STUDY OF SECOND AND THIRD GENERATION BIOFUEL FEED STOCKS

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By

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CERTIFICATE

This is to certify that the work titled **"Study of Second and Third Generation Biofuel Feed stocks"**, submitted by **Zainab** in partial fulfillment for the award of degree of **B. Tech Biotechnology** from Jaypee University of Information Technology, Solan has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or Institute for the award of this or any other degree or diploma.

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Zainab
Date :

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ABSTRACT

The continues use of petroleum based fuels is now recognized as unsustainable because of the decrease in its amount and the harmful effect caused by its use to the environment due to the accumulation of carbon dioxide in the atmosphere. There is a need to find renewable, carbon neutral transport fuels for economic and environmental sustainability. A possible alternative is biodiesel derived from oil crops. It is renewable and carbon neutral. Unfortunately, biodiesel from oil crops possess food vs. fuel dilemma. To overcome this problem, the combined use of different feed stocks for biofuel production can be done. Both second generation feed stock-waste cooking oil and third generation feedstock-microalgae can together help in fulfilling the global demand for energy. The use of waste oil for biodiesel production can be done both on industrial level and even in houses, universities, armed forces premises, small scale and also large scale hotel businesses. Whereas, the production of algal based biofuel is required to be done only on industrial and commercial level in order to utilize it as potential alternative. Therefore, cost-effective technologies are desired to produce biofuels that are suitable for drop-in use in cars, trucks, and jet planes. These advanced biofuels can be sustainably manufactured from waste oil, cellulosic and algal feed stocks. Biomass conversion technologies are also needed to produce chemical intermediates and high-value chemicals.

The aim of this project is to study potential feed stocks for sustainable production of biodiesel, the environment friendly use of a waste product, i.e. waste cooking oil for generating our own source of energy and the screening of methods to enhance the algal based feed stock to generate large amounts of biodiesel. It also focusses on the use of the by-products generated during the reaction of biodiesel production in a valuable and economically benefitting manner.

CHAPTER 1

INTRODUCTION

"Biofuels R&D is like the wild west, there's a lot of hype and everybody is scrambling to get involved- what we need is for people to dig in and support or refute the hype with real-world proof of concept. One challenge seems to be going from laboratory flask to production scale"- Mak Saito, Associate Scientist, WHOI: speaking in reference to grand potential of emerging science and applied efforts in biofuels- considerate of the hype and uncertainty present micro algal biofuel prospects.

The need for new renewable resources to fulfill the emerging energy demands has been gaining great importance in the recent years because of the mounting crude oil prices, a permanent decrease in oil reserves and increasing environmental degradation. Global demand for petroleum is predicted to increase by 40% by 2025 (Ghasemi *et al*.2011). Biomass derived fuels such as methane, ethanol, and biodiesel are well-accepted alter- natives to diesel fuels as they are economically feasible, renewable, environmental-friendly and can be produced easily in rural areas where there is an acute need for modern forms of energy. Edible vegetable oils such as canola, soybean, and corn have been used for biodiesel production and are recognized diesel replacements (B.Freedman *et al*.1986, Lang.X *et al*.2002) Still, a major difficulty in the commercialization of biodiesel production from edible vegetable oils is their high production cost, which is due to the demand for human consumption. Decreasing the cost of the feedstock is required for biodiesel's long- term commercial possibility. One way to reduce the cost of this fuel is to use less expensive feedstock including waste cooking oils and vegetable oils that are non-edible and require low harvesting costs. Waste cooking oil (WCO), which is much less expensive than edible vegetable oil, is a promising substitute to edible vegetable oil. (Canakci. M *et al*.2003)

The disposal of waste cooking oil and fats is a big problem in many parts of the world, which is an environmentally threatening problem. To overcome this problem, the waste cooking oil can be used into both economic and environment friendly way by proper utilization and management of it as a fuel substitute. Many developed countries have set policies that penalize the disposal of waste cooking oil into waste drainage (Kulkarni M G *et al*.2006). The Energy

Information Administration (EIA) in the United States (USA) estimated that around 100 million gallons of waste cooking oil is produced per day in USA, where about 9 pounds of waste cooking oil are generated per person per year (Radich.A, US Energy Information Administration, 2006). The estimated amount of waste cooking oil collected in Europe is about 0.49 - 0.7 million gallons/day (Supple. B *et al*.2002)

The third generation biofuels are also a promising source of energy but still a lot has to be investigated. Their energy output per land is at least 30X higher than second generation biofuels. Biodiesel has various advantages like it reduces emissions of unburned hydrocarbons, CO, sulfates, polycyclic aromatic hydrocarbons and nitrated polycyclic aromatic hydrocarbons. The only source which appears to be capable enough of reaching the global demands of fuel is microalgae. Lipids, carbohydrates and proteins, all the biochemical fractions of microalgae can be converted to fuels. Algae are said to yield about 1200-10000 gallons of oil/acre, compared to 48 and 18 gallons of oil/acre for soy and corn, respectively (Ghasemi *et al*.2011). Commercially microalgae are cultivated for human nutritional products in a number of small and medium scale production systems. The main algae cultivated photosynthetically are *Spirulina, Chlorella, Dunaliella and Haematococcus* (Benmann 2009).

To understand the importance of microalgae, I am quoting this precise abstract by Lie *et al*. "Microalgae are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that grow rapidly due to their simple structure. They can potentially be employed for the production of biofuels in an economically effective and environmentally sustainable manner. Microalgae have been investigated for the production of a number of different biofuels including biodiesel, bio-oil, biosyngas and bio hydrogen. The production of these biofuels can be coupled with flue gas CO_2 mitigation, wastewater treatment, and the production of high value chemicals. Microalgal farming can also be carried out with seawater using marine microalgal species as the producers. Developments in microalgal cultivation and downstream processing (e.g. harvesting, drying and thermochemical processing) are expected to further enhance the cost-effectiveness of the biofuel from microalgae strategy."

Keeping in mind the importance of a need to have renewable resources of energy, the object of the following project is to study and find economically and environmentally favorable methods to utilize two different types of feed stocks for biofuel production. The methods discussed for second generation biofuel production can be utilized in institutes, colleges, army mess, small

restaurants as well as big hotel chains to have their own energy sources. While third generation feedstock still requires cost effective processes for biofuel production.

CHAPTER 2

REVIEW OF LITERATURE

2.1 BIOFUELS:

Fuels that contain energy from geologically recent carbon fixation are called bio fuels. These are made from biomass conversion which can be done by 3 different ways- thermal conversion, chemical conversion, biochemical conversion.

2.1.1 FIRST GENERATION BIOFUELS: (biofuel.org.uk)

Bio fuels which are obtained from sources like sugar, animal fats, starch and vegetable oil. Conventional techniques of production are used to derive the oils. The various popular types of 1st generation bio fuels are:

1. Biodiesel: - Most commonly used in European countries. The process of trans esterification is mainly used to produce biodiesel. Chemically it is called as fatty acid methyl. Biomass is mixed with methanol and sodium hydroxide and the chemical reaction thus taking place forms the biodiesel. This is used along with mineral diesel for various diesel engines.
2. Vegetable oil: - These oils can be used both for cooking as well as a fuel. Quality of the oil determines as to how this will be used. Good quality oil is generally used for cooking. Mostly, vegetable oil is used for the production of biodiesel.
3. Biogas: - Anaerobic digestion of organic matter leads to production of biogas. The residue can be used as manure or fertilizers for crop fields. Methane is the main component of the biogas produced and it can be recovered easily through the use of mechanical biological treatment systems.
4. Bio alcohols: - Fermentation of starches and sugar using enzymes and microorganisms produces these alcohols. The most common one is Ethanol while butanol and propanol are less common. Bio butanol can be directly used in various gasoline engines and is thus sometimes called as the direct replacement of gasoline and is more energy efficient. The process of ABE fermentation is used to produce butanol.

5. Syngas:-the gas produced after the combined process of gasification, combustion and pyrolysis is called syngas. The biofuel produced is converted into CO and then to energy by the method of pyrolysis. Very less oxygen is supplied during this process to control combustion. The last step of gasification involves the conversion of biomass into gases like CO and hydrogen. The resulting gas, Syngas has various uses.

2.1.2 SECOND GENERATION BIOFUELS: (biofuel.org.uk)

The bio fuels which are derived from the inedible parts of plants like wood, agricultural waste etc. are termed as second generation bio fuels. These do not compete with the use of raw materials as food. These use biomass to liquid technology to produce fuels. The main components of herbaceous biomass are cellulose and hemicellulose. These are also sugar polymers and can be used for bioethanol production, but due to their role as structural plant materials, they are less accessible. For second generation bioethanol (or lignocellulosic ethanol) production, the whole plane, including cellulose and hemicellulose, is utilized, which increases hectare yields and reduces the competition with food production. The mail challenge in second generation bioethanol production is the efficient breakdown of cellulose to sugar monomers (hydrolysis). Due to the recalcitrant character of cellulose, feedstock pretreatment and adapted hydrolysis steps are needed to obtain fermentable sugar monomers. These steps are currently cost and energy intensive which still limits the economic viability of second generation biofuel. The development of a cost efficient production process, including improved cellulose utilization, is the main challenge for the future development of second generation biofuels.

An example of pretreatment of feedstock is steam explosion which is used by BIOLYFE (BIOLYFE is co-founded by the European Commission in the 7^{th} Framework Program). It is currently considered one of the most effective and least costly physiochemical pretreatments for biomass. The process uses saturated steam at high pressure to destroy the biomass structure from inside the pores resulting in a physical breakage of the bonds between lignin, cellulose and hemicellulose without the addition of chemicals or high energy input.

Another example of second generation bio fuel can be the production of bio diesel from waste cooking oil. It qualifies for 2nd generation because it has already been used and is no longer fit for human consumption.

2.1.3 THIRD GENERATION BIOFUELS: (biofuels.org.uk)

These are also called as "advanced fuels". These are the fuels derived from algae. Earlier, algae were grouped with second generation bio fuels but when it became apparent that algae is capable of much higher yields with less resource input then it was moved to its own category. There are 2 main advantages of algae as a potential feedstock for fuel production. Firstly, the oil produced by algae can be easily refined into diesel and some components of gasoline. Secondly, it can be genetically modified to produce a range of fuels from ethanol to butanol, gasoline and diesel directly.

The various third generation fuels are biodiesel, butanol, methane, gasoline, jet fuel, ethanol, vegetable oil. Algae are capable of producing outstanding yields. According to the US Department of Energy, yields that are 10 times higher than second generation bio fuels mean that only 0.42% of the U.S. land area would be needed to generate enough bio fuel to meet all of the U.S. needs.

2.1.4 FOURTH GENERATION BIOFUELS:

The technology for fourth generation biofuel combines genetically enhanced feedstock growth, which is aimed to capture higher amounts of carbon with genetically synthesized microbes and can thus make fuels efficiently. The crucial point of the process is the capture and sequestration of CO_2. The company "Synthetic Genomics" plans to combine the processes of feedstock growth and fuel processing by designing organisms that can inhale CO_2 and excrete sugars.

The advantages of fourth generation biofuels is that is can be made using non-arable land and do not require destruction of biomass. They can be made anywhere where CO_2 and water can be found in sufficient amounts and these are environment advocates. The limitations to this technology are that it is in its early days of development, it has high capital costs, use of microbes to do fuel conversion and processing time needs to be improved to make these fuels cost-competitive. Companies such as Amyris, LS9, Joule Unlimited, Algenol can be found working in this area.

2.2. WASTE COOKING OIL AS A SECOND GENERATION BIODIESEL FEEDSTOCK:

The transesterification of fats and oils by lower alcohols like methanol leads to the production of Biodiesel. This fuel is becoming gradually popular as a straight motor fuel or as biodiesel blends by mixing with various amounts of fossil diesel in developed as well as developing countries. The usage of biodiesel has various positive sides to it. It can be used directly in the diesel engines with little or no modification and with similar fuel economy. It is non-toxic and biodegradable. Biodiesel contributes much less to global warming than conventional diesel since most of the carbon found in the oils and fats which is used to create the fuel is originated from CO_2 in the atmosphere. In spite of all advantages of biodiesel, the major drawback in biodiesel production is high production cost, which is due to the high price of the vegetable oils. The cost of the raw material can be reduced tremendously by using non-edible oil or waste cooking oil. With addition to the low cost, there is the environmental advantage of the residue disposal (Patil *et al.* 2012)

"Waste Cooking Oil" (WCO) is vegetable oil which has been used in food production and usually contains free fatty acids (FFA), water and other impurities which determine the quality of biodiesel. It can be obtained from different sources- domestic (from home kitchens), commercial (from restaurants and hotels) and industrial (food factories). (Hassani M *et al*.2013).
To calculate the FFA% in the WCO, titration method is used and the following formula is applied:

FFA%= v*N*28.2/w

Where v=volume of titration solution in ml

N=normality of titration solution

w=weight of oil sample in grams

28.2= molecular weight of oleic acid divided by 10

The molecular weight of oleic acid is taken as it is one of the major fatty acid found in the WCO and is a major constituent in biodiesel production (Sannyasi *et al.* 2013). The presence of other free fatty acid can also be calculated by using the molecular weight of that corresponding fatty acid. (Gerpen.V *et al.* 2004)

2.2.1. TRANSESTERIFICATION:

The most commonly used method for converting oils to biodiesel is through the transesterification reaction. Transesterification is defined as a reaction between vegetable oil or animal fat with an alcohol in the presence of a catalyst to produce monoglycerides and diglycerides as intermediate products and esters (biodiesel) and glycerol as final products (Hassani.M *et al*.2013) The reaction can be carried out in the presence of a catalyst- acid, base or enzyme. In base catalyzed reaction, NaOH and KOH are commonly used. This is the most popular method for biodiesel production but strict feedstock specification is required. Highly refined vegetable oils can be used as a feedstock for this method. Additionally, water and free fatty acid (FFA) contents should be 0.1% and 0.5% respectively (Lotero E *et al*.2005). At high water contents, a decline in biodiesel yield occurs as hydrolysis becomes the dominant reaction. When oils with high FFA contents are used in alkali catalyzed transesterification, it results in the formation of soaps and causes difficulties in the purification of biodiesel, hence increasing the overall production cost. (Saifuddin *et al*.2009). Acid catalyzed transesterification has also been carried out in the presence of H_2SO_4 and HCl. Transesterification reactions with acid catalysts can tolerate high FFA and water contents and hence, unrefined oils can be used as a feedstock for biodiesel production via this process. Nevertheless, high reaction temperatures (100°C or more) and longer reaction time (approx. 48 hours) are needed to achieve high biodiesel conversion rates. (Lotero E. *et al.* 2005).

Both the alkali and acid catalyzed transesterification processes produce large amounts of wastewater which is of environmental concern. To eliminate the inherent problems linked with the use of chemical catalysts, enzymes, i.e. biocatalysts can be used for transesterification of oils. However, the limitation associated with the use of enzymes is that they are easily deactivated in the presence of polar compounds like water, methanol and glycerol due to low solubility of the polar compounds in the oil phase (Royan D *et al.* 2007). The major step in using the enzymatic process lies in the successful improvement of the efficiency of the enzymatic process and the stability of the immobilized enzyme, however, the high cost of immobilized enzymes increases the biodiesel production cost. The repeated use of immobilized enzyme and the high market price of pure glycerol, which is the byproduct of enzyme-mediated transesterification can lead to reduction in the overall cost of biodiesel production (Hass M J *et al*. 2006).

