Investigation of microalgae biomass as a potential source of bio-fuel for Bangladesh.

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ABSTRACT

Fossil fuel energy resources are unsustainable. World reserves are gradually depleting and the greenhouse gas emissions associated with their use are contributing to global warming. Therefore, there are vigorous research initiatives all aimed at developing alternative fuel resources that are both sustainable and carbon neutral. Based on contemporary research, third generation biofuels that are specifically derived from microalgae are considered to be a technically viable alternative energy resource that is free from the major problems associated with the use of fossil fuels.

This project investigated the use of microalgae for biodiesel production, including their cultivation, harvesting, and processing. The microalgae species most suitable for biodiesel production in Bangladesh are mentioned as well.

LITERATURE REVIEW

Chapter One

Background & Introduction

In recent years, energy crisis has turned into a major problem throughout the world. The deposits of conventional fossil fuel are reducing day by day. Moreover, increasing carbon emission is deteriorating the ozone layer rapidly and thus increasing global warming. At this rate, many low-laying islands like the Maldives will be engulfed very soon. So, necessity of finding clean and renewable source of energy has become an important issue. Among different sustainable, clean renewable energy sources, only solar energy and biogas have become prominent in Bangladesh. Other sources like tidal energy, wind energy are not efficiently utilized in our country [1]. Recently some high-lipid containing species of microalgae has been identified in different freshwater bodies in Bangladesh [2], which can be used to produce biofuels in commercial methods. Microalgal biofuel is an excellent replacement of conventional diesel as its properties are almost as same as that of the petroleum [3].Moreover, production of microalgae does not require arable land space, which can easily solve the food versus fuel dispute. Microalgae can also be successfully used in treatment of waste water from different industries.

Microalgae are sources of energy-rich oils and other valuable products. The amount of a desired compound in an algal biomass determines its value. Microalgae have been produced commercially for several decades to obtain high value compounds such as β-carotene, phycobilin, astaxanthin, and long chain polyunsaturated fatty acids. Microalgae are also used as aquaculture feeds and in the future may provide fuels such as biohydrogen and bioethanol; methane; diesel, gasoline, kerosene (jet fuel); and oils obtained via thermochemical conversion of algal crude oil or biomass.

Chapter Two

Strain Selection & Cultivation

2.1. Selection of Strain

Till now, about 35000 species of microalgae have been discovered, although the available species in the world is assumed to be significantly higher. Almost all algal Phyla have microalgae representatives and microalgae can be found in most environments on earth. The first and most critical step in developing a reliable and commercially viable process for production of microalgae to obtain biofuel is selection of algal species and strain. For commercial scale production, a species must demonstrate high productivity in proposed culture system. The main target is to increase the productivity of lipid, but it itself is not sufficient for potential commercial production. The important properties considered during selection of strain are given below.

1) *Optimum Temperature and Temperature tolerance:* Geographical location and its climate is an important factor for production of microalgae. The average temperature of Bangladesh varies from 23.9°C to 31.1°C during summer and from 7.2°C to 12.8°C in winter [4]. It is to be kept in mind that, the lethal temperature for microalgae is generally slightly higher than optimum temperature. Strain with broad optimum range usually shows better growth [5-6]. Respiration during night time causes loss of biomass significantly [7]. So, length of day is also needed to be considered.
2) *CO_2 supply, pH and O_2 tolerance:* For high rates of photosynthesis, efficient uptake of inorganic Carbon by the cell is very important. All microalgae can take

up CO_2 and some can take up carbon in the form of HCO_3^- [8-9] . Generally, inorganic carbon exists in 4 forms in water - CO_2, H_2CO_3, HCO_3^-, CO_3^{2-}. Uncontrolled pH can be fatal for microalgae, as CO_2 is converted into HCO_3^- with increasing pH. Most of the microalgae cannot uptake HCO_3^- as their nutrient. Moreover photosynthesis nearly ceases at pH 9.00.It should be noted that, CO_2 diffusion from air to the medium is greater at more alkaline pH [10-11].

3) *Respiration rate:* Respiration rate is usually dependent on conditions like temperature, nitrogen content, light etc.
4) *Salinity:* For production of lipid enriched microalgae, salinity of water is an important factor. Use of saline water reduces the pressure on fresh water requirement for cultivation [12]. It also reduces the possibility of contamination by local freshwater strains in the medium.
5) *Competitive strain:* The selected strain must outlive other weaker strains for successful cultivation. Some selected species can outcompete contaminating organisms by surviving in high pH and producing DMSO (dimethyl-sulphoxide).This can act as an antibiotic and helps in successful long term outdoor production.

