2016

Comparative study on Bio-diesel Content in Micro-algal Biomass

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University of Rajshahi

M.Phil

Thesis

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SUBMITTED BY

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Roll No.: Shaheed Suhrawardy hall No.11702

Registration No.: 01881

M.Phil Session: 2011-2012

Phycology & Limnology Laboratory
Department of Botany
Rajshahi University
Rajshahi-6205, Bangladesh.

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A Dissertation
Submitted in partial fulfillment of the requirements for the Degree of
Master of Philosophy in Botany,
University of Rajshahi

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“Dedicated to my beloved parents”

DECLARATION

I do have by declare that the entire work submitted as a thesis towards the fulfillment of the degree of Master of Philosophy in Botany of the University of Rajshahi, Bangladesh is the result of my own investigation.

I further declare that the work embodied in this thesis has not already been submitted in substance for any degree and has not been co-currently submitted as a candidature for any degree.

Candidate

Md. Abdul Qayum Sarker

CERTIFICATE

This is to certify that the research work entitled “Comparative study on Bio-diesel Content in Micro-algal Biomass” presented by Md. Abdul Qayum Sakrer, as a thesis towards the fulfillment of the degree of Master of Philosophy in Botany of the University of Rajshahi, Bangladesh, is the result of his own investigation.

It is approved for submission as a M. Phil thesis as to its style and content.

Supervisor

Professor Dr. Sabrina Naz
Department of Botany
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ACKNOWLEDGEMENT

I an very grateful to almighty Allah because Allah has given me the opportunity to complete this research.

The author expresses his deepest sense of gratitude and indebtedness to Professor Dr. Sabrin Naz, Department of Botany, University of Rajshahi, under whose constant guidance thankful to her for her valuable suggestion, Constrictive criticism and company as supervisor during the research work and the course of study.

The author also expresses his deep sense of gratitude and indebtedness to tae chairman, Professor Dr. Rafiul Islam, Department of Botany, University of Rajshahi for kindly providing necessary laboratory facilities to carryout this research work.

The Author: Md. Abdul Qayum Sarker.

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ABBREVIATIONS

μ	:	Micron
et al.	:	et alli and the rest
etc.	:	et cetra and the rest
DW	:	Distilled water
SDW	:	Sterilized distilled water
Fig.	:	Figure
g	:	Gram
i.e.	:	id est-which to say in other words
mg	:	Milligram
mg/l	:	Milligram/liter
min (s)	:	Minutes (s)
ml	:	Milliliter
mm	:	Millimeter
No.	:	Number
pH	:	Negative logarithm of hydrogen
S.N.	:	Serial number
sp.	:	Species
viz.	:	Namely
%	:	Percentage

ABSTRACT

Biodiesel content of the six selected algal strains was highest in *Chlorococcum* sp. (44.59%) followed by *Closterium* sp. (42%), *Oedogonium* sp.(40.82%), *Oscillatoria* sp.(37.52%), *Pithophora* sp.(37.5%) and *Cladophora* sp. (21.66%).

Biomass (after oil extraction) was maximum (46g) in *Cladophora* sp. and minimum (26g) in *Oscillatoria* sp.. Sediment (glycerine, water and pigments) was higher in *Pithophora* sp. (25.92g) and lower in *Oedogonium* sp. (8.17g). The pH range of bio-diesel and byproducts of the selected strains is 7.0-7.4 and 10.7-12, respectively; whereas the Optical Density (OD) range of bio-diesel and byproduct were 0.8-0.95 and 0.8-1, accordingly.

In vitro culture of six different algal strains (viz. *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp.) were established in three different algal media (viz BBM, Fogg's medium and Chu medium). Among them, *Chlorococcum* sp., *Closterium* sp. *and Pithophora* sp. exhibited optimum growth (μ= 0.04 d^{-1}) in all media. Net growth rate (μ= 0.03 d^{-1}) were same incase of the rest three strains in all mediums. Among three media biomass yield *of Cholorococcum* sp. was best in BBM (51.01g$\pm$0.01) followed by Fogg's medium (50.20g $\pm$0.01) and Chu medium (49.20g $\pm$ 0.02). Yield of rest of the five selected strains e.g. *Closterium* sp. (50.45$\pm$0.03), *Cladophora* sp. (29.97$\pm$0.02), *Oedogonium* sp.(31.75$\pm$0.03), *Oscillatoria* sp. (29.01$\pm$0.02) and *Pithophora* sp. (49.75$\pm$0.02) was also highest in BBM. Suitable pH in BBM for growth of all strains was found to be 6.5 while in Fogg's and Chu media the best performance were observed at pH 7.2.

As evident, *Chlorococcum* sp. contains highest biodiesel content (44.59%) and is highest biomass gaining and fast growing (biomass= 51.01g $\pm$0.01 and growth rate, μ= 0.04 d^{-1}) green algae commonly found in the northern region of Bangladesh may be recommended as a potential source of commercial bio-diesel production. Biodiesel content of *Closterium* sp. (42%), *Oedogonium* sp.(40.82%) indicate these two strains are also potential for biodiesel extraction.

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1: INTRODUCTION

Biomass is one of the better source of energy (Kulkarni and Dalai, 2006). Large-scale introduction of biomass energy could contribute to sustainable development in several fronts, environmentally, socially and economic (Turkenburg, 2000). Biodiesel (monoalkyl esters) is one of such alternative fuel, which is obtained by the transesterification of triglyceride oil with monohydric alcohols. It has been well-reported that biodiesel obtained from canola and soybean, palm, sunflower oil, algal oil as a diesel fuel substitute (Lang *et al*. 2002; Spolaore *et al*., 2006). Biodiesel is a nontoxic and biodegradable alternative fuel that is obtained from renewable sources. Among biomass, algae (macro and microalgae) usually have a higher photosynthetic efficiency than other biomass (Shay, 1993). Shay (1993) reported that algae were one of the best sources of biodiesel. In fact algae are the highest yielding feedstock for biodiesel. It can produce up to 250 times the amount of oil per acre as soybeans.

Microalgae are an organism capable of photosynthesis that is less than 2 mm in diameter. Macroalgae, like seaweed, is not as widely used in the production of biodiesel. Microalgae has much more oil than macroalgae and it is much faster and easier to grow (Shay, 1993).

Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass (Spolaore *et al*., 2006), biodiesel derived from microalgal oil (Thomas, 2006; Roessler *et al*., 1994; Banerjee *et al*., 2002) and photobiologically produced biohydrogen (Gavrilescu and Chisti, 2005, Fedorov *et al.*, 2005). The idea of using microalgae as a source of fuel is not new (Kapdan and Kargi, 2006; Chisti, 1980-1981) but it is now being taken seriously because of the escalating price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning fossil fuels (Sawayama *et al*., 1995).

Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives (Metting and Pyne, 1986; Schwartz, 1990; Kay, 1991; Shimizu, 1996; Borowitzka, 1999; Ghirardi *et al*., 2000; Akkerman *et al*., 2002; Banerjee *et al*., 2002; Melis, 2002; Lorenz and Cysewski, 2003; Metzger and Largeau, 2005; Singh *et al*., 2005; Walter *et al*., 2005; Spolaore *et al*., 2006) In addition, these photosynthetic microorganisms are useful in bioremediation applications (Mallick, 2002; Suresh and Ravishankar, 2004; Kalin *et al*., 2005; Munoz and Guieysse, 2006) and as nitrogen fixing biofertilizers (Vaishampayan *et al*., 2001). Biodiesel is produced currently from plant and animal oils, but not from microalgae. This is likely to change as several companies are attempting to commercialize microalgal biodiesel. Biodiesel is a proven fuel. Technology for producing and using biodiesel has been known for more than 50 years (Knothe *et al*., 1997; Fukuda *et al*., 2001; Barnwal and Sharma, 2005; Demirbas, 2005; Van, 2005; Felizardo *et al*., 2006; Kulkarni and Dalai, 2006; Meher *et al*., 2006). In the United States, biodiesel is produced mainly from soybeans. Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil (Felizardo *et al*., 2006; Kulkarni and Dalai, 2006), and jatropha oil (Barnwal and Sharma, 2005). Any future production of biodiesel from microalgae is expected to use the same process. Production of methyl esters, or biodiesel, from microalgal oil has been demonstrated (Belarbi *et al*., 2000) although the product was intended for pharmaceutical use.

Replacing all the transport fuel consumed in the United States with biodiesel will require 0.53 billion m^3 of biodiesel annually at the current rate of consumption. Oil crops, waste cooking oil and animal fat cannot realistically satisfy this demand. For example, meeting only half the existing U.S. transport fuel needs by biodiesel, would require unsustainably large cultivation areas for major oil crops. Using the average oil yield per hectare from various crops, the cropping area needed to meet 50% of the U.S. transport fuel needs is calculated in column 3 (Table 1). In column 4 (Table 1) this area is expressed as a percentage of the total cropping area of the United States. If oil palm, a

high-yielding oil crop can be grown, 24% of the total cropland will need to be devoted to its cultivation to meet only 50% of the transport fuel needs. Clearly, oil crops cannot significantly contribute to replacing petroleum derived liquid fuels in the foreseable future. This scenario changes dramatically, if microalgae are used to produce biodiesel. Between 1 and 3% of the total U.S. cropping area would be sufficient for producing algal biomass that satisfies 50% of the transport fuel needs (Table 1). The microalgal oil yields given in Table 1 are based on experimentally demonstrated biomass productivity in photobioreactors, as discussed later in this article. Actual biodiesel yield per hectare is about 80% of the yield of the parent crop oil given in Table 1.In view of Table 1, microalgae appear to be the only source of biodiesel that has the potential to completely replace fossil diesel. Unlike other oil crops, microalgae grow extremely rapidly and many are exceedingly rich in oil. Microalgae commonly double their biomass within 24 h. Biomass doubling times during exponential growth are commonly as short as 3.5 h. Oil content in microalgae can exceed 80% by weight of dry biomass (Metting,1996; Spolaore *et al*., 2006).

Table 1: Comparison of some sources of biodiesel (Chisti, 2007).

Crop	Oil yield (L/ha)	Land area needed (M ha) [a]	Percent of existing US cropping area [a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae [b]	136,900	2	1.1
Microalgae [c]	58,700	4.5	2.5

[Note: a For meeting 50% of all transport fuel needs of the United States.
b 70% oil (by wt) in biomass. c 30% oil (by wt) in biomass.]

Oil levels of 20–50% are quite common (Table 2). Oil productivity, that is the mass of oil produced per unit volume of the microalgal broth per day, depends on the algal growth rate and the oil content of the biomass. Microalgae with high oil productivities are desired for producing biodiesel.

Table 2: Oil content of some microalgae (Chisti, 2007).

Microalga	Oil content (% dry wt)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

Depending on species, microalgae produce many different kinds of lipids, hydrocarbons and other complex oils (Banerjee *et al*., 2002; Metzger and Largeau, 2005; Guschina and Harwood, 2006). Not all algal oils are satisfactory for making biodiesel, but suitable oils occur commonly. Using microalgae to produce biodiesel will not compromise production of food,

fodder and other products derived from crops. Potentially, instead of microalgae, oil producing heterotrophic microorganisms (Ratledge, 1993; Ratledge and Wynn, 2002) grown on a natural organic carbon source such as sugar, can be used to make biodiesel; however, heterotrophic production is not as efficient as using photosynthetic microalgae. This is because the renewable organic carbon sources required for growing heterotrophic microorganisms are produced ultimately by photosynthesis, usually in crop plants. Production of algal oils requires an ability to inexpensively produce large quantities of oil-rich microalgal biomass.

In Bangladesh (2012), total Consumption of fuel is 114 thousand barrel (ethanol and biodiesel is also included) (BP Statistical Review, 2013). The uses of diesel at various sector of Bangladesh given as bellow:

Electricity sector:

Different types of power plants generate electricity and synchronize it with the national grid. There are some isolated diesel power stations at remote places and islands which are not connected with the National Grid. Terminal voltage of different generators are 11 kV, 11.5 kV and 15.75 kV. In the Eastern Zone (eastern side of river Jamuna), electricity is generated from indigenous gas and a small percentage through hydro power (Energypedia, 2013).

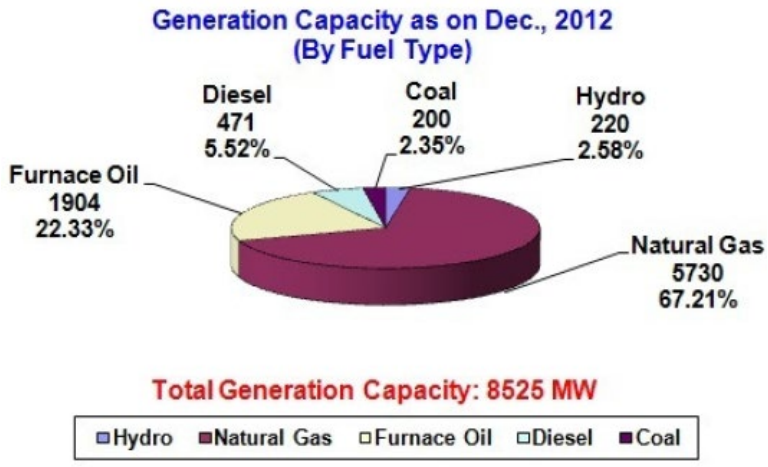

Fig. 1: Bangladesh installed capacity fuel in the production of electricity (http://www.bpdb.gov.bd).

The road sector:

The Road sector diesel fuel consumption (kilotons of oil equivalent) in Bangladesh was 1457 kilotons in 2009, according to a World Bank report, published in 2010. Diesel is heavy oils used as a fuel for internal combustion in diesel engines.

Agricultural sector:

Since, there is no data available for petroleum (diesel) consumption of the machinery used in agriculture, the total diesel energy input to agriculture was calculated from the petroleum consumed by tractors and power tillers. From field investigations, it is found that a 70-hp tractor consumes 8 L diesels per hour and its average use on the field is 1140 hr per year. On the other hand, a 10-hp power tiller consumes 1.75 L diesel per hour with an 80% loading capacity and its average use on the field is 720 hr per year (Ozkan *et al*., 2004). Deep tube-well, shallow tube-well and low lift pump are operated by electricity and diesel. Data on electricity and diesel, used in irrigation were collected from field investigation. It is found that for irrigation, a deep tube-well consumes 1388 KWh electricity per hectare, shallow tube-well and low lift pump consume 266.4 L diesels per hector. Chemical energy input data on individual fertilizer materials (nitrogen, phosphorus, potash and zinc); manure and pesticides (insecticide, herbicide and fungicide) were used on the basis of practices (Ozkan *et al*., 2004).

Taking into considerations the above mentioned crucial facts it is an urgent need to explore the mechanism of transesterification, amount of ester (biodiesel production) and physical properties (yield of ester or biodiesel, glycerine and sediments) of biodiesel from native microalgal strains.

