Bio-diesel production from microalga *Chlorella minutissima* & FAME analysis by GC-MS

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Masters of Technology

In

Industrial Biotechnology

Submitted by

Gaurav Saxena
(DTU/14/M. Tech./087)
Delhi Technological University, Delhi, India

under the supervision of

Dr. Navneeta Bharadvaja
Assistant Professor
In-charge, Plant Biotechnology Laboratory
Department of Biotechnology,
Delhi Technological University, Bawana Road, Delhi

DECLARATION

This is to certify that the major project entitled "**Bio-diesel production from microalga *Chlorella minutissima* & FAME analysis by GC-MS*" (DTU / 14 / M. Tech./087)*** in the partial fulfilment of the requirements for the reward of the degree of Masters of 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.

(**Gaurav Saxena**)
2K14/IBT/05
M. Tech. (Industrial Biotechnology)
Department of Bio-Technology
Delhi Technological University
(Formerly Delhi College of Engineering, University of Delhi)

CERTIFICATE

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(**Dr. Navneeta Bharadvaja**)
In-charge, Plant Biotechnology Laboratory
Department of Biotechnology
Delhi Technological University

(Prof. D. Kumar)
Head of Department
Department of Biotechnology
Delhi Technological University

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GAURAV SAXENA
2K14/IBT/05

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Bio-diesel production from microalga *Chlorella minutissima* & FAME analysis by GC-MS

Gaurav Saxena (2K14/IBT/05)

*Delhi Technological University, Delhi, India
e-mail ID:gau.sdop@gmail.com

ABSTRACT

Microalgae has been considered as best feedstock for the production of biofuels such as bioethanol, biodiesel, bio-hydrogen, etc. Production of biofuel from microalgae is considered as a very important potential process to mitigate the demand of future energy as microalgae can be cultivated year by year. But biofuel such as biodiesel production from microalgae comprised of many steps and considered as multistep process and also previous literature has reported range of methods for each step. Previous studies concluded that no single method for a single step is universal for all micro-algae as there is a difference in the size and cell wall compositions of micro-algae. This gap provides an opportunity to do the research in the area of process optimisation. ***In this study,*** biodiesel production from the microalga *Chlorella minutissima* has been worked out thoroughly and tried to identify potential method of each stage. Focus has been done on the identification of potential biomass yielding culture conditions, evaluation of different harvesting methods, estimation of best cell disruption method, assessment of combination and ratio of high lipid yielding organic solvents, identification of potential lipid yielding culture conditions and fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass by GC-MS. Parameter-wise potential culture conditions have been identified. Flocculation with aluminium sulfate, cell disruption with sonication, lipid extraction with the organic solvent combination of chloroform and methanol in 1:1 were found potential methods of their respective stage. With the help of these potential methods, biomass of 66 culture conditions has been processed and maximum lipid was obtained with the culture condition of carbon concentration at 1.25 g/l. Top three lipid yielding culture conditions have been further used for fatty acid methyl ester (FAME) analysis by GC-MS to get the details of their respective FAME profile. FAME analysis concluded that this microalga is rich in fatty acids of C16 to C18 so it can be a good source of bio-diesel production.

INTRODUCTION

Energy & its concerns

Energy is the most important component of our life and it mainly comes from the non-renewable and renewable sources. The major portion of non-renewable energy is made from fossil fuels such as petrol, diesel and gas. These fossil fuels are the gifts of nature which were produced by the process of millions of years. But due to uncontrolled use of fossil fuels, a threat of their depletion is growing day by day. Also there are various hazardous effects of fossil fuel such as high CO_2 emission to environment as well as green-house effect [EEA 1, 2]. Due to such conditions we were compelled to think about the renewable sources of energy.

Bio-energy, bio-fuels, its generations

Renewable sources of energy include solar, wind, hydro, bioenergy, etc. [Dewulf *et al*., 2006; Gilbert *et al*., 2008]. Bioenergy is a type of energy which has huge potential as it comes from the biomass which is available in our planet in a huge amount as well as it can be regenerated year by year. Biomasses which are utilized for energy production are termed as feedstock of biofuel and there are several generations of biofuel. First generation biofuel includes bio-ethanol which is derived from sugar based crops such as maize, sugarcane, etc. But sugarcane and maize were itself food crops so their use as a fuel was highly criticized. This led us to generate second generation biofuel such as bio-ethanol from ligno-cellulosic feed stock and biodiesel from non-edible oil based crops such as *Jatropha.* Still there are lot of complications with second generation biofuel such as long-time degradation of cellulosic material in case of ligno-cellulosic fuels and huge requirement of land for *Jatropha* [Scarlat *et al.,* 2008]. These critical situations forced us to move to third generation biofuel i.e. biofuel from microalgae. Microalgae have been found most suitable among all possible potential feedstocks [Mata e*t al.,* 2010].

Microalgae and Algal Biofuels

Algae are photosynthetic, prokaryotic or eukaryotic, and micro or macro-organisms. Prokaryotic algae include Cyanobacteria (blue green algae) while eukaryotic algae include green algae and diatoms [Li *et al.,* 2008a; 2008b]. Algae have been classified in four different groups i.e. Bacillariophyceae (Diatoms), Chlorophyceae (Green algae), Cyanophyceae (Blue green algae) and Chrysophyceae (Golden algae). There are 1,00,000 known species of diatoms and mainly found in ocean, fresh and brackish water. Chyrsolaminarin and TAG are the storage material of these algae. Green algae are found in fresh water and there are approximately 8,000 species of green algae. Starch and TAGs are the main storage material in green algae. Blue green algae are very diverse in their habitat. There are around 2000 species in this group. They are similar to green algae as they also have storage material like starch and

TAGs. Golden algae are mainly fresh water algae having carbohydrate and TAGs as storage material. This is the smallest group of microalgae as they have approximately 1000 species.

In India, different institutes like University of Madras, Chennai, Central Food Technological Research Institute (CFTRI), Mysore, Vivekananda Institute of Algal Technology (VIAT), Chennai, Central Rice Research Institute (CRRI), Cuttack, Orissa, Alternate Hydro Energy Centre, Indian Institute of Technology, Roorkee, CSMCRI, Bhavnagar, Synthetic Biology & bio fuel Group (ICGEB, New Delhi) are working on different species of microalgae like *Sargassum, Seaweeds, Botryococcus braunii, Chlorella vulgaris, Gracilaria, Gelidium, Kappaphycus, Chlamydomonas, Desmococcus olivaceous,* etc. Major focus is being done on cultivation, biogas production, cultivation in open raceway pond, isolation and characterization of hydrocarbon, development of technology to treat industrial waste water, biofuel production from diatom species, conversion of micro-algal oil to biodiesel, productivity of open pond micro algae production for algal oil, pulsed magnetic field (PMF) can be suitably integrated with the existing mass cultivation technology to enhance the bio-fuel quality of algal oil, etc. [web-1, web-2, Dayanand *et al.* 2005; 2006*;* Tripathi *et al*. 2001, web-3,4, Rao P.H. 2010, Chinnasamy *et al.* 2009*,* Ramachandra *et al.* 2009, Rajvanshi *et al.* 2012, Rengasamy R. 2008, Sivasubramanian V. 2011]. But still there are lots of complicated issues are related with the processing of algae for the production of biofuel.

On the basis of size, algae are divided into two broader groups i.e. micro-algae (small algae) and macro-algae (large algae). For example: *Chlorella minutissima* belongs to green algae (Chloropyta or Chlorophyceae). It is a spherical shape, no flagella, bearing chlorophyll a and b, synthesize starch and rich in amino acids & polyunsaturated fatty acids. Macro algae like *Fucus, Porphyra* are large in size and also known as see-weed. They are mainly found in marine environment. Since beginning, macro-algae were not considered for the production of biofuel as their size is very large and growth rate is not as fast as microalgae. But now days, macro-algae are also utilized for ethanol production. Microalgae are considered as best feedstock for the production of biofuel due to several reasons such as easy to culture, fast growth rate, can utilize the waste water as media, short life cycle [Sheehan *et al.,* 1998], can be grown anywhere [Aslan & Kapdan 2006, Pratoomyot *et. al.,* 2005, Renaud *et al.,* 1999], higher production yield [Chisti Y. 2007], high lipid content [Mata *et al.,* 2010; Li *et al.,* 2008b; Richmond A. 2004; Renaud *et. al.,* 1999, Chisti Y. 2007; Rodolfi *et al.,* 2009; Spolaore *et al.,* 2006;] in comparison to other lipid bearing feedstocks.

Biodiesel produced from algae has no pollutants of sulfur, particulate matter, CO and hydrocarbons. But they emit large amount of nitrogen pollutants [Delucchi MA. *et al.,* 2003]. They also help in the bio-fixation of carbon di oxide [Singh & Ahluwalia 2013]. *Chlorella minutissima* can tolerate up-to 50% CO_2 level [Singh & Sharma 2014]. Microalgae are also being used in waste water treatment as they utilize the nitrogenous and phosphorus compounds of waste water [Wang *et al.,* 2008].

Some of algae are very much utilized for health food, feed and pharmaceuticals. Due to this feature of such algae, biofuel production can be clubbed with the production of such other commercial products. This can make the process economically more viable. For example, *Chlorella* sp. have been cultivated as alternative and unconventional protein sources [Backer W. 2004; Cornet JF. 1998], also contains proteins, carotenoids, lipids, immuno-stimulator, polysaccharides, vitamins, antioxidants & minerals [Mohan *et al.,* 2009], antimicrobial effects [Gors *et al.,* 2010] etc. The residue that remains after the production of biodiesel can be utilized for the production of other biofuel, fertilizers, animal feed etc. [Wang *et al.,* 2008]. Algae can also be utilized for the production of other high value products simultaneously with the production of biofuel which again make the production process cost effective [Li *et al.,* 2008a; 2008b; Raja *et al.,* 2008].

Objective of this study

Due to the difference in the cell composition, it is very hard to finalise a universal method for processing of all microalgae for the extraction of commercial products. This difference creates a lots of problem to handle microalga. Thus it is necessary to first work-out potential method of processing of the concerned microalga 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 microalga *Chlorella minutissima,* potential method of each stage of biodiesel production was worked out. The objectives of this project were as follows:

- **Identification of potential biomass yielding culture conditions**: Biomass was cultivated in 97 different culture conditions of 16 different parameters to observe the effect on biomass and lipid content.
- **Evaluation of different harvesting methods**: Different harvesting methods such as sedimentation, filtration, flocculation, centrifugation and their combinations were tested to finalise potential method of harvesting.
- **Estimation of best cell disruption method**: Algal biomass was disrupted with autoclave, microwave, sonication, acid and alkali treatment so that best cell disruption method can be ascertained.
- **Assessment of combination and ratio of high lipid yielding organic solvents**: Different lipid extracting organic solvents such as chloroform, methanol, hexane, dichloromethane, propane-2-ol, cyclohexane, etc. were used in different combination and ratio to assess best lipid extracting combination and ratio.
- **Identification of potential lipid yielding culture conditions:** Algal biomass of 66 different culture conditions were used to identify best lipid producing culture conditions.
- **Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass by GC-MS.**

REVIEW OF LITERATURE

Production of biofuels such as bio-diesel or bio-ethanol from microalgae is a multistep process which includes cultivation of microalgae in suitable culture conditions, harvesting of biomass, disruption of algal biomass, extraction of commercial compounds such as lipid in case of biodiesel, analysis of lipid, conversion of lipid into fatty acid methyl ester (FAME) or bio-diesel, characterization of FAME, etc. These multistep process need perfection of each stage i.e. at each stage maximum or optimized yield is essential so that final yield of commercial active compound can be maximum. But different species of microalgae show different results with same method due to difference in their cell compositions. This difference creates a critical problem as we cannot rely properly on existed method to process our own microalga. This situation demands a review of different method being used for each stage of production of bio-diesel from microalgae and based on the results the potential method for own microalga should be tested experimentally. The same thing was done in this study with microalga *Chlorella minutissima.* **Following** is a brief review of each stage of production of biodiesel from microalgae:

1. Culture media & conditions

Three different types of metabolism i.e. photo-autotrophic, heterotrophic and mixotrophic [Chojnacka & Marquez-Rocha 2004] have been shown by microalgae which helps to decide the culture medium of microalgae. Besides the carbon sources, different culture conditions i.e. light (intensity, period and color), temperature, nutrient concentration, oxygen concentration, carbon dioxide concentration, pH, salinity, effect of toxic chemicals and biotic factors such as pathogens, competing algae, operational factors, etc. are also responsible for deciding the cultivation strategy of microalgae.

To get maximum yield of commercial compounds, various research groups have tested different culture conditions. [Singh *et al.,* 2014] and [Sharma *et. al.,* 2011] worked on *Chlorella minutissima* and *Chlorella vulgaris* respectively and reported the effects of different culture conditions on these microalgae such as effect on cell count, chlorophyll a and b production etc. Tang *et al.,* 2011 also studied culture conditions for the growth of microalgae *Chlorella minutissima* for biodiesel production. They examined the effect of light color (red, white and fluorescent light), intensity (100, 200, 350 and 400 $\mu E/m^2 s$), photoperiod (15L:9D, 12L:12D and 24L:0D) and nitrogen starvation on growth of algae. They got maximum biomass productivity in 24L:00D and nitrogen starvation conditions. Different groups found different results in different microalgae which concluded that other culture conditions are yet to be explored and based on the results we can prepare a cultivation strategy for concerned microalgae. In our study, we have also examined different culture conditions and compared

the results with the previous literature and conclude a strategy for microalga *Chlorella minutissima.*

2. Growth measurement

Different methods have been tested for growth measurement by different groups in the previous literature such as counting of algae cells, chlorophyll estimation, optical density measurement, morphology study, etc. Counting of algal cells are being done by Sedgwick-Rafter [Wetzel & Likens 2000], Hemocytometer [Sharma *et al.,*2011] and Coulter Counter Model ZM [Mandalam & Palsson 1998]. Estimation of chlorophyll a is being done by McKinney method [G. Mackinney G. 1941]. Sharma *et al.*, 2011 used UV visible spectrophotometer for chlorophyll a and b estimation by following Parson and Strickland method [Parson & Strickland, 1965]. Hitkins and Baker 1986 method was utilized by Mandalam & Palsson 1998 to extract the chlorophyll. Measurement of Optical Density at 670 nm [Sharma *et al.,*2011] wavelength to measure the growth in *Chlorella vulgaris* in five different media i.e. Juller's, Bold's Basal, Modified Chu-10, N-8 and Kuhl medium was reported. Morphology also reflects the state of the algae cells and thus may be considered as growth parameter. Singh et al. 2011 concluded that in the best medium i.e. CHU-10, the algae cells were healthy, bright green and having intact chloroplast. But this observation was found up-to the fourth week and during fifth week cells were noticed as unhealthy and the chloroplasts were found broken. The growth analysis is the primary analysis to measure whether the algae culture has reached up-to the level of harvesting. Growth analysis by cell counting is tedious & laborious; chlorophyll estimation is a long process & needs good amount of solvent such as methanol; morphology analysis is also laborious process. The growth analysis by taking optical density of the culture media is a quick and reliable method, easy to handle and don't involve any chemical. Additionally, optical density method may be a good method for growth analysis at pilot and industrial level.

3. Culture system

Algae can be grown anywhere but commercially they are grown either in open raceway ponds or in closed photo-bioreactor systems. Three major designs of open pond system are raceway ponds with endless loop, circular ponds and inclined system [Richmond A. 2004, Schenk *et al.,* 2008]. They are easy to construct, economical for large production of biomass but very prone for contamination and difficult to handle for long time. Closed systems or photo-bioreactors have been classified according to their design and mode of operation [Mata *et al.,* 2010]. Main designs are flat, tubular, horizontal, inclined, vertical, spiral, manifold or serpentine, etc. [Mata *et. al.,* 2010, Ugwu *et al.,* 2008) has compared several forms of closed systems. According to them, tubular photo-bioreactors are cheap, having large illumination area and good biomass productivity but there are some problems associated with these photo-bioreactors such as fouling, wall growth, dissolved oxygen and carbon di oxide and pH gradients. Vertical bubble

columns and airlift cylinders have increased fluid movements, greater gas hold up ability, high mass transfer good mixing ability, low shear stress, low energy consumption, high potential for scalability, easy to sterilized, readily tempered, good for immobilization, less photo inhibition and photo oxidation and adequate light-dark cycle. But these photo-bioreactors also have certain disadvantages such as low surface to volume ratio, cost, small illumination surface area, requirement of sophisticated materials for constructions and large numbers of units are required for commercial plants.

Mode of operation of photo-bioreactor is also very important. Continuous mode has several advantages over batch mode [Williams JA, 2002] such as more reliable and reproducible results can be achieved due to the steady state in continuous mode, higher degree of control, regulation and maintenance of growth rate is possible for long time, the concentration of biomass can be regulated at various dilution rates, easy to have desired product quality.

A comparison of yield of open and closed bioreactors has been done by Chisti Y. 2007. According to this analysis, volumetric productivity of photo-bioreactor (1.535 kg m^{-3} d^{-1}) is higher in comparison to raceway ponds (0.117 kg m^{-3} d^{-1}). Biomass concentration of photo-bioreactors was 4 kg m^{-3} which is far beyond the raceway ponds (0.14 kg m^{-3}). Area needed in case of photo-bioreactor in low, oil yield is high, number units required for cultivation is less in comparison to raceway ponds.

Sustainability in the production is also a very important issue. Although algae have been considered as best feedstock for biofuel production but the amount of biomass required to meet the demand of energy is very huge. CIA 2009 [Borowitzka & Moheimani 2013] report has estimated the requirement of various components of production of 100,000 bbl. algal lipid per year. To meet the total energy demand of the world; we need 0.11% of world surface area [Stephens *et al.*, 2010b]. This huge biomass will also demand the basic nutrient such as water, nitrogen, phosphate, CO_2 etc. Due to the requirement of such a huge amount of basic nutrient, there will be a threat of depletion of these nutrients so there is a need to pay attention to develop such as sustainable cultivation system and maximum focus should be given to recover maximum amount of nutrients. We should also consider the alternative source of water, nitrogen, phosphate, CO_2 etc.

Phosphate is provided in the form of inorganic form but algae can utilize both inorganic and organic forms [Kuhl A. 1974]. Phosphate rocks are available for 50-100 years [Steen L. 1998; Cordell *et al*., 2009] so new alternative source of phosphate such as waste water [Tredici *et al.*, 1992; Tan *et al.*, 1994; An *et al.*, 2003] and bone meal [Becker & Venkataraman 1982] may be utilized. Nitrogen is supplied as ammonia, nitrate or urea. It has been estimated by Greenwell *et al.*, 2010 that 2.5 x 10^{12} kg biomass of algae is required for meet the demand of fuel of Europe and to cultivate such biomass 2 x 10^{11} kg nitrogen is required. The nitrogen fixing algae such as *Anabaena* may be utilized for the alternate production of nitrogen source.

One kg of biomass fixes 1.8 kg of CO_2. A large amount of CO_2 is required for such a huge amount of algae cultivation and thus cultivation of algae can be coupled with the CO_2 producing power plants [Benemann 1997; Haiduc *et al.*, 2009].

4. Harvesting of Biomass

Harvesting of biomass means separation of biomass from the culture medium. 20-30% expenditure was recorded due to harvesting process [Mata *et al.,* 2010; Molina *et al.,* 2003; Verma *et al.,* 2010] but in some cases it was increased up-to 50% [Greenwell *et al.*, 2010] and up-to 90% [Amer et al. 2011]. The challenges of harvesting include the small size of the microalgae cells [Molina *et al.*, 2003], similarity in the density of the algae cells and the medium [Reynolds CS. 1984], formation of stable suspensions due to presence of negative charge on the surface of algae cell [Edzwald JK. 1993; Moraine *et al.*, 1979; Packer M. 2009], requirement of frequent harvesting of biomass due to high growth rate [Milledge & Heaven 2012]. To cope-up these challenges, several different methods have been tested for the harvesting of algae biomass [Milledge & Heaven 2011; Mohn F. 1998; Molina *et al.*, 2003; Shen et al. 2009].

a. Sedimentation: Sedimentation is the process in which the liquid or solid particles are separated from the liquid medium (with different density) with the help of gravitational force [Milledge & Heaven 2012]. It has been found that a common round shaped alga, C*hlorella* has achieved a settlement velocity of 0.1 m day^{-1} in fresh water. But there was reported a huge difference in the settlement rate (Reynolds 1984, Smayda TJ 1970) as it depends on factors such as type of microalgae (Cole and Wells 1995), light intensity (Waite et al. 1992), nutrient deficiency and age of cell (Bienfang 1981). Therefore, harvesting of algae biomass is not widely done by sedimentation (Uduman *et al.*, 2010). The recovery of harvesting was also low i.e. 60-65% of biomass (Mata et al. 2010; Shen et al. 2009; Collet et al 2011; Ras et al. 2011).

b. Flocculation: Flocculation is the aggregation of micro algae cells by the use of flocculating agents. It increases the rate of sedimentation (Mata *et al.*, 2010). Flocculation is considered as most reliable but expansive (Benemann *et al.*, 1980) method. Flocculation has been divided in to two types i.e. auto-flocculation and induced flocculation. Due to various environmental factors such as stress, changes in nitrogen, pH and dissolved oxygen (Schenk *et al.*, 2008; Uduman *et al.*, 2010), the cells of microalga get flocculated. But it is a slow and unreliable process. Flocculation may be induced chemically, biologically or physically. The flocculants should be less expensive, non-toxic, highly effective in low concentration (Molina *et al.*, 2003), derived from non-fossil fuel, sustainable and renewable source.

Chemical flocculants may be organic or inorganic. **Inorganic** may be lime (Oswald WJ 1988), ferric chloride, ferric sulfate and aluminum chloride (alum). In case of *Chlorella* and *Scenedesmus,* alum has been found more effective flocculent (Molina *et al.*, 2003). 1 g l^{-1}

concentration of inorganic flocculent was found appropriate by Papazi et al. 2010. These inorganic flocculent can be toxic [Harith *et al.*, 2009] which can damage algal biomass [Molina *et al.*, 2003, Papazi *et al.,* 2010; Schenk *et al.*, 2008]. But the cost of these inorganic flocculent is less in comparison to organic one. So some new inorganic flocculent must be tested which don't have any adverse effect [Milledge & Heaven 2012].

Organic flocculants such as cationic poly-electrolytes (CPE) (Uduman *et al.*, 2010) provides 35 times higher results at the concentration of 2-25 mg l^{-1} (Grandos *et al.*, 2012). **Magnafloc LT 25** {0.5 mg l^{-1} [Knuckey *et al.*, 2006]}, **Magnafloc 1597 and Praestol** {1 mg l^{-1} in *Tetraselmis* and *Spirulina* [Pushapraj *et al.*, 1993] are some of popular organic flocculants. Chitosan is renewable organic flocculant derived from crustacean shells and used for treating food industry wastewater [Harith *et al.*, 2009]. It is a non-toxic flocculent [Vandamme *et al.*, 2010] but its dosage is very high at the range of 20-150 mg l^{-1} [Harith *et al.*, 2009; Molina *et al.*, 2003]. Starch and modified starch are another category of flocculent that is utilized for harvesting of micro algae [Mohn F. 1988]. These modified starches can be utilized more effectively than both inorganic and synthetic organic flocculants [Vandamme *et al.*, 2010; Mohn F. 1988] but they are costly [Vandamme *et al.*, 2010].

Microorganism such as bacteria can be utilized as flocculent [Shelef *et al.*, 1984a] and also reported in case of *Chlorella* [Molina *et al*., 2003] and *Pleurochrysis carterae* but they need a good amount of carbon source to grow which made this process less cost effective.

Therefore, there is no single or universal flocculent that can be utilized for the harvesting of all type of microalgae although flocculation is a potential method of harvesting as the input of energy is very less [Milledge & Heaven 2012]. In our study, we have also tested different inorganic and organic flocculants on microalga *Chlorella minutissima* to identify the potential flocculant.

c. Flotation: Flotation is commercially done by introducing the air bubbles in the culture. Flotation may be classified [Shelef *et al.*, 1984a] as dissolved air flotation, electrolytic flotation and dispersed air flotation. Sparger is used for the preparation of bubbles at gas pressure of 3 atmospheres [Moraine *et al.*, 1979]. The range of size of bubbles is 10 to 100 micrometer [Edzwald JK. 1993]. But high energy is required for small bubble production which is large in comparison to centrifugation that made this process less economic [Mohn F. 1988].

d. Filtration: At a very large scale, filtration gives satisfactory results [Molina *et al.*, 2003). The filters used in the filtration process have a range and can be classified according to pore size as macro filtration (>10 μm), micro filtration (0.1-10 μm), ultra-filtration (0.02-0.2 μm), reverse osmosis (<0.001 μm). Microfiltration seems to be best type of filtration as the average size of the microalgae ranges between 2 and 30 μm [Brennan & Owende 2010; Molina *et al.*, 2003] for example *Chlorella* and *Cyclotella are* 5-6 μm in diameter [Edzwald JK 1993]. It

is less economic than centrifugation at commercial scale (Molina *et al.*, 2003). Ultra-filtration is used for fragile cells and microalgae can't be processed with it as the operating and maintenance costs are very high [Mata *et al.*, 2010; Molina *et al.*, 2003; Purchas 1981). Filter press is also a good method of filtration. Filter presses has several advantages such as simple design, flexibility and capability to handle a wide range of slurries and equipment is relatively cheap. But the labor costs can be high [Brennan *et al.*, 1969; Richardson *et al.*, 2002]. Rotary vacuum filters with simple filter design are utilized for filtration of large micro algae but are not effective for smaller species [Brennan *et al.*, 1969; Richardson *et al.,* 2002; Goh A. 1984]. It has also been concluded by two extensive reviews [Molina *et al.*, 2003; Uduman *et al.,* 2010] that filtration is good for large cells of algae but not very effective for cells with diameter of less than 10 micrometers.

e. Centrifugation: In centrifugation, gravity is replaced as the force driving separation at a much greater rate. Almost all types of micro algae can be separated reliably and without difficulty by centrifugation [Mohn F. 1988]. Disc stacking centrifuges are the most common industrial centrifuge and are widely used in commercial plants for high value algal products and in algal biofuel pilot plants [Molina *et al.*, 2003]. They are ideally suited for separating particle of the size (3-30 micro meter) and concentration (0.02-0.05%) of the algal cells in a growth medium. They can separate solid/liquid, liquid/liquid or liquid/solid on a continuous basis. Disc stacking centrifuges generally have high energy consumption [Uduman *et al.*, 2010].

