AN INTEGRATED APPROACH FOR BIOFUEL AND FERTILIZER PRODUCTION USING MICROALGAE GROWN IN WASTEWATER

This work is submitted in fulfilment of the requirements for the degree of Master of Technology: Biotechnology in the Faculty of Applied Sciences at Durban University of Technology.

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I hereby declare that the thesis herewith submitted for the MAppSci: Biotechnology at the Durban University of Technology has not been previously submitted for a degree at any other Higher educational learning institution/university.

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Pfano Musetsho

DEDICATION

This thesis is dedicated to my beloved family. Thank you so much for all your support and inspiration.

PREFACE

The research outputs of the Masters qualification are as follows:

Published articles in accredited journals/ book chapters:

MUSETSHO, P., RENUKA, N., GULDHE, A., SINGH, P., PILLAY, K., RAWAT, I. AND BUX, F. 2021. Valorization of poultry litter using *Acutodesmus obliquus* and its integrated application for lipids and fertilizer production. *Science of the Total Environment*, *796*, p.149018.

MUSETSHO, P., RENUKA, N., RATHA, S.K., RAWAT, I. AND BUX, F. 2021. Valorization of wastewater via nutrient recovery using algae-based processes. In: Mandotra S.K., Upadhyay A.K., Ahluwalia A.S. (Eds.) *Algae*. Springer, Singapore. ISBN (print) 978-981-15-7517-4, ISBN (online) 978-981-15-7518-1. https://doi.org/10.1007/978-981-15-7518-1_1

APPROVAL

I hereby approve the final submission of the following thesis.

Prof F. Bux Dr N. Renuka Dr A. Guldhe

This 9th day September of 2022, at the Durban University of Technology.

REFERENCE DECLARATION

I, Musetsho Pfano, Dr Nirmal Renuka, Dr Abhishek Guldhe and Prof. Faizal Bux hereby declare that in respect to the following dissertation: An integrated approach for biofuel and fertilizer production using microalgae grown in wastewater. As far as we know and can be confident, no other dissertation like it exists: all references included in the dissertation are full, including all personal communications and published works consulted.

__________________ __2022/09/09________________

Signature of Student Date

ABSTRACT

Microalgae are recognized as potential candidates for resource recovery from wastewater and are projected for biorefinery models. Therefore, this study was undertaken to evaluate the potential of poultry litter and municipal wastewater as nutrient and water sources, for the cultivation of *Acutodesmus obliquus* for lipids production for biodiesel application. The efficacy of lipid extracted biomass (LEA) as fertilizer for mung bean crops was also assessed in microcosm. *A. obliquus* cultivation in acid pre-treated poultry litter extract (PPLE) showed maximum biomass production of 1.90 g L^{-1}, which was 74.67% and 12.61% higher than the raw poultry litter extract (RPPE) and BG11 respectively. Higher NO_3-N, NH_3-N, and PO_4-P removal of 79.51%, 81.82%, and 80.52% respectively were observed in PPLE as compared to RPLE treatment. The highest biomass (140.36 mg L^{-1} d^{-1}), lipids (38.49 mg L^{-1} d^{-1}), and carbohydrates (49.55 mg L^{-1} d^{-1}) productivities were observed in the PPLE medium. The application of LEA as a fertilizer for mung bean crops showed improvement in plant growth and soil microbial activity. A maximum increase in organic carbon (59.5%) and dehydrogenase activity (130.8%) was observed in LEA amended soil which was significantly higher than chemical fertilizer (CF) control in 30 days. Whilst plant fresh weight and leaf chlorophyll in the LEA amended soil was comparable to whole algal biomass (WA) and CF control. The findings of the present study could be a basis for sustainable biorefinery for the valorization of wastewater for the production of microalgae-derived biofuel and byproducts for agricultural applications.

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LIST OF MATHEMATICAL SYMBOLS

Beta - β

Colony forming units per 100 mL - CFU 100 mL^{-1}

Colony forming units per mL – CFU mL^{-1}

Degrees Celsius - ºC

Litre – L

Grams - g

Grams per litre - gL^{-1}

Gram per dry cell weight - g^{-1} DCW

Grams per litre per day -$gL^{-1}d^{-1}$

Hours - h

Intrinsic fluorescence -Fo

Kilowatt hour per kilogram -kWh kg^{-1}

Maximum efficiency of PS II -Fv/Fm

Maximal fluorescence yield- Fm

Maximum fluorescence in a light adapted sample - F′m

Metres per second - $m^{-2}s^{-1}$

Microgram per millilitre - µg mL^{-1}

Microlitre - µL

Micrometre - µm

Wattage in watts -W

Micromole - µmol

Milligram - mg

Milligrams per litre - mgL^{-1}

Milligrams per litre per day - $mgL^{-1}d^{-1}$

Millijoule per kilogram – $mJkg^{-1}$

Millilitre - mL

Molar – M

Nano meter – nm

Percent - %

Per day - d^{-1}

Percentage by weight - wt. %

PS II operating efficiency - F′q/F′m

Relative centrifugal force - × g

Rotations per minute - rpm

Seconds - s

Standard deviation - SD

Variable fluorescence - Fv

LIST OF ABBREVIATIONS

ANOVA - analysis of variance

As - arsenic

B - boron

BG11-blue-green medium

C - carbon

Ca - Calcium

Cr - Cranium

CCM - chlorophyll Content Meter

Cd - cadmium

CF - chemical fertilizer

CFU-colony forming units

Chl- chlorophyll

CHNS-Carbon, Hydrogen, Nitrogen, Sulphur

CO_2- carbon dioxide

COD - chemical oxygen demand

Cu - copper

DCM - dichloromethane

DHA - docosahexaenoic

DIC - dissolved inorganic carbon)

DMB - de-oiled microalgae biomass

DMRT - Duncan's Multiple Range Test

DMSO - dimethyl sulfoxide

DO - dissolved oxygen

EN - European standards

EPA - eicosapentaenoic acid

Etc - etcetera

FA - fatty acid

FAMEs- fatty acid methyl esters

Fe- iron

Fig- figure

Fv/Fm-maximum efficiency of PSII

GC - gas chromatography

H - hydrogen

H_2SO_4 - sulfuric acid

HCl - hydrochloric acid

Hg - Mercury

HPC - heterotrophic plate count

HTL - hydrothermal liquefaction

i.e. - that is

K - potassium

LEA - lipid extracted algae

M-molar

Mg- magnesium

Mn^{2-}- manganese

Mo^{6+} - Molybdenum

MW- molecular weight

N - nitrogen

NA - Not available

Na- sodium

NaOCL - sodium hypochlorite

NaOH - sodium hydroxide

NF - no fertilizer

NH_4-N - ammonia

NO_2-N - nitrite

NO_3-N - nitrate

O-oxygen

Pb - Lead

PCW - post chlorinated wastewater

pH- potential of hydrogen

PLDE - Poultry litter digested extract

PLE - poultry litter extract

PLL - poultry liter leachate

PL - poultry litter

PO_4-P - phosphate

PPLE - pre-treated poultry litter extract

PS II - photosystem II

Pty Ltd - proprietary limited

PUFA - polyunsaturated fatty acids

R-2A agar – Reasoner’s 2A agarrETR -

elative electron transport rate

RPLE - raw poultry litter extract

Rpm - revolutions per minutes

SD - standard deviations

Sp. - species

TL-PBR - Twin-Layer photo bioreactor

TN - total nitrogen

TPF- triphenyl formazan

TP - total phosphate

TSS - total suspended solids

TTC - triphenyl tetrazolium chloride

UK - United Kingdom

WA - whole algae

WB - whole biomass

Zn - zinc

CHAPTER 1

INTRODUCTION

1.1. Context of the study

Inadequate accessibility of fossil fuel resources and environmental apprehensions about greenhouse gases has resulted in increased public and scientific attention toward biofuels (Chisti, 2007, Rawat et al., 2011, Singh et al., 2014, Faried et al., 2017, Shah et al., 2018, Mat Aron et al., 2021, Kandasamy et al., 2022). Biofuels, particularly biodiesel, are a viable alternative to traditional fossil diesel fuels (Deshmukh et al., 2019). Among different potential candidates for biofuels, microalgae are more popular because of some significant advantages such as CO_2 sequestration and nutrient retrieval pertaining to their ability to grow in wastewater streams (Usher et al., 2014, Chiu et al., 2015, Suparmaniam et al., 2019, Siddiki et al., 2022). Most importantly, microalgae do not need arable land as required by other potential terrestrial crops for biodiesel production. Even when grown under extremely unfavourable conditions, microalgae can thrive and produce valuable by-products. Moreover, microalgae contain a substantial amount of lipids per unit of biomass, which act as precursors for biodiesel production (Chen et al., 2018a, Khan et al., 2018).

Microalgae are capable of producing diverse bioproducts of commercial importance that are of interest in industries (Santana et al., 2017). Lipids, proteins, and carbohydrates are some of the biochemical compounds found within microalgal biomass that find commercial applications in biofuel, fertilizer, feed, and nutraceutical sectors (Ramsundar et al., 2017b, Mat Aron et al., 2021). Although microalgae have been recognized as optimistic feedstocks for biodiesel production, the commercial-scale production of microalgae biodiesel is hindered by challenges associated with the requirements of water and fertilizers. The amount of freshwater and fertilisers required for commercial scale microalgal biomass generation are significant impediments to the environmental sustainability and economic feasibility of microalgal biodiesel (Zaimes, 2013a, Ting, 2017, Kalra et al., 2021).

Wastewater is an inexpensive water and nutrient source, comprising numerous organic matters and nutrients, which are needed for microalgal growth, and thus it can be used for cultivation and biomass generation (Ramsundar et al., 2017b). Macronutrients such as phosphorous (P), nitrogen (N), calcium (Ca), magnesium (Mg), and micronutrients such as copper (Cu), iron (Fe), molybdenum (Mo^{6+}), manganese (Mn^{2+}) and zinc (Zn^{2+}) present in wastewater are the crucial elements required by microalgae for growth and reproduction (Guldhe et al., 2017a, Daneshvar et al., 2019). Microalgae have the ability to efficiently remove and utilize nitrogen and phosphorus present in wastewater and assimilate them into their biomass (Cai et al., 2013b, Arora et al., 2021). Subsequently, the biomass and lipids generated can be converted into biodiesel. This cultivation technique can assist in reducing the use of freshwater and minimizing the cost of nutrient addition (Wu et al., 2015). Pacheco et al. (2021) documented that utilizing wastewater for algae cultivation is potentially the most promising system to minimize the production cost related to water and nutrients.

Microalgae possess their own exclusive ability to thrive in a variety of environments under different cultivation modes. These intriguing groups of tiny photosynthetic microorganisms are able to adopt various routes of metabolism for their growth and survival in photoautotrophic, heterotrophic, and mixotrophic conditions (Prathima Devi et al., 2012). The most predominant microalgae cultured are known as photoautotrophic microalgae (Hu et al., 2018). In the photoautotrophic mode of cultivation, CO_2, inorganic nutrients, and light energy are the most crucial elements that are required by microalgae for growth and reproduction purposes (Daneshvar et al., 2019). In heterotrophic cultivation, microalgae are allowed to grow and metabolize in the absence of light energy (under dark conditions), utilizing organic carbon as the source of energy (Hu et al., 2018). Mixotrophic cultivation is an additional potential mode for biomass and lipid production in which light energy and organic carbon as energy sources are supplied concurrently to the algae production system (Patnaik and Mallick, 2021). Wastewater contains organic carbon, which influences the cultivation modes and makes it a suitable substrate for microalgae cultivation. Wastewater streams can be utilized as nutrient sources for microalgal cultivation. However, low biomass productivity, bacterial contamination, water, and nutrient availability remain challenges.

Among the various wastewater streams, domestic, industrial, and agricultural wastewater are the most popular streams which have been examined as water and nutrient substitutes for microalgal cultivation for feasible biomass production (Chiu et al., 2015, Guldhe et al., 2017a, Arora et al., 2021). Each type of wastewater stream has its exclusive characteristics and challenges due to variation in physicochemical characteristics and nutrient load. Combining more than one wastewater stream to improve nutrient availability has been shown to be beneficial for improving nutrient availability for microalgae cultivation (Renuka et al., 2021). Additionally, the use of various pre-treatment procedures prior to microalgae cultivation is found suitable to reduce the microbial load that might compete with the microalgae (Zhang et al., 2021).

Many studies on the utilization of various wastewater streams for microalgae cultivation have been conducted and have shown great potential as a nutrient source and biomass production of algal biomass for biofuel/biodiesel applications. However, there is still a huge concern about the economic feasibility and sustainability of biodiesel production from microalgae because the application of technology for a single purpose may not be a commercially viable strategy (Han et al., 2019). To make the production process of microalgal biodiesel more feasible, an integrated biorefinery approach needs to be implemented (Al Ketife et al., 2019, Almomani et al., 2019, Fan et al., 2020). The biorefinery concept, i.e., the production of multiple products from a single biomass feedstock, has been reorganised as one of the useful strategies to improve the economic viability of microalgae-based products (Silambarasan et al., 2021).

The production of biodiesel from microalgal biomass produces residual biomass, which is generally rich in metabolites and nutrients. Therefore, the production of biodiesel from microalgae must be combined with the production of other useful products to get an energy return on investment (Silambarasan et al., 2021). The transition from a single product strategy (biodiesel production) to multi-product production has the potential to improve the viability and competitiveness of microalgal biofuels. The utilization of lipid extracted biomass can generate extra income, possibly increasing the cost-effectiveness of biodiesel production. The application of residual biomass as fertilizer to assist in the growth and production of crops is of significance. This novel approach can result in the recycling and reuse of microalgal biomass assisting in waste valorization and forming a sustainable biorefinery. Additionally, the use of microalgal biomass residue can help reduce the use of chemical fertilizers in agriculture, which have been reported to have an adverse impact on the environment (Chew et al., 2017). Maurya et al. (2016b) stated that lipid extracted biomass is a rich source of proteins, and carbohydrates and is rich in nutrients such as carbon and nitrogen, which can be utilized in the production of fertilizer, feed, and substrate for bioethanol and biomethane production.

Microalgae even have the potential to stop nutrient losses through the gradual release of N, P, and K (Renuka et al., 2018a). Besides the release of macronutrients, these phototrophic microorganisms also contain trace elements and plant growth-promoting substances like phytohormones, vitamins, carotenoids, amino acids, and antimicrobial substances. Recently, lipid extracted biomass of green algae has been investigated as fertilizers in agriculture due to their nutrient-rich biomass, which provides nutrients mainly through the mineralization process (Maurya et al., 2016b, Silambarasan et al., 2021). Microalgal biomass is also rich in organic carbon and can be useful in the improvement of soil organic carbon and fertility. However, limited literature on the use of lipid extracted biomass as fertilizer is accessible.

The challenges of the availability of nutrients, microbial contamination, and low microalgal biomass productivity in wastewater still need to be addressed. Additionally, the idea of biorefinery approaches for simultaneously producing various products from a single microalgal feedstock is yet to be established and explored. This study thus focused on developing a suitable strategy for the utilization of waste streams for microalgal production while concurrently producing multiple products for biofuels and agricultural applications.

The aim and objectives are as follows:

1.2. Aim and objectives of the study

1.2.1. Aim

Evaluation of wastewater streams as a nutrient source for the production of microalgal biodiesel and fertilizer.

1.2.2. Objectives

- To investigate the suitability of wastewater as a cultivation medium for microalgal biomass production.
- To evaluate the macromolecular composition of wastewater grown microalgal biomass.
- To assess the potential of wastewater grown microalgal biomass for biodiesel production.
- To evaluate the efficacy of whole and residual biomass as fertilizer for agricultural application in a laboratory scale study.

CHAPTER 2

REVIEW OF LITERATURE

2.1. INTRODUCTION

Because of the escalating cost of petroleum-based fuel and irrepressible environmental concerns such as global warming, numerous countries are promoting the use of biodiesel as a fuel for transportation. Renewable feedstocks for biofuel generation have a great potential to eradicate the ever-increasing greenhouse gases to a greater extent. There is a humongous need for biofuel renewable feedstock as alternatives to the fossil fuels (Milano et al., 2016). Biodiesel has been gaining enormous scientific and public interest as a green renewable fuel and a promising substitute for sustainable energy (Chisti, 2007, Rawat et al., 2013). Biodiesel is defined as a mixture of fatty acid monoalkyl esters derived from the reaction of vegetable oils or lipids with or without a catalyst for use in diesel engines (Faried et al., 2017, Zahan and Kano, 2018). Locally, biodiesel is often produced using different feedstocks depending on their accessibility in a particular area providing energy security. At present, oils from animals and plants are used as the main source of biodiesel (Khan et al., 2018, Zahan and Kano, 2018). Oil crops derived from biodiesel could be the potential renewable and carbon neutral substitute for fossil fuels. Regrettably, biodiesel from animal fat, waste cooking oil, and oil crops cannot genuinely meet even half of the transport fuel demand that is already in existence. Therefore, microalgal biodiesel is the centre of attention as an environmentally friendly substitute (Singh et al., 2014, Faried et al., 2017, Shah et al., 2018).

In comparison to other available feedstocks for biofuels, microalgae show some significant advantages including nutrient retrieval and CO_2 uptake (Usher et al., 2014, Chiu et al., 2015). In general, the efficiency of photosynthetic microalgae compared to plants is higher (Raheem et al., 2018). Moreover, they contain a higher amount of lipids per unit biomass, can be grown using waste substrates such as wastewater and their growth rate has been found to be higher (Chen et al., 2018b, Khan et al., 2018). Most importantly, microalgae do not need fertile land for their cultivation as required by other terrestrial energy crops. The biochemical compounds such as lipids, proteins, and carbohydrates within the cells may provide a glimpse into the metabolic and physiological condition of an organism. Even when grown under extremely unfavourable conditions, microalgae are able to flourish their growth and produce valuable by-products.

Regardless of these advantages, the production of microalgal biodiesel is still not feasible enough to be commercialized. Identification of a microalgal strain with high oil productivity, development of cost-effective harvesting, lipids conversion to biodiesel, and growing systems are the main bottlenecks in the production of biodiesel from microalgae (Sharma et al., 2016, Tan et al., 2018). Furthermore, the production of large commercial scale biodiesel has been handicapped by the necessity of water for microalgal cultivation, and the cost and amount of fertilizers for an efficient biomass generation for biodiesel production (Zaimes, 2013b, Gupta et al., 2016, Ting, 2017, Chen et al., 2018a, Guldhe et al., 2019). These challenges can be resolved by using an inexpensive nutrient and water source from waste streams (Renuka et al., 2018a).

The production of biodiesel and fertilizer using microalgal biomass grown using suitable wastewater streams can reduce the use of freshwater and minimize the cost of nutrient addition

(Wu et al., 2015). Guldhe et al. (2017c) suggested that the production process of microalgal biodiesel feasibility can be achieved *via* the implementation of an integrated biorefinery approach. Biorefinery refers to the sustainable production of biomass using inexpensive substrates as well as the sustainable processing of biomass to obtain a wide range of value-added products simultaneously through low waste production (González-Delgado, 2011).

2.2. Wastewater as a cultivation medium for microalgae

Fabrication and utilization of natural resources for the survival and day-to-day activities of humans produces multiple unusable by-products that eventually end up as waste and are eventually released into the environment. Currently, the wastewater streams are increasingly adulterated with these by-products and contaminants. Wastewater types are mostly classified according to their origin or point source, such as agricultural, municipal, agro-industrial, and industrial waste (Lellis et al., 2019, Bhatia et al., 2021, Bhuyar et al., 2021). Wastewater released into the environment from these sources is rich in nutrients and, therefore, promotes eutrophication, which impacts the environment and human lives negatively.

Microalgae have shown to be dynamic in the recovery of nitrogen, phosphorus, and other contaminants from a broad range of wastewater. Several empirical studies have focused on the utilization of wastewater as a substrate for microalgae growth, particularly in agricultural, municipal, and industrial wastewater (Renuka et al., 2014, Garcia-Gonzalez and Sommerfeld, 2016, Garcia et al., 2018, Khalid et al., 2019a, Khalid et al., 2019b). The significance of microalgae cultivation using wastewater in high-rate ponds (HRP) was revealed in the 1950s (Oswald, 1957). Numerous studies have proved the ability of different microalgal species to remove nitrogen, phosphorus, and other nutrients (Markou and Monlau, 2019). Such studies have reported a substantial amount of nutrient removal and algal biomass production in a wide range of wastewater. Microalgal biomass seeks application in various industrial sectors such as feed, food, biofertilizers, and renewable fuels. Various wastewater sources, composition, and their potential as substrates/medium for microalgae cultivation are discussed.

