"ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL MICROALGAE FROM DTU LAKE AND ITS EVALUATION FOR BIOFUEL PRODUCTION"

To be submitted as Major Project in partial fulfilment of the requirement for the degree of

Master of Technology

In

Industrial Biotechnology

Submitted by

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(2K14/IBT/08)
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DECLARATION

I, Lakhan Kumar, hereby declare that the work which is being presented in the project report entitled, ***"Isolation, Identification and Characterization of potential microalgae from DTU Lake and its evaluation for Biofuel production"*** being submitted by me in the partial fulfilment of the requirement for the award of degree of Master of Technology in Industrial Biotechnology, **Delhi Technological University, Delhi, India,** is an authentic record of my own work carried out under the guidance of **Dr. Navneeta Bhardvaja**, Asst. Professor, Department of Biotechnology. The matters embodied in this record have not being submitted by me for the award of any other degree.

DATE: 2016-06-21

LAKHAN KUMAR
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CERTIFICATE

This is to certify that the thesis of minor project entitled ***"Isolation, Identification and Characterization of potential microalgae from DTU Lake and its evaluation for Biofuel production" (DTU/14/MTech./090)*** in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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ABBREVIATIONS:

SEM	**:**	**Scanning Electron Microscope**
FTIR	**:**	**Fourier Transform Infrared Spectroscopy**
UV-VIS	**:**	**Ultraviolet-Visible Spectroscopy**
µg/ml	**:**	**Microgram per Millimetre**
g/l	**:**	**Gram per Litre**
CO_2	**:**	**Carbon di oxide**
Na	**:**	**Sodium**
K	**:**	**Potassium**
SO_4^{2-}	**:**	**Sulphate**
NO_3^-	**:**	**Nitrate**
mm	**:**	**Millimetres**
m	**:**	**Meters**
Km	**:**	**Kilometres**
^0C	**:**	**Degree centigrade**
CPCB	**:**	**Central Pollution Control Board**

"Isolation, Identification and Characterization of potential microalgae from DTU Lake and its evaluation for Biofuel production"

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ABSTRACT:

Due to the increasing demand of energy by leaps and bounds, it is requisite to identify the local solution for the generation of energy to mitigate the future demand. Microalgae have been conferred as a potential source of biomass based energy. Many potential microalgae have been isolated for biofuel production across the globe till date considering their enormous potential and compatibility to be converted into liquid and gaseous fuels. Herewith, we have focussed on the isolation and characterization of a potential microalgae strain from the local water bodies under natural environmental conditions which provides an edge to commercialization of microalgae biofuel production. Microalgae sample obtained from Delhi Technological University's lake (DTU Lake) was used for the isolation of potential microalgae. Light microscope, Scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR) and 28S rDNA analysis were performed for identification of microalgae. After the identification, effect of various abiotic factors such as light (intensity, color and period), pH of medium, shaking period and temperature on algal growth rate and biomass productivity was studied. *Scenedesmus rubescens* provided maximum biomass productivity in white light of 36W intensity under illumination of 16 hours. pH 8, 24 hours shaking period and 26 ± 2^0C temperature were also found to be best for this microalga. The present study also aims to find out the superior source of nitrogen, phosphorous and carbon for the optimum growth rate and biomass productivity (dry cell weight) for freshwater microalgae *Scenedesmus rubescens*. Seven different freshwater media compositions were also tested for a period of 27 days. Among Nitrogen Sources (NH_4Cl, $CO(NH_2)_2$, $NaNO_3$, KNO_3, $Co(NO_3)_2$ and $Ca(NO_3)_2$) Urea followed by Sodium Nitrate was found to be the best source. For Phosphorous Source, K_2HPO_4 showed the optimum yield as compared to $NH_4H_2PO_4$, KH_2PO_4 and Na_2HPO_4. Sucrose was found to be the superior carbon source than Glucose, Sodium Acetate and Glycerol for the optimum growth and biomass yield. Modified HUT medium was found to be best media for the microalga *Scenedesmus rubescens*. Lipid and chlorophyll content was also determined.

1. INTRODUCTION

Microalgae are unicellular photosynthetic autotrophic microorganisms that require relatively simple nutrients and space as compared to plants for their metabolism. They are capable of fixing carbon dioxide from the atmosphere and water to produce biomass as same as plants do but more efficiently and rapidly than plants do. The basic reaction in water is (Darmak et al 2012)

Carbon dioxide + water + light energy = glucose + oxygen + water.

This biomass is composed of various biomolecules such as lipids, carbohydrates, proteins and carotenoids, DHA etc which is used for the food, fuel and medicinal purposes etc. Lipids and carbohydrates extracted from microalgae biomass are the best source for biodiesel and bioethanol production. An algae-based biorefinery approach could potentially incorporate a number of different conversion technologies to produce biofuels including biodiesel, aviation fuel (commercial and military), bioethanol, and biomethane, as well as valuable co-products including inks, paper, Docosahexaenoic acid (DHA) and Diacylglycerol acyltransferase (DAGT) etc. (Singh and Olsen, 2011)

Microalgae accumulate large amounts of storage lipids primarily in the form of triacylglycerols (TAG). The fatty acid profile is important in selecting microalgae as a feedstock for biodiesel production.(Bi & He 2013). Wastewater from industrial and domestic sources, consists various nutrients, is also a potential source for microalgal biomass production which can be utilized in the production of biofuels. Due to fast industrial growth, water and air pollution level has been increased tremendously and it is causing environmental degradation. So, it is required to treat wastewater and various emissions before discharging them into the environment. (Singh *et al.*2013). Microalgae are known to its nutrient removal efficiency from the industrial effluents and wastewater.(Giuliano et al 2010; Chisti 2007) As fossil fuels are depleting at very fast rate, we need to explore newer eco-friendly and economically sustainable sources of energy to meet the global demand. Microalgae grow in most of the natural environments including marine and freshwater sources, rocks and soils. (Schenk *et al* 2012).

The isolation of microalgae from lakes or ponds is a useful strategy for obtaining microalgae tolerant to the conditions prevalent in the area, such an organism tending to have the ability to grow without applying any extra effort with having good biomass productivity. Keeping the

competitive and selective growth condition advantages like the adaptation in local geographical, climatic and ecologic conditions in mind, microalgae thus isolated was cultured in the lab and evaluated for the biodiesel production. (Munir et al 2015). High lipid or carbohydrate producing microalgae are the desired ones for the production of biodiesel and bioethanol respectively. Analysis of the bulk biochemical composition of the microalgae is essential for the understanding of biological processes happening in the cell and abiotic factors affecting the accumulation of various metabolites within the cell. As per required biofuel production, it is desired to know the biomolecules composition of the cell for determination of the optimum use of biomass for biofuel production. The lipid-rich microalgae are suitable feedstock for biodiesel production while carbohydrate-rich microalgae are best sources for bioethanol production.

The biomolecules composition *i.e.* lipid, carbohydrate, protein, chlorophylls etc. of algal biomass is usually investigated and analysed by the conventional chemical extraction method followed by gravimetric determination and spectroscopy. (Flynn *et al* 2013). For rapid determination of the isolated microalgal sample for its biochemical composition, use of FTIR is a highly advanced technique and powerful analytical tool. (Miglio *et al* 2013) ;(Flynn *et al* 2013). FTIR is a non-destructive and rapid method, which helps in identification of the vibrational structure of materials. (Erukhimovitch *et al* 2003). FTIR provides the metabolic fingerprinting of the material by discriminating and identifying the various functional groups present in it. The composition and structure of molecules and functional groups can be determined by the analyzing the position/width and infrared light absorption of the sample.(D' Souza *et al* 2008). Biomolecules such as lipid, carbohydrate and protein etc has their own characteristics IR spectra, which is rich in molecular composition and functional aspects and which allow identification and quantification based upon absorbance. (Yakoob *et al* 2011); (Erukhimovitch *et al* 2003);(Flynn *et al* 2013). For microalgae sample, the sample is analyzed in 4000-400 cm^{-1} wave number range with a resolution of 4 cm^{-1} (Erukhimovitch *et al* 2003). It saves time, the quantity of sample to be analyzed and minimise the use of hazardous reagents for the biochemical composition determination. The only limitation of this method is the practitioner's efficiency. Scanning Electron Microscope provides the absolute morphological details of the material and this property is utilised for the characterization of the microalgae. The light microscope provides other morphological features and cellular details and paves the way for identification of isolated microalgal strain. (Shabudeen *et al* 2013). In examining the physical structures of microalgae, SEM provides

good visual images of possible microalgal structure and morphology. It scans solid samples with a beam of electrons, which interacts with the atoms that make up the sample to produce signals. These signals contain topographic information about the samples. (Bi & He 2013).

Several factors either biotic or abiotic influences microalgal growth. Abiotic factors include the intensity and amount of light, the temperature, salinity and pH of the medium and nutrient concentrations etc. Bacteria, cyanobacteria, fungi, competition by other microalgae and predation by other aquatic organisms come under biotic factors which limit the algal growth. Other operational factors like dilution and shear rate, the process and frequency of harvesting, depth of the tank etc also influence the microalgal growth rate and thus the biomass productivity. The microalgal biomass and the biochemical composition's amount may be influenced and improved by manipulating the surrounding environment.

In the present scenario, where fossil fuels are depleting at very fast rate and about to end in some coming years, every nation, either developed or developing, is looking for an alternative source of energy to meet the energy requirements of their population and microalgae based biofuels production has gained the worldwide attention to fulfil the same. At present algal biofuel production has not been commercially exploited due to the high cost associated with biomass production, harvesting of biomass from culture vessels, dewatering and drying and transesterification for the conversion of algal oil into biodiesel. (Singh *et al.*2013) There is a dire need to optimise the biomass productivity along with the lipid and carbohydrate composition to make microalgae biofuel production economically viable.

Microalgae was isolated from the DTU Lake and identified as *Scenedesmus rubescens* on the basis of morphology and molecular identification. To optimise the biomass productivity, the influence of various abiotic factors such as light intensity, light colour, light period, pH of medium, and shaking period, temperature and % inoculums on algal growth rate has been studied in the present study. Microalgal growth was observed under different light intensity, three colour and three photoperiods for 24 days at 670nm by a spectrophotometer. Effects of pH on growth pattern were determined under a wide range 6-11. Additionally, the effect of temperature on algal growth was also recorded in a period of 24 days. In these experiments, only one factor at a time was variable while all other conditions were kept constant. The culture conditions which were controlled for the algal growth were light, temperature, pH and constant mixing or aeration. Effects of different sources of Carbon, Nitrogen and Phosphorous were also studied by measuring biomass productivity and optical density at the

end of the cultivation period. Seven different media compositions were also tested for the better biomass yield. . This study aims to find out the superior source of nitrogen, phosphorous and carbon for the optimum growth rate and biomass productivity (dry cell weight) for freshwater microalgae *Scenedesmus rubescens*. Biochemical compositions i.e. lipid, carbohydrate, protein and total carotenoids of the microalgae were determined by the conventional biochemical tests and spectrophotometric techniques.

Objectives of the study:

1. *Isolation, Identification and Characterization of microalgae from local freshwater water body: Morphological and Molecular*
2. *Optimization of culture condition and selection for suitable Carbon, Nitrogen and Phosphorous sources for optimum biomass production and growth rate*
3. *Evaluation for biodiesel production*

2. REVIEW OF LITERATURE

Biofuels

Biofuels are liquid or gaseous fuels produced from biomass resources and used in place of, or in addition to, diesel, petrol or other fossil fuels for transport, stationary, portable and other applications. 'Biomass' resources are the biodegradable fraction of products, wastes and residues from agriculture, forestry and related industries as well as the biodegradable fraction of industrial and municipal wastes. Examples include biodiesel, bioethanol and other biofuels such as Biomethanol, Biobutanol and biosynthetic fuels etc. In India, mainly Oil Marketing Companies, OMCs NABARD, SIDBI and IREDA etc like agencies are contributing actively, and financing the biofuel sector. Quality and standards of biofuels, for example biodiesel are governed by The Bureau of Indian Standards, BIS. BIS is Indian adaptation of American standard ASTM D-6751 and European Standard EN-14214. BIS has also published IS: 2796: 2008 which covers specification for motor gasoline blended.

Why microalgae based Biofuel

- Sustainable and renewable source of biomass based biofuel production.
- A microalga, a photosynthetic microorganism, grows 25 times more faster than other terrestrial plants.
- Cell contains high amount of carbohydrates, proteins and lipids which can be converted into biofuels by using suitable conversion techniques.
- High yield and low cost.
- Environment friendly, it sequester CO_2 from the environment and fix it into biomass in presence of sunlight. It helps in reduction in other greenhouse gases.
- Requires less land as compare to other oil producing plants. In case of ethanol production, for producing same amount, microalgae require only 4% of land as compare to corn.
- Main advantage of algae based biofuel is the biomass production from wastewater discharged by domestic and industrial sources as it grows on wastewater by utilizing N, P and other elements present in it.

What are Microalgae?