When the levels of FFA in the waste cooking oil is very high (>3 wt %), it has been reported that direct transesterification becomes undesirable (Canakci M *et al.* 2001). To solve this issue, two-step transesterification, i.e. acid esterification and alkali transesterification is utilized. Initially, esterification is carried out in the presence of H_2SO_4 to reduce the amount of FFA in the WCO. In the second step, the triglycerides (TGs) in the WCO are transesterified to monoesters in the presence of alkali catalyst-KOH. (Hassani M *et al.*2013)

The mechanism of biodiesel production by two-step transesterification (Patil *et al*.2012) is given as follows:

Step 1: Acid Esterification (in the presence of H_2SO_4)

$$RCOOH + CH_3OH \longrightarrow RCOOH_3 + H_2O$$

Step 2: Alkali Transesterification (in the presence of KOH)

$$\begin{array}{lcl} CH_2OOCR_1 & & R_1COOH_3 \quad CH_2OH \\ | & & \quad\quad\quad\quad\quad\quad | \\ CHOOCR_2 + 3CH_3OH & \longrightarrow & R_2COOH_3 + CHOH \\ | & & \quad\quad\quad\quad\quad\quad | \\ CH_2OOCR_3 & & R_3COOH_3 \quad CH_2OH \end{array}$$

Triglyceride Methanol Methyl Ester Glycerol

Table 1. A comparison between 1-step and 2-step transesterification reaction

ONE STEP TRANSESTERIFICATION	**TWO STEP TRANSETERIFICATION**
Direct reaction using a base, enzyme or acid catalyst. No pretreatment is done.	Pretreatment is done, i.e., esterification reaction using acid catalyst.
Due to high FFA content, the use of alkali catalysts leads to high soap formation.	Reduces the soap formation during transesterification.
Low biodiesel yields	The biodiesel yield increases.

Using only acid as a catalyst increases the reaction time to as high as 400 fold.	Acid is used for esterification while alkali is used for tranesterification, this reduces the reaction time.

2.2.2 QUALITY TESTING OF THE BIODIESEL PRODUCED:

The following tests are followed by home brewers of biodiesel. These tests can be used on a small scale, i.e. laboratory levels to test the batches of biodiesel produced.

1. ACID NUMBER

Acid Number is the amount of KOH in mg needed to react with the acid in an amount of oil in grams. The Acid Number value is one of the ASTM (American Society for Testing and Materials) tests for finished biodiesel.

According to ASTM D 664 "Determination of Acid Number by Potentiometric Titration", Max.value for finished biodiesel = 0.50 mg/g

Acid value= v*N*56.1/w

Where v=volume of titration solution in ml

N=normality of titration solution

w=weight of oil sample in grams

2. 3/27 CONVERSION TEST (JAN WARNQUIST TEST, 2006):

This test tells us the degree to which the oil (triglycerides) is converted to biodiesel (fatty acid methyl ester). The degree of conversion is perhaps the most important factor in defining the biodiesel quality. The results obtained correlate closely with the Gas Chromatography test results on many samples of fuel. If a batch of biofuel passes this test, then the fuel is likely to be very close in quality to the ASTM standards. The test can also be used in the middle of the production process to determine how long to run the process and to monitor the conversion at various stages in the process. This is not a quantitative test, it can only tell qualitatively the quality of the biodiesel produced.

The test is based on the fact that biodiesel is quite soluble in methanol, while triglycerides have a low solubility in methanol. Even if a trace of triglyceride (unreacted oil) is present; it can be seen with the test. The presence of triglycerides gives a direct reflection of the degree to which the oil is converted to biodiesel.

2.2.3 BY-PRODUCTS OF TRANSESTERIFICATION REACTION:

The process of transesterification of oil yields two products, biodiesel and glycerol. The main product is biodiesel but glycerin can is useful too. Glycerin has a lot of uses which range from food sweetener to heart medication depending on the quality. The glycerin obtained from biodiesel production is of low quality because it contains many contaminants from the reaction process. The glycerin derived from the biodiesel process is actually a mixture of free fatty acids (FFA) that were neutralized in the course of transesterification, soaps, water, catalyst, methanol and glycerin. The only contaminant that carries a risk is methanol. To obtain a high conversion of oil into biodiesel we use excess of methanol. The excess methanol settles into the glycerin layer and must be removed to make the glycerin safe to handle. This residual methanol must be removed before using glycerin. It can be boiled at temperatures above 68°C. Removal of methanol makes the glycerin safe to handle and becomes suitable for making soap. The remaining contaminants are all required for soap production, which is the easiest way to capture the value of glycerol. The soap made can have multiple uses from hand to stainless steel cleaner.

The limitations of first generation biofuels, the major one being the food vs. fuel problem, are overcome by the second generation biofuels. The shift to third-generation biofuels is determined by the need to integrate biomass-derived fuels more effortlessly into the existing petroleum based infrastructure. Ethanol, whether derived in first-generation processes or from biomass in second-generation methods, has narrow market access due its difference to conventional petroleum-derived fuels. The limitations comprise restrictions on ratios in which ethanol can be blended with gasoline, inability to transport ethanol through existing pipeline network, lack of compatibility with diesel and jet engines, and tendency to absorb water. Though it is clear that biomass can offer a sustainable and renewable source of carbon to replace an important portion of petroleum resources presently used to generate fuel, power, and chemicals

(Raschka, A, and Carus, M,2012), it is also evident that technologies must be established to convert biomass into direct substitutes for petroleum.

2.3 ALGAE AS A THIRD GENERATION BIOFUEL FEEDSTOCK:

One of the most promising feedstock for biodiesel production is unicellular algae (Demirbas, 2009; Pienkos, 2009). In fact, when compared with superior plants microalgae show higher photosynthetic efficiency, higher biomass productivities and faster growth rates. This aspect, together with high intracellular lipid content, can potentially make a number of unicellular algae species among the most efficient producers of lipids of the planet. Moreover unlike traditional oilseed crops, microalgae cultures do not need herbicides or pesticides and can be performed in ponds or photo bioreactors on non-arable lands including marginal areas unsuitable for agricultural purposes, minimizing damages caused to eco and food-chain systems (Chisti, 2007) and without compromising the production of food, fodder and other products derived from crops. Furthermore, unicellular algae physiology can be manipulated to obtain certain desiderated effects. It is well known the possibility of addressing the algal metabolism to the accumulation of lipid in spite of cellular duplication and protein synthesis by imposing nutrient limiting conditions. Some microalgae are capable of use organic substrates in regimen of mixotrophy and heterotrophy.

Algae come in many forms (species) and are primary producers of organic material in aquatic and marine ecosystems. Depending on species and type of algae, this energy can be in the form of lipids as well as carbohydrates. It is the lipid that can be easily converted into a suitable industrial fuel source. Most of the world's fossil fuel and industrial carbon emissions have little value at best, and will take on large costs in the future both environmentally and monetarily. Growing algae, to not only sequester the carbon but to also provide other possible fuel (Melis and Happe, 2001) and food sources, warrants extensive research and development.

In their review of microalgae for biodiesel production, Mata *et al.* (2010) concluded that "a considerable investment in technological development and technical expertise is still needed ... and correct policies and strategies are still needed."

2.3.1 WHAT IS ALGAE?

Algae belong to a very vast and diverse group of organisms which are simple, autotrophic organisms. They can be unicellular or multi cellular. These are called "simple" organisms because they lack many distinct cell and organs found in plants. The largest and complex forms are called seaweeds. Algae reproduce through various modes, from simple asexual division to complex sexual reproduction. Mainly these are phototrophic but some members are also mixotrophic, i.e. they derive energy both from photosynthesis and uptake of organic carbon.

Table 2 Microalgae and their respective lipid contents. (Ghasemi *et al*. 2011)

Microalgae species	Lipid content, % dry weight biomass	Microalgae species	Lipid content, % dry weight biomass
Ankistrodesmus species	28-40	*Euglena gracilis*	14-20
Anabaena cylindrical	4-7	*Ellipsoidion sp.*	27
Botryococcus braunii	25-86	*Haemotococcus pluvialis*	25
Chaetoceros muelleri	33	*Hantzschia species*	66
Chlamydomonas species	23	*Isochrysis galbana*	21.2
Chlorella emersonii	25-63	*Monallantus salina*	20-22
Chlorella minutissima	57	*Nannochloropsis sp.*	20-56
Chlorella protothecoides	14-57	*Neochloris oleoabundans*	35-65
Chlorella sorokiniana	22	*Nitschia closterium*	27.8
Chlorella vulgaris	14-56	*Nitschia frustulum*	25.9
Crypthecodinium cohnii	20-51	*Pavlova lutheri*	35
Cylotella species	42	*Phaeodactylum tricornutum*	20-30
Dunaliella primolecta	23	*Prostanthera ineisa*	62
Dunaliella salina	28.1	*Prymnesium parvum*	22-39
Dunaliella tertiolecta	36-42	*Pyrrosia laevis*	69.1
Skeletonema costatum	13-51	*Spirulina plantensis*	16.6
Scenedesmus dimorphus	16-40	*Stichococcus species*	33
Scenedesmus quadricauda	19.9	*Tetraselmis suecia*	15-23
Schizochytrium sp.	50-77	*Thalassiosira pseudonana*	20
Selenastrum species	21.7	*Zitzschia sp.*	45-47

It can be seen from the above table that the *Chlorella sp.* show high lipid content. That *Chlorella* is a good choice for biodiesel production is a conclusion reached by Mata *et al.* (2010) in their extensive review of microalgae and biodiesel production. They found lipid content measured as percent dry weight biomass ranged from 5.0% to 58.0%, lipid productivity as mg/L/day from 11.2 to 40.0, and biomass productivity as g/L/day from 0.02 to 0.20 for *C. vulgaris*. It is also reported to grow in heterotrophic and mixotrophic (combining auto- and heterotrophic) conditions as well as the typical autotrophic condition. (William R. 2010)

2.3.1.1 Chlorella vulgaris

Chlorella vulgaris is classified in the Division Chlorophyta, Class Chlorophyceae. The Chlorophyta, one of the 10 recognized Algal Divisions, are commonly known as the green algae. They have green chloroplasts that are not masked by other pigments and both chlorophyll a and b are present. In addition they have β- and γ- carotene and several xanthophylls. These characteristics are very similar to higher plants and this similarity may be of significance when investigating green algae nutrient requirements. Starch is the polysaccharide storage product. Green algae as a group range in body type from non-motile single cells, to flagellates, and to colonial multicellular complexes.

The approximately 10 species of genus *Chlorella* are unicellular, coccoid (round) cells, typically two to12 μm. They live in freshwater or on soil and are easy to grow, making them useful in physiological and biochemical laboratory studies. *Chlorella* species are haploid organisms so there is no opportunity for genetic buffering due to recombination. However, they undergo rapid vegetative reproduction that results in exploitation of the advantageous micro mutations.

The growth physiology comparative to major nutrient elements N, P, K, Mg, and S of *Chlorella* species was studied in the late 50's using a synchronous culture technique. The life cycle of the algae was divided into various stages. The first stage was the appearance of new daughter cells followed by the appearance of photosynthetically, chlorophyll-rich active cells, then intermediate stage with less chlorophyll cells, and ongoing to lighter cells just prior to cell division.

2.3.2 CULTIVATION OF MICROALGAE:

Algae can be cultivated in the following ways:

1. Open ponds: The simplest system of growing algae is in ponds in open air. They have the advantage of being low in capital costs but are less efficient. Cross contamination by other species is a major problem which can lead to damage or even kill the algae.
2. Closed loop system: These are similar to ponds with the only difference that they are not exposed to the atmosphere and use sterile source of carbon dioxide. They are efficient because they may be able to direct connect to carbon dioxide and thus use it in a better way.
3. Photo bioreactors: These are the most expensive in terms of capital costs and are the most advanced system. They have advantages in terms of yield and control. These are closed systems.

Table 3: Open pond vs. Photo bioreactors for cultivation of microalgae (Ghasemi *et al*.2011)

Factor	Open Pond	Photobioreactor
Required Space	High	For PBR itself low
Water Loss	Very High, may also cause salt precipitation	Low
CO_2 Loss	High, depending on pond depth	Low
Oxygen Concentration	Usually low enough because of continuous spontaneous outgassing	Build up in the closed system requires gas exchange devices (O_2 must be removed to prevent inhibition of photosynthesis and photo oxidation damage)
Temperature	Highly variable, some control possible by pond depth	Cooling often required (by spraying water on PBR or immersing tubes in cooling baths)
Shear	Usually low(gentle mixing)	Usually high (fast and

		turbulent flows require for good mixing, pumping through gas exchange devices)
Cleaning	No issue	Requires (wall-growth and dirt reduce light intensity), but causes abrasion, limiting PBR lifetime
Contamination Risk	High (limiting the number of species that can grow)	Medium to low
Biomass Quality	Variable	Reproducible
Biomass Concentration	Low, between 0.1 and 0.5g/l	High, generally between 0.5 and 8.0g/l
Production flexibility	Only few species possible, difficult to switch	High, switching possible
Process Control and Reproducibility	Limited (flow speed, mixing, temperature only by pond depth)	Possible within certain tolerances
Weather Dependence	High (light intensity, temperature, rainfall)	Medium (high intensity, cooling required)
Start-up	6-8 weeks	2-4 weeks
Capital Cost	High($100000 per ha)	Very High-PBR plus supporting systems
Operation Costs	Low (paddle wheel, CO_2 addition)	Higher CO_2 addition, oxygen removal, cooling, cleaning, maintenance)
Harvesting Cost	High, species dependent	Lower due to high biomass concentration & better control over species and conditions.

Thus, algae can be grown almost anywhere with the right temperatures (warm). The cultivation of microalgae is not a threat to the farm land. Moreover, algae can be grown in waste water,

which provides additional advantage by helping to take care of the municipal waste. All of this makes the cultivation of algae much easier compared to the cultivation of other traditional bio fuels.

2.3.3 LIPIDS IN MICROALGAE:

The lipids produced by microalgae can be grouped into two categories, storage lipids (non-polar) and structural lipids (polar). The storage lipids are mainly made of predominantly saturated fatty acids and some unsaturated fatty acids which can be transesterified to produce biodiesel. The polar lipids are important structural components of cell membranes and some polar lipids may act as key intermediates in cell signaling pathways (e.g., inositol lipids, and oxidative products). Amongst the non-polar lipids, the triacylglycerides (TAGs) are the abundant storage products which can be easily catabolized to provide metabolic energy. Triglycerides are reacted with alcohol such as ethanol or methanol to give ethyl esters of fatty acids and glycerol. Microalgae survive in diverse and extreme conditions which are seen in the tremendous diversity and sometimes unusual pattern of cellular lipids obtained from microalgae. Some of the microalgae can also modify lipid metabolism efficiently in responses to environmental stress conditions. The occurrence and the extent to which TAGs are produced is species/strain specific and is controlled by the genetic makeup of individual organisms.

