EFFECT OF NUTRIENTS ON ALGAL GROWTH AND BIOGAS PRODUCTION

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By

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CERTIFICATE

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I dedicate this to my father to whom I owe all my success and fulfilments in life. His encouragement has led me pass every hurdle in life with a smiling face.

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Zara Maqsood

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
COD	Chemical Oxygen Demand
mg/l	Milligrams per Liter
NARC	National Agricultural Research Center
OD	Optical Density
PET	Polyethylene Terephthalate
PVF	Polyvinyl Fluoride
TS	Total Solids
VS	Volatile Solids

ABSTRACT

The major problems that humanity faces in the 21st century are water quality issues and energy supply. In Pakistan, there are mostly simple dumping grounds that are designated for waste disposal and the resultant leachate is discharged without any treatment. On the other hand, only 8% of the wastewater generated by country is treated, while the rest is discharged into streams without treatment. Adding to the dilemma, present human systems are artificially supported by heavy inputs of nonrenewable energy (fossil fuels) to sustain the materialistic lifestyles. There has been an increasing interest in coupling wastewater and leachate treatment to bioenergy production. Microalgae is a novel green technology that possess very high potential to remove pollutants from leachate and wastewater. It is also being explored as source for third generation biofuel to combat the increasing energy crisis. Hence providing us two benefits at once i.e. leachate treatment & sustainable energy. The objective of this study was to observe the growth of microalgae strains in wastewater and leachate, along with consumption of nitrate and phosphate. Three strains (S4, S5, S6) were collected from ASAB, NUST, while one strain was isolated from a waste water stream. The four microalgae strains were acclimatized and grown in 100% wastewater and 50% leachate. The strain 3 performed well in wastewater with highest average optical density of 2.56, almost 100% nitrate reduction and 57% phosphate reduction during 15 days of growth period. While strain 4 performed well in leachate with optical density of 2.43 with 98% nitrate reduction and 97% phosphate reduction. The overall results indicated their capability to support microalgae growth. The results clearly indicate the possibility of co digestion of microalgae with wastewater and leachate respectively. The resultant algae was co-digested with wastewater and leachate along with cow dung for biogas production. The co-digestion of strain 6 with leachate gave highest methane content of 61.5% and co-digestion of strain 4 with wastewater gave the highest methane content of 57.6% in biogas. The study concluded that coupling microalgae based treatments to bioenergy production has a potential and can be applied in future for sustainable development.

CHAPTER 1

INTRODUCTION

1.1 Background

Increase in population is putting pressure on natural resources which in result is causing environmental pollution. At present, human systems are artificially supported by heavy inputs of nonrenewable energy e.g. fossil fuels (*i.e.* petroleum, coal and natural gas). As a community, human civilization is artificially supported in both the creation of inputs and the treatment of waste products. However, new options of renewable energy need to be explored which are sustainable, manageable and secures the future of upcoming generation.

In order to sustain various activities associated with daily life in today's world, energy is direly needed. This is extracted and put into use from different conventional sources which include fossil fuels, thermal, hydro-power, wind and nuclear energy. A proportion of energy is extracted from non-conventional resources i.e. biomass energy which is becoming the most popular among all other forms. According to world energy council 2016 report, in the energy mix, the portion of renewables i.e. 18% comprises of 14% bioenergy and it is the largest proportion of renewable energy making it 10% of global energy supply (World Bio Energy Association, 2016). Bioenergy is promoted as more sustainable and alternate source for hydrocarbons in developed countries, especially for transportation fuels, like biodiesel and bioethanol, residential heating and the use of wood in combined heat and power generation. It represent opportunities for domestic industrial development and economic growth in developing countries.

As a general rule energy is a prerequisite for a country's economic development. Pakistan is currently facing a serious energy deficit. This has led to increase in fossil fuel prices. In order to fill this gap, research on developing the alternative biomass for bioenergy has become increasingly important. One of the growing resources of renewable energy is bioenergy, which can provide solutions for environmental concerns arising from fossil fuel and can be produced using agricultural derived biomass quite a successfully (Elliott et al., 2013). But negative sides of this approach include resource depletion and the competition of land usage (Ras et al., 2011). However, according to research studies, microalgae have shown to

convert sunlight energy to biomass along with low land use footprint and a total yield that is high.

1.2 Algae -A Bio resource

Algae is a diverse group of photosynthetic organisms, which includes unicellular as well as multicellular forms. There are three major group of algae namely: cyanobacteria, green algae and red algae. These are abundantly found in water bodies, land environments and are also found in unusual environments, like on snow as well as ice. Photosynthetic algae is known to contribute to the global production of bio resources and bioremediation i.e. the mitigation of anthropogenic wastes.

Currently, algae is in the spotlight and is being considered in research and development globally as an alternate source of renewable energy. It is a viable alternative to conventional fuel. The biomass from algae has many advantages such as carbon dioxide consumption, oxygen production by photosynthesis and uptake of inorganic compounds from leachate and wastewater (Munoz et al., 2006). Similarly, different macromolecules within algae can be converted to biofuels such as bio ethanol, bio diesel and biogas. Using microalgal biomass among biofuel production, biogas production seems promising to exhibit lower environmental impacts and seems less complex as extraction is not required from biomass (Mendez et al., 2016). Algae are excellent for removal of nutrients processes as they show several times higher nitrogen and phosphorus concentrations than other plants, about 10% and 1% of the dry weight, respectively. Because algae produces oxygen as a byproduct of photosynthesis they can increase the concentration of dissolved oxygen in water. Algae have also been shown to have an excellent capability to remove heavy metals from water. The algae use for nutrient removal is beneficial than other methods because it is a continuous treatment process and does not physically disrupt the natural ecosystem (Mendez et al., 2016).

1.3 Nutrient Pollution- An Environmental Concern

Nutrient pollution can be defined as the excessive input of nutrients into surface waters, it can also be referred as a form of nutrient pollution. This is also one of the primary causes of eutrophication. According to the US EPA, water nutrient pollution is one of the most costly and omnipresent problems facing not only the

United States but worldwide. Nutrient pollution in the form of excess nitrogen and phosphorus can have a far-reaching effect on water quality, health, and the economy (Sohi et al., 2010). Human activities have increased the flow of nutrients to estuaries and other coastal marine systems over the last half century and the input is likely to escalate globally as a result of consumption of fossil fuels and use of inorganic fertilizers in agriculture by humans, the two ascendant sources of nutrients, continues to grow on a global basis. Worldwide, the effects of nutrient pollution can be far-reaching because significant portions of the mobilized nutrients are transported to rivers & streams and end up in coastal zones. Excess nitrogen and phosphorus cause an over-enrichment of water bodies and can have detrimental effects on the life of marine species (Zhang & Huang, 2011).

1.4 Leachate and Wastewater – Source of Nutrients

On the other hand, wastewater and leachate are also a rich source of nutrients. As the runoffs from fields and the nutrient rich surface waters wash into the waste streams and contaminate them to the extent that they have rich quantities of nutrients. While the leachate at most sites possess these nutrients along with heavy metals and other harmful substances.

1.4.1 Leachate and its characteristics

A major problem for municipal solid waste (MSW) landfills is leachate generation and it is a cause of significant threat to surface water and groundwater (Raghab et al., 2013). Leachate can be defined as a liquid that percolates through dumped solid waste and takes along dissolved and suspended matter from it. As a result of precipitation entering the landfill or from moisture that exists in the waste when it is composed, it results in leachate formation. It comprises of high concentration of metals and contain some hazardous organic chemicals and is also characterized by different forms nitrogen, phosphorus and potassium.

1.4.2 Wastewater and its characteristics

Most domestic wastewater contains organic carbon, nitrogen, phosphorus and other compounds, which makes them suitable for microalgae cultivation. Wastewater offers ideal conditions for bacterial growth and decomposition of organic matter by oxygenation; however, bacteria are less efficient in the removal of inorganic nutrients such as phosphorus, which is usually the main cause of eutrophication of freshwater

ecosystems. Thus, an additional final process following bacterial treatment must be applied prior to release into natural waterways, which tends to increase the process cost. Typical municipal wastewater contains approximately 350 mg L^{-1} chemical oxygen demand (COD), 50 mg L^{-1} NH_4 and 10 mg L^{-1} PO_4^{3-}. After its treatment, this effluent contains nutrients such as nitrogen and phosphorus and other trace elements that are known to enhance metabolism and growth of microalgae. Due to this reason, secondary and tertiary effluents have been widely applied as culture media (Zhang and Huang., 2011).

1.5 Third Generation Biofuel

Due to increasing energy crisis, the microalgae biomass has been increasingly promoted as third generation biofuel. They are being researched for their potential in biogas production as they have the potential to grow in aquatic environments and have rapid growth rate. Moreover, they do not compete with food crops. Due to concerns about shortage of energy, the developed and developing nations are exploring the options of harvesting energy by biogas production through algae.

1.6 Problem Statement

Cultivating algae on a large scale for bio resources is a comparatively new idea in the agronomic human history. The mass cultivation of algae and its development of techniques started in mid twentieth century. Algae is the requirement of elemental nutrients, many of which are non-renewable (*e.g.* rock phosphate). Utilizing anthropogenic waste nutrients may allow for the dual purpose of remediation and resource production (Rawat et al., 2011), contributing to the foundation of human sustainability. 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. The co digestion of harvested algae with other substrates produce biogas is the area lacking research. That is why we can say that the potential of microalgae to produce biogas still needs to be studied.

1.4 Objectives

The objectives of the study were to

1. To study the effects of nutrients (nitrogen & phosphorus) on growth of algal strains.
2. To evaluate biogas potential of various algal strains in mesophilic conditions.

CHAPTER 2

LITERATURE REVIEW

In this chapter, different aspects related to research topic are presented that support the research argument and already carried out research is also mentioned.

2.1 Algae- Potential for Renewable Energy Resource

Microalgae are photosynthetic microorganisms and have two major types i.e. Prokaryotic and Eukaryotic. Prokaryotic microalgae is termed as cyanobacteria while eukaryotic algae include green algae and diatoms, including other groups. Microalgae's size is very small, usually measured in micrometers, it is normally found in water bodies or ponds. Green algae represents wide range of autotrophic organisms. Moreover, they are robust microorganisms and they are able to grow under the harshest conditions in aquatic as well as terrestrial environments. Since they are characterized by high cell surface/volume ratio resulting in higher nutrient assimilation. The green algae grows 100 times faster than terrestrial plants and their biomass can double in less than one day. The requirements for micro algal growth are minimal that includes sugar, light, nitrogen, carbon dioxide, potassium and phosphorus and hence are able to synthesize large amounts of lipids, carbohydrates and proteins which after processing can be converted into biofuels (Qui et al., 2017).

The rapid depletion of fossil fuels and the increasing energy crisis is one of the biggest problems of 21st century. One of the promising alternative to counteract this energy crisis is the use of microalgae. The key advantage of importance is the short harvesting cycle of microalgae, as compared to other conventional crops having longer harvesting cycle (Rawat et al., 2011). This source of biomass have significant growth rates and can be harvested to produce different biofuels including the biogas. The main requirement for algal growth include a supply of inorganic nutrients, sufficient light and favorable temperatures. Micronutrients required for growth and enzymatic activity include calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), iron (Fe), manganese (Mn), sulfur (S), zinc (Zn), copper (Cu), and cobalt (Co). While macronutrients include nitrogen, phosphorus and carbon. Different algal species have different nutritional needs and vary from specie to specie. Photosynthetic

algae have significant potential to contribute to the global production of bio resources and the mitigation of anthropogenic wastes (Rawat et al., 2011).

2.2 Wastewater – Growth Medium for Algae

The water that has been adversely affected in terms of quality due to anthropogenic activities is termed as "wastewater". The wastewater originates from a combination of domestic, commercial and industrial activities, surface run off or from storm water. The wastewater is characterized by physical and biological pollutants. Wastewater is treated conventionally through a series of treatments i.e. primary, secondary and tertiary which are based on physical, biological and chemical processes. These biological treatments are highly efficient, not dependent on outdoor conditions and requires less space. However, the disadvantages of these treatments outweighs the benefits i.e. constant high electrical energy requirements, the economic costs for design functioning, maintenance, supervision and the general cost of construction and highly skilled workers. The disposal of the waste i.e. sludge is also an issue. However, in most of the developing countries it is discharged into water bodies without treatment. Hence, it contaminates not only the surface waters but also poses serious public health concerns. The untreated wastewater generally compromises of industrial and domestic wastewater (Murtaza and Zia., 2012). The main causes for eutrophication of water can be attributed to discharge of secondary effluents containing nutrients (NH^{4+}, NO_3 and PO_4^{3-}) from wastewater treatment plants. Therefore, the wastewater must receive suitable treatment before being discharged into water bodies (Ruiz-Marin et al, 2010).