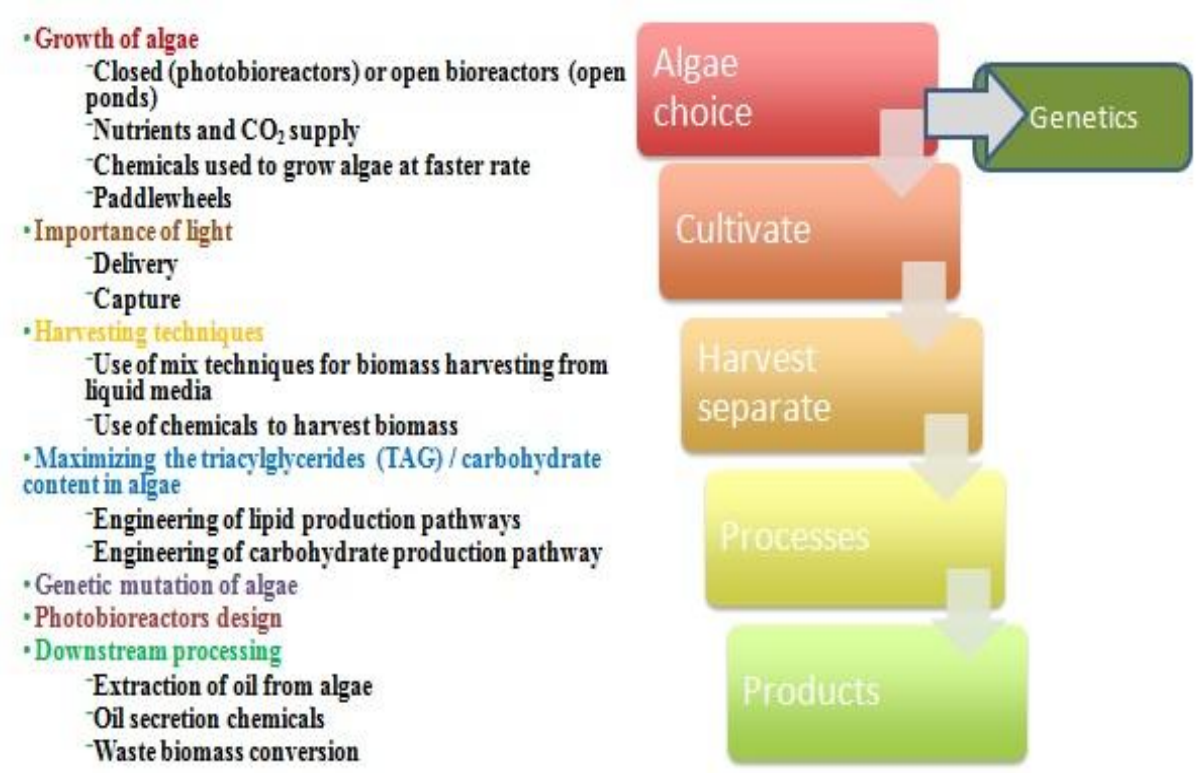
Microalgae are unicellular photosynthetic organisms that require relatively simple nutrients and whose biomass can be used to obtain biocomposites, food supplement for humans and animals and as a source of biofuels (Andrade and Costa, 2008), its homogeneous biomass allows greater recovery than oilseeds in which only parts of the plant are directed to the process of interest. (Morais et al 2009). Algae by definition are an assemblage of a large and diverse group of primitive waterborne organisms, sometimes referred to as "simple plants" because they lack the many distinct organs found in plants on lands, such as roots, stems and leaves. Algae are autotrophic organisms that can range in size from single cells to large multicellular seaweeds that can grow to over 150 feet (45 meters). Autotrophic organisms are the "producers" in a food chain that manufacture complex organic compounds from simple inorganic molecules using photosynthesis, or the energy generated by the sun. (Giuliano *et al.* 2010; Shenk *et al.* 2008)Algae are ubiquitous in the environment and occur in every kind of water habitat, including freshwater, brackish and marine environments. Because the term "algae" is used to define such a large and varied group of organisms, scientists typically subdivide the assemblage into microalgae, the multicellular algal organisms, and microalgae, the microscopic algal organisms. (Brian H. Kiepper, 2013, Giuliano *et al.*2010; Chisti 2007; Lee RE, 2008)

Microalgae are single-cell algal species that can survive individually or in chains or clusters. Microalgae also exist in both suspended forms –species that free-float in a water body – and attached forms, which are species that adhere to a submerged surface. Depending on the species, algae can range from 1 micron (μm) to a few hundred microns in size. As a point of reference, 1 micron (μm) = 1/1000 of a millimetre (mm). Microalgae are one of the most important groups of organisms on the planet. It is estimated that microalgae produce approximately half of the atmospheric oxygen on earth while consuming vast amounts of the greenhouse gas carbon dioxide. The biodiversity of microalgae is also enormous. It has been estimated that as many as 200,000 to 800,000 species exist of which only about 35,000 species have been identified and described. (Brian H. Kiepper, 2013; Van *et al.*2012)

Microalgae are autographs in nature, i.e. they can synthesize nutrients themselves from simple inorganic compounds. Various literature has reported that these have higher photosynthetic activity i.e. they are highly efficient in converting solar energy into biomasses and incorporating nutrients such as nitrogen and phosphorus causing eutrophication. (Ibrahim *et al.* 2012; De la Nou¨ e and De Pauw, 1988)A stoichiometric formula for the most common

elements in an average algal cell is C106H181O45N16P, and the elements should be present in these proportions in the medium for optimal growth. (Oswald, W.J. 1988).High ratios between nitrogen and phosphorus, about 30:1, suggest P- limitation, whereas low ratios of about 5:1 suggest N-limitation. According to the ratios most often found in wastewater, phosphorus is rarely limiting algal growth, but nitrogen may be. (Borowitzka, M.A. 1998). Though, since wastewater often exposes the algae to nutrient concentrations of up to three orders of magnitude higher than under natural conditions, growth is more likely limited by carbon and light. (De *et al.* 1992; Karin Larsdotter 2006) Microalgae, managed in a controlled environment, are a proven low initial capital cost, low operational cost supplement or alternative to mechanically aerated wastewater treatment systems. Microalgae-based wastewater treatment systems are particularly advantageous in low land cost areas where sunlight and warm temperatures are plentiful.(Brian H. Kiepper 2013) To date, although there is mounting interest to develop microalgal biodiesel production, the cost for microalgal biomass production is currently much higher than from other energy crops (Van *et al.*2012, Chisti, 2007)

Figure 1: Major technology aspects involved in biofuel production from microalgae:



Algal growth is limited by the following factors: (Becker, E.W. 1988 and Karin Larsdotter 2006).

- Abiotic factors, physical and chemical: Light (quality, quantity), Temperature, Nutrient concentration, O2, CO2, pH, Salinity, Toxic chemicals
- Biotic factors: Pathogens (bacteria, fungi, viruses), Predation by zooplankton, Competition between species
- Operational factors: Mixing, Dilution rate, Depth, Addition of bicarbonate, Harvesting frequency

Footprint Requirement:

The foremost inconvenience of mass-cultured microalgae systems compared to conventional aerobic wastewater treatment systems is the space requirement, or "footprint." Since microalgae rely on photosynthesis, the availability of sunlight to reach the microalgae is most essential. Most mass-cultured microalgae systems are designed as shallow raceways 6 to 12 inches (15 to 30 cm) in depth, which requires much more space than conventional wastewater treatment oxidation ditches that can be several feet deep. However, the disadvantage of the larger footprint associated with microalgae systems is much greater in highly developed urban areas where land prices are high. This disadvantage is often offset in less developed areas or in urban areas where large amounts of inexpensive land are available and low operation costs are desired. Microalgae wastewater treatment systems also operate much more efficiently in warm weather climates where the little chance of freezing temperatures exists. (Brian H. Kiepper 2013).At present algal biofuel production has not been commercially exploited due to the high cost associated with biomass production, harvesting of biomass from culture vessels, dewatering and drying and transesterification for the conversion of algal oil into biodiesel. (Singh *et al.*2013)

Environmental concern:

With the enormous use of fossil fuels, the pollution level is increasing day by day at a higher pace, affecting the human's health, economy and environment very badly. Increased industrialization brought a rapid economic development, there is no any doubt, but it also brought some serious threats also with it such as global warming, energy crisis and pollution. (Ibrahim *et al.*2012; Singh *et al.* 2013; Giuliano *et al.* 2010). Water and air pollution are major concerns. Wastewater is a home for a wide range of microorganisms. Construction,

transportation and manufacturing like human developmental activities require a vast amount of energy. These activities not only deplete the natural resources but also produce a large amount of wastes that leads to environmental pollution; global warming and acid rains. (Ibrahim *et al.*2012; Singh *et al.*2013)

Untreated or improperly treated waste is a major cause of pollution of water bodies, groundwater and environmental degradation causing a loss in health and loss of crop productivity. Discharge of domestic waste, agricultural surface runoff, land drainage and industrial effluents directly (without treatment) in a water body leads to rapid nutrients enrichment in a water body. The excessive nutrient enrichment in a water body encourages the growth of algae duckweed, water hyacinth, phytoplankton and other aquatic plants.(Giuliano et al 2010; Chisti 2007)The biological demand for oxygen (BOD) increases with the increase in aquatic organisms. As more plants grow and die, the dead and decaying plants and organic matter acted upon by heterotrophic protozoan and bacteria, deplete the water of dissolved oxygen (DO). The decrease in DO result in sudden death of a large population of fish and other aquatic organisms including plants, releasing offensive smell and makes the water unfit for human use. The sudden and explosive growth of phytoplankton and algae impart a green colour to the water is known as water bloom, or "algal blooms". This phytoplankton releases toxic substances in water that causes sudden death of a large population of fishes. This phenomenon of nutrient enrichment of a water body is called eutrophication. Human activities are mainly responsible for the eutrophication of a growing number of lakes and water bodies in the country.

Effects of culture conditions on microalgae:

Algal biochemical composition's amount may be influenced and increased by changes in the surrounding environment such as light, temperature, pH, shaking period etc. The effects of culture conditions at different temperature and light regimes on growth and the contents of chlorophyll-a, chlorophyll-b, total carotenoids, total protein and total free amino acids of *Chlorella vulgaris* were determined. The growth of *C. vulgaris* in terms of optical density (0.42 at 670 nm), cell count (440 x 104 cells/ml) and dry weight (30.2 mg/50 ml), and the amount of chlorophyll- a (2.16%), chlorophyll- b (0.59%) and total protein, was found higher at the temperature 25-30°C and natural day light receiving through the north facing window of the growth room. Although, the amount of total carotenoids (0.440%) and free amino acids (834 μg/gm fresh weight) were found maximum in continuous light at 30-35°C, not much

differences in the amount of carotenoids (0.385%) and free amino acids (822 µg/gm fresh weight) were found at 25-30°C and natural day light. The natural day light at 25-30°C was also proved proficient, as distinct banding pattern with unique polypeptides such as 15KDa, 47KDa and 50KDa, on the other hand, 23KDa, 26KDa and 36KDa appeared in all samples, these bands were not affected by light and temperature. Our results indicate that among all five culture conditions tested, the cultures kept at north facing window receiving natural day light at temperature 25-30°C, show best growth and higher contents of biochemicals that will be beneficial to use *Chlorella* for high nutritive purpose.

Krzemin´ska et al 2013, studied about the Influence of photoperiods on the growth rate and biomass productivity of green microalgae. he effect of different photoperiods: 24 h illumination and a 12:12-h light/dark (12L: 12D) cycle on the growth rate and biomass productivity was studied in five algal species: *Neochloris conjuncta, Neochloris terrestris, Neochloris texensis, Botryococcus braunii and Scenedesmus obliquus. (*Krzemin´ska et. 2014) The quantity and quality of light determines the amount of available energy that is indispensable for the photosynthetic process. Equally important is the dark/light regime, which influences algal growth and biomass production. In the natural environment, light intensity undergoes continuous changes, and the light regimen is not constant (*Khoeyi* et al. 2012). Changes in light quantity induce alterations in the biochemical composition of microalgae. Increased frequencies of the light/dark cycles may considerably enhance productivity and photosynthetic efficiency (*Grobbelaar JU (2009)*). Recently, investigations concerning the influence of the photoperiod on the biomass yield in several freshwater and marine microalgae, e.g., Chlamydomonas reinhardtii, Chlorella sorokiniana, Dunaliella tertiolecta (*Janssen M (2002)*), Chlorella vulgaris (*Khoeyi* et al. 2012). have been carried out. The varied productivity and growth rate of the algal species investigated in the present work were dependent on the photoperiod and were species specific (over the same taxonomic group Chlorophyceae).(Krzemin´ska et. 2014)

Light also plays an important role in enhancing the biomass productivity of microalgae. Parameters such as light source, light intensity and photoperiod have significant effects on biomass productivity. Previous research studies showed that fluorescent light is suitable for microalgae growth (Yeh et al. 2010). Photoinhibition occur when light intensity exceeds 6000 LUX (*Khoeyi* et al. 2012). . Cellular content of protein, carbohydrates and lipids can be varied through variation of photoperiod. Photoperiod is important because photosynthesis process is controlled by a photochemical period which depends on light and biochemical dark phase which is independent of light (*Khoeyi* et al. 2012). Studies showed that dark phase is

beneficial for microalgae growth because certain enzymes of pentose cycle which is used in photosynthesis and carbon dioxide fixation are active during dark phase and inactive during light phase. Kong et al. (2011) also stated that less biomass is depleted during dark phase. The optimum photoperiod for microalgae cultivation varies depending on species.(Hannah et al 2013) The effect of different illumination on S. obliquus was investigated. The species shows a maximum growth rate at 150 μmol m−2 s−1. Above this value, the growth is inhibited, and although algae show substantial biomass accumulation, they exploit light with a lower efficiency. Cultures exposed to pulsed light show reduced growth compared to continuous light, but if cells are allowed the time to acclimate to alteration of dark–light cycles, they strongly enhance their productivity. Finally, it was found that the lipid content of S. obliquus is not affected by the variation of light intensity.(Gris et al. 2014)

It has been mentioned above that natural day light at 25-30°C showed highest concentration of biomass, chlorophyll and protein contents, but carotenoids and amino acids were found little lower from the maximum in natural day light at 25-30°C. A slight stress condition is developed in natural day light due to sun light intensity or photoperiod, which favors the accumulation of carotenoids and free amino acids, without affecting the concentration of biomass, chlorophyll and protein contents. (Sarma et al. 2012)

Light and temperature are the two most important factors that affect algae biomass productivity. The energy for growing algae is provided by light via photosynthesis. Sufficient light energy must be effectively utilized to achieve higher biomass productivity.

Due to its rapid growth, microalgae can produce considerably larger amounts of biomass and lipids per hectare than oilseeds. (Mate et al.2013) Temperature influences the rates of all chemical reactions related to algal growth and its metabolism (Sandnes *et al.,* 2005). Change in temperature affects the biochemical composition of the cells specifically lipids and proteins. Thus light and temperature have a significant effect in the metabolism, enzyme activities and cell composition of algae. Algae cultivation also depends on pH levels and optimum pH influences the carbon availability, metabolism and biochemical composition of cells (Richmond, 2000).