2.2.1. Municipal wastewater

Generally, municipal wastewaters are a combination of domestic (80-95%) and industrial (5-20%) influents. It mainly contains nutrients, microorganisms, and other organic waste, household, and industrial chemicals; however, most significantly, the percentage composition of municipal wastewater differs based on local community activities. The distinctive components of such types of wastewater involve nutrients, COD (chemical oxygen demand), metals, pathogenic microorganisms, and organic and inorganic materials. Ammonia (NH_4-N), phosphate (PO_4-P), and other nutrients essential for microalgal growth are the most dominant nutrients in municipal wastewater. Conversely, in the activities of some localized small-scale factories, a significant level of heavy metals such as cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), zinc (Znad et al., 2018), mercury (Hg), arsenic (As), etc. are also present in raw municipal sewage (Tytla, 2019). It is of great significance to examine wastewater characteristics for the determination of its suitability for microalgal propagation (Renuka et al., 2014). Komolafe et al. (2014) indicated that the characterization of wastewater serves to examine wastewater treatment process during the microalgal cultivation period, and also gives an understanding of the requirement of dilution or nutrient supplementation in wastewater for the growth of microalgae. Research studies based on the usage of municipal wastewater for microalgal cultivation have been done extensively. Different microalgal species have shown a capability to recover wastewater nutrients and production of substantial biomass, which can be exploited for wider applications (Table 2.1).

The research studies on growing microalgae in municipal wastewater for nutrient removal process and biomass production are conducted extensively. Several strains of microalgae have been utilized and have proven to possess the potential of removing nutrients from wastewater. However, nutrient removal efficiency and biomass production potential are prominently dependent on the nature of strains, cultivation conditions, and type of wastewater.

The retrieval of nitrate (NO_3-N) and PO_4-P from wastewater is of great significance in the view of tightened discharge regulations. A study conducted by Ramsundar et al. (2017a) evaluated the suitability of different wastewater streams within a domestic wastewater treatment plant for the cultivation of *Chlorella sorokiniana*. Promising biomass production of 72.5 mg L^{-1} d^{-1} and 77.14 mg L^{-1} d^{-1} was achieved in *C. sorokiniana* with 89.13% and 94.29% of NH_4-N removal in influent and anaerobic centrate wastewater respectively under mixotrophic cultivation conditions. However, *C. sorokiniana* showed a lower NO_3-N removal rate of 19.23% and 28.67% in influent and anaerobic centrate respectively. Higher COD (Chemical oxygen demand) removal was obtained in heterotrophic cultivation as compared to mixotrophic growth. In contrast, Li et al. (2011) conducted a study based on the evaluation of an adapted strain of *Chlorella sp.* to highly concentrated municipal wastewater for its efficiency for nutrient removal and biodiesel production. The results indicated the removal efficiency of 93.9%, 89.1%, 80.9%, and 90.8%, of NH_4-N, TN, TP, and COD correspondingly on day 14 of batch culture. Shi et al. (2014) fabricated a prototype TL-PBR (Twin-Layer photo bioreactor) system with immobilized *Halochlorella rubescens,* microalga for the removal of nitrogen and phosphorus from primary and secondary municipal wastewater. The average growth of *H. rubescens* was reported to be 6.3 g m^{-2} d^{-1}. Phosphorus and nitrate concentrations in the effluents could efficiently be reduced by 70-99% after treatment. Nevertheless, under precise functioning conditions, a pilot scale test should be implemented at a wastewater treatment plant to assess the economic, ecological, and technical insinuations of this innovative technology for efficient nutrient removal from wastewater.

The strategies for diluting highly concentrated wastewater with weak wastewater have been proved promising. Reyimu and Özçimen, (2017) supplemented wastewater with seawater for the cultivation of *Nannochloropsis oculata* and *Tetraselmis suecica* under similar culture conditions (Reyimu and Özçimen, 2017). *N. oculata* had the highest specific growth rate of 0.5430 d^{-1} (75% of wastewater) followed by *T. suecica* with a growth rate of 0.4778 d^{-1} (25% of wastewater). A study conducted by Onay (2018) cultivated *N. gaditana* in various ratios of municipal wastewater for the production of bioethanol. *N. gaditana* showed the highest biomass productivity in 30% wastewater. Furthermore, Zhou et al. (2018) studied the potential application of municipal wastewater mixed with pig biogas slurry as a supplement for the cultivation of *Chlorella zofingiensis.* Results revealed that pig biogas slurry (8%) in municipal wastewater had a substantial outcome on microalgal growth. *C. zofingiensis* obtained 2.5 g L^{-1} biomass yield and removed 93% and 90% of total nitrogen (TN) and total phosphorus respectively (Maurya et al., 2016a). Leite et al. (2019) blended municipal wastewater with piggery wastewater for the cultivation of *C. sorokiniana* in a UASB (Up flow anaerobic sludge blanket reactor) reactor. The UASB reactor showed >90% of organic matter removal with substantial biomass production of 1g L^{-1}, with average removal of 40-60%, 100%, and 46-56%, of PO_4-P, NH_4-N, and DIC (dissolved inorganic carbon) respectively. Municipal and piggery wastewater combination utilized for *C. sorokiniana* cultivation presented the tremendous potential to resolve the wastewater dilution problem from centralized wastewater (Leite et al., 2019). Their findings illustrated a pertinent NH_4-N removal *via* air stripping, reducing the N:P ratio during microalgal cultivation and, hence, improving the nutrient recycling efficiency and biomass production. Further studies on reducing ammonia stripping and improving nutrient assimilation in microalgal biomass could be useful for improving the nutrient recovery process.

Municipal wastewater contains an adequate amount of different nutrients required for algal growth; however, the limiting factors and undesirable components of wastewater should be taken into consideration. Low concentrations of nutrient content and excessive, and/or varying organic load in municipal wastewater are limiting factors that could affect microalgal growth. Raw wastewater with higher concentrations of TSS (Total suspended solids) would hinder light penetration and scattering, which could have a negative influence on microalgal growth under phototrophic conditions. High concentration of heavy metals in municipal/urban wastewater and other emerging organic contaminants are the research areas that still need to be explored in depth. Some metals can act as micronutrients for microalgal growth, whilst others are dispensable and can exert toxic impact at high concentrations (Renuka et al., 2014). Nevertheless, the effect of heavy metals on microalgae can vary among different microalgae species and is extremely dependent on the type of compounds (Kumar, 2015). Microalgal species that possess a high affinity for polyvalent metal ions can be exploited for the remediation of such metals from wastewater.

Table 2.1 Nutrient removal and biomass production by different microalgal species in municipal wastewater

Algal strain	Type of wastewater	Removal Efficiency (%)	Biomass yield (Y) or productivity (P)	Applications	Reference
Halochlorella rubescens	Primary and secondary municipal wastewater	TP-70 TN-99	6.3 g m^{-2} d^{-1} (P)	Nutrient removal, Biomass production	(Shi et al., 2014)
Nannochloropsis gaditana	Municipal wastewater (MW)	NA	0% MW -0.92 g L^{-1} (P) 30% MW -1.87 g L (P) 60% MW -1.44 g L (P) 100% MW -0.94 g l ^{1} (P)	Bioethanol	(Onay, 2018)
Chlorella zofingiensis	Municipal wastewater supplemented with pig biogas slurry	TN-93 TP-90	2.5 g L^{-1} (Y)	Nutrient removal, Biomass and lipid production	(Zhou et al., 2018)
Dunaliella salina	Tertiary treated municipal wastewater	COD-52 NH_4-N-70.7 NO_3-N-88 PO_4-45.7	0.209 g L^{-1} d^{-1} (P)	Nutrient removal efficacy and biomass production	(Liu et al., 2018)
Chlorella sorokiniana	Diluted municipal and industrial wastewater	COD-52.1 TN-57.5 TP-68.8	1.524 g L^{-1} d^{-1} (P)	Biomass production, high-added value products and biofuels	(De Francisci et al., 2018)
Chlorella sorokiniana	Diluted effluent mixed with piggery Wastewater	COD-NA NH_4-N-100 NO_3-N-NA PO_4-P-40-60 DIC-46-56	1.0 g L^{-1}(Y)	Nutrient removal	(Leite et al., 2019)
Wild-algae and *Scenedesmus*	Simulated Municipal wastewater	COD-NA NH_4-N-NA NO_3-N-87 PO_4-P-19	0.278 g L^{-1} (Y)	Nutrient removal efficiency. Lipid Production	(Qu et al., 2019)
Scenedesmus sp. 336 and activated sludge symbiotic system	Synthetic municipal wastewater	COD-100 NH_4-N-87.13 NO_3-N-100 PO_4-P-99.82	0.81g L^{-1} (Y); 0.115 g L^{-1} d^{-1}	Nutrient removal efficiency. Biomass and lipid production	(Chen et al., 2019)
Microalgae (*Scenedesmus*) and bacteria (Sphingobacteria, Flavobacteria, and Proteobacteria) consortium	Municipal wastewater	COD-92 NH_4-N-NA TN-95.8 TP-98.1	1.8 g L^{-1} (Y)	Nutrient removal. Biomass and lipid production	(Lee and Han, 2016)

Chlorella sarokiniana, Nitrosomonas and *Dechloromonas*	Synthetic Municipal wastewater	COD-88 NH_4-N-NA NO_3-N-98 PO_4-P-96	2.5 g L^{-1} (Y)	Nutrient removal	(Fan et al., 2020),
Chlorella vulgaris and activated sludge	Municipal wastewater	COD 5-64 TN-94-95% PO_4-P-NA	1.42 g L^{-1} (Y)	Biomass and lipid productivity	(Leong et al., 2019)
Scenedesmus obliquus and wild yeast	Municipal	COD-95 NH_4-N-100 NO_3-N-100 PO_4-92.6	2.74 g L^{-1} (Y)	Bioethanol	(Walls et al., 2019)
Microalgae-bacteria consortia	Influent (pre-settled municipal wastewater)	COD-87 NH_4 -99 NO_3-N -NA PO_4-P-16	NA	Nutrient removal	(Foladori et al., 2018)
Microalgae-bacteria consortia	Municipal wastewater	COD-50 TN-36 PO_4-P-92 (High irradiance) COD-89 TN-60 PO_4-P-28 (Low irradiance)	NA	Nutrient removal	(Arcila and Buitrón, 2017)
Chlorella zofingiensis	Municipal wastewater	TN-93 TP-90	2.5 g L^{-1} (Y)	Nutrient removal, biomass and lipid productivity	(Zhou et al., 2018)
Chlorella Vulgaris	Municipal wastewater	COD-83.0 TN-96.5 NH_3-N-97.8 TP-99.2	1.62 g L^{-1} (Y)	Nutrient removal and biomass production	(Xu et al., 2019)

*NA – Not available; COD – Chemical oxygen demand; MW – municipal wastewater; NH_4-N –Nitrogen in the form of ammonium; NO_3-N – Nitrogen in the form of nitrate; TN – Total nitrogen; PO_4-P – Phosphorus in the form of phosphate; TP: Total phosphorus; DIC – Dissolved inorganic carbon

Furthermore, abundant indigenous pathogenic microorganisms and bacteria found in municipal wastewater may also affect the growth of microalgae primarily via cell meditated contact and/or secondarily through extracellular compounds (Kouzuma and Watanabe, 2015). These indigenous microorganisms can compete with inoculated microalgae for essential macro- and micro-nutrients. Conversely, microalgae can pose a detrimental effect on microbial growth and activity due to an increase in pH, DO, and/or excretion of growth inhibiting metabolites (Kouzuma and Watanabe, 2015). Additionally, beneficial associations between different microalgal and bacterial species in wastewater treatment processes have also been revealed by various studies (de-Bashan et al., 2004, Ji et al., 2018, Bohutskyi et al., 2019, Leong et al., 2019). Extensive investigations need to be done for the identification of bacteria that possess symbiotic relationships with potential microalgal strains, and exploration of their potential role in such cultivation systems.

2.2.1.1. Cultivation of microalgae in municipal raw wastewater

Raw domestic wastewater which is extensively dispersed in villages and towns is rich in nutrients and several algae strains have been reported to grow well in this wastewater type (Mennaa et al., 2015, Ma et al., 2020b). Cultivation of algae in domestic wastewater does not only provide algae with nutrients but also aids in the process of bioremediation since it contains heavy pollutants. Singh et al. (2017) screened *Chlorella* sp*, Nannochloropsis gaditana, Chlamydomonas reinhardtii,* and *Parachlorella kessleri-I* for municipal wastewater phycoremediation and the production of biodiesel. The authors observed an increased biomass and lipid production in one of the strains (*P. kessleri-I*). Further, *P. kessleri-I* reclaimed major nutrients from the wastewater after 10 days of growth. The biomass obtained was further analyzed for its potential for biodiesel production and it was concluded that green algae (*P.*

kessleri-I) have the effective potential to reclaim nutrients from wastewater and can be utilized to produce biodiesel. Apart from nutrients suitable for microalgae growth, raw municipal wastewater contains different concentrations of toxic compounds that may hinder the growth of microalgae if their concentration is too high (Wang et al., 2017, Han et al., 2019). Lately, researchers have come up with various ways how to overcome the challenges faced when algae are cultivated in raw wastewater. To date, various pre-treatment strategies to minimize the bacterial load have been introduced. Ramsundar et al. (2017a) studied the suitability of a domestic wastewater treatment plant at various stages for microalgal growth. In their study, various pre-treatment methods were assessed to minimize the bacterial load. The results reported showed increased biomass productivity in anaerobic centrate. Furthermore, the filtration pre-treatment method was recorded as the most viable strategy for bacterial load minimization. Based on the findings given in various studies, the use of these pre-treatment methods might be a suitable solution. However, more studies on the pre-treatment of municipal wastewater for microalgae cultivation still need to be conducted. Fernandez-Linares et al. (2017) conducted a study aimed at investigating the viability of microalgae (*Chlorella* CICESE, *Chlorella vulgaris* UTEX, and indigenous consortium) grown in secondary treated wastewater from a domestic treatment plant (Fernandez-Linares et al., 2017). Additionally, the treated secondary wastewater was supplemented with the fertilizer Bayfolan ®. Their results showed enhanced pigments and biomass productivity when secondary treated wastewater was supplemented with fertilizer.

On the other hand, nutrient supplementation for boosting biomass production has gained scientific attention. The commercialization of microalgae produced products is still held back by low biomass productivity. The attempts on cultivating microalgae under different cultivation conditions supplemented with nutrients have been put into practice. *Tetradesmus* *obliquus* PF3 was grown in municipal wastewater supplemented with nitrate to fulfil the nutritional requirement of microalgae. Biomass productivity of 1.75 g L^{-1} was accumulated in 8 days (Ma et al., 2020b). Tripathi et al. (2019) cultivated *Scenedesmus sp.* ISTGA1 for 14 days at different concentrations of filtered (0.22 μm) sterilized wastewater (25%, 50%, and 100%) for biodiesel production. The findings suggested that *Scenedesmus sp.* ISTGA1 can be cultivated in pre-treated wastewater for biodiesel production with additional benefits of nutrient recovery. Further, Ramsundar et al. (2017b) supplemented domestic wastewater with different concentrations of urea under various cultivation conditions (mixo, auto, and hetero) to enhance the productivity of biomass. The results showed an increased biomass yield when 1500 mg L^{-1} urea was added. Additionally, enhanced production of biochemical constituents was observed. Based on their findings, it can be concluded that nutrient supplementation has the ability to increase the productivity of both biomass and biochemical compounds.

2.2.1.2 Anaerobic digestate

Anaerobic digestion is a process involving the use of anaerobic microorganisms for the breakdown of industrial or municipal organic waste materials in oxygen free environment. Mostly when this process occurs, anaerobic bacteria convert wastes into energy. During this process, biogas rich in methane and the recurrent discharge of effluent digestate full of organic and inorganic, metal salts, microorganisms, and undigested solids results. High concentrations of nitrogenous compounds particularly ammonia are the most predominant compounds in anaerobic liquid digestate (Uggetti et al., 2014, Praveen et al., 2018). Moreover, macro and micronutrients contained in anaerobic digestate are essential for microalgal growth. Several previous studies on anaerobic digestate as a source of nutrients for microalgae growth have been reported (Pittman et al., 2011, Ramsundar et al., 2017b). Zuliani et al. (2016) observed increased biomass, lipid productivity, and carotenoid accumulation when *Chlorella* was grown in municipal, agro-waste, and sewage sludge digestate. Though anaerobic digestate substrate supports the growth of microalgae, a few challenges have been reported. Olsson et al. (2018) utilized co-digested municipal wastewater with sewage sludge in mesophilic conditions for growing a natural mix of microalgae. The findings indicated that when microalgae were added, methane yield was reduced from 200 to 168 NmL. Further, they also noted a low digestibility in their batch test.

2.2.2. Agricultural wastewater

Agriculture, one of the main industries around the globe, generates a variety of wastewater rich in N and P. Agricultural wastewater is deliberated as a suitable nutrient source for microalgae cultivation because of the similarity between microalgal nutrition requirements, nutrient concentration, and easy availability. Several microalgal strains have revealed the ability to utilize nutrients and grow in a range of agricultural wastewater (Abou-Shanab et al., 2013, Lowrey et al., 2014). Agricultural wastewaters also contain solid wastes such as animal compost, plant materials, etc. (Chiu et al., 2015). The nutrient composition of these wastewaters however purely depends upon the source of origin (Table 2.2). Wastewaters from the animal husbandry industry are one of the most polluting sources of wastewater because of the high concentration of organics (Olguin, 2012). However, location, age, productivity, animal diet, management, and usage have a great influence on the characteristics of animal wastewater. The N: P ratio in swine, beef feedlot, and dairy wastewater is approximately 2-8 (Cai et al., 2013a). Regardless of high nutrient content, proficient growth of microalgae on agricultural wastewater has been demonstrated in various studies (Table 2.2). Additionally, different studies have also revealed the capability of microalgae in eliminating nitrogen and phosphorus from agricultural wastewater. For instance, Hena et al. (2015) showed that a consortium of native microalgae was capable of eliminating >98% nutrients from dairy wastewater with 153.5 t ha^{-1} $year^{-1}$ and 16.9 % of biomass and lipid production respectively.

Microalgal biomass generation using wastewater from the animal husbandry industry could form a sustainable biorefinery since the generated biomass could be further utilized as a protein supplement in animal feed production. A study by Ansari et al. (2017) investigated the potential of aquaculture wastewater as a nutrient source supplemented with sodium nitrate for the cultivation of *Scenedesmus obliquus, Chlorella sorokiniana,* and *Ankistrodesmus falcatus* and their nutrient removal efficiencies. The biomass acquired in their study demonstrated adequate protein, lipid, and carbohydrate productivities to be utilized as a feed supplement. *A. falcatus* showed the highest biomass and lipid yields among the selected strains. Cultivation of microalgal strains in aquaculture wastewater showed high nutrient removal efficiencies for NO_3-N (> 75%), PO_4-P (> 98%), and NH_4-N (> 86%). The composition of generated biomass was found to be suitable for its use as a feed supplement. Whereas Zhu and Hiltunen (2016) examined the microalgal biomass and lipid production in livestock waste. The livestock waste compost medium provided optimal nutrients concentration for the cultivation of *Chlorella sp*. and obtained 288.84 mg L^{-1} day^{-1} and 104.89 mg L^{-1} d^{-1} of biomass and lipid productivities, respectively. Thus, these biorefinery concepts have great potential to benefit the agriculture sector via the value-addition of wastewater-grown microalgal biomass. Further, a study by Guldhe et al. (2017) revealed the potential of aquaculture wastewater as a substrate for *Chlorella Sorokiniana* cultivation for the generation of biomass for the production of biofuels or feed applications with additional benefits of nutrient reclamation. Sodium nitrate was supplemented in aquaculture in order to increase the productivity of biomass. The strategy of supplementing aquaculture wastewater with sodium nitrate improved the growth of microalgae and the productivity of lipids, proteins, and carbohydrates. The results obtained in their study clearly emphasized the extent of aquaculture wastewater as a medium for microalgae cultivation.

2.2.2.1. Poultry Litter

Production of poultry domesticated livestock produces a massive amount of significant organic wastes (poultry litter) that the poultry producers clean to promote animal health and control the building up of manure to avoid disease outbreaks (Agwa, 2014). Poultry litter (PL) is a rich source of organic and inorganic macronutrients such as phosphorus (P), nitrogen (N), carbon, potassium (K) (Markou et al., 2016), and micronutrients such as Copper (Cu), Iron (Fe), Zinc (Znad et al., 2018), Manganese (Yeomans and Bremner, 2008), Boron (B) and Sodium (Na) which are essential for microalgal growth (Drekeke Iyovo et al., 2010, Markou et al., 2016). The use of PL as a nutrient source for microalgae growth may diminish the reliance on synthetic fertilizers, which could minimize the overall production cost (Markou et al., 2016, Han et al., 2019). In most cases, poultry litter produced from chicken industries is in solid form and therefore requires to be dissolved in an aqueous medium to extract the nutrients. Previous studies indicated the need for diluting nutrient rich wastewater with wastewater containing a low concentration of nutrients to reduce the concentration of nutrients to support the growth of microalgae (Cheah et al., 2018, Garcia et al., 2018, Mishra and Mohanty, 2019). Further, this also helps in minimizing the utilization of fresh water which is already scarce (Guldhe et al., 2017a, Mishra and Mohanty, 2019).

