt. Experimental validation yielded reducing sugars of 15.67 g/L. These findings demonstrate that dairy wastewater and paper and pulp wastewater that could pose a serious environmental nuisance. They could be blended to form a suitable microalgae growth media, consolidating the potency of microalgae as a viable feedstock for fermentable sugars. Also, the outcome of this study supports the microalgal wastewater biorefinery concept, where wastewater remediation is coupled with bioenergy production.

Keywords: Wastewater cultivation, mixture design, lipid, biomass, nutrient removal, **microwave, *Chlorococcum*, raceway pond, fermentable sugar, modelling, optimization**

Chapter One
General Introduction

1.1. Global need for renewable energy sources

The global energy deficit due to rapid human population growth and the increasing need for an improved standard of living remains a critical issue worldwide (Abas *et al*., 2015). Besides urbanisation, escalating human population growth, which is estimated to reach 9.6 billion by 2050, stimulates industrialization, food, water, and energy insecurity (Ismail and Nizami, 2016). The major source of global energy, fossil fuels, which contributes about 86% of global demand, is non-renewable and rapidly depleted (Abas *et al*., 2015). Global fossil fuel reserves are predicted to be depleted by 2045 (Faried *et al*., 2017). Also, the consumption of fossil energy resources results in the emission of greenhouse gases such as CO_2, triggers global warming and climate change (Ouda *et al*., 2016). These situations, therefore, call for the generation of green alternative energy sources such as biofuels (Dutta *et al*., 2016). The production of major biofuels, such as biodiesel, biohydrogen, biogas, and bioethanol, has focused on using biomass feedstocks derived mainly from edible food crops and some inedible plants (Chye *et al*., 2018). Unfortunately, the cultivation of these crop-based biomass requires huge agricultural land area, therefore triggering food insecurity, habitat destruction, water depletion, and air pollution (Brennan and Owende, 2010; Chye *et al*., 2018). Also, the downstream processing of this biomass sometimes faces technical challenges in the form of conversion of the complex biomass to the desired components (Lee and Lavoie, 2013). Overcoming these barriers is challenging, yet this knowledge is required to achieve the implementation of renewable energy applications that can compete with fossil fuels. Such efforts include the use of non-food and renewable substrates like microalgae as feedstock in renewable energy production. Microalgal biomass has gained tremendous interest in recent times as one of the suitable alternative feedstocks for the recovery of various bioproducts (Figure 1.1), such as multi-valuable bioactive compounds and fermentable sugar for biofuel production (Odjadjare *et al*., 2017).

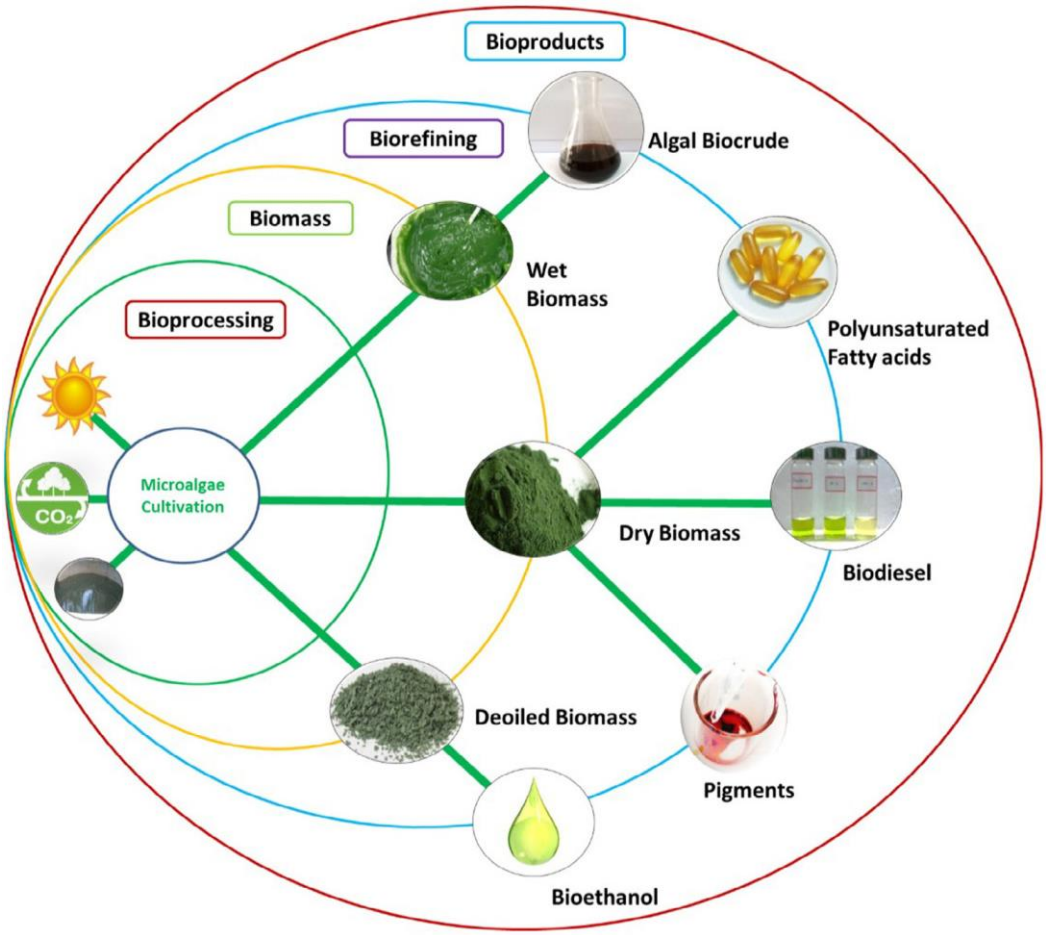

Figure 1.1: Diverse applications of microalgal biomass (Mohan *et al*., 2016).

1.2. Microalgae

Microalgae are unicellular organisms with unique features of phototropism as well as the ability to survive in water. These organisms have received increased scientific interest for their intrinsic capacity to produce various useful metabolic products (Matsuda *et al.*, 2016). Microalgae have better tolerance towards diverse environments and do not compete for arable lands (Cheah *et al*., 2016). These organisms possess high CO_2 sequestration ability and biomass productivity (Cheah *et al*., 2016). Various microalgal species like *Chlorococcum*, *Spirulina*, *Chlorella*, *Chlamydomonas*, *Dunaliella*, and *Scenedesmus* accumulate sufficient biomass concentration. This biomass is made up of lipids (20 – 80%), carbohydrates (10 – 40%), and proteins (10 – 50%) (Chojnacka *et al*., 2012; Yaakob *et al*., 2014; Suganya *et al*., 2016; Rehman & Anal, 2018). The lipid content is composed of triacylglycerol (TAG) and converted to biodiesel via transesterification (Chye *et al*., 2018). The carbohydrates, in the form of reducing sugars, are

usually transformed to bioethanol or biogas via the fermentation pathways or anaerobic digestion (Chye *et al*., 2018). In addition, biohydrogen can be generated through the biodegradation of starch recovered from the microalgal biomass. Besides biofuels, microalgal biomass can further be processed to proteins, polyunsaturated fatty acids, pigments and additives for fish and feeds (Odjadjare *et al*., 2017).

Despite the tremendous potentials of microalgae as a feedstock for bioenergy, it requires specific growth requirement and their cultivation requires huge volumes of freshwater, thus posing a serious threat to commercial-scale production and utilization of the biomass (Cheah *et al*., 2016).

1.3. Sources of wastewater for microalgae cultivation

Wastewater production is of major concern to industries, with the primary issues being biological oxygen demand (BOD), chemical oxygen demand (COD), total suspended solid (TSS); pathogenicity; pH and high nutrient concentration (Alam *et al*., 2007, Shete & Shinkar, 2013). Wastewater streams are generated from a wide range of activities such as manufacturing, sanitation, and heating and cooling operations (Kanu & Achi, 2011). The volume of effluents produced depends on regions and industrial operations. For instance, in developing nations like South Africa, approximately 69 Mm^3/year (69 000 Mℓ/year) of industrial effluent is produced annually (Cloete *et al*., 2010). The largest industrial wastewater producers are the pulp and paper (42%) and petroleum (25%) industries, with mining (10%) and power generation (7%) as the other major wastewater producers (Harrison *et al*., 2016) Untreated wastewater is unfit for reuse and when discharged into receiving environments, results to various environmental hazards. Industrial effluents composition differs depending on the type of industry, raw material processed, and generated pollutants (Mhlanga & Brouckaert, 2013). Also, some industrial wastewaters may be severely nutrient deficient, containing high concentrations of heavy metals (Mhlanga & Brouckaert, 2013). Interestingly, some industrial wastewaters contain macronutrients, including phosphates and nitrates and have been touted as cheap and abundant microalgal growth media (Daneshvar *et al*., 2019). Indeed, some wastewaters generated from

sources such as food processing industries, breweries and animal confinements possess a wide range of inorganic matter such as nitrogen, carbon and phosphorus. These wastewaters could support microbial/microalgae growth, representing highly profitable media for production of high value bioproducts. Consequently, their use in microalgae cultivation will offer both wastewater remediation opportunities and reduction in the channelling of scarce freshwater for microalgae cultivation (Zacharof, 2017). Wastewater with potential to support microalgae cultivation and growth include dairy wastewater, pulp-paper wastewater, fruit and soft drink processing wastewater and poultry wastewater.

The major process of dairy enterprise includes the transformation of raw milk into pasteurised and sour milk, cottage cheese, yoghurt, cream and butter products, milk and whey powders, condensed milk, lactose, as well as various kinds of desserts (Kolev Slavov, 2017). Unfortunately, dairy manufacturing has a significant impact on the environment, releasing large volumes of wastewater with high organic and nutrient content and extreme pH variations (Kolev Slavov, 2017). This industry uses water for all phases of production process, such as, cleaning, sanitisation, heating, cooling and floor washing (Esterhuizen *et al*., 2015). Contaminated water from dairy industries, including sanitary activities, covers about 50–80% of the overall water consumed in the dairy factory, and it is estimated that the wastewater generated is approximately 2.5 times higher than that of processed milk in units of volume (Kolev Slavov, 2017). Processing wastewater emerges from the cooling of milk in special coolers and condensers, as well as condensates from the evaporation of milk (Esterhuizen *et al*., 2015). Drying of milk generates vapours which condense and form the cleanest effluent, which can be reused or discharged together with storm water after a minimal pre-treatment (Kolev Slavov, 2017). Cleaning wastewater is usually derived from washing equipment which is in direct contact with milk or dairy products, milk spillage, equipment malfunction and operational errors (Kolev Slavov, 2017). This effluent comes in large quantities and is highly polluted, thus requiring intensive

treatment. Sanitary wastewater is produced from the lavatories, shower rooms, and can be used as nitrogen source for unbalanced dairy effluents (Kolev Slavov, 2017).

Likewise, pulp-and-paper industry consumes large volume of fresh water and produce high volume of wastewater during different stages of pulping and papermaking activities, presenting various detrimental impacts on the environment and poses a serious threat to the wild and human life (Ashrafi *et al*., 2015). This wastewater is released after various processes including wood debarking or chip making, pulp manufacturing and bleaching, paper manufacturing and fibre recycling (Ashrafi *et al*., 2015). This industry generates the largest amount (42%) of wastewater when compared to other industries. It was reported by Hagelqvist (2013) that an estimated 400 million tonnes of paper and paperboard was manufactured worldwide in 2012 with an estimated 30 to 90 billion tonnes of wastewater generated concomitantly, implying that 150 tonnes wastewater was generated for every tonne of paper produced (Hagelqvist, 2013). Paper and pulp effluent contain significant amount of organic and inorganic contaminants (Ashrafi *et al*., 2015), that may be slightly biodegradable or non-biodegradable due to the presence of complex organic substances such as chlorinated lignosulphonic acids, chlorinated phenols and hydrocarbons in the effluent (Harrison *et al*., 2016). A typical paper and pulp wastewater contain high COD values that ranges 700 mg per litre to 1200 mg per litre (2 100 – 3 600 mg C/l) and an average pH of 6 - 8 that does not present a huge detrimental effect to the environment (Harrison *et al*., 2016). The total suspended solids level is as high as 6 000 mg/L and do pose serious threat to the environment (Harrison *et al*., 2016). Also, it requires huge cost for wastewater treatment, disposal and reclamation of contaminated sites. However, it is deficient in phosphorous and nitrogen due to the low nutrient content in the paper mill wastewater (Harrison *et al*., 2016). Notwithstanding, some wastewater effluent contains very high organic matter, with estimated average carbon, nitrogen and phosphorus of 13200mg/L, 175mg/L and 57.1mg/L, respectively. Hence, these effluents could be optimally blended to obtained suitable mix, that can be use in microalgae cultivation.

The attention of many studies has been on the influence of a single effluent as microalgal growth media. Unfortunately, single wastewater may not provide enough quality and quantity of nutrients needed at the right proportions to sustain microalgae growth (Moreno-Garcia *et al*., 2019). Hence, there is a need for complementary wastewater blend approach, by mixing different wastewaters at optimized proportions for efficient microalgal growth and biomass production.

1.4. Microalgae growth requirements

The environmental factors require by microalgae for growth include light intensity, temperature and pH. Microalgae growth is directly proportional to light intensity and temperature. These increases continue until the optimum values are obtained. The cells can undergo photoinhibition over and above optimal light intensity requirements (Park *et al.*, 2011). For instance, *Chlorella vulgaris* requires a light intensity of 20 W/m^2 to achieve a high specific growth rate. Reduction in specific growth rate was observed with light intensity up to 40 W/m^2 (Yeh *et al.*, 2010). This decrease in specific growth rate was attributed to photooxidation (Yeh *et al.*, 2010). Temperature need of microalgae defers for different species. The impact of temperature on growth medium can affect the water ionic equilibrium, pH and oxygen solubility, consequently, influencing the microalgae growth (Park *et al.*, 2011). High oxygen content could negatively impact the photosynthetic ability of microalgae resulting in low biomass accumulation. While the pH of the medium as a factor in microalgae cultivation could influence microalgae biological processes such as photosynthesis, ammonia toxicity and inorganic carbon accessibility to the cells. Consequently, regulating microalgae growth and biomass accumulation (Mayo, 1997).

Similarly, various nutrient requirements at optimal proportion are required for microalgae cultivation. These growth requirements include nutrients (macro and trace elements), H_2O, and carbon source (often CO_2). Essential macronutrients for most microalgae include nitrogen, iron, phosphorous, and cobalt. On the other hand, trace elements require for microalgae cultivation are manganese, boron, molybdenum and zinc. Nutrient requirements for microalgae growth have

been studied in different regards such as eutrophication of water bodies but not as much in terms of microalgae cultivation using wastewater (Conley *et al*., 2009, Liu *et al*., 2009). Providing sufficient nutrients for microalgal growth is a significant challenge using wastewater as the nutrient source. Trace elements and macro-nutrient supplements, account for the major costs in the use of commercial Blue-Green-11 microalgae media (US Department of Agriculture, 2009). Microalgae, need sources of phosphorus, nitrogen, potassium, iron and sulphur for robust biomass accumulation. Phosphorous of approximately 0.03–0.06% is required in the cultivation medium to ensure algal growth. Nitrogen a limiting macronutrient required by microalgae to be fixed into ammonia, nitrates and similar compounds (Ryther and Dunstan, 1971). Moreover, microalgae require preferably ammonia, as it is less energetically demanding compared to other nitrogen sources (Inokuchi *et al*., 2002). Also, most microalgae preferably use chelated iron for its growth and fortunately, iron is more bioavailable compared to other required nutrients. In addition, sulphur plays a vital role in the electron transport chain, protein synthesis as well as lipid metabolism. To obtain the aforementioned nutrients, many of them may be provided by appropriate blend of nutrient-rich wastewater. Providing a cheap source of fixed nitrogen, iron and sulphur will be desirable for microalgae biofuel or bioproducts production, and the possibility of using wastewater to supply these elements is desirable from economic point of view (Berman-Frank *et al*., 2003). For a multi-media formulation, a suitable wastewater mixture will require a well-designed protocol for the optimization of essential nutrients for microalgal growth.

1.5 Microalgae biomass as feedstock

Microalgae biomass as a feedstock for bioethanol production has been challenged by the downstream processes such as harvesting and product extraction (Karemore & Sen, 2016). Microalgal cell often contains a thick cell wall, comprising of sporopollenin, algaenan, and other materials which are difficult to lyse (Dunker & Wilhelm, 2018). Various physicochemical techniques are widely used for the pre-treatment and extraction of intracellular microalgal

products. However, these methods are usually time-consuming and sometimes cause degradation or detrimental chemical changes to the products (Cravotto *et al*., 2008). Hence, fast and mild process conditions such as microwave-assisted pre-treatment techniques are being used.

Microwave-assisted pre-treatment has gained tremendous interest from researchers as a promising method for cell disruption and extraction of bioactive components of microalgae (Gilbert-López *et al*., 2015; Esquivel-Hernández *et al*., 2017). This pre-treatment technique possesses effective cell wall disruption potential with relatively low energy input, a rapid treatment time, and the utilisation of non-hazardous substances (Al Hattab *et al*., 2015).

1.6. Research motivation

A microalgae-based biofuel economy will contribute to the mitigation of environmental pollution, such as greenhouse gas emissions, and could help build a sustainable energy system. Microalgal biomass as a substrate for biofuels has gained tremendous interest in recent times as one of the suitable alternative feedstocks (Odjadjare *et al*., 2017). Despite the tremendous attributes of microalgae as a potential source of energy, their cultivation requires huge volumes of freshwater, thus posing a serious threat to commercial-scale production and utilization of microalgal biomass (Cheah *et al*., 2016). Besides, huge volumes of fresh water are being directed towards the operation of various industries such as dairy, paper-and-pulp, petroleum, and mining for a wide range of activities. These industries generate a lot of wastewaters that is released sometimes without adequate treatment into the environment, causing contamination of land, rivers and lakes. Interestingly, some industrial wastewaters contain macronutrients, including phosphates and nitrates and have been touted as cheap and abundant microalgal growth media (Daneshvar *et al*., 2019). Microalgal wastewater cultivation could support the microalgal wastewater biorefinery, where wastewater remediation is coupled with energy production (Daneshvar *et al*., 2019). The recovery of microalgal biomass with simultaneous wastewater remediation has environmental and economic. Studies have focused on the influence of a single effluent as microalgal growth media. Unfortunately, the use of only dairy or paper and pulp

wastewater (single wastewater source) may not provide enough quality and quantity of nutrients needed at the right proportions to sustain microalgae growth (Moreno-Garcia *et al*., 2019). This implies that mixing different wastewaters for microalgal cultivation at optimized proportions is crucial for efficient wastewater microalgal growth and biomass production. Hence, the need to evaluate the potential of various industrial wastewaters for microalgae cultivation and biofuel production.

Furthermore, the potential of microalgae biomass as a raw material for biofuel production has been challenged biomass pre-treatment and product extraction (Karemore and Sen, 2016). There is a dearth of knowledge on microwave-assisted pre-treatment of microalgal biomass compared to other physicochemical techniques that have been widely used for the pre-treatment of microalgal biomass (Cravotto *et al*., 2008). Microwave-assisted pre-treatment technique possesses effective cell wall disruption potential with relatively low energy input at rapid treatment time to release fermentable sugars for biofuel production (Al Hattab *et al*., 2015; Gilbert-López *et al*., 2015; Esquivel-Hernández *et al*., 2017). Hence, findings from this research could contribute to the cultivation of microalgae using industrial wastewater and recovery of fermentable sugars for biofuel production.

1.7. Aims and objectives

This study aimed to model and optimized industrial wastewater mixtures for *Chlorococcum* sp. cultivation. Additionally, the modelling of the release of reducing sugars from microwave-assisted chemical pre-treatment of cultivated microalgae was evaluated.

To achieve these goals, this study is guided by the following specific objectives:

(i) Assessment of dairy and paper-pulp wastewaters mixture for *Chlorococcum* sp. cultivation.

(ii) Modelling and optimisation of microwave-assisted acid (HCl) pre-treatment of the obtained microalgae biomass in objective (i) for the release of fermentable sugars.

1.8. Dissertation outline

This dissertation comprises a literature review chapter and two empirical chapters that are presented in research paper format. Each experimental chapter is independent, containing an introduction, materials and methods, results and discussion, conclusion, and references. The blending of industrial wastewater for microalgae cultivation and the pre-treatment of the cultivated microalgae for fermentable sugars is central to all the chapters.

Chapter 2 presents an overview of industrial wastewater generation. It examines the different industrial wastewater and microalgae cultivation for the production of bioproducts such as biofuel.

Chapter 3 focuses on the optimization of complementary dairy and paper-pulp wastewater mixture for *Chlorococcum* sp. cultivation. Moreover, the efficiency of the mixed wastewater media for *Chlorococcum* sp. are assessed for growth performance, lipid accumulation, nutrient utilization, and chemical oxygen demand (COD) removal.

Chapter 4 focuses on the development of a pre-treatment technique that is suitable for the release of fermentable sugars entrapped in microalgae biomass.

The final chapter, Chapter 5**,** integrates the main findings from the experimental chapters and highlights the significant conclusions derived from this study. Recommendations for future research are also provided in this chapter.

