Characterization of Local Algal Strain for Possible Lipids and Bioactive Compounds Production

By

MUNEEB QAYYUM

NUST-2012-60706-MCES-64112-F

Session 2012 – 2014

Supervised by

Dr. Ehsan Ali

MASTERS of SCIENCE

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ENERGY SYSTEMS ENGINEERING

Center for Advanced Studies in Energy (CAS-EN)

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Islamabad, Pakistan

January, 2015

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H-12, Islamabad, 44000, Pakistan

Certificate

This is to certify that the work in this thesis has been carried out by **Mr. Muneeb Qayyum** and completed under my supervision in Biofuels Laboratory, Center for Advanced Studies in Energy, National University of Sciences & Technology, Islamabad, Pakistan.

Supervisor	_____ Dr. Ehsan Ali Center for Advanced Studies in Energy NUST, Islamabad
GEC Member # 1	_____ Dr. Imran Hashmi Institute of Environmental Sciences and Engineering NUST, Islamabad
GEC Member # 2	_____ Dr. Muhammad Faraz Bhatti Atta-ur-Rehman School of Applied Biosciences NUST, Islamabad
GEC Member # 3 (External)	_____ Mr. Shahid Hussain Ansari Center for Advanced Studies in Energy NUST, Islamabad
HoD–CAS-EN	_____ Dr. Zuhair S. Khan Center for Advanced Studies in Energy NUST, Islamabad
Principal/Dean	_____ Dr. M. Bilal Khan Center for Advanced Studies in Energy NUST, Islamabad

Abstract

Green microalgae are potential candidate for fulfilling future energy demands along with conserving nature. Microalgae is good source of lipids and some essential bio active compounds like carotenoids, alkaloids, phycobilins and other proteins etc. but according to a Research under the Department of Energy Aquatic Species Program during 1978-1996 concluded that cultivating algae for biofuels was cost prohibitive at that time and that an integrated approach should be studied that combined algal biofuel production with other goals like environment and other products to make it a viable option. In this research our main goals was first to capture carbon dioxide from algae using local algal strain by large raceway pond system and to cut down the cost of algae products by designing cheap algal media which will definitely help this technology to become more adoptive. Presently algae are being cultured with conventional medias which are available in pure form and are much costly. These conventional medias significantly increase the cost of algal products and even sometimes large scale production becomes impossible due to heavy cost of media. A feasibility study was conducted for installing algae production facility at local industry using Industrial Emissions (CO_2) as a carbon source. To meet the cost issues, raceway pond design were chosen to best fit in local environment. A comprehensive design study of raceway pond was conducted to make it economically viable, and a cheap nutrient media was also designed using commercial grade nutrients., A local algal strain, initially isolated and named as KKL5 and later identified as *Dictyosphaerium 8-6* by Macrogen Inc. was used to carry out all these studies. A number of experiments were performed in series to provide local algae with essential nutrients from cheap sources like agricultural fertilizers, market available multivitamins and egg proteins. The algal growth parameters were checked regularly and effects of cheap nutrient sources onproductivity of lipids, carotenoids and phycobiliproteins were also noted using UV spectrophotometeric techniques. All results were compared with a standard culture (Bold's Basal Medium). A best recipe of media was screened out with results as competitive as Bolds' Basal media.

Keywords: Bio-fuels; Proteins; Lipids; Algae; Bio-Diesel.

Dedication

I would like to dedicate my thesis to my beloved parents and teachers.

Acknowledgements

First and foremost, I would like to extend my sincerest gratitude to my thesis supervisor, Dr. Ehsan Ali, for providing me with a wonderful opportunity to work under his tutelage and guidance. His advices and suggestions had been a constant source of motivation and learning during my stay at Biofuels Labs.

Many thanks to my co-supervisors, Dr. Imran Hashmi and Dr. Muhammad Faraz Bhatti, from Institute of Environmental Sciences and Engineering(IESE) and Atta-ur-Rehman School of Applied Biosciences(ASAB), NUST for their support and encouragement. I would like to mention the effective cooperation of Mr. Shahid Hussain Ansari, Center for Advanced Studies in Energy, NUST, whose well-intentioned comments produced a substantial improvement in the reasoning and correlating the results in thesis write-up.

I would always remain indebted to Dr. Usman Shah Kazmi, CIIT, Islamabad, for his help in designing the experimentation phase for lipids and bioactive compounds determination

I would also like to mention the effective cooperation of Mr. Aleem Uddin and Mr. Yawar Ikram from Attock Oil Refineries, Rawalpindi for allowing us to use their facilities for feasibility study of carbon capture via algae project.

Many thanks to PGP and Research Directorates of NUST for their financial support for the completion of my thesis.

My warm wishes for my fellow lab members; Muhammad Naveed Akbar, Mustafa Anwar, Muhammad Akmal Rana and Sehar Shakir for their continuous support and help. I will cherish the time I have spent with them and hope for their bright future and success in both their careers and lives.

Finally, I owe my deepest gratitude to my family members whose unconditional love, absolute affection, infinite trust and endless confidence inspired me to complete my work.

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List of journal/conference papers from this work

1. Muneeb Qayyum, Abeera Ayaz Ansari, Asif Husain and Ehsan Ali "An Attempt to design Cheap Media for local algal strain KKL-5*(Distyosperium iso 6-8)"* Polish Journal of Environmental Studies.

*Attached in **Annex 1**

Chapter 1

Introduction

1.1 Background

In recent years, algae production has received large attention all over the world due to the wide utilization of algae. They are potential sources of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, pharmaceutical, and fuel industries based on their attractive advantages. These include good growth and formation of large amounts of biomass, non-utilization of arable land, carbon dioxide fixation, high oil accumulation etc. Especially in the biofuel development, algae have been expected to be the most favorable energy "crop" which has the potential to replace fossil fuels in the future. However, algae research on basic biology and species selection and molecular characterization have lasted for a very long time, while the industrial production of algae, also at large scale, for different purposes have only been studied for the last decades. Commercial large-scale algae cultivation has started in the middle of 20^{th} century. In the following decades, large-scale algae production developed very fast in the whole world, mainly for food and nutrients supplement production. Nowadays, with the increased understanding of algal physiology and development of the bioprocess engineering, the research is more focused on the efficient biomass formation for biofuel production. Unfortunately, lots of unwanted problems still challenge scientists. For example, theoretical high lipid productivity has not been validated on a large commercial scale because of the inefficient culturing systems, restricted photosynthetic capability and productivity, and lack advanced technologies for harvesting and oil recovery for an efficient biofuel production [1].

1.2 Algae Basics

Algae constitute a large and diverse group of eukaryotic microorganisms, ranging from unicellular to multicellular forms [1]. According to this, two main types of algae can be distinguished: 1) macro algae, also called seaweed that are normally growing in oceans

and can reach considerable size (up to about 50 meters); 2) microalgae that are microscopic sized microorganisms, which can be found in marine and fresh waters.

Algae can carry out photosynthesis like other plants, but they lack water transporting tissue, leaves, roots and flowers. This makes them distinct from land plants. Several characteristics, such as their pigmentation, life cycle and basic cellular structure, are used to categorize algae into a variety of classes. The presence of chlorophyll is apparently the most important one. Algae contain chlorophyll and are thus mainly green in color. Some kinds of algae contain other additional pigments, such as xanthophyll and carotenoids, which make them appear brown or red [1]. Four divisions have been initially introduced by W.H. Harvey (1811-1866) as green algae (chlorophyta), red algae (rhodophyta), brown algae (heteromontophyta) and diatomaceous [2].

Some algae are autotrophic (photosynthetic) organisms that use light as the energy source and other inorganic compounds such as CO_2 and salts as food. Some are heterotrophic (no photosynthetic) organisms and require additional organic compounds as nutrient and energy sources (fig. 2.1). In addition, some algae are referred to as mixotrophy that can use a complex spectrum of nutritional strategies, combining photo autotrophy and heterotrophy, i.e. They have the ability to both perform photosynthesis and acquire exogenous organic nutrients [3].

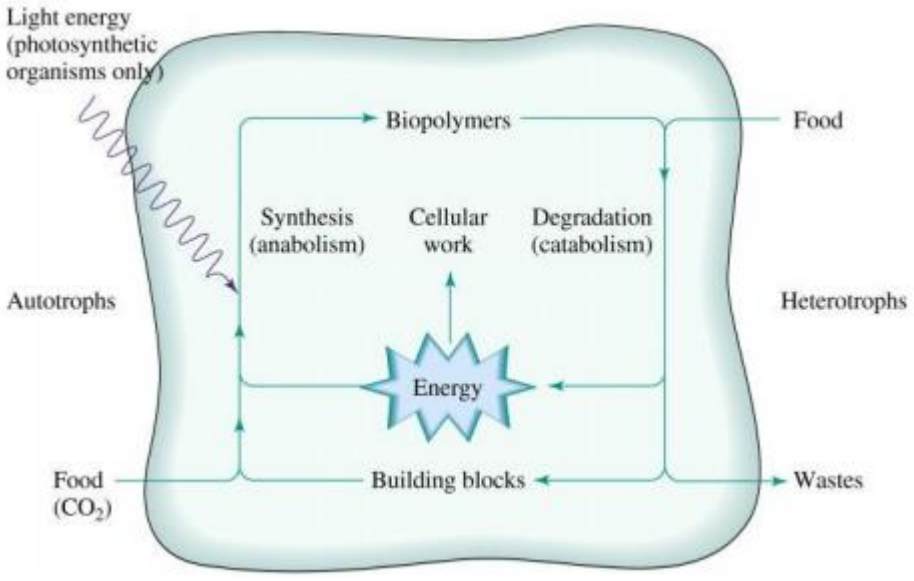

Figure 1.1: Metabolism processes of autotrophs and heterotrophs [4].

1.2.1 Algae and Environment

During the last century, human activities have caused a lot of environmental problems including high global greenhouse gas emissions and declining water quality. It has been reported that since pre-industrial times global greenhouse gas (GHG) emissions have been increased with 70% between 1970 and 2004, of which especially the CO_2 emissions have increased up to about 80% [5].

A climate change has probably been the result of the increased emissions, and thus this trend will continue to grow over the next few decades. In order to mitigate the climate change many strategies have been suggested, such as change of individual lifestyle, behavioral pattern, and industrial management practices, to reduce energy use and GHG emissions [5]. Besides that, some new techniques are developed to eliminate those gases potentially responsible for global warming. Algae biomass production may be a possible way to reach the purpose due to its ability of CO_2 fixation via photosynthesis. By comparison to conventional forestry, agricultural, and aquatic plants, algae mostly present much higher growth rate and CO_2 fixation abilities, and require small growth areas, energy consumption and costs. A more profitable system will be made when combining the algal CO_2 bio-mitigation processes with other processes such as wastewater treatment, biofuel production etc. [6].

1.2.2 Algae and Future Energy

The high level of GHG emissions is mainly attributed to the large scale use of fossil fuels for transport, electricity, and thermal energy generation. With the global climate change, it has become increasingly important to develop effective technologies and unconventional renewable biofuels to reduce the fossil fuel usage. The first-generation biofuels is the liquid biofuel production based on sugar and starch crops (for ethanol) and oilseed crops (for biodiesel). But it is restricted due to the negative impact on global food markets like competitive consumption of crops or other food and competitive requirement of arable land. Thus, the currently developed technologies that use lignocellulose biomass for biofuel production is referred to as the second-generation of biofuels, which can avoid the competition impact of food. The cost of cellulosic feedstock itself is lower than the first generation feed stocks, however, the conversion

technology for converting cellulosic biomass into liquid fuel is difficult and has not yet reached the scales for commercial exploitation [4]. Thus, with many advantages like large oil content (up to 75% of dry weight) [7], low land occupation and efficient photosynthesis, algae have been considered to be an alternative energy resource that can technically and economically overcome the problems associated with production of first and second generation of biofuels, and the liquid biofuels derived from algae is specifically defined as the third generation of biofuels [3] . Especially when combining algae oil production with carbon dioxide elimination, wastewater treatment or other biological processes, there is a great potential that an efficient and economical system can be built for biofuel production [3].

However, after several decades of investigations it is still not possible to produce algae biofuels in an economically efficient way compared to using fossil fuels. Lots of challenges still exist, such as lack economical and efficient culture system, no standard harvesting techniques, and restricted oil recovery process that caused by the oxidation reactions of presented polyunsaturated fatty acids (PUFAs) and high moisture content of algal feedstock. In order to solve those problems, the most important things to work on are the development of screening techniques for selecting better algae strain, as well as the investigation of optimal culture conditions and production systems. Furthermore, some studies have suggested that the harvesting technologies already used in the food or wastewater treatment sector may suitably be used as possible solutions for the harvesting problems in biofuel production, and thermochemical liquefaction and pyrolysis may also be used as the most technically and feasible methods for conversion of algal biomass to biofuels [3].

1.3 Problem Statement

With growing energy demands and environment concerns it is need of hour to explore new horizons for fulfilling our energy needs without doing any harm to our environment.

Coal was past, Fossil fuels are present and renewables are future. So, eyes of the world are on algae as well as it contains a lot of potential to provide with carbon neutral fuels and essential bioactive compounds along with sequestration of carbon dioxide.

Considering these aspects it would be a good idea to cultivate local algae within the vicinity of power plant to capture carbon dioxide and other flue gases and the algal biomass can further be processed for lipids and other bioactive compounds.

1.3.1 Objectives

The objectives of this research were:

- Isolation and Genetic Identification of algal strain.
- Study growth parameters of KKL-5 in ambient conditions using synthetic growth media and optimization of certain factors like pH, light and temperature.
- Design low cost media for large scale production of KKL-5, also comparing it with conventional media for certain growth parameters like cell count, turbidity etc.
- Determination of lipid contents in KKL-5 and study the effect of newly designed media on lipid contents.
- Determine of various bioactive compounds through UV spectrophotometer
    - Chlorophyll a ,b , c
    - Total carotenoids
    - Phycobili proteins
- Propose an algae biomass producing system within locality of ARL to capture carbon dioxide and to produce value added compounds including lipids.

1.4 Thesis Overview/Summary

In the study local algal strain was isolated and further evaluated for its lipids and bioactive compounds potential. Local algal strains should have more growth rate in our environment than foreign strains due to natural habitat conditions.

Chapter 1 (Introduction) contains information about global energy and environment concerns. And algae potential regarding energy and environment is discussed in this chapter. A brief problem statement is also stated.

Chapter 2 (Literature Review) is about literature review. A comprehensive literature review was done and stated regarding our problem statement. Work done by various researcher was explored to get the most up to date information about algae potential.

Chapter 3 (Review of Available Technologies) is about microbiological techniques used in this study.

Chapter 4 (Materials and Methods) is about the technology that is available till now to carry out scientific experimentation. A review of different technologies available was done and a suitable methodology was designed with keeping in mind the available resources. Details of experimentations performed is also stated in this chapter.

Chapter 5 (Results and Discussion) contains the results from the experiments performed during the study. Results were also discussed with reference to available literature.

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Chapter 2

Literature Review

2.1 Introduction on Microalgae

Algae represent a large group of different organisms from different phylogenic groups with no particular taxonomic standing [1]. In context of size and morphology, algae can be ranging from unicellular to filamentous and multicellular. Based on this feature algae is categorized into two groups: 1) macro algae, also known as seaweeds normally found in the ocean and have plant-like appearance due to their considerable size (can grow up to about 50 meters) [1].; and 2) microalgae: the oxygen producing chlorophyll a containing photosynthetic type of algae, which are only visualized under microscope [2]. This group of algae is highly diversified in morphology and usually is found in aquatic environment, including fresh water, brackish water, marine water and moist surroundings (lichen, symbiosis with fungi). Due to the presence of chlorophyll, microalgae are generally green in color. Some types of microalgae contain different pigments, such as xanthophylls and carotenoids, which provide them brown or red appearance [1]. Microalgae used in this study are all green algae grouped under Chlorophyta division [2].

2.2 Algae growth: Autotrophy, heterotrophy and mixotrophy

Knowledge regarding algae growth is one of the key features that are essential to know for proper implementation of algae technology. Microalgae species that are capable of using energy obtained directly from light are referred to as autotrophic microalgae. These microalgae actively sequester carbon dioxide from the atmosphere as carbon source [3]. In addition, some microalgae can also utilize organic compounds as their carbon and energy source, eliminating the need of CO_2 and light. This mode of growth is called heterotrophic growth [3]. When microalgae can use both organic and inorganic carbon source simultaneously, they are growing as a mixotrophic culture [3].

In this study, the selected microalgae are autotrophic microalgae in general. However, some of them are capable of heterotrophic growth: *Chlamydomonas reinhardtii*,

Chlorella sorokiniana, *Chlorella vulgaris* and *Chlorella protothecoides* [4], and some can grow mixotrophically as well: *Chlorella protothecoides* [5], *Chlorella vulgaris*, and *Scenedesmus obliquus* [6]. *Chlorella sorokiniana* is reported to grow heterophically at night and switch to mixotrophic growth during day time [3]. Based on the growth mode, biomass productivity of microalgae may vary. According to Lee [4], heterotrophic culture shows relatively lower maximum specific growth rates compared to photosynthetic growth, but result in higher cell density and productivity.

2.3 Algae Photosynthesis

In general Photosynthesis comprises a series of reactions where carbon, from its maximally oxidized state (CO_2) is converted to reduced compounds (carbohydrates), using light energy. During respiration, these reduced compounds can be re-oxidized to CO_2 [1].

2.3.1 Photosynthetic apparatus

For algae, photosynthetic energy transformation is the basic energy supplying process and it takes place in the chloroplast: an organelle found in plant cells and eukaryotic microalgae [7].Chloroplast consists of three membranes: outer membrane, inner membrane and thylakoids. A semi gel like fluid: stroma is present within the outer and inner membrane, where the thylakoid system is immerged [1].

The photosynthetic unit comprises of photosystem-I (PS-I), photosystem-II (PS-II) and the light harvesting complexes (LHC). A pigment system is integrated into the thylakoid membrane, distributed throughout PS-I and PS-II complex system[8]. The major pigments for photosynthesis are: chlorophylls (chlorophyll a, b, c1 and c2), carotenoids, cytochromes, and phycobiliproteins. Each has distinctive absorption spectrum as a characteristic of different algal divisions [5]. Chlorophyll is a cyclic tetra pyrrole molecule, ubiquitous in most photosynthetic organisms. This pigment molecule is crucial for both the light harvesting and the reaction center complexes of the photosynthetic apparatus [9]. In general, the basic function of pigments is to absorb light energy and transfer it to P680 and P700 [10]. Another important function is protecting the photosynthetic assemblies from photosensitization, especially by secondary carotenoids,e.g. β-carotene, zeaxanthin [8].

2.3.2 Light dependent Reaction

During the light dependent reaction, light energy is captured and transferred to reducing potential and energy molecules, NADPH and ATP. This reaction takes place in the thylakoid membranes of chloroplasts [9].

Once the algal cells are exposed to photons of appropriate energy, the antenna pigments absorb and transfer the energy of photons to the photosynthetic centers. PS-II is the central to the photosynthetic process, as it catalyzes the photo-induced oxidation of water molecule, followed by release of electrons and protons. The electrons are transferred to the stromal side of PS-I to reduce NADP+ to NADPH. The concomitant proton gradient generated across the thylakoid membrane is used to drive the ATP synthesis [1]. However, no ATP is released if the gradient force is too small (pH gradient less than 2). Furthermore, the sequential arrangement of PS-I and PS-II determines the fate of released electrons [1]. The final end products in the light reaction are ATP and NADPH.

2.3.3 Dark Reaction/ Light independent Reaction

During the light independent reaction, the assimilatory power of NADPH and ATP is utilized to fixate CO_2 into carbohydrates [1]. This reaction takes place in chloroplast stroma (eukaryotic algae) or in cytoplasm (prokaryotic algae). Generally, light independent reactions take place simultaneously with the light reactions, except for the fact that these reactions can occur in dark as well [1].

The reaction scheme involves three phases: first, carboxylation, where CO_2 is added to a 5-carbon molecule, Ribulose bisphosphate (RuBP), resulting in a 6-carbon molecule that is later broken down to form 3-phosphoglycerate. The reaction is catalyzed by the enzyme Rubisco. In the second phase, reduction of 3-phosphoglycerate takes place by NADPH2 to form phosphoglyceraldehyde. In the final phase, ribulose bisphosphate is regenerated from triose phosphate, through a series of reactions to bind more CO_2. ATP from the light reaction is utilized in the final step [1]. The products of the dark reaction includes carbohydrates, fatty acids, amino acids, and organic acids [6].

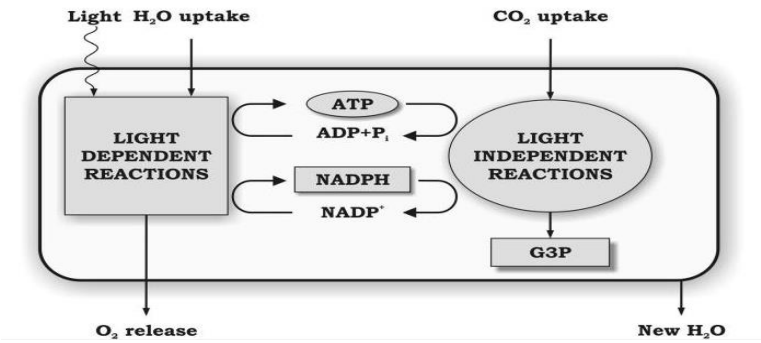

Figure 2.2: Schematic drawing of the photosynthetic machinery, redrawn from [1].