Good harvesting method of algae includes less consumption of time of harvesting, less power consumption, minimum involvement of resources (human and machines), high yield of biomass and low cost. Every method of harvesting of algae has its advantage and disadvantage. In case of time as a parameter, centrifugation seems best method. But centrifugation has higher consumption of electricity. If we consider energy consumption as parameter for best method, sedimentation and flocculation seems good methods in comparison to centrifugation. But sedimentation has low settling speed and flocculation leads to contaminate culture media as well as it restricts us to reuse the culture media as the recovery of flocculent is tedious and cost ineffective. Centrifugation has highest biomass yield but utilizing maximum energy. Filtration has great efficiency but a time consuming process. So there appears no single method suits best for harvesting all microalgae as each alga has its own organization and harvesting methods may be selected according to it. Although combinations of methods such as sedimentation and centrifugation may be utilized as the early sedimentation reduces amount of culture.

5. Drying of biomass

The process of harvesting of algae convert the culture media in to 5-15% of algal culture but still it contains some water which is not good as it leads to the spoilage of biomass [Molina *et*

al., 2003]. So it is necessary to dry the algae so that biomass can be saved and further processed to cell disruption and lipid extraction. Drying can be done by solar, roller, spray and freeze drying methods. In case of solar drying, no need of fossil fuels, and it is least expensive [Brennan & Owende 2010] but it is weather dependent, good amount of degradation of organic compounds and little amount of productivity as only 100g dry biomass is obtained in 1 square meter [Oswald WJ. 1988]. Roller drying provides satisfactory results for food processing [Molina *et al.,* 2003] but not utilized for algal biofuel. Spray drying produce dark green powder [Oswald WJ. 1988] but there is significant degradation of cells [Brennan & Owende 2010; Molina *et al.,* 2003) and it is expensive [Brennan & Owende 2010; Molina *et al.,* 2003, Oswald WJ. 1988). Freeze drying gives intact cells and less damaging than spray drying (JJM) but this kind of drying is also more expensive [Brennan *et al.*, 1969].

6. Cell disruption

To increase the efficiency of lipid extraction, it is must to have appropriate cell disruption method and instruments. Lee *et al.*, 2010 tested different methods on microalgae *Botryococcus sp., Chlorella sp. And Scenedesmus sp.* They have used autoclave at 125^0C with 1.5 MPa for 5 minutes, bead beater of 0.1 mm size at a high speed of 2800 rpm for 5 minutes, microwave at 100^0C at 2450 MHz for 5 minutes, sonication at a resonance of 10 KHz for 5 minutes and osmotic shock by using 10% NaCl solution with a vortex for 1 minute and maintained at 48 hrs. The maximum lipid has been extracted in all three algae with the help of microwave methods. The yield was 10.2, 7.4 and 7.4 mg L^{-1} d^{-1} in case of *Botryococcus sp. Chlorella sp.* and *Scenedesmus sp.* respectively. Conclusively, microwave oven method seems most simple, easy and efficient method and the best part of it is that it can be easily scaled up. Similar investigation was conducted in our study on microalga *Chlorella minutissima* to identify potential cell disruption method for it.

7. Lipid extraction

Two types of lipids i.e. polar lipids and non-polar or neutral lipids have been reported in algae. Phospholipids and glycolipid are major polar lipids while neutral non-polar lipids include mono, di, and tri-acylglycerides and carotenoids [Von & Harder 1953; Greenwell *et al.,* 2010]. A detailed comparative study has been done by Li et al. 2014 on *Tetraselmis sp. (Strain M8)* by using different types of methods of lipid extractions. They have performed their work in two approaches.

In first approach, Soxhlet extraction has been performed with single solvent Hexane (52 ml) and mixture of Hexane and ethanol (39 ml + 13 ml) and further fatty acid analysis was done with GC-MS. In approach 2, different methods such as Bligh and Dyer method (Chloroform: Methanol) [Bligh & Dyer 1959], Cequier-Sanchez method (Dichloromethane: Methanol) [Cequier-Sanchez *et al.,* 2008], Schlechtriem method (Propan-2-ol: Hexane) [Schlechtriem *et*

al., 2003], Direct Saponification (Ethanol: KOH) [Burja *et. al.,* 2007] and super Critical CO_2 [Andrich *et. al.,* 2005] were tested. In terms of lipid yield, the order of extraction efficiency on *Tetraselmis* sp. was found as Dichloromethane: Methanol > Propan-2-ol: Hexane > Chloroform: Methanol > $ScCO_2$ > Ethanol: KOH. But this result differs in different algae [Lee *et. al.,* 2014; Lewis *et. al.,* 2000; Lam & Lee 2012] due to the differences in size and in particular cell wall composition. The extraction of lipid from microalgae is dependent on the method by which the micro algae cell is disrupted and the type of solvent of mixture of solvent is used [Li. *et al.*, 2014; Hamilton *et. al.,* 1992; Lewis *et al.* 2000]. This approach was also taken in to consideration during our study to finalize potential combination and ratio of organic solvents to extract algal lipid from *Chlorella minutissima.*

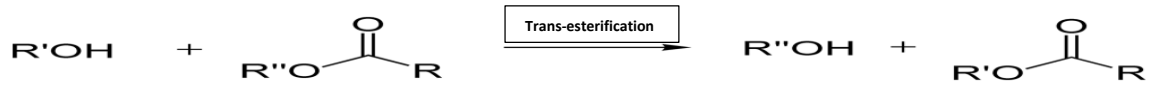
8. Lipid analysis (SSS-NIBE training manual)

After extracting the lipid from microalgae, it is necessary to characterize the lipid so that the further conversion of lipid into oil may be done accordingly. Various parameters of lipid are being characterized. Density (mass per unit volume) is measured by density meter via ASTM D4052. Specific gravity is the ratio of density of a substance with the reference and it is measured by density meter via ASTM D4052. API Density also measured by density meter via ASTM D4052. Viscosity refers to a fluid's resistance at a given temperature. The kinematic viscosity (ν) is the ratio of the dynamic viscosity (μ) to the density of the fluid (ρ) and measured by Kinematic viscometer via ASTM D 446-12. Acid value is number of mg of KOH required to neutralize one gram of oil or fat and measured via AOCS Cd 3d-63. Free Fatty acid content (% FFA) value reflects the age and quality of oil and calculated via acid value. Iodine number measures the unsaturation of the lipid and constant for a particular lipid. It is measured via AOCS Cd 1-25. Saponification number is the amount of alkali (mg) required to saponify 1 gm of oil and it reflects the fatty acid chain length and measured via AOCS Cd 3-25. Ester Value is calculated via saponification value. % of glycerol is determined from ester value.

9. Conversion of Lipid to oil

Lipid obtained from algal biomass generally contains 90-98% triacylglycerides, small amount of mono and diacylglycerides and 1-5% of free fatty acids. They also contain very small amount of phospholipids, phosphatides, carotenoids, tocopherols, sulphur compounds and trace amount of water [Bozbas K. 2008]. Lipid may be converted either into biodiesel or green diesel. The biodiesel is prepared via trans-esterification, esterification and enzyme based catalysis. The green diesel is produced via hydrogenation.

Trans-esterification is simply the conversion of one ester into another. Chemically, it is the process of exchanging the organic group R'' of an ester with the organic group R' of an alcohol.



Alcohol-1 Ester-1 Alcohol-2 Ester-2

Figure-1: Transesterification Reaction

There are acid or base catalyzed reactions where lipid mainly triacylglycerol (TAG) are converted into alkyl ester in the presence of alcohols. Trans-esterification is a three step reaction where triacylglycerides (TAG) are first converted into diacylglycerides (DAG) then into monoacylgyrerides (MAG) and then into fatty acid methyl ester (FAME) and glycerol (as by product) [Mata *et al.,* 2010].

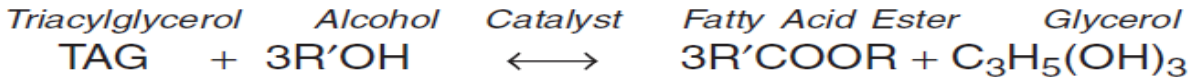


Figure-2: Transesterification Reaction

Before going for trans-esterification it is essential to know the free fatty acid value of the lipid. For this purpose, the titration is performed and the amount of KOH and NaOH (catalyst) is measured. Based on the value of KOH and NaOH the mode of trans-esterification is decided. If 9-15 gram of catalyst is required per liter of lipid the "alkali trans-esterification" is performed. If 15-25 gram of catalyst is required per liter of lipid, the "acid esterification pretreatment" with 1 wt % sulfuric acid followed by alkali trans-esterification is performed. If more than 25 gms catalyst per liter is required, then "acid catalyzed esterification" with simultaneous acid trans-esterification is performed.

In most of the cases the alkali trans-esterification is performed. In this method, 30 ml lipid is mixed with 9 ml of catalyst alcohol solution in a 50 ml centrifuge tube. The mixture is heated up-to 55^{o}C for 1 hour and in between it is mixed with vortex mixer. Then the mixture is centrifuged for 3 min at 3000 rpm. The lipid and glycerol layers are measured for estimation of conversion product.

Green diesel is the next or second generation biodiesel. It has better quality than biodiesel. It is produced via hydro-processing. Hydrogen is used to degrade TAG in to alkane (green diesel), propane and water. For this reaction a high pressure and high temperature (HPHT) reactor is used. It is a new chemical process applied on lipids usually from non-edible seeds. The use of micro algal lipid for hydro-processing was rarely seen in the literature. But this can be explored in the future because algae give high biomass and lipid and this chemical process gives better oil so if we combine both concepts we can achieve a high amount of high quality green diesel. After the process of hydro-processing, crude is obtained which is further processing in true boiling point distillation unit (TBP unit) and fraction of different carbon

compound is achieved at different temperature. This distillation is achieved via ASTM D2892 & ASTM D5236 standard process.

10. Characterization of bio-diesel

After the production of biodiesel from trans-esterification, it is important to characterize it. Various methods are used to characterize the biodiesel so that it can be estimated weather the biodiesel is fit for engine consumption or not. Fatty acid methyl ester (FAME) analysis via gas chromatography (ASTM D6584-10/EN 14103), fatty acid methyl ester (FAME) analysis via Fourier Transformation Infrared Spectroscopy (FTIR), Flash point and Fire point analysis (ASTM D92-12b), Carbon residue analysis by Ramsbottom's carbon residue apparatus (ASTM D524) and Oxidation stability analysis (ASTM D6751-14) are some regular analysis done to characterize the bio-diesel.

11. Theoretically possible maximum production

The high rate of production of algae biomass attracted the researchers of the world to utilize it for biofuel production. But these projections of high production rate are mainly based on the small scale experimental data [Wayer *et al.* 2010]. Thus it is necessary to access maximum possible yield of algae so that industry may design their projects or plant accordingly. A thorough research has been done by Kristina et al. 2009 on this issue. They have done an exhaustive theoretical review to calculate the absolute upper limit of biomass production on the basis of basic physical laws and efficiencies. They have conducted their study in six different sites i.e. Denver, Colorado (40^{o} N), Phoenix, Arizona (33^{o} N), Honolulu, Hawaii (21^{o} N), Kuala Lumpur, Malaysia (3^{o} N), Tel Aviv, Israel (32^{o} N) and Malaga, Spain (37^{o} N) and compared the biomass produced with the theoretically possible production.

Wayer *et al.*, 2010 have formulated an equation for calculating the total yield which was based on 11 terms. The different terms used in this study are full spectrum solar Energy (term 1), photosynthetic portion of spectrum (term 2), number of photon available (term 3), photon transmission efficiency (term 4), photon utilizing efficiency (term 5), quantum requirement (term-6) and carbohydrate energy content (Term-7), biomass accumulation efficiency (term 8), biomass energy content (term 9), cell oil content (term 10), oil density (term-11). The first three terms are combined to give average energy available in the photosynthetic active region (PAR). Terms 3, 6, 7 were combined to calculate maximum photosynthetic efficiency. Photosynthetic efficiency is also a measure of energy stored as biomass. Then first nine terms were combined to calculate growth rate in mass area^{-1} day^{-1}. This analysis finally concluded that maximum theoretically possible yield of annual oil production was 3,54,000 L/ha/year. This amount is very huge in comparison to the oil produced in the six different sites i.e. 40,000, 44,000, 46,000, 48,800, 51,700, 53,200 L/ha/year in Kuala Lumpur, Denver, Malaga, Tel Aviv, Honolulu and Phoenix respectively.

MATERIALS AND MEHODS

The aim of this project is to analyse the fatty acid methyl ester (FAME) profile of high lipid yielding culture conditions of microalga *Chlorella minutissima*. But to get high yield of lipid, there is a need to not only evaluate the potential lipid yielding culture conditions but also to identify best methods of processing of microalgae so that loss of lipid during extraction process can be avoided. So, there is a need to know the potential methods of each stage of production of FAME from microalgae. Thus the project was designed in such a way that potential methods of each stage can be ascertained and with the help of these methods maximum lipid can be extracted and further processed for FAME analysis by GC-MS. Therefore, the project was completed in following successive stages:

- Identification of potential biomass yielding culture conditions.
- Evaluation of different harvesting methods.
- Estimation of best cell disruption method.
- Assessment of combination and ratio of high lipid yielding organic solvents.
- Identification of potential lipid yielding culture conditions.
- Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass.

2 ml culture of microalga *Chlorella minutissima* was obtained from Indian Agricultural Research Institute (IARI), New Delhi, India. The microalga was scaled up in BG-11 medium [Stainer *et al.,* 1971] so that culture for setting experiments can be maintained in the laboratory. Final pH of BG-11 medium was 7.1. Scale up was done from 2 ml eppendorf tube to 5000 ml Erlenmeyer flasks by transferring the culture subsequently to a higher volume on weekly basis. The culture was scaled up at 27-29^0 C with white light of 40 W tube. The culture was kept in 16L: 08D photoperiod and manually agitated twice per day. No carbon source has been provided for the culture during scale up i.e. photo-autotrophic nutrition. Detail of different materials and methods used in above mentioned stages is as follows:

1. Identification of potential biomass yielding culture conditions:

Microalga *Chlorella minutissima* has been cultivated in 97 different culture conditions of 16 different parameters to identify the potential biomass yielding culture conditions. A brief detail of these culture conditions is mentioned in Table-1. Except the variation in the parameter, all other culture conditions were remaining same and as follows: culture medium- BG-11, pH- 7.1, temperature- 27-29^0 C, light colour- white, light intensity- 40 W, light period- 16L: 08D, shaking-manual & twice per day and carbon source-nil. All experiments of cultivation of microalga were performed separately in Erlenmeyer flasks with concerned volume of culture medium and 0.5-1% of inoculum of second week master culture. The cultivation was maintained till the viability of algal biomass. The detail of volume of culture

medium, Erlenmeyer flask and days of cultivation 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 in deep freezer for further analysis.

Table-1: Detail of cultivation of microalga *Chlorella minutissima* in 97 different culture conditions of 16 different parameters. The duration of cultivation in days (X-D) and volumes of culture medium (Y) and Erlenmeyer flasks (Z) in ml. are mentioned in each parameter (X-D; Y/Z).

Sl.no.	Parameter with number of variation, days and volume of medium and flask	Variant/s
1	Media (6 variations) (36-D; 1000/2000)	BG-11 [Stainer *et al.,* 1971], N-11 [Soeder & Bolze 1981], CHU-10 [Stein J. 1973], M-8 [Mandalam & Palsson 1998], BBM [Bischoff & Bold 1963] and Bold 3N [Tang *et al.,* 2011] (Detail of composition of each medium is mentioned in Appendix-I.
2	Media with Nitrogen starvation (6 variations) (36-D; 1000/2000)	
3	Media with Phosphorus starvation (6 variations) (6-7-D; 1000/2000)	
4	Media with Phosphorus and nitrogen starvation (6 variations) (6-7-D; 1000/2000)	
5	Carbon concentration variation (10 variations) (14-D; 1000/2000)	0.5, 0.75, 1.0, 1.25, 2.5, 5.0, 7.5, 10.0, 12.5 and 15 g/L of glycerol.
6	Nitrogen concentration variation (5 variations) (41-D; 1000/2000)	<< Standard (0.375 g/l) (N-1), < Standard (0.75 g/l) (N-2), Standard (1.5 g/l), (N-3), > Standard (3.0 g/l) (N-4), >> Standard 6.0g/l (N-5)
7	Phosphorus concentration variation (5 variations) (13-D; 500/1000)	<< Standard 0.01 (P-1), < Standard 0.02 (P-2), Standard 0.04 (P-3), > Standard 0.08 (P-4), >> Standard 0.16 (P-5)
8	Carbon source variation (4 variations) (20-D; 500/1000)	Glycerol (1g/L), Sucrose (0.93 g/L), Fructose (1g/L) and Maltose (1 g/L)
9	Nitrogen source variation (9 variations) (26-D; 500/1000)	Inorganic [nitrate containing {sodium nitrate (1.5 g/L), calcium nitrate (0.75 g/L), potassium nitrate (1.5 g/L), cobalt nitrate (0.75 g/L)}, {ammonium containing (ammonium molybdate (0.25 g/L), ammonium chloride (1.5 g/L), ammonium meta vendate (1.5 g/L)}, {both ammonium and nitrate containing (ammonium nitrate (1.5 g/L)}] and organic {glycine (1.5 g/L)}

10	Phosphorus source variation (5 variations) (26-D; 500/1000)	Sodium di-hydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4) and ammonium phosphate {$(NH_4)_3PO_4$} at the concentration of 0.04 g/l
11	pH (5 variations) (28-D; 500/1000)	pH 5, 6, 7.1, 8, 10
12	Light intensity (4 variations) (28-D; 400/1000)	8, 15, 20 40 W
13	Light colour (3 variations) (28-D; 500/1000)	White, Red, Blue
14	Light period (3 variations) (28-D; 400/1000)	24L:00D, 16L: 08D, 12L:12D
15	Shaking period (6 variations) (14-D; 150/500)	0, 2, 4, 6, 8, 24 Hours
16	Culture Vessels (14 variation) (45-D)	Tubes {normal (10 ml), culture (20 ml), Pyrex (50 ml), falcon (10 ml) and other tubes (5 ml)}, flask (100 and 250 ml), beakers (200, 400 and 1000 ml) and cylinders (50, 250, 500 and 1000 ml)

2. Evaluation of different harvesting methods: For ascertaining potential method of harvesting for microalga *Chlorella minutissima,* first of all 1000 ml algal culture was cultivated for 45 days in following standard culture conditions:

- **Medium:** BG-11
- **pH:** 7.1
- **Light Intensity:** 40 W
- **Light colour:** White
- **Light period:** 16 L: 8D
- **Organic Carbon source:** Nil
- **Culture Vessel:** Flask (2000 ml.)
- **Shaking:** Manual (twice per day)

After 45 days of cultivation, the culture has been divided for different experiments of harvesting. Triplets of 18 ml have been used for one experiment and following methods of harvesting have been tested:

- Simple sedimentation

- Filtration
- Organic flocculent I (Chitosan)
- Organic flocculent II (Starch)
- Inorganic flocculent I ($FeCl_3$)
- Inorganic flocculent II {$Fe_2(SO_4)_3$}
- Inorganic Flocculent III {$Al_2(SO_4)_3$}
- Centrifugation
- Combination of methods

i. Sedimentation: 18 ml culture has been poured in three test tubes and kept for 24 hours. The biomass settled in 24 hours and supernatant media was removed and sedimented biomass was transferred in pre-weighed eppendorf tubes for drying. After drying, weight of eppendorf with biomass was taken with the help of analytical balance.

ii. Flocculation: Following chemicals were used separately in the triplets of 18 ml of culture:

a. Inorganic flocculants: 20 mg of ferric chloride, ferrous sulphate, aluminium sulphate was used separately in each sample at this stage.

b. Organic flocculants: 4 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 24 hours. After 24 hours, the supernatant was removed and flocculated biomass was transferred in pre-weighed eppendorf tubes for drying. After drying, weight of eppendorf with biomass was taken with the help of analytical balance. The amount of flocculent was subtracted from the final weight of algal biomass.

iii. Filtration: Whatman filter paper 1 with the pore size 11 µm was used for the filtration of microalgae. 18 ml culture has been transferred in three test tubes and used for filtration. The weight of filter paper was taken before the experiment and then the cultures were filtered. The filter papers were dried and weighed with biomass.

iv. Centrifugation: Triplets of 18 ml of culture were used for this experiment. The cultures were centrifuged at 3000 rpm for 5 minutes in falcon tubes. After centrifugation, supernatant was pipetted out and the biomass was transferred in pre-weighed test tubes for drying and final weighing with biomass.

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

a. Sedimentation + Centrifugation: In this setting, triplets of 18 ml of culture were first sedimented for few hours and then sedimented slurry was centrifuged at 3000 rpm for 5 minutes. Then the biomass was transferred and analysed similar to centrifugation experiment above.

b. Sedimentation + Filtration: In this experiment, again the culture was sedimented and then filtered with pre-weighed filter paper. Then the biomass was process as per the filtration experiment.

3. Estimation of best cell disruption method: Cell disruption is pre requisite for the extraction of the biochemical such as lipid and sugars from the cell of microalgae. The extraction of lipid becomes very hard in case of non-disruption of the microalgae cells. 5000 ml of separate culture was cultivated in following lab conditions to obtain 20-25 grams of biomass for the experiments of cell disruption:

- **Medium:** BG-11
- **pH:** 8
- **Light Intensity:** 40 W
- **Light colour:** White
- **Light period:** 16 L: 8D
- **Organic Carbon source:** Glycerol (1g/L)
- **Culture Vessel:** Flask
- **Shaking:** Manual (twice per day)

After harvesting the biomass, following methods [first four methods -Lee. *et al.*, 2010] were tested for concluding best cell disruption method for microalga *Chlorella minutissima:*

- Microwave
- Sonication
- Osmotic shock by 10% NaCl
- Autoclave
- Acid treatment by 4N HCl and 3% H_2SO_4

- Alkali treatment by 0.1 NaOH

i. Microwave: 1.5 gm of harvested biomass was mixed in 60 ml double distilled water and divided into triplets of 20 ml. These triplets were transferred in 50 ml flasks and further microwaved for 2 minutes at 100^{o}C. The disrupted algal biomass culture was transferred in falcon tubes of 50 ml. Then 20 ml mixture of Chloroform and methanol in the ratio of 1:2 (6.66 ml Chloroform and 13.33 ml Methanol) was added and the mixture was vortexed. Further the falcon tube was centrifuged at 2000 rpm for 10 minutes. The organic phase was transferred in to pre-weighed eppendorf tubes. Then the volume of the remaining mixture was make up with mixture of solvents and again centrifuged. After 2-3 cycles almost all organic material came out and the biomass become white. The collected organic phase was kept 2-3 days for natural evaporation. Then the tubes were weighed with lipid with the help of analytical balance.

ii. Sonication: Sonicator is used for disrupting the microalgal cells. 1.5 gm biomass is divided equally in three falcon tubes and further 20 ml double distilled water added in these tubes. This algal mixture was then subjected to sonication for 10 minutes. After 10 minutes, the cells were disrupted. Then, 20 ml mixture of Chloroform and methanol as mentioned above was added in each tube and centrifuged at 2000 rpm for 10 minutes. Then the organic phase was pooled and the cycle of addition of solvents, centrifugation was repeated until the biomass become white. All organic phases were collected in pre-weighed eppendorf tube and kept 2-3 days for drying. The lipid was weighed along with tube by analytical balance.

iii. Osmotic shock: For this method, 10% NaCl solution was used. 1.5 gm biomass was added with 60 ml 10% NaCl. Then this mixture was divided into three falcon tubes equally and kept the mixture for 24 hours for cell disruption. Then after 24 hours, lipid was extracted and weighed with the same process followed for above mentioned methods.

iv. Autoclave:1.5 gm biomass was added with 60 ml of double distilled water and then divided equally in three flasks of 50 ml each. These flasks were kept in autoclave at 121^{0}C for 20 minutes at 15 psi. After autoclave, the cells were disrupted. The disrupted material from each flasks was transferred to falcon tubes and lipid was extracted and weighed with the same process mentioned in above methods.

v. Acid treatment: Two types of acids were tested in this experiment i.e. 4N HCl [Li. *et al.,* 2011] and 3% H_2SO_4. In 4N HCl method, 1.5 gm biomass was mixed with 60 ml 4N HCl and then this mixture was divided in three equal parts in three glass tubes. This triplet was kept for 30 minutes at room temperature. After 30 minutes, the mixture was kept in boiling water for 3 minutes. After 3 minutes, these tubes were rapidly ice cooled by deep freezer. Then the cool mixture was transferred in 50 ml falcon tubes. The mixture was added with 20 ml. another mixture of chloroform and methanol and mixed properly. This mixture was subjected to centrifugation at 2000 rpm for 10 minutes and lipid was collected and weighed with similar method mentioned above.

In H_2SO_4 method, 1.5 gm biomass was disrupted by mixing it with 60 ml 3% H_2SO_4. This disrupted mixture was equally divided into three parts and lipid was extracted and analysed with the help of solvent combination of chloroform and methanol as mentioned in above experiments.

vi. Alkali treatment: The rest of details are same in this experiment except the disrupting agent was 0.1 N NaOH.

After the completion of each experiment of cell disruption method and weighing of lipid amount, it was also confirmed whether the extracted material is actually the desired lipid. For this confirmation, Sudan test was performed at the end of each experiment of cell disruption. In Sudan test, Sudan IV dye was 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, 5 ml 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. Then 20 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.

