TREATMENT OF WASTEWATER COUPLED WITH THE SEQUESTRATION OF CARBON DIOXIDE BY TWIN LAYER CULTIVATION OF MICROALGAE

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A thesis submitted in partial fulfillment of requirements for the degree of
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Dedication

This research is dedicated to my loving, caring, and industrious parents and my friends whose efforts and sacrifice have made my dream of having this degree a reality. words cannot adequately express my deep gratitude to them. "O My Sustainer, Bestow on my parents your mercy even as they cherished me in my childhood".

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LIST OF ABBREVIATIONS

US	United States
WHO	World Health Organization
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
TSS	Total Suspended Solids
TDS	Total Dissolved Solids
RED	Renewable Energy Directive
PSBR	Porous Substrate Bioreactor
POME	Palm Oil Mil Effluent
TP	Total Phosphorous
TN	Total Nitrogen
DOC	Dissolved Organic Carbon
EPS	Extra Polymeric Substances
NIVA	Norwegian Institute for Water Research
LED	Light Emitting Diodes
MWW	Municipal Wastewater
AD	Anaerobic Digestate
TLS	Twin Layer Cultivation System
APHA	American Public Health Association
TKN	Total Kjeldahl Nitrogen
ROS	Reactive Oxidative Stress
AAS	Atomic Absorption Spectrum
IPCC	Intergovernmental Panel on Climate Change
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
RNA	Ribonucleic Acid

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ABSTRACT

Microalgae provides an eco-friendly solution for wastewater treatment, CO_2 fixation and lipid and pigments production. High-nutrient wastewater such as solid waste leachate, anaerobic digestate and poultry wastewater has high turbidity, dark color and high concentration of nutrients. Therefore, it cannot be treated using microalgal systems and need to be diluted prior to treatment. This study assessed the potential of *Tetradesmus obliquus* for treatment of high-nutrient wastewater using twin layer cultivation system. For this purpose, high nutrient wastewater was diluted by wetland treated municipal wastewater prior to microalgal treatment. Results showed that microalgae cultivated in poultry wastewater gave the highest biomass productivity and CO_2 fixation of 5.13 and 9.25 $g/m^2/d$ respectively. Similarly, the maximum nutrient removal rate was achieved from poultry wastewater, which as 31.6, 39.3 and 3.5 mg/d for NH_4^+-N, TKN and PO_4^{-3}-P respectively. In contrast, the highest lipid content of 30% was observed in microalgae cultivated in municipal wastewater. However, lipid productivity was maximum in biomass grown in poultry wastewater, which was 1.48 $g/m^2/d$. Chlorophyll a, b and carotenoid content of microalgae grown in poultry wastewater were 36.6%, 47.3% and 47.4% higher than municipal wastewater. Thus, successful microalgal treatment of high-nutrient wastewater can be performed through its prior dilution.

Keywords: Microalgal Biomass; Lipids; Pigments; Poultry Wastewater; Solid Waste Leachate; Anaerobic Digest

CHAPTER 1

1 INTRODUCTION

1.1 Background

Beginning of the 21st century has directed planet earth towards a population boom (Shahid et al., 2020). It is expected that population count will reach up to 9.7 billion in 2050 (Torres-Tiji et al., 2020). This increase in population has pressurized humans for extravagant industrialization, commercialization and urbanization. These developments along with the beneficiaries have also given birth to many consequences, such as, water depletion, global warming and energy security issues (Thawechai et al., 2016, Shahid et al., 2020). It is expected that human life will face a water deficit of 40% by 2030 coupled with the deficiency in the consumption of fossil fuels, since 85% of our energy requirements are met by it. Numerous types of wastewaters are being released from various sources, for instance, medium strength wastewater (municipal wastewater (MWW)), and high nutrient containing wastewater (anaerobic digestate (AD), landfill leachate, poultry wastewater (PWW)) (Yu et al., 2020, Dogaris et al., 2020, Guldhe et al., 2017).

Landfill leachate is one of the inescapable problems that arises from municipal solid waste disposal into a landfill. In US, merely State of Florida is reported for generating 24 million liters leachate per hectare of landfill annually (Dogaris et al., 2020). Anaerobic digestate is nutrient rich byproduct of anaerobic digestion of organic waste (Fernandes et al., 2020). Similarly, municipal wastewater is also a nutrient rich wastewater originated from municipalities (Guldhe et al., 2017). Not only this, the increase in the consumption of fossil fuel will possess serious threats, such as, greenhouse gas emissions, lack of fossil fuels and variations in crude oil prices (Hosseini et al., 2018). CO_2 is the major greenhouse gas, its concentration has reached up to 400 ppm and imparting 76% in global warming (Verma and Sirvastava, 2018, Fazal et al., 2018). A loss of 2 trillion US dollar has been reported from 1998 to 2017 due to climate change (Feng et al., 2020).

Owing to all the above mentioned problems, certain techniques have been studied and employed for wastewater treatment and biofuel production. A broader range of biofuels have been used depending upon their feedstock, third and fourth generations biofuels are produced by from algae. For the production of microalgal biofuel, 40% of its production cost is required for microalgal cultivation (Sajjadi et al., 2018). For devising microalgal biofuel production economically feasible, wastewater can be employed as a nutrient source. Microalgae has an innate property of absorbing nitrogen (N) and phosphorous (P) for its growth. These nutrients are profoundly present in wastewater. Microalgae can absorb these nutrients from wastewater by the process of assimilation. Nitrogen is utilized by microalgae to produce energy transfer fragments, chlorophylls, enzymes and genetic constituents. Phosphorous is consumed by microalgae for producing amino acid, RNA, DNA, ATP and cell membrane material. Along with this, microalgae can also absorb carbon efficiently (Salama et al., 2017). Use of microalgae for CO_2 uptake is vital since microalgae need CO_2 to perform the most important function required for its growth, i.e., photosynthesis (Hosseini et al., 2018). Its carbon sequestration efficiency has been reported as 1.83 kg of CO_2 per kg biomass (Verma and Sirvastava, 2018).

With this background, it can be suggested that benefits of microalgal cultivation are sky reaching, since it helped in tackling wastewater treatment, carbon dioxide biofixation and biofuel production in an integrated manner. Historically, microalgae can be grown in open ponds (Lu et al., 2020). Unfortunately, ample number of disadvantages, such as, cross culture contamination, improper mixing, temperature fluctuations, lower rates of mass transfer, evaporative losses and unmanageable light intensity with lesser biomass productivity have been reported (Banerjee and Ramaswamy, 2017, Faried et al., 2017, Yadav et al., 2020, Nwoba et al., 2019). Therefore, later on research conducted under the umbrella of microalgal cultivation brings a lot of advancement and various photobioreactors have been fabricated to fill the voids of conventional systems. Both suspended and attached growth photobioreactors have been used for microalgal cultivation (yad et al., 2018). Attached cultivation systems are prior over suspended one due to higher biomass, higher wastewater treatment, higher CO_2 absorption, less contamination and easy harvesting, higher absorption rate and higher biomolecule profile (Padola et al., 2017, Faried at al., 2017, Zhao et al., 2018, Zhuang et al., 2018). It has been reported that for production of microalgal biofuel, 20-30% of its total cost is needed for the harvesting of cultivated microalgae (Sajjadi et al., 2018). Attached cultivation system can be used for cultivating microalgae so that it can be easily harvested by mechanical detachment reducing its cost (Podola et al., 2017). Various studies for comparing suspended and attached cultivation system has been conducted. In a study conducted by Huang et al. (2016) an attached cultivation system was compared to the traditional suspended cultivation mode to determine biomass production in both reactors. The results have shown a 30.4% increase in attached biomass production in comparison to the suspended one under same light intensity and CO_2 supply. In another study, two reactors have been designed one with flexible fiber bundles acting as solid carriers and the other one without extra addition of carriers. Results has reported a 1.44 fold increased biomass production in the presence of solid carriers in comparison to other one (Gao et al., 2015).

A number of attached cultivation system has been designed for the cultivation of microalgae. The basic categories include non-immersed, semi immersed and immersed (Zhuang et al., 2018). Twin layer cultivation system (TLS) is one of the non-immersed cultivation system and it is preferred over other systems due to high light transfer and utilization and higher mass transfer of nutrients and gases (Podola et al., 2016). This system consists of macro porous, source layer and micro porous, substrate layer. Source layer helps for transfer of nutrients whereas, microalgae is attached on substrate layer. A mass number of studies has reported its applications; Shi et al. (2007) has studied the removal of phosphorous and nitrogen from synthetic wastewater using nitrocellulose membrane as a substrate layer and glass fibre fleece as a source layer. Results showed the removal of ammonium, phosphate and nitrate to less than 10%. Naumann et al. (2012) has reported a biomass productivity of 1.8 $g/m^2/d$ using modified f/2 medium. In this study, glass fiber nonwoven was used as source layer, however, news printing paper was used as a substrate layer. In 2014, a pilot scale TLS was fabricated using nylon fiber sheet as substrate layer coupled with reinforced glass fiber sheet as a source layer. Different types of wastewaters such as, effluent from bio-phosphorus tank, denitrification tank and secondary settlement tank were used as nutrient media for the cultivation of microalgae. Bio-phosphorous contained higher ammonium-N and Phosphate content than other effluent types. This treatment resulted in 78.9% total phosphate and 99% ammonium-N removal from bio-phosphorous tank (Shi et al., 2014). Li et al. (2015) has studied the treatment of mine dump leachate for the removal of zinc. High zinc absorption of 15 -19 mg Zn g^{-1} algal dry matter was reported by this study. Afterwards, anaerobically digested effluent has also been studied for removal of total nitrogen, total phosphorous and chemical oxygen demand by using *Chlorella* in presence of phytohormones. The removal capacities for TN, TP and COD were reported as 0.65, 0.25 and 3.31 $g/m^2/d$ with a biomass productivity of 5.66 $g/m^2/d$ (Yu et al., 2020). In 2017, a study has been conducted on anaerobic digestate, livestock wastewater and grey domestic wastewater on an algal biofilm reactor using a consortia of *Phormidium and Chlorella*. High biomass productivity and treatment potential of various wastewaters was tested with accumulation of lipids and proteins. The highest microalgal biomass production of 4 $g/m^2/d$ was reported when cultivated in livestock wastewater. Lipid content of 38% was observed in microalgal biomass grown using domestic grey water and 44% of protein content was recorded in livestock wastewater and anaerobic digestate (Choudhary et al., 2017). Present study adds to existing literature by fabrication of TLS, capable to produce microalgal biomass and compared the nutrient removal of highly problematic wastewaters, such as, solid waste leachate (SWL), PWW, AD and MWW. Furthermore, for the first time the potential of *Tertradesmus obliquus* was explored for its growth, wastewater treatment and lipid production on TLS employing cheaper source and substrate layers. The microalgal biomass produced was also evaluated for finding its CO_2 fixation potential and pigments production.

1.2 Objectives

1. To evaluate the effect of wastewater type on different parameters of wastewater treatment and carbon absorption rate using Twin Layer System.
2. To study the effect of wastewater type on lipid productivity of *Tetradesmus obliquus* using Twin Layer System.
3. To measure the pigments production ability of *Tetradesmus obliquus* cultivated in different wastewaters.

CHAPTER 2

2 LITERATURE REVIEW

A huge number of literature is available regarding growth of microalgae, its nutrient removal efficiency, carbon dioxide biofixation and by products production. A brief overview of this literature is given below in different sections and subsections.

2.1 Major environmental problems

2.1.1 Water pollution/scarcity

Water pollution has become the most prominent issue of this time. It has been estimated that a city having a population of 5 Million using 0.2 ton per day per capita water can on average generate 85 thousand ton per day per capita wastewater (Salama et al., 2017). According to the study conducted in 2018, it has been reported that 2 billion people globally are living in the areas of economic and physical water scarcity (UN Water, 2018). It has been reported that among the 37 world's largest aquifers, 21 has already traversed their sustainability tipping ranges (Hasan et al., 2019). The major contributors of wastewater are human factors, industrial, agriculture, mining and power generation (UN Water, 2001). Considering the origin of wastewater, it has been divided into many major types. Polluted water majorly cause eutrophication, acidification and ecosystem distraction (Shahid et al., 2019). These wastewater types are chiefly responsible for the water-borne diseases, such as, hepatitis A, dysentery, diarrhea and cholera. WHO, 2017 says annually 8,42,000 people die ascribing to diarrhea. Therefore, solutions are required to meet the current water demands (Salama et al., 2017).

2.1.2 Energy crisis

Approximately 88% of the world energy demands are met by fossil fuels. It has been reported that the energy demands will elevate by 53% by 2030. Petroleum demands in USA are expected to be increased by 116 million barrels per day till 2030. Owing to this consumption pattern, a total depletion of fossil fuels is expected to be occurred in next 60 years (Shah et al., 2018). Developing countries such as, Pakistan are already facing energy crisis. Pakistan Electric Power Company (PEPCO) has reported that the energy demand of Pakistan is 18,000 MW, however, the capacity to generate electricity is only 13,240 MW. The daily imports of oil are 346,400 barrels raising its bill to approximately 11 billion (Ali et al., 2017). Additionally, It has been expected that by 2040, the CO_2 emissions will upsurge up to 45,000 mega tons (Adeniyi et al., 2018). At this juncture, various new solutions are required to overcome these problems. In 1997, Kyoto Protocol, stands to overcome these problems by using clean fuel for transportation (Ambat et al., 2017).

2.1.3 Climate change

The CO_2 release from the fossil fuel also act as an important. By the year 2006, 29 billion ton carbon dioxide release by fossil fuel has been reported. A greater number of this CO_2 in the atmosphere is absorbed by oceans causing ocean acidification, resulting in a prominent reduction of marine species (Lam et al., 2012). The areas seriously affected by a tremendous increase in CO_2 concentration are sociological, technological, economical along with natural and human development (Anwar et al., 2018). Intergovernmental Panel on Climate Change (IPCC) has suggested to reduce the concentration of greenhouse gases by 50% till 2050 to prevent humans from these catastrophic episodes due to a change climatic conditions (Pachauri et al., 2014).

2.2 Technologies for reducing water pollution/scarcity

Among the technologies employed to circumvent water scarcity, reuse of water offers environment and finance friendly opportunities. It involves collection of wastewater from various sources, treating it and transporting it to the users. Wastewater comprises of nutrients that can act as a resource for producing energy (Diaz-Elsayed et al., 2020). A number of technologies has been used for treating wastewater. It includes primary and secondary treatment. Primary treatment involves the removal of solid particles from wastewater through settling and filtration etc. Secondary treatment of wastewater is the treatment of wastewater through micro-organisms. It involves the accumulation of organic particles from wastewater. It also includes treatment of wastewater through chemicals. But the major drawback involved is the excessive production of sludge. This sludge further need any treatment or disposal. So, these technologies do not provide the complete treatment of wastewater released (Kalra et al., 2020). To counter these problems, phycoremediation imparts a significant role for wastewater treatment. Microalgae has a property to assimilate nutrients from microalgae. (Faried et al., 2017). For instance, a small scale edible oil industry produces 80-100 tons of waste that has a very higher chemical and biological oxygen demand (COD, BOD), total dissolved and suspended solids (TDS, TSS) along with higher sulphate and phosphate content (Ahmad et al., 2020). Microalgae can be used to reduce the nutrient content of the wastewater released and can efficiently help in wastewater treatment (Hernández-García et al., 2019).