At present, viable strains which are available in Bangladesh are [13]:

Oedogonium: It is widely available in Bangladesh and can be collected from nearby ponds or other still water sources. Its growth rate is also good. It contains 29-49% lipids of its dry weight from which the biodiesel can be extracted.

Spirogyra: It is one of the most common and available algae found in Bangladesh. Its growth rate is very good in Bangladeshi atmosphere. But its lipid content is lower than the Oedogonium. It contains around 18-22% lipids of its dry weight.

Navicola: It is also a single cell alga. It is not readily available but found at some areas. Its Lipid content is around 25-40% of its dry weight.

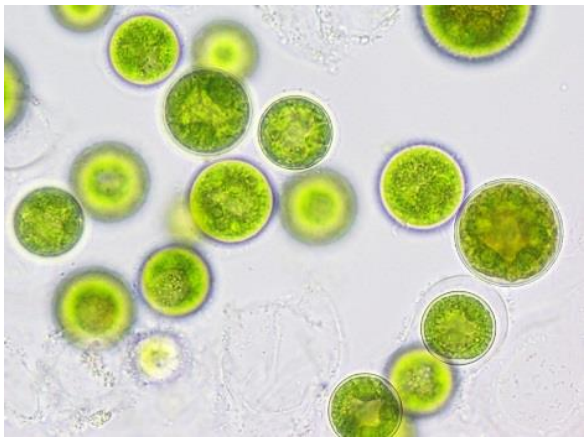

Fig.2.1. Photo-autotrophic microalgae under microscope

2.2. Cultivation

Recent decades have seen tremendous advancement in the field of algal biotechnology. Different methods for greater algal biomass production have been developed including closed photo bioreactors and open pond systems. [14]

2.2.1 Method of Cultivation:

Considering the environmental issues of Bangladesh and economic aspects, raceway ponds have been identified as the most feasible option for commercial cultivation of microalgae. Raceway pond is the most widely used medium for microalgae cultivation because of its low production cost. The pond can be built above or below the ground level and a lining of plastic, such as HDPE geotextile liners is provided. The culture in the ponds should be circulated at about 20-30 cm/s, to keep the algae suspended and provide relatively even illumination to the algae and prevent thermal stratification. For circulation and mixing of microalgae in medium, Archimedes pump, air lift, propeller, pumps etc. are used. Generally an 8-blade paddle wheel is considered optimal. At least one of the blades is always immersed in the culture. [15]

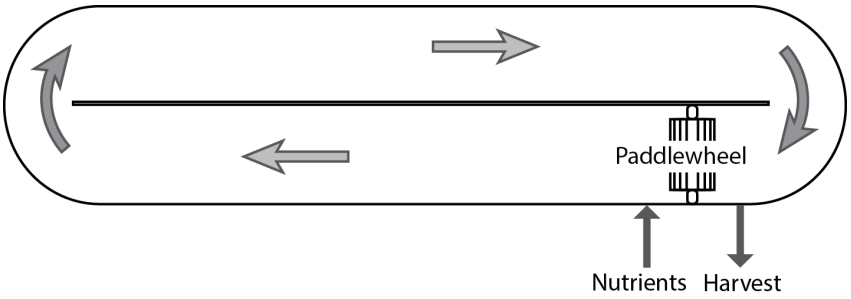

Fig. 2.2. A typical raceway pond used for cultivation.

2.2.2 Management and control of contamination

Open pond systems are severely prone to natural contamination. Unlike laboratory culture, large volume of water used in open pond culture is very difficult to sterilize. Possible contaminating agents and processes are:

- Bacteria, protozoa, fungi and other algae which can blow in by wind or via the water source.
- Culture collapse due to phage-like activity has also been reported occasionally in Arthrospira (Spirulina) pond cultures [16]

Possible ways of reducing the contamination rate are as follows [15]:

1. Culture conditions can be optimized for the selected strain only, so that other strains can't grow well.
2. By controlled use of specific chemicals.
3. Expelling contaminating organisms by netting.