Effect of different Carbon, Nitrogen and Phosphorous on growth and productivity:

Mata et al (2013) studied the influence of a nutrients change in the culture medium of the saltwater microalga *D. tertiolecta* on its growth, biomass and lipid productivities, having in

mind its use for biodiesel production and concluded that, concerning the macronutrients, the addition of nitrogen (in the form of nitrate) increases the biomass productivity and the addition of magnesium reduces the biomass productivity in comparison with the standard medium. The addition of potassium does not significantly affect biomass concentration and productivities when compared to culture with standard medium. (Mate et al. 2013) One possible way to influence lipid synthesis is through the growth medium by varying the nitrogen concentration in the medium. In dense cultures, the assimilation of inorganic carbon is impaired due to high density cell, due to the low availability of light. When microalgae assimilate more carbon has its production of carbohydrates, proteins and lipids increased (Derner, 2006). Cerón García et al. (2006), performing mixotrophic cultivation with microalgae *Phaeodactylum tricornutum* grown with glycerol and CO2 injection, reached biomass of 7.04 g/L, while cultivation without addition of glycerol did not reach 1 g/L. The additional carbon source for these cultures enables higher yield of biomass, because there is greater availability of carbon than the produced via photosynthetic metabolism. The carbon source is a major component in the production of microalgal biomass (Radmann et al., 2009). Glycerol is an interesting carbon source in mixotrophic cultures where the focus is the production of biodiesel due to its availability resulting from the Transesterification process. (Morais et al 2009). The growth of the algae strains C. vulgaris, Scenedesmus sp. and B. braunii under mixotrophic conditions in the presence of different concentrations of crude glycerol was investigated with the objective of increasing the biomass growth and algae oil content. The highest biomass concentration was obtained in the presence of 5 g/L of glycerol and was 39.42%, 60.00% and 57.82% higher than for the autotrophically grown C. vulgaris, Scenedesmus sp. and B. braunii, respectively. The content of TAG in the three species cultured under mixotrophic conditions was also higher than that under autotrophic conditions. In particular, in the biomass obtained in the 5 g/L glycerol-containing medium, the TAG content was 12.20%, 13.11% and 9.30% higher than the respective values for the above species in autotrophic conditions. (Whan Yu (2015)

On the other hand, nitrogen source is also vital for microalgae cell physiology and growth. It is an essential component that contributes to the biomass formed. Lack of nitrogen will cause chlorophyll reduction and increase in carotenoids,(Hannah et al 2013) Arumugam et al. 2011 studied the influence of organic waste and inorganic nitrogen source on biomass productivity of Scenedesmus and Chlorococcum sp. The findings of these experiments revealed that Farm Yard Manure (FYM) extract (50%) along with urea (0.10%) could be a

good cost effective alternative for satisfactory algal biomass growth in open pond condition.(Arumugam et al. 2011)

Carvalho et al evaluated the Influence of Nitrogen and Phosphorus Nutrients in the Culture and Production of biosurfactants by Microalga S*pirulin.* In this study, the variables phosphorus and nitrogen showed significant influence on the maximum biomass concentration and maximum productivity of Spirulina platensisParacas, (Carvalho et al. 2014)

Gim et al (2014) conducted a study, in which growth and total lipid contents of four green microalgae were compared in three different modes of cultivations. Comparing to autotrophic cultivation, higher biomass production was observed in heterotrophic cultivation. Among the various carbon sources tested, glucose was the best carbon source for four microalgal growths, and 1 % glucose was optimum for higher biomass production in three microalgal species except *S. obliquus* R8 (2 % glucose). The *B. braunii* FC124 was found to be a best lipid producing microalga under 80mol m-2 s-1 of light intensity, 1 %of glucose and 20 days incubation. Lipid accumulation increased with increasing light intensity in the mixotrophic cultivation.

3. MATERIALS AND METHODS:

3.1 ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF MICROALGAE FROM LOCAL FRESHWATER WATER BODY: MORPHOLOGICAL AND MOLECULAR

3.1.1 Sample collection and isolation of microalgae

For microalgae isolation, the freshwater sample, collected from Delhi Technological University's lake (DTU Lake) was first filtered for the removal of solid and undesired particles and, then inoculated in BG-11 Medium. For growth, this preparation was incubated at 27^0C in an artificial light chamber under illumination with a white fluorescent light at 300µmol/m^2 s^{-1} on a photoperiod of 16L: 8D. After proper growth (optical density 0.512 at 670 nm on day 20^{th} day) of microalgae was observed in the culture flask, the culture was spread onto BG11 Agar plate, and kept at the same growth conditions. Colonies were sub-cultured repeatedly into the same medium under same growth conditions until a culture dominated by the pure strain was developed. The sub-cultured individual colonies were picked and transferred into the BG-11 medium and scaled up successfully. Thus isolated and established pure culture was obtained.

The isolated microalga was cultivated in the BG11 medium at similar conditions for 24days in a working volume of 200 ml in Erlenmeyer flasks. Flasks were agitated twice or thrice a day manually to prevent any deposition at the base of the flask and to ensure proper mixing. Cultivation lasted for up to 24 days with samples being taken on a period of 24 hours and analysed on spectroscopy as well as by calculating dry cell mass of the sample. The dry cell weight of the microalgal culture was estimated by taking 50ml of culture and centrifuging it at 3000 rpm for 10 minutes and hence separated biomass was dried in an oven for the time till the complete dry condition is achieved. After a substantial period of growth, 100ml of the sample was taken and biomass was harvested and preserved for the identification purpose.

3.1.2 Growth Medium:

For growth of the microalgal isolate, BG11 (ATCC medium 616) media was used. The composition of media consists (gram/litre):Sodium nitrate ($NaNO_3$),1.5; ($K_2HPO_4.3H_2O$) 0.04;Magnesium Sulphate ($MgSO_4.7H_2O$),0.075; Calcium Chloride ($CaCl_2.2H_2O$),0.036; Citric Acid,0.006; Ferric Ammonium Citrate,0.006; EDTA (disodium magnesium salt),0.001;Na_2CO3,0.01; and one millilitre of **A5** Solution which contains

(gram/litre):H_3BO_3,2.86; $MnCl_2.4H_2O$,1.81; $ZnSO_4.7H_2O$,0.222; $Na_2MoO_4.2H_2O$,0.39; $CuSO_4.5H_2O$,0.079; $Co(NO_3)_2.6H_2O$,0.0494. The pH of medium was kept 8.

3.1.3 Identification of isolated microalgae

Morphological Identification

Isolated microalgae species was examined under light microscope and scanning electron microscope (SEM) for cell morphology. SEM was performed for the absolute morphological determination of the microalgae isolate. Scanning Electron Microscope provides the absolute morphological details of the material and this property is utilised for the characterization of the microalgae. FTIR spectroscopy was also performed for the biochemical composition characterization.(Ponnuswamy et al 2013).The biomolecules composition *i.e.* lipid, carbohydrate, protein, chlorophylls etc. of algal biomass is usually investigated and analysed by the conventional chemical extraction method followed by gravimetric determination and spectroscopy. (Flynn et al 2013). For rapid determination of the isolated microalgal sample for its biochemical composition, use of FTIR is a highly advanced technique and powerful analytical tool. (Flynn et al 2013) (Miglio et al 2013). FTIR is a non-destructive and rapid method, which helps in identification of the vibrational structure of materials. *(Erukhimovitch et al* 2003). The IR spectrum of the microalgal sample was recorded on FTIR at room temperature under region 4000-400 cm^{-1}. Analysis of composition, shape and intensity of absorption at specific wave numbers reveals information about functional groups and molecular structure that can then be used for identification (Flynn et al 2013) (Yakoob et al 2011).

Molecular Identification

28S rDNA molecular examination was performed for identification of the isolated microalgae (Eurofins Genomics India Pvt. Ltd. Bangalore, India). DNA was isolated from the culture by using Nucleospin DNA extraction kit. Its quality was evaluated on 2.0% Agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of 28S rDNA gene was isolated and amplified by Merk PCR amplification kit. A single discrete PCR amplicon band of 800 bp was observed when resolved on Agarose. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 28SF (5ACCCGCTGAATTTAAGCATA3) and 28SR (5CCTTGGTCCGTGTTTCAAGA3) primers using BDT v3.1 Cycle sequencing kit on ABI

3730xl Genetic Analyzer (3730xl ABI-96 Capillary machine). Consensus sequence of 28S rDNA gene was generated from forward and reverse sequence data using aligner software. (Dopazo J 1994; Kimura M 1980 ; Rzhetsky A & Nei M 1992; Saitou N & Nei M 1987; Tamura et al 2007)

Experimental Method

1. DNA was isolated from the culture by using Nucleospin DNA extraction kit .Its quality was evaluated on 2.0% Agarose Gel, a single band of high-molecular weight DNA has been observed.
2. 28s rdna molecular identification was done for identification of the isolated microalgae.
3. Fragment of 28S rDNA gene was isolated and amplified by Merk PCR amplification kit. A single discrete PCR amplicon band of 800 bp was observed when resolved on Agarose
4. The PCR amplicon was purified to remove contaminants.
5. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.(3730xl ABI-96 Capillary machine.)
6. Consensus sequence of 28S rDNA gene was generated from forward and reverse sequence data using aligner software.
7. The 28S rDNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI GenBank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.
8. Sample was labelled as Microalgae was found to be Scenedesmus rubescens strain based on nucleotide homology and phylogenetic analysis.

   Table 1: PCR Protocol

PCR protocol		
Temp	Time	Cycles
95°C	5min	
95°C	30sec	
45°C	30sec	
72°C	1min	10cycles
95°C	10sec	
50°C	10sec	
72°C	30sec	25cycles
72°C	5min	

3.2 OPTIMIZATION OF CULTURE CONDITION FOR OPTIMUM BIOMASS PRODUCTION AND GROWTH RATE

Microalgal cells were allowed to grow under different environmental conditions in 1L flask with an operating volume of 800 ml. to see their effect on biomass productivity. The standard conditions for cultivation were same as mentioned in isolation process and only conditions under observation were changed. The different culture condition are six different light intensity regime (10, 15, 25, 36, 60 and 100 Watt), three different light color (red, blue and white), three different light periods (24L: 0D, 16L: 8D and 12L: 12D), six different pH (pH 6 to 11), three different temperature regimes (18°C (±2), 26°C (±2) and 30°C (±2)) and three different shaking periods (24 hours, 18 hours and manual shaking twice-thrice per day). Six different light intensities were provided with the help of different yellow light bulbs and fluorescent tube. Red and blue colour was provided by wrapping the flask with polyethylene sheet of red and blue color. All cultivation was done for 24 days and growth was measured at 670 nm by spectrophotometer. After cultivation, harvesting was done to finalize the biomass productivity with the help of following formulae:

$$\text{Biomass productivity (mg/l-d)} = \frac{\text{Total dry weight (mg)}}{\text{Number of days of cultivation(days) X Volume(ml)}}$$

3.2.1 Effect of Light intensity on biomass productivity: Microalgae isolate was cultivated under six different light intensity regime in BG11 medium having pH 8 and light and dark period of 18h: 6h for 24 days and growth was monitored by spectrophotometer. Reading was noted at a period of two and three days and plotted to derive a growth curve on the basis of absorbance and time.

3.2.2 Effect of Light colour on biomass productivity: Microalgae isolate was inoculated in BG11 medium having pH 8 and growth was monitored for 24 days. Three different colours Red, Blue and White colour light was given to the flask by wrapping flask with red and blue coloured transparent plastic onto the flask. One flask was left unwrapped and kept directly under the white light.

3.2.3 Effect of Light period on biomass productivity: Three different light periods in hours (light: dark) 24:0, 18:6, 12:12 was given to the microalgal culture and growth was monitored. BG11 medium with pH 8 was used as a growth medium. The experiment was done for 24 days and growth pattern was plotted.

3.2.4 Effect of pH on biomass productivity: Microalgal biomass productivity was calculated under different pH regimes. A pH range from 6 to 11 was used under white light with a light period of 18h: 6h at room temperature for the growth of microalgae. BG11 medium was prepared and pH was set and autoclaved before the inoculation.

3.2.5 Effect of temperature on biomass productivity: Three different temperature regimes 18°C(±2), 26°C(±2), and 30°C(±2), were provided to the microalgae culture allowed to grow under white light with a light period of 18h: 6h at room temperature for the growth of microalgae.

3.2.6 Effect of shaking period on biomass productivity: Three different shaking periods were provided to the microalgae culture under white light with a light period of 24h at room temperature for the growth of microalgae. Microalgae culture flask was kept on a shaker for shaking purpose. One flask was kept for 24hours on shaking for the entire growth period. Second flask was given an 18 hours shaking on daily basis. Third flask was manually shaked for twice or thrice a day.

3.3 SELECTION AND OPTIMIZATION OF VARIOUS SOURCES OF CARBON, NITROGEN AND PHOSPHOROUS CONCENTRATION:

Various nutrients, Carbon, Nitrogen, Phosphorous and media composition were investigated for optimum biomass production for the *S. rubescens.* This study aims to find out the superior source of nitrogen, phosphorous and carbon for the optimum growth rate and biomass productivity (dry cell weight) for freshwater microalgae *Scenedesmus rubescens*. Seven different freshwater media compositions were also tested for a period of 21 days. The microalgal culture growth was analyzed on a daily basis by measuring the absorbance at 670nm performed on a spectrophotometer. The dry cell weight of the microalgal culture was estimated by taking definite volume of culture and centrifuging it at 4000 rpm for 15 minutes and hence separated biomass was dried in an oven for the time till the complete dry condition is achieved. Diagrams tell about the biomass productivity (mg/l-day) of different media composition, carbon, nitrogen and phosphorous sources.

3.3.1 Effect of Media composition:

Seven different media compositions, Annexature-1, (BG11, SE (Bristol's solution), Bold's Basal medium (BBM), Synthetic Freshwater medium (SFM), Waris-H Medium (WH

medium), HUT medium, Bold 3N medium) were tested for the maximum biomass production and growth rate. Cultivation lasted for 21 days and biomass productivity was calculated on the 21st days of cultivation by using the formula mentioned below:

$$\text{Biomass productivity (mg/l-d)} = \frac{\text{Total dry weight (mg)}}{\text{Number of days of cultivation(days) X Volume(ml)}} \text{ ----------(1)}$$

Optical density was also recorded for the entire cultivation period at 670 nm at the interval of 3-4 days.

Media composition of all seven media:

3.3.2 Effect of Carbon sources:

Sucrose, Glucose, Sodium Acetate and Glycerol were used as Carbon sources and biomass yield was calculated at the end of 20days. Microalga was cultured in BG-11 media. The concentration of the carbon sources were taken as 0.1M. For biomass productivity analysis formula mentioned in (1) was used.

3.3.3 Effect of Nitrogen sources:

NH_4Cl, $NaNO_3$, KNO_3, $CO(NH_2)_2$, $Co(NO_3)_2$ and $Ca(NO_3)_2$) were used as Nitrogen sources and biomass yield was calculated at the end of 27 days. Microalga was cultured in BG-11 media. The concentration of the nitrogen sources were taken as 1.5 g/litre for the first three N-sources (NH_4Cl, $NaNO_3$, KNO_3) and 0.75g/litre for the rest three $CO(NH_2)_2$, $Co(NO_3)_2$ and $Ca(NO_3)_2$. Biomass productivity was calculated by the same method as per mentioned in the media composition section.

3.3.4 Effect of phosphorous sources:

Four Phosphorous Sources, K_2HPO_4, $NH_4H_2PO_4$, KH_2PO_4 and Na_2HPO_4 were used as phosphorous sources and biomass yield was calculated at the end of 27 days. Microalga was cultured in BG-11 media. The concentration of the nitrogen sources were taken as 0.1mM. Biomass productivity was calculated by the same method as per mentioned in the media composition section.