There are wide ranges of stress conditions which have been studied to induce lipid production including nitrogen and/or phosphorus starvation, light irradiation temperature, pH, peptone addition, heavy metals and other chemicals.it has been observed that nitrogen is the single most critical nutrient affecting lipid metabolism in algae (Kalpesh *et al.* 2012)

2.3.4 LIPID EXTRACTION METHOD- “BLIGH DYER METHOD”:

All the extraction procedures aim to separate cellular of fluid lipids from the other constituents like proteins, polysaccharides, amino acids, sugars and to also preserve these lipids for further analysis. The ideal solvent for lipid extraction would entirely extract all the lipid components from a sample, while leaving all the other components behind. In practice, the efficiency of solvent extraction depends on the polarity of the lipids present paralleled to that of the solvent.

Polar lipids (such as glycolipids or phospholipids) are more soluble in polar solvents (such as alcohols), than in non-polar solvents (such as hexane). On the other hand, non-polar lipids (such as triacylglycerols) are more soluble in non-polar solvents than in polar ones. The fact that different lipids have different polarities means that it is impossible to select a single organic solvent to extract them all. Thus the total lipid content determined by solvent extraction depends on the nature of the organic solvent used to carry out the extraction: the total lipid content determined using one solvent may be different from that determined using another solvent.

The Bligh and Dyer method is a simple version of this method. They do clearly state that for quantitative extraction of lipids, it is necessary to perform a re-extraction of the tissue residue with chloroform alone and add this extract to the filtrate preceding to evaporation of the solvent. This would improve the yield of non-polar lipid.

2.3.5 "NILE RED": A SELECTIVE FLUORESCENT STAIN FOR INTRACELLULAR LIPID DROPLETS:

The formation of cytoplasmic lipid droplets is a normal cellular process. The droplets are neutral lipids, usually triacyl glycerols or cholesteryl esters. Triacyl glycerols serve as fatty acid energy reserves and the cholestery esters function as storage storehouses for excess cellular cholesterol. Abnormal accumulation of the cytoplasmic lipid droplets occurs in a variety of pathological conditions. Fluorescence microscopy is a powerful technique for the analysis of a number of cellular processes. Nile red (9-diethylamino-5H-benzophenoxazine-5-one) is an excellent stain. The dye is present as a minor component of commercial preparations of the nonfluorescent lipid stain nile blue. Nile red is intensely fluorescent and, if correct spectral conditions are taken, it can function as a sensitive vital stain for the detection of cytoplasmic lipid droplets. Nile red shows properties of a near-ideal lysochrome. It is powerfully fluorescent, but only in the presence of a hydrophobic environment. The dye is highly soluble in the lipids it is intended to show, and it does not interact with any tissue component except by solution. Nile red can be applied to cells in an aqueous medium, and it does not dissolve the lipids it is supposed to disclose. (Greenspan *et al.* 1986)

Lipids, fatty acids and FAMEs (Fatty Acid Methyl Esters) can be quantified using Gas Chromatography techniques with Mass Spectrometry (Medina *et al.,* 1998).This method is highly reliable and can generate accurate results.

2.3.6. LIMITATIONS OF USING ALGAE AS A FEEDSTOCK:

With all of the advantages associated with algae as a feedstock for bio fuel, there is one major disadvantage. The growth of algae requires large amounts of water, nitrogen and phosphorous. The amounts required as so high that their production would produce more greenhouse emissions than which were saved by using algae based bio fuel to begin with. And the cost of production of algae based bio fuel is much higher than fuel from other sources.

Another minor drawback is that these bio fuels are less stable compared to other bio fuels. The reason, oil produced by algae is highly unsaturated. Unsaturated oils are more volatile especially at high temperatures and hence more prone to degradation. This problem however, has a potential solution. (biofuel.org.uk)

2.3.7 CONVERSION PATHWAYS FOR BIODIESEL:

The intermediate products (bio oil) that are formed after pretreatment of biomass thermo chemically must be converted to biofuel under different conditions.

Conversion pathways include:

1. Transesterification
2. Biochemical conversion- fermentation, anaerobic digestion
3. Thermo chemical conversion- gasification, pyrolysis, liquefaction
4. Hydro processing

The conversion pathway used for production of biodiesel from algae is Transesterification.

Transesterification is also called as alcoholysis. It is the reaction when a fat or oil reacts with an alcohol and forms esters and glycerol. This reaction can be acid catalyzed, base catalyzed or enzymes can be used. Different alcohols can be used like methanol, ethanol, propanol, butanol and amyl alcohol. The low cost along with the physical and chemical advantages of methanol

makes it the most common alcohol to be used. Various alkali catalysts can be NaOH, KOH, carbonates and acid catalysts can be sulfuric acid, sulfonic acid and HCl. Lipases are also used. Commercially, alkali catalysts are used since the alkali based transesterification process is much faster. The parameters that influence the transesterification reaction are: reaction temperature, type of acyl donor and acceptor, ratio of alcohol to vegetable oil, type and amount of catalyst, mixing intensity and quality of starting material (Ghasemi *et.al* 2011). Recovery of biodiesel is done by repeated washing with water to remove methanol and glycerol.

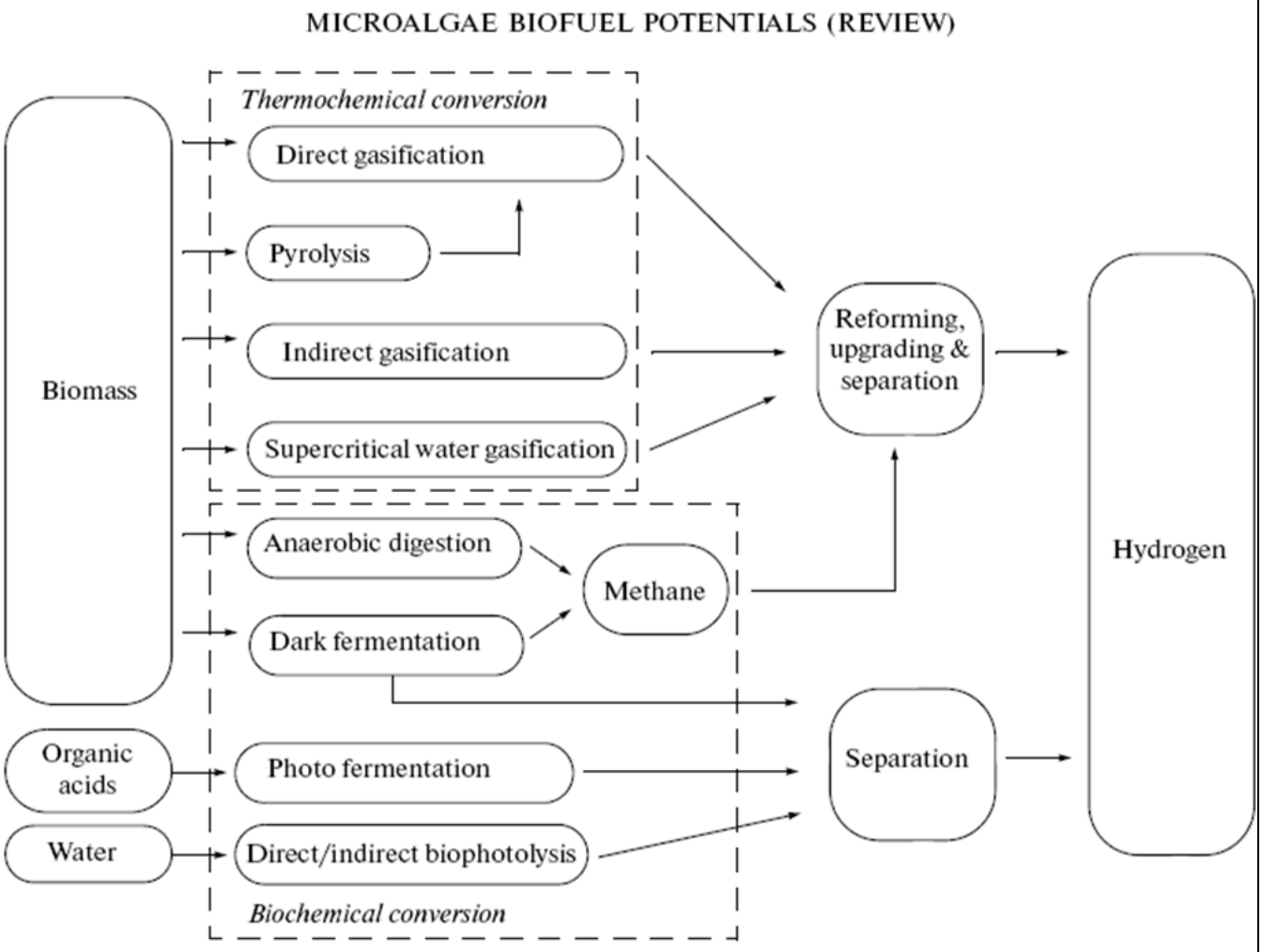


Fig.1 Conversion pathways for biomass (Ghasemi *et al*. 2011)

The problems for commercialization of algal fuels are many: the high cost of production, high demands on certain key resources and the need to attain an energy ratio much greater than unity. Possible solutions to all these issues are being explored. To have a widespread availability of algal fuels is not likely to be seen in the near future. There is an increase in the consumption of petroleum and replacing this demand with algal oil is not possible without developing new and improved technologies. The interest in commercial production of algae based biofuels is very strong which indicates the possibility of an economically viable production and also within a reasonable time frame (Chisti.Y, 2013)

CHAPTER 3

MATERIALS AND METHODS

3.1 SECOND GENERATION BIOFUEL

3.1.1 COLLECTION OF WASTE COOKING OIL (WCO):

Waste cooking oil (used twice or thrice) was collected from the students' mess of Jaypee University of Information Technology, Waknaghat. The oil was filtered to remove the solid food particles using a muslin cloth.

3.1.2 TITRATION TO DETERMINE THE FREE FATTY ACID (FFA) % OF WCO:

For carrying out the titration process, the following were used-

Sodium hydroxides, Isopropyl alcohol, waste cooking oil and phenolphthalein indicator.

a. Alkali solution (titration solution) was prepared by dissolving 1g of NaOH in 1L of water. Fill the burette with this solution
b. In a 100 ml beaker, 10 ml alcohol was added. This was followed by adding 3-4 drops of the indicator. (To blank the solution, the alkali solution is to be added from the burette while mixing until a color change is obtained. Blanking the solution is done only if required).
c. Now 1 ml of oil was added to this titration beaker and mixed well. The alkali solution was slowly added from the burette with constant swirling. The solution was added until a color change was observed and the color change stayed for more than 15 seconds.
d. The amount of alkali solution used for titration step was noted.
e. The process was repeated thrice and a mean titration value was calculated. And further the FFA% was calculated.

3.1.3 TRANSESTERIFICATION OF WCO TO PRODUCE BIODIESEL:

One-Step Transesterification:

a. 500 ml of oil was pre heated at 55°C in a conical Erlenmeyer flask.

b. A solution of KOH (4g) and methanol (100ml) was added to the pre heated oil with proper mixing.
c. The flask was kept in a water bath with shaker at 55°C for 2 hours.
d. The oil was then transferred to a conical flask and kept overnight for the separation of biodiesel and its by-product glycerol.
e. The following day, glycerol was removed and several washing was done with distilled water to remove the foam formation. The washing was continued till the foam or soap formation almost became nil.
f. The biodiesel obtained was then heated at 70°C for 2 hours in a water bath with a shaker.
g. The biodiesel thus produced is then tested for its quality using acid value and 3/27 tests.

Two-Step Transesterification: (Hassani M. *et al* 2013)

a. 100 ml (124g) WCO was added to a 1L conical Erlenmeyer flask.
b. For the acid esterification step, 3ml/100g of sulfuric acid was added slowly to 800 ml of methanol in a separate flask. The methanol: oil ratio for the first step was 8:1.
c. This solution was added to the oil and the flask was kept at 70-80°C in a water bath with constant shaking for 3 hours.
d. For the second step, a solution of 1.24g (1g/100g of oil) KOH in 600 ml of methanol was prepared. The methanol:oil ratio was 6:1. After the completion of the first step of transesterification, this solution was added to the flask containing the oil. The flask was then kept at 60-64°C in a water bath with constant shaking for 1.5 hrs.
e. After completion of the 2-step reaction, the resultant was kept overnight in a separating funnel to separate the biodiesel produced and its by-product, glycerol.
f. Next day, glycerol was separated from biodiesel and the two were collected in different flasks. The biodiesel was heated at 70°C for 1.5 hours to remove the excess methanol (methanol can be collected by using a rotary evaporator). This step should be done in a fume hood as methanol fumes are harmful.
g. And the biodiesel produced was tested for its quality by determining its acid value and by using the 3/27 conversion test.

3.1.4 QUALITY TESTING OF THE FINAL PRODUCT:

The biodiesel produced should meet either the ASTM standards or the EU (European Union) standards. I tested the quality of biodiesel keeping in mind the ASTM standards.

The tests applied are the ones used by home brewers, i.e. these methods can be appropriate to test for biodiesel on the small scale.

Acid value Determination:

a. The acid value of finished biodiesel by both one step and two step transesterification was measured by performing titration. The procedure followed is same as the one done for determining the FFA% of the waste cooking oil.
b. After measuring the titration value, the acid value of finished biodiesel was calculated using the following formula:

   Acid value= v*N*56.1/w

   Where v=volume of titration solution in ml

   N=normality of titration solution

   w=weight of oil sample in grams

The 3/27 Conversion Test:

a. To begin the test, 2 clean and dry vials were taken.
b. 27 ml of methanol was added to both the vials, followed by addition of 3 ml of water free biodiesel from one-step and two- step transesterification in vial no. 1 and 2, respectively, i.e. the ratio of methanol: oil ratio is 27:3.
c. The vials were shaken and then were let to settle down.
d. The vials were then observed for any oily material settling down in the vials for the next 30 minutes.

3.1.5 SOAPS MADE FROM GLYCEROL:

Soap Bars:

a. The glycerol was filtered to remove unwanted impurities. The glycerol was heated at 65°C to remove excess methanol from the transesterification reaction.
b. A mixture of lye (sodium hydroxide) and water was added. The mixture was made by adding 11.55g of lye in 75 ml of water (heated to 37.7°C) and properly dissolved (for 300

ml of glycerol). More the amount of lye, higher the grease cutting property but it dries the skin. Increasing the amount of water with increase the lather formation.

c. Glycerol was heated at 65°C for 10 minutes with mixing, followed by another 10 minutes with slow mixing.
d. The mixture was then poured into containers or glass petri dishes and allowed to cool for 2-3 days; the containers were covered with cardboard.
e. After 2-3 days, the soap was sliced around corners and overturned. The soap was cut into pieces.
f. The bars were allowed to set for 4-7 days at 4°C before using.
g. Essential oils can be used to give fragrance to the soap bars.

