Performance of Microalgae for Treatment of Wastewater, Leachate and Biodiesel Production

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THESIS ACCEPTANCE CERTIFICATE

It is certified that the contents and form of the thesis entitled **"Performance of Microalgae for Treatment of Wastewater, Leachate and Biodiesel Production"** submitted by Mr. Awais Javaid, Reg. No. 00000119401 has been found satisfactory for partial fulfillment of the requirements of the degree of Master of Science in Environmental Science.

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List of Abbreviations

ASAB	Atta-ur-Rehman School of Applied Biosciences
BBM	Bold Basal Medium
COD	Chemical oxygen demand
DCW	Dry cell weight
FFA	Free fatty acid
GC-MS	Gas chromatography–Mass spectrometry
LL	Landfill leachate
lux	SI unit of illumination: 1 lumen per square meter
MLA	Modified Letheen Agar
N/P	Nitrogen/Phosphorus
NEQS	National Environment Quality Standards
NLs	Neutral lipids
NUST	National University of Science & Technology
OD	Optical density
PET	Polyethylene terephthalate
RPM	Revolutions per minute
TLD	Tube Luminescent-Dunn
TN	Total nitrogen
TP	Total phosphorus
USPCAS-E	US-Pakistan Center for Advanced Studies in Energy
$\mu mol.m^{-2}.s^{-1}$	Micromol per square meter per second
WW	Wastewater
XOCs	Xenobiotic organic compounds

Abstract

Issues concerning sustainable wastewater treatment can be found almost in every scientific, social, or political agenda all over the world. Untreated industrial and household wastewater still being dumped in watercourses and leachate from the landfilling sites are the factor leading to dwindling of clean water. The energy demand all over the globe is also increasing continuously. The expected depletion of fossil fuels within a century or so has compelled the global community to seek renewable energy sources. Biofuels produced from biomass are the most promising source and anticipated to satisfy the escalating global energy demands. In this study microalgae, the unicellular robust organisms, which are capable of growing in all types of environment, have been proposed as an alternative biological treatment for wastewater and leachate with the added benefit of lipids extraction for formation of biodiesel. For this purpose, four local microalgal strains S1, S2, S4 and S6 were evaluated for their potential to grow and treat wastewater and leachate. The microalgal biomass collected after the treatment was used for lipids extraction and biodiesel production through transesterification. It was found that all four strains were able to grow well in 100% wastewater and up to 50% leachate concentration. The results indicated more than 83.3% and 91.7% removal of NO_3-N, PO_4 and COD from wastewater and leachate, respectively, with slight variation among all strains. While in case of heavy metals, S1 showed the highest cumulative removal of 63% from wastewater and 52.9% from leachate. Maximum lipids yield, extracted using chloroform: methanol solvent was 20.5%, given by S6. Whereas highest convertible lipids into biodiesel were obtained from S4, with 93% biodiesel yield through direct transesterification by using methanol in the presence of H_2SO_4 as a catalyst. GC-MS analysis showed 89% and 85.5% conversion of microalgal lipids into alkyl esters, by strains S4 and S6 respectively. This study concluded that selected microalgal strains can be used for the treatment of both, domestic wastewater and leachate. Additionally strains S4 and S6 have the potential to provide the added benefit of biodiesel production.

Chapter 1

Introduction

1.1 Background

It is truism nowadays to recognize that pollution associated problems are a major concern of society. Environmental laws are given general applicability and their enforcement has been increasingly stricter. Therefore, in terms of health, environment and economy, the fight against pollution has become a major issue. Today, although the strategic importance of fresh water is universally recognized more than ever before, and although issues concerning sustainable water management can be found almost in every scientific, social, or political agenda all over the world, water resources seem to face severe quantitative and qualitative threats. The pollution increase, industrialization and rapid economic development, impose severe risks to availability and quality of water resources, in many areas worldwide.

Moreover, demand of energy is continuously increasing owing to rapid industrialization and exponential growth of population round the world. The foremost source of energy are fossils, renewables and nuclear power. Since the industrial revolution, fossil fuels such as coal, natural gas and oil were responsible to meet the world's energy demand. Combustion of fossil fuels leads to emissions of SO_2, CO_2, CO, NOx, volatile organic compounds and particulate matter. These compounds collectively lead to atmospheric pollution. The expected exhaustion of fossil fuels within a century or so has compelled scientists to find renewable energy sources. The alternative energy sources mainly comprise of wind, solar, hydro, geothermal, hydrogen, nuclear and biomass. Among these sources, biofuels obtained from biomass are the most promising and anticipated to meet the increasing global energy demands (Demirbas, 2005). Hence, there is a need of renewable and carbon neutral fuels for environmentally clean and sustainable economy.

1.2 Pakistan's Scenario

Water scarcity is the lack of sufficient available fresh water resources to meet water demand. It affects every continent and was listed in 2015 by the World Economic Forum as the largest global risk in terms of potential impact over the next decade (World Economic Forum, 2015). Problems associated with water are amongst the important challenges confronted by Pakistan. Pakistan's status has changed radically from being a water abundant, to a water scarce country. Decline in per capita water availability was observed from 2,172 to 1,306 cubic meters per person between 1990 and 2015. There is a tremendous pressure on renewable water resources as the country extracts nearly 74% of its fresh water from underground sources. In spite of substantial enhancements including improved sanitation and water supply, 27.2 million Pakistanis are still deprived of safe drinking water, and 52.7 million are rundown to adequate sanitation facilities (UNDP, 2016).

Growing demand for water and its erratic supply are together resulting in water shortages. Rapid urbanization, population growth, water intensive farming methods and industrialization, all contribute to Pakistan's growing demand for more water. On the other hand, the supply of water is effected by climatic changes that have made rainfall pattern more erratic, leading to floods in some years and droughts in others. Pollution of available water resources due to contaminated agricultural run-offs and untreated domestic and industrial waste being dumped in watercourses is another important factor leading to dwindling freshwater resources. There is dire need to find new ways to improve to quantity as well as the quality of the available water resources in the country.

1.3 Wastewater

Wastewater is the used water from any combination of domestic, industrial, commercial or agricultural activities, surface runoff or storm water, and any sewer inflow or sewer infiltration. The characteristics of wastewater vary depending on the source. Types of wastewater include domestic wastewater from households, municipal wastewater from communities (also called sewage) or industrial wastewater from industrial activities. There are a number of cases where municipal wastewater is discharged directly into waterways without any treatment. The constituents of domestic and industrial input to water resources are pathogens, nutrients, suspended solids, salts and oxygen demanding materials (Abdel et al., 2012). Water quality degradation is quickly joining water scarcity as a major issue in the region. Scarcity of water, the need for energy and food are forcing us to explore the feasibility of wastewater recycling and resource recovery.

1.4 Leachate- An Environmental Burden

Leachate can be defined as a liquid that passes through a landfill and has extracted dissolved and suspended matter from it. Generation of leachate is the main problem for municipal solid waste (MSW) landfills that results in significant threat to surface and groundwater. Leachate is produced from the precipitation entering the landfill and from the moisture that exists in the waste when it is composed. Leachate generated in municipal solid waste landfill consists of large quantities of inorganic and organic contaminants. As a general rule, leachate is characterized by high values of COD, ammonia nitrogen, pH and heavy metals, as well as a bad order and strong color. At the same time, characteristics of landfill leachate also vary with its composition, volume, and biodegradable contents present in it. All these factors make leachate treatment difficult and complicated (Raghab et al., 2013).

1.5 Public Health Concerns

Results gathered from various surveys and an investigation indicate that water pollution has radically amplified in Pakistan. The pollution altitudes are certainly higher, mostly in and around the enormous cities of the country, where industrial setups have been made. Pakistan has now drained its existing water resources and has become a water deficit country. Public water requirement has increased manifolds, due to day-by-day increase in population. Industry is expanding at the expense of forest area under cultivation to satisfy the growing demand for agriculture yields. Untreated disposal of industrial and municipal wastewater is further worsening the quality of surface and ground water (Bhatti et al., 2009).

Our natural environment and human health are threatened due to untreated wastewater and miserable solid waste and leachate management practices. Both public and private sectors of developing countries like Pakistan are not concentrating on wastewater treatment practices at industrial and domestic levels. This deficiency is thriving with several water borne illness causing health an environmental deterioration. Presently the wastewater is not treated, and with rapid urbanization and improper treatment facilities, it will endure to harmfully affect the environment and public health.

1.6 Algae- An Alternative Treatment

Microalgae are extremely diverse group of eukaryotic organisms that thrive in a wide range of habitats including fresh and salt water, blackish, marine and soil environments. They are unicellular species, which exist individually, or in chains or groups. Microalgae is capable of performing photosynthesis, they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photo-autotrophically.

Microalgae species are capable of growing in all types of waters (salty, brackish, fresh), and temperatures (ranging from polar to tropical and even extremely hot conditions), this property makes them an ideal candidate for environmental remediation. Currently several types of unit processes exist for the removal of nutrients from wastewater but these are costly and produce high sludge content. Microalgae have been proposed as an alternative biological treatment to remove nutrients (Ruiz et al., 2010).

The major hazard of releasing leachate and wastewater, which are rich in organic compounds and inorganic chemicals such as nitrates and phosphates, is mainly eutrophication and ground water contamination by heavy metals. This problem can be solved by the use of microalgae whereby the wastewater is used as feed for micro algal growth. The main advantage is that while the microalgae will be removing excess nutrients in the wastewater, there will be concomitant accumulation of biomass for further processing (Rawat et al., 2011). Other benefits include the low cost of the operation, the possibility of recycling assimilated nitrogen and phosphorus into algae biomass as a fertilizer avoiding a sludge handling problem, and the discharge of oxygenated effluent into the water body (Aslan & Kapdan, 2006).

1.7 Depletion of Fossil Fuels and Energy Crisis

Crude oil, coal and gas are the main resources for world energy supply. The size of fossil fuel reserves and the dilemma that “when non-renewable energy will be diminished”, is a fundamental and doubtful question that needs to be answered (Shafiee & Topal, 2009). Because of global population increase and materialistic lifestyles of the people, energy resources are depleting at an increased rate. Moreover, the increasing energy consumption all over the globe has adverse effects and implications on the ecosystem and environment of the earth. The use of fossil fuels for production of energy is the main cause of environmental degradation. Growing demand and consumption of energy depicts that energy shortages will be one of the most important problem of future world (Sen, 2004).

Pakistan is confronting a severe energy deficiency, the country has very limited indigenous fossil fuel resources and needs to import large quantities of oil to fill this gap (Rafique & Rehman, 2017). Oil contributes about 38.3% of the primary commercial energy supply in the country. Transport and power sectors are the major consumers of oil. These two sectors consume more than 85% of total oil consumption. According to the Ministry of Petroleum and Natural Resources, indigenous crude oil meets 18% of total demand; the remaining 82% of demand is met through imports of crude oil, high-speed diesel, and fuel oil (Memon et al., 2006).

Moreover, about 80% of the electric energy production of Pakistan is fulfilled by utilizing fossil fuels, which includes coal, diesel and natural gas, which are limited and non-renewable resources of energy (Pakistan Energy Year Book, 2012). These conventional resources are already depleting at a rapid rate, which indicates that there is a need to switch from these non-renewable to alternate renewable resources to meet the increasing energy demand, while at the same time to fight against the adverse climatic and environmental problems.

1.8 Algae as a Source of Biodiesel Production

The expected elimination of fossil fuels within a century or so has compelled to search for renewable energy sources. The alternative energy sources consists of hydro, wind, geothermal, hydrogen, solar, nuclear and biomass. Out of these, biofuels derived from biomass are the most promising energy source and are expected to satisfy the increasing global energy demands (Haq et al., 2014). Several types of renewable biofuels can be obtained thourgh microalgae. These comprise of methane formed by digestion of algal biomass in anaerobic environment, biodiesel resulting from micro algal oil and photo-biological exposure results in production of bio-hydrogen (Spolaore et al., 2006). This is not an innovative idea but presently due to the mounting petroleum prices and more importantly global warming, because of the fossil fuels consumption, microalgae are now being preferred to be used as a source of fuel.

Unfortunately, the biodiesel produced from waste cocking oil, oil crops and animal fat cannot realistically fulfill even a small proportion of the increasing demand of transport fuels. In this regard, microalgae appear to be the promising source of renewable biodiesel, which has the potential to meet the global fuel demand. Similar to plants, microalgae utilize sunlight to produce oils, but microalgae do so more efficiently than the crop plants. Potential of oil production of many microalgae species significantly exceeds the oil productivity rates of the best oil producing crops (Chisti, 2007). A variety of algal species has oil yields in excess, that is, up to 60% of their body mass. Other benefits of utilizing algae for oil production include rapid growth rates and higher capacity to absorb CO_2, in addition to diverse habitats. Microalgae can thrive virtually anywhere with sufficient sunlight. Hence, there is also no competition for growth space with agricultural crops (Cowlishaw, 2009).

Pakistan is concurrently facing many challenges such as climate change, lack of financial resources, absence of appropriate government policies and state of art technology, which hinders the commercial production of biodiesel. Various institutions are established by Pakistan's government to develop and promote alternate energy technologies, and to achieve 10% bioenergy share in the energy sector by 2020, but still no marks are reached on concrete grounds with some serious contemplation and practical approaches, Pakistan can maximize its potential for production biodiesels from Jatropha plants seed oil and Microalgae. This will result in making this country self-sufficient for energy production (Shah et al., 2018).

1.9 Significance of the Study

Algae require elemental nutrients for its growth, many of which can be found in wastewaters. Utilizing anthropogenic waste nutrients may allow for the dual purpose of bio-remediation and resource production (Rawat et al., 2011), contributing to the foundation of human sustainability. As it is less expensive and ecologically safer way with the added benefits of resource recovery and recycling (Christenson & Sims, 2011). The growth of microalgae on wastewater has been widely studied in research but the growth of algae in leachate is sparsely studied. The potential of algae to grow in leachate needs to be explored. Moreover, it is a sustainable sources of biomass and oils for fuel, food, feed, and other co-products such as biodiesel, which can be helpful in fulfilling the ever increasing energy demand of the modern world.

1.10 Objectives

The aims and objectives of the study are;

- Comparison of various native microalgae strains performance for leachate and wastewater treatment.
- Extraction of lipids for biodiesel production from these algal strains.

Chapter 2

Literature Review

2.1 Microalgae - General Characteristics

Microalgae are the most abundantly found unicellular primary producers found in all kinds of aquatic environments such as, seawater, freshwater, hypersaline lakes and even in deserts and arctic ecosystems (Raja et al., 2008). These are subdivided into eukaryotic and prokaryotic algae, eukaryotic containing the defined cell organelles such as nuclei, mitochondria and chloroplasts. While prokaryotes (blue green or cyanobacteria) are primitive, having the simple cellular structure like bacteria (Park et al., 2011). The photosynthetic mechanism of algae is similar to land based plants, but because of simple cellular structure, and submergence in an aqueous environment, where they have efficient access to water, CO_2 and other nutrients, they are more efficient in converting solar energy into biomass (Karthikeya, 2012). Over 15,000 novel compounds, originating from algal biomass, have been chemically determined. Most of the microalgae species produce unique products, like, carotenoids, anti-oxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols (Cardozo et al., 2007).