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Chapter Two
Literature Review

2.1. Fossil fuels consumption

Industrialisation, urbanisation, and socio-economic status are closely linked with the availability of energy. The primary source of this energy is fossil fuels (Dean, 2017). These dominant forms of energy contribute about 86% of the global primary demand with oil, gas, and coal, having a share of 36%, 27%, and 23%, respectively (Abas *et al.*, 2015). Unfortunately, fossil fuel resources are ever-depleting (Höök and Tang, 2013) since they are non-renewable and limited. This emanates from the simple fact that the accumulation of fossil fuels requires millions of years while mining of the deposits takes place rapidly, making it impossible for the rate of replenishment to meet up with the rate of extraction (Höök and Tang, 2013). It has been reported that the estimated 1688 billion barrels proved global oil reserves available as of the end of 2013 would be sufficient to meet only 53 years of production (Faloye, 2015). Another problem facing the dependence on fossil energy resources is their uneven distribution worldwide. As shown in Fig 2.1, the Middle East is the principal global fossil fuel supplier, having 47.9% of the total global oil reserves. This region is currently facing civil unrest, political instability, and terrorism that could pose a severe threat to world energy security (Faloye, 2015).

Increasing concerns for the depleting fossil fuel reserves and the resulting environmental concerns from fossil fuel consumption have resulted in the search for renewable and sustainable energy sources such as microalgal as feedstock for biofuel production (Odjadjare *et al.*, 2017). Many microalgae can produce substantial amounts of lipids and carbohydrates that can be converted to biofuel (Nautiyal *et al.*, 2014). Biofuels are mainly energy-rich chemicals produced directly via biological processes or obtained from biochemical transformation (Rodionova *et al.*, 2017). The production of eco-friendly energy sources such as biohydrogen, biogas, biodiesel, and bioethanol, primarily dependent on food crops and non-food lignocellulosic biomass as feedstock (Martín-Juárez *et al.*, 2017). However, the use of these feedstocks might be unsustainable. The use of food crops for bioproducts such as biofuel production requires

extensive arable land and raises food security concerns (Rodionova *et al*., 2017). On the other hand, pre-treatment techniques for non-food lignocellulosic raw materials require severe process conditions and huge costs (Martín-Juárez *et al*., 2017). These drawbacks have led to substantial research interest in seeking another potential feedstock for biofuel production.

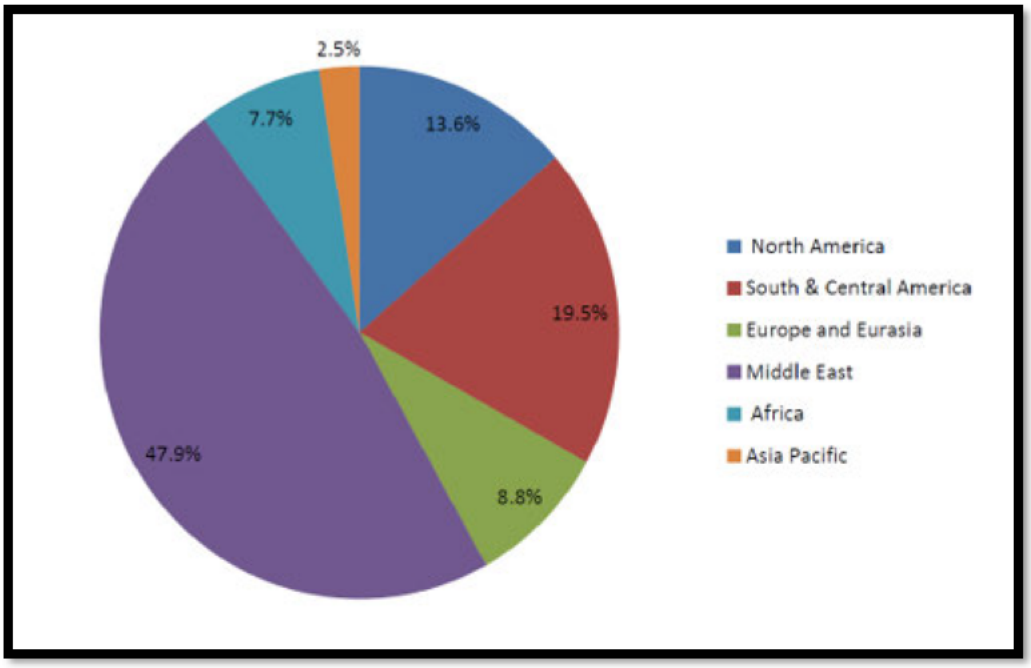

Figure 2.1: The distribution of global oil reserves (BP, 2014)

2.2. Biofuel and other valuable bioproduct production

Biofuels are usually energy-enriched products generated through biological processes or recovered from the biomass of living organisms such as microalgae and lignocellulosic plants (Rodionova *et al*., 2017). Generally, biofuels exist in gaseous, liquid, or solid form; and can be grouped into primary and secondary categories (Rathore *et al*., 2016). The primary category is the natural biofuels directly generated from plants, firewood, forest, and animal wastes (Rathore *et al*., 2016). The secondary biofuels are usually derived from plants and microorganisms. Significant examples of secondary biofuels include bioethanol, biodiesel and biohydrogen

(Rodionova *et al*., 2017). This class of biofuels may be further categorised into three generations based on the source of feedstocks.

The first-generation biofuels such as bioethanol, biodiesel, and biogas are predominantly produced using conventional food crops (Ullah *et al*., 2014). They may be generated either by starch fermentation of feedstocks such as corn, wheat, barley, and potato, or chemically by utilizing soybeans, rapeseed, coconut, and animal fats as feedstocks (Lee and Lavoie, 2013). The use of these feedstocks increases competition between food supply and biofuel production, resulting in socio-economic conflicts and food insecurity (Harrison *et al*., 2016), thus shifting researchers' attention towards the use of second-generation feedstocks (Dodo *et al*., 2017). The second-generation feedstocks are abundant globally and include mainly lignocellulosic materials like sugarcane bagasse, corn stover, and rice hulls (Dodo *et al*., 2017). Therefore, channelling them towards the production of biofuels such as bioethanol, biohydrogen, and biobutanol will not interfere significantly with food security (Dodo *et al*., 2017). Nevertheless, most second-generation feedstocks consist of recalcitrant cellular structures that usually require an expensive disruption process to break their lignin, cellulose, and hemicellulose clusters (Talebnia *et al*., 2010). Third-generation feedstock such as microalgae generates biomass, which can be processed for the recovery of bioenergy such as bioethanol and biodiesel (Gaurav *et al*., 2017). Also, the microalgal biomass requires relatively mild pre-treatment, presenting microalgae as a highly competitive feedstock for bioproduct production (El-Dalatony *et al*., 2017).

Aside production of biofuel form microalgae biomass as feedstock, microalgal biomass can further be processed to other bioproducts such as proteins, polyunsaturated fatty acids, pigments, phycobiliproteins, carotenoids, vitamins and additives for fish (Odjadjare *et al*., 2017). Various microalgae including *Chlorella*, *Dunaliella*, *Chlamydomonas*, *Scenedesmus*, *Spirulina* are known possess a vast amount (>45% of the dry weight) of starch and glycogen, used as substrates for value-added bioproduct production (Odjadjare *et al*., 2017).

2.3. Microalgae as a potential feedstock for valuable bioproducts

Microalgae are mainly photosynthetic microorganisms with better tolerance towards various environmental conditions, including nutrients, pH, and temperatures (Karemore and Sen, 2016). These versatile organisms possess a short generation cycle, with many species achieving a complete growth cycle within a few days (Chisti, 2008). Therefore, they can be cultivated and harvested year-round (Peralta-Ruiz *et al*., 2013; Zhou *et al*., 2013). Microalgae as a potential feedstock for biofuels have many advantages over other feedstocks. These include better tolerance towards diverse environments and, significantly, they do not compete for arable agricultural lands (Cheah *et al*., 2016). Also, microalgae accumulate reasonable biomass consisting of lipids, carbohydrates, and proteins, which could serve as major substrates for producing valuable bioproducts (Yaakob *et al*., 2014). From an economic point of view, most microalgae contain no lignin. Hence, it requires moderate and cost-effective pre-treatment techniques (Günerken *et al*., 2015). Furthermore, microalgae are endowed with enhanced CO_2 sequestration capability and reduce greenhouse gas emissions (Alam *et al*., 2012; Cheah *et al*., 2016). Microalgae possess complex biochemical composition with considerable lipid (20 – 80%), carbohydrate (10 – 40%), and protein (10 – 50%) contents (Suganya *et al*., 2016; Rehman and Anal, 2019). This attribute provides them with a great potential for bioconversion into biofuels and other value-added products (Odjadjare *et al*., 2017). As shown in Table 2.1, the biochemical composition of various microalgae species is strain specific. Prevailing growth conditions can influence these biochemical compositions. Another feature that influences microalgae cellular content is their physiological responses to biotic and abiotic factors, including temperature, photoperiod, and growth phase (Barkia *et al*., 2019). Moreover, features such as fast growth rate, rapid lipid synthesis capacity, and less cultivation land requirement qualify microalgae as a potential alternative feedstock to the conventional biodiesel feedstocks (Alam *et al*., 2012). Microalgae undergo photosynthesis like other plants but possess more solar energy converters due to various factors, including simpler cellular structure, easy rudimentary nutrients, water and CO_2 accessibility, and the ability to grow optimally between 20°C and 30°C

(Alam *et al*., 2012). Consequently, they can double their biomass within 24 h and complete their growth cycle within a few days (Chisti, 2008). This attribute makes microalgae desirable as feedstock for bioproduct production.

Table 2.1: Composition of microalgal biocomponents expressed in % dry weight

Microalgal species	Carbohydrates	Lipid	Protein	Reference
Anabaena cylindrica	25 – 30	4 – 7	43 – 56	Chye *et al*. (2018)
Chlorella pyrenoidosa	26	2	57	Chye *et al*. (2018)
Chlorella vulgaris	12 – 17	14 – 22	51 – 58	Chye *et al*. (2018)
Ellipsoidion sp.	14 – 18	14 – 20	39 – 61	Chye *et al*. (2018)
Porphyridium cruentum	40 – 57	9 – 14	28 – 39	Chye *et al*. (2018)
Pyrmnesium parvum	25 – 33	22 – 39	28 – 45	Chye *et al*. (2018)
Scenedesmus dimorphus	21 – 52	16 – 40	8 – 18	Chye *et al*. (2018)
Scenedesmus obliquus	10 – 17	12 – 14	50 – 56	Chye *et al*. (2018)
Spirulina maxima	13 – 16	6 – 7	60 – 71	Chye *et al*. (2018)
Spirulina platensis	8 – 14	4 – 9	46 – 63	Chye *et al*. (2018)
Synechoccus sp.	15	11	63	Chye *et al*. (2018)
Tetraselmis maculate	15	3	52	Chye *et al*. (2018)
Chloroccum sp.	32.5	–	–	Harun and Danquah, (2011)
Dunaliella bioculata	4	8	49	Chye *et al*. (2018)
Chlorella sorokiniana	10	10	43.6	Selvarajan *et al*. (2015)
Chlorella sp.	73.6	–	–	Zhou *et al*. (2012)
Chlorella protothecoides	–	15 – 58	–	Chye *et al*. (2018)
Micractinium sp.	41.5	28.1	14.3	Selvarajan *et al*. (2015)
Dictyosphaerium ehrenbergianum	40.3	34.1	17.3	Selvarajan *et al*. (2015)

2.4. Microalgae biocomponents

Interestingly, studies have reported microalgae's feasible and cost-effective capabilities as a superior alternative to the second-generation feedstocks for biofuel and bioproduct production (Gaurav *et al*., 2017). Microalgae utilization for third-generation biofuel production ensures efficient land utilization and has minimal effects on food security and sustainability (Vohra *et al*., 2014; Carneiro *et al*., 2017; Chye *et al*., 2018). As identified above, microalgal biomass contains; low lignin, requires relatively mild pre-treatment, significant carbohydrate unit and

easily saccharified (El-Dalatony *et al*., 2017). Microalgae are also rich lipids and fatty acids sources, with some species having oil content > 80% of their dry biomass weight (Chisti, 2008). Compared to other biodiesel feedstocks, microalgae have a better biodiesel production efficiency based on factors such as percentage oil content in dry biomass weight, oil yield per hectare per year, and land consumption. These attributes present microalgae as biomass with exclusive potential to be used as feedstock for bioproduct production (Chisti, 2008). These carbohydrate and lipid components can be converted to valuable bioproducts, thus increasing interest in the propagation and cultivation of microalgae (El-Dalatony *et al*., 2017).

2.4.1. Microalgae protein content

Proteins are complex-structured macromolecules with diverse morphological, physiological, and technological potentials (Barka and Blecker, 2016). Proteins play a crucial role in the microalgal cell structure and metabolism. They form a major component of the membrane and light-harvesting complexes, including diverse catalytic enzymes involved in photosynthesis (Barkia *et al*., 2019). They can be applied as single protein concentrates or in combination with processed foods (Barka and Blecker, 2016). Some microalgal species possess higher protein content compared with conventional protein sources (Table 2.2). For instance, the protein contents of *Chlorella* sp. and *Spirulina* sp. are 50 – 60 and 60 – 70% dry matter, respectively, compared to that of an egg (47%), skimmed milk (36%), chicken (19 – 24%), beef (17,4%). Moreover, microalgal protein can compete qualitatively with conventional plant or animal sources (Graziani *et al*., 2013; Barkia *et al*., 2019). Microalgal proteins constitute various essential amino acids that mammals are unable to synthesize. Furthermore, the amino acid profiles are well-structured like high-quality protein sources, such as egg albumin, lactoglobulin, and soy (Barkia *et al*., 2019).

Table 2.2: Comparison of protein contents of various food sources and microalgae

Food source	Protein content (% dry weight)
Beef	17.4
Fish	19.2 – 20.6
Chicken	19 – 24
Peanut	26
Wheat germ	27
Parmesan cheese	36
Soybean	36
Skimmed milk	36
Beer yeast	45
Whole egg	47
Chlorella sp.	50 – 60
Spirulina sp.	60 – 70

(Barkia *et al*., 2019)

2.4.2 Microalgae carbohydrate content

Carbohydrates are the main energy products of photosynthesis and carbon fixation metabolism (Chen *et al*., 2013). Microalgae accumulate high structurally diverse carbohydrates in the plastids as reserve materials such as starch or as the major constituent of their cell walls (Chen *et al*., 2013; Barkia *et al*., 2019). Starch constitutes a huge, insoluble α-(1-4) polymer of 10^5 – 10^6 glucose units with fewer α-(1-6) branches than glycogen (Martín-Juárez *et al*., 2017). The microalgal cell walls mainly consist of an inner cell wall and outer cell wall layers. Various microalgae species cell walls have a trilaminar outer layer. Some consist of a thin outer monolayer, while others lack an outer layer (Chen *et al*., 2013). The outer cell wall composition is species-specific but primarily consists of specific polysaccharides, including pectin, alginate, and agar (Martín-Juárez *et al*., 2017). The microalgae inner cell wall layer mainly consists of cellulose and hemicellulose (Chen *et al*., 2013). The inner cell wall layer could sometimes contain carbohydrates that are attached to proteins, thus forming glycoproteins (Barkia *et al*., 2019). Microalgae cellulose component is usually composed of multiple β-1-4 glucans, linked

by hydrogen bonds to form a complex and crystalline structure that is resistant to enzymatic degradation (Martín-Juárez *et al*., 2017). Hemicellulose is a form of polysaccharide consisting of various types of monosaccharides like xylose, galactose, arabinose, mannose, and rhamnose, and are connected by β-(1-4), and sometimes β-(1-3) glycosidic bonds (Cheng *et al*., 2015). The presence of cellulose-based cells and the ability to accumulate starch as the main carbohydrate source present microalgae as an attractive feedstock for biofuel production (Chen *et al*., 2013). During the pre-treatment process and microbial fermentation, both starch and most cell wall polysaccharides are transformed into fermentable sugars for subsequent bioethanol production (Chen *et al*., 2013).

2.4.3. Microalgae lipid content

Microalgae accumulate considerable lipid contents that form the major structural constituents of plasma membrane and energy reservoirs (Barkia *et al*., 2019). The microalgae lipid fraction exists mainly in the form of non-polar lipids, like triacylglycerols, and polar lipids, such as glycerophospholipids (Aratboni *et al*., 2019). Polar lipids contain primarily long chains of fatty acids, which are transformed into polyunsaturated fatty acids (PUFAs). These include docosahexaenoic, eicosapentaenoic acid, and docosapentaenoic acids (Aratboni *et al*., 2019). PUFAs play a crucial role in the formation of mitochondrial super complexes (Althoff *et al*., 2011). Polar lipids such as phosphoglycerides and glycosylglycerides, provide imperative structural support to the cell (Chen *et al*., 2018) and enhance the separation of various intracellular compartments (Gopalakrishnan *et al*., 2014). Moreover, these structural lipids have a significant function in the optimal maintenance of membrane fluidity, biosynthetic processes, and various intracellular organelles fusion events (Aratboni *et al*., 2019). Also, polar lipids act as important intermediates in cell signalling pathways and play a key role in sensing changes in the cellular environment (Gopalakrishnan *et al*., 2014; Aratboni *et al*., 2019). Non-polar lipids like triacylglycerols (TAGs) play a key role in energy storage activation. Also, TAGs can aid polar lipid production by transferring a special acyl group to trigger an adaptive rearrangement

of the membrane (Aratboni *et al*., 2019). The lipid content of many microalgal species has been reported, with this fraction representing about 20% – 50% of the dry biomass (w/w). (Chen *et al*., 2018). However, values ranging from 1% – 70% have also been documented (Barkia *et al*., 2019). Lipid accumulation in microalgae is dependent on the species and is greatly influenced by factors including cultivation system, nutrient availability, temperature, salinity, pH, and light intensity (Guschina and Harwood, 2006).

2.5. Potential of microalgae wastewater cultivation

A fundamental phase in microalgal bioproduct production is the cultivation and recovery of its biomass. Despite microalgae's tremendous attributes as a renewable feedstock source, their cultivation requires large volumes of freshwater (Cheah *et al*., 2016). Therefore, channelling the freshwater available for human consumption towards commercial-scale production poses a serious threat to microalgae cultivation sustainability (Odjadjare *et al*., 2017). Moreover, huge volumes of freshwater are already being devoted to the operation of various industries such as dairy, paper and pulp, petroleum, and mining industries for a wide range of activities, thus reducing freshwater availability (Musingafi, 2014). Indeed, these industries generate a lot of wastewaters that is released sometimes without adequate treatment into various environments, thereby causing further harm to the already scarce freshwater resources (Musingafi, 2014). Consequently, the need for suitable, efficient, and cost-effective wastewater management for adequate wastewater treatment and proper disposal. Wastewater management remains a significant concern to the industrial sector. The primary issues are chemical oxygen demand (COD), biological oxygen demand, pH, pathogenicity, and high nutrient concentration (Alam *et al*., 2009). The high nutrient concentration of industrial wastewaters is usually in the form of phosphates and nitrates (Alam *et al*., 2009). However, these pollutants are required as macronutrients for optimum microalgal growth (Daneshvar *et al*., 2019). Hence, the abundant industrial wastewaters can be exploited as potential microalgal growth media. Microalgae cultivation using wastewater produces fit-for-a-purpose water as non-negotiable products, thus,

coupling bioproduct production with wastewater remediation (Daneshvar *et al*., 2019). The environmental and economic benefits linked to this green technology establish a driving force to promote and consolidate the use of microalgae as a viable feedstock for biofuels and other bioproducts. However, information is scarce regarding the suitability of wastewater effluents either singly or in mixed form for microalgae cultivation. The reuse of these effluents is desirable from an economic and environmental point of view.

2.6. Wastewater generation

Huge volumes of freshwater are already being directed towards the operation of various industrial processes. Industries that use large volumes of freshwater include dairy, paper and pulp, petroleum, and mining industries (Harrison *et al*., 2016). High water demand and consumption lead to increases in the volume of wastewater generated. Wastewater has been identified as a potential solution to the large volumes of water required to cultivate microalgae. Moreover, channelling scarce freshwater, otherwise meant for human consumption, towards energy generation will negatively impact sustainability (Odjadjare *et al*., 2017).

Wastewater effluent characteristics are dynamic and dependent on several factors, including wastewater source, composition, and treatment applied before discharge into the environment. Agricultural wastewaters, such as those from the diary, pig slurry, poultry and others, are known to contain very high amounts of nutrients, while nutrient contents vary within municipal wastewater treatment plants (De Godos *et al*., 2009). Similarly, wastewaters from industries endanger aquatic lives, reduce human access to clean drinking water, and spread deadly diseases (Conradie *et al*., 2014).

Wastewaters may contain microorganisms, inorganic and organic compounds; nutrients including phosphates and nitrates, that warrant treatment prior to release to various waterbodies (Conradie *et al*., 2014). However, huge treatment costs have caused the discharge of unfit wastewater into the environment with potential socio-economic and environmental consequences (Naidoo & Olaniran, 2013). Effective use of wastewater could establish a link

between water consumption and management, resulting in the recovery of resources in closed-loop cycles, supporting the concept of a circular economy, where valuable nutrients and components are recovered and reused (Nizami *et al*., 2017). The potential of reusability of wastewater as an approach that integrates wastewater remediation and bioproducts production is desirable. Unfortunately, implementation of this approach has been hindered by various challenges such as high costs of facilities, technological know-how and insufficient information on wastewater beneficiation.

