
# Estimation of Oil Content in Locally Isolated Algal Strains for Prospective Biodiesel Production

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Islamabad – Pakistan

(2013)

# Estimation of Oil Content in Locally Isolated Algal Strains for Prospective Biodiesel Production

Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree of

BACHELORS OF SCIENCE

IN

ENVIRONMENTAL ENGINEERING

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# CERTIFICATE

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towards partial fulfillment for the award of degree of

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embodies original work carried out by the students under my supervision.

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# APPROVAL SHEET

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We dedicate this effort

to

the forgotten and unrecognized legacy of Muslim scientists and researchers;

whose contributions laid the foundations of modern-day science & technology.

The pioneers of scientific knowledge and wisdom;

*Al-Biruni, Al-Zahrawi, al-Haitham, Jabar bin Haiyyan, Al-Khwarizmi*

and many others ...

Unfortunately their names have been lost in history.

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# List of Abbreviations

| | |
|---|---|
| GHG(s) | Greenhouse Gas(es) |
| MJM | Modified Johnson's Medium (J/I) |
| BBM | Bold's Basal Medium (Modified) |
| DS | Dunaliella salina |
| CV | Chlorella vulgaris |
| TC | Tetraselmis chuii |
| NO | Nannochloropsis oculata |
| KKL-5 | Local algal strain isolated from Kallar Kahar Lake Sample – 5 |

# ABSTRACT

The global issues like fossil fuel depletion, increasing levels of $CO_2$ and environmental damages can be addressed by using alternative environment friendly fuels from different feedstock including algae. Algae have a high growth rate (*especially microalgae*); yield more oil than conventional terrestrial crops and can be grown on freshwater, wastewater and saline water. Strategies can be devised to grow algae in Pakistan for targeted products including biomass, animal feed and biofuels.

The study aimed to isolate local algal strains which can resist local environmental conditions for optimum yield of oil and biomass leading to subsequent fuel conversion. Algal samples were collected from Kallar Kahar Lake (Jhelum) during summer season and from Rawal Lake & Sawan River (Rawalpindi) during winter season; enriched in *Bold's Basal Medium (Modified)* at pH 6.8, incubated and illuminated under controlled conditions. Dilution, micro-pipetting, heat exposure, spreading and streaking techniques were employed for isolation of strains. Growth conditions were standardized to get maximum biomass at IESE Chemistry Lab. A single strain was selected out of pure cultures of local isolated strains which showed significant growth rate. The selected strain was represented as KKL-5 *(from Kallar Kahar Lake)*. This local algal strain is yet to be identified. It was found that this strain can withstand temperatures from 30°C up to 48°C and had a maximum cell count of approx. 29 million cells per ml. Cultured strains were harvested using 0.1% alum (100 mg/L), centrifuged at 5000 rpm for 14 min, dried in oven for 24 hours at 85°C temperature and pulverized in pestle-mortar apparatus. Soxhlet extractor was employed to extract underlying lipids by using n-hexane as solvent. Growth rate of KKL-5 was compared with pure cultures of *Chlorella vulgaris, Nannochloropsis oculata, Tetraselmis chuii* and *Dunaliella salina,* grown under the same set of environmental conditions. KKL-5 showed 15 times higher growth rate than the other pure algal strains in local environmental conditions.

*Chapter 1*

# INTRODUCTION

"The life-expectancy of Industrial Civilization is less than one-hundred (100) years. Industrial Civilization doesn't evolve; rather, it rapidly consumes the necessary physical prerequisites for its own existence. It's short-term, unsustainable" (Duncan, 2000).

The entire world's economy is based on oil and other fossil fuels. They provide fuel, lubricants, asphalt, paint, plastics, fertilizer, and many other products. In 1850, before commercial production began, there were about 2 trillion barrels of oil in the ground. By about the year 2010, half of that oil had been consumed, so about 1 trillion barrels remain. At the moment about 30 billion barrels of oil are consumed annually, and that is probably close to the maximum that will ever be possible. By the year 2030, some analysts say, oil production will be down to about half of that amount (Goodchild, 2010).

Since 1751, approximately 337 billion tons of carbon have been released to the atmosphere from the consumption of fossil fuels and cement production. The 2007 global fossil-fuel carbon emission estimate of 8365 million metric tons of carbon represents an all-time high and a 1.7% increase from 2006 (See Figure 1-1).

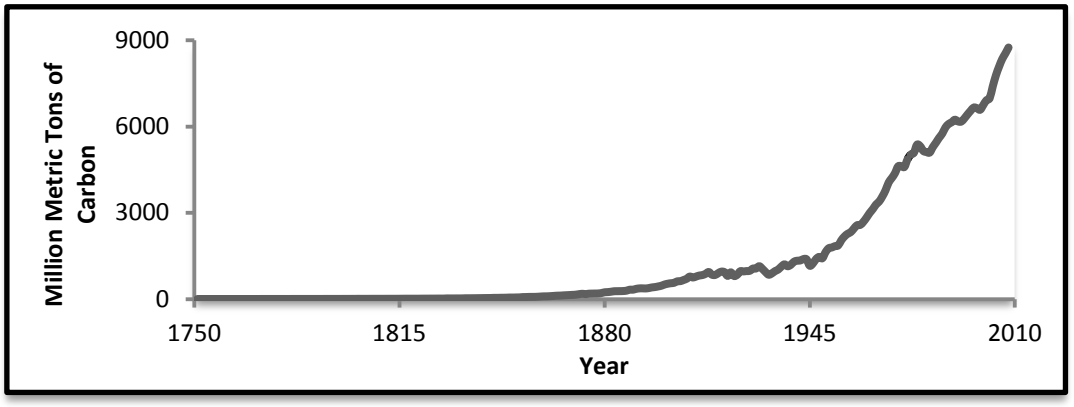


**Figure 1-1: Global carbon emissions' estimate from fossil fuel combustion** [4]

The world's fossil fuel supply has accumulated over vast eons. After we use up this non-renewable energy source, it is gone until natural processes create more over a few hundred million more years. Ever since that first oil well punched into the earth in 1859, demand for crude oil has equalled supply. The more oil that was pumped worldwide (an average 2 – 5 % more each year), the more got used (Emery, 2010). Table 1-1 shows the world fossil fuel depletion trends.

**Table 1-1: World fossil fuel reserves and projected depletion** [31]

| **Global Fossil Fuel Reserves** | **World Petroleum** (Billion Barrels) | **Natural Gas** (Trillion Cubic Feet) | **Coal** (Billion Short Tons) |
|---|---|---|---|
| **World Reserves** (Jan 1, 2000) | 1017 | 5150 | 1089* |
| **World Potential Reserve Growth** | 730 | 3660 | -- |
| **World Undiscovered Potential** | 939 | 5196 | -- |
| ***Total Reserves*** | *2686* | *14,006* | *1089* |
| **Annual World Consumption** | 27.340 | 84.196 | 4.740 |
| **Years of Reserves Left**** | 98 | 166 | 230 |

**World Estimated Recoverable Coal*
***Based on current levels of consumption and estimated total reserves*

According to the National Oceanic and Atmospheric Administration's National Climatic Data Centre the average combined global land and ocean surface temperature for January – September 2012 was the eighth warmest such period on record, at 0.57°C (1.03°F) above the 20$^{th}$ century average. Since the Industrial Revolution began around 1750, human activities have contributed substantially to climate change by adding $CO_2$ and other heat-trapping gases

to the atmosphere (US EPA, 2012). These greenhouse gas emissions have increased the greenhouse effect and caused Earth's surface temperature to rise. The primary human activity affecting the amount and rate of climate change is greenhouse gas emissions from the burning of fossil fuels.

A research study on ocean acidification states that due to increasing atmospheric carbon dioxide, oceans are absorbing $CO_2$ from the atmosphere and this is causing chemical changes by making them more acidic i.e., decreasing the pH of the oceans (The Royal Society, 2005). In the past 200 years, oceans have absorbed approximately half of the $CO_2$ produced by fossil fuel burning and cement production. Calculations based on measurements of oceans' surfaces and our knowledge of ocean chemistry indicate that this uptake of $CO_2$ has led to a reduction of the pH of surface seawater of 0.1 units, equivalent to a 30 % increase in the concentration of hydrogen ions.

## 1.1 What is Biofuel

Biofuel is a fuel produced from renewable resources especially plant biomass, vegetable oils, and treated municipal and industrial wastes.

Biofuels are considered neutral with respect to the emissions of carbon dioxide. When burned/combusted, biofuels release the carbon back into the atmosphere and have no overall effect on atmospheric $CO_2$ levels. In contrast, fossil fuels contain carbon that has been locked up underground for millions of years. Burning a fossil fuel increases the level of $CO_2$ in the atmosphere, but it is not balanced out by photosynthesis (Martin, 2013; Your Dictionary.com, 2013).

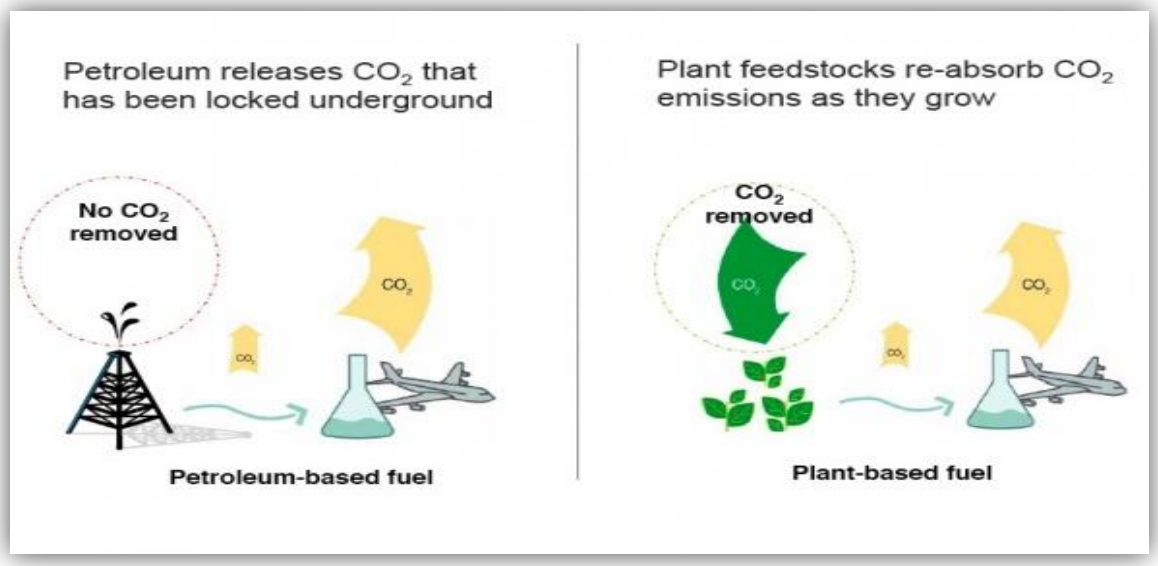


**Exhibit 1-1: Plant-based feedstock naturally removes $CO_2$ from the atmosphere** [23]

## 1.2 Significance of Algae and Algal Biofuels

Oil palm, rapeseed and jatropha can be used as a feedstock for biodiesel production but some experts are of the view that microalgae is set to eclipse all other biofuel feedstock as the cheapest, easiest and the most environment friendly to produce liquid fuel. Some types of algae comprise more than 50 percent oil, and an average acre of algae grown today for pharmaceutical industries can produce 5,000 gallons (19,000 liters) of biodiesel each year. By comparison, an average acre of corn produces 420 gallons (1,600 liters) of ethanol per year, and an acre of soybeans yields just 70 gallons (265 liters) of biodiesel per year (World Watch Institute, 2013).