2.4 Factors affecting microalgal growth

2.4.1 Light

Light is one of the most important factors in aspect of microalgae growth, as it is the fundamental energy source for photosynthesis. In an outdoor cultivation system, natural sun is used to take advantage of the natural provided light, minimizing the cost of production. In an indoor/closed setup, artificial light is supplied uninterruptedly or in light and dark cycles.

Theoretically, only the visible portion of the natural sun light, ranging from 380 nm to 750 nm is utilized during photosynthesis, leading to energy losses while using natural light [8]. Furthermore, availability of sun light is extremely dependent on regional climate, with variation in the light intensity both daily and seasonally. In Sweden, there is a great deal of difference of light hours among summer and winter. In summer during June, the light is available from 160 to 340 hours, whereas, in winter during December the time range of available light is from 0 to 60 hours only [11]. Apart from light hours, the light intensity also varies significantly during these two seasons: light intensity is in June between 140 to 200 kW/m^2 and in December between 0 to 20 kW/m^2 [11]. Therefore, despite the cost efficiency, utilization of sunlight as a growth parameter in Sweden is challenging. From the data it is clear that, winter is not suitable for algae cultivation and Sweden has long winters. An alternative to sun light is using artificial light in a closed cultivation system, confirming a relatively controlled environment for the algal growth.

When exposed to light, there are three possible incidents that may occur: light adaptation, light saturation and light inhibition [12]. At presence of lower irradiance, the onset of the cellular mechanism for increasing the number of photosynthetic units allows more efficient harvesting of light energy. At high irradiance, the numbers of units are declined due to inhibition of the pathway. Regulation of these mechanisms enables the algal cell to adapt to natural variations in irradiance. The event is called light-adaptation or photo-adaptation [7]. Algae become light saturated when the photosynthetic rate is at its maximum. Prior to reach saturation, when light is limiting, the photosynthesis rate is linearly dependent on the light irradiance, meaning algae cells grow faster as the light intensity increases [11]. Once the light saturation point is reached, the cells reduce the number of photosynthetic units leading to photo inhibition [9].

2.4.2 Temperature

Temperature is another major influencing factor for optimal growth of microalgae, as temperature below or above the optimum temperature of the species may lead to different stress responses in the algal cell. This is particularly an important issue when the culturing is conducted to be in a Northern country like Sweden. Sweden has a long winter, when the temperature is between -16 to 4oC during December and in June the temperature is between -2 to 16 °C [11], which is quite lower temperatures comparing with tropical countries. Apart from the lower temperature, while culturing in natural way (outdoor), algae has to face temperature fluctuations as well.

Suboptimal temperature, which indicates very low temperature, combined with other environmental factors like high light or excessive oxygen concentration, may lead to photo inhibition, resulting in impaired algae growth [13]. Additionally, low temperature also reduces the rate of CO_2 fixation, resulting in low algae biomass levels [13].

Fluctuation in temperature leads to onset of stress response in algae cells, which thus counteracts the effects of an increase in temperature. In other words, even though the temperature is increased gradually to an optimum level, the initial exposure of the culture to suboptimal temperature for a short time may result in significant decrease in the photosynthetic parameters [7]. This type of inhibition is more likely to happen in an open culturing system in Sweden, where temperature is lower relatively to the light

irradiance. However this can be avoided in the closed cultivation system by exposing the microalgae to a fixed temperature from the beginning.

Contrarily, lower temperature than optimum level influences the lipid profile of algae species. In order to adapt to the lower temperature, cells begin to synthesize unsaturated fatty acids in the thylakoid membrane to enhance its stability and fluidity, which in turn helps to protect the photosynthetic machinery from photo inhibition [7]. However, the impact of low temperature is more directed to the type of lipid rather than enhancing the total content of lipid [7].

Temperature also has a direct correlation to the nutrient concentration and cell constituent. The algae cell volume and biochemical content increases if the temperature is below or above the optimum level, thus more nutrients is required to grow microalgae compared to growth at optimum temperature [7]. It has been found that, high temperature significantly increased the chlorophyll a content in *S. obliquus* when there was a fairly high amount (10 mg/L) of phosphorus present in the media. In another report [14], it was found that the inhibition of *S. obliquus* growth by excessive phosphorus was highly dependent on the culture temperature. However in this report, the temperature optimum for the phosphorus consumption (25 °C) was found to be different compared to at the optimum temperature for the algal growth (30 °C). Therefore the choice for temperature suitable for an algae culturing system needs to be decided strictly based on the original aim of algae cultivation.

2.4.3 pH

The pH is another environmental factor crucial for microalgae growth. In the algae culture; pH is balanced by the interplay between photosynthesis and respiration. CO_2 fixation during photosynthesis liberates OH^- in the culture, which accumulates while the algae species grow and results in an elevated pH in the system. A similar reaction takes place, when bicarbonate is the inorganic carbon source [3]. During respiration, the opposite reaction takes place, resulting in decreasing the pH in the culture [3].

Besides CO_2 and bicarbonate, nitrate and ammonium also influence the pH level in a culture. When ammonium is the sole nitrogen source of a culture, due to release of H^+

ions during assimilation a decrease in pH is obtained, whereas nitrate as major nitrogen source has the opposite effect [3].

It is very important to maintain the pH level in a culture medium, as very high pH (>9.0) inhibits the photosynthesis. Because at this pH most inorganic carbon is in the carbonate form, thus cannot be taken up by photosynthesis. High pH also influences the overall nutrient uptake, by mediating flocculation [15].

2.4.4 Nutrients

After the environmental parameters are fulfilled, microalgae require presence of nutrients in the water (or habitat) in order to grow. Microalgae have relatively simple nutritional demand, which make them an attractive package for commercial production. Carbon, nitrogen and phosphorous are the major nutrients for survival of microalgae. Among other nutrients some are macronutrients such as sulfur, potassium, calcium, magnesium and some are micronutrients, like manganese, copper, molybdenum, boron, iron, zinc, chloride, nickel etc. [3]. These trace elements has an important role in many enzymatic reactions and biosynthetic pathways [3]. The requirement for nutrients may vary depending on the type of microalgae species. An overall stoichiometric formula for an algae cell including the most common elements is $C_{106}H_{181}O_{45}N_{16}P$ [3][16].

Carbon

The carbon sources can be either organic or inorganic or both, depending on the growth mode. For an autotrophic system microalgae use mainly carbon dioxide and bicarbonate (HCO_3^-) to make up most of the carbon skeleton. Heterotrophic microalgae grows on organic carbon sources such as glucose, fructose, and acetic acid [3].

Nitrogen and Phosphorous

Nitrogen (N) is the second most important nutrient after carbon, contributing to the algae biomass production. Although several inorganic compounds can be utilized by algae, for most of them, nitrate, ammonium, and urea are the sole N-suppliers [3]. However, when both nitrate and ammonium are present in a culture, algae often prefers ammonium over nitrate as their nitrogen source [3].

Phosphorus is another vital nutrient for algal growth, as it is an active participant in many metabolic processes, like energy transfer and different biosynthetic pathways. Algae take up phosphorus mostly as orthophosphate (PO_4^{2-}) in an energy dependent way [3]. Although a low amount of phosphorus is required for algae growth compared to C and N, phosphorus uptake can be limiting because orthophosphates can easily bind to other ions present in the culture (CO_3^{2-}, iron etc.) and precipitate [3]. High pH also mediates precipitation of phosphate, which re solubilizes when the pH drops [15].

Sulfur

Sulfur is an essential macronutrient that is utilized by microalgae, primarily as the inorganic sulfate anion [17]. In microalgae, metabolic changes take place in order to acclimate to any environmental stress including nutrient deficiency. It is known that, generally, microalgae in nutrient deprived conditions tend to accumulate lipids. In case of mineral deprivation, sulfur in particular, has been shown to increase hydrogen production [18]. Morsy [19] reported that culturing *Chlamydomonas reinhardtii* in sulfur limiting condition leads to increase in anaerobic H_2 production. Although both nitrogen and sulfur deficiency cause elevation of lipid content, according to Cakmak *et al*, 2012, (30) tri acyl glycerol (TAG) production in *Chlamydomonas reinhardti*i is larger in a sulfur limiting culture.

2.5 Algae Cultivation

2.5.1 Mode of growth: Batch or Continuous

In a continuous culture, the medium is replenished with new medium, while the existing medium is continuously used up by the algae. Theoretically, new medium is fed in similar rate as the culture removed, thus the culture volume and rate of dilution is maintained [20]. However, in practice, a continuous culture is rather complex to install and run. A major drawback is that it is difficult to maintain the reproducible cultivation set-up and quality of the feedstock [21].

In the batch culture, the culture medium and the algae species are prepared only once at the beginning of the cultivation. No more medium is fed during the growth, but CO_2 and air can be added in order to enhance the growth [20]. Unlike the continuous culturing

mode, in a batch culture the growth rate varies throughout the culturing period because, when cell density becomes high, the light intensity begins to vary with time and location, leading to a light limiting situation. On the other hand, in a continuous culture the light varies only with location [22]. If a small amount of the algae inoculum is added, a lag phase may occur representing the adaptation phase of the cells to the new environment [20]. As the cells adapt to the new culturing condition, the lag phase is followed by an exponential growth phase (log phase), which shows to be linear due to photoautotrophic growth [20]. The growing culture reaches to a state when the cell density, restrain the penetration of light to reach all cells and cause lack of light saturation to those cells. This may result in limited biomass production giving rise to the linear trend of growth [20].

2.6 Algae Cultivation in Low cost Medium

It is well known that freshwaters display a wealth of environments and algal flora. The distribution of algal species in freshwaters depends not only on the selective action of the chemophysical environment but also on the organism's ability to colonize a particular environment. Therefore, various culture media have been developed and used for isolation and cultivation of freshwater algae. Some of them are modifications of previous recipes to meet a particular purpose, some are derived from analysis of the water in the native habitat, some are formulated after detailed study on the nutrient requirement of the organism, and some are established after consideration of ecological parameters [6].

However the cost remained the first question whenever the cultivation of algae is considered for large scale production, and algae culture media comprises the major running cost of any large scale setup for algae. Many attempts are made to reduce the culturing cost. Nutrients from cheap sources are tested by many scientists to lower down the media cost. Nabris *et al. 2012,* Ashraf *et al. 2011,* Basirath *et al. 2006* developed cheap culture media for algal species like *Nannochloropsis sp., Chlorella Vulgaris, Spirulina* from cheap nutrient sources like agriculture grade fertilizers and cattle dung [23][24][25]. The results were satisfactory but not much comparable to synthetic Medias like Bold's Basal media and Zarouk's media for fresh water algal strains. But the overall

economy of the system can be improved significantly. Man *et al. 2012* studied the potential of organic fertilizers for biodiesel production and concluded that outdoor microalgal cultivation is only economically viable if the nutrient sources are cheap enough [26]. And organic fertilizers have good potential to provide algae with essential nutrients like nitrogen , phosphorous, calcium and sulfur [26].

In our study, we designed a low cost media from agriculture grade fertilizers along with egg proteins and vitamins to enhance the algal growth, as algae require vitamins [27] and proteins for its growth so it was perceived that addition of these compounds will enhance its growth significantly.

2.7 Products from algal Biomass

2.7.1 Biofuels

The most prevalent way of making biofuel from microalgae is to produce biodiesel from algal lipids (oil) through transesterification [28]. Compared to plant-based oils, algae oil has relatively high carbon and hydrogen contents and low oxygen content. These characteristics make algae an attractive biodiesel feedstock because they lead to a fuel with high energy content, low viscosity, and low density. In most cases, biodiesel can be directly combusted in a standard diesel engine without the issue of blending with regular diesel. Transesterification of alcohols and lipids are the chemical reaction required to produce biodiesel, with glycerol being produced as a byproduct [29].

Ethanol

Certain strains of algae are capable of producing high levels of carbohydrates, such as starch, which is an ideal fermentative substrate. High ethanol productivity can be accomplished from fermentation of algae biomass [30]. The production of ethanol from algae is done by breaking the cells via mechanical means followed by dissolving carbohydrates with either water or solvent. Once the carbohydrates are extracted, traditional methods, such as scarification with enzymes and fermentation with bacteria or yeasts, are used to produce ethanol. Finally, a distillation step concentrates the ethanol. In addition to producing ethanol via fermentation of algal starch, ethanol can be also be produced directly by algae. This process includes genetically modifying an algae

species so it secretes ethanol into the medium. This process has the advantage of not having to harvest the actual algae cells but rather separating the ethanol from the culture medium. This technique has been adopted by the algae company Algenol claiming that their technology can produce 9,000 gallons of ethanol per acre per year. In comparison, corn ethanol produces approximately 890 gallons per acre per year. They project the production cost to be only $ 0.85 per gallon.

2.7.2 Non-Fuel Products

Animal Feed

In the United States, a majority of livestock feed is derived from corn or soybean. In recent years, algae has received increased attention as an alternative feed source that can reduce the demand for these two crops. Microalgae have the benefit compared to conventional feed crops in that it can be grown on non-fertile land. Another benefit is that many species have protein content that exceeds that of both soybean- and corn-based products.

In 2007, approximately 30% of all cultivated algae were grown for animal feed [31]. In the coming years if large-scale algae cultivation for biofuels is successful, a massive quantity of lipid-extracted algal residue must find a market. The most obvious market is animal feed applications. The most promising algal feed applications are poultry and aquaculture [32]. There is also interest in using it as a cattle, swine, pet, and various other animal feeds.

World aquaculture is expected to dramatically increase in the upcoming decade due to shortages in wild fish populations. As aquaculture becomes more popular, the need for feed will also increase. Algae can serve as an optimal feed for various forms of aquaculture including fish, crustaceans, and molluscs [32]. A common provider of both protein and lipid for aquaculture is fish meal which price is heavily dependent on capture fisheries and wild fish populations. Algae can provide an alternative protein and lipid source for these animals.

Human Consumption

Microalgae were first consumed by the Chinese nearly 2000 years ago to survive during famine [33]. In modern times, humans can consume whole cell algae in tablets, capsules, liquids, and incorporated into various other products, including pastas, drinks, gums and snack foods. The commercial applications are dominated by a few genera of algae. *Spirulina* and *Chlorella* dominate the worlds production in the consumption of whole cell algae [33]. The largest microalgae human food production site is owned by Earthrise Farms. This facility is located in California, USA and stretches over an area of 440,000 m^2.

A majority of the algal products that humans consume are produced from a specific portion of algae, not whole cells. These products are commonly taken as a supplement in a pill or capsule. The most common algal compounds consumed are omega-3 fatty acids and β-carotenes, both of which are produced by special algae at very specific growth conditions. *Dunaliella salina* is the most common species to produce β-carotene because of its ability to produce up to 14% DW of this product. The most common species for producing the omega-3 fatty acids, *eicosapentaenoic acid (EPA)*, are *Nannochloropsis, Phaeodactylum, and Nitzschia* [34].

Summary

Microalgae is comparatively a new option regarding renewable energy resources. And true potential of microalgae is not explored yet as the estimated species range from 30,000 to about 1 million, but only 20-30% are known or identified [35]. But a little fraction of known algal species are oil producing. So, it is need of time to explore new species that can possibly contribute in renewable energy market. In this study, we focused on exploring our local species and characterizing them for lipids and bioactive compounds. Bioactive compounds are other than fuel products from algae and are useful in making algae projects economically viable.

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Chapter 3

Review of Available Technologies

3.1 Algal Species Isolation

Pure samples of algae are required for characterization or any other analytical study of algae. For that reason, algal cells of specific species need to be isolated. Enrichment of algal samples is done and then isolation, number of techniques are available to isolate the algal strains. Some of them are describes below:

3.1.1 Florescence Activated Cell Sorting (FACS)

Flow cytometry is a technology that allows for the analysis of multiple physical characteristics of single particles as they flow in a fluid stream through a beam of light. The properties measured may include particle size, granularity, and fluorescence intensity, among others [2].

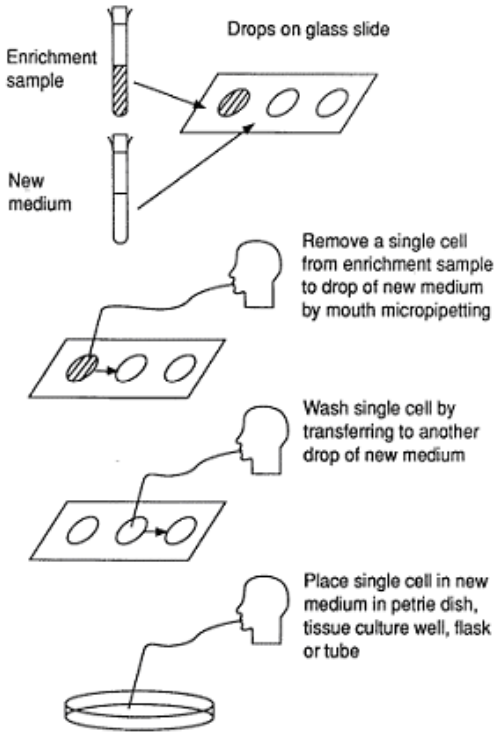

Figure 3.3: Fluore separation based on diff am [1].

Figure 3.4: Micromanipulation of an enrichment sample with a capillary micropipette.

The FACS machine was developed by Bonner [1] following the flow cytometry principle, and it is used to sort a heterogeneous mixture of biological cells, one at a time, based on their specific light scattering and fluorescent characteristics. The cells pass sequentially through a laser beam and cellular fluorescence give rise to electrical signals. Then, the stream is modified into a series of uniform size drops downstream of the laser, each containing one cell. The fluorescent signals are used to give appropriate electrostatic charges to the drops, which pass between two charged plates and are deflected to appropriate containers (as shown in Figure 3.1).

Even though the machine is a very fast and efficient method to isolate specific types of organisms from complex cell mixtures, in general, cell sorters are expensive and need a trained, dedicated technician to run it properly. In many laboratories where isolations are not done repeatedly, the purchase of this equipment is not justified and alternative methods must be practiced.

3.1.2 Micro-manipulation

The micromanipulation method consists in first producing a micropipette by heating and pulling out from both ends of a capillary tube. The narrow end should be at least twice the diameter of the cell to be micro manipulated. A silicone tube is attached to the thick end of the micropipette that will allow the investigator to suck up a single cell from the enrichment media; this procedure is done under microscope [3].

The cell obtained by sucking is transferred to a drop of sterile medium. Then, the micropipette is sterilized, the cell is picked and transferred to a new drop. The process is repeated to 'wash' the cell from bacteria.

To reduce the probability of bacterial contamination, the cell can be washed several times. However, the more times a cell is handled the higher the risk of cell damage. Hence, the optimum number of washes will depend on the type of algae. Finally, the cell is placed in culture medium at appropriate conditions for growth. Axenic cultures should result from this method [3].

3.1.3 Agar plating technique

Agar planting technique is used to isolate different algal species from a consortium or to check the purity of a sample strain.

Agarose solution is made and autoclaved for removal of any contamination. Then petri plates are half filled by the solution and left for solidification. After that sample strain is picked by using loop and streaked on plate. This is left until a uniform growth is achieved. The growth pattern shows number of colonies. It also tells about the contamination rate [4].

3.2 Preservation of Algal Strain

3.2.1 Agar Slant Method

Agar slanting is a preservation technique. The preparation method of slants is same as that of agar platting, the only difference is test tubes are used instead of plates. After pouring agar into the test tubes, test tubes are placed at 45° angle to make slant. After

this pure sample is streaked on slant, covered by lid and stored at room temperature. Agar slants can preserve algal species up to six months [4].

3.2.2 Cryo-preservation

Cryopreservation is based on the ability of certain small molecules to enter cells and prevent dehydration and formation of intracellular ice crystals, which can cause cell death and destruction of cell organelles during the freezing process. Two common cryo-protective agents are dimethyl sulfoxide (DMSO) and glycerol [5].

Most systems of cellular cryopreservation use a controlled-rate freezer. This freezing system delivers liquid nitrogen into a closed chamber into which the cell suspension is placed. Careful monitoring of the rate of freezing helps to prevent rapid cellular dehydration and ice-crystal formation. In general, the cells are taken from room temperature to approximately −90 °C (−130 °F) in a controlled-rate freezer. The frozen cell suspension is then transferred into a liquid-nitrogen freezer maintained at extremely cold temperatures with nitrogen in either the vapor or the liquid phase [5].

3.2.3 Lyophilization

Lyophilization is a technique in which liquid part of sample is sublimed and dry powder is obtained. This dry powder can preserve specie for years.

Lyophilizer or Freeze Dryer is equipment used for lyophilization. A pressure of thousands bar and temperature of -50 °C is obtained for the purpose. Sample is poured into vessels and freeze dried by dipping vessels in liquid nitrogen. After that the vessels are mounted on the Freeze Dryer. Vacuum pump is turned on about half an hour before mounting of vessels to achieve uniform temperature and pressure [6].