Few successful studies on microalgal cultivation in poultry litter have been conducted. Markou et al. (2016) investigated PL as a source of nutrients for the growth of *A. platensis and C.*

vulgaris. It was found that under leachate dilution of 15x and 10x *A. platensis* struggled to survive and the biomass obtained from higher dilutions was half of that in a control media (BG11). However, *A. plantesis* could achieve a high amount of carbohydrates (37- 44%). On the other hand, *C. vulgaris* was able to survive in PPL media and could achieve a high amount of biomass. Singh et al. (2011) utilized anaerobic digested poultry litter effluent for the cultivation of mixotrophic algae (*Chlorella sorokiniana*, *Scenedesmus bijuga,* and *Chlorella minutissima*) for biomass production. Biomass productivity of 76 mg L^{-1} d^{-1} was achieved from microalgae grown in PLDE. Further, anaerobically digested poultry manure was used to cultivate *Chlorella vulgaris* for nutrient removal and biomass production. Two hours (2-h) of electrolysis pre-treated effluent achieved the maximum biomass of 0.53 g L^{-1} (Wang et al., 2015). On the other hand, a study by Agwa, utilized poultry litter dissolved in distilled water to grow *Chlorella* sp. for biomass and lipid production (Agwa, 2014). The results achieved revealed the potential of poultry manure as a nutrient source for microalgae cultivation, since a reasonable amount of biomass was produced as compared to control.

2.2.2.2. Dairy wastewater

Among the various streams of agricultural wastewater, wastewater discharged from dairy industries has a high concentration of phosphorus, nitrogen, and organic matter. The presence of such nutrients makes dairy wastewater a suitable substrate for microalgae cultivation. Previous studies have also reported successful cultivation, nutrient reclamation, and lipid production from microalgae grown in dairy wastewater (Abreu et al., 2012, Choi et al., 2018, Pereira, 2019). A study on raw and recycled dairy wastewater for microalgae cultivation under various cultivation conditions indicated that *Tetraselmis suecica* and *Scenedesmus quadricauda* were able to reclaim nutrients and attain biomass concurrently (Daneshvar et al., 2019). Further, most studies with reasonable biomass productivity accumulation have been reported under mixotrophic cultivation.

Pang et al. (2020) investigated the prospect of integrating extremophilic algae cultivation in anaerobically digested dairy wastewater, from their observation, the highest biomass concentration was achieved together with the improved nutrient removal efficiency. Moreover, *Chlorella vulgaris* was able to tolerate the highest content of ammonia in the utilized media. Mixotrophic cultivation of microalgae in dairy wastewater can be considered a viable approach to minimize microalgal biomass production cost-effective method as it does not require any modification or the addition of costly chemical nutrients to the growth medium (Abreu et al., 2012, Choi, 2016, Choi et al., 2018, Pereira, 2019). Production of biomass under heterotrophic conditions in dairy wastewater is highly dependent on the adaptiveness of the strain. *Chlorella* sp. and *Chlorella vulgaris* were cultivated in highly turbid dairy wastewater under heterotrophic conditions, nevertheless, *Chlorella* sp. performed poorly while *Chlorella vulgaris* was able to grow (Choi, 2016). In particular, *Chlorella vulgaris* (*C. vulgaris*) is known to show high resistance to organic matters and stable growth in various wastewaters (Kwon et al., 2019). Based on the previous reports, more microalgal strains need to be characterized and evaluated for their potential to grow, reclaim nutrients, and produce biomass for lipids and other constituents when grown in dairy wastewater.

2.2.2.3. Aquaculture

Recent aquaculture activities have a deleterious effect on the environment, particularly because of the release of high P (phosphorus) and N (nitrogen) concentrations that can give rise to eutrophication environments (Andreotti, 2017). Aquaculture generates wastewater rich in nutrients. Utilization of microalgae to minimize the nutrients before discharge is a better solution to minimize eutrophication.

Aquaculture wastewater contains nutrients suitable for microalgae growth and development. Cultivation of microalgae in aquaculture wastewater is a biorefinery concept that offers dual benefits of consequent nutrient removal and biomass production (Ansari et al., 2017). Ansari et al. (2017) cultivated *C. Sorokiniana* and *A. falcatus* in aquaculture wastewater supplemented with sodium nitrate and the results obtained were compared to synthetic media results. The highest biomass productivity of 198.46 mg L^{-1} d^{-1} was achieved by *A. falcatus* in aquaculture wastewater supplemented with 400 mg L^{-1} of sodium nitrate, whereas *C. sorokiniana* achieved the highest biomass productivity of 157.04 mg L^{-1} d^{-1} when cultivated in aquaculture wastewater supplemented with 600 mg L^{-1} of sodium nitrate. These results were comparable to that of synthetic media. Furthermore, satisfactory nutrients (ammonia, phosphate, nitrate) and COD reclamation results were observed. Recently, Zhang et al. (2019) cultivated *C. Sorokiniana* in mariculture wastewater with monosodium glutamate supplementation and observed an accumulation of proteins with crucial amino acids, carotenoids, and unsaturated fatty acids. They concluded that *C. sorokiniana* cultivated in modified mariculture wastewaters could be utilized as a protein source in the preparation of feeds. Andreotti compared the potential of three (*Isochrysis galbana, Tetraselmis suecica, Dunaliella tertiolecta)* microalgal species grown in grey mullet Mugil Cephalus wastewater (Andreotti, 2017). Among the three microalgal species, *Tetraselmis suecica* achieved the highest biomass yield. While *Dunaliella tertiolecta* and *Tetraselmis suecica* showed a better potential to remove nutrients from wastewater.

2.2.2.4. Piggery wastewater

The pig industry is becoming a fast-growing industry since there is a high demand for pork due to the increased population (Chen et al., 2020). Piggery wastewater is rich in organic matters as well as phosphorus and nitrogen. As stated in previous literature, piggery wastewater that is anaerobically digested contains ammoniacal nitrogen (1.196-3.141 mg L^{-1}) that is considered to be rarely treatable using biological treatment (Park and Craggs, 2010, Carney et al., 2014, Kwon et al., 2018, Kwon et al., 2019, Kwon et al., 2020). Moreover, this nitrogen present in piggery wastewater is toxic to living organisms. Its discharge into water streams has a high risk of increasing eutrophication. Microalgae have the potential to assimilate nutrients to produce biomass and therefore their cultivation in piggery wastewater may be useful for bioremediation and biomass generation (Kwon et al., 2020). However, not all microalgae species are able to survive in piggery wastewater besides *Spirulina*, *Chlorella*, and *Scenedesmus* which are the most commonly used microalgae species for nutrient reclamation (Kwon et al., 2020).

Various investigations of microalgae growth in piggery wastewater for biomass and nutrient reclamation have been conducted and the findings have been promising. Piggery wastewater has a high concentration of nutrients particularly ammonia, and microalgae perform poorly in concentrated medium, thus the dilution/pre-treatment of piggery wastewater is necessary before cultivation. Kwon et al. (2020) conducted a study aimed at evaluating the prospect of utilizing piggery wastewater (raw and anaerobically digested) for cultivating *C. vulgaris*. Raw piggery wastewater used as a medium did not support the growth of *C. vulgaris* due to high turbidity and high content of ammonia. Therefore, piggery wastewater was treated with sodium hypochlorite (NaOCL) and air stripping to improve the color and minimize ammonia content. The results showed that air stripping pre-treatment enhanced the amount of biomass yield and removed 99.8% TP and 98.7% NH_3-N. Further, no inhibition sign was observed under air stripped pre-treatment. Nevertheless, no significant effects on C*hlorella vulgaris* growth were observed under sodium hypochlorite treatment. Air stripping was therefore reported as an operative technique for microalgae cultivation and nutrient recovery from piggery wastewater. Kuo et al. (2015) cultivated *Chlorella* sp. GD in various piggery wastewater concentrations (0%, 25%, 50%, 75% and 100%) diluted by the medium in search of a cultivation system to produce proficient biomass and oil for biodiesel. The results recorded showed that *Chlorella* cultivated in piggery wastewater grew proficiently and a constant performance growth was achieved for long-term microalgal cultivation in a semi-continuous culture. In this culture, 25-75% wastewater ratios achieved biomass and lipid efficiencies, which were higher than 0.128 and 0.852 g L^{-1} d^{-1} respectively. Wang et al. (2012) utilized diluted piggery wastewater for *Chlorella pyrenoidosa* cultivation under mixotrophic conditions. Efficient removal of nutrients and chemical oxygen demand was also observed along with microalgal biomass and lipid production. Further, Deng et al. (2018) supplemented post-harvest culture broth with swine manure and used it for growing *C. vulgaris* under various recycling ratios. The recycling ratios of 1/4 or 1/6 were observed to have an impact on the biochemical composition of biomass. Fatty acid profiles indicated that this alga could be used as a good-quality biodiesel feedstock with biodiesel productivity of 9.65–40.1 mg L^{-1} d^{-1}.

Table 2.2 Microalgal biomass production and nutrient removal in different types of agricultural wastewater

Algal strain	Type of wastewater	Removal Efficiency (%)	Biomass yield (y) or productivity (p)	Application	Reference
Chlorella pyrenoidosa and *Rhodotorula glutinis*	Piggery wastewater	COD-85 NH_4-N- NA NO_3-N - 83 PO_4-53	1.0 g L^{-1} (Y)	Nutrient removal	(Li et al., 2019)
Chlorella, Klebsiella and *Acinetobacter*	Dairy farm wastewater	COD-90 NH_4-N-NA NO_3-N-84.7 PO_4-P-NA	2.87 g L^{-1} (Y)	Biofuel	(Makut et al., 2019)
Chlorella and *Scenedesmus*	Anaerobically digested piggery wastewater	COD-44 NH_4-N-98 NO_3-N-NA PO_4-P-NA	2.96 g L^{-1} (Y)	Nutrient removal	(Raeisossadati et al., 2019)
Scenedesmus obliquus	Brewery effluent	COD-71 NH_4-N-NA NO_3-N-88 PO_4-P-30	NA	Bioactive compounds, biofuel, biofertilizer	(Ferreira et al., 2019)
Tribonema minus	Tofu whey industry wastewater	COD-86.7 NH_4-N-NA NO_3-N-92.8 PO_4-P-72 Protein-321.2	0.432 g L^{-1} d^{-1} (P)	Biomass and lipid production	(Wang et al., 2018)
Ascochloris sp.	Raw dairy wastewater	COD-95 NH_4-N-NA NO_3-N-78 PO_4-P - 98	0.102-0.107 g L^{-1} d^{-1} (P)	Nutrient recovery, biomass and lipid production	(Kumar et al., 2018)
Parachlorella kessleri	Agro waste	COD 39 NH_4-N-NA NO_3-N->98 PO_4-P-59	0.062 g L^{-1} d^{-1} (P)	Nutrient removal, biomass and lipid production	(Koutra et al., 2018)
Chlorella	Seafood processing wastewater	COD-NA NH_4-N-NA NO_3-N-94.5 PO_4-P-68.4	0.077 g L^{-1} d^{-1} (P)	Nutrient recovery and lipid production	(Gao et al., 2018)
Scenedesmus obliquus	Secondary brewery effluent	COD-74 NH_4-N-NA NO_3-N-94 PO_4-P-NA	0.200 g L^{-1} d^{-1} (P)	Biomass and fatty acids production, nutrient recovery	(Marchão et al., 2017)
Coelastrum sp.	Cattle farm wastewater	COD 42 NH_4-N-NA NO_3-N-80 PO_4-P-100	0.281 g L^{-1} d^{-1} (P)	Nutrient recovery and biomass production	(Mousavi et al., 2018)
Chlorella vulgaris	Aquaculture and pulp wastewater	COD-75.5 NH_4-N-NA NO_3-N-76.5 PO_4-P-92.7	0.187 g L^{-1} d^{-1} (P)	Nutrient recovery and lipid production	(Daneshvar et al., 2019)
Scenedesmus and *Trichoderma ressei*	Seafood processing wastewater	COD-74 TN-44 TP-93	2.17- 6.64 g L^{-1} (Y)	Biofuel	(Srinuanpan et al., 2018)

C. vulgaris and *Yarrowia lipolytica*	Yeast industry	COD-80 NO_3-N-80	1.23 - 1.56 g L^{-1} (Y)	Nutrient recovery and biomass production	(Qin et al., 2019)
Ankistrodesmus falcatus	Aquaculture wastewater	COD-NA NO_3-N-80.85 NH_4-N-86.45 PO_4-P-98.52	0.161 g L^{-1} d^{-1} (P)	Nutrient recovery, biomass and lipid production	(Ansari et al., 2017)
Leptolyngbya and *Ochromonas*	Winery and raisin industry wastewater	COD-93 TN-78 TP-99	1.3 g L^{-1} (Y)	Nutrient recovery, biomass and biodiesel application	(Tsolcha et al., 2018)
Chlorella vulgaris	Dairy wastewater	TN-96 TP-79 Protein-43.4	13.3 g L^{-1}(Y)	Nutrient recovery	(Pang et al., 2020)
Saccharomyces cerevisiae	Dairy wastewater	COD-90 TN-65.5 TP-73 Proteins-15 Carbohydrates - 38	1.4 g L^{-1} (Y)	Nutrient recovery and bioethanol production	(Hemalatha et al., 2019)
Microalgae consortium	Piggery wastewater	COD-90.64 NH_4-N-100 PO_4-P-81.40 Proteins-610.28 mg L^{-1} Carbohydrates -157.80 mg L^{-1}	4.98×10^6 cell/mL	Nutrient recovery, biomass and lipid production	(Fernández-Linares et al., 2020)
Scenedesmus sp.	Municipal wastewater+ Cattle	COD->90 NH_4-N->90 PO_4^{3-}P-79-88	4.65 g L^{-1} (Y)	Nutrient recovery, biomass and lipid production	(Luo et al., 2019)
Chlorella pyrenoidosa	Poultry excreta leachate	COD-N/A NH4-N-53.1 PO_4^{3-}-P-96.2 Carbohydrates -0.64 gL^{-1} Protein-1.02 gL^{-1} lipid- 0.49 gL^{-1}	2.5 g L^{-1} (Y)	Resource recovery, animal feed and supplements	(Mohan Singh et al., 2020)

*NA – Not available; COD – Chemical oxygen demand; NH_4-N –Nitrogen in the form of ammonium; NO_3-N – Nitrogen in the form of nitrate; TN – Total nitrogen; PO_4-P – Phosphorus in the form of phosphate; TP: Total phosphorus; DIC – Dissolved inorganic carbon.

2.3 Different modes of microalgae cultivation in wastewater

Microalgae species can adjust to various environmental conditions, and this is one of the reasons why they have gained so much interest as feedstocks for various industrial and environmental applications. Microalgae can grow under different modes of cultivation namely, photoautotrophic, heterotrophic, and mixotrophic (Whangchenchom et al., 2014), and still, be able to accumulate biomass and lipids. However, their performance is highly dependent on the type of algal strain's adaption to their environment (Zhan et al., 2017). According to literature, an increase in biomass and lipid content in microalgae cultivated in wastewater under various cultivation modes has been observed (Chinnasamy et al., 2010, Jiang et al., 2011, Zhou et al., 2011, Ramsundar et al., 2017b, Li et al., 2020b). Phototrophic cultivation of microalgae is advantageous, as it requires only sunlight and carbon dioxide for growth, although it can be limited when it comes to achieving a high yield of biomass and oil production. Heterotrophic condition offers a possibility of increasing algal biomass yield and productivities in the absence of light. Thus, microalgae that are adapted to grow under heterotrophic mode on cheap carbon substrate are of importance in oil production (Zhang et al., 2013, Zhan et al., 2017). In addition, heterotrophic cultivation using various wastewaters is cost-effective as it drops the usage of expensive inorganic and organic chemicals. However, getting a cheap source of carbon in adequate amounts can be a limiting production factor (Ummalyma and Sukumaran, 2014). On the other hand, mixotrophic mode combines heterotrophic and photoautotrophic conditions by using both, organic and inorganic carbon (Zhan et al., 2017). The biomass and lipid productivity may also vary in different modes of cultivation and is highly dependent on the microalgal species. Generally, wastewater contains sufficient amount of organics and can be suitable for heterotrophic and mixotrophic cultivation of microalgae (Ummalyma and Sukumaran, 2014). It is essential to find out the suitability of different wastewater as cultivation medium for microalgal growth and mode of cultivation to obtain optimum biomass and lipid productivities.

Photoautotrophic cultivation occurs when microalgae utilize light captured from the sun or any source of light as their source of energy and carbon dioxide as a carbon source for photosynthetic reactions (Chen et al., 2011, Li et al., 2020a). It is recognized as the oldest, energy saving and most commonly used cultivation condition for the growth of microalgae. The amount of biomass and lipids produced under this cultivation condition is relatively lower compared to the other cultivation conditions. Nonetheless, the productivity mostly depends on the selected microalgal strain used in the cultivation process. Fortunately for this cultivation condition, lipids may further be increased by either minimizing nitrogen or the amount of nutrients (Gao et al., 2019).

The phototrophic cultivation condition is advantageous in the production of algal lipids as it is a carbon neutral and cost-effective process. Photoautotrophic cultivation contributes to the global reduction of atmospheric carbon dioxide as the algae use carbon dioxide for cell growth (Ling et al., 2019). Precisely the pollution problem and contamination risk are less severe when using the phototrophic cultivation mode than when using other cultivation modes (Ling et al., 2019). It is currently the only technical and economically viable method for large scale production of microalgal biomass and hence, an outdoor scale-up cultivation system for microalgae is always recommended to be done using photoautotrophic cultivation conditions to minimize contamination problems. Most outdoor microalgae cultivations are commonly run under phototrophic conditions. Some researchers have also reported on the success of cultivating microalgae consortia for enhancing the production of the lipid under phototrophic cultivation (Huy et al., 2018) used different types of wastewater for the cultivation of mixed microalgae consortia and the consortia were able to grow and produce biomass. Additionally, this also has had a major impact on the oxidative stability of microalgal biodiesel. Even though this cultivation condition has been widely used, it is nonetheless economically unfriendly as it provides little biomass and lipid productivity that is not sufficient in terms of commercial oil production. In addition, the cost of harvesting biomass increases as the concentration of microalgae is constantly low under phototrophic mode (Chew et al., 2018).

The heterotrophic cultivation mode involves the growth of microalgae on organic carbon in absence of light. In heterotrophic cultivation, the photosynthesis process of algae is inhibited due to the absence of light, and alternative organic substrates are used as an energy source converted to starch which is converted to lipid depending on a number of factors (Chew et al., 2018, Salama et al., 2017, Di Caprio et al., 2019).

Heterotrophic cultivation has shown great potential in improving both lipid and biomass productivity (Chew et al., 2018, Di Caprio et al., 2019) while offering the potential for minimizing production costs through simplified bioreactor designs. In addition, this type of cultivation could offer the likelihood that microalgal biomass concentration and the yield on an industrial scale would greatly increase. *Chlorella protothecoides* is one of the algal species that has been reported to have the potential to obtain a 40% increase in lipid content when cultivated under heterotrophic mode (Chen et al., 2011, Choi et al., 2018) Previous studies focused on the viable microalgal biomass and lipid production on slightly diluted and undiluted dairy effluent using indigenous *Chlorella sp*. indicated the best performance under heterotrophic conditions.

Even though the heterotrophic mode of cultivation has been reported to be the best method for higher lipids and biomass production, the sugar-based organic carbon source is frequently prone to contamination which might contaminate the microalgal culture and thus limit its potential as a large-scale cultivation strategy (Di Caprio et al., 2019). Adding carbon sources and contamination risks are the main drawbacks to commercial scale applications of microalgae grown under heterotrophic cultivation mode (Chew et al., 2018). Moreover, the utilization of organic compounds as both carbon and energy source increases the substrate cost required for microalgae growth and thus increases the total cost of heterotrophic cultivation conditions. The cost of glucose as the main carbon substrate has accounted for at least 80% of the total medium cost, according to previous estimates (Gao et al., 2010, Mitra et al., 2012). Waste substances containing organic carbon such as wastewater, organic acid from the end product from fermentation, and molasses of sugar cane may be substituted as carbon sources. Because of its organic carbon load, the use of wastewater streams for heterotrophic cultivation may be feasible for large scale microalgae production. The reuse of this carbon from wastewater through microalgae assimilation can significantly minimize microalgae cultivation costs.