Microalgae have the ability to grow in extreme environments; it can be grown on agricultural and non-agricultural lands. It can also be grown in fresh, brackish, saline, wastewater, industrial effluents and municipal sewage. Many species of microalgae are capable of switching from phototrophic to heterotrophic growth mechanisms. As heterotrophic organisms, microalgae rely mainly on glucose or other carbon sources for carbon metabolism and energy production while some algal species can also grow mixotrophically (Raja et al., 2014). There are about 100,000 different types of microalgae living not only in the oceans but also in fresh water (lakes, ponds, and rivers) (Sumi, 2009).

2.2 Factors Affecting Microalgae Growth

Researchers have identified several key limitations factors for the algal growth systems. In both natural and artificial systems, algae has to deal with a variety of environmental conditions, which affect their cellular composition and growth rate.

2.2.1 Light

Light is one of the most important parameters affecting algal growth. Light intensity and photoperiod plays an important role in algal growth and distribution but the requirements vary greatly with the species, culture conditions and the density of the algal culture. Light regime, including fluctuations in intensity and photoperiod, is one of the main factors influencing growth and biochemical composition of microalgae (Wahidin et al., 2013). Green algae contains major light harvesting pigments (chlorophyll-a and b), which are sensitive to wavelengths of blue and red light. An improved growth of green algae was observed in this region. Increasing light intensity to a certain limit promotes algal growth.

The effect of phosphorus concentration and light intensity was observed on composition and growth of benthic algae. It was found that light effects were much stronger as compared to phosphorus effects. Around ten-fold increase in algal biomass over 10 to 400 µmol m^{-2} s^{-1} irradiances was observed (Hill et al., 2009). Rai and Gupta in 2017 reveals that with increase in light intensity (27–40.5 µmol m^{-2} s^{-1}) biomass and lipid production of microalgae *Scenedesmus abundans* increases but decreases with further increase of light intensity (54 µmol m^{-2} s^{-1}). Maximum biomass of 1.342 g/L and lipid production of 0.644 g/L were obtained at light intensity of 40.5 µmol m^{-2} s^{-1} (Rai & Gupta, 2017).

2.2.2 pH

In the cultivation of micro algae, pH is a crucial environmental factor affecting the accessibility and solubility of many nutrients and CO_2. It also has a significant effect on microalgal metabolism (Moheimani, 2012). The pH of microalgal cultures gradually rises during the cultivation period because of the inorganic carbon uptake by microalgae. But higher pH limits the CO_2 availability, which inhibits the cell growth. On the other hand, cultivation of microalgal at high pH levels can suppress undesired biological contaminants. Famous methods for controlling the pH of culture include CO_2 injection, buffer addition, and acid/base adjustment. The former two are more commonly used in cultivation of algae. (Spolaore et al., 2006; Bartley et al., 2013).

The effect of pH on the growth of unidentified species of *Chlorella* was investigated using CO_2 addition by Moheimani in 2012. For microalgae grown in a 5 L open glass aquarium the highest specific growth rate observed was at pH 7. The growth rates reduced with increasing pH from 7 to 9 (Moheimani, 2012). Qiu and his coworkers in 2017 reported that maximum biomass productivity of *Chlorella sorokiniana* was observed at pH 6 (0.140 g/L^{-day}), the productivity was statistically the same as at pH 5.8. Also, they found that production of biomass decreased with increasing pH from 6 to 9. Practically the productivity was much lower at pH 9 (0.071 g/L^{-day}), which was almost half of the value at pH 6 (Qiu et al., 2017).

2.2.3 Temperature

Temperature effects al the metabolic processes of any organism. The optimum temperature for a specific algal specie will have a significant influence on the desrired biomass productivity of the culture (Borowitzka, 2016). It is important to maintain the microalgae suspension temperature always close to the optimum temperature so that maximum growth can be ensured. Beyond the optimum temperature, the decrease of the growth rate becomes linear and depending on the species reaches to the lethal temperature (Deb et al., 2017). The different culture environmental conditions, such as seasonal fluctuation, result in low and high temperatures and can cause variable growth rates and lipid accumulation of microalgae (Ippoliti et al., 2016). The rate of microalgal photosynthesis, growth and respiration decline when optimum temperatures are exceeded because of the imbalances between production of adenosine triphosphate (ATP), energy demand, denaturation and inactivation of necessary proteins for photosynthesis or stress on photosystem-II activities (Sheng et al., 2011; Ras et al., 2013).

The optimum water temperature needed for cultivation of microalgae ranges from 15 to 30 °C beyond this temperature range micro algal cell damage or death may occur. *Chlorella vulgaris* can grow well in temperature range 25–30 °C, but can also survive in extreme environmental conditions (30–35 °C). While *Scenedesmus* species are able to grow in wide temperature range from 10 to 40 °C. *Spirulina* species also has the ability to grow well in higher temperature ranging from 20 to 40 °C. However, the extreme temperatures affect the carbohydrate and protein levels. (Singh & Singh, 2015). Park and his coworkers in 2011 studied that the optimum temperature range for maximum growth of microalgae under optimal light and nutrient conditions varies from one species to another. For the maximum growth rate of most algal species, the optimum temperature range is between 28 to 35 °C (Park et al., 2011).

2.2.4 Nutrients

A critical factor in the sustainability of photosynthetic resource production via algae is the requirement of elemental nutrients, many of which are renewable. Carbon, oxygen and hydrogen are required as non-mineral nutrients for growth of algae. While macronutrients mainly include phosphorus, nitrogen, magnesium, potassium and sulfur. Certain micronutrients like manganese and iron are also required in small quantities (2.5 to 30 ppm) while some other elements including zinc, copper, cobalt, molybdenum and boron are also essential trace elements (2.5 to 4.5 ppm). Phosphorus and nitrogen are two most critical macronutrients for metabolism and growth of microalgae. Nitrogen is a building block for production of nucleic acids and proteins. Phosphate is another very important nutrient, being a part of essential molecules such as ATP, which are the source of energy in cells. Phosphate is the backbone of RNA and DNA structures, which are fundamental macromolecules in all living cells. Phosphorus is also an integral part of phospholipids. It is not uncommon for microalgae to become nutrient limited (i.e. nitrogen and phosphorus limited) in natural environments (Juneja et al., 2013).

Alketife and coworkers in 2016 investigated the optimal concentrations of nutrients (P, N and C), required not for only increasing the growth rate of microalgae but also for the removal efficiencies of total nitrogen and phosphorus, which are very important when wastewater effluent is used as a culture medium. They found that increasing the initial total phosphorus concentration (0, 1.2, 2.7, 7 and 19 mg/L) with constant total nitrogen (70 mg/L) gave an increase in growth rate of alga biomass but with a less consistent trend in removal efficiency. It was also reported that *C. vulgaris* could not survive without enough N and P. A balanced N/P ratio is very important for biomass growth and nutrient removal. (Alketife et al., 2016).

2.3 Leachate Production and Characteristics

Landfill leachate is commonly characterized as polluted water that has percolated through the landfill, where the water can come from precipitation, groundwater seepage or from the wastes in the landfill (Renou et al., 2008). The water passing through the waste masses is polluted with nutrients and toxic substrates like heavy metals, loosened from their origins through biodegradation or other chemical processes (Jones et al., 2006). Landfill leachate is thus considered an environmental hazard as the pollutants spread into the surroundings, affecting the local biota and in cases of transport via groundwater or other aquatic systems even farther away (Abbas et al., 2009).

Landfill leachate (LL), is a liquid waste generated by solid waste landfill sites. It is an environmental burden because of its high nutrient content and other harmful constituents, but it may have an intrinsic value as an algal culturing medium. Landfills continue to generate leachates throughout their lifetimes, even after closure. These leachates must be managed to prevent environmental contamination (Edmundson & Wilkie, 2013). The main ingredients of leachate are organic hazardous substances such as aromatic and chlorinated aliphatic compounds, phenols, phthalates and pesticides. leachate concentration of leachate depends on various factors, such as waste composition of waste and other pollutants, all of which have accumulative, threatening, and detrimental effects on the survival of aquatic life forms, ecology, and food chains that lead to public health crisis including carcinogenic effects, acute toxicity, and genotoxicity (Shariatmadari et al., 2018).

Despite of many changes of landfilling technology from uncontrolled solid waste dump-site to a highly engineered landfill for the ultimate disposal of solid waste, production of highly contaminated leachate still remains an inevitable problem (Cortez et al., 2011). Inefficient and poorly managed landfilling methods have been seen as the major factors leading to hydro geological contamination, which originates from leachate that infiltrates into soil and groundwater. Moreover, leachate is a heterogeneous mixture consisting of high strength inorganic and organic contaminants such as ammonia nitrogen, xenobiotic organic compounds (XOCs), humic acids, heavy metals and many other inorganic salts (Honjiang et al., 2009). If not properly treated and securely disposed off, leachate may cause serious pollution of surface and groundwater as it can into soils and sub-soils. Hence, leachate treatment is necessary before its discharge into the environment in order to safeguard the surrounding populations and ecosystems (Tatsi et al., 2003; Zamri et al., 2017).

2.4 Wastewater Production and Characteristics

Wastewater is generated as a result of various human activities, such as industrial, commercial and domestic uses. The composition and quantity of urban wastewater are also influenced by multiple factors, such as the lifestyle and living standard of inhabitants, the proportion of industrial and domestic effluents, and the design of the sewerage and treatment systems (Becerra-Castro et al., 2015). Urban wastewater mainly consists of dissolved and particulate organic matter, and many inorganic substances (e.g., N, P, Na, Ca, K, Mg, B and Cl). It also contains microorganisms, including pathogens and antibiotic resistant bacteria (Varela & Manaia, 2013).

Additionally, toxic, recalcitrant and bioaccumulative chemicals (e.g., trace metals, xenobiotics and natural or semi-synthetic compounds) are normally present, although representing minor components, often designated as micro-pollutants or microcontaminants (Henze & Comeau, 2008). The wide range of trace chemical contaminants persisting in municipal wastewater also includes heavy metals, persistent organic pollutants like endocrine disrupting compounds, pharmaceutically active compounds, disinfection by-products, and many other complex compounds (Fatta-Kassinos et al., 2011).

In Pakistan, domestic and industrial wastewater is either discharged directly to a sewer system, a natural drain or water body, a nearby field or an internal septic tank. Mostly, this wastewater is not treated and none of the cities have any biological treatment process except Islamabad and Karachi, and even these cities treat only a small proportion (<8%) of their wastewater before disposal. Estimates reveal that total quantity of wastewater produced in Pakistan is 962,335 million gallons per year, including 674,009 million gallons from municipal and 288,326 million gallons from industrial use. The total wastewater discharged to the major rivers is 392,511 million gallons per year (1/3rd of all wastewater), which includes 316,740 million gallons of municipal and 75,771 million gallons of industrial effluents (Murtaza & Zia, 2012). Municipal sewage is important contributor to water pollution with an estimated discharge to surface water bodies of 7.57×10^6 m^3/day. Less than 10% of this wastewater is currently being treated in municipal wastewater treatment plants (Ali et al., 2018).

2.5 Phycoremediation Potential of Microalgae

Phycoremediation generally refers to a type of biological treatment of wastes in which algae remove inorganic and simple organic compounds for their growth while some more complex substances can undergo a certain degree of biotransformation. The studies having assessed the viability of such technology have mostly been conducted in countries with plentiful supply of light and in warm climates (Paskuliakova et al., 2018a).

In addition to biofuels and other bio-product applications, largescale methods of producing and harvesting algae have uses in wastewater treatment. While chemical and physical based technologies are available to remove these nutrients, they consume significant amounts of energy and chemicals, making them costly processes. Compared to physical and chemical treatment processes, algae based treatment can potentially achieve nutrient removal in a less expensive and ecologically safer way with the added benefits of resource recovery and recycling (Christenson & Sims, 2011). This is especially important since the microalgal biofuel applications appear to be strongly economically convenient only in conjunction with wastewater treatment (Samori et al., 2013).

2.6 Wastewater and Leachate - As Microalgal Growth Medium

Domestic wastewater having high concentrations of all necessary nutrients is favorable for algal growth. It is the metabolic ability of microalgae to uptake the and utilize nutrients (e.g. phosphorus and nitrogen) from polluted agricultural, industrial and municipal wastewater. This ability allows the use of polluted water for microalgal growth and simultaneously provides a promising and sustainable method for bioremediation of wastewater (Qiu et al., 2017).

There is a lot of on-going research on the treatment of industrial, municipal and agricultural wastewaters and leachate by microalgae culture systems (Zhang et al., 2012; Ji et al., 2013). It was found that when cultivating the *Arthrospira platensis* in olive-oil mill wastewater, the maximum removal of chemical oxygen demand (COD) was 73.18%, while phenols, phosphorus and nitrates in some runs were completely removed (Markou et al., 2012). Alketife and coworkers (2016) reported that the algal strain *C. vulgaris* cultivated in MLA medium with 7 mg/L phosphorus and 70 mg/L nitrogen (N/P=10) completely utilized the nutrients (N and P) at the end of 13 days cultivation period with maximum biomass production of 1.58 g/L. However, the nitrogen removal efficiency reduced significantly from 100% to 28%, when the N/P ratio increased from 10 to 58 (Alketife et al., 2016).

Edmundson and Wilkie (2013) evaluated the potential of solid waste landfill leachate (LL) as an algal cultivation medium to minimize the water and nutrients demands of algae strains *Scenedesmus cf. rubescens* and *Chlorella cf. ellipsoidea*. Their results indicate that LL can be utilized as a culture medium for the growth of microalgal biomass. It was found that *S. cf. rubescens* grew well in 100% LL, with average growth rate and cell yield 91.2% and 92.8% respectively, more than those observed in BBM. *S. cf. rubescens* was also found more adaptable than *C. cf. ellipsoidea* to the LL. Cultivating algae in LL will undoubtedly provide a remediation benefit in the removal of total ammoeniacal-nitrogen and orthophosphate in LL (Edmundson & Wilkie, 2013). Toxicity reduction of landfill leachate subsequent to phycoremediation was investigated by Paskuliakova and his colleagues. Ammonia nitrogen in the diluted landfill leachate containing up to 158 mg/L NH_4^+-N (60% dilution of the original) was reduced by 83% during the microalgal treatment by using strain *Chlamydomonas sp. SW15aRL* (Paskuliakova et al., 2018b).

2.7 Biodiesel - An Emerging Biofuel

Biofuel is any solid, liquid or gaseous fuels derived from organic biomass, which is any living matter such as field crops, wood products, water plants and municipal solid waste etc. that is converted into energy. As an alternative for fossil fuel in the transportation part, biofuel can become critical for solving environmental troubles as it minimizes greenhouse gases (GHG) emission (Tseten & Murthy, 2014).

The International Energy Agency (IEA) suggests that by 2050, biofuels could meet about 27% of total global transport fuel demand, as well as save 2.1 giga tonnes (109 tonnes) of CO_2 emissions per year that would otherwise have been produced from fossil fuels. This claim has been reflected in the amounts of biofuels traded globally (IEA, 2011).