About 90% of wastewater produced globally are released without adequate treatment into aquatic environments such as rivers and lakes (Khan *et al*., 2017b). Sometimes, they are disposed in landfills and seep into nearby aquatic environments, resulting in contamination of rivers and seas (Musingafi, 2014). Water contamination endangers the lives of aquatic organisms and reduces human access to clean drinking water, that could stimulate the spread of deadly diseases (Naik, 2017). Wastewater properties is dynamic and are influenced by a range of factors such as wastewater source, location of treatment plant, treatment method, population density and climate (Odjadjare *et al*., 2017). Generally, these waste streams contain nutrients like phosphorus, potassium and nitrogen; pathogenic microorganisms and heavy metals including nickel, chromium, copper, mercury, lead and zinc (Iloms *et al*., 2020). Other constituents include organic pollutants such as polyaromatic hydrocarbons, polychlorinated biphenyls, pesticides, cleaning agents, cosmetics and medicines (Iloms *et al*., 2020). This organic matter, designated by chemical oxygen demand (COD), biochemical oxygen demand (BOD) and total organic carbon (TOC), alter oxygen levels, causing death of some aquatic organisms and air pollution due to the anaerobic decomposition (Zacharof, 2017). Extreme alkalinity or acidity, specified by the pH, impacts negatively on the solubility of ions and heavy metal content, thus escalating water toxicity that affect plant and animal growth (Zacharof, 2017).

Additionally, high salt content can raise sodium adsorption ratio of soil and prompt breakdown of soil structure, resulting in surface crusting, which in turn causes low infiltration and hydraulic

conductivity (Conradie *et al*., 2014). Moreover, high nutrient content such as N, K and P stimulates eutrophication and algal blooms, and exposes humans to nitrite and nitrate toxicity (Conradie *et al*., 2014). Furthermore, high salinity indicated by electrical conductivity and total dissolved solids affects the uptake of water by crops, the flora and wellbeing of fauna (Zacharof, 2017). Nevertheless, high total solids and total suspended solids, reduce light transmission, endangering the ecosystem's health and smothering its habitants (Conradie *et al*., 2014).

2.6.1. Municipal wastewater discharge

Municipal wastewater associated with domestic, or household activities represents one of major sources of wastewater. Domestic effluent is usually characterised by huge amount of organic matter, phosphate, nitrate, detergents, inorganic salt, and oil (Rathore *et al*., 2014). According to Huang and Gu (2010), the dominant components of the organic matter include fibre, proteins and sugar, with each contributing 20.64%, 12.38% and 10.65%, respectively. Due to large volume of highly concentrated domestic wastewater being discharged into drainage, river, or lake, this waste effluents present a serious challenge to the global water security. Apart from eutrophication, other problems partly attributed to improper municipal wastewater discharge include the rise of water treatment cost, reduced recreational value of water, health risks to livestock and humans, loss of oxygen and detrimental fluctuation of aquatic ecosystem (Wijaya and Soedjono, 2018).

2.6.2. Industrial effluents discharge

Wastewater discharge is of significant concern to industries, with the primary issues being BOD; COD; total suspended solid (TSS); pathogenicity; pH and high nutrient concentration (Alam *et al*., 2009). These waste streams are generated from various activities such as manufacturing, sanitation, and heating and cooling operations (Kanu and Achi, 2011). The volume of effluents produced depends on regions and industrial processes (Cloete *et al*., 2010). As illustrated in Table 2.3**,** the largest industrial wastewater producers are the pulp and paper (42%) and petroleum (25%) industries, with mining (10%) and power generation (7%) as the other major wastewater producers.

Industrial effluents composition differs depending on the type of industry, raw, materials processed, and generated pollutants (Mhlanga and Brouckaert, 2013). Based on the general classification into organic and inorganic (Naidoo and Olaniran, 2013), these wastewaters may possess a range of constituents, including high concentrations of readily biodegradable and non-biodegradable organic matter, inorganic matter, and potentially inhibitory or toxic substances (Mhlanga and Brouckaert, 2013).Organic matter (BOD, COD, TOD and TOC) contamination is increasing at an alarming rate, posing serious threat on fisheries, food security and livelihoods of poor rural communities that rely on freshwater as their primary water source (Connor *et al*., 2017). Some industrial wastewaters may be severely nutrient deficient such as paper and pulp, containing high concentrations of heavy metals (Mhlanga and Brouckaert, 2013). Indeed, waste streams generated from food processing industries, breweries and animal confinements possess a wide range of inorganic matter such as nitrogen, carbon, and phosphorus. These waste liquids support microbial and microalgae growth, thus representing highly potential media for producing high-value products. (Zacharof, 2017).

Table 2.3: Proportion of South African industrial wastewaters (Cloete *et al*., 2010)

Sector	Effluent Volume %
Pulp and paper industry	42.0
Petroleum industry	25.5
Mining industry	10.0
Food and beverage industry (animal-based and plant-based)	8.0
Power generation	7.0
Other industries (Organics-based & non-organics-based)	7.5

2.6.3. Dairy industry wastewater

The major process of dairy enterprise includes the transformation of raw milk into pasteurised and sour milk, cottage cheese, yoghurt, cream and butter products, milk and whey powders, condensed milk, lactose, as well as various kinds of desserts (Kolev Slavov, 2017). This industry uses water for all phases of production process (Esterhuizen *et al*., 2015). Contaminated water, including sanitary activities, covers about 50–80 % of the overall water consumed in the dairy

factory, and it is estimated that the wastewater generated is approximately 2.5 times higher than that of processed milk in units of volume (Kolev Slavov, 2017). Drying generates vapours that condense and form the cleanest effluent, which can be reused or discharged together with storm water after the minimal pre-treatment (Kolev Slavov, 2017). This effluent comes in large quantities and is highly polluted, thus requiring intensive treatment. Sanitary wastewater is produced from the lavatories and shower rooms and can be used as a nitrogen source for unbalanced dairy effluents (with lesser nitrogen content) in wastewater microalgae cultivation (Kolev Slavov, 2017). A typical dairy wastewater is whitish in colour, turbid and unpleasant (Shete and Shinkar, 2013). It is characterised by large variations in pH, TSS, BOD, COD, total nitrogen (TN), total phosphorus (TP), fat, oil and grease (Kolev Slavov, 2017). Dairy effluent contains suspended solids, and high organic components that contribute immensely towards their high BOD and chemical oxygen demand COD (Shete and Shinkar, 2013). Also, the effluents have low alkalinity of approximately 2.5 g/L expressed as $CaCO_3$ in milk permeate (Kolev Slavov, 2017). Study by Du Preez (2010) reported that dairy effluent contains 20 g-COD/ℓ (60 000 mg-C/ℓ) (unfiltered) and 10 g/ℓ COD (30 000 mg-C/ℓ) (filtered), 350 mg-N/ℓ total nitrogen and 40 mg-P/ℓ total phosphorous. Table 2.4 summarizes the volume, concentration and complexity for a typical primary dairy wastewater.

Table 2.4: Typical dairy industry wastewater (Harrison *et al*., 2016)

Total estimated effluent volume (ML/year)	4 547
Estimated average carbon content (mg/L)	45 000
Estimated average nitrogen content (mg/L)	350
Estimated average phosphorus content (mg/L)	40
pH	8.2

2.6.4. Pulp and paper industry wastewaters

Pulp and paper industry uses large volume of fresh water as well as release huge volume of wastewater during different stages of pulping and papermaking activities. The released wastewater poses various detrimental threats to the environment and human life (Ashrafi *et al*., 2015). Wastewater is released in various processes including wood debarking or chip making, pulp manufacturing and bleaching, paper manufacturing and fibre recycling (Ashrafi *et al*., 2015). This industry generates the largest amount (42%) of wastewater when compared to other industries such as dairy, poultry and fruit and soft drink processing industries. Paper and pulp effluent contain significant amount of organic and inorganic contaminants (Ashrafi *et al*., 2015), that may be slightly biodegradable or non-biodegradable due to the presence of complex organic substances such as chlorinated lignosulphonic acids, chlorinated phenols and hydrocarbons in the effluent (Harrison *et al*., 2016). Table 2.5 shows that a typical paper and pulp wastewater contains high COD values that ranges 700 mg per litre to 1200 mg per litre and an average pH of 6 - 8 (Harrison *et al*., 2016). The total suspended solids level could be as high as 6 000 mg/L (Harrison *et al*., 2016). However, it is deficient in phosphorous and nitrogen (Harrison *et al*., 2016).

Table 2.5: South African pulp and paper industry (Harrison *et al*., 2016)

Total estimated effluent volume (ML/year)	339 300
Estimated average carbon content (mg/L)	2850
Estimated average nitrogen content (mg/L)	9.04
Estimated average phosphorus content (mg/L)	1.30
pH	6-8
Total suspended solids (mg/L)	6000

2.6.5. Fruit and soft drink processing wastewater

Fruit processing including canning, juicing, winemaking, and fruit drying consumes large volume of water (7–10.7 m^3/tonne of raw produce) and generates wastewater containing, suspended solids, various cleaning solutions and softening or surface-active additives, and particulate organics including reducing sugars (Khan *et al*., 2015). In the soft drink processing

industry, the amount of wastewater generated at the production sites differs significantly, depending on the type of drink (carbonated drinks, bottled water or fruit, juices), annual production volume and parts of the entire process that are included on site (Isla *et al*., 2013). The average specific water intake for carbonate drinks, bottled water and fruit drinks are 1.6 l/l, 1.4 l/l and 2.2 l/l, respectively (Pollution Research Group, 2015). An estimated 3 700 ML/year and 4 070 ML/year of soft drink and fruit juice effluents, respectively, are produced (Table 2.6). Generally, the effluent is high in COD and TDS and contains nitrates, sodium, phosphates and potassium. The pH varies widely with different process levels and can fluctuate between 2.8 and 12.2; 6.1 and 11, for carbonated and fruit drinks, respectively (Harrison *et al*., 2016). The high pH range is evidence of cleaning with caustic soda (Pollution Research Group, 2015).

Table 2.6: Typical soft drink industry (Harrison *et al*., 2016)

Total estimated effluent volume (ML/year)	4 070
Estimated average carbon content (mg/L)	18 262
Estimated average nitrogen content (mg/L)	-
Estimated average phosphorus content (mg/L)	-
pH	2.8-12.2
Total suspended solids (mg/L)	-

Value not reported in literature (-)

2.6.6. Poultry wastewater

Poultry waste streams are highly complex, with the high concentration of organic load, high COD, proteins (70%) and suspended solids (15-30 mg/L) (Cristian, 2010). Poorly treated poultry abattoir wastewater is unfit for reuse if not properly recycled and when discharged into receiving environments, results to various environmental hazards such as eutrophication, groundwater contamination, nutrient leaching and land degradation (Matheyarasu *et al*., 2014). This category of wastewater brings about loss of aesthetic value observed in a majority of the disposal sites and industrial zones (Matheyarasu *et al*., 2014). Also, it requires huge cost for wastewater treatment, disposal and reclamation of contaminated sites (Matheyarasu *et al*., 2014). Likewise, Molapo (2009), noted that this effluent contains very high organic matter, with estimated average

carbon, nitrogen and phosphorus of 13200 mg/L, 175 mg/L and 57.1 mg/L, respectively. Furthermore, Molapo (2009), stated that the poultry abattoir wastewater possesses estimated COD (1300-7500 mg/L), BOD (700-4000 mg/L) and pH range of 7.0-7.2 (Table 2.7).

Table 2.7: Data on typical poultry abattoir industry (Harrison *et al*., 2016)

Total estimated effluent volume (ML/year)	5400
Estimated average carbon content (mg/L)	13200
Estimated average nitrogen content (mg/L)	175
Estimated average phosphorus content (mg/L)	57.1
pH	7.0-7.2
Suspended solids	fat, blood, feathers and faeces

Untreated or partially treated wastewater contains a large number of mineral nutrients, including ammonia (NH^{4+}), nitrates (NO^{3-}) and phosphates (PO^{3-}), which, if released into receiving water bodies, results in eutrophication of these natural water bodies as well as degradation of the environment (Odjadjare *et al*., 2017). Consequently, there is need for proper wastewater management.

2.7. Wastewater management

Despite the huge resources being invested on the conventional wastewater management processes, these investments have proved insufficient for the recovery of water to sustain the rapidly growing global population (Wang *et al*., 2012). Wastewater management is confronted by challenges such as deplorable infrastructure (Wang *et al*., 2014) and poor water quality monitoring (Re *et al*., 2011), especially in developing nations. For instance, Wang *et al.* (2014) reported the terrific state of the pump station in Kisumu district in Kenya, which caused the overflow of sewage at manholes upstream of the pump stations and direct discharge of the sewage to Lake Victoria (Wang *et al*., 2014). Also, in some parts of Africa, effluents disposal does not always comply with pre-treatment standard. To illustrate, the typical influent COD of municipal WWTPs is often higher than 2000 mg/L in many pond systems in Africa, while it is approximately 400 mg/L in many developed countries (Wang *et al*., 2014). Wastewater

management crisis is further intensified by sub-standard quality control in wastewater treatment plants, with only few parameters such as, pH, alkalinity and turbidity being evaluated (Re *et al*., 2011). Utilizing wastewater for microalgal cultivation can reduce the amount of freshwater required for large-scale microalgae cultivation. At the same time, excess nutrients in wastewater can be reduced at a lower cost with the added benefit of generating wastewater fit for discharge into receiving waterbodies. Mahapatra *et al.* (2014) reported using mixotrophic algal consortia for the bioremediation of municipal wastewater and simultaneous nutrient removal (as high as 90%) and lipid accumulation (reaching 28.5% of dry algal biomass).

2.7.1 Wastewater management and microalgae wastewater cultivation

Despite the potential of microalgae as a bioproduct feedstock, their cultivation requires huge volumes of freshwater, thus posing a serious threat to commercial-scale production and the utilization of algal biomass (Cheah *et al*., 2016). To address this challenge, wastewater is being employed as one of the approaches to minimise the amount of freshwater required for microalgae cultivation. Industries generate a lot of wastewaters that is sometimes released without adequate treatment into the environment, thus causing the contamination of land, rivers, and lakes (Musingafi, 2014). Consequently, there is a need for holistic wastewater management (Alam *et al*., 2007). This wastewater management approach could involve microalgal wastewater biorefinery, where wastewater remediation is coupled with energy production (Daneshvar *et al*., 2019). Although single wastewater as a microalgae cultivation medium has been implemented in microalgae cultivation, there is a lack of data in the literature on the application of mixtures of different wastewaters in microalgae cultivation. The reason for mixed wastewater microalgae cultivation is because single wastewater may not provide enough nutrients needed in the good quality and proportions to sustain optimal microalgae growth (Moreno-Garcia *et al*., 2019). Despite the merits of wastewater microalgae cultivation, several challenges still plague its implementation. These include obtaining single nutrient-rich wastewater microalgae, mixed

complementary wastewater media for microalgae cultivation, reusability, process optimization, scalability, and efficient microalgae pre-treatment.

2.8 Bioprocess Modelling and optimization

Wastewater such as industrial effluents contains varying amounts of macronutrients such as phosphates and nitrates that are required by microalgae for growth (Daneshvar *et al*., 2019). Wastewater, like paper and pulp effluent, is known to be independently deficient in many important microalgae growth nutrients. This single wastewater may not provide the required quality and quantity of nutrients at suitable proportions to sustain microalgae growth (Moreno-Garcia *et al*., 2019). The attention of past studies has been on the potential of single effluent (industrial, agricultural, or municipal) as a microalgal growth medium (Ummalyma and Sukumaran, 2014; Gurumoorthy and Saravanan, 2016). Nutrient limitation in single wastewater, as seen in paper and pulp industry wastewater, suggests the need for nutrient supplementation to ensure optimum growth performance (Slade *et al*., 2004). Hence, this supplementation could be achieved by blending paper and pulp wastewater with other nutrient-rich effluents. A suitable mixture design will be required to model and optimize different wastewater for microalgae cultivation. Various process mixture design strategies have been reported for such mixture purpose to obtain the most suitable and complementary wastewater mixture for microalgae growth (Nouadjep *et al*., 2019). A common tool used for such design, among others, is the Simplex lattice mixture design, a Surface Response Methodology (RMS). The technique implies commutation of set points; the relationship between factors and target variables, and then selecting the optimal configuration. The Simplex lattice mixture design creates the design by imposing a grid on the design scope. Afterward, the design is complemented by inner points and replicates of points to boost capabilities (Nouadjep *et al*., 2019).

Process optimization is an important factor in the development of economically feasible bioprocess, owing to their impact on the process (Faloye, 2015). Bioprocess optimization is vital to industrial production processes since slight improvements can be essential for the

commercialisation of a process such as industrial wastewater microalgae cultivation and microalgae pretreatment. The conventional method of one-factor-at-a-time is an approach that examines one variable singly, maintaining the other parameters constant. The result is represented on a graph to describe the effects of the single factor on the process output (Faloye, 2015). Moreover, the one variable at a time (OVAT) technique is usually not preferred because many influential factors may be involved in the process, and their interactive effects might not be accounted for (Faloye, 2015). Modelling and optimization techniques are being employed with the aim to improve the process efficiency and productivity. Due to the limitations of other optimization methods such as OVAT, statistical modelling and optimization techniques such as Response Surface Methodology (RSM) are progressively being adopted (Nikzad *et al*., 2015; Izmirlioglu and Demirci, 2016).

The RSM model allows for the recognition of many factors and their interactive influences on the process yield and has been reported in the modelling and optimization of various bioprocesses (Sanusi *et al*., 2020). RSM combines stepwise mathematical, statistical, and experimental techniques developed to improve and optimize processes. The merits of Response Surface Methodology include minimum experimental runs, shorter process time, closer confirmation of the output response to the objective requirements, evaluation of relations existing between experimental factors and the target responses (Talasila and Vechalapu, 2015). Experimental design optimization is of immense importance in pre-treatment processes due to the complexity and influence of many process factors. Hence, a suitable experimental design will be required to assess the effects of these parameters. Similarly, the model could provide valuable suggestions for the analysis, design, and operation at a large scale (Izmirlioglu and Demirci, 2016).

2.9. Microalgae biomass pretreatment

Microalgae accumulate starch in the cells, which can be processed to generate biofuel following an appropriate protocol. The harvested microalgae biomass can be disrupted to make the cells

susceptible to enzymatic attack (Rehman and Anal, 2019). This ensure the embedded carbohydrates that are locked up in the cells. Different physicochemical-mechanical pre-treatments such as bead milling, chemical, microwave radiation, and thermal methods have been employed in microalgae biomass pre-treatment (Martín-Juárez *et al*., 2017). Chemical pre-treatment entails the exposure of biomass to certain reagents to release fermentable sugars based on variables including reagent concentration, exposure time, temperature, and biomass concentration (Harun and Danquah, 2011; Martín-Juárez *et al*., 2017). Acid and alkali are the most widely used solvents for chemical pre-treatment. Chemical pre-treatment techniques are fast and relatively inexpensive, with acids showing higher sugar yields (up to 100%) compared to alkali (Harun *et al*., 2011). However, they have some disadvantages, such as equipment corrosion, generation of degradation compounds, and high operational and preservation costs when used in higher concentrations (Martín-Juárez *et al*., 2017). The pre-treatment step is usually followed by enzymatic liquefaction and simultaneous saccharification of pre-treated biomass. The hydrolysed starch is broken down into simple sugars via saccharification using the enzyme amyloglucosidase. Thereafter, the recovered sugars are fermented to produce biofuel such as ethanol by a suitable microbial strain (Odjadjare *et al*., 2017).

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Chapter Three
Optimization of mixed wastewater *Chlorococcum* sp. cultivation for biomass production and lipid accumulation

Abstract

This study modelled and optimized biomass and lipid accumulation using mixed wastewater for the cultivation of *Chlorococcum* sp. The simplex lattice mixture design was used to optimize the complementary wastewater mixture ratio (25:75, 50:50, 100:0) of dairy and paper-pulp wastewater. The coefficients of determination (R^2) of 0.91 and 0.98 were obtained for biomass concentration and lipid accumulation models, respectively, demonstrating the fitness of the models for process optimization. The developed optimization model predicted biomass concentration and lipid accumulation of 1.17 g/L and 0.39 g/g, respectively. It suggested 64.69% DWW and 35.31% PWW mixture for biomass concentration, 34.21% DWW and 65.79% PWW for lipid accumulation. Experimental validation generated 0.94 g/L and 0.39 g/g of biomass concentration and lipid accumulation, respectively. These findings demonstrate the optimal complementary mixture of DWW and PWW as an excellent media for *Chlorococcum* cultivation; a source of feedstock for bioproduct production.

Keywords: Wastewater cultivation, mixture design, lipid, biomass, nutrient removal

3.1. Introduction

The ever-increasing depletion of the dominant global form of energy fossil fuel calls for the development of sustainable and green alternative energy sources (Dutta *et al*., 2016). The production of the major biofuels relies on first-generation (such as corn and soybean) and second-generation (examples include, sugarcane bagasse and corncob) biomass feedstocks (Chye *et al*., 2018). Unfortunately, the cultivation of the first-generation edible food crops for biofuel production requires huge agricultural land area, triggering food insecurity, habitat destruction, water depletion, and air pollution (Brennan and Owende, 2010; Chye *et al*., 2018). Similarly, the downstream processing of the second-generation biomass faces technical challenges in the breakdown of the complex lignocellulosic structures to their respective components (Lee and Lavoie, 2013).