3.3 Genetic Identification

Genetic identification is necessary to categorize specie and allocating a specific position to it. There are several methods of DNA extraction to identify specie genetically.

Ctab and phenol extraction are mostly used with slight modification according to the type of specie.

3.3.1 CTab Method of DNA extraction

Preheated (65˚C) 2% CTAB and β-mercaptoethanol is added to each ground sample. The mixture is gently shaken to get homogenate.

An incubation of an hour at 65 °C is observed to extract DNA.

Eppendorf tubes are taken out of incubator and allowed to cool for couple of minutes.

1. Chloroform: IAA is added to each tube, mixed softly and transferred to shaking incubator at 100 rpm for 30 minutes.
2. The tubes are then centrifuged at 13,000 rpm for ten minutes and supernatant is removed and shifted to new eppendorf tubes.
3. Above steps are repeated to get better results.
   DNA is precipitated by adding ice cold isopropanol. At this stage DNA is left overnight in freezer at 4 °C.
4. DNA precipitate pellet is obtained on following day by centrifugation at 13,000 rpm for 10 minutes.
5. Supernatant is removed and 1 ml of wash buffer is added and vigorously agitated to remove the pellet from bottom. This mixture is left for 30 minutes at room temperature until this is centrifuged at 13,000 rpm for 5 minutes.
6. Supernatant is discarded and DNA pellet is allowed to dry. Pallet is dissolved in TE.

RNAase is supplemented to each extracted DNA dissolved in TE, and incubated at 37 °C for 30 minutes to remove RNA from sample.

Summary

This chapter gives an overview of different available technologies for algal species isolation, preservation and genetic identification of algal strains. However, the techniques used in our study is elaborated in next chapter.

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Chapter 4

Materials and Methodology

This chapter will focus of the methodology followed to carry out our research. Whole research was divided into four phases and was worked accordingly to obtain positive results. Microalgal strains were isolated from Kallar Kahar Lake and was further characterized for lipids and bioactive compounds potential.

4.1 Phase 1

In phase 1 the initial experimentation was to be performed for the isolation and selection of local algal strain. Samples of algae were collected from Kallar Kahar Lake and were isolated, identified and evaluated for their lipids and bioactive compounds potential.

4.1.1 Strain Revival and Enrichment

A consortium of algal strains were collected from Kallar Kahar Lake and was preserved in Biofuels Lab. This consortium was used for further studies. This mixture contains more than 1 algal species and was subjected to revival. The revival and enrichment was done with growing them in Bold's Basal Media in lab at ambient conditions for 15 days. Room temperature remained between 20 to 25 °C, light was provided by placing samples near window, air was supplied via small air pumps and pH was initially adjusted to 7.

4.1.2 Algal species Isolation

Absolutely pure algal samples are required for characterization and evaluation of any algal strain. For that purpose, after enrichment, algae samples can be isolated and purified using different techniques like Florescence Activated Cell Sorting (FACS) [1], Micro-manipulation [1][2], Serial dilution [3] and Agar plating technique[4].

4.1.2.1 Serial Dilution

Serial dilution technique [3] was used for isolation. 9 ml of culture medium (BBM) was dispensed into ten test tubes with sterile 10 ml pipettes using aseptic technique. The

tubes were labeled 10^{-1} to 10^{-10} indicating dilution factor and then aseptically 1 ml of enrichment sample was added to the first tube (10^{-1}) and mixed gently. After this 1 ml from this dilution was taken and added to the next tube (10^{-2}). The procedure was repeated for the remaining tubes (10^{-3} to 10^{-10}) after which the test-tubes were incubated under ambient temperature (20 ± 3 °C) and natural light conditions.

The cultures were examined microscopically after 2—3 weeks by withdrawing a small sample aseptically from each dilution tube. A uni-algal culture was observed in one of the higher dilution tubes e.g. 10^{-6} to10^{-10}. The procedure was repeated till we were able to isolate different strains. The resulting uni-algal isolates were transferred aseptically to 5 mL BBM medium to increase the cell number and concentration.

4.1.3 Preservation of algal strains

After isolation, algal strains are preserved for backup and for further studies in future. Preservation is important for optimum viability and genetic stability. Different preservation techniques are used, most usually used techniques are Agar Slant Method [4], Cryo-preservation [5], lyophilization [6], Buffered glutraldehyde method [6] and Lugol's solution [7].

Short term preservation of about 2 to 3 months were required and that's why agar slant method were used as it just required Inoculation of culture onto test tubes containing agar slants and incubating till growth appeared on slants.

Screw cap test tubes (15 ml, Borosilicate) and agar medium (300 mL BBM and 1% pure agar) were first autoclaved at 121 °C for 15 minutes and then agar slants were prepared under Laminar flow hood after which test tubes were incubated at 37 °C for overnight. Subcultures were made next day by inoculation of 50 µl algae culture solution onto agar slants via spreading technique. The procedure was repeated for each of the other isolated algae strains and then all test tubes were incubated at ambient temperature under natural illumination (via windows) till growth appeared [4]. Colonies were observed after 20-30 days. This preservation technique was repeated after every three months to ensure fresh, contamination free algae strain backup for research purposes.

4.1.4 Genetic identification

Theoretical analysis

The strains of microalge were observed under microscope. They appeared green, sphere to oval, with chloroplast and mucilaginous sheath around them. Usually they were free floating but sometimes appeared in colony.

Experimental analysis

0.5 grams of dried microalgae powder was taken and ground in a pistol mortar using liquid nitrogen and a pinch of Polyvinylpyrrolidone (PVP) [35]. To extract DNA of microalgae for molecular identification CTAB method was used [36], with some modifications.

Reagents and solutions

CTAB/mercaotoethanol buffer: 1 M Tris-HCl pH 8.0; 5 M NaCl; 0.5 M EDTA; CTAB (cetyltrimethyl ammonium bromibe)
1×TE buffer: 1 M Tris-HCl pH 8.0; 5 M NaCl
Wash Buffer: Ethanol; ammonium acetate
Chloroform: Isoamylalcohol (IAA) (24:1); Isopropanol (chilled)

DNA Isolation

1. 1 ml of preheated (65 °C) 2% CTAB and 2 µl of β-mercaptoethanol was added to each ground sample. The mixture was gently shaken to get homogenate.
2. An incubation of an hour at 65 °C was observed to extract DNA.
3. Eppendorf tubes were taken out of incubator and allowed to cool for couple of minutes.
4. 500 µl of Chloroform: IAA was added to each tube, mixed softly and transferred to shaking incubator at 100 rpm for 30 minutes.
5. The tubes were then centrifuged at 13,000 rpm for ten minutes and supernatant was removed and shifted to new eppendorf tubes.
6. Step 4 and 5 were repeated to get better results.
7. DNA was precipitated by adding 600 cµl of ice cold isopropanol. At this stage DNA was left overnight in freezer at 4 °C.

8. DNA precipitate pellet was obtained on following day by centrifugation at 13,000 rpm for 10 minutes.
9. Supernatant was removed and 1ml of wash buffer was added and vigorously agitated to remove the pellet from bottom. This mixture was left for 30 minutes at room temperature until this was centrifuged at 13,000 rpm for 5 minutes.
10. Supernatant was discarded and DNA pellet was allowed to dry.
11. Pallet was dissolved in 100 µl TE.
12. 10µl RNAase was supplemented to each extracted DNA dissolved in TE, and incubated at 37 °C for 30 minutes to remove RNA from sample.

Concentration and Quality of DNA

DNA quality was checked on 1% (w/v) agarose gel for electrophoresis. Agarose gel was prepared by dissolving 0.1 g of agarose in 100 ml of TAE buffer and adding, stained with 10 µl of ethidium bromide. Warm solution of gel was poured in gel casting tray and allowed to solidify. Prior to load sample in wells, every sample was mixed with 10 µl of DNA loading dye to give fluorescent under UV light. Gel was placed in tank with TAE buffer. 500 mA current and 100 V was set for 20 minutes to track the gel. Nucleic acid quantification was carried out by placing 1.5 µl DNA solution in Nanodrop spectrophotometer. The provided absorbance ratios were A268/A280 and A260/A230. These ratios can be used to access the presence of protein and polyscharide/polyphenolic contamination [17].

PCR amplification and DNA sequence

Extracted DNA was packed according to requirements and sent to Macrogen Inc. Seoul, South Korea, for further procession of PCR amplification and DNA sequencing. Universal primers for microalgae sequencing ITS1 and ITS4 were used for amplification. 3́ to 5́ sequence for ITS1 and ITS4 were TCCGTAGGTGAACCTGCGG and TCCTCCGCTTATTGATATGC, respectively

4.1.5 Growth Studies

Growth Studies were the basis for selection of algal strain for further evaluation. All the six isolated strains were cultured in Biofuels Lab. The algae was cultivated in conical

flasks and reagent bottles containing 10% inoculum and autoclaved Bold's Basal Medium [8]. CO_2 was provided via aeration pumps and flasks were covered to avoid contamination. The cultures were maintained for 15-20 days initially and after stationary phase was achieved they were further scaled up. Growth parameters like cell counting, turbidity, pH, light and temperature were recorded daily. Growth Kinetics were also done at the end to compare growth parameters of 6 algal strains.

4.1.5.1 Cell Counting

The growth properties of algae can be determined by measuring the cell number per unit volume of cell suspension. Thus, the number of cells in the algal suspension should be counted to calculate the cell concentration by using for example a device called haemocytometer counting chamber. In the past, the haemocytometer was developed for counting cells in blood samples. But now it is widely used for determining the growth of algae and other microorganisms. It consists of a mirror-like polished surface, which has a grid etched upon it, and a special thick cover slip. When counting cells, the cover slip is placed on top of the surface giving a defined height and a drop of well-mixed algal suspension is introduced beneath the cover slip by using a pipette. Then, the chamber is placed under a microscope and the counting grid is brought into focus at low power [9].

In this study, cell counts of all algae cultures were determined daily using Haemocytometer protocol by Castellanos et al [3]. 50 µL of algae sample was introduced on 0.1 mm depth improved neubauer bright line Haemocytometer (Marienfed, Germany) and observed under light microscope (Model XS2-107BN). The cells present on five specific squares were counted and the cell concentration per milliliter was determined using the equation:

Cell concentration per ml = Total cell count in 5 squares × 50,000 × dilution factor *(4.1)*

4.1.5.2 Dry Biomass Weight

The biomass concentration of algae can be determined by measuring either dry mass or wet mass. However wet mass is not frequently used due to the lower accuracy in the results resulting from the water content. Dry mass is given as total dry weight of biomass per volume of culture. Cell separation should be done by membrane filtration or

centrifugation. The filter membrane or centrifuge tubes should be pre-weighed. After separation, the filter membrane or centrifuge tubes are dried at high temperature around 100 °C, and then weighed immediately to avoid air-moisture absorption [10].

In our experiments dry biomass weight (g/L) was calculated after every 3 days using protocol by Irving T.E [11]. A representative aliquot of algae culture (10 ml) was filtered through pre-weighted and preheated (105 °C, 2 hr.) glass fiber filters (Whitman GF/F, 47 mm, nominal pore size 0.45 µm) using GAST vacuum pump filtration assembly (Model DOA-P104-BN.,USA). Wet filters (containing algae) were weighted using digital electronic balance (Model ATY224, SHIMADZU) and then transferred to oven for drying till constant weight (105 °C, 2 hr.). The procedure was repeated for blank (10 ml BBM medium) and other respective algae cultures. The dried filter was weighted and used to determine the dry algae biomass weight using the equation below:

$$\text{Dry Biomass Weight} = \frac{\text{DWA-DWC}}{\text{V}} \qquad (4.2)$$

Where,

DWA is the average dry weight retained on algal filter

DWC is the average dry weight retained on control filter

V is volume of algal culture filtered

4.1.5.3 pH

pH is another important growth factor for algae. Different algal species have different favorite pH ranges and outside the optimal range the growth is affected resulting in slower specific growth rates. *Chlorella* and *Botryococcus* are growing very well at around pH 6. *Scenedesmus* prefer alkaline medium between pH 9 to 10. In addition, algae can also change the pH of the medium during cultivation. When using CO_2 as carbon source, rapid growth of algae can cause the pH to rise due to photosynthetic uptake of inorganic Carbon.

pH values of our culture media were checked daily as it is important for coagulation, precipitation, neutralization and biological treatment processes. A hand held pH meter and calorimetric method were used to measure the pH [12].

4.1.6 Synthetic growth media preparation

Bold's Basal Medium (BBM), a fresh water algae culture medium was used for revival and growth of algae in lab. BBM was made by following standard recipe[13]. BBM is highly enriched medium with low salinity is considered ideal for culturing fresh water algae. Elemental analysis of BBM is given below:

Table 4.1: Bold's Basal Medium Elemental Analysis.

Macronutrients	**Compound**	**Concentration (mmol/L)**
Sodium	$NaCl$, $NaNO_3$	3.37
Nitrogen	$NaNO_3$	2.94
Phosphorus	KH_2PO_4, 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_2EDTA	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
Molybdenum	$Na_2MoO_4.2H_2O$	0.02

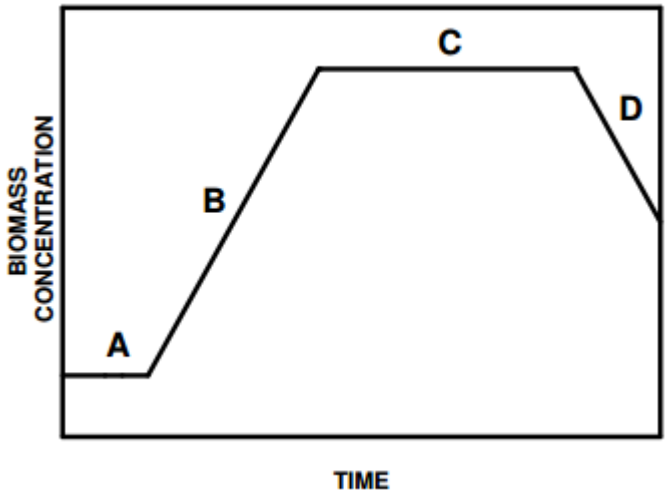

Figure 4.5: Microalgae batch growth profile.

4.1.7 Growth Kinetics

To estimate growth rates, one must have a series of measurements, at different times, that will permit the calculation of the rate of change in biomass concentration. Cell number should be counted, either through a direct method, as through light microscopy with a hemacytometer, or indirectly through biomass concentration (as dry weight) or optical density, as long as this measurements correlate linearly with the number of cells[14].

Under a typical homogenous batch regime (in a closed system), microalgae will pass through the following growth phases, illustrated in Figure:

A is adaptation (lag phase).

B is exponential growth phase (log phase).

C is stationary phase.

D is logarithmic death phase.

The individual phases, shown in Figure 2.4, are not always clearly defined; their length or slope might change according to the culture conditions. During lag phase (phase A) the microalgae cells are adapting to the new environment conditions, at the end of the lag phase, the cells are well adjusted and then start to multiply rapidly, this is the exponential phase (phase B), in this phase, the number of living cells, doubles regularly with time [15][16]. During this period, equations (a) and (b) describe cell growth.

$$\frac{dn}{dt} = \mu n \text{ or } \frac{1}{n}\frac{dn}{dt} = \mu \qquad (4.3)$$

$$n = n^\circ \text{ or } t = t^\circ$$

Where n and n° are the number of cells (or cell concentration) at different time's t and t°, where t° is the initial time and μ is the specific growth rate.

From the integrated form of equation "a" the following is obtained:

$$\ln\frac{n}{n^\circ} = \mu(t-t^\circ) \text{ Or } \mu = \frac{\ln {}^{n}/_{n^\circ}}{t-t^\circ} \qquad (4.4)$$

From above equation it can deduced that the time required for cell population to double (t_d) is given by:

$$t_d = \frac{\ln 2}{\mu} \qquad (4.5)$$

During the exponential phase t_d or μ can be used to characterize the state of the cell population.

4.2 Phase 2

The phase 2 experimentation were designed to evaluate lipids and bioactive compounds potential in algae. Lipids are responsible for biodiesel

4.2.1 Lipids and Bioactive Compounds Determination

4.2.1.1 Lipids (Bligh and Dyer Method)

The Enssani [17] adaptation of the Bligh and Dyer procedure was used to extract lipids from the algae for further study.

$$\text{Relative centrifugal field} = \frac{\omega 2r}{g} \qquad (4.6)$$

Where ω is the angular velocity in radians per second, based on a rotational frequency of 4,000 rpm. The value r is the distance between the center of the centrifuge tube and the center of rotation, 8.8 cm, and g is the acceleration of gravity.

Due to the limitations of test tube capacity, the centrifugation process was undertaken 20 mL at a time. After 20 mL had been centrifuged for four minutes, the supernatant liquid was poured off from each sample and replaced with 20 mL of sample water. This process was repeated until the solids from 200 mL of sample water had been collected in a pellet at the bottom of each test tube.

A room temperature table-top centrifuge was used. The centrifugation of each pellet required approximately 25 minutes and the pellets were centrifuged four at a time.

After the samples had been pelleted in the centrifuge tubes, they were either used for extraction immediately or were flushed with nitrogen and placed in a freezer until needed.

The first step of the extraction was to add 5 mL of chloroform, 10 mL of methanol and 4 mL of deionized water to each tube containing a pelleted sample of algae. The methanol, which is miscible in the water layer, is included to amend the polarity of the water layer to limit the concentration of polar lipids such as chlorophyll and phospholipids in the chloroform layer. Once the tubes had all reached room temperature, a sonicator was used to disrupt the algae cells in the suspended mixture. A Branson Ultrasonic sonicator was operated at a constant duty cycle on power level 8. Each tube was sonicated for one minute. The sonicator tip was rinsed between each sonication to remove any residual material. The samples were then placed horizontally on a shake table with a single-axis motion of 6 cm oscillations at 2 cycles per second for between 6 and 8 hours. The

purpose of the shaking step was to promote the complete exposure of intracellular products to the solvents.

The tubes were removed from the shake table and an additional 5 mL of chloroform and 5 mL of deionized 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 4,850 g for four minutes to separate the contents into layers.

The green layer at the bottom, comprised primarily of chloroform, contains lipophilic material. The upper layer contains methanol and water. A thin layer of cell debris separates the two layers.

The lower, lipid-rich chloroform layer was removed using a glass Pasteur pipette for additional testing. This extract was pipetted into a 60 mL Luer-lock syringe made of solvent-resistant polypropylene. The syringe was used to force the extract through a 0.2 μm, nylon syringe-driven filter. Depending on whether the extract was to be used for lipid quantity analysis or lipid identification, it was injected into either a tared aluminum weighing dish (4 cm diameter) or a glass test tube (50 mL).

After the extract had been pipetted and filtered, an additional 10 mL of chloroform were added to each of the Teflon centrifuge tubes. The tubes were again vortex mixed for 30 seconds each and centrifuged at 4,850 g for four minutes. The chloroform layer was again pipetted from the bottom of each tube into the same vessel as had been used during the prior set of extractions.

4.2.1.2 Chlorophyll a, b and total carotenes

Chlorophyll is a green pigment and is an extremely important biomolecule, critical in photosynthesis, which allows plants to absorb energy from light for carbon dioxide fixation. The most green leaf material contains about 0.3% chlorophyll and on extraction using conventional methods yields about only 5%. Cheap sources, like grass and lucerne (alfalfa), are usually selected as chlorophyll source [18]. In more esoteric extractions, leaves of nettles and elder are preferred, whilst algae and silk worm droppings have also been used commercially [19][20].

Chlorophyll has also been investigated as potential source of pigments in cosmetics; the brown and red algae are mostly used in the cosmetic industries [5]. Furthermore, in food industry, chlorophyll is used as natural pigment ingredient in processed foods. Because of its strong green pigment and consumer demand for natural foods, chlorophyll is gaining importance as food additive. This in turn is encouraging food processors to switch from artificial pigments to chlorophyll-based natural coloring. However, a downstream process needs to be developed to purify chlorophyll a and b from algae [20].

The determination of chlorophyll a, b and c were done using spectrophometrically as proposed by Sukran DERE *et. al.* 1980 [21].The weighed samples, having been put in 100% acetone (50 ml for each gram), were homogenized with the B-Brawn type homogenizer at 1,000 rpm for one minute. The homogenate was filtered through two layer cheese cloths, and was centrifuged at 2500 rpm for ten minutes. The supernatant was separated and the absorbance were read at 400-700 nm on UV 2800, BMS, and Canada spectrophotometer. It was recorded that Chlorophyll a showed the maximum absorbance at 662 nm, chlorophyll b at 646 nm and total carotenes at 470 nm and the amount of these pigments was calculated according to the formulas of *Lichtentaler and Wellburn (1985)* [22]. The formulas are given below for chlorophyll a, b and total carotenes.

$$C_a = 11.75\ A_{662} - 2.350\ A_{645} \qquad (4.7)$$

$$C_b = 18.61\ A_{645} - 3.960\ A_{662} \qquad (4.8)$$

$$C_{x+c} = 1000\ A_{470} - 2.270\ C_a - 81.4\ C_b/227 \qquad (4.9)$$

C_a= Chlorophyll a, C_b= Chlorophyll b, C_{x+c}= Total carotene

Instead of the wave-lengths determined by these researchers, 662, 646 and 470 nm at which we observed maximum absorbance were used. The experiments were repeated three times. And corrected values were noted.