Previous studies have proved that a heterotrophic cultivation strategy might increase the microalgal lipid yield and overcome the light dependency when cultivated in wastewater. Cheng et al. (2013) heterotrophically cultivated three microalgal strains namely *Chlorella sp.* ZTY4, *Scenedesmus sp.* ZTY2 and *Scenedesmus sp*. ZTY3 in domestic water in the absence of light. The three species showed great potential for growth in domestic wastewater under heterotrophic conditions since an increase in algal densities of 203.0% and 60.5% was observed. Therefore, this could possibly mean that these algal species could be grown under heterotrophic cultivation for both biomass and lipid production in the absence of light. Furthermore, the highest lipid productivity of 79.2%, 69.1%, and 55.3% was also respectively achieved by the 3 microalgal strains. Shen et al. (2020) examined the effects of supplying phosphorus and nitrogen on the production of biodiesel from *Scenedesmus obliquus* in soya wastewater under heterotrophic conditions with glucose as the source of carbon. It was found that under nitrogen starvation, an adequate amount of phosphorus could further increase the development of biodiesel. Additionally, the productivity of fatty acids reached 99.3 mg L^{-1} d^{-1} after day 8 of cultivation and this was 1.15 times higher than the maximum output with a glucose culture. Moreover, Nzayisenga et al. (2018) investigated the production of biomass and variations in the biochemical composition of *Chlorella* sp. under 3 different cultivation conditions in municipal wastewater supplemented with glucose or glycerol as carbon sources. Heterotrophically grown microalgae with glycerol resulted in the highest lipid content and good biodiesel quality.

However, heterotrophic microalgae growth performance is highly dependent on the conditions of the culture and microalgae (Chew et al., 2018). This type of cultivation may avoid problems associated with limited light during phototrophic cultivation that impede the high density of cells in large scale photobioreactors. However, there are only a few industrial heterotrophic processes that have been carried out due to the inhibition of microalgal growth when the organic substrate is supplied at a lower concentration. Moreover, there are only a limited number of microalgal species with the potential to survive under heterotrophic conditions (Lowrey et al., 2014, Di Caprio et al., 2019).

Mixotrophy is simultaneous utilisation of inorganic (Phototrophy and/or autotrophy) and organic carbon (Chemotrophy and/or heterotrophy). Generally, in case of algae and cyanobacteria, it is simultaneous utilisation of inorganic and organic carbon by photoautotrophy and heterotrophy. Light is the primary source of energy in mixotrophic mode, nonetheless, supplementation of inorganic or organic carbon to the culture medium is done concurrently (Perez-Garcia et al., 2011, Wang et al., 2012, Sajadian et al., 2018, Gao et al., 2019). Both light and carbon sources have substantial effects on metabolites accumulation and growth of mixotrophic microalgae (Li et al., 2020a). Because of the simultaneous utilization of light, organic and inorganic carbon sources, mixotrophic cultivation offers great tractability in microalgal production in a short period of time (Gao et al., 2019, Patel et al., 2020). Hence, the mixotrophic culturing of microalgae could be a promising sustainable practice in achieving large quantities of biomass merged with high growth rates with photosynthetic metabolite production as an additional benefit (Perez-Garcia et al., 2011)

Microalgae grown under mixotrophic cultivation have a higher growth rate and offer high biomass production yield over a short period of time than those cultivated under heterotrophic and phototrophic conditions. Additionally, biomass loss under this cultivation is minimized due to dark hours of respiration (Mitra et al., 2012, Gao et al., 2019, Patel et al., 2020, Li et al., 2020a). In comparison to other cultivation modes, the accumulation of lipids under mixotrophic conditions has also been reported to be higher, nonetheless, heterotrophic algae on the other hand can produce a comparable amount of lipids. However, even though this might be the case, lipids content also depends on the type of microalgal strain (Gao et al., 2019). Wang et al. (2012) cultivated *C. pyrenoidosa* mixotrophically in piggery wastewater with the main aim of treating piggery wastewater and producing lipids and their findings showed that *C. pyrenoidosa* had the potential to treat piggery wastewater and accumulates lipids. In a study by Gao et al. (2019) the ability of *T. bernardii* to produce lipid and biomass productivity under 3 commonly used modes of cultivation were investigated. And the highest biomass and lipid yield were observed under mixotrophic cultivation. Moreover, Roostaei et al. (2018) accumulated 2-10 times higher lipids and 2-3 times higher biomass productivity. Likewise, Sajadian et al. (2018) have shown an increase of 158% in the amount of biomass when *C. vulgaris* was cultivated mixotrophically, which was higher as compared to heterotrophic and photoautotrophic cultivation.

2.4. Application of wastewater-grown microalgal biomass

Microalgal biomass quests potential applications in the production of bioenergy, feed, biofertilizer, bioactive compounds, pigments, and synthesis of various compounds for the nutraceutical, cosmetics, and pharmaceutical industries (Elrayies, 2018, Renuka et al., 2018a). Production of microalgal biomass for various commercial applications using different wastewater has both environmental and economic benefits. However, more emphasis has mainly been given to the production of bioenergy from wastewater grown algal biomass. Owing to concerns related to possible heavy metal and microbial contamination in wastewater, wastewater-grown microalgal biomass remains untapped for other applications such as food, feed, and biofertilizer production, which needs comprehensive studies.

2.4.1. Bioenergy

Bioenergy production in the form of biodiesel, biocrude, biomethane, biochar etc. from microalgal biomass feedstock has attracted strong interest among researchers over the past decade (Roles et al., 2020). Successful usage of microalgae biofuels in the transportation sector and power generation has the potential to improve energy security and may help to minimize serious environmental concerns related to the use of fossil fuels. Extensive studies on the production of biofuels from wastewater-grown biomass are available. Among different biofuels, biodiesel production combined with wastewater treatment is proved as a promising approach to reducing the cost of production. The combination of wastewater remediation with the processing of algal biomass is potentially one of the most economically and environmentally safe ways of producing bioenergy and bio-products. Research studies on the use of wastewater to produce microalgal biomass for biodiesel production have revealed that wastewater provides a cheap source of nutrients and could increase the accumulation of lipids under natural stress conditions of wastewater; however, it can vary depending upon the cultivation conditions (Elshobary et al., 2019, Marella et al., 2019). Marella et al. (2019) reported that lipid productivities in wastewater-grown microalgal biomass varied seasonally. The highest lipid productivity of 9.2 g m^{-2} d^{-1} was observed in the summer season along with superior biodiesel quality with high cetane number. Lipids, C18:3 in high amounts are not favourable since they have an adverse effect on the biodiesel oxidative stability, therefore, the maximum limit of 12% for C18:3 have been set by the European standards for biodiesel. Soydemir et al. (2015) utilized secondarily treated domestic wastewater effluent for the cultivation of mixed cultures for biodiesel production, which gave a 26.2% lipid yield. The fatty acid profile analysis showed that FAME (fatty acid methyl ester) composition mainly consisted of stearic, oleic, palmitic, linoleic, palmitoleic, and linoleic acid methyl esters that had FAME composition and biodiesel conversion efficiency comparable to pure cultures and monoculture grown in a synthetic culture media. Moreover, a study conducted by Mata analyzed the possibility of cultivating *Scenedesmus obliquus* in brewery wastewater as a prospective candidate for biodiesel production (Mata, 2013). In their study, the results revealed an average lipid content of 27 % of dry-weight biomass and average biomass and lipid yield of 0.90 and 0.24 g L^{-1} respectively. They concluded that oil extracted from *S. obliquus*, containing 56.4 % of saturated esters (C16:0) and less than 12 % of the linolenate unsaturated ester (C18:3), could be used to produce biodiesel. Another study by Guldhe et al. (2019) reported that the fatty acid profile and biodiesel properties of wastewater-grown microalgal biomass were comparable to the microalgal biomass grown in standard culture medium.

Microalgal carbohydrates are also an important feedstock for a variety of commercial products. The processing and recovery of carbohydrates are dependent upon their solubility. Martin Juarez et al. (2021) illustrated the feasibility of microalgae biomass grown in domestic, piggery, and synthetic medium for the production of fermentable monosaccharides. They revealed that bacterial content in the wastewater-grown microalgal biomass did not exert an adverse effect on carbohydrates solubilization, however, improved the degradation of carbohydrates in piggery and domestic wastewater-grown biomass as compared to the synthetic medium. This study revealed the efficacy of wastewater-grown biomass for the production of biofuels through the fermentation process.

Thermochemical conversion is an important method for biomass up-gradation for bioenergy production, which include different processes such as gasification, pyrolysis, and direct liquefaction (Jena et al., 2011, Chiaramonti et al., 2015). Biomethane is an important form of bioenergy, which could be produced through the anaerobic digestion of wastewater-grown microalgal biomass. An algae-bacteria consortium was reported to contain 34-38% of protein and 18-28% of lipids in biomass grown in wastewater in an open raceway pond (Bohutskyi et al., 2018). The methane production through the anaerobic digestion of the biomass varied significantly (by 30%) with the highest value of 0.34 L g VS^{-1} depending upon the lipid content. Net Energy Ratios and Net Energy Efficiency ranged from 1.6-2.2 and 60%-70% respectively for large-scale anaerobic digestion processes, and an optimal hydraulic retention time of 20-30 days (Bohutskyi et al., 2018). However, Bohutskyi et al. (2018) demonstrated that the biomethane production from lipid extracted wastewater-grown microalgal biomass through anaerobic digestion can be improved through the hydrothermal-pre-treatment and co-digestion with sewage sludge, which can lead to 30-50% increase in methane yield and approximately 4 folds increase in the energy output. A study by Shahid et al. (2019) demonstrated the potential of *Chlorella* sp. and *Bracteacoccus* sp. for the treatment of urban wastewater and biomass production. The wastewater-grown biomass showed significant potential for energy production through the pyrolysis process with 159-190 kJ mol^{-1} of Gibbs free energy and 43-81 J mol^{-1} of entropy values.

Among different technologies of algal biomass conversion, the hydrothermal liquefaction (HTL) process has been found to be energy efficient method, since it minimizes the dewatering requirements and has higher conversion rates as compared to other technologies. HTL process has been reported to contain a biocrude yield of approximately 60% with HHV (high heating value) of 36-40 MJ Kg^{-1} (Deng et al., 2018). Recent studies on the biocrude formation from wastewater-grown microalgal biomass through the HTL process have revealed the significant biocrude conversion efficiency (Arun et al., 2018, Cheng et al., 2019). Cheng et al. (2019) demonstrated the potential of two microalgal strains (*Galdieria sulphuraria* algae, 5587.1 and SOOS) grown in municipal wastewater. However, the biocrude oil yields were lower in the municipal wastewater as compared to biomass grown in synthetic medium, which showed the need for further studies on the optimization of HTL conditions for enhancing the production of biocrude. Recently, Naaz et al. (2019) compared the bioenergy production from wastewater grown microalgal biomass (*Chlorella pyrenoidosa* and *Phormidium* grown in municipal wastewater through anaerobic digestion and the HTL process. HTL process showed 43% conversion into biocrude with a net energy value of 0.08, which was significantly higher than the anaerobic digestion process (0.007). HTL process has been found an energy-efficient process for bioenergy production; however, research in this direction is in the early stage, which needs further optimization and life cycle assessment studies.

2.4.2. Biofertilizer

Microalgal bioinoculants and/or fertilizers have been reported to improve the quantity and quality of agricultural crops (Renuka et al., 2018a). The usage of biofertilizers reduces the requirements of synthetic N fertilizers for agricultural lands, therefore assisting in the mitigation of harmful effects of chemical fertilizers and increasing the yield of agronomic crops. N, P, and potassium (K) are the most important nutrients needed for crop nutrition. Microalgae cultivation coupled with the removal of excess nutrients in wastewater could improve the economic viability of algae-based bioinoculants. Microalgae have the potential to assimilate wastewater nutrients into their biomass, such generated biomass can be utilized as biofertilizer/ organic fertilizer for agronomic crops. Wuang et al. (2016) examined the technical viability of growing *Spirulina platensis* using aquaculture wastewater for algae fertilizer production. The use of algae-based fertilizer showed higher amounts of iron (Fe), zinc (Znad et al., 2018), calcium (Ca), and magnesium (Mg), however, low amount of NPK in comparison with chemical fertilizer. They reported that wastewater grown *S. plantensis* biomass exhibited the ability to improve the growth of leafy plants (*Ameranthus gangeticus, Brassica rapa ssp. Chinensis, Eruca sativa*) and enhance the seed germination of Kai Lan (*Brassica oleracea alboglabra*) and the Chinese Cabbage (*B. rapa ssp. Chinensis*). Another study by Renuka et al. (2016) demonstrated a significant improvement in the productivity and yield of wheat crops with the inoculation of two wastewater grown microalgal consortia of filamentous and unicellular strains as compared to recommended synthetic fertilizer. A significant increase in the N, P and K contents of shoots, grains, and roots was also obtained with wastewater-grown microalgal bioinoculant. Another approach is the use of residual nutrient rich microalgal biomass (after the extraction of compounds and digested biomass) as organic fertilizer. The use of residual biomass has been reported to act as a soil conditioner (Doğan-Subaşı and Demirer, 2016). These studies revealed the efficiency of such wastewater-grown microalgae as bioinoculant and/or fertilizer for enhanced plant growth, yield, and soil fertility. This can also assist in integrated nutrient management of agronomic practices (Tripathi et al, 2008). The presence of toxic metals and pathogenic microorganisms in different types of wastewater could have a detrimental effect on the quality of microalgal biofertilizers. Moreover, there are limited studies on assessing the effect of wastewater-grown algal biomass as biofertilizer in agronomic crops and its ecological implications. Most of studies are conducted either at lab-scale or under controlled conditions. Therefore, field level investigations on wastewater-grown biomass as bioinoculant are required to prove their practical and economic feasibility.

2.4.3. Food and feed

Microalgae find potential applications in human food supplements and animal feed, since they are a rich bioresource of pigments, proteins, and essential fatty acids (Apandi et al., 2019, Yarnold et al., 2019). Polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are naturally produced by microalgae could boost the immune system and are also used in treating inflammatory and heart diseases (Sims, 2019). While in aquaculture, these organisms can be used as a live feed and/or feed supplement. Microalgae also assist in boosting the immune system and improving overall health. The protein and fatty acid content in many microalgal species have been found to be substantial and comparable to the conventional sources of fish feed (Sims, 2019). Microalgae-based aquaculture feed has been found to increase organism health, productivity, and yield. Therefore, there is an increased interest in the production of microalgae-based aquaculture feed in recent times. However, studies on the evaluation of wastewater-grown microalgal biomass as human food supplements and aquaculture (fish) feed are very limited (Apandi et al., 2019, Ahmad Ansari et al., 2020).

There are few studies on the production of microalgal biomass in aquaculture wastewater, which could act as a basis for setting up microalgae-aquaculture wastewater based biorefinery for aqua feed production (Tossavainen et al., 2018, Apandi et al., 2019). Tossavainen et al. (2018) studied the growth, fatty acid (FA) profile, tocopherol content, and nutrient recycling in a consortium of *Euglena gracilis* and *Selenastrum* cultivated in aquaculture wastewater from catfish (*Clarias anguillaris*) and pikeperch (*Sander lucioperca*). They revealed that the highest biomass yield of (1.5 g L^{-1}) with substantial EPA and DHA content, and efficient nutrient removal were achieved in sludge amended aquaculture wastewater. However, the tocopherol and arachidonic acid content were higher than the required standard for the fish feed. Tocopherol at high amount may result in poor growth, toxic liver reaction and death. Therefore, in their study microalgae proved to be a promising substitute for eradication of nutrients from wastewater while offering an alternative for fish oil. Ahmad Ansari et al. (2020) recently investigated the use of whole and lipid extracted biomass of *Scenedesmus obliquus* for Nile tilapia growth. In their study, the economic feasibility of algal-assisted aquaculture wastewater involving the direct utilization of algal biomass for fish production and the use of algae to produce biodiesel trailed by the usage of LEA (Lipid extracted algal biomass) in fish feed. Nile tilapia was able to ingest and use both LEA and whole algae biomass, the substitution of 7.5% LEA in fish feed showed the highest fish growth. However, there are limited studies on the evaluation of wastewater-grown microalgal biomass as aqua feed. In this context, extensive studies on the field scale evaluation of such wastewater-grown feed on model organisms. The challenges related to microbial loading and heavy metal contamination in wastewater-grown biomass need to be addressed, which needs field scale life cycle assessment studies.

2.5. Lipid extracted Biomass and its potential application

The use of microalgal biomass for biodiesel production is well established (Ramsundar et al., 2017b, De Francisci et al., 2018), however, the commercialization has not been yet achieved due to the economic challenges. To make the microalgal biorefinery method more innovative and economically feasible, the use of de-oiled biomass to generate various energy sources and the extraction of high-value co-products has been recommended (Abomohra et al., 2018). The process of producing biodiesel from microalgae involves the extraction of the lipids from microalgal biomass with the use of appropriate techniques such as chemical solvents, physicochemical, supercritical CO_2, biochemical, and direct trans-esterification (Chisti, 2007). A large amount of de-oiled microalgal biomass remains after lipid extraction, which is rich in macro- and micronutrients (Maurya et al., 2016b, Abomohra et al., 2018).

Various studies have shown that lipid extracted algae biomass is rich in carbon and other essential micro- and macronutrients such as nitrogen (N), phosphorous (P), copper (Cu), iron (Fe), etc. and can be used for agriculture as a nutrient source (Patterson and Gatlin, 2013). Residual algal biomass after lipid extraction can be explored for a wide range of applications (Figure 2.1). Ansari et al. (2015) studied lipid extracted biomass as a source of protein and reduced sugar and their findings showed that LEA offered similar protein and reduced sugar yields to those of algae. Ansari et al. (2015) revealed that the extraction method and extraction solvent system plays a vast role in determining the quality and quantity of lipid extracted biomass. They suggested the use of a dichloromethane/methanol solvent system particularly if the de-oiled biomass is going to be used for feed, food, or bioenergy application.

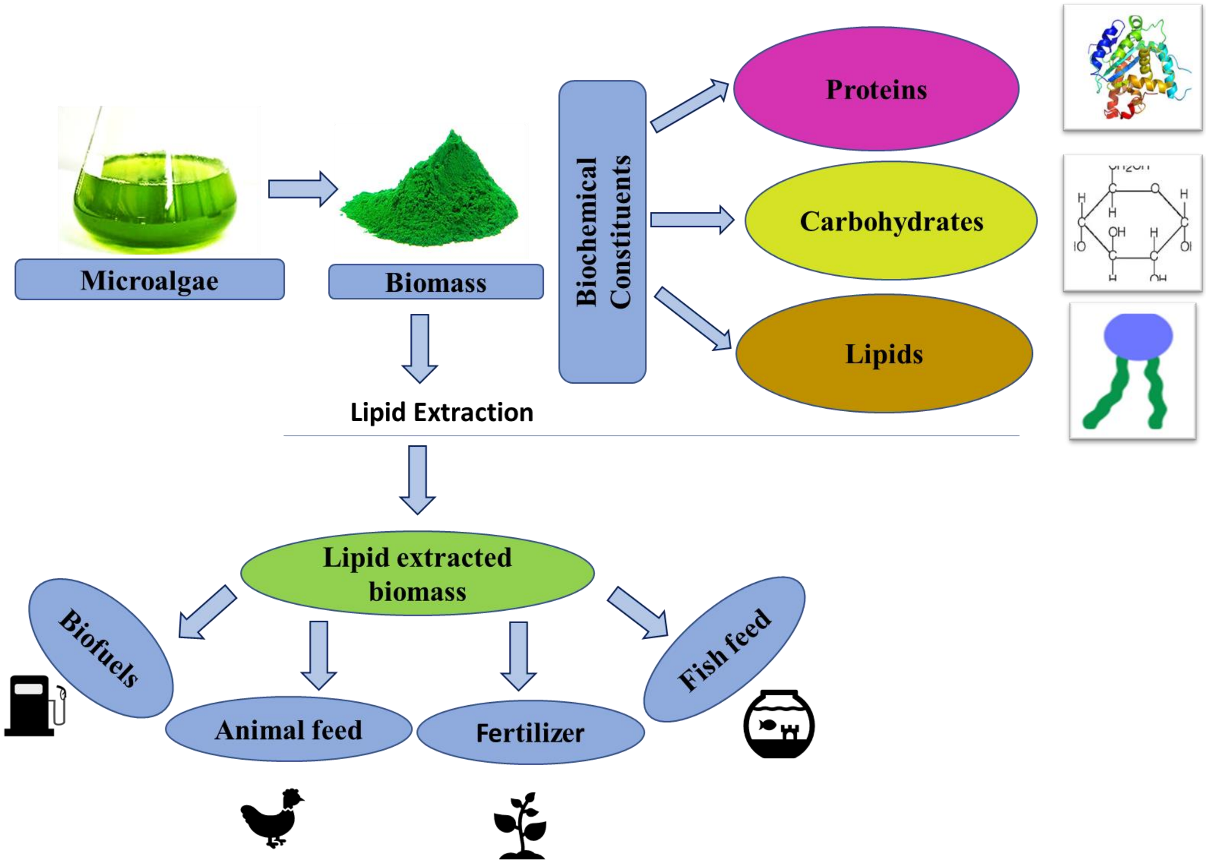

Figure 2.1 Schematic representation of potential applications of lipid extracted biomass.