Algae are the best $CO_2$ sequestration agents when compared to terrestrial photosynthetic plants. It is estimated that algae fix more than 90 giga-tons of carbon per year (Rasmussen, et al., 2009). Some of the perceived advantages of microalgae are that they grow rapidly, yield significantly more biofuel per hectare than oil plants (15-30 times more oil production as compared to terrestrial plants like oil palm or jatropha), utilize less water in closed systems as

their terrestrial counterparts, can grow on wastewater and saline water, can sequester excess carbon dioxide, produce a fuel that contains no sulphur with low toxicity that is highly biodegradable, does not compete significantly with food, fibre or other crops and does not involve destruction of natural habitats (Lone, et al., 2013; Dragone, et al., 2010).

Apart from biomass to produce biofuels, microalgae can be used to achieve other substances of great industrial value in various sectors, such as food, pharmaceuticals or cosmetics. Depending on the crop species, they can get antibiotics, polyunsaturated fatty acids, enzymes, proteins, vitamins, triglycerides or antioxidants (RUVID, Asociación, 2013).

## 1.3 Advantages of Microalgae for Biofuel Production

The utilization of microalgae for biofuels production offers the following advantages over higher plants (Dragone, et al., 2010):

i. Microalgae synthesize and accumulate large quantities of neutral lipids (20 – 50 % dry weight of biomass) and grow at high rates
ii. Microalgae are capable of all year round production, therefore, oil yield per area of microalgae cultures could greatly exceed the yield of best oilseed crops
iii. Microalgae need less water than terrestrial crops, therefore reducing the load on freshwater sources
iv. Microalgae cultivation does not require herbicides or pesticides application
v. Microalgae sequester $CO_2$ from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas (1 kg of dry algal biomass utilizes about 1.83 kg of $CO_2$)
vi. Wastewater bioremediation by removal of $NH_4^+$, $NO_3^-$, $PO_4^{---}$ from a variety of wastewater sources (e.g. agricultural run-off, concentrated animal feed operations, and industrial and municipal wastewaters)

vii. Combined with their ability to grow under harsh conditions and their reduced needs for nutrients, microalgae can be cultivated in saline/brackish water, coastal seawater on non-arable land, and do not compete for resources with conventional agriculture.

## 1.4 Objectives of the Research

The proposed study was designed to search for those species of algae which are able to thrive in local climatic conditions (e.g. high temperatures, arid conditions, etc.) and at the same time, yield high lipid content. The species, once isolated and identified, could later be checked for their biodiesel production potential.

Following objectives were set forth for this research;

i. Isolation of the strain with high oil/lipid content

ii. Determination of oil/lipid content in the isolated strain
*(as percentage of dry biomass)*

iii. Biodiesel production potential of the strain
*(by comparison with other known strains)*

iv. Identification of the isolated strains
*(using visual/morphological/genetic identification, if possible)*

*(See Section 2.5 for details about the scope of study).*

*Chapter 2*

# LITERATURE REVIEW

Biofuel can be produced from different types of feedstock. Microalgae are a type of feedstock which can be used for biofuel production. Studies have been conducted on different microalgal strains to check their biofuel yield and feasible temperature ranges. This chapter will present a brief review of the biofuel production potential of different microalgal strains reported in literature.

## 2.1 Potential of Microalgae for Biofuel Production

Continued use of petroleum sourced fuels is now widely recognized as unsustainable because of depleting supplies and their contribution to the accumulation of carbon dioxide in the environment. Renewable carbon neutral transport fuels are necessary for environmental and economic sustainability (Chisti, 2007).

**Table 2-1: Common biodiesel feedstock(s) and their oil yield in US gallons/acre** [35]

| CROP | OIL YIELD (US Gallons per Acre) |
|---|---|
| Corn | 18 |
| Soybeans | 48 |
| Jatropha | 202 |
| Coconut | 287 |
| Canola | 127 |
| Oil Palm | 636 |
| Microalgae | 6283 – 14641 |

With energy prices reaching historical highs, biodiesel as an alternative fuel is increasingly attracting attention. Microalgae have long been recognized as potentially good sources for biofuel production because of their high oil content and rapid biomass production. Microalgae grow very quickly compared to terrestrial crops (Table 2-1). They commonly double in number every 24 hours. During the peak growth phase, some microalgae can double every 3.5 hours (Wen, et al., 2009). Recent studies have shown that microalgal biomass is one of the most promising sources of renewable biodiesel that is capable of meeting the global demand for transport fuels. Biodiesel production by microalgae will not compromise the production of food, fodder and other products derived from crops.

Microalgal biomass contains three main components: proteins, carbohydrates, and lipids (oil).

**Table 2-2: Protein, carbohydrate and lipid content of *Chlorella vulgaris* and *Dunaliella salina*** [10]

| STRAIN | UNDERLYING CONTENT | | |
|---|---|---|---|
| | Proteins | Carbohydrates | Lipids |
| *Chlorella vulgaris* | 41-58 | 12-17 | 10-22 |
| *Dunaliella salina* | 57 | 32 | 6 |

## 2.2 Microalgae Strain Selection

Selection of a suitable microalgal strain is one of the most important criteria for biodiesel feedstock production. Out of the estimated 100,000 strains of microalgae which exist in nature, only a few of them are characterized for their potential for feedstock production. Microalgae are naturally acclimatized to a range of aquatic habitats, and it is sensible to use strains for feedstock production which are isolated from native environments (Sheehan, et al.,

1998). When choosing the algal species for an algae production system, it is important to know what characteristics of algae are wanted. When the purpose is to use the algae as biofuel, the following characteristics are important: high lipid productivity, high carbon dioxide sinking capacity, limited nutrient requirements, tolerance to a wide range of temperatures, and a fast productivity cycle (Edberg, 2010).

### 2.2.1 Chlorella vulgaris

*Chlorella vulgaris* can be found both in freshwater as well as marine water habitats. *Chlorella vulgaris* has a simple life cycle and simple nutritional requirements. *Chlorella vulgaris* produces a total lipid content of about 11 % of the dry biomass, in an environment with 10 % $CO_2$ (Edberg, 2010). The optimum temperature for biomass production from *Chlorella vulgaris* is shown to be 30°C (Chinnasamy, et al., 2009). It has been shown that *Chlorella vulgaris* is a very effective organism in sequestering carbon dioxide and 1 kg of its dry biomass utilizes about 1.83 kg of carbon dioxide (Edberg, 2010).

### 2.2.2 Dunaliella salina

Dunaliella is a halo tolerant green alga that belongs to the phylum Chlorophyta (Shariati, et al., 2011). The total lipid content of *Dunaliella salina* is 6 - 21.2 % of its dry biomass (Devi, et al., 2012; Dragone, et al., 2010). Optimum temperature for the highest growth of *Dunaliella salina* is in the range of 21 – 22°C (Abu-Rezq, et al., 2010).

### 2.2.3 Nannochloropsis oculata

*Nannochloropsis oculata* is well known for its high biomass and lipid productivity; thus it is suitable for biodiesel production (Das, 2010). The total lipid content in *Nannochloropsis oculata* is 27.5 % of its dry biomass (Devi, et al., 2012).

### 2.2.4 Tetraselmis chuii

*Tetraselmis chuii* is a marine unicellular alga. *Tetraselmis chuii* culture is maintained in the algal-culture room under controlled conditions of temperature (25°C) in culture flasks having 32 – 34 % salinity (Gopinathan, 1986). Lipid content of this phototrophic algal strain is 17.23 – 23.5 % (Liu, et al., 2011).

## 2.3 Biodiesel Yield

The direct trans-esterification method results in the maximum biodiesel yield of 68.5 % in *Nannochloropsis oculata* followed by *Dunaliella salina,* for which the reported biodiesel yield is 66.6 %. However, the biodiesel yield of oil crops such as jatropha, coconut, groundnut, white soy bean, castor and red soy bean are 62.2 %, 57.4 %, 51.2 %, 37.8 %, 33.4 % and 9.4 % respectively as reported (Devi, et al., 2012).

## 2.4 Biofuel Production

The fatty acid fraction of the intracellular lipid generated via microalgae metabolism can be converted into fatty acid methyl esters (FAME) i.e. biodiesel via a chemical process known as trans-esterification (Ma, et al., 1999). Fatty acids are present in microalgae as free fatty acids (FFA), triglycerides (TG), diglycerides (DG) and monoglycerides (MG) (Das, 2010). Biodiesel consists of fatty acid methyl esters (FAME) (lipids) originating from vegetable oils and animal fats. Most attempts at making biodiesel from algae are done by first extracting the algal oil (lipids), then treating the algal oil similar to any other seed oil, and subsequently employing the trans-esterification process to produce biodiesel. Trans-esterification is the process where the triglycerides from vegetable oils and animal oils are converted into fatty acid alkyl esters, and glycerol using an alcohol. The reaction requires heat and a strong base catalyst. Free fatty acids can also be converted to biodiesel using an alcohol and a strong acid

catalyst. This is a well understood way used to produce biodiesel from vegetable oils and works equally well for algal oil (Edberg, 2010).

## 2.5 Algal Biofuel in Pakistan

Studies on algal strains are mostly conducted in the optimum temperature range of 20 – 35°C. The algal species mentioned above have the following maximum temperatures (Table 2-3):

**Table 2-3: Maximum thriving temperatures of algal species**

| SERIAL | ALGAL STRAIN | MAXIMUM TEMPERATURE |
|---|---|---|
| 1 | *Chlorella vulgaris* | **38 °C** (Virthie, et al., 2011; Attilio, et al., 2009) |
| 2 | *Nannochloropsis oculata* | **20 °C** (Attilio, et al., 2009) |
| 3 | *Dunaliella salina* | **24 °C** (Rathinam, et al., 2007) |
| 4 | *Tetraselmis chuii* | **20 °C** (San, et al., 2013; Sibel, et al., 2007) |

Pakistan however has severe climatic conditions ranging from very hot to very cold climates, i.e. from 45°C to -29°C (45°C in Jacobabad – Sindh, -29°C in Kallat – Balochistan). The proposed study was designed to screen local algal strains with sufficient oil content from hot climate (42°C) and cold climate (15°C) to be used for mass biofuel production in Pakistan.