Liquid Soap:

a. 100 ml of waste cooking oil was measured into a 500 ml beaker.
b. The beaker was placed onto a hot plate and the oil was heated to 75°C.
c. While the oil was heated, KOH solution was prepared by dissolving 20 g of KOH in 60 ml of water (the amount KOH can be decreased to lower the pH of soap to 10).
d. The solution was added to the heated oil along with stirring. A hot plate with a magnetic stirrer was used.
e. The temperature was maintained between 65-70°C with constant stirring using a magnetic bead.
f. Stirring was continued till the mixture became thick. This took about 30-45 minutes. The "soap paste" was now ready (Stirring should not be stopped and temperatures should not exceed 75°C, otherwise the mixture starts to boil and splash out).
g. The soap paste was allowed to cool. And then the paste was added to 500 ml of water in a 1L beaker.
h. This was heated to 80-85°C with stirring until the soap clumps were segregated and the soap paste dissolved completely in the water. A basic liquid soap was now ready.
i. 55 g of glycerol was added while the soap base was still hot. Essential oils can also be added at this stage. But care should be taken.
j. The soap was mixed well and allowed to cool overnight.

k. The following day, the soap was tested for its pH using pH strips. The usable pH range is from 6-10. In case the pH exceeds 10, the amount of NaOH can be reduced for the next batch. Citric acid or boric acid can also be used to lower the pH as these are weak acids.
l. The soap was then tested on hands to check its grease cutting property.

3.2 THIRD GENERATION BIOFUEL

3.2.1 SAMPLING AND ISOLATION OF ALGAL CULTURES:

a. Samples were collected from different parts of Himachal Pradesh, India and various species of algae were identified like *Scenedesmus quadricauda, Scenedesmus dimorphus* and *Chlorella sp.*
b. Isolation of *Chlorella sp.* was done by successive dilutions, plating and maintaining cultures using BG-11 growth medium. The isolated cultures were maintained on agar plates and test tubes (liquid medium).

Table 4. Composition of BG-11 Media (Stainer *et al.* 1971)

Stocks	Components	Per 1000ml
Stock 1	Sodium nitrate	15.0g
Stock 2	K_2HPO_4	4.0g
Stock 3	$MgSO_4.7H_2O$	7.5g
Stock 4	$CaCl_2.2H_2O$	3.6g
Stock 5	Citric acid	0.6g
Stock 6	Ammonium ferric citrate	0.6g
Stock 7	SDTA	0.1g
Stock 8	Na_2CO_3	2.0g
Stock 9	Trace elements:	
	H_3BO_3	2.86g
	$MnCl_2.4H_2O$	1.81g
	$ZnSO_4.7\ H_2O$	0.22g
	$Na_2MoO_4.2\ H_2O$	0.39g

	$CuSO_4.5\ H_2O$	0.08g
	$Co(NO_3)_{2.}6\ H_2O$	0.05g

c. For preparing the BG-11 media, the stocks were added in the following ways:
   Stock 1- 100 ml/L
   Stocks 2 to 8- 10 ml/L
   Stock 9- 1 ml/L
   The media was then autoclaved for use.

3.2.2 IDENTIFICATION OF ALGAL SPECIES:

Initial identification was on the basis of morphological features followed by strain identification through molecular method.

a. Isolated colonies from the agar plates of the algal cultures were picked and added on a glass slide.
b. 3-4 drops of BG-11 media was added to the glass slide and covered with a cover slip.
c. The slide was observed under 100X oil immersion might microscope.
d. Once the species were identified, BG-11 was again added to the slides and then this was used to inoculate test tubes containing 2 ml of BG-11 media for further cultivation of the microalgae. The inoculated test tubes were then incubated at 25°C, circadian cycle of 16:8 for 5-6 days with daily shaking of the tubes.

3.2.3 CULTURING ON AGAR PLATES:

a. Petri plates were prepared by adding 14g/L of agar in BG-11 media.
b. Isolated colonies were picked from already existing algal culture plates and further streaked by using various streaking methods.
c. The plates were then kept for incubation at 25°C with circadian cycle of 16:8.
d. A few agar plates of mixed cultures were also prepared by the spread plate method for isolation of various other species of microalgae from the mixed cultures.
e. These plates were also kept for the same incubation conditions.

3.2.4 MOLECULAR ANALYSIS OF ISOLATED ALGAL SP. (*Chlorella sp*):

DNA extraction by CTAB method:

a. 1 g of sample (algal biomass) was grinded in liquid nitrogen.
b. 10 ml of CTAB buffer at 60°C was added. The contents were mixed by inversion and incubated at 60°C for 60 minutes.
c. After removing from the heat, the contents were cooled at room temperature for 5 minutes.
d. Freshly prepared 12 ml of Chloroform: isoamyl alcohol was added and mixed by inversion to form an emulsion.
e. The vials were spun at 6000rpm for 10 minutes at room temperatures.
f. The aqueous solution was transferred to a fresh vial.
g. 2 volumes of chilled isopropanol (-20°C) was added to the aqueous phase and mixed by inversion. The vials were kept at -20°C overnight for precipitation.
h. The next day, the vials were spun at 6000 rpm for 10 minutes.
i. The supernatant was discarded and the pellet was washed with cold 70% ethanol. The pellet was then air dried.
j. The pellet obtained was dissolved in TE buffer overnight at 4°C
k. 3 µl of RNase A was added (10 mg/ml) and kept for incubation at 37°C for 1 hour.
l. Freshly prepared phenol:chloroform:isoamyl alcohol was added (150 µl)
m. The contents were vortex briefly and spun at 14000 rpm for 15 minutes. The upper layer was collected in fresh vials.
n. 2 volume of chilled isopropanol was added.
o. The vials were kept at -20°C for 1-2 hours for DNA precipitation.
p. The vials were spun at 14000 rpm for 15-20 minutes. The pellet obtained was washed twice with cold 70% ethanol.
q. The pellet was air dried.
r. TE buffer was added and then left overnight at 4°C for resuspension.
s. The DNA was then checked for purity using Nano Drop and by running on 0.8% agarose gel.

DNA Quality Check:

1. By Nano Drop instrument which gives the concentration of DNA and the ratio of absorbance at 260 nm and 280 nm to check the purity of the DNA obtained.

2. By running the DNA sample on the agarose gel.
   a. 0.8% of agarose gel was prepared in 1X TAE buffer and EtBr was added (5 µl/100ml)
   b. The gel was casted using a gel tray and comb.
   c. The samples were loaded (1µ dye+ 5 µl DNA sample) in the wells.
   d. The gel was run at 50V for 1 hour.
   e. Then the gel was exposed to UV light using a Gel-Doc and a photograph of the gel was taken.

PCR:

A PCR reaction was setup using the following primers:

Table 5 Primer Combinations Used for the PCR amplification of the 18S rRNA.

Primers ID	Primers Sequence	Temperature (Tm)	Source of Primers
Chloro F	TGGCCTATCTTGTTGGTCTGT	56.1C	Moro.C.V, *et al.*
Chloro R	GAATCAACCTGACAAGGCAAC	54.3C	
CV1 F	TACCTGGTTGATCCTGCCAGT	58.1C	Senousy Hoda.H, *et al.*
CV2 R	CCAATCCCTAGTCGGCATCGT	59.1C	

The PCR reaction was as follows for 35 cycles:

Initial denaturation 94°C for 5 minutes

Denaturation 94°C for 30 seconds

Annealing 52°C for 1 minute

Extension 72°C for 30 seconds

Final extension 72°C for 5 minutes

The PCR products were run on 1.5% agarose gel with a 100bp DNA ladder.

The products were sent for sequencing, the results of which are awaited.

3.2.5 GROWTH KINETICS OF *Chlorella sp.:*

a. Using 50 ml Tarson centrifuge tubes, algal culture from glass tanks was centrifuged at 6000rpm for 10 minutes.

b. The pellet was washed with distilled twice by centrifuging at 6000 rpm for 10 minutes each time.

c. The pellets were collected and the volume of the inoculum was raised to 1000 ml by adding distilled water. The color of the inoculums should be medium green.

Setting up of Bioreactors:

a. 500 ml bioreactors with 150 ml working volume and 350 ml head space were set up for three different conditions- full nitrogen source as per BG-11 media (15g/L), reduced nitrogen source (7.5g/L) and no nitrogen source.

b. In each bioreactor, 150 ml of BG-11 growth medium with corresponding nitrogen conditions was added.

c. Followed by the addition of 20 ml inoculum.

d. The bioreactors were then set up in the green house at 30-32° with aeration pumps attached.

Sampling:

a. Every alternate day, a small sample was collected from each bioreactor.

b. The sample was diluted to a factor of 10.

c. The samples were analyzed using a cell haemocytometer for cell count.

d. A growth curve was then plotted and various parameters were calculated. The parameters are specific growth rate and doubling time of *Chlorella sp.*

1. Specific Growth Rate, $\mu = \text{Ln}\ (N_t/N_o)/\ T_t - T_o$

   Where N_t is the number of cells at the end of log phase

   N_o is the number of cells at the beginning of log phase

   T_t is the end of log phase

   T_o is the start of log phase

2. Doubling time, $t = 0.6931/\mu$

   Where μ is specific growth rate

3.2.6 OPEN PONDS FOR GENERATION OF ALGAL BIOMASS:

For scaling up of the cultivation of algal biomass, 2 green tanks were set up

a. In each tank, 35 L of BG-11 media (made in tap water) was added and the tanks were inoculated with algal cultures from test tubes.
b. Aeration pumps were attached. These tanks were also maintained in the green house area.

3.2.7 CHLOROFORM: METHANOL METHOD: (Bligh and Dyer. 1959)

This method is used to determine the % of lipids in the algal biomass

a. For 300-400 mg of sample, 4 ml of methanol, 2 ml of chloroform and 0.4 ml of distilled water was added.
b. The contents were vortex for 30 seconds followed by sonication for 15 minutes.
c. Then 2 ml each of chloroform and water was added.
d. The vials were centrifuged at 4000 rpm for 5 minutes.
e. The upper of methanol and water was removed using pipettes and the middle layer of lipids was transferred to fresh vials.
f. The above steps were repeated according to the need.
g. Using a funnel and Watmann Filter paper, 2 cm thick layer of anhydrous sodium sulphate was made and the lipid layer was made to pass through this.
h. The filtered solution was transferred in a round bottom flask and subjected to a rotary evaporator.
i. Before transferring the solution to an RBF, the weight of the empty RBF was taken.
j. After using the rotary evaporator, the weight of the RBF was again measured and the % of lipid present was calculated as follows:

✓ Lipid content (%) = amount of lipid extract (gram unit of lipid sample)

3.2.8 LIPID INDUCTION IN *Chlorella Sp.* UNDER VARIOUS STRESS CONDITIONS:

a. Using 50 ml Tarson centrifuge tubes, algal culture from glass tanks was centrifuged at 6000rpm for 10 minutes.

b. The pellet was washed with distilled twice by centrifuging at 6000 rpm for 10 minutes each time.

c. The pellets were collected and the volume of the inoculum was raised to 1000 ml by adding distilled water. The color of the inoculums should be medium green.

Setting up the bioreactors:

Bioreactors were set up with aeration pumps attached to each of them. 40 ml (10%) inoculum was added to each bioreactor.

The stress conditions were as follows:

Table 6 Various stress conditions for inducing lipid production in *Chlorella sp.*

Culture Condition	Media Used	Stress Applied	Sunlight	Aeration
Autotrophic	BG-11	Nil	Yes	Yes
Mixotrophic	BG-11	Dextrose added (1%w/v)	Yes	Yes
Heterotrophic	BG-11	Dextrose added (1%w/v) and no sunlight	No	Yes
Protein addition	BG-11	Peptone added (1g/L)	Yes	Yes
Nitrogen limitation	BG-11 (without nitrogen source)	$NaNO_3$–0.6g/L was added	Yes	Yes

Sampling:

a. Every alternate day a small sample was taken from each bioreactor.

b. To check the growth of cells, absorbance was measured at 680 nm for each sample. This was continued till the stationary was achieved.

c. A graph of biomass concentration (g/L) vs. time (days) was plotted for all the stress conditions.

3.2.9 FLOURESCENCE MICROSCOPY USING NILE RED FOR QUALITATIVE ANALYSIS OF LIPID PRODUCTION IN *CHLORELLA SP.* (Elumalai.S., *et al,* 2011):

a. The fluorescence Nile red was used to detect lipid bodies in samples of micro algae species.
b. Micro algal cells were obtained by centrifugation at 5000rpm for 5 minutes of 1 ml sample from each bioreactor.
c. The cells were washed with 0.9% NaCl solution (1 ml) 3-5 times.
d. The cells were then resuspended in saline solution and were stained by adding Nile red solution (0.1 mg/ml in acetone) in 1:100 v/v proportions and incubated for 10 minutes.
e. The stained micro algal cells were then observed under a fluorescent microscope (Olympus) using 450-490 nm excitation, 515 nm emission filter and 40X objective lens. The images were taken with color CCD digital camera (DP12, Olympus) and the software used was Image Pro Plus.
f. The images of various samples were then compared for the level of lipid bodies in the cells and the best stress condition for lipid production in micro algal cells was determined.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 SECOND GENERATION BIOFUELS

4.1.2 TRANSESTERIFICATION OF WASTE COOKING OIL (WCO):

In making biodiesel, triglycerides (in WCO) are reacted with methanol in a reaction known as transesterification, which produces methyl esters of fatty acids (biodiesel) and glycerol. (Chisti 2007)

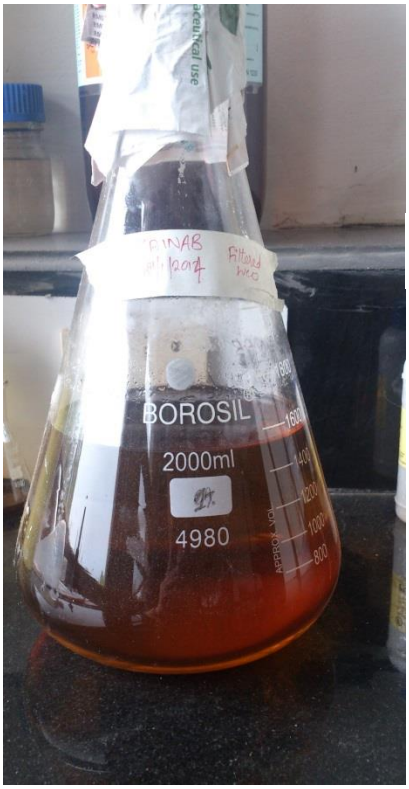


Fig.2 Waste Cooking oil from the students mess of JUIT

TRANSESTERIFICATION

Fig.3 Separation of Biodiesel and its by-products in a separating funnel

According to Hassani.M *et al*. 2013, the oils with FFA % greater than 1% w/w will result in large soap formation when only base catalysts are used. Hence, two-step transesterification is a better choice to avoid saponification. The acid esterification step is a pre-treatment to avoid

high levels of soap formation during the process. The same was reported by various other groups (Endalew *et al.* 2011 and Tahira *et al*. 2012).