2.3 Technologies for circumventing energy crisis

Various techniques have been employed to circumvent the issue of energy crisis. European Union Renewable Energy Directive (RED) has suggested to meet at least 15% of energy demands in UK by renewable resources (Adeniyi et al., 2018). Renewable resources means the use of solar, geo thermal, wind, hydro and feedstock. Depending upon the feedstock required for biofuel production, different generations are defined. First generation (G1) biofuels includes the synthesis of biofuels from edible crops. Second generation (G2) biofuels comprises of the used of lignocellulose waste and forest residues (Sajjadi et al., 2018). However, first and second generation biofuels have some sustainability and ethical concerns (Rashid et al., 2014). Third and Fourth generation (G3, G4) biofuels are the use of microalgae for bio fuel production. Production of bio-oils by microalgae is 10 times more than oil produced by one of most efficient vegetable oil crop, palm tree (Sajjadi et al., 2018). The space required by microalgae to grow is 132 times lower than microalgae (Mathimani and Pugazhendhi, 2018). An abundant number of studies has been reported for production of biofuels from microalgae. In a study conducted by Shahi et al. (2020) *Dunaliella sp.* was cultivated in open pond. Later on, hydrothermal liquefaction of the harvested algal biomass was done at 350 ºC by maintain a pressure of 200 bar for the synthesis of bio-oil. This results in the production of 11.81 w/w% yield of bio-oil. In another study conducted by Vassalle et al. (2019) microalgae was cultivated in a High Rate Algal Ponds, then Co-digestion of sewage waste and microalgae was performed to enhance the biogas production in an anaerobic sludge blanket reactor. It has been observed that during the co-digestion methane yield enhanced from 156 to 211 $NLCH_4$ $Kg^{-1}VS$.

2.4 Technologies for reducing climate change

Many technologies have been employed to combat the issue of climate change. One of them is the capturing of CO_2 through, pre, post and oxy fuel consumption (Anwar et al., 2018). Other than this, the most important is the upgrading of CO_2 captured from various sources. This captured carbon can be used for the synthesis of various chemicals, sea water desalination and algal biofuel production (Anwar et al., 2020). Using microalgae for carbon sequestration has many benefits. The capacity of microalgae for carbon assimilation is 10-50 folds enhanced than terrestrial plants. Microalgae can grow in few hours leading to higher biomass and higher carbon absorption. Flue gas release from various industries can also be used as a carbon feed to microalgae for growth (Xu et al., 2019). Many studies have been conducted for using flue gas as a source of CO_2 to the microalgae. In Hawaii, USA, energy produced by a power plant was used to provide mixing to 67 culture tanks used for the cultivation of *spirulina* and *hemococcus*. The waste gases release from this power plant were used to provide inorganic carbon to the strains. The utilization efficiency of power plant was recorded as 75%. In China an experiment was conducted to compare the carbon fixation ability of microalgae grown under the CO_2 release from a power plant in comparison to terrestrial plant. For this experiment, 25 ton CO_2 was absorbed by plants in the time span of one year in comparison to microalgae which has shown a 2-3 folds better trend by absorbing 58-90 ton CO_2 in a year (Xu et al., 2019).

2.5 Microalgal cultivation and its affecting factors

Microalgae is a group of photosynthetic unicellular microscopic organisms that can either be eukaryotic or prokaryotic. Prokaryotes lack nuclei, mitochondria and chloroplast. Microalgae, on the basis of taxonomy are grouped as, yellow-green, red, golden, blue-green, green, brown microalgae and diatoms (Rashid et al., 2014, Phwan et al., 2018). Microalgae can also be divided depending upon its carbon demand. Autotrophs need inorganic carbon to thrive, whereas, heterotrophs need carbon in organic form. Those algal species that can uptake carbon in both organic and inorganic forms are known as heterotrophs (Rashid et al., 2014, Dogaris et al., 2020). Microalgae has the capacity to fix nitrogen as a nutrient to grow in different forms. Prokaryotic microalgae can absorb nitrogen in molecular form and convert it into NH_4^+-N for the synthesis of protein. On the other hand, eukaryotic microalgae use nitrogen in fixed form such as, nitrite, nitrate and NH_4^+-N (Dogaris et al., 2020).

Microalgae have a higher growth rate couple with the high photosynthetic ability. They can live in brackish water, freshwater, marine water, even in wastewater depending upon its specie (Rashid et al., 2014, Dogaris et al., 2020). Some microalgal species have the ability of tolerate extreme conditions. After growth, microalgae can be turned into various value added products, such as, feed for animals, pharmaceuticals, fertilizers, cosmetics and biofuels Different factors can affect the growth of microalgae, such as, carbon source, pH, light intensity, light duration, temperature and nutrients (Bibi et al., 2017, Rashid et al., 2019, Zhuang et al., 2018, Faried et al., 2017). In case of attached growth, support media can also affect the growth of microalgae (Zhuang et al., 2018).

2.5.1 Light intensity and photoperiod

Light intensity is one of an important factor for microalgal growth as, microalgae needs light for photosynthesis. Several experiments has been performed for the authentication of its dependency. In a study conducted by Liu et al. (2013) the growth of *Scenedesmus obliquus* was observed using porous substrate bioreactor (PSBR) at different distances from illumination source. The biomass productivity kept on increasing as the light intensity reaching the biomass was increasing, however, for *Scenedesmus obliquus* the light saturation point –the point after which microalgal growth start inhibition, was reported as 150 µmol photon $m^{-2}s^{-1}$. Ji and his colleagues in 2013 elucidated the effect of light on *Pseudochlorococcum sp.*. Microaclgae was grown on different filter membranes with a pore size of 0.45µm using agar as a nutrient source.

The biomass productivity was increasing by increasing light intensity from 10 to 100 µmol photon $m^{-2}s^{-1}$. However, 100 µmol photon $m^{-2}s^{-1}$ was reported as light saturation point for *Pseudochlorococcum sp.*. Along with light intensity, photoperiod as plays an important role in the cultivation of microalgae. In a study conducted by Wahidin et al. (2013*)* *Nannochloropsis sp.* was cultivated to find the optimum light conditions for its growth. Different light intensities, such as, 50, 100 and 200 µmol $m^{-2}s^{-1}$ were selected with the various photoperiods of 12:12, 18:6 and 24:0 h light:dark. Results indicated a higher growth of 6.5 107 cells m/L under the light intensity of 100 µmol $m^{-2}s^{-1}$ and 18:6 h. Gao et al. (2022) studied the effect of light intensity on the growth of *Chlorella vulgaris*.

The results indicated 8000-12000 lux as an optimum light intensity range for the growth of *Chlorella vulgaris*. In a study conducted by Iasimone et al. (2018) studied the effect of different light intensities on a polyculture of microalgae grown under wastewater. A maximum biomass productivity of 227 mg/L with an average growth rate of 58.7 mg/L/d was observed in 100 µmol $m^{-2}s^{-1}$ light intensity. Yan et al. (2016) cultivated microalgae in photobioreactor to find the optimum light intensity and photoperiod for its CO_2 fixation. The results demonstrated that a light intensity of 300 µmol $m^{-2}s^{-1}$ with a photoperiod of 16 h light: 8 h dark resulted in highest CO_2 removal efficiency of 85.46 ± 6.25%. Additionally, George et al. (2014) cultivated *Ankistrodesmus falcatus* in a photobioreactor to find its maximum biomass production at an optimum light intensity and photobioreactor. A maximum biomass production of 7.9 mg/L/d was achieved at a light intensity of 60 µmol $m^{-2}s^{-1}$ with a photoperiod of 12 h light: 12 h dark.

2.5.2 Nutrients and growth media

Microalgae needs several nutrients for its growth such as, nitrogen and phosphorous (Zhuang et al., 2018). Various forms of nitrogen can be uptake by microalgae especially molecular nitrogen, nitrate, nitrite and NH_4^+-N nitrogen. These nitrogen forms are incorporated into the cell by active transport to form amino acid in microalgae. Nitrate, nitrite and molecular nitrogen are usually reduce into NH_4^+-N and then into amino acids, whereas, NH_4^+-N is directly converted into amino acids, as shown in Figure 1 (Gonçalves et al., 2016). Phosphorous is usually used by microalgae in its inorganic form i.e. orthophosphate (Khanzada et al., 2020). It is one the basic nutrient needed for various purposes such as, synthesis of nucleic acid, Adenosine triphosphate (ATP), Adenosine diphosphate (DNA), Ribonucleic acid (RNA), lipids, proteins and an intermediate for the metabolism of sugars.

2.5.3 Temperature

Temperature is one of the prominent factor that effects the growth of microalgae. Because temperature effects the mechanism of enzymes, nutrient assimilation, cell cycle division and utilization efficiency of CO_2 (Xu et al., 2019). Attached microalgal cultivation systems are more subjected to temperature fluctuations than the suspended ones due to lower interaction of water and microalgae (Rosli et al., 2020). The optimum temperature range observed for microalgal growth is reported as 15-30 ºC (Xu et al., 2019). It has been reported that increase in temperature can increase the biomass productivity of microalgae because increase in temperature increases the metabolic activity of microalgae (Gonçalves et al., 2016). It has been reported that microalgae can tolerate a maximum temperature of 30 ºC above this temperature only a certain microalgal species can grow (Rosli et al., 2020). A research conducted by Fica and Sims, 2016 has shown an increase in the biomass productivity from 4.55 to 7.57 $g/m^2/d$ by increase in the temperature from 7 to 27 ºC on a rotatory algal biofilm.

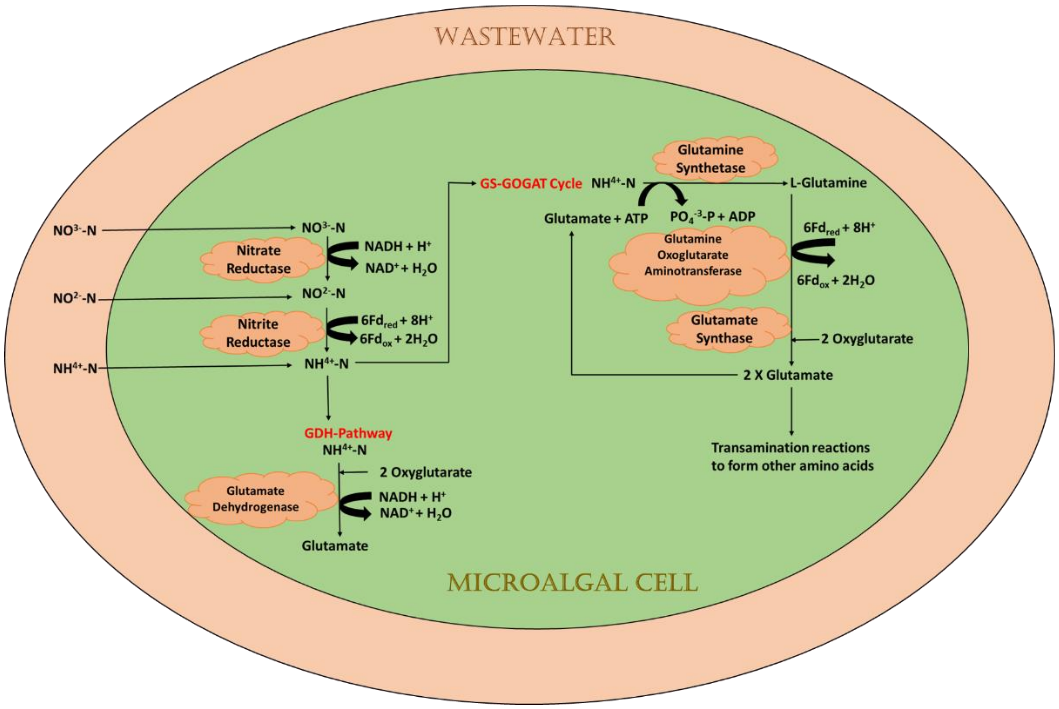

Figure 2. 1 Assimilation of Nitrogen by Microalgae

2.5.4 pH

pH plays a crucial role for the growth of microalgae because it imparts a huge role in maintaining the physiological activities of microalgae coupled with the support medium, such as, electron donor-acceptor properties, polarity, hydrophobicity and chemical group composition (Katarzyna et al., 2015). The optimum pH range of microalgae to grow is reported as 7-9. But this is also specie dependent, some microalgae are alkalophilic and some are acidophilic. *Spirulina platensis* is reported to give highest biomass productivity under the higher pH of 9-10. However, *Chlorococcum littorale* has grown best in acidic environment having the pH ranges 5-6 (Gonçalves et al., 2016). pH of the nutrient media plays an important role in the formation of biofilm, in fact, the pH of the layers of biofilm differs with the surrounding media. It has been reported that best attachment of *Nitzschia amphibian* to substrate layers of glass and titanium has been found in neutral environment

(Katarzyna et al., 2015). Composition of substrate layer also plays a significant role in attachment of microalgae to substrate material. It has been reported that *Nannochloropsis oculata,* freshwater *Chlorella sp*. and marine *Chlorella sp*. when attached on a glass substratum, their attachment went on decreasing by increasing pH (5.5., 6.8 and 8.1). The initial attachment of the cell was found to be subjugated by electrostatic forces, where pH effects the zeta potential of support media and microalgae. But when attached on PVC material, marine *Chlorella sp*. and *Nannochloropsis oculat* was found to be more attached in case of higher pH, in this case the most dominant force in initial attachment was Lewis acid-base forces, in which the cell's surface energy was affected by pH (Yuan et al., 2019).

2.5.5 Aeration and mixing for CO_2 supply

Mixing imparts a significant role in increasing microalgal biomass. It can help in various ways such as, distribution of light evenly in the photobioreactor, increasing mass transfer of CO_2 and medium nutrients (Wang et al., 2014). Baffles can be used for proper mixing within a photobioreactor (Yang et al., 2016). Carbon is a crucial component for the growth of microalgae it is consumed by microalgae in two forms, either directly in the form of CO_2 or by converting into HCO_3^- and CO_3^{+2} using carbonic anhydrase activity into free CO_2 (Salama et al., 2017). López-Pacheco et al. (2021) reported a 446 ± 150 mg CO_2 L^{-1} day^{-1} sequestration of CO_2 by providing 10% CO_2 to *Scenedesmus sp.* grown ander 50% industrial wastewater. Zheng et al. (2020) reported a highest biomass production of 3.08 g/L by providing 5% CO_2 to *Chlorella vulgaris* grown in swine slurry.

2.5.6 Support media

Factors involved in the attached cultivation of microalgae are same but attached cultivation needs a support media to grow. Support media needs various qualities for microalgae to grow. These are selected on the basic of biotoxicity, cost, porosity, hardness, hydrophobicity/hydrophilicity, texture and porosity (Zhuang et al., 2018). Now a days most of the substrate used for the study are directly bought from the market, such as, plastic foam, polycarbonate, stainless steel, cotton rope, concrete slab, micro filters, printing and newspaper (Johnson and Wen, 2010, Liu et al., 2013, Ozkan et al., 2012, Naumann et al., 2013). The basic driving force of adhesion of microalgae is the interaction between microalgal cell and the substratum. Two types of interactions are involved in the microalgal biofilm production (Figure 2). First is the interaction of cell to substratum and secondly cell to cell interaction. Initially, the cells are attached to substratum by hydrodynamic and gravitational forces. After attachment, many biochemical reactions occurs, such as the release of soluble algal products, these attach the cell on the substratum permanently (Wang et al., 2018). So basically, many factors are responsible for both the substratum and microalgae to form a biofilm (Wang et al., 2017).