Pakistan has an extreme climate in terms of temperature; it is especially very hot in summer months. Currently collected and investigated algal strains (for large scale biofuel production) survive at moderate temperatures (20 – 30°C), since algal culture research is being carried out in western countries with lesser average temperatures. Local strains can

withstand the local climatic and environmental conditions better than foreign strains. In order to explore biofuel production by algae as an option for warmer climates, locally isolated algal strains will be checked for their potential to be used for large-scale biofuel production in Pakistan.

*Chapter 3*

# MATERIALS AND METHODS

Algal samples were collected from different locations and enriched with growth media. Strains isolated from these samples were cultured, harvested and dried prior to oil extraction. The sampling procedures; culture methods; isolation, harvesting & drying techniques; and the methodology used for the extraction of oil are discussed in detail in the subsequent sections.

## 3.1 Sample Collection

Samples of local algae were collected from different water bodies. Samples from Kallar Kahar Lake were provided by Centre for Energy Systems (CES) – NUST while the rest of the samples were collected by the students. All of the samples were collected according to the standard algal sampling procedures. Sampling was carried out on the following dates from the locations as shown in Table 3-1.

**Table 3-1: Sampling details**

| DATE | LOCATION | TYPE OF ALGAE | COLLECTED BY |
|---|---|---|---|
| 22 JUL 2012 | Lake Kallar Kahar, Jhelum | Micro and Macro | Dr. Ehsan Ali |
| 10 DEC 2012 | Rawal Lake, Islamabad | Micro only | Liaqat Karim |
| 12 DEC 2012 | Sawan River, Rawalpindi | Micro and Macro | Syeda Asma |
| 2 JAN 2013 | Sedimentation Basin in Environmental Chemistry Lab, IESE – NUST, Islamabad | Macro only | Anum Masood, Liaqat Karim, Jamal Shahid & Syeda Asma |
| 16 FEB 2013 | Sawan River, Rawalpindi | Micro and Macro | Syeda Asma |

Besides these indigenous strains, some foreign strains were also sought to carry out a comparative study of growth, oil yield and other parameters, under local conditions. These strains have already been reported in various studies (*mentioned below*) to have a high oil yield. These strains were;

1. *Chlorella vulgaris* (Van, et al., 2012)
2. *Dunaliella salina* (Balavigneswaran, et al., 2013)
3. *Nannochloropsis oculata* (Chio, et al., 2009)
4. *Tetraselmis chuii* (Kyndt, et al., 2013)

*Chlorella vulgaris* is a freshwater strain, while rests of the three are marine strains.

## 3.2 Enrichment

*Chlorella vulgaris* and the strains which were obtained as samples were enriched in Bold's Basal Medium (Modified) *(See Appendix B)*. BBM is a highly enriched medium with low salinity and is used for culturing a variety of freshwater algal strains. BBM had a salinity of 0.6 % and pH was adjusted to 6.8. Since all the samples were collected from freshwater sources, so they were enriched in BBM.

The marine strains, i.e. *Dunaliella salina, Nannochloropsis oculata* and *Tetraselmis chuii* were enriched in Modified Johnson's Medium *(See Appendix A)*. Modified Johnson's Medium is a universal medium used for culturing marine strains. The salt concentration (NaCl) of MJM was adjusted to 2.5 % and pH was maintained at 7.5.

$CO_2$ was also provided to the algal cultures by aeration through air pumps normally used in aquariums. The enrichment was carried out at a temperature of 23 ± 1°C regulated by an incubator.

## 3.3 Isolation

Local algal samples were not pure samples i.e., they were not consisting of a single algal strain, and rather they were containing consortiums of different algal strains. The objective of the study was to isolate a local algal strain with high oil content. Isolation of the strains was carried out using the following techniques (Algal Culturing Techniques, 2005 pp. 83-100);

### 3.3.1 Microscopy

This technique involved isolating a strain using microscope. A single algal cell was picked up from the slide under observation in a microscope using micro-pipettes. This cell was then carefully transferred to sterile culture tube containing a minute quantity (often 1-2 ml) of the growth medium (i.e. BBM).

In cases, when it was not possible to isolate a single cell, a whole colony was picked up from the slide. It was then diluted before being transferred to the culture tube.

### 3.3.2 Spreading and Streaking

1 % BBM and 1 % MJM agar plates were prepared. The inoculum from algal samples was streaked and spread on the plates. Isolated colonies started appearing on the plates after about 20-30 days. Cells from isolated colonies were picked up using sterile wired loop and then transferred to the culture tubes containing 4-5 ml growth medium.

### 3.3.3 Temperature-based Isolation

Temperature was increased beyond 38°C for one of the samples of Kallar Kahar Lake. Only the species which was able to thrive at such a high temperature survived, while the rest died off. In this way, isolation of a single species was achieved. This was also confirmed by the microscopy of the sample.

## 3.4 Growth Conditions

Algae are photosynthetic organisms. They require $CO_2$ and a source of light to carry out the process of photosynthesis.

The algal strains were cultured indoors in Environmental Chemistry Lab – IESE in different vessels.

Following growth conditions were provided in the lab scale setup (Exhibit 3-1);

**Exhibit 3-1: Growth conditions provided to the culture vessels in IESE lab**

1. Continuous supply of $CO_2$ to the cultures was ensured via filtered aeration
2. Natural sunlight was provided to culture vessels via windows
3. Temperature was maintained at 25°C (±7°C) for all strains via incubator
4. Nutrients were provided via autoclaved Bold's Basal Medium (Modified) and Modified Johnson's Medium

By maintaining these growth conditions, the volume of algal cultures was increased in subsequent stages as explained below:

- Stage 1: 2 ml – 20 ml
- Stage 2: 20 ml – 250 ml
- Stage 3: 250 ml – 1 liter
- Stage 4: 1 liter – 5 liters
- Stage 5: 5 liters – 20 liters
- Final Stage: 20 liters – 40 liters

## 3.5 Growth Rates

The growth rates of both local and foreign strains needed to be measured in order to carry out a comparative study. Standard/calibration curves of all the strains were established by measuring their cell counts in order to measure growth rates under local conditions.

The turbidity of a culture sample was determined followed by its cell count. The cell counts' values were plotted against the respective turbidity values to achieve the calibration curves. Cell counting was carried out by the standard method of haemocytometry using a nebular haemocytometer (Moheimani, et al., 2013).

Once these calibration curves were established, the cell count of any strain could easily be determined by finding out its turbidity value (in NTUs) and locating the corresponding cell

count value (in Million Cells per ml) from the plot *(See Section 4.1 for details)*.

## 3.6 Visual Identification

Algal strains from the samples collected were identified visually under bright field microscopy at magnifications ×400 and ×1000 (Bellinger, et al., 2010). Some of the algal strains which were successfully identified are discussed below.

### 3.6.1 Scenedesmus acuminates

This species was found in abundance in samples taken from Sawan River on 16 February 2013. This species of the genus Scenedesmus does not bear spines or projections 1000 (Bellinger, et al., 2010).

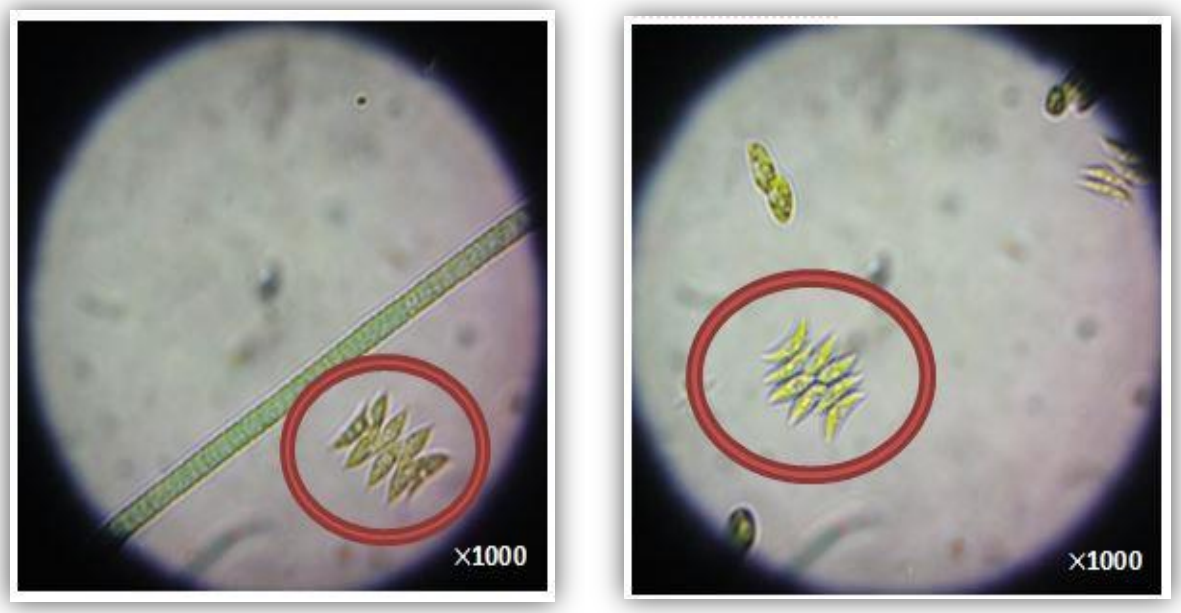


**Exhibit 3-2: Images of *Scenedesmus acuminates* from Sawan river sample**

Documented images of the strain can be viewed in the book "Freshwater Algae – Identification and Use as Bioindicators" (Bellinger, et al., 2010 p. 189). Exhibit 3-2 shows the images of the strain from local samples and Exhibit 3-3 shows the image obtained from

Protist Information Server (PIS) (Saitama, 2003).