4.2.1.3 Phycobiliproteins

The phycobiliproteins (PBPs) are antennae-protein pigments involved in light harvesting in cyanobacteria (blue-green algae, prokaryotic), rhodophytes (red algae, eukaryotic), crypto monads (biflagellate unicellular eukaryotic algae) and cyanelles (end symbiotic plastid -like organelles) [23]. The three common phycobiliproteins are phycoerythrin (PE) with phycoerythrobilin chromophores, and PC and allophycocyanin (APC), with phycocyanobilin chromophores [24]. Purified C-phycocyanin (C-PC) has nutraceutical and pharmaceutical potentials. The antioxidant and radical scavenging activities of CPC from different cyanobacteria are well documented [25]. Allophycocyanin was found to inhibit enterovirus 71- induced apoptosis [26][27]. Phycobiliprotein has been utilized commercially as natural dyes and has a variety of applications in pharmaceutical industry. It is classified according to UV-visible absorption maxima as phycocyanin (blue pigment), phycoerythrins (red pigment), and allophycocyanin (pale-blue pigment). The annual market of phycocyanin was around US$5-10 million [28][20].

The methodology of Bryant *et. al* 1979 [29] were followed to determine water soluble proteins. The water soluble Phycobiliprotein pigments including C-phycocyanin (CPC), Allophycoyanin (APC) and C-phycoerytherin (CPE) were extracted from fresh algal sample (1 g) with 0.05 M phosphate buffer (10 ml, pH 6.8). The absorbance (A) of the solution was measured at 650 nm, 620 nm and 565 nm and the concentrations calculated using the equation as:

$$\text{CPC (mg/ml)} = A620-0.72xA650/6.29 \qquad (4.10)$$

$$\text{APC (mg/ml)} = A650-0.191xA620/5.79 \qquad (4.11)$$

$$\text{CPE (mg/ml)} = A565-2.41x\ (\text{Conc. of CPC } x1.4(\text{Conc. of APC})/13.02) \qquad (4.12)$$

4.3 Phase 3

The main objective of phase 3 experimentation were to design low cost media for algal growth. Algae require nutrients for its growth that are provided in lab or in larger cultures through synthetic media. The growth of algae in synthetic media is good but the cost of these synthetic culture media is also much high [30]. For larger cultivations of

algae the cost of media is about 40-60% of the total cost [31] that should be reduced to make it a viable option. In this phase of experimentation we replaced the expensive source of essential nutrients to low cost sources. An attempt were made to provide algae with essential nutrients via agriculture grade fertilizers, market available multivitamins and egg proteins.

4.3.1 Low Cost Media Constituents

The experimentation of low cost media were carried out in three steps. In first step agriculture grade fertilizers were used in different ratios. In second and third steps the effect of vitamins and egg proteins were studied respectively on growth rate of algae.

Low cost media are made by agriculture grade fertilizers. DiAmmonium Phosphate, Urea and Calcium Ammonium Nitrate (Fauji Fertlizer Company) were brought from local market. Revitale Multi tablets (GlaxoSmithKline), multivitamins were also brought from local market. Egg White was dissolved in water with the help of 10% NaOH Solution and was then used as protein source for algae.

4.3.2 Experimentation

The experiments to design the media was conducted in three steps to evaluate cheap nutrient sources. The final results of last phase were compared with synthetic media BBM as control. The results include Algae growth parameters, Bioactive Compound Analysis and Growth Kinetic Parameters of KKL-5 in Cheap Media and its comparison with Synthetic Media (BBM).

4.3.2.1 Step 1

In step one of experiments the Urea, DAP and CAN (agriculture grade fertilizers) were tested for KKL-5 with different composition ratios, Five samples (i.e. S1,S2,S3,S4,S5) were prepared with different Ratios of Urea, DAP and CAN (i.e. 6:1:3, 5:2:3, 4:3:3, 3:4:3, 2:5:3 respectively), these five samples were then introduced in five flasks (1 L) with water and 10% algal inoculum thus making 1L of algae culture in each bottle. Each bottle was put to aeration with the help of aeration pumps to provide with carbon dioxide. pH was maintained at 7.5. Growth parameters were calculated daily for about

30 days and growth kinetics study were performed on the results of all samples and their values were compared. The best sample were again evaluated with addition of further nutrients.

4.3.2.2 Step 2

Step 1 provided the algae with essential major nutrients of Nitrogen, Phosphorous and Calcium, but the results were not as good as results from synthetic media. Algae require Vitamins for its growth, Three vitamins—vitamin B1 (thiamine · HCl), vitamin B12 (cyanocobalamin), and vitamin H (biotin)—are usually used for culture of microalgae.[14]. Many algae need only one or two of the vitamins, but there seems to be no harm caused by adding a nonessential vitamin [32]. In addition to the three common vitamins, some recipes call for other vitamins. For example, nicotinamide (nicotinic acid amide, niacinamide) is added to the culture medium for *Phacotus lenticularis* (Ehrenberg) Stein (see N-Hs-Ca medium,Schlegel et al. 2000) [33].

In this step we introduced vitamins and minerals in our culture in the form of market available *Revital Multi Multivitamins and Minerals Tablet.* Each tablet weigh 0.90 g was crushed into powder and then used. Best sample from step 1 was prepared in five flasks (1 L) and each flask was introduced with MV tablets of different weight amount, i.e. 10 g, 20 g, 30 g, 40 g, 50 g, and was further provided with aeration and other growth conditions were same as in step 1. Growth parameters were measured daily for about more than 30 days. Growth Kinetics study were performed on the results of all samples and their values were compared. The best sample were again evaluated and tested with egg proteins in step 3.

4.3.2.3 Step 3

Algae have recently been explored for their potential as a novel protein expression system. There are many reasons why algae would make a more efficient and robust, expression system. First, algae are able to carry out many of the post translational mechanisms that are needed for the production of many complex proteins and are relatively easy and cheap to culture. They require very basic media to grow on such as minimal or acetate based media with a few trace elements, as well as the ability to use sunlight as an energy source, hence cost of goods are low [34].

In this phase we try to introduce proteins in culture to see their effect on algae growth and production. Egg proteins were selected to be used in culture. Egg white is usually insoluble in water and make clumps when added or stirred in water. Egg white was made soluble in water with the help of NaOH solution.

The best sample out of five from phase 2 was now tested with egg proteins. Five solutions were prepared each with different percentage of egg proteins (i.e. 1%, 2%, 3%, 4%, and 5%) and previous constituents of phase 2. All samples were given same ambient conditions as in phase 1 & 2. Growth parameters were measured daily for about more than 30 days. Growth Kinetics study were performed on the results of all samples and their values were compared. The results of the best sample were compared with control synthetic culture (BBM). Lipids, chlorophyll, carotenoids and water soluble proteins were determined in both experiment and control cultures and was compared.

4.4 Phase 4

Biofuels lab, NUST is in collaboration with Attock Oil Refineries, Rawalpindi, initiated a project of carbon dioxide sequestration and biofuels production using algae. For this purpose a feasibility study was done and submitted to ARL. This work was done in phase 4. The types of algae growth systems in accordance with ARL scenario was addressed. The output from this study is discussed in next chapter.

Summary

Different experimental techniques available were discussed in this chapter. Different experimental techniques gives different level of accuracy for same type of results. The techniques are chosen based on nature of work, degree of precision required and availability of resources. In phase 1 and phase 2 of experimentation different techniques and guidelines from literature were followed. Results from strain revival, species isolation and preservation were satisfactory. Ctab method for DNA extraction required many trials as was successful after 5, 6 attempts because the algal specie was unknown and the standard protocols were proving insufficient. Growth studies were done in triplicates to reduce the error and improve the accuracy. Data for growth studies were collected round the year. Experimentation for phase 2 required UV spectrophotometer. High quality lab grade solvents were used for UV spectrophotometer to enhance the accuracy of data. Phase 3 required a little hit and trial method and it also required different experimental conditions with different parameters. The results from phase 3 was satisfactory.

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Chapter 5

Results and Discussions

5.1 Phase 1

Consortium of algae that was isolated from Kallar Kahar contains variety of algal species. Only six species were isolated and was first subjected to grow in lab at ambient conditions, because the specie to be evaluated will be used in large scale algal growth systems that's why growth at ambient conditions were critical. Out of six algal strains, strain 4 showed maximum growth than rest of the strains at ambient conditions. As strain 4 showed more growth so it was further identified genetically and evaluated for lipids and bioactive compounds potential. A low cost media was also designed for strain 4*Dictyosphaerium 8-6*) to make it viable for large scale cultivation in open ponds.

5.1.1 Genetic Identification

Results established by Macrogen Inc. developed the consensus that microalgae derived from fresh water belong to genus *Dictyosphaerium*. PCR amplification and BLAST results described species of the genus in detail.

Fig:5.1 shows the phylogenetic tree for 1st strain. It clearly demonstrated internode that is splitting the pedigree to genus *Dictyosphaerium* species 1-6, 8-6. Now for further confinement of the specie between these two species, BLAST and coting report for the strain was consulted.

BLAST report analysis showed Score of the blast 1133, 613 bits there were 6 gaps out of 655 amino acids and 642 identities for *Dictyosphaerium 8-6,* while same gaps but lesser number of bits and score was observed for *Dictyosphaerium 1-6*. So a general consensus developed for specie was that it is *Dictyosphaerium 8-6.*Result analysis was supported by Macrogen Inc. as well.

Similarly Fig:5.2 shows phylogenetic relation of 2nd strain with specie *Dictyosphaerium Iso 6.*

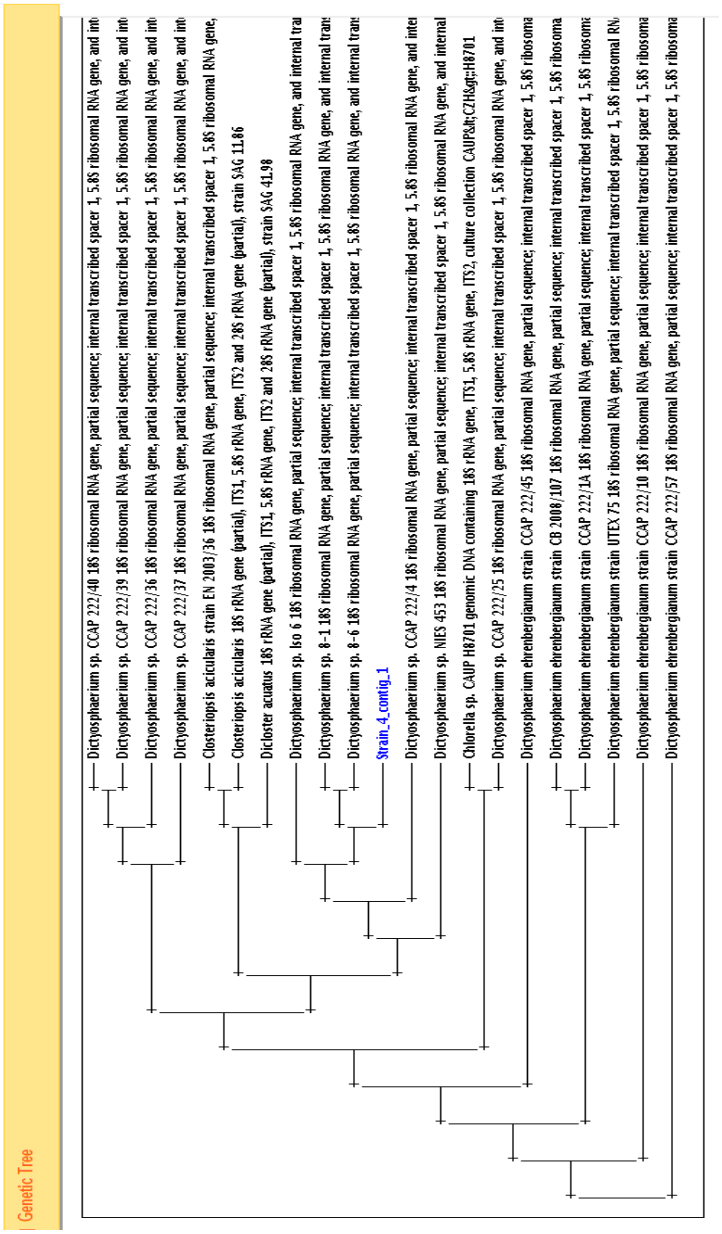

Fig:5.1 Pylogenetic tree representation for 1st strain (4).

Analysis Report

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
Strain_4_contig_1	882	538	530	51.13378684807256
Strain_4_F	792	593	558	50.505050505050505
Strain_4_R	530	424	406	51.698113207547166

Fig:5.2 GC contact analysis report for 1st strain (4).

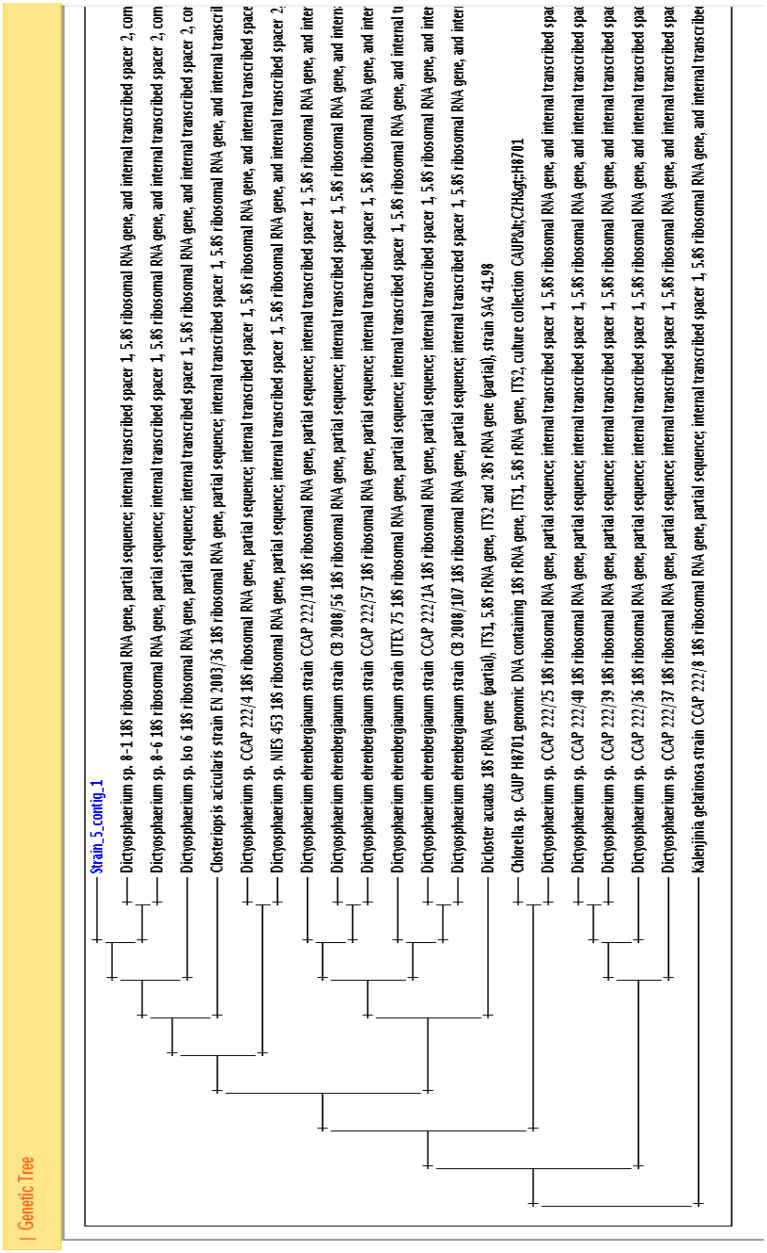

Fig: Shows phylogenetic relation of 2nd strain (5).

Analysis Report

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
Strain_5_contig_1	666	551	540	52.4024024024024
Strain_5_F	593	452	428	52.951096121416526
Strain_5_R	557	373	347	53.50089766606823

Fig: Shows GC content analysis report for 2nd strain (5).

As it is evident from the phylogenetic tree representation that strain 5 belongs to genus *Dictyosphaerium*, for specie identification BLAST reports were consulted. Starin showed a Score of 1114, 603 bits, 10 gaps and 650 identities for 671 amino acids.
For other species score, bits, and identities were less in number.

5.1.2 Growth Studies

Initially all six strains were grown in biofuels lab at ambient conditions. Standard curves were drawn and results from growth studies were compared. The cell count of all six strains isolated from consortium is given in Figure 5.1.

Strain 4 showed growth of 27 million cells per ml at 24th day of its growth, which is much more than rest of the strains. This results showed that strain 4 is much more compatible to grow in our local environment than many other available strains so this strain was further evaluated for its growth potential followed by genetic identification. In the further experiments strain 4 potential to grow in large scale ponds were also evaluated by making it prone to different pH, temperature conditions, this was done by growing strain 4 in lab round the year without any controlled conditions.

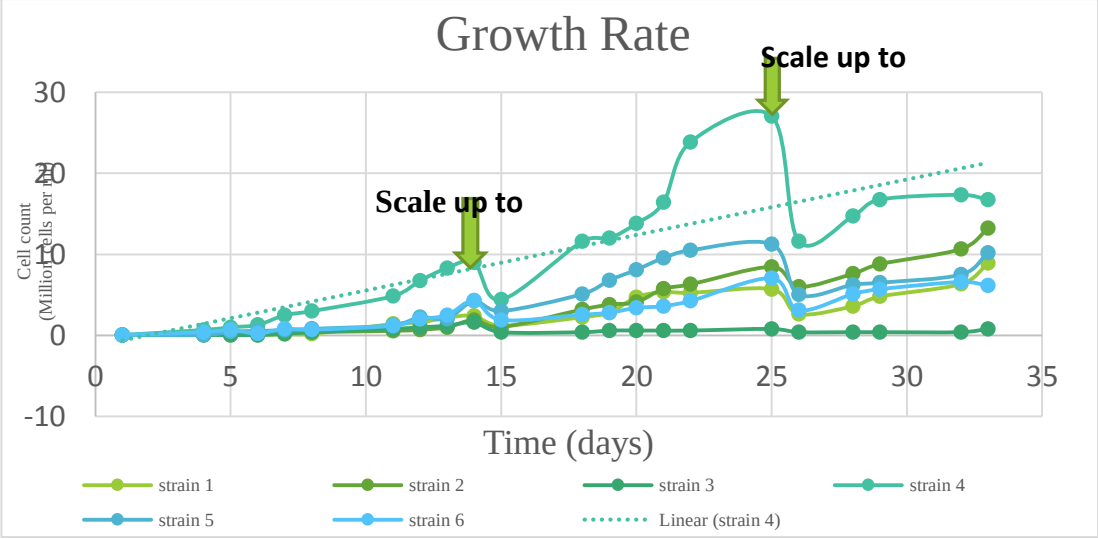

Figure 5.6: Cell count comparison [1].

5.1.2.1 pH

pH is important growth factor for algae. Different algal species have different favorite pH ranges and outside the optimal range the growth is affected resulting in slower specific growth rates. Algae can also change the pH of the medium during cultivation. When using CO_2 as carbon source, rapid growth of algae can cause the pH to rise due to photosynthetic uptake of inorganic C.

In this study we tested strain 4 at 6, 8 and 9 pH ranges. It's already been tested at 7.5 during initial growth studies. All experiments were done in triplicates to minimize the error. Strain 4 showed best growth at pH 9. The results from experiment are given below:

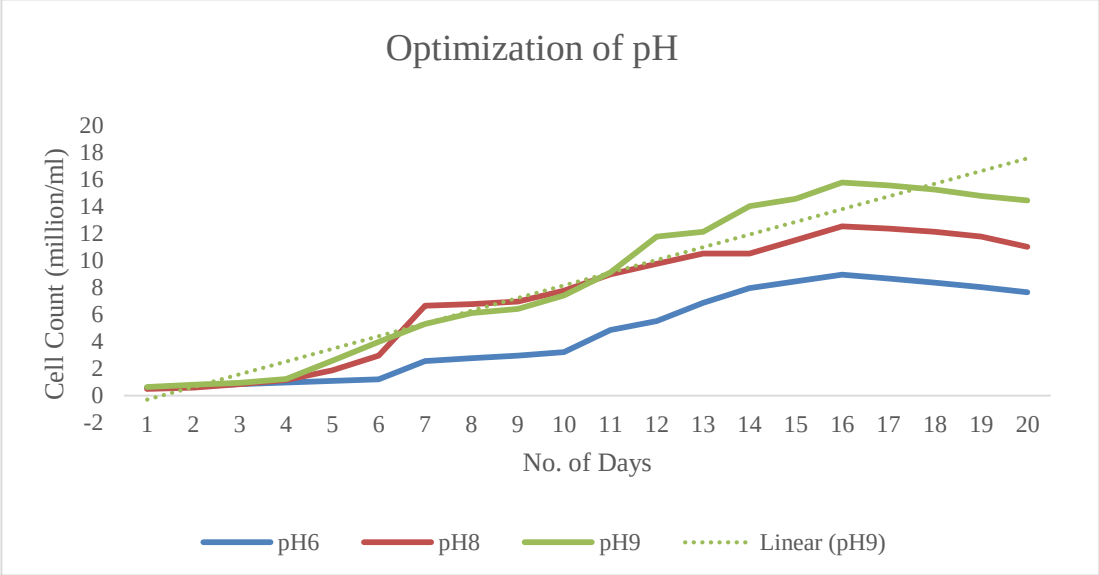

Figure 5.7: Optimization of pH

5.2 Phase 2 (Lipids and Bioactive compounds determination)

Lipid potential of any algal specie means its potential of being classified as fuel producing specie. The lipids are first extracted from dry algal biomass using techniques as discussed in previous chapter. After lipids are extracted it is converted into biodiesel by Tran esterification reaction. Usually lipids to biodiesel conversion is 100%. So our one objective was to evaluate the lipid potential of selected species to measure their potential to produce biodiesel because till now no local specie is evaluated for its

biodiesel production, only foreign species are available but their growth rate is not that good in our local environment, so it was decided to evaluate our local species for lipids potential that obviously will grow much better in their natural habitat.