Chen et al. (2019) studied the production of bioethanol using *S. dimorphus* deoiled biomass and their findings revealed that *S. dimorphus* deoiled biomass produced significant bioethanol yield. On the other hand, Chen et al. (2020) investigated the potential of *Dunaliella* and *Thermococcus eurythermalis* A501's lipid extracted biomass as a source of biohydrogen production. Their findings showed that lipid extracted biomass of *Dunaliella* could greatly enhance the H_2 production. More studies on the exploration of potential methods for the valorization of lipid extracted biomass may assist in improving the economic feasibility of microalgae-based products and forms a sustainable biorefinery.

2.6. Motivation of the study

Presently, the commercial viability of microalgae-based biodiesel production is hindered by high biomass production costs. This is due to the requirement of expensive chemical fertilizers and excess freshwater resources, and therefore more novelties for green production are needed. Municipal and agricultural wastewater contains an adequate amount of different nutrients required for microalgal growth and biomass production. Nutrient unavailability, excessive, and/or varying organic load, and undesirable components of wastewater are some of the limiting factors that affect microalgal growth. These challenges can be overcome by using the combination of low strength and high strength wastewater sources and employing pre-treatment procedures. Additionally, the biorefinery concept, i.e., the production of multiple products from a single feedstock, offers a viable solution to improve the economic feasibility of microalgal biodiesel production. Microalgal biomass residue (also referred to as the surplus byproduct) left after the extraction of lipids is rich in other metabolites and nutrients. These residues can be exploited for applications such as aquaculture, nutraceuticals, animal feeds, fertilizers, etc. to make the process economically feasible and environmentally sustainable. Therefore, the present study was undertaken to evaluate the competence of municipal wastewater (post-chlorinated wastewater) and agricultural waste (poultry litter) as water and nutrient sources for microalgae cultivation for biodiesel production. Residual biomass after lipid extraction was also assessed for its efficacy in being used as fertilizer for agricultural applications.

CHAPTER 3

MICROALGAE CULTIVATION IN WASTEWATER AND BIODIESEL PRODUCTION

MUSETSHO, P., RENUKA, N., GULDHE, A., SINGH, P., PILLAY, K., RAWAT, I. AND BUX, F. 2021. Valorization of poultry litter using *Acutodesmus obliquus* and its integrated application for lipids and fertilizer production. Science of the Total Environment, 796, p.149018.

3.1. Introduction

Limited availability of fossil fuels, energy security, and increased greenhouse gas emissions are some of the primary reasons behind the pursuit of alternative and renewable energy resources (Hussain et al., 2021). Microalgal biofuels have gained extensive attention as a promising substitute for fossil fuels due to their renewable and environmentally friendly nature (Milano et al., 2016, Chen et al., 2018b). However, the commercialization and production of microalgal biomass at a large scale for various purposes has only been studied for the last decade and there are major research gaps that still need to be addressed (Chen et al., 2018b). Sufficient microalgal biomass production at an industrial level for commercial scale production of biofuel requires a colossal amount of freshwater along with chemical fertilizers (mainly nitrogen and phosphorus). The utilization of economical and sustainable nutrient sources that are considered waste, is one of the ways to overcome the dependence on synthetic fertilizers and reduce overall production costs (Han et al., 2019, Fernandes et al., 2020, Hussain et al., 2021, Scarponi et al., 2021). Additionally, the use of various wastewaters (domestic, industrial, agricultural, etc.) for microalgal growth could assist to overcome the challenges associated with the use of freshwater.

Municipal wastewater streams are one of the more extensively explored waste streams for microalgae cultivation. Treated municipal wastewater is generally discharged into the environment, and sometimes contains residual nutrients (Noguchi et al., 2021). However, this treated wastewater may not contain an adequate amount of nutrients for microalgae growth and can be improved using nutrients from high-strength waste streams. High-strength waste streams can act as low-cost nutrient sources. For example, in the agriculture sector, domesticated poultry livestock produces a massive amount of organic waste as poultry litter (Plaza et al., 2018), which could be a nutrient resource if used prudently (Markou et al., 2016). Disposal and treatment of PL is an environmental challenge due to its high nutrient load, and its soil application may cause eutrophication and degradation in the environment (Haynes et al., 2008).

PL is a rich source of organic and inorganic macronutrients such as phosphorus (P), nitrogen (N), carbon (C), potassium (K), and micronutrients such as zinc (Znad et al., 2018), iron (Fe), manganese (Yeomans and Bremner, 2008), copper (Cu), boron (B), sodium (Na), etc., that are essential for microalgal growth (Markou et al., 2016, Ferreira et al., 2018, Mohan Singh et al., 2020). The use of PL as a nutrient source for microalgae growth may lessen or even completely negate the reliance on synthetic fertilizers, minimizing the overall microalgal biomass production cost. Few studies have been successfully conducted on microalgal cultivation in poultry litter wastewater (Markou et al., 2016, Ferreira et al., 2018, Han et al., 2019, Agarwal et al., 2020, Mohan Singh et al., 2020). However, PL is a solid substrate that cannot be used directly for microalgae cultivation and therefore requires an external water source for nutrient retrieval. Most reported studies used distilled water as an aqueous medium for nutrient release and water source for microalgae cultivation using PL (Markou et al., 2016, Ferreira et al., 2018, Han et al., 2019, Agarwal et al., 2020, Mohan Singh et al., 2020). Municipal wastewater is one of the most inexpensive water sources, which is continuously produced and easily available. The use of post-chlorinated effluent for microalgal cultivation would assist in minimizing the usage of freshwater for microalgae cultivation for biodiesel production (Ramsundar et al., 2017b). Therefore, supplementation of PL in post chlorinated wastewater from municipal wastewater treatment plants could also be a great strategy for nutrient release/retrieval from PL for microalgae cultivation.

Algae cultivation in wastewater could serve the dual role of treating wastewater by nutrient recovery and production of useful biomass in the downstream process (Laurens et al., 2017). The feasibility of wastewater grown microalgal biomass for biodiesel production is well documented in the literature (Rosli et al., 2020, Fazal et al., 2021, Zhang et al., 2021). During microalgal biodiesel production, a huge amount of lipid extracted microalgal biomass remains after the lipid extraction process (Abomohra et al., 2018). LEA contains nutrients and other valuable metabolites in abundance which can be used to produce other useful products such as aquaculture feed, animal feeds, energy, and fertilizers (Zhu, 2015, Renuka et al., 2018a, Fan et al., 2020).

Acutodesmus Obliquus is a freshwater green microalga that has shown tolerance to various environmental conditions, ability to consume nutrients over a wide range of concentrations, adaptability towards wastewater cultivation, and adequate lipid content for biodiesel production (Singh et al., 2016, Renuka et al., 2018b). The feasibility of different municipal and wastewater streams is extensively explored as a medium for microalgae cultivation and biomass production for various applications. However, each wastewater stream has its own challenges in nutrient composition and availability. Limited studies focused on the utilization of a combination of wastewater streams to improve the physicochemical and biological characteristics.

Therefore, this study was carried out to assess the feasibility of valorization of agricultural waste and municipal wastewater using microalgae. The potential of poultry litter extract prepared using post chlorinated effluent was evaluated as a growth medium for *A. obliquus* cultivation for lipids production for biodiesel application.

3.2. Materials and methods

3.2.1. Microalgae strain

Microalga *Acutodesmus obliquus* isolated from Kwazulu-Natal, South Africa was selected for the present study (Singh et al., 2016). *A. obliquus* was maintained in standard BG11 medium in a culture room at an irradiance of approximately 80 µmol photons m^{-2} s^{-1}, light: dark-cycle of 16:8 h, and temperature of 25 °C ± 2 °C.

3.2.2. Preparation of poultry litter extract

Raw poultry litter was obtained from NG poultry farm located in Newlands West in Durban, South Africa. Post chlorinated effluent was collected from the Kingsburgh wastewater treatment plant located in Kwazulu-Natal, South Africa. Wastewater samples were transported to the laboratory and stored in a cold room at 4°C until use. The characteristics of the post chlorinated effluent are shown in table 3.1. Before use, PL was sun-dried, large particles of chicken bedding, solids, and feathers were manually removed and the remaining litter was ground using a laboratory-scale blender (Waring Commercial Blender, HGB250, USA) and sieved (710 µm) to obtain a powder form.

Poultry litter extract (PLE) was prepared by dissolving 2g (dry weight basis) of PL into 100 mL of post-chlorinated effluent. The concentration of poultry litter extract was selected based on preliminary experiments. The mixture was subjected to orbital shaking at 200 rpm overnight and then allowed to settle for approximately 2 h (Ramsundar et al., 2017b). The supernatant was filtered with Whatman no.1 filter paper and used for further analysis.

Table 3.1 Characteristics of post-chlorinated wastewater

Parameter	Value
pH	7.54
Temperature (°C)	20.6
Total suspended solids (mg L^{-1})	33.62
Chemical oxygen demand (mg L^{-1})	46.86 ± 0.58
NO_3- N (mg L^{-1})	1.25 ± 0.07
NO_2-N (mg L^{-1})	N.D.
NH_4-N (mg L^{-1})	2.7 ± 0.14
PO_4-P (mg L^{-1})	0.92 ± 0.04

3.2.3. Pre-treatment and characterization of PLE

Poultry litter extract was pre-treated chemically to minimize the microbial load that may interfere with the growth of microalgae, and done as described by (Ramsundar et al., 2017b). The pH was decreased to pH 2 using 5 M HCl dropwise, and the changes in pH were monitored with a pH meter (Thermo Scientific, UK). The pH of pre-treated PLE was adjusted to 7.2-7.5 using 5 M NaOH or 5 M H_2SO_4 before microalgae cultivation. Table 3.2 describes the details of PLE pretreatment.

Table 3.2 Pre-treatment of PLE in the final effluent

Treatment	Pre-treatment	Conditions	References
RPLE	Raw	Orbital shaking at 200 rpm overnight	(Ramsundar et al., 2017b)
PPLE	Acid pre-treatment	Orbital shaking at 200rpm overnight + Addition of 5 M HCl (pH_2)	(Ramsundar et al., 2017b)

PLE- Poultry litter extract; RPLE- Raw poultry litter extract; PPLE- Pre-treated poultry litter extract

The RPLE and PPLE were characterized for physicochemical parameters and bacterial load prior to and post- microalgal cultivation. The pH and temperature were measured using a pH meter (Thermo Scientific™ Orion™ DUAL STAR™ Meter, UK). Nitrate (NO_3-N), ammonia (NH_4-N), nitrite (NO_2-N), and phosphate (PO_4-P) concentrations were determined using the Hach Kits (Hach, USA). Chemical oxygen demand (COD) was determined following the standard colorimetric method. Total suspended solids were determined according to the standard methods (Fernandes et al., 2020). Heterotrophic plate count (HPC) in PLE was analyzed using the spread plate method on R-2A agar (Reasoner's 2A agar) plates with incubation at 28°C for 5 days.

3.2.4. Microalgae cultivation in PLE and lipid analysis

Microalgae cultivation was carried out in RPLE and PPLE, while standard BG11 medium was used as the control. The experiment was conducted in 1 L Erlenmeyer flasks with a working volume of 500 mL in triplicate in a culture room at an irradiance of approximately 80 µmol photons m^{-2} s^{-1}, temperature of 25 ± 2 °C and light: dark-cycle of 16:8 h. Growth was analyzed at 2 days interval for 14 days by measuring optical density using a spectrophotometer.

3.2.4.1. Microalgal growth analysis

Microalgal growth was monitored daily by taking absorbance at 680 nm using a spectrophotometer (Spectroquant® Pharo 300, Merck, Germany). Biomass was determined gravimetrically at 2 days interval and biomass productivity (mg $L^{-1}d^{-1}$) was calculated as the dry cell weight produced at the late-log phase using equation 1:

$$Biomass\ productivity\ = \frac{Amount\ of\ biomass\ (mg\ L^{-1})}{number\ of\ days} \qquad \text{Equation 1}$$

Non-invasive fluorescence was measured to evaluate the photosynthetic efficiency of the microalga using a Dual-PAM 100 chlorophyll fluorometer (Heinz Walz Gmbh, Effeltrich, Germany). Samples were kept under dark conditions for 20 minutes before measurements to open all photo system-II (PS-II) reaction centres. The quantum efficiency of PS-II charge separation (Fv/Fm) was calculated as per the equation described by (Ramanna et al., 2014).

3.2.4.2. Biochemical analyses

Microalgal biomass grown in PPLE and control synthetic medium was harvested at the late log phase and analyzed for lipids, proteins, and carbohydrates Total lipids were extracted from microalgal biomass obtained using microwave-assisted solvent (methanol: chloroform, 2:1) extraction. Dried biomass (1 g) was mixed with solvent (20 mL) and then subjected to microwave (Milestone S.R.L., 1200W; Italy) treatment at 1000 W at 100°C for cell disruption for 10 min (Guldhe et al., 2014). Solvent and extracted lipids were centrifuged; biomass residues were filtered using Whatman no.1 filter paper. The organic layer was then collected and dried in an oven at 70°C for lipid recovery. Total lipids were quantified using gravimetric analysis, and the lipid content was expressed as a percentage of lipid per dry weight of biomass (% w/w). The lipid productivity was calculated using equation 2:

$$Lipid\ productivity\ (mgL^{-1}\ d^{-1}) = Biomass\ productivity \times \frac{Lipid\ content(\%)}{100} \quad \text{Equation 2}$$

Where biomass productivity is in mg L^{-1} d^{-1}, and lipids content is in percentage per biomass weight.

For protein quantification, a known amount of dried algal biomass was mixed with 1 M NaOH. The mixture was incubated at 80ºC for 10 min and 900 µL of distilled water was added to the hydrolysed sample to bring the volume up to 1 mL. The mixture was subjected to centrifugation at 12000 g for 10 min and the supernatant was utilized to determine the protein concentration by the Bradford method using bovine serum albumin as standard. Absorbance readings were measured at 595 nm using a spectrophotometer (Spectroquant® Pharo 300, Merck, Germany). Protein productivity was determined using equation 3.

$$Protein\ productivity\ (mgL^{-1}\ d^{-1}) = Biomass\ productivity\ \times \frac{Protein\ content(\%)}{100} \quad \text{Equation 3}$$

Where biomass productivity is in mg L^{-1} d^{-1}, and protein content is in percentage per biomass weight

Total carbohydrates in microalgal biomass were analyzed by the phenol sulfuric acid method (Dubois et al., 1956). Briefly, 500mg of dried biomass was mixed with 50 mL of H_2SO_4 (2% v v^{-1}) and autoclaved at 121°C for 30 min. The mixture was neutralized using 0.1M NaOH or 0.1M H_2SO_4 and centrifuged at 3000 × g for 5 min. An aliquot of the supernatant was diluted to 1mL and then mixed with 1mL phenol (5% w v^{-1}). Further, 5 mL of H_2SO_4 (96% w w^{-1}) was added. The mixture was maintained at 25 - 30 °C in a water bath for 10 min. Absorbance was read at 490 nm using a spectrophotometer (Spectro quant Pharo300, Merck) using glucose as a standard. Carbohydrate productivity was determined using equation 3.

$$Carbohydrate\ productivity\ (mgL^{-1}\ d^{-1}) = Biomass\ productivity \times \frac{Carbohydrate\ content\ (\%)}{100}$$ Equation 4

Where biomass productivity is in mg L^{-1} d^{-1}, and carbohydrate content is in percentage per biomass weight.

3.2.5. Fatty acid profile

Fatty acid profiling was performed by converting the lipids to fatty acid methyl esters (FAMEs). Extracted lipid was dissolved in 1 mL of hexane and subjected to simultaneous esterification and transesterification using a 10% concentration of H_2SO_4 as a catalyst and methanol as an acyl acceptor for its conversion to FAMEs. The reaction conditions were methanol to oil molar ratio: 30:1; temperature: 60°C; catalyst concentration: 10% w w^{-1} of oil; reaction time: 4 h and stirring rate: 200 rpm (D'Oca et al., 2011). The hexane layer was separated and analyzed for fatty acid methyl esters using gas chromatography (GC) with Flame Ionization Detector (Shimadzu, Japan) as described by (Guldhe et al., 2014).

3.2.6. Statistical analysis

The statistical analysis of data was carried out using the Statistical Package for Social Sciences (SPSS Version 16.0). Significant differences among treatments were evaluated using one-way analysis of variance (ANOVA). The triplicate set of data was analyzed using a completely randomized design ($p \leq 0.05$). Standard deviations are depicted as error bars in the graphs (Rosemarin et al., 2020).

3.3. Results and discussion

3.3.1. Wastewater characterization

Municipal and agricultural waste streams are a renewable resource of water and nutrients, which can be efficiently valorized using microalgae. However, wastewater characteristics such as the availability of nutrients, microbial load, etc. directly impact the feasibility of microalgae biomass production in these wastewaters (Ling et al., 2019, Noguchi et al., 2021). In the present study, poultry litter extracts (2%) prepared by dissolving grounded poultry litter into post-chlorinated effluent (PCW) from a municipal wastewater treatment plant were characterized for physicochemical and biological parameters (Table 3.3). As depicted in Table 3.2, PCW showed a low concentration of nutrients. Nitrogen is one of the most vital macronutrient components for microalgae growth and significantly impacts biomass productivity (Noguchi et al., 2021). Results revealed that subjecting the mixture of poultry litter (Plaza et al., 2018) and PCW to orbital shaking at 200 rpm overnight showed significant potential in releasing nutrients ((NO_3- N (nitrate), NH_4-N (ammonia), PO_4-P (phosphate), and COD (chemical oxygen demand)) into PCW. An increased nutrient concentration was detected after the addition of PL into PCW (Table 3.3).

Generally, wastewater is a reservoir of numerous microorganisms, which may hamper the growth of microalgae of choice by competing for nutrients (Montemezzani et al., 2015, Salama et al., 2017). Different pre-treatment methods (physical and chemical) have been reported to be beneficial for improving the release of nutrients, reducing microbial load, and suspended solids in wastewater for improving microalgal biomass productivity (Ramsundar et al., 2017b, Salama et al., 2017). In the present study, acid pretreatment was employed to reduce bacterial load in the selected wastewater.

Table 3.3 depicts the physico-chemical and biological characteristics of raw (RPLE) and pretreated poultry litter extracts (PPLE). The concentrations of NO_3- N in RPLE and PPLE were 13.25 ± 0.35 mg L^{-1} and 13.75 ± 0.35 mg L^{-1} respectively. The ammonia (NH_4-N) concentration was observed to be higher in PPLE (117.00 ± 1.41 mg L^{-1}) as compared to RPLE (108.00 ± 2.83 mg L^{-1}) (Table 3.3). While concentrations of PO_4-P were found to be comparable in RPLE (21.05 ± 0.07 mg L^{-1}) and PPLE (21.95 ± 0.07 mg L^{-1}). The total suspended solids concentration was lower in PPLE (20.00 ± 1.12 mg L^{-1}). The occurrence of the essential nutrients in PLE showed its potential to be used as a medium for microalgae cultivation. Additionally, acid pre-treatment showed the potential to significantly reduce the bacterial load in PPLE (7.5×10^1 CFU mL^{-1}) as compared to RPLE (9.2×10^5 CFU mL^{-1}). Therefore, PPLE was used for the cultivation of microalga *Acutodesmus obliquus* and was compared with RPLE and standard BG11 medium.