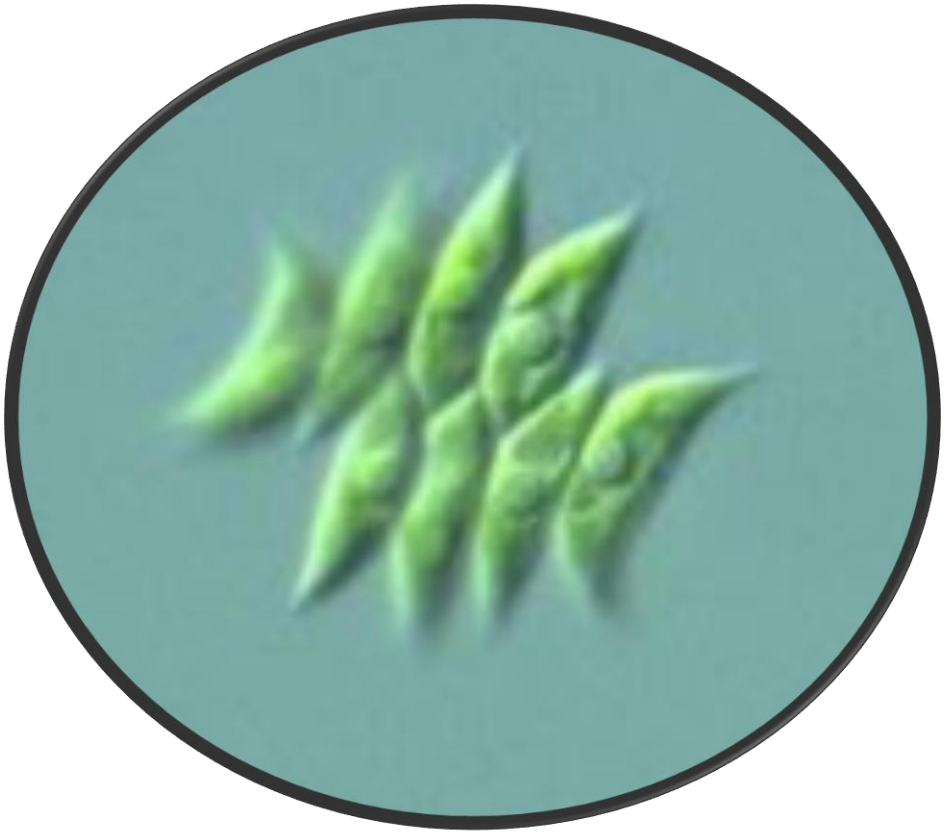

**Exhibit 3-3: An image of *Scenedesmus acuminates* from PIS** [27]

### 3.6.2 Stigeoclonium

This species of algae is macroscopic as a single strand and is visible by the naked eye. This algal species has a branched structure and is usually attached to surfaces via basal cells. It is also found in free flowing waters (Bellinger, et al., 2010). *Stigeoclonium* was found abundantly in the samples taken from Lake Kallar Kahar on 22 July 2012. Documented images of the strain can be viewed in the book "Freshwater Algae – Identification and Use as Bioindicators" (Bellinger, et al., 2010 pp. 145-146).

Exhibits 3-4 and 3-5 show the images of this strain from local samples and from other researches respectively:


**Exhibit 3-4: Images of *Stigeoclonium* from Kallar Kahar lake sample**

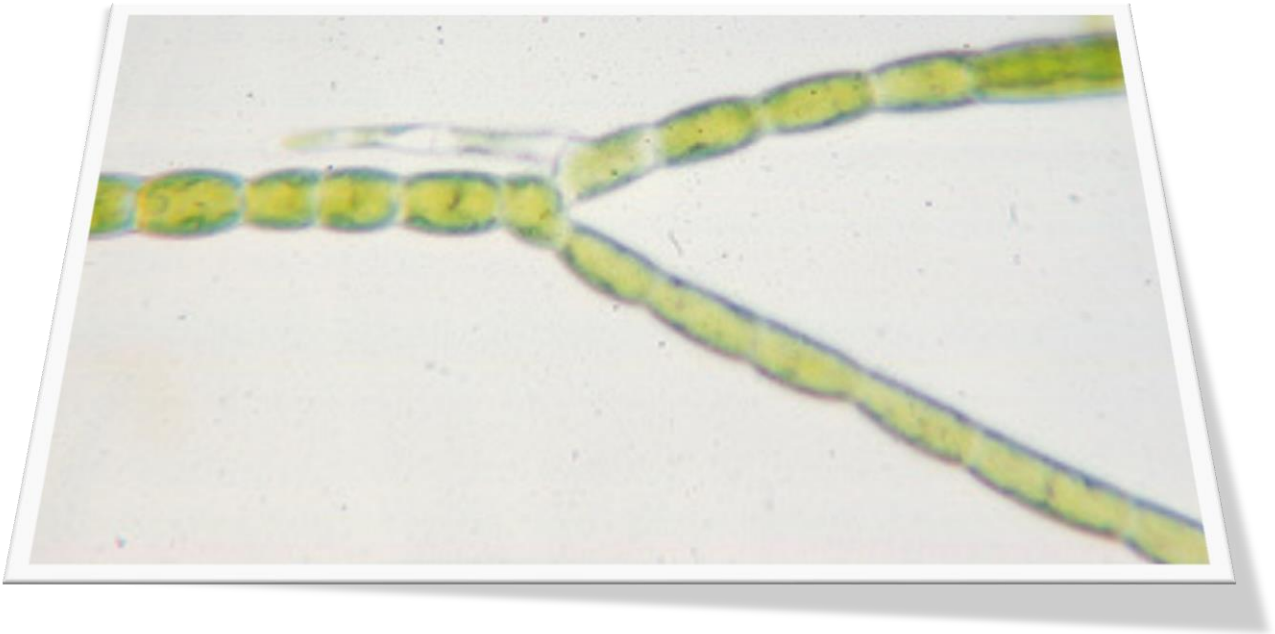

**Exhibit 3-5: Image of *Stigeoclonium* from DiscoverLife.com** [30]

### 3.6.3 Other Algal Strains Isolated

Some other algal strains which were successfully isolated from the samples are shown in Exhibit 3-6.

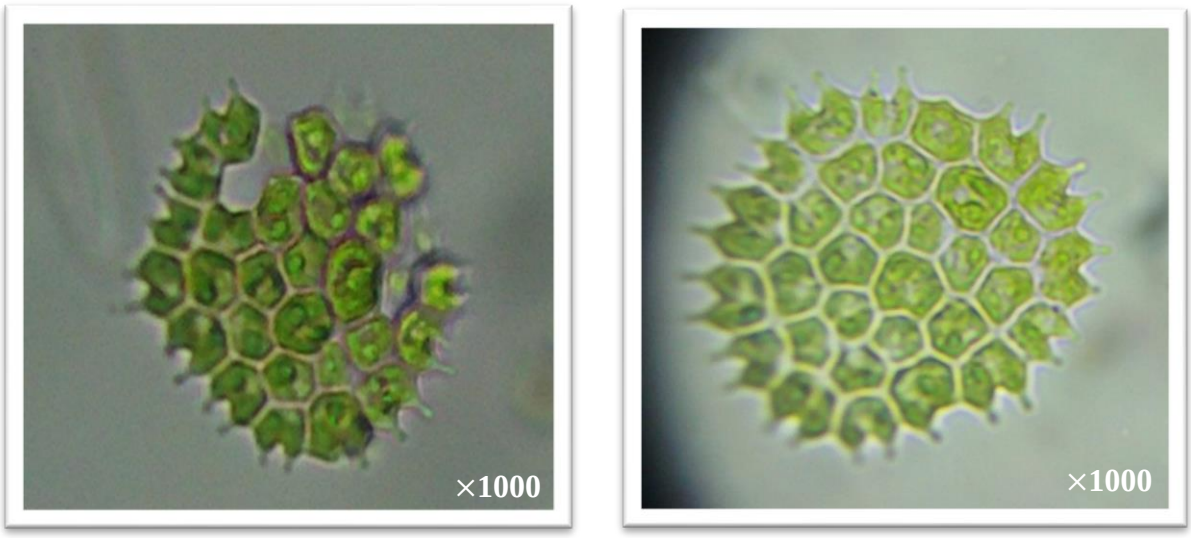


**Exhibit 3-6: Images of other algal strains from Sawan river sample**

Only the images of those isolated strains are included in Exhibit 3-6 which showed a significantly high growth rate. They can be further researched upon.

### 3.6.4 Foreign Algal Strains

The four algal strains namely *Chlorella vulgaris, Dunaliella salina, Nannochloropsis oculata* and *Tetraselmis chuii* were also observed under the light microscope to ensure that they are the same species. This precaution was undertaken to ensure the validity of results and nullify any possibility of contamination; and also to enable the comparison of growth rates and oil content of these species with local strains.

### 3.6.5 KKL-5

A local algal strain was isolated from the samples of Lake Kallar Kahar (sampling date: 22

July 2012). This local strain showed a very high growth rate as compared to other algal strains being researched upon in this study. The strain was represented as *KKL-5* and was also observed under bright field microscopy. However, identification of the genus and species of this particular strain requires further analyses and can be done by applying different staining and microscopy techniques. Following images in Exhibit 3-7 were taken from the isolated algal strain *KKL-5*.

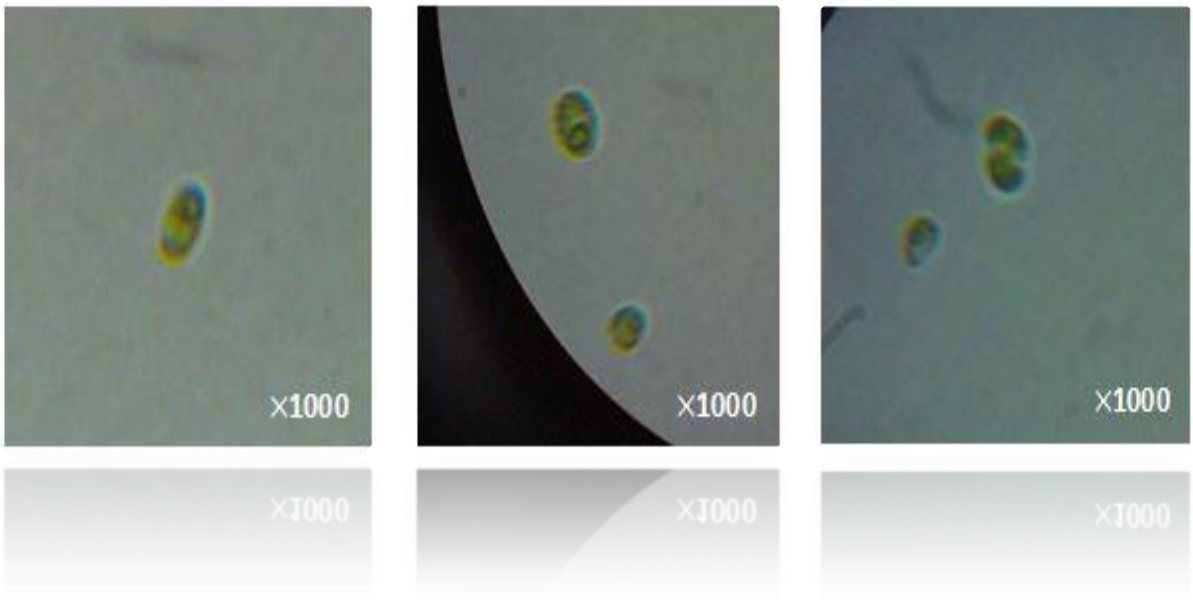


**Exhibit 3-7: Images of *KKL-5* strain from Kallar Kahar lake sample**

## 3.7 Selection of KKL-5 Strain

The proposed study had a limited span of time. So the growth rate of the strain was selected as limiting factor in the study. In this context, the local strain exhibiting the highest growth rate under local conditions was selected for further study, based on the results from growth curves. It was represented as *KKL-5*.