4. Assessment of combination and ratio of high lipid yielding organic solvents: This study was inspired by the previous investigation of Li *et al.,* 2014. The efficiency of this stage was enhanced combining the best cell disruption method i.e. sonication with it. Following combinations and ratio of different organic solvents for lipid extraction have been tested:

i. Hexane: Ethanol combinations (modified soxhlet method): Three different ratio of hexane and ethanol i.e. 1:1, 1:2 and 2:1 were tested. 200 mg dried algal biomass was mixed in the 5.2 ml mixture of hexane and ethanol. The volume of these solvents in three different ratios was as follows:

a. Ratio 1:1 - Hexane 2.6 ml + Ethanol 2.6 ml
b. Ratio 1:2 - Hexane 1.74 ml + Ethanol 3.46 ml
c. Ratio 2:1 - Hexane 3.46 ml + Ethanol 1.74 ml

After mixing in the mixture of organic solvents, the biomass was subjected to sonication for 10 minutes. After sonication, the disrupted material was centrifuged at 10,000 rpm for 10 minutes. The upper organic layer was pipetted out in a pre-weighed eppendorf tube and kept at room temperature for 2-3 days for evaporation of organic solvent. After evaporation, the lipid remains in the tube. The tube was weighed for the assessment of amount of lipid. Sudan test was conducted for the confirmation of lipid.

ii. **Chloroform: Methanol combination {Bligh and Dyer Method [Bligh & Dyer 1959] (modified)}:** Three different ratio of chloroform and methanol i.e. 1:1, 1:2 and 2:1 were tested. 200 mg algal biomass was mixed with 5 ml mixture of organic solvents. The volume of these solvents in three different ratios was as follows:

a. Ratio 1:1 - Chloroform 2.5 ml + methanol 2.5 ml
b. Ratio 1:2 - Chloroform 1.67 ml + methanol 3.33 ml
c. Ratio 2:1 - Chloroform 3.33 ml + methanol 1.67 ml

The mixture was sonicated for 10 minutes and then 2 ml chloroform and 3.6 ml water was added in the disrupted material. The mixture was mixed properly and centrifuged at 10,000 rpm for 10 minutes. Then the upper organic phase was pipetted out and subjected for evaporation at room temperature for 2-3 days. After evaporation, the amount of lipid was assessed and confirmation of lipid was done with Sudan test.

iii. **Dichloromethane: Methanol combinations {Cequier Sanchez method [Cequier-Sanchez *et al.,* 2008] (modified)}:** Three different ratio of dichloromethane and methanol i.e. 1:1, 1:2 and 2:1 were tested. 200 mg algal biomass was mixed with 7 ml mixture of solvents. The volume of these solvents in three different ratios was as follows:

a. Ratio 1:1 - Dichloromethane 3.5 ml + methanol 3.5 ml
b. Ratio 1:2- Dichloromethane 2.33 ml + methanol 4.66 ml
c. Ratio 2:1- Dichloromethane 4.66 ml + methanol 2.33 ml

The mixture was sonicated for 10 minutes and centrifuged at 10,000 for 5 minutes. The organic phase was pipetted out into a new tube and 1.25 ml 0.88 w/v aq. KCl was added in it. The mixture was vortexed and then subjected to centrifugation at 10,000 rpm for 10 minutes. The organic phase was pipetted out and subjected for evaporation. After evaporation, the lipid amount was measured and confirmation was done with Sudan test.

iv. **Propan-2-ol & Cyclohexane combination {Schlectriem method [Schlechtriem *et al.,* 2003] (modified)}:** Three different ratio of propan-2-ol and cyclohexane i.e. 1:1, 1:1.25 and 1.25:1 were tested. 200 mg algal biomass was mixed with 9 ml mixture of solvents. The volume of these solvents in three different ratios was as follows:

a. Ratio 1:1 - Propan-2-ol 4.5 ml + Cyclohexane 4.5 ml
b. Ratio 1:1.25 - Propan-2-ol 4 ml + Cyclohexane 5 ml
c. Ratio 1.25:1 - Propan-2-ol 5 ml + Cyclohexane 4 ml

The mixture was vortexed for 30 seconds and then subjected to sonication for 10 minutes. After sonication, 5.5 ml water was added and vortexed for 30 seconds. The mixture was then subjected to centrifugation at 10,000 rpm for 10 minutes. The upper organic phase was

pipetted out and kept at room temperature for 2-3 days for evaporation of solvents. Then the lipid was measured and Sudan test was performed for confirmation.

5. Identification of potential lipid yielding culture conditions: Based on the results of potential method of cell disruption and lipid extracting combination & ratio of organic solvents, the biomass cultivated in 66 different conditions (detail mentioned in Table-2) were processed. The reason for processing of biomass of only 66 out of 97 culture condition is that out of 97 culture conditions no biomass was obtained in 17 culture conditions (detail mentioned in point 1 of discussion) and 14 conditions or variation of parameter culture vessel were not found suitable for this study. Sonication was found potential method for cell disruption and combination of chloroform and methanol in 1:1 was found potential method for lipid extraction so these potential methods were utilised for the processing of biomass of 66 culture conditions. 50 mg algal biomass was mixed with a mixture of 1.25 ml chloroform and 1.25 ml methanol (1:1 ratio). This mixture was further subjected to sonication for 10 minutes. After sonication, 0.5 ml chloroform and 0.9 ml water was added and the mixture was subjected to centrifugation at 10,000 rpm for 10 minutes. The organic phase was pipetted out in pre-weighed eppendorf tube and kept at room temperature for 2-3 days for evaporation. After evaporation amount of lipid was measured and confirmation of lipid was done with Sudan test. This method was used for processing the biomass of all 66 conditions.

Table-2: Detail of biomass of 66 culture conditions of microalga *Chlorella minutissima* which was used to identify potential lipid producing conditions.

Sl.no.	Parameter with number of lipid extracting variant/s
1	Media (6 variations)
2	Media with Nitrogen starvation (6 variations)
3	Carbon concentration variation (10 variations)
4	Nitrogen concentration variation (5 variations)
5	Phosphorus concentration variation (5 variations)
6	Carbon source variation (4 variations)
7	Nitrogen source variation (5 variations)
8	Phosphorus source variation (5 variations)
9	pH (4 variations)
10	Light intensity (4 variations)
11	Light colour (3 variations)
12	Light period (3 variations)
13	Shaking period (6 variations)

6. 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. Following three culture conditions provided maximum amount of lipid in previous stage:

a. Carbon concentration variation- 1.25g/l
b. Carbon concentration variation- 5.0g/l
c. Nitrogen source variation- Sodium Nitrate (1.5g/l)

Biomass of above mentioned three culture conditions and BG-11 medium as standard were used for the preparation of FAME. 250 mg algal biomass was transferred in a glass tube. Then 2.5 ml 2% methanolic HCl was added in biomass. This mixture was incubated at 90^{o}C for 1 hour. After incubation, 2.5 ml 0.9% NaCl solution and 5 ml hexane were added. The mixture was centrifuged at 10,000 rpm for 10 minutes and upper organic layer was pipetted out in another glass tube. The organic phase was further subjected to GC-MS analysis.

7. Data Analysis: All experiments of each stage were done in triplicates and mean of the triplets and standard deviation were calculated and used for further analysis.

RESULTS

The result of each stage mentioned in materials and methods section is as follows:

1. Identification of potential biomass yielding culture conditions: The potential culture conditions which provided maximum biomass productivity are mentioned parameter-wise in Table-3.

Table-3: Parameter-wise detail of potential culture conditions which provided maximum biomass productivity (mg L-1 d-1).

Sl. no.	Parameter with number of variation, days and volume of medium and flask	Variations	Potential variant/s with biomass productivity (mg L^{-1} d^{-1})
1	Media (6 variations) (36-D; 1000/2000)	BG-11, N-11, CHU-10, M-8, BBM, Bold 3N (Detail of composition of each medium is mentioned in Appendix-I.	N-11 (66 mg L^{-1} d^{-1}), BBM (62 mg L^{-1} d^{-1}) and BG-11 (mg L^{-1} d^{-1})
2	Media with Nitrogen starvation (6 variations) (36-D; 1000/2000)		BBM (79 mg L^{-1} d^{-1}) and BG-11 (77 mg L^{-1} d^{-1})
3	Media with Phosphorus starvation (6 variations) (6-7-D; 1000/2000)		No variation was found suitable as the microalga died in each variation.
4	Media with Phosphorus and nitrogen starvation (6 variations) (6-7-D; 1000/2000)		
5	Carbon concentration variation (10 variations) (14-D; 1000/2000)	0.5, 0.75, 1.0, 1.25, 2.5, 5.0, 7.5, 10.0, 12.5 and 15 g/L	12.5 g/L (461 mg L^{-1} d^{-1})
6	Nitrogen concentration variation (5 variations) (41-D; 1000/2000)	<< Standard (0.375 g/l) (N-1), < Standard (0.75 g/l) (N-2), Standard (1.5 g/l), (N-3), > Standard (3.0 g/l) (N-4), >> Standard 6.0g/l (N-5)	<< Standard (0.375 g/l) (N-1) (200 mg L^{-1} d^{-1})
7	Phosphorus concentration variation (5 variations) (13-D; 500/1000)	<< Standard 0.01 (P-1), < Standard 0.02 (P-2), Standard 0.04 (P-3), > Standard 0.08 (P-4), >> Standard 0.16 (P-5)	>> Standard (0.16 g/l) (P-5) (107 mg L^{-1} d^{-1})
8	Carbon source variation (4 variations) (20-D; 500/1000)	Glycerol (1g/L), Sucrose (0.93 g/L), Fructose (1g/L) and Maltose (1 g/L)	Glycerol-1g/L (361 mg L^{-1} d^{-1})

9	Nitrogen source variation (9 variations) (26-D; 500/1000)	Inorganic [nitrate containing {sodium nitrate (1.5 g/L), calcium nitrate (0.75 g/L), potassium nitrate (1.5 g/L), cobalt nitrate (0.75 g/L)}, {ammonium containing (ammonium molybdate (0.25 g/L), ammonium chloride (1.5 g/L), ammonium meta vendate (1.5 g/L)}, {both ammonium and nitrate containing (ammonium nitrate (1.5 g/L)}] and organic {glycine (1.5 g/L)}	Sodium nitrate-1.5 g/L (66 mg L^{-1} d^{-1}) and Potassium nitrate-1.5 g/L (64 mg L^{-1} d^{-1})
10	Phosphorus source variation (5 variations) (26-D; 500/1000)	Sodium di-hydrogen phosphate (NaH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), potassium di-hydrogen phosphate (KH_2PO_4), di-potassium hydrogen phosphate (K_2HPO_4) and ammonium phosphate {$(NH_4)_3PO_4$} at the concentration of 0.04 g/l	Di-potassium hydrogen phosphate-0.04 g/L (149 mg L^{-1} d^{-1}) and Potassium di-hydrogen phosphate-0.04 g/L (144 mg L^{-1} d^{-1})
11	pH (5 variations) (28-D; 500/1000)	pH 5, 6, 7.1, 8, 10	pH 8 (99 mg L^{-1} d^{-1}), 7.1 (98 mg L^{-1} d^{-1})
12	Light intensity (4 variations) (28-D; 400/1000)	8, 15, 20 40 W	20 W (110 mg L^{-1} d^{-1})
13	Light colour (3 variations) (28-D; 500/1000)	White, Red, Blue	White (140 mg L^{-1} d^{-1})
14	Light period (3 variations) (28-D; 400/1000)	24L:00D, 16L: 08D, 12L:12D	24L:00D (169 mg L^{-1} d^{-1})
15	Shaking period (6 variations) (14-D; 150/500)	0, 2, 4, 6, 8, 24 Hours	2 (787 mg L^{-1} d^{-1})
16	Culture Vessels (14 variation) (45-D)	Tubes {normal (10 ml), culture (20 ml), Pyrex (50 ml), falcon (10 ml) and other tubes (5 ml)}, flask (100 and 250 ml), beakers (200, 400 and 1000 ml) and cylinders (50, 250, 500 and 1000 ml)	Tube {normal (10 ml)} (114 mg L^{-1} d^{-1})

2. Evaluation of different harvesting methods: Result of each method of harvesting is as shown in Table-4:

Table-4: Detail of biomass obtained in different methods of harvesting. Inorganic flocculent 1-$FeCl_3$; Inorganic flocculent 2-$Fe_2(SO_4)_3$; Inorganic flocculent 3-$Al_2(SO_4)_3$; Organic flocculent 1-Chitosan; Organic flocculent 2-Starch.

Sl. No.	Harvesting method	Sample Number	Amount of biomass (mg)	Amount of biomass (mg) (Mean ± SD)
1	Sedimentation	1	37.70	38.70±1.2
		2	38.30	
		3	40.10	
2	Filtration	1	1.20	2.17±0.9
		2	2.90	
		3	2.40	
3	Organic Flocculent 1	1	49.70	54.57±8.3
		2	49.90	
		3	64.10	
4	Organic Flocculent 2	1	38.20	41.37±3.8
		2	40.30	
		3	45.60	
5	Inorganic Flocculent 1	1	54.20	48.57±6.0
		2	42.20	
		3	49.30	
6	Inorganic Flocculent 2	1	59.70	56.03±6.6
		2	48.40	
		3	60.00	
7	Inorganic Flocculent 3	1	85.00	64.10±19.4
		2	60.50	
		3	46.80	
8	Centrifugation	1	51.90	47.90±3.5
		2	46.50	
		3	45.30	
9	Sedimentation + Centrifugation	1	39.10	43.50±4.0
		2	44.40	
		3	47.00	
10	Sedimentation + filtration	1	5.40	4.40±1.0
		2	4.30	
		3	3.50	

(a) **(b)**

Figure-3: Biomass of different methods of harvesting (a) before and (b) after processing.

3. Estimation of best cell disruption method and Sudan Test: Results of this stage is mentioned in Table-5 as follows:

Table-5: Detail of Lipid obtained in different methods of cell disruption. ET-Eppendorf tube.

Sl. No.	Cell disruption method	Sample	Biomass used in mg	Initial weight of ET (mg)	Final weight of ET (mg)	Amount of lipid in mg	Mean amount of lipid
1	Microwave	1	500	1187.4	1223.9	36.5	25.60
		2		1190.1	1197.1	7	
		3		1182.2	1215.5	33.3	
2	Sonication	1	500	1093.1	1197.5	104.4	48.33
		2		1188.6	1208.7	20.1	
		3		1200.1	1220.6	20.5	
3	Osmotic shock	1	500	1188.1	1222.1	34	25.65
		2		1189.9	1207.2	17.3	
4	Autoclave	1	500	1193.5	1219.2	25.7	21.33
		2		1191.6	1210.4	18.8	
		3		1191.8	1211.3	19.5	
5	4 N HCl	1	500	1188	1237.9	49.9	30.67
		2		1186.3	1209.2	22.9	
		3		1192.3	1211.5	19.2	
6	3% H2SO4	1	500	1190.5	1210.8	20.3	22.37
		2		1189.6	1211.3	21.7	
		3		1030.9	1056	25.1	
7	0.1 NaOH	1	500	1189.1	1199.7	10.6	13.73
		2		1180.9	1195	14.1	
		3		1181.5	1198	16.5	

3.1 SUDAN IV Test: The result of Sudan test of best cell disrupting method is shown in figure-4.

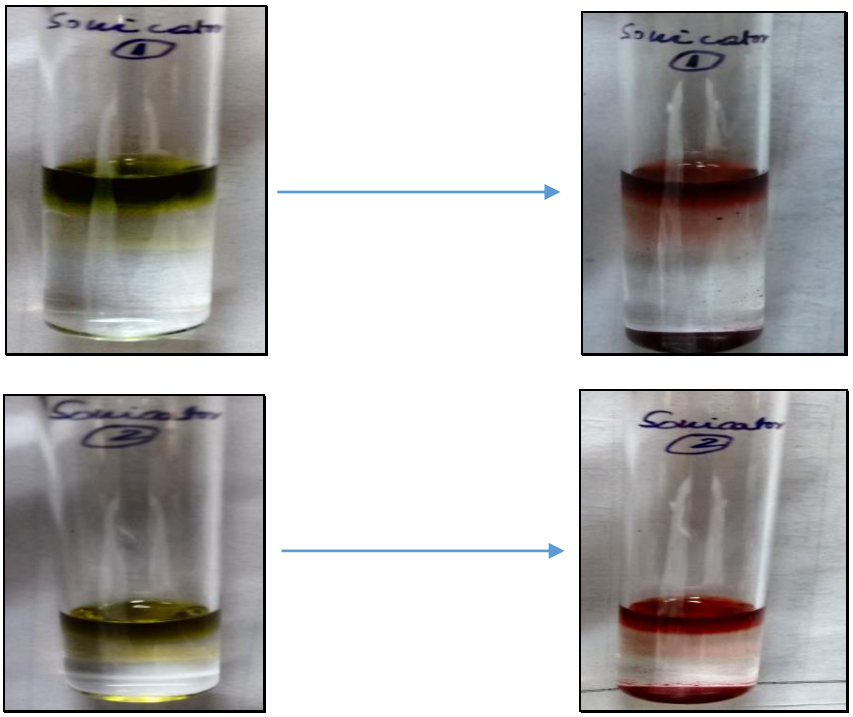


Figure-4: Confirmation of lipid by Sudan test in Sonication experiment.

4. Assessment of combination and ratio of high lipid yielding organic solvents:

The result of this experiment is mentioned in Table-6 as follows:

Table-6: Detail of lipid obtained with the help of different combination and ratio of different organic solvent. ET-Eppendorf tube.

Combination of organic solvent	Ratio	volume of solvent mixture	Sample number	Weight of empty ET	Weight of ET with lipid	Weight of lipid	Total lipid in 200 mg of biomass (mg)	Mean of lipid with SD	Lipid in 100 mg Biomass (mg) (Projected)	Lipid in 1 ml of Solvent mixture (mg) (Projected)
Chloroform: Methanol	01:01	5 ml	1	1029.77	1047.15	17.38	44.49	43.64±0.77	21.82	4.36
				1032.2	1046.34	14.14				
				1020.22	1033.19	12.97				
			2	1024.73	1042.07	17.34	42.98			
				1022.65	1039.81	17.16				
				1026.38	1034.86	8.48				
			3	1019.31	1034.24	14.93	43.46			
				1030.38	1045.68	15.3				
				1021.64	1034.87	13.23				
Chloroform: Methanol	01:02	5 ml	1	1031.43	1047.29	15.86	32.79	30.41±2.29	15.21	3.04
				1020.79	1037.72	16.93				

				1022.12	1022.12	0				
			2	1031.71	1047.75	16.04	28.21			
				1023.76	1035.93	12.17				
				1026.31	1026.31	0				
			3	1032.39	1048.23	15.84	30.24			
				1031.38	1045.78	14.4				
				1030.49	1030.49	0				
Chloroform: Methanol	02:01	5 ml	1	1027.12	1042.69	15.57	40.85	36.70±5.71	18.35	3.67
				1031.16	1045.15	13.99				
				1026.84	1038.13	11.29				
			2	1020.23	1034.06	13.83	39.06			
				1023.48	1037.34	13.86				
				1026.43	1037.8	11.37				
			3	1022.46	1035.44	12.98	30.18			
				1024.37	1036.23	11.86				
				1031.78	1037.12	5.34				
Hexane: Ethanol	01:01	5.2 ml	1	1026.48	1029.85	3.37	10.08	10.71±0.55	5.355	1.029808
				1031.53	1035.01	3.48				
				1019.95	1023.18	3.23				
			2	1031.15	1034.87	3.72	11.04			
				1026.65	1030.29	3.64				
				1025.93	1029.61	3.68				
			3	1031.16	1034.85	3.69	11.01			
				1022.91	1026.42	3.51				
				1029.9	1032.21	2.31				
				1022.7	1024.2	1.5				
Hexane: Ethanol	01:02	5.2 ml	1	1021.98	1025.12	3.14	9.86	9.80±0.19	4.90	0.94
				1025.19	1028.58	3.39				
				1019.68	1023.01	3.33				
			2	1022.56	1025.79	3.23	9.95			
				1026.09	1029.6	3.51				
				1031.5	1034.71	3.21				
			3	1019.66	1022.99	3.33	9.59			
				1023.34	1026.94	3.6				
				1026.82	1029.48	2.66				
				1031.43	1033.32	1.89				
Hexane: Ethanol	02:01	5.2 ml	1	1019.4	1024.54	5.14	15.67	16.55±1.29	8.28	1.59
				1027.35	1032.57	5.22				
				1026.59	1031.9	5.31				
			2	1019.83	1025.18	5.35	15.96			
				1031.03	1036.31	5.28				
				1030.69	1036.02	5.33				
			3	1026.42	1032.16	5.74	18.03			

				1031.23	1036.13	4.9				
				1027.08	1031.4	4.32				
				1023.63	1026.7	3.07				
Dichloromethane: Methanol	01:01	7 ml	1	1024.22	1036.2	11.98	44.93	39.99±7.72	19.99	2.86
				1029.2	1041.24	12.04				
				1025.36	1036.61	11.25				
				1029.35	1039.01	9.66				
			2	1023.7	1032.82	9.12	35.52			
				1026.24	1034.65	8.41				
				1030.65	1039.64	8.99				
				1026.85	1035.85	9				
			3	1031.64	1042.18	10.54	39.51			
				1032.11	1042.98	10.87				
				1023.44	1032.86	9.42				
				1022.76	1031.44	8.68				
Dichloromethane: Methanol	01:02	7 ml	1	1027.08	1032.35	5.27	19.51	17.87±3.40	8.93	1.28
				1031.53	1036.86	5.33				
				1027.09	1031.58	4.49				
				1023.94	1028.36	4.42				
			2	1020.33	1024.92	4.59	20.13			
				1032.64	1037.83	5.19				
				1023.72	1029.86	6.14				
				1022.78	1026.99	4.21				
			3	1020.83	1023.32	2.49	13.96			
				1021.75	1024.28	2.53				
				1031.46	1034.24	2.78				
				1025.95	1028.5	2.55				
				1031.17	1034.78	3.61				
Dichloromethane: Methanol	02:01	7 ml	1	1032.36	1040.59	8.23	33.17	29.58±4.15	14.79	2.11
				1030.23	1038.6	8.37				
				1030.4	1039.04	8.64				
				1022.49	1030.42	7.93				
			2	1022	1030.07	8.07	30.54			
				1023.71	1031.76	8.05				
				1031.61	1038.8	7.18				
				1031.55	1038.79	7.24				
			3	1021.58	1030.62	9.04	25.03			
				1031.16	1039.43	8.27				
				1028.47	1036.14	7.67				
				1021.17	1021.22	0.05				
Propane-2-ol: Cyclohexane		9 ml	1	1027.74	1032.2	4.46	8.28	20.94±25.68	10.47	1.16
				1027.84	1031.66	3.82				
			2	1031.59	1035.64	4.05	4.05			

			3	1021.06	1071.55	50.49	50.49			
Propane-2-ol: Cyclohexane		9 ml	1	1021.14	1026.97	5.83	9.8	9.75±1.83	4.88	0.54
				1031.12	1035.09	3.97				
			2	1030.29	1034.14	3.85	7.9			
				1030.56	1034.61	4.05				
			3	1021.2	1025.33	4.13	11.56			
				1026.6	1030.45	3.85				
				1031.17	1034.68	3.51				
				1022.27	1022.34	0.07				
Propane-2-ol: Cyclohexane		9 ml	1	1030.08	1034.81	4.73	9.33	8.38±1.96	4.19	0.47
				1025.55	1030.15	4.6				
			2	1027.53	1032.16	4.63	9.68			
				1020.98	1026.03	5.05				
			3	1031.53	1036.55	5.02	6.12			
				1027.36	1028.46	1.1				

5. Identification of potential lipid yielding culture conditions: The lipid production by biomass of 66 different culture conditions is mentioned in Table-7 in descending order:

Table-7: Descending order of lipid production in 66 different culture conditions. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

Sl. No.	Culture condition	Mean of lipid in 50 mg biomass with SD	Projected amount of lipid in 100 mg biomass (mg or %)
1	CCV 1.25g/l	18.74±2.02	37.48
2	CCV 5.0g/l	12.36±0.93	24.71
3	NSV- Sodium Nitrate	10.98±0.38	21.96
4	Nitrogen Starvation-CHU-10	9.54±2.46	19.08
5	NSV-Potassium Nitrate	9.54±0.37	19.07
6	NSV-Calcium Nitrate	9.14±1.07	18.27
7	NSV-Ammonium Nitrate	9.11±0.81	18.21
8	NSV-Ammonium Meta Vendate	8.94±0.21	17.87
9	Media-CHU-10	8.84±0.32	17.67
10	CCV 7.5g/l	8.82±0.41	17.63
11	NCV (>> Standard)	8.69±0.27	17.38
12	Light Period (12L:12D)	8.23±0.69	16.46
13	Light Intensity (15 W)	7.95±0.64	15.91
14	CCV 15.0g/l	7.55±0.90	15.10
15	PSV-DSHP	7.36±1.56	14.72
16	Media-BG-11	7.21±0.36	14.43
17	PCV (Standard)	7.17±2.53	14.35
18	Shaking period- 4 Hours	7.14±0.16	14.29

19	Light period 24L:00D	7.14±0.15	14.29
20	Carbon Source- Sucrose	6.92±0.63	13.83
21	Carbon Source-Maltose	6.90±0.35	13.81
22	CCV 10.0g/l	6.77±0.28	13.54
23	Light Intensity-20 W	6.71±1.27	13.43
24	CCV 12.5g/l	6.70±0.23	13.39
25	Media- BBM	6.68±1.38	13.35
26	NCV (> Standard)	6.68±0.32	13.35
27	CCV 0.5g/l	6.60±0.71	13.19
28	Light Intensity (8 W)	6.55±0.19	13.09
29	NCV (< Standard)	6.50±0.73	13.01
30	Shaking period- 8 Hours	6.50±0.59	13.01
31	CCV 0.75g/l	6.43±0.10	12.87
32	NCV (<< standard)	6.39±0.29	12.78
33	Light Colour-Blue	6.34±0.81	12.68
34	NCV (Standard)	6.22±0.32	12.43
35	PSV-AP	6.19±0.40	12.39
36	Carbon Source- Fructose	6.02±0.62	12.05
37	PCV (< Standard)	5.77±0.49	11.53
38	CCV 1.0g/l	5.46±0.08	10.92
39	CCV 2.5g/l	5.42±0.58	10.84
40	pH-10	5.40±0.56	10.80
41	Light Colour- Red	5.37±4.82	10.75
42	Light colour- White	5.34±0.32	10.67
43	Carbon Source-Glycerol	5.32±0.91	10.63
44	Media-N-11	5.30±0.16	10.61
45	PSV-PDHP	5.28±0.37	10.55
46	Light Intensity- 40 W	5.19±0.75	10.37
47	pH-7.1	5.09±1.45	10.18
48	Light Period 16L:00D	4.96±0.17	9.93
49	Nitrogen Starvation-N-11	4.82±0.30	9.65
50	PSV-SDHP	4.66±4.47	9.32
51	PCV (<< Standard)	4.61±0.07	9.21
52	PSV-DPHP	4.61±0.31	9.21
53	PCV (> Standard)	4.52±1.36	9.04
54	PCV (>> Stndard)	4.51±0.21	9.01
55	Nitrogen Starvation-BBM	4.48±0.79	8.95
56	pH-6	4.46±1.51	8.93
57	pH-8	4.31±0.69	8.62
58	Nitrogen Starvation-BG-11	4.08±0.81	8.15
59	Nitrogen Starvation-M-8	4.05±0.77	8.10
60	Nitrogen Starvation-Bold 3N	3.95±0.83	7.90
61	Shaking period- 6 Hours	3.93±0.07	7.85
62	Shaking period- 2Hours	3.74±0.60	7.49
63	Shaking period- 0 Hours	3.62±0.59	7.24
64	Media-Bold 3N	3.37±0.19	6.74
65	Media-M-8	3.33±0.17	6.65
66	Shaking period- 24 Hours	2.43±0.39	4.86

6. Fatty Acid Methyl Ester (FAME) analysis of potential lipid yielding algal biomass: The GC-MS result of FAME sample of three culture conditions and standard BG-11 is mentioned in figure-5 (a) to (l) as follows:

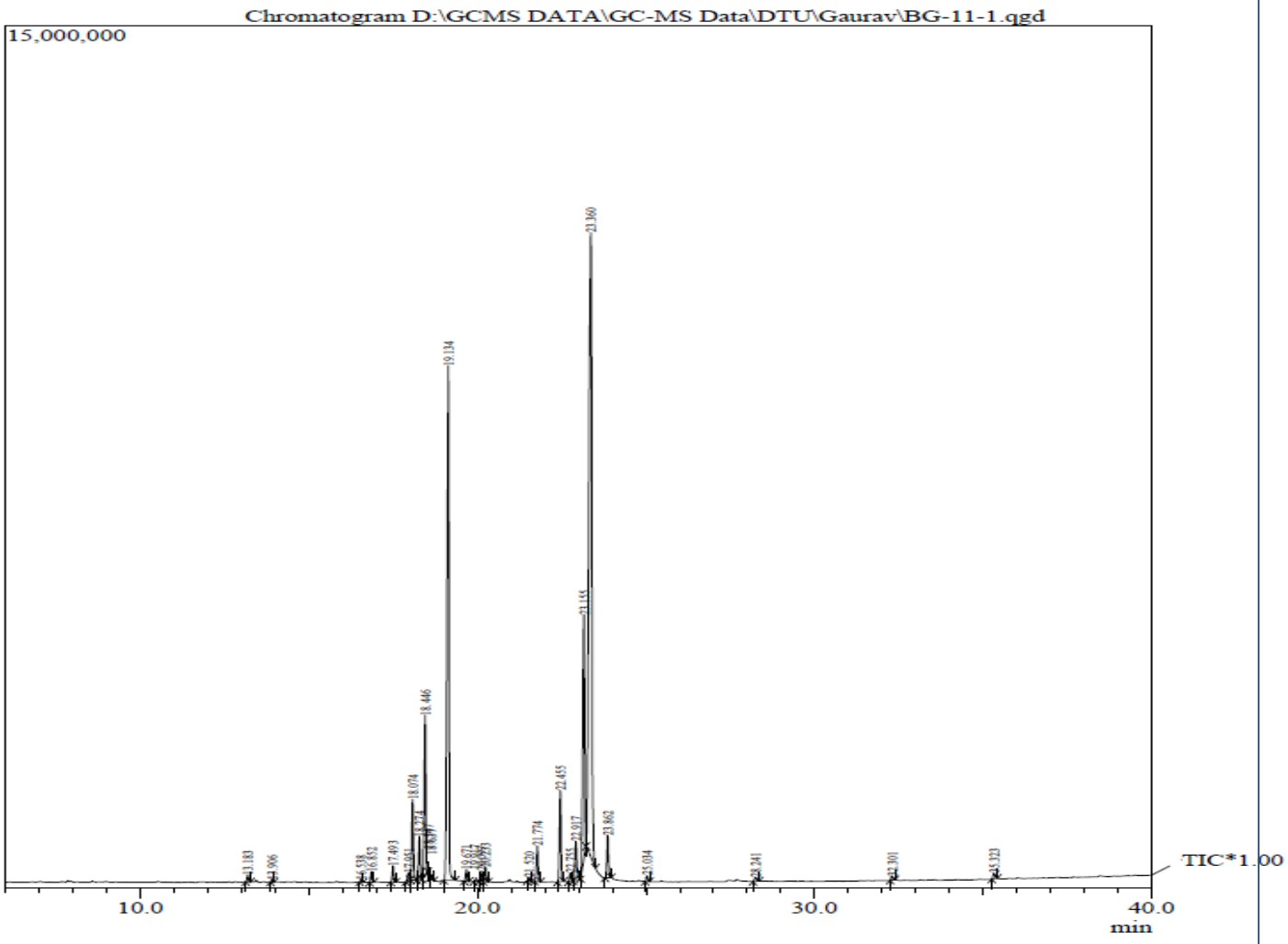


Figure-5 (a): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in standard conditions i.e. BG-11 medium (Sample-1)

Peak#	R.Time	Area	Area%	Name
2	13.906	112569	0.07	TETRADECANOIC ACID, METHYL ESTER
3	16.538	97326	0.06	HEPTADECANOIC ACID, METHYL ESTER
8	18.274	2553238	1.64	7,10-HEXADECADIENOIC ACID, METHYL ESTER
9	18.446	9479657	6.09	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
10	18.547	163494	0.11	9-OCTADECENOIC ACID, METHYL ESTER
11	18.637	177686	0.11	6-OCTADECENOIC ACID, METHYL ESTER
12	19.134	35626825	22.89	HEXADECANOIC ACID, METHYL ESTER
13	19.671	700138	0.45	Phosphoric acid, monododecyl ester
17	21.520	245003	0.16	HEXADECANOIC ACID, METHYL ESTER
20	22.755	515842	0.33	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
21	22.917	2707073	1.74	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTER
22	23.155	16983802	10.91	9,12-OCTADECADIENOIC ACID, METHYL ESTER
23	23.360	66420922	42.67	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
24	23.862	2470820	1.59	OCTADECANOIC ACID, METHYL ESTER
26	28.241	314273	0.20	HENEICOSANOIC ACID, METHYL ESTER
27	32.301	268091	0.17	HENEICOSANOIC ACID, METHYL ESTER
		155646297	100.00	

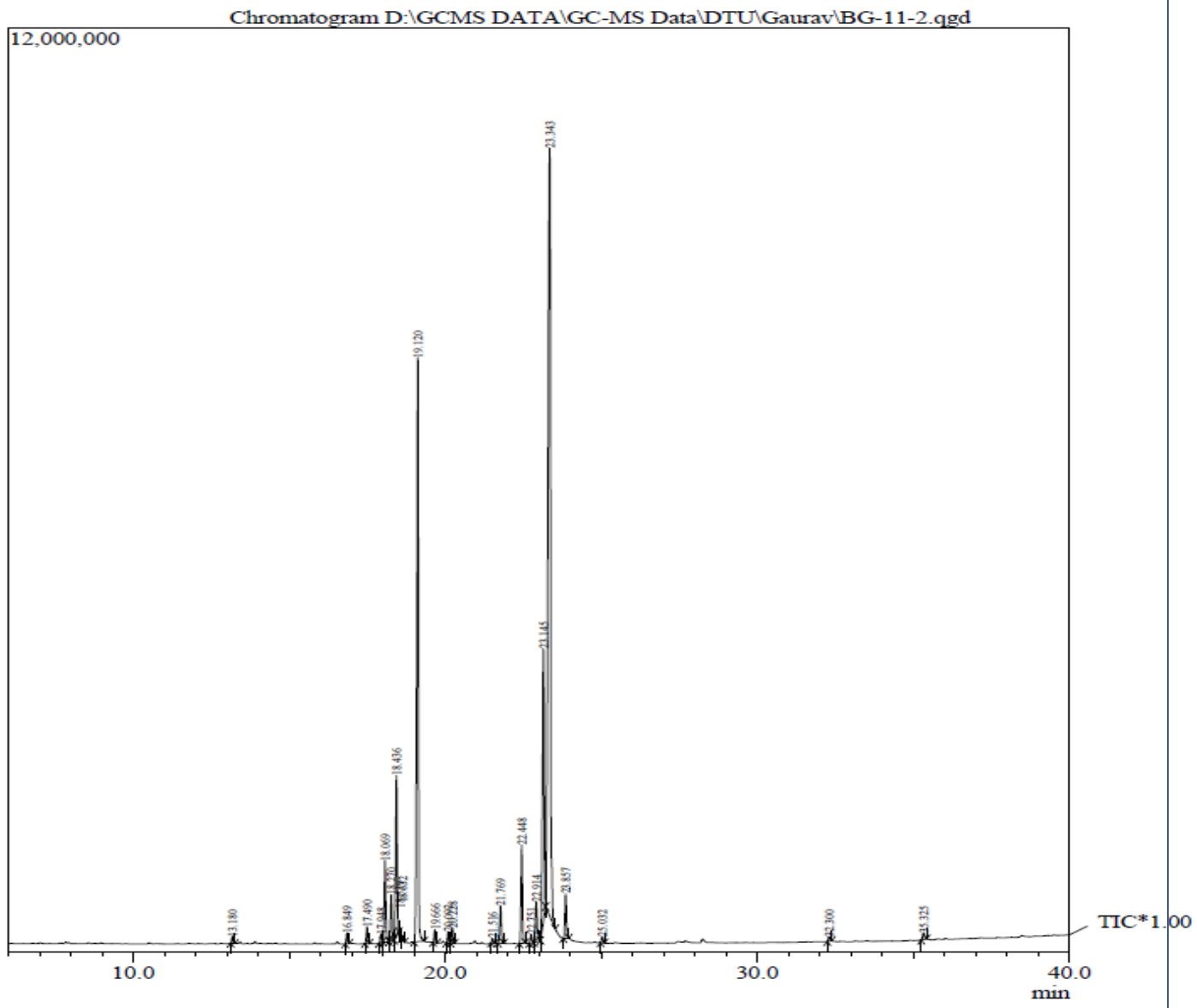


Figure-5 (b): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in standard conditions i.e. BG-11 medium (Sample-2)

Peak#	R.Time	Area	Area%	Name
6	18.270	1981572	1.56	7,10-HEXADECADIENOIC ACID, METHYL ESTER
7	18.436	7096010	5.59	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
8	18.539	85784	0.07	13-DOCOSENOIC ACID, METHYL ESTER0
9	18.632	124280	0.10	9-OCTADECENOIC ACID (Z)-, METHYL ESTER
10	19.120	28432011	22.39	HEXADECANOIC ACID, METHYL ESTER
11	19.666	545792	0.43	Phosphoric acid, monododecyl ester
14	21.516	202453	0.16	HEXADECANOIC ACID, METHYL ESTER
17	22.751	403729	0.32	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
18	22.914	2118977	1.67	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTE.
19	23.145	13734328	10.82	9,12-OCTADECADIENOIC ACID, METHYL ESTER, (E
20	23.343	56824221	44.75	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
21	23.857	1982169	1.56	OCTADECANOIC ACID, METHYL ESTER
23	32.300	228354	0.18	HENEICOSANOIC ACID, METHYL ESTER
		126985747	100.00	

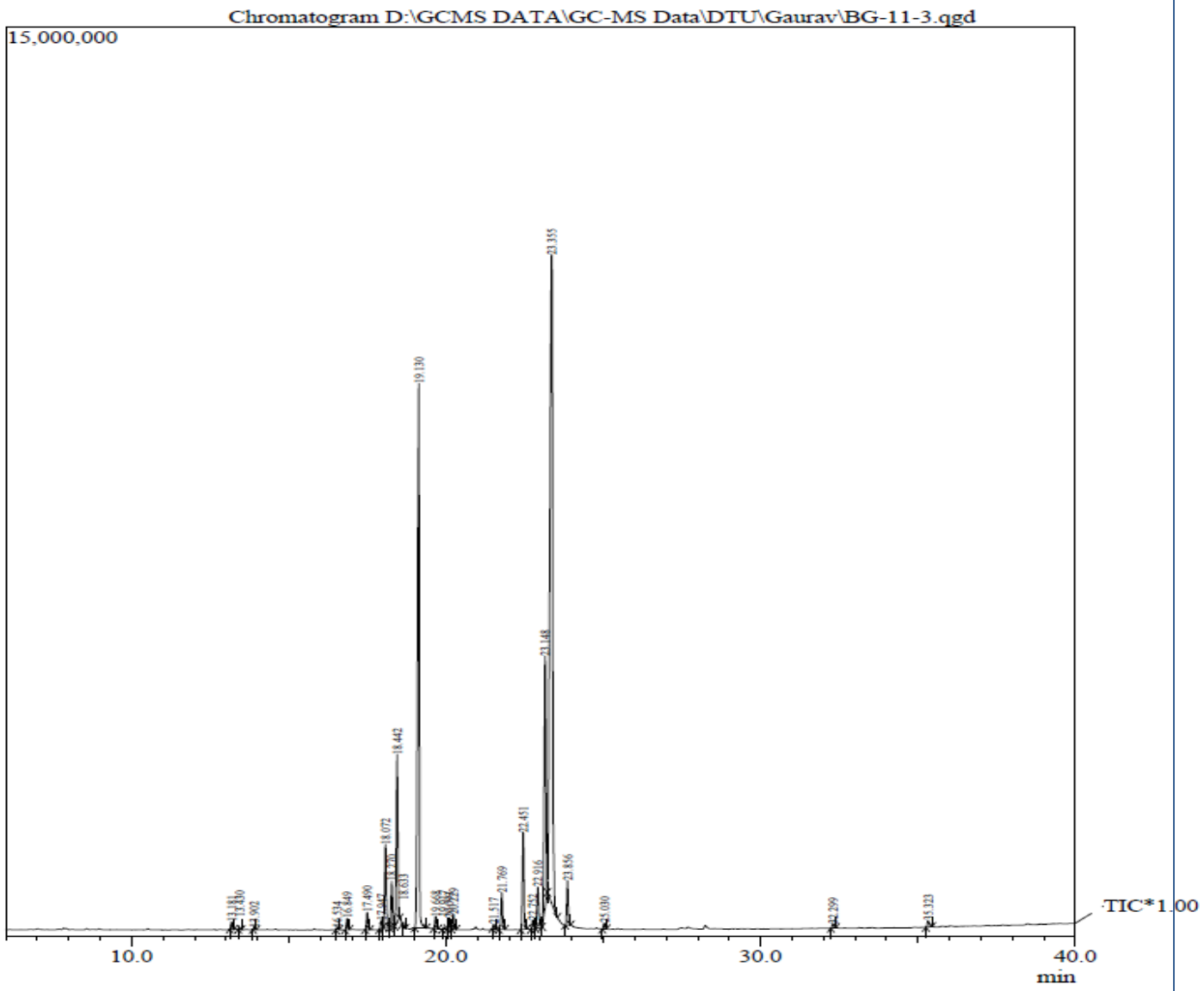


Figure-5 (c): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in standard conditions i.e. BG-11 medium (Sample-3)

Peak#	R.Time	Area	Area%	Name
3	13.902	115665	0.07	TETRADECANOIC ACID, METHYL ESTER
4	16.534	106294	0.07	HEPTADECANOIC ACID, METHYL ESTER
9	18.270	2575358	1.65	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.442	9482040	6.09	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.633	163591	0.11	9-OCTADECENOIC ACID, METHYL ESTER
12	19.130	35881528	23.06	HEXADECANOIC ACID, METHYL ESTER
13	19.668	707588	0.45	Phosphoric acid, monododecyl ester
17	21.517	241959	0.16	HEXADECANOIC ACID, METHYL ESTER
20	22.752	506281	0.33	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
21	22.916	2705221	1.74	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTE.
22	23.148	16916762	10.87	METHYL OCTADECA-9,12-DIENOATE
23	23.355	66269807	42.59	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
24	23.856	2478331	1.59	OCTADECANOIC ACID, METHYL ESTER
26	32.299	280395	0.18	HENEICOSANOIC ACID, METHYL ESTER
		155614018	100.00	

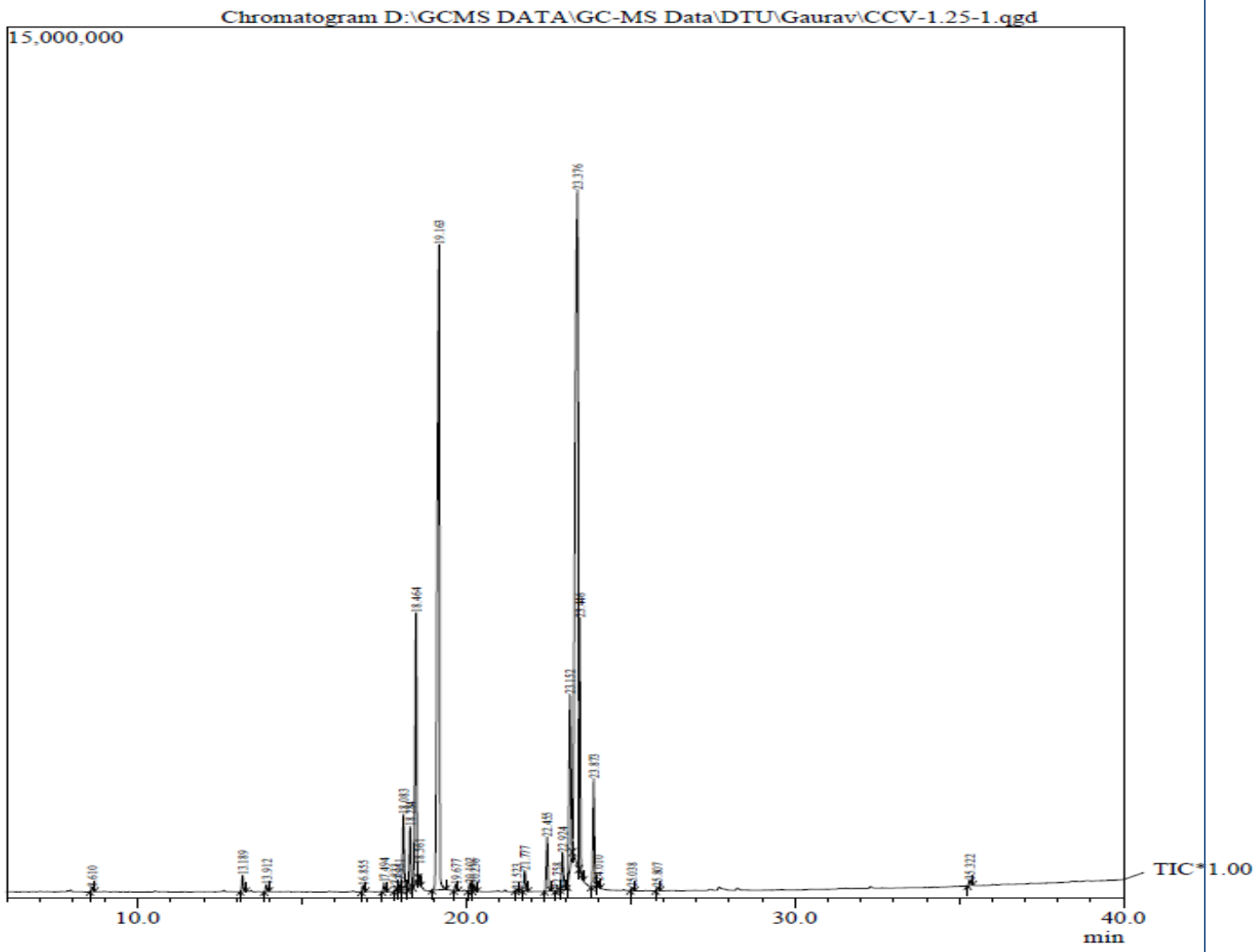


Figure-5 (d): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 1.25 g/l (Sample-1)

Peak#	R.Time	Area	Area%	Name
1	8.610	281552	0.14	DODECANOIC ACID, METHYL ESTER
3	13.912	415051	0.21	HEPTADECANOIC ACID, METHYL ESTER
6	17.833	93401	0.05	TETRADECANOIC ACID, 3-HYDROXY-, METHYL EST.
9	18.284	3767817	1.92	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.464	15944170	8.11	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
11	18.561	237802	0.12	METHYL 9-OCTADECENOATE
12	19.163	54214462	27.56	HEXADECANOIC ACID, METHYL ESTER
13	19.677	380219	0.19	Phosphoric acid, monododecyl ester
16	21.523	103210	0.05	HEXADECANOIC ACID, METHYL ESTER
19	22.758	204872	0.10	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
20	22.924	2599428	1.32	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTER
21	23.152	12371048	6.29	METHYL OCTADECA-9,12-DIENOATE
22	23.376	77464409	39.38	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
23	23.446	9271383	4.71	16-OCTADECENOIC ACID, METHYL ESTER
24	23.873	5928591	3.01	OCTADECANOIC ACID, METHYL ESTER
27	25.807	239369	0.12	METHYL 9-OCTADECENOATE
		196710617	100.00	

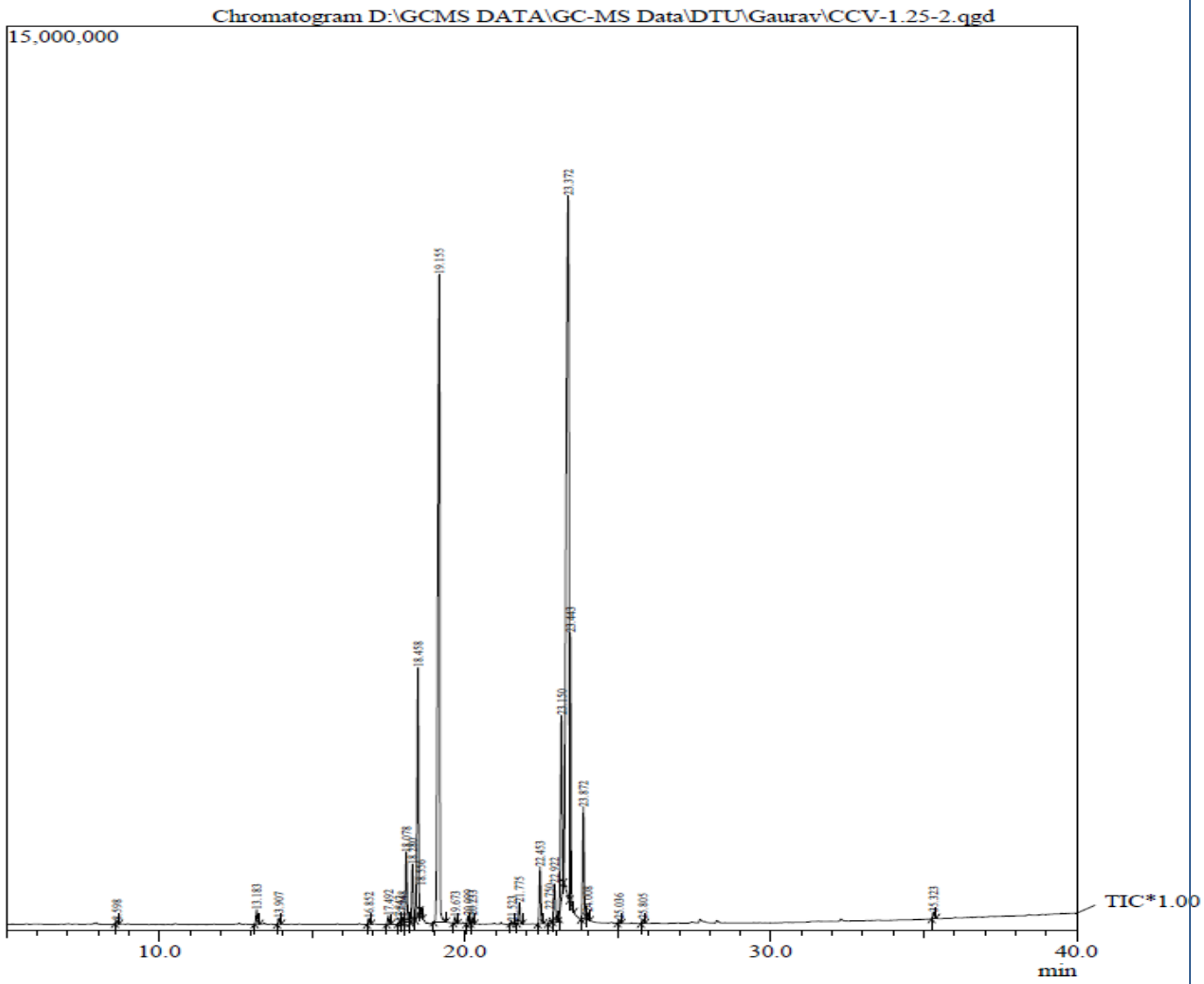


Figure-5 (e): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 1.25 g/l (Sample-2)

Peak#	R.Time	Area	Area%	Name
1	8.598	181608	0.10	TETRADECANOIC ACID, METHYL ESTER
3	13.907	341529	0.18	HEPTADECANOIC ACID, METHYL ESTER
6	17.842	58719	0.03	TETRADECANOIC ACID, 3-HYDROXY-, METHYL ESTER
9	18.280	3428543	1.82	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.458	14181198	7.51	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
11	18.556	184745	0.10	METHYL 9-OCTADECENOATE
12	19.155	50500258	26.76	HEXADECANOIC ACID, METHYL ESTER
13	19.673	368211	0.20	Phosphoric acid, monododecyl ester
16	21.523	95024	0.05	HEXADECANOIC ACID, METHYL ESTER
19	22.750	172949	0.09	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
20	22.922	2553640	1.35	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER
21	23.150	12129235	6.43	METHYL OCTADECA-9,12-DIENOATE
22	23.372	75645660	40.08	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
23	23.443	10190389	5.40	16-OCTADECENOIC ACID, METHYL ESTER
24	23.872	6086013	3.22	OCTADECANOIC ACID, METHYL ESTER
27	25.805	249163	0.13	METHYL 9-OCTADECENOATE
		188733917	100.00	