3.4 EVALUATION FOR THE BIOFUEL PRODUCTION

Microalgae culture thus isolated is then subjected to a process of biochemical characterization for the biofuel production. The biochemical constituents of the microalgal cell, lipid, carbohydrate and protein was estimated by performing lipid extraction by Bligh and Dyer method, Anthrone method and the Lowry method respectively. (P. Rajasulochana *et al* 2012). Chlorophyll content was also measured for the total carotenoids determination. (NREL protocol)

3.4.1 Lipid estimation for biodiesel production

For lipid extraction, the 150ml culture was taken and centrifuged at 4000rpm for 10 minutes. Supernatant was discarded and the pellet was resuspended in 1ml distilled water. A known volume of chloroform: methanol (2:1 v/v) was added to this preparation and subjected to sonication for 30 min at maximum power. After this, the solution was incubated overnight at 27^0C at 100 rpm. On the next day, 1.25 ml chloroform was added and again sonicated for 30minutes. Chloroform and methanol layer was separated and 1.25 ml of dH_2O was added and centrifuged at 4000rpm for 10minutes. The bottom layer was removed and fresh chloroform was added and second round of extraction procedure was performed. The chloroform containing portion was collected and washed with 5ml, 5% NaCl. Thus obtained preparation was kept in the oven to evaporate the chloroform, methanol and water residues if any. Crude lipid thus obtained was weighed and estimation was accomplished. (Anitha &Narayanan 2012).

3.4.2 Total carotenoids estimation:

Carotenoids estimation was done by 80% acetone extraction method.(Vaidyanathan and Chen, 2013) A slight modification was exercised in the protocol provided by NREL for total carotenoids estimation and chlorophyll measurement. 2 ml culture of microalgae was taken and subjected to centrifugation for 10 minutes at 4000 rpm. The harvested biomass was washed twice with distilled water and resuspended in 0.4 ml of 0.1 M phosphate buffer (PH 7). Cell disruption was performed by sonication of the preparation. After sonication, sample was allowed to cool and 1.6 ml of pure acetone was added in it to make the solution of 80% acetone. Sample was kept on vortex for 2 minutes and placed in dark for 15 minutes. After incubation, the sample was subjected to centrifugation and supernatant was collected. The

absorbance of collected supernatant was taken for chlorophyll a, chlorophyll b and total carotenoids estimation. The following formula was used for the estimation:

$C_a = 12.23A_{663} - 2.81A_{646}$

$C_b = 20.13\ A_{646} - 5.03\ A_{663}$

$C_t = \frac{1000A470 - 3.27Ca - 104Cb}{198}$

Where C_a, C_b, and C_t are chlorophyll a, chlorophyll b and total carotenoids respectively in µg/ml.

4. RESULTS AND DISCUSSIONS:

Freshwater microalga was isolated and its morphology was determined by Light microscope, SEM and FTIR. Characteristics and morphological features of the isolate have demonstrated its close similarity with microalgae *Scenedesmus sp*. The microalgae isolate was finally identified as *Scenedesmus rubescens* based on 28s rDNA molecular identification.

4.1 Isolation and identification of microalgae

Light Microscope Studies

Inverted light microscope (Magnus) determined that cells are spherical in shape. The colour of colony is green and it can be easily perceived by the Figure-1.

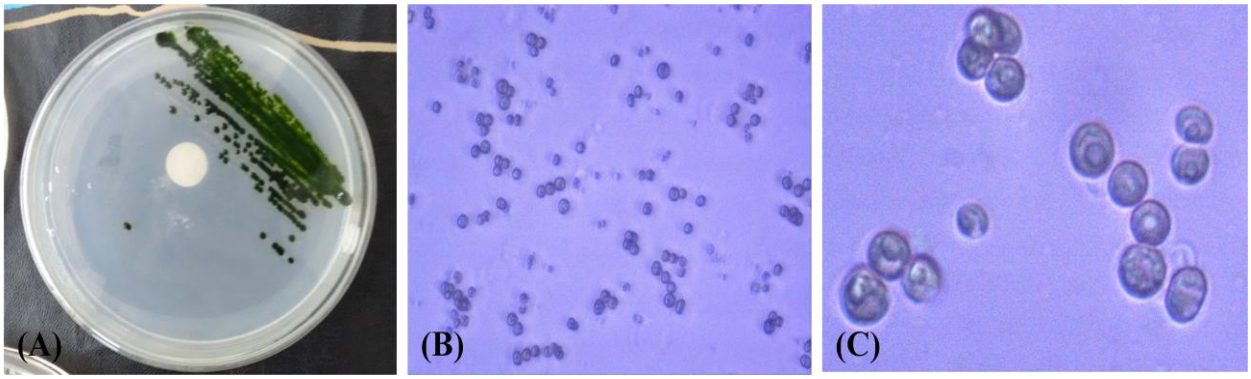
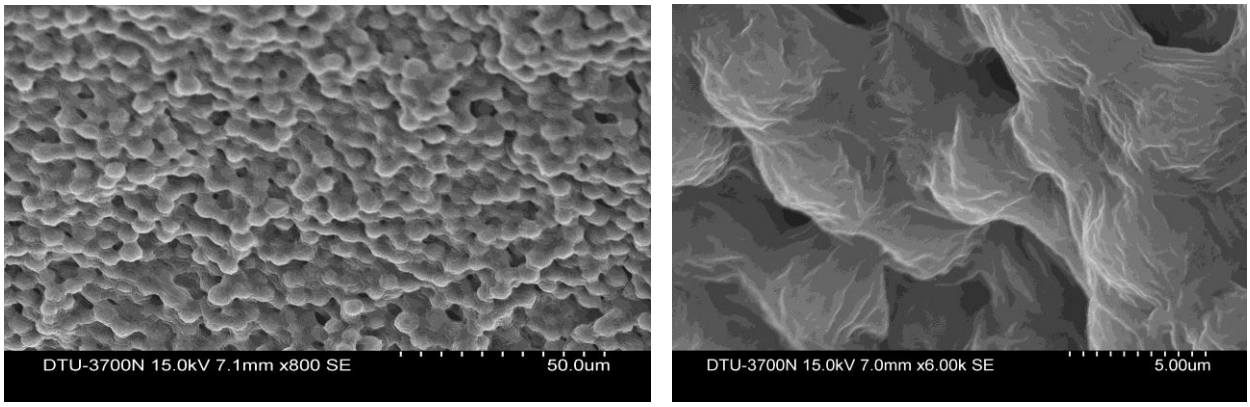


Figure-2: Isolated colonies of microalga *Scenedesmus rubescens* in agar plate (a) and under inverted light microscope (20X) (b) and 40X (C) enriched with BG-11 media. The isolated colonies were further used to establish pure culture, morphological, molecular and biomass productivity analysis.

Scanning Electron Microscope Studies:

On examining the physical structures of microalgae, SEM provides good visual images of possible microalgal structure and morphology. It scans solid samples with a beam of electrons, which interacts with the atoms that make up the sample to produce signals. These signals contain topographic information about the samples (Bi & He 2013). The average cell size was in the range of 5.6-5.8µm.



(a) (b)

Figure-3: Morphological determination of microalga *Scenedesmus rubescens* by scanning electron microscope (S-3700) (a) depicts the resolution of microalga at 800 SE and (b) at 6000 SE. With the help of these resolutions average size of 5.8 microns has been confirmed. DTU: Delhi Technological University; 3700N

Fourier Transform Infrared Spectroscopy Studies

FTIR provides the metabolic fingerprinting of the material by discriminating and identifying the various functional groups present in it. The composition and structure of molecules and functional groups can be determined by analysing the position/width and infrared light absorption of the sample. Biomolecules such as lipid, carbohydrate and protein etc. has their own characteristics IR spectra, which is rich in molecular composition and functional aspects and which allow identification and quantification based upon absorbance For microalgae sample, the sample is analyzed in 4000-400 cm^{-1} wave number range with a resolution of 4 cm^{-1}. (Flynn et al 2013);(*Erukhimovitch et al* 2003);(Yakoob et al 2011);(D' Souza et al 2008). Following FTIR chromatogram (Figure-3) and table (Table-1) shows the presence of various functional groups attributed the protein; lipid and carbohydrate present in the *Scenedesmus rubescens***.**

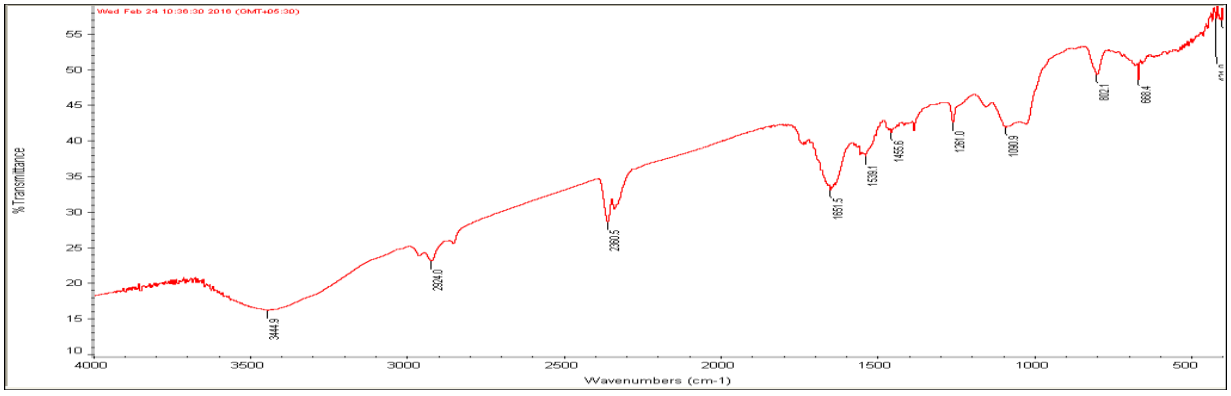


Figure-4: FTIR Spectra (4000-400 cm^{-1}) of *Scenedesmus rubescens.* Characteristic functional groups contributing to the formation of % transmittance bands at specific wave numbers.

Table-2: Tentative assignment of bands found in FTIR spectra of *Scenedesmus rubescens.* (*Erukhimovitch et al* 2003); (D' Souza et al 2008); (Yakoob et al 2011); *(Duygu et al 2012)* (Ponnuswamy et al 2013)

S. No.	**Typical band assigned to lipid and carbohydrate**	**Wave number range cm $^{-1}$**	**Main peak cm^{-1} *(Scenedesmus rubescens)***
1	Water V(O-H) stretching Protein V(N-H) stretching	3029-3639	3444.9
2	Lipid –carbohydrate mainly Vas (CH2) and Vs(CH2) stretching	2809-3012	2924
3	Protein amide I band mainly V(C=O) stretching	1583-1709	1651.5
4	Protein amide II band mainly σ(NH)bending V(C-N) stretching	1481-1585	1539.1
5	Protein σas (CH2) and σ s(CH3) bending of methyl lipid as (CH2) bending of methyl	1425-1477	1455.6
6	Nucleic acid (other phosphate containing compounds) Vas> P=0 stretching of phosphodiesters	1191-1356	1261.0
7	Carbohydrate V (-O-C) of polysaccharides. Nucleic acid (other phosphate containing compounds) Vas> P=0 stretching of phosphodiesters	1072-1099	1090.9
8	Carbohydrate V(C-O-C) of polysaccharides	980-1072	1026

Molecular Identification studies: DNA was isolated from the culture by using Nucleospin DNA extraction kit .Its quality was evaluated on 2.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. 28s rDNA molecular identification was done for

identification of the isolated microalgae. Fragment of 28S rDNA gene was isolated and amplified by Merk PCR amplification kit. A single discrete PCR amplicon band of 800 bp was observed when resolved on Agarose

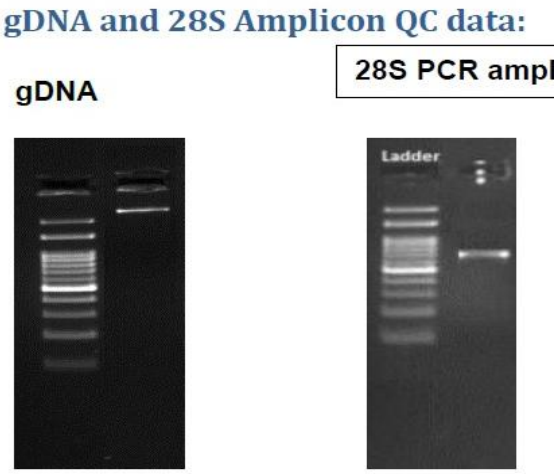


Figure 5: Gel electrophoresis of microalgal gDNA

The 28S rDNA gene sequence was used to carry out basic Local Alignment Search Tool (BLAST) with the nrdatabase of NCBI genebank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Scenedesmus rubescens isolate EP1H8 28S ribosomal RNA gene, partial sequence	778	778	99%	0	94%	JX289896.1
Acutodesmus obliquus partial 28S rRNA gene, strain KGE18	773	773	99%	0	93%	HE965014.1
Asterarcys sp. MS3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	761	761	99%	0	93%	KM893430.1
Scenedesmus obliquus partial 28S rRNA gene, isolate YSL009	761	761	99%	0	93%	FR751197.1
Scenedesmus obliquus partial 28S rRNA gene, isolate YSL012	761	761	99%	0	93%	FR751168.1
Scenedesmus obliquus partial 28S rRNA gene, isolate YSR007	761	761	99%	0	93%	FR751165.1
Scenedesmus obliquus isolate YSW14 large subunit ribosomal RNA gene, partial sequence	761	761	99%	0	93%	HM103381.1
Scenedesmus obliquus partial 28S rRNA gene, isolate YSR016	758	758	99%	0	93%	FR751169.1
Scenedesmus obliquus isolate YSL02 28S ribosomal RNA gene, partial sequence	758	758	99%	0	93%	GU732415.1
Acutodesmus obliquus partial 28S rRNA gene, strain KGE31	756	756	99%	0	93%	HF536545.1

Table-3: Sequences producing significant alignments. Nearest relatives (Accession Number, % Identicity) observed in GenBank when BLAST was performed with microalgae consensus sequence. Microalgae displayed the maximum identity with Scenedesmus rubescens, JX289896.1

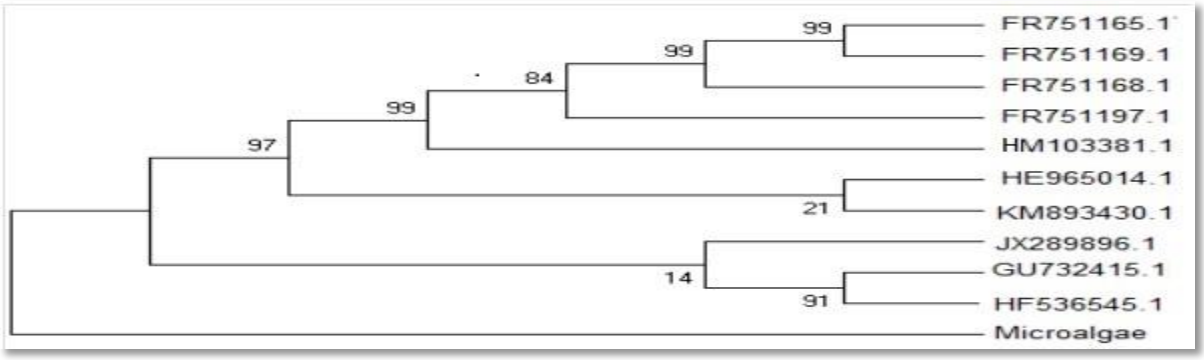


Figure-6: The evolutionary history was inferred using the Neighbour-Joining method. The phylogenetic tree was constructed using MEGA 4.