Since recent years, the energy produced for sustaining the world's economy depends mainly on fossil fuels, which are not only causing environmental degradation but are also depleting rapidly. This issue has gained the attention of many countries all around the globe to develop the alternative of petroleum fuels, the biofuels as substitute. Biodiesel is a biofuel made up of esters of fatty acids, which is produced through the chemical reaction of vegetable oils and animal fats with an alcohol in the presence of a catalyst. It has gained top priority for its production from non-edible, edible and microalgal lipids as feed stocks. Its properties are almost similar to the petroleum diesel except the cold flow properties and oxidation stability (Kumar & Sharma, 2015). Its environmental benefits are already known to the point of it being considered an advanced fuel, since it reduces the emission of greenhouse gases (GHG) by at least 57% compared to its direct competitor, diesel, making it one of the most practical and cost-effective ways to combat the climate change process and energy problem (Martins & Carneiro, 2017).

Various studies have indicated that the biofuels obtained through agricultural crops have inauspicious economic and social effects on population of world and has resulted in “food versus fuel” conflict in the society. (Liew et al., 2014). It is estimated that approximately 95% of biodiesel produced globally is obtained through edible vegetable oils (Sajjadi et al., 2016). Use of edible vegetable oils for biodiesel production not only increases its cost but also the cost of food due to the reduced availability of vegetable oils. Whereas the biofuels produced from nonedible sources, for example, Pongamia Pinnata, Jatropha Curcas and Microalgae etc. bypass this “food versus fuel” dilemma. Due to this fact, non-edible feed stocks have gained attention as the most suitable biodiesel sources, as the demand of edible oils greatly exceeds the domestic supply rate while many developing countries are net importers of edible oils (Agarwal et al., 2017).

2.8 Potential of Microalgae As a Biodiesel Source

In current practices, most of the biodiesel is produced through oils obtained from oleaginous seed plants, e.g. soybean, sunflower, palm and rapeseed. However, a potentially efficient and better alternative of these oil crops is photosynthetic microalgae, which can produce 10 to 20 times higher oil yields per unit land area and also have the additional benefit of no competition for agricultural land (Converti et al., 2009). Biodiesel obtained from microalgae is a sustainable renewable energy source, which might completely substitute the conventional diesel without effecting the human food supply. In fact, microalgae, as compared to crop plants, have much higher oil yields as a biodiesel feedstock. The annual yield of algal oil is 7 to 31 times more than palm oil, given the same land area. This is because of their very high actual photosynthetic yield and their ability to accumulate more lipids (El-Sheek et al., 2017).

Biodiesel produced through crop based plant oils has many drawbacks. Such as, high water and land requirements, low oil yields, negative impacts on food supplies and associated extensive deforestation to clear the land for agriculture, is posing threats to ecosystem functions, native biodiversity, goods and services (Sharma et al., 2012). These negative impacts can be avoided by the use of next generation microalgae based biofuels. In comparison to agricultural crops, the major benefit of microalgal systems are that they have high efficiency for photon conversion, microalgae can be cultivated batch wise all year around, they can use brackish and wastewater streams for their growth. Most importantly, these systems can couple CO_2 neutral fuel production along with high CO_2 sequestration rate, producing highly biodegradable and non-toxic biofuels (Cobos et al., 2017).

2.9 Lipid Content of Microalgae

Microalgae can be grown in either open ponds or closed reactors of various designs. The actual oil production yields of microalgae are very controversial and different results can be found in literature. Besides, several factors affecting microalgal cultivation and processing, lead to quite different results and it is possible to obtain oil yields of the same microalgal strain varying from 5 to 70% (Pinho et al., 2017). However, the oil content of some microalgae species exceeds 80% of the dry weight of algal biomass according to Oilgae (2010), while some have about 15 to 40% of dry weight. In comparison, the oil content of some best known oil crops such as copra has 60%, sunflower contains 55% and palm kernel has about 50%. In fact, microalgae give the highest oil yields as compared to various plants. It can yield up to 100,000 L of oil per hectare per year, on the other hand palm, coconut, castor and sunflower are reported to produce 5950, 2689, 1413 and 952 litter per hectare per year, respectively (Shah et al., 2018).

Apandi and her colleagues in 2017 grown *Scenedesmus* sp. in four different concentrations of wet market wastewater (10%, 15%, 20% and 25%) in comparison with the Bold Basal Medium (BBM) as a control. The result shows that the highest lipid concentration was obtained in the 25% wastewater with the value of 26.7% compared to other concentrations and BBM (Apandi et al., 2017).

2.10 Pre-Treatment of Algal Biomass

The lipids extraction at low cost and their environmental friendly nature are the most significant advantages of the commercial generation of biodiesel from microalgae. The microalgal cells normally have sturdy cell walls that prevents the extraction of intracellular products, while breaking them is an energy intensive process. Therefore, it is important to rupture these cell walls by using pre-treatment methods, in order to liberate the intracellular products for lipids extraction (Mubarak et al., 2016).

The pre-treatment or disruption techniques can be categorized into mechanical and non-mechanical methods. The mechanical techniques are further divided as solid and liquid shear methods. While non-mechanical methods are categorized as desiccation and lysis. The liquid shear methods are further sub-categorized into high pressure homogenization, ultra-sonication and microwaving, while solid shear techniques are sub-divided as freeze press, glass grinding, sand grinding, and bead mill. Whereas the non-mechanical techniques of cell disruption are the use of alkalis, acids, autoclaving and enzymes (Chisti & Moo-Young, 1986). Pre-treatment techniques such as, microwaving, bead beating, autoclaving, electro floatation by alternating current, osmotic shock, laser treatment, manual grinding with liquid nitrogen and ultra-sonication are widely reported in literature for different algal biomasses like mixture of microalgae e.g. *Chlorella vulgaris, Botryococcus braunii* and

Nanochloropsis oculata (McMillan et al., 2013; Florentino de Souza et al., 2014). According to Suganya and Renganathan (2012), ultra-sonication methods consume minimum time and solvents, and results in better extraction yields of lipids from marine macroalgae *Ulva lactuca*. Mubarak et al., (2016) also found experimentally that the lipid content was maximum (19.97%) from dried *Salvinia molesta,* through the use of ultra-sonication.

2.11 Microalgal Lipids Extraction

The efficient lipid extraction from microalgal biomass is an important step in biodiesel production. The lipids extraction from the microalgae can be carried out by using polar organic solvents such as ethanol, acetone and methanol, as well as low-polarity organic solvents such as ethyl acetate, diethyl ether, hexane, chloroform, toluene and benzene. However, organic solvents having low polarity, alone are not able to extract lipids effectively from the biomass, because they cannot separate those lipids which have strong hydrogen bonding with the polar lipids attached with the cell walls (Tang et al., 2016). In order to extract these neutral lipids (NLs), some polar organic solvent must be used along with a low-polarity organic co-solvent. The polar solvent is used to disrupt the complexes of neutral polar lipid, while the low-polarity solvent intends to dissolve the intracellular NLs. Thus, solvent systems having a mixture of polar organic solvent and a non-polar organic solvent usually maximize the extraction efficiency of NLs *(*Harris et al., 2018).

According to Balasubramanian and colleagues (2013) the disruption of microalgal cell by using a micro bead beater followed by extraction through chloroform/methanol solution (2:1 v/v) has been found most effective and efficient as compared to other lipid extraction methods. Ansari and his fellows in 2017 studied the effect of six different solvents and their mixtures on the lipid yields of wet and dry biomass of microlagae *Scenedesmus obliquus.* Solvent/solvent mixtures were as follows: (1) isopropanol, (2) dichloromethane/methanol (2:1 v/v), (3) hexane/ isopropanol (1:2 v/v), (4) chloroform/ethanol (1:1 v/v) (5) chloroform/methanol, (2:1 v/v) and (6) hexane. Lipid yields in dry biomass varies from 2.85% to 19.25% by using these six solvent mixtures. Among all, chloroform/methanol mixture produced the maximum lipid yield whereas hexane gave minimum lipid yield. In case of wet algal biomass, lipid yield was significantly lower in comparison to the dried biomass and ranged from 1.35% with hexane to 10.08% with chloroform/ethanol solvent mixtures (1:1 v/v) (Ansari et al., 2017).

2.12 Transesterification of Microalgal Lipids

The transesterification reaction (fig. 2.1) takes place in the presence of a suitable homogeneous catalyst i.e. base catalyst like potassium hydroxide (KOH) or sodium hydroxide (NaOH) and acidic catalyst such as sulfuric acid, or heterogeneous catalysts such as carbonates and metal oxides. Among these, sodium hydroxide is a well known and mostly used catalyst due to its high product yield efficiency and low cost (Lotero et al., 2005; Teo et al., 2014). Most of the biodiesel produced all around the world is through base catalyzed transesterification reaction because it is a low pressure and temperature process, having high conversion efficiencies without intermediate steps. Moreover, it also requires less costly materials for construction of the system. Type of catalysts (enzymatic, acidic or alkaline), concentration of catalysts, alcohol to oil molar ratio, moisture content of reactants, reaction temperature and free fatty acid (FFA) content of oil are the major factors influencing the biodiesel (ester) yield from the transesterification process (Agarwal et al., 2017).

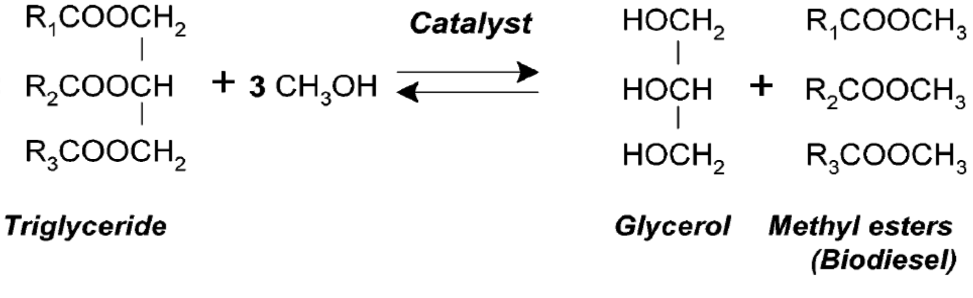
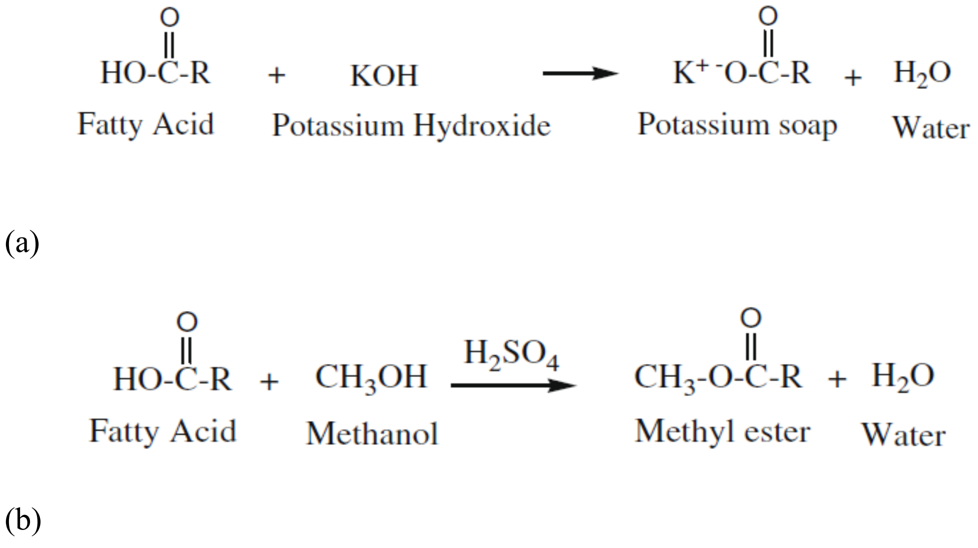


Figure 2.1 Typical transesterification reaction using methanol for biodiesel production (Lotero et al., 2005)

2.13 Acidic vs Basic Catalysts

Traditional industrial processes of biodiesel production currently give priority to use of homogeneous basic catalysts, which includes alkaline methoxide ($NaOCH_3$)

and alkaline hydroxides (KOH, NaOH). However unfortunately, even if moisture free oil feeds tocks and alcohols are used, these basic catalyzed reactions still produce some water due to the chemical reaction between alcohol and hydroxide (Yee et al., 2011). The presence of moisture will cause hydrolysis of esters and thus subsequent saponification occurs as shown in fig 2.2 (a), which may reduce the fatty acid methyl esters' yield and makes the downstream separation processes difficult; hence, increasing the process costs. On the other hand, acidic-catalyzed reactions (fig. 2.2 b) do not result in saponification, due to which the development of heterogeneous acid catalysts has attracted a great deal of attention recently. Moreover, the use of these acid catalysts also give the benefit of easier downstream product separation processes and increased ability to recover and reutilize the catalyst (Huang et al., 2010). Another important advantage of the use of heterogeneous acid catalyzed reaction is the ability to catalyze the transesterification of animal fats and vegetable oils having high levels of free fatty acid (FFA) (Tran et al., 2013).



$$\mathrm{HO\text{-}\overset{\overset{O}{\|}}{C}\text{-}R} + \mathrm{KOH} \longrightarrow \mathrm{K^{+}\,{}^{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}R} + \mathrm{H_2O}$$

$$\mathrm{HO\text{-}\overset{\overset{O}{\|}}{C}\text{-}R} + \mathrm{CH_3OH} \xrightarrow{\mathrm{H_2SO_4}} \mathrm{CH_3\text{-}O\text{-}\overset{\overset{O}{\|}}{C}\text{-}R} + \mathrm{H_2O}$$

Figure 2.2 (a) Transesterification by alkali catalyst. (b) Transesterification by acidic catalyst (Huang et al., 2010)