OBJECTIVE OF THE RESEARCH:

This research has been undertaken with the following objectives:

1. Identifying the suitable algal strains growing in fresh water habitats of Rajshahi for biodiesel extraction.
2. Bio-diesel extraction from locally available different algal strains.
3. Qualitative and quantitative characterization of extracted bio-diesel.

2: REVIEW OF LITERATURE

Sustainable production of renewable energy is being hotly debated globally since it is increasingly understood that first generation biofuels, primarily produced from food crops and mostly oil seeds are limited in their ability to achieve targets for biofuel production, climate change mitigation and economic growth. These concerns have increased the interest in developing second generation biofuels produced from non-food feedstocks such as microalgae, which potentially offer greatest opportunities in the longer term. This paper reviews the current status of microalgae use for biodiesel production, including their cultivation, harvesting, and processing. The microalgae species most used for biodiesel production are presented and their main advantages described in comparison with other available biodiesel feedstocks. The various aspects associated with the design of microalgae production units are described, giving an overview of the current state of development of algae cultivation systems (photo-bioreactors and open ponds). Other potential applications and products from microalgae are also presented such as for biological sequestration of CO_2, wastewater treatment, in human health, as food additive, and for aquaculture (Teresa *et al.*, 2010).

The transportation and energy sectors are the major anthropogenic sources, responsible in European Union (EU) for more than 20% and 60% of greenhouse gas (GHG) emissions, respectively (EEA, 2004). Agriculture is the third largest anthropogenic source, representing about 9% of GHG emissions, where the most important gases are nitrous oxide (N_2O) and methane (CH_4) (EEA, 2007). It is expected that with the development of new growing economies, such as India and China, the global consumption of energy will raise and lead to more environmental damage (International Energy Agency, 2007). GHG contributes not only to global warming (GW) but also to other impacts on the environment and human life. Oceans absorb approximately one-third of the CO_2 emitted each year by human activities and as its levels increase in the atmosphere, the amount dissolved in oceans will also increase turning the water pH gradually to more acidic. This pH decrease

may cause the quick loss of coral reefs and of marine ecosystem biodiversity with huge implications in ocean life and consequently in earth life (Ormerod *et al*., 2002). As Global Warming is a problem affecting different aspects of human life and the global environment, not only a single but a host of solutions is needed to address it. One side of the problem concerns the reduction of crude oil reserves and difficulties in their extraction and processing, leading to an increase of its cost (Laherrere, 2005). This situation is particularly acute in the transportation sector, where currently there are no relevant alternatives to fossil fuels. To find clean and renewable energy sources ranks as one of the most challenging problems facing mankind in the medium to long term. The associated issues are intimately connected with economic development and prosperity, quality of life, global stability, and require from all stakeholders tough decisions and long term strategies. For example, many countries and regions around the world established targets for CO_2 reduction in order to meet the sustainability goals agreed under the Kyoto Protocol. Presently many options are being studied and implemented in practice, with different degrees of success, and in different phases of study and implementation. Examples include solar energy, either thermal or photovoltaic, hydroelectric, geothermal, wind, biofuels, and carbon sequestration, among others (Dewulf and Van, 2006; Gilbert and Perl, 2008). Each one has its own advantages and problems and, depending on the area of application, different options will be better suited. One important goal is to take measures for transportation emissions reduction, such as the gradual replacement of fossil fuels by renewable energy sources, where biofuels are seen as real contributors to reach those goals, particularly in the short term.

Biofuels production is expected to offer new opportunities to diversify income and fuel supply sources, to promote employment in rural areas, to develop long term replacement of fossil fuels, and to reduce GHG emissions, boosting the decarbonisation of transportation fuels and increasing the security of energy supply. The most common biofuels are biodiesel and bio-ethanol, which can replace diesel and gasoline, respectively, in today cars with little or none modifications of vehicle engines. They are mainly produced from

biomass or renewable energy sources and contribute to lower combustion emissions than fossil fuels per equivalent power output. They can be produced using existing technologies and be distributed through the available distribution system. For this reason biofuels are currently pursued as a fuel alternative that can be easily applied until other options harder to implement, such as hydrogen, are available.

Although biofuels are still more expensive than fossil fuels their production is increasing in countries around the world. Encouraged by policy measures and biofuels targets for transport, its global production is estimated to be over 35 billion liters (COM, 2006). The main alternative to diesel fuel in EU is biodiesel, representing 82% of total biofuels production (Bozbas, 2008) and is still growing in Europe, Brazil, and United States, based on political and economic objectives. Biodiesel is produced from vegetable oils (edible or non-edible) or animal fats. Since vegetable oils may also be used for human consumption, it can lead to an increase in price of food-grade oils, causing the cost of biodiesel to increase and preventing its usage, even if it has advantages comparing with diesel fuel. The potential market for biodiesel far surpasses the availability of plant oils not designated for other markets. For example, to fulfill a 10% target in EU from domestic production, the actual feedstocks supply is not enough to meet the current demand and the land requirements for biofuels production, would be more than the potential available arable land for bio-energy crops (Scarlat *et al*., 2008). The extensive plantation and pressure for land use change and increase of cultivated fields may lead to land competition and biodiversity loss, due to the cutting of existing forests and the utilization of ecological importance areas (Renewable Fuel Agency, 2008). Biodiesel may also be disadvantageous when replacing crops used for human consumption or if its feedstocks are cultivated in forests and other critical habitats with associated biological diversity. Current policies at regional and national levels and the expected cost and difficulties in obtaining fossil fuels will necessarily lead to an increase in biodiesel production and of other types of renewable energy. To become a more viable alternative fuel and to survive in the market, biodiesel must compete

economically with diesel. The end cost of biodiesel mainly depends on the price of the feedstocks that accounts for 60–75% of the total cost of biodiesel fuel (Canakci and Sanli, 2008). In order to not compete with edible vegetable oils, the low-cost and profitable biodiesel should be produced from low-cost feedstocks such as non-edible oils, used frying oils, animal fats, soap-stocks, and greases. However the available quantities of waste oils and animal fats are not enough to match the today demands for biodiesel. Thus transition to second generation biofuels, such as microalgae, can also contribute to a reduction in land requirements due to their presumed higher energy yields per hectare as well as to their non-requirement of agricultural land.

Additionally, biodiesel needs to have lower environmental impacts and ensure the same level of performance of existing fuels (Reinhardt *et al*., 2008). Albeit the growing interest and fast growth of this area, it is still on its infancy. A large investment in research and development (R&D) and correct policies and strategies are still needed, for all stages of the biofuels value chain, from raw materials production to delivery and final consumption. Among the various possibilities currently being investigated and implemented at pilot scale or even at industrial scale concerning potential feedstocks, the more interesting ones are microalgae. Besides their cultivation is not directly linked to human consumption, they have low space requirements for its production. This review focuses its attention on microalgae and how they can be used for biodiesel production. Questions associated with production and processing of microalgae are considered in detail, not only those directly related with biofuels production but also the possibilities of combining it with pollution control, in particular with biological sequestration of CO_2 emissions and other greenhouse gases, or wastewater treatment.

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure. Examples of prokaryotic microorganisms are Cyanobacteria (Cyanophyceae) and eukaryotic microalgae are for example

green algae (Chlorophyta) and diatoms (Bacillariophyta) (Li *et al*. 2008a, Li *et al*. 2008b). Microalgae are present in all existing earth ecosystems, not just aquatic but also terrestrial, representing a big variety of species living in a wide range of environmental conditions. It is estimated that more than 50,000 species exist, but only a limited number, of around 30,000 have been studied and analyzed (Richmond, 2004). During the past decades extensive collections of microalgae have been created by researchers in different countries. An example is the freshwater microalgae collection of University of Coimbra (Portugal) considered one of the world largest, having more than 4000 strains and 1000 species. This collection attests to the large variety of different microalgae available to be selected for use in a broad diversity of applications, such as value added products for pharmaceutical purposes, food crops for human consumption and as energy source. A bit all over the world, other algae collections attest for the interest that algae have risen, for many different production purposes. For example, the collection of the Goettingen University, Germany (SAG), that started in the early 1920s and has about 2213 strains and 1273 species. About 77% of all the strains in the SAG collection are green algae and about 8% cyanobacteria (61 genera and 230 strains). Some of them are freshwater red algae and others from saline environments. The University of Texas Algal Culture Collection is another very well known collection of algae cultures that was founded in 1953. It includes 2300 different strains of freshwater algae (edaphic green algae and cyanobacteria), but includes representatives of most major algal taxa, including many marine macrophytic green and red algae species. In the Asian continent, the National Institute for Environmental Studies Collection (NIES), in Ibaraki, Japan, holds a collection of about 2150 strains, with around 700 species of different algae. The CSIRO Collection of Living Microalgae (CCLM), in Australia, holds about 800 strains of different algae, including representatives from the majority of classes of marine and some freshwater microalgae, being the majority of the strains isolated from Australian waters.

Many research reports and articles described many advantages of using microalgae for biodiesel production in comparison with other available feedstocks (Li *et al*. 2008a, Li *et al*. 2008b, Sheehan *et al*., 1998; Chisti, 2007; Hossain *et al*. 2008; Hu *et al*., 2008; Rodolfi *et al*., 2009; Rosenberg *et al*., 2008; Schenk *et al*., 2008; Tsukahara and Sawayama, 2005). From a practical point of view, they are easy to cultivate, can grow with little or even no attention, using water unsuitable for human consumption and easy to obtain nutrients. Microalgae reproduce themselves using photosynthesis to convert sun energy into chemical energy, completing an entire growth cycle every few days (Sheehan *et al*., 1998). Moreover they can grow almost anywhere, requiring sunlight and some simple nutrients, although the growth rates can be accelerated by the addition of specific nutrients and sufficient aeration (Aslan and Kapdan, 2006; Pratoomyot *et al*., 2005; Renaud *et al*., 1999). Different microalgae species can be adapted to live in a variety of environmental conditions. Thus, it is possible to find species best suited to local environments or specific growth characteristics, which is not possible to do with other current biodiesel feedstocks (e.g. soybean, rapeseed, sunflower and palm oil). They have much higher growth rates and productivity when compared to conventional forestry, agricultural crops, and other aquatic plants, requiring much less land area than other biodiesel feedstocks of agricultural origin, up to 49 or 132 times less when compared to rapeseed or soybean crops, for a 30% (w/w) of oil content in algae biomass (Chisti, 2007). Therefore, the competition for arable soil with other crops, in particular for human consumption, is greatly reduced.

Microalgae can provide feedstock for several different types of renewable fuels such as biodiesel, methane, hydrogen, ethanol, among others. Algae biodiesel contains no sulfur and performs like petroleum diesel, while reducing emissions of particulate matter, CO_2, hydrocarbons, and SOx. However emissions of NOx may be higher in some engine types (Delucchi, 2003). The utilization of microalgae for biofuels production can also serve other purposes. Some possibilities currently being considered are listed below:

_ Removal of CO_2 from industrial flue gases by algae bio-fixation (Wang *et al*., 2008), reducing the GHG emissions of a company or process while producing biodiesel (Directive 2003/30/EC).

_ Wastewater treatment by removal of NH_4+, NO_{3-}, PO_{4-}, making algae to grow using these water contaminants as nutrients (Wang *et al*., 2008).

_ After oil extraction the resulting algae biomass can be processed into ethanol, methane, livestock feed, used as organic fertilizer due to its high N:P ratio, or simply burned for energy cogeneration (electricity and heat) (Wang *et al*., 2008);

_ Combined with their ability to grow under harsher conditions, and their reduced needs for nutrients, they can be grown in areas unsuitable for agricultural purposes independently of the seasonal weather changes, thus not competing for arable land use, and can use wastewaters as the culture medium, not requiring the use of clean water.

_ Depending on the microalgae species other compounds may also be extracted, with valuable applications in different industrial sectors, including a large range of fine chemicals and bulk products, such as fats, polyunsaturated fatty acids, oil, natural dyes, sugars, pigments, antioxidants, high-value bioactive compounds, and other fine chemicals and biomass (Li *et al*. 2008a, Li *et al*. 2008b; Raja *et al*., 2008).

_ Because of this variety of high-value biological derivatives, with many possible commercial applications, microalgae can potentially revolutionize a large number of biotechnology areas including biofuels, cosmetics, pharmaceuticals, nutrition and food additives, aquaculture, and pollution prevention (Rosenberg *et al*. 2008; Raja *et al*., 2008).

For the past 50 years, extensive research has been performed on microalgae and how they can be used in a wide variety of processes or to manufacture many practical and economic important products. The first large-scale culture of microalgae started in the early 1960s in Japan by Nihon *Chlorella* with the culture of *Chlorella* (Spolaore *et al*., 2006). The interest in using microalgae

for renewable energy increased in 1970s during the first oil crisis (Spolaore *et al*., 2006). The U.S. National Renewable Energy Laboratory (NREL) through the Aquatic Species Program (ASP), launched a specific R&D Program dedicated to alternative renewable fuels, including biodiesel from microalgae that lasted from 1978 to 1996. One of its main objectives was to study the biochemistry and physiology of lipid production in oleaginous microalgae. From 1987 to 1990, an ‘‘Outdoor Test Facility’’ of two 1000 m^2 high-rate ponds was operated in Roswell, New Mexico. It was concluded that the use of microalgae for the low-cost production of biodiesel was technically feasible, but still needs considerable long term R&D to achieve the high productivities required. Other objective of this NREL R&D program was to produce improved algae strains by looking for genetic variability between algal isolates, attempting to use flow cytometry to screen for naturally occurring high lipid individuals, and exploring algal viruses as potential genetic vectors. However in 1995 the Department of Energy reduced the budg*et al*located to funding this program and it was discontinued before these experiments could be carried out beyond the preliminary stages (Sheehan *et al*., 1998). The recent price volatility of crude oil and the expected future price increase, tied with the urge to reduce pollutant emissions and greenhouse gases, have created a new interest in the production of biodiesel using microalgae. For example, several companies were created or have entered this market niche, selling either entire processes or key process units, such as photo-bioreactors with optimized designs to cultivate microalgae for biodiesel production and other applications (Barclay , 2005; Behrens *et al*., 2007; Kanel and Guelcher, 1999). Torrey (2008) presents links to 37 companies that are currently exploring algae as a fuel source. Nowadays, microalgae are seen as an alternative feedstock for biodiesel production, being the target of a large number of consortiums, private and public organizations’ investments in R&D, aiming to use the most effective and cheap technology to produce large amounts of oil. They are considered to be a second generation feedstock together with other biomass sources, such as Jatropha, lignocellulosic materials, agricultural residues, and systematically grown energy crops, with

high potential yields of biofuels and that are not used as food source for human consumption. Though it is not cost effective yet to compete with fossil diesel without additional support (for example government subsidies) research is being done to turn it economically viable, both in academia and in industry (Kanel and Guelcher, 1999; Bijl *et al*., 2004; Yokochi *et al*., 2003). In a long term, as crude oil reserves diminish and price per barrel increases in a daily basis, other alternatives must become available, and thus, it is now the time to search, develop and implement them. Recent research efforts have concentrated on applying metabolic engineering and genetic methods to microalgae in order to develop organisms optimized for high productivity and energy value, in order to achieve their full processing capabilities (Rosenberg *et al*., 2008; Raja *et al*., 2008). Since microalgae represent a much simpler system than plants, usually with no cell differentiation, genetic manipulations to increase its content of higher value compounds is very tempting. Nevertheless, progress in the genetic engineering of algae was extremely slow until recently. Also, these promising advances should be viewed with caution because transgenic algae potentially pose a considerable threat to the ecosystem and thus will most likely be banned from outdoor cultivation systems (Pulz and Gross, 2004).