In Pakistan, since there is no check on the kind of effluents being discharged into waste streams and open dumping of waste is leading to pollution of surface and ground waters. Other than that domestic waste water is usually discharged into streams without any treatment and it is hardly subjected to biological treatment in any cities other than Islamabad and Karachi. The previous suggests that through treatment pants, a negligible portion of 8% urban waste water is treated (Murtaza & Zia. 2012).

2.3 Leachate – Source for Nutrients for Algae

The disposal of solid waste either in landfill or in open dumps, both result in production of leachate which is a liquid that in course of passing through solid waste dissolves environmentally harmful substances. It is becoming one of the major concerns in rural and urban areas around the globe. If the waste generated will not be handled effectively and properly than there will be serious public health and environmental concerns. In order to avoid these problems, there should be proper understanding about the waste generation and handling which varies from area to area. The different waste disposal practices include engineered or secured landfills and the second is open dumping (Foo & Hameed, 2009).

Open dumping is broadly practiced all over the world because of the less cost incurred in terms of capital and management for waste (Renoua et al., 2008). But the downside of this method is the production of leachate which is highly concentrated with pollutants. Consequently, it has environmental drawbacks of ground water pollution and ultimately leading to public health concerns (Aziz et al., 2004). Landfill leachates are typically characterized by excessive and concentrated levels of chemical oxygen demand (COD), dissolved metals, VOCs, ammonia-nitrogen and xenobiotic organic compounds (Kjeldsen et al., 2002).

On the other hand, both wastewater and leachate can be seen as a source for cultivating algae without additional cost and with right functional parameters. Microalgae is a diverse group of photosynthetic organisms and they are well known to be able to grow in wide range of conditions including aquatic environments, hence reducing the terrestrial land use. Moreover, they are also being considered for bioremediation and for our interest as a source of biofuel and biogas production. Not only they can remove excessive nutrients but also help in mitigating climate change.

2.4 Phytoremediation

Bioremediation using microalgae, macroalgae and cyanobacteria (phytoremediation) for removal or biotransformation of xenobiotics from wastewater or pollutants from gaseous effluents is an emerging technology that shows great promise due to its economic viability and environmental sustainability. The tertiary treatment of urban wastewater in maturation or facultative ponds requires microalgae due to its metabolic capabilities of removing

heavy metals, nutrients and producing secondary metabolites that inhibit growth of pathogens.

2.5 Factors Affecting Growth of Algae

2.5.1 Nutrients

A critical factor in the sustainability of photosynthetic resource production via algae is the requirement of elemental nutrients, many of which are non-renewable (*e.g.* Rock phosphate). Utilizing anthropogenic waste nutrients may allow for the dual purpose of remediation and resource production (Rawat et al., 2011), contributing to the foundation of human sustainability

The high potential of microalgae to eliminate inorganic nutrients using wastewater as cultivation medium has been studied. The study investigated the growth and nutrient uptake of fresh water microalga *Scenedesmus* which resulted from nitrogen and phosphorus concentrations in the medium. The studied showed that initial nitrogen or phosphorus concentration resulted in increased maximum algal density. Phosphorus could be almost 100% eliminated at provided N/P ratios conditions, however nitrogen uptake was affected by the N/P ratios. 83–99% nitrogen and 99% phosphorus could be removed from in the nitrogen/phosphorus ratio of 5:1–12:1 (Li et al., 2010).

Hai Xu and coworkers (2010) studied the effect of nitrogen and phosphorus loading on phytoplankton growth in lake Taihu situated in China. The results showed that in winter the growth and biomass was increased significantly, with no primary effects from N, with additions of P suggesting P limitation of phytoplankton growth and that availability of N is a key growth-limiting factor during summer for the growth and maintenance of toxic *Microcystis* spp. Blooms (Xu et al.,2010).

The effect of different nitrogen sources on biomass productivity of green alga *Neochloris oleoabundans* was studied. The different sources of nitrogen were urea, ammonium bicarbonate and sodium nitrate. A modified soil extract was used as a medium along with cultivation conditions as 30^{+-} temperature with continuous illumination of 360 umol/(m^2.s^1). Ammonium bicarbonate under the investigated conditions can only support poor growth of *N. oleoabundans* while the best nitrogen source was sodium nitrate with the maximum biomass obtained i.e.

2.5g/l. The sodium nitrate concentration varied from 3, 5, 10, 15 and 20mM and showed that cell growth of alga improved 3 to 10 mM and was impacted negatively as it increased from 10 mM to 20 mM. The highest values for biomass productivity and biomass concentration were reached at 10 mM (Yanqun et al., 2008).

Meng and his fellow workers (2010) studied the effect of nutrients on growth of green algae *Dunaliella tertiolecta*. Of the elements measured, phosphorus exhibited a drastic decrease, during the course of the culture resulting in 84% reduction. As phosphorus is involved in multiple metabolic processes, in the form of phosphate, all organisms require it in relatively high amounts and for biomass production. Medium concentrations of ammonium were tolerated and high levels inhibited growth while high concentrations increased maximum cell densities of nitrate.

2.6 Biogas Production

Biogas is defined as a gas that is produced in the absence of oxygen by the action of microorganisms (Hessami et al., 1996). It can be produced from sewage sludge, municipal waste, animal dung and crop residue as well as biomass. Many factors affect the digestion rate and biogas production including temperature, pH, carbon/nitrogen ratio, water/solids ratio, particle size of the material being digested, mixing of the digesting material and retention time (Vindis et al., 2009). The composition of biogas varies depending upon the composition of substrates, and conditions within the anaerobic reactor such as temperature, substrate concentration and pH. Biogas produced in anaerobic digesters consists of methane (CH_4) 50–70%; carbon dioxide (CO_2) 30–35%; nitrogen (N_2) 1%; hydrogen (H_2) 0.1–0.5%; carbon monoxide (CO) 0.1%; hydrogen sulphide (H_2S) Traces. Biogas can be produced by two different ways i.e. anaerobic digestion and anaerobic co-digestion.

2.6.1 Anaerobic digestion

It can be defined as the biological process of biogas making in the absence of oxygen, which is characterized by breakdown of organic matter and stabilization off these materials. The end product consists of biogas i.e. methane & carbon dioxide and of nearly stable residue i.e. the slurry that is

often used as rich fertilizer source. Basically microorganisms digest organic materials to produce biogas. Biogas has a typical composition of 50 to 65 % (volume) CH_4, 35 to 50 % (volume) CO_2 and trace gases (Sahito et al., 2015).

2.6.2 Anaerobic co-digestion

In the conventional process, single substrate from single source was used in anaerobic digestion. However, in anaerobic digestion process, two substrates can be used simultaneously for digestion. It is supported by literature that co-digestion has the most promising results for biogas production (Mata-Alvarez et al., 2000).

In co-digestion methane yield is higher as compared to the digestion of single substrate. There is a basic substrate and contains major portion of feed which is mixed with minor quantity of single or multiple substrates. In a single system combination of multiple microbes increase the methane yield (Carucci et al., 2005). The opportunity for balancing nutrients efficiently is provided by co-digestion (Montusiewicz et al., 2008). In research related to microalgae, it is found that microalgae biomass has a lower carbon to nitrogen ratio (C:N) than optimal for anaerobic digestion, so focus has been placed on enhancing the C:N ratio for improving the algae digestion (Samson & LeDuy, 1983).This technique relies on the hypothesis that the low C:N ratio present in typical algae biomass produces compounds inhibitory to the digestion process, mainly ammonia, when digested. By co-digesting algae with low-cost, high-carbon wastes, the ammonia nitrogen concentration can be diluted, potentially decreasing ammonia inhibition. Yen and Brune (2007) co-digested waste paper with algae and found an optimum C:N ratio for methane production of 20:1-25:1, similar to the optimum for other substrates.

2.7 Methane Production Mechanism

In a process of anaerobic digestion various sets of different microorganisms act in four different phases a series. The organic material is decomposed in four important steps of anaerobic digestion named as hydrolysis, acidogenesis, acetogenesis and methanogensis (Jarvis, 2004).

2.7.1 Hydrolysis

In hydrolysis change in insoluble organic compounds that has high molecular mass, i.e., carbohydrate, fats, protein and lipids are facilitated by enzymes (Yadvika et al., 2004). The strong chemical bond between large

molecules that contains numerous small molecules that are tightly linked. The bond must be broken down before they enter through the wall of cell of bacteria. The process of hydrolysis is carried out by Various different facultative and anaerobic bacteria (Yadvika et al., 2004).

2.7.2 Acidogenesis

In the phase of acidogenesis, dissolvable compounds that were produced in the hydrolysis are more degraded by an assortment of facultative anaerobes in various procedures of methane formation. H_2, natural nitrogen mixes CO_2, natural, alcohols, sulfur mixes and other natural acids are the consequences of fermentation procedure. Here at this stage, acetic acid generation is the most critical as it is the significant acid utilized as a feed for methane-producing microbes (Gerardi, 2003).

2.7.3 Acetogenesis

It can be defined as a process in which acetate is produced from CO_2 and an electron source through anaerobic bacteria. In this stage, there is no reasonable refinement between Acetogenesis and Acidogenesis response. In this progression, the Acetogenesis microorganisms degrade the hydrogen sinks acids like propionic, butyric and valeric acids into formate, acetic acid derivation, CO_2 and hydrogen(Gerardi, 2003).

2.7.4 Methanogenesis

Methanogenesis is the final and last stage, in which methanogenic microorganisms convert acetic acid, H_2 and CO_2 to methane and carbon dioxide called as biogas. Remaining substrate composition like natural nitrogen, alcohols and so forth that are left finished and can't be changed over by methanogens are gathered as digestate (Gerardi, 2003).

2.8 Functional Parameters for Production of Biogas

There are various functioning parameters that are important for biogas production. Significant parameters that have a major effect on biogas generation are temperature, pre-treatment, pH, agitation, rate of organic load, retention time, particle size, etc. The parameters, if changed suddenly can affect dangerously the production of biogas (Yadvika et al., 2004).

2.8.1 Temperature

One of the most important factors is temperature inside the digester which have extreme effect on biogas production process. There are three

temperature ranges at which methane is produced naturally which comprises of psychrophlic (<25 °C), mesophilic (25–45 °C), and thermophilic (50–70 °C) (Yadvika et al., 2004). In mesophilic and thermophilic temperature ranges anaerobic microorganisms are more energetic. Higher the temperature range results in higher the methane yield and more degradation of organic matter that is helpful to reduce the amount of any particular substrate required. At higher temperatures, anaerobic digestion kills harmful microorganisms thus helps in better sanitation. Since degradation is highest at thermophilic temperature range as a result any change like ammonia inhibition is very sensitive. The operational and upkeep cost for thermophilic process are higher when contrasted with mesophilic process because of their heat requirements (Yadvika et al., 2004). Whereas higher retention time is required by mesophilic digestion from 30 to 40 days as compared to thermophilic process requiring only 15 to 25 days as temperatures are higher. In mesophilic range gas production is delayed than in thermoplic (Jarvis, 2004).

2.8.2 pH

The pH range between 6.0 – 8.0 inside the digester is the ideal pH for better execution of microorganisms. So microorganisms and the enzymes produced by them can best survive in the previously mentioned conditions of pH and any adjustment in pH range which is either above or underneath this range can seriously restrain the procedure of anaerobic assimilation. Amid the procedure of anaerobic degradation of natural issue at times a circumstance emerges in which pH is unfavorably influenced by various reasons like high estimations of VFA (Volatile Fatty Acid), CO_2, acids, and smelling salts. Any pH change because of these elements contrarily influences bacterial movement and can control the absorption procedure (Yadvika et al., 2004)

2.8.3 Carbon to nitrogen (C/N) ratio

Influent substrate must contain carbon to nitrogen ratio in the preferred range for effective functioning of biogas plant as bacterial growth and its activity is affected by balance in nutrient composition. The nutrients required by anaerobic microorganisms for digestion are carbon and nitrogen. The carbon value should always be 20-30 times more than nitrogen for the anaerobic digestion. In order for best working of microbes C/N ratio should be 20-30:1 with the significant portion of carbon which can be degraded easily.

The deviation from ratio results in low efficiency of biogas production. In a single digestion process mixing numerous different substrates together enhances the nutrient balance and complete the requirements of missing nutrients and results in high biogas production (Nijaguna, 2002). Attributed to this reason cow dung is mixed with other organic waste usually to optimize the process of digestion and increase the generation of biogas (IEA, 2005, Nijaguna, 2002). C/N ratio of cow manure ranges from 16 – 25.

2.8.4 Water content

Water standout amongst the most critical variables for microbial action and their growth. The measure of water present in digester, decides the portability and additional cell enzymatic movement of microorganism. For better execution dampness substance ought to be kept up in the scope of 60 – 95% for processing. The perfect moisture content is distinctive for various feedstock, it generally relies on their substance and natural qualities (Nijaguna, 2002).