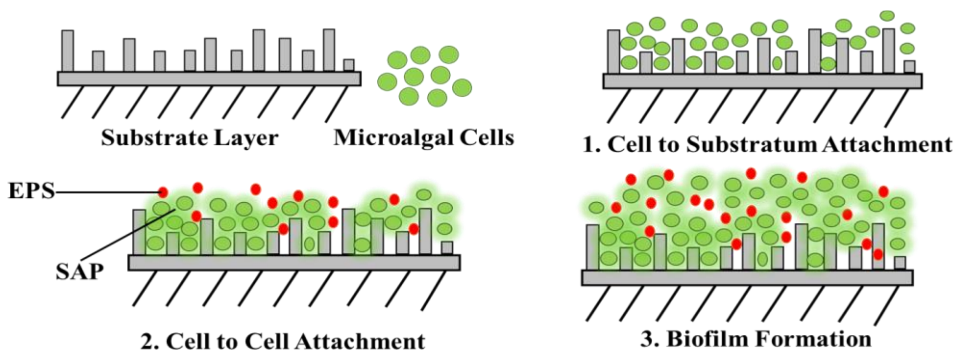

Figure 2. 2 Biofilm formation of microalgae on a substratum

A research was conducted to evaluate the biomass areal density of microalgae on different substratum. Various, hydrophilic and hydrophobic membranes were tested to find the more suitable substratum. Results have shown that mixed cellulose substrate layer has shown more biomass than other substrates tested (Ji et al., 2013). Another study was conducted by Gross et al. (2016) has observed the biomass productivity of microalgae on 30 different substratum and have found that polypropylene mesh and nylon are the best substratum for microalgal productivities. These cells for detachment needs several treatments, such as, ultrasound, strong flushing, chemical treatment and mechanical scraping. Once the cells are firmly attached on the substratum the biofilm starts forming by extracellular polymeric substances and soluble algal products to strengthen the biofilm thickness (Wang et al., 2018).

2.6 Types and significance of wastewaters for microalgal cultivation

Our waterbodies have soaring concentrations of nitrogen and phosphorus arising the issue of eutrophication. It has become a prominent cause of undesirable imbalance of organisms and water quality in water bodies (Guldhe et al., 2017). Wastewater is a cheaper and readily available nutrient medium for the cultivation of microalgae. These nutrients are abundantly present in wastewater from several sources most commonly agricultural waste, industrial waste and municipal waste. Several studies have been conducted for the treatment of various wastewater type (Salama et al., 2017). Industrial wastewater can be efficiently used for cultivation of microalgae. Various type of wastewaters can be used, such as, palm oil mill effluent, piggery wastewater, rubber wastewater, textile wastewater etc. Palm oil mill effluent (POME) is the wastewater released from palm oil processing industry and is known as a dominant agro-industrial wastewater. For the production of one ton of fresh fruit bunch approximately 1.5 m^3 of water is required and half of the water is released as effluent. However, for the production of sago-starch 30-50 m^3 water is used (Udaiyappan et al., 2017). Piggery wastewater is also an agro-industrial wastewater, it has been estimated that in Korea 49 million tons of piggery wastewater has been released (Salama et al., 2017). Industrial wastewater has been globally used for the cultivation of microalgae. In a study conducted on tannery wastewater it has been observed that *Tetraselmis sp.* have removed 100% NH_4^+-N however, total phosphorous, biological oxygen demand, total organic carbon, chemical oxygen demand and total nitrogen were 97.64%, 20.68%, 31.35%, 56.70 and 71.74% removed (Pena et al., 2020). Approximately 88 trillion gallon tons -50%, of industrial wastewater is widely used for cultivating 247 million tons of microalgae. This huge amount of microalgae can efficiently produce 37 million tons of lipids (Guldhe et al., 2017).

Leachate is also one of the most prominent type of liquid waste that is generated by the storage of solid waste and usually contains higher amount of organic and inorganic substances (Quan et al., 2019). It can be used as a good source of nutrients for microalgal growth (Hernández-García et al., 2019). It contains various nutrients that are essential for the growth of microalgae such as, nitrite, nitrate, NH_4^+-N and phosphorous. NH_4^+-N is one of the prominent nitrogen source and its average range varies from 626 mg/L to 2589 mg/L. The value of nitrate and nitrite also varies depending upon the source of leachate. Concentration of nitrate usually varies from 327mg/L to 1471 mg/L whereas, for nitrite the value varies from 229 mg/L to 712 mg/L. However, phosphorous usually occurs in lesser dominance it varies from 19 mg/L to 270 mg/L (Dogaris et al., 2020). The higher NH_4^+-N concentration can directly effects the growth of microalgae but the effect of growth inhibition varies among different species. Microalgal species belonging to Chlorophyceae was found to be least affected by higher NH_4^+-N concentrations in wastewater (Chen et al., 2020). Cheng and Tian (2013) has used *Scenedesmus sp.* for the treatment of landfill leachate by considering following parameters, Chemical Oxygen Demand (COD), Total Nitrogen (TN), NH_4^+-N, pH and Total Phosphorous (TP). Leachate used in this study contained very higher concentration of the above mentioned nutrients so different dilutions (2, 5, 10, 20%) were made to evaluate the algal biomass production and nutrients removal in 20 days experimental period. Higher algal biomass has been observed in higher dilutions i.e. 2, 5 and 10%. However, 20% dilutions caused an inhibitory effect in the algal biomass production. It has been reported that higher amount of NH_4^+-N in leachate causes toxic effects in algal biomass production. Removal efficiency of NH_4^+-N, total nitrogen, COD and total phosphorous are 72, 70, 16 and 95% respectively. El-Ouaer and his colleagues in 2019 studied the effect of NH_4^+-N and COD on two microalgal species, *Chlorella sp. and Scenedesmus sp.* using landfill leachate. Different dilutions of leachate were used such as, 10, 30, 50, 80 and 100%. The initial concentrations of NH_4^+-N and COD were 2570 and 21,475 mg/L. Among these species *Scenedesmus sp.* was unable to grown even in highest dilution of 10%. Though in the same dilution, biomass productivity of *Chlorella sp.* was twice than that of the control –using Bold Basal Media (BBM) as a nutrient source. The removal of NH_4^+-N and COD using *Chlorella sp.* were reported as 100 and 60% respectively.

Many pretreatment processes of leachate can be used before phycoremediation because it has higher concentration of various heavy metals and NH_4^+-N (El-Ouaer et al., 2019, Dogaris et al., 2020). Many technologies can be combined to deal with the complex nature of leachate such as, an integration of physcio-chemical and biological treatment processes

(Dogaris et al., 2020). Nair et al. (2019) has used phycoremediation for the tertiary treatment of landfill leachate. Leachate was pretreated with coagulation and air stripping to reduce its nutrients up to the tolerance limit of microalgae. Different dosages of potash alum were used to reduce the concentration of different parameters of leachate. However, potash alum was not that efficient to reduce the concentration of NH_4^+-N from the leachate, that is why, air stripping was the need of the demand to lessen the concentration of NH4+-N. After the 8 hours air stripping the NH_4^+-N concentration reduced to 77 mg/L. Furthermore, *Chlorella pyrenoidosa* was used for the tertiary treatment. Very prominent reduction in dissolved organic carbon (DOC), total nitrogen (TN), NH_4^+-N and phosphates were observed. *Chlorella pyrenoidosa* has removed 96% phosphates, 90% NH4+-N, 86% TN and 91% DOC from the leachate in the cultivation time of 20 days. The release of municipal wastewater has been increasing day and day. In Mexico, the municipal wastewater release has been increased by approximately 62%, from 2003 to 2015 (Hernández-García et al., 2019). In a study conducted by Jeong et al. (2020) *Chlorella sorokiniana* was used for the treatment of municipal wastewater. The result have reported the highest nitrogen and phosphorous removal. Statistically, nitrogen removal rate was 8.1 mg TN/L/d, however, phosphorous removal was 1.6 mg TP/L/d. Zhao et al. (2018) has used microalgae for the treatment of municipal wastewater using rotating algal bioreactor. A total assimilation of orthophosphate and NH_4^+-N along with 80 and 87%, total phosphorous and total kjeldhal nitrogen removal has been observed.

Moreover, anaerobic digestate is the byproduct of anaerobic digestion conducted by using bacterial communities for converting carbon enriched organic waste into biogas (Wang et al., 2019). With a rapid increase in the production of biogas, a higher liquid digestate is produced. China has reported to produce 385 million tons of liquid digestate annually (Huang et al., 2014). Fernandes et al. (2020) treated the anaerobic digestate produced from the anaerobic digestion of kitchen and food waste. Liquid digestate was pre filtered from micro, ultra and nano filtration for the maximum recovery of total nitrogen –approximately 94%. This nitrogen rich digestate was then used for the cultivation of *Chlorella vulgaris* at pilot scale and has achieved a biomass productivity of 0.86 g/L with a prominent decrease in the concentration of nitrogen from digestate. In another study, liquid digestate was pretreated with activated sludge process and then *C. sorokinian*a was cultivated with a biomass productivity of 250-500 mg/L/d was achieved with pretreated liquid digestate (Wang et al., 2019). Choudhary et al. (2016) conducted a comparative study using different wastewaters, such as, livestock wastewater, domestic grey water and anaerobic digestate for the cultivation of microalgal consortia (*Chlorella and Phormidium*). The biomass productivity obtained was reported as 4, 3.64 and 3.10 $g/m^2/d$ in livestock wastewater, domestic grey water and anaerobic digestate.

Livestock wastewater has reported a highest removal efficiencies of total NH4+-N, nitrate and total dissolved phosphorous as 98%, 91% and 93%. Very higher removal efficiencies of NH_4^+-N is subjected to volatilization, nitrification and consumption by microalgae. Zhu et al. (2019) observed the growth of *Chlorella vulgaris* in different dilutions of liquid digestate i.e. 25%, 50%, 75% and 100%. Higher dilutions such as, 100, 75 and 50% has higher concentration of chemical oxygen demand ammonium concentration that caused inhibition in microalgal cultivation. 25% has shown a higher biomass concentration of 0.69 per day with higher lipid productivity of 113 mg/L/d. Poultry litter has also been used for the cultivation of microalgae. In a study conducted by Agarwal et al. (2020) three types of poultry farm manures were tested for the cultivation of Ulva lactuca and Scenedesmus obliquus. Black gold manure, poultry litter and organic farm litter were used. The results indicated that poultry farm litter has given the highest biomass productivity. Han et al. (2019) has used anaerobically digested poultry litter wastewater for the carotenoid extraction and biomass production. Results has shown a positive trend in both. Total phosphorous, total nitrogen and organic carbon removal were 87.2, 63.8 and 64.1%. A biomass production of 678 mg/L with β-carotene yields of 4.02 mg/L. Singh et al. (2020) has used different dilutions of poultry litter excreta for the cultivation of *Chlorella pyrenoidosa*. Different dilutions such as, 25, 50, 75 and 100% of the wastewater were made. Results indicated that 25% dilution has given the highest biomass production of 2.5 g/L with the lipid production of 0.49 g/L.

2.7 Systems for microalgal cultivation

Various systems have been designed for the cultivation of microalgae. These are grouped into open systems, closed systems and hybrid systems. However, mostly the commercial cultivation of microalgae occurs in open systems (Hosseini et al., 2018). The basic purpose involved in the photobioreactors is the upgrading of light energy into organic compounds. The conversion efficiency depends upon the capacity of microalgae to undergo the process of photosynthesis (Padola et al., 2017). Additionally, microalgae can grow in both suspended and attached systems explained below (Katarzyna et al., 2015).

2.7.1 Suspended cultivation of microalgae

Open cultivation involves the cultivation of microalgae in tanks, circular ponds, natural ponds, raceways and shallow ponds (Faried et al., 2017, Lam et al., 2018, Hosseini et al., 2018). Ample number of advantages has been observed in it, such as, low cost, easier design consideration and biomass production (Faried et al., 2017). However, some of the major disadvantages of open systems are cross culture contamination, improper mixing, temperature fluctuations, lower rates of mass transfer, evaporative losses and unmanageable light intensity with lesser biomass productivity (Banerjee and Ramaswamy, 2017, Faried et al., 2017, Yadav et al., 2020, Nwoba et al., 2019). In 1953, closed photobioreactors were constructed for the pollutant removal (Vo et al., 2019). Closed systems are superior then open systems because of many strong reasons, such as, these are controllable -many significant parameters such as, light, temperature, pH can be regulated, CO_2 transfer and higher biomass productivity (Lu et al., 2019). Various types of closed systems can be used, such as, tubular photobioreactor, bubble column photobioreactor, flat plate photobioreactor etc. (Figure 2.3). These photobioreactors gives 5-6 times more biomass than in open systems (Bose et al., 2019). There are usually four important phases in microalgal cultivation, liquid phases, solid phases, gaseous phase and light radiation field. Liquid phases is basically the nutrient media, solid phase comprises of microalgal cells and gaseous phase is the mass transfer of O_2 and CO_2 (Gupta et al., 2015). Many closed systems are reported such as, plastic bags, tubular photobioreactors, flat plate photobioreactors and column photobioreactors (Bose et al., 2019).

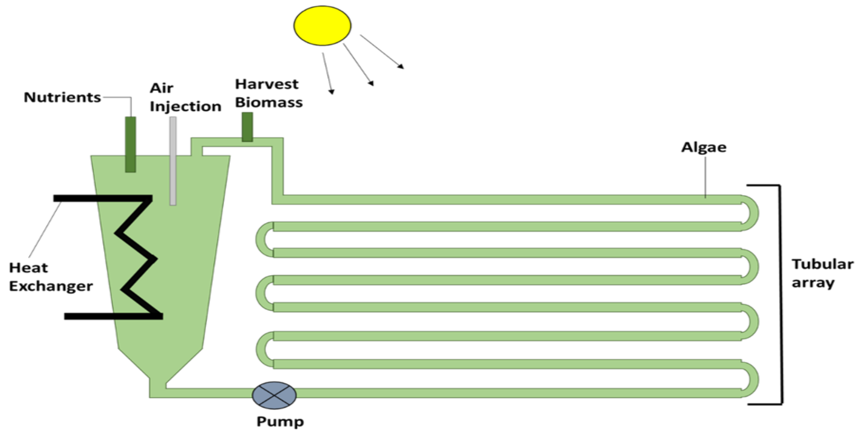

Figure 2. 3 Diagrammatic representation of tubular photobioreactor

2.7.2 Attached cultivation of microalgae

In attached cultivation, microalgae grows in the form of a biofilm. Costerton with this colleagues firstly used the word biofilm to name the consortia of microorganism surrounded by extracellular matrix (EPS) to form a complex structure on a solid substratum providing nutrients (Mantzorou and Ververidis, 2019). Besides extracellular substances there are more polymers named as, chitosan and alginate and soluble algal products (SAP's) are released. These substances act as glue for attaching microalgae on the surface of the substratum and reducing its mobility. Nutrients from the media or pollutants get stored in these polymeric substances and are slowly released to microalgae making attached cultivation system more robust (Wang et al., 2018, Zhuang et al., 2020). After the initial attachment of microalgae on the substrate layer, the microalgal biofilm will start growing by microalgal reproduction. A higher microalgal biofilm thickness was observed in the case of microalgal consortium rather than a single microalgal strain (Wang et al., 2018). Various types of attached cultivation systems has been reported, such as, rotatory algal biofilm reactor, moving-bed biofilm reactor, enclosed biofilm tubular reactor, vertical submerged biofilm reactor and twin layer cultivation bioreactor (Podola et al., 2017, Katarzyna et al., 2015).