One suitable feedstock with great potential as a source of biofuel production for the replacement of the first- and second-generation feedstocks is the third-generation microalgal biomass. Microalgae have better tolerance towards diverse environments and do not compete for arable lands (Cheah *et al*., 2016). These versatile organisms possess high CO_2 sequestration ability and biomass productivity (Cheah *et al*., 2016). Microalgal biomass contains a reasonable amount of lipids, carbohydrates, and other valuable components (Chojnacka *et al*., 2012; Yaakob *et al*., 2014; Odjadjare *et al*., 2017).

Despite the tremendous potentials of microalgae as a source of biofuel, their cultivation requires huge volumes of freshwater, thus posing a serious threat to commercial-scale production and utilization of the biomass (Cheah *et al*., 2016). Moreover, channelling the scarce freshwater for human consumption towards energy generation will negatively impact sustainability (Odjadjare *et al*., 2017). Additionally, huge volumes of fresh water are being directed towards the operation of various industries such as dairy, paper-and-pulp, petroleum and mining for a wide range of activities. These industries also generate a lot of wastewaters that is released sometimes without adequate treatment into various environments, causing contamination of land, rivers, and lakes

(Musingafi, 2014). The major concern to the industrial sector wastewater disposal is the chemical oxygen demand (COD), biological oxygen demand, pH, high nutrient concentration, and pollution (Alam *et al*., 2007). Wastewaters, especially from industries, endanger the lives of aquatic organisms, reduce human access to clean drinking water, and stimulate the spread of deadly diseases (Conradie *et al*., 2014). The organic matter in wastewater, designated by COD, may alter oxygen levels, thereby causing the death of some aquatic organisms and air pollution due to anaerobic decomposition (Zacharof, 2017). Extreme alkalinity or acidity, specified by the pH, negatively impacts the solubility of ions and heavy metal, thus raising water toxicity that adversely affects plant and animal growth (Zacharof, 2017). Nevertheless, high nutrient content such as N, K and P stimulates eutrophication, algal blooms and exposes humans to nitrite and nitrate toxicity (Conradie *et al*., 2014).

Of the various industries, the paper-and-pulp industry has been presented as the largest wastewater producer, generating about 42% of the total industrial wastewater (Cloete *et al*., 2010). This industry consumes large quantities of freshwater and produces high volumes of wastewater during the different stages of pulping and papermaking activities (Ashrafi *et al*., 2015). Typical paper-and-pulp wastewater (PWW) is brownish in colour with an average pH of 6 – 8 (Singh *et al*., 1994; Harrison *et al*., 2016). It has high COD values that range between 700 – 1200 mg/L (Harrison *et al*., 2016). However, it is deficient in phosphorous and nitrogen (Harrison *et al*., 2016). This nutrient limitation in PWW implies that nutrient supplementation may be required to ensure the conventional wastewater treatment processes (Slade *et al*., 2004). Nonetheless, this supplementation could be achieved by blending paper and pulp wastewater with other nutrient-rich effluents such as wastewater from the dairy industry.

Another major source of wastewater is the dairy industry. This industry uses fresh water for all phases of its production processes, such as cleaning, sanitisation, heating, and cooling (Esterhuizen *et al*., 2015). Also, it generates large volumes of wastewater with high organic load, nutrient content, and a broad pH range (4.7 – 11) (Kolev Slavov, 2017; Daneshvar *et al*., 2019).

Contaminated water, including sanitary activities, covers about 50 – 80 % of the overall water consumed in the dairy factory. It is estimated that the wastewater generated is approximately 2.5 times higher than that of processed milk (Kolev Slavov, 2017). Regular dairy wastewater is whitish, with a turbid character and an unpleasant smell (Shete and Shinkar, 2013). It is characterised by large variations in biochemical oxygen demand (BOD), COD, total nitrogen (TN) and total phosphorus (TP) (Kolev Slavov, 2017).

Interestingly, industrial wastewaters contain macronutrients including phosphates and nitrates and have been touted as cheap and abundant microalgal growth media (Daneshvar *et al*., 2019). Wastewater cultivation of microalgae supports the microalgal wastewater biorefinery concept, where wastewater remediation is coupled with energy production (Daneshvar *et al*., 2019). This technological concept complements conventional biorefineries by providing additional resources to recover value-added products (Harrison *et al*., 2016). The environmental and economic benefits linked to the recovery of microalgal biomass with simultaneous wastewater remediation establish a driving force to promote and consolidate the use of microalgae as a viable feedstock for biofuels.

Various studies have evaluated microalgae cultivation using wastewaters with focusing on both wastewater remediation and cost-effective microalgae biomass production (Ummalyma and Sukumaran, 2014; Gurumoorthy and Saravanan, 2016; Arora *et al*., 2016; Paskuliakova *et al*., 2018). However, the attention of these studies has been on the influence of a single effluent as microalgal growth media. For instance, Ummalyma and Sukumaran (2014) evaluated the use of dairy wastewater to cultivate microalgae *Chlorococcum* sp. RAP13. Their study revealed a maximum biomass yield of 1.94 g/L, lipid accumulation of 42% and 93% of COD removal. Similarly, Gurumoorthy and Saravanan (2016) investigated biodiesel production using microalgae *Nannochloropsis oculata* cultivated in PWW. They reported a maximum biomass yield and lipid accumulation of 7.7 g/L dry weight and 42%, respectively.

Unfortunately, single wastewater may not provide enough quality and quantity of nutrients needed at the suited proportions to sustain microalgae growth (Moreno-Garcia *et al*., 2019). This

implies that the complementation of the cultivation medium by mixing different wastewaters at optimized proportions is crucial for efficient and sustainable microalgal biomass production. Formulation of microalgal growth medium involves different approaches, and these include single algae cultures and multi-algae media. A suitable multi-algae media will require a good design protocol for an appropriate blend is apt for microalgal cultivation. Various process mixture design strategies have been reported for such mixture purposes (Nouadjep *et al*., 2019). The 'one-factor at a time' method is not only time consuming and do not account for the interactive effects of the process inputs, thus, the obtained optimal process parameters are not reliable. On the other hand, the Response Surface Methodology (RSM) is an optimization tool that combines mathematical and statistical functions to ascertain the relationship between empirical factors and the observed outcomes. A suitable tool used for such RSM design, is the Simplex lattice mixture design. The technique implies commutation of parameters, the relationship between factors and target variables, and then selecting an optimal configuration. However, there is a dearth of literature on mixed wastewater media for microalgal cultivation using Simplex lattice mixture design. Knowledge of the appropriate wastewater mixture design will facilitate its application in an industrial scale. Thus, the main objectives of the present work are (i) to determine the most suitable and complementary wastewater mixture for microalgae growth, (ii) assess the lipid accumulation, and (iii) to evaluate the wastewater remediation efficiency of *Chlorococcum* sp cultivation.

3.2. Materials and methods

3.2.1. Microalgal species

The *Chlorococcum* sp. used in this study was kindly provided by the Discipline of Microbiology, University of KwaZulu-Natal, Westville Campus, South Africa. The enrichment medium was composed of 10% BG-11 solution, purchased from Sigma Aldrich (Germany), 1% trace metals prepared based on standard protocols (Mutanda *et al*., 2011), and 89% distilled water was used

to maintain the microalgae. The inoculum for the study was obtained under UV illumination (54.36 μmol/m^2s^{-1}) with shaking speed of 150 rpm for 14 days.

3.2.2. Wastewater sample collection

The dairy wastewater (DWW) used in this study was collected from the Fairfield dairy industry's wastewater storage tank in Howick, Pietermaritzburg, KwaZulu-Natal province, South Africa. The tank contains processing and cleaning wastewaters. Processing wastewaters emanated from heating and cooling activities. Cleaning wastewaters were derived from the cleaning of equipment that has been in contact with milk products, milk spillage, and whey processing. Also, it contained cheese, cream, and clarifier dairy waters coupled with dilute yoghurt wastewaters due to operational errors and equipment malfunctions.

Paper-and-pulp wastewater (PWW) was obtained from Mondi limited Richards Bay mill, KwaZulu-Natal province, South Africa. The mill consumes potable water of about 75.0 mL/ day and generates effluent of about 65 mL/day. The wastewater sample was collected from the secondary effluent treatment plant. The major constituent of this effluent is lignin and its derivatives (Sharma *et al*., 2014). Analysis of various physicochemical parameters of the wastewaters was conducted with the characteristics presented in Table 3.1.

The wastewater samples (20 L) were subsequently filtered using Whatman No.1 filter paper to remove solid particles. Thereafter, they were used in various proportions for preliminary screening to establish a wastewater mixture formulation ratio suitable for the microalgae's growth.

3.2.3. Screening of wastewater for the growth of microalgae

A preliminary screening was undertaken to assess the potential of the wastewater as a single or mixed medium and treated or untreated to support the growth of *Chlorococcum* sp. Treated wastewater was adjustment to pH of 7.1 and autoclave at 121 ℃ for 15 min. Flask cultivation experiments were carried out at controlled process set points of initial pH of 7.1, agitation speed of 150 rpm and cultivation period of three weeks. Different proportions of DWW and PWW

supplemented with BG11 medium were used to prepare *Chlorococcum* sp. cultivation media. The most suitable ratio was therefore modelled and optimized (Table 3.2).

3.2.4. Development of process model and optimization

The simplex lattice mixture design was used to generate eight experimental runs with varied input compositions based on the results from the preliminary study. The details of these inputs are shown in Table 3.3.

3.2.4.1. Experimental mixture design

The experimental mixture design aimed at obtaining the best mixed wastewater, while studying the influence of the individual wastewater on the hybrid wastewater. An appropriate approach for such as experimental conception based on two segments is the Design Expert software (Nouadjep *et al*., 2019). The simplex mix mixture design (Design-Expert software) creates the network by enacting a grid on the model scope. The design model is supplemented by inner loci and replicas of loci to improve evaluation capabilities (Brown and Brown, 2012). The executed experimental plan on a simplex mixture design network is represented in Table 3.3. The try-out components are PWW and DWW. The rating value of each component in the whole portion ranges from 0 to 100%. Regression analysis and analysis of variance (ANOVA) were carried out using design of experiment software. The aim of these analyses was to determine the shaping component that fundamentally impacts ($p < 0.05$) the mixing features. Step by step correlation was achieved continually and appropriate regression models were obtained, and the contour lines were obtained from the experimental data. The accurate outcomes for each of the two response variables were finally obtained for the two types of wastewater fractions. The ultimate pattern of the blended wastewater was analytically confirmed.

Table 3.1: Physico-chemical characteristics of DWW and PWW in both single and mixed

Parameter	Unit	Dairy wastewater	Paper and pulp wastewater
pH	-	2.87	6.94
Colour	-	White	Brown
COD	mg/L	876	955
TN	mg/L	736.25	562.25
TP	mg/L	27.07	1.20
Na	mg/L	237.73	1153.73
K	mg/L	27.73	68.40
Ca	mg/L	50.80	48.00
Mg	mg/L	5.47	18.13
Fe	mg/L	0.24	0.04
Cu	mg/L	0.01	0.004
Zn	mg/L	0.13	0.04
Mn	mg/L	0.04	0.76
Al	mg/L	0.15	0.52

TN-Total nitrogen, TP- Total phosphorous

Table 3.2: The selected mixtures for the optimization model

Mixture	BG11 (%)	PWW (%)	DWW (%)	BG11+PWW+DWW (%)
DWBG25 (A)	25	0	75	100
DWBG50 (B)	50	0	50	100
DWPWBG25 (C)	25	25	50	100
DWPWBG50 (D)	50	25	25	100
A+B+C+D	150	50	200	400

DWBG25: Dairy wastewater (75%) and blue-green algae 11 (25%); DWBG50: Dairy wastewater (50%) and blue-green algae 11 (50%); DWPWBG25: Dairy wastewater (50%), paper and pulp wastewater (25%) and blue-green algae 11 (25%); DWPWBG50: Dairy wastewater (25%), paper and pulp wastewater (25%) and blue-green algae 11 (50%)

Table 3.3: Simplex lattice design for the DWW and PWW mixture design

Run	A:DWW	B:PWW	Response 1: Biomass (g/L)	Response 2: Lipid yield (g/g)
1	50.00	50.00	1.07	0.40
2	50.00	50.00	1.05	0.40
3	100.00	0.00	0.88	0.50
4	25.00	75.00	0.68	0.38
5	100.00	0.00	0.90	0.50
6	75.00	25.00	1.16	0.40
7	0.00	100.00	0.91	0.36
8	0.00	100.00	0.91	0.36

Table 3.4: Analysis of variance (ANOVA) for biomass and lipid yield models

Source	Sum of squares	df	Mean squares	F-value	P-value	R^2
Biomass concentration model	0.14	3	0.046	14.30	0.0132	0.9147
Lipid accumulation model	0.022	3	7.322E-003	76.21	0.0006	0.9828

df: degree of freedom, F-value: Fisher-Snedecor distribution value, P-value: probability value, R^2: coefficient of regression

Table 3.5: Model's coefficient of estimates with standard errors

Component	Biomass concentration coefficient estimates	Biomass concentration standard error	Lipid yield coefficient estimates	Lipid yield standard error
A	0.88	0.040	0.50	6.897E-003
B	0.90	0.040	0.36	6.897E-003
AB	0.48	0.18	-0.15	0.031
AB(A-B)	2.61	0.46	-0.27	0.078

3.2.5. Photobioreactor configuration

The *Chlorococcum* sp was subsequently cultivated in a laboratory-scale transparent photobioreactor lit with four fluorescence bulbs for optimal illumination for 21 days (Fig.3.1). The photobioreactor was obtained from the Department of Microbiology, University of KwaZulu-Natal, Pietermaritzburg Campus, South Africa. The photobioreactor is made up of 15 wells, each having a maximum volume capacity of 1 L. The wells are uniformly separated into three rows, with each row having five wells. Each well consists of a length, breadth, depth, and working volume of 27 cm, 10 cm, 7.5 cm, and 800 mL, respectively. Mixing of the culture was provided by a submerged paddle (43 rpm) while the mixing speed was monitored and controlled using sensors and actuators.

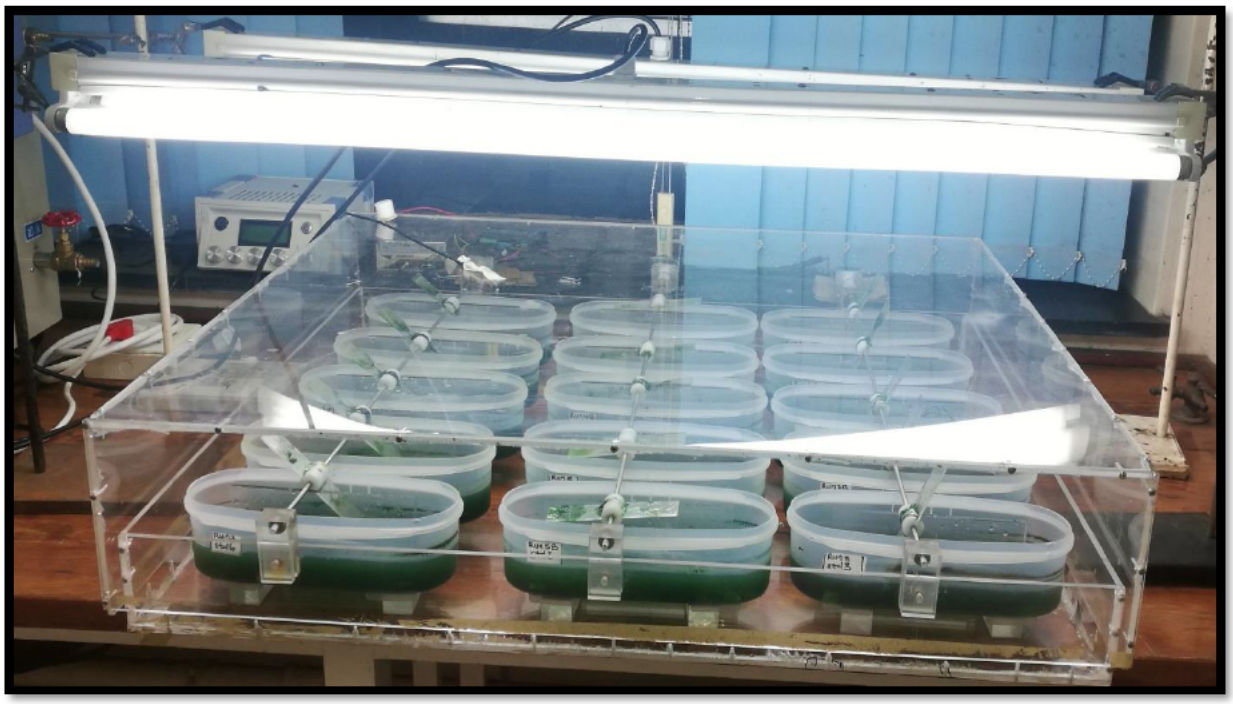

Figure 3.1: The microalgae photobioreactor (obtained from Buhlebenkosi Msweli research project)

3.2.6. Analytical methods

The physicochemical characteristics of both wastewater samples were determined using American Public Health Association (APHA) protocols (APHA, 1998). Chemical oxygen demand (COD) was measured using the Spectroqaunt® COD cell test kit (Merck, Darmstadt, Germany). The microalgae biomass concentration was obtained by measuring the optical density at 680 nm using SpectroVis® plus Spectrophotometer (Vernier Software & Technology, USA). The dry weight was then computed using a calibration curve, a correlation dependence on dry biomass weight as a function of optical density (Griffiths *et al*., 2011).

Then, the nutrient removal efficiency from the mixed wastewater was calculated using equation 1:

$$P_R = \frac{P_0 - P_1}{P_0} \times 100\% \qquad (1)$$

Where P_R is the efficiency of parameter removed, P_0 and P_1 are initial and final concentrations of the parameter, respectively.

The lipid content of samples was determined using the solvent extraction protocol described by Bligh and Dyer (1959). Dry microalgal biomass (1 g) with 80 mL distilled water was homogenised and heated at 2450 MHz for 5 min using a 1000 W capacity Samsung microwave oven (Model: ME9114S1, South Korea). Thereafter, 100 ml and 200 ml of chloroform and methanol, respectively, were added to the disrupted cells and vortexed for 30 seconds. Chloroform (100 ml) was further added to the mixture. Afterward, the mixture was homogenised for 30 seconds then, 100 ml of distilled water was added and vortexed for additional 30 seconds. The final mixture was filtered using a pre-weighed Whatman No.1 filter paper. After filtration, chloroform was evaporated from the layer containing chloroform and lipid. Then the lipid content was determined gravimetrically.

3.3. Results and discussion

3.3.1. Physico-chemical characteristics of the wastewaters

The physicochemical parameters of the dairy wastewater (DWW) and paper and pulp wastewater (PWW) used in this study are presented in Table 3.1. It was observed that both DWW and PWW were turbid with whitish and brownish colouration, respectively. Turbidity could affect light penetration and, subsequently, the growth performance of microalgae. Shete and Shinkar (2013) describe typical dairy wastewater as turbid, whitish, and unpleasant compared to the present observation. Similarly, Sharma *et al*. (2014) suggested that the brownish colour of PWW and the turbidity could be attributed to the presence of its major constituent, that is, lignin and its derivatives. The deep brownish colour of PWW compared to the white colouration of DWW could have resulted in lesser penetration of light. Microalgae require light for photosynthesis and growth. This could account for the lower biomass and lipid yield observed in the present study with the PWW. The pH of DWW and PWW were 2.87 and 6.94, respectively. These pH values could not support *Chlorococcum* growth. Microalgae growth, like other cellular activities, is known to be pH dependent. Significant *Chlorococcum* growth was observed after pH adjustment to 7.1. Moreover, higher concentrations of nutrients such as total nitrogen (TN) and total phosphorus (TP) were observed in DWW compared to PWW (Table 3.1). The high nutrient concentration in dairy wastewater could be ascribed to its high organic load and high concentrations of nitrogen, phosphorous, protein, and dissolved sugars (Ummalyma and Sukumaran, 2014). These macronutrients are required in the cultivation of microalgae. Besides these macronutrients, the wastewaters used in the present study also contained a variety of metal elements such as Fe, Ca, Mn, Mg, and Zn in small quantities (Table 3.1). The presence of these nutrients in microalgae cultivation influences the culture performance and consequently plays an important role in improving microalgae biomass concentration and lipid yield. Furthermore, both DWW (876 mg/L) and PWW (955 mg/L) contained considerable COD levels, with PWW having a greater concentration. This is comparable to observations in another study by Harrison *et al*. (2016), where they reported COD values between 700 – 1200 mg/L for PWW. They ascribed

these COD values to lignin and its derivatives that are not readily biodegradable and eventually increase organic loading in the wastewater (Sharma *et al*., 2014).