2.2.3 Productivity of outdoor open pond system

For economic and sustainable production of biofuels from algae high and reliable annual average biomass and lipid productivity is essential. Productivity of microalgae varies from species to species. Using approach based on available solar energy and a realistic microalgae photosynthetic efficiency, it has been calculated that the maximum achievable biomass productivity is no more than 10 g(C)/ m^2 /day.[17] Depending on average solar irradiation and other factors, average biomass productivity in open ponds may vary from 20-25 gm (dry weight)/m^2 /day. In winter (lower light, short days and lower temperatures) productivities can be up to five times or lower than summer productivities.

2.2.4 Limitations of growth

Extensive studies have revealed that, efficiency of using external nutrient sources for growth is limited for any specific species. It may depend on cell size, genetic factors like rRNA gene copy number [18] etc. generally, heterotrophic organisms have faster growth rate than photolithotrophic or chemolithotrophic organisms.

(i) Metabolism products

For specific trophic mode, metabolism products will have important effect on growth rate. Generally, oil producing species have lower specific growth rate than similar sized other cells without intracellular oil. Even in case of extracellular oil producing species, production reduces as photosynthetic products are divided up rather than producing more catalysts.

(ii) Light attenuation

Mass algal culture requires optically dense medium. In outdoor culture, challenge of supra-optimal incident irradiation is faced. About 90% of radiation is generally absorbed by first 10mm of water with algae suffering severe light inhibition. The rest portion of algae uses the photon very efficiently, but lacks the required light. So, for higher growth rate, arrangements should be made to avoid self-shading. Efficiency of production can be increased by reducing the optical pathway [17]. By reducing the size of light harvesting antenna, the penetration of photon into deeper water can be increased. It has been found that, the production rate of *Chlamydomonous* can be increased significantly by reducing antenna size at high irradiance.[19]

(iii)Nitrogen content

Nitrogen content of culture medium affects the lipid content of microalgae. When there is nitrogen deficiency in the medium, protein production is reduced. As a result, the unused carbon from photosynthesis is stored as triglyceride. Study by Thomas et al shows that nitrogen deficient condition increases the lipid content of microalgae. But this is not verified for a great number of algal species.

Chapter Three

Harvesting of biomass

3.1 Harvesting

In simple terms, harvesting is separating the algae from its supporting medium and/or concentrating the algal biomass. The harvesting technology that needs to be chosen, depends on the type of algae being cultivated. e.g. characteristics of the microalgae, size, density etc. [20] The most common harvesting methods are screening, coagulation, flocculation, flotation, sedimentation, filtration and centrifugation [21]. The choice of technology for algae harvesting should be energy efficient and inexpensive for feasible biofuel production.

In various algae-harvesting technologies such as sedimentation, flotation, filtration and centrifugation, coagulation & flocculation of algal cells is carried out as an upstream treatment. [22]

Coagulation and *flocculation* are two distinct processes. Coagulation is used to condition suspended, colloidal and dissolved matter to help in subsequent processing. It involves the addition of a chemical coagulant(s). The aggregation of the destabilised particles and the precipitation products that are formed by one or more coagulants, into larger particles is known as flocculation. Coagulation-flocculation causes algal cells to become aggregated into larger clumps, which are more easily filtered and/or settle more rapidly to facilitate harvesting. Coagulation and flocculation may be initiated through the use of inorganic coagulants, organic coagulants (often polymers) or by using autoflocculation, bioflocculation, ultrasound and electrocoagulation procedures.

Inorganic coagulants can include Alum ($Al_2(SO_4)_3 \cdot 18H_2O$), Ferrous Sulphate ($FeSO_4.7H_2O$) etc. each having a particular optimal pH range for proper functioning. Organic coagulants may include Chitosan, Praestol® etc.

Coagulation and flocculation improves the ease of solid-liquid separation processes involved later. However, all microalgae may not require flocculation to be removed effectively.

Solid-liquid separation techniques constrain either the liquid or the solid.

3.2 Liquid constrained systems

Liquid constrained systems include gravity thickening, centrifugation, sedimentation and flotation [23].