This local strain, i.e. *KKL-5* was studied along with the foreign strains i.e. *Chlorella vulgaris, Dunaliella salina, Nannochloropsis oculata* and *Tetraselmis chuii.*

## 3.8 Algal Harvesting

Algae are cultured in aqueous conditions. The concentration of algal biomass which is achievable in algal cultivation systems is relatively low. Oil extraction from algal biomass cannot be carried out unless it is harvested and dried. The separation of algae from growth medium is known as harvesting. Harvesting increases the biomass concentration and is considered a major bottleneck towards industrial processing of algae for biofuel production (Dragone, et al., 2010).

There are several techniques available for harvesting of algae, i.e. flocculation, centrifugation, filtration, ultra-filtration, air-flotation, auto-flotation, etc. Generally, harvesting of micro-algae is a two-stage process, i.e. bulk harvesting followed by thickening (Dragone, et al., 2010). However, following methodology was adopted for harvesting of the algal strains under study:

### 3.8.1 Jar Testing

The purpose of jar testing is to find out the optimum coagulant dosage required for effective flocculation/coagulation.

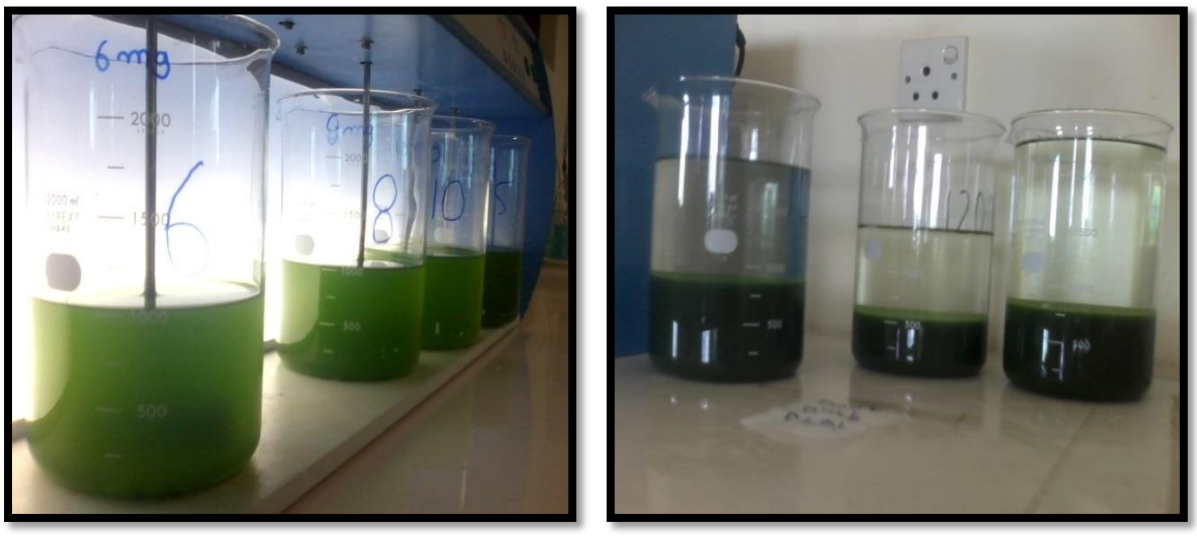


**Exhibit 3-8: Jar testing**

Jar testing was carried out in four beakers, each containing 1 liter culture of *Chlorella vulgaris* and having the same cell count (approx. 5.4 million cells/ml) (Exhibit 3-8). The turbidity of the culture before jar testing was 147 NTUs. Values of final turbidity of clear water over the settled algal biomass were compared to determine the optimum alum dosage (Table 3-2).

**Table 3-2: Jar testing results**

| SERIAL | ALUM DOSAGE (mg) | TURBIDITY (NTUs) | | | |
|---|---|---|---|---|---|
| | | I | II | III | Average |
| 1 | 250 | 2.48 | 2.49 | 2.54 | 2.5 |
| 2 | 400 | 1.49 | 1.5 | 1.47 | 1.49 |
| 3 | 500 | 0.668 | 0.63 | 0.636 | 0.64 |
| 4 | 600 | 1.51 | 1.58 | 1.53 | 1.54 |

Jar test yielded the following result: 500 mg of alum dosage required to harvest one liter of algal culture (Figure 3-1).

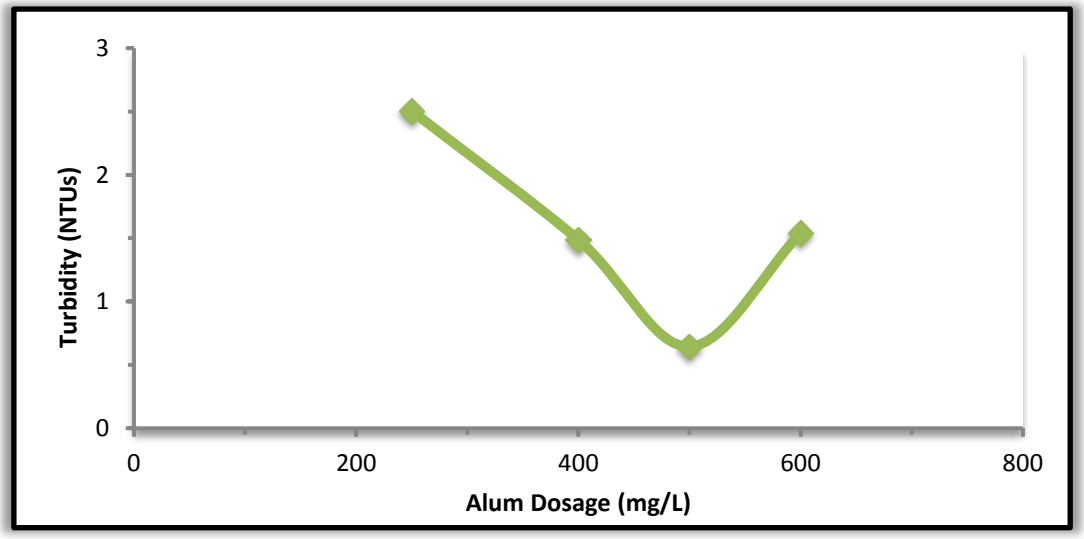


**Figure 3-1: Optimum alum dosage**

This optimal alum dosage was used for harvesting cultures of all strains, i.e. *Tetraselmis chuii, Dunaliella salina, Nannochloropsis oculata* and *KKL-5*.

### 3.8.2 Flocculation

The process of flocculation can be employed as a predecessor to the extraction of algal biomass from nutrient broth (i.e. the growth medium). The purpose is to achieve algal aggregates known as flocs. Since microalgal cells carry a negative charge, hence they do not self-aggregate. Addition of flocculants such as ferric sulphate, aluminum sulphate, etc. causes the microalgal cells to form aggregates without causing a significant change in composition (Dragone, et al., 2010).

Aluminum sulphate [$Al_2(SO_4)_3$] was added to the cultures as flocculent. The addition of flocculent was followed by rapid mixing or coagulation (at 20 rpm) for 1 minute and slow mixing or flocculation (at 8 rpm) for 20 minutes.

### 3.8.3 Sedimentation

The flocs formed by the flocculation process were allowed to settle for 24-48 hrs under gravity (Exhibit 3-9).


**Exhibit 3-9: Algal flocs after settling**

### 3.8.4 Thickening

The aim of this process is to concentrate the slurry achieved after the initial dewatering processes (i.e. flocculation, sedimentation, flotation, etc.). Methods commonly employed include centrifugation, filtration, and ultrasonic aggregation (Dragone, et al., 2010).

In this case, centrifugation was employed as a thickening technique. The dewatered algal slurry was subjected to centrifugation at 5000 rpm for 15 minutes. Dark green, thickened algal biomass was achieved after the process.

## 3.9 Drying

Algal biomass, harvested and thickened was dried in a hot air oven at 85°C for 24 hours. Drying removes all the residual moisture still present in the biomass. Presence of even minute quantity of moisture can disrupt the production of biodiesel at later stages.

All the algal cultures namely *KKL-5, Chlorella vulgaris, Dunaliella salina, Nannochloropsis oculata* and *Tetraselmis chuii* were subjected to harvesting and drying.

## 3.10 Pulverization

Pulverizing (grinding) was carried out using pestle-mortar apparatus and n-Hexane as a lubricant. Only the biomass from cultures of *KKL-5* and *Chlorella vulgaris* was pulverized.

The purpose of pulverization is to break/open the algal cell wall, since it is the hard part of the cell. Unless and until the cell wall is broken, access to underlying lipids is not possible.

Apart from pestle-mortar, other methods such as high pressure homogenizing, autoclaving, addition of hydrochloric acid, etc. have also been reported to be used as a substitute for pulverization. (Mendes-Pinto, et al., 2001).

## 3.11 Lipid Extraction

The extraction of algal oil i.e. lipids from *KKL-5* and *Chlorella vulgaris* was carried out by solvent extraction technique using Soxhlet Extraction apparatus. n-Hexane was used as solvent.

Several other organic solvents can also be used for the extraction of lipids but hexane has been reported to have a better extraction efficiency and low cost (Harun, et al., 2010).

## 3.12 Trans-esterification

After the extraction process, the microalgal oil can be treated further by a process known as trans-esterification to yield biodiesel. Trans-esterification is a process in which triglycerides from plant/algal oils are converted into fatty acid alkyl esters after treatment with alcohol (i.e. methanol or ethanol) in the presence of a catalyst, such as an alkali (NaOH or KOH) *(see Section 2.4 for details)*. Glycerol is obtained as a by-product. These long chain methyl esters are essentially the biodiesel molecules (Vasudevan, et al., 2008).

$$\text{Triglycerides} + 3\ \text{Alcohol} \rightleftharpoons 3\ \text{Methyl esters} + \text{Glycerol}$$

To achieve biodiesel from the extracted microalgal oil, sodium hydroxide was dissolved in methanol (by keeping it on an auto-stirrer for 24 hours). The extracted microalgal oil from *KKL-5* and *Chlorella vulgaris* was treated with methanol-sodium hydroxide solution to yield biodiesel and glycerol. Appearance of viscous fluid (glycerol) at the bottom confirmed that the reaction has proceeded in the forward direction.

