Influence of various carbon and nitrogen sources on Biomass and Lipid productivity of *Chlorella minutissima* and *Scenedesmus* sp. and FAME analysis by GC-MS

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

Master in Technology

In

Industrial Biotechnology

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DECLARATION

This is to certify that the major project entitled "Influence of various carbon and nitrogen sources on Biomass and Lipid productivity of *Chlorella minutissima* and *Scenedesmus* sp. and FAME analysis by GC-MS" done by Tushita Attre (2k15/IBT/14) in the partial fulfilment of the requirements for the reward of the degree of Masters in Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the my own work carried out under the guidance of my project supervisor Dr. Navneeta Bharadvaja, Assistant Professor, Plant Biotechnology laboratory, Department of Biotechnology, DTU. The information and data enclosed in this report is original and has not been submitted elsewhere for honouring of any other degree.

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CERTIFICATE

This is to certify that the Major project entitled "Influence of various carbon and nitrogen sources on Biomass and Lipid productivity of *Chlorella minutissima* and *Scenedesmus* sp. and FAME analysis by GC-MS*"* done by Tushita Attre (2K15/IBT/14) is in the partial fulfilment of the requirements for the reward of the degree of Masters of Technology in Industrial Biotechnology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her 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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Influence of various carbon and nitrogen sources on Biomass and Lipid productivity of *Chlorella minutissima* and *Scenedesmus* sp. and FAME analysis by GC-MS

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Abstract

Green fuels are getting greater attention day by day due to increase in energy demands and environmental problems. In the current study, *Chlorella minutissima* and *Scenedesmus sp*. were cultivated in BBM medium. The impact of various nitrogen (Sodium Nitrate, Potassium Nitrate, Yeast Extract, Glycine and Urea) and natural carbon (Glucose, Glycerol, Fructose, Maltose, Starch, Sodium acetic acid derivation and Sucrose) sources were broke down for development and lipid collection on these green growth. The most noteworthy biomass development in *Chlorella minutissima* and *Scenedesmus sp.* in nitrogen sources was found in Urea (2577.27 mg/L), (1201.3 mg/L) respectively while in natural carbon sources, the biomass development and profitability for *Chlorella minutissima* was discovered greatest in Fructose (1268.58 mg/L) and for Scenedesmus was observed to be in Starch (1151.33 mg/L). The lipid content was analyzed utilizing Folch strategy by changing dissolvable framework and Chloroform: Methanol (2:1) was seen demonstrating best outcomes and was utilized for extraction. Maximum lipid was found in Potassium nitrate nitrogen source (50.08%) for *Chlorella minutissima* and for *Scenedesmus sp* Urea as nitrogen source (79.05 %). Among organic carbon sources, the maximum lipid yield for *Chlorella minutissima* (36.79% and lipid productivity 2577.27mg/L/day were found in case of Glucose) and for *Scenedesmus* sp. (27.4 % and lipid productivity 1690.18 mg/L/d were found in Maltose). Further it has been observed both the algae contain Fatty acid from C:16 to C:18 which are essential for biodiesel production. Along with lipid extraction harvesting of biomass is also an important factor which will indicate higher biomass means higher lipid content and it was seen Centrifugation is the best method among various methods tested. Various other analyses done were Chlorophyll estimation, Protein estimation and Carbohydrate estimation to test their presence and quantify it.

Keywords: Biodiesel, Biomass growth, *Chlorella minutissima*, *Scenedesmus sp*., Lipid extraction, Harvest, Chlorophyll, Protein, Carbohydrate

INTRODUCTION

Power is backbone of each United States's financial improvement and prosperity. India is the sector's 5th largest primary energy patron and fourth largest petroleum consumer after U.S.A, China and Japan (Sharma P. et al., 2015). With an outlook for slight to robust monetary boom and a growing population, growing infrastructural and socio-financial improvement will stimulate an boom in power intake throughout all main sectors of the Indian economic system (Muhit I.B, et al.,2014).Transportation is one of the fastest growing sectors the usage of 27% of the primary electricity (Antoni D,et al.,2007). At the present notable rates of utilization, the world fossil oil holds are probably going to be depleted in below forty five years (BP Statistical overview of the world vitality,2008). In this way, the growing energy (gas) emergency and ecological debasement have represented an intense problem to our doable improvement and survival. So attention has now moved to the choice powers like biodiesel, bio-ethanol and biogas. Biodiesel is synthesis of unsaturated fat of ethyl or methyl ester produced the usage of virgin or utilized vegetable oil (either palatable or non-consumable).

The most of the biodiesel manufacturing are primarily based on fit to be eaten oil like soybean oil, rapeseed oil, canola oil or sunflower oil in developed countries (Sharma P. et al.,2015). In India the palatable oil request is better than its actual technology. So there's no chance of occupying this oil for generation of bio diesel. The precept item warm spots for bio diesel can be non-consumable oils obtained from vegetation that may supplant the neighborhood oil makers. there are numerous species, which bear seeds wealthy in oil content and out of those some encouraging tree species had been distinguished. those are Jatropha curcas (Ratanjyot), Pongamia pinnata (Karanja), Calophyllum inophyllum (Nagchampa or Polanga), Mahua, Castor, Seemarouba and etc (Sharma P. et al.,2015).

Utilizing the ebb and waft yields, mammoth measures of land and crisp water might be expected to create sufficient oil to absolutely supplant petroleum spinoff use. it'd require double the land vicinity of america to be dedicated to soybean technology, or sixty six% to be devoted to rapeseed advent, to meet modern US warming and transportation needs. Microalgae are an emerging source for biodiesel manufacturing in recent years as because of higher biomass and lipid productiveness, and the dearth of opposition with meals plants for agriculture lands and sparkling water resources as they may be develop on non-arable land using saline or waste water (Abdelazi A.E. et al., 2013 Amaro H.M. et al., 2011 Leite G.B. et al., 2013). Microalgae are photoautotrophic sunlight-driven mobile factories which can convert carbon dioxide to

diverse merchandise along with lipids, carbohydrates, proteins, fatty acids, nutrients, antibiotics, and antioxidants (Chisti Y. 2007).

The lipid content has been elevated in many microalgae as a response to excessive way of life conditions which include CO2, nitrogen awareness and mild depth [Yoo et al., 2010]. For the fast accumulation of lipid, microalgae had been cultured in increase-restricting environment such as nitrogen depletion, (Illman AM.et al., 2000 Takagi M. et al., 2000 Li Y. et al., 2008), high mild depth (Khotimchenko SV. et ., 2005), low temperature (Renaud SM. et al., 2002), excessive salt awareness (Takagi M. et al., 2006) and high iron awareness (Liu ZY. et al., 2008]. It has been seen that carbon and nitrogen source changes highly influence the biomass and lipid technology of microalgae.

Various carbon sources including Glucose, Sucrose, Fructose, Starch, Maltose, Sodium Acetate and Glycerol and nitrogen sources such as Urea, Yeast Extract, Glycine, Potassium Nitrate, Sodium Nitrate, and Malt Extract had been used. The growth of these resources becomes recorded by taking absorbance at 680nm on exchange days and additionally through recording dry cell weight on alternate days. The biomass content material, lipid content and productiveness have been additionally calculated for each source. For improved biomass it's far vital to look how this higher biomass awareness may be executed. Various harvesting strategies had been used consisting of centrifugation, filtration, sedimentation, aggregate of these techniques and additionally the usage of natural and inorganic flocculants. Although it's miles recognized that *Chlorella minutissima* has high amount of oil present in it however it is also rich in other components. So different biochemical evaluation had been additionally accomplished along with Protein, Carbohydrate and Chlorophyll estimation. Eventually FAME evaluation confirmed how much percent of reputation become present in each the algae while various unique carbon and nitrogen assets have been used.

Objectives of this study:

Due to the difference in cell composition, it is very hard to finalise the universal method for processing of all microalgae for the extraction of commercial products. The difference creates a lot of problem to handle microalgae. Thus it is necessary to first work out potential method of processing of the concerned microalgae which will be utilised for the production of any commercial product in future. Based on this

concept, before the production and analysis of bio-diesel from microalgae *Chlorella minutissima* and *Scenedesmus* sp., potential method of each stage of biodiesel production was worked out. The objectives are:

- Identification of potential biomass yielding condition: using various carbon and nitrogen sources.
- Evaluation of different harvesting methods: Sedimentation, Filtration, Centrifugation, Flocculation
- Assessment of combination organic solvents using Folch method
- Growth analysis for algae in all culture conditions
- Biochemical analysis: Protein, Carbohydrate and Chlorophyll estimation
- Kinetic studies of the biomass utilized and lipid produced under all conditions
- Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass using GC-MS

REVIEW OF LITERATURE

Algae are huge, various organization of easy, autotrophic organisms that exist as unicellular and multi cell bureaucracy (Makareviciene Violeta. et al., 2011, Mayur M. et al., 2011). Their presence in nature and enormous role in fossil fuels formation is obvious from evolutionary research (Hannon Michael. et al., 2010). it's far into this era, that mankind is exploring algal power saved in the shape of triglycerides, to convert it into biodiesel (Chader S. et al., 2011, Makareviciene Violeta. et al., 2011). meals versus fuel conflicts with admire to agricultural plants (Chisti Y. et al., 2007), growing demand of fossil fuels (Sharma P. et al., 2013) and an alarming problem of worldwide warming are few of the fundamental challenges that can be addressed via algae.

Microalgal feedstock serves as an eco pleasant, carbon impartial, smooth and green gasoline (Barghbani R. et al., 2012). special algal species are being studied for fatty acid composition, (Hempel N. et al., 2012, Elumalai S. et al., 2011) dietary dietary supplements, antioxidants, natural dyes and animal feed (Sharma P. et al., 2013). Chlorella, The earth's oldest living organism, is a certainly present, pure entire food. it is recognized to reproduce asexually by using dividing 4 times each 20-24 hours. Transportation makes use of a giant degree of power and as the petroleum by-product make use of is expanding at a speedier pace the stores are draining quickly and may be depleted in below 45 years. in this way, the growing energy (gasoline) emergency and ecological debasement have represented an intense trouble to our viable development and survival.

So attention has now moved toward optional fills like biodiesel, bio-ethanol and biogas. amongst various resources of renewable electricity, microalgae have become more interest as liquid (biodiesel and bioethanol) and gaseous (biogas and hydrogen) resources of renewable fuels (Prajapati SK. et al.,2014). Microalgal feedstock serves as an eco pleasant, carbon impartial, clean and inexperienced fuel (Barghbani R. et al., 2012). excessive lipid profitability, no ordinary adjustments, much less water and land necessity and greater noteworthy photosynthetic productiveness, are points of hobby of microalgae which make it more sensible for bio gasoline creation. Improvement kingdom of microalgae affects both the biomass and lipid profitability along cost-adequacy. Biodiesel is company of unsaturated fats of ethyl or methyl ester produced the use of virgin or utilized vegetable oil (both palatable and non-eatable). basically utilized parts for biodiesel generation are soybean, rapeseed, canola or sunflowers which can be applied as a part of created countries.

In India the consumable oil request is higher than its actual technology. So there's no plausibility of redirecting this oil for era of biodiesel. The primary ware hotspots for biodiesel may be non-eatable oils gotten from vegetation which could supplant the local oil makers. there are numerous species, which endure seeds rich in oil content and out of those a few encouraging tree species had been identified. Those are Jatropha curcas (Ratanjyot), Pongamia pinnata (Karanja), Calophyllum inophyllum (Nagchampa or Polanga), Mahua, Castor, Seemarouba and so on (Sharma P. et al., 2015). making use of the ebb and go with the flow yields, huge measures of land and new water would be anticipated to deliver sufficient oil to absolutely supplant petroleum derivative use. it might require double the land location of the united states to accept to soybean creation, or 66% to be dedicated to rapeseed technology, to satisfy modern US warming and transportation wishes. utilizing the ebb and go with the flow yields from these plants, big measures of land and crisp water would be required to create sufficient oil to absolutely supplant the momentum petroleum product use.

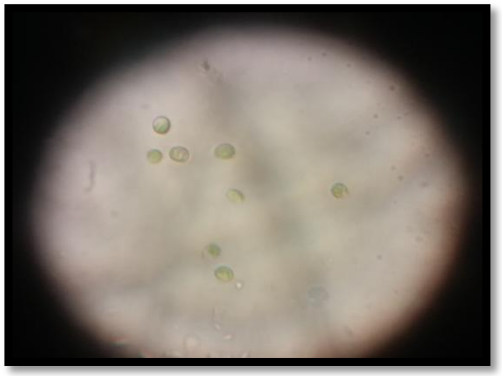
For this expanded quantity of biomass and lipid productiveness additional carbon and nitrogen resources have to be brought. Algae are companies of autotrophic and easy organism which are gift each in unicellular and multi-mobile bureaucracy. Majority of the microalgae are photoautotrophic in nature and may be cultivated either in open ponds or closed system consisting of image bioreactors the use of CO2 and mild found in ecosystem as carbon and electricity sources, respectively and also can grow in reasonably-priced carbon sources such as waste water in heterotrophic mode however this cultivation mode poses numerous dangers consisting of low biomass productivity, low lipid content and lengthy intervals of cultivation. Heterotrophic and mixotrophic cultures have been proposed as possible options for the production of biomass and cell lipid accumulation (Yu H. et al., 2009).

Heterotrophic development of microalgae includes the usage of herbal mixes as sole carbon and power resources. Mixotrophic cultures of microalgae have an edge over photoautotrophic cultures as they have got energy assets as natural carbon supply and light, they could concurrently drive photoautotrophic and heterotrophic to utilize both inorganic (CO2) and natural carbon substrates (Sun N. et al., 2008, Ip PF. et al., 2005). Consequently, microalgae cultivated below mixotrophic way of life synthesize compounds characteristic at high production rates of both photosynthetic and heterotrophic metabolisms of organic substrates are impartial of every other (Ogawa T. et al., 1981). Various carbon and nitrogen sources used were Glucose, Sucrose, Maltose, Fructose, Starch , Glycerol and Sodium acetate ; Urea, Potassium nitrate, Glycine , Yeast extract and Malt extract. Among the carbon assets Glucose is stated to show a

high quantity of biomass and lipid manufacturing and Urea is visible as a nitrogen source having high biomass and lipid production. on this have a look at the, various carbon and nitrogen assets are examined on Chlorella minutissima and Scenedesmus sp., if you want to acquire a high biomass and lipid producing mixotrophic medium. Several harvesting techniques have been additionally tested to increase the biomass accumulation such as sedimentation, centrifugation, filtration, etc. and all these experiments were done in batch flask cultures.

Chlorella minutissima

Chlorella is a genus of single-cell green algae belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 μm in diameter, and is without flagella. *Chlorella* contains the green photosynthetic pigments chlorophyll-a and -b in its chloroplast. It multiplies rapidly using carbon dioxide, light, water and minerals.