The FFA% of the waste oil was measured by titration method and it was calculated to be 2.007%. The oil was subjected to both one step and two step transesterification and the results were compared. The FFA% reported by Hassani.M *et al*. 2013 for their feedstock was 9.8 wt% using a similar method of titration. Since, the amount of FFA were lesser in our feedstock, we first studied one-step transesterification and in fact did witness a significant amount of soap formation while the biodiesel formed had a turbid appearance.

For the next batch, the waste oil was pre-treated by acid esterification and then followed by alkali catalyzed transesterification. The soap formation levels went down and the biodiesel produced was much clearer with no turbid appearance.

In the separating funnel, glycerol which is the by-product settles down as it is heavier than biodiesel produced.

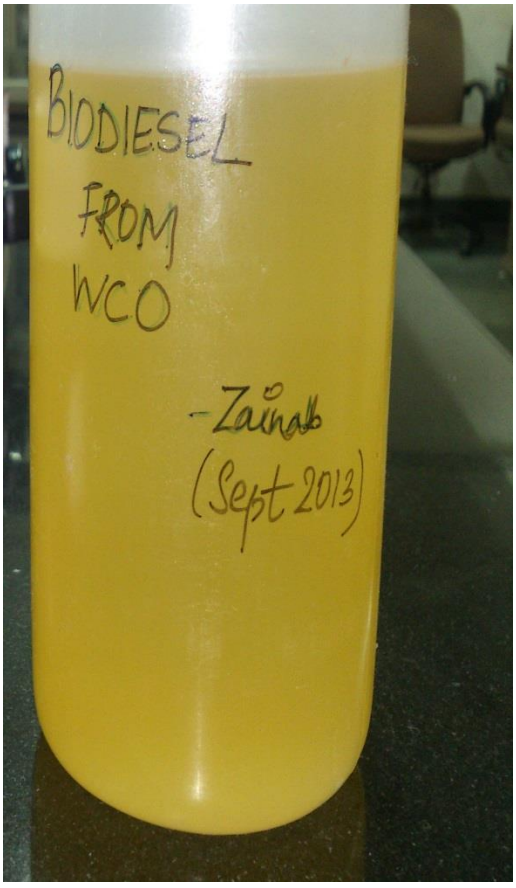


Fig.4 Biodiesel produced by one-step transesterification reaction.

Fig.5 Biodiesel produced by two-step transesterification reaction.

4.1.3 QUALITY TESTING OF FINISHED BIODIESEL:

The quality of biodiesel was tested by the following two methods:

4.1.3.2 Acid value determination.

According to the ASTM standards D664, the maximum acid value of finished biodiesel should be 0.50 mg/g. The acid value indicates the amount of alkali required to react with the acid in an amount of oil.

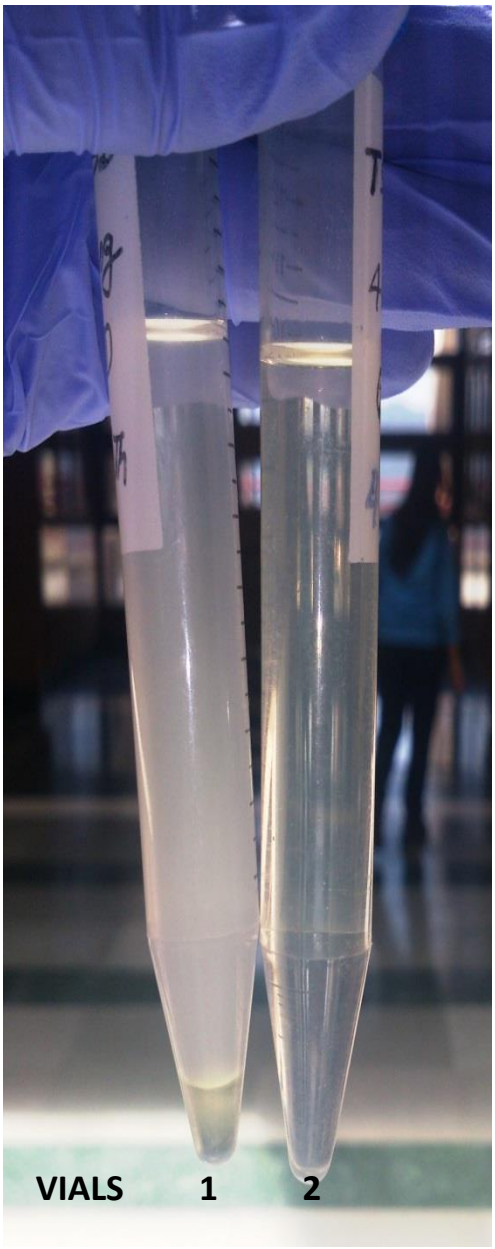
The acid value was measured by titration against alkali solution, and calculated to be 20.94 mg/g and 0.82 mg/g for one-step and two-step production methods, respectively.

The acid value clearly indicates that the biodiesel produced without the pretreatment of the feed stock does not comply with ASTM standards and is not of good quality. It indicates that a high amount of alkali is required for reacting with the acid present, i.e. the biodiesel is highly acidic in nature. Whereas, the acid value of finished product from two-step method is very near to the ASTM standards indicating the good quality of biodiesel produced.

4.1.3.2 The 3/27 conversion test:

This test indicates the presence or absence of unreacted triglycerides in biodiesel. If the biodiesel dissolves completely in methanol, no unreacted triglycerides are present and the entire reactants have been converted to the product. If some part of the oil settles down, it shows the presence of unreacted triglycerides in the finished biodiesel. This test correlates with the testing of biodiesel using Gas Chromatography for presence/ absence of unreacted fatty acids.

The following photos clearly show that presence of some amount of settled oil in vial 1 whereas no oil can be seen in vial 2. The biodiesel added to vial 1 was produced using one-step reaction while the sample added to vial 2 was of two-step reaction. This is a clear indication that no triglycerides are present in biodiesel produced by using two-step transesterification, and the conversion rate to biodiesel was high in this case.



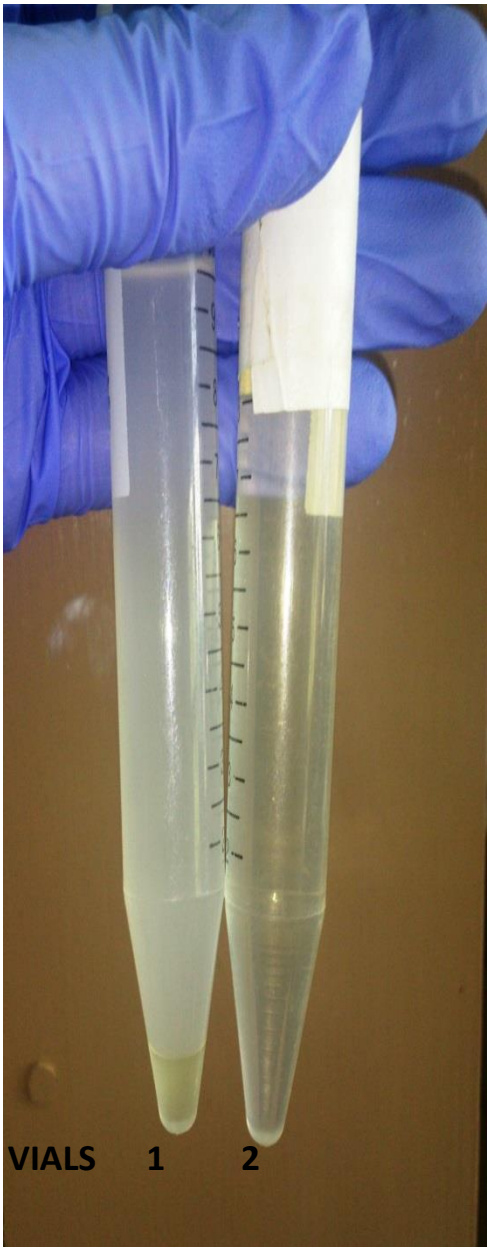


Fig.6 Vials showing the 3/27 conversion test for biodiesel

Vial 1- One-step transesterification biodiesel and Vial 2- Two-step transesterification biodiesel

Table 7 Comparison between the product (biodiesel) made by the two types of transesterification reactions.

SAMPLES	WEIGHT (gm) of 1ml of sample	TITRATION VALUE(ml)	FFA%	ACID VALUE(mg/g)	3/27 CONVERSION TEST PASSED
Waste cooking oil	0.871	3.1	2.007	-	-
Biodiesel (1-step transesterification)	0.871	16.26	-	20.94	No
Biodiesel (2-step transesterification)	0.824	4.03	-	0.824	Yes

4.1.4 SOAPS MADE FROM GLYCEROL:

Our research group works on zero waste technology. Hence, glycerol is referred as the by-product of the biodiesel reaction.

Glycerol was utilized to make soap with grease cutting property. Both the forms of soap were prepared- soap bars and liquid soap.

The soaps were tested on our hands. The bars have high grease cutting property while the lather formation was less due to low levels of water used during its preparation. The liquid form of soap was of pH 10. The pH can be changed by either keeping the amount of lye (NaOH) low in the beginning or by adding citric acid after the soap has been made. The safe pH range is from 6-10.

Fig.7 The by-product of transesterification of WCO- Glycerol

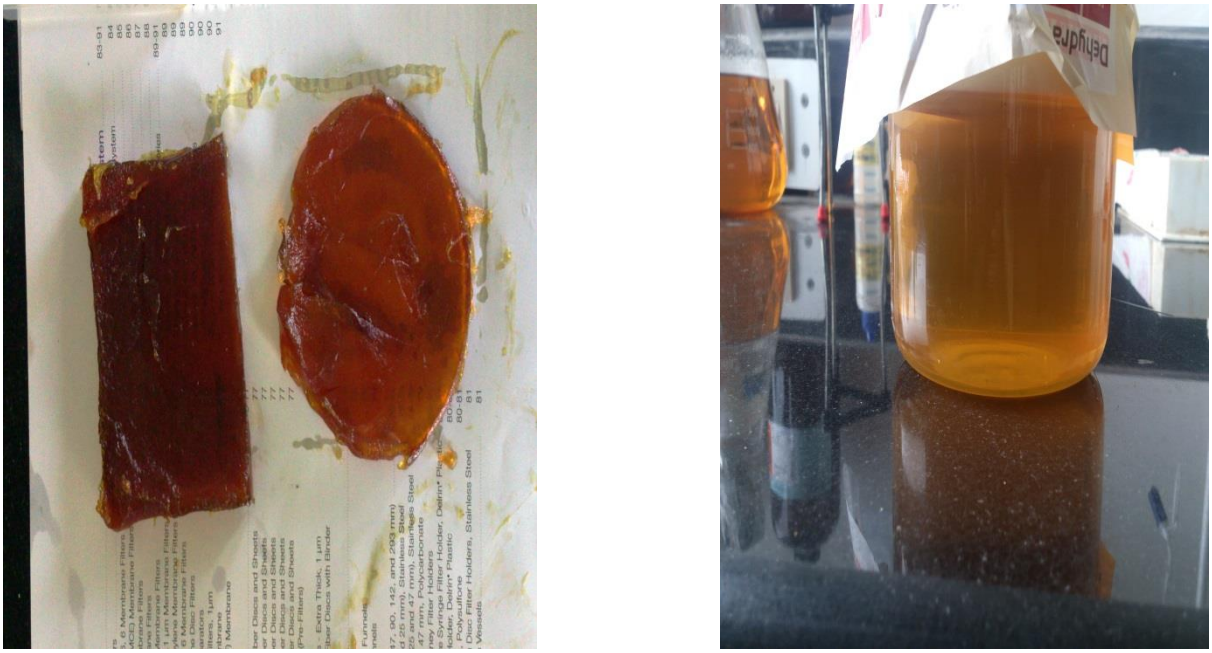


Fig.8 Soaps made from glycerol. On the left are soap bars and on the right is liquid soap.

4.1.5 CONCLUSION:

Large amount of Waste cooking oil is generated in institutes' student mess, cafeterias, army mess, jails' mess, restaurants and hotels. Utilizing this waste oil to produce biofuel can help people generate their own energy sources and also provide a solution for the disposal of waste

oil. The by-product, glycerol can be utilized for making further products or can be sold to industries that use glycerol to made valuable products, thus helping them to recover the cost of production of biofuel, generate profits and also protect the environment.

4.2 THIRD GENERATION BIOFUELS

Microalgae are presently being considered as an ideal third generation biofuel feedstock due to their CO_2 fixation ability, rapid growth rate and high production capacity of lipids. Additionally, they also do not compete with food crops, and can be produced on non-arable land (Dragone.G *et al.* 2010)

4.2.1 SAMPLING AND ISOLATION OF ALGAL CULTURES:

The samples collected from various areas of Himachal Pradesh were first observed under 100X oil immersion light microscope to morphologically identify the different algal species in the samples.

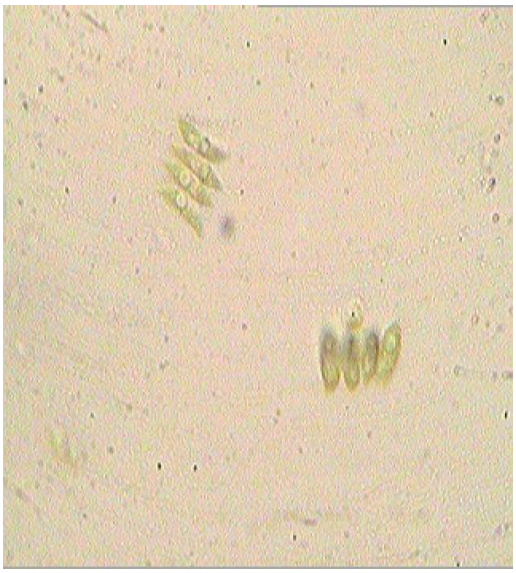


Fig.9 Microscopic view of mixed culture showing *Scenedesmus dimorphus*

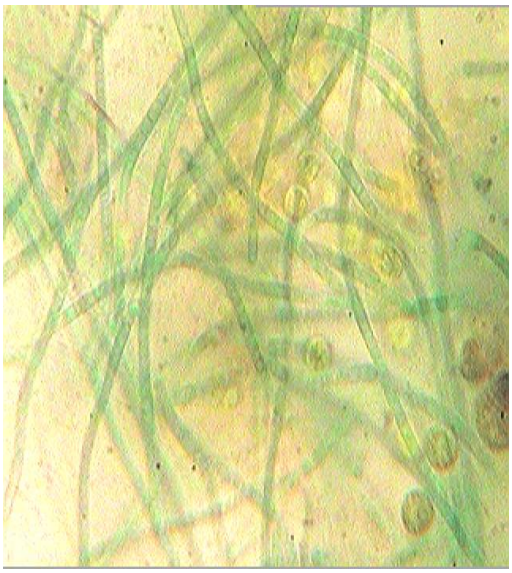


Fig.10 Microscopic view of mixed culture showing diatoms and *Chlorella sp.*

Isolation of algal species was done by successive dilutions and by sub culturing on agar plates of BG-11 media. The isolated colonies were observed under 100X oil immersion light

microscope, morphologically identified as *Chlorella sp.* and then further cultured to get isolated pure cultures of *Chlorella sp.*