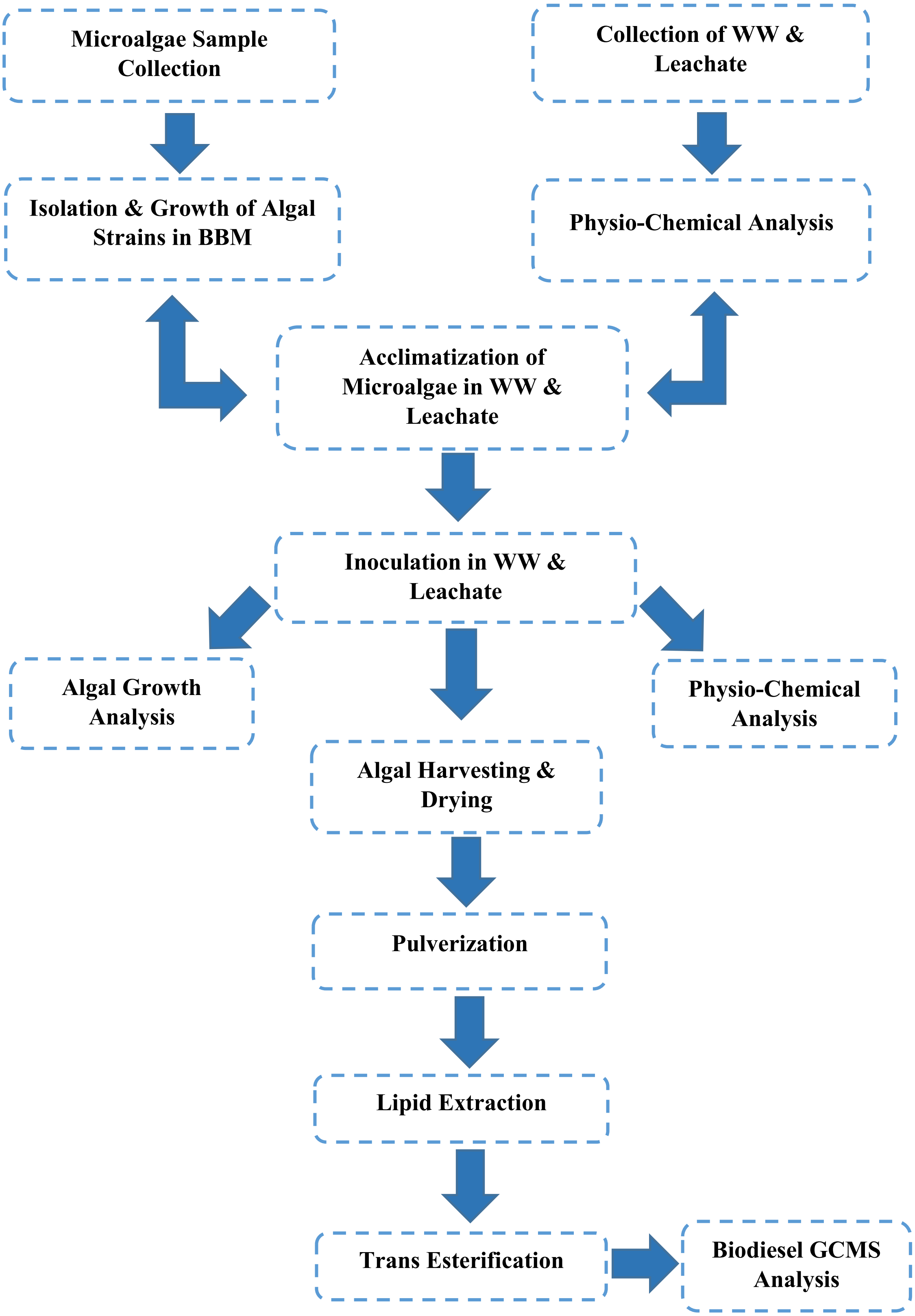
Pinho and coworkers in 2017, observed only 75% of conversion of *Chlorella sp.*'s lipids into biodiesel by direct esterification. Four consecutive esterification steps were carried out to maximize the conversions up to 96.5%. It was also reported that the different compositions of the algal oils occurs because of the different growth medium of microalgae and also due to the environmental interferers, which effect the fatty acids composition of the algal oil as well as the presence of other non-fatty acid lipids (Pinho et al., 2017). Rahman and fellows in 2017 developed a two-step method (esterification and transesterification) for biodiesel production from microalgae S*pirulina maxima* to determine the best operating conditions. In first step the optimum conditions for maximum yield of esterified oil were observed at alcohol to oil molar ratio of 12:1, 1% by wt. H_2SO_4 as catalyst, with mixing speed of 400 rpm and temperature 60 °C, for the reaction duration of 90 min. For the second step of transesterification for maximum biodiesel yield (86.1%), the optimum conditions were observed as the alcohol to oil molar ratio of 9:1, catalyst concentration 0.75% by wt. KOH, stirring speed of 600 rpm and temperature 65 °C for the reaction duration of 20 min (Rahman et al., 2017).

Chapter 3

Materials & Methods

In this chapter, the procedures and methods used during the research experiment are discussed. The simplified sketch diagram of the steps, which were carried out during the lab work are as follows.

Figure 3.1 Simplified flow chart of research methodology



3.1 Microalgae Collection

Four Microalgae strains were used for the experimentation.

- Strain 1 (S1) was collected from a pond located near the G-11/1 signal at Kashmir Highway, Islamabad.
- Strain 2 (S2) was collected from the lake located in NUST, H-12, Islamabad.
- Strain 4 (S4) and Strain 6 (S6), later identified as *Dictyosphaerium* sp. and *Pectinodesmus* sp. respectively (Khalid et al., 2017), were acquired from Nano Biotechnology Lab, Atta-ur-Rehman School of Applied Biosciences, NUST, Islamabad.

3.2 Synthesis of Growth Media

Bold's Basal Medium (BBM) was used as growth media for the microalgae strains. It is an inorganic salts medium widely used for the culture of free-living planktonic freshwater algae. Klinger and Garoma in 2018, analyzed the potential of Bold's Basal Medium (BBM), Bristol's Medium, Sueoka Medium, MiracleGro All Purpose Water Soluble Plant Food Media and Highly Assimilable Minimal Growth Medium (HAMGM) for the growth of *Chlorella vulgaris*. They found the Bold's basal medium as the best medium for biomass production. The BBM was made by following the modified recipe as given in the table 3.1 (Peña-Castro et al., 2004):

Table 3.1 Elemental composition of Bold Basal Medium

Ingredients	**Concentrations (mg/L of DW)**
$NaNO_3$	95.2
$CaCl_2.2H_2O$	25
$MgSO_4.7H_2O$	75
K_2HPO_4	75
KH_2PO_4	175
NaCl	25
EDTA	50
$FeSO_4.7H_2O$	4.98
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.22
$MnCl_2.7H_2O$	14.4
MoO_3	0.71
$CuSO_4.5H_2O$	1.57

$Co(NO_3)_2.6H_2O$	0.49
KOH/NaOH	31
H_2SO_4	0.001 (ml)

3.3 Isolation of Algal Strains

After initial cultivation of the mixed cultures, collected from NUST lake and G-11 pond, in BBM, unicellular algae were isolated from flasks showing growth. The algae were subjected to purification by serial dilutions followed by inoculation onto Petri plates containing BBM supplemented with 1.5% (w/v) of agar (Tale et al., 2014). The petri plates were placed in incubator with an incandescent bulb having the light intensity of 400 lux, at 27°C, for 1 week. Single colonies appearing on plates were picked up and purified by streaking again on the nutrient agar plate. Distinctive colonies were then picked up and cultivated in BBM. The strains that showed good growth (S1 and S2) were further chosen for the experiment, along with S4 and S6, obtained from ASAB, NUST.

3.4 Inoculation in Bold Basal Medium

The microalgae strains were inoculated in 1 liter glass bottles, containing the Bold Basal Medium. The growth was aided by provision of CO_2 and continues mixing by means of constant aeration at the rate of 3.5 L/min. Illumination was also provided through 36W TLD fluorescent lamps (≈800 lux) continuously, to accelerate the photosynthesis process. The setups were sustained for 15 days and then iterated several times with sub-cultures. The pH was maintained at 7.

3.5 Biomass Determination

The growth of the microalgae was determined by measuring its biomass through optical density (OD) using spectrophotometer at 680 nm, daily (Menezes et al., 2016). In order to measure the biomass through OD, standard curves were plotted representing the relation between OD and dry algal biomass. The algae samples were diluted in different ratios with distilled water and OD was measured. Samples were then filtered and centrifuged at 4000 rpm for 10 minutes at 4°C. After centrifugation, the biomass samples were washed with distilled water and the pellet were dried at 70°C for four hours in china dish. The dry biomass was calculated gravimetrically and graph was plotted between the OD and the dry weight of samples. The algal growth was expressed in terms of the dry cell weight (DCW) per liter (g/L) (Li et al., 2008; Zhu et al., 2013).

3.6 Collection of Wastewater and Leachate

- **Wastewater**

  The wastewater was collected from the wastewater influent tank near the Membrane Bioreactor Plant (MBR) installed in NUST, H-12, Islamabad. The sample were collected in 1.5 L PET bottles, which were pre washed with distilled water and stored in refrigerator for further analysis.

- **Leachate**

  The leachate was collected from I-12 solid waste dumping site, Islamabad. The samples were randomly collected in pre washed 1.5 L PET bottles and transferred to laboratory where they were mixed and stored in refrigerator until further experimentation.

3.7 Physio-Chemical Analysis

3.7.1 Nitrate determination

The concentration of nitrates in wastewater and leachate samples was determined by Cadmium Reduction Method using NitraVer® 5 Nitrate Reagent Powder Pillows (HACH-8039, 2014). Cadmium metal used in the method, reduces nitrate in the sample to nitrite. The nitrite ion reacts in an acidic medium with sulfanilic acid to form an intermediate diazonium salt. The salt couples with gentisic acid to form an amber colored solution, which indicate the concentration of nitrates. The absorbance wavelength is 500 nm for spectrophotometers. Standard curve was developed to determine the concentration of nitrate ions in the sample from the corresponding absorbance value.

3.7.2 Phosphates determination

The phosphates in samples were determined by Vanadomolybdate Phosphoric Acid Colorimetric Method as orthophosphates (APHA 4500-P. C, 2005). This method includes two general procedural steps; (a) conversion of the phosphorus from different forms to dissolved orthophosphate, and (b) colorimetric determination of dissolved orthophosphate at the wavelength of 470 nm. Ammonium molybdate reacts under acid conditions to form a heteropoly acid, molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow color is proportional to phosphate concentration. To determine the exact concentration, standard curve was developed.

3.7.3 Chemical oxygen demand

Chemical Oxygen Demand (COD) is often used as a measurement of pollutants in wastewater and natural waters. To determine the COD of wastewater and leachate, Closed Reflux Titrimetric Method was used (APHA 5220-C, 2005). In this method the sample is refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). For this purpose, 2.5 ml of sample was taken, diluted and digested with Standard Potassium Dichromate digestion solution and oxidized with 3.5 ml of Sulfuric acid solution in COD vial. The sample was then refluxed for 2 hours standard time at 150 ^{o}C. After complete digestion, the remaining unreduced $K_2Cr_2O_7$ is titrated with Ferrous Ammonium Sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

3.7.4 Heavy metals analysis

The presence of hazardous heavy metals and metalloids in water is an important environmental and social problem. As many of these elements are persistent and bio-accumulative. The heavy metals Lead (Pb), Chromium (Cr), Copper (Cu), Iron (Fe), Nickle (Ni), Zinc (Zn), Cadmium (Cd) and Mercury (Hg), present in wastewater and leachate were analyzed by using Atomic Absorption Spectroscopy at Fatima Jinnah Women University, Rawalpindi. To assess the heavy metals removal efficiency of microalgae strains, concentrations of these hazardous metals were analyzed prior to the treatment and at the end of treatment. Both concentrations were compared to determine the percentage removal of these metals.

3.8 Setup for Acclimatization of Microalgae

Microalgae strains were acclimatized to different increasing concentrations of leachate and wastewater as shown in table 3.2. Firstly, all strains were inoculated in 0.5 L transparent PET bottles containing 20% wastewater and 20% leachate, separately. The growth was observed regularly by measuring optical density at 680 nm (Menezes et al., 2016). Upon significant growth after every 4 days of observations, the concentration of wastewater was increased at the rate of 20% and leachate by 10%, until the decline in microalgae growth was observed. During the process, aeration and illumination source were provided through aeration pumps and TLD fluorescent lights respectively.

Table 3.2 Concentration (%) of wastewater and leachate for acclimatization of algae

	1st Day	5th Day	9th Day	13th Day	17th Day	21st Day
Wastewater %	20	40	60	80	100	100
Leachate %	20	30	40	50	60	60

3.9 Treatment of Wastewater and Leachate

In order to assess the treatment efficiency of microalgae, 25 ml of acclimatized microalgae samples were inoculated in 1.5 L transparent PET bottles containing diluted leachate (50%) and undiluted wastewater (100%), separately. The bottles were illuminated with TLD 36W fluorescent lamps continuously (≈800 lux) and an air flow rate of 3.5 L/min was maintained. The setup was sustained for 15 days, during which pH was maintained at 7. OD, Nitrates and Phosphates concentrations and COD were observed regularly. While heavy metals were analyzed before and after the treatment.

3.10 Harvesting of Microalgae

After the removal of aeration and illumination setup, microalgae were filtered by membrane filter to obtain wet biomass. The wet algal slurry was subjected to sonication for 10 minutes. The sonicated residue was transferred to 50 ml Eppendorf tubes. The tubes were subjected to centrifugation at 4000 rpm for 10 min at 4 ˚C and followed by washing with distilled water to get rid of excessive salts. Algal biomass was then transferred to petri dishes and dried at 70 °C for 4 hours to remove the moisture content (Zhu et al., 2013; Lu and Zhang, 2016). The dried biomass was weighted and stored in air-tight plastic bags for lipids extraction.

3.11 Extraction of Microalgal Lipids

3.11.1 Sonication of algal biomass

Before drying, the harvested biomass was subjected to high intensity sonication for 10 minutes using ultrasound sonicater. During lipid extraction from biomass, the physical effects of ultrasonication can significantly enhance the lipid yield. Sonication generates high pressure in the medium, which can disrupt microbial cellular structures. This cause the lysis of microalgal cells. Ultrasound also generate intense local turbulence in the medium, pushing the lipids away from the surface of the microbial cells, and thus, maintaining a constant concentration gradient for continuous diffusion of lipids from the cells (Naveena et al., 2015).

3.11.2 Lipids extraction

Modified Bligh and Dyer Method (1959) was used for the extraction of lipids from the dried algal biomass. This method uses a solvent system of chloroform, methanol and water to extract the lipids (Qayyum, 2015; Chatsungnoen & Chisti, 2016).

- The first step of the extraction was to add 5 mL of chloroform, 10 mL of methanol and 4 mL of distilled water to each tube containing 1g of dried microalgae.
- Once the tubes had all reached room temperature, a sonicator was again used to disrupt the algae cells in the suspended mixture. Each tube was sonicated for 5 minutes.
- The samples were then placed on a shake table at the rate of 250 rotations per minute for 8 hours, at room temperature. Purpose of the shaking step was to promote the complete exposure of intracellular products to the solvents.
- After 8 hours, tubes were removed from the shake table and an additional 5 mL of chloroform and 5 mL of distilled water were added to each sample. Each tube was vortex mixed for 30 seconds to mix the newly added solvents.
- The tubes were then centrifuged at 5000 rpm for 10 minutes to separate the contents into three layers. The green layer at the bottom was collected,

comprised primarily of chloroform, containing the lipophilic material. The upper layer containing methanol and water was discarded. A thin middle layer separating the two layers was the residual algal cell debris.

- To extract the lipids from larger quantity of biomass, the process was repeated several times for each strain. Amount of lipids obtained was measured by using the equation (Abbah et al., 2016; Rai & Gupta, 2017):

$$\text{Lipids yield (\%)} = \frac{\text{Mass of lipids extracted}}{\text{Mass of microalgae used}} \text{ X } 100 \qquad \text{Eq. 1}$$

3.13 Transesterification of Lipids

In order to avoid the subsequent saponification as a result of hydrolysis of esters due to the water generation from the reaction between hydroxide and alcohol during basic-catalyzed reactions, Acid Catalyst H_2SO_4 was used for the one step transesterification of the algal lipids. Another benefit of the heterogeneous acidic-catalyzed process is its ability to catalyze the transesterification of lipids with high levels of free fatty acids (FFAs). As one of the main problems of microalgae lipids is their high content of free fatty acids, which creates problems of soap formation during homogeneous alkali transesterification (Tran et al., 2013; Veillette et al., 2017).