1) *Gravity Thickeners:* Gravity thickeners rely provide a final solids concentration of generally 2–3%. Most commonly used gravity thickeners are circular in shape. The influent is pumped up through the middle section of the thickener and is released. With time the particles settle, and several zones are created. The top zone consists of relatively clear media and the lower zones consist of particles. Gravity thickeners frequently have a sloped base and scrapers to collect the settled solids for removal [23].
2) *Centrifugation:* Centrifugal processes generate a centrifugal force which acts radially and accelerates the movement and separation of particles based on a density difference between the particle and the surrounding medium. If the particle is denser than the media the particle migrate outwards otherwise it migrates inwards. The maximum discharge concentration typically achieved in continuous centrifugation processes are 10–20% solids. The decanter centrifuge and the disk stacked centrifuge seem to be the most promising centrifugal devices for the recovery of microalgae. In Table I, the two centrifuges are compared [23].

Table 1.0

Comparison of Two Centrifugal Equipment (Adapted from [23])

Device	**Energy Requirements (kWh/m^3)**	**Biomass concentration in solid discharge (%)**	**Reliability**
Disc stacked and nozzle	0.7–1.3	2–15	Very good
Decanter	8	22	Very good

3) *Flotation:* Flotation is a separation process which uses air or gas bubbles. The bubbles adhere to the particles, which are then carried to the liquid surface where they can be separated, usually by skimming. The gas bubbles can be generated within the flotation cell or it can be introduced in to it. The most common gas used for flotation is air. Dispersed air flotation, dissolved air flotation (DAF), bio-flotation and electrolytic flotation are the most popular flotation mechanisms [23].

3.3 Particle constrained systems

Particle constrained systems include cake filtration, screening, attachment etc.

1) *Filtration:* All filtration processes need a filter media. The processes generally operate under gravity, pressure or vacuum [23]. Algal suspension is forced to pass across the filter medium by a suction pump. The medium retains the algae biomass. Filtration process is able to harvest microalgae or algal cells of very low density. But the deposited cells often cause fouling/clogging of the medium [22].
2) *Attachment:* Sometimes the microalgae cells can be removed by physical, chemical or electrical attachment to other materials, which can be directly submerged into the culture medium. Magnetic particles can also be added to separate microalgae by magnetic separation techniques [23].

The harvested biomass slurry consists of 5–15% dry solid content and needs to be processed rapidly after harvest.

Dehydration or drying is commonly carried out after harvesting. Sun drying, low-pressure shelf drying, spray drying, drum drying, fluidised bed drying and freeze drying are some of the methods that have been used.

Sun drying is the cheapest dehydration method but brings certain disadvantages into the scenario, e.g. long drying times, loss of material etc. Spray drying is relatively expensive. Freeze drying is equally expensive but extraction of oils is easier from freeze-dried biomass [20].

Chapter Four

Extraction of lipids

4.1 Extraction Process

In simple terms, extraction is taking out the lipid oil from the harvested microalgae where oil is pressed in between the cell wall and the plasma membrane.

Extraction processes can be physical (screw press methods) or chemical (organic solvents) and where the latter has proved to be more workably approached.

4.2 Types of lipids present

Lipids present in algae can be classified as:

(i) Neutral lipids (NLs) [triacylglicerols (TAGs), wax esters, hydrocarbons, fatty acids (FA)s, and sterols];

(ii) Phospholipids (PLs) (phosphatidylcholine, PC,phosphatidylethanolamine(PEA), phosphatidylserine(PS)

(iii) Phosphatidylglycerol(PG),and phosphatidylinositol(PI);glycolipids(GLs)(sulfoquinovosyldiacylglycerol(SQDG), monogalactosyldiacylglycerol(MGDG), and digalactosyldiacylglycerol (DGDG).

Autoxidation, the spontaneous free radical reaction of organic compound with oxygen, is the responsible for the degradation of lipids during processing and storage. It results in deteriorated lipid macromolecules which cannot be used as biodiesel [24].Algae which harvested in the increasing growth rate will contain more polar lipids (GLs and PLs) where as those harvested in a late steady phase contains more neutral lipids. (TAGs).