4.2 OPTIMIZATION OF CULTURE CONDITION FOR OPTIMUM BIOMASS PRODUCTION AND GROWTH RATE

Microalgal growth depends upon the various environmental factors mainly light, pH, temperature, and availability of nutrients as they not only influence the photosynthetic activity of the microalgae but also affects the biochemical composition, cellular metabolism and morphology. Other factors such as shaking and species richness are also important factors influencing microalgal growth and metabolism. As a whole, these environmental factors significantly affect the CO2 fixation and photosynthesis, thus resulting into alteration of carbon fixation and biochemical composition (Morris et al 1974); (Sukenik et al 1989); (Thompson et al 1992); (Chen et al 1994); (Kilhan et al 197);(Gordilo et al 1998); (Visviki et al 2000)

The isolated microalgal sample was inoculated in BG11 medium and incubated at room temperature under a photoperiod of 16L: 8D for growth. Microalgal growth was monitored on a regular basis on spectrophotometer by observing absorbance at 670nm. A growth curve was plotted for a period of 24 days between absorbance and number of days. Figure-7 shows the absorbance noted on various days of culture. This isolated sample was then used as inoculums in the various experiments.

Proper light intensity is a requisite condition for the photosynthesis as it affects the algal growth through its impact on photosynthesis reaction. reaction (Stockenreiter et al 2013) Growth rate is maximum at optimum intensity and the rate decreases as the intensity increases or decreases in comparison to optimum condition. (Sorokin and Kraus 1958). At light intensity more than the optimum, photo-inhibition reaction occurs and thus reduction in

light utilization efficiency (Gordillo et al 1998); (Wu and Merchuk 2001); (Long et al 1994);(Brody and Vatter 1959). This efficiency can be enhanced by allowing the culture to remain in longer dark period while using high light intensities. During this prolonged dark period photosynthesis system works efficiently by optimally utilising captured photons and converting this light energy into chemical energy by avoiding photo-inhibition effects (Long et al 1994). This happens because high light intensity disrupts the chloroplast lamellae (Brody and Vatter 1959) and inactivation of enzymes involved in carbon dioxide fixation. (Iqbal and Zafar 1993). However, algal growth rate under increasing light intensities depends upon the types of algal species as well as other culture conditions like temperature and pH etc. (Sorokin and Kraus 1958)

In the present study, microalgal growth was allowed in six different light intensities 10, 15, 25, 36, 60 and 100 Watt in BG11 medium having pH 8 and light period of 16L: 8D for 24 days and growth was monitored by spectrophotometer at 670nm. All the intensities were provided by yellow light bulb except one, 15watt. For 15watt light intensity, a white fluorescent light was used. Initially a high growth rate was noticed in the flask maintained at 100 W intensity conditions. Later on, the growth rate was found high in the flask maintained at 36 W. Colour of light has a very significant effect on the biomass productivity. The fact established well by higher growth observed in flask with the 15 W flasks in this experiment. Reading was recorded at a period of two or three days and plotted to derive a growth curve on the basis of absorbance and time (Figure-8). Biomass productivity was recorded on spectrophotometer at 670 nm when allowed to grow under three different light colours white, blue and red light. During first two weeks, high growth rate was observed in white light condition, after that a significant change was observed in algal growth. Blue and red light both conditions showed the comparable growth rate to the white in third week onwards of microalgae culture. In an experiment done on light intensity, it was found that 15 W white fluorescent lights gives high biomass productivity in comparison to yellow light with having intensity 100 watt. The maximum biomass productivity was achieved in white light condition (1.56 mg/ml) followed by red (1.37mg/ml) and blue light (0.83mg/ml) (Figure-9).

Light period is important mainly because of dark reactions as well as CO_2 fixation. Studies showed that dark phase is beneficial for microalgae growth because certain enzymes of pentose cycle which is used in photosynthesis and carbon dioxide fixation are active during dark phase and inactive during light phase (Kong et al 2011). Colours of light for

providing illumination to microalgae culture has substantial effect on growth rate due to the wavelength spectral energy of various light colours. Wu and Merchuk 2001 studied the effect of light and dark periods on the microalgal growth and found that with increasing photon flux density, specific growth increases up to a certain value and then it decreases with increased photon flux density. When cultured under three different photoperiods, microalgal growth rate was observed highest in a photoperiod of 16 L: 8D hour's dark with biomass productivity of 1.39mg/ml at the end of 24 days of cultivation. When grown under 24 hours' light supply the microalgal culture shown the biomass productivity of 1.33mg/ml while under 12 hours' photoperiod the biomass productivity was 1.07 mg/ml (Figure-10).

pH is one of the important factors for algal growth as it can affect the activity of different enzymes. Studies on the effect of pH on microalgae demonstrate that different algal species have a different level of tolerance to pH. The pH of the media has also the significant effect on the morphology of the algal cells. It influences the carbon availability, metabolism and thus the biochemical composition (Hargreaves & Whitton 1976). The pH determines the solubility and accessibility of CO_2 and essential nutrients, and because it can have a significant impact on algal metabolism (Mate et al. 2013);(Goldman, J.C. (1973). The pH of the culture media rises significantly during cultivation due to the uptake of inorganic carbon. Highest microalgal biomass productivity was observed at a pH 8 with biomass productivity of 1.50mg/ml among six different media pH, 6 (0.94 mg/ml), 7(1.22 mg/ml), 8(1.50 mg/ml), 9(1.28 mg/ml), 10 (1.31 mg/ml) and 11(1.26 mg/ml) when tested in BG-11 for highest growth rate and biomass productivity (Figure-11).

Photo-inhibition also depends upon the temperature of the culture medium. Low temperature enhances the light inhibition and thus affects the nutrients uptake rate as well as decrease the fluidity in the cell membrane. Temperature beyond the optimum affects the protein synthesis and thus results into reduction of growth rate.(Konopka and Brock 1978). It influences the CO_2 assimilation and O_2 evolving activity of the PS II system of photo-synthesis. It has been reported that it influences microalgal growth rate, cell morphology, various biomolecules composition and nutrient compositions. Various studies based on temperature dependent photo-inhibition suggest that at low temperature, electron transport decreases at a particular photon flux due to decreased rate of CO_2 assimilation. Low temperature causes inhibition of reactive oxygen species and therefore resulting in reduced photo-inhibition by protecting photo system II of photosynthesis system (Vonshak and

Torzillo 2004.). Morris et al 1974 reported that protein synthesis rate increases at lower temperature with marine microalgae *Phaeodactylum tricornutum* (Cuhel et a 1984). Very similar to this, Rhee and Gotham (1981) also reported a significant increment in protein concentration in Scenedesmus sp. at lower temperature. Out of three different temperature regimes 18°C(±2), 26°C(±2), and 30°C(±2), the maximum biomass yield (1.51 mg/ml) was observed in the flask maintained at 26°C(±2). Microalgal culture has shown very less growth at a temperature or below 18°C (±2) with biomass productivity (0.57 mg/ml), while there was very less difference in growth was observed at 30°C(±2) (1.22 mg/ml), when to compare to high biomass yielding temperature regime (figure-12).

In high dense culture, self-shading occur which results in decrease in light absorption capacity of photosynthetic antenna. Thus shaking is essential for mixing of nutrients and to prevent the settling of biomass. Agitation or shaking provides proper mixing which plays an important role in the increasing of microalgal biomass productivity (Goldman, J.C. (1973).
In this experiment, out of three shaking conditions 24hours on shaking, 18 hours a day on shaking and manual shaking, 24 hours on shaking gave the highest biomass productivity 1.56 mg/ml while 18 hours shaking and manual shaking resulted in to 1.45 mg/ml and 1.40mg/ml biomass productivity respectively (figure-13).

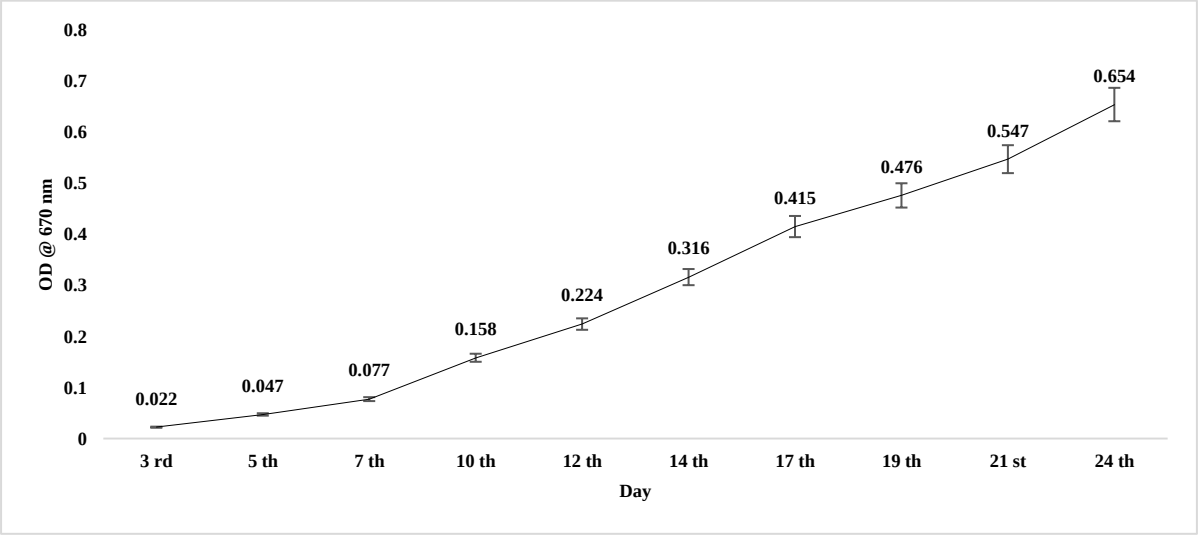


Figure 7: Growth curve of isolated microalga *Scenedesmus rubescens* for a period of 24 days. OD: optical density; 670 nm.

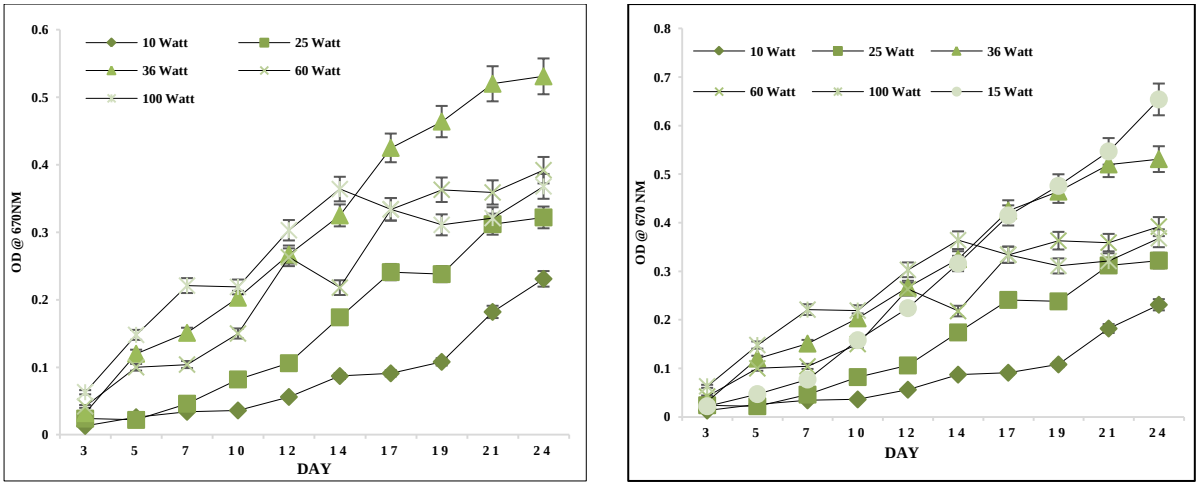


Figure 8: Effect of light intensities on biomass growth and productivity of microalga. (a) Compares the biomass productivity of all intensities of yellow colour light where 36 W provided maximum productivity while (b) compares the productivity with 15 W fluorescent lights. The biomass productivity of 15 W fluorescent lights is better than 36 W yellow lights. OD: optical density; 670 nm.