2.8 Twin layer cultivation

Porous Substrate Bioreactor (PSBR) is the most advanced attached cultivation method, it is also known as Twin-Layer Cultivation System (Figure 4). The improvement from submerged attached cultivation system to PSBR is the relocation of nutrient media towards the opposite side of gas bulk and illumination. It involves two layers that, a substrate layer -microporous and a source layer -macroporous (Podola et al., 2017). Microalgal cells are attached on hydrophilic substrate layer (Ji et al., 2013). Nutrients are supplied to microalgal cell by macroporous source layer (Shi et al., 2014). The nutrients are evenly distributed on the source layer by gravitational flow to reach the microalgal biofilm (Podola et al., 2017). This source layer also acts as a carrier for the substrate layer having microalgal biofilm attached over it. The basic mechanism of nutrient transfer is diffusion from source layer to substratum and then finally to immobilized microalgal biofilm (Figure 2.4). In this bioreactor, microalgal layer will be in direct contact with atmospheric gases, such as, CO_2 (Shi et al., 2007). Various researches have been conducted using twin layer cultivation system to treat wastewater, to sequester carbon dioxide and to produce lipids. Shi et al. (2014) marked 99% and 77% nitrogen and phosphorous from municipal wastewater in pilot scale experiment conducted on Twin layer cultivation system.

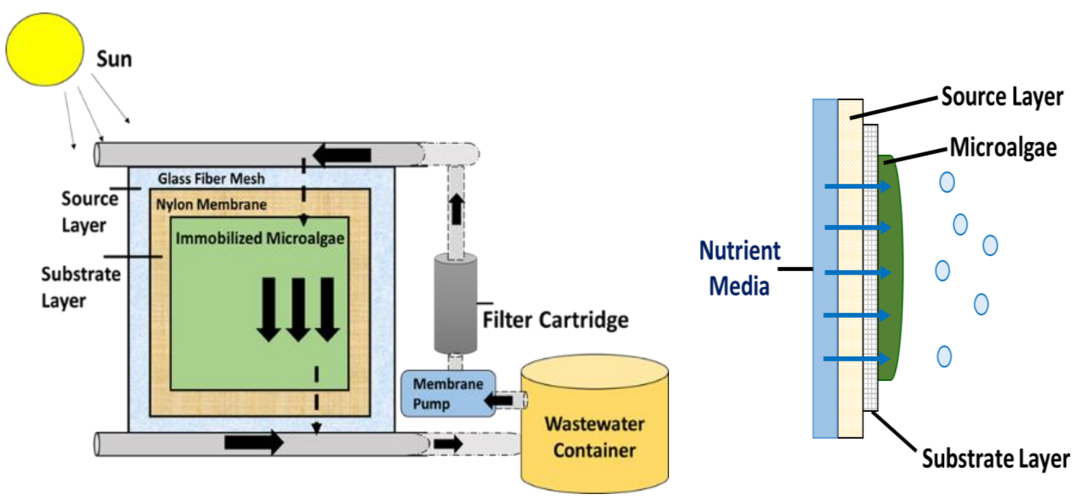

Figure 2. 4 Diagrammatic representation of porous substrate bioreactor (PSBR)

Table 2. 1 Nutrient removal of different wastewaters by attached cultivation of microalgae

Wastewater Type	Rector Used	Specie Used	Experimental Conditions Used			Nutrient Removal		Biomass Productivity	References
			Light Intensity	Temperature	Time	P	N		
			µmol m^{-2} s^{-1}	°C	days	%	%	g/m^2/d	
Anaerobic Digestate	Inclined algal biofilm photobioreactor	*Scenedesmus* and *Chlorella*	60	25	10	80	60	3.66 and 5.66	Yu et al., 2020
Swine Wastewater	Biofilm Attached Culture	*C. pyrenoidosa*	80 ± 5	25	8	84.3	94.1[b]	4.21	Cheng et al., 2020
Municipal Wastewater	Revolving Algal Biofilm	Algal Consortium	Sunlight	10-30	7	100[a]	87[c]	7	Zhao et al., 2018
Anaerobically Digested Slurry	Algal Biofilm Reactor	*Chlorella* and *Phormidium*	Sunlight	---	6	88	93[b]	3.10	Choudhary et al., 2017
Municipal Wastewater	Rotating Algal Biofilm Reactor	--	200	25	2	97[a]	100	5	Shayan et al., 2016
Municipal Wastewater	Twin Layer PBR's	*Halochlorella rubescens*	22 to 220	18 to 32	32	70	99	6.3	Shi et al., 2014
Municipal Wastewater	Twin Layer PBR's		20 to 120	30	7	90	96[b]	--	Shi et al., 2007

Orthophosphate[a]; Ammonium-Nitogen[b]; TKN

2.9 Comparison between suspended and attached cultivation

Various research has been conducted showing the benefits of attached cultivation over conventional suspended cultivation systems (Table No. 1). Attached cultivation of microalgae circumvent many lope holes of suspended bioreactors (Shi et al., 2014). The most important between them are, less water consumption, high productivity, less space usage and ease in harvesting of microalgae (Zhuang et al., 2018). It has been studied that attached microalgal cultivation utilized less water for the production of huge microalgal biomass. A comparison done for attached and suspended cultivation explains it clearly with the requirement of 2857 L water per kg growth of biomass whereas 1618 L water was required for attached cultivation of one kg microalgal biomass (Zhuang et al., 2018). It has been estimated that for the cultivation of 1 ton microalgae in suspension, 200 ton of water is required, however, for cultivating the same amount of biomass in attached only 17 ton of water is required (Katarzyna et al.,2015). Additionally, Microalgae from a substrate layer can be easily harvesting by simple scarping and the substrate layer can be reused (Katarzyna et al., 2015). Higher biomass productivity has been reported in attached cultivation in comparison to suspended one. Table 2.2 shows the biomass productivities of various attached cultivation systems.

Liu et al. (2013) cultivated *Scenedesmus* on an attached bioreactor and reported a biomass productivity of 50-80 $g/m^2/d$. In another study conducted by Schultze et al. (2015) reported similar biomass productivity (50 $g/m^2/day$) on a twin layer cultivation system. Shen et al. (2016) cultivated 55 $g/m^2/d$ of microalgal biomass using rotating drum bioreactor. However, a very less biomass productivity of 10-30 $g/m^2/d$ using open ponds and various suspended photobioreactors. A mass number of reasons are associated with this fact, such as, higher CO_2 and O_2 transfer. For instance, in attached cultivation system the water layer between microalgae and ambient gases has been efficiently reduced to a thin layer making the consumption of CO_2 by microalgae from the ambient air and the release of O_2 by microalgae into the ambient air easier (Wang et al., 2017). Huang et al. (2016) reported a higher CO_2 affinity by microalgal biofilm than suspended one due to lower transfer resistance between gas and solid surface (CO_2 and microalgal cells) than gas, liquid and solid (CO_2, nutrient media and microalgal cells). In case of attached cultivation, microalgal biofilm is highly illuminated in comparison to suspended cultivation. Huang et al. (2016) has reported a 16 times higher illuminated cell portion than in suspended cultivation.

2.10 Major products of microalgal cultivation systems

2.10.1 Lipid production

Microalgal biochemical composition comprises of four imperial groups of molecules: lipids, carbohydrates, nucleic acid and proteins. The most energetic group between these is lipids (37.6 kJ g^{-1}). Microalgae has the ability to generate various kinds of lipids, such as, wax esters, sterols, hydrocarbons, neutral lipids, polar lipids and prenyl derivatives (Sajjadi et al., 2018). An ample number of research has been conducted to produce lipids from microalgae. *Botryococcus brauni* was cultivated on an algae biofilm photobioreactor. The total harvested biomass was 96.4 kg/m^3 couple with a lipid productivity of 0.71 $g/m^2/d$ (Ozkan et al., 2012). It has been reported in number of studies that microalgae can produce a tremendous amount of lipids when exposed to “Fattening” conditions. These conditions are nutrient limitation or salinity. Majorly studies have reported an elevated lipid content in the nutrient starved condition. Nitrogen starved conditions leads to lower proteins content in the microalgae as the carbon absorbed by the microalgae shifts from synthesis of proteins and peptides towards the synthesis of lipids and carbohydrates (Ho et al., 2012). In general, nitrogen limitation activates three functions in microalgae, activation of acyl hydrolase, hydrolysis of phospholipids and decrease in the cellular content of microalgal thylakoid membrane. These changes upgrade the fatty acid acyl-CoA content of microalgae. And eventually the diacylglycerol acyltransferase converts the acyl-CoA to triglyceride (Sajjadi et al., 2018). Additionally, it has been reported that lipid accumulation in microalgae increases the size of microalgal cell and decreases the thickness of its cell wall (Ju et al., 2020). In a study conducted by Poh et al. (2020) *Chlorella vulgaris* was cultivated under nutrient starved conditions. Under the nutrient starved conditions, completely dark cycle and salinity of 6.0 g/L, the lipid content of microalgae was raised up to 40.28%. In another study conducted by Liu et al. (2020) has reported a lipid content of 30.8% by *S. dimorphu*. Low nitrogen concentrations can increase lipid content of most microalgae from 30-70 %, depending upon the specie (Sajjadi et al., 2018).

2.10.2 Carotenoid production

Carotenoids are natural pigments that are lipophilic in nature. They are found in various colors, such as, red, yellow and orange (Gong and Bassi, 2016). Carotenoids are located in thylakoid membrane of the organism and are firmly bounded to the light harvesting complexes (LHC's) (Nisar et al., 2015). Carotenoids are segregated into two major classes, such as, xanthophyll and carotenes. Carotenes are hydrocarbons in nature and are devoid of oxygen atom, whereas, xanthophyll are oxygen containing derivatives (Kalra et al., 2020). Carotenoids are very diverse but merely 30 of them are imparts a significant role in photosynthesis (Gong and Bassi, 2016). Major sources of carotenoids are plants, bacteria, fungi and algae. The main function performed by carotenoids are photo protection and harvesting of light in photosynthetic organisms (Kalra et al., 2020). Carotenoids brooders the light spectrum for microalgae (Gauthier et al., 2020). Moreover, their anti-oxidant nature aid microalgae to tolerate reactive radicals, promote stability, prevent peroxidation of lipids and helps in keeping membranes integrated (Gong and Bassi, 2016). Carotenoids can be used as food, coloring agent, animal feed, nutraceuticals and medications (Eismann et al., 2020).

2.11 Summary

Microalgae can efficiently treat various types of wastewaters. Along with this, it can sequester CO_2 from the atmosphere and produce lipids. Several cultivation conditions, such as, temperature, light, pH, support media and nutrient media are required for its growth. It can be cultivated in suspended and attached cultivation systems. Attached cultivation system, such as, TLS is the most advanced cultivation system. This study focuses on treatment of various wastewater types using *Tetradesmus obliquus* for nutrient removal, carbon dioxide fixation and production of lipid and pigments.

Table 2. 2 Lipid contents of various microalgal species

Microalgal Specie	Stress Provided	Growth Medium	Incubation Time (days)	Lipid Content (%)	Reference
Chlorella	Phytohoromes	Anaerobic Digestate	10	50	Yu et al., 2020
Scenedesmus dimorphu	Nutrient Deficiency	Municipal Wastewater	5	30.8	Liu et al., 2020
Chlorella vulgaris	Salinity and Light	Organic Fertilizer	4	38.8	Poh et al., 2020
Chlorella and Phormidium	Nutrient Deficiency	Domestic Grey Wastewater	6	38	Choudhary et al., 2017
Chlorella sorokiniana	Nutrient Deficiency	Raw Sewage	16	22.7	Gupta et al., 2016
Scenedesmus sp.	Light	Domestic Wastewater	11	69.1	Zhang et al., 2013

CHAPTER 3

3 MATERIALS AND METHODS

Based upon the introduction and literature review done for the cultivation of *Tetradesmus obliquus* on TLS for nutrient removal, carbon dioxide fixation and lipid and pigments production. Following materials and methods were used.

3.1 Cultivation of microalgal strain

Tertradesmus obliquus was acquired from the Norwegian Culture Collection of Microalgae, NORCCA, Oslo, Norway. It was pre-cultured in Erlenmeyer flask of 50 mL using autoclaved Z8 media under the light intensity of 75 µmol /m^2/s with a 14;10 light/ dark cycle at 25±3 ºC. For this purpose, LED lights of 9 W were used for providing light from the bottom of the flask. Filtered air was provided using 0.22 µm cellulose acetate syringe filters. For further use in the immobilization of microalgae on the substrate layer, this culture was upgraded to 2 L.

3.2 Wastewater collection and preparation

Solid waste leachate was collected from the transfer station of a waste management company, Albayrak located in Rawalpindi. Poultry wastewater was collected from a local poultry farm, located at Japan road, Rawalpindi. Anaerobic digestate was collected from a biogas plant. The biogas plant was using cow manure as a substrate for the production of biogas installed at Haloki Industrial Estate, Lahore. Municipal wastewater of NUST, Islamabad was used as a control. All wastewater types were filtered through Whattman filter paper 1 and stored at 4 ˚C before use. Cheng et al. (2013) reported that *Tetradesmus obliquus* cannot tolerate an NH_4^+-N concentration above 100 mg/L thereby promoting the need to dilute high-nutrient wastewater. Therefore, high-nutrient wastewater was diluted using wetland treated wastewater (WTWW) which had a very low ammonium nitrogen, TKN and orthophosphate concentrations as given in Table 1.

Table 3. 1 Composition of Z8 nutrient media

STOCK SOLUTION 1	
$NaNO_3$	46.7 g
$Ca(NO_3).4H_2O$	5.9 g
$MgSO_4.7H_2O$	2.5 g
Dissolve all the chemicals separately and raise the volume up to 1 Liter.	
STOCK SOLUTION 11	
K_2HPO_4	3.1 g
Na_2CO_3	2.1 g
Dissolve all the chemicals separately and raise the volume up to 1 Liter.	
STOCK SOLUTION 111	
Fe-Solution	
$FeCl_3.6H_2O$	2.80 g
Dissolve it in 100 ml 0.1 N HCl.	
EDTA-Solution	
EDTA-Na_2	3.90 g
Dissolve it in 100 ml 0.1 N NaOH.	
10 ml of the Fe-Solution was dissolved in 900 ml distilled water. Afterward, 9.5 ml of EDTA solution was added then the solution was raised to 1 L.	
STOCK SOLUTION IV	
$Na_2WO_4.2H_2O$	0.330 g/100 ml
$(NH_4)6M_{O7}O_{24}.4H_2O$	0.880 g/100 ml
KBr	1.20 g/100 ml
KI	0.83 g/100 ml
$ZnSO_4.7H_2O$	2.87 g/100 ml
$Cd(NO_3)_2.4H_2O$	1.55 g/100 ml
$Co(NO_3)_2.4H_2O$	1.46 g/100 ml
$CuSO_4.5H_2O$	1.25 g/100 ml
$NiSO_4(NH_4)_2SO_4.6H_2O$	1.98 g/100 ml
$Cr(NO_3)_3.9H_2O$	0.410 g/100 ml
V_2O_5	0.0890 g/1000 ml
$KAl(SO_4)_2.12H_2O$	4.74 g/100 ml
H_3BO_3	3.10 g
$MnSO_4.H_2O$	1.60 g/1000 ml

For final media preparation, add 10 ml from stock 1, 2 and 3 and 1ml from stock for and dilute it to 1 L. Now, maintain the pH to 7.