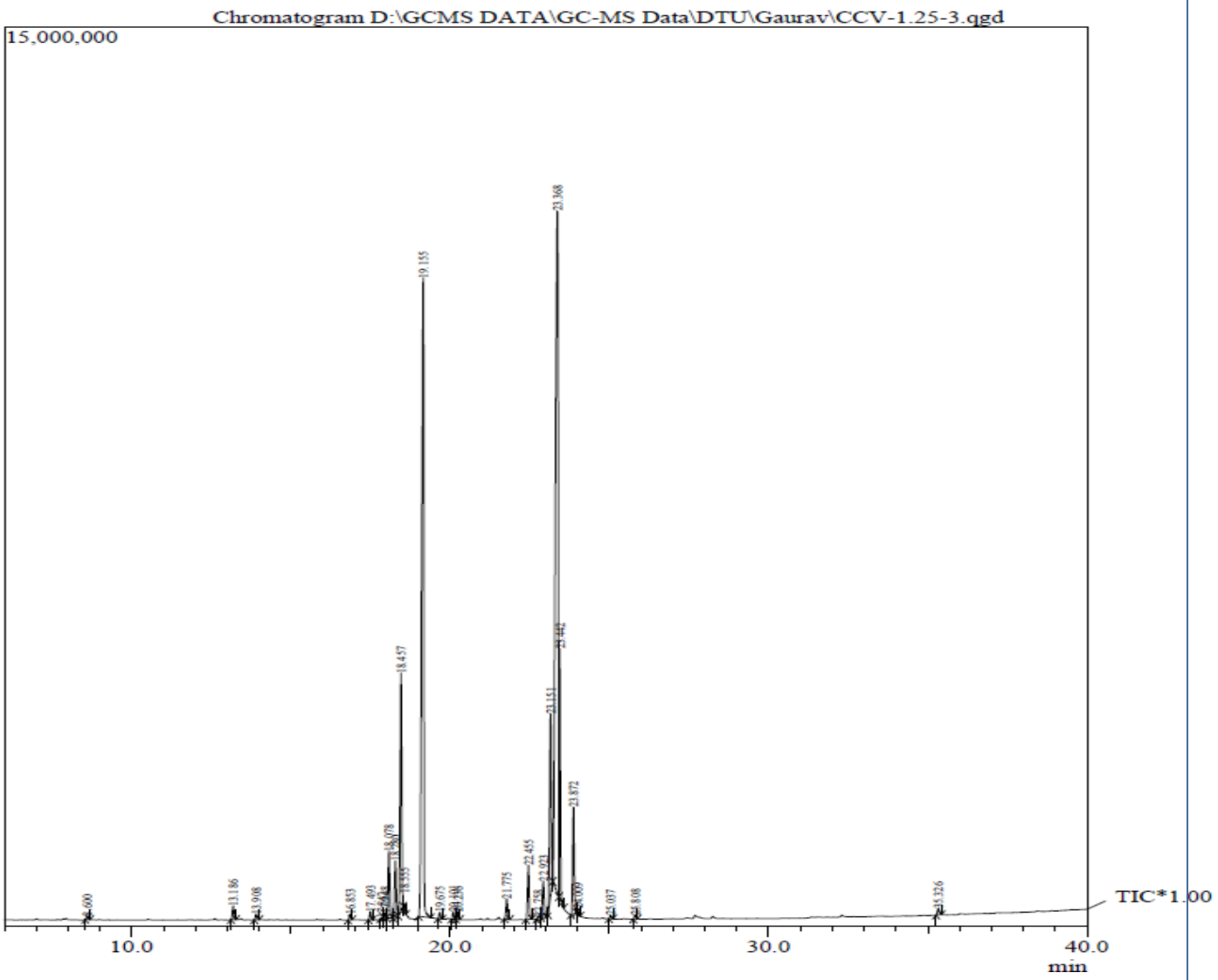


Figure-5 (f): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 1.25 g/l (Sample-3)

Peak#	R.Time	Area	Area%	Name
1	8.600	222660	0.12	DODECANOIC ACID, METHYL ESTER
3	13.908	345439	0.19	HEPTADECANOIC ACID, METHYL ESTER
6	17.842	32196	0.02	TETRADECANOIC ACID, 3-HYDROXY-, METHYL ES.
9	18.280	3324803	1.83	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.457	13563427	7.45	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.555	169389	0.09	METHYL 9-OCTADECENOATE
12	19.155	48426353	26.59	HEXADECANOIC ACID, METHYL ESTER
13	19.675	365009	0.20	Phosphoric acid, monododecyl ester
18	22.758	174324	0.10	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
19	22.923	2441173	1.34	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
20	23.151	11788216	6.47	METHYL OCTADECA-9,12-DIENOATE
21	23.368	73552536	40.38	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
22	23.442	9624624	5.28	16-OCTADECENOIC ACID, METHYL ESTER
23	23.872	5853417	3.21	OCTADECANOIC ACID, METHYL ESTER
26	25.808	235717	0.13	METHYL 9-OCTADECENOATE
		182150134	100.00	

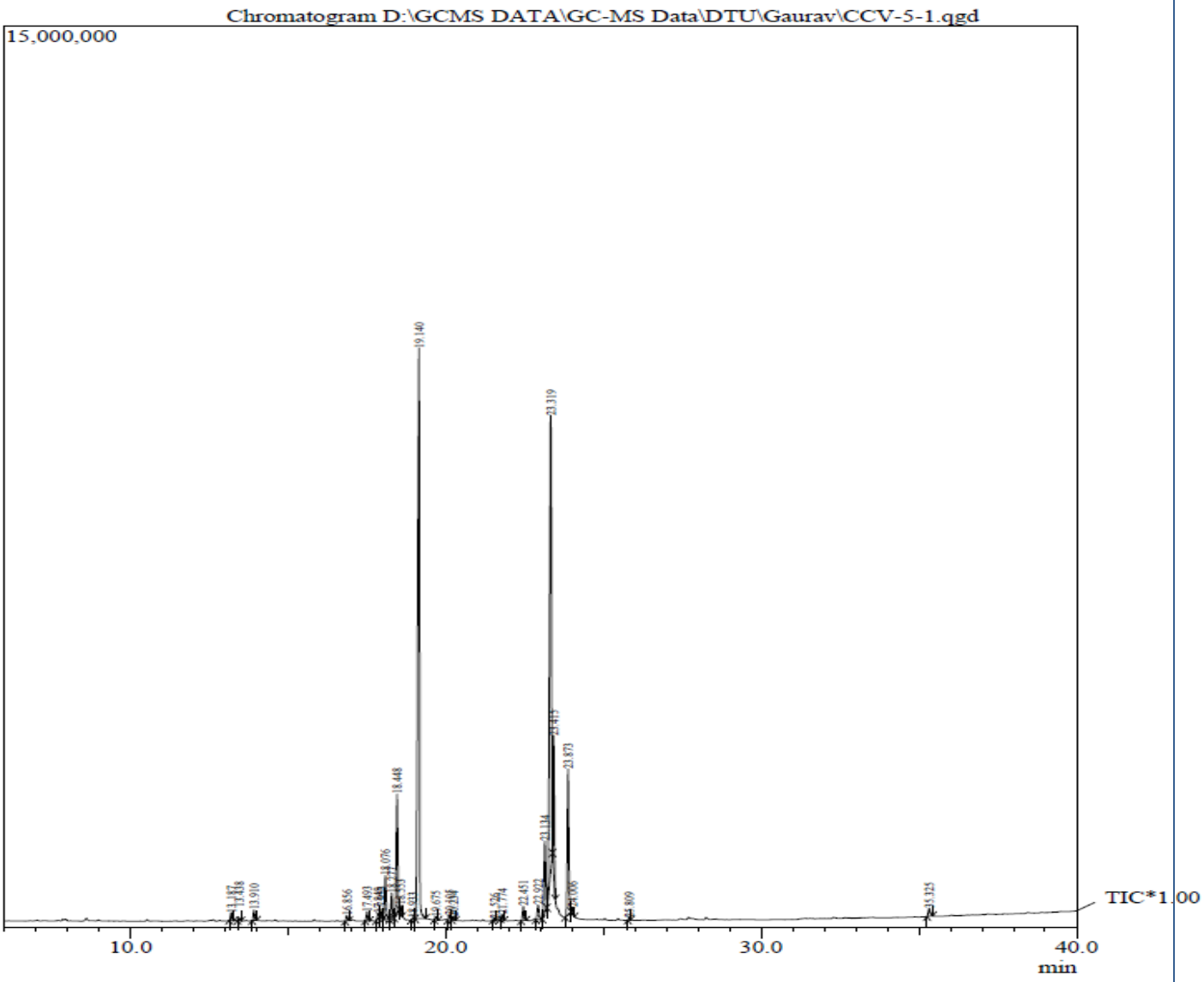


Figure-5 (g): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 5 g/l (Sample-1)

Peak#	R.Time	Area	Area%	Name
3	13.910	589167	0.55	HEPTADECANOIC ACID, METHYL ESTER
6	17.848	303848	0.28	TETRADECANOIC ACID, 3-HYDROXY-, METHYL ES.
9	18.277	1454676	1.35	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.448	6937415	6.46	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.553	250217	0.23	METHYL 9-OCTADECENOATE
13	19.140	38540519	35.88	HEXADECANOIC ACID, METHYL ESTER
14	19.675	188651	0.18	Phosphoric acid, monododecyl ester
17	21.526	94100	0.09	HEXADECANOIC ACID, METHYL ESTER
20	22.922	1018325	0.95	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
21	23.134	4352412	4.05	9,12-OCTADECADIENOIC ACID, METHYL ESTER
22	23.319	33049305	30.77	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
23	23.415	5520337	5.14	16-OCTADECENOIC ACID, METHYL ESTER
24	23.873	8239456	7.67	HEXADECANOIC ACID, 15-METHYL-, METHYL EST.
26	25.809	116677	0.11	9-OCTADECENOIC ACID (Z)-, METHYL ESTER
		107406980	100.00	

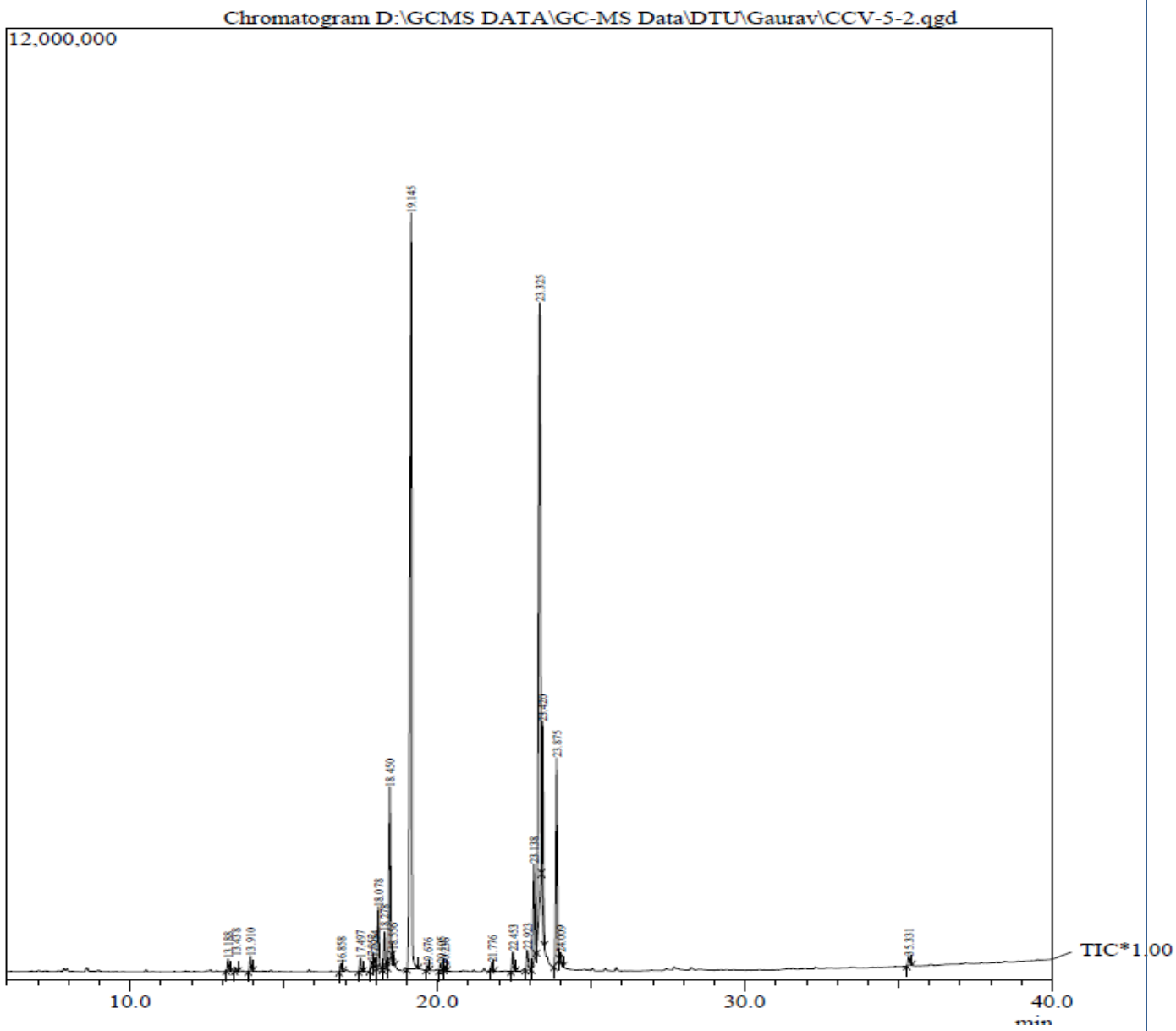


Figure-5 (h): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 5 g/l (Sample-2)

Peak#	R.Time	Area	Area%	Name
3	13.910	654425	0.58	HEPTADECANOIC ACID, METHYL ESTER
6	17.852	289842	0.26	TETRADECANOIC ACID, 3-HYDROXY-, METHYL ES.
9	18.278	1574130	1.40	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.450	7478137	6.67	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.556	274973	0.25	METHYL 9-OCTADECENOATE
12	19.145	40518442	36.15	HEXADECANOIC ACID, METHYL ESTER
13	19.676	171609	0.15	Phosphoric acid, monododecyl ester
18	22.923	1051717	0.94	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
19	23.138	4628546	4.13	9,12-OCTADECADIENOIC ACID, METHYL ESTER
20	23.325	34360907	30.66	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
21	23.420	5471998	4.88	16-OCTADECENOIC ACID, METHYL ESTER
22	23.875	8635665	7.70	HEXADECANOIC ACID, 15-METHYL-, METHYL EST
		112088293	100.00	

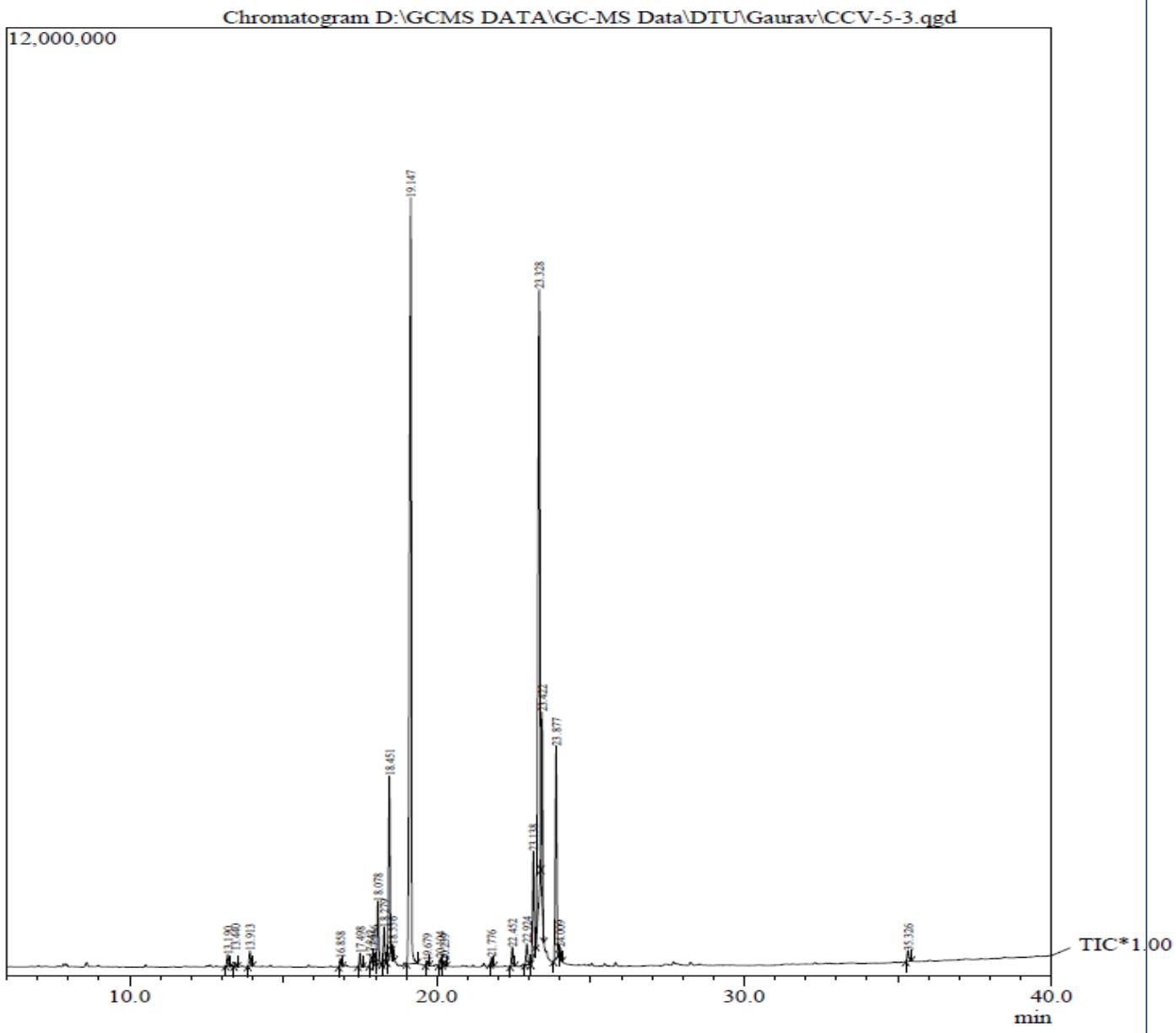


Figure-5 (i): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in carbon source glycerol concentration 5 g/l (Sample-3)

Peak#	R.Time	Area	Area%	Name
3	13.913	671842	0.58	HEPTADECANOIC ACID, METHYL ESTER
6	17.842	294320	0.26	HEXADECANOIC ACID, 3-HYDROXY-, METHYL EST.
9	18.279	1607944	1.39	7,10-HEXADECADIENOIC ACID, METHYL ESTER
10	18.451	7722055	6.70	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.556	314299	0.27	METHYL 9-OCTADECENOATE
12	19.147	41169330	35.72	HEXADECANOIC ACID, METHYL ESTER
13	19.679	203678	0.18	Phosphoric acid, monododecyl ester
18	22.924	1110050	0.96	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
19	23.138	4852714	4.21	9,12-OCTADECADIENOIC ACID, METHYL ESTER
20	23.328	35233924	30.57	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
21	23.422	5804110	5.04	16-OCTADECENOIC ACID, METHYL ESTER
22	23.877	9021972	7.83	HEXADECANOIC ACID, 15-METHYL-, METHYL EST.
		115271159	100.00	

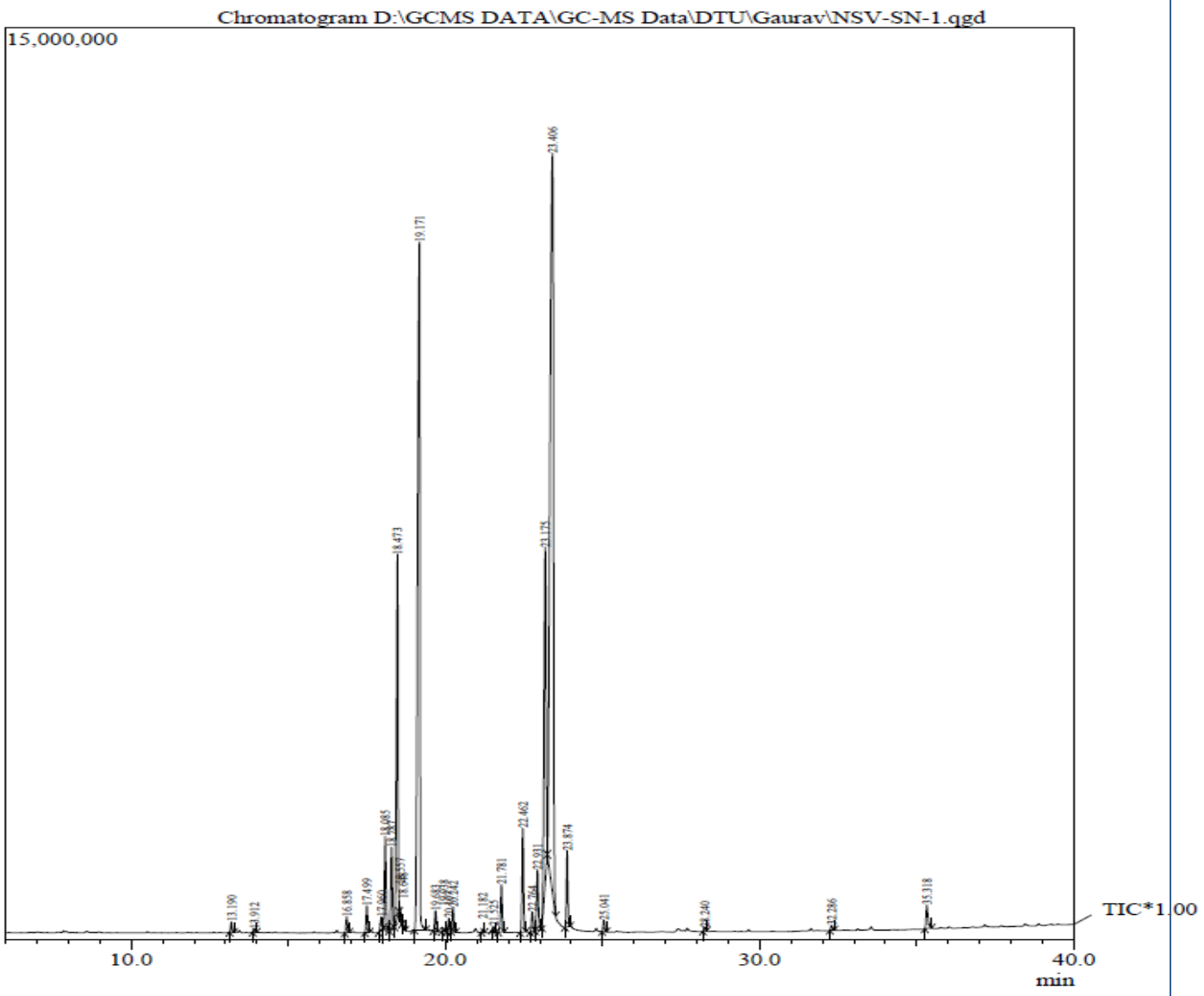


Figure-5 (j): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in nitrogen source variation - Sodium Nitrate (Sample-1)

Peak#	R.Time	Area	Area%	Name
2	13.912	208033	0.09	HEPTADECANOIC ACID, METHYL ESTER
7	18.287	4795087	2.05	7,10-HEXADECADIENOIC ACID, METHYL ESTER
8	18.473	20606498	8.79	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
10	18.648	218504	0.09	9-OCTADECENOIC ACID
11	19.171	57745383	24.63	HEXADECANOIC ACID, METHYL ESTER
12	19.683	1143657	0.49	Phosphoric acid, monododecyl ester
17	21.525	220460	0.09	HEXADECANOIC ACID, METHYL ESTER
20	22.764	1216749	0.52	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
21	22.931	3998200	1.71	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTE.
22	23.175	25715104	10.97	9,12-OCTADECADIENOIC ACID (Z,Z)-, METHYL EST
23	23.406	91355431	38.97	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
24	23.874	3930602	1.68	OCTADECANOIC ACID, METHYL ESTER
26	28.240	242305	0.10	HENEICOSANOIC ACID, METHYL ESTER
27	32.286	299637	0.13	TETRACOSANOIC ACID, METHYL ESTER
		234427793	100.00	

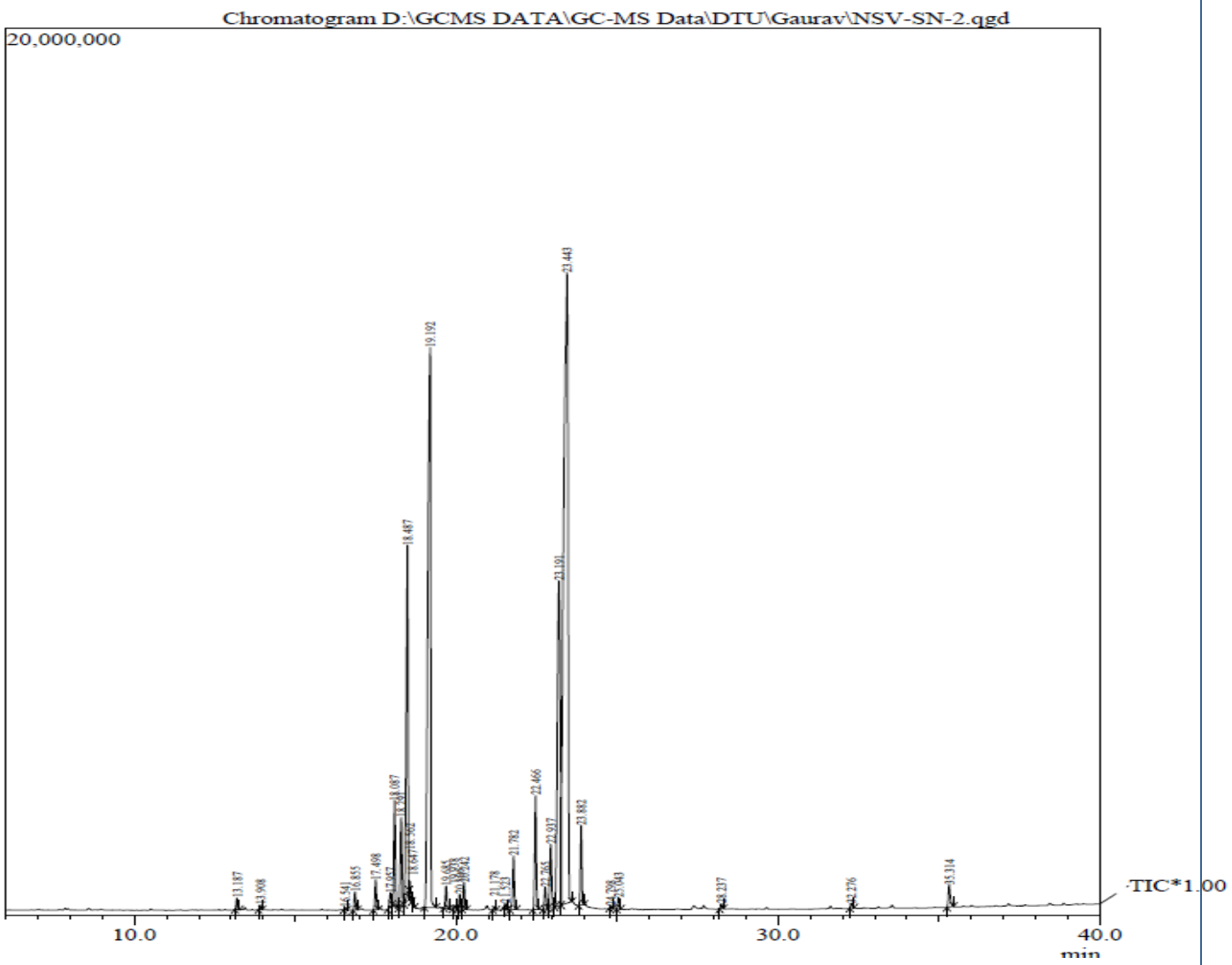


Figure-5 (k): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in nitrogen source variation - Sodium Nitrate (Sample-2)