Chlorella minutissima is rich in amino acids and PUFA, especially Eicosapentaenoic corrosive which makes it valuable for wellbeing items and pharmaceuticals and furthermore has a quick development and a high PUFA content. In the past *Chlorella minutissima* has been thought to be utilized as a protein source to the total populace to tackle the issue of appetite because of two World Wars and the expanding total populace. It has popular uses such as:

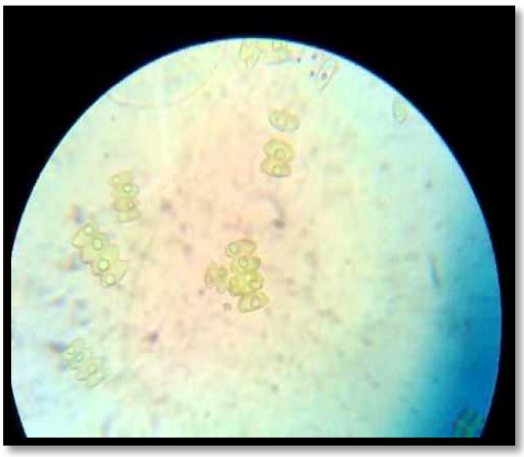
Health foods and pharmaceuticals

High substance of PUFA, especially Eicosapentaenoic which is vital for well evolved creatures as an operator to counteract blood platelet accumulation.

Reduction of Carbon Dioxide Pollution

Microalgae has substantially higher development rate than higher plants and can develop under high carbon dioxide fixations that is unacceptable for higher plants. *Chlorella minutissima* can develop under outrageous carbon dioxide fixations (0.036–100%). There is a solid increment in the miniaturized scale algal biomass amid photosynthesis when CO2 is high. Be that as it may, microalgae biomass increment additionally requires high light force. To maintain high intensity high cost is involved so it is used in small scale industries only.

Scenedesmus sp.



Scenedesmus is a family of green growth, in the class Chlorophyceae. They are frontier and non-motile. Scenedesmus is a standout amongst the most widely recognized freshwater green growth genera. They have different uses, for example:

- Bio fuel production

In spite of the fact that *Scenedesmus* is fit for delivering numerous sorts of bio fills, for example, bio-hydrogen, biodiesel, bio-ethanol and drop-in powers, most broad research has been done on the utilization of Scenedesmus for bio-diesel generation.

- Bio-hydrogen (H_2) production
- Bio-diesel production

In current investigations it has been seen that Scenedesmus lipid yield after improvement has come to ~60% dry cell weight, lower than some other green growth and is more proficient in catching CO2 than other green growth.

- Bio-ethanol

Scenedesmus, and other microalgae, for example, *Chlorella, Dunaliella, Chlamydomonas, and Spirulina,* contain a lot of sugar (>50% of the dry weight), which improve them alternatives for bio-ethanol creation.

- Drop-in fuels

Isoprenoids are viewed as imperative metabolites that can be used as drop-in fills, frequently as alkane chains. Scenedesmus conducts a pyruvate/glyeraldehyde 3-phosphate non-mevalonate pathway to incorporate isoprenoids.

- Wastewater management

In an examination the alkali and phosphorous expulsion proficiency by *Chlorella vulgaris* and *Scenedesmus dimorphus*. Scenedesmus from agro mechanical waste was thought about and it showed better productivity of evacuating smelling salts in tube shaped bioreactor while both green growth expelled phosphorus from the wastewater to a similar degree. Algal Turf Scrubber (ATS) is one of advancements that use green growth for treating assortment of squanders and contaminated waters.

Harvesting techniques

1. Centrifugation

Centrifugation is a procedure in which a divergent power is applied to enhance the partition of solids. Turning the suspension makes the weight distinction this is essential for molecule partition from the fluid suspension. maximum microalgae can be recuperated from the fluid juices making use of centrifugation. Radiating restoration can be rapid, but it's miles vitality concentrated. by using the with the aid of, centrifugation is a favored method for recuperating algal cells, especially to produce elevated time span of usability concentrates for aquaculture. There are five crucial styles of rotators to be precise, circle stack axes, punctured wicker bin axes, imperforated bushel axes, decanters (or parchment axes) and hydro typhoon. Centrifugation advancements are extra power critical and required significant introductory capital assignment.

2. Sedimentation

Sedimentation is a machine which is in most cases connected for isolating microalgae in water and waste water treatment. This procedure for the maximum element yields a wet, voluminous ooze, due to moderate settling restriction. Upgraded miniaturized scale algal reaping by means of sedimentation may be executed thru lamella separators and sedimentation tanks. Flocculation is as frequently as viable used to extend the skillability of gravity sedimentation. Talent of sedimentation is based on upon the thickness of algal debris. Milledge (Milledge JJ et al., 2013) reported a settlement rate of 0.1 m/d for Chlorella species in freshwater

3. Filtration

Filtration is a typically applied strategy for sturdy fluid partition. Filtration harvests small scale algal biomass through sifting the algal cells and shaping a thick glue and permitting the water to go through the channel. Filtration manner might be persistent or damaged. Filtration frameworks may be named microfiltration (pore length of zero.1–10 m), big scale filtration (pore size of >10 m), ultra filtration (pore length of zero.02–2 m), and switch osmosis (pore length of <0.001 m). Standard filtration might be lacking due for biomass recuperation. Layer microfiltration and ultra filtration are manageable contrasting alternatives to everyday filtration. Microfiltration, or smaller scale screening, is an crucial method for biomass recovery where algal cells are sifted via miniaturized scale displays to be isolated from the improvement way of life. This device calls for a weight comparison over the channel which can be pushed by way of vacuum, weight or gravity. the weight required to compel the liquid over the film diminishes because the pore size of the layer is extended. Milledge and Heaven (Milledge JJ et al., 2013) stated that the macro-filtration membranes can be used for large microalgae cells or if the algae cells are flocculated together. Uduman (Uduman N et al, 2010.) reported that the vacuum filtration harvesting technique is most suited for large microalgae cells (greater than 10 μm).

4. Flocculation

it's far a process, often related with the help of flocculating operators or flocculants (chemical compounds of feature or synthetic root), that causes the coagulation of algal cells into little clusters, called herds, enabling them to settle down effects in order that they can be evacuated. this is the principle degree within the mass reaping method this is proposed to total the smaller scale algal cells retaining in thoughts the cease goal to extend the powerful expulsion. There are two kinds of flocculation, automobile flocculation

and, synthetic flocculation. auto flocculation happens due to precipitation of carbonate salts with algal cells whilst in synthetic flocculation chemical substances (herbal and also inorganic) are brought to microalgae tradition to incite flocculation. Auto-flocculation does no longer manifest in all small scale inexperienced boom species and may moderate and inconsistent method. Compound flocculation takes place while exclusive natural and inorganic flocculants are applied. Inorganic flocculants carries Aluminum sulfate Al2(SO)3, Ferric sulfate Fe2(SO4)3, Ferric chloride FeCl3, Lime Ca(OH)2 though natural flocculants carries okra adhesive, chitosan, changed cationic chitosan-polyacrylamide, Greenfloc a hundred and twenty, blend of starch and chitosan. Aragon (Aragon AB et al., 1992) used aluminium sulfate to harvest a culture made up of Scenedesmus acutus (80%) and Chlorella vulgaris (20%) using a dosage of 30-50 mg/L at a pH of 6-6.5. Inorganic flocculants have detriments, for instance, a large convergence of inorganic flocculants is expected to motive sturdy fluid partition of the microalgae, sooner or later creating an extensive quantity of slop. a great flocculant have to be modest, secure, inexhaustible and compelling in low fixations.

Lipid extraction methods

There are various methods for lipid extraction and some have been listed below.

In the Folch strategy (Folch J. et al., 1957), microalgal glue was homogenized in a 2:1 chloroform:methanol (v/v) blend and cell trash was expelled by filtration. The homogenizer and gathered cell trash were flushed with new dissolvable blend and the wash was pooled with the past filtrate before the expansion of a 0.73% NaCl water arrangement, delivering a last dissolvable arrangement of 2:1:0.8 chloroform:methanol:water (v/v/v).

In the strategy for Bligh and Dyer (Bligh EG. et al., 1959) microalgal glue was blended with deionized water, chloroform, and methanol to achieve 1:2:0.8 sections chloroform:methanol:water (v/v/v) and homogenized. One section of chloroform was included and the blend was additionally homogenized. At that point, one section deionized water was added to the homogenate giving a last proportion of 2:2:1.8 chloroform:methanol:- water (v/v/v); the homogenate was re-homogenized lastly separated to expel cell flotsam and jetsam.

In the Selstam and O quist technique (Selstam E. et al., 1985), microalgal glue was homogenized in chloroform and a 4:1 blend of methanol and 0.73% NaCl water arrangement delivering a 1:2:0.5

chloroform:- methanol:water (v/v/v) framework. The homogenate was sifted and the homogenizer and cell flotsam and jetsam washed with crisp methanol-water blend and chloroform bringing about 2:3.6:0.9 sections chloroform:- methanol:water (v/v/v) as the flush was gathered to the past filtrate. At long last, more chloroform and 0.73% NaCl water arrangement were added to give a proportion of 1:1:0.8 chloroform:methanol:water (v/v/v).

Protein estimation from algae

Protein is an essential segment of green growth and records to up to a large portion of the dry weight of green growth. Miniaturized scale algal proteins can possibly be utilized as creature nourish or human utilization, recombinant protein innovation and to be utilized as a part of bio fuel generation. Regardless of this potential, issues exist no sweat at which algal proteins can be removed from a few strains, for example, Chlorella, which has hard cell dividers and makes it hard to separate lipids. Procedures that can be utilized to quantify protein content quickly.

In lyophilized material incorporate Dumas and Kjeldahl strategy to gauge N-content. Protein can likewise be evaluated from add up to amino acids by HPLC.

Carbohydrate estimation from algae

Starches comprise of a vast bit of the algal biomass constituents and are hence a critical computation for algal estimations. Thus a precise strategy is required which could evaluate the measure of starch content in green growth. Most as often as possible utilized strategy for its estimation is phenol sulfuric corrosive that is thought to hydrolyze and respond with the sugars in the arrangement. However, there is issue that every one of the starches won't not act similarly to this extraction system. Different techniques that can be utilized are Anthrone strategies, consecutive hydrolysis of sugar polymers in green growth and distinguishing proof and evaluation of monomers through fluid HPLC or gas chromatography which is a precise strategy and starch estimation can be utilized by measuring alpha 1,4 connected glucan from a particular enzymatic hydrolysis step yet this is absent in all strains of green growth.

Chlorophyll and carotenoid estimation from algae

Photosynthetic Pigments are the materials with very distinctive chemical structure; they're gift inside the shape of porphyrin pigments (chlorophyll a, b and c), carotenoids, anthocyanins and flavones (Britton G. et al., 1983 Brown S.B. et al., 1991 Costache M.A. et al., 2012). general leaf pigment includes chlorophyll-a, chlorophyll-b and carotenoids which might be important for photosynthesis process. range in leaf sunglasses (chlorophylls and carotenoids) and its connection can be due to internal additives and natural situations. For extracting those pigments it is vital to choose the satisfactory solvent so that amount of pigments may be extracted and can be analyzed spectrophotometrically (Nayek S. et al., 2014).

Acetone offers very sharp chlorophyll absorption peaks and has remarkable merit because the solvent for assay of chlorophylls (Ritchie R.J. et al., 2006). but acetone is not an appropriate solvent for extraction; and from time to time a bad extractant of chlorophyll from many vascular plants and a few algae together with Scenedesmus, Chlorella and Nannochloris (Sartory D.P. et al., 1984 Jeffrey S.W. et al., 1977). Acetone is volatile, exceedingly inflammable, is narcotic in high concentrations and is a pores and skin irritant (erythema).

Methanol is a very good extractant for chlorophylls, specifically from recalcitrant vascular plant and algae (Porra R.J. et al., 1991, Porra R.J. et al., 1989, Porra R.J. et al., 2002). it is less risky and flammable than acetone but is notoriously toxic. it's far an insidious poison because it's miles with no trouble absorbed by using inhalation and thru the pores and skin and so must no longer be used in a teaching laboratory. Methanol slowly fogs polystyrene spectrophotometer cuvettes main to fake readings and can't be used in any respect with polystyrene and polymethylacrylates (PMMA) cuvettes.

Ethanol is taken into consideration as drastically greater at ease dissolvable than both CH3)2CO or methanol however isn't always applied all the time for the measure of chlorophylls. Albeit flamable it isn't extraordinarily deadly and is suitable for use in an teaching research facility. Ethanol does now not attack polystyrene for that reason polystyrene plastic spectrophotometer cuvettes can be make use of Ethanol is considered as much more secure solvent than both acetone or methanol however isn't used very often for the assay of chlorophylls although equations for chlorophyll-a and chlorophyll-b are available (Lichtenthaler H.k. et al., 1987, Wright S.W. et al., 1977, Rowan et al., 1989). although

flammable it isn't always very poisonous and is appropriate to be used in a coaching laboratory. Ethanol does not assault polystyrene and so polystyrene plastic spectrophotometer cuvettes can be used.

MATERIALS AND METHODS

The aim of this project is to analyse the Fatty acid methyl esters (FAME) profile, biomass and lipid productivity of high lipid yielding culture conditions of microalgae *Chlorella minutissima* and *Scenedesmus* sp. But in order to a get a high yield of lipid, we need to evaluate not only the potential lipid yielding conditions but also to identify the best methods of processing of microalgae such as change in solvent for lipid extraction and harvesting methods so that loss of lipid during extraction process can be avoided or to some extent reduced. Thus the project was designed in such a way that potential methods can be used for maximum lipid extraction and further processed for FAME analysis by GC-MS.

Therefore the project was designed as:

- Culture collection and growth
- Identification of potential biomass yielding culture conditions
- Evaluation of different harvesting methods
- Assessment of combination organic solvents using Folch method
- Growth analysis for algae in all culture conditions
- Biochemical analysis
- Kinetic studies of the biomass utilized and lipid produced under all conditions
- Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass

The microalgae were scaled up in BBM medium so that culture for setting various experiments can be maintained in the laboratory. Final pH of BBM medium was 6.8. Scale up was done using 10 ml sample in 1000ml Erlenmeyer flasks by transferring the culture subsequently to a higher volume on weekly basis. The culture was scaled up at 27-29˚ Celsius with a white light of 40 W three tubes. The culture was kept in 12L: 12D photoperiod and manually agitated twice per day. No carbon source has been provided during scale up i.e. photoautotrophic nutrition. Details of different materials and methods used in above mentioned stages are as follows:

1. Culture collection and growth:

Pure cultures of *Chlorella minutissima*, *Scenedesmus* sp., were collected from IARI and The Energy and Resources Institute (TERI), Delhi (India). These species after collection were sub cultured on BBM agar media plates under laboratory conditions (with initial pH of 6.8) and at 25 ˚Celsius under (~ 1935 lux)

light intensity and 12/12 light dark cycle. All the strains were transferred from agar plates to the medium and incubated under the same conditions of temperature and light as in a reactor. Liquid cultures were used as the inoculums for the designed experiments.

2. **Identification of potential biomass yielding conditions**:

Microalgae *Chlorella minutissima* and *Scenedesmus* sp. has been cultivated in 2 different culture conditions. A brief detail of these culture conditions is mentioned in Table -1. Except the variation in parameter, all the culture conditions were same and as follows : Culture medium-BBM, pH-6.8, temperature – 24-26^{o}C, light color- white, 1935 lux, light period- 12L:12D, shaking – manual and twice per day and carbon source- Nil. All experiments of cultivation of microalgae were performed separately in Erlenmeyer flasks with concerned volume of culture medium and 0.5-1% of inoculums of second week master culture. The culture was maintained till the viability of algal biomass. The details of cultivation of microalgae *Chlorella minutissima* and *Scenedesmus* sp. in different Nitrogen and Carbon sources are mentioned in Table-1. After the completion of cultivation, biomass of each experiment was harvested separately and after drying it was measured and stored for further analysis.