Figure 3. 1 Collection and filtration of wastewaters

Similarly, WTWW was collected from the outlet of wetland constructed in NUST, Islamabad for MWW treatment. To adjust ammonium-nitrogen concentration to the tolerable limit of 100 mg/L, 10% SWL, 15% PWW and 25% AD were taken and the remaining volume was filled with WTWW. pH of the diluted wastewater was adjusted to neutral before use.

3.3 Design of a lab-scale Twin Layer Cultivation System

Tertradesmus obliquus was cultivated in a TLS as shown in Figure 1. This system comprised of a 1.5 cm thick masonite base covered with a layer of melamine. This horizontal base was mounted on a steel frame and titled at an angle of 15º for proper wastewater flow and efficient transfer of ambient air to the biofilm provided through aerator. A macroporous source layer of duck cloth having a weight of 272 g/m^2, length of 75 cm and width of 15 cm was placed on the base. Upon the source layer 30 microporous substrate layers made up of newspaper (weight: 57.6 g/m^2) each having an area of 12.56 cm^2 were attached for the immobilization of microalgae. Five cool LED bulbs of 18 W were used from the top of the system for providing white light to the microalgae. These LED lights were attached to a digital timer to maintain light/dark cycle. System was covered with a plastic sheet to avoid air contamination.

3.4 Immobilization of microalgae and operation of the system

Tertradesmus obliquus with an initial biomass density of 3 g/m^2 was evenly immobilized using filtration assembly on newspaper having an area of 12.56 cm^2. For treating each wastewater type, 30 substrate layers were attached on one source layer.

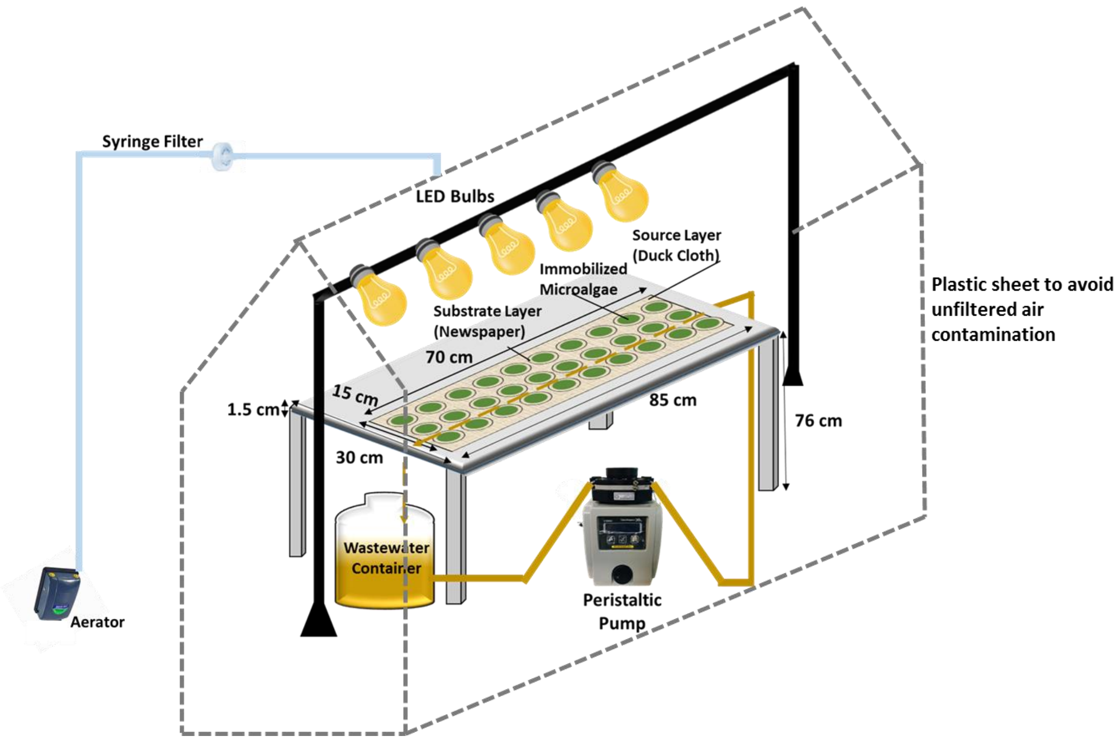

Figure 3. 2 Diagrammatic representation of the laboratory scale twin layer cultivation system (TLS)

Microalgae was acclimatized on the system for 4 days using 4L Z8 media. Afterwards, Z8 media was replaced with 4L of the dilute,kd wastewater which was recirculated for 14 days. Wastewater and Z8 media were provided using a peristatic pump at a flow rate of 220 mL/h. An acid wash container was used for the collection and recirculation of wastewater. White light was provided at a light intensity of 80 ± 5 µmol/m^2/s by LED bulbs which were positioned at the top of the system and were set for light/dark cycle of 14:10 h. Ambient CO_2 concentration (0.04%) was used through aerator. All experimental sets were conducted under same operational conditions.

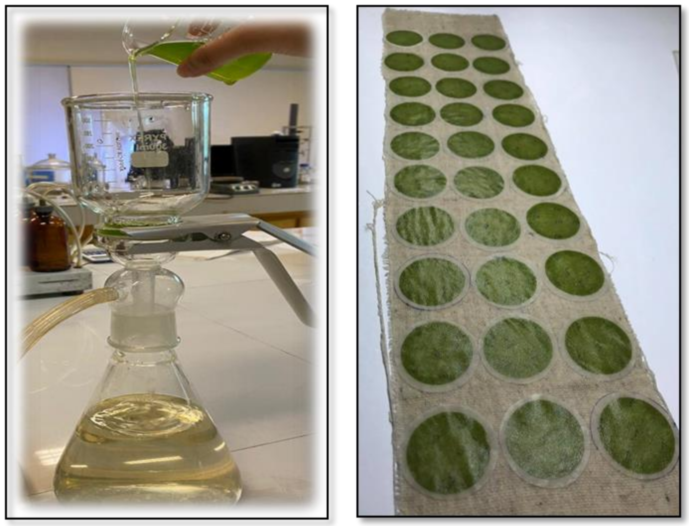

Figure 3. 3 Immobilization of microalgae on the substrate layer and placement of substrate layers on the source layer

3.5 Analytical procedures

Prior to wastewater analysis it was filtered through a cellulose acetate filter membrane of0.45 µm pore size. Ammonium-nitrogen, orthophosphate, nitrite nitrogen and Total Kjeldahl Nitrogen (TKN) were measured after every 24 hours by considering following methods. Heavy metals were assessed using Atomic Absorption Spectrophotometer (Agilent Technologies, 55 AA, US).

3.5.1.1 Ammonium-Nitrogen

NH_4^+-N was estimated by titrimetric method using distillation apparatus. For this, 25 mL wastewater sample was poured in the distillation tube. An erlenmeyer flask containing 50 mL boric acid was placed at the end of delivery tube. The sample was automatically buffered with the borate buffer solution. The distillate was then titrated using 0.02 N H_2SO_4 after adding 5 drops of mixed indicator (APHA et al., 2017). Following equation was used;

$$mg/L\ Ammnoium - Nitrogen\ = \frac{(A - B) \times 280}{mL\ of\ Sample} \tag{1}$$

3.5.1.2 TKN

Total Kjeldhal Assembly was used for the estimation of TKN in wastewater samples. For this, 50 mL sample was digested in the heating device -providing the temperature of 375-380 ºC for 30 minutes. After digestion, the samples were distillated in the distillation unit and titrated. (APHA et al., 2017). Following equation was used;

$$mg/L\ TKN\ = \frac{(A - B) \times 280}{mL\ of\ Sample} \tag{2}$$

3.5.1.3 Nitrite Nitrogen

Colorimetric method was used for the determination of nitrite in wastewaters. After the preparation of standard curve, 50 mL sample was used containing 2mL color reagent. Prepared sample was left for 10 minutes and absorbance was measured at 543 nm using spectrophotometer (APHA et al., 2017).

3.5.1.4 Orthophosphate

Colorimetric method was used for the determination of orthophosphate in wastewaters. After the preparation of standard curve, 10 mL sample containing 2 mL vanadate-molybdate reagent. Prepared sample was left for 10 minutes and absorbance was measured at 470 nm using spectrophotometer (APHA et al., 2017).

The nutrient removal efficiency (RE), nutrient removal rate (RR) and nutrient removal capacity (RC) of microalgae were calculated by using equation (3) (4) and (5), respectively.

$$\text{RE}\ (\%) = \frac{(C_{in} - C_{eff})}{C_{in}} \times 100 \tag{3}$$

$$\text{RR}\ (\frac{\text{mg}}{\text{d}}) = (C_{in} - C_{eff}) \times V/t \tag{4}$$

$$\text{RR}\ (\frac{\frac{\text{mg}}{m^2}}{\text{d}}) = (C_{in} - C_{eff}) \times \frac{V}{A\ /\ t} \tag{5}$$

In these equations, C_{in} and C_{eff} is the initial and final concentration of the nutrient. "V" indicates the volume of the wastewater used, "x" is the specific nutrient and "t" is the total time required. Whereas, "A" represents the area of a substratum (Yu et al., 2020).

3.5.1.5 Biomass production and productivity

The growth of microalgae was characterized by biomass production and productivity. For its determination, substrate layers were randomly removed after every second day from the source layer, placed in an oven (Memmert, UNB-400, Germany) for 24 hours and weighted. Following equations has been used.

$$\text{DW}\ (\frac{\text{g}}{m^2}) = \frac{DW_1 - DW_0}{\text{area}\ (\text{m}^2)} \tag{6}$$

$$P_x (\frac{\frac{\text{g}}{m^2}}{\text{d}}) = \frac{DW_1 - DW_0}{\text{area}\ (\text{m}^2)\text{time (d)}} \tag{7}$$

In this equation, DW and P_x are the biomass production and productivity. DW_0 and DW_1 are the dry weight of the substratum at the start and on the harvesting day.

3.5.1.6 Growth kinetics and carbon dioxide biofixation

The specific growth rate (μ) and doubling time (T_d) of microalgal biomass was calculated by equation (8) and (9), respectively (De Assis et al., 2019). Carbon dioxide biofixation rate was calculated by using equation (10) (Almomani et al., 2019).

$$\mu\ (d^{-1}) = \frac{\text{lnX}_f - \text{lnX}_\text{i}}{\text{T}_\text{f} - \text{T}_\text{i}} \tag{8}$$

$$T_d(d) = \ln 2/\ \mu \tag{9}$$

$$\text{Carbon Dioxide Biofixation Rate } (\frac{\frac{\text{g}}{m^2}}{\text{d}}) = \text{C}_\text{c}\ \text{P}_\text{x}\ \frac{\text{M}_{\text{CO2}}}{\text{M}_\text{c}} \tag{10}$$

In equation (6) X_f and Xi are the biomass production on the final and initial day of the experiment, respectively. Where T_i and T_f are the initial and final time of the experiment, respectively. In equation (10) C_C is the percent mass of carbon whereas, M_{CO2} and Mc are molar mass of CO_2 and carbon, respectively. Percent mass of the carbon (C_C) was measured by equation (11) (Adams et al., 1951).

$$C_C\ (\%) = \frac{\text{VS}(\%\text{TS})}{1.8} \tag{11}$$

3.5.1.7 Lipid production

For the estimation of lipids in microalgae, Bligh and Dyer Method was used (Cui et al., 2020). Lipid production and productivity were measured by equation (13) and (14), respectively (Ye et al., 2020).

$$\text{Lipid Content } (\%) = \frac{\text{W}_\text{t}}{\text{B}} \times 100 \tag{12}$$

In this equation, "W_t" is final weight of oil whereas, "B" is initial weight of microalgae.

$$\text{Lipid Production } (\frac{\text{g}}{m^2}) = \text{Lipid Content} \times \text{Biomass Production} \tag{13}$$

$$\text{Lipid Productivity } (\frac{\frac{\text{g}}{m^2}}{\text{d}}) = \frac{\text{Lipid Production}}{\text{Time}} \tag{14}$$

3.5.1.8 Pigments production

Chlorophyll a, b and carotenoids were measured by spectrophotometric method. One gram of microalgae was taken and 50 mL 100% acetone was added into it. The sample was then homogenized using homogenizer (Diahan, HG-150, Korea) at 1000 rpm for one minute. The homogenate was later on filtered using K.T 4000 (35% cotton and 65% polyester) and centrifuged (Hermle, Z 206 A, Germany) at 2500 rpm for ten minutes. The supernatant was than separated and its absorbance at 662, 645 and 470 nm. Following equations were used to estimate pigments (Şükran et al., 1998).

$$\text{Chlorophyll a } = 11.75\ A_{662} - 2.350\ A_{645} \quad (15)$$

$$\text{Chlorophyll b } = 18.61\ A_{645} - 3.960\ A_{662} \quad (16)$$

$$\text{Carotenoids } = \frac{1000 \times A_{470} - 2.270\ C_a - 81.4\ C_b}{227} \quad (18)$$

3.6 Statistical analysis

All the experiments and tests were performed in triplicates and their standard deviations were calculated. The average values of the experimental results were presented. One way-Analysis of variance was applied in order to assess the differences between the tested groups by using Origin 8 (OriginLab Cooperation, USA). Tuckey's multi comparison test (t-test) was employed by considering the significant level of 5% (p-value < 0.05).

CHAPTER 4

4 RESULTS AND DISCUSSION

By using the above discussed materials and methods for the cultivation of *Tetradesmus obliquus* on TLS for nutrient removal, carbon dioxide fixation and lipid and pigments production. Following results were obtained and discussed under different sections and sub sections.

4.1 Characterization of wastewater

Table 4.1 presents the physiochemical properties of the high-nutrient wastewater used in this study. Wastewater types used were alkaline in nature and had an unpleasant odor. High-nutrient wastewater had a higher turbidity ranging from 100-7000 NTU and dark brown color in comparison to MWW having lower turbidity of 16 NTU and brown color. Alkalinity in wastewater reduces the growth of *Tetradesmus obliquus* –neutral pH was reported as an optimum growth condition for *Tetradesmus obliquus (*Breuer et al., 2013). However, other characteristics such as, ammonium-nitrogen, TKN and orthophosphate concentrations were higher in high-nutrient wastewater in comparison to MWW. The assessed metals present in these wastewater types were (Calcium), Mg (Magnesium), Pb (lead), Cu (Copper), Fe (Iron), Zn (Zinc) and Mn (Manganese). Additionally, the concentration of NH_4^+-N in PWW, SWL and AD ranged from 400-1000 mg/L. This higher concentration of NH_4^+-N inhibits the photosynthetic activity of *Tetradesmus obliquus* (Hernández-García et al. 2019). The NH_4^+-N concentrations were 95.3%, 93.4% and 88.4% higher in SWL, PWW and AD respectively compared to MWW. This is why, the dilutions of high nutrient wastewater were prepared using a low strength wastewater that is wetland effluent. The orthophosphate concentration was dominant in SWL, although it was under the admissible limit for microalgal growth, which is 150 mg/L (Su, 2020). High-nutrient concentrations in SWL, PWW and AD were consistent with the previous studies conducted on these wastewater types (Luo et al., 2019, Wang et al., 2019, Oliveria et al., 2018). It has been reported that Zn, Fe, Mn and Cu act as a micronutrient for the growth of *Scenedesmus* (Shahid et al., 2020). However, Pb is reported as a toxic metal for microalgae to grow. The presence of 0.18 mg/L Pb observed in SWL was under the permissible limit for the growth of *Scnedesmus sp.* (Pham et al., 2020). Mixing low nutrient wastewater with high nutrient containing wastewater was reported as a useful solution for treating wastewater by using microalgae (Hernández-García et al. 2019).