2.9 Leachate as Growth Medium

Very few studies have examined the effectiveness of leachate as a growth medium for micro algal growth (Pittman et al., 2011; Rawat et al., 2011). Lin and his coworkers in 2007 investigated the nutrient removal from different dilutions of leachate. They studied the growth and nutrient removal rates of *Chlorella pyrenoidosa* and *Chlamydomonas snowiae*. The leachate concentrations used were 10%, 30%, 50%, 80% and 100%. The cell densities i.e. algal population growth in diluted leachate increased by 81.6 and 3.66 times respectively for tests with 10% leachate. While negative growth rates were observed in 30%, 50%, 80% and raw leachate (Lin et al., 2007).

2.10 Biomass Productivity of Microalgae

Over the last few decades, the depletion of fossil fuels and the large energy supply and demand gaps has led to the development of new stable energy techniques in the form of renewable energy. The microalgae has shown to have higher biomass productivity of up to 40–80 dry weight ha^{-1} y^{-1} as compared to conventional agricultural crops The production of biogas from

algae on large scale is hindered by three main problem. The first is the cost incurred for biomass production is high (Wijffels et al., 2013). Secondly, many species have shown resistance towards degradation by microorganisms. Another major obstacle is due to presence of high amounts of protein in microalgae which leads to lower carbon to nitrogen ratios which are detrimental due to release of ammonia during fermentation which in turn is toxic for methanogenic bacteria (Zhong et al., 2012).

2.11 Digestion of Microalgae

In studies conducted earlier, anaerobic digestion of algae alone showed lower biogas yield resulting from recalcitrance of algae sludge high nitrogen content from ammonia toxicity and to hydrolysis (Yen & Brune, 2007). Different substrates have been co digested and have shown to increase the biogas yield and methane content.

Wang and coworkers investigated the co-digestion of algae with waste activated sludge and reported the improvement of biogas yield (Wang et al., 2013). Yen and Brune added waste paper as a cosubstrate to algae digestion reported an increase of 50% in biogas yield (Yen & Brune, 2007). To run anaerobic co-digestion with algae, selection of suitable co-substrate for biogas production can have a major environmental and economic impact on the future bioenergy industry.

A comparison study conducted between cyanobacteria and microalgae strain chlorella vulgaris showed that chlorella vulgaris biomass grows in any temperature and it does not have temperature preference. During cultivation in waste water, within 4 days ammonium and phosphate were completely removed by the micro algal strain. However biogas yield was low for microalgal strain as compared to cyanobacteria because of the low biodegradability of cell wall microalgae during digestion (Mendez et al., 2016).

2.12 Septic Sludge

Dignan Lu and coworkers (2016) investigated the effectiveness of using septic sludge as a co-substarte to digest microalgae. The conditions of digestion setup included 30 days of co-digestion at the temperature of 35C , the different co-digestion groups (25% algae, 50% algae, and 75% algae)

delivered an average biogas production of 547.3 35.6 mL/gVS_{fed}, the amount that was three times more than biogas production from the 100% algae group. More favorable initial carbon to nitrogen ratios (11:1 to 27:1) resulted from the addition of septic sludge to the microalgae and improved digestion of algal biomass, and decreased hydrogen concentrations, that were directly related to the increased quality and quantity of methane produced. The results demonstrated the effectiveness of using a co-substrate such as septic sludge as well as anaerobically digest microalgae *Chlorella sp*. and increase the biogas production (Lu & Zhang. 2016).

2.13 Paper Waste

This study have shown that adding high carbon content of waste paper in algal sludge feedstock provides a balanced C/N ratio. This helped in reducing the problem of unbalanced nutrients in algal sludge i.e. low C/N ratio which is an major limiting factor in anaerobic digestion process. The results showed that the combination of 50 % waste paper with algal sludge enhanced the rate of methane production to 1170 mL/l day in contrast to 573 ml/l day of algal sludge digestion alone. Both the digesters were operated at 4g VS/l day at 35 °C and 10 days retention time. The study resulted in suggestion of an optimum C/N ratio for co-digestion of algal sludge and waste paper with in the range of 20–25/1 (Yen & Brune, 2007).

2.14 Sewage Sludge

Selenastrum capricornutum, freshwater microalgae species were co-digested with sewage sludge under mesophilic and thermophilic conditions. The substrates and the temperatures majorly influenced biogas production. Under mesophilic conditions, the digestion of sewage sludge produced 451 mL Biogas/gSV. While *S. capricornutum* produced 271 mL Biogas/gSV and the mixtures comprising of sludge produced moderate values between sludge and microalgae production. The highest biogas yield was achieved by sludge digestion under thermophilic conditions reported to be 566 mL Biogas/gSV. When the microalgae content increased during co-digestion, biogas production decreased, for *I. galbana* and minimum values were reached for *S. capricornutum*, 261 and 185 mL Biogas/gSV, respectively. But there was neither evidence of inhibition found and the low yields were consequence of microalgae species characteristics. The methane content in biogas resulted in

similar values, independently from the digested substrate (Coporgno et al., 2015).

2.15 Waste Water Sludge

This study confirmed the possibility of producing biogas from *Nannochloropsis sp.* The hydrolyzed and oil-extracted algae, mixed with wastewater sludge, generated significant amounts of biogas in excess of the quantities generated by sludge alone (Adam & Shanableh., 2016).

2.16 Brief Overview of Literature

Microalgae are robust photosynthetic microorganisms. Although they are small in size but they grow rapidly in aquatic as well as terrestrial environments. Current research on microalgae species have proved them to be a viable and promising alternative to fight energy crisis of present and future generations due to their short harvesting cycle. The growth requirements include micronutrients and macronutrients, sufficient light and favorable temperatures. Different species of algae have significant potential to contribute to global production of bio resources (Rawat et al., 2011). Untreated wastewater and leachate are a major cause of pollution of ground and surface water as well as different diseases. There are different conventional treatments but they are costly. One of the unconventional treatment that are in focus of researchers is phycoremediation i.e. treatment of wastewater and leachate through microalgae. The nutritional requirements of algae mimics to those nutrients which are regarded as pollutants in these mediums. Another advantage of microalgae is that it can be harvested and used for production of biogas through anaerobic codigestion. However, the conditions to grow microalgae needs to be optimized for optimal growth and factors to produce biogas needs to be improved for maximum yield, in order to make it a feasible alternative to combat energy crisis.

CHAPTER 3

MATERIALS & METHODS

In this chapter, the methodology and procedure used during the experimental phases are discussed. The analysis during the research phase were carried out in environmental chemistry teaching lab, IESE, NUST.

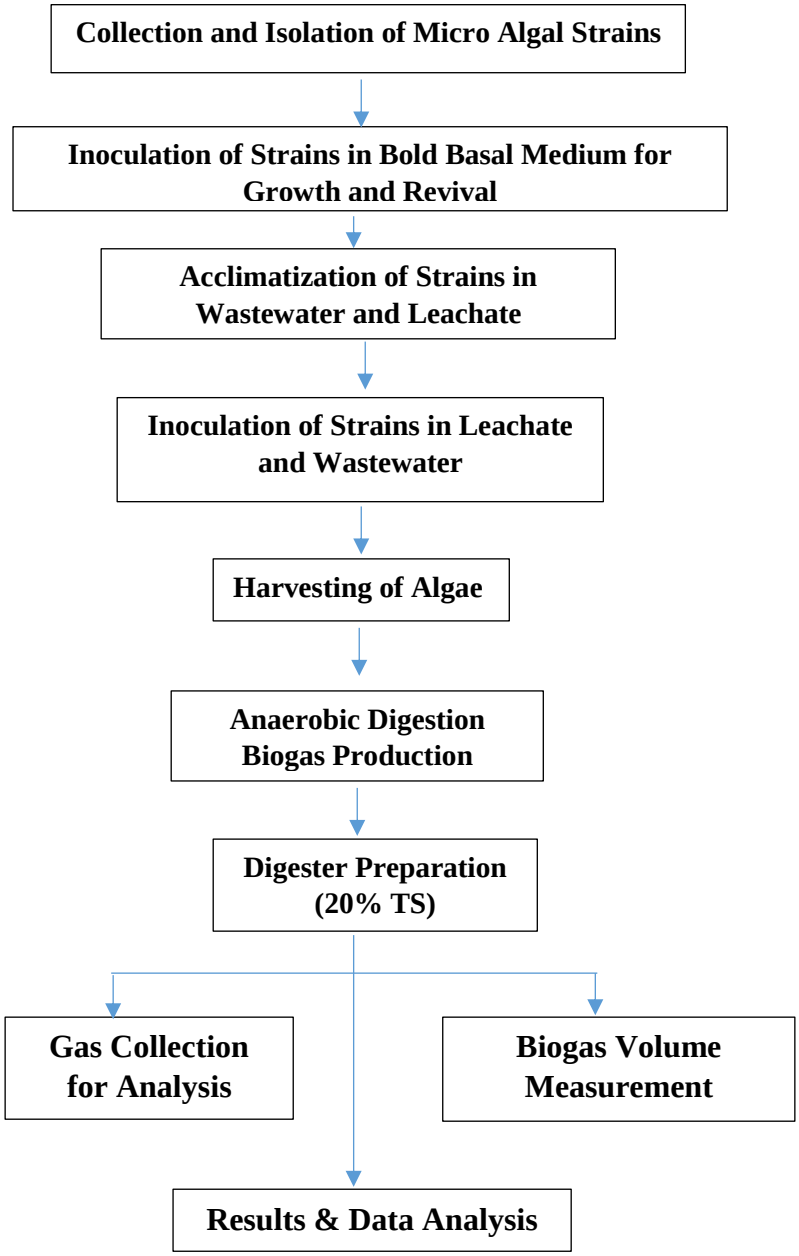

Figure 3.1 Flow diagram showing the sequence of experiment

3.1 Collection of Microalgae

3.1.1 Sampling location

Microalgae samples were collected from two locations as given below.

1) ASAB

   The *Dictospharium* strain i.e. s4 and s5 and *Pectinodasimus* strain s5 were collected from Nano Biotechnology Laborotary, Atta-Ur-Rehman School of Applied Biosciences, NUST, Islamabad.

2) Unidentified strain s3 from pond near G11 Kashmir Highway.

   Water samples containing green microalgae were collected from the pond. These samples were cultivated in bold basal medium.

3.2 Preparation of Synthetic Growth Media

Bold's Basal Medium (BBM), a fresh water algae culture medium was used for revival and growth of algae in lab. BBM is highly enriched medium with low salinity and is considered ideal for culturing fresh water algae. BBM was made following standard recipe having an elemental analysis as given below in table 3.1: (Leslie & Summerel., 2007).

Table 3.1 Bold basal medium composition used for growth of algal strains

Macronutrients	**Compound**	**Concentration (mmol/L)**
Sodium	NaCl	3.37
Nitrogen	$NaNO_3$	2.94
Phosphorus	K_2HPO_4	1.73
Calcium	$CaCl_2.2H_2O$	0.17
Magnesium	$MgSO_4.7H_2O$	0.30
Iron	$FeSO_4.7H_2O$	0.02
EDTA (chelating agent)	Na_2. EDTA	0.03
Boron	H_3BO_3	0.13
Manganese	$MnCl_2.4H_2O$	0.5
Zinc	$ZnSO_4.7H_2O$	0.05
Copper	$CuSO_4.5H_2O$	0.02
Cobalt	$Co(NO_3)_2.6H_2O$	0.02

3.3 Inoculation of Microalgae in BBM

The samples of microalgae were cultivated in BBM and allowed to grow for two weeks. The unidentified sample from G11 pond was streaked on BBM and 1.5% agar plates using plate streaking method. The petri plates were placed in incubator with light having an intensity of 400 lux. After the time period of 7 days, single colonies were picked up from plates and inoculated into BBM for growth. The strain that showed growth was used in further experiments.

3.4 Collection of Leachate and Waste Water

3.4.1 Leachate

The leachate for growing the strains was collected from I- 12 Islamabad waste dumping site and is shown in figure 3.2. The sample was collected in 1.5 L PET bottles and transferred to laboratory where it was stored at 4˚C until further analysis. The pH, COD, Nitrate and Phosphate content of the sample were determined.

Figure 3.2 Leachate collection from I - 12 Waste Dumping Site, Islamabad

3.4.2 Wastewater

The sample of wastewater was collected from the site of MBR plant installed in NUST, Islamabad. The point of collection was the influent tank of waste water. The sample was collected in 1.5 L PET bottles and stored in laboratory for further analysis.

Figure 3.3 Wastewater collection from MBR plant wastewater inlet

3.5 Acclimatization of Microalgae Strains

Microalgae strains were acclimatized in different concentrations of leachate and waste water by increasing the amount as shown in table 3.2. The concentration was increased after every 4 days. The growth of algae was observed by spectrophotometer i.e. optical density at wavelength of 680nm (Li et al., 2008).