Table 1: Details of cultivation of microalgae *Chlorella minutissima* and *Scenedesmus* sp. in different Nitrogen and Carbon sources

S.NO	PARAMETER	VARIANT/S
1	Media with carbon source variation	Glycerol, Sucrose, Glucose, Sodium acetate, Fructose, Starch, Maltose all in 0.5 g/L concentration with Nano3 , as a nitrogen source with concentration same as in BBM.
2	Media with nitrogen source variation	Glucose (0.5/L) as a carbon source with Urea,KNo3,NaNo3,Glycine,Yeast extract, Malt extract all having 0.25 g/L concentration

Table 2: BBM media composition

COMPONENT	CONCENTRATION
$NaNo_3$	0.25g/L
$MgSo_4.7H_2O$	0.075g/L
NaCl	0.025g/L
K_2HPO_4	0.075g/L
KH_2PO_4	0.175g/L
$CaCl_2.2H_2O$	0.025g/L
COMPONENT	**CONCENTRATION**
$ZnSO_4.7H_2O$	8.82g/L
$MnCl_2.4H_2O$	1.44g/L
MoO_3	0.71g/L
$CuSO_4.5H_2O$	1.57g/L
$Co(NO_3)_2.H_2O$	0.49g/L
COMPONENT	**CONCENTRATION**
Vitamin B_1(Thiamine HCl)	0.1g/100ml
Biotin	0.025g/100ml
Vit B_{12}	0.015g/100ml
H_3Bo_3	1.14g/100ml

3. Evaluation of different harvesting methods:

For finding the potential method of harvesting for microalgae *Chlorella minutissima* and *Scenedesmus* sp., first all 1000ml, algal culture was cultivated for 45 Days in following standard culture conditions:

- **Medium:** BBM
- **Light intensity:** 1935
- **Light colour:** White
- **Light period:** 12L:12D
- **Organic carbon source:** Nil
- **Culture vessel:** Flask (2000ml)

- **Shaking :** Manual Shaking(thrice per day)

After 45 days of cultivation, the culture has been divided for different experiments of harvesting. Triplets of 12 ml have been used for one experiment and following methods of harvesting have been tested:

- Sedimentation
- Filtration
- Organic flocculent I(Chitosan)
- Organic flocculent II(Starch)
- Inorganic flocculent I(Ferric Chloride)
- Inorganic flocculent II(Ferrous Sulphate)
- Inorganic flocculent III(Aluminium Sulphate)
- Inorganic flocculent IV (Ferric Sulphate)
- Centrifugation
- Combination of methods

1. Sedimentation: 12 ml of culture has been taken into three 15ml falcons and kept for 24 hours. The biomass settled in 24 hours and the supernatant media was removed and the sedimented biomass was transferred in a pre-weighted Petri plate for drying. After drying, weight of the Petri plate with biomass was taken with the help of analytical balance.

2. Flocculation : Following chemicals were used separately in triplets of 12 ml culture:

- Inorganic Flocculants: 14mg of Ferric Chloride, Ferrous Sulphate, Ferric Sulphate, Aluminium Sulphate and Aluminium Chloride was used separately in each sample at this stage.
- Organic Flocculants: 3 mg of Chitosan and Starch were used separately in each sample at this stage.

After addition of flocculants in separate tubes, the cultures were kept for 7-8 hours(if kept more than that, the biomass cells start to die). After 7-8 hours, the supernatant was removed and flocculated biomass was transferred in pre-weighted Petri plate for drying. After drying, weight of the Petri plate with biomass was taken with the help of analytical balance. The amount of flocculant was subtracted from the final weight of the biomass.

Filtration: Whatman filter paper 1 with the pore size of 11μm was used for the filtration of microalgae. 12ml of culture was taken in three falcons and used for filtration. The filter paper was pre-baked in the

hot air oven for 10 min at 70 degree Celsius and then the weight was taken. The cultures were filtered and the filter paper was dried and weighed with biomass.

3. Centrifugation: Triplets of 12 ml of culture were used for this experiment. The cultures were centrifuged at 4000 rpm for 5 minutes in 15ml falcon tubes. After centrifugation, supernatant was pipette out and the biomass was transferred in pre-weighted test tubes for drying and final weighing with biomass.

4. Combination of methods: For expectation of better results, different methods have been combined. Following two such combinations were tested:

- Sedimentation + Centrifugation: In this combination, triplets of 12ml of culture were first sedimented for few hours and then the sedimented slurry was centrifuged at 4000 rpm for 5 minutes. Then the biomass was transferred and analysed similar to centrifugation experiment mentioned above.
- Sedimentation + Filtration: In this experiment, again the culture was sedimented and filtered with pre-weighted filter paper. Then the biomass was taken same as experiments mentioned above.

4. Assessment of combination organic solvents using Folch method:

Lipid was extracted by using Folch method (Folch et al. 1957). As per this method, a weighted amount of biomass i.e. around 250 mg was taken and 5 ml chloroform/methanol (2:1 v/v) was added and vortex for 30 s. This was followed by keeping the mixture for 15–20 min at room temperature. The mixture is then centrifuged at 4000 rpm for 10 min to isolate cell trash from supernatant. This supernatant was washed by 0.9 % NaCl arrangement/water and vortex for few moments. The mixture was centrifuged at 3000 rpm for 5 min. Lower chloroform layer with lipid was evacuated deliberately and gathered in 20 ml pre-weighted glass vial. The biomass was re-extricated with 2.5 ml chloroform/methanol (1:1 v/v) same as expressed above until biomass is white. The supernatant was gathered in same vial. The supernatant was then air dried until consistent weight of lipid was accomplished. The lipid content was figured gravimetrically. The standard solvent system was changed with different solvent systems such as:

1. Solvent system 1: Dichloromethane: Methanol (Bligh and Dyer Method)
2. Solvent system 2: Chloroform: Methanol (Folch method)
3. Solvent system 3: Hexane: Isopropanol

4. Solvent system 4: Cyclohexane: Isopropanol
5. Solvent system 5: Hexane: Ethanol
6. Solvent system 6: Petroleum Ether

Dry biomass calculation:

- Bake the Petri dish in oven at 80˚C for 1 hour
- Weight the dish once it is at normal temperature
- Centrifuge sample at 4000 rpm, 5 minutes
- Pour the centrifuged biomass (free from media) into the petri dish
- Bake the dish with biomass again at 60-70 ˚ C until it is dry.
- Posts weigh the dish once biomass is dry.
- Calculate biomass weight by subtracting Pre-weight from Post weight.

5. Sudan Test :

After completion of extraction of lipid, it was also confirmed whether the extracted material is actually the desired lipid or not. For this conformation, Sudan test, Sudan IV dye is used. This dye is not soluble in water however soluble in lipids. 100 ml stock solution of 1mg/ml concentration of Sudan dye was prepared. Lipid sample was prepared by dissolving the extracted lipid in ethanol for each case of above mentioned experiments. For confirmation of lipid, 5ml water is added in a test tube and then the lipid sample dissolved in ethanol was added in to it very slowly. Due to difference in chemical nature , two different phases were formed. Upper phase was lipid phase and lower phase was water. The n20 drops of Sudan IV dye were added slowly with the help of micropipette. The dye was absorbed by the lipid available in upper phase and rest of it was settled in the bottom of the test tubes. This retention or absorbance of dye by the upper phase confirmed that the extracted material was lipid.

6. Growth analysis for algae in all culture conditions

The growth analysis of algae was done by taking reading on alternate days using spectrophotometer and also by calculating biomass weight of the algae on alternate days.
The spectrophotometric reading was taken at 680nm for 2 weeks and the biomass mass weight was taken accordingly:

Biomass weight: W_2-W_1

W_2= post weight after drying the biomass at 80ºC

W_1= pre weight without the biomass at 80˚C

7. Biochemical analysis

Various analyses such as Protein estimation, Carbohydrate estimation and Chlorophyll estimation were done using various standardized methods.

Chlorophyll estimation:

Accurately weighted 0.2g of fresh algae sample (dry biomass) was taken and different extraction solvents (5ml) were added to it. This sample mixture was centrifuge at 4500 rpm for 10 min at room temperature. Repeat this step until the biomass is white. If the solution is concentrated, then 0.5 ml of sample and 0.5 ml of solvent is added. The solution mixture was analyzed for Chlorophyll-a, Chlorophyll-b and Carotenoids content in spectrophotometer (Eppendorf Bio-spectrophotometer). The equation used for the quantification of Chlorophyll-a, Chlorophyll-b, and Carotenoids by different extractant solvents are given in Table 3

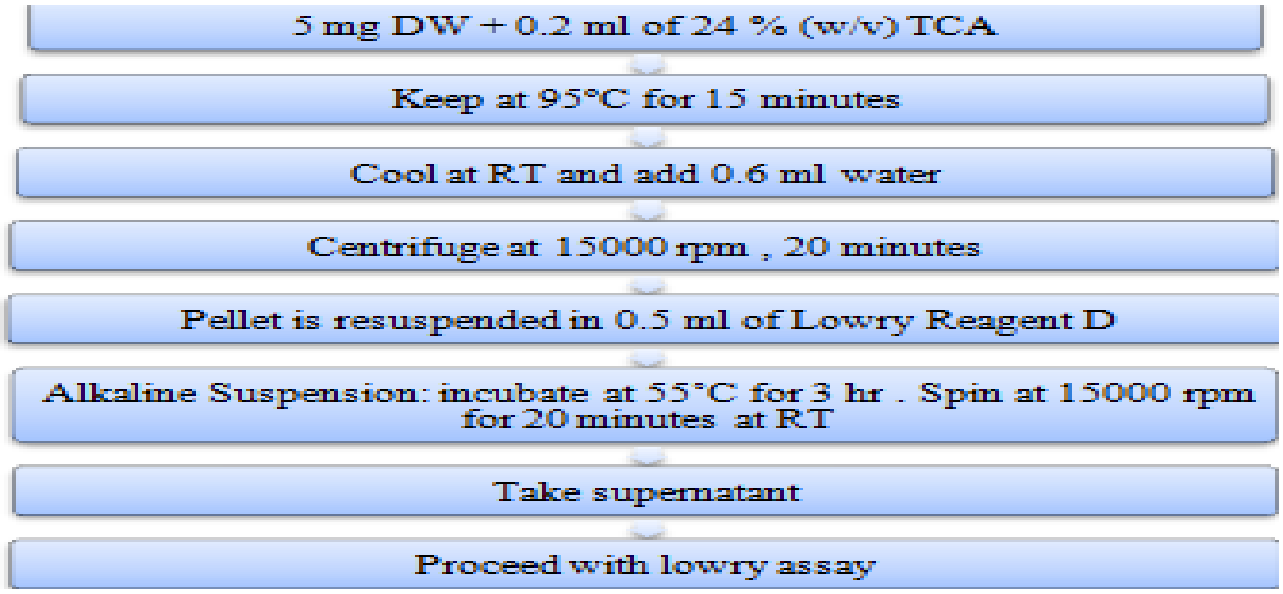
Table-3 Equations to determine concentrations (µg/ml) of chlorophyll a (Ch-a), chlorophyll b (Ch-b) and total Carotenoids (C x+c) by different extractant solvents in spectrophotometer

Solvents	Equations/Formula
80% Acetone	$C_{h\text{-}a}=12.25A_{663.2}-2.79A_{646.8}$ $C_{h\text{-}b}=21.5A_{646.8}-5.1A_{663.2}$ $C_{x+c}=(1000A_{470}-1.82C_a-85.02C_b)/198$
95% Ethanol	$C_{h\text{-}a}=13.36A_{664}-5.19A_{649}$ $C_{h\text{-}b}=27.43A_{649}-8.12A_{664}$ $C_{x+c}=(1000A_{470}-2.13C_a-97.63C_b)/209$
Dimethyl-sulphoxide (DMSO)	$C_{h\text{-}a}=12.47A_{665.1}-3.62A_{649.1}$ $C_{h\text{-}b}=25.06A_{649.1}-6.5A_{665.1}$ $C_{x+c}=(1000A_{480}-1.29C_a-53.78C_b)/220$
Methanol	$C_{h\text{-}a}=16.72A_{665.2}-9.16A_{652.4}$ $C_{h\text{-}b}=34.09A_{652.4}-15.28A_{665.2}$ $C_{x+c}=(1000A_{470}-1.63C_a-104.96C_b)/221$

Protein estimation:

Protein estimation is done using TCA method followed by Lowry's method.

TCA method is explained below:



Lowry reagent A: 0.1 N NaOH + 2% Na_2CO_3

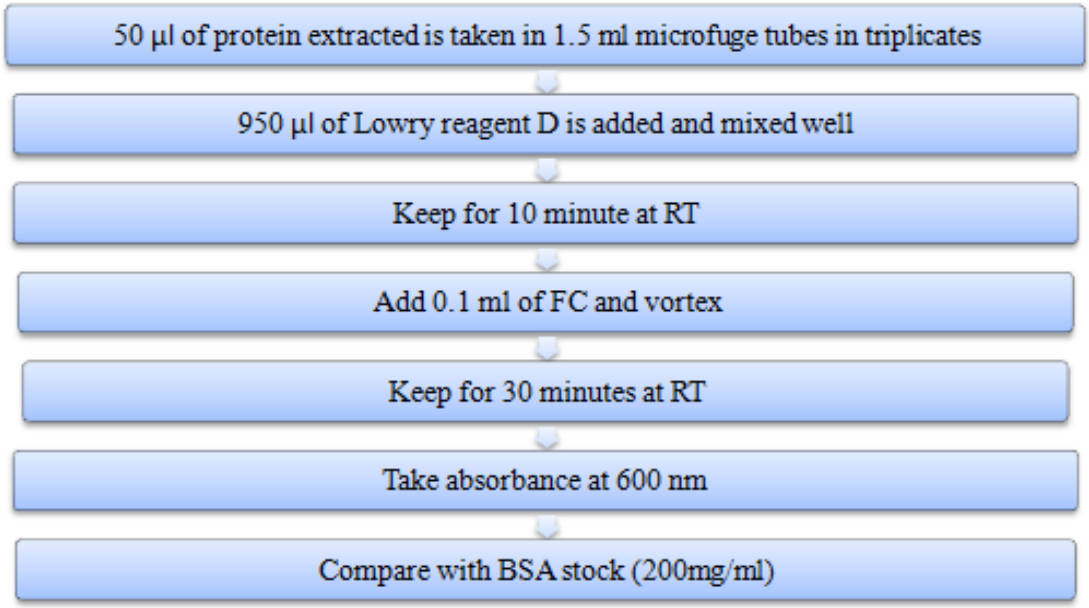
Lowry reagent B :1% sodium potassium tartarate

Lowry reagent C: 0.5 % Copper sulphate

Lowry reagent D: 48(A) + 1(B) + 1(C)

Folins reagent: 1:1

Lowry assay:



Carbohydrate estimation:

This is done by Anthrone method. The sample is centrifuged and the biomass is collected. 100 mg of sample in 5 ml of 2.5N HCl is kept for 3 hr in water bath. Further it is kept to cool at RT. Sodium carbonate is added into the sample until effervescence ceases. Make up to 100 ml and centrifuge at 4000 rpm for 5 min. Take supernatant in following volumes (0.2, 0.4, 0.6, 0.8 and 1.0 ml)

Stock solution: Glucose standard (1mg/ml)

Standard preparation: 0.2 ml – 1.0 ml of working standard

- To this working standard water is added upto 1 ml.
- Then add 4 ml Anthrone reagent and keep in boiling bath for 8 minutes.
- Keep for cooling and take absorbance at 630 nm.
- Compare with standard

8. Kinetic studies of the biomass utilized and lipid produced under all conditions:

Microalgae growth was observed by measuring the optical density at 680nm (OD680) using UV-visible spectrophotometer (Eppendorf) alternatively and related to algal biomass (g/l). For biomass estimation, 6 ml sample containing algae is centrifuged at 4000rpm for 5 minutes and the pellet is washed to remove the media. Petri plate is baked initially in order to weigh the biomass and dry wt. of algae is determined gravimetrically. 120 ml of sample was taken for measuring the lipid yield, productivity, biomass yield and productivity.