- To initiate the process, lipids were heated at the hot plate for 10 minutes at 60 ^{o}C to remove any residual moisture.
- Methanol (40% v/v) and Sulfuric acid (5% v/v) were mixed separately and pre-heated algal oil was then transferred to the methanol sulfuric acid solution.
- The whole mixture was allowed to react for 2 hours at 60 ^{o}C, with continuous stirring at 400 rpm.
- After the reaction was completed, samples were cooled to room temperature and time was given for phase separation. The lower phase which contained the biodiesel was collected and measured by using the equation (Tariq et al., 2011; Abbah et al., 2016; Gandure et al., 2017):

$$\text{Biodiesel yield (\%)} = \frac{\text{Amount of biodiesel produced}}{\text{Amount of lipids oil used}} \text{ X } 100 \qquad \text{Eq. 2}$$

- Biodiesel was then transferred to glass tubes for the analysis of esters content via gas chromatography–mass spectrometry (GC-MS) (Johnson & Wen, 2009; Pinho et al., 2017).

3.14 Quantification of Alkyl Esters by GC-MS Analysis

Biodiesel is primarily consisted of mono alkyl esters of long chain fatty acids (Bajpai & Tyagi, 2006; Abbah et al., 2016). To quantify the alkyl esters in the biodiesel, GC-MS was used, as it is a highly recommended tool for monitoring organic compounds and is exclusively used for the analysis of esters, fatty acids, alcohols, aldehydes, terpenes etc. (Al-Rubaye et al., 2017).

Analysis was carried out in Combined Lab, USPCAS-EN, NUST, Islamabad. Shimadzu GC-MS QP2020 with SH-Rxi-5Sil MS silica based capillary column was used (L=30m, ID=0.25, DF=0.25). Ethyl acetate was used as solvent. GC-MS was equipped with an automatic split injector at 250 ^{o}C. Helium gas was used as a carrier gas at flow rate of 1.78 ml/min. The oven initial temperature for each run was started at 40 ^{o}C for 5 min, then raised to 300 ^{o}C and maintained for 5 min. The rate of increase in temperature was set at 7 ^{o}C/min. All the compounds were identified by means of inbuilt NIST library and added together to determine the total percentage of alkyl esters present in the sample (Rahman et al., 2017).

Results & Discussion

As discussed in methodology, after the collection of S4 *(Dictyosphaerium sp.)* and S6 *(Pectinodesmus sp.),* and isolation of two unidentified microalgal strains S1 and S2, all four strains were assessed for the growth, acclimatization in wastewater and leachate, treatment and biodiesel production potential. The results of these studies are discussed in this chapter.

4.1 Growth of Microalgae in BBM

Strains S1 and S2, which were isolated from G-11 and NUST Lake, respectively, and strains S4 and S6, collected from ASAB were inoculated in Bold Basal Media, as discussed in methodology. The growth was observed by means of optical density (OD) at 680nm and biomass determination. The strains when enriched in bold basal media started to show significant growth around 6th day, except S4, which started showing growth at 4th day of inoculation as shown in figure 4.1. Highest performance was observed for S4, whereas the S6 also showed competitive growth.

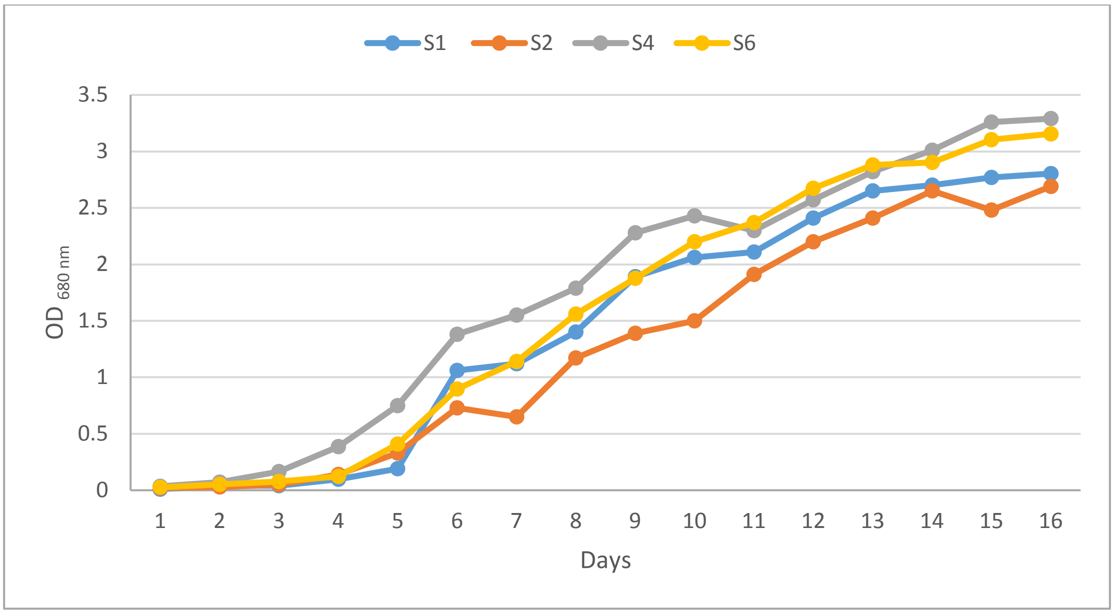


Figure 4.1 Growth of microalgae strains in BBM, determined by OD at 680 nm

Figure 4.2 represents the biomass growth of the algae strains in bold basal media, expressed in g (dry cell weight)/L. The highest biomass was achieved by S4 at 16th day, which was 4.94 g/L, which was 28.5% more than the lowest biomass produced by S2. It was followed by S6 (4.63 g/L), S1 (3.8 g/L) and S2 (3.53 g/L). Menezes and fellows in 2016 studied the growth of *Choricystis minor var. minor* in BBM for 8 days and found the average biomass of 0.984 g/L. The biomass concentrations at 8th day, as shown in figure 4.2, are seems to be in agreement with this value (Menezes et al., 2016). The biomass concentration of 2 to 5 g/L was reported by Yeh and Chang in 2012, for microalga *Chlorella vulgaris,* grown in BBM and Modified Bristol's medium, which is in line with our results (Yeh & Chang, 2012).

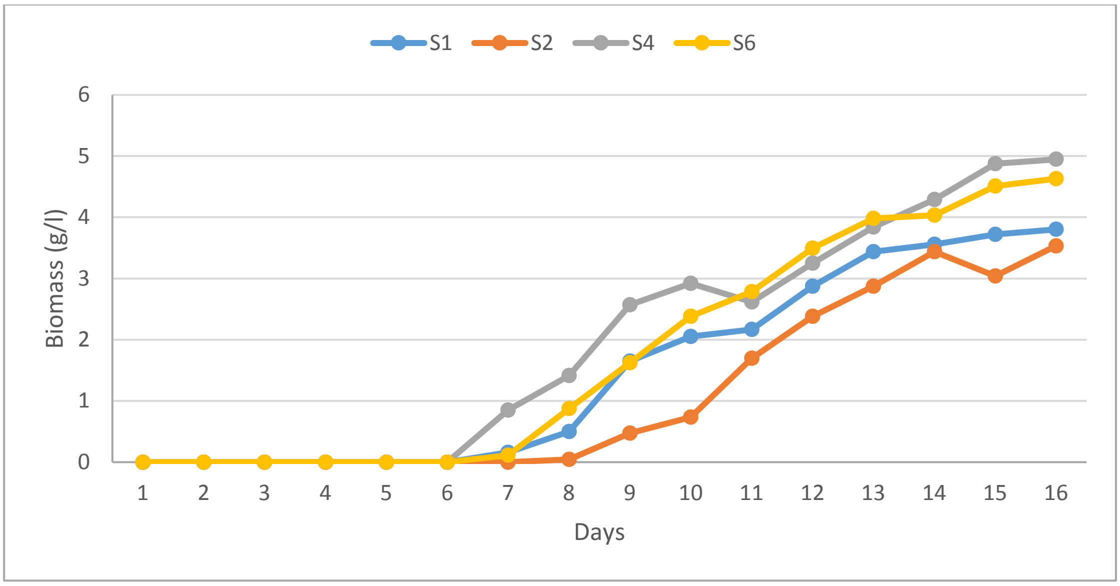


Figure 4.2 Biomass growth of microalgae strains in bold basal media

4.2 Characteristics of Wastewater

The wastewater collected from the inlet tank near the MBR plant located in NUST, was analyzed for COD, nitrates, phosphates and heavy metals, values of which are given in table 4.1. The composition of wastewater varies widely according to the sources and types of the wastes, it carries. For example, the typical composition of municipal wastewater, characterized by Boelee and coworkers was 350 mg/L of COD, 50 mg/L NH_4 and 10 mg/L of PO_4 (Boelee et al., 2014). Whereas Wang and his fellows (2010) reported the pollutant values in urban wastewater as 231 mg/L COD, 16.95 mg/L nitrates- nitrogen and 5.66 mg/L phosphates (Wang et al., 2010).

4.3 Leachate Characteristics

The leachate was collected from I-12 waste dumping site, which contains the mixed domestic and commercial solid waste of Islamabad. The concentrations of COD, nitrates, phosphates and heavy metals found in diluted leachate (50% dilution) are given in table 4.1. Munir and her fellows in 2014 reported the values of COD 2310 mg/L, nitrates 49.7 mg/L, phosphates 88.7 mg/L, Ni 0.001 mg/L, Pb 0.74 mg/L, Cr 2.17 mg/L, Cu 1.13 mg/L, Cd 0.001 mg/L, Mn 29.1 mg/L and pH 7.8 for the leachate collected from Mehmood Booti solid waste dumping site Lahore (Munir et al., 2014). Where as Kumari and her colleagues in 2016 assessed undiluted leachate from Ghazipur landfill site India and found the concentration of COD as 29200 mg/L. High levels of Ni (0.63 mg/L), Pb (0.40 mg/L), Cr (1.5 mg/L), Zn (2.5 mg/L) and Fe (9.5 mg//L) were also found (Kumari et al., 2016).

Table 4.1 Characteristics of wastewater (100%) and leachate (50%), collected from NUST and I-12

Characteristics	Concentration in Wastewater	Concentration in Leachate (50%)	NEQS (2016)
COD (mg/L)	302	8880	150
NO_3-N (mg/L)	22.2	98.41	10 (US EPA)
PO_4 (mg/L)	29	57.5	0.1 (US EPA)
Pb (mg/L)	0.030	0.081	0.5
Cr(6) (mg/L)	0.263	1.052	1.0
Cu (mg/L)	0.107	0.544	1.0
Fe (mg/L)	6.851	131.58	8.0
Ni (mg/L)	0.176	1.888	1.0
Zn (mg/L)	0.132	0.337	5.0
Cd (mg/L)	0.037	0.088	0.1
Hg (mg/L)	0.00072	0.00526	0.01
pH	7.1	7.5	6-9

4.4 Acclimatization of Microalgae in Wastewater

The acclimatization of algal strains in wastewater was started with initial concentration of 20%. After every 4 days, the concentration of wastewater was increased by 20%. All the strains showed good growth and were able to grow in real wastewater (100% concentration), as shown in figure 4.3. Maximum growth was of S4 *(Dictyosphaerium sp*). Ruiz and coworkers were also able to grow two algal species *Scenedesmus obliquus* and *Chlorella vulgaris* in 100% urban wastewater through acclimatization, they also found that *Scenedesmus obliquus* performed better than *Chlorella vulgaris* (Ruiz et al., 2010).

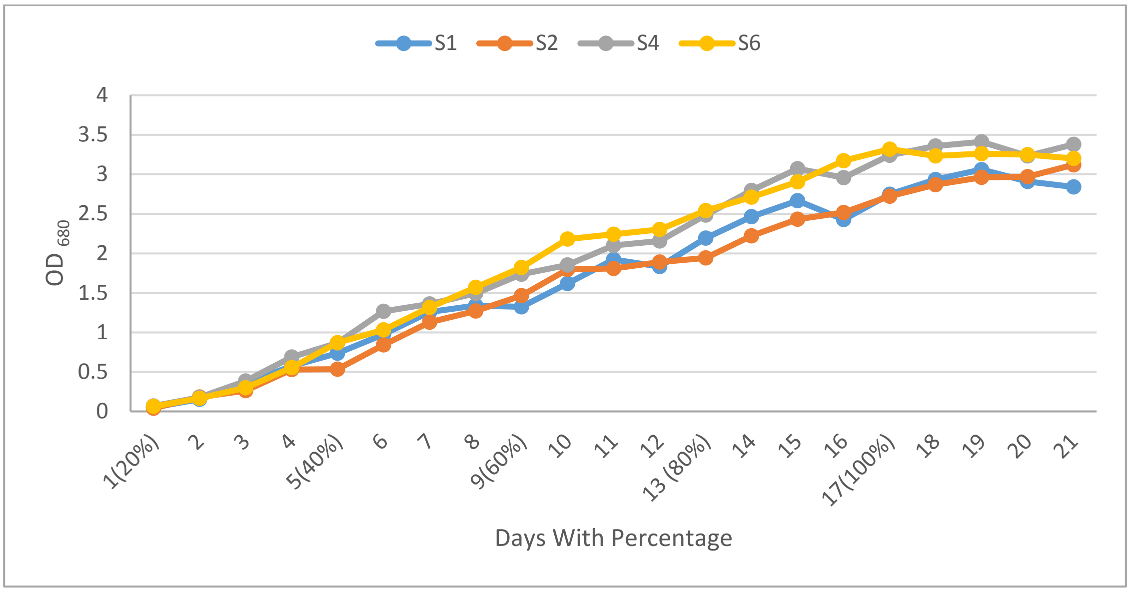


Figure 4.3 Acclimatization of microalgae strains in wastewater

4.5 Acclimatization in Leachate

In leachate, the strains were first acclimatized in 20% concentration. After words, the concentration of leachate was increased by 10% after every 4th day, as mentioned in methodology. The algae strains took nearly a week to start significant growth, as represented in figure 4.4. All strains showed satisfactory growth up to 50% leachate concentration, however it was less than the growth observed in BBM. After that, the growth of microalgal strains started to decline. Strain S6 showed maximum growth in leachate, which was 55% more than least growth shown by S1. Mustafa and fellows (2012) also reported the similar trend in their study. Out of the five microalgae species tested, only *A. convolutus, E. gracilis* and *S. quadricauda* grew well in the medium containing up to 50% leachate. They found that due to toxicity, the specific growth rates of the algae species started to decrease with the increasing leachate concentration (Mustafa et al., 2012).

Three microalgae, *Chlorella pyrenoidosa*, *Chlamydomonas snowiaea* and *C. pyrenoidosa* were grown in landfill leachate by Lin and his mates. Their results indicated the growth of all three algae was inhibited by high leachate concentrations (>30%). They linked the growth inhibition due to high concentrations of ammonia nitrogen, usually present in leachate, as it inhibits the photosynthesis process and carbohydrates assimilation in microalgae (Lin et al., 2007). Pereira and fellows also evaluated *Chlorella vulgaris* for biomass production and nutrients removal from different compositions of a landfill leachate. The results have shown that *C. vulgaris* was able to grow in the different leachate compositions assessed. However, microalgal growth was higher in the cultures presenting the lowest N–NH_4^+ concentration (Pereira et al., 2016).