Table 3.2 Concentration of leachate and wastewater for acclimatization

Sr. No	Waste Water Concentration (%)	Leachate Concentration (%)
1.	100	100
2.	80	90
3.	60	80
4.	40	70
5.	20	60
6.	10	50
7.	-	40
8.	-	30
9.	-	20
10.	-	10

The microalgae strains were inoculated in 1 L transparent PET bottles with leachate and wastewater. The concentration was increased after every 4 days. Aeration and illumination source were provided through aeration pumps and TLD fluorescent light respectively as shown in the following figure 3.4.

Figure 3.4 PET bottles containing leachate and wastewater

3.6 Growth Performance of Microalgae in Leachate and Wastewater

The 25 ml of microalgae samples were inoculated in 1.5 L transparent PET bottles comprising of 50% leachate and 100% waste water. Algae cultures were set up in 1.5 L PET bottles, 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.

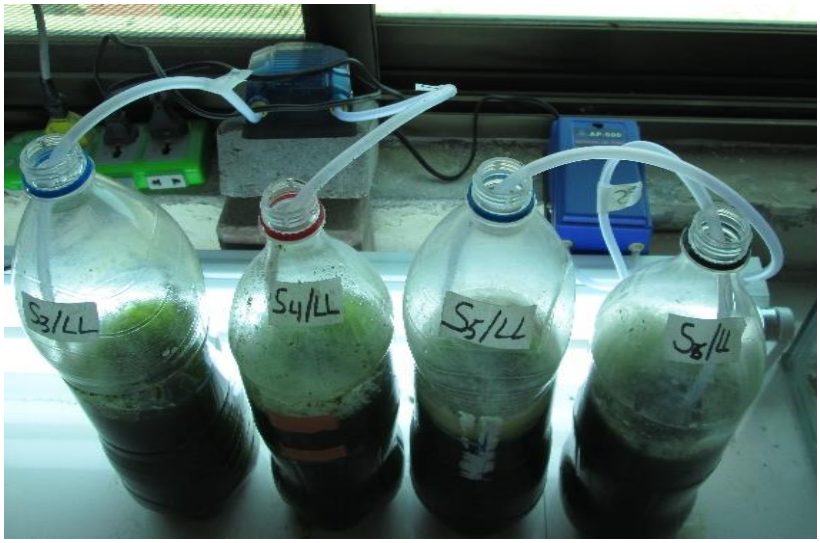

Figure 3.5 Microalgal Strains in leachate with illumination by TLD fluorescent light and air flow provided by aeration pumps

3.7 Harvesting of Algae

After allowing the various strains to grow in leachate and waste water for 15 days, the harvesting process was started. The algae was removed from aeration and illumination setup and centrifuged to obtain wet biomass. The suspended algae was centrifuged so that it can be separated from the medium. The residue was then transferred to four 50ml 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 than transferring the biomass into petri dishes. This procedure was repeated for all the strains (Lu and Zhang., 2016).

3.8 Preparation of Digesters for Biogas Production

To evaluate the biogas potential of each strain, the anaerobic digestion was carried out in a series of 500 mL serum bottles with a 100 mL head space. Different volumes of algal strains, cow dung were combined on the basis of 20% total solid concentration. The batch digestion was performed by placing the bottles in water bath and temperature was maintained by aquarium heater at 40 ^{O}C as shown in figure 3.6. The composition of digesters was defined on the basis of 20% total solids. The table 3.3 and 3.4 shows the composition used for the different digesters separately for each microalgae strain.

Figure 3.6 Digester bottles dipped in water bath and the temperature is maintained by aquarium heater

Table 3.3 Digester Preparation on the basis of 20% TS for co digestion with inoculum and waste water

Sr. no	Digester ID	Algae (g)	Cow dung (g)	Waste water (mL)	Distilled water (mL)
1.	S3W	40	40	320	According to requirement
2.	S4W				
3.	S5W				
4.	S6W				
5.	Blank	-			

Table 3.4 Digester Preparation on the basis of 20% TS for co digestion with inoculum and leachate

Sr. no	Digester ID	Algae (g)	Cow dung (g)	Waste water (mL)	Distilled water (mL)
1.	S3L	40	35	320	According to requirement
2.	S4L				
3.	S5L				
4.	S6L				
5.	Blank	-			

3.9 Analysis Performed

3.9.1 Nitrate determination

The nitrate concentration of samples were determined by cadmium reduction method using a spectrophotometer. The development of amber color in the sample indicates the presence of nitrate. The absorbance of samples were measured in spectrophotometer at 500 nm wavelength. The value of absorbance gives the corresponding concentration of nitrate in a sample from the nitrate standard curve that is developed prior to experiments (HACH 8039, 2014).

3.9.2 Phosphate determination

The phosphate in samples were determined by Vanadomolybdate Phosphoric Acid colorimetric method (4500-P C APHA). It was used to measure the absorbance by UV- Visible Spectrophotometer. The absorbance of samples is measured at 470nm wavelength.

3.9.3 Optical density and biomass determination

The spectrophotometer was used to determine the growth of algae. Optical density was measured at wavelength of 680 nm (Li et al., 2008). When the strains started showing constant OD, six samples of 5 ml each were collected from a bottle. The samples were diluted in different ratios with distilled water. The OD of the diluted samples was measured. Than samples were centrifuged at 4000 rpm for 15minutes. Wash the samples with distilled water and transfer completely in the china dish. Now dry them for 4 hours at 70 C and measure the weight. a graph was plotted between the OD and the dry weight of sample

3.9.4 pH

During the experiments, pH was determined using pH meter HI-8520. Its gives us the measure of the acidity or basicity of a sample.

3.9.5 Total solids

To determine solids in the sample gravimetric method was used. First, the evaporation dish was weighed for TS to the nearest 0.1 mg. volume of sample was measured accurately in the evaporation dish and placed in the oven at 105^{o} C. After evaporation of water, desiccator was used to cool the dish, than the dish plus the remaining solids were weighed. Total Solids is the term used for the material residue left in the vessel (APHA, 2005).

3.9.6 Volatile solids

The ignition of sample in muffle furnace at 550°C for an hour and then cooling in desiccator gives VS. The weight lost on ignition is the volatile solids while the leftover solids represent the fixed total, dissolved, or suspended solids while. The determination presents a rough approximation of the amount of organic matter present in the solid fraction of activated sludge, wastewater and industrial wastes (APHA, 2005).

3.9.7 COD

The closed reflux titrimetric method was used to determine COD in the samples. The digestate sample was first centrifuged and then diluted up to 10 % in 100 ml distilled water before COD analysis. 10 ml of it was oxidized by 3.5 ml of sulfuric acid in COD vial. The sample was then refluxed in strongly acidic solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After digestion, the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter was calculated in terms of oxygen equivalent. The standard reflux time was 2 hours. The samples were analyzed in duplicate and their average value was taken (APHA, 2005).

3.9.8 C/N ratio

The organic carbon content of the substrates were determined by the rapid titration procedure of Walkley-Black method involving chromic acid wet oxidation. Oxidisable matter in the sample was oxidised by 1 N $K_2Cr_2O_7$ solution. The reaction was assisted by the heat generated when two volumes of H_2SO_4 were mixed with one volume of the dichromate. The remaining dichromate was titrated with ferrous sulphate. The titre was inversely related to the amount of C present in the sample. The nitrogen in samples was measured by total kjeldhal nitrogen. The analysis was done by following standard methods in ICARDA manual (1996). The cow dung used as inoculum had a C/N ratio of 22.7. According to literature, it serves as carbon rich substrate as well as inoculum providing anaerobic bacterial community. The C/N ratio of microalga strains ranged between 4 and 10. The microalgae species are known to have lower C/N ratio. That is why co-digestion with a carbon rich substrate is preferred.

Table 3.5 C/N ratio of substrates

Sample ID.	C/N Ratio
Cow Dung	**22.7**
Strain 3	**4.5**
Strain 4	**5.6**
Strain 5	**10.4**
Strain 6	**4.3**

3.12 Gas Measurement and Collection

3.12.1 Water displacement method

The biogas was measured every day by using water displacement method (Ayu & Aryati., 2010). In the experiment, the assembly was self-constructed using a plastic tub and a cylinder. The tub was filled with water. The cylinder was filled with water and inverted in the tub so that no water leaks out. The outlet of the digester was than connected to a pipe whose head was opened in the inverted cylinder. As the valve was opened, the produced gas replaced the water in cylinder. The water displaced in the cylinder gave the volume of gas produced.

Figure 3.7 Water displacement assembly for measurement of gas

3.12.2 Biogas collection in tedlar bags

The biogas for the purpose of analysis was collected on the 15th day of the experiment. The gas was collected in Cel Scientific 1 litre- Gas Sampling Tedlar bags. The bag is made of PVF film and a polypropylene fitting that combines both the

septum and the valve as shown in figure 3.8. They are considered chemically inert to wide range of compounds as well as tough.

Figure 3.8 Gas Sampling using Tedlar bags attached to digester bottles

Figure 3.9 Gas Sampling Tedlar Bag made of PVF

3.12.3 Biogas analysis

The samples collected in the bags were analyzed by a portable BIOGAS 5000 gas analyzer (GEOTECH) at NARC, Islamabad. The device have different sensors that detects different concentrations of methane and carbon dioxide.

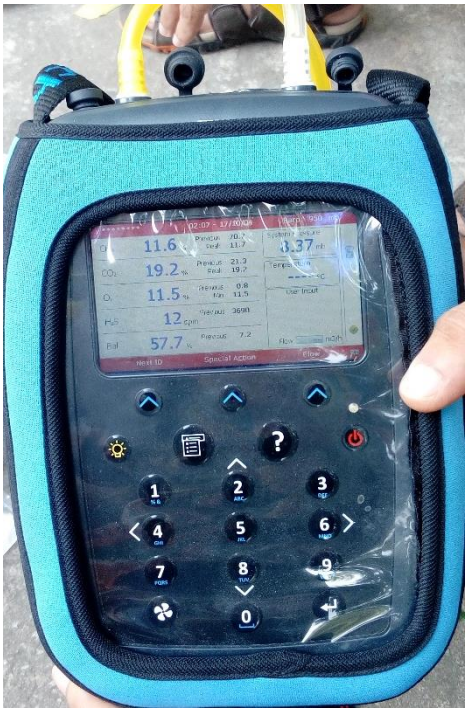

Figure 3.10 Gas sample in tedlar bags analyzed by biogas analyzer at NARC, Islamabad

CHAPTER **4**

RESULTS AND DISCUSSION

4.1 Isolation of Microalgae

After growth occurred on few petri plates on incubation, colonies that were distinctive were picked up and cultivated in bold basal media. The strain that showed maximum growth was further selected for experiment.

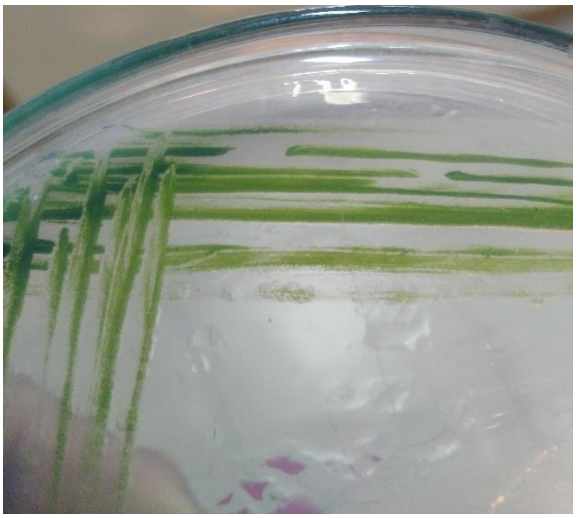

Figure 4.1 Microalgae growth on 1.5% agar plate after 10 days incubation

4.2 Growth in Bold Basal Media

Bold basal medium is fresh water medium, which is highly nutrient rich and supports the growth of microalgae. The strain 3 isolated from the wastewater pond, when enriched in bold basal medium started to show growth in the second week. The strain adjusted to the growth conditions provided in the set up. The strain 4 and strain 5 representing *Dictyosphaerium* species and strain 6 from *Pectondasimus* species showed significant growth as shown in the figure 4.2. All the strains were maintained in media throughout all the experiment phases. Xin et al (2010) studied the growth of *Scenedesmus* sp. LX1 in BG 11 and reported the maximum algal density in the medium (Xin et al., 2010). The cultivation of *Monoraphadium sp., chlorella sp and scenedesmus sp* in BBM during their study showed greater growth rates than any other mediums used (Guerrero et al., 2014).