Initially FTIR [4] was used to study the presence of lipids in strain 4, FTIR showed peaks of N-H stretching which indicated the presence of lipids and proteins. After that when reasonable biomass was produced in lab further testing for lipids and bioactives were evaluated by further testing. Bligh and Dyer [2] method were used for lipid testing while UV spectrophotometric [5] methods were used for determination of chlorophyll, carotenoids and phycobiliproteins. The above methods are elaborated in previous chapter. The algal strain are grown in synthetic media [9] and low cost medias, the bioactive compounds were determined at different experimental conditions.

5.2.1 Lipids

Bligh and Dyer [2] method is used to determine lipids and the results of lipids test are given below:

Table 5.2: Lipids determination in Strain 4.

Samples	Sample 1[1]	Sample 2[2]	Sample 3[3]	Sample 4[4]	Sample 5[5]
Lipids	14	13	12	13	12

Where,

[1]Bold's Basal Medium (1 L)

[2]Low Cost Media (1 L)

[3]Bold's Basal Medium (100 L)

[4]Low Cost Media (100 L)

[5]Low Cost Media (200 L)

Strain 4 contains about 13% lipids of dry biomass, this is near to 16% of lipids in *Chlorella Vulgaris*, which is renowned oil producing species in international market. However strain 4's lipids contents are less than *chlorella vulgaris* but its growth is much

faster than any foreign strain (fig 5.5). Although the lipids formation in algae grown in synthetic media was higher than in low cost media, but the overall variation in results were negligible.

5.2.2 Chlorophyll a, b and total carotenes

Chlorophyll a, b and total carotenes were measured by the method of *Sukran et al.* [5] which used UV spectrophotometer for determination of above bio molecules. This method involves calculating the UV absorbance at 662 nm, 644 nm and 470 nm for Chlorophyll a, b and total carotenes respectively. The detailed methodology is stated in previous chapter, however the results are:

Table 5.3: Chlorophyll a, b and total carotenes determination.

Pigment	S1[1]	S2[2]	S3[3]	S4[4]	S5[5]
Chlorophyll a (µg/gfw)	32	29	33	30	31
Chlorophyll b (µg/gfw)	17	14	15	13	13
Carotenes (µg/gfw)	16	15	16	12	11

Where,

[1]Bold's Basal Medium (1 L)

[2]Low Cost Media (1 L)

[3]Bold's Basal Medium (100 L)

[4]Low Cost Media (100 L)

[5]Low Cost Media (200 L)

About average of 30 µg/gfw chlorophyll a were present in strain 4, 15 µg/gfw of chlorophyll b and about average of 13 µg/gfw of total carotenes were present in strain 4. The extraction of these bioactive compounds is usually costly process and require sophisticated equipment however at biodiesel from algae facility the remaining biomass after lipids extraction can be used to extract various bioactive compounds. This can improve the overall economics of the process thus helping it to be a viable option for future.

5.2.3 Phycobiliproteins

The water soluble proteins or phycobiliproteins were determined by using the method of Bryant *et. al* 1979 [3] as explained in previous chapter. The three common phycobiliproteins are phycoerythrin (PE) with phycoerythrobilin chromophores, and PC and allophycocyanin (APC), with phycocyanobilin chromophores [8]. The results from experiments were as follows :

Table 5.4: Water soluble proteins

Pigment	CPC (mg/ml)	APC (mg/ml)	CPE (mg/ml)	Total Phycobiliproteins (mg/ml)
S1[1]	0.22	0.15	0.09	0.46
S2[2]	0.20	0.12	0.08	0.4
S3[3]	0.22	0.13	0.09	0.44
S4[4]	0.19	0.11	0.07	0.37
S5[5]	0.19	0.11	0.07	0.37

Where,

[1]Bold's Basal Medium (1 L)

[2]Low Cost Media (1 L)

[3]Bold's Basal Medium (100 L)

[4]Low Cost Media (100 L)

[5]Low Cost Media (200 L)

As stated in previous chapter the purified water soluble proteins have nutraceutical and pharmaceutical importance. So it can add up the value of strain 4 from commercial point of view.

5.3 Phase 3 (Low-cost media)

Design of low cost media were done in phase 3. The results from all three steps are shown below.

5.3.1 Step 1

The first phase of the experiments included the optimization of agriculture grade fertilizers which include Urea, Di-ammonium phosphate and Calcium ammonium nitrate.

Five samples were prepared with different ratios of the constituents as shown in table below:

Table 5.5: Fertilizers with different compositions.

Sample	Ratio (Urea:DAP:CAN)
S1	6:1:3
S2	5:2:3
S3	4:3:3
S4	3:4:3
S5	2:5:3

All the five samples were subjected to experimentation and were tested for their potential as algae growth media. Algae growth parameters were recorded on daily basis

and results were compared and best sample's combination was further improved with addition of vitamins and proteins.

The results initially included cell count, turbidity, and dry biomass for evaluation of best fertilizers combination.

The cell count and turbidity of all five samples is shown below:

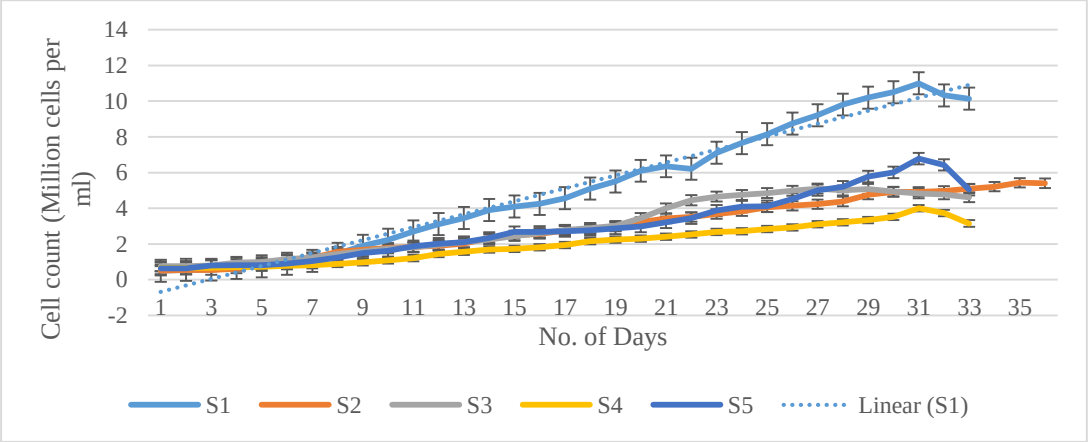

Figure 5.8 Cell count

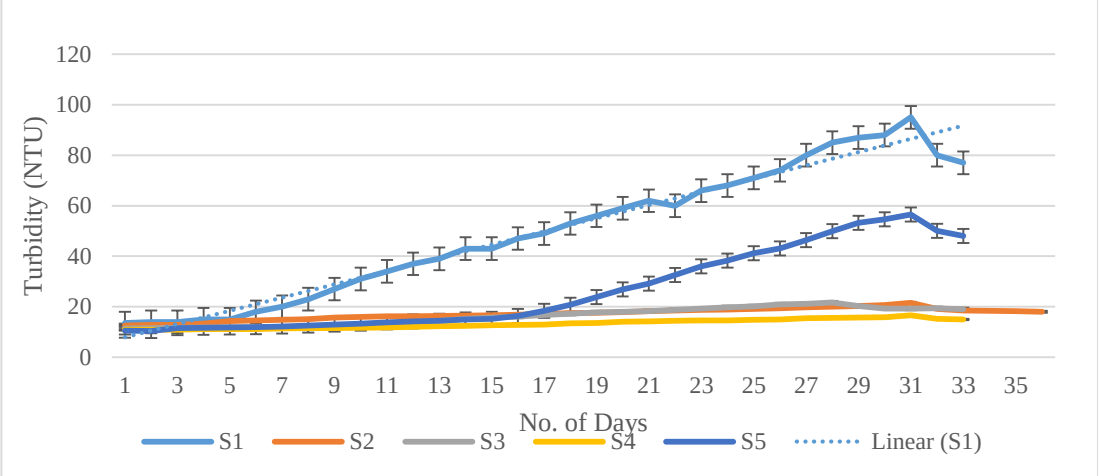

Figure 5.9: Turbidity

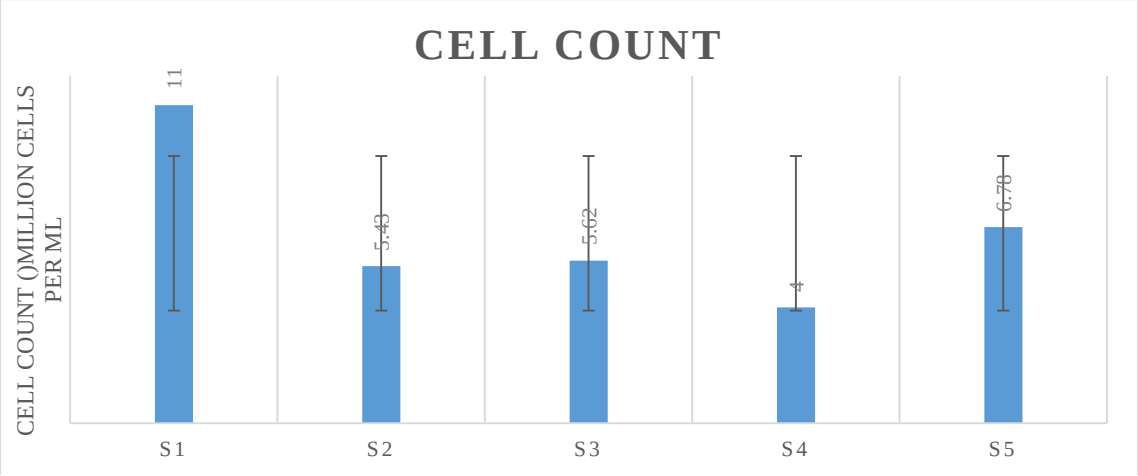

Figure 5.10: Comparison of cell count.

Table 5.6: Growth kinetics.

Cultivation in agricultural fertilizers					
Media Samples	S1	S2	S3	S4	S5
Ambient conditions					
Temperature (°C)	26±5				
pH	9±0.5				
Light intensity (LUX)	700±50				
Working volume (L)	1				
Inoculum Conc.	10%				
%CO_2	0.02% (via air pump)				
Growth kinetics					
Max. cell count (million cells/ml)	11	5.43	5.08	4	6.78
Max. turbidity (NTU)	95	18.21	20.43	16	56.46
Specific growth rate (d^{-1})	0.106	0.051	0.058	0.061	0.072
Doubling time	2.94	3.66	3.54	3.49	3.32
Max. biomass conc. (g)	0.32	0.25	0.23	0.19	0.28

Sample 1 showed the growth rate of 0.106 $d^{-1,}$ doubling time of 2.94 days and maximum biomass concentration of 0.32 g which is higher than all other samples, so this sample was further evaluated in next phase.

5.3.2 Step 2

Here S1 was further tested with five compositions (10 g, 20 g, 30 g, 40 g, and 50 g) of multi vitamin tablet with objective of improving the overall cell count and biomass of algae. Addition of multi minerals improved the results, again the data was calculated and growth kinetic studies were performed. Five samples were prepared, as shown in table below:

Table 5.7: Multi-vitamins concentration.

Samples	Multi Vitamin Conc. (g/L)
V1	10
V2	20
V3	30
V4	40
V5	50

All five samples were given ambient conditions and data were collected for about thirty days until decline phase of algae sample is achieved.

Below graphs shows the cell count, turbidity of samples.

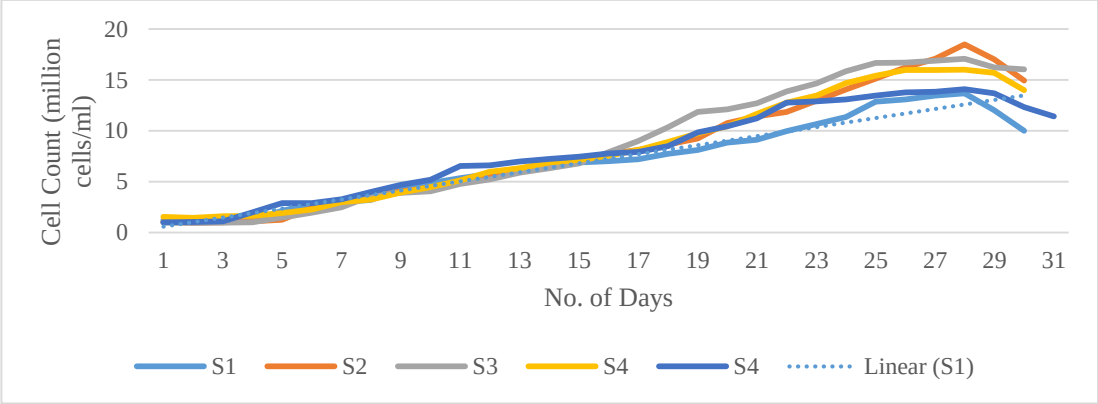

Figure 5.11: Cell count.

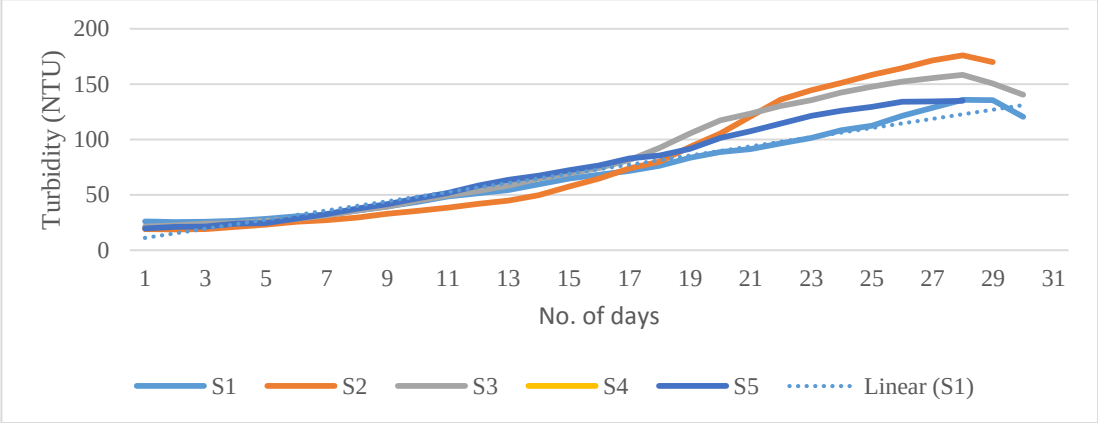

Figure 5.12: Turbidity.

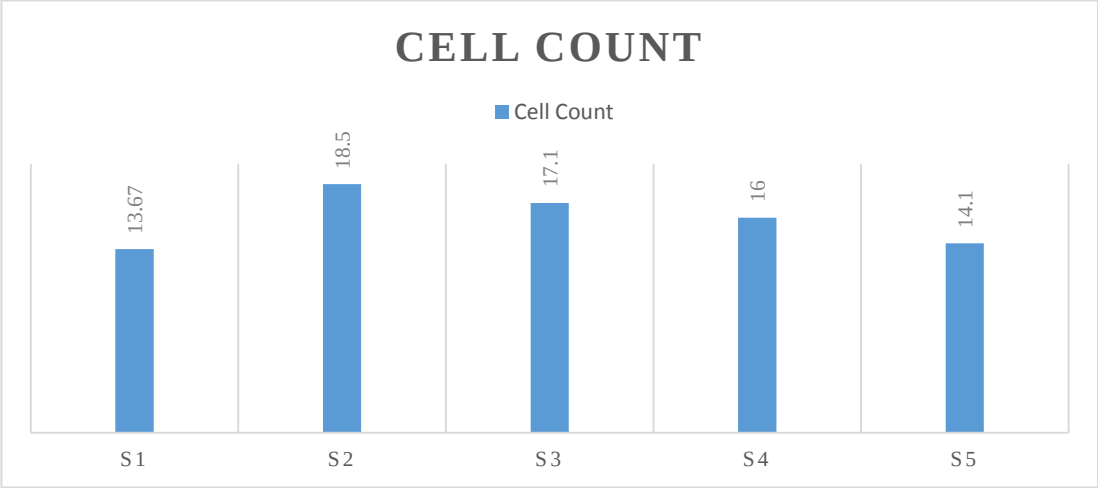

Figure 5.13: Cell count comparison.

Table 5.8: Growth kinetics.

Cultivation in agricultural fertilizers					
Media Samples	V1	V2	V3	V4	V5
Ambient conditions					
Temperature (°C)	26±5				
pH	9±0.5				
Light Intensity (LUX)	700±50				
Working Volume (L)	1				
Inoculum Conc.	10%				
$\%CO_2$	0.02% (via air pump)				
Growth Kinetics					
Max. cell count (million cells/ml)	13.67	18.5	17.1	16	14.1
Max. turbidity (NTU)	159.85	199.98	165.87	158.23	135
Specific growth rate (d^{-1})	0.077	0.089	0.081	0.088	0.06
Doubling time	3.25	3.17	3.10	3.12	3.50
Max. biomass conc. (g)	0.35	0.26	0.14	0.21	0.16

The above graphs and kinetic study shows that results improved significantly in phase 2, so we conclude that multi vitamins have positive effect on algae growth.

Among all five samples sample V2(fertilizers(6:1:3) + Revitale 20g) showed best results but still the results were not that satisfactory to compete with conventional fertilizers and to improve it further sample V2 which contains best fertilizer combination along with best multi vitamin combination and now in phase 3 proteins were tested along with this best combination.

5.3.3 Step 3

Till third phase of experiments we already had achieved 75% of the target results. Algae not only require nitrates, phosphates and silicates it also requires certain metals, vitamins and proteins.

In third phase of experiments we tried to feed our algae with some protein rich media. To make media protein sufficient we tried to dissolve egg white in media with the help of NaOH, and made five different Solutions, as shown below:

Table 5.9: Sodium albumate samples.

Samples	Sodium albumate
P1	1%
P2	2%
P3	3%
P4	4%
P5	5%

Now the third phase was intended to study the effect of proteins on growth of algae. All five protein samples were tested with S1 and V1, experiment conditions were same and the same growth parameters were measured.

Below are the results from phase 3 experiments:

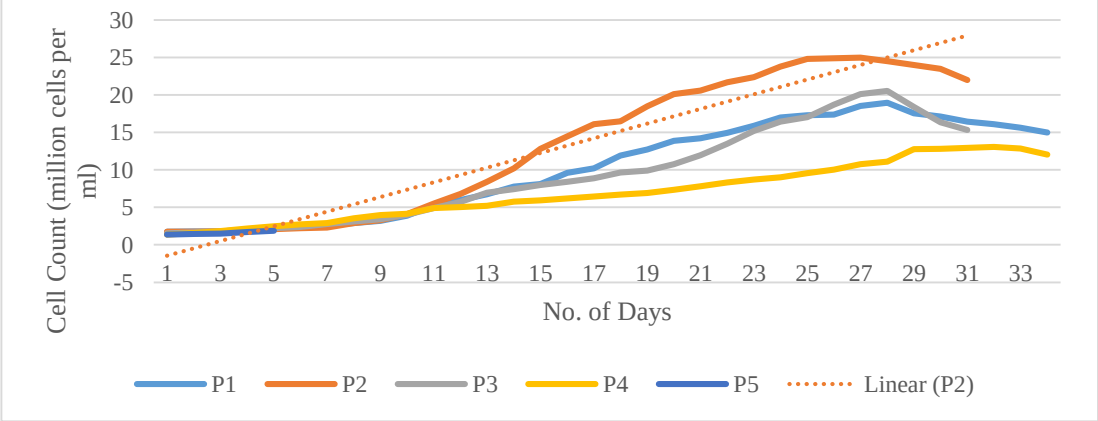

Figure 5.14: Cell count.