4.2 Biomass production and productivity

The growth of *Tetradesmus obliquus* on TLS is shown in Figure 4.1. Among various types of wastewater used for the cultivation of *Tetradesmus obliquus*, PWW, SWL and AD having higher nutrient concentrations produced a higher biomass than MWW. *Tetradesmus obliquus* cultivated in PWW had a higher biomass production of 92.39 g/m^2 which was due to higher ammonium nitrogen, TKN and orthophosphate content in it than MWW (Oliveria et al., 2018). Algal biomass cultivated in AD and SWL showed a biomass production of 87.7 and 84.5 g/m^2, respectively, however, MWW produced a biomass of 67.8 g/m^2. Though SWL and PWW had a comparable concentration of ammonium-nitrogen and orthophosphate in them yet the higher concentrations of micronutrients, for instance, Zn^{+2} and Mn^{+2} in PWW in comparison to SWL could be the reason of high microalgal biomass production by PWW (Shahid et al., 2020). A lower growth of microalgae in AD in comparison to PWW could be attributable to lower concentrations of orthophosphate, Mn, Zn and Fe in AD. The statistical analysis of high-nutrient wastewater with MWW marked no significant variability between groups ($p > 0.05$). Consistently, the highest biomass productivities were observed in PWW. *Tetradesmus obliquus* grown under PWW, AD and SWL achieved biomass productivities of 5.13, 4.87, 4.69 $g/m^2/d$, respectively in comparison to MWW which was 3.48 $g/m^2/d$. Biomass productivity in PWW, AD and SWL was 36.2%, 29.3% and 24.6% higher as compared to MWW. The biomass productivity observed in the present study was higher than 3.6 $g/m^2/d$ obtained in inclined algal biofilm photobioreactor used by Yu et al. (2020). The higher NH_4^+-N, orthophosphate and TKN concentration in PWW, AD and SWL might mainly contributed to the higher productivity in the system used. Regarding the statistical analysis, high-nutrient wastewater

Table 4. 1 Characteristics of different types of wastewater used in this study

Parameters	Units	Wastewater Type				
		Municipal Wastewater	Solid Waste Leachate	Poultry Wastewater	Anaerobic Digestate	Wetland Effluent
Color		Brown	Dark Brown	Dark Brown	Dark Brown	Pale Yellow
pH		8.1	8.3	7.7	8.4	7.9
Turbidity	**NTU**	16	102	6660	4315	10
EC	**mS/cm**	1.5	15.0	12.0	9.8	1.1
NH_4^+-N		47 ± 0.57	989 ± 4	707 ± 2.8	405 ± 3	12 ± 3.95
TKN		99 ± 0.9	1050 ± 3.95	832 ± 3.9	601 ± 4	25 ± 4.92
PO_4^{3-}-P		9.1 ± 0.03	142.0 ± 3	12.0 ± 0.06	34.3 ± 0.02	1.6 ± 0.11
Ca		72	324	71	108	72
Mg		45	216	46	36	54
Pb	**mg/L**	N/D	0.18 ± 0.8	N/D	N/D	N/D
Zn		0.30	0.26	1.78	0.36	0.38
Mn		0.01	0.15	0.44	0.14	0.02
Fe		0.11	4.36	0.96	0.35	0.13
Cu		0.05	0.10	0.11	0.54	0.06

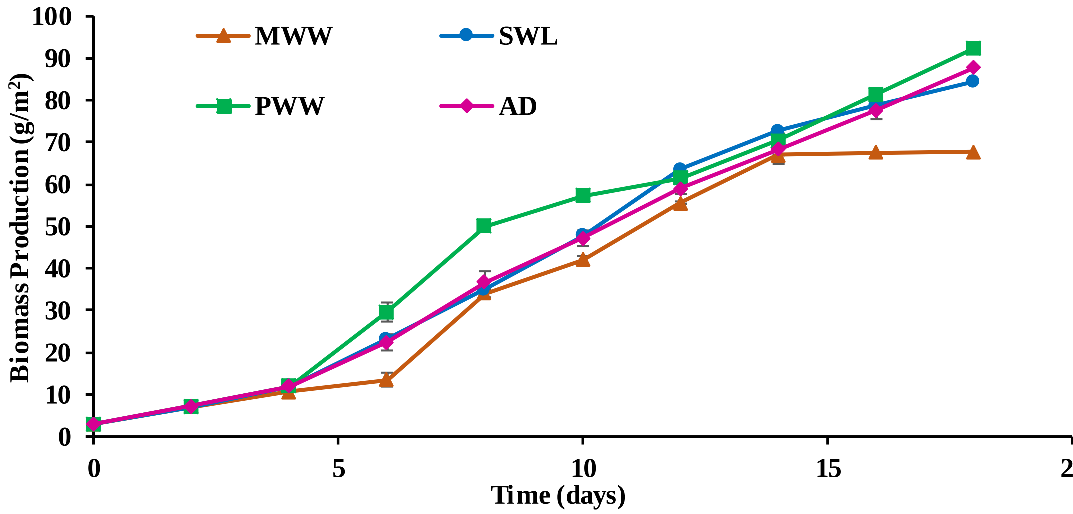

Figure 4. 1 Effect of wastewater type on biomass production of *Tetradesmus obliquus*

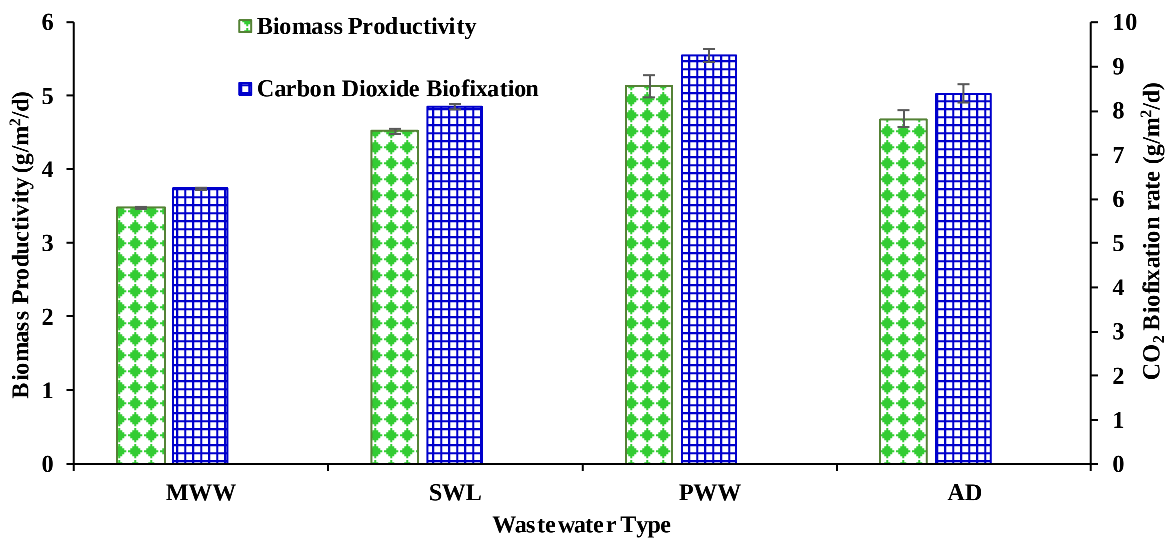

Figure 4. 2 Effect of wastewater type on biomass productivity and carbon dioxide fixation rate of *Tetradesmus obliquus*

marked significant differences in comparison to MWW ($p < 0.05$).. However, AD and SWL had no significant differences among them ($p > 0.05$). *Tetradesmus obliquus* exhibited substantial biomass productivity in TLS using high nutrient wastewater types, marking this method feasible for their treatment.

4.3 Growth kinetics and CO_2 biofixation

Table 4.2 shows the specific growth rate and doubling time of *Tetradesmus obliquus* in under different types of high-nutrient wastewater. Microalgae grown in PWW showed the highest specific growth rate of 0.19 d^{-1} in comparison to other types of wastewater used. Biomass grown in SWL and AD showed the same specific growth rate of 0.18 d^{-1} whereas, microalgal biomass grown in MWW achieved a specific growth rate of 0.17 d^{-1}. The observed specific growth rate was higher than 0.12 d^{-1} reported by De Assis et al. (2019) who cultivated microalgae on a biofilm reactor in domestic wastewater. Higher ammonium nitrogen, TKN and orthophosphate content producing a higher biomass in PWW contributed in achieving higher specific growth rate. *Tetradesmus obliquus* grown in MWW showed a highest doubling time of 4.05 days in comparison to PWW, SWL and AD.

It was observed due to lesser ammonium-nitrogen, TKN and orthophosphate content of MWW in comparison to other wastewater types used. *Tetradesmus obliquus* grown in SWL and AD observed higher doubling time of 3.83 days in comparison to *Tetradesmus obliquus* grown in PWW which was 3.63 days. This might be attributable higher toxic levels of SWL and AD in comparison to PWW (Luo et al., 2019, Wang et al., 2019). Figure 2b represents the CO_2 biofixation of *Tetradesmus obliquus* in various types of high-nutrient wastewater. Algal biomass in PWW achieved a higher carbon biofixation of 9.20 $g/m^2/d$ in comparison to MWW which was 6.23 $g/m^2/d$.

The higher biomass productivity in PWW increased the CO_2 fixation in microalgae, accordingly (Verma et al., 2018). This result marked 47.6%, 34.9%, and 29.8% increase in the carbon biofixation of microalgae in PWW, AD and SWL due to higher photosynthetic activity compared to MWW (Hosseini et al., 2018). Similar trend of CO_2 biofixation was observed in a study conducted by Lopez-Pacheco et al. (2021) who reported a higher CO_2 fixation by *Scenedesmus sp.* grown in industrial wastewater collected from a local industrial wastewater treatment plant in Mexico.

4.4 Nutrient removal performance

Removal of NH_4^+-N, NO_2^--N and TKN from high-nutrient wastewater and MWW is shown in Figure 4.3, 4.4 and 4.5. High -nutrient wastewater types, such as PWW, AD and SWL showed a higher removal rate of NH_4^+-N, NO_2^--N and TKN in comparison to MWW (Figure 4.3b, 4.4b and 4.5b). The higher NH_4^+-N removal in PWW, SWL and AD was 31.6, 29.6 and 29.7 mg/d respectively in comparison to MWW which was 13.4 mg/d.

Table 4. 2 Specific growth rate and doubling time of *Tetradesmus obliquus* cultivated in different types of wastewater

Wastewater Type	Specific Growth (μ)	Doubling Time (T_d)
	(d^{-1})	(d)
Poultry Wastewater	0.19	3.63
Anaerobic Digestate	0.18	3.83
Solid Waste Lechate	0.18	3.83
Muncipal Wastewater	0.17	4.05

Similarly, a higher TKN removal rate in PWW, AD, SWL was observed as 39.3, 36.8, 34.2 mg/d respectively in comparison to MWW which was 26.2 mg/d. Moreover, the removal rate of TKN in high nutrient wastewater showed a significant difference with MWW ($p < 0.05$). The lower NO_2^--N removal in comparison to other nutrients was obtained in PWW, SWL, AD and MWW, which was 0.17, 0.082, 0.071 and 0.017 mg/d respectively. Regarding its statistical significances, high nutrient wastewater showed statistical variability with MWW ($P < 0.05$). However, AD and SWL observed no statistical differences in their NO_2^--N removal rate ($p > 0.05$). Nutrient levels in all wastewater types started decreasing instantly since the experiment started. The highest removal of nitrogenous compounds in PWW as compared to other wastewater types was attributable

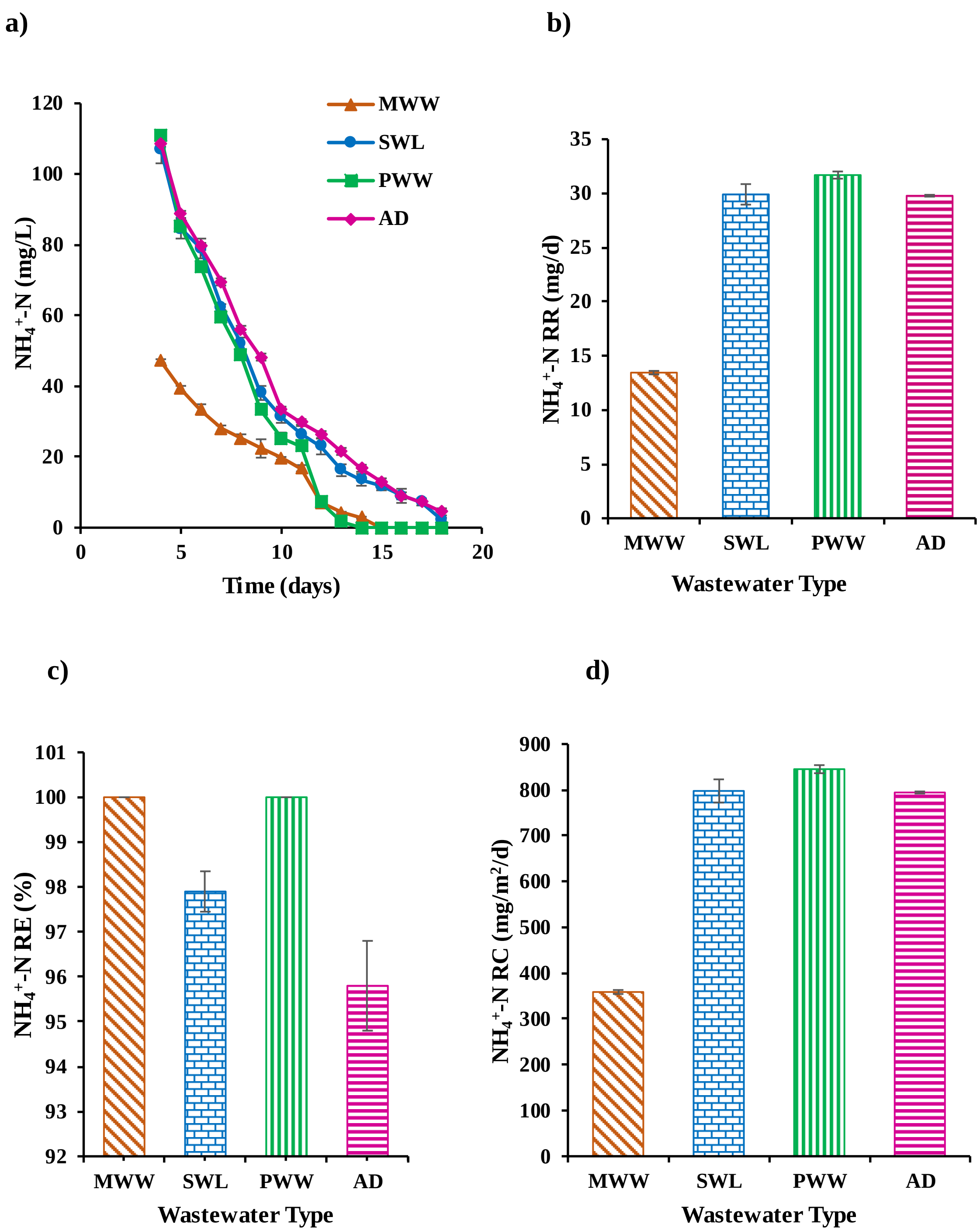

Figure 4. 3 Effect of wastewater type on removal (a) removal rate (b) removal efficiency (c) and removal capacity (d) of NH_4^+-N by *Tetradesmus obliquus*

to the higher biomass productivity observed in *Tetradesmus obliquus* cultivated in PWW (Salama et al., 2017). Additionally, the nutrient present in wastewater are also reported as an important source for bacteria to grow. This might have also contributed to the decrease in nutrient concentration in wastewater (Zhang et al., 2018). Apart from these, volatilization of NH_4^+-N by an increase in the pH of wastewater might have also **c**ontributed to the removal of NH_4^+-N from wastewater (Hernández-García et al., 2019). It was also well depicted from this study that lower NH_4^+-N, NO_2^--N and TKN supply exhibit a lower removal of them whereas higher supply led towards a higher removal (Beuckels et al., 2015).