The relationship developed between OD680 and biomass (g/l) is given as follows:

$y=0.3942\times OD680 +0.0188$ $(R2=0.997)$

Where y is algal biomass in g/l and OD680 is optical density of culture at 680 nm

The maximum specific growth rate (μmax day-1) was calculated as follows:

$\mu max\ (day\text{-}1) = (\ln X2 - \ln X1)/ (t2-t1)$

Where X1 and X2 were the dry biomass weight (g/l) at time t1 and t2 respectively.

The doubling time (TD, days) was calculated as follows:

$TD\ (days) = \ln(2)/ \mu max$

Lipid content (C_{lipid}) = (Weight of lipid/ Weight of sample) x 100

Lipid productivity (mg/L/day) = (C_{lipid} x DCW)/t

Lipid yield (mg/L) = [Weight of lipid/Volume of sample] x 1000

Biomass yield or Dry Cell Weight (mg/L) = [weight of dry sample (mg)/weight of culture (ml)] x 1000

Biomass Productivity (mg/L/day)= [(final weight initial weight)/Volume of sample(ml) x Days] x 1000

9. Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass

Based on the results of potential lipid yielding culture conditions experiment was set for FAME production and analysis.

5-10ml of algae grown under various conditions is taken and centrifuged in order to obtain the biomass and remove all the media from it. Further for FAME analysis the procedure followed was:

- Harvest 5-10 ml microalgae to the screw cap glass tube in accordance to the density of your material.
- Add 1ml of 2% Methanolic-HCl
- Cap tube with Teflon lined caps tightly
- Incubate at 80 degree Celsius for 1 hr.
- Add 1 ml of 0.9% NaCl in water
- Vortex
- Add 2ml Hexane (GC or HPLC Grade)
- Vortex(1min)
- Spin at 2000 rpm for 2 min for phase separation
- Pull hexane (upper phase) in fresh tube using pipette
- Dry under N_2 flow
- Add 50 μl Hexane

- Inject 1 µL into GC

Once the analysis is done all fatty acids are calculated and tabulated in order to find out the percentage of MUFA, PUFA and Saturated fatty acids.

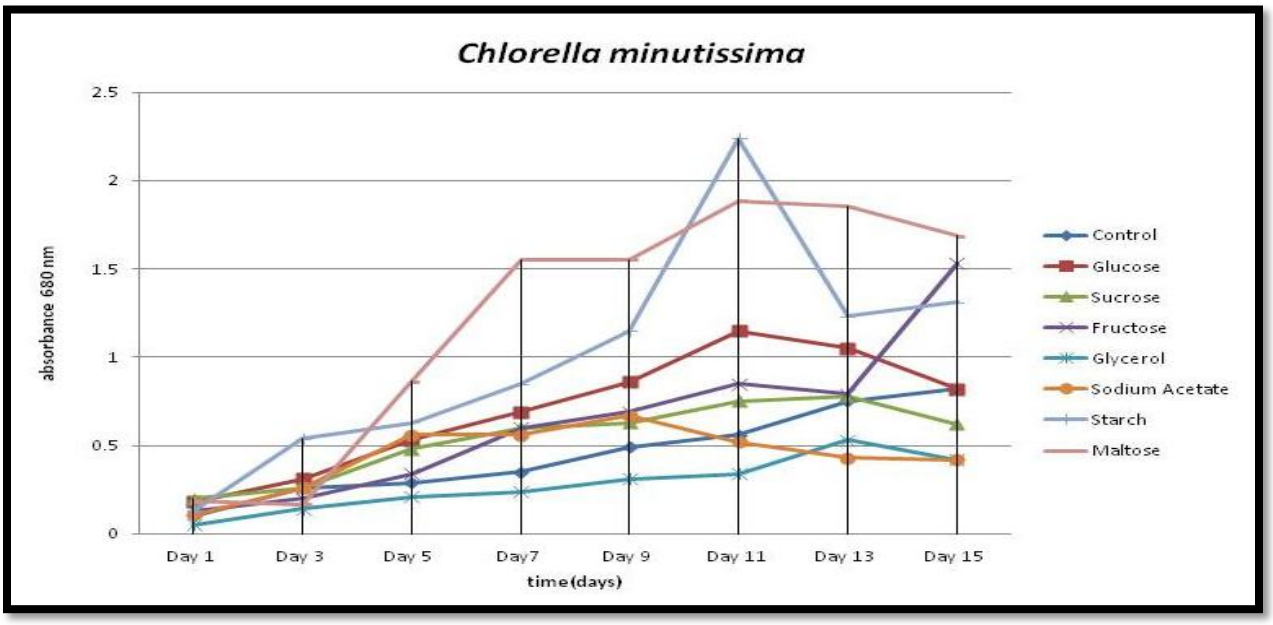
RESULTS AND DISCUSSION

GROWTH CURVE

Potential of microalgae for the production of high and low value commercial products is very huge but effect of environmental stress effect of environmental conditions on productivity is a great hurdle. The environmental stress affects the biomass productivity as well as final productivity of commercial product. So identification of potential culture conditions is primary requirement of algal cultivation. Keeping this in mind various Carbon and Nitrogen sources were used to see which source is better for lipid production.

Chlorella minutissisma

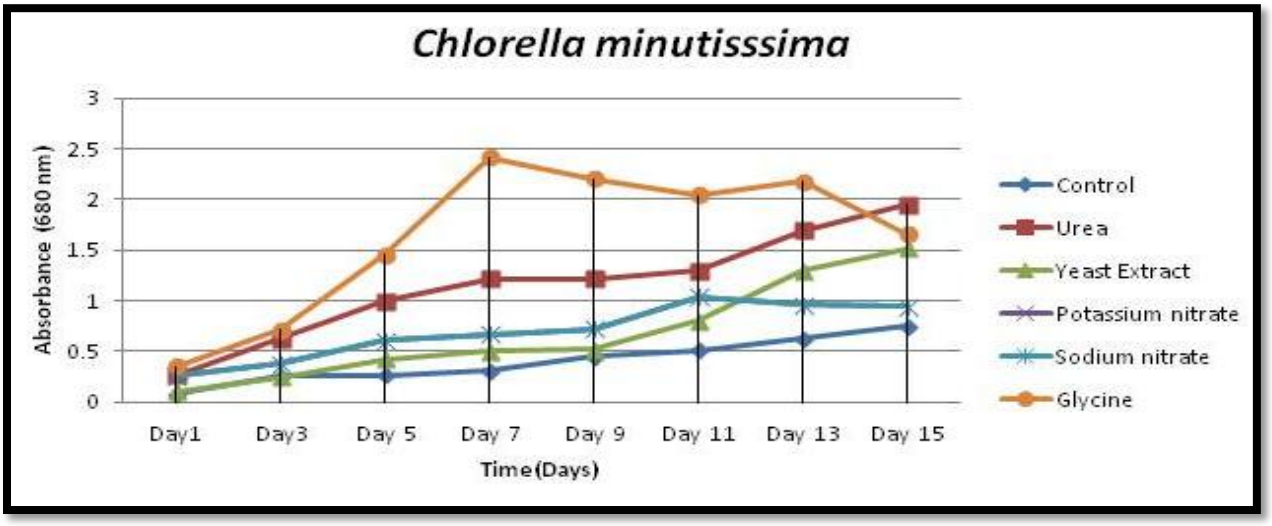
Carbon Sources Figure 1. *Chlorella minutissima* growth curve using Carbon sources



In case of carbon sources used in *Chlorella minutiisma* Maltose, Glucose, Sucrose and Fructose show a better growth than the control but Maltose and Glucose show the best growth curve. Sodium acetate did show good results upto 7 days but after that it started to decline.

Nitrogen sources

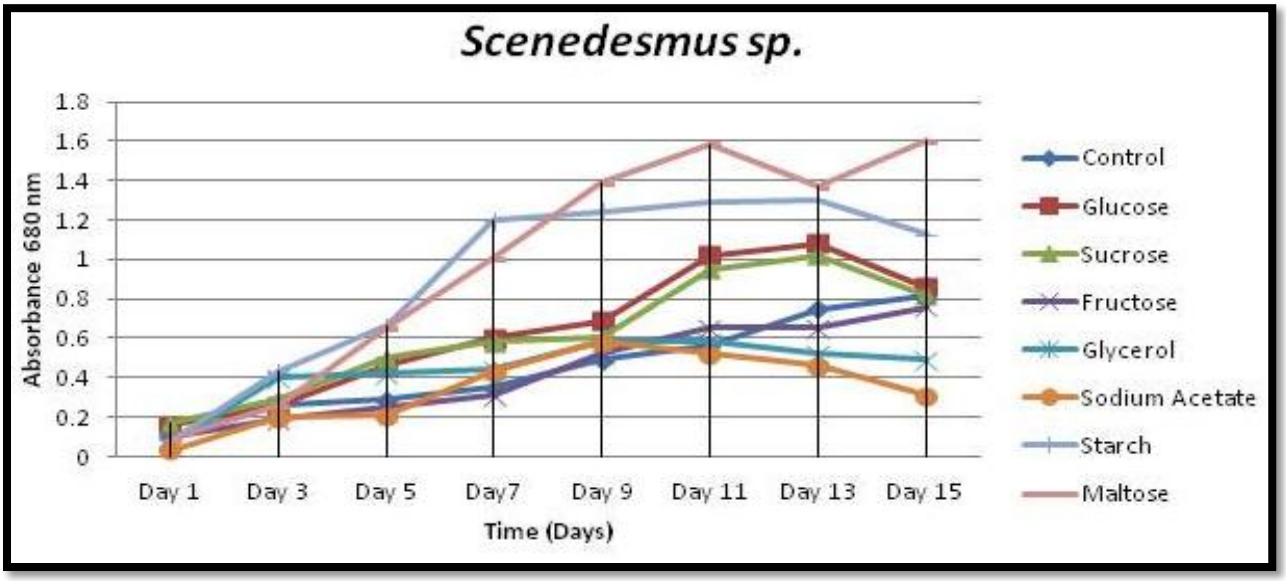
Figure 2. *Chlorella minutissima* growth curve using Nitrogen sources



In case of nitrogen sources, every component shows a better and increased growth than the control but Urea and Yeast extract have the proper growth curve and tend to increase even after 14 days.

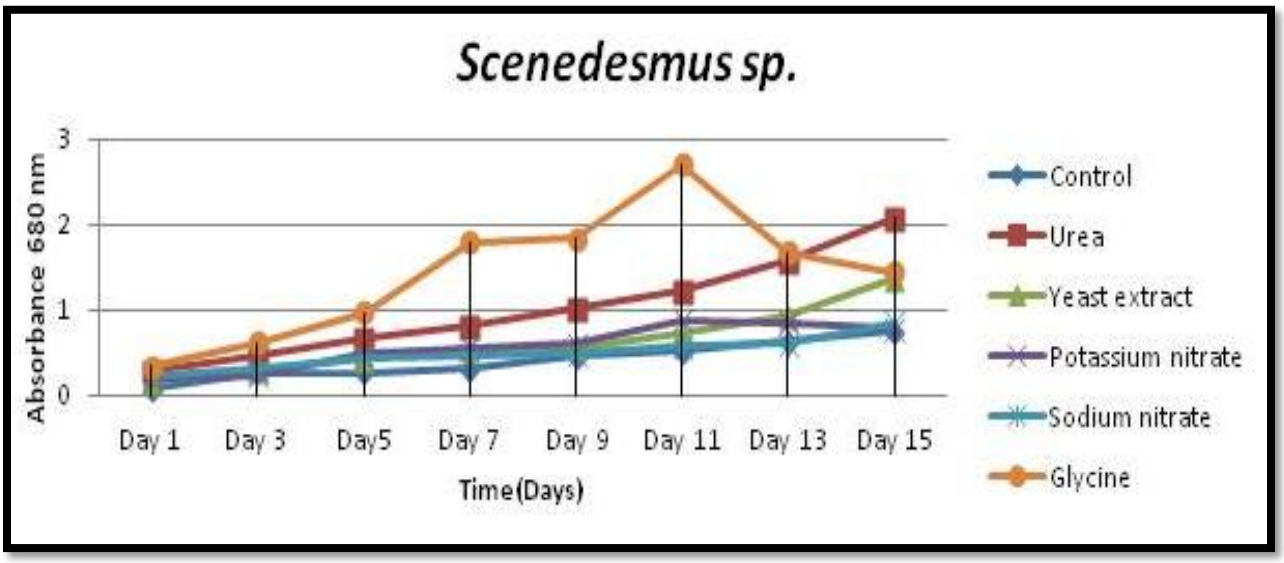
Growth curve of *Scenedesmus sp.*

Carbon Sources Figure 3.*Scenedesmus sp*. growth curve using Carbon sources



In case of carbon sources, all except Sodium acetate Glycerol and Fructose show a better and increased growth in algae than control. But best growth is seen in Maltose, Glucose and Sucrose.

Nitrogen sources Figure 4. *Scenedesmus sp.* growth curve using Nitrogen sources



In case of nitrogen sources Urea and Yeast extract show and increasing growth and tend to increase even after a time frame of 7 days.