Table 3: Lipid content and productivities of different microalgae species (Teresa *et al*., 2010).

Marine and freshwater microalgae species	**Lipid content (%dry weight biomass)**	**Lipid productivity (mg/L/day)**	**Volumetric productivity of biomass (g/L/day)**	**A real productivity of biomass ($g/m^2/day$)**
Ankistrodesmus sp.	24.0–31.0	–	–	11.5–17.4
Botryococcus braunii	25.0–75.0	–	0.02	3.0
Chaetoceros muelleri	33.6	21.8	0.07	–
Chaetoceros calcitrans	14.6–16.4/39.8	17.6	0.04	–
Chlorella emersonii	25.0–63.0	10.3–50.0	0.036–0.041	0.91–0.97
Chlorella protothecoides	14.6–57.8	1214	2.00–7.70	–
Chlorella sorokiniana	19.0–22.0	44.7	0.23–1.47	–
Chlorella vulgaris	5.0–58.0	11.2–40.0	0.02–0.20	0.57–0.95
Chlorella sp.	10.0–48.0	42.1	0.02–2.5	1.61–16.47/25
Chlorella pyrenoidosa	2.0 – 2.90	–	3.64	72.5/130

Chlorella	18.0–57.0	18.7	–	3.50–13.90
Chlorococcum sp.	19.3	53.7	0.28	–
Crypthecodinium cohnii	20.0	–	51.1 – 10	–
Dunaliella salina	6.0–25.0	116.0	0.22–0.34	1.6–3.5/20–38
Dunaliella primolecta	23.1	–	0.09	14
Dunaliella tertiolecta	16.7	–	71.0 – 0.12	–
Dunaliella sp.	17.5–67.0	33.5	–	–
Ellipsoidion sp.	27.4	47.3	0.17	–
Euglena gracilis	14.0	–	20.0 – 7.70	–
Haematococcus pluvialis	25.0	–	0.05–0.06	10.2–36.4
Isochrysis galbana	7.0–40.0	–	0.32–1.60	–
Isochrysis sp.	7.1–33	37.8	0.08–0.17	–
Monodus subterraneus	16.0	30.4	0.19	–

Monallanthus salina	20.0	–	22.0 – 0.08	12
Nannochloris sp.	20.0–56.0	60.9–76.5	0.17–0.51	–
Nannochloropsis oculata.	22.7–29.7	84.0–142.0	0.37–0.48	–
Nannochloropsis sp.	12.0–53.0	37.6–90.0	0.17–1.43	1.9–5.3
Neochloris oleoabundans	29.0–65.0	90.0–134.0	–	–
Nitzschia sp.	16.0–47.0	8.8	–	21.6
Oocystis pusilla	10.5	–	–	40.6–45.8
Pavlova salina	30.9	49.4	0.16	–
Pavlova lutheri	35.5	40.2	0.14	–
Phaeodactylum tricornutum	18.0–57.0	44.8	0.003–1.9	2.4–21
Porphyridium cruentum	9.0–18.8/60.7	34.8	0.36–1.50	25
Scenedesmus obliquus	11.0–55.0	–	0.004–0.74	–
Scenedesmus quadricauda	1.9–18.4	35.1	0.19	–

Scenedesmus sp.	19.6–21.1	40.8–53.9	0.03–0.26	2.43–13.52
Skeletonema sp.	13.3–31.8	27.3	0.09	–
Skeletonema costatum	13.5–51.3	17.4	0.08	–
Spirulina platensis	4.0–16.6	–	0.06–4.3	1.5–14.5/24–51
Spirulina maxima	4.0–9.0	–	0.21–0.25	25
Thalassiosira pseudonana	20.6	17.4	0.08	–
Tetraselmis suecica	8.5–23.0	27.0–36.4	0.12–0.32	19
Tetraselmis sp.	12.6–14.7	43.4	0.30	–

Currently a lot of research effort is being focused on the algal cultivation unit, as in most cases it represents the key step that ultimately determines the economic viability of the process. According to Maxwell *et al*. (1985), for the implementation of an algae cultivation unit a site selection and resource evaluation have to be performed considering several criteria: (i) the water supply/demand, its salinity and chemistry; (ii) the land topography, geology, and ownership; (iii) the climatic conditions, temperature, insulation, evaporation, precipitation; (iv) the easy access to nutrients and carbon supply sources. One needs also to decide if the algal cultivation unit will be operating in batch or continuous mode and if the production units will be open or close systems. This depends on the microalgae species selected, the expected environmental conditions, availability of nutrients and even the possibility to combine the microalgae growth with a pollution control strategy of other industry, for example for the removal of CO_2 from flue gas emissions or the removal of nitrogen and phosphorus from a wastewater effluent.

Besides the equipment needed for microalgae growth, it is essential to pay close attention to the selection of the most appropiate species and strains, their cultivation conditions and nutrients available for their growth. In most cases the production of biodiesel will rely on already available species and strains that have shown to be adequate due to either their lipid content or productivity. Literature information (as the one summarized in Table 3) and obtained from companies supplying production units can also be used. However, in some cases this approach may not yield an adequate solution and thus a search for more convenient microalgae species has to be done. Typically sources of microalgae include existing collections of microalgae, commercially available either from Universities or other national and international foundations (such as the ones previously mentioned) or from companies specifically devoted to algae growth (Torrey, 2008), or water and soil samples obtained from diverse environments. As microalgae will have to live and thrive in a wide range of environmental conditions, especially of

nutrients scarcity and other adverse conditions, algae samples obtained in harsh environments such as thermal springs or industrial wastewaters can be used. This way it is ensured that if any viable option is found it will be robust and possibly better adapted to specific conditions. The sampling and selection process is well established, although it requires specialized equipment and may be time consuming (Richmond, 2004). A multicriteria strategy has to be considered in this process, considering factors such as follows:

_ Growing rate, normally measured by total amount of biomass accumulated per unit time and unit volume;

_ Lipid content, not total amount but the distribution of free fatty acids and triglycerides, a factor that may be relevant in biodiesel production;

_ Resistance to environmental conditions changes, in particular of temperature, nutrients input, light, competition from other microalgae species and/or bacterial;

_ Nutrients availability, in particular of carbon dioxide sources when the goal of carbon sequestration is also deemed relevant;

_ Ease of biomass separation and processing;

_ Possibility of obtaining other valuable chemicals.

The listed criteria consider not only the microalgae themselves but also their cultivation units. Thus, all experiments should mimic as much as possible the real conditions where the microorganisms will be used and include the biomass processing stages before the production of biodiesel itself. Even when the species listed in literature or obtained from the environment are not adequate, the utilization of genetic engineering may be a solution (Gressel, 2008). This way it is possible to adjust the characteristics of microalgae to the process and desired product(s) and to improve the productivity and lipid yield and composition. However, the fears of biological contamination, restrictive legislation and viable natural options still hinder the broader utilization of

genetically engineered organisms. Although probably lengthy in time and cost intensive, obtaining an organism that better suits the specific operating conditions has some advantages. Firstly, it frees the company that produces biodiesel from microalgae to be dependent from suppliers, and generates in house expertise that can be advantageous in future. Secondly, the identification of new species or the development of new strains represents a business opportunity and source of income from the possible royalties resulting from its intellectual property.

Therefore, it is crucial to understand how to select the right algae species, create an optimal photo-biological formula for each species, and build a cost-effective cultivation unit that can precisely deliver the formula to each individual algae cell, no matter the size of the facility, or its geographical location.

Microalgae are adapted to scavenge their environments for resources, to storage them, or increase their efficiency in resource utilization. In general for biomass growth (consisting of 40–50% carbon) microalgae depend on a sufficient supply of a carbon source and light to carry out photosynthesis (Moheimani, 2005; Kaewpintong, 2004). Yet they can adjust or change their internal structure (e.g. biochemical and physiological acclimation), whilst externally they can excrete a variety of compounds to amongst others, render nutrients available or limit the growth of competitors (Richmond, 2004).

Microalgae may assume many types of metabolisms (e.g. autotrophic, heterotrophic, mixotrophic, photoheterotrophic) and are capable of a metabolic shift as a response to changes in the environmental conditions. For example some organisms can grow (Gressel, 2008):

_ Photoautotrophically, i.e. using light as a sole energy source that is converted to chemical energy through photosynthetic reactions.

_ Heterotrophically, i.e. utilizing only organic compounds as carbon and energy source.

_ Mixotrophically, i.e. performing photosynthesis as the main energy source, though both organic compounds and CO_2 are essential. Amphitrophy, subtype of mixotrophy, means that organisms are able to live either autotrophically or heterotrophically, depending on the concentration of organic compounds and light intensity available.

_ Photoheterotrophically, also known as photoorganitrophy, photoassimilation, photometabolism, describes the metabolism in which light is required to use organic compounds as carbon source. The photoheterotrophic and mixotrophic metabolisms are not well distinguished, in particular they can be defined according to a difference of the energy source required to perform growth and specific metabolite production. The metabolism involved can also be distinguished according to pH changes that depend on the microalgae growth stoichiometry. *Chlorella vulgaris*, *Haematococcus pluvialis*, *Arthrospira* (*Spirulina*) *platensis* are examples of strains found to grow under photoautotrophic, heterotrophic, as well as under mixotrophic conditions. Other strains such as *Selenastrum capricornutum* and *Scenedesmus acutus* can grow either photoautotrophically, heterotrophically, or photoheterotrophically (Chojnacka and Marquez-Rocha, 2004). Not only organic carbon or substract (a carbon source such as sugars, proteins and fats), vitamins, salts and other nutrients (nitrogen and phosphorous) are vital for algal growth, but also equilibrium between operational parameters (oxygen, carbon dioxide, pH, temperature, light intensity, and product and byproduct removal) (Williams, 2002).

When considering algal use for biodiesel production, it is yet important to quantitatively define the influence of these operational parameters and their interrelation to be able to manipulate them. This way one may succeed in obtaining a certain control over the composition of microalgae populations, even on a large scale. As stated by De Pauw *et al*. (1984), experience has

repeatedly shown that properly managed algal cultures are quite resistant and that infections are often an indication of poor culture conditions. Under suitable climatic conditions and sufficient nutrients, microalgae can grow profusely. Commonly they double their biomass within 24 h or within 3.5 h during the exponential growth phase (Chisti, 2007). Generally algal cultures in the exponential growth phase contain more protein, while cultures in the stationary phase have more carbohydrates and glycogen. For example, according to De Pauw *et al*. (1984) oysters fed by algae of the former type usually grow less. A major handicap in the large-scale cultivation of algae is our inability to grow selected species in substantial volumes of hundreds of cubic meters. Exceptions are for example *Chlorella* and *Spirulina* used in aquaculture (De Pauw *et al*. 1984). A premature collapse may occur when up-scaling cultures of algae strains to larger volumes, in an artificially protected environment of semi-sterility, or other species better adapted to outdoor conditions can take-over. This means that they were developed under unbalanced growth conditions and should be better defined, in the exponential growth phase, for high-density cultures. There are several factors influencing algal growth: abiotic factors such as light (quality, quantity), temperature, nutrient concentration, O_2, CO_2, pH, salinity, and toxic chemicals; biotic factors such as pathogens (bacteria, fungi, viruses) and competition by other algae; operational factors such as shear produced by mixing, dilution rate, depth, harvest frequency, and addition of bicarbonate. Temperature is the most important limiting factor, after light, for culturing algae in both closed and open outdoor systems. The temperature effects for many microalgae species in the laboratory are well documented, but the magnitude of temperature effects in the annual biomass production outdoors is not yet sufficiently acknowledged. Many microalgae can easily tolerate temperatures up to 15 °C lower than their optimal, but exceeding the optimum temperature by only 2–4 °C may result in the total culture loss. Also, overheating problems may occur in closed culture systems during some hot days, where the temperature inside the reactor may reach 55 °C. In this case evaporative water cooling systems may be economically used to decrease the temperature to around 20–26 °C

(Moheimani, 2005).Salinity, in both open and closed systems, can affect the growth and cell composition of microalgae. Every alga has a different optimum salinity range that can increase during hot weather conditions due to high evaporation. Salinity changes normally affect phytoplankton in three ways (Moheimani, 2005): (1) osmotic stress (2) ion (salt) stress; and (3) changes of the cellular ionic ratios due to the membrane selective ion permeability. The easiest way for salinity control is by adding fresh water or salt as required. Mixing is another important growth parameter since it homogenizes the cells distribution, heat, metabolites, and facilitates transfer of gases. Also, a certain degree of turbulence, especially in large-scale production, is desirable in order to promote the fast circulation of microalgae cells from the dark to the light zone of the reactor (Barbosa, 2003). In other hand high liquid velocities and degrees of turbulence (due to mechanical mixing or air bubbles mixing) can damage microalgae due to shear stress (Eriksen, 2008). The optimum level of turbulence (above which cell death occurs) is strain dependent and should be investigated in order to avoid decline in productivity (Barbosa, 2003). Common biological contaminants observed include unwanted algae, mould, yeast, fungi, and bacteria. Attempts made to cultivate some microalgae species in raceway ponds failed, since cultures collapse due to predation by protozoa and contamination by other algal species.

As referred by Moheimani (2005) a way to decrease contaminants and improve yield is after removing the unwanted organism to subject the culture to a temporarily extreme change of the environmental factors such as temperature, pH, or light. The closed environment, the higher degree of control over culture parameters, and the higher cell concentration attainable in closed cultivation reactors effectively protect the culture from contamination and make cultivation of some important microalgae feasible. The effects of different cultivation factors on algal growth have been examined by various authors:

Moheimani (2005) analyzed the effects of a reduction in the medium pH, resulting from CO_2, which may inhibit the algal growth. This author determined that for *Pleurochrysis carterae* the pH range for maximum productivities in a plate photobioreactor is pH 7.7–8.0 and in an outdoor raceway pond is pH 9.1–9.6. This author also determined a best operational depth for the outdoor raceway pond between 16 and 21 cm.