3.3.2. Effects of wastewater sterilization on microalgae growth

The cultivation of *Chlorococcum* using unsterilized and non-supplemented DWW and PWW were assessed. The results obtained showed that none of the unsterilized wastewaters could support the growth of the microalgae, both individually and when mixed. Instead, the growth of other organisms presumed to be bacteria, zooplanktons or fungi were observed. It has been previously reported that most wastewaters (unsterilized) contain bacteria, fungi, and zooplanktons that generate biotic pollution (Wang *et al*., 2013; Chiu *et al*., 2015). Biotic pollution usually inhibits the growth of microalgae (Chiu *et al*., 2015). Unlike the result of cultivation with unsterilized effluents, no contamination was observed when cultured with sterilized wastewaters. However, none of the individual nor mixed wastewaters were able to promote the growth of *Chlorococcum.* These results, therefore, highlight the need for supplementation of the sterilized wastewaters with microalgae formulated growth medium. As the wastewaters could not support the growth of the microalgae *Chlorococcum* individually and in mixed form, it became crucial that they be supplemented with formulated microalgae growth nutrients as a growth initiator.

3.3.3. Nutrient supplementation and wastewater blending

DWW and PWW were supplemented with BG11 to formulate a microalgal growth medium that supports *Chlorococcum* cultivation. The results of the mixtures are summarised in Table 3.2. It was observed that blending consisting of (75 and 25%), (50 and 50%), and (25 and 75%) DWW and BG11, respectively, supported the growth of *Chlorococcum.* The experiment for the cultivation of the microalgae using a mixture of PWW and BG11 requires a minimum of 50% formulated BG11 for the growth of the organism. These results present DWW as a better nutrient source than PWW for microalgae cultivation. The combination of DWW, PWW, and BG11 revealed that (50, 25, and 25%), (25, 50, and 25%), and (25, 25, and 50%) of DWW, PWW, and BG11, respectively formed suitable growth media for *Chlorococcum* cultivation (Fig. 3.4). In

Fig. 3.4, the growth pattern of *Chlorococcum* in blended wastewater media was similar to that of the conventional BG11 growth media without any significant difference. This shows the mixed wastewater consist of sufficient growth nutrient for *Chlorococcum* cultivation. Moreover, these observations demonstrated that the hybridity support *Chlorococcum* growth compared to the potential of the individual wastewater to support the microalgae cultivation. Thus, it is vital to ensure their optimum complementary blend to obtained maximum *Chlorococcum* growth due to individual nutrient deficiency. The obtained growth media shows the DWW and PWW can be beneficiated for microalgae cultivation. This is desirable from the economic point of view, as the cost of treating the wastewater is eliminated and simultaneously the wastewater is used for microalgae cultivation. Biomass feedstock for this cultivation can be pre-treated for biofuel production. The suitable microalgae growth blend was thereafter optimized using a mixture design model (simplex lattice mixture design) (Table 3.2).

3.3.4. Process optimization

The data obtained from DWW and PWW mixtures cultivation were used to establish the model equations for responses of biomass concentration and lipid accumulation. Analysis of variance (ANOVA) was carried out to examine the fitness of the models. The result is shown in Table 4. The regression coefficient (R^2) for biomass concentration and lipid accumulation models was 0.91 and 0.98, respectively, indicating that these models could account for 91% and 98% of variations in the observed data. The model's significance is further indicated by the low p-values of 0.0132 and 0.0006 as well as high F-values of 14.30 and 76.21 of the responses of biomass concentration and lipid accumulation, respectively (Table 3.5). The final equations of the models in terms of coded factors were:

$$\text{Biomass concentration} = 0.88 * A + 0.9 * B + 0.48 * A * B + 2.61 * A * B * (A - B) \quad (2)$$

$$\text{Lipid accumulation} = 0.5 * A + 0.36 * B - 0.15 * A * B - 0.27 * A * B * (A - B) \quad (3)$$

Where A and B are the various components of the mixture.

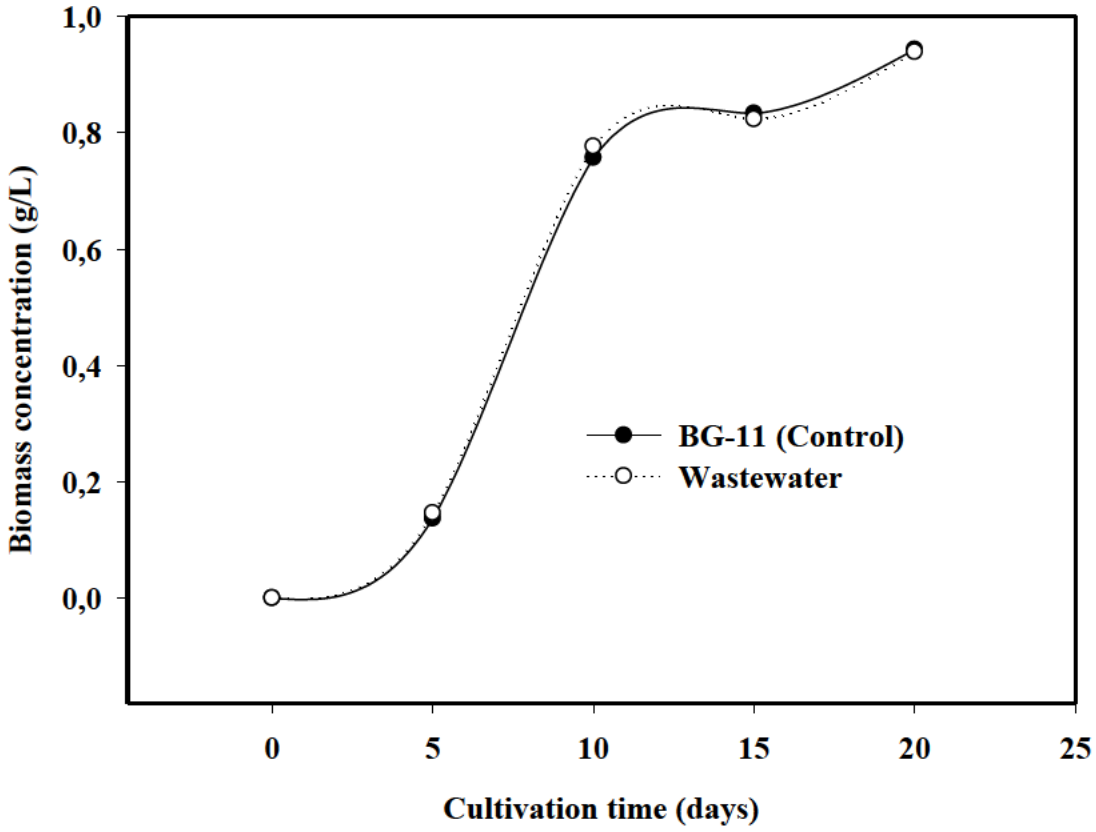

Figure 3.4: Growth curve of *Chlorococcum* cultivation in BG11 (control) and mixed DWW and PWW

3.3.5. Interactive effects of mixing design on biomass concentration and lipid yields

The biomass concentration and lipid yield obtained for each experimental run is shown in Table 3.3. The biomass concentration and the lipid yield ranged from 0.68 g/L to 1.16 g/L and from 0.36 g/g to 0.50 g/g, respectively. The process gave higher responses for biomass concentration and lipid yield when DWW and PWW were blended at their median values compared to using the wastewater individually and at high concentration of PWW. When the mixtures were blended at 50% each of DWW and PWW (Runs 1 and 2), biomass concentrations 1.07 and 1.05 g/L were obtained respectively. Similarly, with Runs 1 and 2 lipid accumulation of 40 g/g was obtained for both runs. Furthermore, the mixed wastewater containing 75% of DWW and 25% of PWW resulted to 1.16 g/L of biomass concentration. While, at a higher percentage of PWW (75%), the lowest biomass concentration of 0.68 g/L was obtained. The noticeable influence of DWW on

high biomass concentration in the *Chlorococcum* cultivation may be ascribed to the higher total nitrogen (736.25 mg/L) and total phosphorous (27.07mg/L) presence in DWW (Table 1), which is also bioavailable for *Chlorococcum* uptake and growth. Nitrogen and phosphorous are essential growth elements required for microalgae cultivation (Cai *et al*., 2013; Razzak *et al*., 2013). Hence, the higher biomass concentration in mixed wastewater with median (50%) DWW or high DWW (75%) was expected.

The interactive effects of the process inputs on biomass concentration and lipid yield using the two-dimensional plots are shown in Fig. 3.2 and Fig. 3.3 for the developed process models. Shown in Fig. 3.2 is the interactive effect of the mixture of DWW and PWW on biomass concentration. It was observed that DWW content percentages (0, 25, 50, 75 and 100%) had a linear relationship on biomass concentration obtained. When DWW content percentage increase with simultaneous decrease PWW content from 0 to 75% and 100 to 0% respectively, an increase in microalgal biomass concentration from 0.90 to 1.19 g/L (Fig. 3.2) was observed. An additional increase in DWW contents from 75 to 100% resulted in a sharp decrease in biomass concentration from 1.19 to 0.95 g/L.

Similar responses were obtained for the mixture of DWW and PWW on lipid accumulation. It was also observed that DWW content percentages had a direct correlation on lipid accumulation obtained (Fig. 3.3). As PWW content percentage decreases with concurrent increase DWW content percentage from 100 to 0% and 0 to 100% respectively, an increase in *Chlorococcum* lipid content was obtained from 0.36 to 0.50 g/g (Fig. 3.3). From these observations, wastewater blend that is highly concentrated in DWW will result in a higher biomass concentration. In the same manner, a higher proportion of DWW in the mixed wastewater considerably increased the lipid yield. These productivities can be attributed to the nutrient constituents of DWW, which were in sufficient quantities (Table 1) and bioavailable for *Chlorococcum* cultivation and growth (Cai *et al*., 2013; Razzak *et al*., 2013).

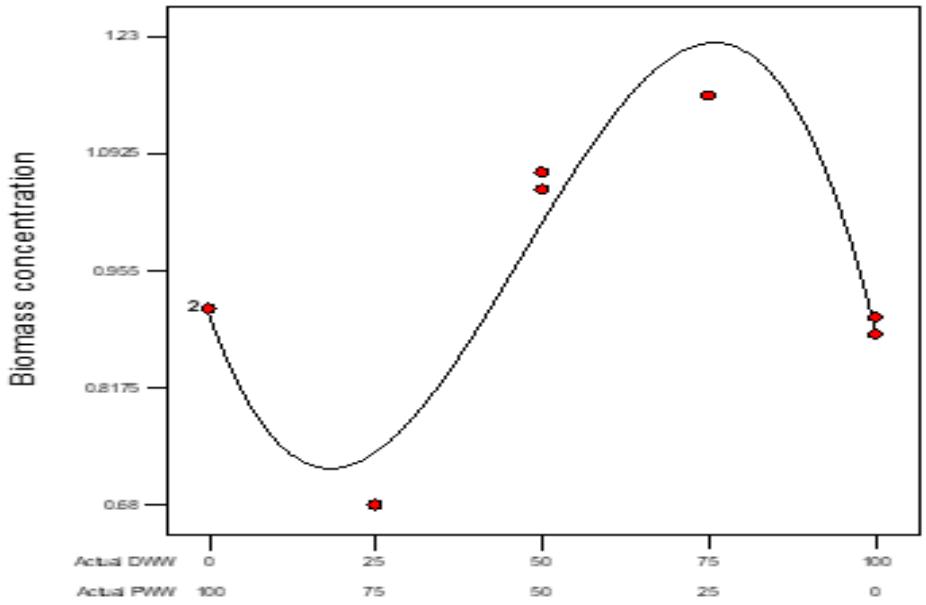

Figure 3.2: A 2-Dimensional plot showing the interactive effect of mixed DWW and PWW on algal biomass concentration

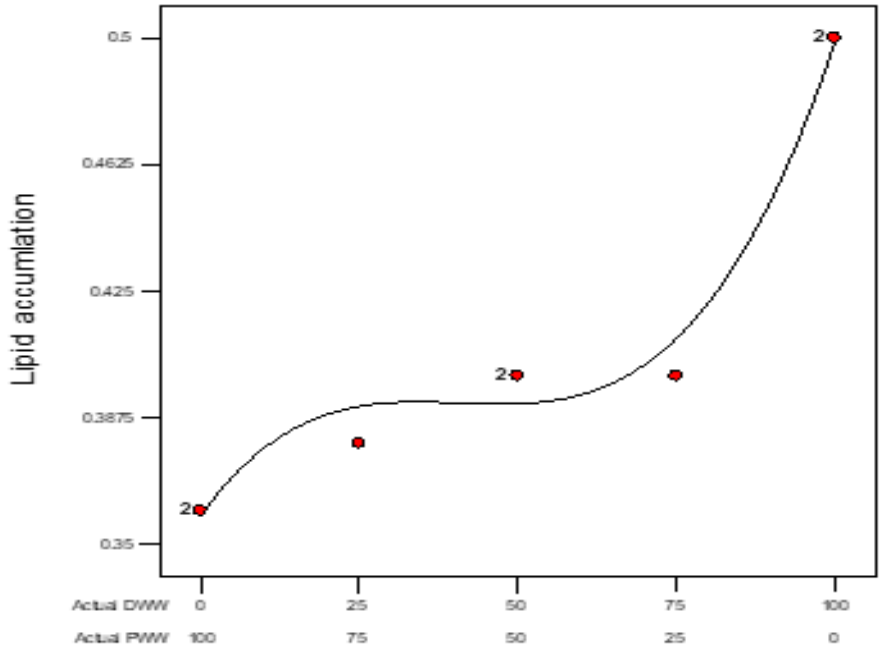

Figure 3.3: A 2-Dimensional plot showing the interactive effect of blended DWW and PWW on lipid yield

3.3.6. Validation experiments

The developed model predicted biomass concentration of 1.17 g/L for 64.69% DWW and 35.31% PWW mixture, while lipid accumulation of 0.39 g/g for 34.21% DWW and 65.79% PWW blend was predicted for the lipid model (Table 3.6). The results obtained in the experimental validation for biomass concentration and lipid accumulation were 0.94 g/L and 0.39 g/g, respectively (Table 3.6). The biomass concentration (g/L) increased rapidly in the first 5 – 15 days of cultivation and then progressed slightly until the 20^{th} day (Fig. 3.4). The microalgae *Chlorococcum* grow effectively in the DWW and PWW wastewater blended. Hence, mixed DWW and PWW could be a suitable substitute for commercial microalgae media. These observations highlight the potential of optimized wastewater mixtures in microalgae cultivation for large-scale biomass cultivation and lipid production. It has been previously reported that besides the type of strain, microalgae lipid accumulation could be affected by the type of cultivation medium, and solvent used for lipid extraction (Huerlimann *et al*., 2010, Abou-Shanab *et al*., 2011, Wang *et al*., 2014; Sharma *et al.*, 2016). The high lipid yield could be attributed to the higher proportion of PWW compared to the DWW in the formulated wastewater mixture. Paper and pulp wastewater are deficient in nitrogen (Harrison *et al*., 2016). Nitrogen limitation has been previously reported to enhance microalgae lipid accumulation (Vitova *et al*., 2015; Zhu *et al*., 2016). In the current study, the percentage composition of DWW and PWW (35% and 65%, respectively) forms approximately the ratio (1:2) of DWW and PWW blend used for *Chlorococcum* cultivation. In a related study, Gentili (2014) employed a different mixture of PWW with DWW and municipal wastewater. A lower lipid content (32%) was observed with a mixture of PWW and DWW (2:1) compared to the lipid content in the present study. Gentili (2014) reported that the higher lipid accumulation could be linked to nitrogen limitation as all the available nitrogen was used up after a few days of cultivation. High nitrogen concentration has been revealed to inhibit lipid production in microalgae (Abou-Shanab *et al*., 2011). However, this factor is usually determined by the nitrogen source (Kim *et al*., 2016). It has been noted that

various microalgae accumulate higher lipid concentration with ammonium nitrogen sources compared to nitrate, nitrite, and organic forms like yeast and urea (Kim *et al*., 2016). These nitrogen sources are usually reduced to the ammonium form via various pathways before assimilation into amino acids (Cai *et al*., 2013).

Additionally, the high TN content in the DWW used in the present study could have contributed to the high lipid content. The TN is composed mainly of NH_4-N, and the assimilation of ammonia into amino acid requires less energy than other nitrogen sources and has, therefore, been suggested as the preferred nitrogen source by microalgae (Chen *et al*., 2011; Ruangsomboon, 2015). Furthermore, a study by Sharma *et al.* (2016) investigated the effectiveness of various culture media, including Blue green-11(BG-11), Fog's medium, Bold basal medium, and Basal medium on microalgae growth and lipid productivity. Although *Chlorococcum* cultivation in BG11 resulted in high lipid accumulation (38 % dry biomass weight), it was 1.03-fold lower than this study's outcome. This supports the fact that establishing the ideal wastewater formulation is a feasible way to promote the integration of remediation and bioenergy production using various microalgal species. Also, the mixture of DWW and PWW in the ratio 1:2 can be considered a suitable replacement for BG11 growth medium in the cultivation of *Chlorococcum* for cost-effective optimization of lipid production.

Table 3.6: Outcomes of experimental validation

	Components (%)		**Response values**	
Response	**DWW**	**PWW**	**Predicted value**	**Observed value**
Biomass concentration	64.69	35.31	1.17 g/L	0.94 g/L
Lipid accumulation	34.21	65.79	0.39 g/g	0.39 g/g

3.3.7. Nutrient and COD removal efficiencies

In this study, nutrients such as TN, NH_4-H, phosphorus (P), metals (magnesium and calcium) and trace elements (zinc, manganese, copper, and iron) were reduced in biomass concentration, and lipid accumulation mixed wastewater media. The nutrient profiles in both experimental mixtures during the 20-day cultivation are presented in Fig. 3.5 – 3.9. The variation in TN concentration is depicted in Fig. 3.5. The total nitrogen removal efficiencies of 30.86% and 10.13% were obtained for biomass concentration and lipid accumulation models, respectively. High nitrogen uptake implies the nitrogen or its complex present in the mixed wastewater was bioavailable for *Chlorococcum* sp growth. The observed results were higher when compared to a previous report by Ding *et al*. (2015), where they noted that no significant change in TN concentration was observed when a microalgae sp. was cultivated in DWW. The study inferred the inability of the microalgae to remove nitrogen could be due to large amounts of complex organic nitrogen sources in the DWW. On the other hand, the observed TN removal efficiency observed in the present study was lower compared to those recorded in previous studies. For instance, Yao *et al*. (2015) evaluated the TN removal efficiency in the cultivation of *Chlorella sorokiniana* and *Desmodesmus communis* using a 1:3 ratio of swine wastewater to secondary treated municipal wastewater with 5% CO_2. The study recorded high TN removal efficiencies of 88.05% and 83.18% for *C. sorokiniana* and *D. communis*, respectively. Total nitrogen removal from wastewaters via nutrient assimilation depends largely on the nitrogen source. Studies have shown that microalgae prefer ammonia and simple organic nitrogen such as yeast extract and urea that require less energy for reduction to ammonia (Cai *et al*., 2013; Razzak *et al*., 2013). Unlike yeast extract and urea, complex organic forms of nitrogen require a huge amount of energy to reduce to ammonia; thus, microalgae uptake of nitrogen from these sources is difficult (Perez-Garcia *et al.*, 2011). Therefore, it could be suggested that the wastewater blends used in the current study contained some complex nitrogen sources that *Chlorococcum* sp. could not easily assimilate.

The observed changes in NH_4-N concentration during the cultivation of *Chlorococcum* for biomass concentration and lipid accumulation are presented in Fig. 3.6. From Fig. 3.6a, it is evident that there was a rapid NH_4-N reduction within the first five days of cultivation for biomass concentration. It could be deduced that within the first five days, the *Chlorococcum* sp. required a nitrogen source with lower energy demand for its acclimatisation and growth in the wastewater environment. This observation aligns with the studies by Chen *et al*. (2011) and Ruangsomboon (2015). They demonstrated that most microalgae prefer ammonium compound as a nitrogen source as it does not require a huge amount of energy for assimilation into amino acids. After the first five days, NH_4-N removal became slower progressively until day 15 of cultivation, reaching the maximum removal efficiency of 87.21%. The decline in NH_4-N removal could be due to the *Chlorococcum* sp. having taken up its optimum NH_4-N requirement, thus becoming adapted to the new environment. Towards the end of the cultivation period, a slight increase in NH_4-N concentration was noticed. This increase could be an indication of a reduction of other nitrogen sources to NH_4-N. This observation is consistent with Cai *et al*. (2013) study, who noted that microalgae usually reduce other nitrogen sources to ammonia before assimilation into amino acids. Like the biomass concentration model, there was a rapid NH_4-N reduction within the first five days of cultivation of *Chlorococcum* sp. for lipid accumulation (Fig. 3.6b). However, the NH_4-N removal continued progressively even after the 15 days of cultivation when no NH_4-N was detected in the medium, resulting in 100% NH_3-N removal efficiency. The NH_4-N removal observed in the present study is comparable with previous studies. In a study by Wang *et al*. (2010), up to 100% NH_4-N removal efficiency was observed when a *Chlorella* sp. was cultivated in a medium supplemented with digested dairy manure. In another study Gentili (2014), microalgal cultivation in mixed municipal, dairy, pulp and paper wastewater for biomass and lipid production resulted in 99% NH_4-N removal efficiency.