Peak#	R.Time	Area	Area%	Name
2	13.908	335026	0.10	HEPTADECANOIC ACID, METHYL ESTER
3	16.541	196680	0.06	HEXADECANOIC ACID, METHYL ESTER
8	18.291	7371288	2.13	7,10-HEXADECADIENOIC ACID, METHYL ESTER
9	18.487	30540050	8.84	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
11	18.647	387330	0.11	9-OCTADECENOIC ACID
12	19.192	77656805	22.47	HEXADECANOIC ACID, METHYL ESTER
13	19.685	1676661	0.49	Phosphoric acid, monododecyl ester
18	21.523	324367	0.09	HEPTADECANOIC ACID, METHYL ESTER
21	22.765	1835908	0.53	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
22	22.937	5987445	1.73	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTE.
23	23.191	46340969	13.41	9,12-OCTADECADIENOIC ACID (Z,Z)-, METHYL EST
24	23.443	132321094	38.29	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
25	23.882	5619554	1.63	OCTADECANOIC ACID, METHYL ESTER
26	24.798	200588	0.06	HEXADECANOIC ACID, 3-HYDROXY-, METHYL EST
28	28.237	375282	0.11	HENEICOSANOIC ACID, METHYL ESTER
29	32.276	403324	0.12	TETRACOSANOIC ACID, METHYL ESTER
		345596016	100.00	

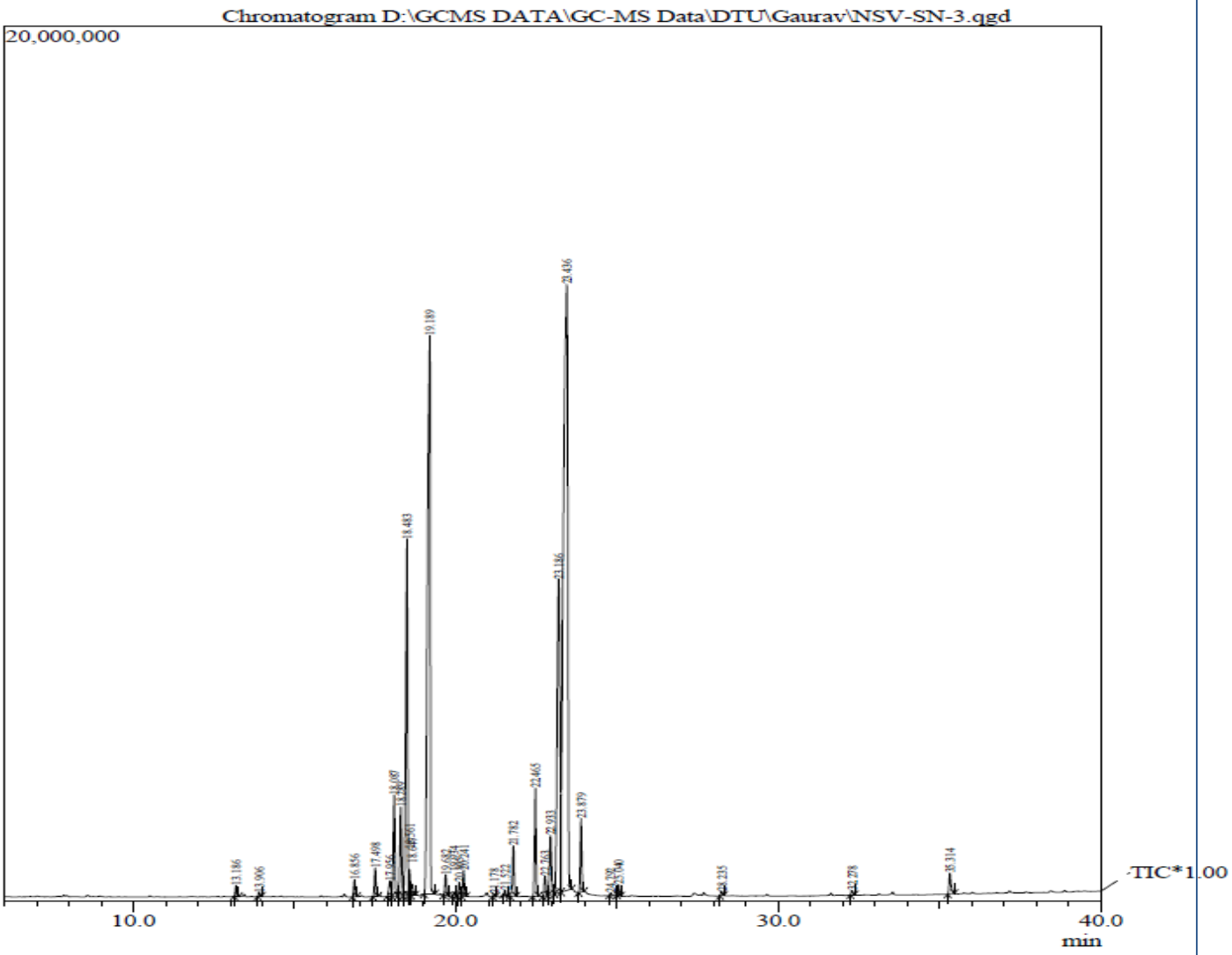


Figure-5 (l): Chromatogram of FAME of lipid prepared from microalga *Chlorella minutissima* cultivated in nitrogen source variation - Sodium Nitrate (Sample-3)

Peak#	R.Time	Area	Area%	Name
2	13.906	310698	0.09	HEPTADECANOIC ACID, METHYL ESTER
7	18.289	7640413	2.25	7,10-HEXADECADIENOIC ACID, METHYL ESTER
8	18.483	31983148	9.43	9,12,15-OCTADECATRIENOIC ACID, METHYL ESTE.
10	18.647	907690	0.27	9-OCTADECENOIC ACID
11	19.189	75661031	22.30	HEXADECANOIC ACID, METHYL ESTER
12	19.682	1554420	0.46	Phosphoric acid, monododecyl ester
17	21.522	349259	0.10	HEPTADECANOIC ACID, METHYL ESTER
20	22.763	1743165	0.51	6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER
21	22.933	5725045	1.69	7,10,13-HEXADECATRIENOIC ACID, METHYL ESTE.
22	23.186	44965706	13.25	9,12-OCTADECADIENOIC ACID (Z,Z)-, METHYL EST
23	23.436	127645572	37.62	8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER
24	23.879	5335221	1.57	OCTADECANOIC ACID, METHYL ESTER
25	24.792	1897	0.00	HEXADECANOIC ACID, 3-HYDROXY-, METHYL EST
27	28.235	354785	0.10	HENEICOSANOIC ACID, METHYL ESTER
28	32.278	365286	0.11	TETRACOSANOIC ACID, METHYL ESTER
		339268342	100.00	

DISCUSSION AND FUTURE PERSPECTIVES

Processing of algal biomass is very essential to obtain better yield of desired products such as lipids, carbohydrates, etc. In this project, efforts have been made to identify potential methods of each stage of bio-diesel production so that maximum possible lipid can be extracted and further processed and analysed by GC-MS.

1. Potential Culture Conditions: Potential of microalgae for the production of high and low value commercial products is very huge but 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 fact into the consideration, 19 different parameters were set separately with a range of 97 variation of culture conditions so that basic culture conditions can be finalized.

N-11 (66 mg L^{-1} d^{-1}), BBM (62 mg L^{-1} d^{-1}) and BG-11 (61 mg L-1 d-1) showed almost similar results among six different media used in the study of best medium with nitrogen source. So we can use these media for the cultivation of microalga *Chlorella minutissima* although due to simple preparation of N-11 medium, it can be favoured. If we desire to grow this microalga under nitrogen starvation conditions, BBM (79 mg L^{-1} d^{-1}) and BG-11 (77 mg L^{-1} d^{-1}) medium can be preferred. Ördög *et al.,* 2012 also worked on *Chlorella minutissima* under three different nitrogen concentrations i.e. 7, 70 and 700 mg L^{-1} and the study concluded that nitrogen limitation or starvation are good for biomass productivity up-to a certain limit.

Sodium nitrate (66 mg L^{-1} d^{-1}) and potassium (64 mg L^{-1} d^{-1}) nitrate were found potential source of nitrate. Li. *et al.,* 2011 [39] also reported that potassium nitrate is second best source of nitrate in similar alga. In case of source of phosphorus, di potassium hydrogen phosphate (149 mg L^{-1} d^{-1}) and potassium di hydrogen phosphate (144 mg L^{-1} d^{-1}) provided good results. Biomass productivity of glycerol as carbon source (361 mg L^{-1} d^{-1}) was very high in a very short time of cultivation (14 days) and Li. *et al.* 2011 also reported the same. Gautam *et al.,* 2013 also concluded that glycerol was second best source of carbon after glucose.

The potential concentration of nitrogen which provided maximum biomass productivity was 0.375 g/L (200 mg L^{-1} d^{-1}). In case of phosphorus, higher concentration of salt favoured more biomass production. In case of glycerol, higher production of biomass was found at specific concentrations i.e. 12.5, 2.5, 1.0 and 5.0 g/L.

Microalga *Chlorella minutissima* showed high biomass production at alkaline pH. pH ranges from 7.1 to 10 provided very less difference in biomass production so it was concluded that this range of pH was favoured by microalga *Chlorella minutissima*. Similar results were found in a study conducted by Cao *et al*. 2014 and it was concluded by them that microalga *Chlorella minutissima* provided maximum biomass at pH 7-8.

There was an increase in the biomass production, as we increased the light intensity. Similar results were recorded by Tang *et al.,* 2011 where fluorescent light was used. But higher intensity beyond 20 W (110 mg L^{-1} d^{-1}) disrupted the trend of increasing of biomass production which supports the fact of use of higher intensity of light for gearing up the biomass production is good but only up-to a certain extent.

There was in increase in biomass production in case of light colour or source, as we move from blue to red to white. Tang *et al.,* 2011 found similar results where they used three light sources i.e. fluorescent, white and red colour. Light period has shown significant effect on final production of biomass as we increased the time of illumination from 0 to 8 to 24 hours. 24 hours of illumination provided maximum biomass production. Tang *et al.,* 2011 reported earlier that although cell densities were high in the photoperiods of 12 H light: 12 H dark and 15 H light: 9 H dark but final biomass production was highest in 24 h light: 0 h dark.

Shaking of culture vessel is almost similar to the flow of water in natural habitat of microalgae. The biomass yield decreased on increasing the time of shaking.

Details of biomass productivity of different culture conditions are mentioned in Figure-6 and7. Figure-6 shows the biomass productivity parameter-wise while figure-7 provides information of biomass production of all culture conditions in descending order. These figures provide valuable information that there are several culture conditions which can help to get higher biomass of *Chlorella minutissima* in comparison to normal or standard condition i.e. cultivation in BG-11 medium.

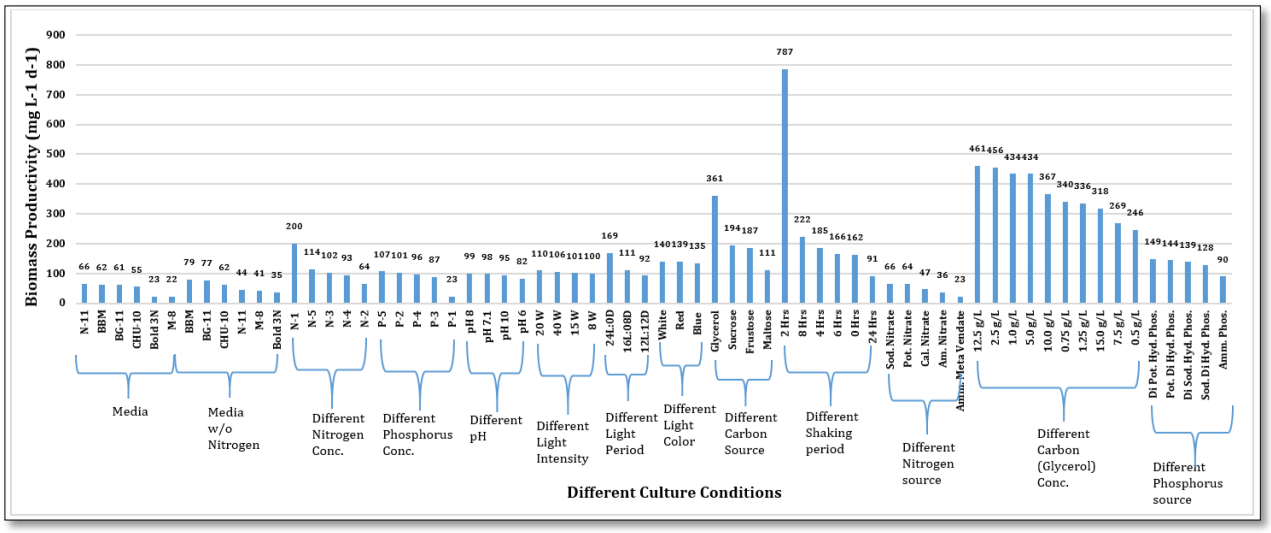


Figure-6: Biomass productivity (mg L^{-1} d^{-1}) of different culture conditions in parameter-wise descending order.

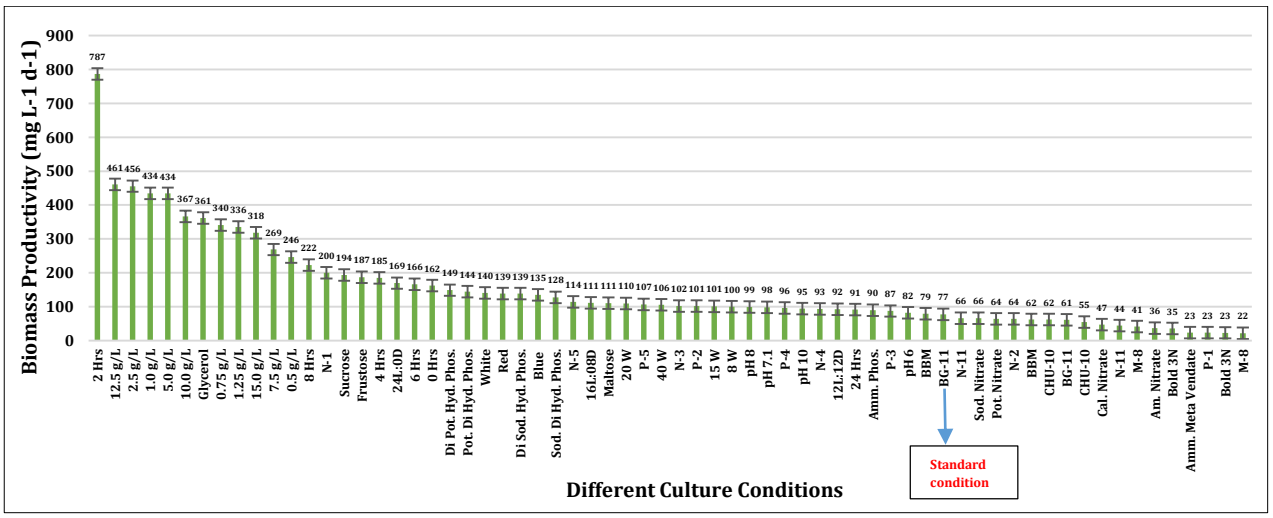


Figure-7: Biomass productivity (mg L^{-1} d^{-1}) of different culture conditions in descending order.

Out of 97 culture conditions, biomass was only harvested in 80 culture conditions because there was no growth in rest of 17 conditions. These 17 culture conditions were either not favourable for the growth or lethal to this microalga. The detail of rest of 17 culture conditions is as follows:

a. Starvation of phosphorus - 6 conditions (All six media)
b. Starvation of both phosphorus and Nitrogen - 6 conditions (All six media)
c. Source of Nitrogen - 4 conditions out of nine (cobalt nitrate, ammonium molybdate, ammonium chloride, glycine)
d. pH - 1 (pH-5)

There are different other parameters which were not taken into consideration during this stage but the same may be explored in future.

2. Potential methods of harvesting: In this work, maximum biomass has been harvested with the help of inorganic flocculant aluminum sulfate. The biomass productivity was 3.57 gm/L. Other methods such as inorganic flocculant ferrous sulfate and organic flocculant chitosan also provided good results. Amount of organic flocculent used is less in comparison to inorganic flocculent but they took more time to act on biomass which inorganic flocculent showed their effect very early. The final results of harvesting experiment in descending order is mentioned in Table-8. This study concluded that flocculation has good potential for the harvesting of biomass and within different flocculants, inorganic flocculants are high yielding in comparison to organic flocculants. This study also concluded that there are four different methods of harvesting which have high yield of biomass in comparison to traditional means of harvesting i.e. centrifugation. Although flocculation uses different flocculant so effect of these chemicals on viability and chemical composition of biomass must

be ascertained. This study didn't look deep in to this issue although it may be taken up in future so that the picture may be more clear. Papazi *et al.,* 2010 also reported that salts of iron have good potential for the harvesting of microalga *Chlorella minutissima.* Although in their study of effect of twelve different salts on harvesting of microalga *Chlorella minutissima,* salts of aluminum provided best results but they destroyed the algal cell so salts of iron were found more potential flocculant.

Table-8: Biomass productivity of different harvesting methods in descending order. Inorganic flocculent 1-$FeCl_3$; Inorganic flocculent 2-$Fe_2(SO_4)_3$; Inorganic flocculent 3-$Al_2(SO_4)_3$; Organic flocculent 1-Chitosan; Organic flocculent 2-Starch.

Sl. No.	Harvesting method	Culture volume (ml)	Days of culture	Mean of amount of biomass (mg) (Mean ± SD)	Projected Biomass in 1000 ml culture (mg)
1	Inorganic Flocculent 3	18	45	64.10±19.4	3561.11
2	Inorganic Flocculent 2	18	45	56.03±6.6	3112.96
3	Organic Flocculent 1	18	45	54.57±8.3	3031.48
4	Inorganic Flocculent 1	18	45	48.57±6.0	2698.15
5	Centrifugation	18	45	47.90±3.5	2661.11
6	Sedimentation + Centrifugation	18	45	43.50±4.0	2416.67
7	Organic Flocculent 2	18	45	41.37±3.8	2298.15
8	Sedimentation	18	45	38.70±1.2	2150.00
9	Sedimentation + filtration	18	45	4.40±1.0	244.44
10	Filtration	18	45	2.17±0.9	120.37

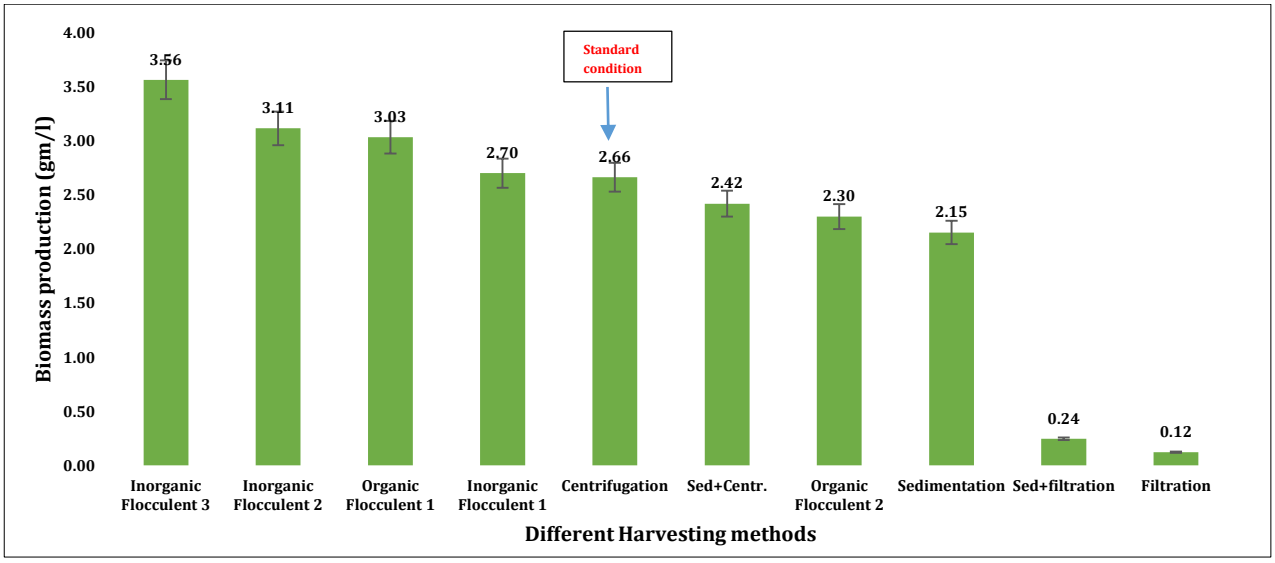


Figure-8: Biomass productivity of different harvesting methods in descending order. Inorganic flocculent 1-$FeCl_3$; Inorganic flocculent 2-$Fe_2(SO_4)_3$; Inorganic flocculent 3-$Al_2(SO_4)_3$; Organic flocculent 1-Chitosan; Organic flocculent 2-Starch.

3. Potential Cell disruption method: Among all cell disruption methods tested, sonication provides maximum % of lipid i.e. 9.67%. Sonication is also very easy to use method and involves no chemicals so there is also no chance of contamination. It is also quick and reliable than other methods. The % of lipid obtained in different cell disruption methods is mentioned in descending order in Table-9. Lee *et al.*, 2010, also conducted a similar comparative study on three different microalgae i.e. *Botryococcus sp., Chlorella vulgaris, and Scenedesmus* sp. to identify potential method of cell disruption in these three microalgae. They have compared five different method of cell disruption such as autoclaving, bead beating, microwave, sonication and osmotic shock by 10% NaCl. They concluded that efficiency of lipid extraction method depends on species of microalgae. However, in their study, microwave provided potential results in all three species and in our case of *Chlorella minutissima,* sonication extracted maximum lipid and found to be most suitable method of cell disruption among different methods tested.

Table-9: % of lipid obtained in different cell disruption methods in descending order.

Sl. No.	Cell disruption method	Amount of lipid in 500 mg of biomass (mg)	Amount of lipid in 1000 mg of biomass (mg) (Projected)	% of lipid i.e. amount of lipid in 100 mg of biomass (mg) (Projected)
1	Sonication	48.33	96.67	9.67
2	4 N HCl	30.67	61.33	6.13
3	Osmotic shock	25.65	51.3	5.13
4	Microwave	25.60	51.2	5.12
5	3% H_2SO_4	22.37	44.73	4.47
6	Autoclave	21.33	42.67	4.27
7	0.1 NaOH	13.73	27.47	2.75

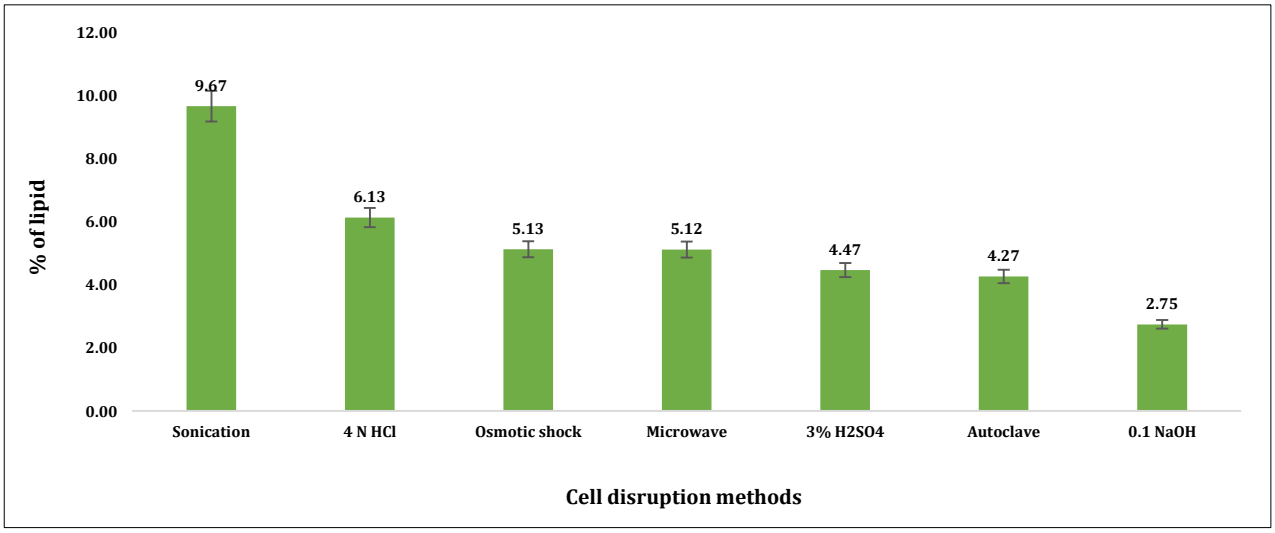


Figure-9: % of lipid in different methods of cell disruption in descending order

4. Potential combination and ratio of lipid extracting solvents: Among the various combinations and ratio of lipid extracting organic solvents tested, combination of Chloroform and Methanol in the ratio of 1:1 was found most suitable for microalga *Chlorella minutissima.* The procedure of extracting lipid utilises different volume of organic solvent so for easy comparison, the results were projected in 1 ml of organic solvents. This extraction process via organic solvents was also combined with best method of cell disruption i.e. sonication which helped in the increase of lipid yield. 21.82 % lipid was extracted when we combined sonication with organic solvent combination chloroform and methanol in the ratio of 1:1. Few other combinations and ratio of organic solvents were also found potential for lipid extraction from microalga *Chlorella minutissima* i.e. dichloromethane: methanol-1:1 (19.99 %), chloroform: methanol-2:1 (18.35%), chloroform: methanol-1:2 (15.21%) and dichloromethane: methanol-1:2 (14.79). If we consider the organic solvent combination of chlorofom and methanol in the ratio 1:2 as standard (Bligh and Dyer method) which is generally used by the researchers, we have recorded more yield of lipid in other ratio. The yield of combination of chloroform and methanol were among the top 4 number in the final results which clearly indicate that this combination is good for extracting lipid from our microalga. Li *et al.,* 2014 also conducted similar kind of comparative study on marine microalga *Tetraselmis* sp. M8. They utilised combination such as chloroform and methanol (1:2), dichloroform: methanol (2:1), propane-2-ol: cyclohexane (1:1.25), ethanol (96%): 3nM KOH, supercritical CO_2 method and soxhlet method {hexane alone and hexane: ethanol (3:1)}. They found dichloromethane: methanol as potential method as it provideed highest yield of lipid. So it can be conclded that due to difference in cell compositions, there is a difference in the efficincy of lipid extraction methods and combinations and ratio of organic solvent. To get maximum yield of lipid in the concenred microalga separate study should be conducted so that it may not be affected by the results of previos study on different microalgae.

Table-10: Descending order of lipid extration by different combination and ratio of organic solvents.