Table 3.3 Physicochemical characteristics of raw and pre-treated wastewater

Parameter	Raw poultry litter extract (RPLE)	Pre-treated poultry litter extract (PPLE)
pH	7.45	7.05
Temperature (°C)	22.8	23.2
TSS* (mg L^{-1})	34.40 ± 0.05	20.00 ± 1.12
COD* (mg L^{-1})	482.2 ± 4.01	456.7 ± 8.02
NO_3- N* (mg L^{-1})	13.25 ± 0.35	13.75 ± 0.35
NH_4-N* (mg L^{-1})	108.00 ± 2.83	117.00 ± 1.41
PO_4-P* (mg L^{-1})	21.05 ± 0.07	21.95 ± 0.07
Bacterial Load (CFU mL^{-1})	$9.2x10^5$	$7.5x10^1$

*TSS- Total suspended solids; COD- Chemical oxygen demand; NO_3- N- Nitrogen in the form of nitrate; NH_4-N- Nitrogen in the form of ammonia; PO_4-P- Phosphorus in the form of phosphate

3.3.2. Microalgae cultivation in selected wastewater

3.3.2.1. Effect on growth, biomass production, and photosynthetic performance of *A. obliquus*

In the present study, biomass concentration obtained in PPLE treatment in mid-log (0.625 g L^{-1}) and late-log phase (1.965 g L^{-1}) was significantly higher ($p \leq 0.05$) than that of RPLE and BG11 medium (Figure 3.1). The accumulated biomass yields at the late log phase were 140.36 mg L^{-1} d^{-1}, 124.64 mg L^{-1} $d^{-1,}$ and 80.36 mg L^{-1} d^{-1} for PPLE, BG11, and RPLE, respectively

(Figure 3.2). The biomass concentration attained in PPLE was 12.6% and 74.67% higher in comparison to BG11 and RPLE medium respectively. In most previous studies, the biomass productivities of microalgae grown in wastewater were reported to be considerably higher than that of synthetic media (Ferreira et al., 2018). Moreover, Agarwal et al. also demonstrated that poultry litter manure could be a submissive nutrient source for microalgal growth and biomass production (Agarwal et al., 2020). They obtained substantial biomass productivity of 410 $gm^{-2}d^{-1}$ and 334 $gm^{-2}d^{-1}$ in *Scenedesmus obliquus* and *Ulva lactuca* cultivated in poultry litter extracts respectively. Biomass productivity results obtained under PPLE in the current study were higher compared to the findings of Singh et al. (2011). Singh et al. (2011) reported biomass productivity of 76 mg L^{-1} d^{-1} in *Chlorella sorokiniana* in untreated poultry litter effluent under mixotrophic cultivation mode.

Markou et al. (2016) investigated PL as a source of nutrients for the growth of *Arthrospira platensis and C. vulgaris* and it was found that *A. platensis* struggled to survive even under poultry litter leachate (PLL) dilutions of 15x and 10x, and the biomass obtained from higher dilutions (20 × and 25 × dilutions) was half as compared to the control medium. On the other hand, *Chlorella vulgaris* was able to grow well in poultry litter leachate and could achieve higher biomass productivity, which showed that microalgal growth and biomass productivity in PLL was species specific. Poultry litter extracts may contain undesirable components such as pharmaceuticals, and other organic contaminants along with containing organic carbon and essential nutrients (Teglia et al., 2017, Bedin et al., 2021). Generally, different species of microalgae have a differential ability to utilize organic carbon during heterotrophic and mixotrophic growth. Additionally, the response of microalgae also differs with the presence of contaminants (Markou et al., 2016). In the present study, *A. obliquus* showed its ability to utilize PLE as a growth medium for biomass production.

Additionally, the photosynthetic performance of *A. obliquus* was evaluated by measuring the quantum efficiency of PS-II (Fv/Fm). The Fv/Fm ratios are considered one of the indicators for stress response in microalgae. Fv/Fm of below 0.5 signifies that the culture is undergoing physiological stress while an Fv/Fm of above 0.5 indicates optimistic adaptability of the microalgae in a growth media (Ramanna et al., 2014). The highest Fv/Fm of 0.577 was observed in the BG11 medium followed by PPLE (0.565) in the mid-log phase i.e., 6th day of cultivation (Figure 3.2b). A similar trend of Fv/Fm ratio was also observed in the late log phase, whereby the BG11 medium recorded higher values followed by PPLE. While RPLE treatment recorded the lowest Fv/Fm in mid-log (0.458) and late-log phase (0.252) of growth, indicating the physiological stress in *A. obliquus* in the respective treatment. This reduction in the photosynthetic efficiency may be due to the turbidity in the PPLE and RPLE which interfere with the light intensity. Agarwal et al. (2020) also reported low light intensity in the poultry litter extracts and revealed that despite low photosynthetic efficiency, PPLE treatment recorded a substantial biomass yield. This may be because microalgae are capable to utilize organic carbon available in wastewater media, and hence undergo mixotrophic growth.

In the presence of an external carbon source, mixotrophic growth results in the additional pool of reducing power through an alternate electron sink, which may result in improved growth and biomass productivity (Mhatre et al., 2019). Therefore, a reduction in the available light intensity may not be the sole parameter to determine microalgal growth and productivity in the presence of an external C source (Mhatre et al., 2019, Agarwal et al., 2020,). High biomass productivities achieved in pre-treated poultry litter could be due to the mixotrophic growth of microalga due to the availability of sufficient COD in the medium as compared to autotrophic growth in the synthetic medium. The organic matter in PL likely stimulated *A. obliquus* biomass production, resulting in reasonable biomass productivity in PPLE treatment. Further efforts can be directed to the utilization of the developed strategy at large scale cultivation of *A. obliquus* to prove its feasibility for implementation at the demonstration scale.

3.3.3. Effect on biochemical composition of *A. obliquus*

The biochemical composition of microalgal biomass is a key factor that determines its suitability for applications in the biofuels sector. The biochemical composition of microalgal biomass could be strain-specific and may be subjected to change with varying media composition and cultivation conditions (Melo et al., 2018). Figure 3.2c illustrates the lipid, carbohydrate, and protein productivity in PPLE and standard BG11 biomass, harvested at the late log phase of growth (14^{th} day of cultivation). The biochemical composition varied in different treatments, indicating the substantial impact of cultivation media on different metabolites. PPLE treatment showed higher lipid content (27.42%) and productivity (38.49 mg $L^{-1}d^{-1}$) as compared to standard BG11 medium (22.45% and 27.98 mg $L^{-1}d^{-1}$). These results were in coherence with previous studies, which recorded higher lipid productivity in microalgae grown in wastewater as compared to standard growth media (Ling et al., 2019). A study by Markou et al. (2016) also reported higher lipid productivity in *C. vulgaris* cultivated in wastewater under mixotrophic cultivation than the photoautotrophic growth in a synthetic medium.

Generally, microalgae tend to generate more lipids under stressed conditions. Species competition and non-availability of nutrients are some of the parameters responsible for the stress occurring during cultivation in wastewater. Under stressed cultivation conditions, microalgae tend to accumulate energy-rich compounds such as lipids and carbohydrates.

Results in the present study showed that PPLE also recorded 35.98% higher carbohydrate productivity as compared to the BG11 medium. However, maximum protein productivity of 56.50 mg L^{-1} d^{-1} was obtained in standard growth medium BG11. Ferreira et al. (2018) also reported higher carbohydrate productivity in *Scenedesmus obliquus* in poultry litter wastewater as compared to swine, dairy, brewery, and cattle wastewater. Similar observations on poultry litter extracts were also demonstrated by (Markou et al., 2016). They revealed that high carbohydrate content could be due to the sufficient carbon contained in the poultry litter media.

Ma et al. (2020a) showed that the external supply of carbon in wastewater also assists in the production of carbohydrate-rich microalgal biomass. While higher content of protein accumulated under autotrophic cultivation in synthetic media may be due to the availability of nitrogen, which is normally utilized by microalgae to synthesize proteins (Ferreira et al., 2019). Venckus et al. (2017) obtained maximum protein productivity of 432 mg L^{-1} d^{-1} when *C. vulgaris* was cultivated in undiluted mechanically treated municipal wastewater. The data in their study illustrated that microalgae could accumulate a higher amount of proteins and a lower amount of carbohydrates when cultivated in nitrogen-rich conditions. The economic feasibility of biodiesel from microalgae is highly dependent on lipid content and productivity. The present study showed improvement in the lipid and carbohydrate productivities in the PPLE as compared to the standard growth medium, which reveals the suitability of the biomass generated for biofuels applications.

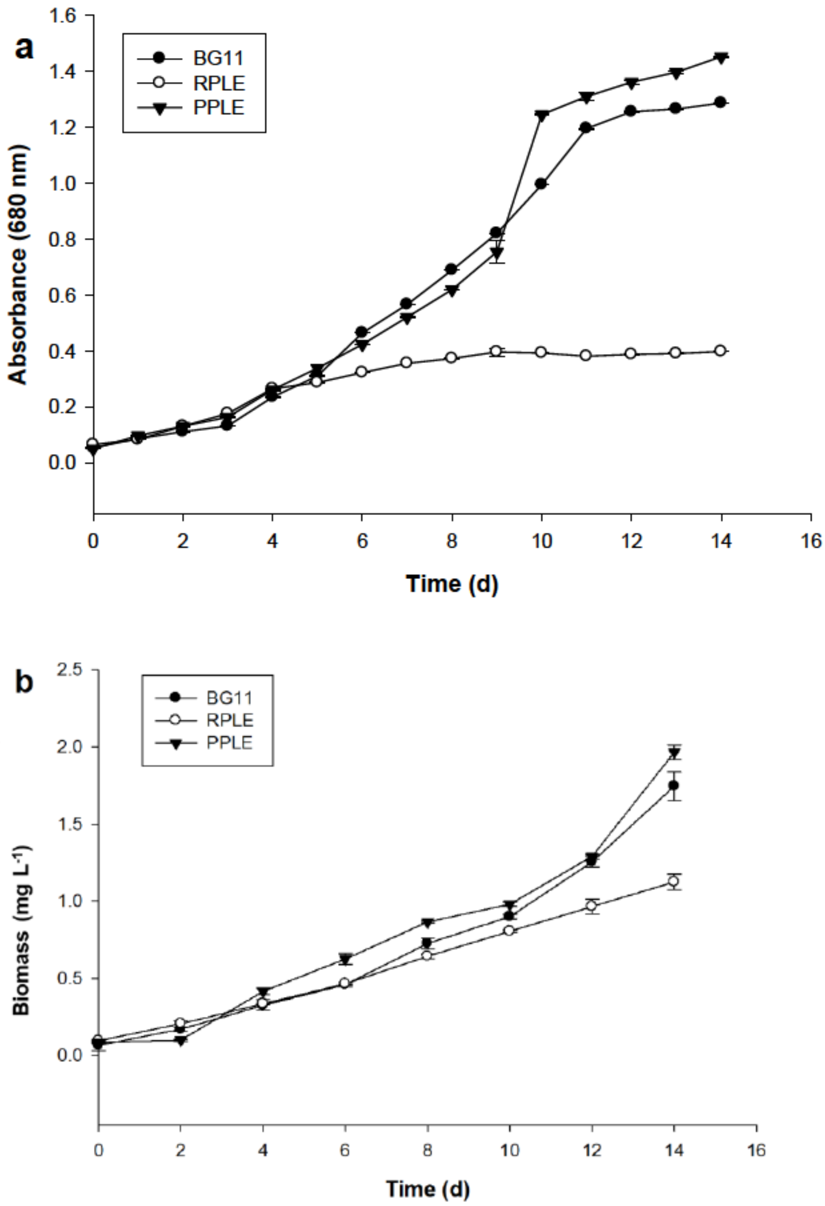

Figure 3.1 Growth profile of *Acutodesmus obliquus* in different treatments (a) Optical density (b) Biomass

3.3.4. Effect on fatty acid profile

Fuel properties are greatly influenced by the fatty acid methyl ester composition. Fatty acid production is significantly impacted by the type of algal strain and cultivation conditions (Jay and Hamilton, 2018, Guldhe et al., 2019). Fatty acid profile of lipids from PPLE grown microalgal biomass was compared with standard BG11 medium (Figure 3.3). Gas chromatography-mass spectrometry was used to evaluate the fatty acids composition to confirm the appropriateness for biodiesel production. The predominant fatty acids in the lipids from *A. obliquus* grown in wastewater and BG11 standard medium were palmitic acid (C16:0), oleic acid (C18:1), linoleic (C18:2) acid, and linolenic acid (C18:3). Palmitic acid (C16:0) concentration (30.3%) was slightly higher in PPLE as compared to standard medium BG11 (28.0%). A slight difference in the concentration of oleic acid was observed between the two cultivation conditions. Palmitoleic acid (16:1) concentration was significantly higher ($p \leq 0.05$) in PPLE (9.6%) as compared to BG11 (6.5%).

Fatty acids such as C20:0 (1.11%; 0.92%), C20:1 (3.23%; 1.64%), C20:2 (5.29%; 3.18%), C22:0 (1.07%; 1.74%), C22:1 (1.42%; 1.60%) and C24:0 (0.18%; 0.56%) were obtained in smaller proportions in both BG11 and PPLE respectively. A decrease in the stearic acid (18:0) concentration was observed in the PPLE medium. However, the total saturated fatty acid concentration in PPLE and BG11 medium was comparable to each other. The fuel properties of microalgae must match the criteria set by the European norms, the European Committee for Standardization, and the American Society for Testing and Materials standard agencies for it to be considered a viable source of biodiesel fuel. Based on the saturated fatty acids obtained, it can be concluded that fatty acids obtained from *A. obliquus* grown in PPLE could be a suitable feedstock to produce biodiesel fuel. Although the fatty acids profile of *A. obliquus* grown PPLE meets criteria on the basis of saturated fatty acids, the linolenic acid content is above the limit (12%) imposed by European standards (EN14214) (Knothe, 2006).

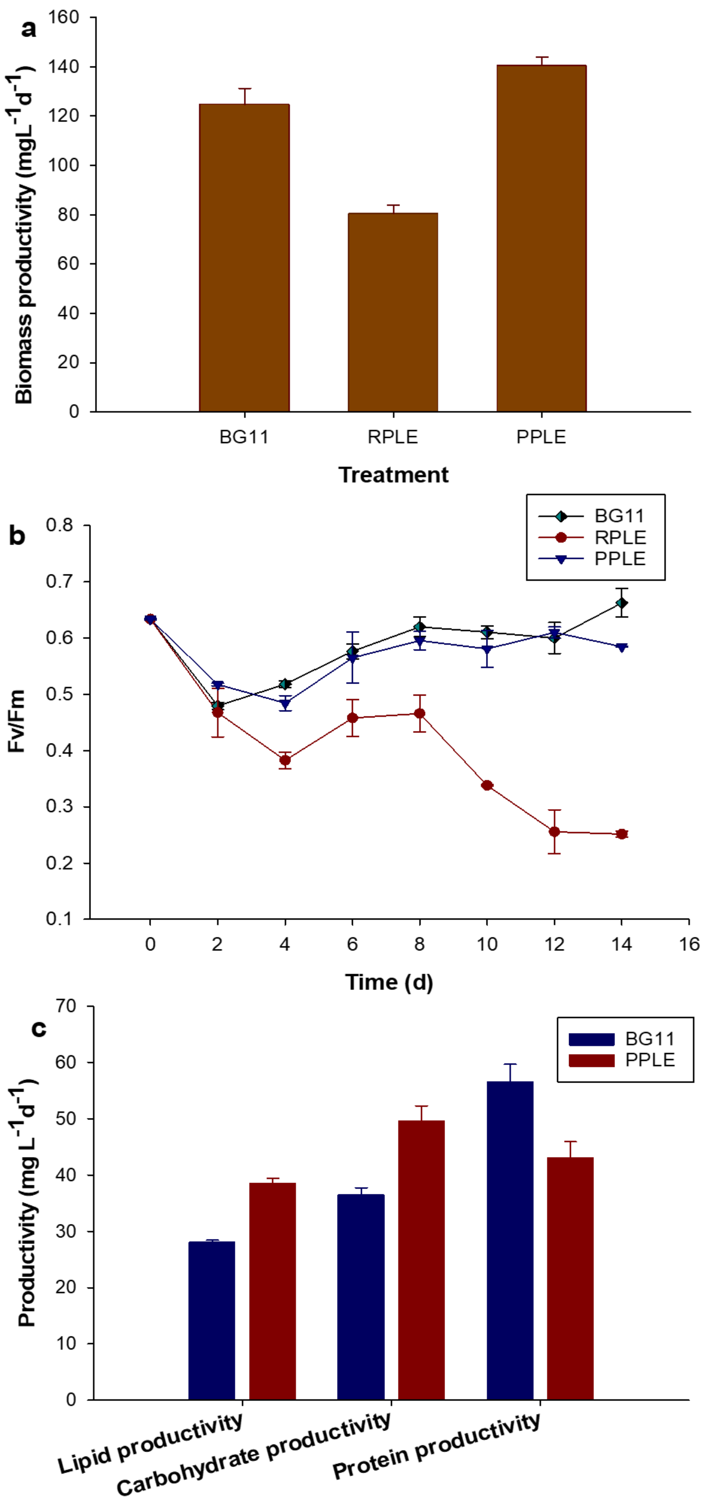

Figure 3.2 Growth characteristics of *Acutodesmus obliquus* in different wastewater treatments and standard growth medium (a) Biomass productivity (b) Quantum efficiency (Fv/Fm ratio) (c) Productivity of different metabolites. RPLE: Raw poultry litter extract; PPLE: Pre-treated poultry extract

The concentration of linolenic acid (C18:3) was observed to be 14.66% and 16.49% in BG11 and PPLE media respectively, thus further optimization is required to improve the suitability of the lipids for biodiesel application. Additionally, future investigations on the downstream processing of the biodiesel from such wastewater grown microalgae biomass, and fuel properties could be useful for the further refinement of the technology.

3.4. Nutrient recovery efficiency of microalgae in wastewater

Poultry industries produce a massive amount of waste, generally rich in ammonia, which could impose a high risk of eutrophication (Markou et al., 2016). Green microalgae possess the potential to utilize different sources of nitrogen and therefore can be used for the recovery of nutrients from such waste (Pham and Bui, 2020). The nutrient uptake efficiency of *A. obliquus* in PPLE was evaluated and compared with the RPLE medium (Figure 3.4).

The nutrient recovery efficiency of NO_3-N was found to be higher in PPLE (79.51%) as compared to RPLE (56.90%) (Figure 3.4). *A. obliquus* was able to effectively remove NH_4-N in PPLE and RPLE at rates of 81.82% and 68.29% (Figure. 3.4) respectively. Nitrogen and phosphorus recovery rates by *A. obliquus* in the present study were lower than achieved in a previous study by Ling et al. (2019) where *S. obliquus* showed a nutrient recovery rate of over 99%. Additionally, 99% of N recovery efficiency was reported from municipal wastewater (Activated sludge leachate in municipal wastewater) using *Parachlorella kessleri* NKG021201 (Aketo et al., 2020). A nutrient recovery rate of 93% NH_4-N, 84% NO_3- N, and 97% PO_4 –P was recorded when *Scenedesmus* sp. were cultivated in fertilizer plant wastewater (Pham and Bui, 2020). This could be due to the difference in the nutrient loading and the efficiency of microalgal species used in the different studies.

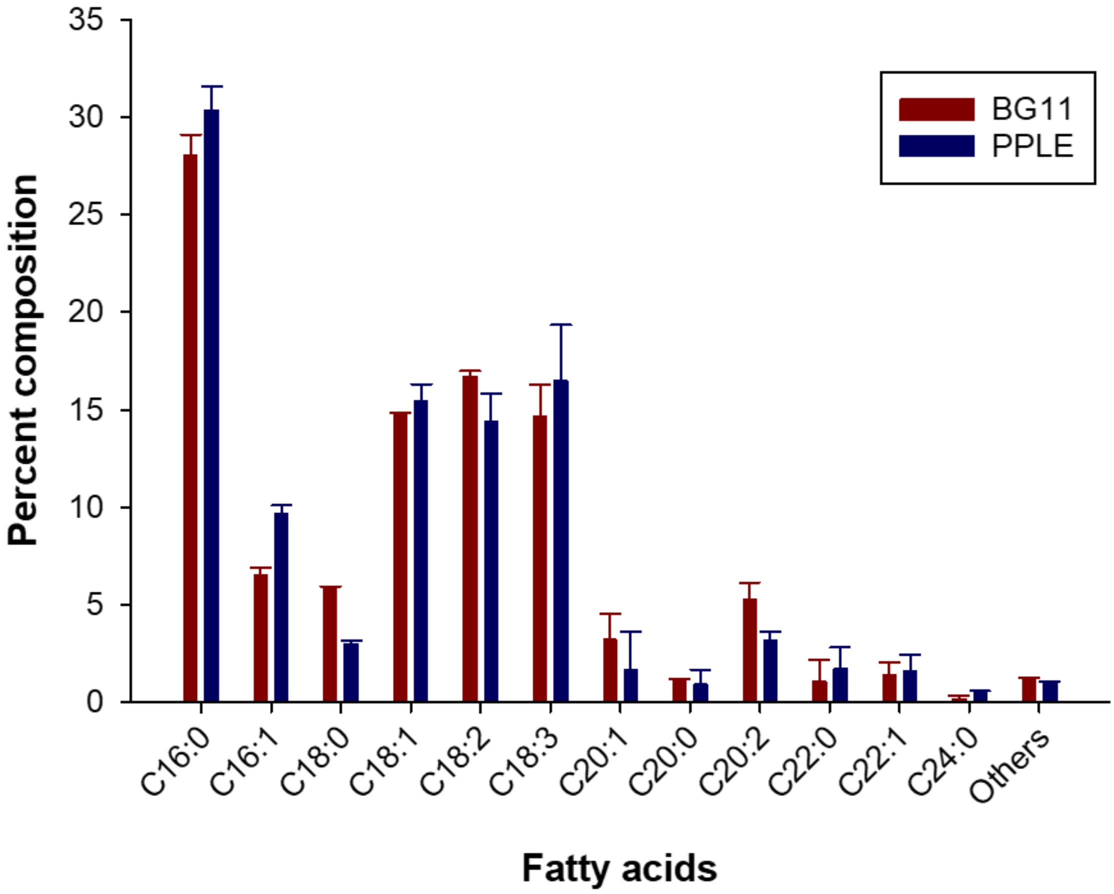

Figure 3.3 Fatty acid profile of *Acutodesmus obliquus* grown in wastewater and standard BG11 medium. PPLE: Pre-treated poultry extract

The nutrient recovery efficiency results achieved in PPLE comply with the results obtained by (Yao et al., 2015). In their study, the recovery rate of total nitrogen by *C. sorokiniana* and *D. communis* was 88.05% and 83.18% respectively. As depicted in Figure 3.4, the higher PO_4-P recovery efficiency of 80.52% was obtained in PPLE as compared to RPLE (66.51%). It is apparent that *A. obliquus* has a great potential for PO_4-P recovery as a significant amount of PO_4-P can also be absorbed into the microalgal cell wall and retained as a reserve. However, a slightly higher COD recovery efficiency of 50.80% was recorded in RPLE as compared to PPLE (40.47%), which could be due to the growth of heterotrophic microorganisms/bacteria in the RPLE medium. The results obtained illustrate that *A. obliquus* showed comparatively higher nutrient recovery potential in PPLE as compared to RPLE.