The depletion in P nutrient is illustrated in Fig. 3.7. The changes in P concentration in both biomass concentration and lipid accumulation cultivation mixtures followed similar removal

patterns, with P being efficiently removed. However, higher removal efficiency of 84.62% was observed in the lipid accumulation culture medium, while in the biomass concentration medium, 59.34% of P was removed. It could be suggested that P removal efficiencies obtained in the present study were due to a considerable amount of P that was utilised by *Chlorococcum* sp. for growth and lipid accumulation. Phosphorus represents one of the essential elements in adenosine triphosphate (ATP), deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and biomembranes. Furthermore, it is required for various cell metabolisms such as chlorophyll synthesis and fatty acid metabolism (Luo *et al*., 2019; Ren *et al*., 2019). Other nutrients that were considerably reduced include Ca and Mg (Fig. 3.8). In the biomass concentration media, 78.91 and 92.11% removal efficiencies were recorded for Ca and Mg, respectively. On the other hand, Ca and Mg were reduced by 52.31 and 90.24%, respectively, in the lipid accumulation culture medium. Both Ca and Mg play essential roles in microalgal chlorophyll synthesis and growth (Luo *et al*., 2019). Hence, the observed high removal efficiencies of these macronutrients by the *Chlorococcum* sp. could have contributed to the substantial biomass and lipid yields achieved in this study. This is in line with observations by McGinn *et al*. (2012), who documented that Mg ion could enhance the activity of the critical enzyme for fatty acid synthesis (acetyl-coenzyme A carboxylase) and stimulate the synthesis of neutral lipid in microalgal cells. Moreover, the profiles of trace elemental components of the mixed wastewater media are represented in Fig. 3.9.

The removal patterns for the trace elements were similar in both media, with significant removal efficiencies. For instance, a 100% removal efficiency was observed with Zn and Mn in both media. The observed removal efficiencies could be largely attributed to their uptake by *Chlorococcum* sp. The ionic form of zinc (Zn^{2+}) is an important component of microalgal cells in which it facilitates photosynthetic efficiency (Dou *et al*., 2013). On the other hand, the ionic form of manganese (Mn^{2+}) act as co-enzyme in microalgal cell and is crucial for activating enzyme activities in glycolysis and tricarboxylic acid cycle (Dou *et al*., 2013). The efficiencies of trace element removal obtained in this study could suggest that optimum concentration of

these elements is indispensable in microalgal growth and lipid accumulation. These observations are consistent with Dou *et al*. (2013), who noted a rise in lipid accumulation of *Nannochloropis oculata* when the concentration of Zn^{2+} and Mn^{2+} in the culture medium was increased.

In the present study, lower COD removal by *Chlorococcum* from the mixed wastewater was observed. In the experiment for biomass accumulation, in which the growth medium contained 65% DWW and 35% PWW mixtures, COD was reduced from 579 to 482 mg/L, accounting for 16.75% removal efficiency. In comparison, *Chlorococcum* cultivation in 35% DWW and 65% PWW blend for lipid accumulation led to a reduction of COD from 611 to 444 mg/L, which translates into a 27.33% removal efficiency. The COD removal efficiency can be used to identify the potential of microalgae species to tolerate and grow under certain COD levels (Wang *et al*., 2012). Therefore, the observations in the present study suggested that *Chlorococcum* sp. was less tolerant to COD and assimilated a substantial amount of other organic constituents from the mixed wastewaters for its energy requirement.

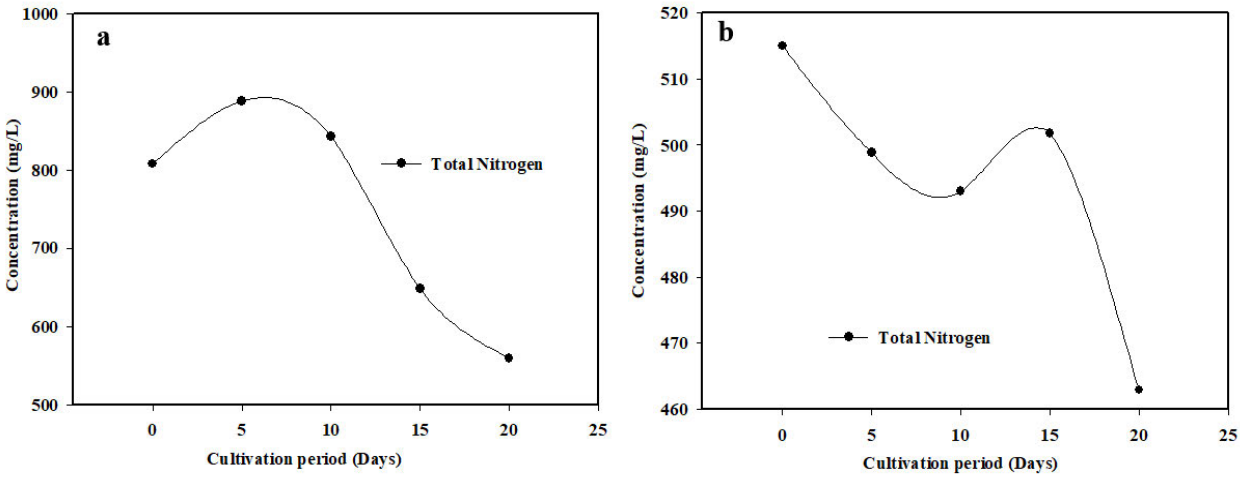

Figure 3.5: Variations of TN concentration in (a) biomass accumulation mixture model and (b) lipid accumulation mixture model

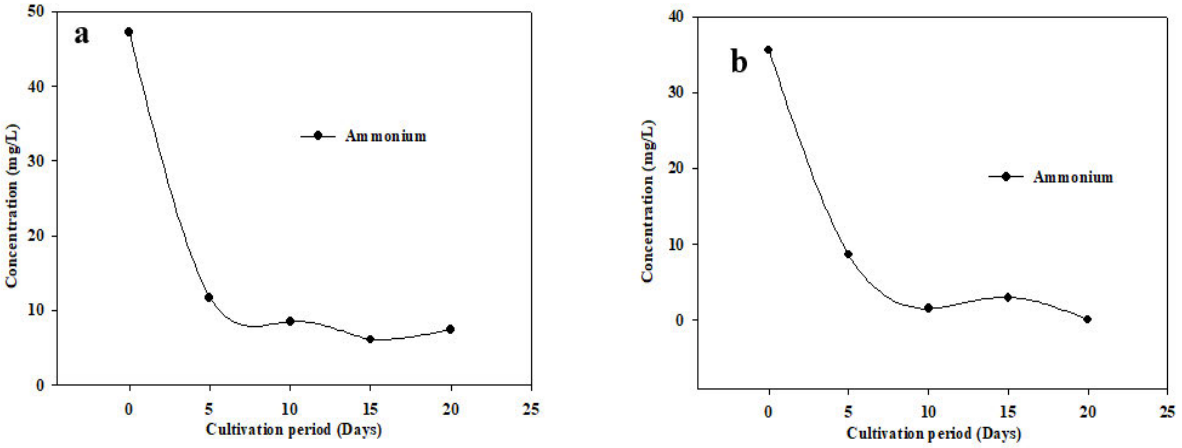

Figure 3.6: The variation of NH_4-N concentration in (a) biomass accumulation mixture model and (b) lipid accumulation mixture model

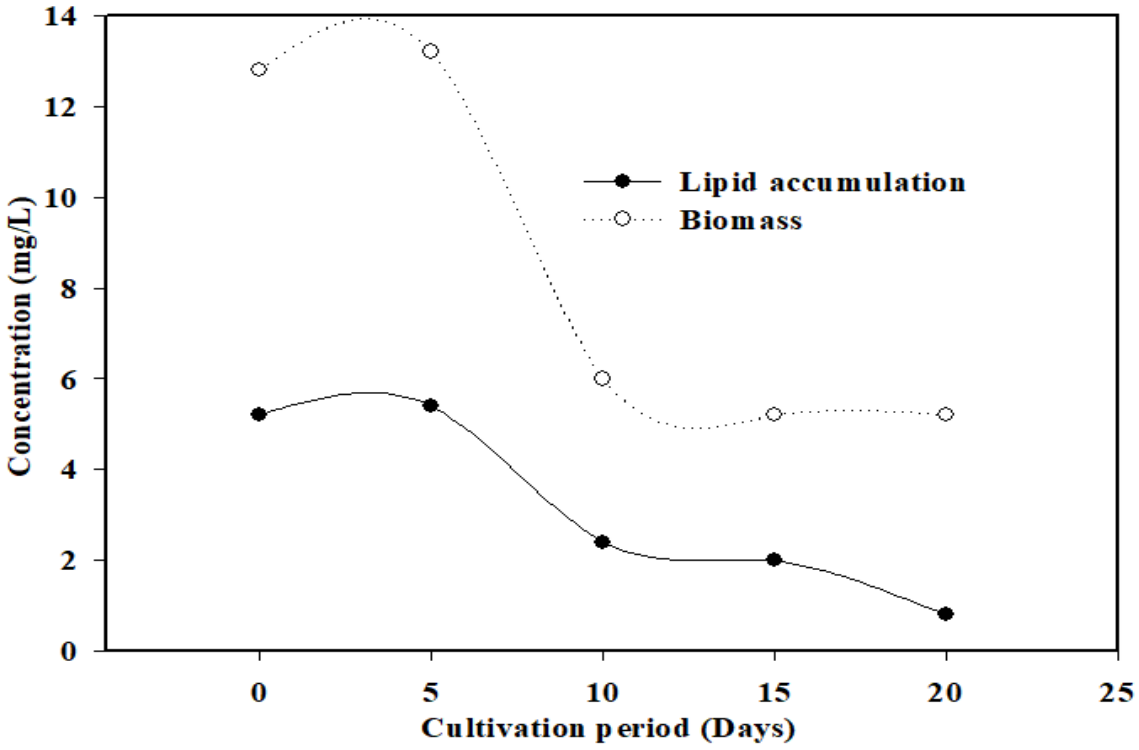

Figure 3.7: The changes in P concentration in biomass concentration and lipid accumulation mixture models

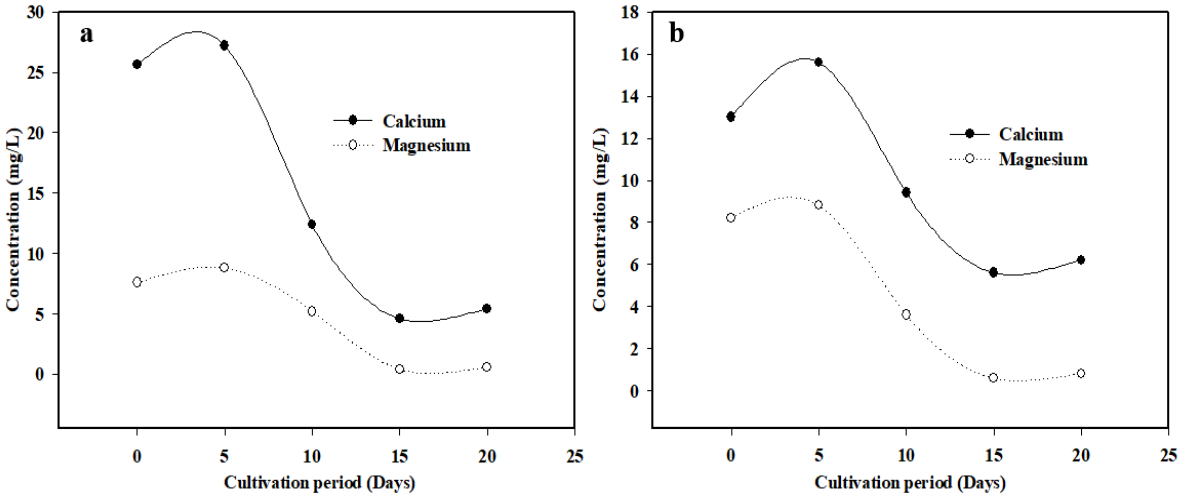

Figure 3.8: The changes of Ca and Mg concentrations in (a) biomass concentration and (b) lipid accumulation mixture models

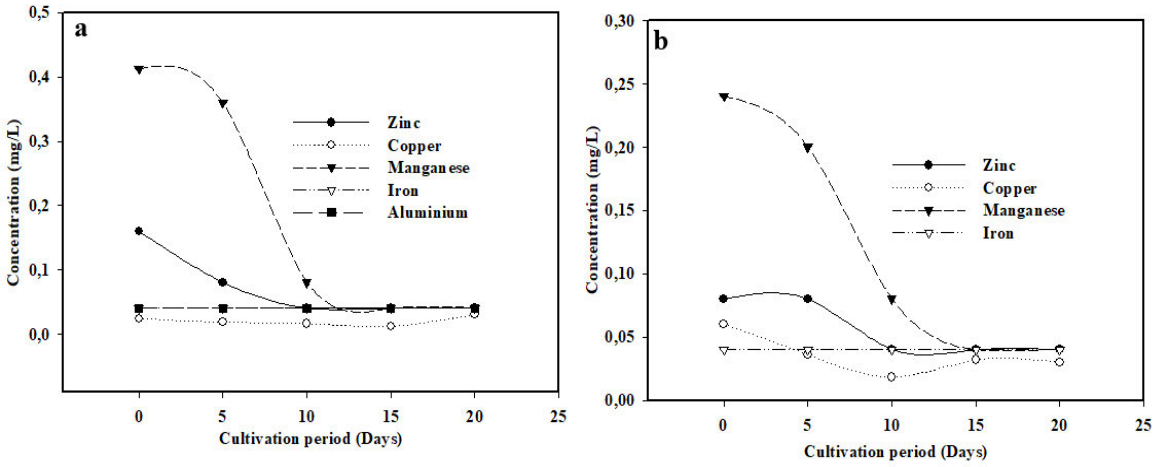

Figure 3.9: The variation of trace element concentrations in (a) biomass concentration and (b) lipid accumulation mixture design models

3.3.8. Comparison of the growth performance and lipid yield with previous reports

In this study, a biomass concentration of 0.94 g/L was obtained from *Chlorococcum* cultivated in a mixture of DWW and PWW (Table 3.7). This value is lower than the biomass concentration of 1.2 g/L observed previously by Guruvaiah *et al*. (2014) in *Scenedesmus SBC39* using DWW. The higher biomass yield obtained by Guruvaiah *et al*. (2014) could be attributed to the nutrient composition of the DWW medium used. However, the observed biomass in this study is similar to those reported in other studies that used mixed wastewater as cultivation media (Gentili, 2014; Yao *et al*., 2015). Gentili (2014) obtained a biomass concentration of 0.86 g/L using *Selenastrum dimorphus*, which was grown in a mixture of DWW and PWW. They also observed a similar biomass concentration of 0.83 g/L when *Selenastrum minutum* was cultivated in a blend of municipal wastewater and PWW. These values were 1.09- and 1.13-fold, respectively, lower than what was obtained in this study present study. Furthermore, Yao *et al*. (2015) reported a biomass concentration of 0.84 g/L when *Desmodesmus communis* was grown in a medium composed of swine and treated municipal wastewaters. The current study showed a 1.12-fold increase in the biomass concentration compared to the one report by Yao *et al*. (2015). This high biomass concentration value obtained in this study can be attributed to the optimal wastewater (DWW and PWW) blended medium and the cultivation technique employed, which positively impacts the microalgae growth. These maximized the nutrient composition in the medium while the cultivation approach improved the distribution of nutrients, air, and light penetration efficiency in the system, and in turn, enhanced microalgae photosynthetic and metabolic activities which led to an improved biomass concentration.

On the other hand, a maximum lipid concentration of 0.39 g/g corresponding to 39% dry weight of *Chlorococcum* biomass was obtained in this study using a mixture of DWW and PWW as cultivation medium (Table 3.7). Guruvaiah *et al*. (2014) obtained a lipid concentration of 0.28 g/g corresponding to 28% of *Scenedesmus* SBC39 dry biomass using DWW as the sole growth medium. Similarly, Brar *et al*. (2019), using DWW as the growth medium, obtained a 16.93%

lipid of dry weight using *Scenedesmus abundans*. These values are 1.39 and 2.29 times, respectively, lower compared to the present study. Equally, the current study is 2.29-fold higher than the study by Yao *et al*. (2015). Yao *et al*. (2015) obtained a 17.04% lipid yield from *Chlorella sorokiniana* biomass cultivated in a mixture of swine and treated municipal wastewaters. These variations in lipid yield could be attributed to media composition, microalgae strain, and the cultivation approach employed. Mixed wastewater media containing PWW, which had lower nutrient constituents, resulted in nutrient deprivation, thus enhancing lipid production. Studies have shown that nutrient deprivation, medium composition, and environmental stress contribute to the induction of high lipid composition.

Table 3.7: Comparison in the microalgae biomass and lipid accumulation with previous studies

Wastewater source	Microalgae strain	Biomass (g/L)	Lipid	Reference
DWW (65%) and PWW (35%)	*Chlorococcum* sp	0.94	ND	This study
DWW (35%) and PWW (65%)	*Chlorococcum* sp	ND	0.39 g/g	This study
Dairy	*SBC39* (*Scenedesmus* sp)	1.20	28%	Guruvaiah *et al*. (2014)
Dairy	*Scenedesmus abundans*	ND	16.93%	Brar *et al*. (2019)
Swine wastewater (25%) and treated municipal wastewater (75%)	*Chlorella sorokiniana*	1.22	17.04%	Yao *et al*. (2015)
Swine wastewater (25%) and treated municipal wastewater (75%)	*Desmodesmus communis*	0.84	ND	Yao *et al*. (2015)
Municipal (50%) and PWW (50%)	*S. minutum*	0.83	37.2%	Gentili (2014)
DWW (33%) and PWW (67%)	*Selenastrum dimorphus*	0.86	25.70%	Gentili (2014)

ND-Not determined

3.4. Conclusion

This study demonstrated the feasibility of cultivating *Chlorococcum* using complementary mixture of DWW and PWW as growth medium. *Chlorococcum* growth was substantial and desirable in the established mixed wastewater. The observed nutrient removal rate/efficiency from the wastewater and biomass growth and lipid accumulation were significant. This study has demonstrated the blending of different types of wastewaters to achieve high microalgae biomass yield with a substantial lipid content, and at the same time, the remediation of the wastewater used. Therefore, the wastewater mixture strategy can be considered as a suitable replacement for commercial BG11 growth medium in the cultivation of *Chlorococcum* for cost-effective optimization of biomass accumulation and lipid production.

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Chapter Four

Microwave-assisted pre-treatment of microalgal biomass for the release of fermentable sugars: An optimization and enzymatic study

Abstract

This study presents the optimization of microwave pre-treatment of microalgal biomass (*Chlorococcum*) using response surface methodology (RSM). Microwave power (100 – 700 W), pre-treatment time (1 – 7 min), and acid-liquid ratio (1 – 5%) were selected as independent variables for the RSM optimization study. The optimum conditions were achieved at microwave power, pre-treatment time, and the acid-liquid ratio of 700 W, 7 min, and 32.33:1, respectively. Optimum conditions provided the highest amount of reducing sugars of 10.73 g/L. The coefficient of determination (R^2) of 0.92 was obtained for the reducing sugar model. The validation experiment yielded a reducing sugar of 15.67 g/L as against predicted reducing sugars of 11.14 g/L. These findings demonstrate that *Chlorococcum* microalgal biomass can be a suitable source of fermentable sugars that is obtainable within a short recovery time for use in the production of biofuels.

Keywords: Microwave, *Chlorococcum*, raceway pond, fermentable sugar, optimization

4.1. Introduction

The rapid growth of the human population and industrialisation with the attendant challenges of food, energy, and water insecurity remains a major concern worldwide (Owusu and Asumadu-

Sarkodie, 2016; Ouda *et al*., 2016; Mohan *et al*., 2016; Amulya *et al*., 2016). The required to balance the standard of living of this ever-growing population has centred on fossil fuel resources (Owusu and Asumadu-Sarkodie, 2016). The continuous consumption of these non-renewable energy resources results in the constant emission of greenhouse gases (GHGs) that promote climate change (Ouda *et al*., 2016). It is, therefore, imperative to break away from dependence on fast-depleting fossil fuels and move towards renewable, environmentally friendly, and sustainable alternatives such as biofuels.

Bioethanol is gaining tremendous global attention as one of the most eminent biofuels for replacing petroleum-based fossil fuels due to its high-octane number, clean, green, and renewable attributes (Thangavelu *et al*., 2016). Bioethanol production has been mainly from first-generation feedstocks such as wheat, sugarcane, corn, and sugar beet (Voloshin *et al*., 2016). However, the use of these food crops requires huge portions of land for cultivation, thus raising food insecurity, ethical and economic concerns (Chye *et al*., 2018). Consequently, the second-generation lignocellulosic feedstocks have been devoted to replacing first-generation feedstocks (Dodo *et al*., 2017). These agricultural residues, including rice hulls, sugarcane bagasse, and corn stover, are abundant globally; therefore, channelling them towards biofuel production will not interfere significantly with food security (Dodo *et al*., 2017). Nonetheless, as a recalcitrance feedstock, lignocellulose requires expensive cell disruption processes to break its matrix content of lignin, cellulose, and hemicellulose (Talebnia *et al*., 2010). Hence, further research on potential substitutes such as microalgal biomass is needed.