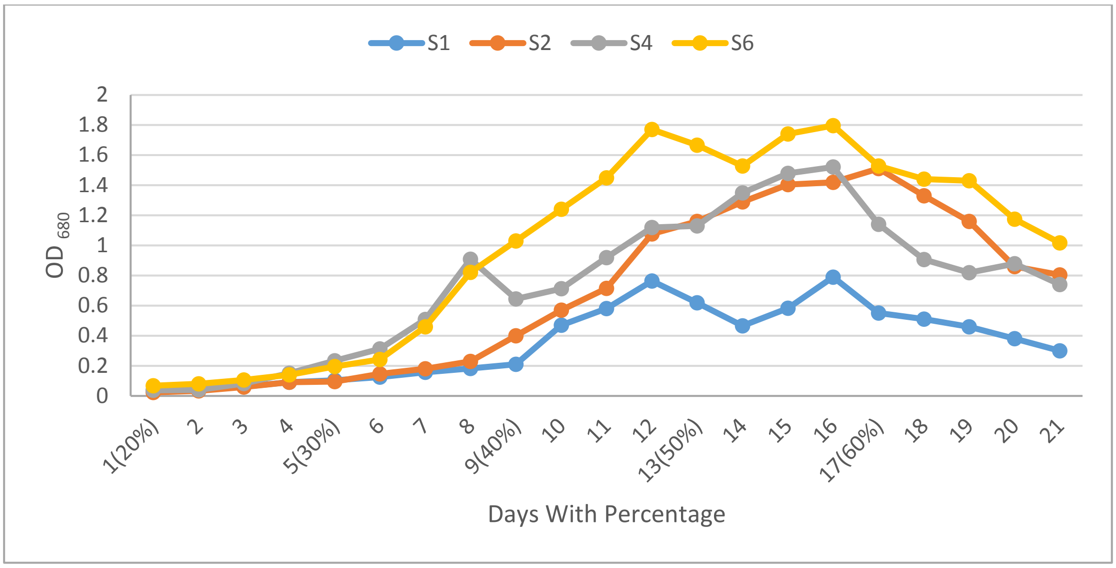


Figure 4.4 Acclimatization of microalgae strains in leachate

4.6 Treatment of Wastewater and Leachate

After the acclimatization, acclimatized algae strains were cultivated in wastewater and leachate. As discussed in methodology, nitrates, phosphates, COD, heavy metals concentrations and biomass growth were determined.

4.6.1 Nitrates removal

4.6.1.1 Nitrates removal from wastewater

All the selected microalgae strains were grown in wastewater medium under similar conditions. The initial concentration of nitrates (NO_3-N) in wastewater was 22.2 mg/L. All the strains showed efficient removal of nitrates from the wastewater during the 15 days growth period (figure 4.5). Strains S6 and S4 showed 100% and 99% removal of nitrates by 12th day, respectively. Whereas all the nitrates were consumed by S1, S2 and S4, during the 15 days incubation period. This result is supported by Sayadi et al, (2016). They grown microalgae *Spirulina platensis* and *Chlorella vulgar* in 1 L of municipal wastewater. The highest nitrate removal after 8 days of growth for *Chlorella vulgaris* was 89.80% and for *Spirulina platensis* it was 81.49%. Moreover, Franchino and his fellows observed nutrient removal from an agro-zootechnical digestate and reported more than 99% of ammonia nitrogen removal during 14 days study for *C. vulgaris* and *N. oleoabundans* regardless how high initial concentration was (Franchino et al., 2013).

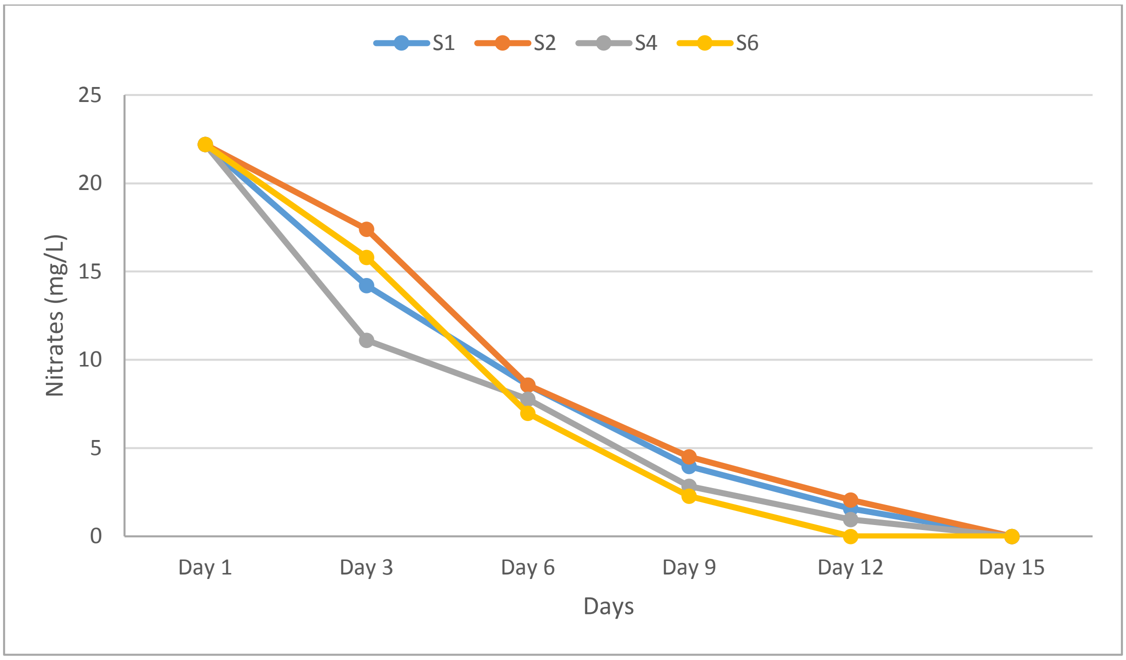


Figure 4.5 Removal of nitrates from wastewater by microalgae strains

4.6.1.2 Nitrates removal from leachate

Removal of nitrogen from leachate is depicted in fig. 4.6, having the initial concentration of 98.41 mg/L. During 15 days of treatment, the nitrate concentration in leachate showed an almost linear decreasing trend. In case of leachate, all the strains showed nearly similar nitrates removal efficiency of 98.3 ±0.4%, however S1 and S4 showed a relatively rapid decrease of nitrogen concentration as compared to S2 and S6. These results are supported by Aisien et al., (2010). They reported 93.8% of nitrates removal form leachate by the phycoremediation through algae. Mustafa and colleagues in 2012, screened a consortium of five species of microalgae *Chlorella vulgaris, Scenedesmus quadricauda, Euglena gracilis, Ankistrodesmus convolutus* and *Chlorococcum oviforme*, for their ability to grow and treat 50% diluted landfill leachate and observed 99.9% removal of ammonia nitrogen from the leachate (Mustafa et al., 2012).

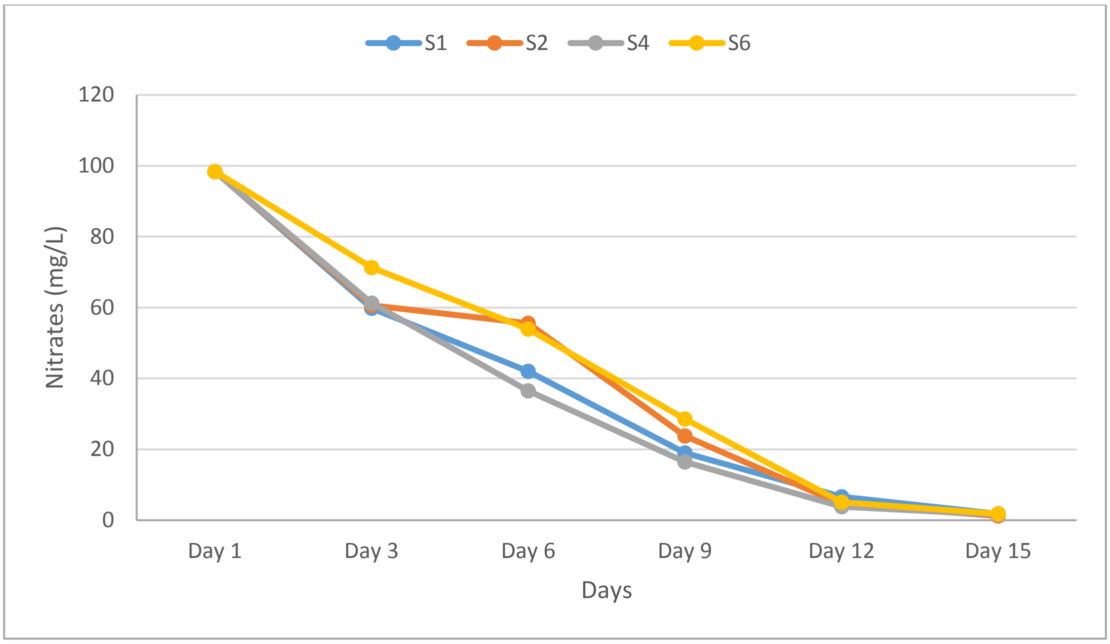


Figure 4.6 Removal of nitrates from leachate by microalgae strains

4.6.2 Phosphates removal

4.6.2.1 Phosphates removal from wastewater

Similar to the nitrate decrease, phosphate concentration also showed a decreasing trend in wastewater by the microalgal treatment. The initial concentration of phosphate (PO_4) in wastewater was found to be 29 mg/L. The highest reduction was given by S4, which was 96.3% reduction of the initial concentration, as shown in figure 4.7. Whereas least reduction of 92% was given by S2. S1 and S6 also showed good phosphates reduction of 95% and 94% respectively. Rasoul-Amini and her fellows in 2014 also reported the similar trend. They found the initial concentration of 19.11 mg/L orthophosphate in the wastewater and it decreased to the minimum value with microalgae *Chlamydomonas sp. (YG04 and YG05)* with the approximate removal efficiency of 100%, while for *Chlorella sp. (YG02)* it was 99% over 14 days (Rasoul-Amini et al., 2014).

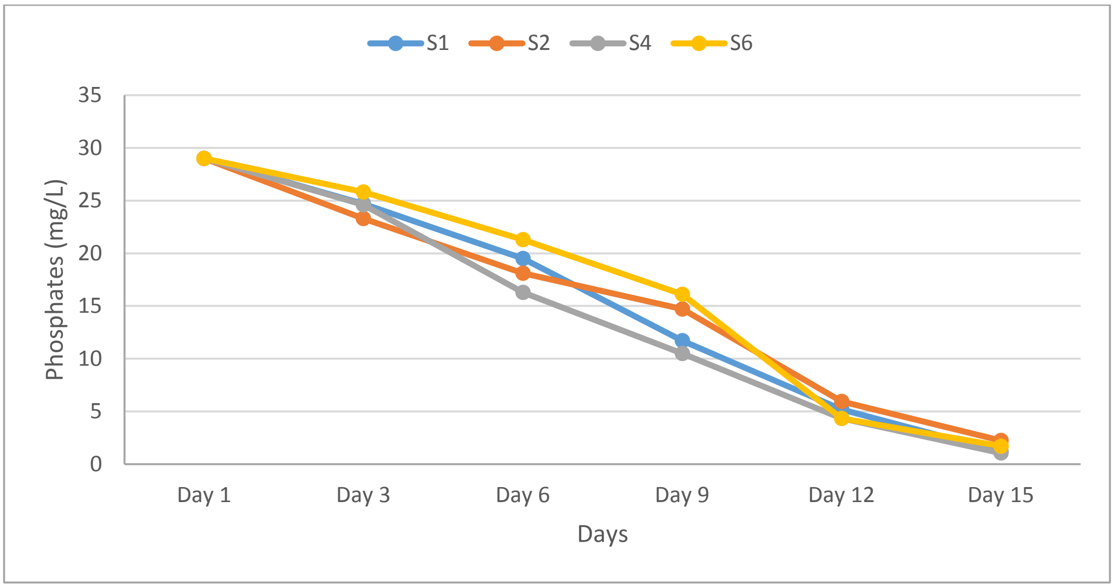


Figure 4.7 Removal of phosphates from wastewater by microalgae strains

4.6.2.2 Phosphates reduction in leachate

The reduction of phosphates from leachate by using microalgae strains is shown in fig. 4.8. The concentration of phosphates 57.5 mg/L was efficiently reduced up to 96.2% by strain S6. Other strains also showed the similar trend, with least removal of

94.3% by S4. It is interesting to note that nearly half of the phosphates were removed from the leachate in first three days of treatment. Pereira and coworkers evaluated the potential of *Chlorella vulgaris* for biomass production and nutrients removal from different compositions of a landfill leachate. They reported 92% phosphates removal for N/P ration of 23:1 and 100% removal with N/P ratio 35:1 over 12 days (Pereira et al., 2016).

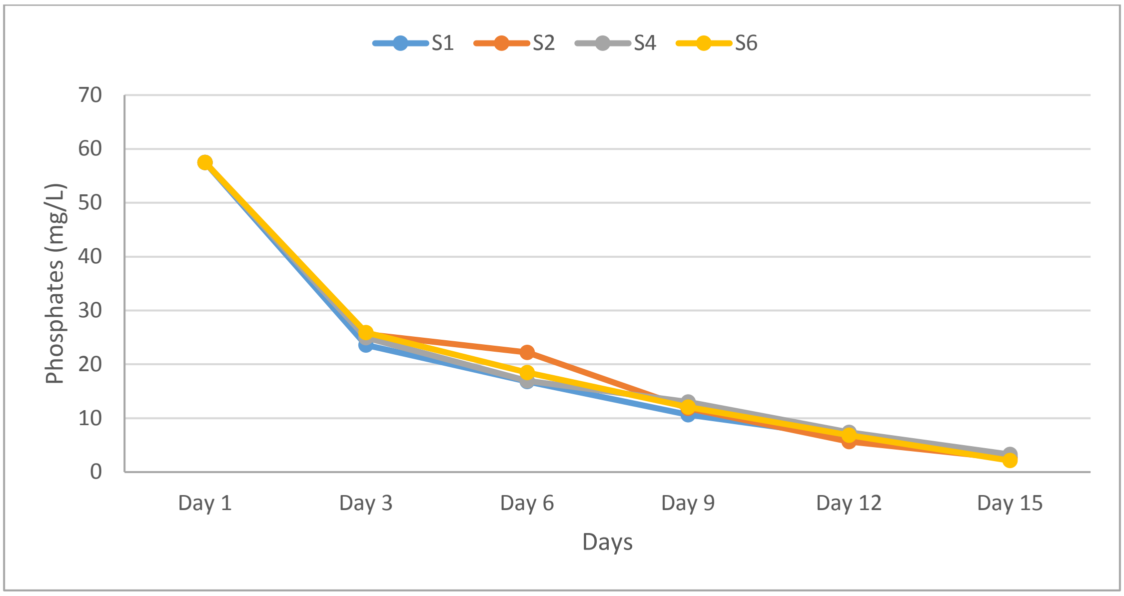


Figure 4.8 Removal of phosphates from leachate by microalgae strains

4.6.3 Reduction of chemical oxygen demand

4.6.3.1 COD reduction in wastewater

Chemical oxygen demand of the wastewater was also monitored regularly as an indicator for the treatment of organic and inorganic contaminants by microalgal strains. During 15 days treatment period, all the strains showed slightly different COD removal efficiencies as depicted in figure 4.9. However, at the end of treatment process all the strains achieved the COD reduction of more than 80%. S4 and S1 showed maximum reduction of 87.7% and 87.4% respectively. Choi and Lee (2012) also evaluated the efficiency of nutrients removal from wastewater, having the initial COD of 270.3 mg/L, by *Chlorella vulgaris*. They found the COD removal efficiency ranging from 78.33% to 82.30%, observed during five runs for 8 days each. In 2017, nutrients and COD removal of swine wastewater with microalgae *Neochloris aquatic* was studied by Wang and his fellows. The result indicated the highest COD removal of 81.7% (Wang et al., 2017).