4.3 Thermodynamics of solvent extraction

One endothermic and two exothermic processes are involved in dissolution process. Firstly, separation of lipid molecules into individual molecules is an endothermic process and its energy is small for nonpolar TAGs and slightly higher for polar lipids (i.e. PLs and GLs) [24].These are next dispersed into solvent and energy is required to dissociate the solvent molecules. Higher the intermolecular attraction; higher the energy required: non-polar solvent < polar solvent < hydrogen-bonded solvent. Thirdly, which is exothermic process, the lipid molecules interact with close-by solvent molecules. The following order represents shows the increasing of energy released: *both solvent and lipid molecules are non-polar < one is polar and the other is non-polar < both molecules are polar < lipid molecules are dissolved by solvent molecules***.** The overall result whether exothermic or endothermic is determined by the difference in energy released in lipid-lipid, solvent-solvent interaction and energy gained lipid-solvent interaction. When solvent molecules are highly inter- associated, as with water, the dissolution is only possible if the resulting solution results in stronger lipid-solvent interaction. In short, it can described by the principle of '*like dissolve like',*i.e. polar lipid dissolves in polar solvent. When solubility parameters (polarity index, solubility parameters and dipole moments) for solvent and lipid are similar, the solvent will be a good candidate for a given application. [24]

4.4 Ideal solvent characteristics

Some of the factors that need to be consider when selecting an ideal solvent: they should be volatile (for easy removal later), free from toxic or reactive impurities (to avoid reaction with the lipids), form a two-phase system with water (to remove non-lipids), and be ineffective towards undesirable components (e.g., proteolipid protein, small molecules) [24]. A solvent with high solubility at elevated temperature and low solubility at ambient temperature may be desirable, because the oil can be separated from the solvent without evaporation.

4.5 Cell wall and plasma membrane

The cell wall (CW) gives cells rigidity and strength, offering protection against stresses. The CW is an extracellular polymeric structure composed of polysaccharides, proteoglycans, peptides, proteins, and associated inorganic elements [24] Electron-microscopy studies have shown that the CW is made up of two major components: (1) an arranged fibular structure (2) a continuous matrix [24]

Plasma membrane (PM) is a light membrane which surrounds the protoplasm directly beneath the CW and a layer of mucilage (a viscous secretion or bodily fluid). [24]

4.6 Biomass Pre-treatment

In order for a rapid and smooth extraction of lipids from microalgae; it may often be extracted in the wet state directly after harvesting. The cells do not need to be deteriorated since they are broken by the extracting solvent. The breakage of the CW and PM may be necessary to: 1. Time for extraction can be reduced 2. Use of high temperatures and pressure is not necessary to make the lipid–solvent interaction occur 3.less solvent required 4. To permit the solvent to easily penetrate into the cell and release cell contents into the bulk for increasing the lipid yield. [24] This may be accomplished by using one of the well-established cell-disruption techniques such as sonication, homogenization in a tissue grinder, blender or in high-pressure flow device, freezing and grinding with a pestle and mortar, autoclaving microwaves and osmotic shock. Generally, bead mill may be the pre-treatment choice for microalgae cells with strong cell wall.

4.7.1 Extraction of lipid from microalgae:

All the linkages between the lipid and other nonpolar lipid cells must be diminished without causing any disruption on the lipid. There are three ways in which lipid bond between each other :(i) van der Waals or London forces can be found in neutral or non -polar lipids, such as triacylglycerol's are bound by relatively weak forces through their hydrocarbon chains. (ii) Hydrogen bonding and electrostatic association are the strongest of interaction and it is by which lipids are bound to proteins, and (iii) the less frequent covalent association. [24]

For extracting lipid having ionic bonds as the mode of association the pH value should be shifted out of the neutral region [25]. In case of poor cell wall strength to solvents the recovery yield of lipid can be elevated by adding water because it causes swelling of the cellular structure [25]

A method explained in [26] uses a technique to quantitatively extract lipid using a combination of chloroform: methanol: water in a ratio 1:2:0.8 v/v/v. It has been the most commonly used technique for the extraction of lipids from microalgae at analytical level in the last 50 years (over 23,500 journal citations currently [27])

4.7.2 Fatty Acid extraction by direct saponification of dry microalgae biomass:

Direct saponification of microalgae biomass enables fatty acids to be obtained as potassium or sodium salts instead of as crude lipids (impure lipids) in a first step. This process of direct saponification is cost effective and less time consuming. Direct saponification of biomass could ideally be induced in two stages, first lipid extraction

with a solvent, and second alkaline hydrolysis of the extracted lipids to get rid of fatty acid salts. Three major conclusions can be drawn lipid or fatty acid extraction when using dry microalgae biomass:

(1) Higher the polarity of the solvent; higher the extraction efficiency.