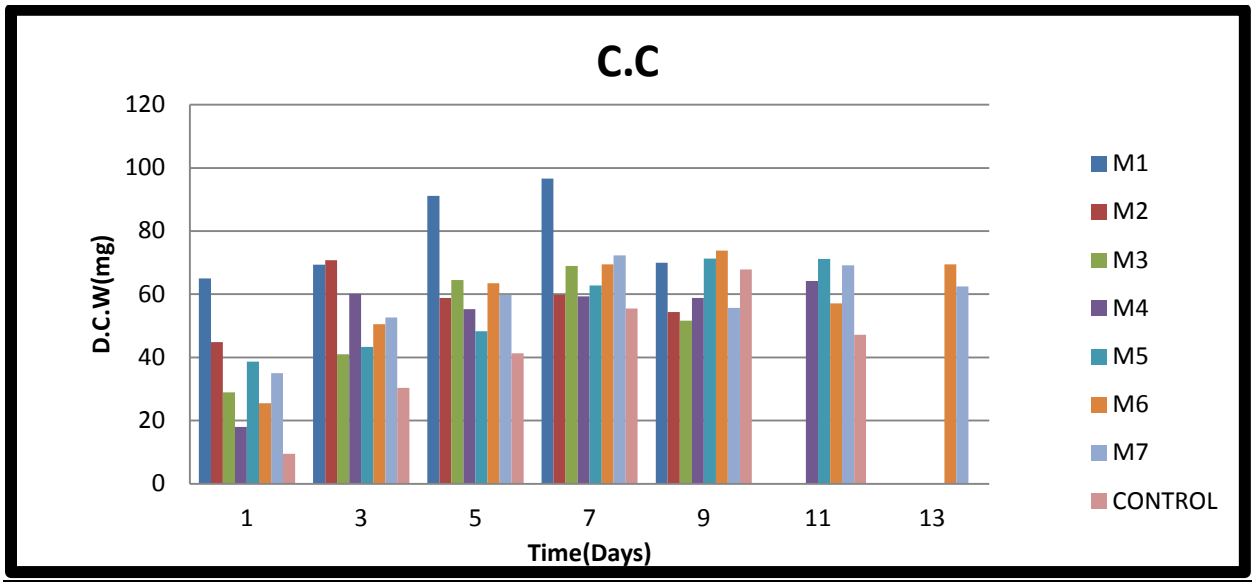
Malt extract was also considered as a nitrogen source but it showed no growth after 3 days, i.e the algae grew only for 3 days and after that it started to go into death phase. This was repeated in triplicates and still the same result.

Figure 5: Algae cultures of *Chlorella minutissima* and *Scenedesmus* using various sources

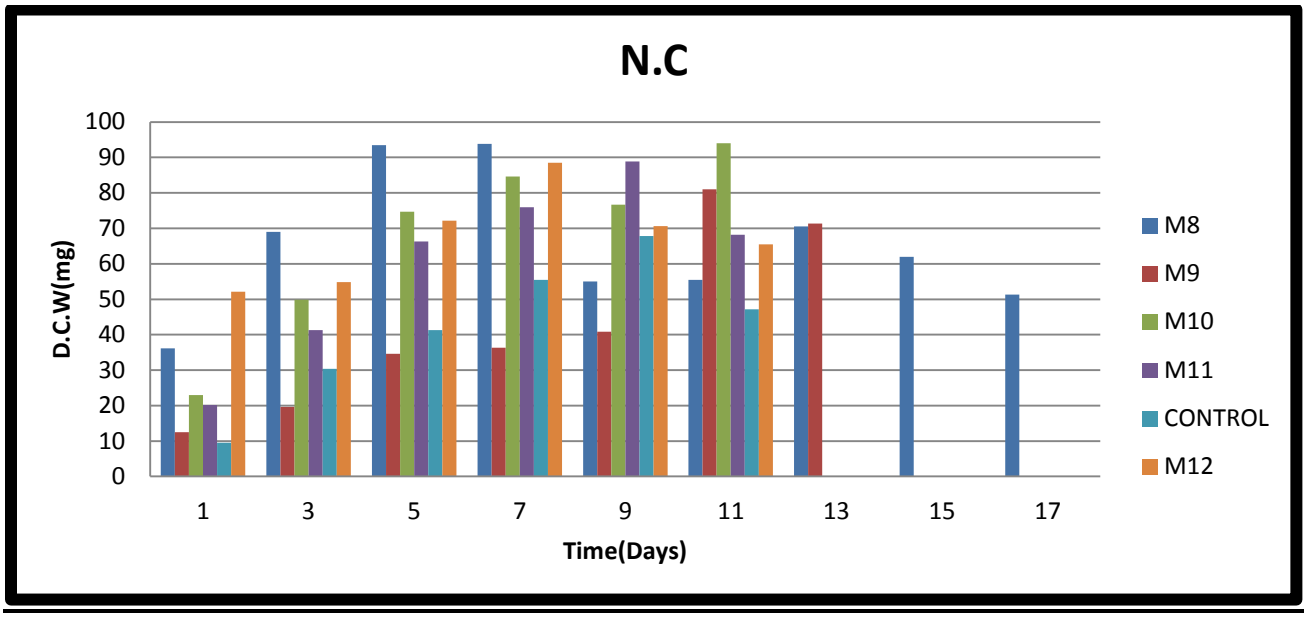
BIOMASS YIELD

Chlorella minutissima

Carbon Sources Figure 6 Biomass yield in *Chlorella minutissima* using Carbon sources

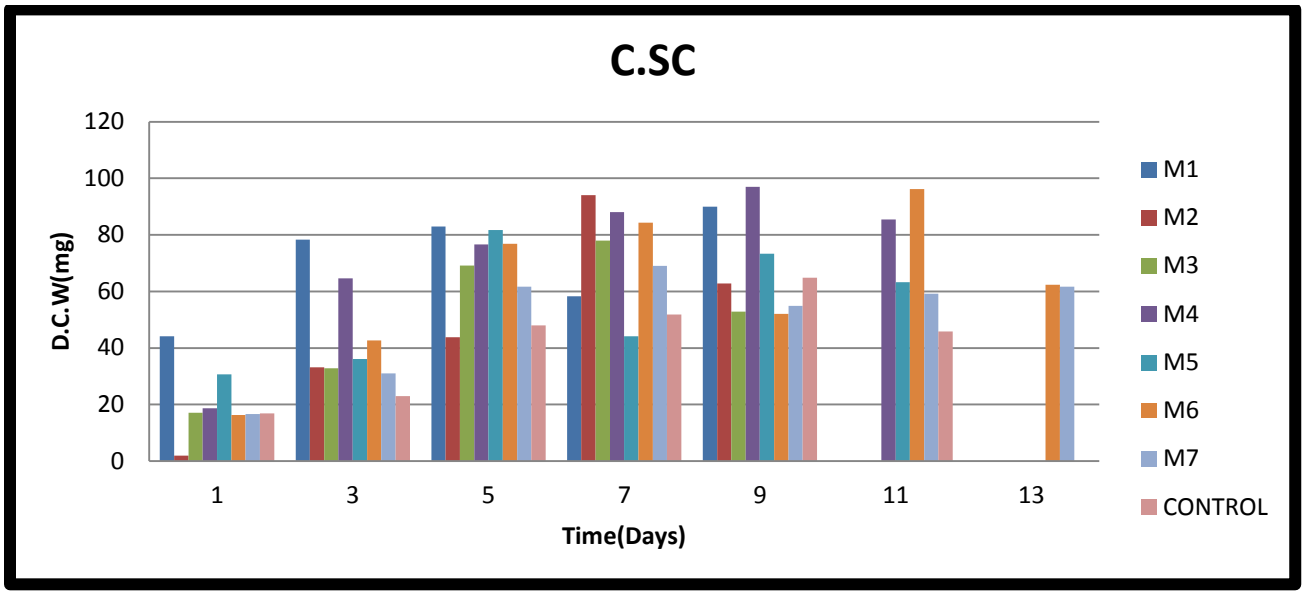


Nitrogen sources Figure 7 Biomass yield in *Chlorella minutissima* using Nitrogen sources

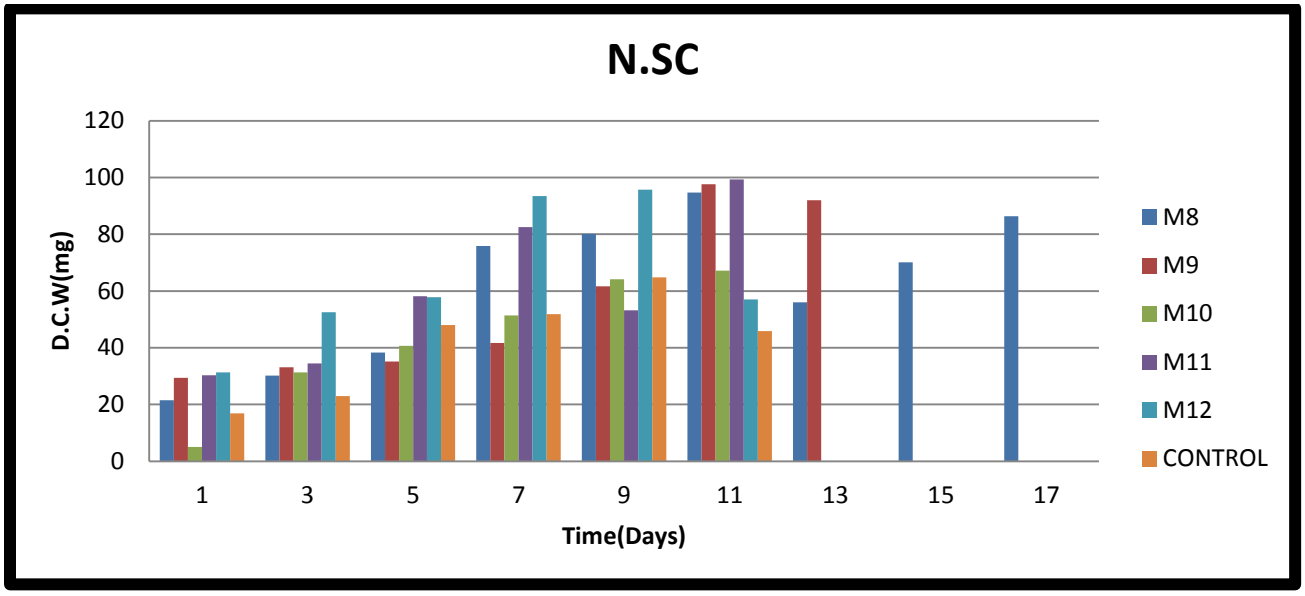


Scenedesmus sp.

Carbon sources Figure 8 Biomass yield in *Scenedesmus sp.* using Carbon sources



Nitrogen sources Figure 9 Biomass yield in *Scenedesmus sp.* using Nitrogen sources



Where, M1= Sucrose

M2= Glucose

M3=Starch
M4=Fructose
M5=Maltose
M6=Glycerol
M7=Sodium Acetate
M8=Urea
M9=Potassium Nitrate
M10=Glycine
M11=Yeast Extract
M12=Sodium Nitrate

LIPID YIELD AND PRODUCTIVITY

Firstly the lipid productivity for *Chlorella minutissima* and *Scenedesmus sp*. was checked for the best solvent and the further lipid extraction was done accordingly.

DRY BIOMASS *Chlorella sp.*

Solvent System	Lipid Content (%)
S1	8.42
S2	9.42
S3	8.5
S4	8.65
S5	8.038
S6	1.64

Scenedesmus sp.

Solvent System	Lipid Content,(%)
S1	9.8
S2	11.58
S3	4.66
S4	8.65
S5	7.03
S6	3.6

WET BIOMASS *Chlorella sp.*

Solvent System	Lipid Content (%)
S1	7.4
S2	12.08
S3	11.28
S4	12.01
S5	11.09
S6	1.8

Scenedesmus sp.

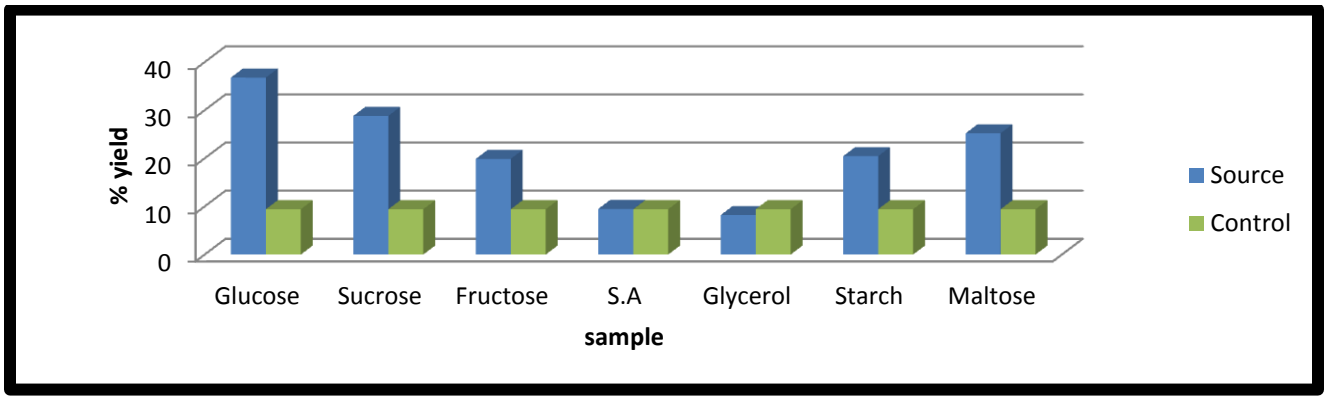
Solvent System	Lipid Content(%)
S1	8.9
S2	12.8
S3	7.6
S4	11.2
S5	10.4
S6	3.8

Looking at this data it can be seen that Chloroform:Methanol (2:1) is the most appropriate solvent system for lipid extraction both using dry and wet biomass . Though wet biomass shows better results but it poses problem of having high moisture content and the results are not that accurate, so instead of that dry biomass is used.

Chlorella minutissima

Carbon Sources

Figure 10 Lipid content using Carbon sources in *Chlorella minutissima*



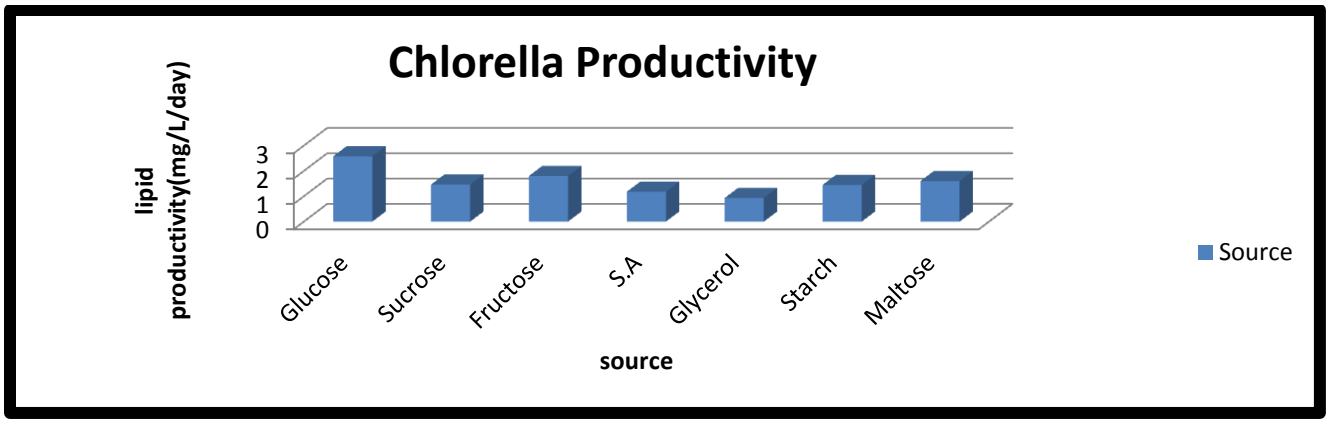


Figure 11 Lipid productivity using Carbon sources in *Chlorella minutissima*

Glucose shows the highest amount of lipid yield and productivity followed by fructose and Sucrose.