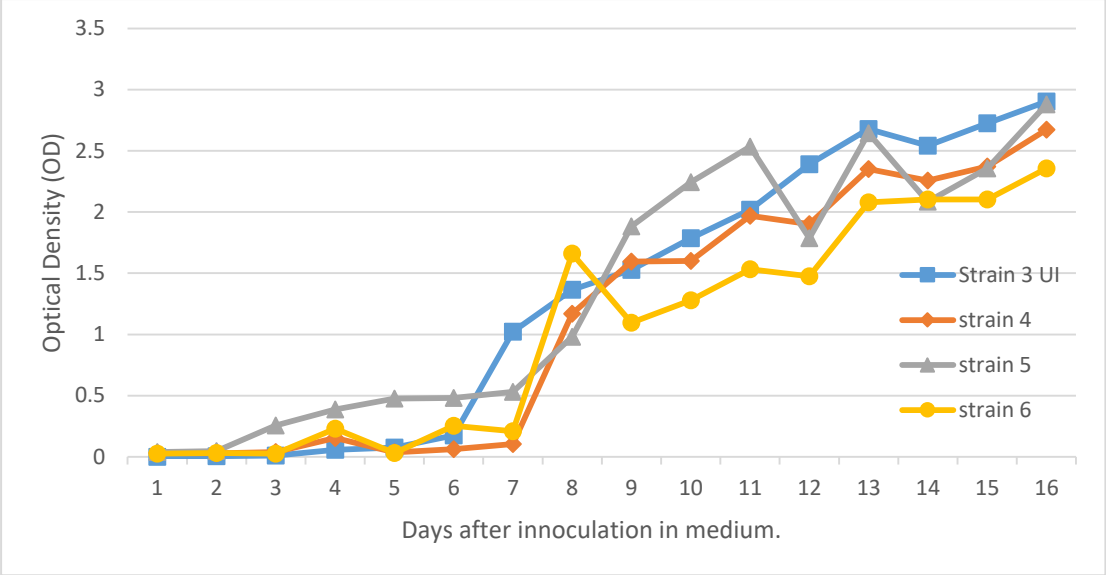

Figure 4.2 Performance of micro alga strains growth in bold basal medium determined by using spectrophotometer at 680nm wavelength

4.3 Characteristics of Wastewater and Leachate

The wastewater was collected from inlet tank of MBR plant constructed in NUST. It had a COD of 302mg/L. Although the composition of waste water from different area varies according to location and environmental conditions. The typical wastewater is characterized by approximately 350 mg L^{-1} chemical oxygen demand (COD), 50 mg L^{-1} NH^{4+} - N and 10 mgL^{-1} PO_4^{-3} (Boelee et al., 2014).

The leachate collected showed a COD of 17760 mg/L. Lin et al.,2007 reported the physicochemical characteristics of the leachate sample they collected from Li Keng Landfill Leachate , sample was basic with a pH of 7.6 , characterized by relatively low level of nitrate (68.4 mg L^{-1}) and phosphate (51.3mg L^{-1}) (Lin et al., 2007).

Table 4.1 Characteristics of wastewater and leachate used during experiments

Sr. no	Characteristics/Parameters	Wastewater	Leachate (50%)
1.	COD (mg/L)	302	8880
2.	Nitrate-nitrogen (mg/l)	22.2	98.41
3.	Phosphate – phosphorus (mg/L)	29	57.5
4.	pH	7.1	7.5

4.4 Acclimatization of Strains

All four strains were acclimatized in wastewater and leachate. The concentration of wastewater was increased by 20 %, after every four days and results are shown in figure 4.3. The strains were able to show growth with concentration was increased up to 100% (original wastewater). The strains adjusted with the medium and did not show any decrease in growth, hence negating the presence of toxicity in wastewater.

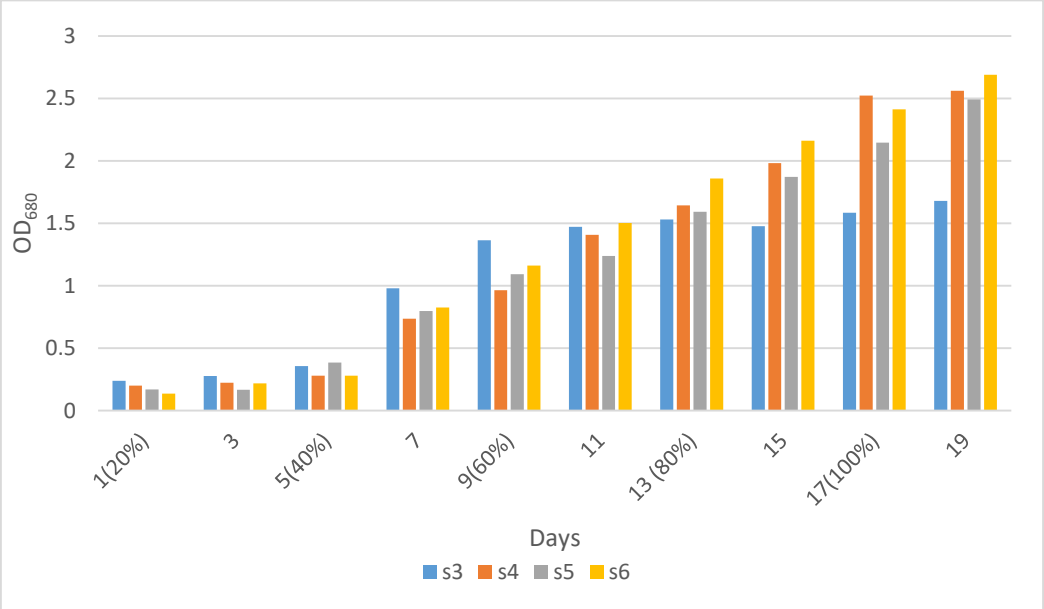

Figure 4.3 Acclimatization of strains in different concentrations of wastewater monitored by measuring optical density at 680nm wavelength.

In case of acclimatization of strains in leachate its concentration was increased by 10 % in each step as given in methodology in section 3.5. The strains were able to grow upto 50% leachate with a COD concentration i.e. COD of 8880 mg/L. The growth of micro algae strains declined when exposed to concentration higher than 50%. The highest growth was observed with strain 5. The growth of all strains began to decline when the concentration of leachate was increased. The possible cause for the inhibition of growth at >50% concentration may be the presence of high ammonia nitrogen. Although algae utilizes ammonia nitrogen for growth but excess can cause inhibitory effects as reported by Przytocka-Jusiak et al., 1984. Another reason can be

the increase of pH which is due to micro algal growth and CO_2 assimilation suggested by Abeliovich and Azov (1976). They also found that ammonia inhibits photosynthesis and growth of *Scendesmus obliquus* at concentrations over 34 mg L^{-1} (Abeliovich & Azov., 1976).

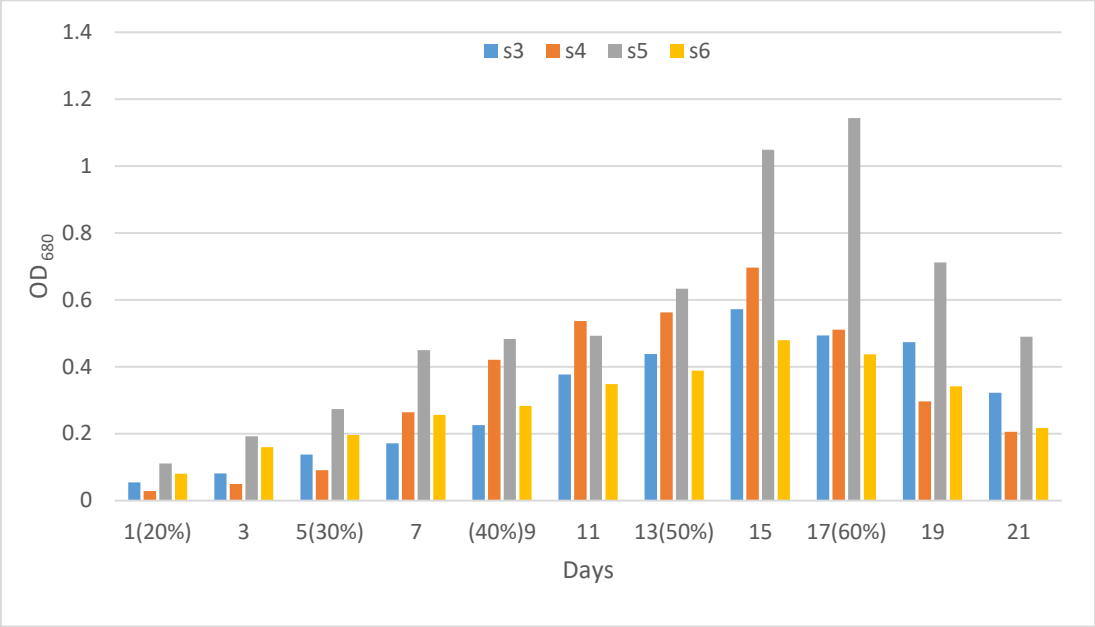

Figure 4.4 Acclimatization of strains in different concentrations of leachate monitored by measuring optical density at 680 nm wavelength.

4.4.1 Performance of microalgae strains

After the acclimatization of strains, the algae cultures were cultivated in wastewater and leachate. The nitrate and phosphate uptake was also monitored in every cultivation medium.

4.4.1.1 Growth in wastewater

Microalgae strains showed considerable growth in wastewater as shown in figure 4.5. The strain 3 that was isolated from wastewater pond showed highest growth and the least average growth was observed in case of strain 6. The domestic wastewater mostly contains organic carbon, nitrogen and other compounds and are considered suitable for microalgae cultivation. Cultivation of microalgae in wastewater is influenced by a number of factors including critical variable such as pH, availability of light, temperature, O_2, CO_2 and availability of nutrients (Pittman et al., 2011). Similar results were

obtained by Boelee et al., 2011 when microalgae *nitzschia* species was cultivated in secondary effluent obtained from wastewater plant in a flask. The retention time was 15 days. Aslan and Kapdan., 2006 investigated the use of synthetic waste water as culture medium and used microalgae species *Chlorella vulgaris* in 1000 ml flasks for 10 days of retention time, (Aslan & Kapdan ., 2006). Huo et al., 2014 used dairy treated wastewater to cultivate *Chlorella zofingiensis* in bench scale outdoor ponds that also prospered very much.

The strain 5 and strain 6 almost performed similarly in wastewater. The growth of strain 5 was 39% slower than that of strain 3 and 36% slower than strain 4. While strain 6 growth was 41% slower than strain 3 and 36% slower than strain 4.

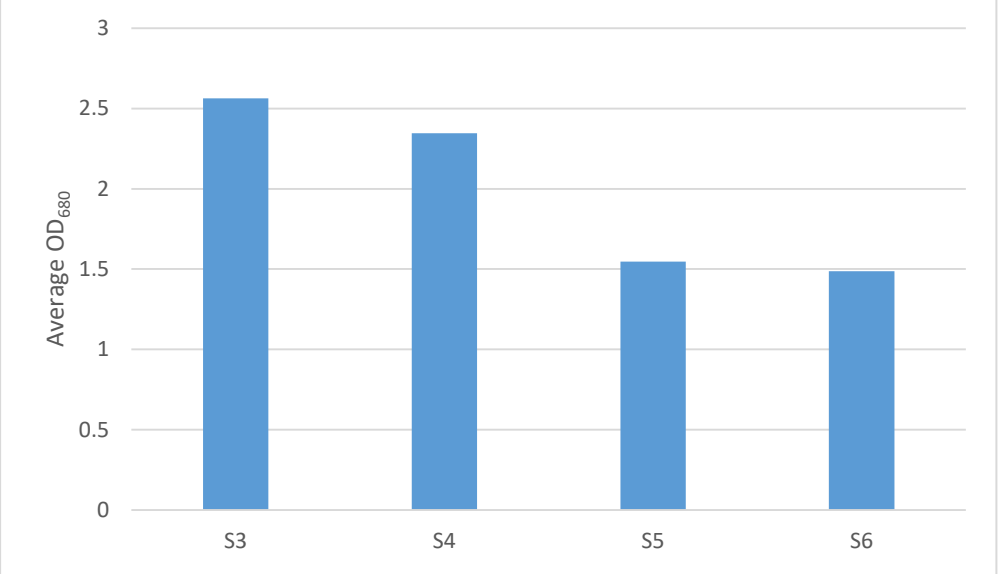

Figure 4.5 Average growth of microalgae strains in wastewater during the time period of 15 days

4.4.1.2 Growth of microalgae strains in leachate

After the acclimatization of strains, the algae cultures were cultivated in 50% leachate. The growth of algae strains were monitored along with the nitrate and phosphate uptake. All the strains showed variable growth but overall growth showed increasing trend as shown in figure 4.6. This growth can be due to intercellular stores of nitrates and phosphates already present inside the microalgae strains. Lin and

colleagues (2007) studied the removal of nutrients from algae strains in leachate, the results showed substantial removal and a positive relationship between nutrient removal and algae growth in the algae treated leachate.