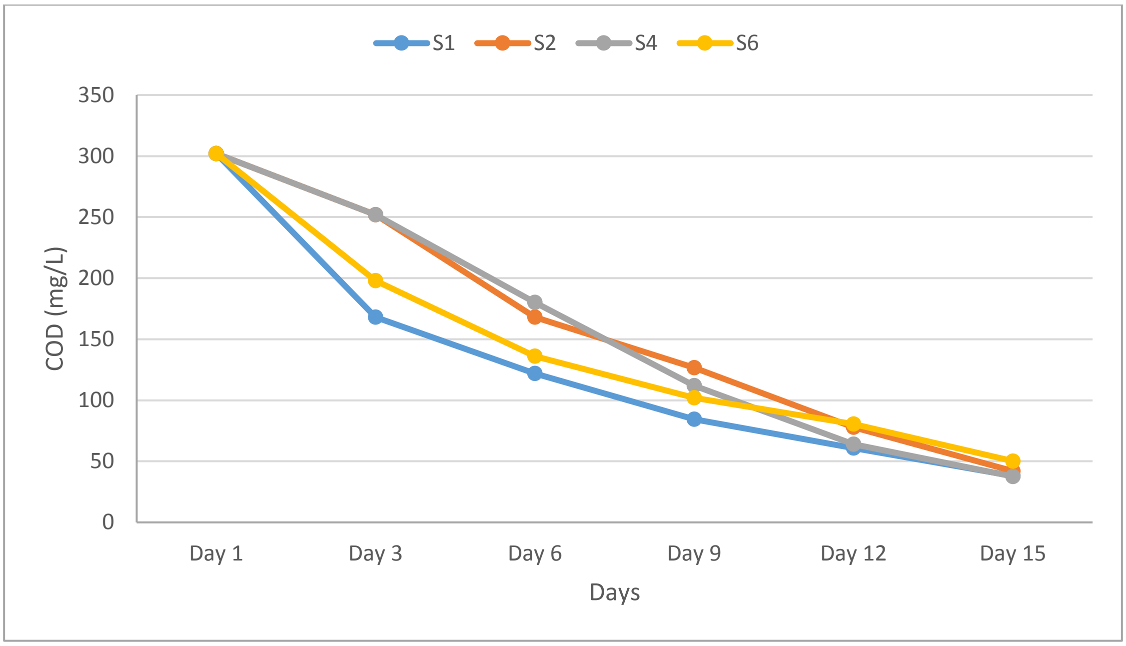


Figure 4.9 COD reduction in wastewater by microalgae strains

4.6.3.2 COD reduction in leachate

In case of leachate, all the strains showed nearly similar COD reduction trend initially, as represented in figure 4.10. The COD concentration, which was 8880 mg/L, was reduced up to 97.5% by strain S4, which is the highest reduction among all four microalgal strains. The least COD removal over 15 days of treatment was 91.7% given by S2. Similar results were found by Mustafa and colleagues in 2012, they screened a consortium of five species of microalgae *Chlorella vulgaris, Scenedesmus quadricauda, Euglena gracilis, Ankistrodesmus convolutus* and *Chlorococcum oviforme*, for their ability to grow and treat 50% diluted landfill leachate and observed 91% of COD reduction in their studies (Mustafa et al., 2012).

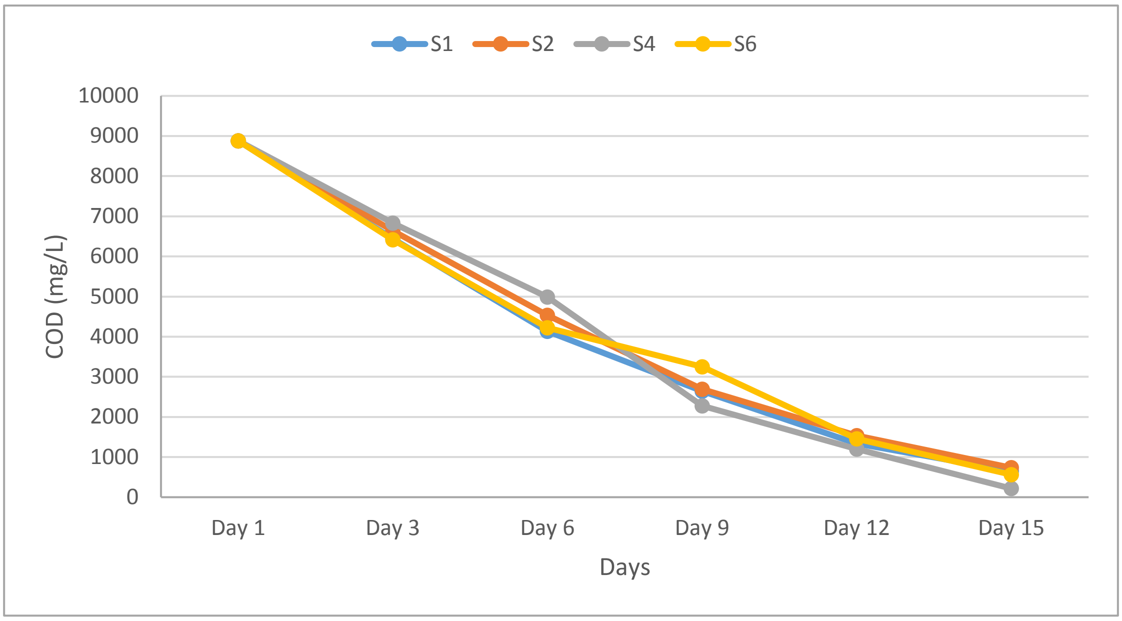


Figure 4.10 COD reduction in leachate by microalgae strains

4.6.4 Uptake of heavy metals

4.6.4.1 Reduction in wastewater

The percentage removal of various heavy metals from wastewater by different microalgal strains, over 15 days of treatment is graphically depicted in fig. 4.11. The initial concentrations of the eight toxic heavy metals are represented in table 2. In wastewater of NUST, none of the observed heavy metal's concentration was above the permissible limits of National Environmental Quality Standards (NEQS) for Municipal and Liquid Industrial Effluents (2016), Pakistan. Microalgae strain S1 and S2 showed the maximum ability for heavy metal's removal, except in the case of zinc, in which S4 showed the maximum performance. As it can be seen in table 4, 96.3% of lead was removed my S1, whereas more than 80% of nickel was reduced by S1, S2 and S4. Mercury metal was also reduced up to 83.2% by S2. Out of all the selected heavy metals, copper and zinc were least removed from wastewater. Highest overall removal efficiency of 63% was shown by S1, while least 39.2% was observed for S6 as shown in table 4.2. However, different removal efficiencies for different heavy metals by each strain can be seen in figure 4.11. This is because the mechanism of the effectiveness in removing heavy metals from wastewater by microalgae is related to their large surface area and high binding affinity. Different algal species have different sizes, shapes, and cell wall compositions, which affect their metal binding efficiency, and the cell wall, in particular, is the main binding site for metals (Wang et al., 2010).

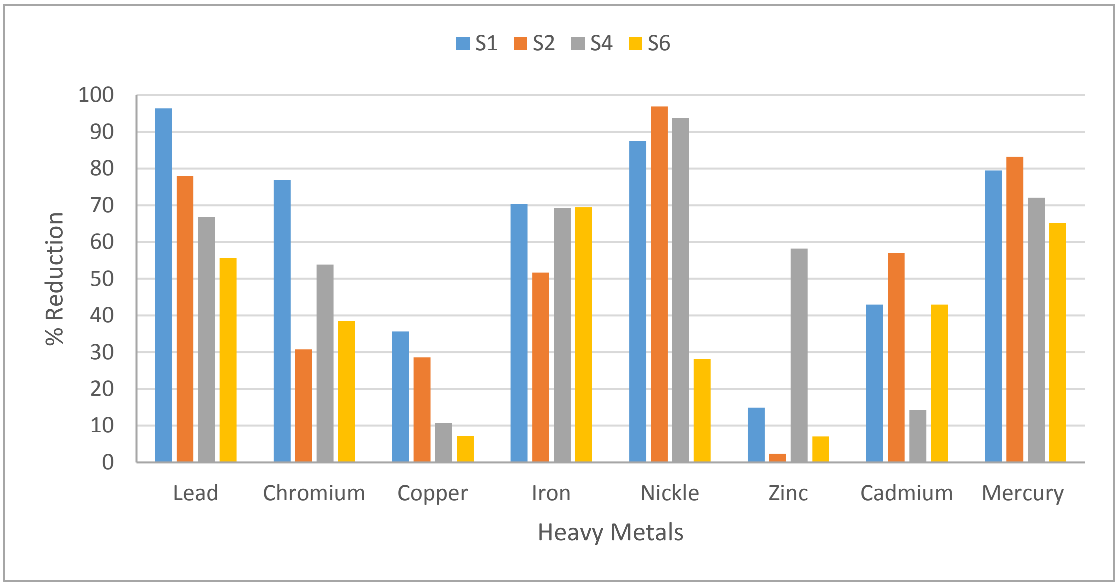


Figure 4.11 Removal of heavy metals from wastewater by microalgae

Wang and coworkers reported also similar results. They cultivated *Chlorella sp.* in four different wastewaters from municipal wastewater treatment plant. The levels of Cd, Cr, and Pb, both before and after algal treatment, were under the detectable limits, while Al, Fe, Mg, Mn, and Zn were found to be removed from all the four wastewaters very efficiently, with removal rates ranging from 56.5% to 100% (Wang et al., 2010). Oleaginous microalgae was used for the heavy metals removal from biogas digestates by Yang and fellows in 2017. During cultivation, the removal efficiencies for 12 metals varied widely from 17% to 97%. Their results also indicate that removal efficiency was different in different trials even for the same metal by same strain. Because heavy metals can be eliminated through a combined process of biosorption and bioaccumulation, but the removal capacity of microalgae may be affected by metal concentration, metal species, culture medium conditions, pH and metabolic requirements, among others factors (Yang et al., 2017).

Table 4.2 Heavy metals reduction (%) by microalgae strains in wastewater

	S1	S2	S4	S6
Lead	96.38	77.96	66.77	55.59
Chromium	76.93	30.77	53.87	38.44
Copper	35.69	28.61	10.71	7.17
Iron	70.33	51.69	69.2	69.44
Nickle	87.49	96.88	93.77	28.12
Zinc	14.89	2.33	58.23	7.07
Cadmium	42.97	57.02	14.32	42.97
Mercury	79.45	83.20	72.09	65.20
Cumulative Reduction	63.02	53.56	54.87	39.25

4.6.4.2 Reduction in leachate

In case of heavy metals reduction from leachate, all the strains showed good removal efficiencies, however similar to removal in wastewater, S1 was found best for the removal of heavy metals (Figure 4.12). Whereas S4 was found least effective for the removal of chromium and cadmium. Maximum removal of more than 90% was observed for the iron metal by all microalgae strains. In leachate, chromium, iron and nickel concentrations were found more than the permissible limits of NEQS 2010 of Pakistan. Which were efficiently brought below the limits by microalgal strains, except for chromium in case of S4. Overall heavy metals removal percentages of all four strains after 15 days of treatment are shown in table 4.3. The highest overall removal efficiency was observed for S1 (52.9%), whereas the least was shown by S4 (42%).

Richards and Mullins in 2013 evaluated the metal removal using consortium of four common marine microalgae species. All of the metals were observed to decrease in concentration compared to their initial concentration. In two reactors, the concentrations of Ce and La were observed to decrease to zero while Fe and Al decreased by over 95% compared to their initial concentrations (Richards & Mullins, 2013). Kumari and her colleagues used bacterial and microalgal co-culture for the treatment of landfill leachate. After bacto-algal treatment for 10 days, the reductions in

Zn, Cr, Fe, Ni and Pb was found to be 92%, 91.5%, 83.6% and 69.2% and 74.9% respectively (Kumari et al., 2016).

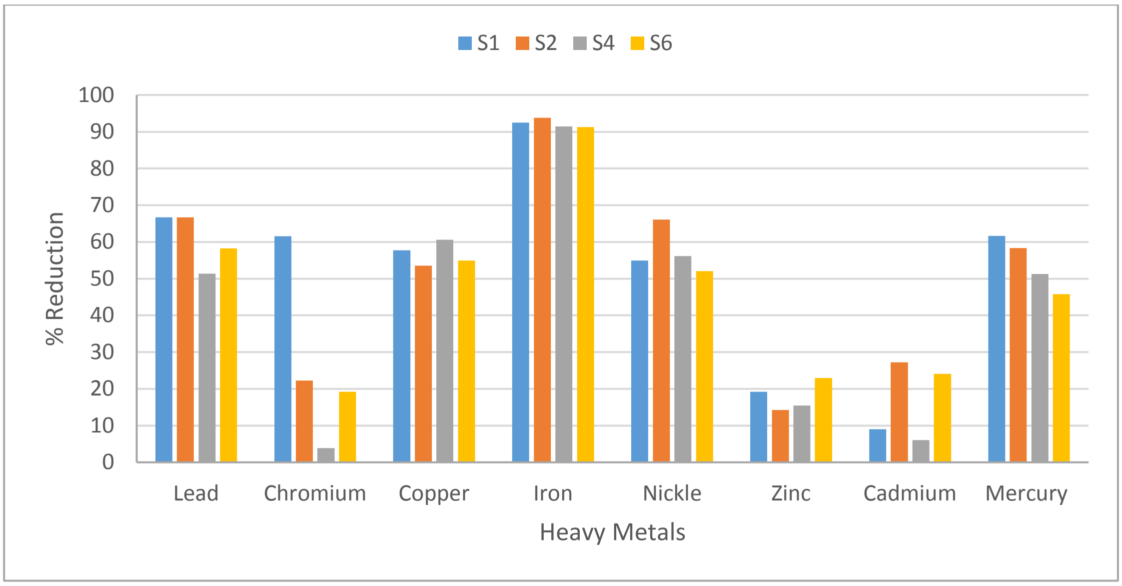


Figure 4.12 Removal of heavy metals from leachate by microalgal strains

Table 4.3 Heavy metals reduction (%) in leachate by microalgal strains

	S1	**S2**	**S4**	**S6**
Lead	66.66	66.66	51.35	58.27
Chromium	61.55	22.22	3.85	19.24
Copper	57.75	53.52	60.55	54.92
Iron	92.45	93.77	91.45	91.24
Nickle	54.97	66.08	56.13	52.04
Zinc	19.22	14.24	15.48	22.96
Cadmium	9.00	27.25	6.08	24.09
Mercury	61.68	58.32	51.26	45.74
Cumulative Reduction	52.91	50.26	42.02	46.06

4.7 Microalgae Growth

4.7.1 Biomass in wastewater

The growth of four selected microalgae strains in wastewater during its treatment is depicted in terms of their biomass, as shown in figure 4.13. All the strains showed significant biomass generation. However, maximum average biomass was produced by S4, which was 2.79 g/L, while S6 produced only 1.9 g/L, the least among all four strains. It was 22% less S4. The average biomass produced by S1 and S2 was 2.53 mg/L and 2.77 mg/L, respectively. The variations in the biomass productivity of microalgae strains can be due to some heavy metals and organic and inorganic compounds in wastewater that have toxicity for some microalgae (Jias et al., 2017). These results are supported by Zhu and fellows (2013). They used freshwater microalgae *Chlorella zofingiensis* for different concentrates of piggery wastewater treatment, the biomass produced over 10 days of treatment of different concentrations of wastewater ranged from 1.06 mg/L to 2.96 mg/L (Zhu et al., 2013).