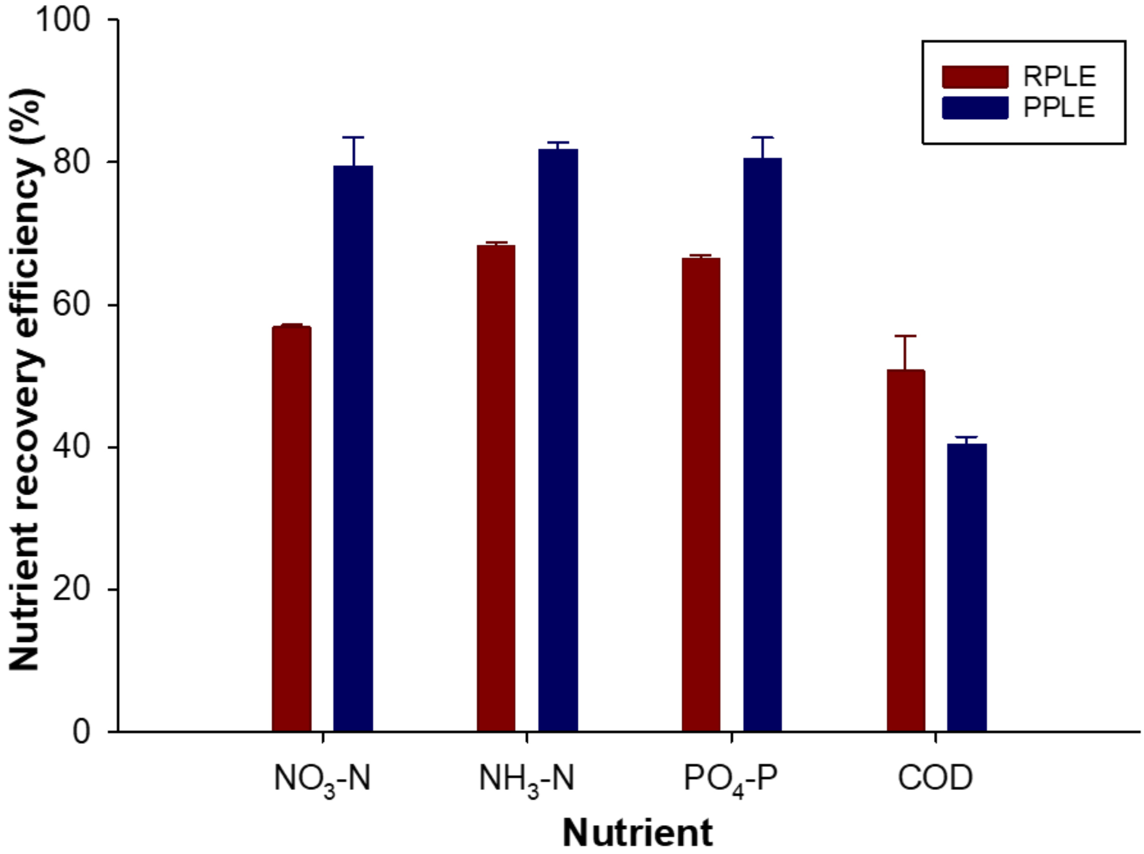

Figure 3.4 The nutrient recovery efficiency of *Acutodesmus obliquus* in poultry litter extract prepared using post-chlorinated effluent from a municipal wastewater treatment plant. RPLE: Raw poultry litter extract; PPLE: Pre-treated poultry extract

3.5. Conclusion

The need for large volumes of water and synthetic fertilizers for microalgal biomass production challenge the economic viability and sustainability of algae biomass derived products such as biofuels and agricultural fertilizers. The present study demonstrated the potential of agricultural waste (poultry litter) and municipal wastewater (post-chlorinated effluent) to be used as nutrient and water sources for microalgal biomass production, respectively. *A. obliquus* exhibited 12.61% - 74.67% higher biomass production and efficient nutrient recovery in pre-treated poultry litter extract (prepared in post-chlorinated effluent, PPLE) as compared to standard growth medium and raw poultry litter extract. Fatty acid profile of PPLE grown microalgal biomass was found to be comparable with standard growth medium, and suitable for lipid production. The approach used in this study can be further studied at pre-pilot and pilot scale to explore its feasibility for large scale microalgae biomass production for biodiesel application. Future investigations can also be focused on exploring different solid and liquid waste streams of domestic, agricultural, and industrial origin as nutrient and water sources for microalgae cultivation.

CHAPTER 4

APPLICATION OF LIPID EXTRACTED MICROALGAE BIOMASS AS FERTILIZER

MUSETSHO, P., RENUKA, N., GULDHE, A., SINGH, P., PILLAY, K., RAWAT, I. AND BUX, F. 2021. Valorization of poultry litter using *Acutodesmus obliquus* and its integrated application for lipids and fertilizer production. Science of the Total Environment, 796, p.149018.

4.1. Introduction

Microalgae biomass has been recognized as a potential feedstock for biodiesel production and many other applications of industrial interest. Microalgal biodiesel production involves the energy intensive procedures in the upstream i.e., biomass production, and downstream i.e., biomass processing (Patnaik and Mallick, 2021). Therefore, there is a huge concern about the economic feasibility and sustainability of biodiesel production from microalgae (Han et al., 2019). To achieve an energy return on investment, the production of biofuels/biodiesel from microalgae needs to be coupled with the generation of other useful products.

The biorefinery concept i.e., the production of multiple products from a single biomass feedstock has been reorganised as one of the useful strategies to improve the economic viability of microalgae-based products (Al Ketife et al., 2019, Almomani et al., 2019, Fan et al., 2020). During microalgal biodiesel, the excess amount of biomass residues left after lipid extraction is regarded as waste (Maurya et al., 2016b). The valorization of lipid extracted biomass (LEA) from wastewater-grown microalgae into other valuable products can be a useful way to improve the sustainability and feasibility of the biodiesel production process (Garcia-Gonzalez and Sommerfeld, 2016, Renuka et al., 2018a). This can be achieved by using the residual biomass for other applications such as aquaculture, nutraceuticals, animal feeds, etc. (Garcia-Gonzalez and Sommerfeld, 2016, Maurya et al., 2016b).

Recently, there is increased global interest in the use of organic fertilizers for improving plant growth and soil fertility due to the harmful environmental implications of the chemical fertilizers such as the continuous decrease in agricultural soil carbon and fertility (de Paula Pereira et al., 2021). Green algae have been investigated as fertilizers in agriculture due to their nutrient-rich biomass, which provides nutrients mainly through the mineralization process (Garcia-Gonzalez and Sommerfeld, 2016, Loganathan et al., 2020). The major nutrients applied most frequently as agricultural fertilizers include phosphorous (P), nitrogen (N), and potassium (K) (Renuka et al., 2016). Besides, organic carbon is the main constituent of soil, which directly influences the soil microbial activity, fertility, and availability of nutrients to the plant. Studies revealed that LEA is rich in carbon and other essential macro-and micronutrients. Carbon (C), N, P, Cu, Fe, etc., can be utilized as a nutrient source in agriculture (Patterson and Gatlin, 2013). Zhu et al. (2013) reported that lipid extracted algae biomass was rich in nutrients such as C (49%), H (6.96%), N (5.76%), and O (26.30%), which can be explored as a nutrient source in agriculture. The utilization of nutrient rich algae waste such as LEA in agriculture as fertilizer could be an inexpensive method of improving soil organic carbon and fertility. Therefore, it could be prudent to evaluate the potential of nutrient-rich LEA biomass to be used as fertilizer in agronomically important crops.

The shift from a single product strategy (manufacturing of biodiesel) to multi-product approaches has the potential to enhance the feasibility and sustainability of algal biofuels.

However, studies on assessing biorefinery approaches for the production of multiple products from a single microalgal feedstock are yet to be established and explored (Maurya et al., 2016b, Rothlisberger-Lewis et al., 2016). Despite microalgal biodiesel production generating a huge amount of nutrient LEA biomass, the usage of LEA in agricultural applications is scarcely explored.

Therefore, this study was carried out to assess the feasibility of using LEA produced from the lipid extracted microalgae biomass grown in wastewater (Chapter 3) for agricultural applications. A pot experiment was carried out to assess the potential of LEA by-products as fertilizer for an agriculturally and economically important legume, mung bean (*Vigna radiata*).

4.2. Materials and Methods

4.2.1. Application of lipid extracted microalgal biomass (LEA) as fertilizer

A laboratory scale experiment was carried out to evaluate the potential of LEA as fertilizer for mung bean (*Vigna radiata*) plant. The experiment was conducted in a greenhouse in pots of 17 cm length and 15 cm diameter containing garden soil purchased from a local nursery. Average daylight intensity and temperature in the greenhouse were 210.0±167.17 $\mu molm^{-2}s^{-1}$ and 20±1.63 °C respectively. Two different soil amendments were prepared by mixing PPLE grown whole algae biomass and lipid extracted algae biomass with soil in separate pots; designated as WA and LEA, respectively. The amount of whole and lipid extracted biomass was mixed with soil based on their N content equivalent to standard chemical fertilizer control (Culterra Pty Ltd). Pots containing soil with no added fertilizer (NF) and standard chemical fertilizer (CF) (Culterra Pty Ltd) were used as controls. Mung bean (*Vigna radiata*) seeds were surface sterilized with 70% ethanol and repeatedly washed with distilled water. The sterilized seeds were sown with equal spacing in between. Water was added to maintain the 60% water holding capacity of the soil. The pots were observed on daily basis for 30 days and all trials were performed in triplicates. Plant and soil samples were withdrawn for analysis on the 15th and 30th day of cultivation.

4.2.2. Soil analysis

The effect of WB and LEA on soil organic carbon, soil chlorophyll, and dehydrogenase activity was analyzed on the 15th and 30th days of the growth of mung bean plants. The quantification of organic carbon in soil was done using the dichromate oxidation method (Jackson, 2005) and equations as described by Yeomans and Bremner, (2008). Soil chlorophyll was estimated spectrophotometrically using acetone: dimethyl sulphoxide (DMSO) mixture (1:1) as extractant. The chlorophyll concentration was determined by taking absorbance readings at 663, 645, and 630 nm, and calculated by the equation described by Swarnalakshmi et al. (2013). Chlorophyll content was expressed as mg (chl) g^{-1} (soil). Dehydrogenase activity of soil was estimated by the method of Casida et al. (1964). The reduction of triphenyl tetrazolium chloride (TTC) (3%) to triphenyl formazan (TPF) was observed by taking absorbance at 485nm using TPF as a standard (Srivastava et al., 2020).

4.2.3. Plant analysis

Plant growth parameters such as leaf chlorophyll, plant fresh and dry weight, plant height, shoot, and root length were measured on the 30th day of cultivation. Leaf chlorophyll was measured spectrophotometrically (Opti-Sciences, CCM-200) using methanol extraction as described Swarnalakshmi et al. (2013).

4.3. Statistical analysis

The statistical analysis of data was carried out using the Statistical Package for Social Sciences (SPSS Version 16.0). Significant differences among treatments were evaluated using one-way analysis of variance (ANOVA). The triplicate set of data was analyzed using a completely randomized design ($p \leq 0.05$). Standard deviations are depicted as error bars in the graph (Rosemarin et al., 2020).

4.3. Results and Discussion

4.3.1. Application of lipid extracted algal biomass (LEA) as fertilizer

The use of chemical fertilizers is one of the most important nutrient management practices for improving crop yield in agriculture. In recent decades, the continuous overuse of chemical fertilizers has led to serious environmental concerns such as an increase in greenhouse emissions, a decrease in soil microbial diversity and fertility (Dai et al., 2021). The use of organic fertilizers in agricultural practices has been found beneficial in improving plant growth, and soil fertility and alleviating the harmful impact of chemical fertilizers *via* improving soil microbial activity and organic carbon (Dai et al., 2021; Li et al., 2021). Microalgae biomass has been found a promising organic fertilizer due to the presence of an adequate amount of different metabolites, nutrients, and organic matter (Renuka et al., 2018a, de Paula Pereira et al., 2021). However, the production of multiple products from a single microalgae feedstock is gaining great interest; because this approach ensures the economic feasibility of derived products and abates the production of waste (Zhu, 2015). Lipid extraction for microalgal biodiesel production generates lipid extracted biomass, which is generally rich in nutrients (Table 4.1). In the present study, the presence of major macronutrients *viz*. C and N in LEA showed its potential usage as organic fertilizer in agriculture. Therefore, a pot plant experiment was conducted to evaluate the potential of LEA as fertilizer in mung bean plants. The effect of LEA amended soil was compared with whole algae biomass (WA) and chemical fertilizer control (CF) on the basis of plant growth and soil characteristics.

4.3.2. Effect of LEA amendments on plant growth

The effect of soil amendment with lipid extracted biomass of *A. obliquus* was also evaluated for its potential effect on plant growth in mung bean plants on the 30th day of inoculation (Figure 4.1). Data obtained from the current study revealed that LEA and WA of *A. obliquus* played a role in influencing mung bean plants' growth in relation to plant parameters *viz*. fresh and dry weight, plant height, and the number of leaves. However, no significant difference ($p>0.05$) was observed in plant height in LEA, WA and CF amended treatments. Nevertheless, it was significantly higher as compared to NF control (Figure 4.2a). LEA treatment recorded a maximum plant height of 29.25cm on the 30th day of inoculation.

The maximum fresh and dry weight of 2.58g and 0.262g was recorded in LEA on the 30th day respectively (Figure 4.2b). There was no significant variation between CF and WA plant fresh and dry weight. Similarly, no significant difference was observed in the number of leaves in LEA, WA, and CF treatments. However, the results indicated that supplementation of microalgae in soil increased plant leaf chlorophyll (Figure 4.2c).

Table 4.1 Carbon and nitrogen content of microalgal biomass used for pot plant experiment

Nutrient	Lipid-extracted algal biomass	Whole algal biomass
C (%)	38.9	40.7
N (%)	6.8	6.0

C: Carbon; N: Nitrogen

Figure 4.1 Mung bean plants on the 15th and 30th day of cultivation period in the different treatments. T1: LEA Lipid extracted algal biomass; T2: WA Whole algal biomass; T3: CF Chemical fertilizer; T4: NF No fertilizer

The highest leaf chlorophyll of 1.55 mg g^{-1} was recorded in LEA followed by WA with 1.36 mg g^{-1}. An increase in the number of leaves, roots, plant height, and weight of agricultural crops grown in microalgae amendment soil or foliar spray as fertilizer and/or biofertilizer has been previously well documented (Plaza et al., 2018, Win et al., 2018, Rachidi et al., 2020). Castro et al. (2017) demonstrated that the soil inoculation of *Chlorella vulgaris* dominated microalgal biofilm increased the leaf and shoot dry matter in *Pennisetum glaucum* plants as compared to commercial urea fertilizer and control with no fertilizer. This was achieved due to a decrease in ammonia volatilization, an increase in cation exchange capacity, and an increase in organic matter in the soil, which further improved plant growth and nitrogen content (Castro et al., 2017; de Paula Pereira et al., 2021).

4.3.3. Effect of LEA amendments on soil characteristics

Soil organic carbon serves as a major source and sink of atmospheric carbon and plays a major role in maintaining and sustaining the quality of the agroecosystem (Sandeep et al., 2016). Further, organic carbon is the main constituent of soil, which directly influences the soil microbial activity, fertility, and availability of nutrients to the plants. LEA has been documented as a rich source of organic carbon and can therefore be useful in the improvement of soil organic carbon and fertility (Maurya et al., 2015, Mohan Singh et al., 2020). In this study, the effect of LEA amendments on soil organic carbon was evaluated on the 15^{th} and 30^{th} days of inoculation of mung beans.

Figure 4.2d depicts the percent (%) increase in the organic carbon content in different microalgae treatments and controls from the initial soil organic carbon at the time of inoculation. Microalgae (LEA and WA) amended treatments showed a higher increase in organic carbon as compared to control treatments.

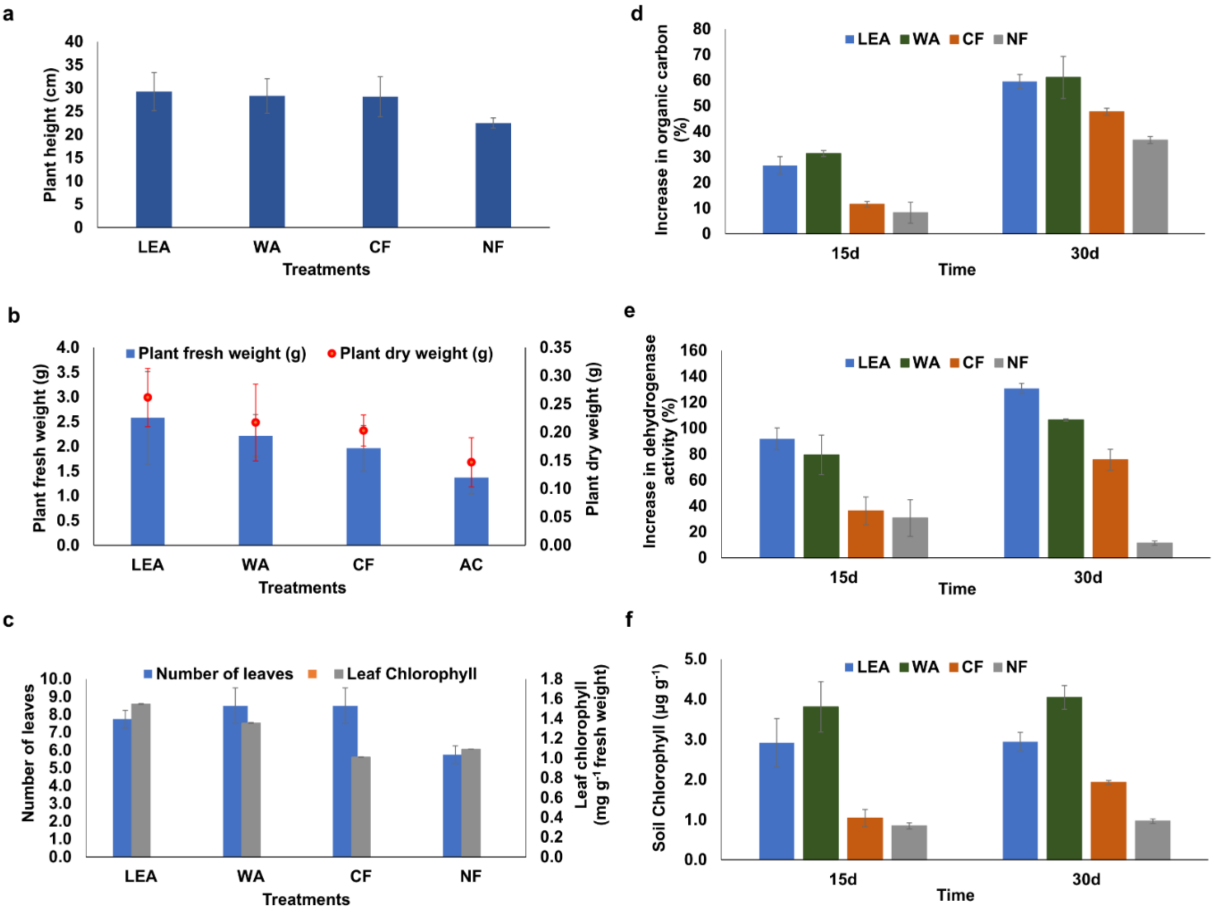

Figure 4.2 Effect of different soil amendments on mung bean plant characteristics on the 30th day of cultivation (a-c) and soil characteristics at the different time intervals of the cultivation period (d-f). LEA: Lipid extracted algal biomass; WA: Whole algal biomass; CF: Chemical fertilizer; NF: No fertilizer.