Nitrogen sources

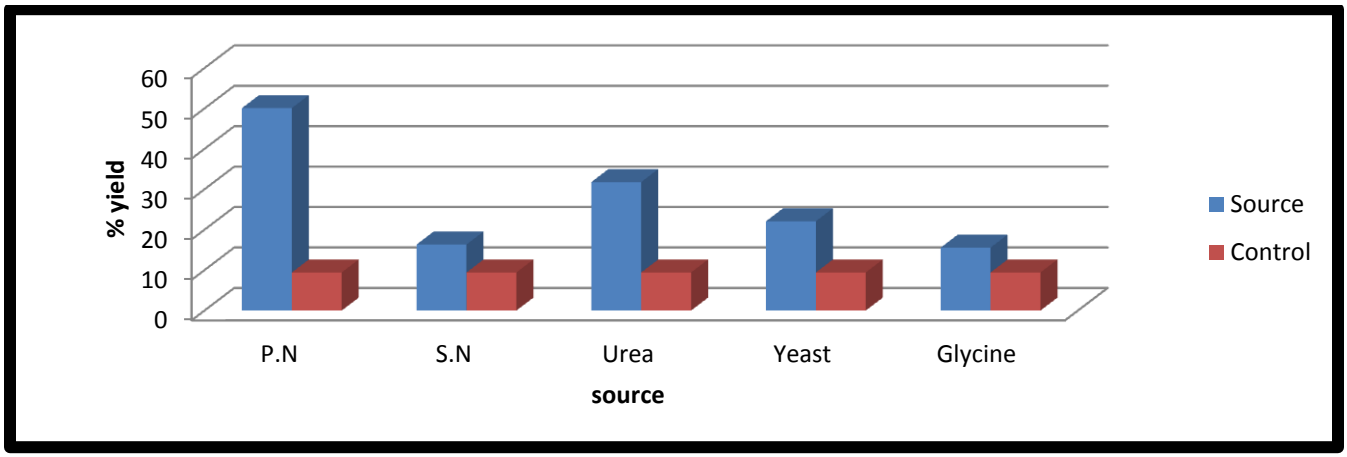


Figure 12 Lipid content using Nitrogen sources in *Chlorella minutissima*

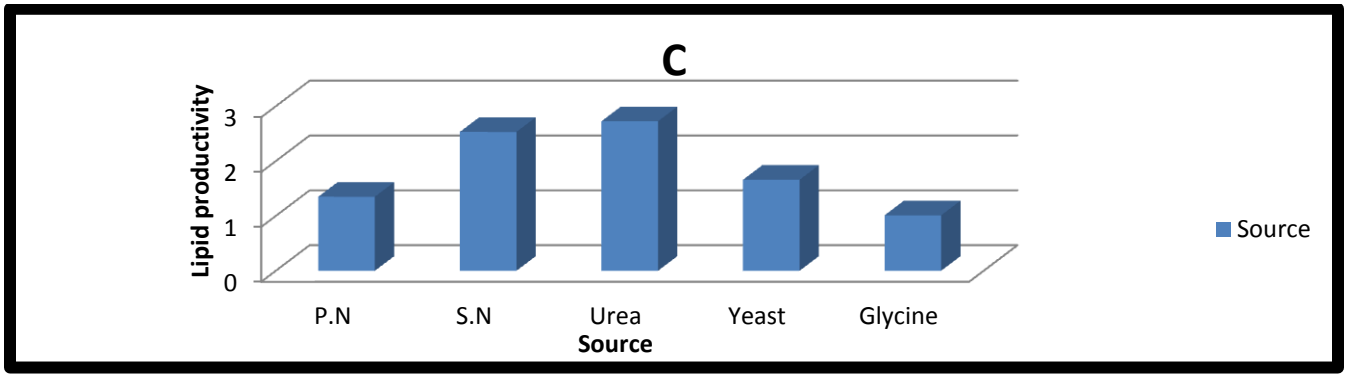


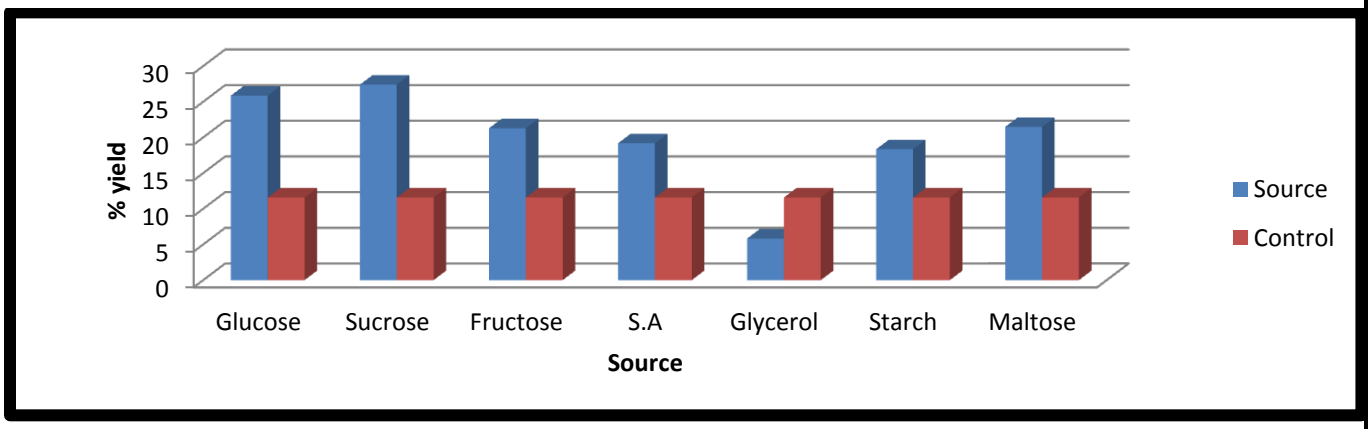
Figure 13Lipid productivity using Nitrogen sources in *Chlorella minutissima*

Urea shows best lipid productivity followed by Potassium nitrate and Sodium nitrate.

Scenedesmus sp.

Carbon Sources

Figure 14 Lipid content using Carbon sources in *Scenedesmus sp*.



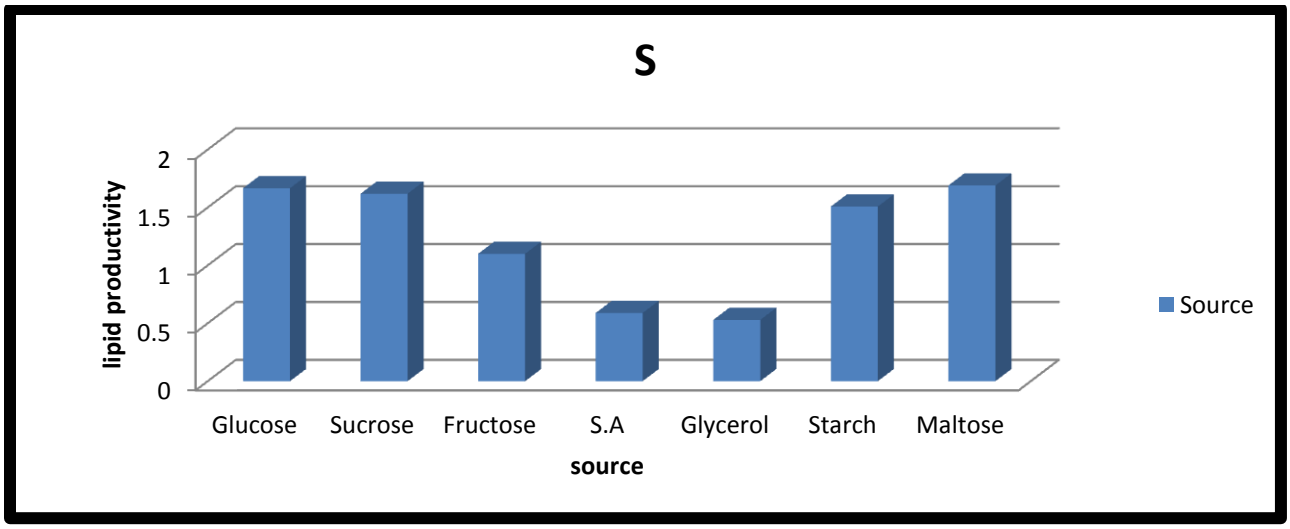


Figure 15 Lipid productivity using Carbon sources in *Scenedesmus sp*.

Glucose, sucrose show better lipid content and productivity for carbon sources in Scenedesmus sp.

Nitrogen sources

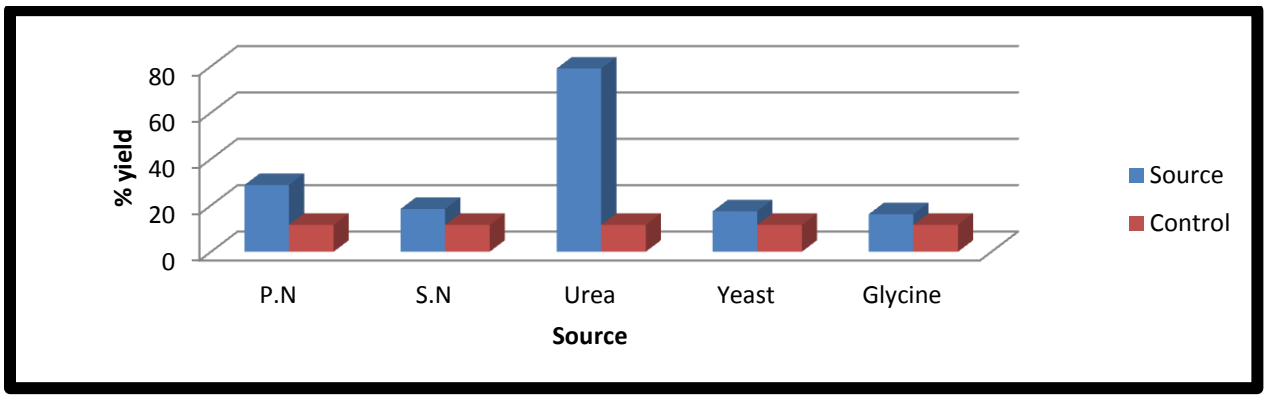


Figure 16 Lipid content using Nitrogen sources in *Scenedesmus sp*.

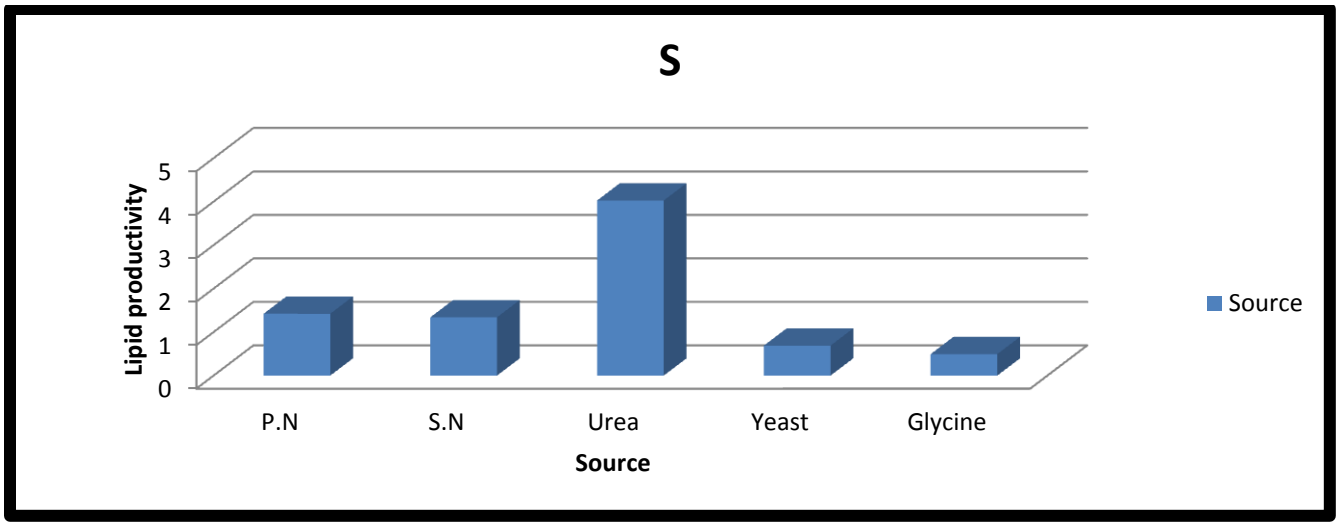


Figure 17 Lipid productivity using Nitrogen sources in *Scenedesmus sp*.

Urea showed maximum lipid productivity and content.

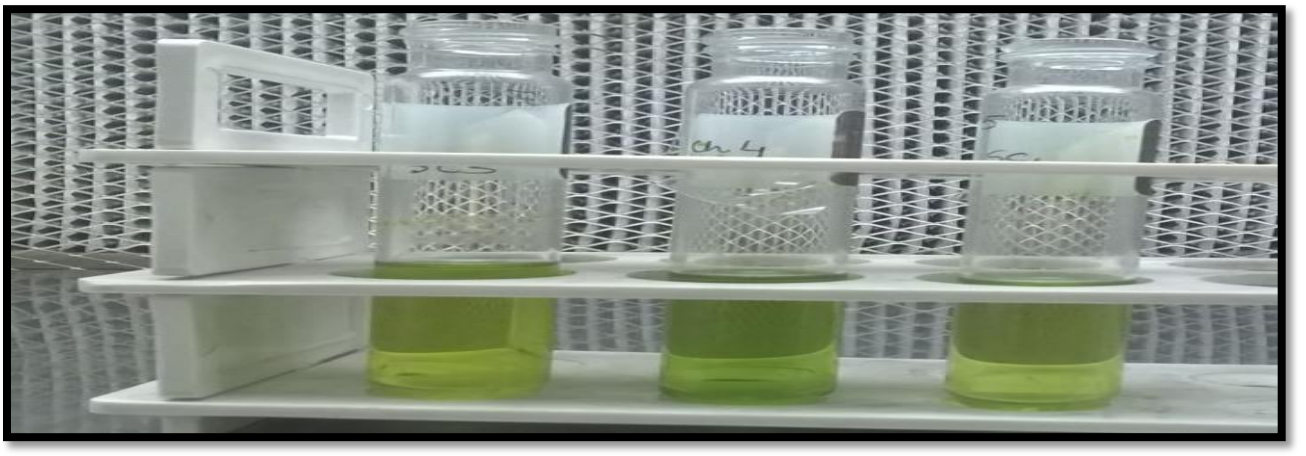


Figure 18 Lipid extraction

Among the various combinations of solvent tested, combination of Chloroform: Methanol in the ratio of 2:1 was found most suitable for microalgae *Chlorella minutissima* and *Scenedesmus* sp. The procedure of extracting lipid utilises different volume of organic solvent so for easy comparison, the results were projected in 5 ml of organic solvents. This extraction process via organic solvents was also combined with the best method of cell disruption i.e. sonication which helped in increase of lipid yield.