*Chapter 4*

# RESULTS AND DISCUSSION

In order to analyze the growth rates of algal strains, cell counts were collected daily; standard curves & growth rate curves were established; and finally oil content was determined.

## 4.1 Standard Curves

The first part comprised of establishing standard curves for each strain. These curves were obtained by plotting the turbidity values (NTUs) of the algal culture against its cell count (million cells per ml). The cell count of each algal strain was obtained by the standard method of haemocytometry and the turbidity was measured using a turbiditimeter/nephelometer. The turbidity was adjusted by mixing autoclaved growth medium in a pure culture of the algal strain. The media used were BBM and MJM for freshwater and marine strains respectively. Standard curves established for each strain are shown in Figures 4-1 through 4-5.

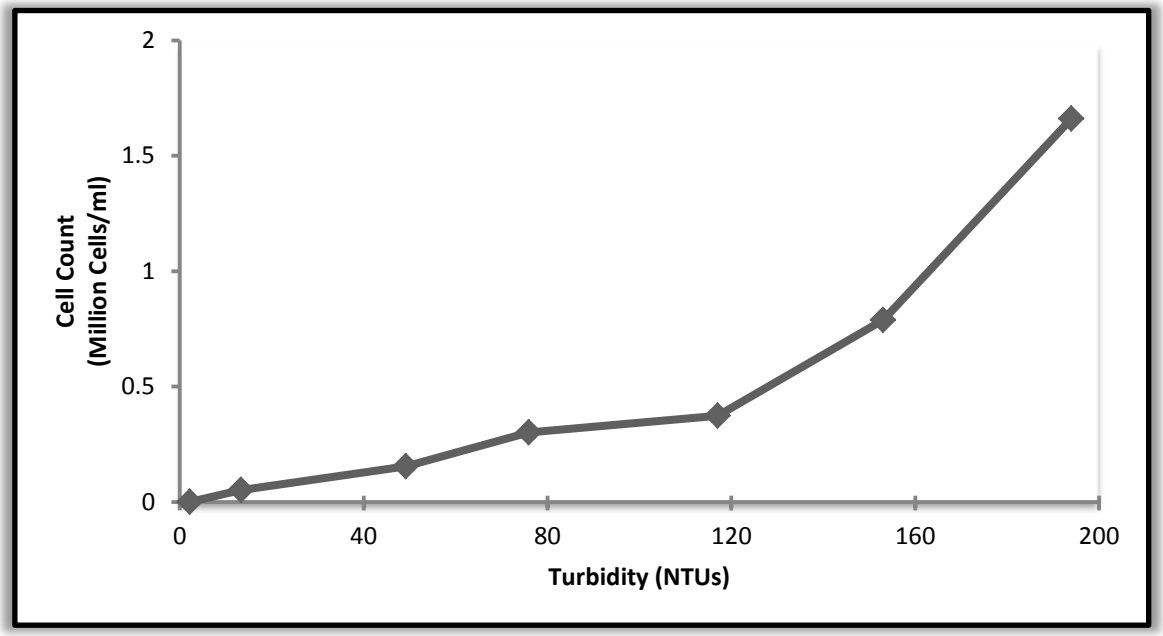


**Figure 4-1: Standard curve for *KKL-5***

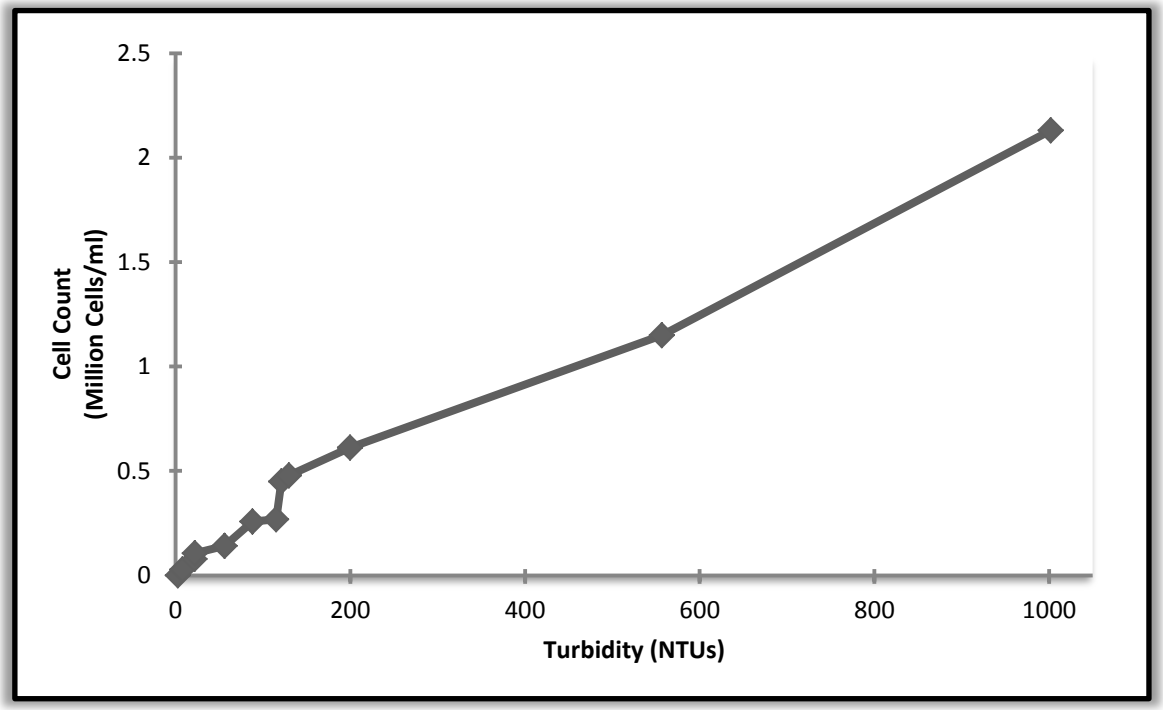


**Figure 4-2: Standard curve for *Chlorella vulgaris***

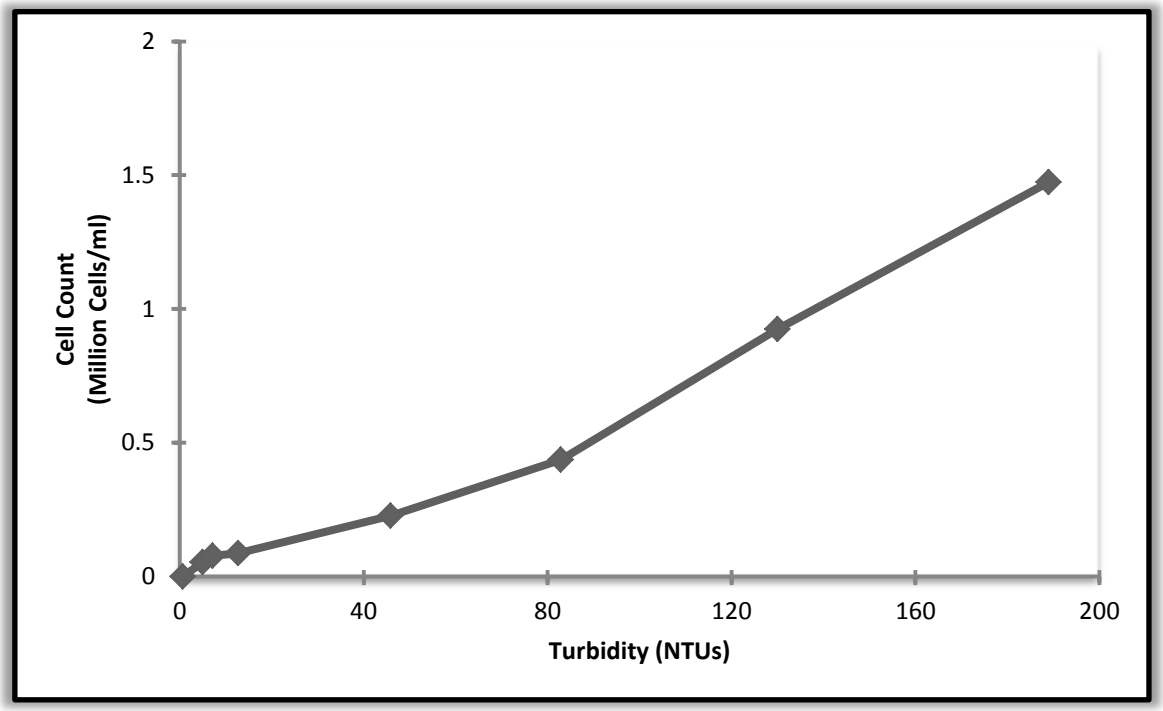


**Figure 4-3: Standard curve for *Dunaliella salina***

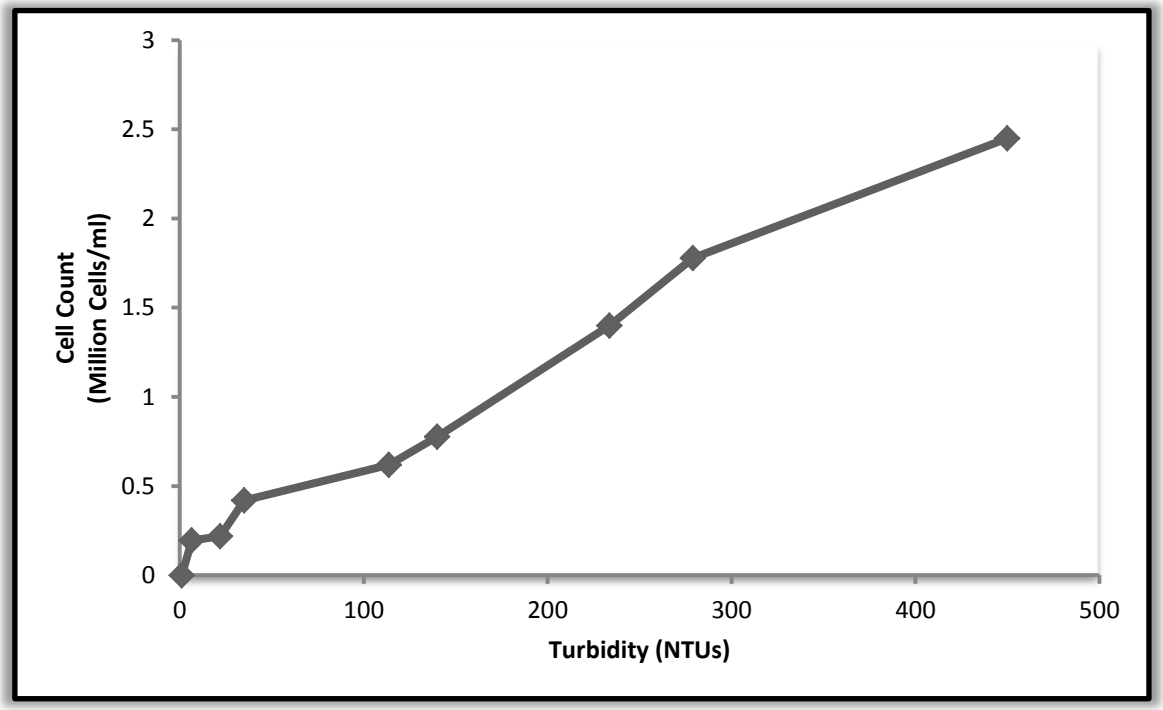


**Figure 4-4: Standard curve for *Nannochloropsis oculata***

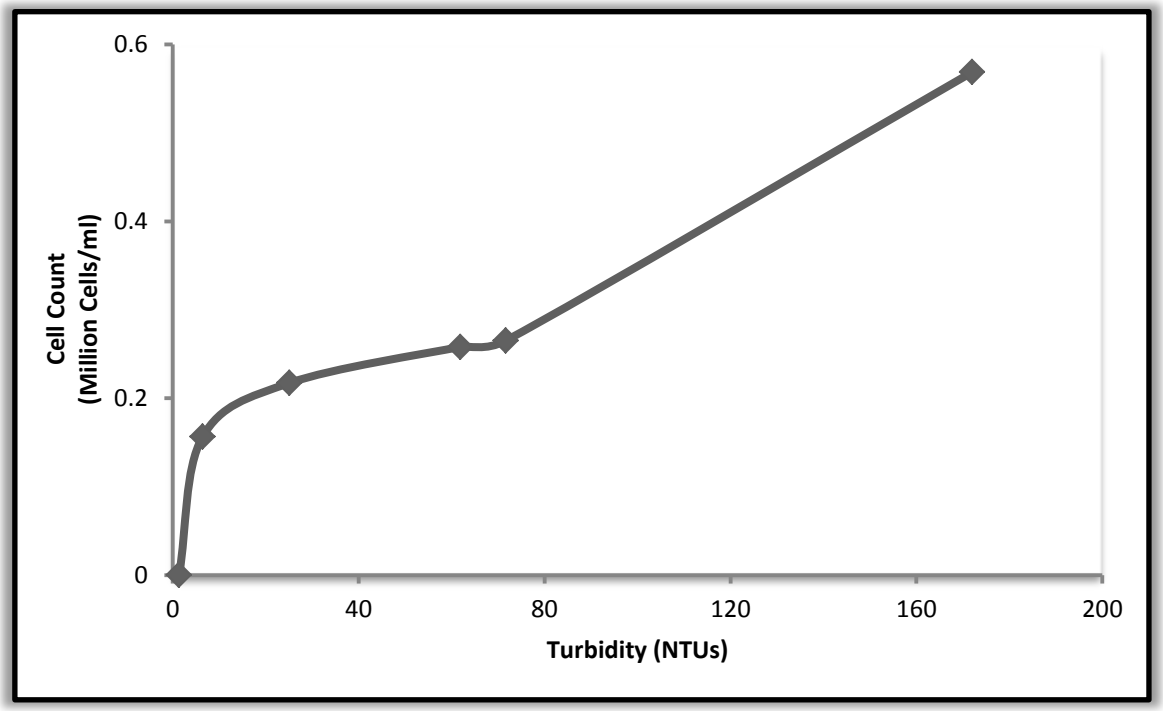


**Figure 4-5: Standard curve for *Tetraselmis chuii***

## 4.2 Growth Curves

The growth rates of the algal strains were compared by plotting their cell counts (million cells per ml) against time (in days) for a total of 15 days. 100 ml of each algal culture was inoculated into 100 ml fresh, autoclaved medium. BBM (Modified) was used for freshwater strains, *KKL-5* and *Chlorella vulgaris*, with pH maintained at 6.8 and salt concentration at 0.064 %. MJM was used for marine