Richmond (2004) reported that supplying CO_2 in shallow suspensions at near neutral pH is difficult to control, since the bubbles residence time is insufficient to complete the absorption, resulting in great CO_2 losses to the atmosphere.

Weissman and Goebel (1987) explain that the absorption of CO_2 into alkaline waters may be accelerated by one of two major uncatalyzed reaction paths, the hydration of CO_2 and subsequent acid-base reaction to form bicarbonate ion and the direct reaction of CO_2 with the hydroxyl ion to form bicarbonate. The rate of the former reaction is faster at pH values below 8; while the second dominates above pH 10. Between 8 and 10 both can be important.

Chiu *et al*. (2009) results show an increase in the biomass production and lipid accumulation with a CO_2 concentration increase in the aeration of *Nannochloropsis oculata* cultures.

Similar results were obtained by Morais and Costa (2007) for *Scenedesmus obliquus* and *Chlorella kessleri* cultures, isolated from a treatment pond of a coal fired thermoelectric power plant in Brazil, concluding that these microalgae have the potential for bio-fixation of CO_2 in thermoelectric power plants.

Other important factors are aeration and light intensity. Kaewpintong (2004) reported a better growth for an airlift bioreactor than for an unaerated column. This is explained because aeration lead to a better mixing of the microalgal culture, which prevents sedimentation, maintains homogeneous conditions,

and helps for a better contact between cells and nutrients. They further added an increase in cell density and specific growth rate with an increase in the light intensity up to a certain limit, above which the growth was inhibited.

Thomas *et al*. (1984) investigated photosynthetic algae species that grow autotrophically on a mineral medium containing carbon dioxide (and bicarbonate) as a carbon source and nitrate as a nitrogen source. The main purpose of this study is to determine the effect of nitrogen as well as the salt stress on the proximate chemical compositions of the algae. The general conclusion is that the species tested differ in their basic cellular composition when they are exposed to environmental stress. On growth under non-stressed conditions, *Botryococcus* contained the highest concentration of lipids the greatest proportion of which is hydrocarbon in nature. All other algae contained lower levels of lipids with an average of 23% per organic weight for the green algae, 12% for *Nitzschia* sp. and 7% for Isochrysis.

Liu *et al*. (2008) showed that high iron concentration could also induce considerable lipid accumulation in marine strain *C*. *vulgaris*. This suggests that some metabolic pathways related to the lipid accumulation in *C. vulgaris* are probably modified by high level of iron concentration in the initial medium.

Illman *et al*. (2000) found that the reduction in nitrogen in the medium increases the lipid content in all five investigated *Chlorella* strains, among which *C. emersonii*, *C. minutissima* and *C. vulgaris* gained an increase in lipid content of 63%, 56% and 40% biomass by dry weight respectively.

Thomas *et al*. (1983) also studied the effect of nitrogen stress on algae lipid fraction, concluding that cultivation under nitrogen deficient conditions did indeed increase the neutral lipid contents, but it cannot be summarized as a single trend. This is observed for *Botryococcus*, *Isochrysis* and *Dunaliella* species grown under nitrogen stress. In *Botryococcus* the neutral lipids comprised a major proportion of the total lipids. However, the greatest neutral lipid production occurred in the resting stage and the greatest amount is formed in the conversion of the algae from the green to the brown growth phase. In contrast to the 10% increase in the *Botryococcus* lipids, there was a

drop in the lipid fraction in *Dunaliella bardawil* and *Dunaliella salina* to about 10% of the organic weight. These halotolerant green algae shifted towards carbohydrate storage under nitrogen stress. On the other hand, *Isochrysis* accumulated higher fractions of lipids and carbohydrates under nitrogen deficiency, with lipids comprising about one-fourth of the algal organic cell weight following 10 days of nitrogen starvation. In general, the effects of nitrate deficiency were that the protein content and the chlorophyll level decreased while carbohydrate and lipids exhibited a species-specific change. The neutral lipid content is expressed in the algae that shift to lipid storage when under environmental stress. These neutral lipids are not predominantly straight chain saturated hydrocarbons but multibranched and/or polyunsaturated components.

Macedo and Alegre (2001) demonstrated that the *Spirulina* lipids content increase approximately 3 times with the decrease of nitrogen content and temperature decrease, being the nitrogen concentration decrease more effective.

Algal harvesting consists of biomass recovery from the culture medium that may contribute to 20–30% of the total biomass production cost (Grima *et al*. 2003). In order to remove large quantities of water and process large algal biomass volumes, a suitable harvesting method may involve one or more steps and be achieved in several physical, chemical, or biological ways, in order to perform the desired solid–liquid separation. Experience has demonstrated that albeit a universal harvesting method does not exist, this is still an active area for research, being possible to develop an appropriate and economical harvesting system for any algal species. Most common harvesting methods include sedimentation, centrifugation, filtration, ultra-filtration, sometimes with an additional flocculation step or with a combination of flocculation–flotation. Flocculation is used to aggregate the microalgal cells to increase the effective particle size and hence ease sedimentation, centrifugal recovery, and filtration (Grima *et al*. 2003). Weissman and Goebel (1987)

studied four primary harvesting methods for the purpose of biofuels production: microstraining, belt filtering, flotation with float collection, and sedimentation. These methods discriminate on a size and density basis in performing the biomass separation. Microstrainers are an attractive harvesting method because of their mechanical simplicity and availability in large unit sizes. The recent availability of very fine mesh polyester screens has revived interest in their use for microalgae harvesting. Subsequent studies concluded that it would be necessary to flocculate the cells prior to microstraining. Filter presses operating under pressure or vacuum can be used to recover large quantities of biomass, but for some applications filtration can be relatively slow which may be unsatisfactory. Also filtration is better suited for large microalgae such as *Coelastrum proboscideum* and *S. platensis* but cannot recover organisms with smaller dimensions such *Scenedesmus*, *Dunaliella*, or *Chlorella* (Cravotto *et al*., 2008). Alternatively, membrane microfiltration and ultra-filtration are other possible alternatives to conventional filtration for recovering algal biomass, which are more suitable for fragile cells and small scale production processes. Furthermore these filtration processes are more expensive especially because of the need for membrane replacement and pumping. Richmond (2004) suggested one main criterion for selecting a proper harvesting procedure, which is the desired product quality. In one hand for low value products, gravity sedimentation may be used, possibly enhanced by flocculation. Sedimentation tanks or settling ponds are also possible, e.g. to recover biomass from sewage-based processes. In other hand for high-value products, to recover high quality algae such as for food or aquaculture applications, it is often recommended to use continuously operating centrifuges that can process large volumes of biomass. Albeit at considerable cost, centrifuges are suitable to rapidly concentrate any type of microorganisms, which remain fully contained during recovery. Additionally, these devices can be easily cleaned or sterilized to effectively avoid bacterial contamination or fouling of raw product. Another basic criterion for selecting the harvesting procedure is its potential to adjust the density or the acceptable level of moisture in the resulting concentrate right to the optimum subsequent

process (Richmond, 2004; Cravotto *et al.*, 2008). Gravity sedimented sludge is generally more diluted than centrifugally recovered biomass, which substantially influence the economics of product recovery further downstream. Since costs of thermal drying are much higher than those of mechanical dewatering, in order to reduce the overall production cost, a concentrate with higher solids content is required after harvest to easy biomass dehydration (e.g. in a drum drying). In this case a combination of methods can also be used, e.g. a pre-concentration with a mechanical dewatering step such as microstrainer, filtration, or centrifugation and then, a post concentration by means of a screw centrifuge or a thermal drying. After separation from the culture medium algal biomass (5–15%dry weight) must be quickly processed at least it should get spoiled in only a few hours in a hot climate.

Processing represents a major economic limitation to the production of low cost commodities (fuels, feeds and foods) and also to higher value products (b-carotene, polysaccharides). It is difficult to discuss processing, since it is highly specific and strongly depends on the desired products.It is common to apply dehydration of biomass that also increases its shelf-life and of the final product. Several methods have been employed to dry microalgae such as *Chlorella*, *Scenedesmus* and *Spirulina*, where the most common include spray drying, drum drying, freeze drying and sun drying (Richmond, 2004). Because of the highwater content of algal biomass sun-drying is not a very effective method for algal powder production and spray-drying is not economically feasible for low value products, such as biofuel or protein. After drying it follows the cell disruption of the microalgae cells for release of the metabolites of interest. Several methods can be used depending on the microalgae wall and on the product nature to be obtained either based on mechanical action (e.g. cell homogenizers, bead mills, ultrasounds, autoclave, and spray drying) or non-mechanical action (e.g. freezing, organic solvents and osmotic shock and acid, base and enzyme reactions).Taking the example

of the astaxanthin recovery, although different methods have been studied the best results were obtained from autoclaved and mechanically disrupted biomass, with yield 3 times higher than with other methods (Richmond, 2004). Lyophilization breaks up the cells and turns the algal material into a loose and fine powder, making other treatment unnecessary. For biodiesel production, lipids and fatty acids have to be extracted from the microalgal biomass. For lipids a solvent extraction is normally done directly from the lyophilized biomass, being a quick and efficient extraction method that slightly reduces the degradation. Several solvents can be used such as hexane, ethanol (96%), or a hexane–ethanol (96%) mixture, being possible to obtain up to 98% quantitative extraction of purified fatty acids (Richmond, 2004). Although ethanol is a very good solvent it can also extract some cellular contaminants such as sugars, amino acids, salts, hydrophobic proteins and pigments, which is not desirable if the purpose of the extraction is just the lipids. Extraction methods such as ultrasound and microwave assisted were also studied for oil extraction from vegetable sources. Cravotto *et al*. (2008) compared oil extraction times and yields using these methods with those resulting from conventional procedures. For that purpose these authors research team developed ultrasound devices working at frequencies of 19, 25, 40 and 300 kHz and multimode microwave oven operating with both open and closed vessels, as well as combined extraction with simultaneous double sonication at 19 and 25 kHz and simultaneous ultrasound/multimode microwave irradiation achieved. These results indicate that compared with conventional methods these new methods can greatly improve oil extraction with higher efficiency. Extraction times were reduced and yields increased by 50–500% with low or moderate costs and minimal added toxicity.

In case of marine microalgae *Crypthecodinium cohnii*, ultrasound worked best as the disruption of the tough algal cell wall considerably improved the extraction yield from 4.8% (in soxhlet) to 25.9%.

Biodiesel is a mixture of fatty acid alkyl esters obtained by transesterification (ester exchange reaction) of vegetable oils or animal fats. These lipid feedstocks are composed by 90–98%(weight) of triglycerides and small amounts of mono and diglycerides, free fatty acids (1–5%), and residual amounts of phospholipids, phosphatides, carotenes, tocopherols, sulphur compounds, and traces of water (Bozbas, 2008).

Transesterification is a multiple step reaction, including three reversible steps in series, where triglycerides are converted to diglycerides, then diglycerides are converted to monoglycerides, and monoglycerides are then converted to esters (biodiesel) and glycerol (by-product). The overall transesterification reaction is described in Fig. 1 where the radicals R_1, R_2, R_3 represent long chain hydrocarbons, known as fatty acids. For the transesterification reaction oil or fat and a short chain alcohol (usually methanol) are used as reagents in the presence of a catalyst (usually NaOH). Although the alcohol:oil theoretical molar ratio is 3:1, the molar ratio of 6:1 is generally used to complete the reaction accurately. The relationship between the feedstock mass input and biodiesel mass output is about 1:1, which means that theoretically, 1 kg of oil results in about 1 kg of biodiesel. A homogeneous or heterogeneous, acid or basic catalyst can be used to enhance the transesterification reaction rate; although for some processes using supercritical fluids (methanol or ethanol) it may not be necessary to use a catalyst (Warabi *et al*., 2004).

Most common industrial processes use homogeneous alkali catalysts (e.g. NaOH or KOH) in a stirred reactor operating in batch mode. Recently some improvements were proposed for this process, in particular to be able to operate in continuous mode with reduced reaction time, such as reactors with improved mixing, microwave assisted reaction (Cravotto *et al*., 2008; Azcan and Danisman, 2008), cavitation reactors (Gogate, 2008; Gogate and Kabadi, 2009) and ultrasonic reactors (Kalva *et al*., 2008; Deshmane *et al*., 2009).

Microalgae cultivation can be done in open-culture systems such as lakes or ponds and in highly controlled closed-culture systems called photo-bioreactors (PBRs). A bioreactor is defined as a system in which a biological

conversion is achieved. Thus, a photo-bioreactor is a reactor in which phototrophs (microbial, algal or plant cells) are grown or used to carry out a photobiological reaction. Although this definition may apply to both closed and open-culture systems, for the purpose of this article we limit the definition to the former ones. Open-culture systems are normally less expensive to build and operate, more durable than large closed reactors and with a large production capacity when compared with closed systems. However, according to Richmond (2004) ponds use more energy to homogenize nutrients and the water level cannot be kept much lower than 15 cm (or 150 Lm^{-2}) for the microalgae to receive enough solar energy to grow. Generally ponds are more susceptive to weather conditions, not allowing control of water temperature, evaporation and lighting. Also, they may produce large quantities of microalgae, but occupy more extensive land area and are more susceptible to contaminations from other microalgae or bacteria. Moreover, since atmosphere only contains 0.03–0.06% CO_2 it is expected that mass transfer limitation could slow down the cell growth of microalgae. PBRs are flexible systems that can be optimized according to the biological and physiological characteristics of the algal species being cultivated, allowing one to cultivate algal species that cannot be grown in open ponds. On a PBR, direct exchange of gases and contaminants (e.g. microorganisms, dust) between the cultivated cells and atmosphere are limited or not allowed by the reactor's walls. Also, a great proportion of light does not impinge directly on the culture surface but has to cross the transparent reactor walls. Depending on their shape or design, PBRs are considered to have several advantages over open ponds: offer better control over culture conditions and growth parameters (pH, temperature, mixing, CO_2 and O_2), prevent evaporation, reduce CO_2 losses, allow to attain higher microalgae densities or cell concentrations, higher volumetric productivities, offer a more safe and protected environment, preventing contamination or minimizing invasion by competing microorganisms. Despite their advantages it is not expected that PBR have a significant impact in the near future on any product or process that can be attained in large outdoor raceway ponds. PBRs suffer from several drawbacks

that need to be considered and solved. Their main limitations include: overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, the high cost of building, operating and of algal biomass cultivation, and cell damage by shear stress and deterioration of material used for the photo-stage.

The cost of biomass production in PBRs may be one order of magnitude higher than in ponds. While in some cases, for some microalgae species and applications it may be low enough to be attractive for aquaculture use, in other cases, the higher cell concentration and the higher productivity achieved in PBR may not compensate for its higher capital and operating costs.