However, 100% nutrient removal efficiency of NH_4^+-N and NO_2^--N with 92.6% TKN were observed in MWW due to its lower initial nutrients concentrations in comparison to high nutrient wastewater. Interestingly, similar NH_4^+-N removal efficiency was observed by Zhao et al. (2018) who treated sludge thickening supernatant on a rotating algal biofilm photobioreactor. Continuous reductions in the concentrations of NH_4^+-N and TKN were observed in all wastewater types with time. It was observed that 110.92, 104.68 and 104.24 mg/L NH_4^+-N were removed from PWW, SWL and AD respectively during experimental period (Figure 4.3a, 4.4a and 4.5a). Additionally, TKN removal of 137.66 129.01 and 119.74 mg was observed in PWW, AD and SWL during the experimental period. The concentration of nitrite in wastewater types such as PWW, SWL and AD went on increasing instead of decreasing on day 12th, 11th and 9th respectively. It can be attributable to the mutualistic relationship between microalgae and bacteria that might helped in the conversion of organic nitrogen and NH_4^+-N (TKN) into oxides of nitrogen by providing oxygen to nitrifying bacteria and heterotrophic bacteria (Petrini et al., 2018). This oxidation led to an increase in the NO_2^--N concentration in high-nutrient wastewater. The NH_4^+-N removed in MWW was observed as 47.07 mg/L in comparison to other wastewater types which showed a higher removal rates.

This depicted a 57.6%, 54.4% and 54.3% increase in NH_4^+-N consumption in PWW, SWL and AD as compared to MWW. TKN removal in PWW, AD, SWL and MWW was observed as 137.6, 129.01, 119.74 and 85.96 mg/L, respectively. However, the nitrite consumption of 0.62, 0.29, 0.25 and 0.062 mg/L was recorded in PWW, SWL, AD and

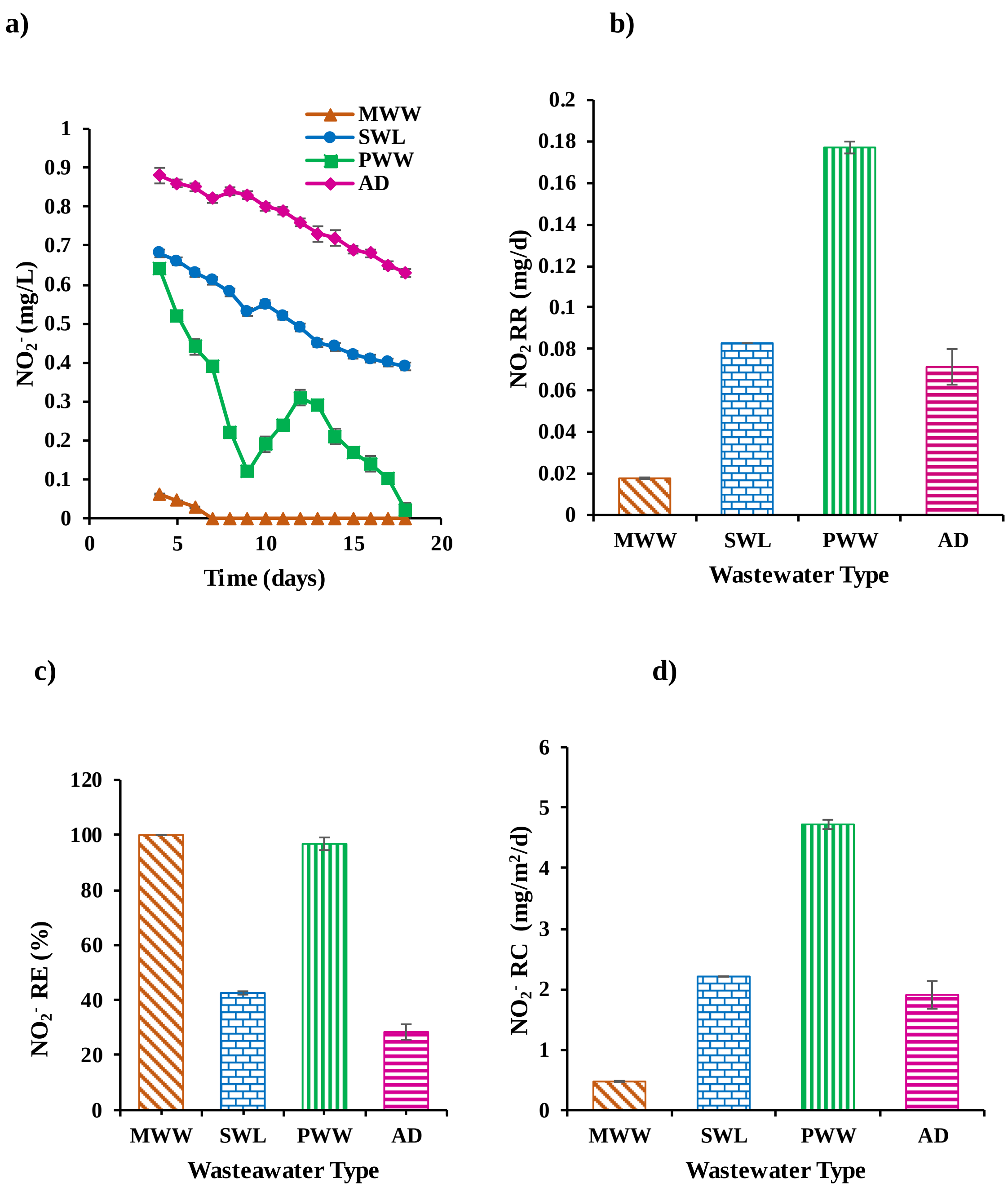

Figure 4. 4 Effect of wastewater type on removal (a) removal rate (b) removal efficiency (c) and removal capacity (d) of nitrite by *Tetradesmus obliquus*

MWW, respectively. Wastewater types such as PWW, AD and SWL showed a 37.6%, 33.4% and 28.3% increased TKN removal compared to MWW. Nitrite removal in PWW, SWL and AD was 90%, 78.7% and 75.2% higher than MWW, respectively. The nutrient removal capacity of TLS for high-nutrient wastewater was also higher than MWW. The

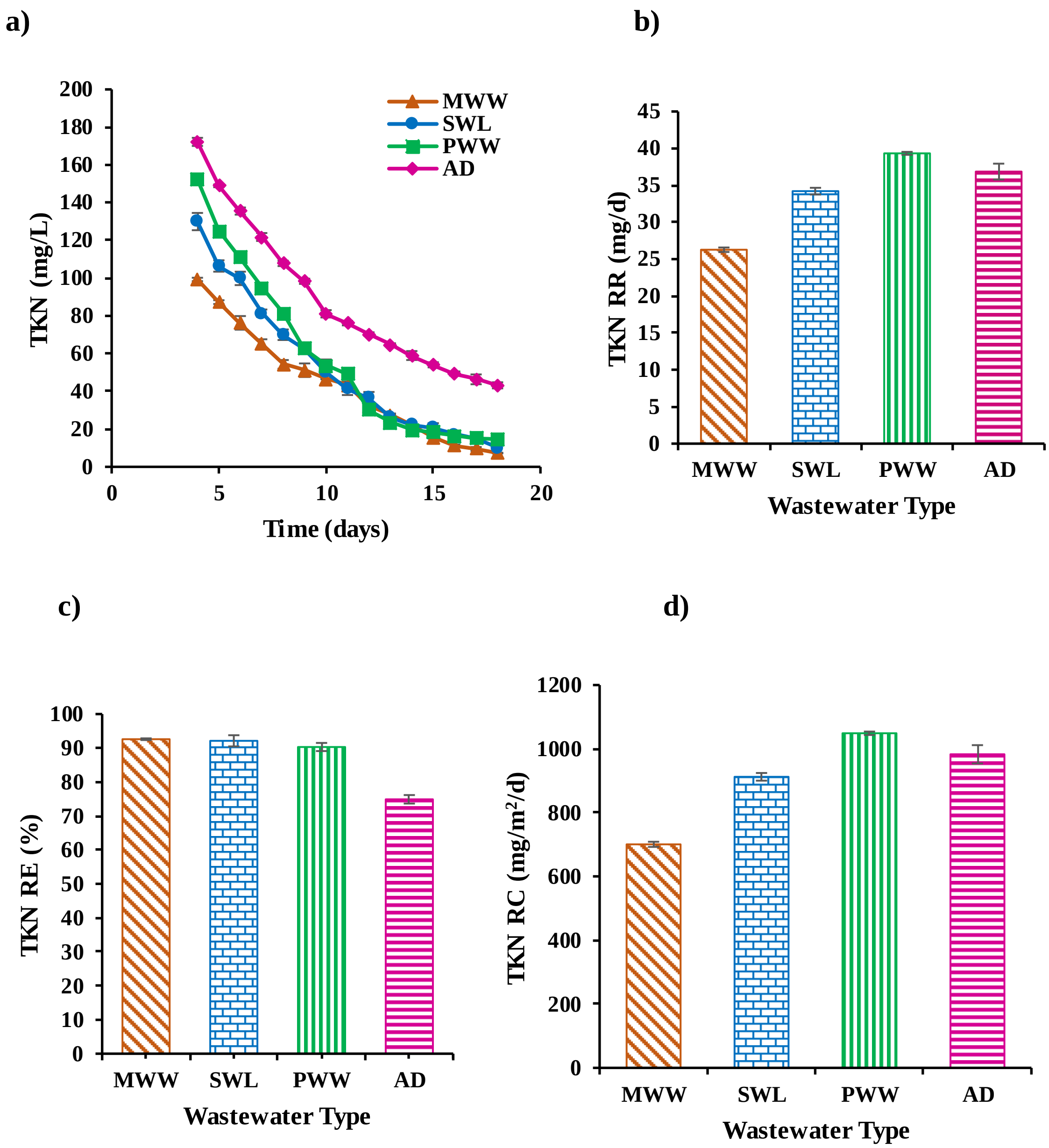

Figure 4. 5 Effect of wastewater type on removal (a) removal rate (b) removal efficiency (c) and removal capacity (d) of TKN by *Tetradesmus obliquus*

highest removal capacity for TKN, NH_4^+-N, and NO_2^--N in PWW were observed as 1038, 845 and 4.7 mg/m^2/d in comparison to MWW which were observed as 358.6, 1048.83 and

0.47 mg/m^2/d. The statistical analysis for removal capacities of TKN, NH_4^+-N, and NO_2^--N marked significant variability between high nutrient wastewater and MWW ($p < 0.05$). However, for the case of NH_4^+-N, and NO_2^- -N, no statistical variability was observed in AD and SWL ($p > 0.05$).

b)

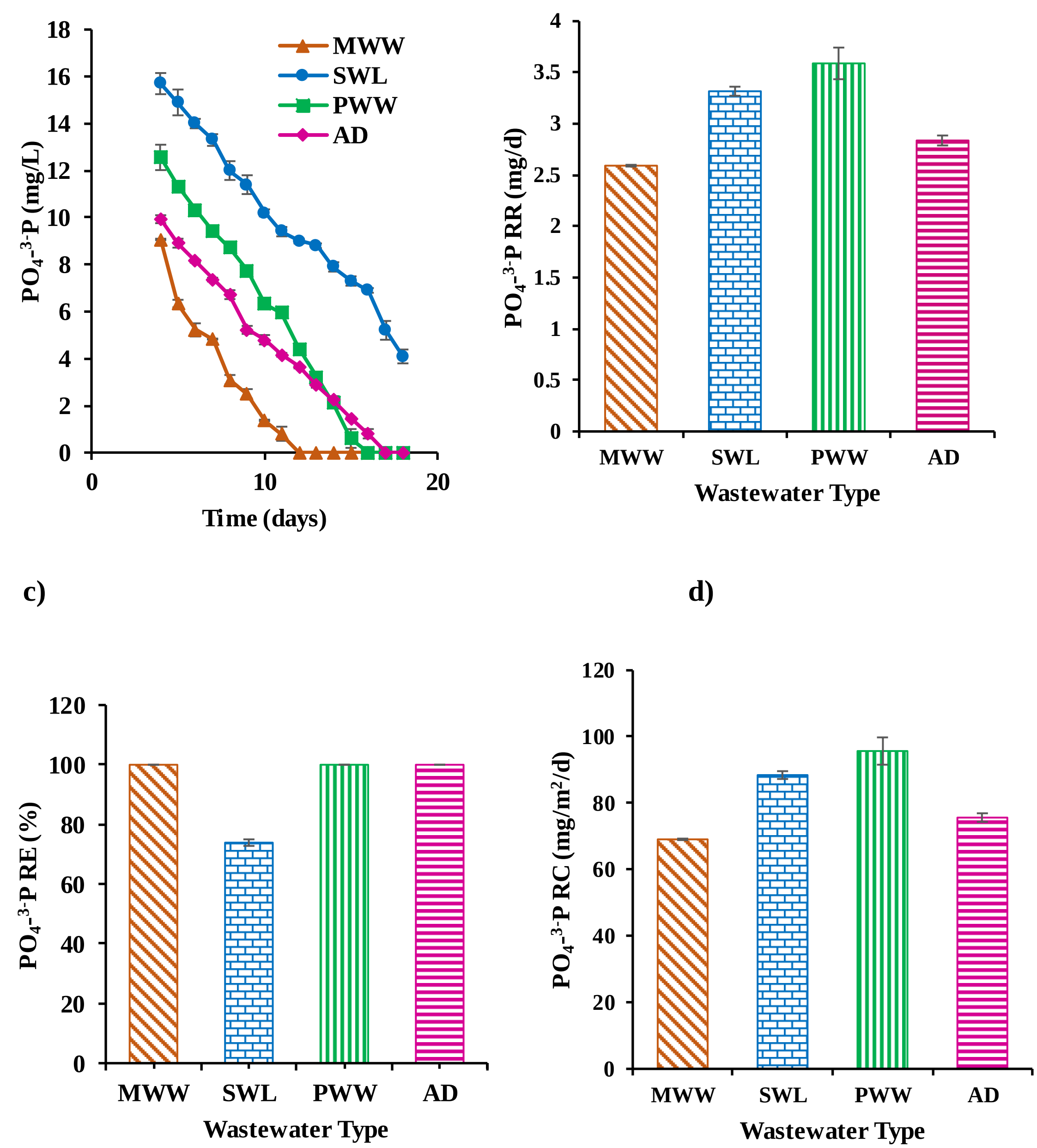

Figure 4. 6 Effect of wastewater type on removal (a) removal rate (b) removal efficiency (c) and removal capacity (d) of PO_4^{-3}-P by *Tetradesmus obliquus*

Among all the nitrogenous nutrient, the highest removal of NH_4^+-N was observed, the main reason behind this removal could be less energy demand by microalgae for assimilation of NH_4^+-N than NO_2^--N and organic nitrogen (Su, 2020). Regarding the daily removal of nutrients, the statistical analysis in NH_4^+-N gave no significant differences between groups ($p > 0.05$). For the statistical analysis of TKN, all groups marked no statistical differences ($p > 0.05$) except for AD and MWW ($p < 0.05$). All groups were statistically different for the case of nitrite ($p < 0.05$). The utilization pattern of orthophosphate is presented in Figure 4.6. An orthophosphate removal of 12.56, 11.61, 9.93 and 9.07 mg/L with a daily removal of 3.58, 3.31, 2.83 and 2.59 mg/d was achieved in PWW, SWL, AD and MWW respectively. It depicted 27.8%, 16.5%and 2.4 % increase in the orthophosphate removal of PWW, SWL and AD in comparison to MWW. The removal rate of high nutrient wastewater tested marked statistical differences with the MWW ($p < 0.05$).