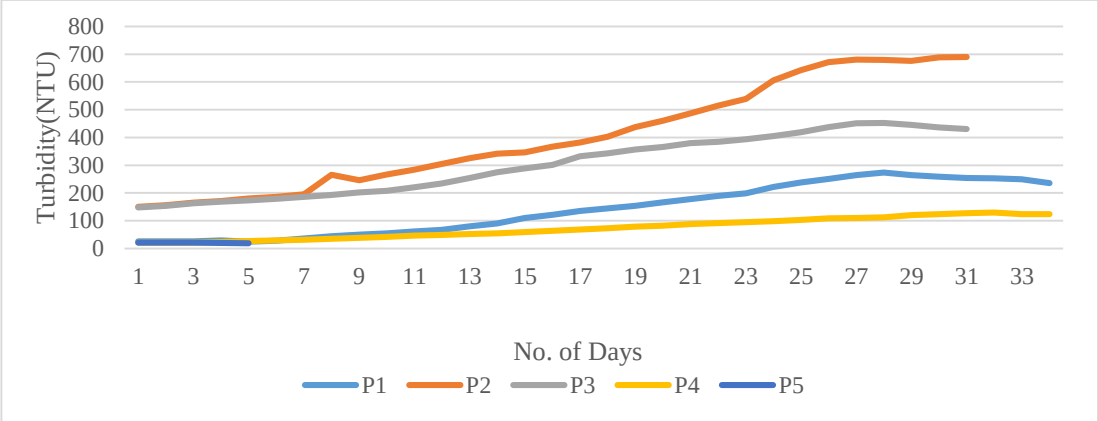

Figure 5.15: Turbidity.

Table 5.10: Growth kinetics.

Cultivation in Agricultural Fertilizers					
Media Samples	P1	P2	P3	P4	P5
Ambient conditions					
Temperature (°C)	26±5				
pH	9±0.5				
Light intensity (LUX)	700±50				
Working volume (L)	1				
Innoculum conc.	10%				
$\%CO_2$	0.02% (via air pump)				
Growth Kinetics					
Max. cell count (million cells/ml)	18.98	25	20.56	13.05	1.9
Max turbidity (NTU)	274.02	680.94	452.54	127.1	18.5
Specific growth rate (d^{-1})	0.094	0.11	0.096	0.063	-
Doubling time	3.05	2.90	3.03	3.45	-
Max. biomass conc. (g/l)	0.61	0.78	0.71	0.53	-

Sample “P2” which contains Fertilizers in ratio of 6:1:3, 200 g of multivitamins and 2% egg albumate gave the maximum Cell count of 25 million cells per mm, maximum specific growth rate of 0.11 d^{-1} and this sample gave maximum biomass concentration of 0.78 grams per liter. These results were much satisfactory and are comparable with conventional Media (BBM) not just in algal growth output but also economically.

5.3.4 Comparison with “Control”

The sample “P2” showed the best results and these results were verified in further experiments with same conditions. Now these the results of P2 were compared with the result of synthetic media for growth parameters, water soluble proteins and bioactive compounds. Both P2 and control were given same ambient conditions and same parameters were measured as before.

5.3.4.1 Cell Count

Cell Count Data of both Experiment and Control is as shown in graph below:

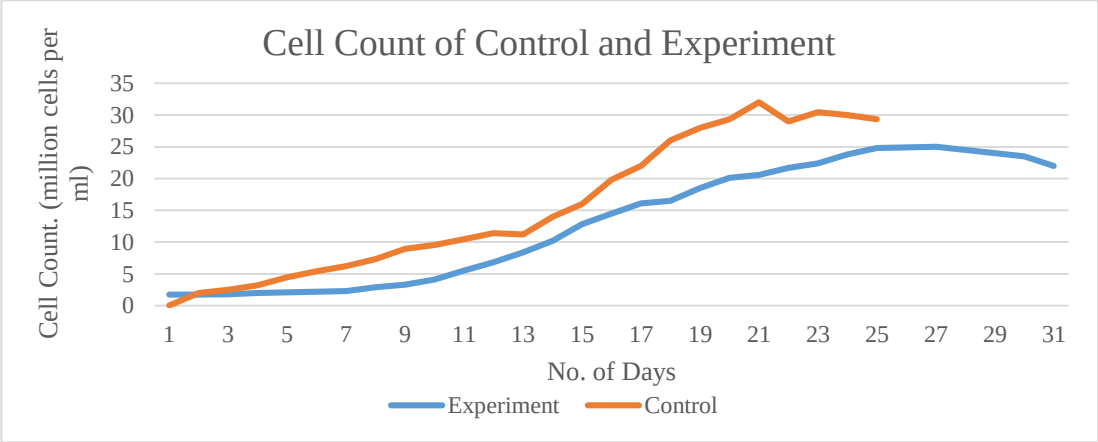

Figure 5.16: Cell count

In above graph it can be clearly perceived that the cell count in BBM on 22nd day is 32 million cells per ml while in cheap fertilizer media it is 25 million cells per ml on 26th day.

5.3.4.2 Dry Biomass Concentration

The dry weight comparison of Experiment and Control is shown below in graph:

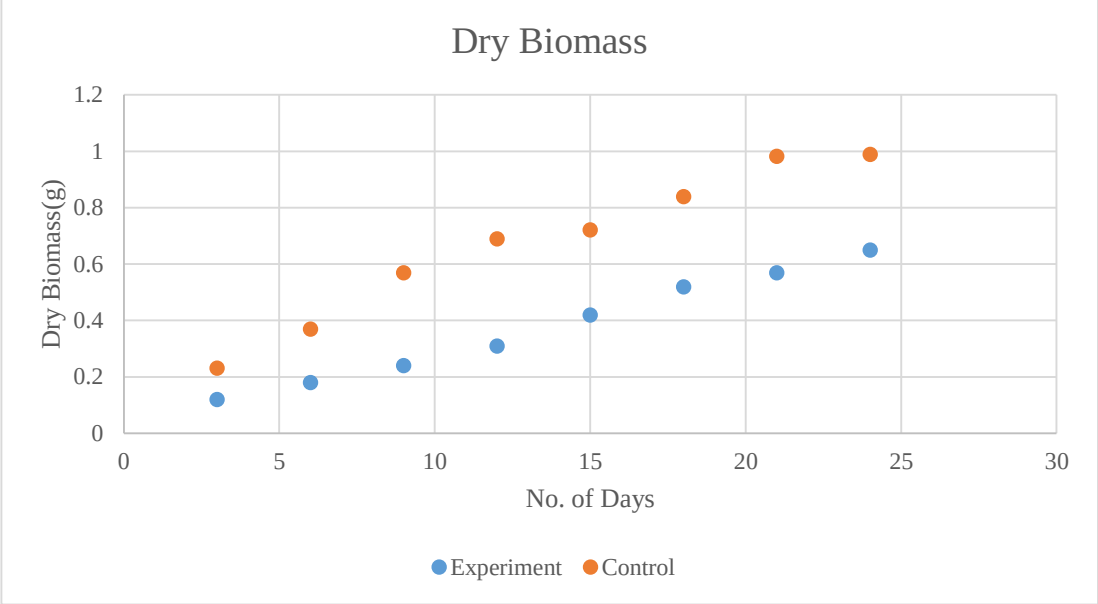

Figure 5.17: Dry bio-mass comparison.

5.3.4.3 Lipid Analysis

Bligh & Dyer method [2] was used to determine lipids contents. The lipid contents of both samples are:

Experiment	*Control*
13g	16g

5.3.4.4 Chlorophyll and Carotenoids Determination

Chlorophyll a, b and carotenes was determined by UV spectrophotometer technique[6].UV absorption at 662, 644 and 470nm was measured respectively. And the results are shown below.

Table 5.11: Comaparison with Control

	Experiment	Control
Chlorophyll a (µg/gfw)	48	53

Chlorophyll b (µg/gfw)	18	21
Carotenes (µg/gfw)	21	23

5.3.4.5 Water soluble proteins (Phycobiliproteins)

Water soluble pigments (Phycobiliproteins). The water soluble Phycobiliprotein pigments including C-phycocyanin (CPC), Allophycoyanin (APC) and C-phycoerytherin (CPE) were extracted from fresh algal sample (1g) with 0.05M phosphate buffer (10ml, pH 6.8). The absorbance (A) of the solution was measured at 650nm, 620nm and 565 nm and the concentrations calculated[7]:

Table 5.12: Phycobiliproteins

	CPC (mg/ml)	APC (mg/ml)	CPE (mg/ml)	Total Phycobiliproteins (mg/ml)
Experiment	0.12	0.01	0.02	0.15
Control	0.13	0.00	0.05	0.18

The water soluble protein pigments in both control and experiment has a difference of 0.3 mg/ml which is negligible. The overall results of KKL-5 algal specie in both Fertilizer Media and BBM is shown below in table:

Table 5.13: Growth kinetics.

Comparison of Control and Experiment		
Media Samples	Control (BBM)	Experiment (FM)
Ambient conditions		
Temperature (°C)	21±5	
pH	9±0.5	
Light intensity (LUX)	700±50	
Working volume (L)	1	
Inoculum conc.	10%	

$\%CO_2$	0.02% (via air pump)	
Growth Kinetics		
Max. cell count (million cells/ml)	25	32
Max turbidity (NTU)	680.94	710
Specific growth rate (d^{-1})	0.11	0.21
Doubling time	2.90	2.01
Maximum biomass conc. (g/l)	0.78	1.1
Lipids (g/L)	16	13
Chlorophyll a (µg/gfw)	48	53
Chlorophyll b (µg/gfw)	18	21
Carotenes (µg/gfw)	21	23
Total Phycobiliproteins (mg/ml)	0.18	0.15
Cost ($)	3.4 [9]	0.5

Local algal strain KKL-5 showed good growth in both Bold's Basal Medium and our new designed Fertilizer media. However the results in FM are a little less than BBM but the less cost overlook the results and made Fertilizer media viable choice for large scale production of Algae in raceway ponds or in photo bioreactors. Cost of fertilizer media is about half dollar while the synthetic Bold's Basal medium cost about $ 3.4 [9].

5.4 Phase 4

5.4.1 High Rate algal pond design Proposal for CO_2 Sequestration and Biodiesel Production

Biofuels lab in collaboration with Attock Oil Refineries, Rawalpindi, initiated a project for sequestration of carbon dioxide. For this job microalgae was selected to capture CO_2 as microalgae uses CO_2 to grow along with other nutrients, the biomass produced was also be proposed to be used for lipid extraction to produce biodiesel. For this matter

algae growth parameters were studied in Biofuels lab to propose a best feasibility of microalgae with all the conditions required for its growth. Finally a High rate raceway

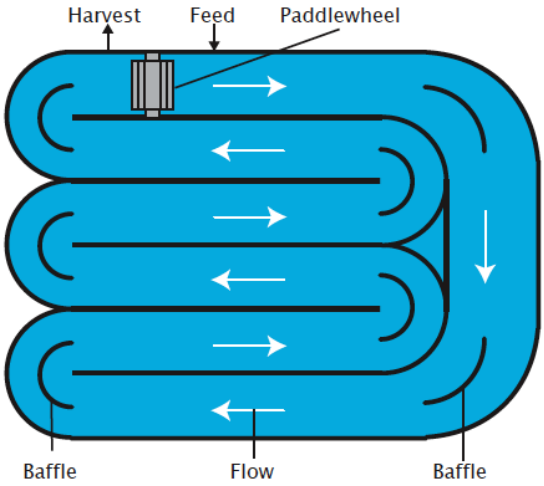

Figure 18 Schematic Diagram of raceway Pond

pond system was selected for the job. High rate pond system have low initial cost then closed systems available and also its operations and maintenance cost was according to available resources.

5.4.2 Introduction to algae growth systems

Microalgae can be cultivated in different types of systems, mainly in open ponds or raceways and in enclosed photobioreactors. Open cultures are usually located outdoors and rely on direct sunlight, while closed photobioreactors can be either indoors or, preferably, outdoors to use free sunlight [1]. Primarily there are two types of algae cultivation systems namely closed systems (photobioreactors) and open systems (raceway ponds).

5.4.3 Open Systems

Open ponds are simple cultivation tanks that are largely obsolete, having been replaced by more efficient raceway ponds. Raceway ponds as shown in Figure 5.13 form closed

Figure 5.19: Raceaway pond.

circuits approximately 0.25 m wide and 0.4 m deep through which the water is circulated using a paddle wheel [2]. They are shallow to maximize light penetration. Where land and water are inexpensive, raceways are extremely cost effective to construct and operate. They require no cooling and do not experience oversaturation with oxygen, which can threaten biofuel production in closed systems. However, they have lower productivity per unit area and volume than closed photobioreactor systems because of the low light-to volume ratio [3].

Additionally, these systems can be easily contaminated by other microorganisms that can compete with the cultivated algal strain [4]. They must be kept highly alkaline to prevent contamination; this high pH limits their suitability to only a few species. The possibility of contamination is often cited as a serious limitation of open systems [5] and it is true that most of the species cultured in such systems currently do grow in selective environments, i.e. *Arthrospira (Spirulina)* (high alkalinity), *Dunaliella salina* (high salinity), and Chlorella (high nutrients) [6]. However, other species with 'normal' growth requirements have also been grown successfully in open ponds [5], either in batch mode e.g., *Haematococcus pluvialis* [7]. Or continuously for very long periods e.g. *Phaeodactylum tricornutum, Nannochloropsis* and *Pleurochrysis carterae*, without significant contamination problems.

Although they have some constraints but still high algae biomass production rates are achievable with open pond systems. However, there are still inconsistencies in the production rates reported in literature (Table 2). Jiménez et al. [8] extrapolated an annual dry weight biomass production rate of 30 tonnes per hectare using data from a 450 m^2 and 0.30 m deep raceway pond system producing biomass dry weight of 8.2 g m^{-2} per day in Malaga, Spain. Using similar depth of culture, and biomass concentrations of up to 1 g l−1, Becker [9] estimated dry biomass productivity in the range of 10–25 gm^{-2} per day. However, the only open pond system for large-scale production that has achieved such high biomass productivity is the inclined system developed by Setlik et al [10] for the production of Chlorella. In this system, a biomass concentration of higher than 10 g/1 was achieved, with extrapolated productivity of 25 g m^{-2} per day. Weissman and Tillett [11] operated an outdoor open pond (0.1 ha) in New Mexico, USA, and attained

an average annual dry weight biomass production rate of 37 tonnes per hectare with a mixed species culture (four species), highest yields were confined to the 7 warmest months of the year.

5.4.4 Closed Systems

Microalgae production based on closed photo-bioreactor technology is designed to overcome some of the major problems associated with the described open pond production systems. For example, pollution and contamination risks with open pond systems, for the most part, preclude their use for the preparation of high-value products for use in the pharmaceutical and cosmetics industry [3]. Also, unlike open pond production, photo-bioreactors permit culture of single-species of microalgae for prolonged durations with lower risk of contamination [12]. Closed systems include the tubular, flat plate, and column photo-bioreactors. These systems are more appropriate for sensitive strains as the closed configuration makes the control of potential contamination easier. Owing to the higher cell mass productivities attained harvesting costs can also be significantly reduced. However, the costs of closed systems are substantially higher than open pond systems.

Photo-bioreactors consist of an array of straight glass or plastic tubes as shown in Fig. 3[13] The tubular array captures sunlight and can be aligned horizontally, vertically, inclined or as a helix [14], and the tubes are generally 0.1 m or less in diameter [12]. Algae cultures are re-circulated either with a mechanical pump or airlift system, the latter allowing CO_2 and O_2 to be exchanged between the liquid medium and aeration gas as well as providing a mechanism for mixing [15]. Agitation and mixing are very important to encourage gas exchange in the tubes.

Some of the earliest forms of closed systems are flat-plate photo-bioreactors [16] which have received much research attention due to the large surface area exposed to illumination [3] and high densities of photoautotrophic cells (>80 g/l) observed [17]. The reactors are made of transparent materials for maximum solar energy capture, and a thin layer of dense culture flows across the flat plate and [17], which allows radiation absorbance in the first few millimeters thickness. Flat-plate photo-bioreactors are

suitable for mass cultures of algae due to low accumulation of dissolved oxygen and the high photosynthetic efficiency achieved when compared to tubular versions [18].

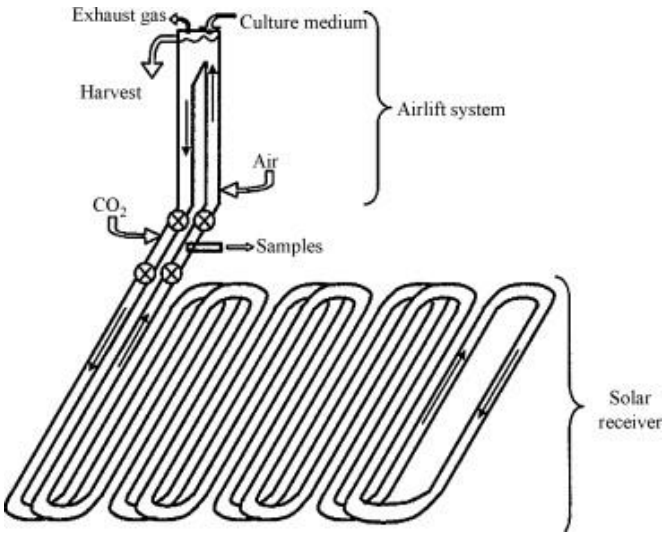

Figure 5.20 Basic design of a horizontal tubular photo-bioreactor (adapted from Becker [9]*). Two main sect*ions: airlift system and solar receiver; the airlift systems allow for the transfer of O_2 out of the systems and transfer of CO_2 into the system as well as providing a means to harvest the biomass. The solar receiver provides a platform for the algae to grow by giving a high surface area to volume ratio.

Tubular photo-bioreactors have design limitations on length of the tubes, which is dependent on potential O_2 accumulation, CO_2 depletion, and pH variation in the systems [15]. Therefore, they cannot be scaled up indefinitely; hence, large-scale production plants are based on integration of multiple reactor units. However, tubular photo-bioreactors are deemed to be more suitable for outdoor mass cultures since they expose a larger surface area to sunlight. The largest closed photo-bioreactors are tubular, e.g. the 25 m^3 plant at Mera Pharmaceuticals, Hawaii [19], and the 700 m^3 plant in Klötze, Germany [20].

Column photo-bioreactors offer the most efficient mixing, the highest volumetric mass transfer rates and the best controllable growth conditions [15]. They are low-cost, compact and easy to operate. The vertical columns are aerated from the bottom, and illuminated through transparent walls [15], or internally [21]. Their performance compares favorably with tubular photo-bioreactors [22].

Closed photo-bioreactors have received major research attention in recent years. The noted proliferation of pilot-scale production using closed photo-bioreactors compared to

open raceway ponds could be attributed to more rigorous process control and potentially higher biomass production rates, hence, potentially higher production of biofuel and co-product production. But still the cost remains the issue in closed system as they are much costly than open pond systems.

5.5 Conclusion and Recommendation

- Identified specie *Dictyosphaerium 8-6* grows best in basic media (pH 9) with cell count of 25million cells/ml in about 25 days.
- *Dictyosphaerium 8-6* were cultured round the year in Biofuels lab and it showed best growth in the months of spring (Feb-April).
- Low Cost Media proved good in indoor conditions.
    - However it is not tested in outdoor conditions.
- *Dictyosphaerium 8-6* showed more growth than Chlorella Vulgaris a renowned oil producing specie and even have same lipids potential.
- *Dictyosphaerium 8-6* has 13% lipids (Dry Biomass) which make it potential candidate to be nominated as oil producing specie.
    - Quantification of lipids via GC or some sensitive equipment need to be done.
- *Dictyosphaerium 8-6* also have good potential of carotenoids and Phycobiliproteins specially C-Phycocianins.

Summary

The designed study was performed at biofuel lab maintaining the ambient conditions for growth. Algal specie *Dictyosphaerium 8-6 (Formerly named as KKL5)* was isolated from consortium along with five other species. All species were subjected to enhance growth rate at room temperature in fresh water and artificially synthesized Bold Basal Media (BBM). *Dictyosphaerium 8-6* tended to show maximum growth of 27 million cells per ml in 25 days, which was apparently higher rate of growth in relevance to other four algae strains. On the basis of highest biomass production quality *Dictyosphaerium 8-6* was advanced for assessment of potential bioactive compounds and lipids.

Dictyosphaerium 8-6 appeared green, sphere to oval, with chloroplast and mucilaginous sheath around them, as studied under microscope initially. For most of the time, they were free floating but sometimes appeared to aggregate and formed colonies. To ensure the specie of the selected strain molecular identification was indispensable. The sequencing analysis of the strain through genetic DNA evaluation revealed the identity of strain as "*Dictyosphaerium 8-6".* Growth pattern of *Dictyosphaerium 8-6* indicated the robust growth rate and tolerance of the strain against harsh environmental conditions. It was subjected to grow annually and its survival alongside different degrees of temperature (min. temp. 5°C to max. 45°C) and environmental fluctuations proved it to be the best candidate for research purpose with economical high yield of biomass.

The lipids analysis was done via FTIR study. Bligh and Dyer method of lipid extraction was adopted for extraction of lipids and results were 13% lipid in this strain as compared to 16% in *Chlorella Vulgaris.* Further determination of Carotenoids, Chlorophyll, and Phycobiliproteins was done with spectrophotometer.