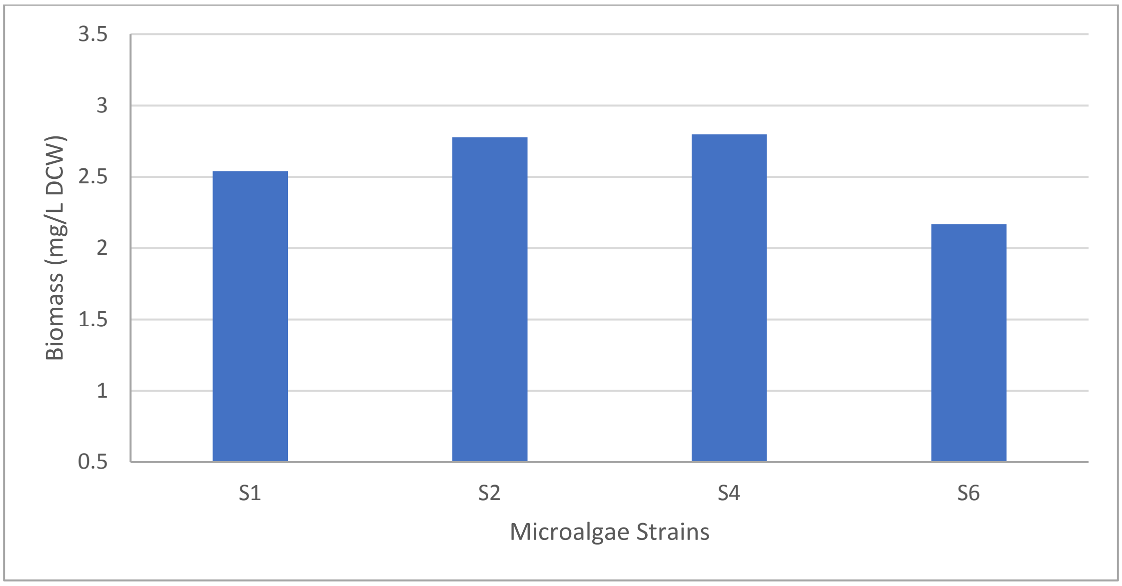


Figure 4.13 Average biomass produced in wastewater by microalgal strains

4.7.2 Biomass in leachate

The average biomass produced by the microalgal strains in leachate was higher than the biomass produced in wastewater. This is due the higher concentrations of essential nutrients, present in the leachate as shown previously in table 3 (Juneja et al., 2013). Maximum average biomass was given by S2 (3.23 mg/L) which was 7% more than the lowest. Other strains S4 (3.21 mg/L), S6 (3.1 mg/L) and S1 (3 mg/L) also indicated good biomass production rate as depicted in figure 4.14. These results are in compliance with Mustafa et al, (2012). They found that the mean biomass of *C. vulgaris* and *Spirulina sp.* grown in landfill leachate with different loading rates ranges from 2 mg/L to 5.5 mg/L (Mustafa et al., 2012).

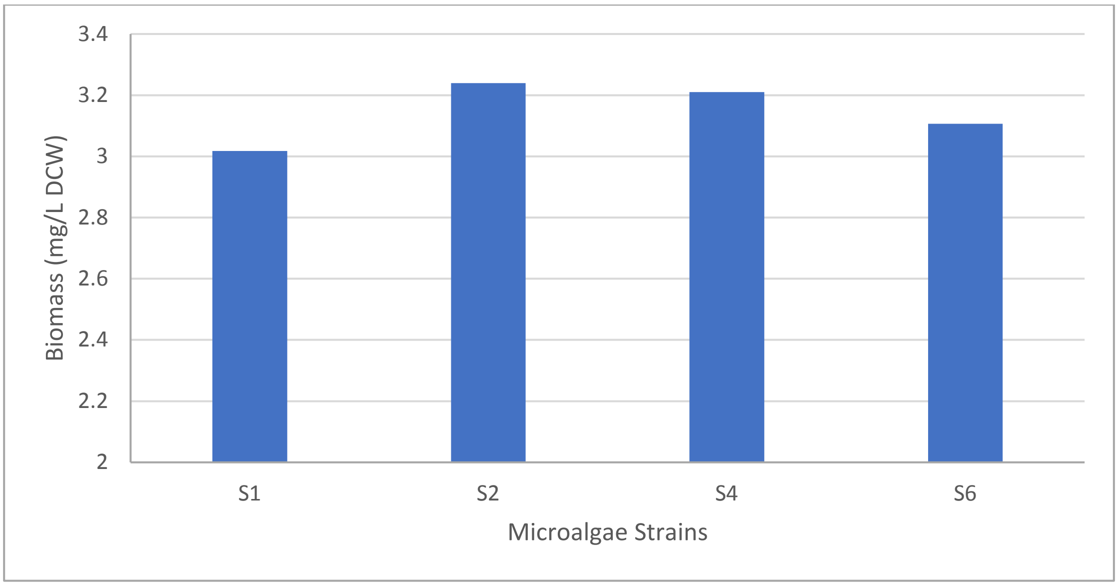


Figure 4.14 Average biomass produced in leachate by microalgal strains

4.8 Evaluation of Lipids Yields

Microalgal lipids were extracted using modified bligh and dyer method, as explained in methodology section. The maximum lipid content of 20.5% was obtained from S6, which was 42% more than the least lipid content of 11.7%, as extracted from S2. The lipids yield of S1 and S4 are within the moderate range of 12.1% and 14.1%, respectively as shown in figure 4.15. The results indicated that the lipid contents of selected microalgae ranged from low to moderate level. As the lipid content mentioned in literature, in microalgae varies from about 1–85% of the dry weight (Chisti, 2007; Chisti, 2008; Rodolfi et al., 2009) and it is interesting to note that, among other factors, lipids quantity is affected by the nutritional composition of the medium. Lipid accumulation in algae typically occurs during periods of environmental stress, including growth under nutrient-deficient conditions (Chen et al., 2011), whereas the wastewater, leachate and BBM media used in this study were nutrient rich mediums.

However, the lipid yields of microalgae, grown in nutrient rich medium showed the results similar to this study. In 2011, Yeesang and Cheirsilp isolated four green microalgae strains (TRG, KB, SK, and PSU) identified as *Botryococcus sp*., when grown in nutrient rich medium the strains achieved a lipid content of 25.8%, 17.8%, 15.8% and 5.7%, respectively (Yeesang & Cheirsilp, 2011).

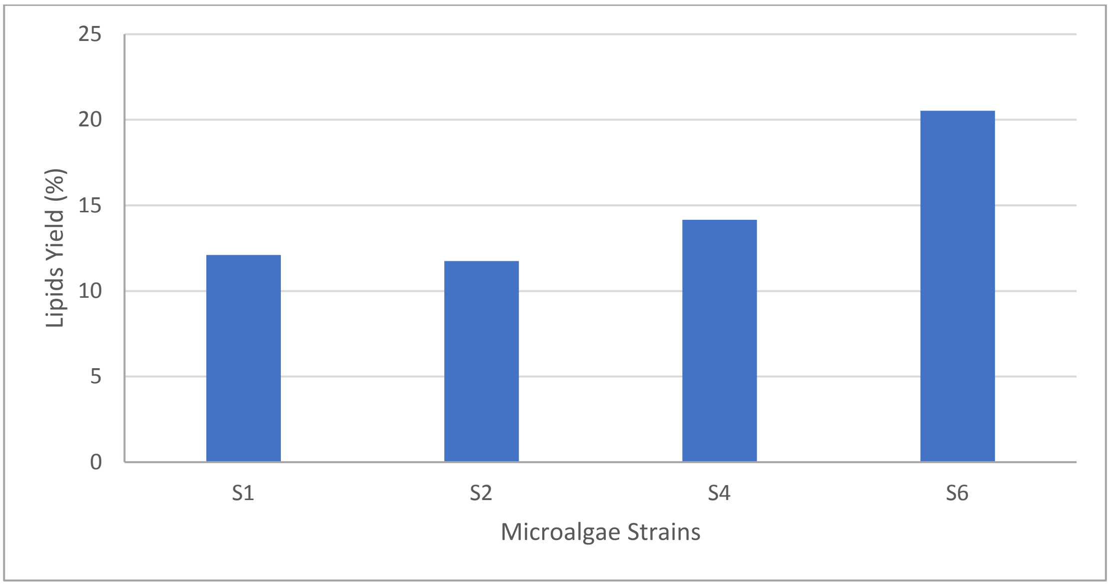


Figure 4.15 Lipid yields obtained from dried biomass of microalgal strains

4.9 Biodiesel Production

The amount of biodiesel and alkyl esters present in biodiesel in percentage, produced from the lipids of microalgae strains through esterification are represented in fig 4.16. It can be seen from the figure that highest biodiesel yield was given by S4, which was 93%, while 89% S4's lipid were also converted to esters, making it the best strain for biodiesel production among all four strains. Whereas the least convertible lipids were found of S2. Under the same conditions, S2 produced 60.6% esters, which is almost 32% lower than the highest yield given by S4, whereas the biodiesel yield of S2 was 88%. S6 produced 89% biodiesel while the lowest yield was given by S1, which was 82%. Strain S1 also showed low ester content of 65.1%. Whereas after S4, S6 achieved the good ester yield of 85.6% as well. Li et al, (2010) reported the biodiesel yields of *Chlorella pyrenoidosa,* ranging from 69.4% to 94.3%, with respect to different methanol ratios used in transesterification.

Although percentages of alkyl esters produced from the lipids of all four strains do not meet the international standards for biodiesel (ASTM) for commercial automobile uses, which require 96.5% alkyl esters in 100% blend. However, the yields of this study are comparable to the results reported in literature. Pinho and coworkers in 2017, observed 75% of conversion of *Chlorella sp*.'s lipids into biodiesel by single step direct esterification by H_2SO_4 and methanol (Pinho et al., 2017). While Rahman and fellows in 2017 developed a two-step process for the production of biodiesel from microalgae S*pirulina maxima* and achieved maximum biodiesel yield of 86.1% under best operating conditions (Rahman et al., 2017).

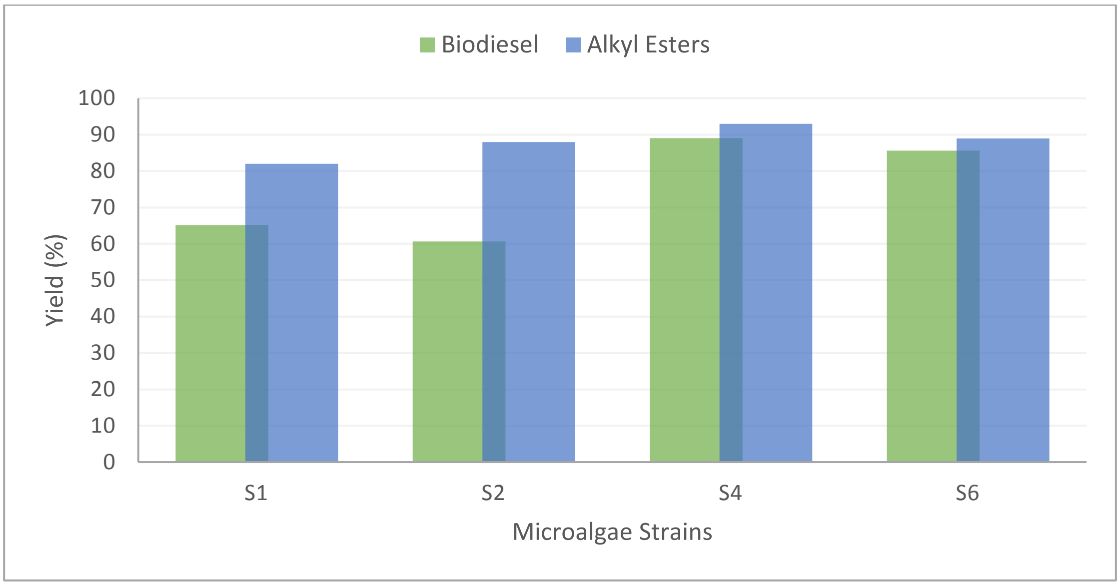


Figure 4.16 Biodiesel and alkyl esters yields obtained from microalgal lipids

Chapter 5

Conclusions & Recommendations

5.1 Conclusions

Microalgae species are capable of growing in all types of waters including municipal, industrial wastewater and leachate, this property makes them an ideal candidate for environmental remediation. Currently several types of unit processes exist for the removal of nutrients from wastewater but these are costly and produce high sludge content. Microalgae can be proposed as an alternative biological treatment to remove nutrients. The major effect of releasing wastewater and leachate, rich in organic compounds and inorganic chemicals such as phosphates and nitrates is mainly eutrophication and ground water contamination by heavy metals. This problem can be solved by the use of microalgae. The main advantage is that while the microalgae will be removing excess nutrients in the wastewater, there will be concomitant accumulation of biomass for energy production such as biodiesel. From the research conducted, it can be concluded that:

a) All four microalgae strains were able to grow in 100% concentrated wastewater and 50% concentrated leachate.

b) All the strains were able to treat wastewater and leachate (50%) with more than 83% overall reduction in nitrates-N, phosphates and chemical oxygen demand over 15 days of treatment.

c) Among the selected strains, S1 was found best for heavy metals removal efficiency with 63% removal from wastewater and 52.9% removal from leachate.

d) The biodiesel yields of more than 82% were obtained from the microalgal strains, with the highest, 93% yield from S4.

e) For alkyl esters, S6 (*Pectinodesmus sp.*) and S4 (*Dictyosphaerium sp.*) gave the maximum yields of 85.6% and 89%, respectively, thus have the potential to be used as biodiesel.

5.2 Recommendations

Following recommendations are made to be pursued for further research:

a) Locally isolated strains S1 and S2 should be identified as they have potential to be used for Phycoremediation.
b) The effect of microalgae consortium, for the treatment of wastewater and leachate could be studied.
c) Upscale setup for Phycoremediation of different wastewaters could be made and studied for dual benefits of treatment and energy production.
d) Other methods (chemical or mechanical) for microalgal lipids extraction, for comparing and enhancing biodiesel yields could be evaluated.

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