As shown in figure 4.6, strain 4 showed highest average growth while the strain 6 showed least average growth. The low growth by strain 6 may due to the inhibition caused by any toxic component present in the leachate or can be attributed to pH rise above 8.0. The fluctuation during growth in leachate can be attributed to the presences of substances in leachate that can cause toxicity and hence leading to decline in growth of micro algal strains. Since pH was not adjusted during the growth, the carbon dioxide assimilation during algal growth can raise pH to 8, which can also contribute to affect growth in some strains i.e. s6.

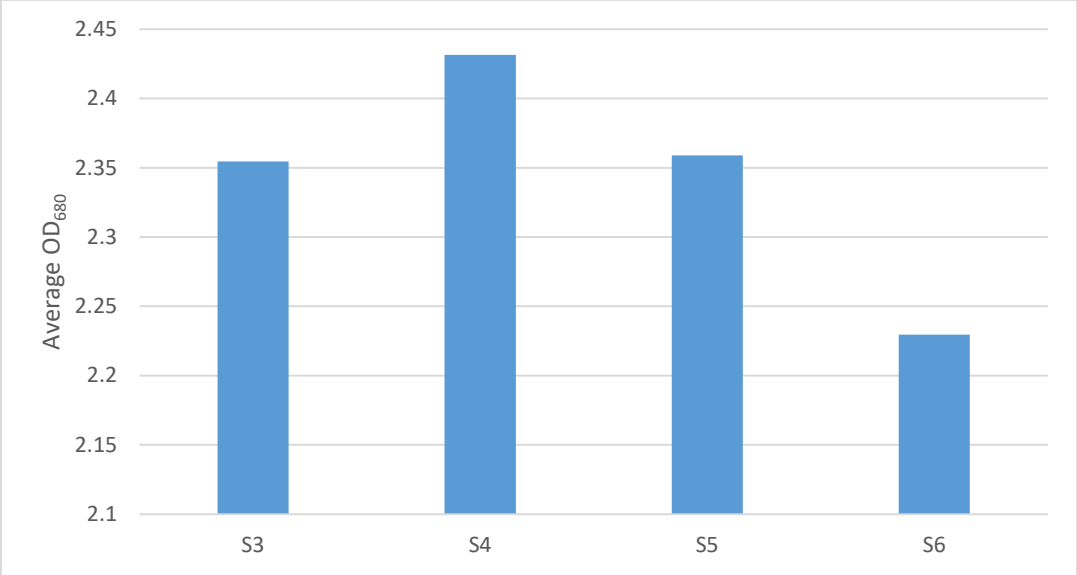

Figure 4.6 Average growth of microalgae strains in leachate during a period of 15 days

4.4.2 Nitrate removal by microalgae strains

4.4.2.1 Nitrate removal from wastewater

The figure 4.7 shows removal of nitrate by microalgae strains in the wastewater medium. The initial concentration of nitrate in wastewater was 22.2 mg/L. Four strains were grown in wastewater and had shown different responses to nitrogen removal. The s4 and s6 showed 100 % removal of nitrate by 8th day. While rapid decrease in nitrate concentration in medium was observed in case of strain 5 and then

strain 3. However, all nitrate (100%) was consumed by 11th day in mediums containing s3 and s5. Boelee et al (2011) cultivated microalgae species (*Nitzschia*) in a flask containing secondary effluent from wastewater plant as medium and initial nitrate 10mg/L was reduced to 0.15mg/L i.e. 98 % reduction. Aslan and Kapdan (2006) studied the growth of *chlorella vulgaris* in synthetic wastewater as medium (1000ml flasks) for 10 days and reported 100% consumption of nitrate.

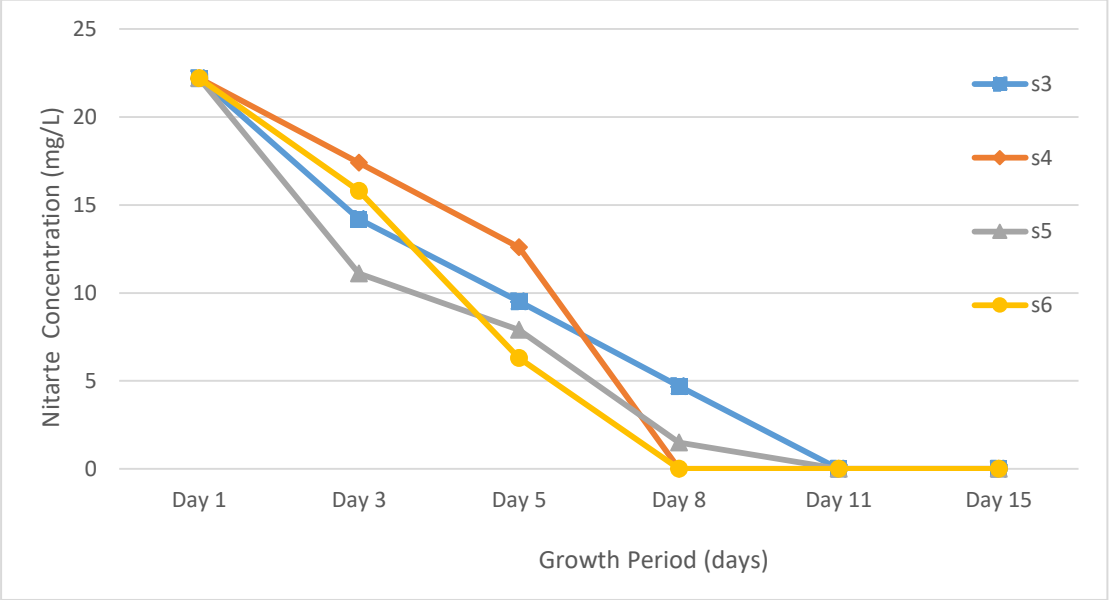

Figure 4.7 Removal of nitrate from wastewater during algal growth over a period of 15 days

4.4.2.2 Nitrate removal from leachate

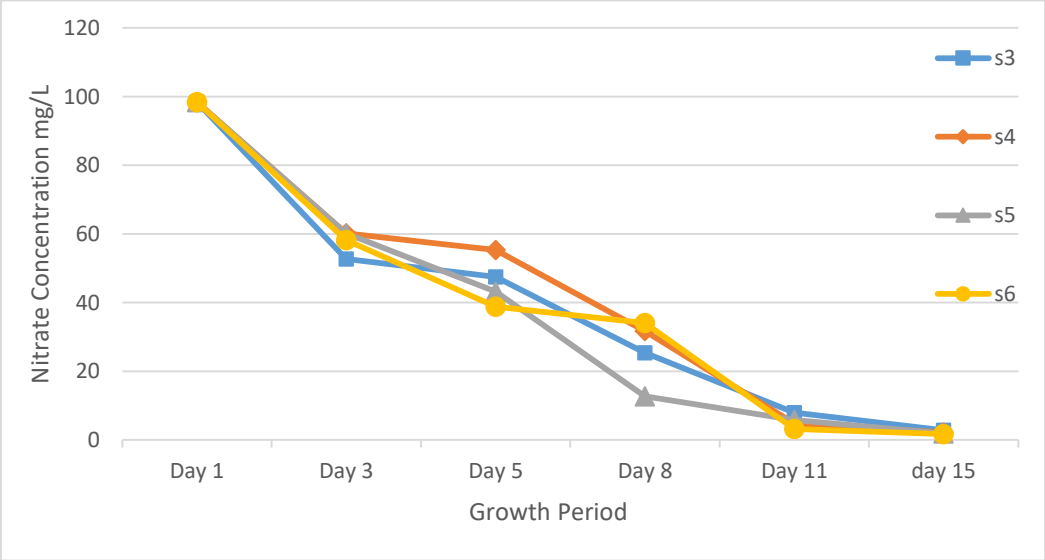

Figure 4.8 Removal of nitrate from the leachate medium during the algal growth

The graph 4.8 shows recommended uptake of nitrate by various strains in leachate medium. Initial concentration of nitrate in leachate was 98.41 mg/L. The nitrate concentration in leachate showed a decreasing trend with all micro algal strains. Strain 3 consumed nitrate faster and showed almost linear drop in concentration. During the 8th day, 74% of the nitrate was removed by strain 3 and 97% was of it by the end of retention time. Strain 4 showed slower decline in nitrate removal as compared to other strains but by the end of retention period, it was able to uptake 97% of the nitrate. While strain 5 showed a rapid decrease in uptake of nitrate i.e. 87% from leachate during the 8th day and 98% by the end of experiment. Sergio et al (2016) studied the removal in different leachate compositions (supplied with phosphorus and no phosphorous) by *Chlorella vulgaris* and reported 21% removal of nitrate per day.

4.4.3 Phosphate removal by microalgae strains

4.4.3.1 Removal from wastewater

The phosphate concentration in medium by all strains showed decreasing trend as shown in figure 4.9. This can be compared with growth rates indicating a positive relation between the growth of strains and their uptake of nutrients. The strain 6 removed 71% of phosphate at the end of experimental period. While the least uptake was observed in case of strain 4. Strain 3 showed a decrease of 57 % and strain 5 showed a decrease of 66 % from the original concentration. Boelee et al (2007) reported 86% removal of phosphate from microalgae *nitzschia* species cultivated in secondary effluent from wastewater plant in 1000 mL flask. Aslan and Kapdan (2006) reported 78% removal of phosphate by microalgae (*Chlorella vulgaris*) cultivated in synthetic wastewater as culture medium (1000 mL flask).

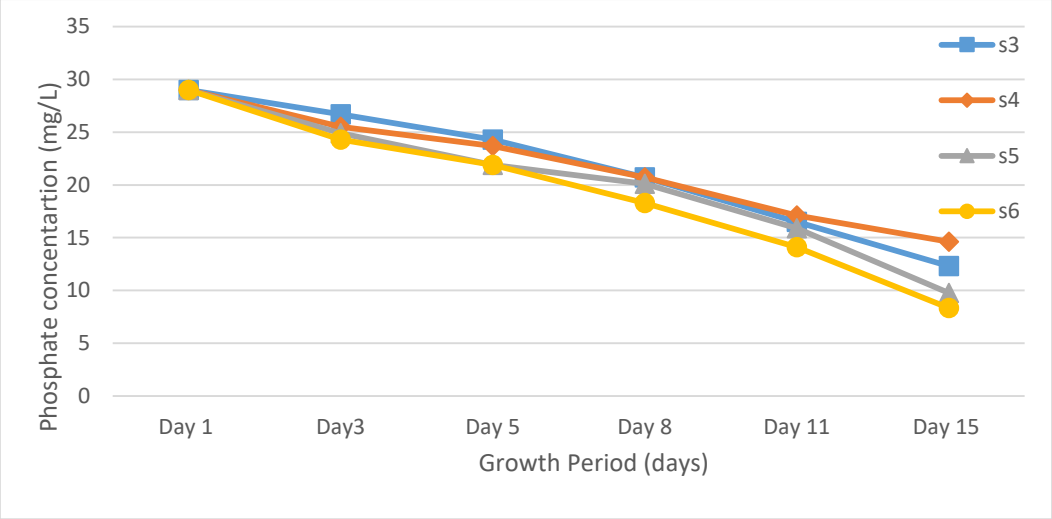

Figure 4.9 Removal of phosphate from wastewater during algal growth

4.4.3.2 Phosphate removal from leachate

Behavior of various strains for uptake of phosphate from leachate is shown in figure 4.10. The decrease of phosphate in leachate medium was rapid i.e. 97% by strain 3 and strain 5. Gradual decrease was noted in leachate for strains containing strain 4 i.e. upto 65% at 8th day and 92% at the end of growing period (15days). Sergio et al (2016) reported 63% removal of phosphate from leachate (35:1 NP ratio) by microalgae *Chlorella vulgaris*.