(2) For each solvent system the yield of lipid extraction is slightly higher than the yield of fatty acid extraction and

(3) Attention should be centred on selecting and optimum solvent mixture because saponification is an instantaneous process. [24]

In Bangladesh, documented extraction of lipids from microalgae has been few and far between although most of the processes are sustainable taking in the climate, environment available technology into account. In [13] extraction was done by mechanical process using ball mill as well as chemically using a soxhlet extractor.

RESEARCH METHODOLOGY

Chapter Five

Research Methodology

5.1 Isolation techniques

In order to explore any algal species, it is necessary to grow algae as a pure culture. That is, a culture of only a single species (called a unialgal culture). To obtain a unialgal culture, isolation of one species from all the rest needs to be done.
Three methods to purify contaminated algal cultures and /or to produce single cells are available.

- The agar plate method,
- Successive dilutions of the original contaminated culture
- Picking up of single cells from the original culture by using the capillary method.

All techniques are also applied when new algal species are isolated from the wild.

Among the available techniques, both the agar plate and successive dilution method were followed. Successive dilution showed better growth of uniculture and easy isolation was possible through it. A brief description of the technique is given below.

1. Equipment:

   - Culture tubes.
   - Test tube racks.
   - Culture media (Chu 10).
   - 10 ml sterile pipettes.
   - Bunsen burner.

2. Method

- Using aseptic technique, 9 ml. of media was dispensed into each of the 0 test tubes with sterile 10 ml pipette. The tubes were numbered from 1 to 10, indicating dilution factor of 10^{-1} to 10^{-10} .
- 1 ml of enrichment sample was added to the first tube. The sample was allowed to mix with the medium thoroughly.
- From the first tube, 1 ml diluted media was taken and was added to the second tube. This process was followed until the last tube was filled.
- The test tubes were nurtured under controlled temperature and light condition inside an incubator.
    1. Temperature was kept fixed at 35.8^0C.
    2. Photoperiod of 10 hours was maintained.
    3. A steady luminous flux of approximately 320 luxes was maintained.

The samples were checked in every 4 days. Noticeable growth was observed at the 16th day. On 24th day, the samples showed enough growth for preparation of mass culture.

In all the 4 samples, test tube no. 2, 3 and 4 had shown good growth. 4th test tubes were selected for further experimentation, as it signified better chance of obtaining unialgal culture.