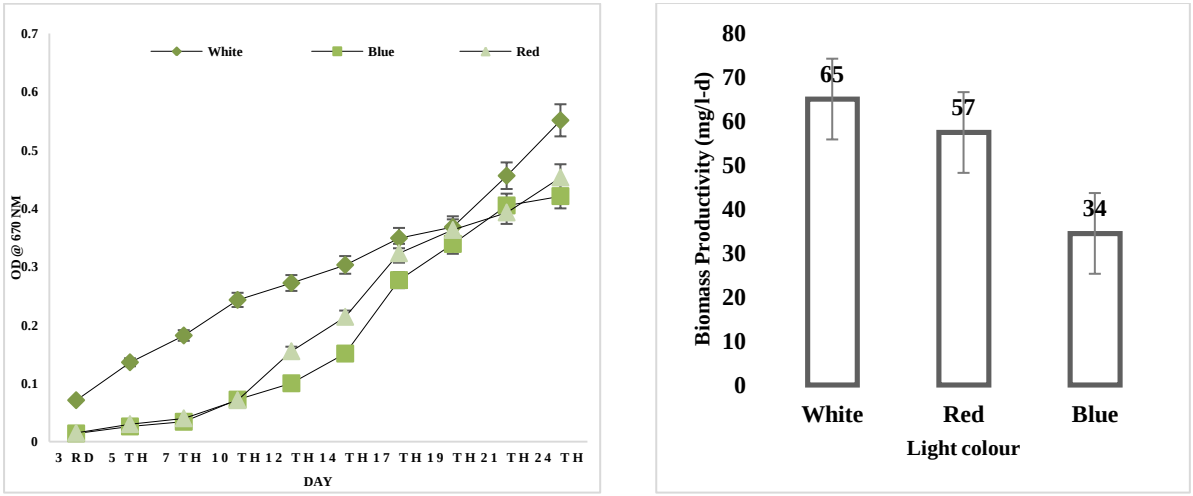
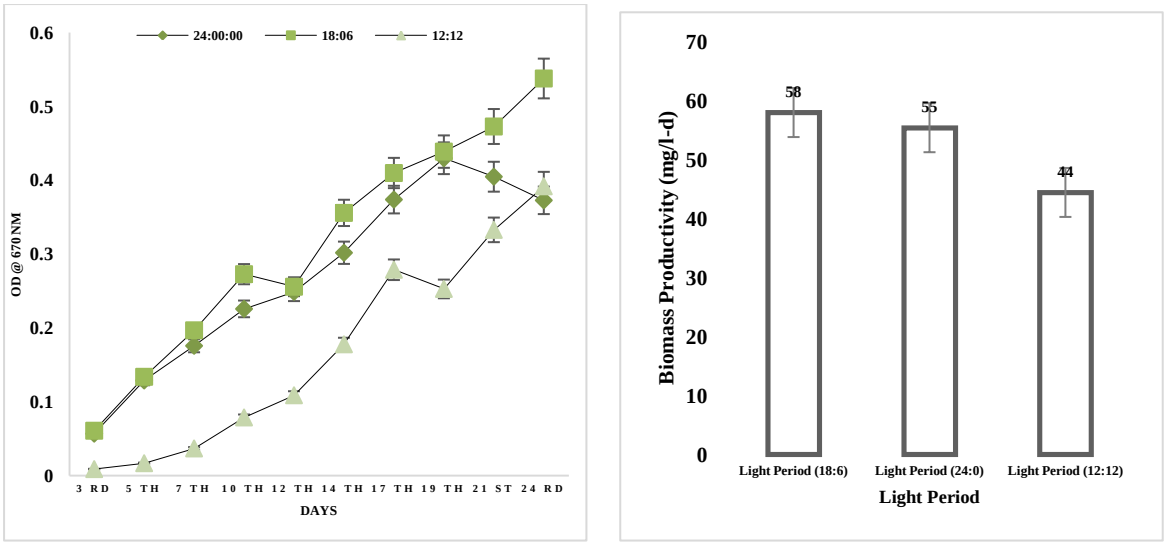
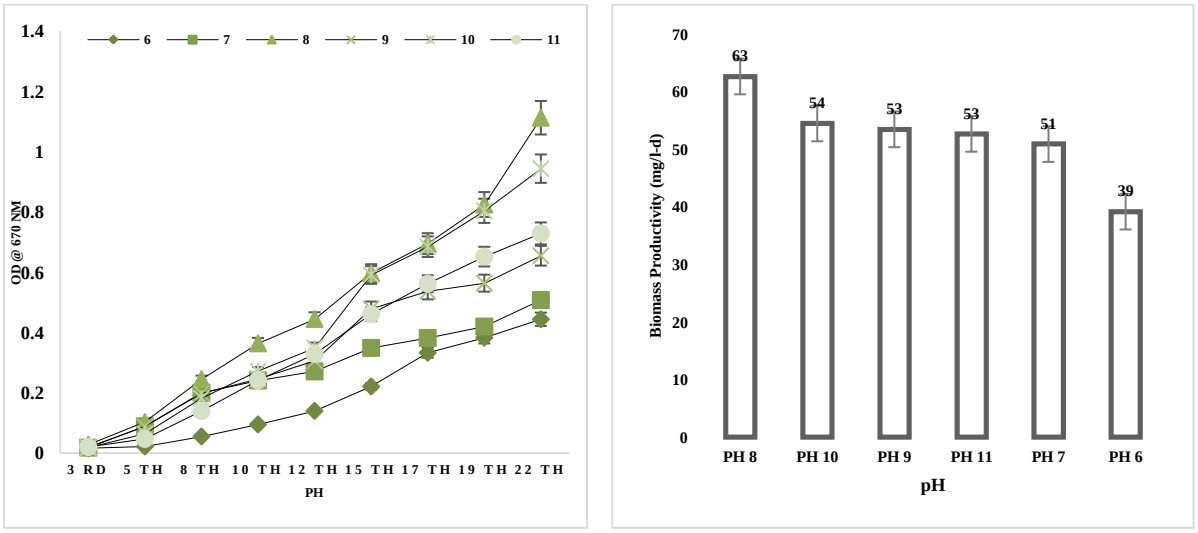


Figure 9: Effect of light colour on biomass growth and biomass productivity of microalga. (a) Compares the growth of microalga in three different light colour and white colour provides maximum growth. (b) Also supports the fact that white colour light is suitable for microalga as it provides maximum biomass productivity. OD: optical density; 670 nm.



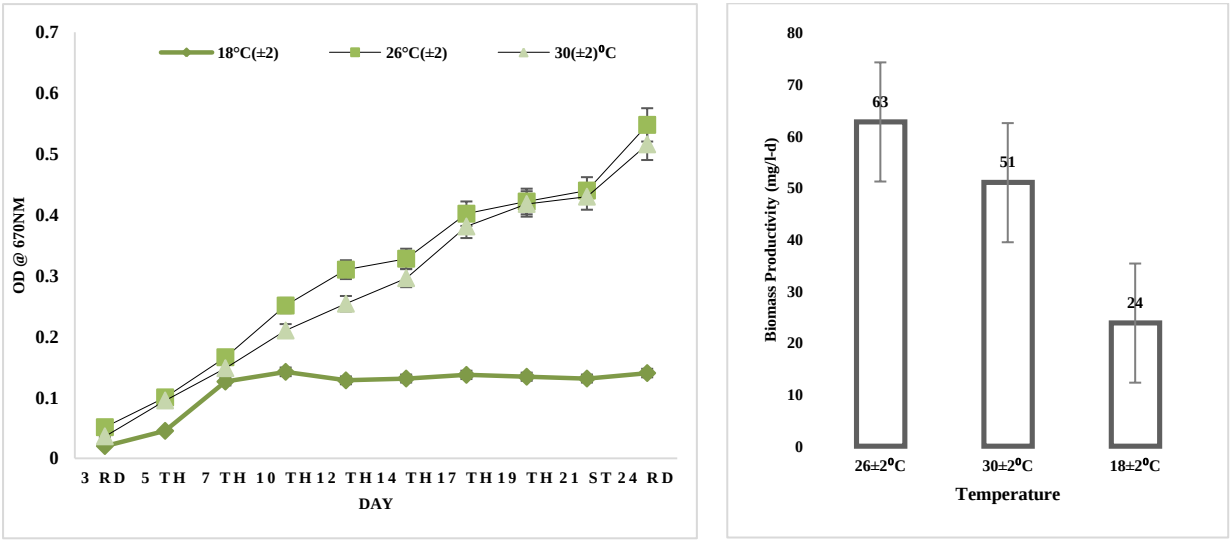
(a) **(b)**

Figure 10: Effect of light period (light: dark) on biomass growth and biomass productivity of microalga. (a) represents a comparison of growth in three different light periods and 16L:08D shows maximum growth. (b) 16L:08D also provided maximum biomass productivity. OD: optical density; 670 nm.



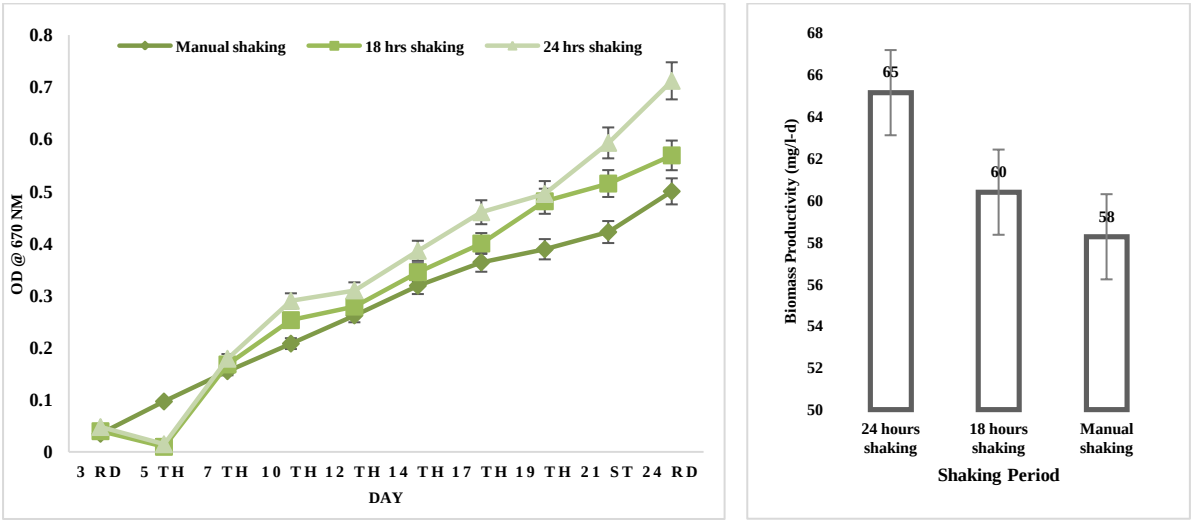
(a) **(b)**

Figure 11: Effect of pH on biomass growth and biomass productivity of microalga. (a) & (b) both represent maximum growth and biomass productivity at pH 8. Alga favours alkaline pH for its growth and biomass productivity. OD: optical density; 670 nm.



(a) **(b)**

Figure 12: Effect of temperature on biomass growth and productivity of microalgae. (a) And (b) both represent that maximum growth and productivity was found at temperature 26°C (±2). Growth was almost same at 30°C (±2) which represents that this microalgae can be grown within a range of temperature from 26-30°C (±2). OD: optical density; 670 nm.

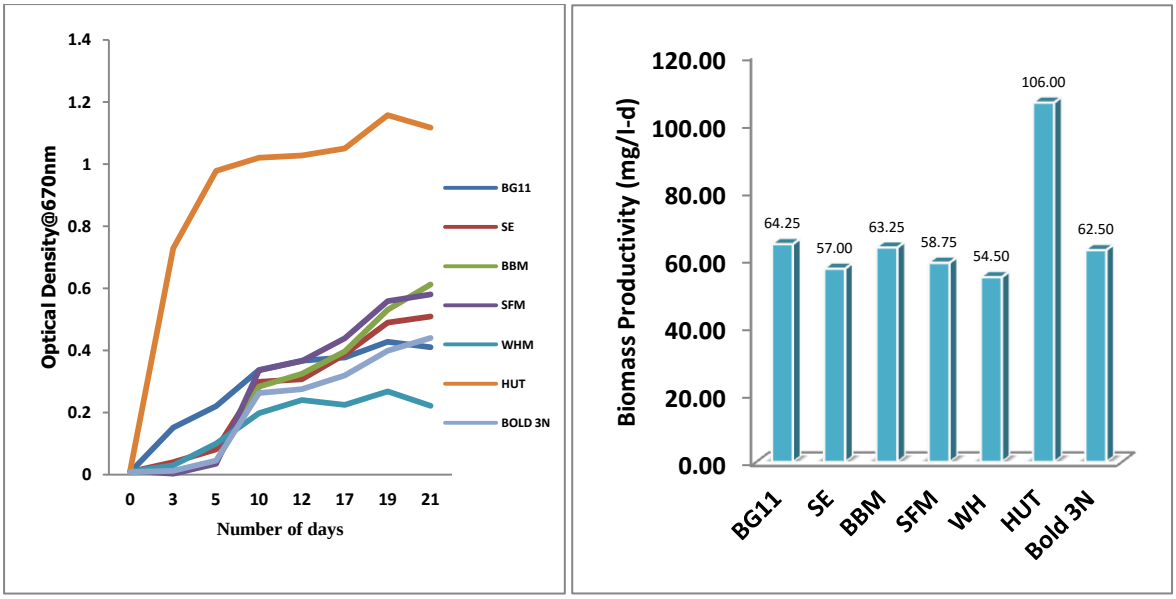


a) **(b)**

Figure 13: Effect of shaking period on biomass growth and productivity of microalga. (a) Represents comparative growth of microalga in different shaking period while (b) provides information of biomass productivity. Shaking of 24 hours is found best for both growth as well as biomass productivity of microalga. OD: optical density; 670 nm.

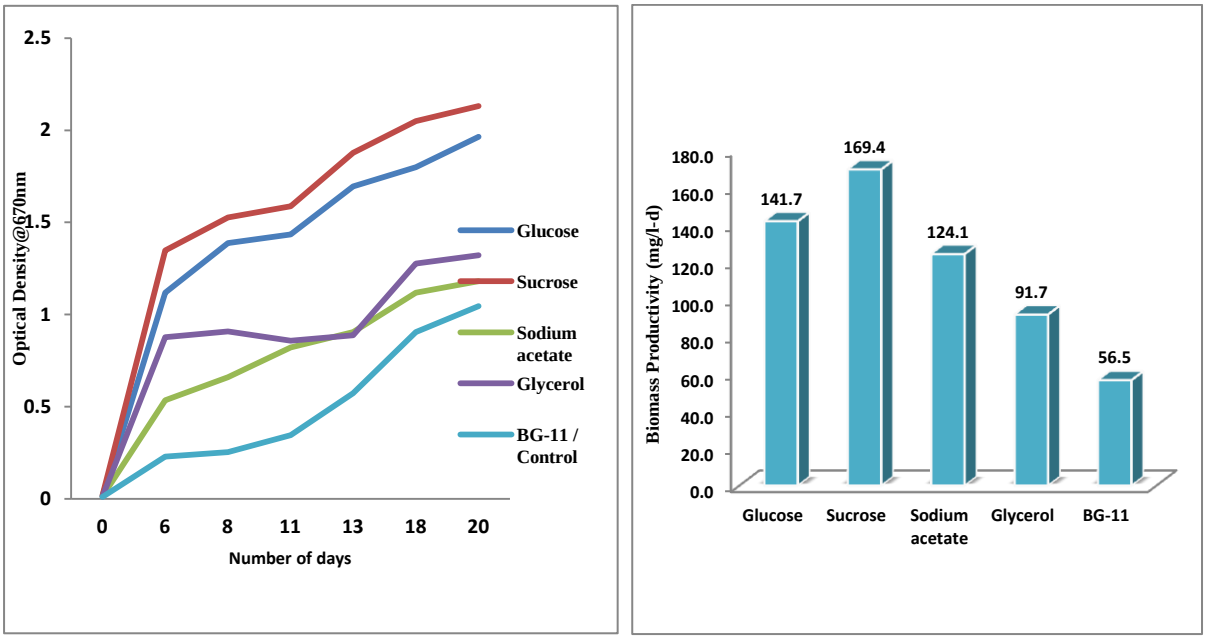
4.3 SELECTION AND OPTIMIZATION OF VARIOUS SOURCES OF CARBON, NITROGEN, PHOSPHOROUS AND MAGNESIUM CONCENTRATION:

Out of seven media composition (BG11, SE (Bristol's solution), Bold's Basal medium (BBM), Synthetic Freshwater medium (SFM), Waris-H Medium (WH medium), HUT medium, Bold 3N medium) HUT media has shown the highest growth and biomass productivity. HUT medium has the total biomass productivity of 106 mg/l-d at the end of 21days. It was the only medium which contains yeast extract and peptone among all seven different mediums. Modified HUT medium was found to be best media for the microalga *Scenedesmus rubescens* with biomass productivity of 106 mg/litre on 21st day of cultivation. Lowest biomass productivity was achieved in SE bristol's solution. BG11 media has the biomass productivity of 64.25 mg/l-d which was greater than rest media excluding HUT medium. BG-11 media was preferred as the best suitable medium for the further work because it is easy to prepare and handle than the other abovementioned compositions. Four carbon sources (Glucose, Sucrose, Sodium acetate, and Glycerol) were evaluated for the maximum biomass productivity for a period of 27days. Among four, sucrose showed the highest biomass productivity with 169.4mg/l-d. Sucrose (169.4 mg/l on 27th day of cultivation) was found to be the superior carbon source than Glucose, Sodium Acetate and Glycerol for the optimum growth and biomass yield. Among Nitrogen Sources (NH_4Cl (85.2 mg/l-d), $CO(NH_2)_2$ (116.7 mg/l-d), $NaNO_3$ (100 mg/l-d), KNO_3 (92.6 mg/l-d), $Co(NO_3)_2$ (9.3 mg/l-d) and $Ca(NO_3)_2$ (57.4 mg/l-d)) Urea followed by Sodium Nitrate was found to be the best source at the end of 27 days of cultivation. For Phosphorous Source, K_2HPO_4 (65 mg/l-d) showed the optimum yield as compared to $NH_4H_2PO_4$ (46.35 mg/l-d), KH_2PO_4 (43.65mg/l-d) and Na_2HPO_4 (45.19 mg/l-d) at the end of 27 days of cultivation. For K_2HPO_4, absorbance was recorded as 0.478 on 27th day of cultivation which was maximum as compared to the rest phosphorous sources.