HARVESTING METHODS

Chlorella minutissima

Table : 4 Various harvesting method results with Mean±Standard deviation in *Chlorella minutissima*

METHOD	SAMPLE	PRE WEIGHT	POST WEIGHT	WEIGHT(mg/12mL)	MEAN ±S.D
Sedimentation	C1	40.41577	40.42218	6.41	4.91666±1.36287
	C2	46.34948	46.35322	3.74	
	C3	35.85991	35.86451	4.60	
Filtration	C1	1.02652	1.01419	12.33	13.7866±2.9937
	C2	1.00877	1.02600	17.23	
	C3	1.05331	1.06511	11.80	
Centrifugation	C1	5.46087	5.61477	153.9	132.38±20.5759
	C2	5.42637	5.54927	112.9	
	C3	5.44406	5.57440	130.24	
S+C	C1	36.32568	36.32757	1.89	2.8833±0.8831
	C2	43.56006	43.56324	3.18	
	C3	36.54887	36.55242	3.58	
S+F	C1	1.02556	1.02755	1.99	2.0766±0.1331
	C2	1.03314	1.03537	2.23	
	C3	1.03499	1.03700	2.01	
A.S	C1	35.10423	35.11394	9.71	11.6866±2.2082
	C2	45.23158	45.24286	11.28	
	C3	38.99103	39.00051	14.07	
F.C	C1	37.89168	37.89865	6.97	6.70333±0.56897
	C2	48.39477	48.40186	7.09	
	C3	34.71722	34.72327	6.05	
Ferrous sulphate	C1	35.12190	35.13509	13.19	11.53±1.59031
	C2	35.72242	35.73380	11.38	

	C3	35.10479	35.11481	10.02	
Ferric sulphate	C1	48.39366	48.40657	12.91	14.52333±1.52857
	C2	37.69339	37.70810	14.71	
	C3	45.28488	45.30083	15.95	
Chitosan	C1	45.22947	45.23651	7.04	6.77±0.761774
	C2	46.34910	46.35646	7.36	
	C3	35.12441	35.13032	5.91	
Starch	C1	35.401635	35.40554	4.19	3.98±0.87504
	C2	35.50636	35.51104	4.68	
	C3	35.40158	35.40456	2.98	

Scenedesmus sp.

Table 5 : Various harvesting method with Mean±Standard deviation in *Scenedesmus sp.*

METHOD	SAMPLE	PRE WEIGHT	POST WEIGHT	WEIGHT(mg/12ml)	MEAN ±S.D
Sedimentation	S1	35.23124	35.23447	3.23	2.4666±0.84417
	S2	37.01712	37.01973	2.61	
	S3	45.65756	45.65912	1.56	
Filtration	S1	1.05467	1.06381	9.14	9.57±2.71070
	S2	1.00540	1.01787	12.48	
	S3	1.02757	1.03467	7.10	
Centrifugation	S1	5.42975	5.53350	103.75	90.7366±15.180
	S2	5.53084	5.60490	74.06	
	S3	5.44860	5.54730	94.40	
S+C	S1	35.50459	35.50993	1.89	3.22±1.9567
	S2	37.89185	37.89453	2.68	
	S3	35.12217	35.12376	1.59	
S+F	S1	1.05730	1.05983	2.53	3.1766±0.5727
	S2	1.2382	1.02744	3.62	

	S3	1.05065	1.05406	3.38	
A.S	S1	35.24580	35.25492	9.12	8.43333±0.6132971
	S2	35.12032	35.12856	8.24	
	S3	34.84361	34.85155	7.94	
F.C	S1	35.10362	35.10766	4.04	4.25±0.281602
	S2	45.28749	45.29163	4.14	
	S3	43.55914	43.56371	4.57	
Ferrous sulphate	S1	34.72187	34.73768	15.81	14.246666±1.36931
	S2	40.41427	40.42753	13.26	
	S3	35.85826	35.87193	13.67	
Ferric sulphate	S1	48.39266	48.40657	13.91	14.856666±1.027878
	S2	37.69339	37.70810	14.71	
	S3	48.28488	45.30083	15.95	
Chitosan	S1	34.91286	34.91660	3.74	4.4266±0.79657
	S2	34.84389	34.84919	5.30	
	S3	35.10457	35.10881	4.24	
Starch	S1	43.55930	43.57793	18.63	9.15333±8.254612
	S2	35.12506	35.13036	5.3	
	S3	37.98128	37.98481	3.53	

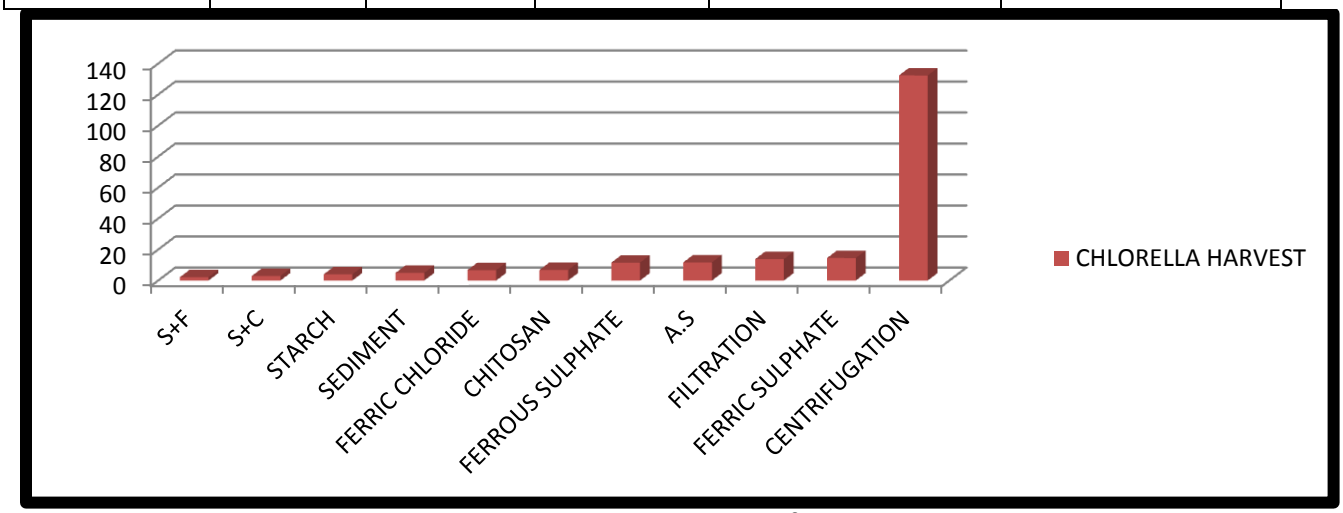


Figure 19 Harvesting method graph of *Chlorella minutissima*

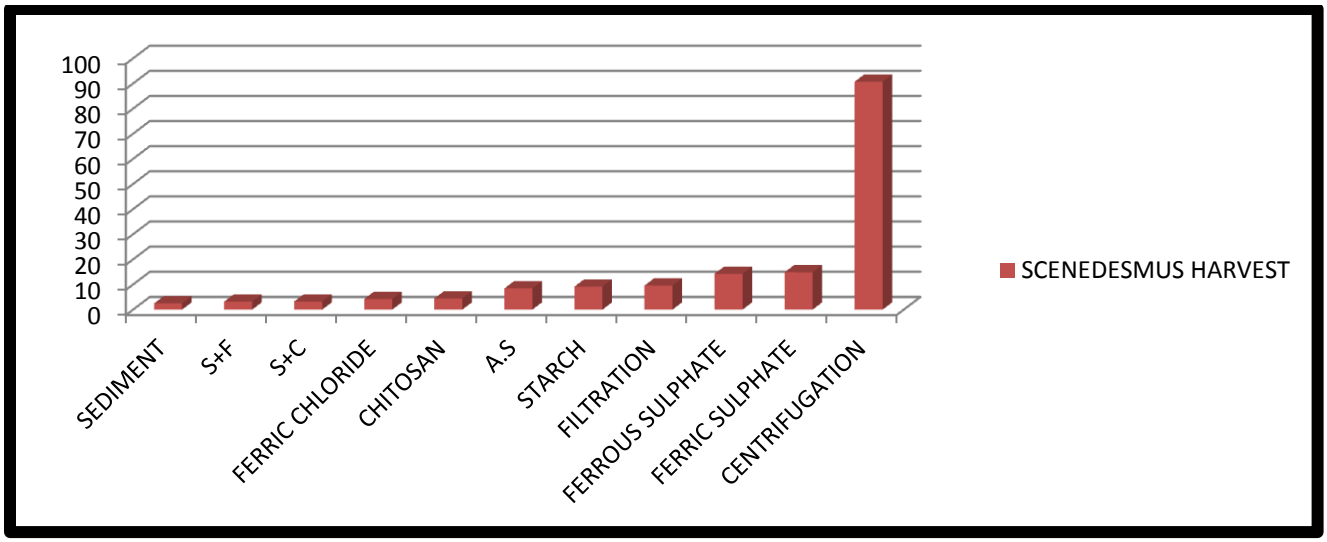


Figure 20 Harvesting method graph of *Scenedesmus sp*.

In this work, maximum biomass has been harvested with the help of centrifugation. The biomass yield is 7.560g/L in *Scenedesmu*s sp. and 11.031 g/L in *Chlorella minutissima.* Other methods such as Organic and Inorganic flocculants also provided good results. Amount of organic flocculent used is less as compared to inorganic flocculent but they took more time to act on biomass which inorganic flocculent showed their effect very early. This study did not look deep in to this issue although it may be taken up in future so that the picture may be clearer. Papazi et al., 2010 also reported that the salts of iron have good potential for the harvesting of microalga *Chlorella minutissima* , salts of aluminium provided best results but they destroyed the algal cell so salts of iron were found more potential flocculent. Same experiments were performed on Scenedesmus sp also in order to check the flocculation efficiency that alga. 36.79% and 25.86 % lipid was extracted using glucose as a carbon source from *Chlorella minutissima* and *Scenedesmus* sp. respectively and 50.08 % and 79.05 % lipid was extracted using Potassium nitrate and Urea as a nitrogen source from *Chlorella minutissima* and *Scenedesmus* sp. respectively when we combined sonication with organic solvent combination Chloroform: Methanol in the ratio 2:1.

Sudan Test

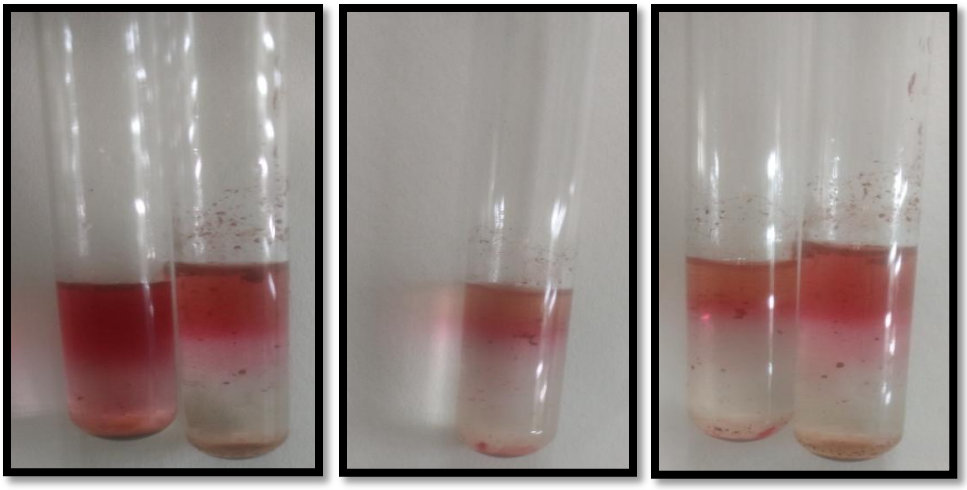


Figure 21 Sudan test for lipids

BIOCHEMICAL ANALYSIS

Chlorophyll a,b and Carotenoid content

Solvents	Equations/Formulae
95% Ethanol	$Ch_a = 13.36A_{664} - 5.19\,A_{649}$ $Ch_b = 27.43A_{649} - 8.12\,A_{664}$ $C_{x+c} = (1000A_{470} - 2.13Ca - 97.63C_b)/209$
Methanol	$Ch_a = 15.65A_{666} - 7.34A_{653}$ $Ch_b = 27.05A_{653} - 15.28A_{666}$ $C_{x+c} = (1000A_{470} - 2.86Ca - 129.8C_b)/221$
DMSO	$Ch_a = 12.19A_{665} - 3.45A_{649}$ $Ch_b = 21.99A_{649} - 5.32A_{665}$ $C_{x+c} = (1000A_{480} - 2.14C_a - 70.16C_b)/220$
80% Acetone	$Ch_a = 12.21A_{663} - 2.81A_{646}$ $Ch_b = 20.13A_{646} - 5.04A_{663}$ $C_{x+c} = (1000A_{470} - 3.27C_a - 104C_b)/198$

Chlorella minutissima

Table 6: Chlorophyll estimation using various solvents in Chlorella minutissima

Pigments / Solvents	Cha	Chb	Carotenoid
95% ethanol	0.0473	0.43185	0.108
Methanol	0.590	0.128	0.337
DMSO	0.259	0.119	0.105
Acetone	20.37059	1.2613	9.766

Scenedesmus sp.

Table 7: Chlorophyll estimation using various solvents in Scenedesmus sp.

Pigments / Solvents	Cha	Chb	Carotenoid
95% ethanol	0.031	0.514	0.118
Methanol	0.130	0.037	0.321
DMSO	0.575	0.488	0.336
Acetone	4.4113	12.95097	1.231

Protein estimation

The protein content estimated in *Chlorella minutissima* was 0.18mg/ml while the protein estimated in *Scenedesmus* sp was 0.19 mg/ml approximately.

Carbohydrate estimation

The carbohydrate estimated in *Chlorella minutissima* was 2.1 mg/ml while in *Scenedesmus* it was 1.7 mg/ml approximately.