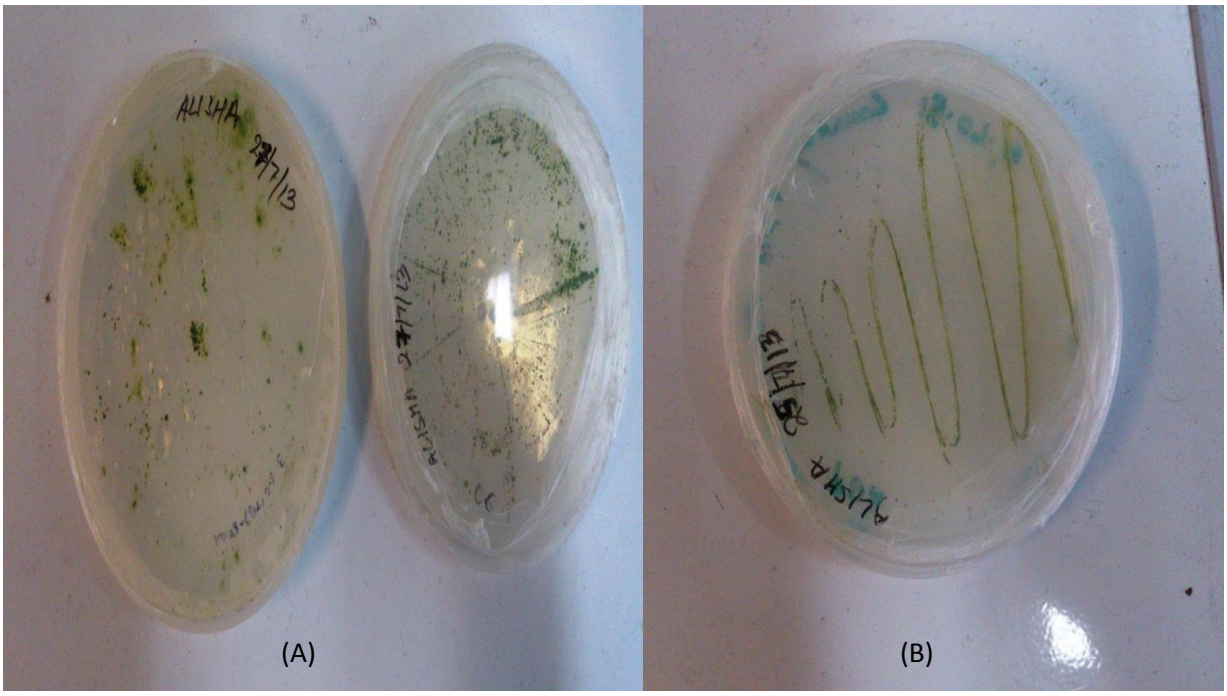


Fig. 11 Agar plates inoculated with mixed cultures to get isolated colonies (A) and streaked with isolated algal colonies (B)

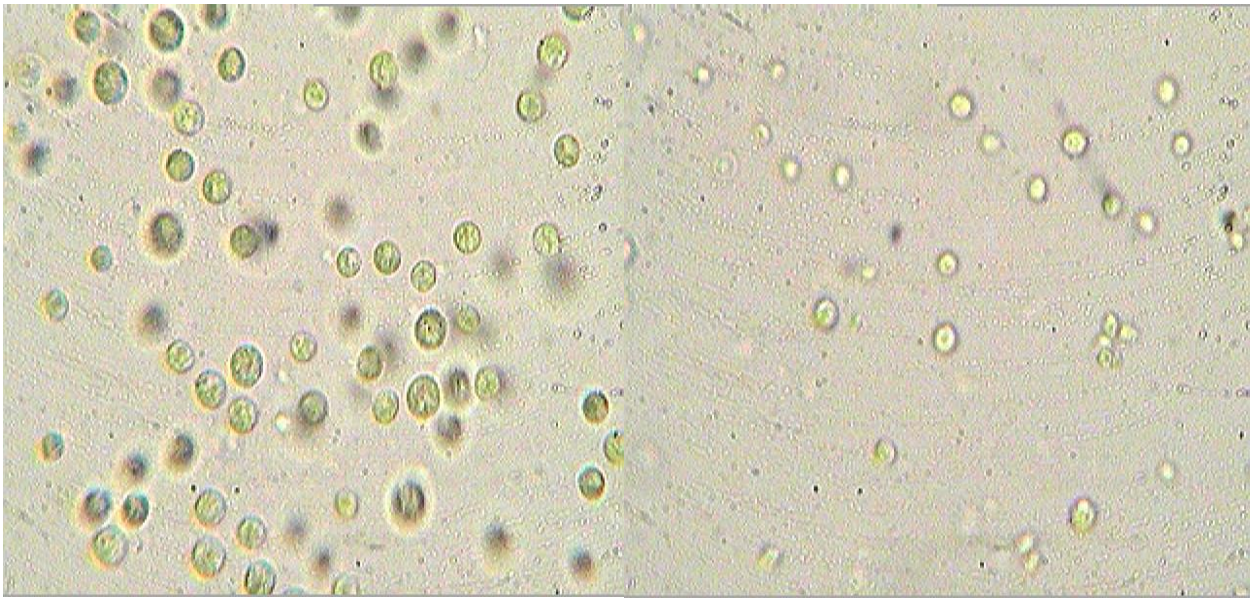
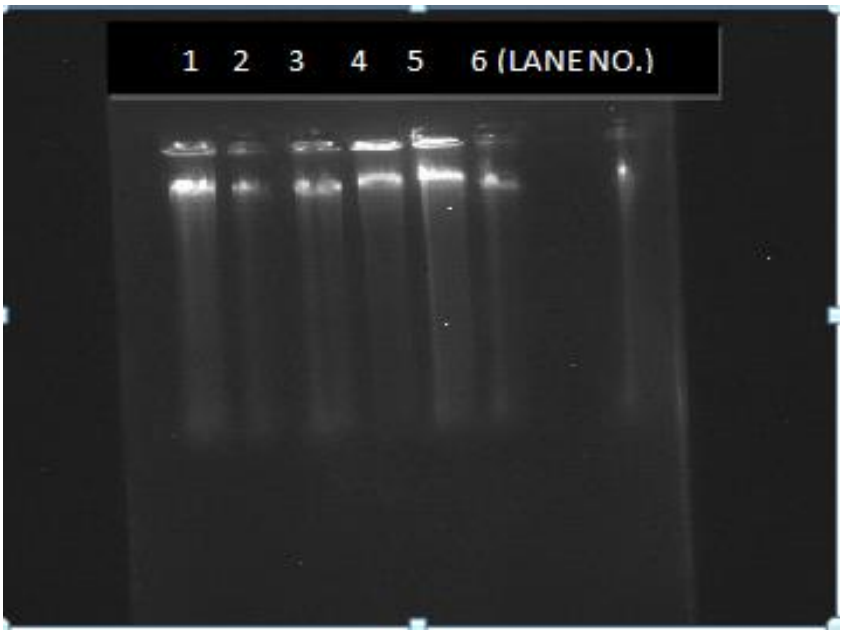


Fig. 12 Microscopic view of isolated *Chlorella sp.*

4.2.2 DNA EXTRACTION AND PCR PRODUCTS:

The DNA extraction procedure was carried out in triplicates. The purity of extracted was checked by running the samples on 0.8% agarose gel and by using Nano Drop.



LANE 1-DNA sample from vial 2

LANE 2-DNA sample from vial 3

LANE 3-DNA sample from vial 1

LANE 4-DNA sample from vial 3

LANE 5-DNA sample from vial 2

LANE 6-DNA sample from vial 1

Fig. 13 DNA samples run on 0.8% agarose gel to check for purity

The gel picture taken in Gel-Doc shows a clear band for each of the sample (triplicates) run on 0.8% agarose gel. This indicates an intact DNA with no or very less RNA or protein contamination.

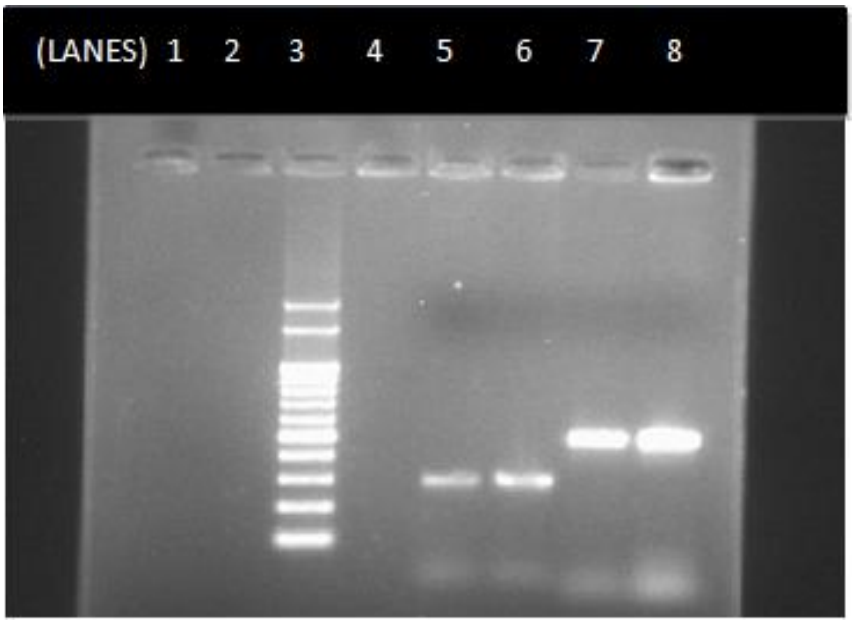
The amount of DNA isolated and the presence of RNA or protein contamination in the samples was checked using the Nano Drop. The Nano drop instrument gives the ratio 260/280nm absorbance. The ratio in the range of 1.8-2 is suggestive of an intact and pure DNA. Below 1.8 means the sample is contaminated with proteins and above 2 will be due to RNA presence.

Table 8 Nano Drop readings

Vial no.	DNA conc. (ng/µl)	Absorbance ratio (260nm/280nm)
1	319.7	1.82
2	411.3	1.77
3	364.8	1.66

The above table shows that the DNA sample from vial 1 is pure while the samples from vial 2 and 3 are contaminated with protein as their ratios are lower than 1.8.

An appreciable amount of *Chlorella sp*. DNA was isolated.



LANE 3- 100bp DNA ladder

LANE 5&6- PCR products of DNA amplified using primers with ID name-Chloro

LANE 7&8- PCR products of DNA amplified using primers with ID- CV 1&2.

Fig. 14 PCR products run on 1.5% agarose gel.

The DNA amplified using primers Chloro have PCR products of size 300bp while the PCR products using primers CV 1&2 are of size 500 bp.

4.2.3 GROWTH KINETICS OF *Chlorella sp.* :

To study the growth kinetics of Chlorella sp. , the 500 ml bioreactors were set up with BG-11 media (Stainer et al. 1971) with various nitrogen levels and 10% algal inoculum.

Fig.15 Experimental setup to study the growth kinetics of *Chlorella sp.*

After sampling every alternate day for 15 days and doing a cell count using a haemocytometer, a graph of Log values of the cell count vs. the no. of days was plotted using MatLAb software.

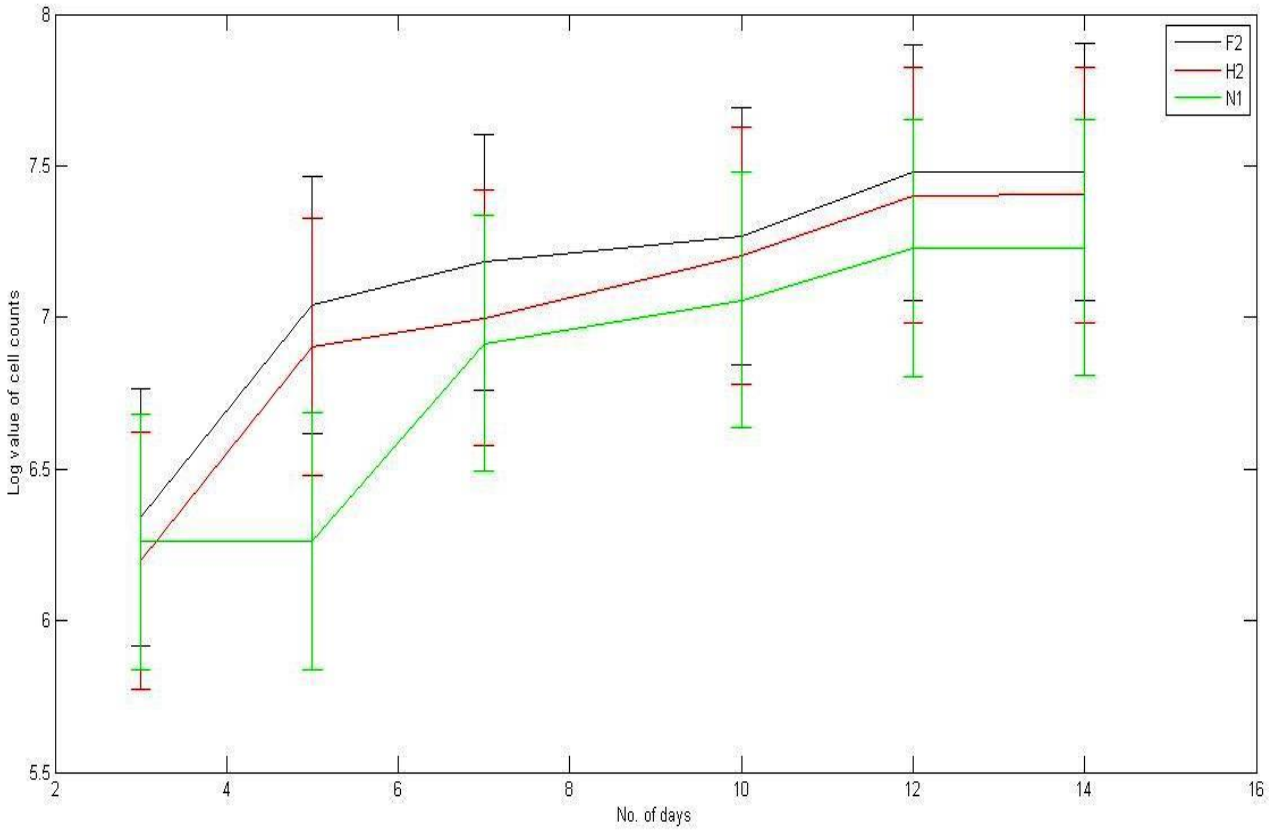


Fig.16 Graph showing the growth kinetics of *Chlorella sp*. at three different nitrogen levels.