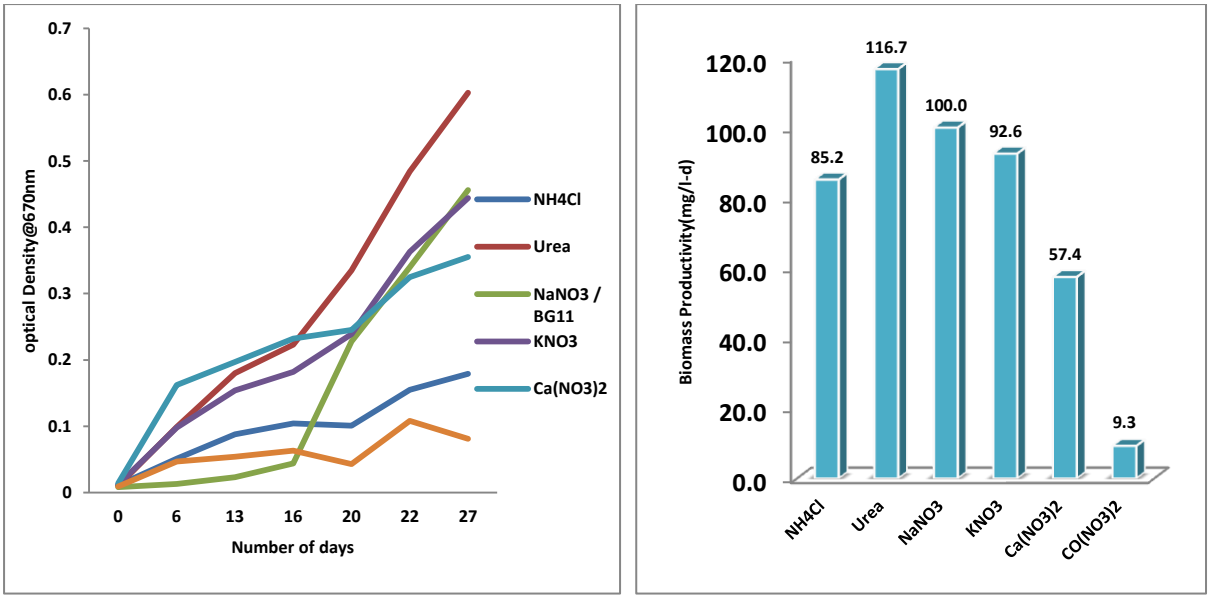
A) B)

Figure 14: Effect of different Media composition on A) algal growth and B) Biomass productivity of the microalga. HUT medium was found to be the best medium among all seven tested composition. OD: optical density; 670 nm. Biomass Productivity (mg/l-d).



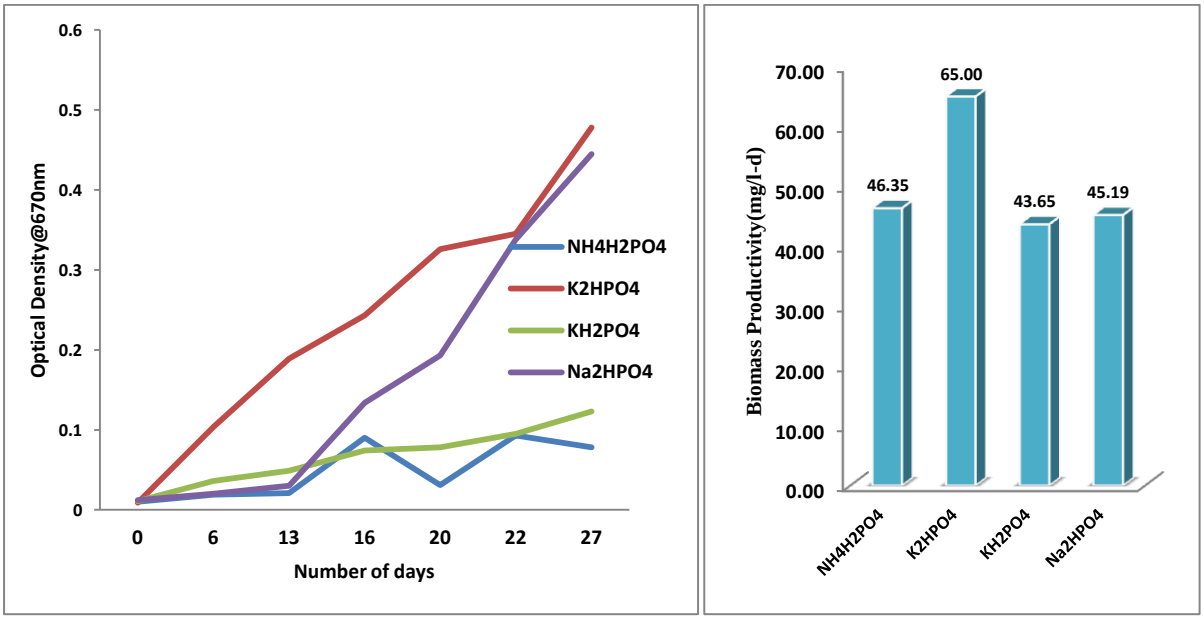
A) **B)**

Figure 15: Effect of different Carbon sources on A) algal growth and B) Biomass productivity of the microalga. Sucrose was found to be the best carbon source among all four tested carbon sources. OD: optical density; 670 nm. Biomass Productivity (mg/l-d)



A) B)

Figure 16: Effect of different Nitrogen sources on A) algal growth and B) Biomass productivity of the microalga. Urea was found to be the best nitrogen source among all six tested nitrogen sources. OD: optical density; 670 nm. Biomass Productivity (mg/l-d)



A) B)

Figure 17: Effect of different phosphorous sources on A) algal growth and B) Biomass productivity of the microalga. K_2HPO_4 was found to be the best phosphorous source among all four tested phosphorous sources. OD: optical density; 670 nm. Biomass Productivity (mg/l-d)

4.4 EVALUATION FOR THE BIOFUEL PRODUCTION

4.4.1 Lipid estimation for biodiesel production:

Biomass yield was calculated by using 10ml of the culture in triplicate by the help of following formula:

$$\text{Biomass yield (mg/l)} = \frac{\text{Total dry weight (mg)}}{\text{Volume(litre)}}$$

$$= 1657.6 \text{ mg/l}$$

Table 4: Biomass Yield of microalga

Sample	Volume(ml)	Blank(g)	Average Biomass+ Blank (g)	Biomass(g)	Biomass(mg)	mg/l
1	10	24.856	24.871	0.01514	15.14	1514
2	10	25.930	25.948	0.01778	17.78	1778
3	10	24.945	24.961	0.016806667	16.80666667	1680.667
Average					16.57555556	1657.556

Table 5: Lipid estimation from microalga

Volume(ml)	Blank(mg)	Blank+ Lipid (mg)	Lipid (mg)	lipid (mg/l)	mg Lipid/ mg Biomass	% Lipid
150	20239.49	20259.3	19.81	132.0666667		
150	20320.25	20339.1	18.85	125.6666667		
150	20381.5	20399.6	18.1	120.6666667		
Average				126.1333333	0.076095991	7.609599142

Average Biomass yield (mg/l): 1657.6 mg/l

Average Lipid yield (mg/l): 126.13 mg/l

$$\% \text{ Lipid} = \frac{\text{Lipid yield } \left(\frac{\text{mg}}{\text{l}}\right)}{\text{Biomass yield } \left(\frac{\text{mg}}{\text{l}}\right)} * 100$$

$$= 7.61$$

4.4.2 Total Carotenoids estimation: Chlorophyll estimation was evaluated by 80% acetone method. Chlorophyll a, Chlorophyll b and Total carotenoids were estimated by the following formula:

$C_a = 12.23A_{663} - 2.81A_{646}$

$C_b = 20.13\ A_{646} - 5.03\ A_{663}$

$$C_t = \frac{1000A470 - 3.27Ca - 104Cb}{198}$$

Where C_a, C_b, and C_t are chlorophyll a, chlorophyll b and total carotenoids respectively in µg/ml.

Table 6: Effect of sonication period on Chlorophyll content

Sonication Period	A663	A646	A470	C_a(µg/ml)	C_b(µg/ml)	T_c(µg/ml)	Average T_c(µg/ml)
3 min sonication A	0.027	0.0137	0.0333	0.291	0.140	0.090	
3 min sonication B	0.0333	0.0182	0.0413	0.356	0.198	0.099	0.094
5 min sonication A	0.0602	0.0312	0.0693	0.648	0.325	0.169	
5 min sonication B	0.0575	0.0306	0.0688	0.616	0.326	0.166	0.167
10 min sonication A	0.2464	0.1151	0.2647	2.685	1.077	0.727	
10 min sonication B	0.2473	0.1119	0.2624	2.705	1.009	0.750	0.739
15 min sonication A	0.2606	0.1236	0.2772	2.835	1.176	0.735	
15 min sonication B	0.2464	0.1151	0.2647	2.685	1.077	0.727	0.731

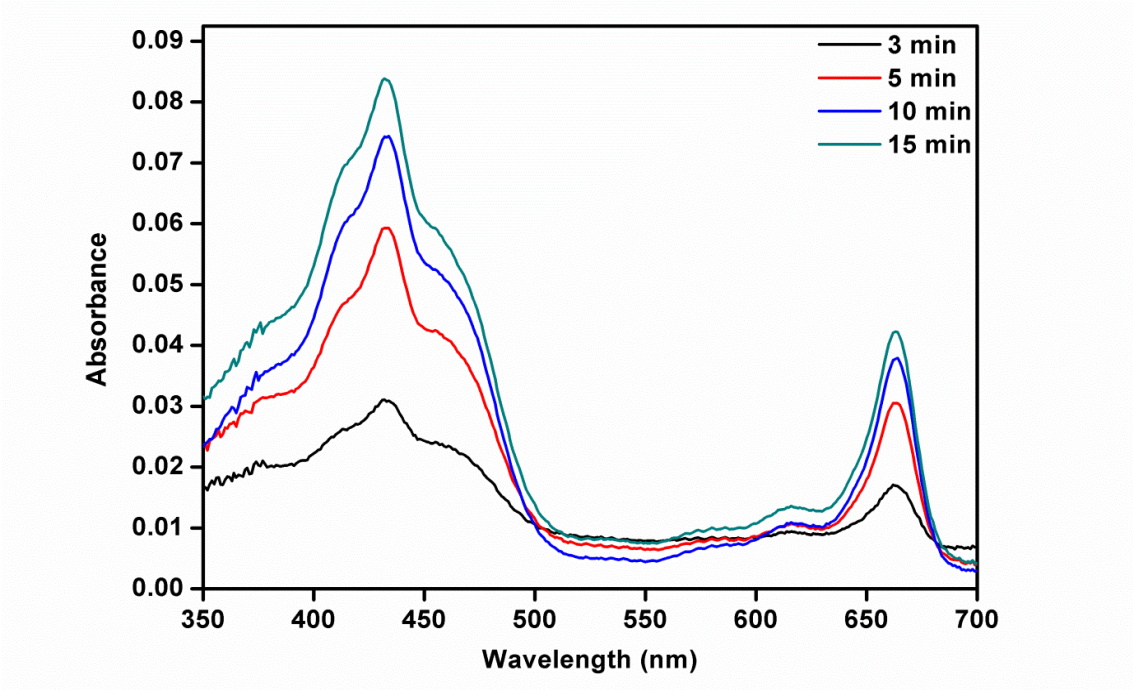


Figure 18: UV-VIS graph (PerkinElmer UV WinLab 6.0.4.0738 / 1.61.00 Lambda 900)

5. CONCLUSION:

For efficient use of microalgae as a source of biodiesel it is very important to focus on the native algal species and to select that algal species which not only has a high growth rate but has greater lipid content. Identification of local microalgal species, optimization of conditions for native algal species and selection of superior carbon, nitrogen and phosphorous source is the focus of this study. The isolation of microalgae from lakes or ponds is a useful strategy for obtaining microalgae tolerant to the conditions prevalent in the area, such an organism tending to have the ability to grow without applying any extra effort with having optimum biomass productivity. Keeping the competitive and selective growth condition advantages like the adaptation in local geographical, climatic and ecologic conditions in mind, microalgae thus isolated from DTU Lake was cultured in the lab and evaluated for the biodiesel production. Based on morphology (SEM, Light microscope and FTIR examination) and molecular examination (28s rDNA), microalgae isolated were identified as *Scenedesmus rubescens.* Effects of various culture conditions on green microalgae isolate *Scenedesmus rubescens* has been studied and thus concluded that the maximum growth rate and biomass productivity can be achieved by providing light having intensity, color and photoperiods of 36W, White and 16 hours respectively. Alkaline pH especially pH 8 supports the maximum growth rate and thus maximum biomass productivity. The optimum growth rate was observed in the 24h shaking period and 26 (±2) ^{0}C temperatures. Among Seven Media compositions, HUT medium (65 mg/l-d) was found to be best for the *Scenedesmus rubescens.* Sucrose (169.4 mg/l-d), Urea (116.7 mg/l-d) and K_2HPO_4 (65 mg/l-d) was found as a superior among tested Carbon, Nitrogen and Phosphorous sources. Lipid yield of Scenedesmus rubescens was found to be 7.61% of total dry weight. Total carotenoids were estimated by 80% acetone method and found to be best as 0.739µg/ml when the sonication was provided for a period of 15 minutes. The amount of total carotenoids varies as per the duration of sonication period. This study deals with the method of isolation identification and characterization of microalgae from local water bodies and evaluate for the biodiesel production. Hence, present study is a significant step forward in utilization of microalgae as a source of renewable energy.