strains i.e., *Dunaliella salina*, *Tetraselmis chuii* and *Nannochloropsis oculata*, with pH maintained at 7.5 and salt concentration at 2.5 %. The cultures were provided aeration through aerators. Natural light (i.e. sunlight) was provided by placing the cultures on a window sill of the Environmental Chemistry Teaching Lab at IESE. The temperature was maintained at 30 ± 5°C. The turbidity of each culture was measured daily by a turbiditimeter, and the time at which the reading was taken, was recorded. The turbidity was used to calculate the cell count of the particular algal strain for that specific day by using the standard curves established before (Figures 4-1 through 4-5).

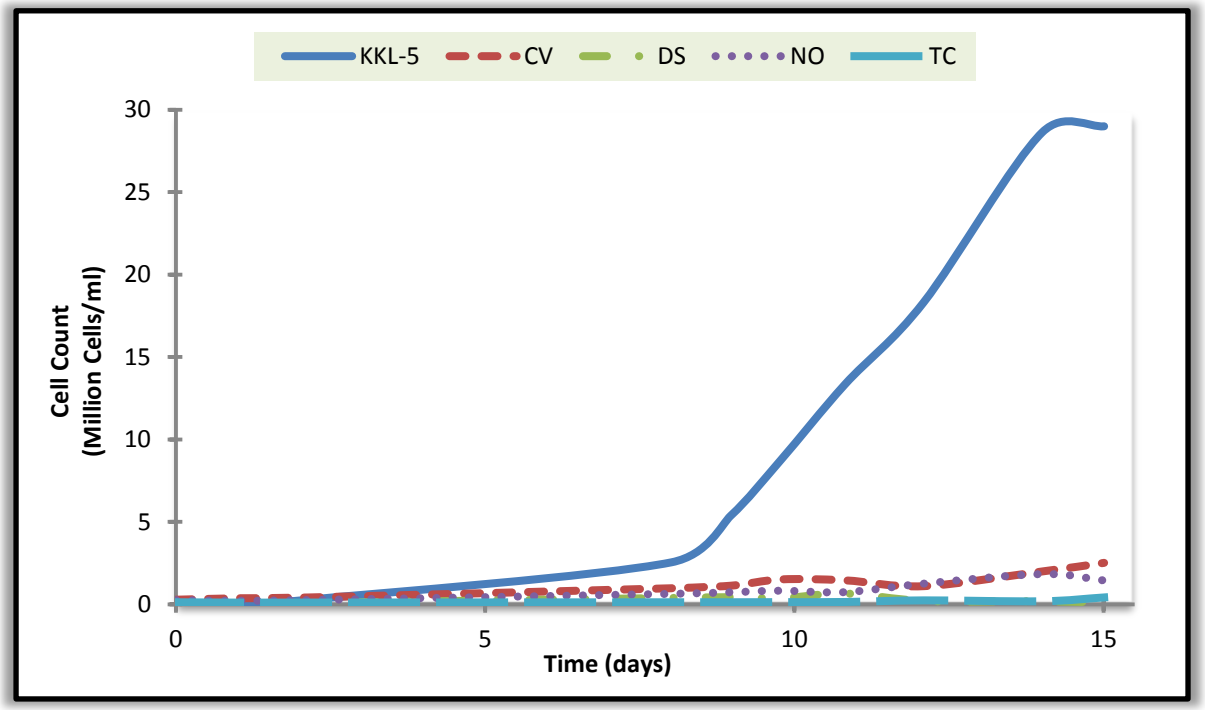


**Figure 4-6: Comparison between growth rates of local and foreign strains**

Figure 4-6 shows a comparison between the growth rates of the local and foreign strains. The graph clearly depicts that the local strain i.e. *KKL-5* is more acclimatized to thriving in the local environment as compared to the foreign strains. It is also evident from the graph that *KKL-5* reached a maximum cell count of approximately 29 million cells per ml within 15 days' time period, which is almost 15 times higher than the foreign strains at provided temperature.

The local strain i.e. *KKL-5* was highly resistant to local conditions however, other pure cultures showed a varying cell count as depicted by the growth curves in Figure 4-7.

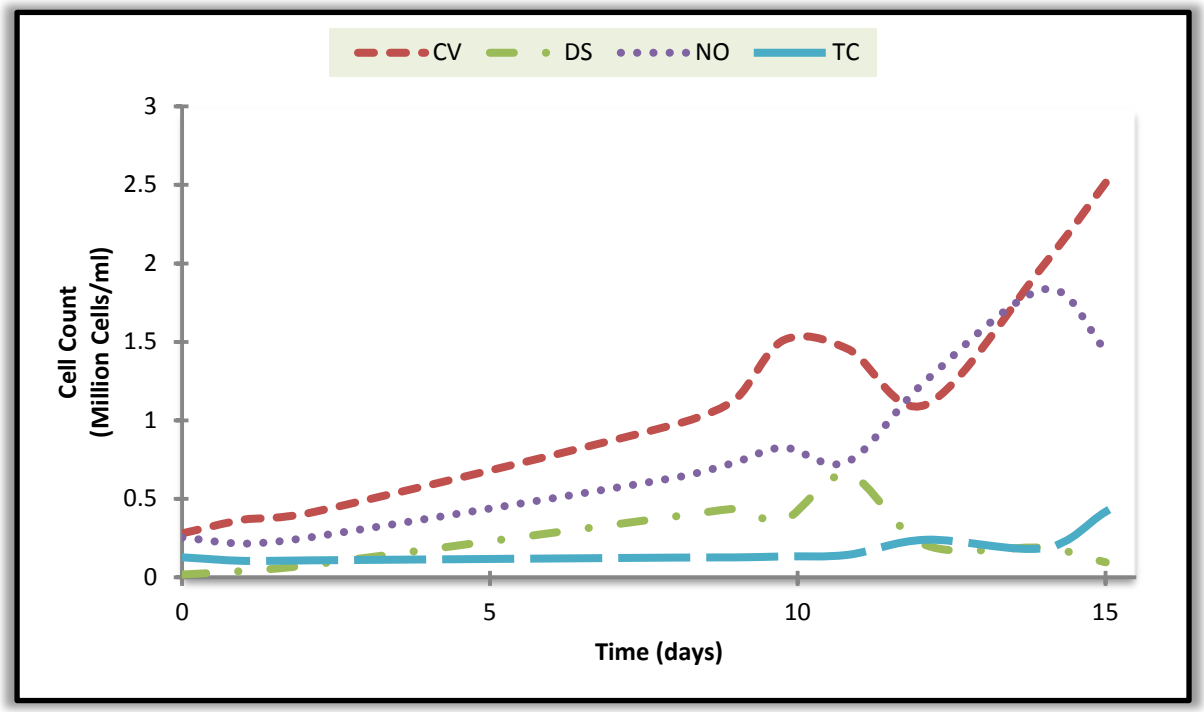


**Figure 4-7: Fluctuations in growth rates of foreign strains**

The fluctuations in the cell counts of foreign strains show that they are not suitable for large-scale growth in the local environment. However, the local strain *KKL-5* formed a straight line during its log phase of growth.