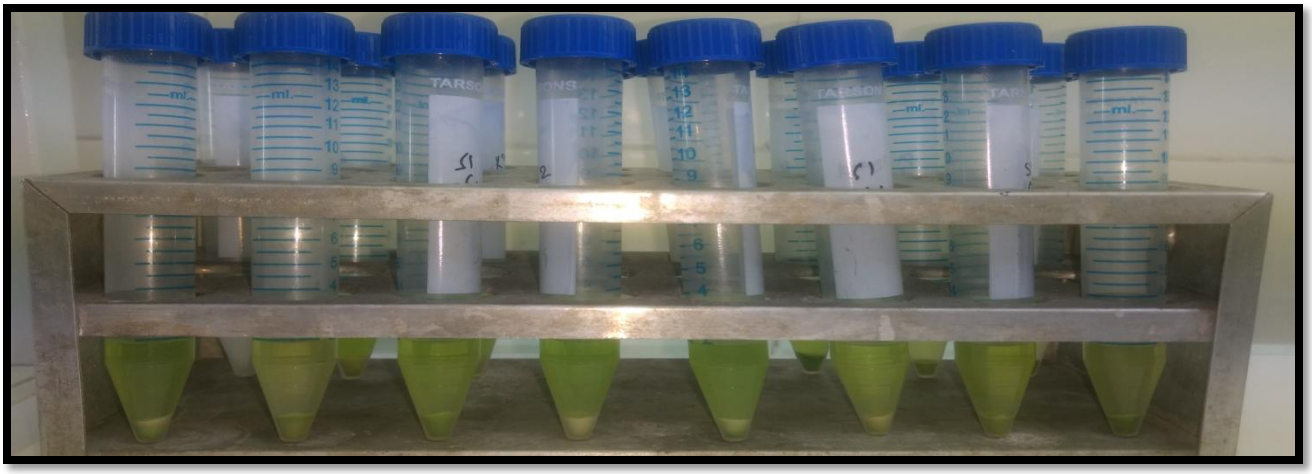


Figure 22 Carotenoid estimation

KINETIC PARAMETERS

Chlorella minutissima Table 8 : Kinetic study

Media	Specific Growth Rate (day-1)	Doubling Time (day)	Biomass Yield (mg/L)	Volumetric Biomass Productivity (mg/L/d)	Lipid Content (%)	Lipid Yield (mg/L)	Volumetric Lipid Productivity (mg/L/d)
Glucose	0.2801	2.47	982.41	5.654	36.79	360.83	2577.27
Sucrose	0.2990	2.31	711.1	2.976	28.82	205	1463.98
Fructose	0.2939	2.35	1268.58	27.476	19.89	255.66	1802.29
Glycerol	0.2995	2.31	1591.41	26.190	8.21	130.75	933.25
Sodium acetate	0.2911	2.38	1739.75	16.369	9.49	165.25	1179.30
Starch	0.2769	2.50	991.25	13.488	20.45	261.33	1447.93
KNo3	0.3022	2.29	377.66	35.017	50.08	189.16	1350.96
NaNo3	0.2942	2.35	2169.8	21.630	16.34	354.75	2532.50
Urea	0.2761	2.51	1201.3	33.714	31.78	381.5	2726.83
Yeast	0.2983	2.32	1052.2	28.570	22.08	232.41	1659.41
Glycine	0.3220	2.15	909.83	23.145	15.56	340.15	1001.95

Kinetic parameters *Scenedesmus sp*. Table 9: Kinetic study

Media	Specific Growth Rate (day -1)	Doubling Time (Days)	Biomass Yield (mg/L)	Volumetric Biomass Productivity (mg/L/d)	Lipid Content (%)	Lipid Yield (mg/L)	Volumetric Lipid Productivity (mg/L/d)
Glucose	0.2943	3.35	902.25	36.208	25.86	233.33	1666.58
Sucrose	0.3183	2.17	827.25	27.285	27.4	226.66	1619.04
Fructose	0.3152	2.19	724.33	39.785	21.28	154.16	1100.98
Glycerol	0.2918	2.37	1274.66	27.380	5.82	74.25	529.89
Sodium acetate	0.2910	2.38	430	26.785	19.18	82.5	589.1
Starch	0.2794	2.48	1151.33	21.232	18.34	78.24	1508.24
KNo3	0.3203	2.16	692	37.303	28.78	199.16	1422.55
NaNo3	0.2843	2.43	1017.66	15.279	18.44	187.75	1340.41
Urea	0.3158	2.19	713.25	38.589	79.05	566	4027.31
Yeast	0.3259	2.12	552.33	21.071	17.48	96.58	689.62
Glycine	0.2987	2.32	426.16	21.348	16.21	92.45	493.44

STATISTICAL ANALYSIS

For harvesting method in *Chlorella minutissima*

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6.41	2	8.34	4.17	0.3698
12.33	2	29.03	14.515	14.74245
153.9	2	243.14	121.57	150.3378
1.89	2	6.76	3.38	0.08
1.99	2	4.24	2.12	0.0242
9.71	2	25.35	12.675	3.89205
6.97	2	13.14	6.57	0.5408
13.19	2	21.4	10.7	0.9248
12.91	2	30.66	15.33	0.7688
7.04	2	13.27	6.635	1.05125

4.19	2	7.66	3.83	1.445

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	23888.68	10	2388.868	150.867	2.71E-10	2.853625
Within Groups	174.177	11	15.83427			
Total	24062.85	21				

F value is greater than Fcrit and Pvalue is less than 0.05 , indicating that this method is efficient.

For harvesting method in *Scenedesmus sp.*

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
3.23	2	4.17	2.085	0.55125
9.14	2	19.58	9.79	14.4722
103.75	2	168.46	84.23	206.8578
1.89	2	4.27	2.135	0.59405
2.53	2	7	3.5	0.0288
9.12	2	16.18	8.09	0.045
4.04	2	8.71	4.355	0.09245
15.81	2	26.93	13.465	0.08405
13.91	2	30.66	15.33	0.7688
3.74	2	9.54	4.77	0.5618
18.63	2	8.83	4.415	1.56645

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11299.48143	10	1129.948143	55.08945831	6.17E-08	2.853625
Within Groups	225.62265	11	20.51115			

Total	11525.10408	21

F value is greater than Fcrit value and Pvalue is less than 0.05 indicates that this method is efficient.

Where,

SS= sum of squares

Df = Degree of freedom

MS= Mean square= Sum of squares/degree of freedom

F= Mean square (between group)/ Mean square (within group)

F= value of the probability of obtaining the F ratio by chance alone

F tables also usually include the mean squares, which indicate the amount of variance (sum of squares) for that " effect" divided by the degrees of freedom for that "effect".

FAME ANALYSIS

The GC-MS result of FAME sample of both algae are given in below figures 23 and 24.

Chlorella minutissima Figure 23 FAME analysis for *Chlorella minutissima*

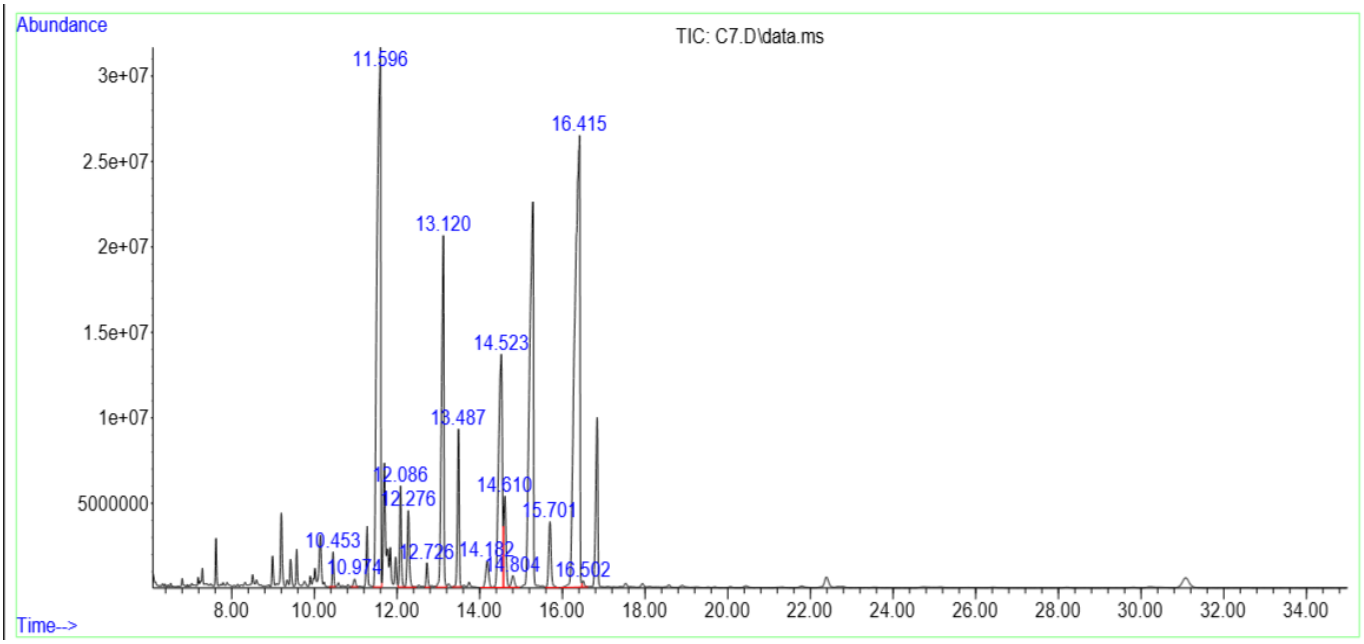


Table 10: Percentage of Methylated fatty acids in C7 sample *: Chlorella minutissima*

Fatty acid	Common name of	Carbon number and	Relative %age

	Fatty Acid	bonds	Content of Fatty acid
Pentadecanoic acid	Pentadecylic acid	C15:0	0.657
Hexadecanoic acid	Palmitic acid	C16:0	27.285
9-Hexadecanoic acid	-	C16:1	2.351
7,10 hexadecadienoic acid	-	C16:2	2.301
Heptadecanoic acid	Margaric acid	C17:0	0.592
7,10,13-Hexadecatrienoic acid	-	C17:3	11.490
Methyl stearate	-	C19:0	1.105
9-Octadecenoic acid, methyl ester	Oleic acid	C18:1	11.886
gamma.-Linolenic acid	-	C18:3	2.269
9,12,15-Octadecatrienoic acid	Alpha linolenic acid	C18:3	32.238
Total			92.174
Saturated Fatty Acid Total			29.639
Monounsaturated Fatty Acid (MUFA) Total			14.237
Polyunsaturated Fatty Acid (PUFA) Total			48.298

Scenedesmus sp. Figure 24 FAME analyses for *Scenedesmus sp*.

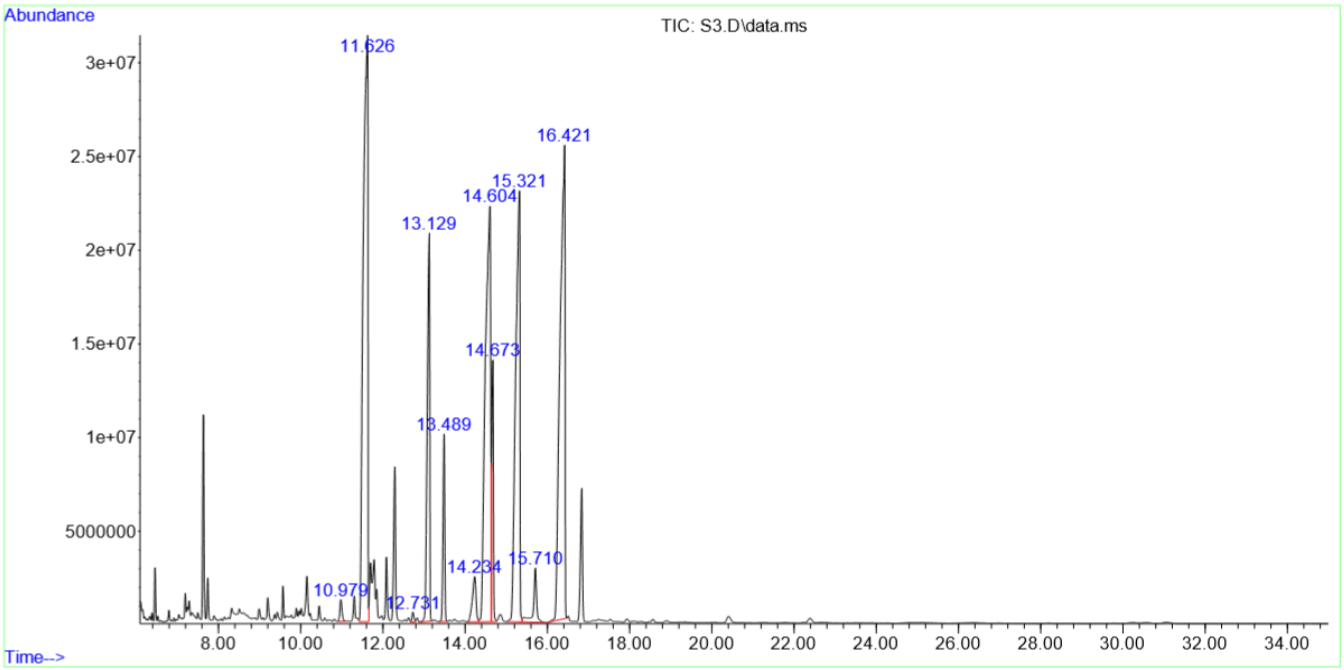


Table 10 : Percentage of Methylated fatty acids in S3 sample *: Scenedesmus sp.*

Fatty acid	Common name of Fatty acid	Carbon number and bonds	Relative %age Content of Fatty acid
Pentadecanoic acid	-	C15:0	0.421
Hexadecanoic acid	Palmitic acid	C16:0	24.330
9,12-Octadecadienoic acid	Linolenic acid	C18:2	16.267
Heptadecanoic acid	Margaric acid	C17:0	0.174
7,10,13-Hexadecatrienoic acid	Roughanic acid	C16:3	8.627
Methyl 4,7,10,13-hexadecatetraenoate	-	C17:3	2.830
Methyl stearate	-	C19:0	1.484
9-Octadecenoic acid, methyl ester	Oleic acid	C18:1	19.469
gamma.-Linolenic acid	-	C18:3	1.556
9,12,15-Octadecatrienoic acid	Alpha-linolenic acid	C18:3	20.791

Total	95.949
Saturated Fatty Acid Total	26.409
Monounsaturated Fatty Acid Total	19.469
Polyunsaturated Fatty Acid Total	50.071

The analysis of fatty acid from different Carbon and nitrogen sources in algae species: *Chlorella minutissima* and *Scenedesmus* sp. by GC-MS showed that they contain various bioactive constituents including Pentadecanoic acid, Octadecadienoic acid, Hexadecanoic acid, Octadecatrienoic acid in major concentration. Heptadecanoic acid and Methyl stearate are present in less concentration. Unsaturated fatty acid Octadecadienoic and Octadecatrienoic acid are the most important essential fatty acids as our body cannot synthesise these fatty acids.

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CONCLUSION AND FUTURE PROSPECTS

This study investigates the effect of various Carbon and nitrogen sources both organic and inorganic on biomass and lipid production potential in *Chlorella minutissima* and *Scenedesmus* sp. The results revealed that Sucrose, Fructose, Maltose, Glucose, Urea showed higher biomass and lipid productivity in both the species. However, maximum lipid productivity was observed for *Chlorella minutissima* (2577.27 mg/L/d in Glucose and 2776.83 mg/L/d in Urea) and for *Scenedesmus* sp. (4027.31 mg/L/d in Urea and 1666.58 mg/L/d in Glucose) among all the sources tested. Highest biomass growth in *Chlorella minutissima* in nitrogen sources was found in Urea (1201.3 mg/L) and in *Scenedesmus sp* was found in Urea (713.25 mg/L) .However in case of organic carbon sources, the biomass growth was found maximum in Fructose (1268.58 mg/L) and for S*cenedesmus* was found to be in Starch (1151.33 mg/L). the Fatty Acid Methyl Ester (FAME) analysis by GC-MS of various conditions revealed that both the algae are rich in fatty acid C:16 to C:18 which is required for biodiesel production. The various harvesting methods used for harvesting maximum biomass indicate that Centrifugation is the best method for harvesting. Further biochemical analyses revealed that these species do have a high protein and carbohydrate content but that can also be increased using external components to increase it so that these algae can be used not only for biodiesel production but also for other component production.

- In order to increase the biodiesel production up to a large scale level C: N: P ratio is to be optimized along with comparing and analysing the fuel properties of the algae with conventional fuels present today.
- Further analysis include analysing the Fuel properties of the algal species such as Cetane number, Saponification value, Iodine Value, Cloud point, Oxidation point etc in the best lipid producing media and comparing them with the conventional fuels available in market.
- Use of microalgae for wastewater treatment, Bio-Plastics production, Nanoparticle synthesis, Biopolymer/Bio glass production etc.

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