6. FUTURE PERSPECTIVE

Till now, the main objective of work, Isolation, Identification and characterization of a freshwater microalgae species, has been accomplished. The second aim of biomass optimization and culture condition optimisation and selection of superior media composition, carbon, nitrogen and phosphorous sources for isolate has been also investigated. Future research area is wide open to various direction and promising aspects such as

- Biochemical composition profiling i.e. FAME analysis (lipid profiling) and carbohydrate profiling of the Scenedesmus sp. under different culture conditions as mentioned in the second aim of the study.

- Biomass to biodiesel production, biogas production (C/N ratio analysis and its optimisation), Biomass to bioethanol production, Biomass to biohydrogen production

- Use of microalgae for wastewater treatment, Bio-plastics production, Nanoparticle synthesis, Biopolymer/ bio-glass production Etc. etc.

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Annexure-I : Media Composition

BG11 Medium

(ATCC Medium 616)

#	Component	Stock solution ($g . L^{-1} dH_2O$)	Addition per 1 Litre culture medium
1	$NaNO_3$		1.5 g
2	$K_2HPO_4 \cdot 3H_2O$	40.00	1 ml
3	$MgSO_4 \cdot 7H_2O$	75.0	1 ml
4	$CaCl_2 \cdot 2H_2O$	36.0	1 ml
5	Citric Acid	6.0	1 ml
6	Ammonium Ferric Citrate	6.0	1 ml
7	$MgNa_2EDTA \cdot H_2O$	1.0	1 ml
8	Na_2CO_3	20	1 ml
9	Trace Metals		1 ml

Preparation of Trace Metal Solution

#	Metal	Addition per 1 litre bidistilled water
1	H_3BO_3	2.86 g
2	$MnCl_2 \cdot 4H_2O$	1.81 g
3	$ZnSO_4 \cdot 7H_2O$	0.22 g
4	$Na_2MoO_4 \cdot 2H_2O$	0.39 g
4	$CuSO_4 \cdot 5H_2O$	0.08 g
6	$Co(NO_3)_2 \cdot 6H_2O$	0.05 g

Preparation of Culture Solution

Add chemicals and stock solutions as indicated above to 1000 ml of bidistilled water. Adjust pH to 7.4 with NaOH and autoclave.

HUT

HUT

#	Component	The amount of	Stock concentration
1	KH_2PO_4	0.02g/L	
2	Peptone	0.6g/L	
3	$MgSO_4 \cdot 7H_2O$	0.025g/L	
4	Yeast extract	0.4g/L	
5	Sodium acetate	0.4g/L	
6	Vitamin B12	0.5ug/L	
7	Potassium citrate	0.04g/L	
8	Vitamin B1	0.4mg / L	

PH 6.4

SE (Bristol's solution)

#	Component	The amount of	Stock concentration
1	$NaNO_3$	1 mL / L	25 g / 100mldH_2O
2	K_2hpo_4	1 mL / L	7.5g/100 mL dH_2O
3	$MgSO_4 \cdot 7H_2O$	1 mL / L	7 .5g/100 mL dH_2O
4	$CaCl_2 \cdot 2H_2O$	1 mL / L	2 .5g/100 mL dH_2O
5	KH_2PO_4	1 mL / L	17.5 g/100 mL dH_2O
6	NaCl	1 mL / L	2.5 g/100 mL dH_2O
7	$FeCl_3 . 6H_2O$	1ml / L	0.5 g/100 mL dH_2O
8	EDTA-Fe *	1 mL / L	
9	A5 （Trace mental solution） **	1ml / L	
10	Soil extract * * *	40ml / L	

*** EDTA-Fe**

1N HCl: take 4.1ml of concentrated hydrochloric acid was diluted to 50ml with distilled water.

0.1N EDTA-Na 2 Weigh 0.9306g dissolved to 50ml of distilled water.

Weigh FeCl3. 6H2O 0.901g were dissolved in 10ml of the above steps have been completed formulated in 1N HCl, then with 10ml been formulated 0.1N EDTA-Na 2 mixed completed, distilled water was added to 1000ml.

****A5 (Trace mental solution)**

Component	concentration
H_3BO_3	2.86g/L dH_2O
$MnCl_2 \cdot 4H2O$	1.86g/L dH_2O
$ZnSO_4 \cdot 7H_2O$	0.22g/L dH_2O
$Na_2MoO_4.2H_2O$	0.39g/L dH_2O
$CuSO_4 . 5 H_2O$	0.08g/L dH_2O
$Co(NO_3)_2.6 H_2O$	0.05g/L dH_2O

Bold's Basal Medium (BBM)
(Bold 1949, Bischoft & Bold 1963)

#	Component	Stock solution ($g \cdot L^{-1}$ dH_2O)	Addition per 1 Litre culture medium
1	$NaNO_3$	25.00	10 ml
2	$CaCl_2 \cdot 2H_2O$	2.50	10 ml
3	$MgSO_4 \cdot 7H_2O$	7.50	10 ml
4	K_2HPO_4	7.50	10 ml
5	KH_2PO_4	17.50	10 ml
6	NaCl	2.50	10 ml
7	Alkaline EDTA solution EDTA (Titriplex III) KOH	50 31	1 ml
8	Acidified Iron solution $FeSO_4 \cdot 7 H_2O$ H_2SO_4	4.98 1 ml	1ml
9	Boron solution H_3BO_3	11.42	1ml
10	Trace Metals solution $ZnSO_4 \cdot 7H_2O$ $MnCl_2 \cdot 4H_2O$ MoO_3 $CuSO_4 \cdot 5H_2O$ $Co(NO_3)_2 \cdot 6H_2O$	8.82 1.44 0.71 1.57 0.49	1ml

Preparation of Culture Solution

Add chemicals and stock solutions as indicated above to 1000 ml of bidistilled water. The final pH should be 6.6.

SFM Medium
(Synthetic Freshwater Medium)

#	Component	Stock solution Addition per 1 Litre stock solution	Addition per 1 Litre culture medium
1	$Ca(NO_3)_2 \cdot 4\ H_2O$	100.00 g	0.5 ml
2	$MgSO_4 \cdot 7\ H_2O$	20.00 g	2.5 ml
3	$K_2HPO_4 \cdot 3\ H_2O$ + $NaNO_3$ + Na_2CO_3	5.00 g 50.00 g 32.00 g	0.6 ml
4	Vitamins		1 ml
5	HEPES	238.31 g	1 ml
6	Trace Metals		1 ml

Preparation of Vitamins Solution

#	Vitamin	Stock solution concentration Addition per 1 Litre stock solution
1	Vitamin B_{12}	0.20 mg
2	Biotin	1.00 mg
3	Thiamine-HCl	100.00 mg
4	Niacinamide	0.10 mg

Preparation of Trace Metal Solution

#	Component	Stock solution ($g \cdot L^{-1}$)	Addition per 1000 ml bidestilled water
1	$Na_2EDTA \cdot 2\ H_2O$		4.36 g
2	$FeCl_3 \cdot 6\ H_2O$		3.15
3	K_2CrO_4	1.94	1 ml
4	$CoCl_2 \cdot 6\ H_2O$	11.90	1 ml
5	$CuSO_4 \cdot 5\ H_2O$	2.50	1 ml
6	$MnCl_2 \cdot 4\ H_2O$	178.10	1 ml
7	$Na_2MoO_4 \cdot 2\ H_2O$	19.90	1 ml
8	$NiSO_4 \cdot 6\ H_2O$	2.63	1 ml
9	H_2SeO_3	1.29	1 ml
10	Na_3VO_4	1.84	1 ml
11	$ZnSO_4 \cdot 7\ H_2O$	23.00	1 ml

Preparation of Culture Solution

Add stock solutions 1-6 to 1000 ml of bidistilled water; adjust the pH to 7.0 and autoclave.

Waris-H Medium

(McFadden G.I & Melkonian M., 1986)

#	Component	Stock solution Addition per 1 Litre stock solution	Addition per 1 Litre culture medium
1	KNO_3	100.00 g	1 ml
2	$MgSO_4 \cdot 7\ H_2O$	20.00 g	1 ml
3	$(NH_4)_2HPO_4$	20.00 g	1 ml
4	$Ca(NO_3)_2 \cdot 4\ H_2O$	100.00 g	1 ml
5	HEPES	238.31 g	1 ml
6	P-II Metals		1 ml
7	Fe-EDTA		1ml
8	Vitamins		1 ml
9	Soil extract		10 ml

Preparation of PII Metals Solution

#	Component	Stock solution Addition per 1 Litre stock solution
1	EDTA (Titriplex III)*	3.00 g
2	H_3BO_3	1.14 g
3	$MnCl_2 \cdot 4\ H_2O$	0.144 g
4	$ZnSO_4 \cdot 7\ H_2O$	0.021 g
5	$CoCl_2 \cdot 6\ H_2O$	0.004 g

Preparation of Fe-EDTA Solution

#	Component	Stock solution concentration Addition per 1 Litre stock solution
1	EDTA (Titriplex II)*	5.22 g
2	$FeSO_4 \cdot 7\ H_2O$	4.98 g
3	1 N KOH	54.00 ml

EDTA (Titriplex II) and $FeSO_4$ x 7 H_2O is heated for 30 min (100^{o}C); KOH is added to the cooled mixture

Preparation of Vitamins Solution

#	Vitamin	Stock solution concentration Addition per 1 Litre stock solution
1	Vitamin B_{12}	0.20 mg
2	Biotin	1.00 mg
3	Thiamine-HCl	100.00 mg
4	Niacinamide	0.10 mg

Preparation of Culture Solution

Add 1 ml of stock solutions 1-8 to 1000 ml of bidistilled water. Add 10 ml of thawed soil extract (= stock solution 9), adjust the pH to 7.0 and autoclave.

* Titriplex II - Ethylenediaminetetraacetic acid - $C_{10}H_{16}N_2O_8$
* Titriplex III - Ethylenediaminetetraacetic acid disodium salt dihydrate - $C_{10}H_{14}N_2Na_2O_8 \times 2\ H_2O$

Bold 3N Medium
Bold 3N Medium

#	Component	The amount of	Stock concentration
1	$NaNO_3$	30 mL / L	10 g / 400mldH_2O
2	$CaCl_2 \cdot 2H_2O$	10 mL / L	1 g / 400 mL dH_2O
3	$MgSO_4 \cdot 7H_2O$	10 mL / L	3G / 400 mL dH_2O
4	K_2HPO_4	10 mL / L	3G / 400 mL dH_2O
5	KH_2PO_4	10 mL / L	7 g / 400 mL dH_2O
6	NaCl	10 mL / L	1 g / 400 mL dH_2O
7	PIV *	6mg / L	
8	Soil extract * *	1 mL / L	
9	Vitamin B_{12} * * *	1ml / L	

*** PIV (Trace mental solution)**

Component	Concentration
Na2EDTA	0.75g / L dH2O
MnCl2 · 4H2O	0.041g / L dH2O
ZnCl2 · 7H2O	0.005g / L dH2O
Na2MoO4 · 2H2O	0.004g / L dH2O
FeCl3 · 6 H2O	0.097g / L dH2O
CoCl2.6 H2O	0.002g / L dH2O

***** Vitamin B_{12}**

Join 2.4gHPEPS buffer in 200mL distilled water. PH value adjusted to 7.8. Add to Vitamin B_{12} 0.027 g. Stored in a refrigerator.

When added to the medium to be added after sterilization in the clean stage filtration with 0.22um of Millpore disposable filter.

ANNEXURE-II

Optical density recorded on 670 nm for different media composition

Days	BG11	SE	BBM	SFM	WHM	HUT	BOLD 3N
0	0.008	0.009	0.007	0.012	0.01	0.015	0.008
3	0.152	0.04	0.011	0.003	0.03	0.729	0.012
5	0.221	0.081	0.041	0.035	0.1	0.978	0.045
10	0.337	0.299	0.283	0.336	0.198	1.02	0.263
12	0.367	0.307	0.325	0.366	0.24	1.028	0.275
17	0.377	0.388	0.397	0.439	0.225	1.05	0.32
19	0.428	0.49	0.531	0.559	0.268	1.158	0.399
21	0.41	0.509	0.612	0.58	0.222	**1.117**	0.44

Optical density recorded on 670 nm for different Carbon sources

Days	Glucose	Sucrose	Sodium acetate	Glycerol	BG-11 / Control
0	0.015	0.018	0.013	0.012	0.009
6	1.119	1.347	0.535	0.877	0.228
8	1.387	1.527	0.66	0.909	0.253
11	1.434	1.587	0.821	0.858	0.346
13	1.695	1.877	0.905	0.888	0.572
18	1.8	2.05	1.118	1.276	0.904
20	1.965	**2.132**	1.181	1.321	1.045

Optical density recorded on 670 nm for different Nitrogen sources

	NH_4Cl	Urea	$NaNO_3$ / BG11	KNO_3	$Ca(NO_3)_2$	$Co(NO_3)_2$
0	0.011	0.01	0.008	0.012	0.014	0.009
6	0.051	0.099	0.013	0.098	0.162	0.047
13	0.088	0.18	0.023	0.154	0.197	0.054
16	0.104	0.223	0.044	0.182	0.232	0.063
20	0.101	0.335	0.228	0.239	0.245	0.043
22	0.155	0.484	0.34	0.363	0.325	0.108
27	0.179	**0.603**	0.456	0.444	0.355	0.081

Optical density recorded on 670 nm for different Phosphorous sources

	$NH_4H_2PO_4$	K_2HPO_4	KH_2PO_4	Na_2HPO_4
0	0.01	0.009	0.011	0.012
6	0.019	0.104	0.036	0.02
13	0.021	0.189	0.049	0.03
16	0.09	0.243	0.074	0.134
20	0.031	0.326	0.078	0.193
22	0.093	0.345	0.095	0.338
27	0.078	**0.478**	0.123	0.445