As stated by Richmond (2004) despite closed systems offer no advantage in terms of a real productivity, they largely surpass ponds in terms of volumetric productivity (8 times higher) and cell concentration (about 16 times higher).

In conclusion, PBR and open ponds should not be viewed as competing technologies, but the real competing technology will be genetic engineering (Richmond, 2004; Gressel, 2008).

PBR can be operated in batch or continuous mode. There are several advantages of using continuous bioreactors as opposed to the batch mode (Williams, 2002):

Continuous bioreactors provide a higher degree of control than do batch;

Growth rates can be regulated and maintained for extended time periods and biomass concentration can be controlled by varying the dilution rate;

Because of the steady-state of continuous bioreactors, results are more reliable and easily reproducible and the desired product quality may be more easily obtained;

Continuous reactions offer increased opportunities for system investigation and analysis.

There are yet inherent disadvantages that may make this process unsuitable for some types of bio-reaction. For example, one challenge lies in controlling the production of some non-growth related products. For this reason, the continuous process often requires feed-batch culturing, and a continuous nutrient supply.

Wall growth and cell aggregation can also cause wash-out or prevent optimum steady-state growth. Another problem is that the original product strain can be lost over time, if it is overtaken by a faster-growing one. The mixture viscosity and its heterogeneous nature can make it difficult to maintain filamentous organisms. Long growth periods not only increase the contamination risk, but also dictate that the bioreactor must be extremely reliable and consistent, incurring a potentially larger initial expenditure in higher-quality equipment. Depending on the local conditions and available materials it is possible to design different culture systems with variations in size, shape, construction materials, inclination and agitation type, which influence their performance, cost and durability (resistance to weathering). Among the various sizes and shapes of ponds operated at a relatively large scale the three major designs include (Richmond, 2004; Schenk *et al*., 2008): (1) raceway ponds constructed as an endless loop, in which the culture is circulated by paddle wheels; (2) circular ponds with agitation provided by a rotating arm; (3) inclined systems where mixing is achieved through pumping and gravity flow. Raceway ponds and also natural ponds may be the most commonly used for commercial production of microalgae. Normally open ponds are relatively economical, easy to clean up after cultivation and good for mass cultivation of algae. However they allow little control of culture conditions, their productivity is poor, occupy large land area, cultures are easily contaminated, are limited to few strains of algae, and have difficulty in growing algal cultures for long periods (Ugwu *et al*., 2008). PBRs can be classified on the basis of both design and mode of operation. Many different

designs have been developed (serpentine, manifold, helical, and flat), where the main categories include (Richmond, 2004): (1) flat or tubular; (2) horizontal, inclined, vertical or spiral; and (3) manifold or serpentine. From these, elevated reactors can be oriented and tilted at different angles and can use diffuse and reflected light, which plays an important role in productivity. Generally tubular reactors are suitable for outdoor cultures, are relatively cheap, have a large illumination surface area and have fairly good biomass productivities. Disadvantages include fouling, some degree of wall growth, dissolved oxygen and CO_2 along the tubes, and the pH gradients (Ugwu *et al*., 2008). Vertical bubble columns and airlift cylinders can attain substantially increased radial movement of fluid that is necessary for improved light–dark cycling. These reactor designs have a low surface/volume, but substantially greater gas hold-ups than horizontal reactors and a much more chaotic gas–liquid flow (Richmond, 2004; Ugwu *et al*., 2008). Other prospects include high mass transfer, good mixing with low shear stress, low energy consumption, high potential for scalability, easy to sterilize, readily tempered, good for immobilization of algae. Consequently, cultures suffer less from photoinhibition and photo-oxidation, and experience a more adequate light–dark cycle. Limitations include their cost, small illumination surface area, their construction requires sophisticated materials, shear stress to algal cultures, and the fact that, since diameter and height cannot be much increased, a large number of units are needed to build a commercial plant. Vertical plate photo-bioreactors mixed by air bubbling seem even better than bubble columns in terms of productivity and ease of operation. Flat-plates allow large illumination surface area, are suitable for outdoor cultures, are good for algae immobilization, relatively cheap, easy to clean up and readily tempered (Ugwu *et al*., 2008). It has been shown that vertical flat plates of 1000–2000 L in volume can be successfully operated for long periods, hence having potential for scale up (Richmond, 2004). Packed flat panels mixed by air bubbling can potentially achieve very high overall ground- a real productivities through lamination of solar light. Limitations include difficulty in controlling culture temperature, some degree of wall growth,

Scale-up require many compartments and support materials, and possibility of hydrodynamic stress to some algal strains (Ugwu *et al*., 2008). PBR can be built with various light paths and can be mixed by different types of pumps or by air bubbling, key issues to reach very high productivities and efficiencies of solar energy utilization. The type of material used for the photo-stage is of fundamental importance for a suitable PBR construction. Materials such as plastic or glass sheets, collapsible or rigid tubes, must lack toxicity, have high transparency, high mechanical strength, high durability, chemical stability and low cost (Richmond, 2004). The ease of cleaning and loss of the plastics transparency exposed outdoors are operational issues to consider. According to Richmond (2004) the construction materials to build the side walls and bottom of a pond can vary from simple sand or clay, to brick or cement, and to expensive plastics like PVC, glass fiber or polyurethane. For the lining most commercial plants use long-life plastic membranes (e.g. 1–2 mm thick, UV-resistant, PVC or polyethylene sheets). Sometimes unlined ponds are used to reduce costs, but they suffer from silt suspension, percolation, heavy contamination, and their use is limited to a few algal species and to particular soil and environmental conditions.

3: MATERIALS AND METHODS

3.1: Collection of Sample:

Systematic collection was carried out for locating fast growing and frequently occurring algal strains from different kinds of natural habitats e.g. ponds, ditches, drains, paddy fields from Rajshahi University area. Collection of algal samples have been taken in sterilized glass bottle for further work.

3.2: Preparation of inoculants:

At first, the samples have been washed by distilled water. After that, mounted on a slide and set under the light microscope for observation. After identification, isolated fresh algal species have been used as inoculants for algal monoculture.

3.3: *In vitro* Algal culture:

In vitro algal culture has been done in BBM (Bold 1942), Fogg's (Fogg 1949) and Chu 10D (Sinclair and Whitton, 1977) culture medium under the condition given as below:

i. Screw cap glass vessel (500ml) has been used for *In vitro* algal culture.

ii. At 30°C temperature with a light intensity of 2000 lux, provided from overhead cool light while fluorescent tubes (16L+8) for a total duration of 90 days.

iii. The pH was adjusted by using buffer 7, NaOH (0.01 N) and HCL (0.01 N) by using pH meter (Model: LIDA pH 25, Shanghai, china).

iv. In all cases, the medium and glass wares were autoclaved at 120°C and 15lb/square inch for 15 minutes for sterilization purpose.

v. All cultures were shaking twice daily to prevent cells from clumping.

vi. Algal growth has been measured by the weight gain of algal biomass and algal growth rate calculated according to Morten and Borum (1996). The weight was taken by an electric balance machine (Model: BL-200S).

3.4: Preparation of *In vitro* algal culture medium:

Preparation of BBM (Bold's Basal Medium) medium:

The medium needs six stock solutions and four trace elements solutions. Finally to make the BBM 10 ml of each stock solution and 1 ml of each trace element solution were added to 940 ml distilled water. The pH was adjusted to 7.0 for blue green algae and 6.0-6.5 for green algae with prepared NaOH solution. The composition of BBM medium given as bellow (Table 4 and table 5):

Table 4: Stock Solutions for BBM medium:

Chemical Compounds	Amount in gm/100 ml DW
$NaNo_3$	2.50
$CaCl_2$. .$2H_2O$	0.25
K_2HPO_4	0.75
KH_2PO_4	1.75
NaCl	0.25
$MgSO_4$.$7H_2O$	0.75

Table 5: Trace Elements Solutions for BBM medium:

Compounds	Amount in gm	Dissolve in DW (ml)
EDTA	0.5	100 ml
KOH	3.1	
$FeSO_4 .7H_2O$	0.498	100 ml acidified water (0.1 ml H_2SO_4 added to 99 ml distilled water)
H_3BO_3	1.14	100 ml
$ZnSO_4 .7H_2O$	0.882	100 ml
$MnCl_2 .4H_2O$	0.144	
MoO_3	0.071	
$CuSO_4 .5H_2O$	0.157	
$Co (NO_3)_2 .6H_2O$	0.049	

Preparation of Fogg medium:

The Fogg medium contains two parts of solutions (viz. stock solutions and trace elements solution). To 1L of distilled water, 1.0 ml of trace element solution and 1.0 ml of each stock solution were added (Table 6 and Table 7). The pH was adjusted to 7.0 using prepared NaOH solution.

Table 6: Stock Solutions for Fogg's medium:

Chemical Compounds	Amount in gm/100 ml DW
$MgSO_4 .7H_2O$	20.0
K_2HPO_4	20.0
$CaCl_2 . .2H_2O$	13.7
$FeCl_3.6H_2O$	0.0667
NaCl	0.5

Table 7: Trace Element Solutions for Fogg's medium:

Compounds	Amount in gm (Dissolve in 100 ml DW)
$MnCl_4 .4H_2O$	0.0360
$NaMoO_4.2H_2O$	0.0252
H_3BO_3	0.0572
$CuSO_4 .5H_2O$	0.0039
$ZnSO_4 .7H_2O$	0.0044

Preparation of Chu 10D medium:

The Chu 10D medium contains two parts of solutions i.e. six stock solutions and one trace element solutions solution (Table 8 and Table 9):

Table 8: Stock Solutions for Chu 10D medium:

Chemical Compounds	Amount in gm/100 ml DW
KH_2PO_4	1.56*
$MgSO_4 .7H_2O$	2.50**
$Ca(NO_3)_2.4H_2O$	5.76**
$CaCl_2..2H_2O$	3.584**
$NaHCo_3$	1.585***
Na EDTA	1.27****
$FeCl_3.6H_2O$	0.97****

[**Note:** *,**,***,****, chemical compounds were added in the ration prepare final culture solution.]

Table 9: Trace Element Solutions for Chu 10D medium:

Compounds	Amount in mg (Dissolve in 1L DW)
$CuSO_4 .5H_2O$	19.6
$ZnSO_4 .7H_2O$	44.0
$CoCl_2.6H_2O$	20.0
$MnCl_2.4H_2O$	36.0
$NaMoO_4.2H_2O$	12.6
H_3BO_3	618.4

Finally to prepare culture solution the above mentioned stock solutions and trace element solution were added in the following proportion:

* 0.5 ml	
** 1.0 ml	
*** 1.5 ml	in 1L of DW
**** 0.25 ml	
Trace element solution (0.25 ml)	

The pH was adjusted to 7.2 using buffer 7 and NaOH 0.1N solution.

3.5: Determination of algal growth:

Algal growth has been measured by the weight gained of algal biomass. The weight has taken by an electronic balance machine (Model: BL-200S, China). According to Morten and Borum (1996), the algal growth rate was calculated using the formula Net growth rate, $\mu = (\ln B_t - \ln B_0)\, t^{-1}$.

3.6: Effect of pH at *In vitro* algal culture:

Four pH values (i.e., 5.5, 6.5, 7.2 & 8.0) have been used to find out the optimum pH value for six algal (viz. *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp.) growths in three different culture Medium (viz. BBM, Fogg's medium and Chu medium) at ±30°C.

3.7: Algal oil extraction:

Cultured algae were ground with motor and pestle as much as possible. The ground algae were dried for 20 min at 80°C in an incubator for releasing water. Hexane (20ml) and ether (20ml) solution were mixed with the dried ground algae (20g) to extract oil. Then the mixture was kept for 24h for settling.

3.8: Bio-diesel conversion of algal oil by Transesterification:

Biomass collection:

The biomass was collected after filtration through Teton cloth filter and weighted.

Evaporation:

The extracted oil was separated by evaporating hexane and ether solutions using rotary evaporator.

Mixing of catalyst and methanol:

For the transesterification reaction, 0.25 g NaOH was mixed with 24 ml methanol and stirred properly for 20 min. After then, that mixture has stored in a glass reagent bottle.

Biodiesel production:

The mixture of catalyst and methanol was poured into the extracted algal oil in a conical flask and following the reaction and steps given as below (Hossain 2008).

Transesterification:

For transesterification the conical flask containing solution was shaken for 3h by electric shaker at 300 rotations per minute.

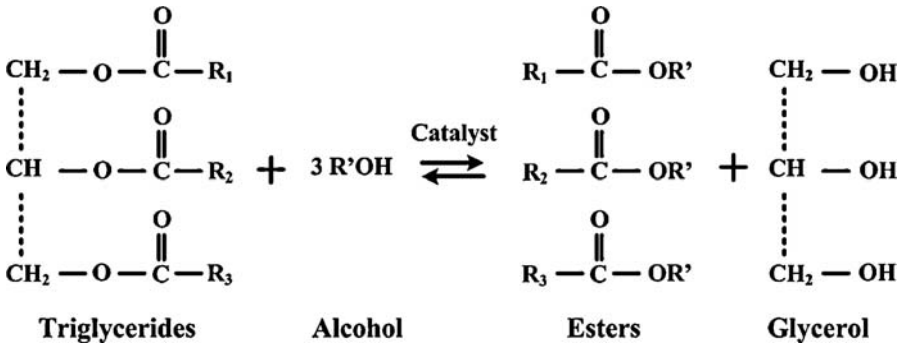

Fig. 2: Transesterification of triglycerides (overall reaction).

Setteling:

After shaking the solution was kept for 16h to settle the biodiesel and sediment layers clearly.

Seperation of biodiesel:

The biodiesel was separated by flask separator carefully. Quantity of the sediment (glycerine, pigments, etc.) was measured.

Washing:

Biodiesel was washed by 5% water until it became clean.

Drying:

Biodiesel was dried by using dryer and finally kept under the running fan for 12 h.

Storage of Biodiesel:

Biodiesel production was measured by using measuring cylinder; pH was measured and stored for analysis.

3.9: Measurement of algal bio-diesel and byproducts:

For quality assessment pH and optical density have been measured.

3.10: Quality test of algal bio-diesel and byproducts:

In that case two parameter i.e., pH and Optical density (OD) have been tested.

Measurement of pH:

A pH meter (Model: LIDA pH 25, Shanghai, china) has used for the measurement of pH of algal bio-diesel and byproducts and noted.

Optical density (OD):

Optical density (OD) of algal bio-diesel and byproducts has been taken by Shengheng Bench top Colorimeter (Model: AE-11M, Zhejiang, China) and noted.

4: RESULTS

4.1: *Isolation and culture of micro-algae:*

Algal isolation was performed using microscopic technique. Six algal strains have been isolated, identified, cultured (*in vitro*) and maintained in three culture medium viz. BBM, Fogg's and Chu 10D. These are *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp. (Fig. 3 and Fig. 4).