F2- Full nitrogen source (15g/L $NaNO_3$)

H2- Nitrogen Depletion (7.5g/L $NaNO_3$)

N1- Nitrogen deprived (no $NaNO_3$)

The following parameters were calculated from the growth kinetics curve:

Table 9 Growth Parameters

Nitrigen levels	Specific growth rate (μ)	Generation time (t)
Full (15g/L $NaNO_3$)	0.290 day^{-1}	2.4 hours
Half (7.5g/l $NaNO_3$)	0.308 day^{-1}	2.2 hours
Nil	0.198 day^{-1}	3.5 hours

The above growth curve shows that the growth of *Chlorella sp*. is highest when the media provides full nitrogen source to the microalgal cells. The same was also reported by Kalpesh *et al.* 2012. They also suggest that nitrogen levels in the media is one of the growth limiting factors in algal cultivation. As the level of nitrogen was reduced, the algal biomass production also decreased, with the lowest being when no nitrogen source was given in the growth media. Additionally, the cells also take longer to adapt to the surrounding when there is nitrogen deprivation, which is seen from a comparatively longer lag phase.

4.2.4 OPEN PONDS FOR ALGAL BIOMASS GENERATION:

Our group set up an open pond to generate higher amounts of algal biomass for harvesting purposes. The pond was initially a pure culture of *Chlorella sp*. but as time moved on and the media was exhausted, we observed the growth of diatoms and fillamentous algae in the pond, which indicated a cross species contamination. This possed a smiliar threat within the bioreactors located near by. Ghasemi.Y. *et al.* 2012 also reported high risk levels of contamination in open pond cultivation.

The major limitaton of harvesting algal biomass on a large scale is cross species contamination. Maintaining a pure isolated culture is a tricky job. We also observed that the closed bioreactors kept nearby also had cross species contamination and this caused a hindrance in studying the growth of microalgae and its various properties.

We suggest that the use of closed bioreactors is a better choice for studying microalage as it is easier to control such contamination problems in a bioreactor set up. The start up cost is high (Ghasemi.Y. *et al.* 2012), but the loss of resources can be avoided and chances of getting positive outcomes are increased.

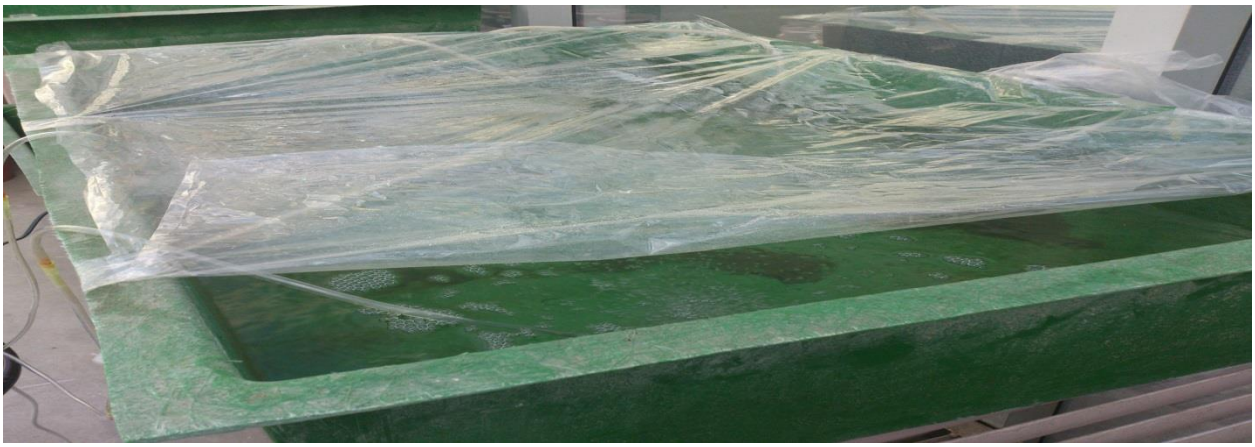


Fig. 17 Open Pond Cultivation of Algal biomass

4.2.5 THE % OF LIPIDS IN ALGAL BIOMASS:

The cultures of microalgae (*Chlorella sp.)* were grown in BG-11 growth medium under optimum conditions mentioned earlier with no stress given. Under optimal growth conditions, large amounts of algal biomass are produced but with relatively low lipid contents, about 5–20% of their dry cell weight (DCW). (Kalpesh *et al*. 2012, Ramasamy.S *et al.* 2011, Schuhmann. H *et al* 2011).

The algal biomass grown was harvested and lipids were extracted using Chloroform: methanol method (Bligh and Dyer,1959). The biomass showed the presence of 6% lipids, which is very low for biodiesel production. It is the presence of stress conditions which induces higher levels of lipid production. (Kalpesh *et al.* 2012)

4.2.6 LIPID INDUCTION IN *Chlorella Sp*. UNDER VARIOUS STRESS CONDITIONS AND QUALITATIVE ANALYSIS USING NILE RED:

The triacylglycerides (TAGs) serve the function of energy storage in microalgae. Extracted TAGs can be easily converted to biodiesel through transesterification (Chishti.Y 2008, Fukuda.H *et al.* 2001). When unfavourable or stress conditions arise, many microalgae alter their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids (20–50% DCW), mainly in the form of TAG, enabling microalgae to withstand these hostile conditions (Schuhmann. H *et al.* 2011)

Various stress conditions were applied to the *Chlorella sp*. to induce lipid production.

1. The microalgae were grown under autotrophic condition, i.e. optimum conditons with no stress applied.. These microalgal cells served as a control for our experiment. Kalpesh *et al*. 2012 reported highest biomass productivity when full nitrogen source is given while the levels of lipid production is low.
2. Heterotrophic conditons were applied, i.e. dextrose was given as a carbon source and no sunlight was provided. Heterotrophic cultivation allows higher algal growth rate and enables microalgae to accumulate higher biomass and amounts of lipid using less time in the absence of light, which is critical for reducing the microalgal biomass production cost (Cheirsilp.B *et al.* 2012). However, only a few microalgae species adjust to heterotrophic cultivation, and most of them belong to the genus *Chlorella*. According to a study conducted by Isleten-Hosoglu.M *et al*. 2012, heterotrophic *Chlorella* cells were rich in

lipid compunds whereas autotrophic cells had accumulated little lipid in the entire cultivation process.

3. The next condition given was mixtrophic environment. Dextrose was given as a carbon source and sunlight was also provided. According to Yanna.L *et al.* 2009, *Chlorella vulgaris* strains are mixotrophic and have highest lipid productivity compared to autotrophic or heterotrophic conditions.
4. Peptone was added as a stress condition to the microalgal cells. Along with $NaNO_3$, which is a component of BG-11 media as a nitrogen source, peptone was given as an additional nitrogen source.

   In a study by Wan.MX *et al.* 2012 on *Scenedesmus strain*, the maximum total lipid content of 44.8% occurred when peptone was utilized as the nitrogen source, but the biomass concentration and specific growth rate had decreased. It might be due to that inefficient utilization of peptone could result in the N-starvation of algal cells which induced higher total lipid content of algae.
5. Nitrogen is the most critical nutrient affecting lipid metabolism in microalgae. Hu et al. 2006 conducted a study on nitrogen stress responses of several green microalgae and all tested species showed a significant rise in lipid production. Hence, nitrogen starvation is the most successful lipid inducing technique at present (Kalpesh *et al.* 2012). However, high lipid production due to nitrogen stress may take 2–5 days and is accompanied with slow growth rates and low cell counts and thus finally effecting the total biomass and lipid productivity as discussed by Widjaja *et al.* 2009. We reduced the level of $NaNO_3$ in the media to 0.6g/L as opposed to 15g/L (full nitrogen source in BG-11 media).

By studying the above stress conditions in *Chlorella sp.*, we compared and thus determined the most appropriate and best method for enhancing lipid metabolism, keeping in mind the importance of high biomass productivity. The entire cultivation process was carried on for 16 days. Samples were collected everyday and absorbance (OD) was measured at 680nm to check for biomass concentration. To compare the biomass concentration for all the conditions, graph of biomass concentration (g/L) vs. number of days was plotted using MatLab.

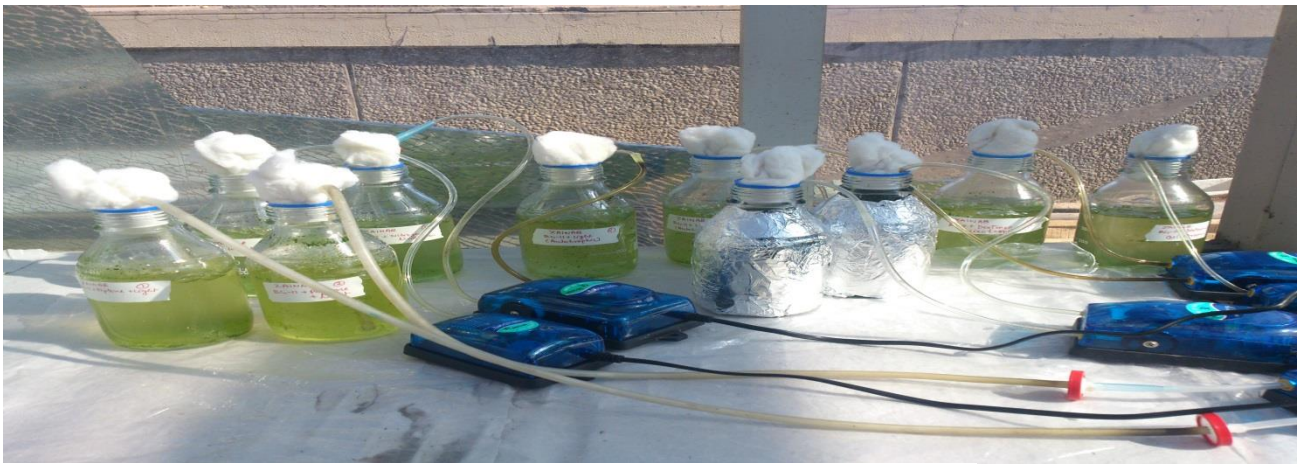


Fig. 18 Experimental setup for study of lipid induction in *Chlorella sp.*

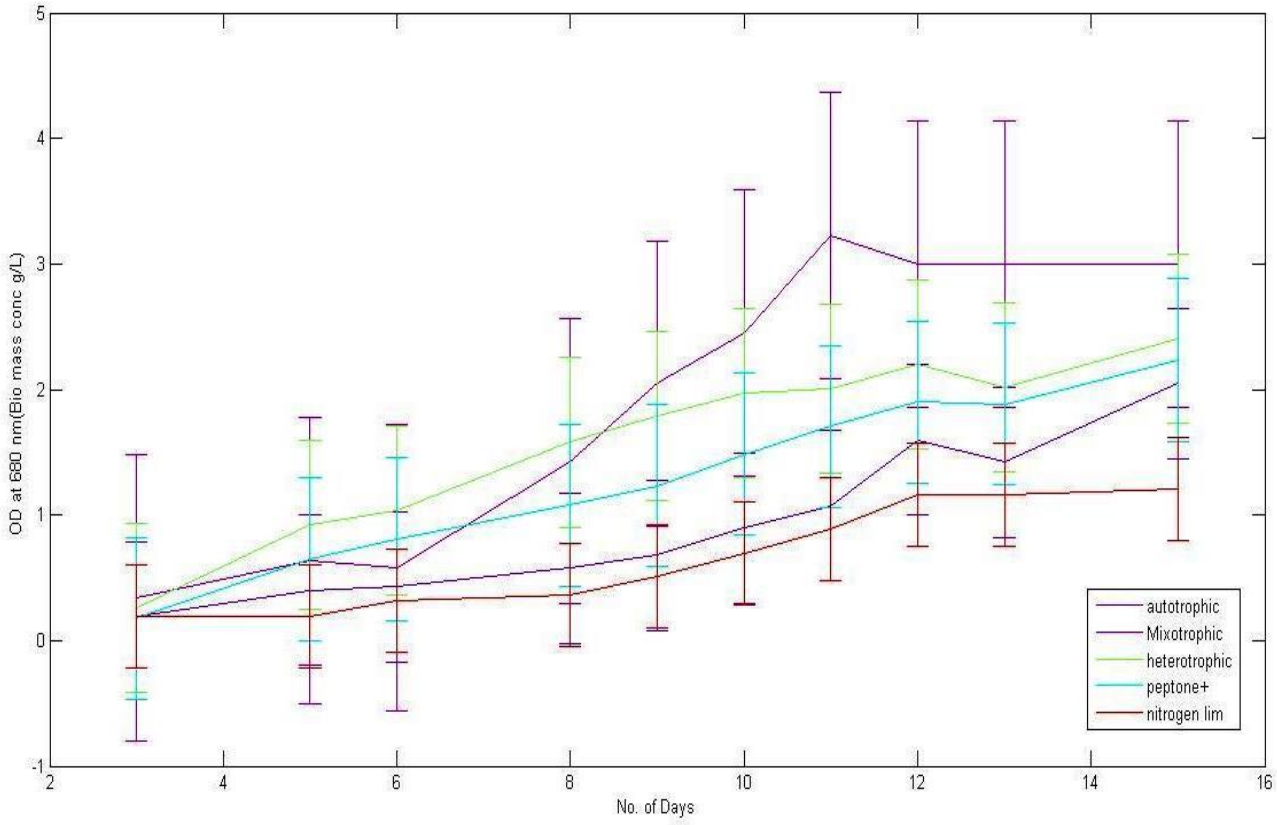


Fig. 19 Graph between biomass concentration (g/L) and number of days for different stress conditions.

The above figure suggests that the biomass production is highest for mixotrophic conditions and the lowest for nitrogen depleted conditions. Cultivating microalgae under mixotrophic, heterotrophic and additional nitrogen source (peptone) conditions, all increase the biomass productivity when compared to the optimum conditions (autotrophic mode). The findings are in agreement with Cheirsilp.B *et al.* 2012 and Kalpesh *et al.* 2012,who reported that heterotrophic conditions increase the biomass growth and nitrogen depletion lower the biomass productivity, respectively. But the work done by Wan.MX *et al.* 2012 suggested that providing peptone as the nitrogen source lowers the growth rate in *Scenedesmus sp*. whereas the graph indicates an increase in the biomass concentrartion when peptone was added as an additional nitrogen source for *Chlorella sp.*

The increasing order of biomass productivity for different stress conditions is- nitrogen limitation, autotrophic conditions, peptone addition, heterotrophic conditions and highest for mixotrophic conditions.

To detemine the levels of lipid production in the microalgal cells qualitatively, nile red dye was used and the cells were viewed under a Flourescence Microscope (Olympus, Tokyo) and the software used was Image Pro Plus (Elumalai, S., *et al* 2011)

Nile red is a lipid-soluble fluorescent dye and has been used in algal biodiesel production to detect the lipid content of many microalgal strains (Ren *et al*.2013). Also, some researchers further alter the Nile red fluorescence technique to develop the lipid staining efficiency and obtain satisfactory results. However, until now, the information about using the modified Nile red method for selection of lipid-rich microalgal species or strains is still inadequate. (Ren *et al*.2013).

All the samples were screened for the lipid bodies formation using this technique.