Figure 4-6 further confirms that the isolated local strain *KKL-5* has a high growth rate and it

can be grown commercially on a large scale without experiencing fluctuations in the cell count due to slight changes in the physical conditions of the environment. The duration of the log phase of growth for *KKL-5* can also be deduced from the graph, which comes out to be 6 days (144 hours). This means that *KKL-5* will grow exponentially for 6 days (144 hours) after the provision of fresh medium and then enter stationary phase. So nutrients have to be added every 6 days to keep a culture of *KKL-5* in its exponential phase of growth.

## 4.3 Oil Content

The lipid content of the local strain *KKL-5* and *Chlorella vulgaris* was compared by quantifying the amount of oil extracted (using Soxhlet Extractor) from the strains per unit of dry mass. The oil content (as a percentage of dry mass) was calculated using the following formula;

$$\text{Oil Content (\%)} = \frac{\text{Mass of Oil}}{\text{Dry Mass of Algae}} \times 100\%$$

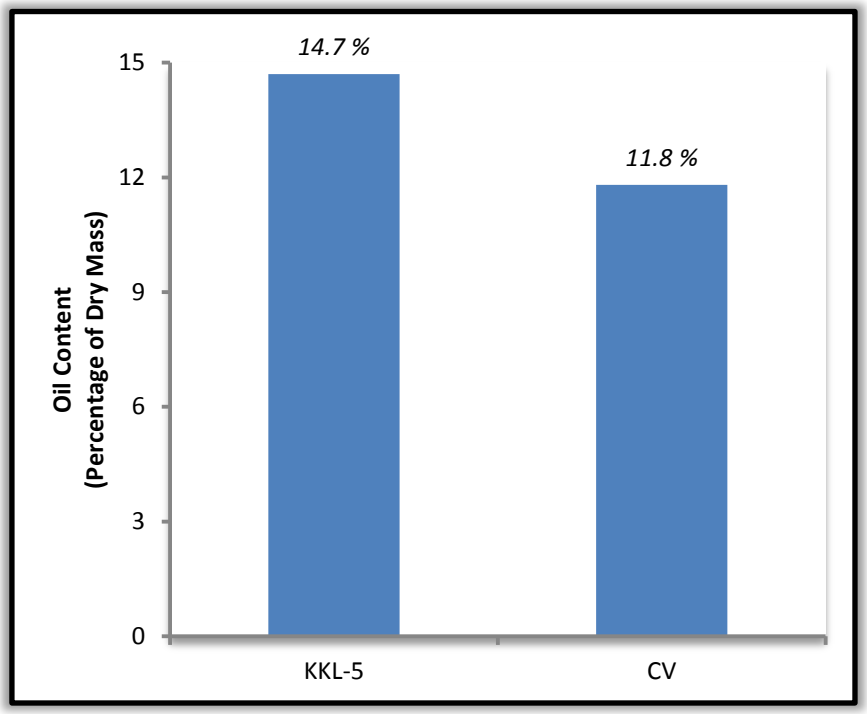


**Figure 4-8: Percentage of oil content in *KKL-5* and *Chlorella vulgaris***

The oil content of *KKL-5* was 14.7 %, which is almost 3 percent greater than the oil content of *Chlorella vulgaris* (11.8 %), as shown in Figure 4-8. By comparing these values it can be deduced that the local strain *KKL-5* contains more extractable lipids than *Chlorella vulgaris*. The percentage of oil extracted can be increased further by applying more efficient extraction techniques, e.g. sonication, CTF, etc.

*Chapter 5*

# CONCLUSIONS

There is a large potential for utilizing algae for biodiesel production in Pakistan to improve the availability of low-cost fuel that can be used in industries and automobiles/transportation sector. This can help reduce oil imports and can impact the Pakistan economy significantly. Utilization of solar energy and carbon sequestration by algae makes this project cost-effective and environmentally sustainable.

The microalgae *KKL-5* isolated from the Kallar Kahar Lake, is a promising strain of algae which has a high growth rate in Bold's Basal Medium (Modified) with a pH of 6.8 and a salt concentration of 0.06%. The growth rate of *KKL-5* is higher than other algal species being used for biodiesel production including *Chlorella vulgaris*, *Nannochloropsis oculata* and *Dunaliella salina* (under local conditions). The algal strain *KKL-5* can thrive at temperatures 20°C to 40°C and can withstand temperatures up to 58°C. This strain can also withstand changes in the physical environment without any impact on growth rate. Apart from that, oil extracted from the strain undergoes trans-esterification to produce biodiesel. These properties enable the researcher to categorize *KKL-5* as a robust strain that can be used for biodiesel production on a commercial scale in Pakistan.

*Chapter 6*

# RECOMMENDATIONS

Depleting fossil fuel reserves and environmental damages are the big challenges to the existing world, and need to be addressed using alternative/renewable energy resources including algae. It has been observed that local algal strains have the potential to be utilized for biofuel and/or biomass purposes. A series of research projects are required to be conducted by local institutes to establish algal strain bank at local level from freshwater and marine sources. The algal species have the potential to produce biofuel, antioxidants, vitamins and a number of bioactive compounds. In addition to this, algae are designated as the best sequestering agents for carbon dioxide to remedy the greenhouse effect. There is also a view that if it had been possible to capture carbon dioxide from flue gases from power stations and industries to grow algae for biofuel/biomolecule production, it might have caused value addition to the products cost-effectively.

Carbon credits is another incentive to be obtained by growing algae, however the permissible carbon credits arise from the flue gas that is captured to replace fossil fuels. In other words, the carbon credits arise as a result of the displacement of the fossil fuel that would have been used if the biofuel had not become available.

More research can be carried out in order to determine the efficiency of the local strain *KKL-5*, in producing biodiesel as compared to other algal strains. The topics which need further consideration include:

- Identification of the algal species *KKL-5*
- Maximization of oil content (by starvation technique)
- Maximization of amount of oil extracted (by sonication)

- Optimization of growth conditions (temperature, light, $CO_2$)
- Optimization of the type of growth medium and salinity & pH
- Potential for growth on wastewater and saline water
- Testing of properties of biodiesel produced for flash point, density and viscosity

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# APPENDIX A: Modified Johnson's Medium – Recipe

| To 980 ml of distilled water *add*: | |
|---|---|
| 1. NaCl | as needed to obtain desired salinity |
| 2. $MgCl_2.6H_2O$ | 1.5 g |
| 3. $MgSO_4.7H_2O$ | 0.5 g |
| 4. KCl | 0.2 g |
| 5. $CaCl_2.2H_2O$ | 0.2 g |
| 6. $KNO_3$ | 1.0 g |
| 7. $NaHCO_3$ | 0.043 g |
| 8. $KH_2PO_4$ | 0.035 g |
| 9. Fe-solution | 10 ml |
| 10. Trace-element solution | 10 ml |
| Adjust pH to 7.5 with HCl | |

| 9. Fe solution *(for 1 liter)* | |
|---|---|
| A. $Na_2EDTA$ | 189 mg |
| B. $FeCl_3.6H_2O$ | 244 mg |

| 10. Trace-element solution *(for 1 liter)* | |
|---|---|
| A. $H_3BO_3$ | 61.0 mg |
| B. $(NH4)_6Mo_7O_{24}.4H_2O$ | 38.0 mg |
| C. $CuSO_4.5H_2O$ | 6.0 mg |
| D. $CoCl_2.6H_2O$ | 5.1 mg |
| E. $ZnCl_2$ | 4.1 mg |
| F. $MnCl_2.4H_2O$ | 4.1 mg |

(Borowitzka, 1990)

# APPENDIX B: Bold's Basal Medium (Modified) – Recipe

## a. Preparation of Stock Solutions

| Stock | Solution Concentration |
|---|---|
| 1. $KH_2PO_4$ | 8.75 g/500 ml |
| 2. $CaCl_2.2H_2O$ | 1.25 g/500 ml |
| 3. $MgSO_4.7H_2O$ | 3.75 g/500 ml |
| 4. $NaNO_3$ | 12.5 g/500 ml |
| 5. $K_2HPO_4$ | 3.75 g/500 ml |
| 6. NaCl | 1.25 g/500 ml |
| 7. $Na_2EDTA.2H_2O$ | 10 g/L |
| 8. Fe Solution | See below |
| 9. Trace Metal Solution | See below |
| 10. $H_3BO_3$ | 5.75 g/500 ml |
| KOH | 6.2 g/L |

| 8. Fe Solution *(for 1 liter)* | |
|---|---|
| a. $FeSO_4.7H_2O$ | 4.98 g/L |
| b. $H_2SO_4$ concentrated | 1 ml/L |

| 9. Trace Metal Solution *(for 1 liter)* | |
|---|---|
| a. $H_3BO_3$ | 2.86 g/L |
| b. $MnCl_2.4H_2O$ | 1.81 g/L |
| c. $ZnSO_4.7H_2O$ | 0.222 g/L |
| d. $Na_2MoO_4.2H_2O$ | 0.390 g/L |
| e. $CuSO_4.5H_2O$ | 0.079 g/L |
| f. $Co(NO_3)_2.6H_2O$ | 0.0494 g/L |

## b. Preparation of Medium

| To 900 ml of distilled water *add:* | |
|---|---|
| 1. $KH_2PO_4$ | 10 ml |
| 2. $CaCl_2.2H_2O$ | 10 ml |
| 3. $MgSO_4.7H_2O$ | 10 ml |
| 4. $NaNO_3$ | 10 ml |
| 5. $K_2HPO_4$ | 10 ml |
| 6. NaCl | 10 ml |
| 7. $Na_2EDTA.2H_2O$ | 1 ml |
| 8. Fe Solution | 1 ml |
| 9. Trace Metal Solution | 1 ml |
| 10. $H_3BO_3$ | 0.7 ml |
| Add more distilled water to complete 1 L volume of medium | |
| Adjust pH to 6.8 using KOH solution | |

(Handbook of Phycological Methods, 1980 pp. 17-24)