Microalgae are mostly photosynthetic microorganisms that can flourish in various environmental conditions, including temperature, pH, and nutrients (Karemore and Sen, 2016). Therefore, they possess a short generation cycle, t and can be cultivated and harvested all year round (Zhou *et al*., 2013; Peralta-Ruiz *et al*., 2013). Additionally, microalgae can accumulate considerable biomass consisting of lipids (20 – 80%), carbohydrates (10 – 40%), and proteins (10 – 50%)

(Chojnacka *et al*., 2012; Yaakob *et al*., 2014; Suganya *et al*., 2016; Rehman and Anal, 2018). Their lipids consist of triacylglycerol (TAG) that can be transformed into biodiesel via transesterification (Chye *et al*., 2018). Besides, microalgal biomass can be processed into proteins, pigments, and additives for fish and feeds (Odjadjare *et al*., 2015). Moreover, the carbohydrate component of microalgal biomass contains considerable amounts of fermentable sugars that can serve as feedstock for bioethanol production (Harun *et al*., 2010; Chye *et al*., 2018).

Despite the beneficial attributes of microalgae as raw material for bioethanol production, its potential has been limited by the associated downstream processing (Karemore and Sen, 2016). Downstream processes contribute about 70 – 80% of the total processing cost and account for the most weight in terms of energy consumption (Kapoore *et al*., 2018). One of the most important upstream processes in bioethanol production from microalgae biomass is the extraction of carbohydrate molecules embedded in the microalgal cells (Martín-Juárez *et al*., 2017). Microalgae often store these biomolecules in the alginate of the outer cell wall, in the inner cell wall as cellulose and hemicellulose, and inside the cell as storage products such as starch (Martín-Juárez *et al*., 2017). Therefore, effective utilisation of microalgal biomass for bioethanol production warrants the disruption of the cells, making them susceptible to the subsequent process of hydrolysis (Eldalatony *et al*., 2017). Various pre-treatment techniques have been established microalgal cells to release their polysaccharides and hydrolyse them to simple sugars (Harun and Danquah, 2011; Karemore and Sen, 2016; Eldalatony *et al*., 2017). However, some of these methods are usually high energy and time-consuming and can cause product degradation (Cravotto *et al*., 2008), and sometimes make no distinction among various biomass fractions (Martín-Juárez *et al*., 2017). Consequently, one technique that has attracted significant research interests for disrupting microalgal cells is the microwave-assisted (MW) pre-treatment (Esquivel-Hernández *et al*., 2017; Gilbert-López *et al*., 2017). Microwave-assisted pre-treatment is a non-contact process in which the heating of the sample occurs simultaneously

via the rotation of ions and dipolar molecules in a solution in an electromagnetic field (Biller *et al*., 2013). Eventually, there is uniform heat distribution in the entire biomass, hence effective cell disruption (Iqbal and Theegala, 2013). Besides the effective cell disruption for fermentable sugar release, the use of this technique minimises product degradation, operational time, and costs (Al Hattab *et al*., 2015). Various studies have investigated the effects of the MW technique on the pre-treatment of microalgal biomass for the release of fermentable sugars for bioethanol production (Kassim *et al*., 2019; Theofany *et al*., 2019). In the study by Kassim *et al*. (2019), the effect of microwave-alkaline-assisted pre-treatment on the hydrolysis of *Tetraselmis suecica* was evaluated. The result showed a maximum sugar release of 9.83 ± 0.24 mg/mL, corresponding to a conversion yield of up to 85.58% of the carbohydrate content of the biomass. The efficiency of MW pre-treatment was also investigated by Hernández *et al*. (2015). The study obtained a sugar yield of 21 mg/g dry weight from *Chlorella sorokiniana* at 150 W for 40 s.

Although pre-treatments have been established in the literature as a crucial process for the release of fermentable sugars from lignocellulosic biomass, the optimization of the most suitable pre-treatment methods for a specific type of biomass is difficult and depend on a combination of intrinsic features of the biomass and the pre-treatment set points applied (Zabed *et al*., 2017). The pre-treatment efficiency is influenced by several factors, such as the reactant concentration, time, temperature, solid to liquid ratio, and pressure (Rezende *et al*., 2018). Therefore, establishing an adequate experimentation strategy to address the above problem is essential (Wahid and Nadir, 2013). A considerable number of researchers have focused on the conventional one variable at a time strategy (OVAT) (Sanusi *et al*., 2020). In the OVAT technique, one variable is separately evaluated while keeping the other parameters constant until an optimum experimentation condition is obtained (Sanusi *et al*., 2020). Unfortunately, the OVAT does not account for the interactive effects between the independent variables, thus making the techniques less efficient and unreliable (Wahid and Nadir, 2013). An alternative to the OVAT is the design of experiment strategy (DOE). Unlike the OVAT, DOE examines

multiple variables, and their interactive effects on one or more outputs are simultaneously investigated in a single or several experimental runs (Rezende *et al*., 2018). Similarly, the DOE examines the influence of various input variables simultaneously and identifies their interactive effects, which cannot be attained by the conventional OVAT approach (Rezende *et al*., 2018). The DOE applies various tools, such as response surface methodology (RSM), factorial design, fractional factorial design, etc., in the modelling and optimisation of experimental designs (Wahid and Nadir, 2013). The use of these tools has been documented in various bioprocesses (Betiku and Ajala, 2014; Sanusi *et al*., 2020). Of these techniques, the RSM has received significant attention as it combines statistical and empirical data to develop an optimized model for a set of experiments and their observed results (Ghosh, 2012; Kushwaha *et al*., 2017). A wide range of studies has employed RSM to optimize pre-treatment conditions to release fermentable sugars (Zambare and Christopher, 2012; Thangavelu *et al*., 2018; Tripathi, 2018). However, the focus of these studies has been mainly on lignocellulosic feedstock, while the use of microalgal biomass has been scantily documented. Furthermore, there is a shortage of knowledge on the interaction and optimization of irradiation time, microwave power, and liquid to solid operational parameters on microalgal biomass pre-treatment. Understanding the interactive dynamics of these pre-treatment parameters on *Chlorococcum* biomass will promote the utilization of *Chlorococcum* biomass for fermentable sugar release, and ultimately, bioethanol production. Thus, this study aims to investigate the effect of MW pre-treatment on *Chlorococcum* biomass. The RSM will be employed to establish the optimum condition for the multivariate interaction of pre-treatment parameters, including irradiation time, microwave power, and liquid to the solid ratio for optimum release of reducing sugars. Thereafter, the use of these fermentable sugars for fermentative bioethanol production will be evaluated.

4.2. Materials and methods

4.2.1. Microalgal species

The *Chlorococcum* sp. used in this study was obtained from the Discipline of Microbiology, University of KwaZulu-Natal, Westville Campus, South Africa. The microalgae were sustained

in the enrichment medium, which is composed of 10% BG-11 solution, purchased from Sigma Aldrich (Germany), 1% trace metals prepared based on standard protocols (Mutanda *et al*., 2011), and 89% distilled water. Sub-culturing was carried out under UV illumination (54.36 μmol m^{-2} s^{-1}) with shaking at 150 rpm for 14 days at room temperature to obtain the inoculum needed for subsequent setup.

4.2.2. Preparation of microalgal biomass

The *Chlorococcum* sp. was further cultured in a laboratory-scale transparent photobioreactor (Locally designed) under four fluorescence bulbs for optimal illumination for 21 days to generate biomass required for subsequent experiments (Blackburn and Lee-Chang, 2018). The photobioreactor is made up of 15 wells, each having a 1 L volume capacity. The wells are uniformly separated into three rows, each row containing five wells. Each well comprises a length, breadth, depth, and working volume of 27 cm, 10 cm, 7.5 cm, and 800 mL, respectively. A submerged paddle provided mixing at a mixing speed of 43 rpm and was controlled and maintained using actuators and sensors.

4.2.3. Microalgal biomass harvesting

Harvesting of the *Chlorococcum* sp. biomass was carried out after 21 days of cultivation (Singh and Patidar, 2018). The culture was centrifuged at 4500 *g* for 10 min at 10 ℃ using a Heraeus Multifuge 3S-R, Germany. The supernatant was decanted, and after that, the pellet was air-dried and stored at room temperature (Singh and Patidar, 2018). Characterisation of various constituents of the harvested biomass was conducted using the protocol described by Templeton *et al*. (2012) and the results are presented in Table 4.1.

4.2.4. Experimental modelling and optimization

The response surface methodology (RSM) was used to develop 17 empirical runs on input parameters of microwave power (400 – 800 W), pre-treatment time (1 – 5 min), and liquid ratio (1 – 10% v/v). The ranges of the input parameters were selected based on previous studies (Onumaegbu *et al*., 2018; Feng *et al*., 2019). All experiments were conducted in replicates, and the empirical data was used to fit the polynomial model equations using the Design Expert

software (Stat-Ease Inc., USA). These model equations relate the input parameters to the response variables of total sugar production, fermentable sugar release, and bioethanol yield. The general form of the model is shown in Eq. (1).

$$Y = \alpha_0 + \alpha_1X_1 + \alpha_2X_2 + \alpha_3X_3 + \alpha_{11}X_1^2 + \alpha_{22}X_2^2 + \alpha_{33}X^2_3 + \alpha_{12}X_1X_2 + \alpha_{13}X_1X_3 + \alpha_{23}X_2X_3 \quad (1)$$

Where Y represents the response output, α_0 is the intercept, α_1X_1 to α_3X_3 are the linear coefficients, $\alpha_{11}X^2_1$ to $\alpha_{33}X^2_3$ are the quadratic coefficients and $\alpha_{12}X_1X_2$ to $\alpha_{23}X_2X_3$ illustrates the interaction of coefficients.

The model will be evaluated using the analysis of variance (ANOVA), while the optimum pre-treatment conditions for reducing sugars yield was achieved by solving the equations according to the method documented by Myers and Montgomery (1995). Thereafter, these conditions were experimentally validated in duplicate.

4.2.5. Enzymatic saccharification of the pre-treated microalgae biomass

The enzymes used in this study were cellulase, α-amylase, and amyloglucosidase (AMG), all of which were obtained from Sigma-Aldrich, South Africa. Enzymatic hydrolysis was carried out in a 100 mL Erlenmeyer flask, incubated in a shaking incubator at 100 rpm. The pre-treated microalgae hydrolysate was admixed with the appropriate enzyme. Firstly, the addition of cellulase at 55°C, and pH 5.5 for 2 h, then the liquefaction stage; α-amylase at 90°C, pH 7 for 1 h (enzyme denaturing by incubating the mixture at 95°C for 10 min). Next was the saccharification stage, which progressed with the amyloglucosidase at 60°C, pH 4.5 for a day (24 h) (then incubated at 96°C for 10 min for enzyme denaturation). The obtained hydrolysate was centrifuged (at 5000 rpm for 5 min) to obtain a supernatant for reducing sugars analysis.

4.2.6. Scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) analysis of microalgae biomass

The microalgae biomass (untreated and optimally pre-treated) was examined under Scanning Electron Microscopy (SEM). Dried and gold sputter-coated samples were mounted on SEM-aluminium specimen mounts and examined using SEM (Zeiss Evo LS 15).

The functional nature and changes of the microalgae were determined using Fourier Transform Infrared Spectroscopy (FTIR) (Spectrum 100, PerkinElmer, USA). The FTIR spectra were recorded between 450 and 4000 cm^{-1} for the microalgae samples.

4.2.7. Analytical methods

Reducing sugar content was quantified with 3,5-dinitrosalicylic acid (Sanusi *et al*., 2019). *Chlorococcum* sp. structural content such as cellulose, hemicellulose, lignin, and total carbohydrates were analysed using previously established protocols (Amezcua-Allieri *et al*., 2017).

Table 4.1: Chemical composition of untreated and pretreated microalgae

Sample	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Untreated	0.86	9.01	0.33
Pretreated	0.98`	17.16	3.15

Table 4.2: Box-Behnken design for microwave assisted pretreatment on variables of microwave power, acid ratio, and pretreatment time

Run	A: MW Power (Watt)	B: Acid ratio (v/v)	C: Pretreatment time (min)	Response 1: Reducing sugar (g/L)
1	700.00	5.00	4.00	9.006
2	400.00	5.00	1.00	7.728
3	700.00	1.00	4.00	10.626
4	700.00	3.00	1.00	10.104

5	400.00	3.00	4.00	9.287
6	100.00	5.00	4.00	8.532
7	400.00	1.00	1.00	6.852
8	100.00	3.00	7.00	7.404
9	400.00	3.00	4.00	9.287
10	400.00	3.00	4.00	9.287
11	400.00	5.00	7.00	8.180
12	700.00	3.00	7.00	10.734
13	400.00	1.00	7.00	6.510
14	100.00	3.00	1.00	6.798
15	100.00	1.00	4.00	7.338

Table 4.3: Analysis of variance (ANOVA) for reducing sugar models

Source	Sum of squares	Df	Mean squares	F-value	P-value	R^2
Reducing sugar model	8.28	3.00	2.76	6.02	<0.0410	0.92

Df: degree of freedom, F-value: Fisher-snedecor distribution value, P-value: probability value, R^2: coefficient of determination

4.3. Results and discussion

4.3.1. Composition of *Chlorococcum* microalgae biomass

The analysis of raw microalgae biomass indicated that the hemicellulose, cellulose, and lignin components were 9.01%, 0.86%, and 0.33% respectively (Table 4.1). The lignin content increased by 98.52%. This could be due to the pseudo-lignin formation (Moodley and Kana, 2015). Similarly, this can be correlated with earlier reports on pre-treatment of lignocellulosic

biomass composition (Moodley and Kana, 2015). Moreover, the compositional analysis of the pre-treated microalgae revealed an increase in hemicellulose and cellulose content up to 47.49% and 12.24%, respectively. The proportionate increase in hemicellulose, cellulose and lignin after chemical hydrolysis of sugarcane bagasse and sorghum straw have been reported (Dussan *et al*., 2014, Rorke *et al*., 2016). The increase in cellulose content could be as a result of cellulose availability from the hemicellulose solubilization process. Likewise, Ruangmee and Sangwichien (2013) reported a percentage decrease and simultaneous increase in hemicellulose and cellulose, respectively. This was obtained after alkali pre-treatment of leaf cattail. This is beneficial for sugar recovery that will contribute to improving the release of fermentable sugar.

4.3.2. Modelling of reducing sugar release in the acid-microwave pretreatment

The experimental data (Table 4.2) from the acid-microwave pretreatment conditions were used to develop a polynomial equation that related reducing sugar concentrations to HCl concentration, microwave intensity, and microwave time (Eqn. 1). The model's fitness was evaluated using analysis of variance (ANOVA). The outcome of ANOVA is presented in Table 4.3. The coefficient of determination (R^2) for the reducing sugar model was 0.92, suggesting the model could account for over 92% of variations in the observed data. The relatively low p-values of <0.0410 and the high F values of 6.02 further elucidate the significance of these polynomial models (Table 4.3). The polynomial equation modelled in terms of encrypted factors were:

Reducing sugar (g/L) = 9.29 + 1.30A + 0.27B + 0.17C – 0.70AB + 6.000E-0.003AC + 0.20BC + $0.52A^2 – 0.93B^2 – 1.04C^2$ (1)

Table 4.4: Model coefficient of estimates with standard errors

Factor	Reducing sugar coefficient estimate	Degree of freedom	Reducing sugar standard error
Intercept	9.29	1	0.39
A	1.30	1	0.24
B	0.27	1	0.24
C	0.17	1	0.24
AB	-0.70	1	0.34

AC	6.000E-003	1	0.34
BC	0.20	1	0.34
A^2	0.52	1	0.35
B^2	-0.93	1	0.35
C^2	-1.4	1	0.35

Table 4.5: Optimum levels of variables during microwave-assisted pretreatment

Independent variables		**Predicated optimum levels**
Microwave power		700 W
Acid ratio		2.52% (v/v)
Pretreatment time		4.06 min
Response	**Predicted value**	**Observed value**
Reducing sugar	11.14 g/L	15.67 g/L

4.3.3. Effect of pre-treatment variables on reducing sugar release

The reducing sugar concentration ranged from 6.51 g/L to 10.73 g/L, thus indicating the sensitivity of reducing sugar release to the considered input variables (microwave power, acid-liquid ratio, and pre-treatment time). As shown in Table 4.2, the microwave pre-treatment carried out at 5% HCl concentration showed a low yield of reducing sugars (7.72 g/L) but a high yield of reducing sugar (10.73 g/L) at 3% acid concentration, whereas pre-treatments at an even lower acid concentration (1%) gave a relatively low yield of reducing sugar (6.51 g/L). Similar outcomes from the effects of pre-treatment acid concentration on fermentable sugar (glucose, xylose, and galactose) release from cellulosic plant biomass such as wheat straw were reported by Sindhu *et al.* (2014) and Saha *et al.* (2005). In addition, acid-microwave pre-treatment at low power (100 W) gave a very low yield of reducing sugar (6.80 g/L) compared to pre-treatment at higher power (700 W) which gave a reducing sugar yield of 10.73 g/L. A similar trend has been reported in the release of xylose and glucose from the pre-treatment of sugarcane leaves (Moodley and Kana, 2015). Correspondingly, low pre-treatment time (1 min) resulted in a lower yield of reducing sugar (6.80 g/L), while pre-treatment with higher process time (7 min) showed

an increase in the reducing sugar released (10.73 g/L). The observations can be attributed to an effective biomass fractionation facilitated by the interaction of the acid and the microwave power. Also, the data show that microwave-assisted acid pre-treatment effectively removes hemicellulose and lignin of the microalgae biomass, thus enhancing enzymatic digestibility (Moodley and Kana, 2019).

4.3.4. Interactions of experimental variables on reducing sugar yield

The two-factor interactive effect of microwave pre-treatment parameters was assessed using the three-dimensional response surface graphs (Fig. 4.1 – 4.3). When these factors were adjusted from lower to higher level setpoints, the reducing sugar concentration increased from 6.51 to 10.73 g/L. As illustrated in Fig. 4.1, if the acid concentration was maintained at 4 (v/v) and microwave power was increased from 100 to 700 W, the reducing sugar concentration also increased from 8.50 to 10.99 g/L. Likewise, as shown in Fig. 4.2, if the pre-treatment time was maintained at 5.50 min, an increase in microwave power from 100 to 700 W resulted in an increase in reducing sugar that was released from 7.55 to 10.85 g/L. The interactive effects of pre-treatment time and acid concentration, when the pre-treatment time was kept at its median value, are shown in Fig. 4.3. Higher concentrations (>7.00 g/L) could be attained using lower acid concentrations (<5 v/v) while maintaining a higher pre-treatment time (7.00 min). These observations also show that low reducing sugar can be obtained when pre-treatment time is increased from 5.5 to 7 min. Similarly, the reducing sugar concentration peaked at 8.80 g/L at a low acid concentration (4%) with a higher pre-treatment time (5.50 min), as shown in Fig. 4.3. A declining trend in reducing sugar was observed when the acid concentration was increased from 4 to 5%. Comparable trends have been reported by Dussan *et al*. (2014), who observed reducing sugar concentration as the acid concentration increased in the pre-treatment of sugarcane bagasse waste. Considering the aforementioned factors that influenced the release of fermentable sugar in the pre-treatment approach, the patterns of sugar release was expected. Similarly, the implemented pre-treatment strategies influenced the cleavage of the cell wall

structure to different extend leading to the varying fermentable sugar release as evident in Fig. 4.1 - 4.3.