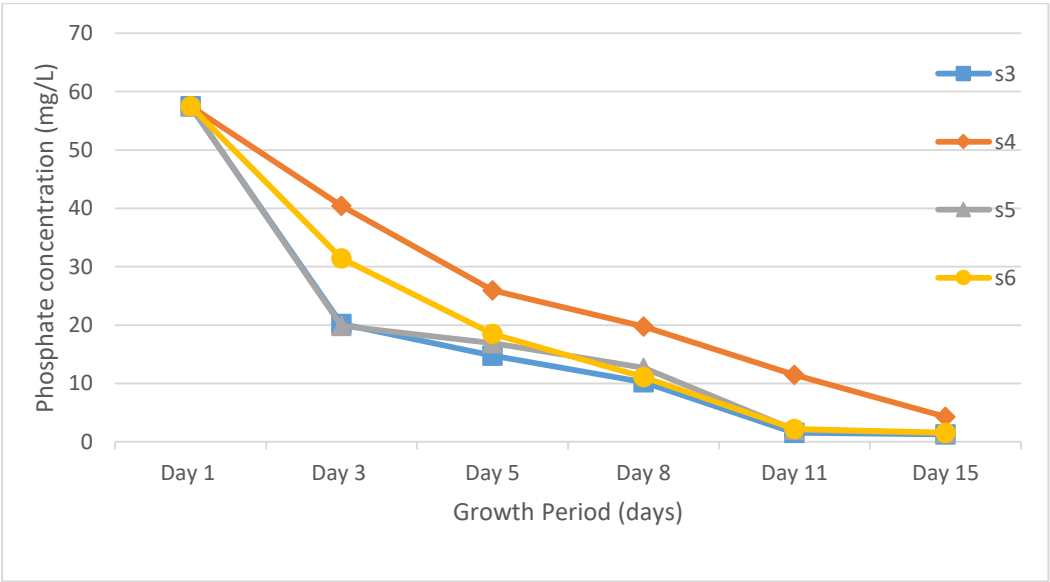

Figure 4.10 Removal of phosphate in leachate during the algal growth

4.5 Anaerobic co digestion of micro algal strains

In the batch experiment, 500 ml serum bottles were used as reactors as discussed in section and figure. The caps were also sealed by silicon glue to avoid any leakage. The bottles were filled up to 400ml with substrates mixture. The ratios of substrates were based on 20 % total solids. The batch experiment was carried out for a period of 31 days, during which pH and COD at the start and end of the experiment for each digester was measured. The biogas volume was observed every day and the biogas samples collected on the 15th for its composition.

4.5.1 Co-digestion with wastewater

4.5.1.1 Biogas production

Figure 4.11 showing biogas production using algal strains and control are shown. The maximum biogas production by 3 strains (S3, S4, S6) were observed in from 10th to 20th day of batch digestion and their production begin to decrease afterwards. This decrease can be attributed to reduction in volatile solids or low biodegradable substrate availability. However, the digestion of the strain 5 reached the maximum production at the end of the batch digestion i.e. 29th day. This strain 5 might have taken longer to adjust in the conditions and the pH might not have been suitable for bacteria to aid in biogas production The strain 4 gave the highest biogas volume of 810 ml.

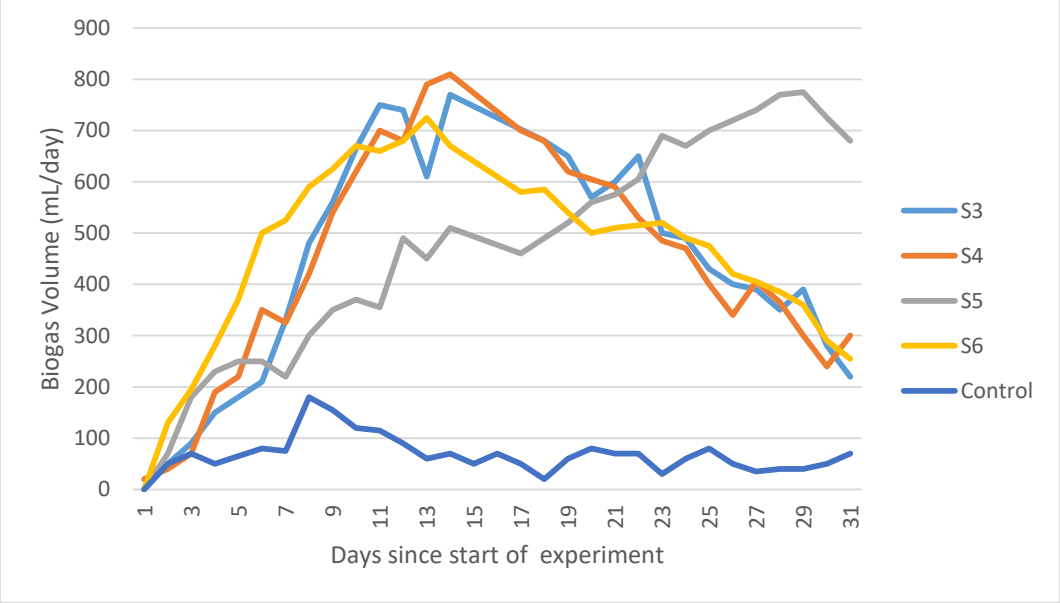

Figure 4.11 Daily volume of biogas produced over the period of experiment in mesophilic condition

4.5.1.2 Cumulative biogas production

Cumulative biogas production from five digesters containing different micro algal strains as shown in figure 4.12. In the beginning, highest cumulative biogas production was noted for strain 6. The strain 5 showed increasing cumulative production during the last few days of batch digestion. From the figure 4.10, it is evident that strain 5 showed highest cumulative biogas production of 13.7 L during batch digestion of 31 days. Gas production from strain 3 showed lower cumulative biogas production i.e. 12.1 L.

Lu and Zhang studied the effectiveness of using septic sludge as co-substrate with microalgae in co digestion. In the different co digestion groups of 25%, 50% and 75% microalgae, the gas production was high as compared to 100% microalgae group. The study also reported that adding septic sludge improved the digestion due to better C/N ratio (Lu and Zhang., 2016).

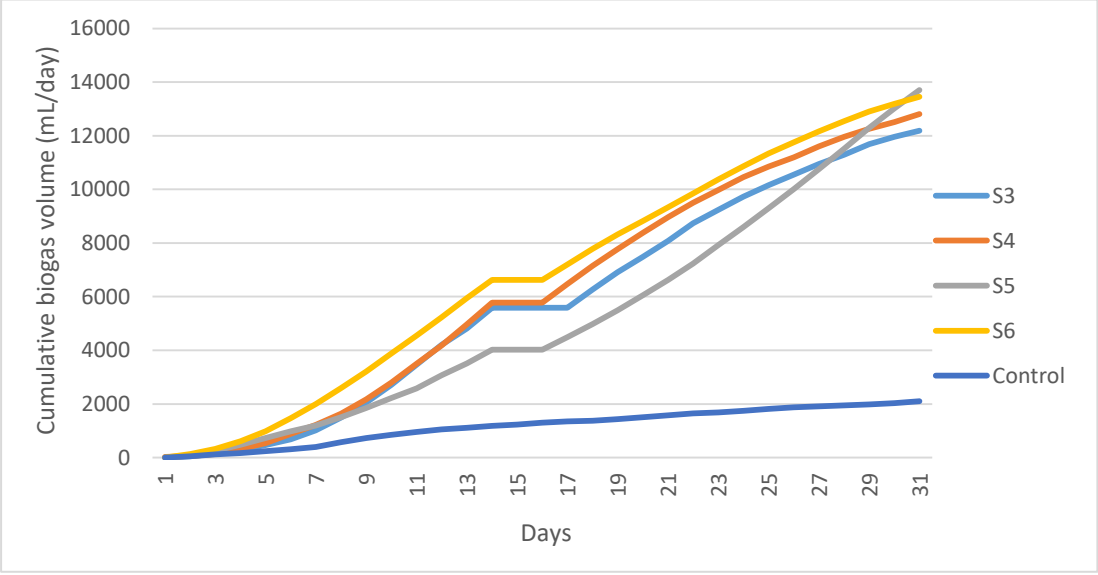

Figure 4.12 Cumulative biogas production from co-digestion with wastewater in experiment with mesophilic conditions

4.5.1.3 Methane content in biogas

The highest methane yield was obtained from co digestion of strain 4 with wastewater. The methane concentration was 57.6%. This stain also gave the maximum biogas volume of 810ml and cumulative biogas production of 12805 ml. The higher biogas volumes corresponds to higher methane yield Similarly, the digestion of strain 3 gave a methane yield of 41.7%, strain 5 gave 50.3% and strain 6 co-digestion resulted in 47.5% methane. Lu and Zhang studied effectiveness of using septic sludge as co-substrate with microalgae in co digestion. In the different co digestion groups of 25%, 50% and 75% microalgae, gas production was high as compared to 100% microalgae group. The study also reported that adding septic sludge improved the digestion due to better C/N ratio (Lu and Zhang., 2016).

Figure 4.13 Concentration of methane and carbon dioxide in the biogas samples on 15th day

4.5.2 Co-digestion with leachate

4.5.2.1 Biogas production

During the batch digestion of experiment which continued for 31 days, the highest biogas volume was observed after the 10th day i.e. by S4 and S6. The biogas volume of strain 4 started to drop after the 11th day and this can be attributed to the presence of extra ammonia nitrogen in either of the substrate or inoculum. The strain 3 showed steady increase and the biogas volume begin to drop by the 24th day, but still had high biogas volume at the end of digestion period compared to that of the

other strains. While the control was struggling below 100 mL/day throughout the experiment. The reason might be due to presence of single substrate.

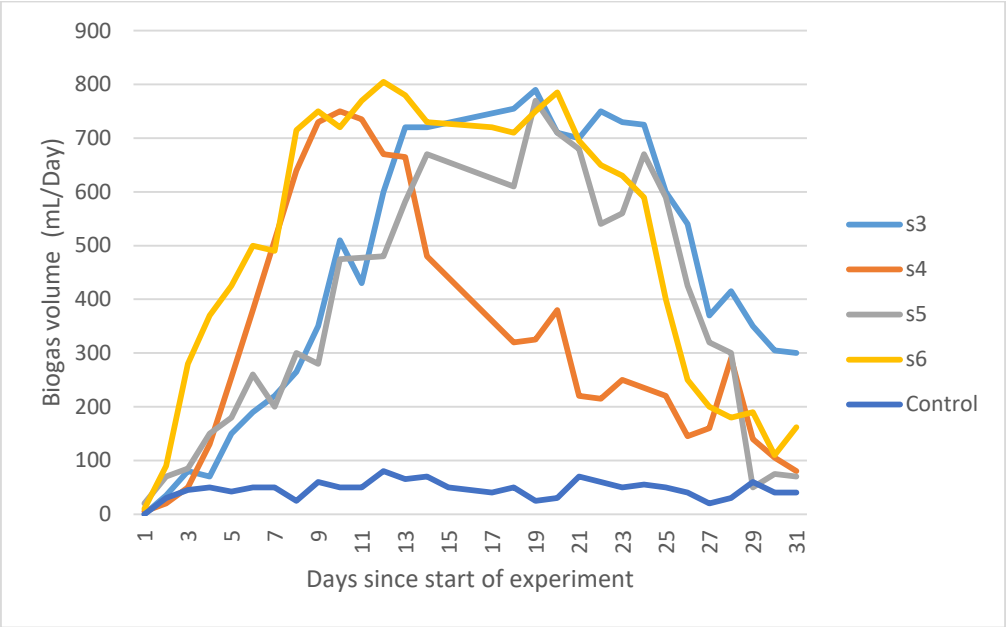

Figure 4.14 Daily volume of biogas produced everyday over the period of experiment in mesophilic conditions

4.5.2.2 Cumulative biogas

As shown in figure 4.15, the highest cumulative biogas production during batch digestion was by strain 6 i.e. 14.4 L. The lowest cumulative biogas production was by strain 4 which exhibited amount of 9 L only by the end of co digestion period. The strain 3 produced cumulative biogas up to 12 L and strain 5 produced up to 10 L. While, the control had the lowest cumulative production. The strain 3 from wastewater pond performed well in comparison to two of the strains i.e. s4 and s5. While strain 6 appeared to be the best performing strain overall.

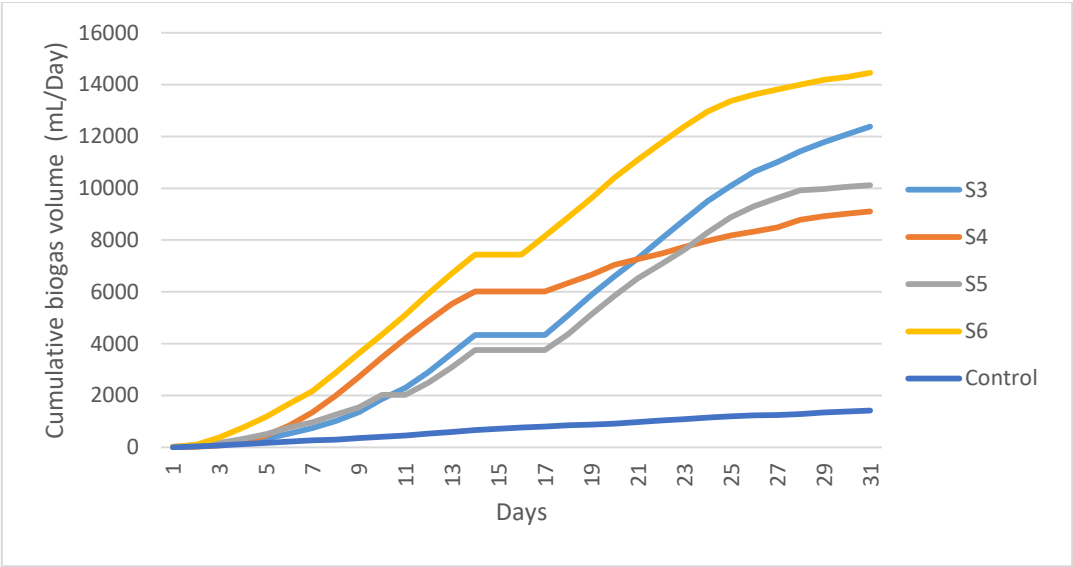

Figure 4.15 Cumulative biogas production from co-digestion with leachate

4.5.2.3 Methane Content in Biogas

Methane content was measured for samples collected on the 15th day of experiment as shown in figure 4.14. Analysis was made using biogas analyzer. Biogas of the alga co-digestion with leachate varied. However highest methane yield was achieved by strain 6 and strain 4 with a concentration of 61.5% and 53.7%. Whereas results of the samples i.e. strain 3 and strain 5 showed the lowest methane yield i.e. 35.8% and 39.2% respectively.