Sl. No.	Detail of combination and ratio	volume of solvent mixture	Mean of lipid with SD in 200 mg of biomass (mg)	Lipid in 100 mg Biomass (mg)	Lipid in 1 ml of Solvent mixture (mg)
1	Chloroform:Methanol (1:1)	5 ml	43.64±0.77	21.82	3.12
2	Dichloromethane:Methanol (1:1)	7 ml	39.99±7.72	19.99	2.86
3	Chloroform:Methanol (2:1)	5 ml	36.70±5.71	18.35	2.62
4	Chloroform:Methanol (1:2)	5 ml	30.41±2.29	15.21	2.17
5	Dichloromethane:Methanol (2:1)	7 ml	29.58±4.15	14.79	2.11
6	Hexane:Ethanol (2:1)	5.2 ml	16.55±1.29	8.28	1.59
7	Dichloromethane:Methanol (1:2)	7 ml	17.87±3.40	8.93	1.28

8	Propane-2-ol:Cyclohexane (1:1)	9 ml	20.94±25.68	10.47	1.16
9	Hexane:Ethanol (1:1)	5.2 ml	10.71±0.55	5.35	1.03
10	Hexane:Ethanol (1:2)	5.2 ml	9.80±0.19	5.21	1.00
11	Propane-2-ol:Cyclohexane (1:1.25)	9 ml	9.75±1.83	4.88	0.54
12	Propane-2-ol:Cyclohexane (1.25:1)	9 ml	8.38±1.96	4.19	0.47

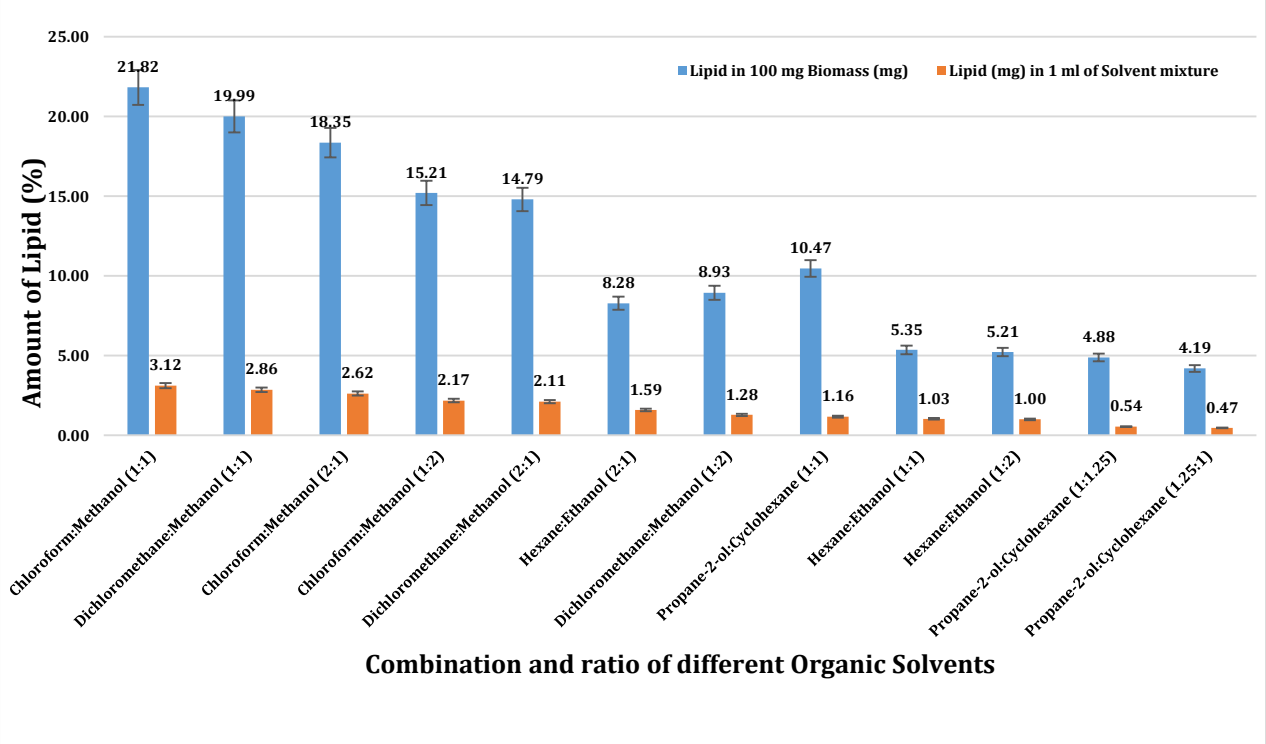
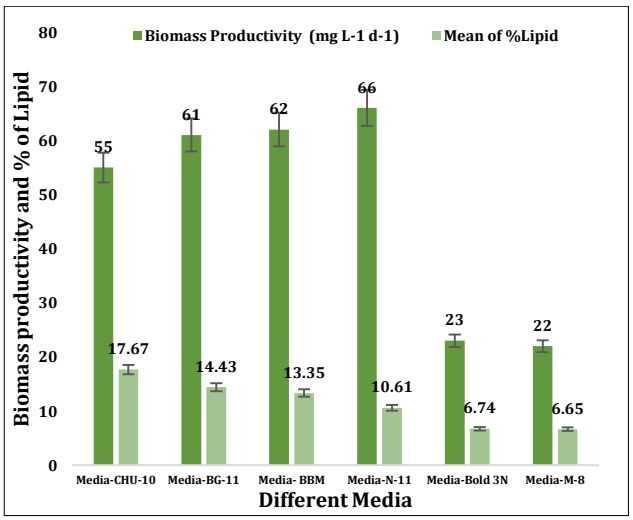


Figure-10: Descending order of yield of lipid extraction combination and ratio of organic solvents as per their 1 ml volume.

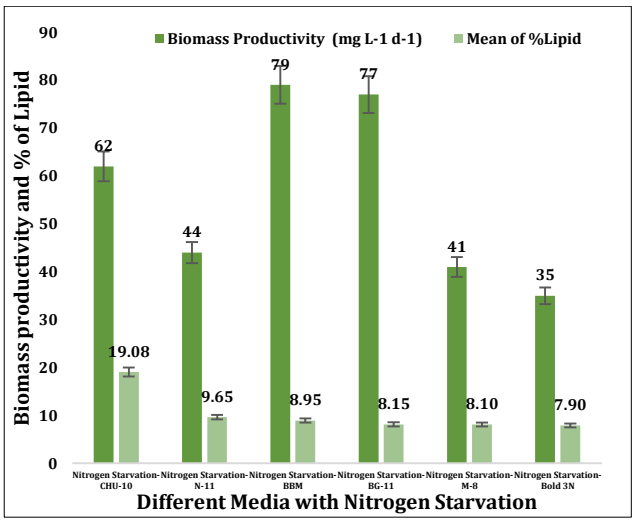
5. Potential culture conditions for high yield of lipid production:

The analysis of result of lipid production of biomass of 66 different culture conditions in different aspects is follows:

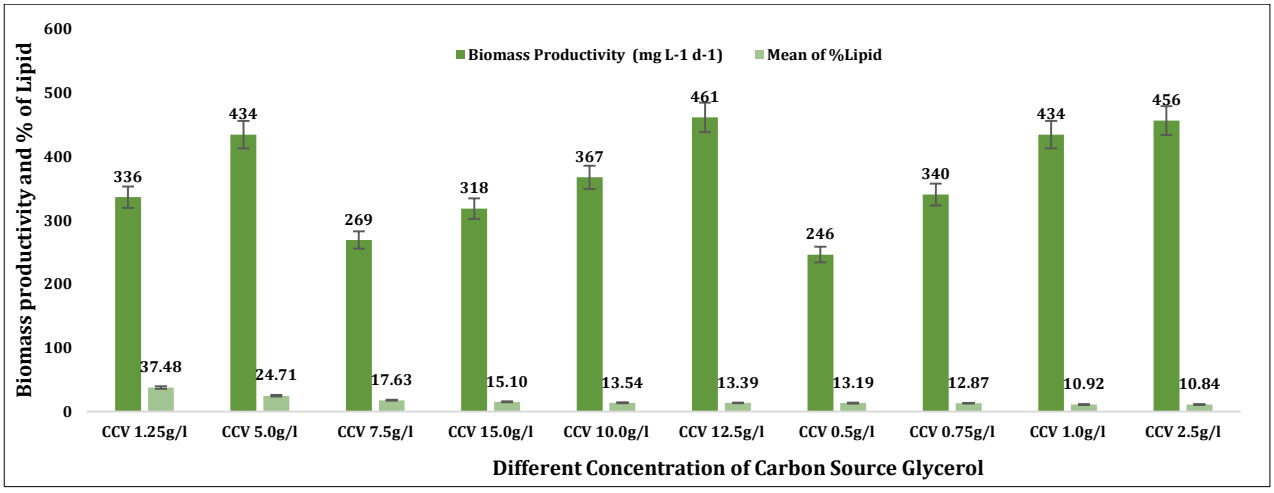
5.1 Parameter-wise detail of lipid production: Biomass cultivated in CHU-10 media with and without nitrogen provided maximum percent of lipid (17.67% with nitrogen & 19.08% w/o nitrogen) among the six different media tested in both parameters. In case of concentration of basic elements such as carbon, nitrogen and phosphorus maximum lipid production was found at concentration of 1.25 g/l (37.48%), 6 g/l (17.38%) and 0.4 g/l (14.35%) respectively. Sucrose (13.83%), sodium nitrate (21.96%) and disodium hydrogen phosphate (14.72%) were found potential source of lipid production in case of source of carbon, nitrogen and phosphorus. In case of light conditions, blue colour (12.68%), 15 W intensity (15.91%) and 12L:12D photoperiod (16.46%) were found most suitable as these condition yielded maximum lipid. pH 10 (10.80%) and shaking period of 4 hours (14.29%) were best variants among their parameter.


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
80
70
60
50
40
30
20
10
0
55
17.67
61
14.43
62
13.35
66
10.61
23
6.74
22
6.65
Media-CHU-10
Media-BG-11
Media- BBM
Media-N-11
Media-Bold 3N
Media-M-8
Different Media

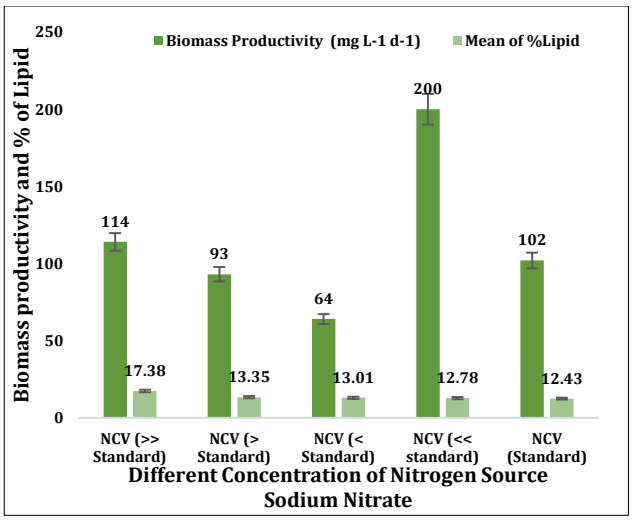
(a)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
90
80
70
60
50
40
30
20
10
0
62
19.08
44
9.65
79
8.95
77
8.15
41
8.10
35
7.90
Nitrogen Starvation-CHU-10
Nitrogen Starvation-N-11
Nitrogen Starvation-BBM
Nitrogen Starvation-BG-11
Nitrogen Starvation-M-8
Nitrogen Starvation-Bold 3N
Different Media with Nitrogen Starvation

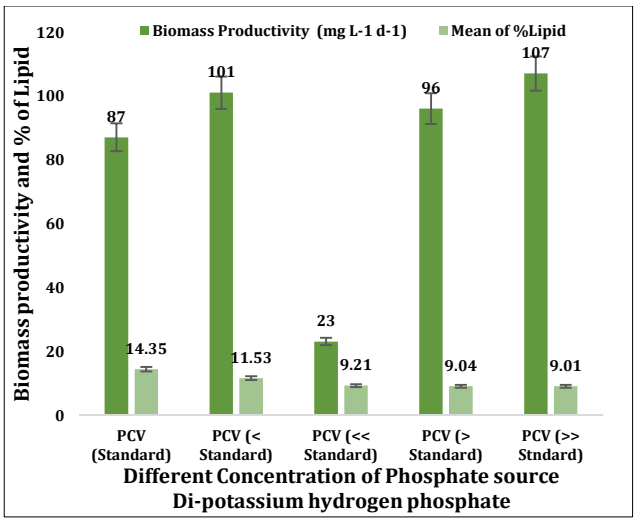
(b)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
600
500
400
300
200
100
0
336
37.48
434
24.71
269
17.63
318
15.10
367
13.54
461
13.39
246
13.19
340
12.87
434
10.92
456
10.84
CCV 1.25g/l
CCV 5.0g/l
CCV 7.5g/l
CCV 15.0g/l
CCV 10.0g/l
CCV 12.5g/l
CCV 0.5g/l
CCV 0.75g/l
CCV 1.0g/l
CCV 2.5g/l
Different Concentration of Carbon Source Glycerol

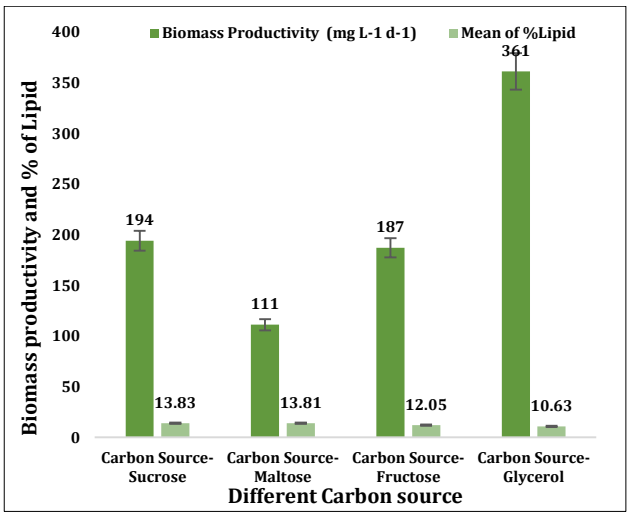
(c)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
250
200
150
100
50
0
114
17.38
93
13.35
64
13.01
200
12.78
102
12.43
NCV (>> Standard)
NCV (> Standard)
NCV (< Standard)
NCV (<< standard)
NCV (Standard)
Different Concentration of Nitrogen Source
Sodium Nitrate

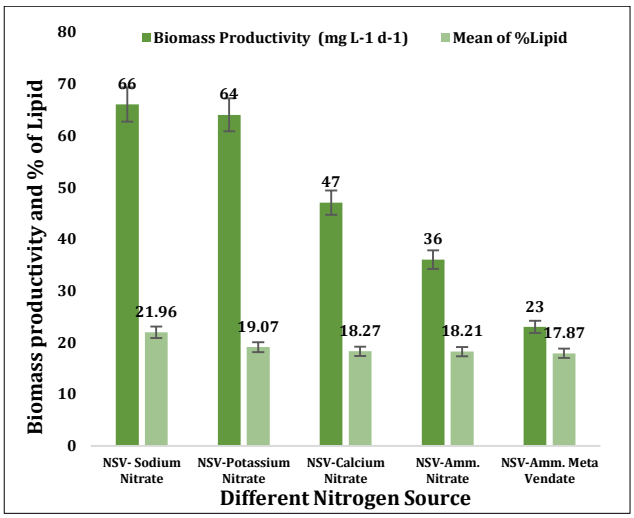
(d)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
120
100
80
60
40
20
0
87
14.35
101
11.53
23
9.21
96
9.04
107
9.01
PCV (Standard)
PCV (< Standard)
PCV (<< Standard)
PCV (> Standard)
PCV (>> Stndard)
Different Concentration of Phosphate source
Di-potassium hydrogen phosphate

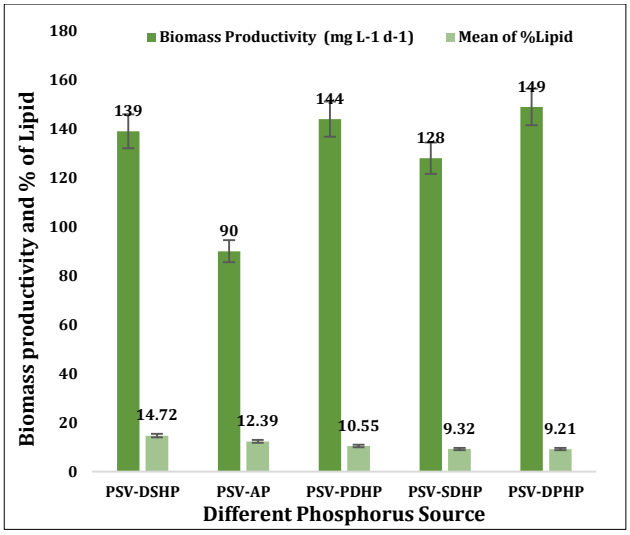
(e)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
400
350
300
250
200
150
100
50
0
194
13.83
111
13.81
187
12.05
361
10.63
Carbon Source-Sucrose
Carbon Source-Maltose
Carbon Source-Fructose
Carbon Source-Glycerol
Different Carbon source

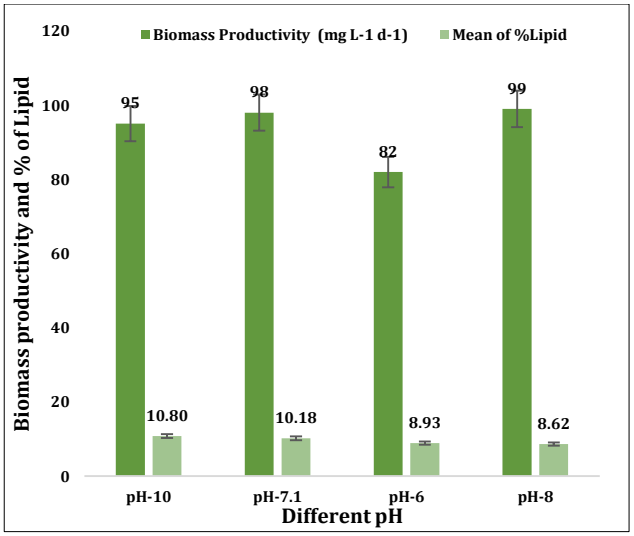
(f)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
80
70
60
50
40
30
20
10
0
66
21.96
64
19.07
47
18.27
36
18.21
23
17.87
NSV- Sodium Nitrate
NSV-Potassium Nitrate
NSV-Calcium Nitrate
NSV-Amm. Nitrate
NSV-Amm. Meta Vendate
Different Nitrogen Source

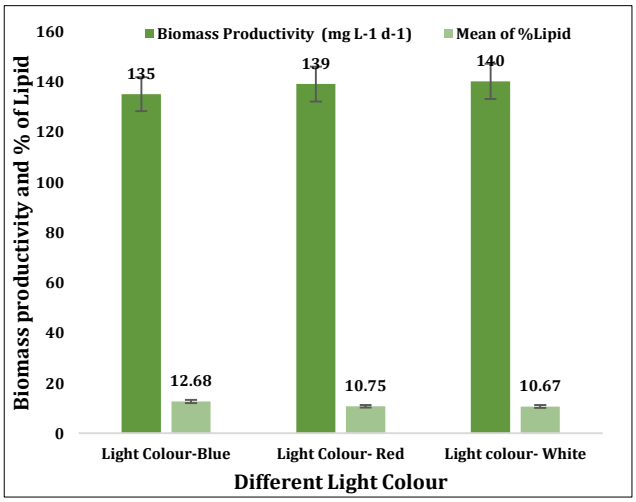
(g)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
180
160
140
120
100
80
60
40
20
0
139
14.72
90
12.39
144
10.55
128
9.32
149
9.21
PSV-DSHP
PSV-AP
PSV-PDHP
PSV-SDHP
PSV-DPHP
Different Phosphorus Source

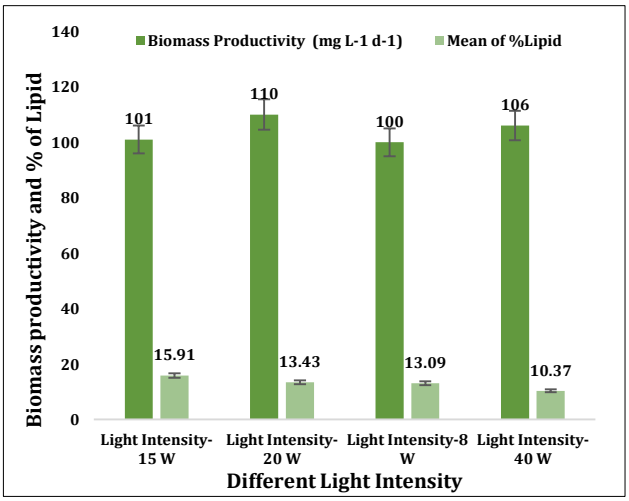
(h)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
120
100
80
60
40
20
0
95
10.80
98
10.18
82
8.93
99
8.62
pH-10
pH-7.1
pH-6
pH-8
Different pH

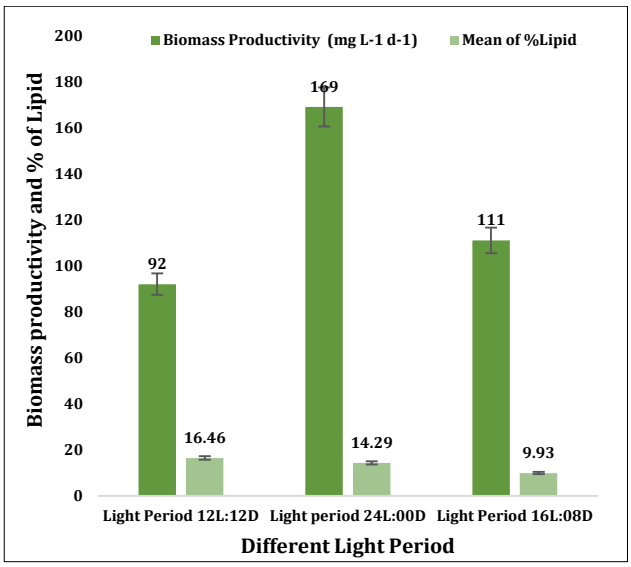
(i)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
160
140
120
100
80
60
40
20
0
135
12.68
139
10.75
140
10.67
Light Colour-Blue
Light Colour- Red
Light colour- White
Different Light Colour

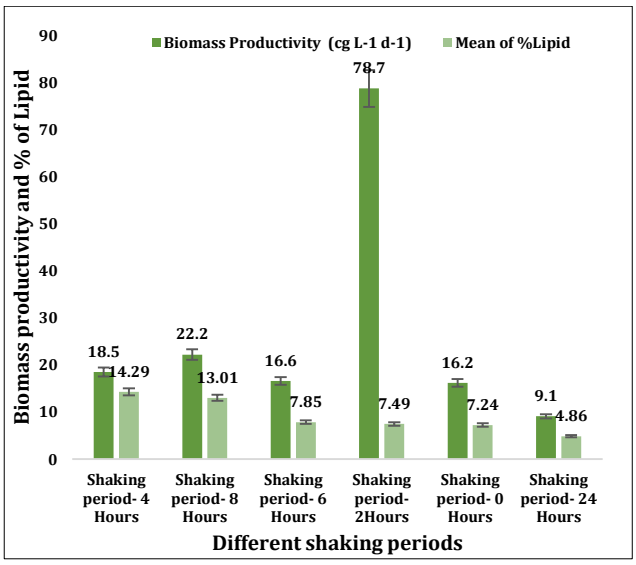
(j)


Biomass Productivity (mg L-1 d-1)
Mean of %Lipid
Biomass productivity and % of Lipid
140
120
100
80
60
40
20
0
101
15.91
110
13.43
100
13.09
106
10.37
Light Intensity-15 W
Light Intensity-20 W
Light Intensity-8 W
Light Intensity-40 W
Different Light Intensity

(k)



(l)



(m)

Figure-11 (a)-(m:) Detail of lipid production along with connected biomass productivity of parameter meter wise 66 culture conditions. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

5.2 Comparison of high lipid producing culture conditions with standard BG-11 medium: If we consider BG-11 medium as standard composition for the cultivation of microalgae, there are total 14 variation of culture conditions except NSV-sodium nitrate (similar to BG-11 culture condition) which have more potential to provide better yield of lipid. Table-11 provides a brief information of such culture conditions. These culture conditions itself or in combined state can be used to get better yield of lipid in case of microalga *Chlorella minutissima.*

Table-11: Comparison of lipid yield of different culture conditions with the yield of standard BG-11 medium. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

Sl. No.	Culture condition	Mean of lipid in 50 mg biomass with SD	Projected amount of lipid in 100 mg biomass (mg or %)
1	CCV 1.25g/l	18.74±2.02	37.48
2	CCV 5.0g/l	12.36±0.93	24.71
3	NSV- Sodium Nitrate	10.98±0.38	21.96
4	Nitrogen Starvation-CHU-10	9.54±2.46	19.08
5	NSV-Potassium Nitrate	9.54±0.37	19.07
6	NSV-Calcium Nitrate	9.14±1.07	18.27
7	NSV-Ammonium Nitrate	9.11±0.81	18.21
8	NSV-Ammonium meta Vendate	8.94±0.21	17.87

9	Media-CHU-10	8.84±0.32	17.67
10	CCV 7.5g/l	8.82±0.41	17.63
11	NCV (>> Standard)	8.69±0.27	17.38
12	Light Period (12L:12D)	8.23±0.69	16.46
13	Light Intensity (15 W)	7.95±0.64	15.91
14	CCV 15.0g/l	7.55±0.90	15.10
15	PSV-DSHP	7.36±1.56	14.72
16	Media-BG-11	7.21±0.36	14.43

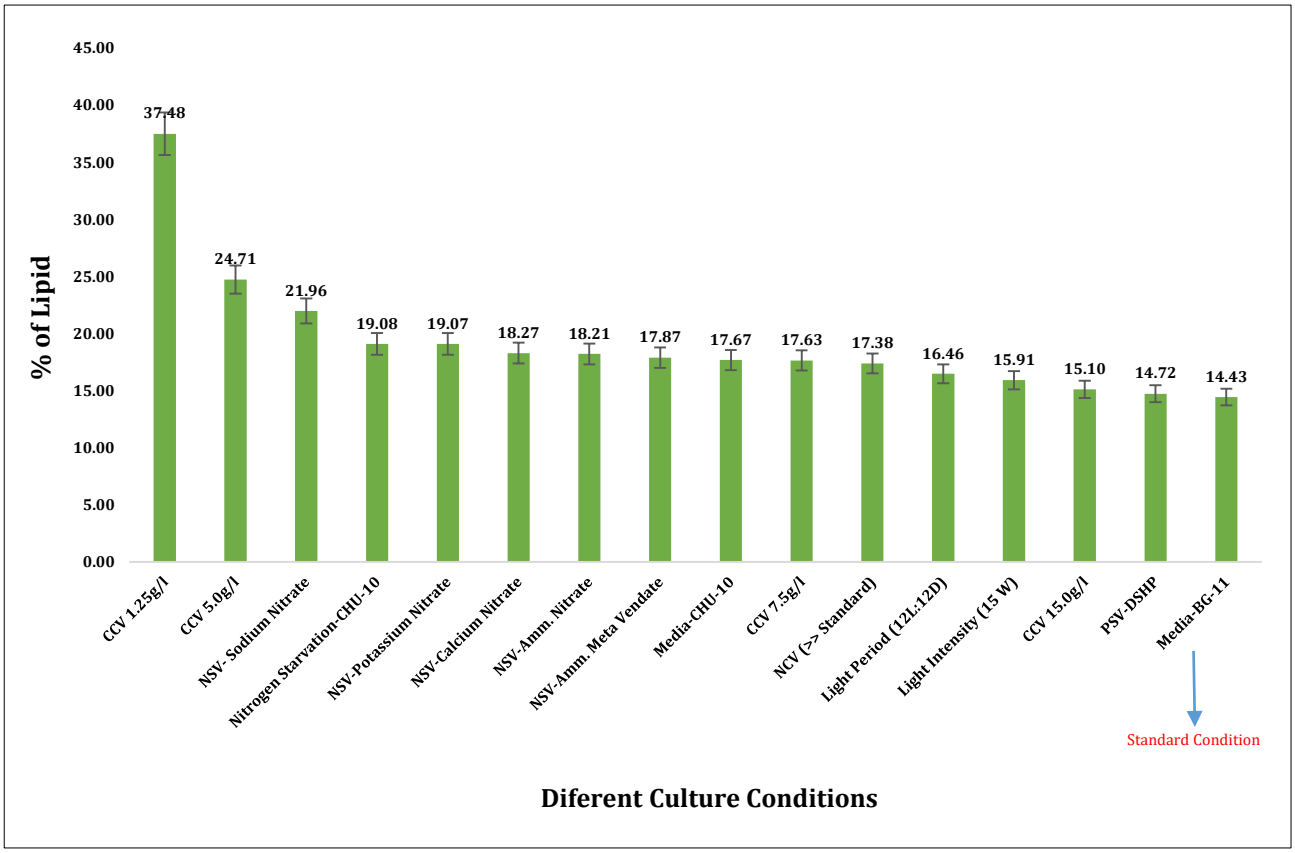


Figure-12: Comparison of lipid yield of different culture conditions with the yield of standard BG-11 medium. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

5.3 Relationship between biomass productivity and lipid production of all culture conditions (Except culture vessel parameter): An analysis was also done to find a relation between the biomass productivity and lipid production so that this relationship can be further explored. But there is no relation between the trend of biomass productivity and lipid production. There are only few conditions such as concentration of carbon source glycerol and nitrogen source sodium nitrate where high biomass production and lipid production was found.