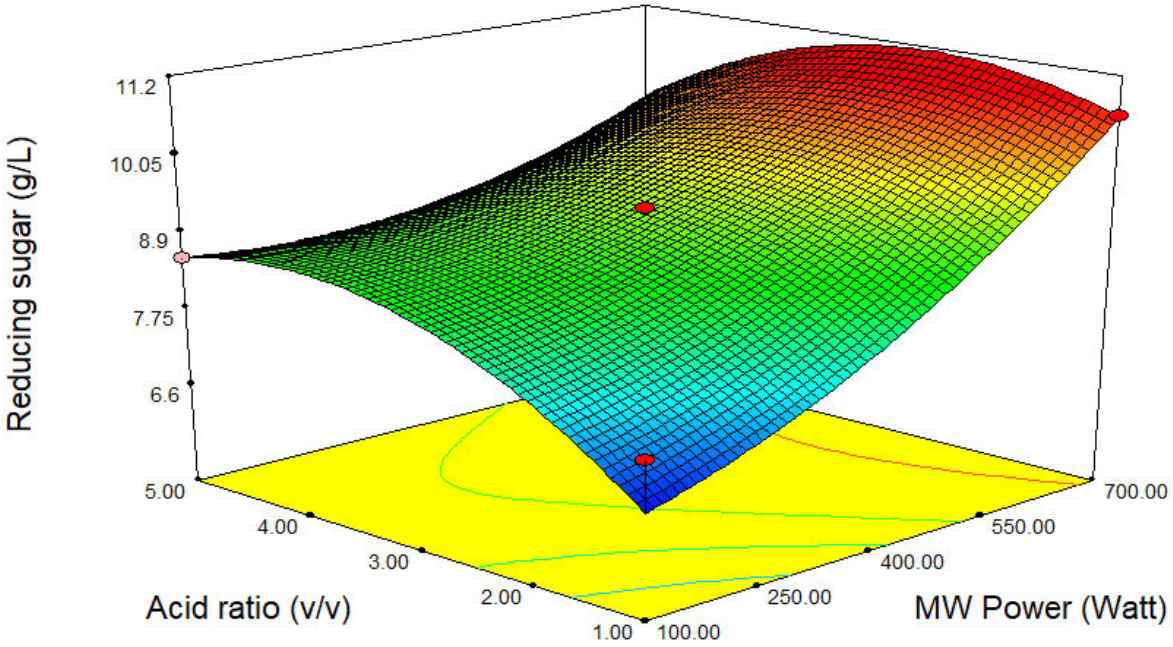

Fig. 4.1: 3-D response surface plot showing the interaction of acid ratio and microwave power on reducing sugar yield

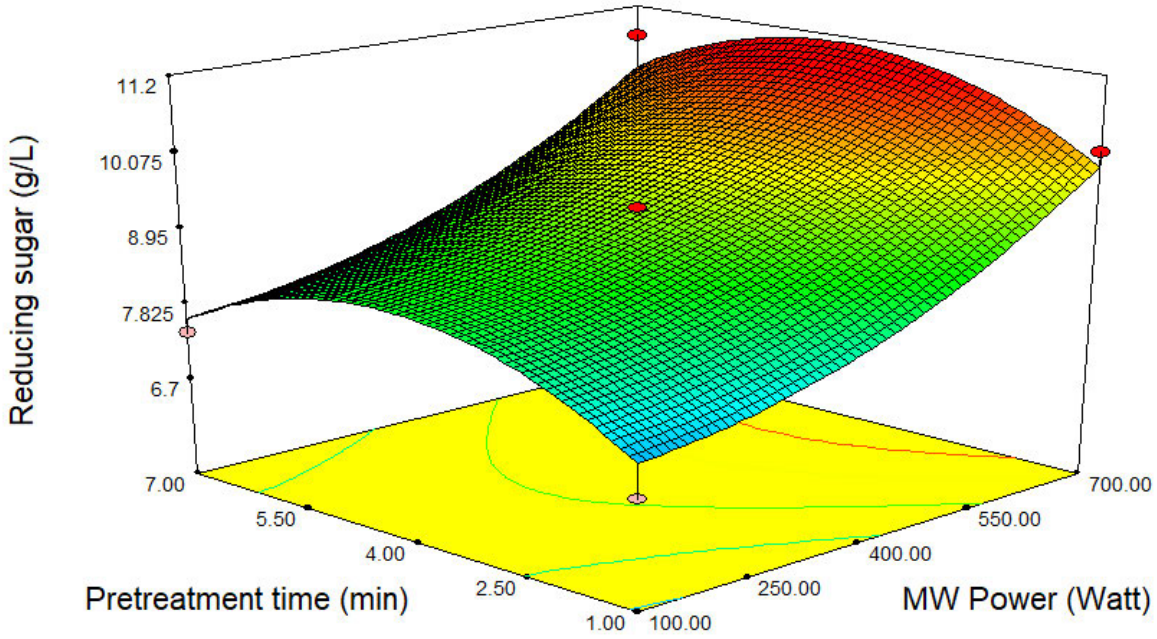

Fig. 4.2: 3-D response surface plot showing the interaction of pretreatment time and microwave power on reducing sugar yield

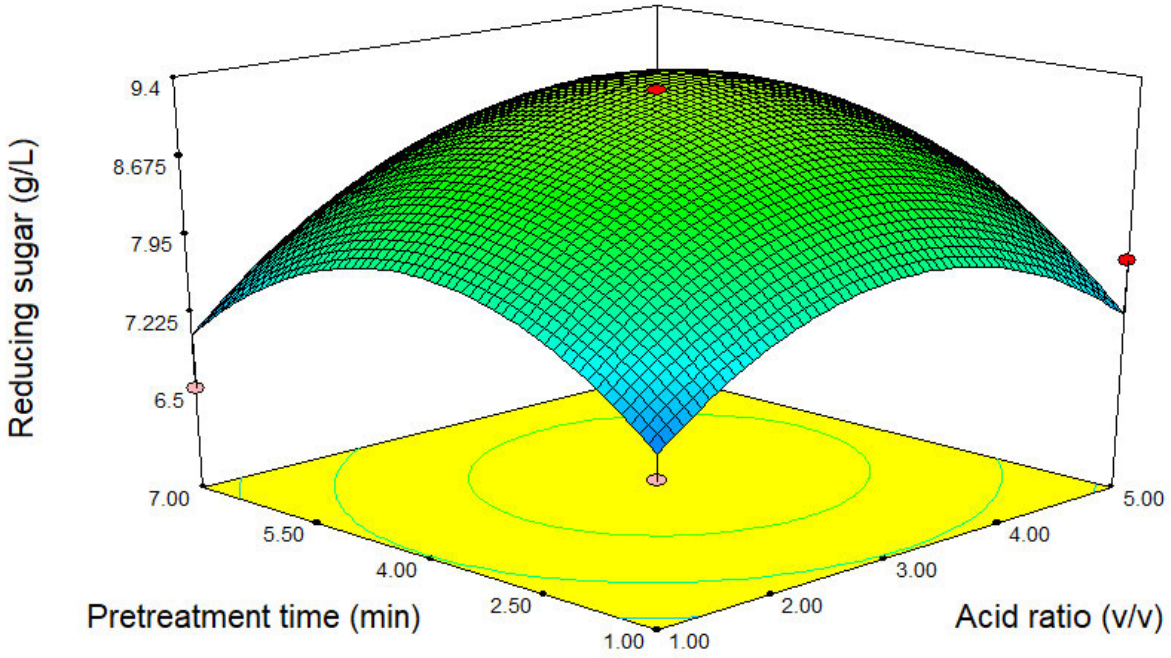

Fig. 4.3: 3-D response surface plot showing the interaction of pretreatment time and microwave power on reducing sugar yield

4.3.5. Optimization of microalgae pre-treatment on reducing sugar production

The optimized microwave pre-treatment conditions based on the developed process model predicted reducing sugar production of 11.14 g/L using optimal set points of microwave power at 700 W, the acid concentration at 2.52%, and pre-treatment time of 4.06 min (Table 4.5). The experimental validation gave a reducing sugar yield of 15.67 g/L (Table 4.5). The study shows high microwave intensity to low acid ratio concentration favours the degradation of the microalgae biomass. This promotes the cleavage of the cell wall structure during pre-treatment leading to higher enzymatic hydrolysis efficiency. The pre-treatment technique implemented effectively released fermentable sugars, and it is comparable to other studies. Shokrkar *et al.* (2017) obtained 13.30 g/L fermentable sugar from dried microalgal biomass by enzymatic hydrolysis. The disparity in fermentable sugar obtained is probably due to differences in the pre-treatment methods implemented in both studies.

Furthermore, three enzymes were employed for the hydrolysis and saccharification of pre-treated microalgae biomass in two experimental probing. The first probing involved using three enzymes (cellulase, amylase, and amyloglucosidase) in the hydrolytic saccharification. This resulted in 15.67 g/L of reducing sugars. The second probing was carried out to assess the effect of eliminating the cellulase enzymatic step. Reducing sugar yield of 15.60 g/L was obtained when the cellulase enzymatic step was excluded in the hydrolytic saccharification. There was no significant difference in the yield of reducing sugars obtained in the two experimental probing. Hence, from economic considerations, eliminating the cellulase enzymatic step is recommended for industrial implementation.

4.3.6. SEM and FTIR analysis

The electron micrograph of pre-treated microalgal biomass showed that the optimal microwave pre-treatment degraded the surface and the architectural structure of the microalgal biomass with exposed inner materials (Fig. 4.4) when compared to untreated microalgal biomass sample, which had a relatively intact architectural structure (Fig. 4.5). Major structural mutilations such

as the pre-treatment induced alteration to cellulose crystallinity can be observed in Fig. 4.4. This substantially enhanced the solubilization of the inner components of the microalgal biomass.

The microalgae biomass obtained after pre-treatment was subjected to an FTIR analysis. The result is presented in Fig. 4.6. Noticeable changes in the peaks for the band 800 – 3800 cm^{-1} were observed for the microalgae sample that was subjected to microwave-assisted pre-treatment. The polysaccharide and C-O-C adsorption is represented by the bands at 900 cm^{-1} and 1200 cm^{-1}, respectively. This indicates the cleavage of the cell wall structure during pre-treatment using microwave treatment techniques. On the other hand, there was a reduction in the protein peaks as represented by the band between 1040 cm^{-1} and 1760 cm^{-1} which signifies the degradation of protein units in the microalgae biomass during the pre-treatment process. Moreover, the FTIR spectrum demonstrated that the microwave treatment also caused alterations in lipid content. This is represented in the band between 1500 cm^{-1} to 1720 cm^{-1}. The spectra characterize the N-H stretching for amine I and II protein (Surendhiran and Vijay, 2014). The decrease in the band within these ranges shows reduced protein content in the pre-treated microalgae biomass. Removal of protein units from the microalgal biomass during microwave-assisted pre-treatment is most probably due to degradation and deterioration during the process (Kapoore *et al*., 2018). Based on the FTIR spectra obtained, it is evident that pre-treatment of the microalgal biomass had an effect on functional groups, thus leading to higher enzymatic hydrolysis efficiency.

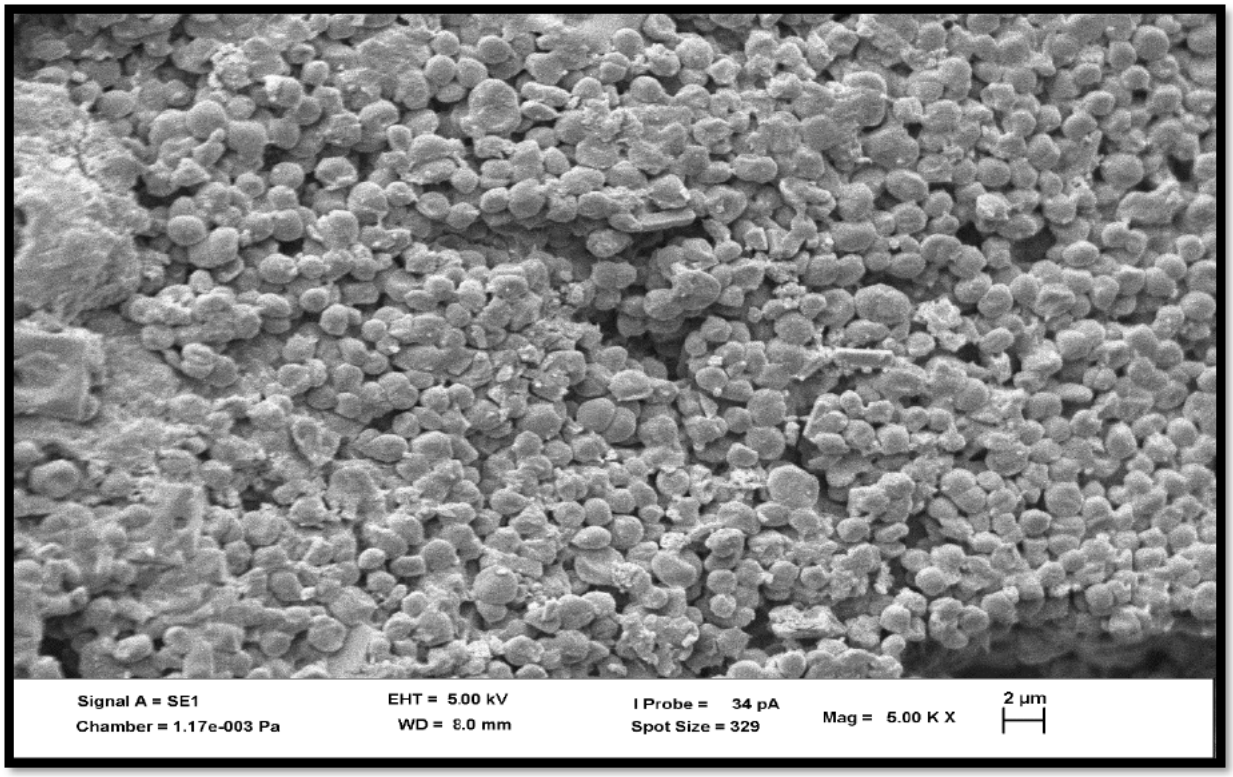

Fig. 4.4: Scanning electron micrograph of the optimally pretreated microalgae

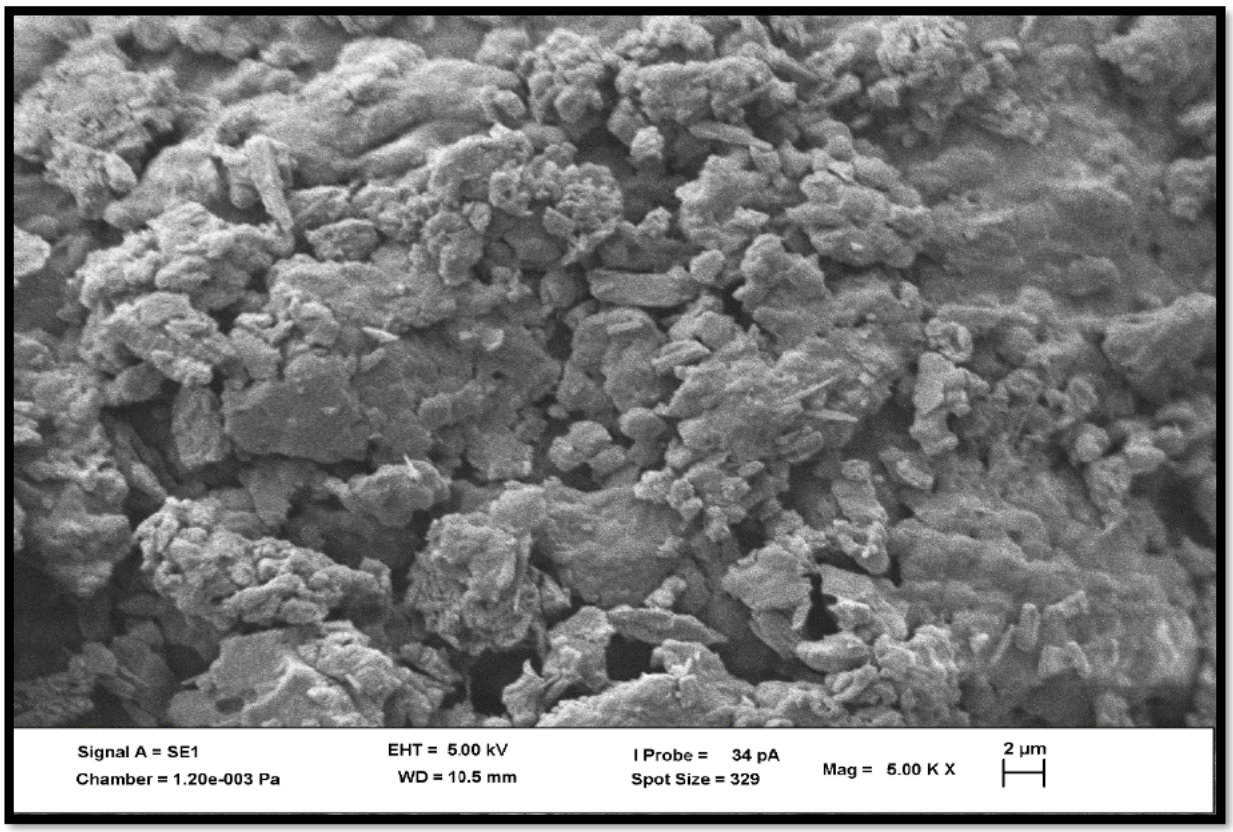

Fig. 4.5: Scanning electron micrograph of the untreated microalgae

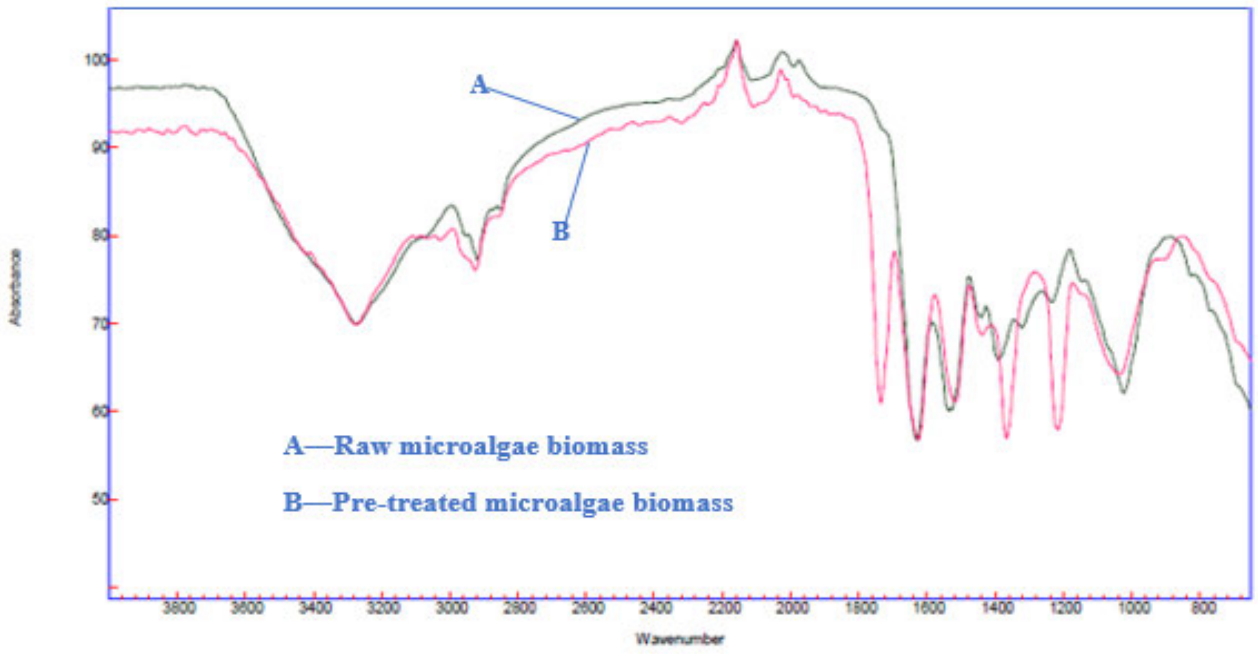

Fig. 4.6: FTIR micrograph of untreated and pre-treated microalgae biomass

4.3.7. Conclusion

An effective microwave-assisted acid pre-treatment strategy of the microalgal biomass was evaluated in this study. A significant model (R^2>0.92) was developed and optimized for HCl pre-treatments. Maximum fermentable sugar yield (15.67 g/L) was obtained under optimal set points of 2.52% (v/v) at 700W for 4.06 min. Considerable structural alterations of pretreated samples were observed after SEM and FTIR analysis with the pre-treatment strategy showing modifications that resulted in the release of reducing sugar. Pre-treatment of microalgae biomass under the optimal conditions showed enhanced fermentable sugar release compared to previous reports. This study demonstrates the potentials of microalgae biomass as a suitable feedstock that could replace crops feedstock as fermentable sugar sources for biofuel production.

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Major research findings and their implications

In this study, the potential of mixed wastewater for microalgae cultivation process modelling and optimization was demonstrated. Additionally, the development of a suitable and fast technique for microalgae pre-treatment was examined. The primary outcomes and their implications are summarised as follows:

i. A maximum biomass and lipid yield of 0.94 g/L and 0.39 g/g, respectively, is achievable with raceway pond using the mixture of dairy wastewater (DWW) and paper and pulp wastewater (PPW) at an optimum percentage mixture volume of 64.69% DWW and 35.31% PWW for biomass concentration; 34.21% DWW and 65.79% PWW for lipid accumulation. These results demonstrate that proper optimization of a mixture of different wastewater is a critical step for complementary wastewater mixture for microalgae growth.

ii. In addition to mixed wastewater microalgae cultivation, microwave-assisted chemical pre-treatment of microalgae biomass revealed the successful optimal release of reducing sugars. The microwave-acid pre-treatment was optimum at 700 W, 2.52% and 4.06 min for microwave power, acid concentration, and pre-treatment time, respectively, for reducing sugars (15.67 g/L) release. Thus, microwave-assisted HCl pre-treatment was efficient for microalgae biomass degradation and reducing sugar release. These, therefore, illustrate microalgae biomass as a potential low-cost feedstock for biofuel production.

Recommendations for future work

To realize the potential of industrial-scale mixed wastewater microalgae cultivation, the following recommendations are proposed outlook studies:

i. Employing other nutrient-rich wastewater for microalgae cultivation will significantly improve its process economics by lowering production costs since they are produced in large quantities and costless.

ii. Integration of wastewater microalgae cultivation into biorefinery concept has been projected to be a practical and feasible approach for wastewater management and simultaneous production of multiple valuable bioproducts at a lower cost. However, techno-economic cost analysis for these processes must be considered and carried out for scaling up.

iii. Multifactorial experimentations will also be needed to generate reliable bioprocess data using pre-treated microalgae biomass as feedstock which is adaptable into practicable intelligence for bioproduct production and scale-up. This requires novel multifunctional bioreactor configurations with a high level of parallelization linked with on-line monitoring systems.