Co digestion of microalgae with leachate has not be widely studied yet. However the digestion of microalgae with other carbon rich substrates have been under consideration. Ayhan and Aysenur studied biogas production from microalgae in a two stage anaerobic bioreactor system. They used a set of waste activated sludge and spirulina platensis biomass and resultant methane content of 35% in acid reactor and 75% methane in methane reactor and biogas volume 2880ml/day. The paper also concluded that low amount of total solids mixture always result in more biogas volume and hence proved that co digestion have a better biogas production potential (Varol and Ugurlu., 2016).

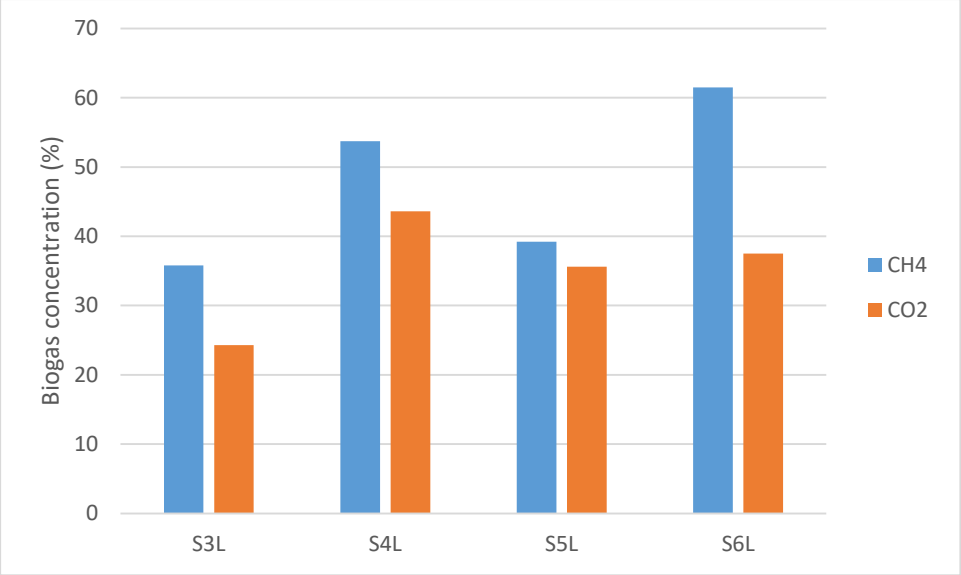

Figure 4.16 Concentration of methane and carbon dioxide in the biogas samples on 15th day

4.6 Analysis of Substrates

The TS and VS of the digesters were measured at the start and end of the experiment with HRT of 31 days. Both of them were significantly reduced. Table 4.2 shows the initial and final total solids of each digester. The least total solids were observed n digester S6W and S5L.

Table 4.2 Initial TS and final TS of digesters at the start and end of experiment

Sr. no	Digester ID	Initial TS (g/L)	Final TS (g/L)
1.	S3W	19.1	10.36
2.	S4W	18.54	12.55
3.	S5W	19.86	10.03
4.	S6W	18.63	9.76
5.	S3L	19.62	9.37
6.	S4L	19.27	7.85
7.	S5L	17.23	9.6
8.	S6L	18.91	10.76

Table 4.3 shows VS of the digesters, where were reduced and indicated that the substrates had been degraded. The highest VS reduction was showed by the digester S6W and the least by digester S5W. While in co digestion of leachate and algae the highest VS reduction was shown by S3L and the least was shown by S4L.

The anaerobic digestion of *Sprirulana plantesis* showed 89% reduction in VS with TS feed of 5% (Varol and Ugurlu., 2016) .

Table 4.3 Initial and final VS along with the %VS reduced during the experiment

Sr. no	Digester ID	Initial VS (g/L)	Final VS (g/L)	% VS reduced
1.	S3W	15	6.35	57.6
2.	S4W	13	6.39	50.84
3.	S5W	13.03	7.2	44.74
4.	S6W	14.32	3.94	72.48
5.	S3L	15.88	6.38	59.82
6.	S4L	13.45	7.43	44.75
7.	S5L	15.29	5.93	61.21
8.	S6L	15.82	7.27	54.04

4.6.1 pH of digesters

The pH of the digesters were measured at the beginning and then at the end of the co-digestion period. No buffer was added in between the retention time. The pH of the digesters showed little fluctuation however, the pH must have changed during the different phases of digestion. Figure 4.15 shows the initial and final pH of digesters during co digestion with wastewater and it was almost in the neutral stage. As during the stages of acidogenisis and hydrolysis, the efficient pH range is 4.5-7 (FNR, 2016). Methanogens that are responsible for methane production are highly pH sensitive. Therefore, it is suggested by literature to maintain the pH of single stage digesters between 7-8 (Bohutskyi & Bouwer., 2013).

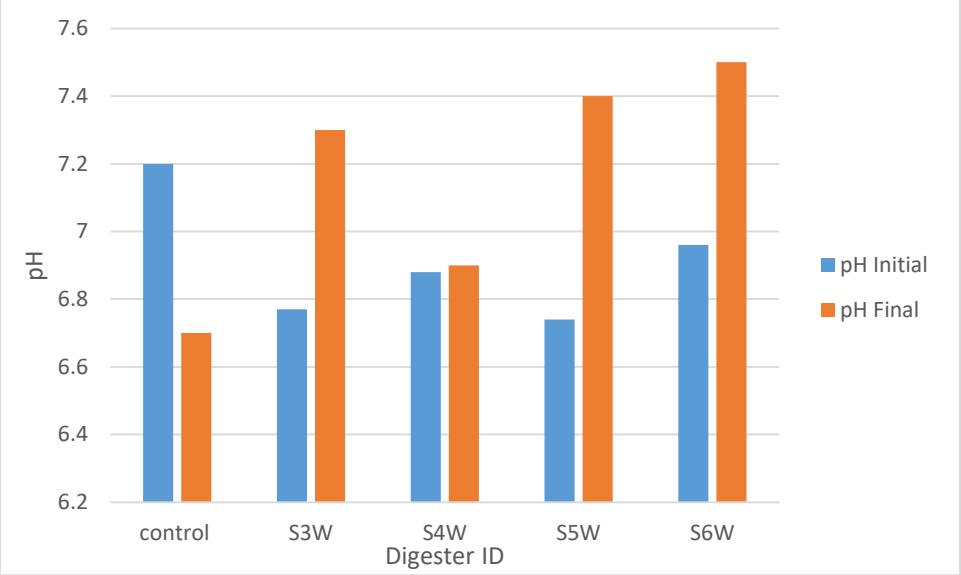

Figure 4.17 Initial and final pH of digesters used for co digestion with waste water

Figure 4.18 shows the initial and final pH of digesters with leachate and algae co digestion. The pH of the digesters were almost in neutral range.

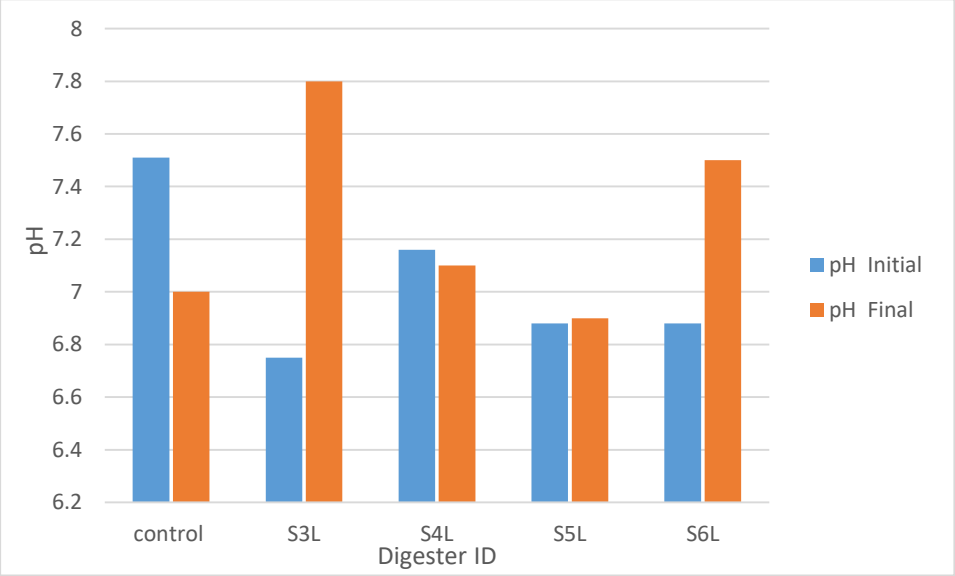

Figure 4.18 Initial and final pH of digesters used for co digestion with leachate

4.6.2 COD of digesters

The table 4.4 shows initial and final COD of the digesters during the experiment period the COD of digesters were measured at the start and end of the co

digestion period. It gives the estimate of how much of the substrate mixture has been utilized and biodegraded during anaerobic digestion process. COD of digesters were significantly reduced. The control digester during wastewater co-digestion observed maximum COD reduction of 96%. The S4W digester gave maximum COD reduction i.e. 78.2. While the least reduction was performed by S6W digester. The S6W digester may have performed well if anaerobic digestion period had been increased.

Table 4.4 Initial and final COD of digesters during experiment in mesophilic conditions

Sr. no	**Digester ID**	**Initial COD**	**Final COD**	**% Reduction in COD**
1	S3L	7379	1844	75
2	S4L	11912	5534	53.5
3	S5L	14758	3689	75
4	S6L	10146	3689	63.6
5	CL	1862	89	95
6	S3W	36890	9224	74.9
7	S4W	21215	4612	78.2
8	S5W	6456	2767	57.1
9	S6W	11068	5534	50
10	CW	1925	72	96

The results suggested that COD was significantly reduced in all digesters, which indicates that substrates were utilized during anaerobic digestion.

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

The "third generation biomass" i.e. the unicellular microalgae is known for growing easily in aquatic environment. This benefit of being able to grow in aquatic environment makes it suitable for growth in wastewater and leachate. Research has also proved them to be able to treat these concentrated contaminated waters. The utilization of algae grown on these waters harbors the benefits of not only treating them but also includes using them for energy production such as biogas production.

The conclusion drawn from the results of the research conducted are as follows.

(a) The wastewater and leachate samples showed nitrate and phosphate concentrations, which were capable of supporting microalgal growth.
(b) The four microalgal strains were acclimatized and grown in 100 % waste water and 50% leachate.
(c) The co digestion of microalgal strains with leachate and wastewater along with cow dung seeding proved to be suitable mixture (20% total solids) for biogas production.
(d) The co digestion of strain 6 with wastewater gave highest methane concentration i.e. 61.5%. While, the co-digestion of strain 4 with leachate gave highest methane concentration i.e. 57.6%.

5.2 Problems Faced

The problems faced during research are listed below:

(a) The biomass growth required for biogas production was hard to achieve in PET bottles.
(b) The digesters had to be regularly checked for gas leakages.
(c) Due to absence of algae screening tests, the tests were unable to determine that all nitrate and phosphate were solely consumed by algae.

5.3 Recommendations

To improve the efficiency of overall process, following recommendations are made to be pursued in further research:

(a) The locally isolated strain S3 showed great potential. Further research should be done using this strain.
(b) The relationship of algae-bacteria consortium, while being grown in leachate and wastewater should be studied.
(c) The growth of algae in leachate with pH control should be studied.
(d) The pH of the digesters should be monitored regularly in order to get better biogas results and it should be tested on a regular basis to check methane concentration.
(e) The digester feed should be optimized based on C/N ratio and further investigations needs to be done to find optimal operation conditions (loading rates, proportions, and retention times) for optimal biogas production.

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