As *Dictyosphaerium 8-6* exhibit lipids potential so it can be used in large scale production of lipids. Most important challenge for large scale production is cost of nutrients media. To encounter the issue a culture media was designed to provide essential nutrients from cheap sources and named as low cost media for *Dictyosphaerium 8-6.*

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Annex 1

An Attempt to design Cheap Media for local algal strain KKL-5

Muneeb Qayyum[1], Abeera Ayaz Ansari[1], Asif Hussain[1] and Ehsan Ali[1]*

[1] Centre for Energy Systems, National University of Sciences and Technology, Islamabad, Pakistan

*Corresponding Author: Ehsan Ali
Email: ihsnalidr@gmail.com
Phone: +92-5190855272

Abstract

Green microalgae is a potential candidate for fulfilling future energy demands along with conserving nature. Microalgae is good source of lipids and some essential bio active compounds like carotenoids, alkaloids, phycobilins and other proteins etc. but according to a Research under the Department of Energy Aquatic Species Program during 1978-1996 concluded that cultivating algae for biofuels was cost prohibitive at that time and that an integrated approach should be studied that combined algal biofuel production with other goals like environment and other products to make it a viable option.

In this research our main goals was to cut down the cost of algae products by designing cheap algal media which will definitely help this technology to become more adoptive. Presently algae is being cultured with conventional Medias which are available in pure form and are much costly. These conventional Medias significantly increases the cost of algal products and even sometimes large scale production becomes impossible due to heavy cost of media.

During this research a number of experiments were performed in series to provide local algae (Chlorella sp. Named KKL-5) with essential nutrients from cheap sources like agricultural fertilizers, market available multivitamins and egg proteins. The algal growth parameters were checked regularly and effects of cheap nutrient sources on lipids productivity, carotenoids and phycobiliproteins were also noted with UV spectrophotometer technique. All results were compared with a standard culture Bold's

Basal Medium. A best recipe of media was screened out with results as competitive as Bolds' Basal media.

Keywords: *Microalgae, Energy, Biofuels, Cheap Nutrient sources*

1. Background

Micro-algae are a large and diverse group of aquatic organisms that lack the complex cell structures found in higher plants. They can be found in diverse environments, some species thriving in freshwater, others in saline conditions and sea water [1] and [2]. Most species are photoautotrophic, converting solar energy into chemical forms through photosynthesis. Micro-algae have received considerable interest as a potential feedstock for biofuel production because, depending on the species and cultivation conditions, they can produce useful quantities of polysaccharides (sugars) and triacylglycerides (fats). These are the raw materials for producing bioethanol and biodiesel transport fuels. Micro-algae also produce proteins that could be used as a source of animal feed, and some species can produce commercially valuable compounds such as pigments and pharmaceuticals[1].

Microalgae have been promoted as one of the more promising third generation biofuels for their ability to accumulate substantial amounts of lipids, divide rapidly, grow in low quality water, absorb CO2, and grow on non-arable land.[3]. There is a wealth of literature that documents the commercial scale growth of various microalga species for natural products as well as the progression of both basic and applied biological research, improvements to photo bioreactor (PBR) and pond design, and lifecycle analyses of algal biofuels.[4][5].

When cultivating algae in an artificial environment (e.g., outdoor pond), it is essential that growth factors are plentiful in order to maximize growth rates.[6]. While CO2can be acquired from the atmosphere, it is commonly fed into algae media to improve production.[7]. In addition to CO2, nitrogen and phosphorus are the major nutrients required for algae growth. It has become an accepted measure that marine plankton have relatively constrained elemental ratios of 106:16:1(C: N: P).[8].

In this research we focused on cheap algal culture for mass production of local algal strain. Algae require phosphates, Nitrates and silicates along with certain vitamins, proteins and other trace elements for its growth[9]. Conventional culture Medias are being used for lab scale culturing of algae but these expensive conventional Medias are not suitable for mass culturing of algae. For large scale culturing raw sources of nutrients are used as they are cheap but it significantly reduces the yield of the algae.[9]. In this study we made an attempt to make a cheap algae culture media by providing compulsory nutrients to algae from cheap sources like agricultural fertilizers and market available multivitamins and egg proteins. New fertilizer media was tested on KKL-5(Chlorella sp.) algal specie and then compared with conventional Media (BBM). The Cell Count in new Fertilizer Media was 25million cells/ml after 30 days as compared to 32 million cells/ml in 22 days with BBM. Lipid contents, chlorophyll a, b, c, carotenes and phycobiliproteins were analyzed and compared. Results showed very negligible difference between both Medias.

2. Materials Required

2.1.Algal Strain (KKL-5):

Algae strains were isolated and preserved at Biofuel Lab at Centre for Energy Systems, NUST. These were local algal strains from Kallar Kahar Lake located in Jhelum, Pakistan. The Strains were revived prior to isolation in Bold Basal Media (BBM) at ambient temperature (20±3^{o}C) with constant CO_2 supply at 0.3L/min flow rate using aquarium air pump. Illumination was done by natural sunlight and pH was maintained at 7. The sample was then serially diluted and cultured on agar plates. The isolation protocol and regular observations under microscope continued until unialgal culture was acquired.[10].The resulting isolated strains were identified but one specie which we named KKL-5 was morphologically classified as Chlorella Genus but species was not confirmed yet was further evaluated with our new Media.

2.2.Synthetic or Control Media Preparation:

Bold's Basal Media was selected as control media for evaluating our new media. Synthetic growth medium used in this study was Bold's Basal Medium (BBM) which was prepared using recipe by [11] having the following composition: In 940ml Distilled water, 10ml each of K_2HPO_4 (75 mg/L), $MgSO_4$ (75 mg/L), NaCl (25 mg/L),

$CaCl_2$ (25 mg/L), $NaNO_3$ (250 mg/L), KH_2PO_4 (105 mg/L), and 1 mL each of trace metal solution: $FeCl_3$ (0.194 g/L), $MnCl_2$ (0.082 g/L), $CoCl_2$ (0.16 g/L), $Na_2MoO_4.2H_2O$ (0.008 g/L), and $ZnCl_2$ (0.005 g/L) was added. Initial pH was 6.6 which was brought in 7 before the commencement of experimentation. BBM is a highly enriched medium with low salinity ideally used for culturing a wide variety of freshwater algal strains. This growth medium is similar to the standard microalgae media 3N-Basal Bold medium except that 3N-BBM has higher nitrate concentrations [7].

2.3.Cheap Media Constituents:

New cheap media are made by agriculture grade fertilizers. DiAmmonium Phosphate, Urea and Calcium Ammonium Nitrate (Fauji Fertlizer Company) were brought from local market. Revitale Multi tablets (GlaxoSmithKline) were also brought from local market. Egg White was dissolved in water with the help of 10% NaOH Solution and was then used as protein source for algae.

3. Methodology

3.1.1. Algae Growth Parameters

Samples were taken from inoculated Experiment and Control media cultures daily for pH (pH meter), Turbidity (Turbidity meter) and cell count which was measured using Hemacytometer [12] while dry biomass weight and chlorophyll a were determined after every 3days. Chlorophyll a (mg/L) was determined spectrophotometrically after extraction by 90% acetone as reported by Arar E.J [13]. Dry biomass weight (g/L) was calculated using protocol by Irving T [14] in which samples (10mL) of algal culture were filtered through pre-heated, pre-weighed glass microfiber filters (Whatman GF/C, 47 mm). After filtration, the filters containing algae suspension were dried at 103°C to a constant weight, cooled in a desiccator and weighed on an electronic balance.

3.1.2. Lipids Determination

Protocol by Bligh and Dyer[13] was used to extract lipids from algal biomass. Algae culture was centrifuged (4000g, 10min) to obtain wet pellet which was mixed in 4ml distilled water and extraction solution (MeOH/chloroform, 1:2 v/v) and left for overnight on shaker at moderate speed. The following day, distilled water and chloroform (1:1 v/v) were introduced in sample and allowed to mix for 5-6 hours. Finally the sample was centrifuged (4000g, 10 min) to produce a biphasic layer in which

the bottom layer contained lipids dissolved in chloroform. This layer was extracted using micropipette and dried at 50^{o}C, 2hrs in oven to obtain lipid content.

3.1.3. Bioactive Compounds Analysis

- **Carotenoids**

Carotenoid Contents were measured by UV spectrophotometer by method of Jaspers.[14].

- **Phycobiliproteins**

The water soluble Phycobiliprotein pigments including C-phycocyanin (CPC), Allophycoyanin (APC) and C-phycoerytherin (CPE) were extracted from fresh algal sample (1g) with 0.05M phosphate buffer (10ml, pH 6.8). The absorbance (A) of the solution was measured at 650nm, 620nm and 565 nm and the concentrations calculated[15]:

CPC (mg/ml) = A620-0.72xA650/6.29

APC (mg/ml) = A650-0.191xA620/5.79

CPE (mg/ml) = A565-2.41x (Conc. of CPC x1.4 (Conc. of APC)/13.02)

- **Chlorophyll a, b, c**

Chlorophyll a, b, c was determined spectrophotometrically by dissolving in 90% acetone (v/v) by the method of Parson and Strickland method [16].

3.1.4. Growth Kinetics

The calculated dry biomass weight (g/L) from the exponential growth phase of each growth study was used to determine the growth kinetics of established experimentation. The specific growth rate (μ, d^{-1}) was calculated using equation $\mu = (\ln X_2 - \ln X_1)/(t_2 - t_1)$, where X_2 and X_1 were the dry biomass weight (g/L) at time t_2 and t_1, respectively. The maximum specific growth rate (μ_{max}, d^{-1}) was determined from all the different μ values calculated while the maximum biomass obtained was designated as X_{max} (g/L). Cell doubling time (t_d, d) was estimated using equation t_d (d) = $\ln 2/\mu_{max}$ [15].

3.1.5. Experimentation

The experiments to design the media was conducted in three phases to evaluate cheap nutrient sources and was than the final results of third phase were compared with synthetic media BBM as control. The results include Algae growth parameters, Bioactive Compound Analysis and Growth Kinetic Parameters of KKL-5 in Cheap Media and its comparison with Synthetic Media (BBM).

1) Phase 1

In phase one of experiments the Urea, DAP and CAN (Agriculture grade fertilizers) were tested for KKL-5 with different composition ratios, Five samples (i-e S1,S2,S3,S4,S5) were prepared with different Ratios of Urea, DAP and CAN(i-e 6:1:3, 5:2:3, 4:3:3, 3:4:3, 2:5:3 respectively), these five samples were then introduced in five flasks (1L) with water and 10% algal inoculum thus making 1L of algae culture in each bottle. Each bottle was put to aeration with the help of aeration pumps to provide with carbon dioxide and pH was maintained at 7.5. Growth Parameters were calculated daily for about 30 days. Growth Kinetics study were performed on the results of all samples and their values compared. The best sample were again evaluated with addition of further nutrients.

2) Phase 2

Phase 1 provided the algae with essential major nutrients of Nitrogen, Phosphorous and Calcium, but the results were not as good as results from synthetic media.

Algae require Vitamins for its growth, Three vitamins—vitamin B1 (thiamine · HCl), vitamin

B12 (cyanocobalamin), and vitamin H (biotin)—are usually used for culture of microalgae.[9]. Many algae need only one or two of the vitamins, but there seems to be no harm caused by adding a nonessential vitamin.[17]. In addition to the three common vitamins, some recipes call for other vitamins. For example, nicotinamide (nicotinic acid amide, niacinamide) is added to the culture medium for Phacotus lenticularis (Ehrenberg) Stein (see N-Hs-Ca medium,Schlegel et al. 2000).[18].

In this phase we introduced vitamins and minerals in our culture in the form of market available *Revital Multi Multivitamins and Minerals Tablet.* Each tablet weigh 0.90 g was crushed into powder and then used. Best sample from phase 1 was prepared in five flasks (1L) and each flask was introduced with MV tablets of different weight amount. (i-e 10g,20g,30g,40g,50g). And was further provided with aeration and other growth conditions were same as in phase 1. Growth parameters were measured daily for about more than 30 days. Growth Kinetics study were performed on the results of all samples and their values were compared. The best sample were again evaluated and tested with egg proteins in phase 3.

3) Phase 3

Algae have recently been explored for their potential as a novel protein expression system. There are many reasons why algae would make a more efficient and robust, expression system. First, algae are able to carry out many of the post translational mechanisms that are needed for the production of many complex proteins and are relatively easy and cheap to culture. They require very basic media to grow on such as minimal or acetate based media with a few trace elements, as well as the ability to use sunlight as an energy source, hence cost of goods are low.[19].

In this phase we try to introduce proteins in culture to see their effect on algae growth and production. Egg proteins were selected to be used in culture. Egg white is usually insoluble in water and make clumps when added or stirred in water. Egg white was made soluble in water with the help of NaOH solution.

The best sample out of five from phase 2 was now tested with egg proteins. Five solutions were prepared each with different percentage of egg proteins (i-e 1%, 2%, 3%, 4%, 5%) and previous constituents of phase 2. All samples were given same ambient conditions as in phase 1 & 2. Growth parameters were measured daily for about more than 30 days. Growth Kinetics study were performed on the results of all samples and their values were compared. The results of the best sample were compared with control synthetic culture (BBM).Lipids, Chlorophyll, Carotenoids and water soluble proteins were determined in both experiment and control cultures and was compared.

4. Results and Discussion

Phase 1

The first phase of the experiments included the optimization of agriculture grade fertilizers which include Urea, Di-ammonium phosphate and Calcium ammonium nitrate.

Five samples were prepared with different ratios of the constituents as shown in table below:

Sample	Ratio (Urea:DAP:CAN)

S1	6:1:3
S2	5:2:3
S3	4:3:3
S4	3:4:3
S5	2:5:3

Table 14Diff. Fertilizers Comp.

All the five samples were subjected to experimentation and were tested for their potential as algae growth media. Algae growth parameters were recorded on daily basis and results were compared and best sample's combination was further improved with addition of vitamins and proteins.

The results initially included cell count, turbidity, and dry biomass for evaluation of best fertilizers combination.

The Cell Count and turbidity of all five samples is shown below:

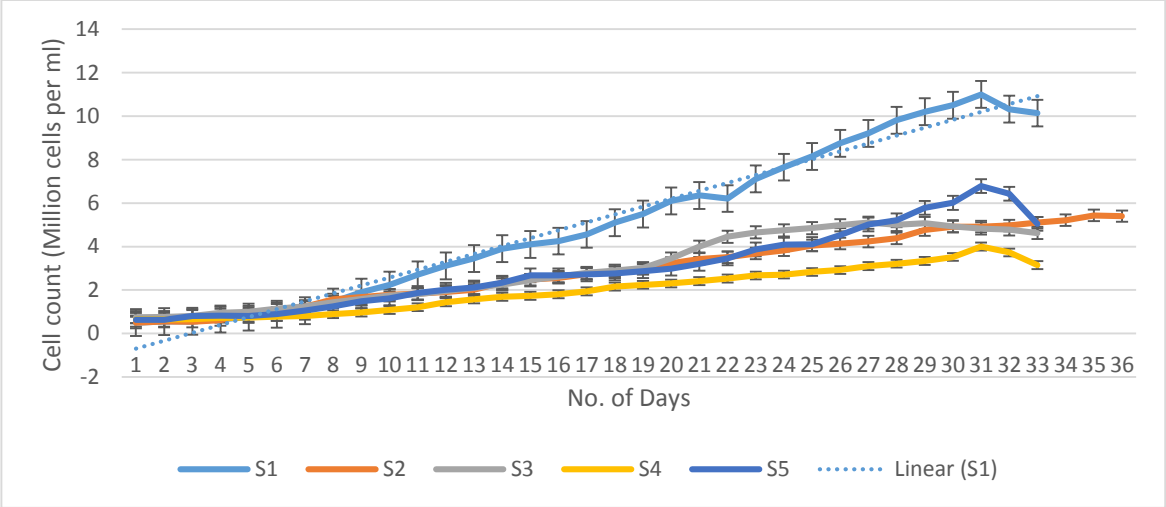
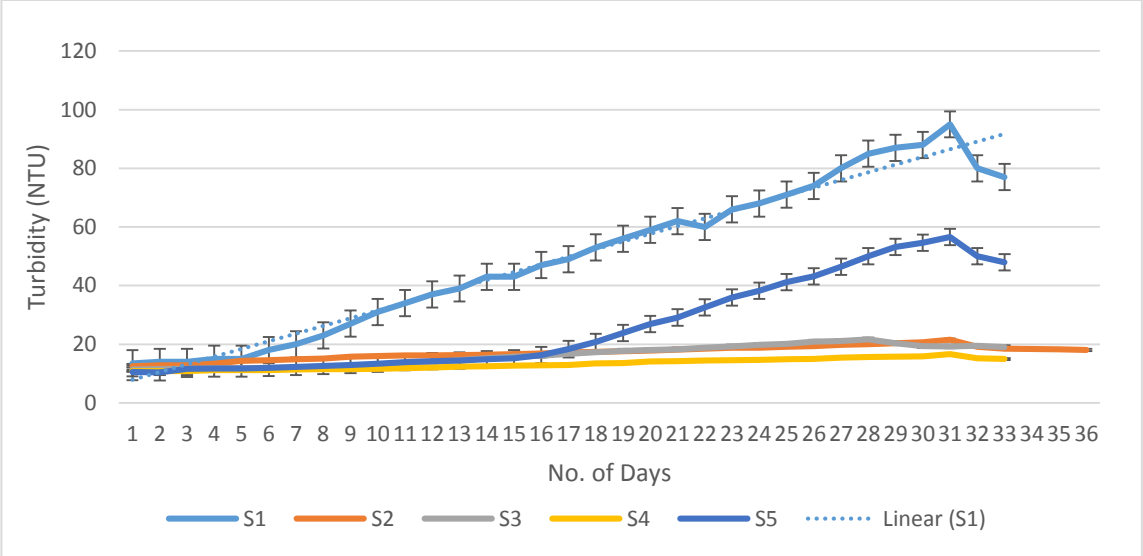


Figure 21Cell Count



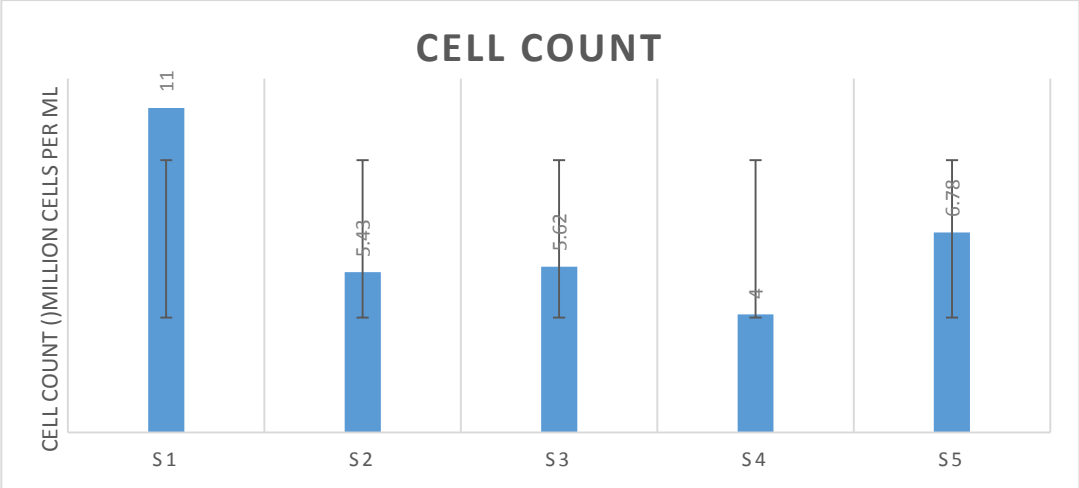


Figure 23Cell Count Comparison

Figure 22Turbidity

The sample 1 with 6:1:3 ratio of Urea, DAP, CAN respectively showed the cell count of about 11 million cells per ml after at about 30th day of experiment which is much higher than other 4 samples. The gowth kinetics of all five samples are given below:

Cultivation in Agricultural fertilizers					
Media Samples	S1	S2	S3	S4	S5
Ambient conditions					
Avg. Temperature(C)	26±5				

Avg. pH	9±0.5				
Light Intensity(LUX)	700±50				
Working Volume(L)	1				
Inoculum Conc.	10%				
%CO_2	0.02% (via air pump)				
Growth Kinetics					
Max. Cell count (million cells/ml)	11	5.43	5.08	4	6.78
Max Turbidity (NTU)	95	18.21	20.43	16	56.46
Specific Growth rate(d^{-1})	0.106	0.051	0.058	0.061	0.072
Doubling time	2.94	3.66	3.54	3.49	3.32
Maximum Biomass Conc.(g)	0.32	0.25	0.23	0.19	0.28

Table 15 Growth Kinetics (Phase 1)

Sample 1 showed the growth rate of 0.106$d^{-1,}$ doubling time of 2.94 days and maximum biomass concentration of 0.32g which is higher than all other samples, so this sample was further evaluated in next phase.

Phase 2

Here S1 was further tested with five compositions (10g, 20g, 30g, 40g, and 50g) of multi vitamin tablet with objective of improving the overall cell count and biomass of algae.

Addition of multi minerals improved the results, again the data was calculated and growth kinetic studies were performed.

Five samples were prepared, as shown in table below,

Samples	Multi Vitamin Conc. (g/L)
V1	10
V2	20
V3	30

V4	40
V5	50

Table 16 Multivitamins Conc.