Additionally, the daily removal of orthophosphate during the experimental time period, all groups showed statistically differences ($p < 0.05$) except PWW and MWW, AD and MWW and PWW and AD ($p > 0.05$). Though, PWW and SWL observed no statistical differences in their orthophosphate removal rate ($p > 0.05$). The highest removal rate of orthophosphate was observed in PWW due to the higher biomass productivity of microalgae grown in it (Salama et al., 2017). A 100% removal of orthophosphate was observed in MWW, PWW and AD achieved a removal efficiency of 73.9%.

Orthophosphate removal efficiencies observed in the present study were in accordance with the study conducted by Zhao et al. (2018). Microalgae traditionally have only 0.5-3.3% phosphorous content (Zhang et al., 2018). Therefore, higher removal of orthophosphate in the present study might be due to the presence of 72, 71, 324 and 108 mg/L Ca and 45, 46, 216 and 36 mg/L Mg in MWW, PWW, SWL and AD resulting in chemical precipitation (Salama et al., 2017). The highest orthophosphate removal capacity was recorded as 95.6 $mg/m^2/d$ for PWW in comparison to MWW which was 69.1 $mg/m^2/d$. The statistical analysis for the removal capacities of orthophosphate marked statistical differences between high nutrient wastewater and MWW ($p < 0.05$). However, the overall removal of nutrients from PWW, AD and SWL marked the efficacy of TLS for successful nutrient removal from high nutrient wastewater having a higher turbidity and a darker color.

4.5 Lipids and pigments production

Figure 4.7 shows the lipid content of *Tetradesmus obliquus* cultivated in different wastewater types. Compared to microalgal biomass grown in high-nutrient wastewater a higher lipid content of 30.3% was yielded in biomass cultivated in MWW. However, microalgal biomass grown in PWW, SWL and AD achieved a lipid content of 29.6%, 28.8% and 28.4% respectively. The nutrient deprivation in MWW mainly attributed to lipid accumulation in microalgae cultivated in MWW (Sajjadi et al., 2018). In addition, the lipid content among biomass grown in wastewater types studied varied which was from 30.3%-28.4%, indicated that along with the nutrient deprivation, other factors might also raise the lipid content of microalgae in high-nutrient wastewater. The presence of higher concentrations of metals (Pb, Cu, Fe, Zn, Ca, Mg and Mn) in high-nutrient wastewater was also a prominent reason for increasing the lipid content in microalgae (Sajjadi et al., 2018). However, biomass grown in MWW indicated a 2.5%, 5.3% and 6.8% increase in lipid accumulation than biomass cultivated in PWW, SWL and AD respectively.

The lipid content in this study was comparable to Liu et al. (2020) who reported a lipid content of 30.8% in the biomass of *Scenedesmus dimorphus* cultivated under effluent from wastewater treatment plant as a nutrient medium. High nutrient wastewater marked statistical difference in comparison to MWW ($p < 0.05$) except for the case of AD and SWL ($p > 0.05$). Figure 4.8 demonstrates the lipid production and productivity of microalgal biomass in different wastewater types. Higher lipid productivities were obtained in the biomass grown in PWW, AD and SWL that were 1.48, 1.36 and 1.31 $g/m^2/d$ respectively in comparison to MWW which was 1.12 $g/m^2/d$. This was mainly due to larger biomass production observed under PWW, AD and SWL. The values yielded in this study were significantly higher than 0.18 $g/m^2/d$ which was obtained by cultivating *Scenedesmus obliquus* in secondary treated wastewater (Schnuur et al., 2013). Higher biomass production observed by high-nutrient wastewater might be the reason of higher lipid productivity. Lipid production and productivity of high nutrient wastewater showed statistical differences in comparison to MWW ($P < 0.05$). However, AD and SWL were not statistically different ($p > 0.05$).

Figure 4.9 shows the photosynthetic efficiencies of the biomass cultivated in wastewater types. Biomass grown in PWW achieved a chlorophyll a and b content of 150.15 and 78 µg/g, whereas, microalgae grown in AD, SWL and MWW exhibited a chlorophyll a and b content of 144, 57.45 > 133.52, 57.45 > 91.3, 30.3 µg/g respectively. A higher chlorophyll content of microalgae in PWW was due to its higher photosynthetic activity. Carotenoid

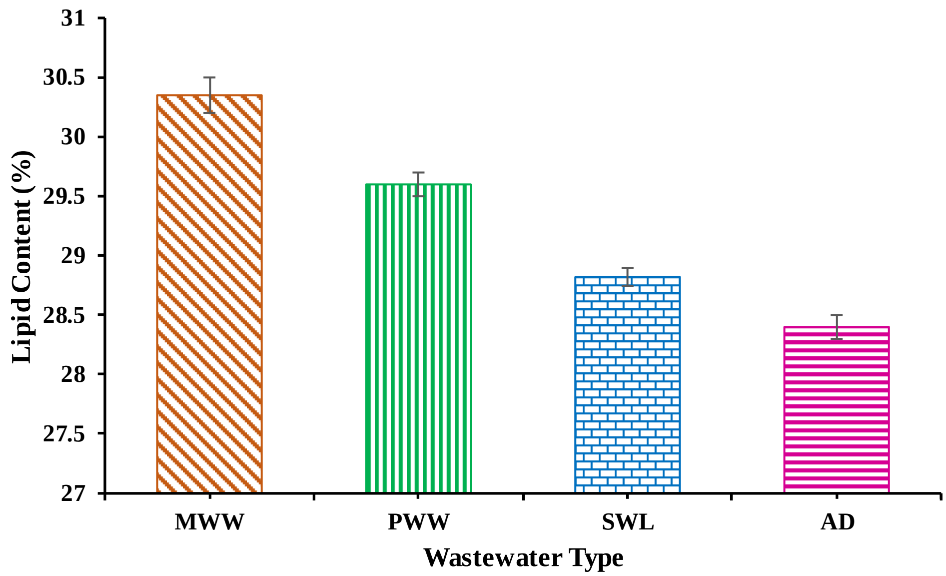

Figure 4. 7 Effect of Wastewater Type on Lipid Content of Tetradesmus obliquus

contents of 60.37, 59.42 and 58.35 µg/g were obtained by PWW, AD and SWL, respectively. However, the carotenoid content in microalgal cells grown in MWW was observed as 31.85 µg/g**.** Many reasons could be ascribed to the observed trend, importantly nutrient limitation and presence of Pb, Cu, Fe and Zn (Sajjadi et al., 2018). MWW achieved nutrient deprived conditions earlier than high-nutrient wastewater. Orthophosphate, NH_4^+-N and NO_2^--N were depleted on day 12th, 14th and 7th in MWW, respectively. PWW, SWL, AD took time for nutrient depletion but had higher heavy metal concentrations than MWW. Moreover, a prolonged nutrient deprivation in MWW might decreased the carotenoid concentration of microalgae (Pourkarimi et al., 2020). Carotenoid content of all high nutrient wastewater were statically significant from MWW ($p < 0.05$). However, the statistical analysis of PWW with SWL and AD marked no differences ($p > 0.05$).

4.1 Upscaling and future prospects of TLS

In the present study, an excellent nutrient removal, biomass and lipid productivity were achieved by cultivation of *Tetradesmus obliquus* on TLS during the treatment of various wastewater types, marking the feasibility of phycoremeditaion for the treatment of highly troublesome industrial wastewater. Using wastewater as a nutrient media could reduce the cultivation cost of microalgae at a greater rate. As it was a lab scale study, it can be further upgraded to reap the full benefits of the system. By considering the experimental data of the present study, a TLS system having an area of one hector could achieve an average nutrient removal rate of about 244.6, 197.1, 1.13 and 22.31 kg/ha/d from TKN, NH_4^+-N, NO_2^--N, and PO_4^3-P concentrations, respectively. Along with the nutrient removal, a microalgal biomass of 51.3 kg/ha/d could be produced. This biomass during its growth could sequester approximately 90.2 kg/ha/d CO_2 from the ambient air. However, the biomass production of microalgae and nutrient removal efficiency of wastewaters might

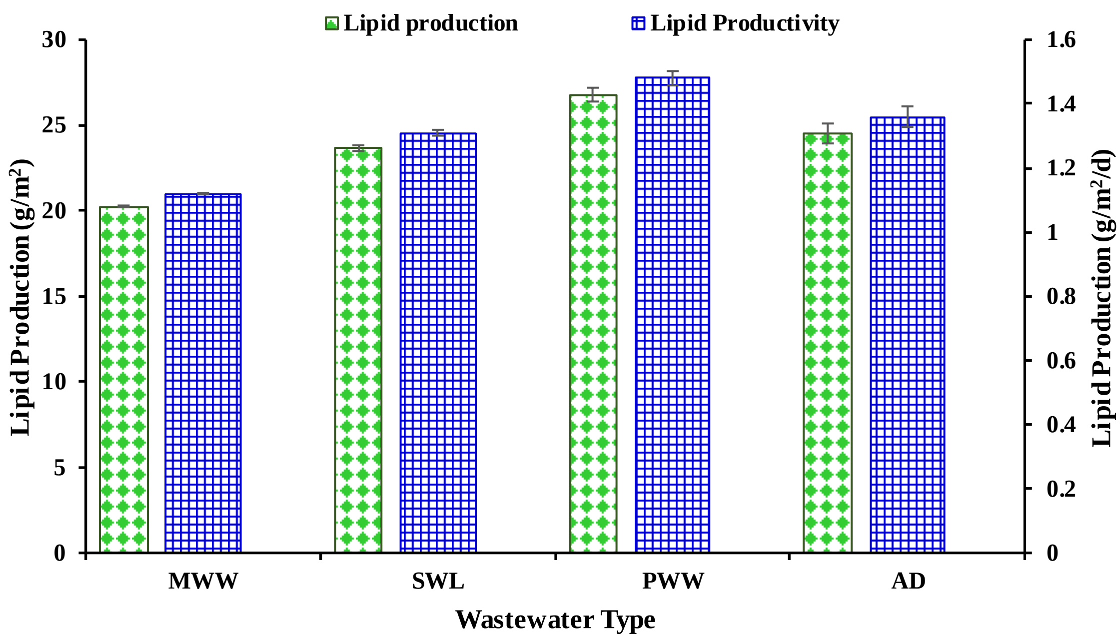

Figure 4. 8 Effect of Wastewater Type on Lipid Production and Productivity of *Tetradesmus obliquus*

report variations in scale-up depending upon the initial concentrations of nutrients in the wastewater used and ambient conditions. In the present study, the harvesting of the algal biomass was performed by mechanical detachment i.e.

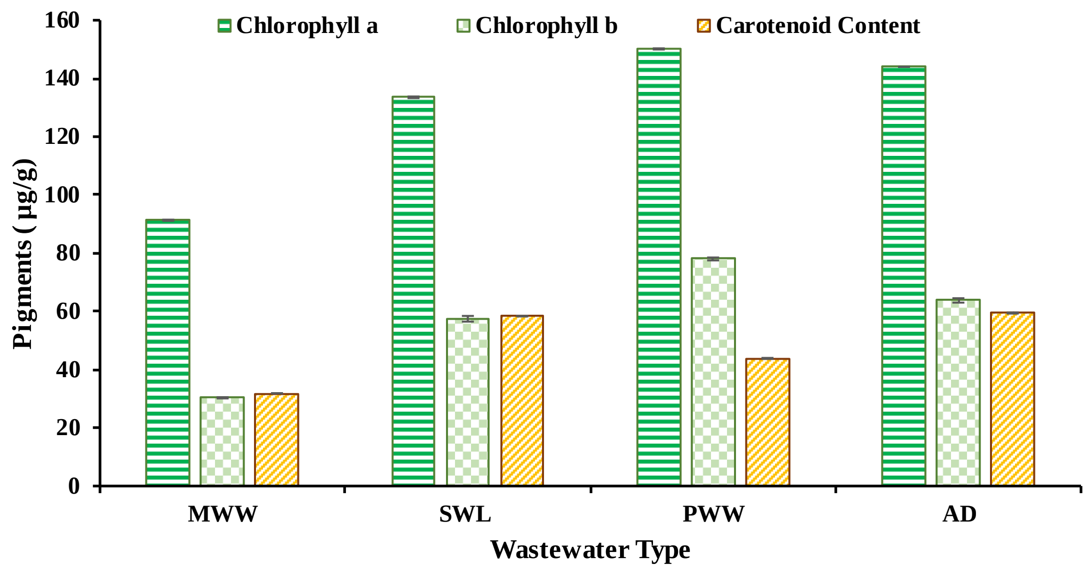

Figure 4. 9 Effect of Wastewater Type on Pigments Production of Tetradesmus obliquus

scraping. This method of harvesting also reduced the harvesting cost demanded for microalgal byproduct synthesis (Padola et al., 2017). In the present study, the lipid production potential of *Tetradesmus obliquus* was also evaluated. After upscaling the current system, the lipid production rate of microalgae could be observed as 14.8 kg/ha/d by using high nutrient containing wastewater type, such as, PWW. In summary, TLS could provide an integrated approach for environmental, social and economic benefits.

CHAPTER 5

5 CONCLUSIONS AND RECOMMENDATIONS

This chapter elucidates the conclusions drawn by the research done. Based upon the conclusions certain recommendations has been given in this umbrella of research.

5.1 Conclusions

Effect of wastewater type on the cultivation of *Tetradesmus obliquus* was studied using TLS for nutrient removal, biomass production, CO_2 fixation and lipid and pigments production. PWW showed the maximum uptake capacities of 845, 1038 and 69.1 mg/m^2/d for NH_4^+-N, TKN and PO_4^{-3}-P respectively. Furthermore, biomass productivity, CO_2 fixation and lipid productivity of 5.13, 9.25 and 1.48 g/m^2/d respectively were observed in case of PWW. Therefore, this study strongly commends to use microalgae for the treatment of high strength wastewaters, CO_2 fixation and lipid production on TLS.

5.2 Recommendations

Certain recommendations can be made by this study. In this study, dilutions of wastewaters were made to lessen their toxicity. Multi-gene manipulations in *Tetradesmus obliquus* should be introduced to make it more tolerant against toxicity. Moreover, the lipids extracted by the microalgae can be upgraded to biodiesel to meet the energy needs of the time. Various other important products could be made with the microalgal biomass, such as, hydrocolloids, animal feeds, cosmetics and pharmaceutical products. Flue gas release from various industries can be captured by using microalgae as it requires CO_2 from it to grow.

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