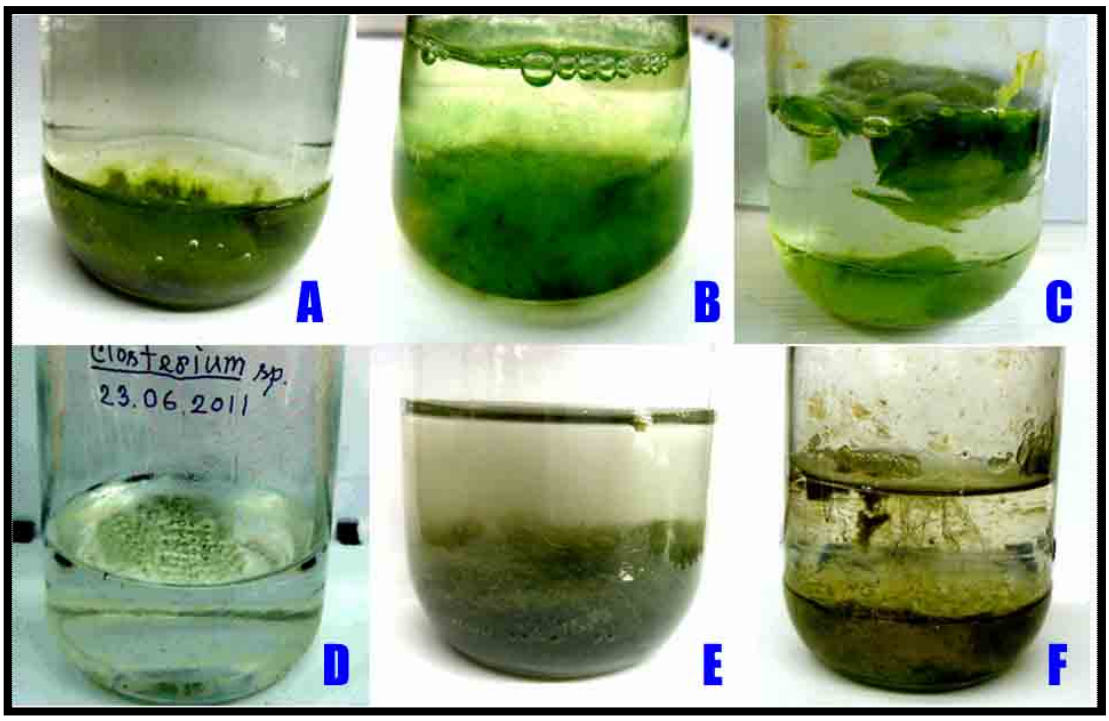

Fig. 3: *In vitro* Cultured algae: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp. and F. *Pithophora* sp.

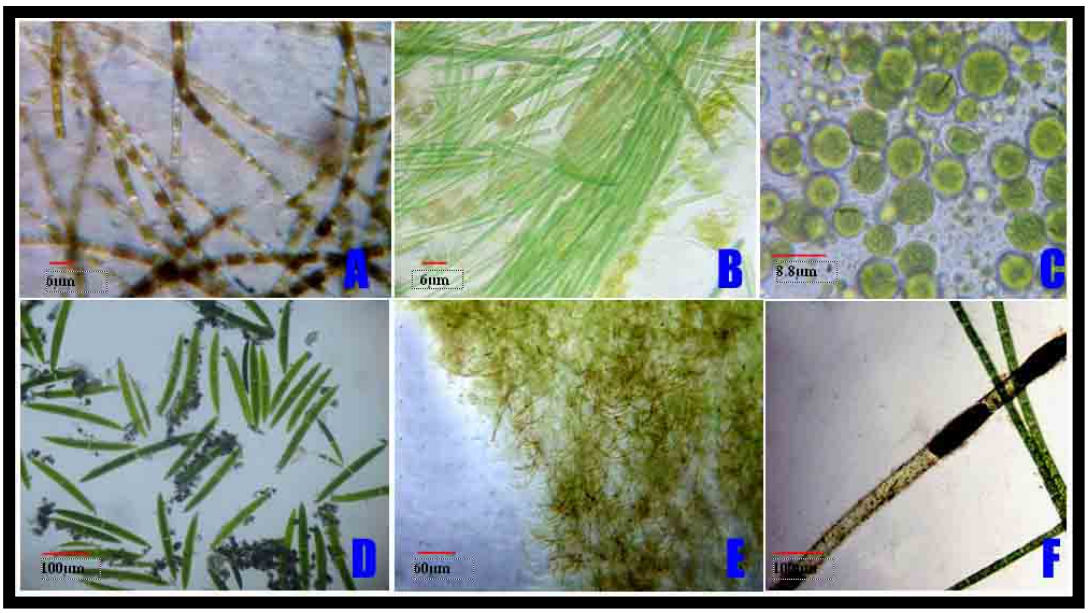

Fig. 4: Microscopic photograph of cultured algae: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

4.2: Measurement of *in vitro* micro-algal net growth and biomass in three different culture Medium:

Algal biomass of the selected strains were measured in all three medium. Net growth rate of the selected strains were measured during the study period. *Chlorococcum* sp., *Closterium* sp. and *Pithophora* sp., Exhibited similar growth rate in three selected culture medium; whereas *Cladophora* sp., *Oscillatoria* sp. and *Oedogonium* sp. Showed similar net growth pattern in the selected medium (Table 10). Six algae have been growing in BBM medium at pH 6.5 (30°C) and increase their biomass according to day's interval. Algal growth has been measured by the weight gain of algal biomass. The weight was taken by an electric balance machine. Significant differences were observed. After 90days *Chlorococcum* sp. exhibited best growth (51.01g±0.01) and minimum growth was obtained in case of *Oscillatoria* sp. (29.01g±0.02) in BBM (Table 11 and Fig. 5). On the other hand, *Chlorococcum* sp. exhibited best growth (50.20g ±0.01) and minimum growth was obtained in case of *Cladophora* sp. (28.70g±0.03) in Fogg's medium (Table 12 and Fig. 6). Whereas, *Chlorococcum* sp. showed best growth (49.20g±0.02) and minimum growth was obtained in case of *Cladophora* sp. (26.76g±0.1) in Chu medium (Table 13 and Fig. 7).

Table 10: Net growth rate of selected algal strains in three culture media.

S.N.	Algae	Net growth rate, $\mu = (\ln B_t - \ln B_0)\, t^{-1}$		
		BBM	**Fogg**	**Chu 10D**
1	*Oedogonium* sp.	0.03	0.03	0.03
2	*Oscillatoria* sp.	0.03	0.03	0.03
3	*Chlorococcum* sp.	0.04	0.04	0.04
4	*Closterium* sp.	0.04	0.04	0.04
5	*Cladophora* sp.	0.03	0.03	0.03
6	*Pithophora* sp.	0.04	0.04	0.04

[**Note**: B_0= initial biomass, B_t= Final biomass],

Table 11: *In vitro* algal biomass (at 30°C) in Bold Basal Medium (BBM).

Culture Medium	Algae	Algal growth (fresh weight in g/day)			
		Initial weight	**After 30days**	**After 60days**	**After 90days**
BBM	*Oedogonium* sp.	1.00	10.05 ±0.02	25.50 ±0.02	31.75 ±0.03
	Oscillatoria sp.	1.00	10.90 ±0.076	20.75 ±0.03	**29.01** ±0.02
	Chlorococcum sp.	1.00	20.05 ±0.04	40.79 ±0.01	**51.01** ±0.01
	Closterium sp.	1.00	20.50 ±0.03	35.45 ±0.02	50.45 ±0.03
	Cladophora sp.	1.00	11.75 ±0.001	24.57 ±0.01	29.97 ±0.02
	Pithophora sp.	1.00	19.05 ±0.02	40.54 ±0.03	49.75 ±0.02

Table 12: *In vitro* algal biomass (at 30°C) in Fogg's medium.

Culture Medium	Algae	Algal growth (fresh weight in g/day)			
		Initial weight	After 30days	After 60days	After 90days
Fogg's	*Oedogonium* sp.	1.00	9.06 ±0.11	20.30 ±0.2	30.06 ±0.02
	Oscillatoria sp.	1.00	8.70 ±0.03	15.54 ±0.11	29.02 ±0.02
	Chlorococcum sp.	1.00	12.02 ±0.01	30.60 ±0.01	**50.20** ±0.01
	Closterium sp.	1.00	18.45 ±0.02	30.40 ±0.0	48.35 ±0.01
	Cladophora sp.	1.00	10.65 ±0.035	20.56 ±0.02	**28.70** ±0.03
	Pithophora sp.	1.00	15.01 ±0.002	36.50 ±0.002	47.65 ±0.03

Table 13: *In vitro* algal biomass (at 30°C) in Chu 10D medium.

Culture Medium	Algae	Algal growth (fresh weight in g/day)			
		Initial weight	After 30days	After 60days	After 90days
Chu	*Oedogonium* sp.	1.00	8.60 ±0.1	18.94 ±0.02	29.16 ±0.01
	Oscillatoria sp.	1.00	8.60 ±0.1	13.50 ±0.1	28.12 ±0.1
	Chlorococcum sp.	1.00	10.01 ±0.03	29.50 ±0.02	**49.20** ±0.02
	Closterium sp.	1.00	15.40 ±0.1	29.30 ±0.02	47.35 ±0.1
	Cladophora sp.	1.00	11.60 ±0.1	18.56 ±0.01	**26.76** ±0.1
	Pithophora sp.	1.00	13.02 ±0.1	33.50 ±0.02	42.55 ±0.1

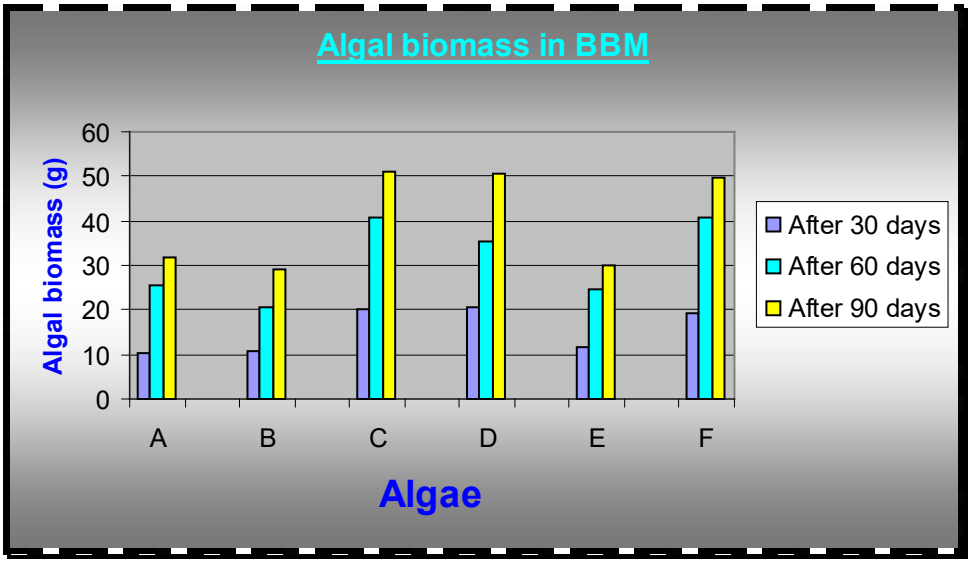

Fig. 5: Comparative graph of *in vitro* algal biomass content in BBM: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

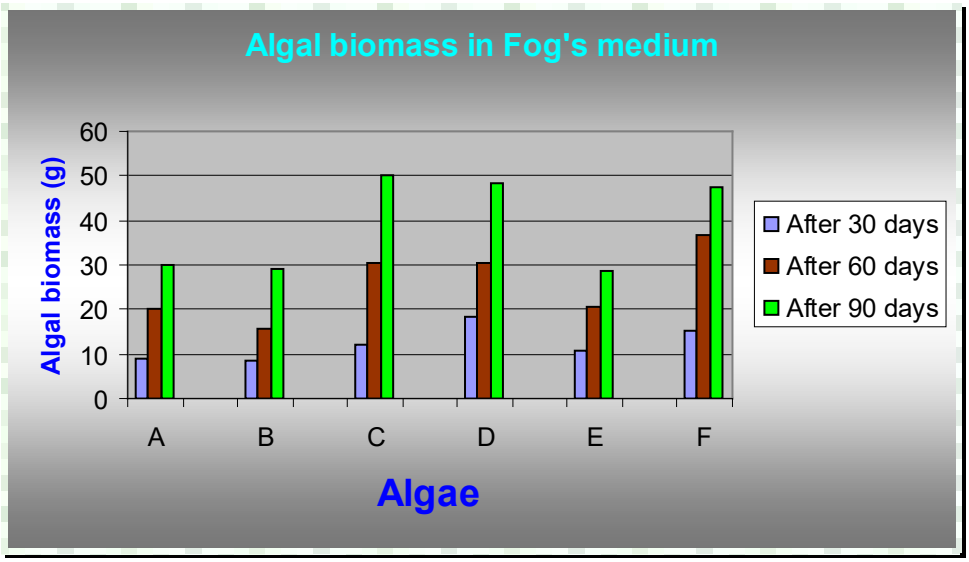

Fig. 6: Comparative graph of *in vitro* algal biomass content in Fogg's medium: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

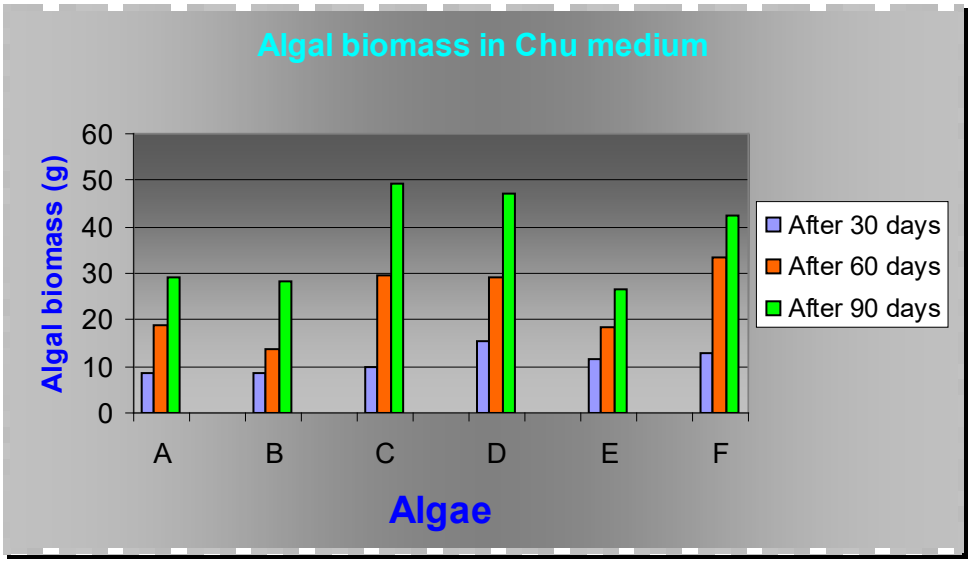

Fig. 7: Comparative graph of *in vitro* algal biomass content in Chu medium: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

4.3: Effect of pH on *In vitro* algal biomass (at 30°C) in three different culture Medium:

In this experiment, four different pH values (i.e, 5.5, 6.5, 7.2 and 8.0) had been used to find out the optimum pH value for six algal growths in three different culture Medium (viz. BBM, Fog's medium and Chu medium) at 30°C. Six algal strains exhibited varying growth response on various pH values of three different culture medium.