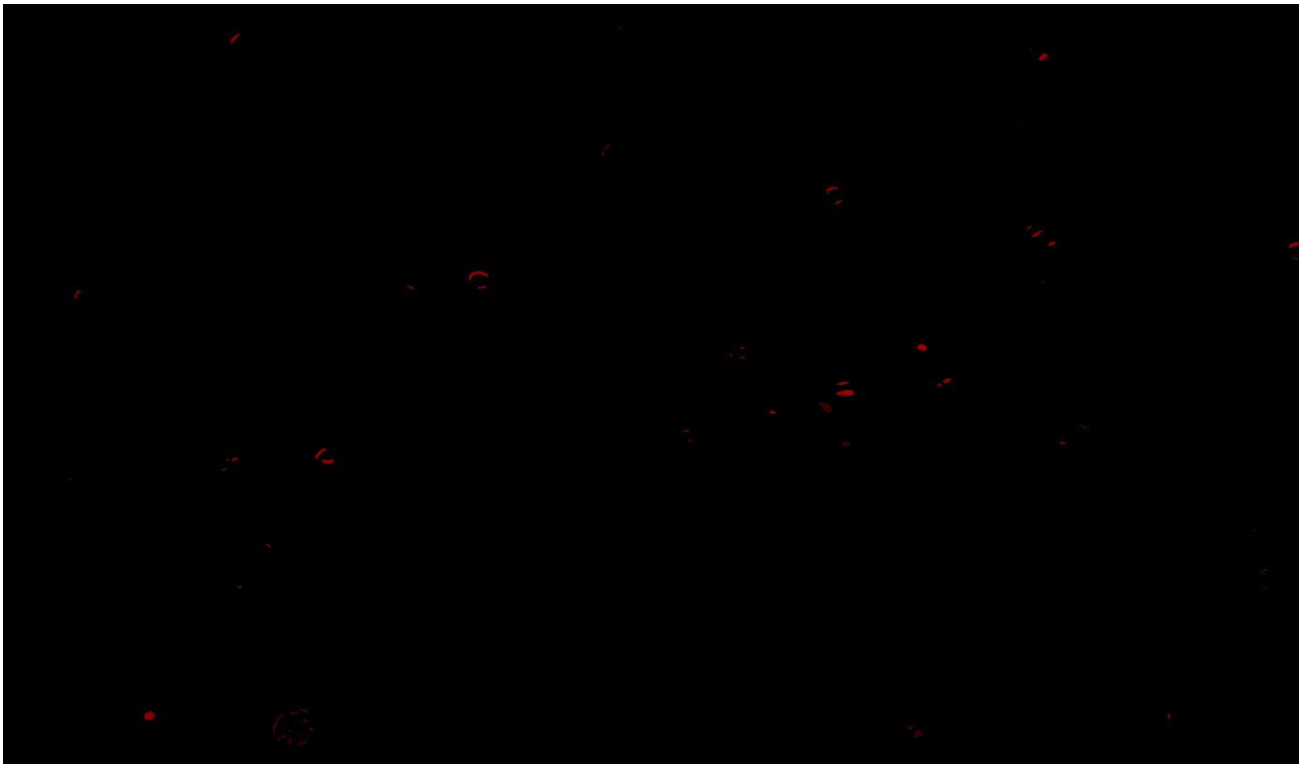


Fig.20 Microscopic view of microalgae cells grown under autotrophic conditions (control) showing lipid bodies emitting red fluorescence.

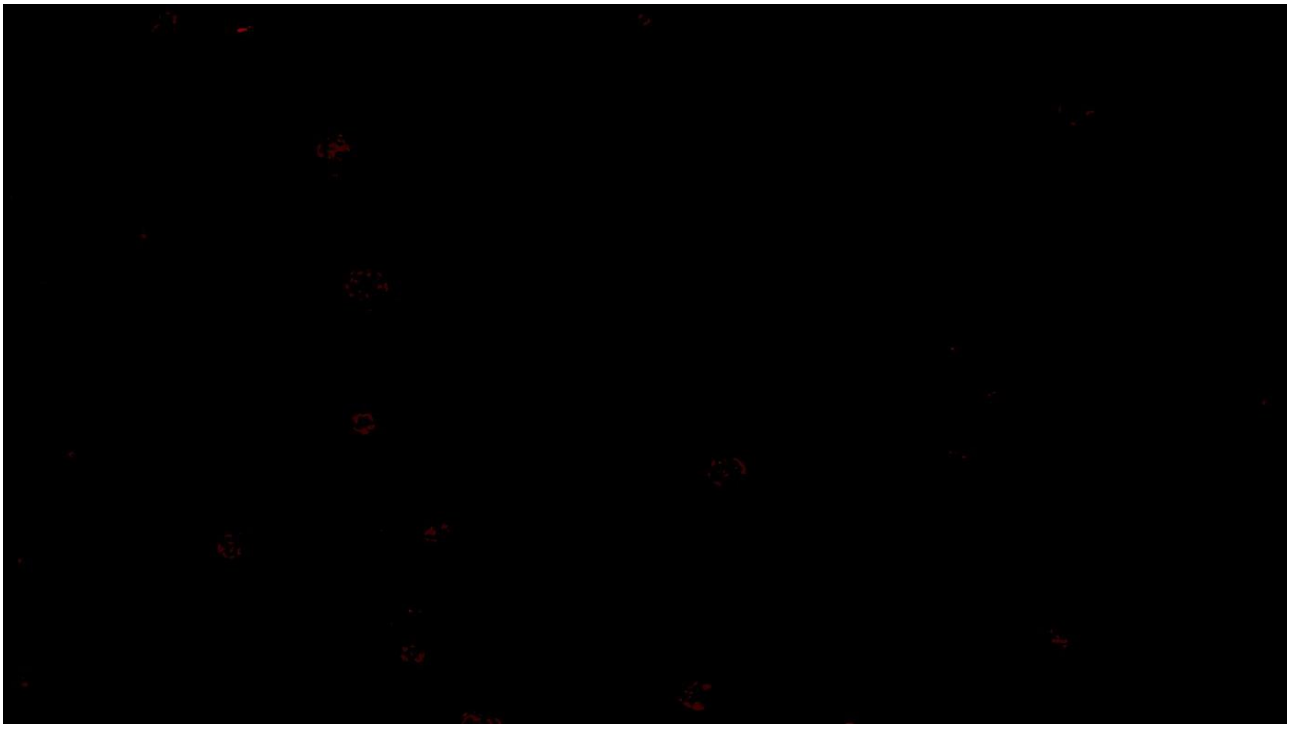


Fig.21 Microscopic view of microalgae cells grown under Heterotrophic conditions showing lipid bodies emitting red fluorescence.

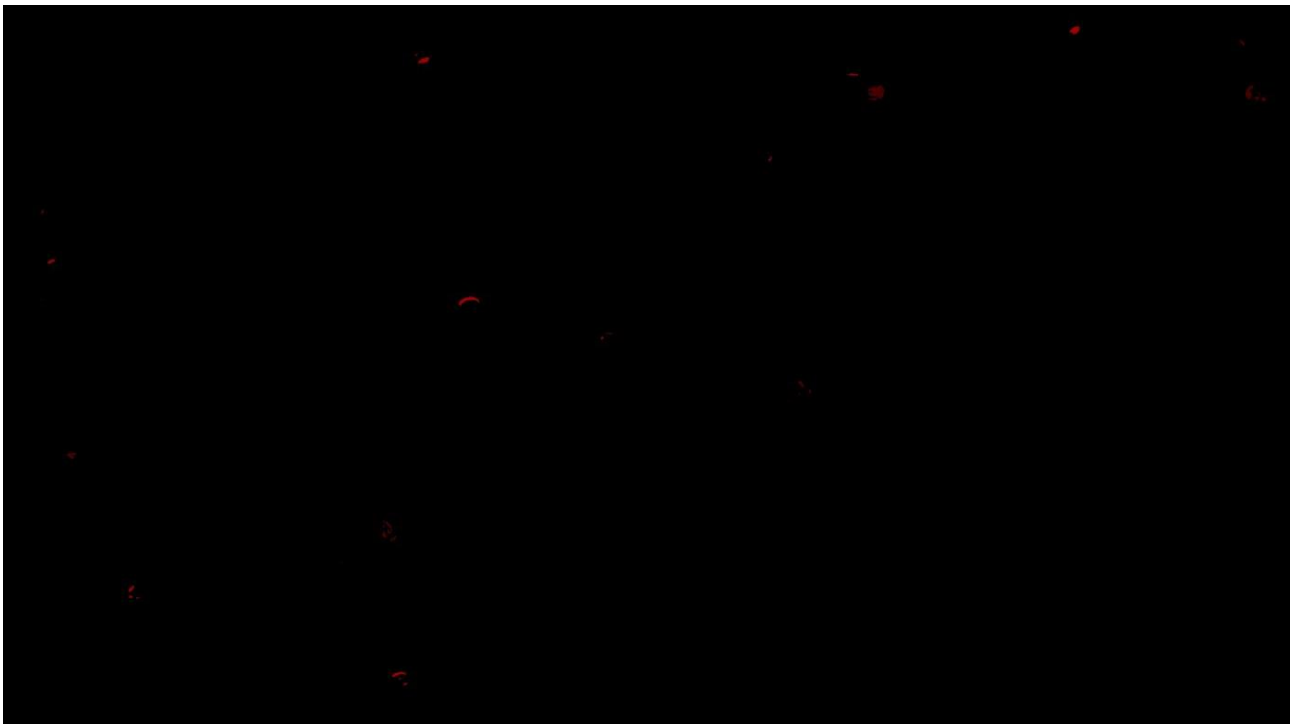


Fig.22 Microscopic view of microalgae cells grown with peptone addition showing lipid bodies emitting red fluorescence.

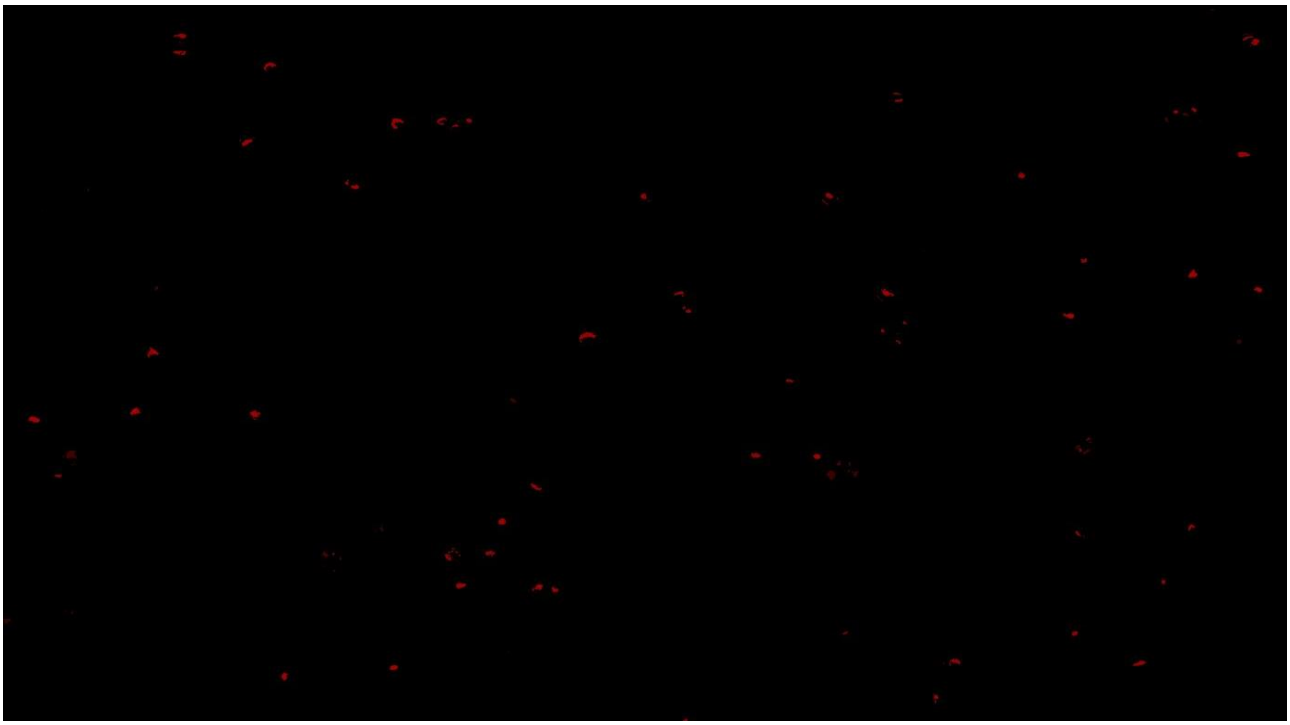


Fig.23 Microscopic view of microalgae cells grown under nitrogen deprived conditions showing lipid bodies emitting red fluorescence

By studying the flourescence emitting bodies in the above photos, we can qualitatively analyse the differnce in lipid body production in algal cells grown under various stress conditions. In agreement with Kalpesh *et al*. 2012, it can be seen that limiting the nitrogen source for algal cells tremendously increases the lipid metabolism of the cells. Comparing with the cells grown under autrotrophic conditions (Fig.19), the lipid production decreased when cells were given dextrose as the carbon source with no sunlight (heterotrophic) (Fig.20) and also when addition nitrogen source was given (peptone) (Fig.21). This is in contrast to the findings of Isleten-Hosoglu.M *et al*. 2012. Making peptone the nitrogen source for *Scenedesmus sp*. increased their lipid production (Wan.MX *et al.* 2012) whereas for *Chlorella sp.,* the addition of peptone did not increase lipid induction.

4.2.7 CONCLUSION:

To use *Chlorella* species as an efficient feedstock for third generation biofuels, we can first increase the biomass productivity by providing mixotrophic conditions, then to induce lipid production in the micro algal cells; we can shift to nitrogen deprived condition. Hence, the cultivation process can be a two stage process, in the first stage, dextrose or glucose can be provided as a carbon source to increase the biomass concentration and in the second stage, when appropriate biomass levels are achieved, the addition of dextrose can be stopped and media containing limited nitrogen source can be supplied for high levels of lipid productivity.

CHAPTER 5

CONCLUSION

The feed stocks required for second generation biofuels are mostly less expensive biomass such as forest, agricultural, animal or municipal waste, in terms of acquiring these feed stocks. Quite a lot of techniques are being studied around the world for the production of second-generation biofuels. Second generation biofuels overcome the limitations of first generation biofuels, mainly the food vs. fuel dilemma. But most of the feedstocks like lignocellulosic biomass, waste require pre-treatment step which can be hazardous to the environment. The use of large amounts of methanol and acid for pretreatment of waste oil can also increase cost inputs. But nevertheless, the use of waste oil is a potential method for biofuel production as it is a renewable source of energy and the problem associated with disposal of waste oil can be handles. The major difference between the second and third-generations is the feedstock. Algae are known to produce biomass quicker and on reduced land surface as compared with lignocellulosic biomass. But, production of algal biomass presents technical challenges such as lipid extraction and dewatering, as well as geographical trials in areas like Canada where temperature are below freezing for long durations (Lee and Lavoie, 2013)

The future of biofuels may not depend on only on one generation, but may be a combination of the three generations to survive with increased worldwide demand which is due to the decrease in the existing fuel resources.

CHAPTER 6

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