Nevertheless, there was an insignificant difference ($p>0.05$) between LEA and WA supplemented treatments. WA treatment recorded the highest organic carbon of 0.96% and 1.18% on the 15th and 30th days respectively (Figure 4.3a). LEA amended treatment showed a 26.73% and 59.54% increase in organic carbon on the 15th and 30th days from the initial soil organic carbon. On the 15th day, LEA and WA treatments recorded 13.61% and 17.75%, and 17.07% and 21.34% higher soil organic carbon content as compared to CF and NF control respectively. While on the 30th day, microalgae inoculated treatments (LEA and WA) showed 8.02% - 9.09% and 16.76% - 17.92% higher soil organic carbon content as compared to CF and NF control respectively. This indicates that microalgae biomass as fertilizer has great potential in the enhancement of soil organic carbon.

An earlier study by Rothlisberger-Lewis et al. (2016) also reported an increase in organic carbon, when lipid extracted biomass of *Nannochloropsis salina* DMB was inoculated into the soil under laboratory conditions (Rothlisberger-Lewis et al., 2016). Previous studies on the improvement of soil organic carbon after the addition of algae have been carried out and the findings were optimistic (Renuka et al., 2017, Yilmaz and Sönmez, 2017 Barone et al., 2018b). However, Maurya et al. (2015) reported that there was no increase in soil organic carbon when lipid extracted biomass was supplemented into the soil. Further, they mentioned that even though soil organic carbon may not change, an unceasing use of carbon-rich LEA could have a substantial benefit in the long run, hence, the application of LEA might have an advantage in preventing nutrient loss through volatilization or leaching. Microalgae offer the most valuable attribute of enhancing soil organic carbon which cannot be attained when using chemical fertilizers (Castro et al., 2017).

Dehydrogenase enzymes are the most vital enzymes, which occur in living microbial cells, and thus are used as overall indicators of soil microbial activity (Barone et al., 2018a, Barone et al., 2018b). Dehydrogenase activity is also one of the key attributes for evaluating and monitoring the fertility and health of the soil. Their main role in the soil is to represent microbial oxidative activities. Dehydrogenase enzyme activity was analyzed to evaluate the soil microbial activity in the soil. The effect of different soil amendments on the dehydrogenase activity at different time intervals is presented in Figure 4.2e. According to the results achieved, dehydrogenase activity was found to be directly proportional to the number of days.

Microalgae (LEA and WA) inoculated treatments recorded higher dehydrogenase activity as compared to CF and NF controls (Figure 4.2e, Figure. 4.3b). Figure 4.2e illustrates a percent increase in the dehydrogenase activity in different treatments from the initial soil dehydrogenase activity on the day of inoculation. It was evident that there was an increase in the dehydrogenase activity with time. However, microalgae (LEA and WA) supplemented treatments showed a higher % increase in dehydrogenase activity on the 15th and 30th day of inoculation as compared to CF and NF controls (Figure 4.2e). Maximum dehydrogenase activity of 61.28 and 73.71 µg TPF g^{-1} soil dry weight d^{-1} was observed in LEA amended treatments followed by WA (57.32 and 65.95 µg TPF g^{-1} soil dry weight d^{-1}) on 15th and 30th day of inoculation respectively (Figure. 4.3b).

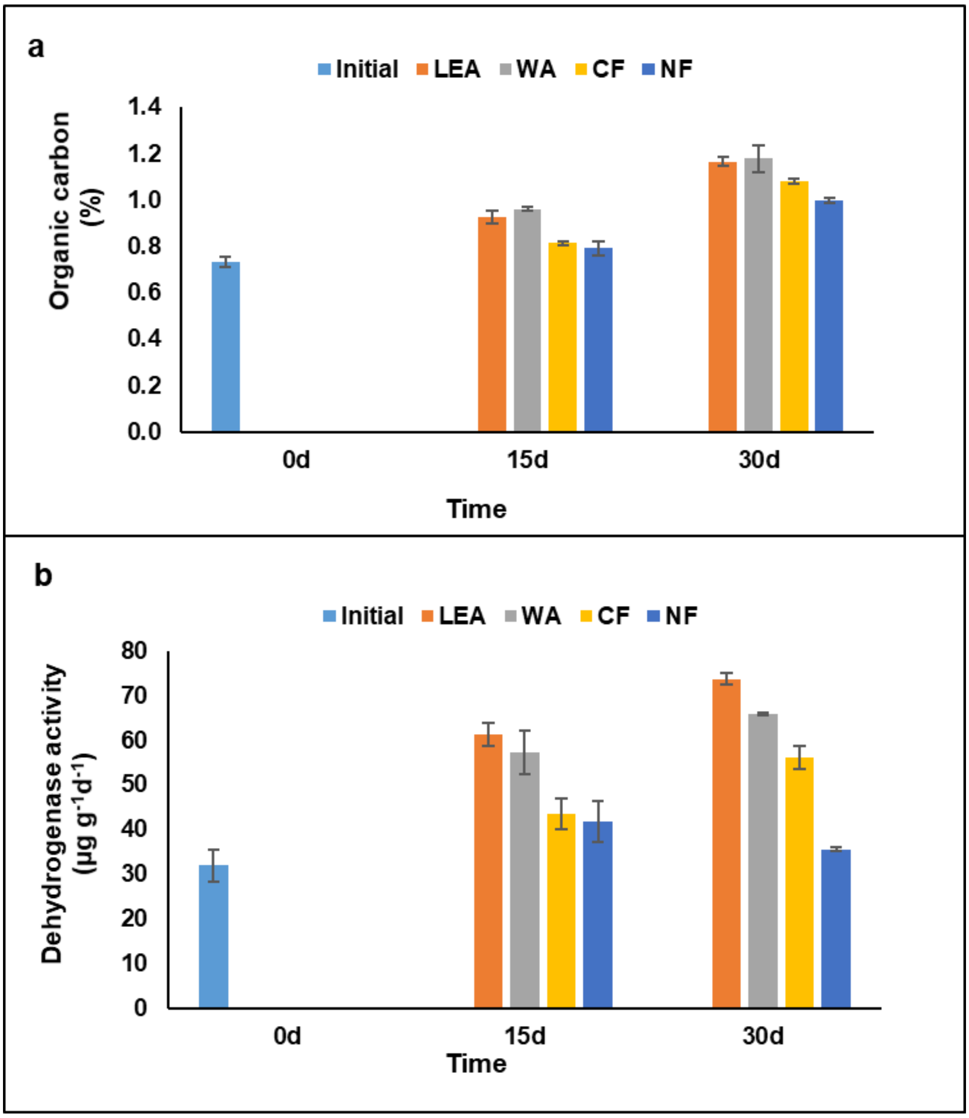

Figure 4.3 Soil characteristics viz. (a) organic carbon and (b) dehydrogenase activity at the time of inoculation (initial) and different time intervals of cultivation period of mung bean plants. LEA: Lipid extracted algal biomass; WA: Whole algal biomass; CF: Chemical fertilizer; NF: No fertilizer

Increased dehydrogenase activity indicates that microalgal inoculation has a positive impact on the respiratory cell cycle (Grzesik, 2014). Similarly, significant variances in the dehydrogenase enzyme activities between microalgae inoculated soil and the control were reported by (Barone et al., 2018a). However, LEA is processed microalgal biomass, which generally does not contain live microflora. This nutrient-rich biomass contains microalgal cells with the disrupted cell wall. Therefore, fast mineralization of the LEA nutrients could be a possible reason for increased dehydrogenase activity in the soil. Substantially, the results obtained in the current study showed that LEA amendment in soil could improve soil microbial activity, which may further assist in enhancing the fertility, functioning, and structure of soil (Bidyarani et al., 2016, Prasanna et al., 2017).

Soil chlorophyll is considered a measure of photosynthetic biomass in the biological soil crusts (Lan et al., 2013). The estimation of soil chlorophyll was conducted on day 15^{th} and 30^{th} days of inoculation. As observed in Figure 4.2f, microalgae (LEA and WA) inoculated treatments showed an improvement in soil chlorophyll, which was significantly higher ($p \leq 0.05$) as compared to CF and NF treatments. However, no significant change in chlorophyll concentration was observed from the 15^{th} to 30^{th} day of inoculation in LEA and WA treatments. A Maximum chlorophyll concentration of 4.05 $\mu g\ g^{-1}$ was observed in WA treatment followed by LEA (2.94 $\mu g\ g^{-1}$) on the 30^{th} day. Similarly, an increase in the soil chlorophyll on microalgae amended soil was observed in a wheat crop system and legume crops (Babu et al., 2015). Results in the present study showed the efficacy of LEA in improving organic carbon, microbial activity, and photosynthetic biomass of soil, in mung bean crops, indicating its potentiality to be used as fertilizer. However, future studies on nutrient dynamics and metagenomics are required to prove its further potential at the field scale level.

4.4. Conclusion

The application of lipid extracted algal biomass as fertilizer in mung beans showed significant improvement in plant growth, and soil organic carbon and dehydrogenase activity as compared to no fertilizer control, which was comparable with the whole algae (WA) and standard chemical fertilizer (CF) control. However, future field-scale evaluation and in-depth studies on nutrient and microbial dynamics are required to prove the potential of LEA as fertilizer at large scale in different crops. The present study thus forms a basis for future studies for developing novel approaches for waste valorization to form a sustainable biorefinery using microalgae.

CHAPTER 5

GENERAL CONCLUSIONS AND RECCOMENDATIONS

Currently, biofuels from microalgal biomass have gained increased public and scientific attention as an alternative substitute to depleting fossil fuel resources. However, challenges such as the high fertiliser and freshwater requirements for microalgae growth and biomass production limit the commercialization of microalgae biomass-derived products.

The utilization of municipal and agricultural waste streams as water and nutrient sources for microalgal cultivation instead of synthetic media has been perceived as a conceivable alternative for viable biomass generation. Treated wastewater (post-chlorinated wastewater (PCW)) from the wastewater treatment plants contains residual nutrients and, therefore, offers a genuine solution to be utilized as a water source for microalgae cultivation. Low nutrient availability in PCW, on the other hand, can limit biomass productivity, which can be overcome by supplying an external nutrient source. The addition of synthetic fertilizers could again challenge the economic viability and sustainability of algal biomass and biofuel production. Therefore, the utilization of waste nutrient sources can be a useful approach for improving the nutrient content of PCW. Utilization of agricultural solid waste such as poultry litter (PL) as a nutrient source may lessen the reliance on synthetic fertilizers, which could, therefore, minimize the overall microalgal biomass production cost. Hence, the strategy of growing microalgae in wastewater offers a proficient way of nutrient recovery from waste resources and simultaneously produces valuable microalgal biomass for various applications.

In the current study, instead of utilizing freshwater, municipal PCW (post-chlorinated wastewater) was used as an aqueous medium. The addition of PL to PCW followed by acid pre-treatment improved the nutrient content in PCW. Chemical pre-treatment of poultry litter (pH 2 acidification) was observed to be the most feasible pre-treatment method for reducing the bacterial load and releasing nutrients from PL into PCW.

Cultivation and biomass production of *A. obliquus* was successfully achieved in pre-treated poultry litter extract (PPLE). PPLE recorded higher biomass yield, biochemical constituent productivity, and photosynthetic performance as compared to raw poultry litter extract. The biochemical composition of wastewater-grown *A. obliquus* biomass revealed the tremendous potential for use in the energy and fertiliser industries. *A. obliquus* showed the highest lipid and carbohydrate productivity in PPLE. The fatty acid profile of wastewater-grown biomass was found to be comparable to that of standard growth medium BG11, which shows its potential to be used for biodiesel production.

Microalgal biodiesel production produces lipid extracted algae biomass (LEA) as waste, which is generally rich in metabolites and nutrients. Hence, LEA could be used for other reasonable purposes like energy, feed, and fertilizer production. This can be considered as one of the novel approaches that can lead to waste valorization and form a sustainable biorefinery. Moreover, the utilization of LEA makes the biodiesel production process more sustainable by simultaneously producing multiple products. In the present study, LEA was found to be rich in carbon and nitrogen. Application of LEA as a fertilizer for mung bean crop in a pot experiment showed an improvement in plant growth, soil organic carbon and microbial activity. Maximum increase in soil organic carbon and dehydrogenase activity in a 30-day interval was observed in LEA amended soil, which was significantly higher than the commercial chemical fertilizer (CF) control. Furthermore, plant fresh weight and leaf chlorophyll in the LEA amended soil was comparable to whole algal biomass (WA) and CF control. The developed strategy could thus be a basis for a sustainable biorefinery for nutrient recovery from wastewater and simultaneously producing microalgae derived biofuels and fertilizer for agricultural application.

Recommendations:

- At the laboratory scale, both liquid (post-chlorinated wastewater) and solid waste streams (poultry litter) demonstrated enormous potential as a water and nutrient source for microalgae cultivation for biomass and biodiesel production. Future research can be directed to explore a wide range of waste streams and identify the most suitable combinations and most economical pre-treatment methods for improved physicochemical and biological characteristics, nutrient availability, and ratio. It is also recommended that the proposed approach should be evaluated at a pre-pilot scale followed by a pilot scale to examine its viability and feasibility.

- Further investigations can be carried out to improve the microalgal biomass productivity and biochemical composition as well as the improved recovery of metabolites from the wastewater grown biomass.

- Lipid extracted biomass (LEA) showed potential to be utilized as fertilizer for mung beans under greenhouse conditions. However, long-term studies at a field scale are needed to test the economic feasibility of utilizing LEA in agricultural crops. In-depth studies on studying the effects of such biomass on soil and plant health should be undertaken.

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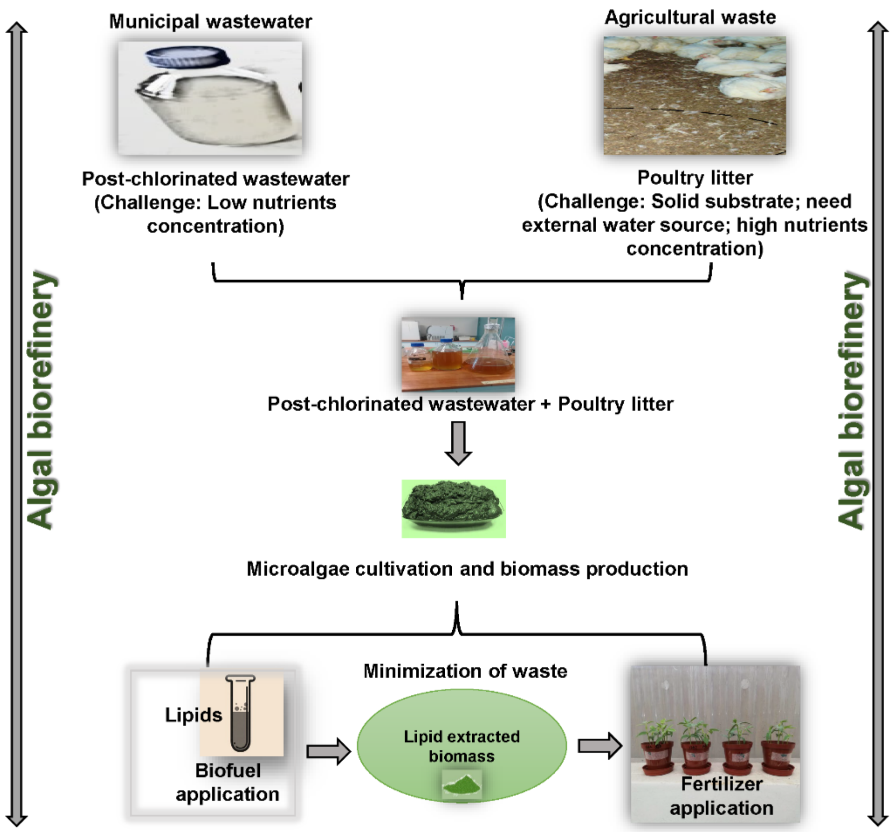
APPENDICES

APPENDIX A: Graphical abstract: Schematic representation of the work undertaken



APPENDIX B: Blue-green (BG11) medium

Macronutrients

Components	Stock solution (g L^{-1})
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
$NaCO_3$	0.02 g
Trace metal mix A5	1.0 mL
Distilled water	1.0 L

Trace metal solution

Components	Quantity
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co\ (NO_3)_2 \cdot 6H_2O$	0.049g
Distilled water	1.0 L

BG11 components were added to 1 L distilled water. After sterilization (autoclaving), pH was adjusted to 7.1.

APPENDIX C: Publication: Valorization of poultry litter using *Acutodesmus obliquus* and its integrated application for lipids and fertilizer production

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Valorization of poultry litter using *Acutodesmus obliquus* and its integrated application for lipids and fertilizer production

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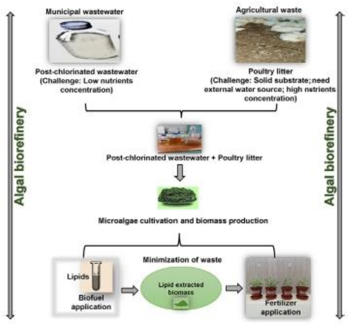
HIGHLIGHTS

- Microalgae mediated resource recovery from poultry litter and municipal effluent.
- Pre-treated poultry litter extract showed improved biomass and lipid productivity.
- Wastewater grown microalgae showed potential for biofuel and fertilizer application.
- Lipid-extracted algae improved mung bean plant growth and soil fertility.
- The study forms a basis for zero-waste process and biorefinery using microalgae.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae are recognized as potential candidates for resource recovery from wastewater and projected for biorefinery models. This study was undertaken to evaluate the potential of poultry litter and municipal wastewater as nutrient and water sources, for the cultivation of *Acutodesmus obliquus* for lipids production for biodiesel application. The efficacy of lipid extracted biomass (LEA) as fertilizer for mung bean crops was also assessed in microcosm. *A. obliquus* cultivation in acid pre-treated poultry litter extract (PPLE) showed maximum biomass production of 1.90 g L^{-1}, which was 74.67% and 12.61% higher than the raw poultry litter extract (RPPE) and BG11 respectively. Higher NO_3-N, NH_3-N, and PO_4-P removal of 79.51%, 81.82%, and 80.52% respectively were observed in PPLE as compared to RPLE treatment. The highest biomass (140.36 mg L^{-1} d^{-1}), lipids (38.49 mg L^{-1} d^{-1}), and carbohydrates (49.55 mg L^{-1} d^{-1}) productivities were observed in the PPLE medium. The application of LEA as a fertilizer for mung bean crops showed improvement in plant growth and soil microbial activity. A maximum increase in organic carbon (59.5%) and dehydrogenase activity (130.8%) was observed in LEA amended soil which was significantly higher than chemical fertilizer (CF) control in 30 days. Whilst plant fresh weight and leaf chlorophyll in the LEA amended soil was comparable to whole algal biomass (WA) and CF control. The strategy developed could be a basis for sustainable biorefinery for the valorization of wastewater for the production of microalgae-derived biofuel and byproducts for agricultural application.

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APPENDIX D: Publication: Valorization of wastewater *via* nutrient recovery using algae-based processes

Valorization of Wastewater via Nutrient Recovery Using Algae-Based Processes

1

Pfano Musetsho, Nirmal Renuka, Sachitra Kumar Ratha, Ismail Rawat, and Faizal Bux

Contents

Abstract

Increasing anthropogenic activities have amplified the unregulated wastewater discharge into water bodies, leading to the accumulation of nutrients and subsequent destruction of freshwater resources and aquatic habitats. Exploration of energy competent, cost-effective systems, which are able to sequester nutrients in wastewaters, has become a requisite. Recently, microalgae–bacterial consortia are gaining attention as an environmentally friendly and economically viable option for wastewater treatment and biomass production. Co-cultivation of these microorganisms promotes mutual growth and enhances nutrients removal from wastewater. Furthermore, several studies have projected the potential applications of wastewater-grown microalgal biomass for the production of renewable energy. Recently, wastewater-grown microalgae biomass has also been explored for its

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