In Bold Basal Medium (BBM), the selected all strains showed suitable growth in pH 6.5 and medium growth in pH 7.2. On the other hand, no growth was observed in pH 5.5 and 8.0 (Table 14).

In Fogg's medium, all six strains showed suitable growth in 7.2 pH and medium growth in pH 6.5. On the other hand, no growth was observed in pH 5.5 and 8.0 (Table 15).

In Chu medium, the selected all strains showed suitable growth in 7.2 pH and medium growth in pH 6.5. On the other hand, no growth was observed in pH 5.5 and 8.0 (Table 16). Here "No growth" has been used to define where algal biomass content increased less than 0.5g in 90 days.

Table 14: Effect of pH on *in vitro* algal biomass (at 30°C) in Bold Basal Medium (BBM).

S.N.	Micro-algae	pH			
		5.5	**6.5**	**7.2**	**8.0**
1	*Oedogonium* sp.	-	+++	+	-
2	*Oscillatoria* sp.	-	+++	+	-
3	*Chlorococcum* sp.	-	+++	+	-
4	*Closterium* sp.	-	+++	+	-
5	*Cladophora* sp.	-	+++	+	-
6	*Pithophora* sp.	-	+++	+	-

[Note: -=No growth, +=medium growth, and +++=maximum growth.]

Table 15: Effect of pH on *in vitro* algal biomass (at 30°C) in Fogg's medium.

S.N.	Micro-algae	pH			
		5.5	**6.5**	**7.2**	**8.0**
1	*Oedogonium* sp.	-	+	+++	-
2	*Oscillatoria* sp.	-	+	+++	-
3	*Chlorococcum* sp.	-	+	+++	-
4	*Closterium* sp.	-	+	+++	-
5	*Cladophora* sp.	-	+	+++	-
6	*Pithophora* sp.	-	+	+++	-

[Note: - =No growth, +=medium growth, and +++=maximum growth.]

Table 16: Effect of pH on *in vitro* algal biomass (at 30°C) in Chu medium.

S.N.	Micro-algae	pH			
		5.5	**6.5**	**7.2**	**8.0**
1	*Oedogonium* sp.	-	+	+++	-
2	*Oscillatoria* sp.	-	+	+++	-
3	*Chlorococcum* sp.	-	+	+++	-
4	*Closterium* sp.	-	+	+++	-
5	*Cladophora* sp.	-	+	+++	-
6	*Pithophora* sp.	-	+	+++	-

[Note: - =No growth, +=medium growth, and +++=maximum growth.]

4.4: Bio-diesel measurement and quality test:

4.4.1: *Measurement of Bio-diesel*

This result showed the proper transesterification, amount of biodiesel production (ester) and physical properties of biodiesel to compare among six algae viz. *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp. Bio-diesel production was higher in *Chlorococcum* sp. (44.59%) and lower in *Cladophora* sp. (21.66%) among those six algae (Table 18, Fig. 7 and Fig. 8). Sediments (glycerine, water and pigments) production was higher in *Pithophora* sp. (25.92g) and lower in *Oedogonium* sp. (8.17g) (Table 19 and Fig. 10). On the other hand, biomass (after oil extraction) was higher in *Cladophora* sp. (64g) and lower in *Oscillatoria* sp. (26g) (Table 17 and Fig. 11).

Fig. 8: Extracted bio-diesel, glycerine and pigment, and residual biomass (after extraction) from Algae:
A. *Oedogonium* sp. (A1: biodiesel, A2: Glycerine & pigments and A3: Biomass)
B. *Oscillatoria* sp. (B1: biodiesel, B2: Glycerine & pigments and B3: Biomass)
C. *Chlorococcum* sp. (C1: biodiesel, C2: Glycerine & pigments and C3: Biomass)
D. *Closterium* sp. (D1: biodiesel, D2: Glycerine & pigments and D3: Biomass)
E. *Cladophora* sp. (E1: biodiesel, E2: Glycerine & pigments and E3: Biomass) and
F. *Pithophora* sp. (F1: biodiesel, F2: Glycerine & pigments and F3: Biomass)

Table 17: Measurement of Micro-algal residual biomass after oil extraction.

S.N.	Micro-algae	Residual biomass (g)	
		Dry weight before oil extraction	**Weight after oil extraction**
1	*Oedogonium* sp.	29.40	27.87
2	*Oscillatoria* sp.	**26.65**	**26.00**
3	*Chlorococcum* sp.	60.55	58.90
4	*Closterium* sp.	50.00	49.00
5	*Cladophora* sp.	**64.65**	**64.00**
6	*Pithophora* sp	40.00	39.50

Table 18: Measurement of bio-diesel content in algal biomass.

S.N.	Micro-algae	Biodiesel content (ml)	% of Biodiesel content (Biodiesel content x100 / Dry Weight of biomass before oil extraction)
1	*Oedogonium* sp.	12	40.82
2	*Oscillatoria* sp.	10	37.52
3	*Chlorococcum* sp.	27	**44.59**
4	*Closterium* sp.	21	42
5	*Cladophora* sp.	14	**21.66**
6	*Pithophora* sp	15	37.5

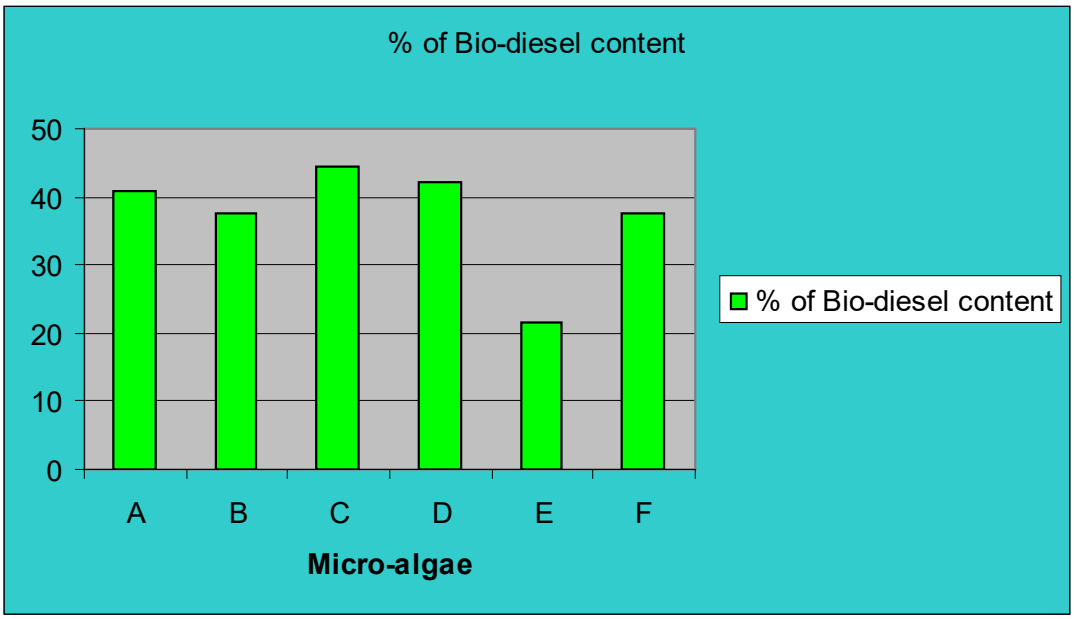

Fig. 9: Graphical presentation of bio-diesel content (%) in micro-algae: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

Table 19: Measurement of byproduct after oil extraction from micro-algal biomass.

S.N.	Micro-algae	Byproduct (g)
1	*Oedogonium* sp.	**8.17**
2	*Oscillatoria* sp.	8.2
3	*Chlorococcum* sp.	23.70
4	*Closterium* sp.	14.5
5	*Cladophora* sp.	9.7
6	*Pithophora* sp	**25.92**

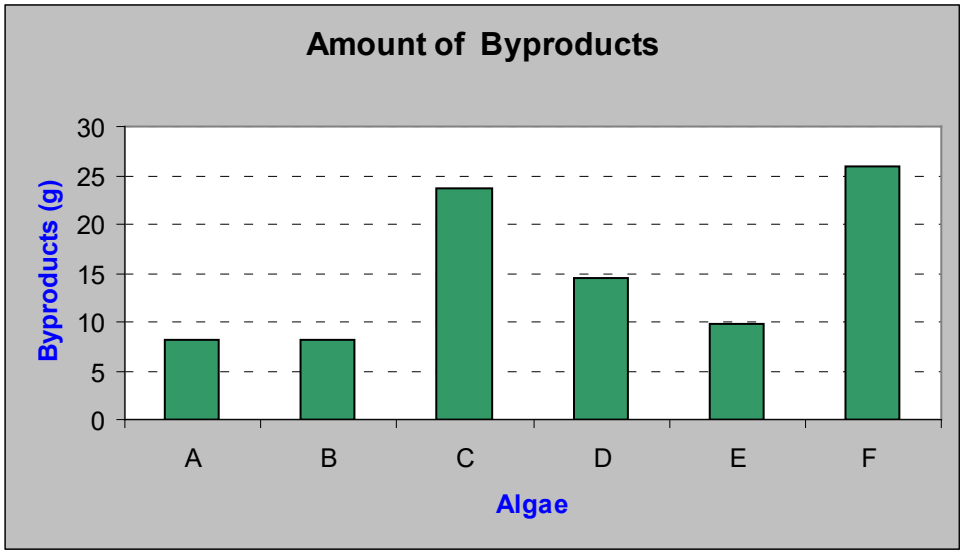

Fig. 10: Graphical presentation of byproduct (Glycerin, water and pigments) after oil extraction of Micro-algal biomass: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

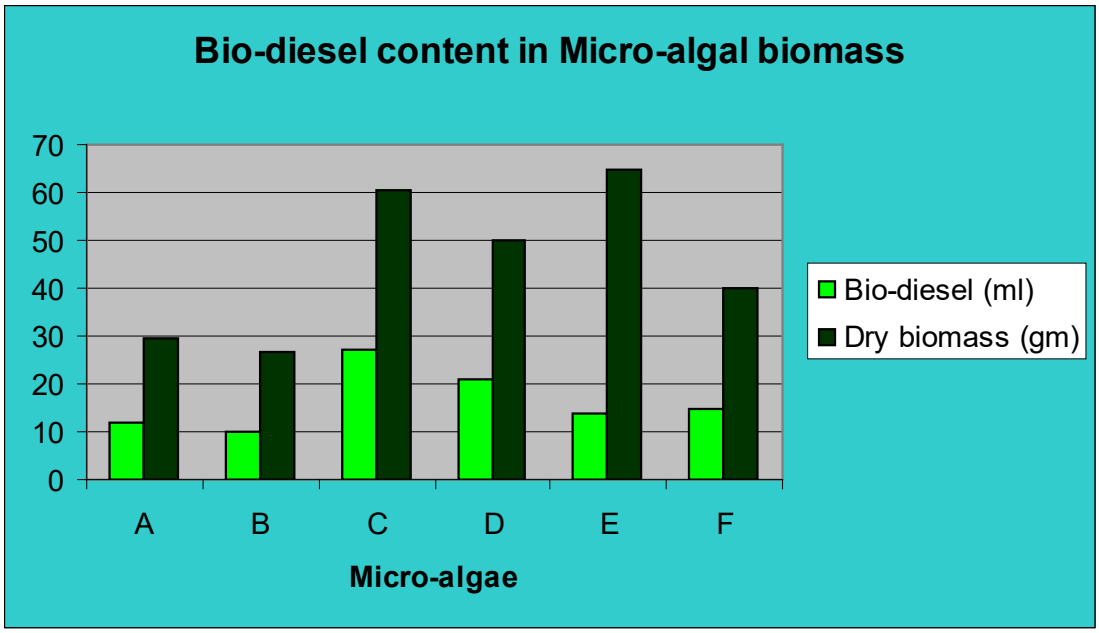

Fig. 11: Comparative study on bio-diesel content (%) in micro-algae according to the bio-mass: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

4.4.2: Quality test of algal bio-diesel and byproducts (Glycerin, water and pigments):

The measured pH and OD of extracted algal bio-diesel and byproducts given as below:

Measurement of pH:

The pH range of Bio-diesel and byproduct is 7-7.4 and 10.7-12, respectively (Table 20 and Fig. 12).

Table 20: pH of extracted micro-algal Bio-diesel and its byproduct (Glycerin and pigments).

S.N.	Micro-algae	pH	
		Biodiesel	Byproduct
1	*Oedogonium* sp.	7.40	12
2	*Oscillatoria* sp.	7.38	11.1
3	*Chlorococcum* sp.	6.98	10.8
4	*Closterium* sp.	6.78	11.6
5	*Cladophora* sp.	6.90	10.8
6	*Pithophora* sp	7.00	10.7

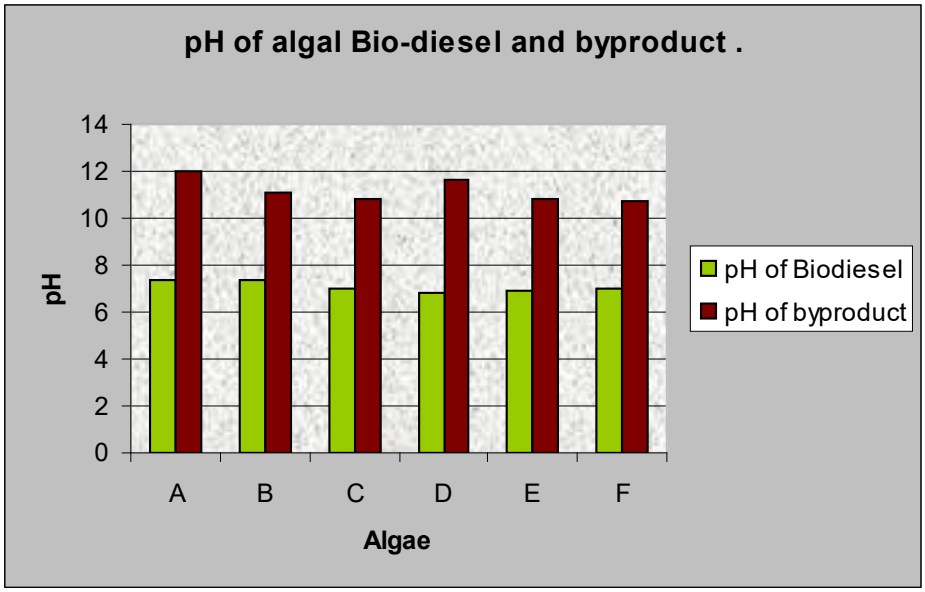

Fig. 12: pH of extracted micro-algal bio-diesel and its byproduct (Glycerin and pigments) : A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

Optical density (OD):

The Optical Density (OD) range of bio-diesel and byproduct is 0.8-0.95 and 0.8-1, respectively (Table 21, Fig. 13 and Fig. 14).