Table-12: Comparison of biomass and lipid productivity of biomass cultivated in 66 different culture conditions. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

Sl. No.	Culture Condition	Biomass Productivity (mg L-1 d-1 & cg L-1 d-1 only in case of shaking period)	%Lipid
1	CCV 1.25g/l	336	37.48
2	CCV 5.0g/l	434	24.71
3	NSV- Sodium Nitrate	66	21.96
4	Nitrogen Starvation-CHU-10	62	19.08
5	NSV-Potassium Nitrate	64	19.07
6	NSV-Calcium Nitrate	47	18.27
7	NSV-Amm. Nitrate	36	18.21
8	NSV-Amm. Meta Vendate	23	17.87
9	Media-CHU-10	55	17.67
10	CCV 7.5g/l	269	17.63
11	NCV (>> Standard)	114	17.38
12	Light Period 12L:12D	92	16.46
13	Light Intensity-15 W	101	15.91
14	CCV 15.0g/l	318	15.10
15	PSV-DSHP	139	14.72
16	Media-BG-11	61	14.43
17	PCV (Standard)	87	14.35
18	Shaking period- 4 Hours	18.5	14.29
19	Light period 24L:00D	169	14.29
20	Carbon Source- Sucrose	194	13.83
21	Carbon Source-Maltose	111	13.81
22	CCV 10.0g/l	367	13.54
23	Light Intensity-20 W	110	13.43
24	CCV 12.5g/l	461	13.39
25	Media- BBM	62	13.35
26	NCV (> Standard)	93	13.35
27	CCV 0.5g/l	246	13.19
28	Light Intensity-8 W	100	13.09
29	NCV (< Standard)	64	13.01
30	Shaking period- 8 Hours	22.2	13.01

31	CCV 0.75g/l	340	12.87
32	NCV (<< standard)	200	12.78
33	Light Colour-Blue	135	12.68
34	NCV (Standard)	102	12.43
35	PSV-AP	90	12.39
36	Carbon Source- Fructose	187	12.05
37	PCV (< Standard)	101	11.53
38	CCV 1.0g/l	434	10.92
39	CCV 2.5g/l	456	10.84
40	pH-10	95	10.80
41	Light Colour- Red	139	10.75
42	Light colour- White	140	10.67
43	Carbon Source-Glycerol	361	10.63
44	Media-N-11	66	10.61
45	PSV-PDHP	144	10.55
46	Light Intensity-40 W	106	10.37
47	pH-7.1	98	10.18
48	Light Period 16L:08D	111	9.93
49	Nitrogen Starvation-N-11	44	9.65
50	PSV-SDHP	128	9.32
51	PCV (<< Standard)	23	9.21
52	PSV-DPHP	149	9.21
53	PCV (> Standard)	96	9.04
54	PCV (>> Standard)	107	9.01
55	Nitrogen Starvation-BBM	79	8.95
56	pH-6	82	8.93
57	pH-8	99	8.62
58	Nitrogen Starvation-BG-11	77	8.15
59	Nitrogen Starvation-M-8	41	8.10
60	Nitrogen Starvation-Bold 3N	35	7.90
61	Shaking period- 6 Hours	16.6	7.85
62	Shaking period- 2Hours	78.7	7.49
63	Shaking period- 0 Hours	16.2	7.24
64	Media-Bold 3N	23	6.74
65	Media-M-8	22	6.65
66	Shaking period- 24 Hours	9.1	4.86

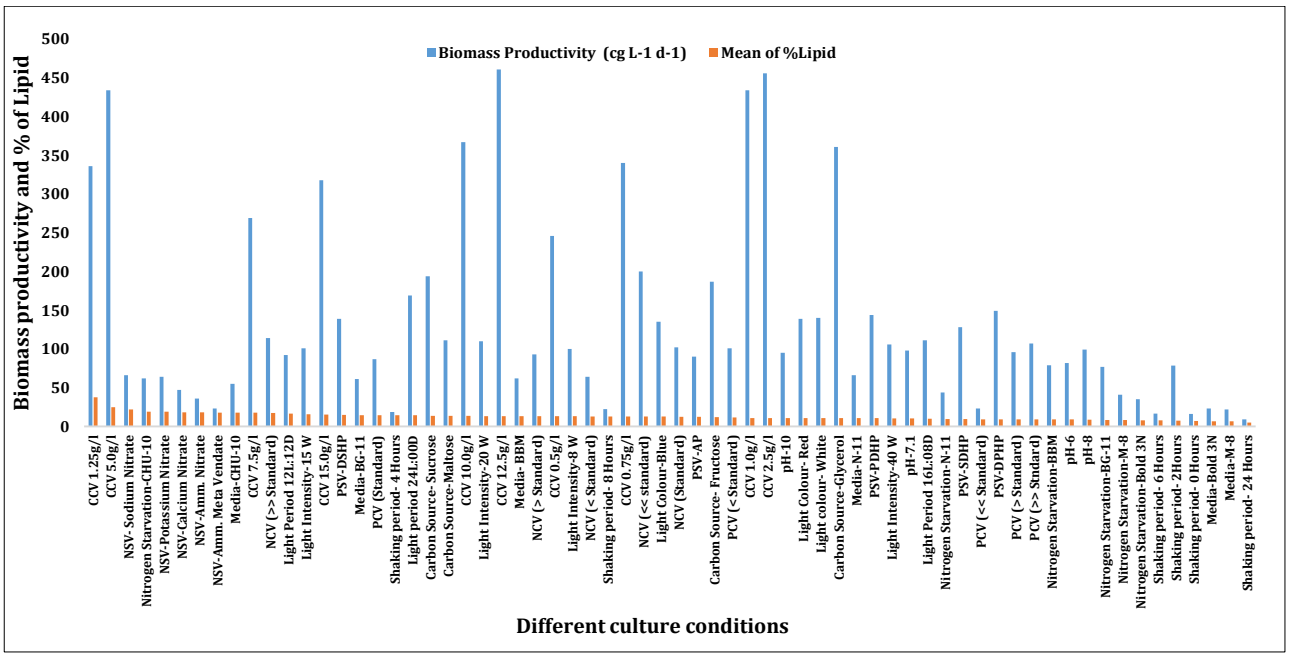


Figure-13: Comparison of biomass and lipid productivity of biomass cultivated in 66 different culture conditions. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

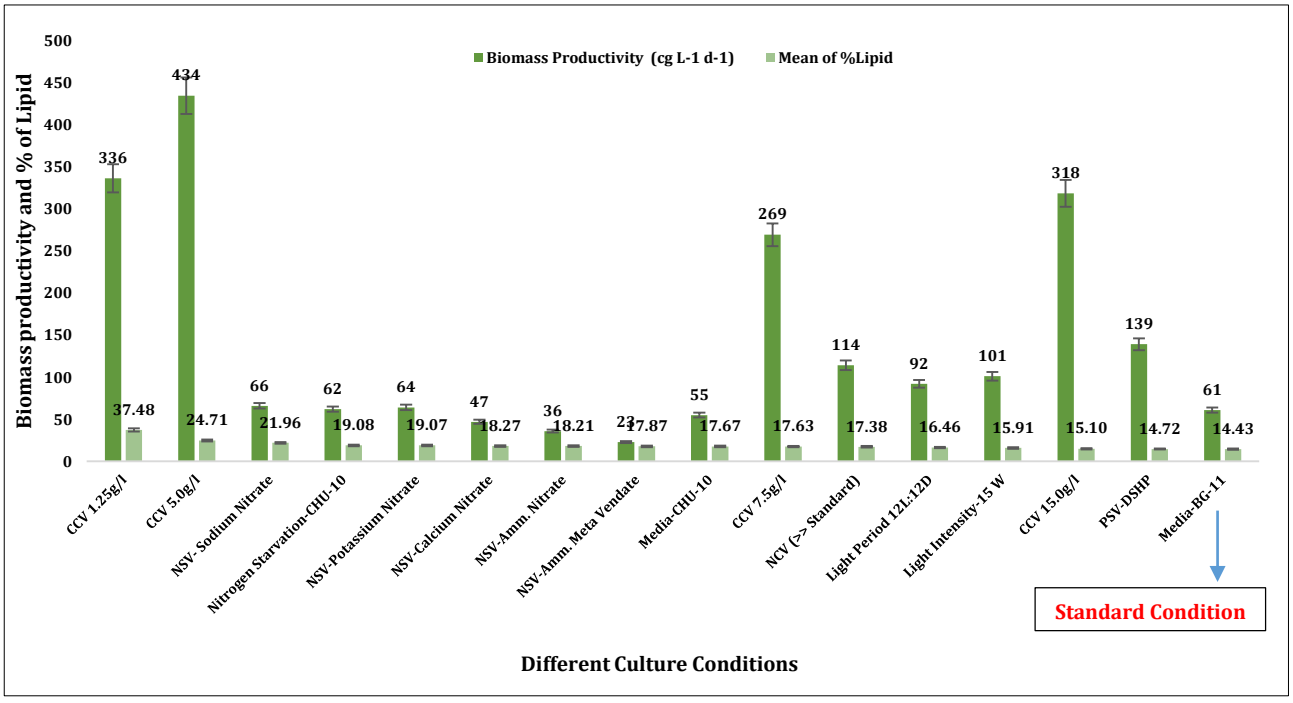


Figure-14: Comparison of biomass and lipid productivity of biomass cultivated in different culture conditions with Standard BG-11 medium. CCV-Carbon concentration variation; NSV-Nitrogen source variation; PSV-Phosphorus source variation; NCV-Nitrogen concentration variation; PCV-Phosphorus concentration variation.

6. Comparison of Fatty Acid Methyl Ester (FAME) profile of high lipid yielding culture conditions with standard BG-11: Fatty acid methyl ester (FAME) profile of both three culture conditions and one standard in BG-11 medium show that the algal biomass of *Chlorella minutissima* is rich in both saturated and unsaturated fatty acids but two fatty acids showed their dominance in saturated and unsaturated profiles i.e. hexadecanoic and 8,11,14-docosatrienoic acid respectively in all culture conditions. Total area % of saturated, mono and poly-unsaturated fatty acid in four different culture conditions are BG-11 (24.90, 0.29, 63.79), CCV-1.25 g/l (30.50, 5.32, 58.20), CCV-5.0 g/l (44.52, 5.31, 43.74) and NSV-SN (25.21, 0.16, 64.23) respectively. The culture condition of carbon concentration increased the % of FAME in comparison to BG-11 from 88.97 to 94.02 and 93.56 in case of CCV-1.25 and CCV-5.0 g/l respectively. This increase in mainly due to the high % of saturated fatty acids as we increased the carbon concentration there is an increase in the saturated fatty acids main of C:16. Fatty acids of C:16 to C:18 were concluded more preferable in several previous studies for the production of bio-diesel [Lee *at al.,* 2010, Li *et al.* 2011, Tang *et al.,* 2011]. The biomass cultivated in nitrogen source variation-sodium nitrate and BG-11 showed a very balanced profile of fatty acids with high content of unsaturated fatty acids. These two conditions are similar as the nitrogen source in BG-11 was sodium nitrate so we can consider them as one condition. Thus this study concluded microalga *Chlorella minutissima* has a balanced profile of fatty acids ranging from C:16 to C:18. This study also concluded that variation in the culture condition such as change in the concentration of carbon is also helpful for enhancing the % of FAME as well as % of saturated fatty acids of C:16. This study may be further explored for identifying those culture conditions which help in the balancing of FAME profile of microalga *Chlorella minutissima* so that potential of this microalga for bio-diesel production may be further increased.

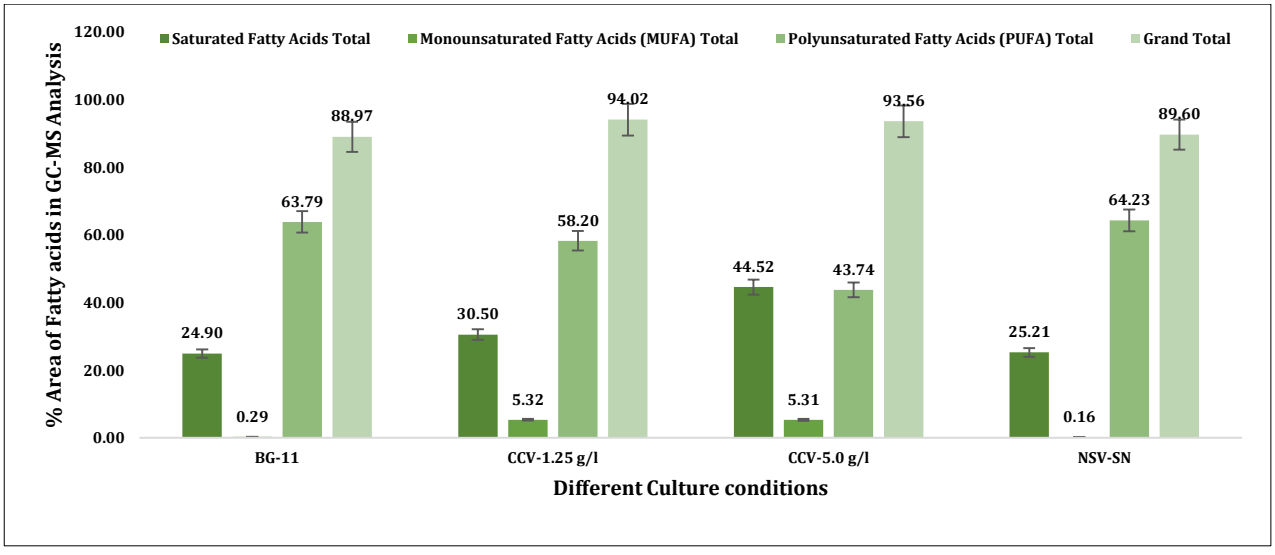


Figure-15: Comparative analysis of different fatty acids in three different culture conditions with standard BG-11 medium. CCV-carbon concentration variation; NSV-nitrogen source variation.

Table-13: Comparative analysis of Fatty acid methyl ester (FAME) profile of three most potential lipid yielding culture conditions with standard BG-11 medium.

Fatty Acid Methyl Ester	Common name of Fatty Acid	Carbon number and Bonds	% Area of fatty acid methyl ester			
			BG-11	CCV-1.25 g/l	CCV-5.0 g/l	NSV-SN
Saturated Fatty Acids						
DODECANOIC ACID, METHYL ESTER	Lauric Acid	C12:0	0.00	0.09±0.08	0.00	0.00
TETRADECANOIC ACID, METHYL ESTER	Myristic Acid	C14:0	0.07±0.04	0.07±0.06	0.27±0.01	0.00
HEXADECANOIC ACID, METHYL ESTER	Palmitic Acid	C16:0	22.94±0.35	27.00±0.54	35.95±0.22	23.18±1.34
HEXADECANOIC ACID, 15-METHYL-, METHYL EST.	-	C16:0	0.00	0.00	7.73±0.09	0.00
HEXADECANOIC ACID, 3-HYDROXY-, METHYL EST	-	C16:0	0.00	0.00	0.00	0.02±0.03
HEPTADECANOIC ACID, METHYL ESTER	Margaric Acid	C17:0	0.07±0.04	0.19±0.02	0.57±0.02	0.16±0.06
OCTADECANOIC ACID, METHYL ESTER	Stearic Acid	C18:0	1.58±0.02	3.15±0.12	0.00	1.63±0.06
HENEICOSANOIC ACID, METHYL ESTER	Heneicosylic Acid	C21:0	0.24±0.1	0.00	0.00	0.10±0.01
TETRACOSANOIC ACID, METHYL ESTER	Lignoceric Acid	C24:0	0.00	0.00	0.00	0.12±0.01
Un-Saturated Fatty Acids						
7,10-HEXADECADIENOIC ACID, METHYL ESTER	-	C16:2	1.62±0.05	1.86±0.06	1.38±0.03	2.14±0.1
7,10,13-HEXADECATRIENOIC ACID, METHYL ESTER	-	C16:3	1.72±0.04	1.32±0.76	0.00	1.71±0.02
6-OCTADECENOIC ACID, METHYL ESTER	-	C18:1	0.11±0.06	0.00	0.00	0.00
9-OCTADECENOIC ACID, METHYL ESTER	Oleic Acid	C18:1	0.11±0.01	0.19±0.06	0.29±0.05	0.16±0.1
16-OCTADECENOIC ACID, METHYL ESTER	-	C18:1	0.00	5.13±0.37	5.02±0.13	0.00
9,12-OCTADECADIENOIC ACID, METHYL ESTER	Linolenic Acid	C18:2	10.87±0.05	6.40±0.09	4.13±0.08	12.54±1.36
6,9,12-OCTADECATRIENOIC ACID, METHYL ESTER	Y-Linolenic Acid	C18:3	0.33±0.01	0.00	0.00	0.52±0.01
9,12,15-OCTADECATRIENOIC ACID, METHYL ESTER	α-Linolenic Acid	C18:3	5.92±0.29	8.68±0.41	7.56±0.13	9.02±0.36
13-DOCOSENOIC ACID, METHYL ESTER	-	C22:1	0.07±0.04	0.00	0.00	0.00
8,11,14-DOCOSATRIENOIC ACID, METHYL ESTER	-	C22:3	43.34±1.22	39.95±0.51	30.67±0.10	38.29±0.68
Saturated Fatty Acids Total			**24.90**	**30.50**	**44.52**	**25.21**
Monounsaturated Fatty Acids (MUFA) Total			**0.29**	**5.32**	**5.31**	**0.16**
Polyunsaturated Fatty Acids (PUFA) Total			**63.79**	**58.20**	**43.74**	**64.23**
Grand Total			**88.97**	**94.02**	**93.56**	**89.60**

CONCLUSION

This study concluded that variation in culture conditions are helpful in the achievement of higher production of algal biomass and lipid. N-11, BBM and BG-11 media were found suitable for this microalga among six different media tested although BBM and BG-11 were found to be the potential media with nitrogen starvation. Potential source of nitrogen, phosphorus and carbon were sodium & potassium nitrate, di-potassium hydrogen phosphate & potassium di hydrogen phosphate and glycerol respectively. The suitable concentration of nitrate, phosphate, and glycerol were 0.375 g/l, 0.16 g/l and 12.5 g/l. Alkaline pH was found suitable for this microalgae to provide higher biomass. *Chlorella minutissima* has shown better biomass production in 24 hours of illumination of white light of 20 W tubelight and shaking period of 2 hours in Erlenmeyer flasks. Flocculation with aluminium sulfate, cell disruption with sonication, lipid extraction with the organic solvent combination of chloroform and methanol in 1:1 were found potential method of their respective stage. With the help of these potential methods, biomass of 66 culture conditions has been processed and maximum lipid was obtained with the culture condition of carbon concentration at 1.25 g/l. The fatty acid methyl ester (FAME) analysis by GC-MS of top three lipid yielding culture conditions and standard BG-11 revealed that the *Chlorella minutissima* is rich in fatty acid of C:16 to C:18 which is required for bio-diesel production. This study also concluded that variation in carbon concentration is helpful in the increase of total % of FAME and fatty acid of C:16.

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APPENDIX-I

The composition of media used in identification of potential culture conditions in g/l is as follows: **BG-11 Medium (pH-7.1)** [$NaNO_3$ (1.5), $K_2HPO_4.3H_2O$ (0.04), $MgSO_4.7H_2O$ (0.075), $CaCl_2.2H_2O$ (0.036), $C_6H_8O_7$ (0.006), $C_6H_8FeNO_7$ (0.006), EDTA (0.001), Na_2CO_3 (0.02), A5 Solution {1 ml from mixture of 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)}], **N-11 Medium (pH-6.8)** [KNO_3 (1.0), Na_2HPO_4 (0.083), KH_2PO_4 (0.052), $MgSO_4.7H_2O$ (0.05), $CaCl_2.2H_2O$ (0.01), FeEDTA (1 ml of 10g/l), SAZ solution {1 ml from mixture of $MnCl_2.4H_2O$ (0.099), $NiSO_4.7H_2O$ (0.236), $ZnSO_4.7H_2O$ (0.063), $CuSO_4$ .H_2O (0.005), $CoSO_47H_2O$ (0.0028), NH_4VO_3 (0.0029), $(NH_4)_6Mo_7O_{24}.4H_2O$ (0.0018)}], **Modified CHU-10 Medium (pH-6.4)** [$Na_2SiO_3.9H_2O$ (10 ml of 5 g/l), $Ca(NO_3)_2.4H_2O$ (1 ml of 57.56 g/l), K_2HPO_4 (1 ml of 10g/l), $MgSO_4.7H_2O$ (1 ml of 25g/l), Na_2CO_3 (1 ml of 20g/l), F/2 Vitamins {1 ml mixture of following vitamins: Vitamin B-12 (0.5 ml of 5mg/ml), Biotin (0.5 ml of 1mg/10 ml)}, Na_2 EDTA.$2H_2O$ (1 ml of 1g/l), Trace metal mix {1 ml from mixture of following chemicals H_3BO_3 (2.86), $MnCl_2.4H_2O$ (1.81), $ZnSO_4.7H_2O$ (0.222), Na_2 $MoO_4.2H_2O$ (0.390), $CuSO_4.5H_2O$ (0.079), $Co(NO_3)_2.6H_2O$ (0.0494)}, 1 ml mixture of Ferric citrate (6) and Citric acid (6)], **M-8 Medium (pH-5.9-6.0)** [(KNO_3 (3), KH_2PO_4 (0.74), $Na_2HPO_4.2H_2O$ (0.26), $CaCl_2.2H_2O$ (0.013), FeEDTA (0.01), $FeSO_4.7H_2O$ (0.13), $MgSO_4.7H_2O$ (0.4), Micronutrient {1 ml from mixture of $Al_2(SO_4)_3.18H_2O$ (3.58), $MnCl_2.4H_2O$ (12.98), $ZnSO_4.7H_2O$ (1.83), $CuSO_4.H_2O$ (3.2)}], **BBM Medium (pH-6.4-6.8)** [Distilled water (850 ml), $NaNO_3$ (2.5), $MgSO_4.7H_2O$ (0.75), NaCl (0.25), K_2HPO_4 (0.75), KH_2PO_4 (1.75), $CaCl_2.2H_2O$ (0.25), Solution 1 {1 ml from mixture of $ZnSO_4.7$ H_2O (8.82), MnCl2.$4H_2O$ (1.44), MoO_3 (0.71), $CuSO_4.H_2O$ (1.57), $Co(NO_3)_2.6H_2O$ (0.49)}, Solution 2 {4 ml (1 ml each) of following chemical H_3BO_3 (1.14), Sodium EDTA+KOH (5) & (3.1), $FeSO_4.7H_2O$ (4.98), Conc. H_2SO_4}, Vitamin Mix {1 ml from mixture of Vitamin B (0.1), Vitamin H (0.025), Vitamin B_{12} (0.015)}], **Modified Bold 3 N Medium (pH-6.2)** [Distilled water (850 ml), P-IV metal solution {6 ml from mixture of $Na_2EDTA.2H_2O$ (0.75), $FeCl_3.6H_2O$ (0.097), $MnCl_2.4H_2O$ (0.041), $ZnCl_2$ (0.005), $CoCl_2.6H_2O$ (0.002), $Na_2MoO_4.2H_2O$ (0.004)} , $NaNO_3$ (30 ml of 10g/400 ml), $CaCl_2.2H_2O$ (10 ml of 1g/400 ml), $MgSO_4.7H_2O$ (10 ml of 3g/400 ml), K_2HPO_4 (10 ml of 3g/400 ml), KH_2PO_4 (10 ml of 7g/400 ml), NaCl (10 ml of 1g/400 ml), Soil water (40 ml in 200 ml DI H2O), Vitamin B12 {1 ml (0.1 mM vitamin B12 in 200 ml of 50 mM HEPES buffer at pH 7.8)), Biotin (1 ml (0.1 mM biotin in 200 ml of 50 mM HEPES buffer at pH 7.8)), Thiamine (1 ml (6.5 mM Thiamine in 50 ml of mM HEPES buffer at pH 7.8)}].