All five samples were given ambient conditions and data were collected for about thirty days until decline phase of algae sample is achieved.

Below graphs shows the cell count, turbidity of Samples.

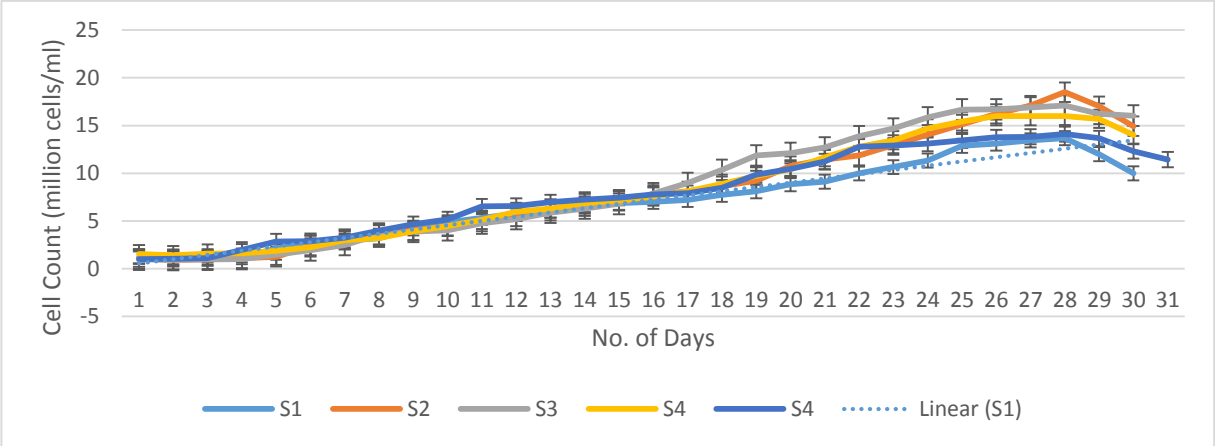


Figure 24 Cell Count

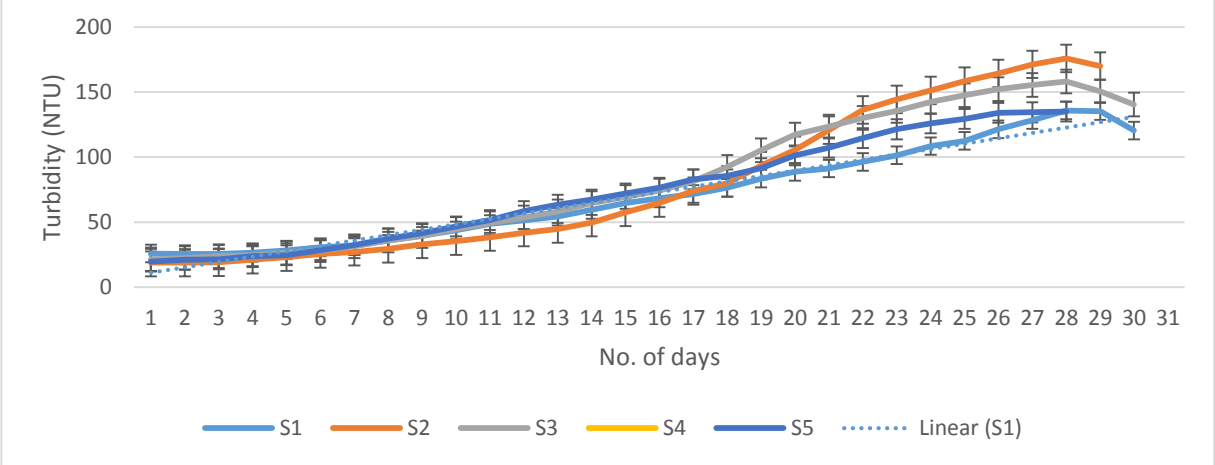


Figure 25 Turbidity

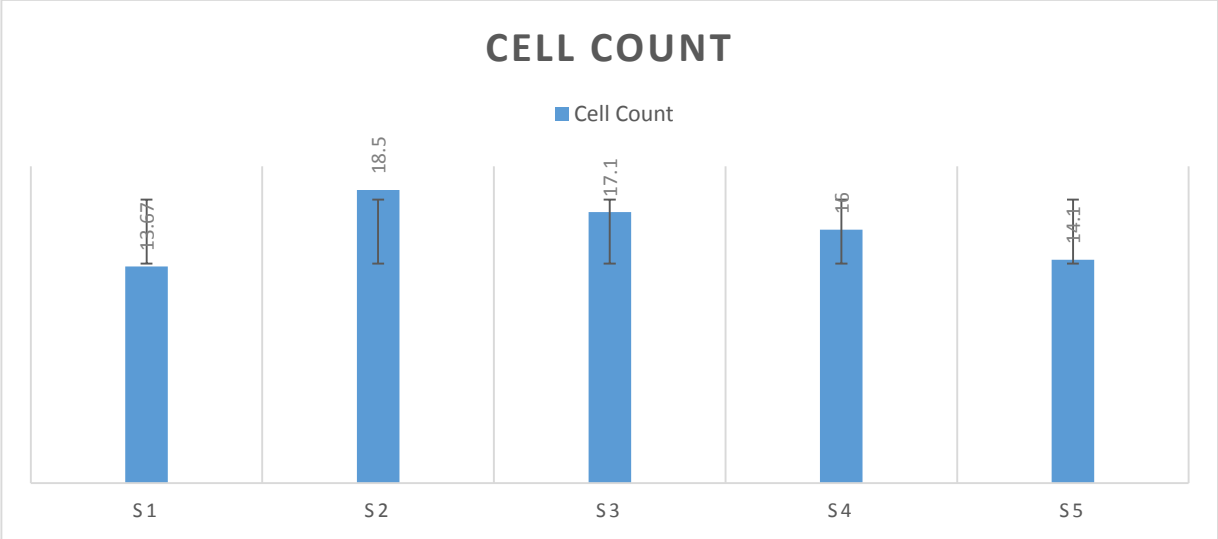


Figure 26 Cell Count Comparison

Cultivation in Agricultural fertilizers					
Media Samples	V1	V2	V3	V4	V5
Ambient conditions					
Avg. Temperature(C)	26±5				
Avg. pH	9±0.5				
Light Intensity(LUX)	700±50				
Working Volume(L)	1				
Inoculum Conc.	10%				
%CO_2	0.02% (via air pump)				
Growth Kinetics					
Max. Cell count (million cells/ml)	13.67	18.5	17.1	16	14.1
Max Turbidity (NTU)	159.85	199.98	165.87	158.23	135
Specific Growth rate(d^{-1})	0.077	0.089	0.081	0.088	0.06
Doubling time	3.25	3.17	3.10	3.12	3.50
Maximum Biomass Conc.(g)					

Table 17 Growth Kinetics (Phase 2)

The above graphs and kinetic study shows that results improved significantly in phase 2, so we conclude that multi vitamins have positive effect on algae growth.

Among all five samples sample V2(fertilizers(6:1:3) + Revitale 20g) showed best results but still the results were not that satisfactory to compete with conventional fertilizers and to improve it further sample V2 which contains best fertilizer combination along with best multi vitamin combination and now in phase 3 proteins were tested along with this best combination.

Phase 3

Till third phase of experiments we already had achieved 75% of the target results. Algae not only require nitrates, phosphates and silicates it also requires certain metals, vitamins and proteins.

In third phase of experiments we tried to feed our algae with some protein rich media. To make media protein sufficient we tried to dissolve egg white in media with the help of NaOH, and made five different Solutions, as shown below:

Samples	Sodium albumate (%)
P1	1%
P2	2%
P3	3%
P4	4%
P5	5%

Table 18Proteins Comp's for Phase 3

Now the third phase was intended to study the effect of proteins on growth of algae. All five protein samples were tested with S1 and V1, experiment conditions were same and the same growth parameters were measured.

Below are the results from phase 3 experimentation:

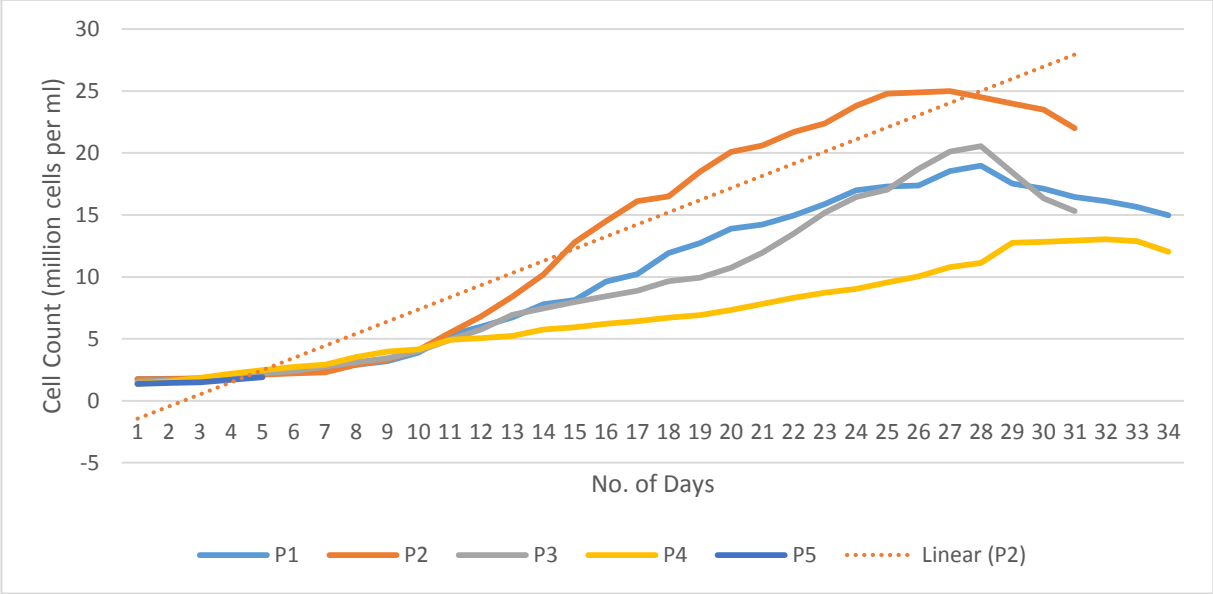


Figure 27 Cell Count

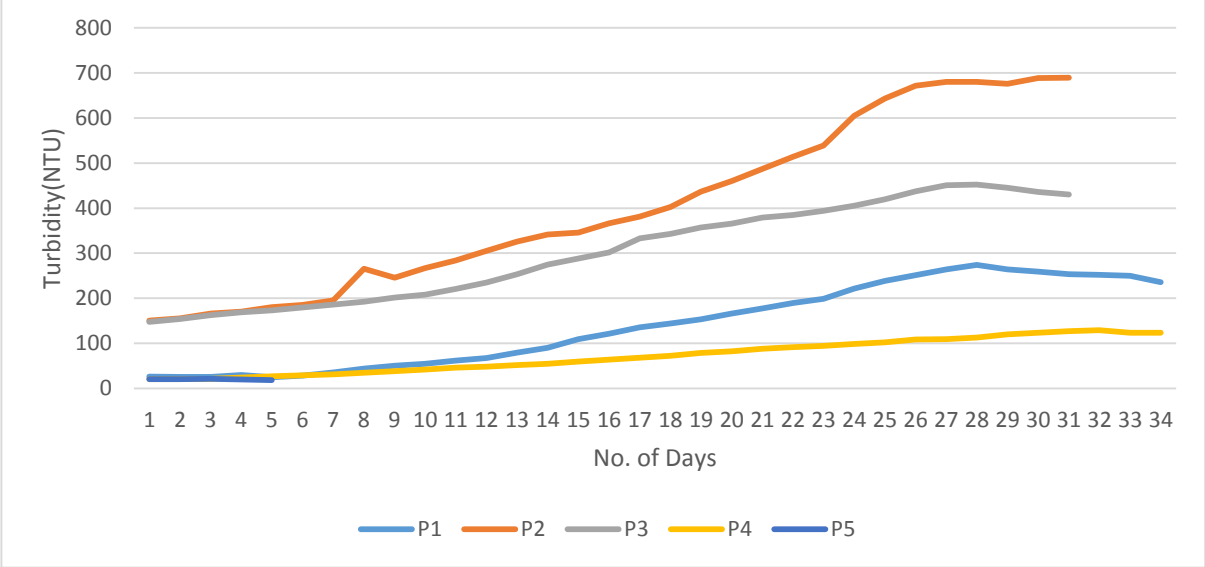


Figure 28Turbidity

Cultivation in Agricultural fertilizers

Media Samples	P1	P2	P3	P4	P5
Ambient conditions					
Avg. Temperature(C)	26±5				
Avg. pH	9±0.5				
Light Intensity(LUX)	700±50				
Working Volume(L)	1				
Innoculum Conc.	10%				
%CO_2	0.02% (via air pump)				
Growth Kinetics					
Max. Cell count (million cells/ml)	18.98	25	20.56	13.05	1.9
Max Turbidity (NTU)	274.02	680.94	452.54	127.1	18.5
Specific Growth rate(d^{-1})	0.094	0.11	0.096	0.063	-
Doubling time	3.05	2.90	3.03	3.45	-
Maximum Biomass Conc.(g/l)	0.61	0.78	0.71	0.53	-

Table 19 Growth Kinetics Phase 3

Sample "P2" which contains Fertilizers in ratio of 6:1:3, 200g of multivitamins and 2% egg albumate gave the maximum Cell count of 25 million cells per mm, maximum specific growth rate of 0.11 and this sample gave maximum biomass concentration of 0.78 grams per liter. These results were much satisfactory and are comparable with conventional Media (BBM) not just in algal growth output but also economically.

Comparison with Control

The sample "P2" showed the best results and these results were verified in further experiments with same conditions. Now these the results of P2 were compared with the result of synthetic media for growth parameters, water soluble proteins and bioactive compounds. Both P2 and control were given same ambient conditions and same parameters were measured as before.

Cell Count

Cell Count Data of both Experiment and Control is as shown in graph below:

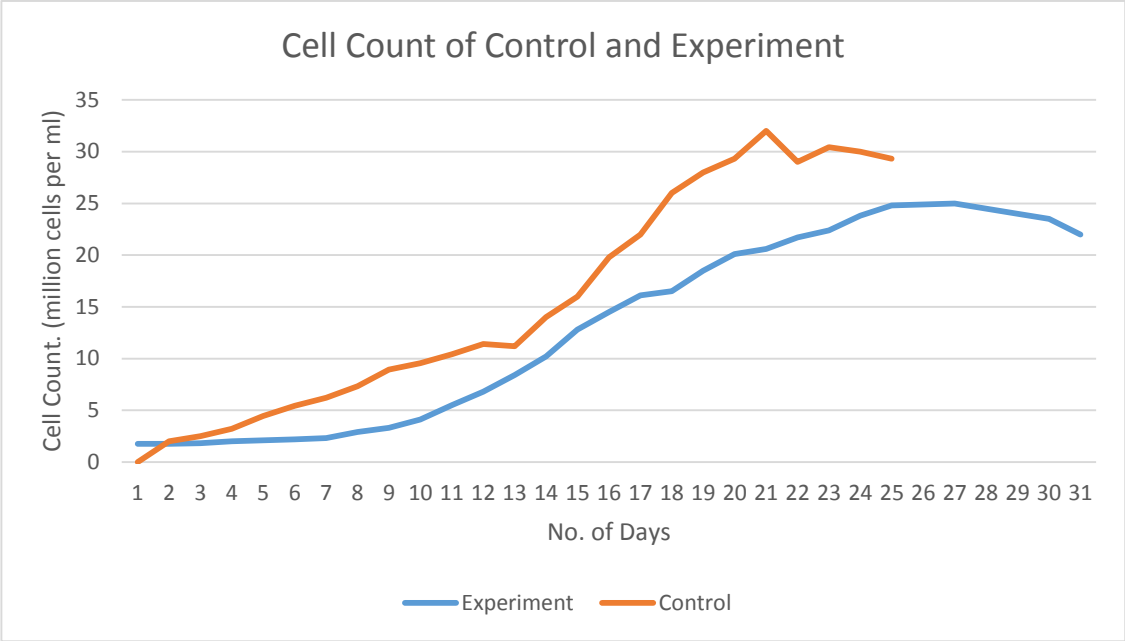


Figure 29Cell Count in Control and Experiment

In above graph it can be clearly perceived that the cell count in BBM on 22nd day is 32 million cells per ml while in cheap fertilizer media it is 25 million cells per ml on 26th day.

Dry Biomass Concentration

The dry weight comparison of Experiment and Control is shown below in graph:

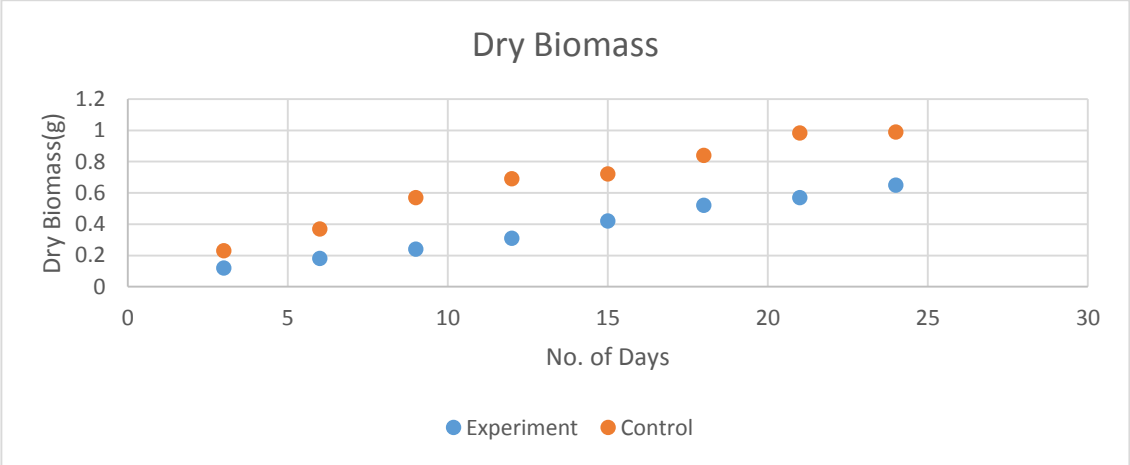


Figure 30Dry Biomass

Lipid Analysis

Bligh & Dyer method[13] was used to determine lipids contents. The lipid contents of both samples are:

Experiment	*Control*
13g	16g

Chlorophyll and Carotenoids Determination

Chlorophyll a, b and carotenes was determined by UV spectrophotometer technique[20].UV absorption at 662, 644 and 470nm was measured respectively. And the results are shown below.

	Experiment	Control
Chlorophyll a(µg/gfw)	48	53
Chlorophyll b(µg/gfw)	18	21
Carotenes (µg/gfw)	21	23

Table 20

Water soluble proteins (Phycobiliproteins)

Water soluble pigments (Phycobiliproteins). The water soluble Phycobiliprotein pigments including C-phycocyanin (CPC), Allophycoyanin (APC) and C-phycoerytherin (CPE) were extracted from fresh algal sample (1g) with 0.05M phosphate buffer (10ml, pH 6.8). The absorbance (A) of the solution was measured at 650nm, 620nm and 565 nm and the concentrations calculated[21]:

	CPC(mg/ml)	APC(mg/ml)	CPE(mg/ml)	Total Phycobiliproteins(mg/ml)
Experiment	0.12	0.01	0.02	0.15
Control	0.13	0.00	0.05	0.18

Table 21

The water soluble protein pigments in both control and experiment has a difference of 0.3 mg/ml which is negligible.

The overall results of KKL-5 algal specie in both Fertilizer Media and BBM is shown below in table:

Comparison of Control and Experiment		
Media Samples	Control(BBM)	Experiment(FM)
Ambient conditions		
Avg. Temperature(C)	21±5	

Avg. pH	9±0.5	
Light Intensity(LUX)	700±50	
Working Volume(L)	1	
Innoculum Conc.	10%	
%CO_2	0.02% (via air pump)	
Growth Kinetics		
Max. Cell count (million cells/ml)	25	32
Max Turbidity (NTU)	680.94	710
Specific Growth rate(d^{-1})	0.11	0.21
Doubling time	2.90	2.01
Maximum Biomass Conc.(g/l)	0.78	1.1
Lipids(g/L)	16	13
Chlorophyll a(µg/gfw)	48	53
Chlorophyll b(µg/gfw)	18	21
Carotenes(µg/gfw)	21	23
Total Phycobiliproteins(mg/ml)	0.18	0.15
Cost($)	3.4[22]	0.5

Table 22 Final Results

5. Conclusion

Local algal strain KKL-5 showed good growth in both Bold's Basal Medium and our new designed Fertilizer media. However the results in FM are a little less than BBM but the less cost overlook the results and made Fertilizer media viable choice for large scale production of Algae in raceway ponds or in photo bioreactors. Cost of fertilizer media is about half dollar while the synthetic Bold's Basal medium cost about 3.4 US dollars[22].

6. Acknowledgement

The authors acknowledge the services of Mr. Yawar Ikram and Mr. Aleemudin from Attock Group of Companies, Rawalpindi, Pakistan for providing with necessary cooperation required for the fulfilment of this research.

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