Table 21: Optical Density (OD) of extracted micro-algal bio-diesel and its byproduct (Glycerin and pigments).

S.N.	Micro-algae	Optical Density (OD)	
		Bio-diesel	Byproduct
1	*Oedogonium* sp.	0.8	0.8
2	*Oscillatoria* sp.	0.79	0.8
3	*Chlorococcum* sp.	0. 79	1.00
4	*Closterium* sp.	0.95	1.00
5	*Cladophora* sp.	0.81	0.8
6	*Pithophora* sp	0.82	1.8

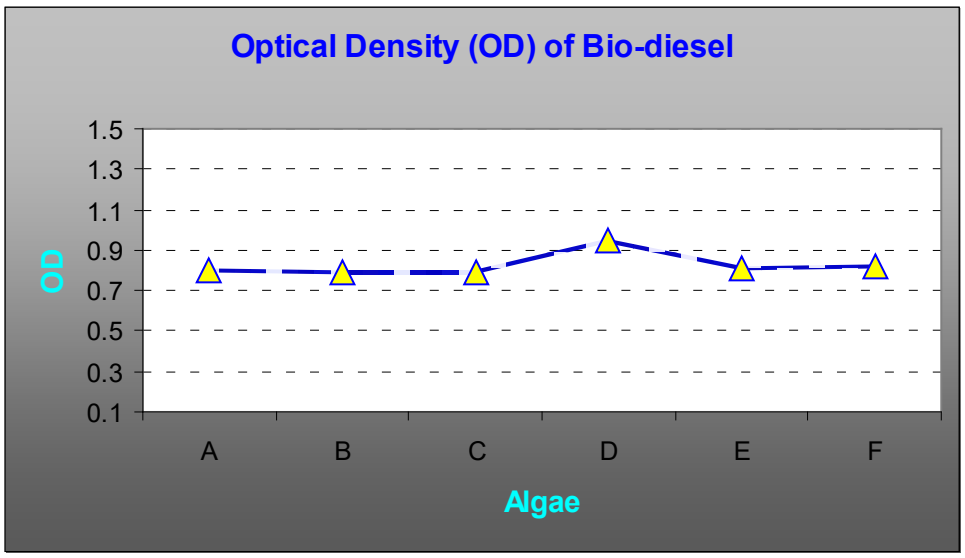

Fig. 13: Optical Density (OD) of extracted micro-algal bio-diesel: A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

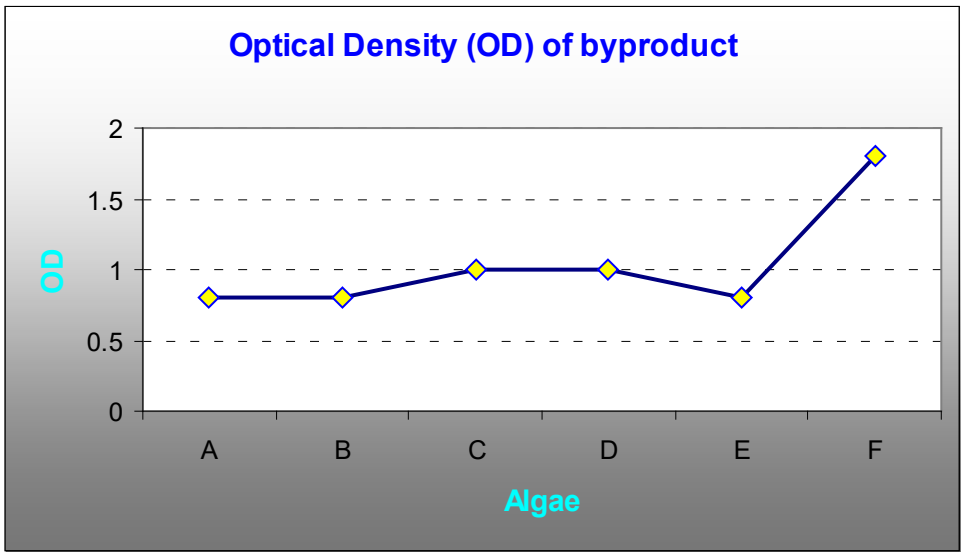

Fig. 14: Optical Density (OD) of extracted micro-algal bio-diesel's byproduct (Glycerin and pigments) : A. *Oedogonium* sp., B. *Oscillatoria* sp., C. *Chlorococcum* sp., D. *Closterium* sp., E. *Cladophora* sp.and F. *Pithophora* sp.

5: DISCUSSION

Algal Culture:

In this study six different algal strains (viz. *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp.) were collected from natural habitats of Rajshahi University area. With careful step by step algal strains were isolated. After isolation and identification *In vitro* algal monoculture has been established. Three culture medium viz. BBM Fogg's and Chu 10D were used for *In vitro* algal monoculture.

At *In vitro* condition, *Chlorococcum* sp. showed best growth (51.01g±0.01) and lowest growth by *Oscillatoria* sp. (29.01g±0.02) among the selected algae in BBM. Kirrolia (2012) isolated *Chlorococcum* sp. grown at 25 ^{0}C, to study the lipid content and chlorophyll contents. He further studied the effect of various concentrations of NaCl on the isolated algal species of *Chlorococcum* sp. and found increased biomass yield at 0.2mM NaCl concentration as compared to control. Initial increase of NaCl concentration from 0.0-0.2 mM decreased the lipid accumulation. According to him BG-11 and BBM are the best suited media for the growth of this species. Microalgal growth does not only depends on the temperature, light and nutrient availability, but is also highly affected by the salinity and culture media composition. Lee and Kim (2002) stated, the physiological factors like pH and salinity or chemical factors like media composition influence the growth rate, physiological status and biochemical composition of cyanobacteria in culture condition. Shruthi and Rajashekhar (2014) showed that, the growth rates ranged between 0.37 and 1.02 per day for all four selected species with varying pH. Protein was the major component observed in all four species and it was maximum in *Oscillatoria tenuis* (33.7% of dry weight). *Lyngbya aestuarii* showed higher concentration of total carbohydrate (18.4% of dry weight) and total lipid was highest in *Plectonema boryanum* (17.2% of dry weight).

In Fogg's medium, *Chlorococcum* sp. showed best growth (50.20g ±0.01) and lowest by *Cladophora* sp. (28.70g±0.03). On the other hand, six algae have been growing in Chu medium at ±30°C and increase their biomass according

to days. In Chu medium, *Chlorococcum* sp. exhibited best growth (49.20g±0.02) and lowest by *Cladophora* sp. (26.76g±0.1) among the selected strains. Shilpkar and Sundaramoorthy (2010) findings showed that, the carbohydrate comes out to be maximum in Chu 10D (0.065 mg ml^{-1}) followed by Chu 10D medium (0.055 mg ml^{-1}), whereas BG-11 supports the best protein content (0.025 mg ml^{-1}) followed equally by BBM and Allen media (0.16 mg ml^{-1}). Munir *et al* (2015) studied the effect of growth media, temperature, pH level, light intensity and aeration on growth of *Spirogyra* sp. and *Oedogonium* sp. and *Chlorella* sp.. It was observed that both *Oedogonium* sp. and *Chlorella* sp. grow well in Blue green medium while *Spirogyra* sp. showed better growth in BBM. Optimum temperature for algal growth was between 24-28 ^{o}C. They observed that neither very low nor very high pH is suitable for algal growth and at pH 7.5 algae were able to grow at maximum with fresh weights of 4.89±0.091 g and 4.79±0.021 g for *Spirogyra* sp. and *Oedogonium* sp. respectively. They further added, artificial fluorescent light resulted in an increased growth of algae as compared to sunlight or when algae placed near window and exposed to indirect sunlight.

In this experimental study, four pH values (i.e., 5.5, 6.5, 7.2 & 8.0) have been used to find out the optimum pH value for more *In vitro* growth of algae in three different culture Medium (viz. BBM, Fog's medium and Chu medium) at 30°C. All algae were varying in their growth response on various pH values of three different culture Medium.

In BBM, six algae exhibited suitable growth when pH is 6.5 and medium growth in pH 7.2. On the other hand, there was no growth of six algae in pH 5.5 and 8.0. In Fog's medium, six algae have been showed suitable growth in 7.2 pH and medium growth in pH 6.5. On the other hand, there was no growth of six algae in pH 5.5 and 8.0. In Chu medium, six algae showed suitable growth in 7.2 pH and medium growth in pH 6.5. On the other hand, there was no growth of six algae in pH 5.5 and 8.0. These result indicated that *in vitro* algal growth is influenced by the pH value, especially when the pH is higher

than 7.2 and lower than 6.5. According to Somchai *et al* (2008), 7.5-9.0 pH were suitable for the growth of *Oscillatoria* sp. and *Microcystis* sp. Shruthi and Rajashekhar (2014) used four isolates of cyanobacteria namely, *Oscillatoria tenuis*, *O. splendida*, *Lyngbya aestuarii* and *Plectonema boryanum* which showed fast growth under laboratory condition were selected to study their optimum salinity and pH requirement to get high biomass. In their study, the culture media varying pH (6, 6.5, 7, 7.5, and 8) were used. Susana (1991) reported, the optimum condition for the growth of *Spirulina platensis* at light intensity 2,500-10,000 lx, pH 6.5-9.0, 30-35°C temperature and 0-7.6% chlorinity. El-Aty *et al* (2014) said, the cultured media were incubated at 30 ± 2 °C without aeration and under continuous illumination of fluorescent lamps with intensity 2500 lux. Shruthi and Rajashekhar (2014) showed that, *Oscillatoria* tenuis and *Plectonema boryanum* got maximum biomass production between pH 6.5 and 7.5. During present *Oscillatoria* sp. maximum growth at pH 7.2.

Biodiesel production:

This study was undertaken to know the proper transesterification, amount of biodiesel production (ester) and physical properties of biodiesel to compare among six algae viz. *Oedogonium* sp., *Oscillatoria* sp., *Chlorococcum* sp., *Closterium* sp., *Cladophora* sp. and *Pithophora* sp.. Biodiesel production was higher in *Chlorococcum* sp. (44.59%) and lower in *Cladophora* sp. (21.66%) among the six selected algae. Spolaore *et al*. (2006) evaluated that microalgae can provide several different types of renewable biofuels. Many researchers (Thomas, 2006; Roessler *et al*., 1994; Banerjee *et al*., 2002; Chisti, 1980-1981) reported biodiesel can be derived from microalgal oil. Chisti (2007) reported that, the oil content (% dry wt) of some microalgae viz. *Botryococcus braunii*, *Chlorella* sp. *Schizochytrium* sp., *Neochloris oleoabundans*, *Nannochloropsis* sp. *Nitzschia* sp.. was 25–75%, 28–32%, 50–77%, 35–54%, 31–68% and 45–47%, respectively. Pankaj. (2011) found that, the oil content percentage of *Tolypothrix*, *Pithophora*, *Spirogyra*, *Hydrodictyon*, *Cladophora* was 12.78%, 10.37%, 14.82%, 13.58%,11.76%,

and their pH was 7, 7, 7 6, 6, respectively. Hossain (2008) found that, biodiesel production in *Spirogyra* sp. and *Oedogonium* sp. was 93% and 95%, and suitable pH for growth was 8, respectively. Munir *et al* (2015) observed, the oil content of the three algal species is in order *Chlorella* sp. > *Oedogonium* sp. > *Spirogyra* sp.

Chisti (2007) stated that biodiesel derived from oil crops is a potential renewable and carbon neutral alternative to petroleum fuels. Microalgae appear to be the source of renewable biodiesel that is capable of meeting the global demand for transport fuels. Like plants, microalgae use sunlight to produce oils but they do so more efficiently than crop plants. Oil productivity of many microalgae greatly exceeds the oil productivity of the best producing oil crops.

Biomass (after oil extraction) was higher in *Cladophora* sp. (64g) and lower in *Oscillatoria* sp. (26g). On the other hand, the sediments (glycerine, water and pigments) were higher in *Pithophora* sp. (25.92g) and lower in *Oedogonium* sp. (8.17g) among the selected strains. Hossain (2008) found the biomass production in *Spirogyra* sp. and *Oedogonium* sp. was 43.3% and 33.6%, respectively. The higher value of lipid content might be because of lower level of nitrates in the media as similar results of higher lipid contents in green algae *Chlorella vulgaris* at lower $NaNO_3$ and KNO_3 were reported by Tornabene *et al*. 1983.

The pH range of Bio-diesel and byproduct is 7-7.4 and 10.7-12, respectively. This result is similar with Pankaj *et al*. (2011) for *Pithophora* sp. and *Cladophora* sp.. The Optical Density (OD) range of Bio-diesel and byproduct is 0.8-0.95 and 0.8-1.0, respectively.

It appears that *Chlorococcum* sp. is higher biodiesel containing algae than rest of five. Apart *Chlorococcum* sp., *Closterium* sp. and *Oedogonium* sp. also do have their potentiality for biodiesel. For a country like Bangladesh where

every drop of oil/ fuel have to be imported algal biodiesel can change the scenario. If proper long term planning and investment is done in this sector.

Round the world, current efforts and business investment are driving attention and marketing efforts on the promises of producing algal biodiesel and superior production systems. Producing algal biodiesel requires large-scale cultivation and harvesting systems, with the challenging of reducing the cost per unit area. At a large scale, the algal growth conditions need to be carefully controlled and optimum nurturing environment have to be provided. Such processes are most economical when combined with sequestration of CO_2 from flue gas emissions, with wastewater remediation processes, and/or with the extraction of high value compounds for application in other process industries. Current limitations to a more widespread utilization of this feedstock for biodiesel production concern the optimization of the microalgae harvesting, oil extraction processes, and supply of CO_2 for a high efficiency of microalgae production. Also, light, nutrients, temperature, turbulence, CO_2 and O_2 levels need to be adjusted carefully to provide optimum conditions for oil content and biomass yield. It is therefore clear that a considerable investment in technological development and technical expertise is still needed before algal biodiesel is economically viable and can become a reality. This should be accomplished together with strategic planning and political and economic support.

Bangladesh being a developing country must take an initiative to patronize algae based bio-diesel production system from governmental level to ensure it’s better future.

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