Fig.5.1. Culture test-tubes on Day 0

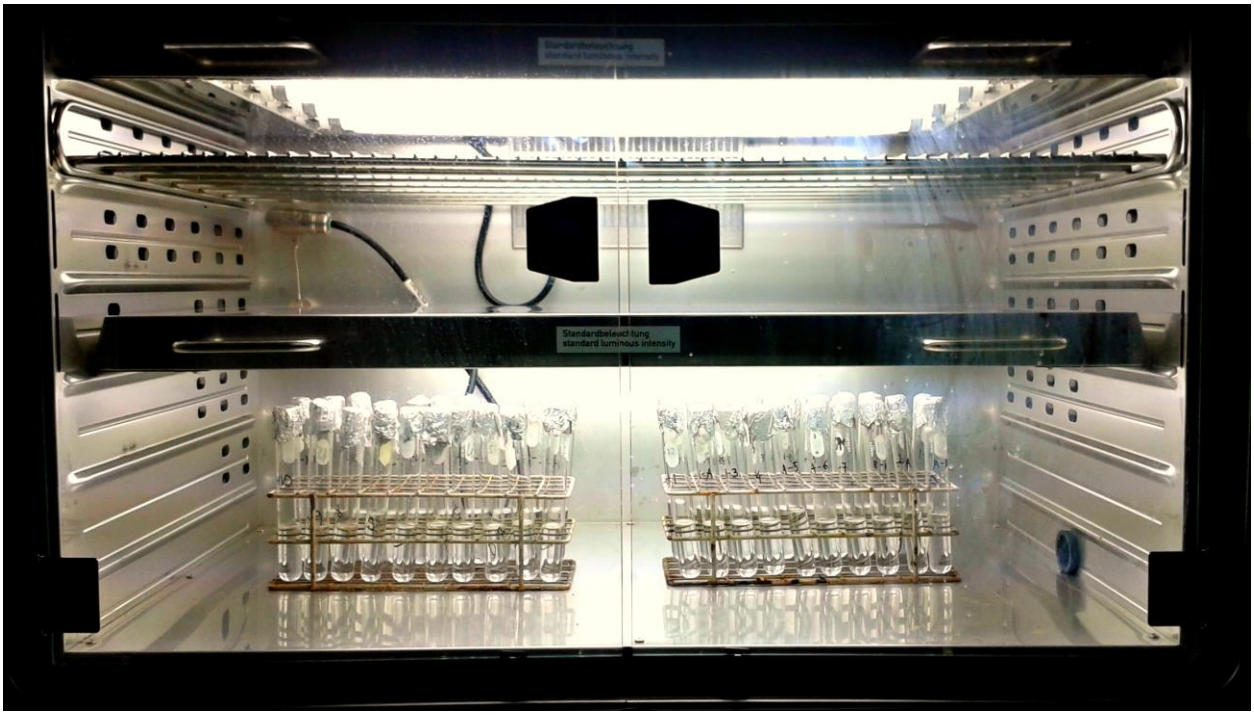

Fig.5.2. Culture test-tubes placed inside growth chamber.

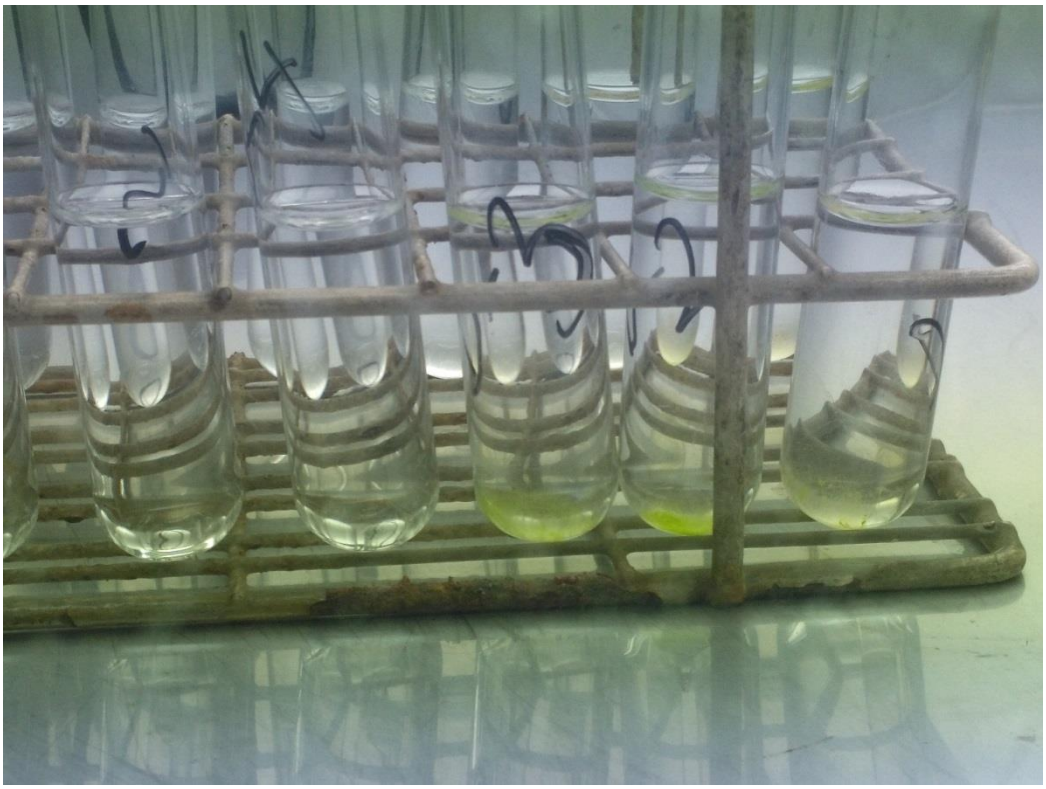

Fig.5.3. Culture test-tubes on Day 24, showing microalgae deposits at the bottom.

5.2 Preparation of culture medium

Culture medium is required for isolation of dominant strain and initial batch production of microalgae. Different researchers have used different types of culture media for isolation purpose. The main objective of this project was to investigate the availability of microalgal species in freshwater bodies. By reviewing several research studies, the culture medium prepared by Chu et al. has been found to be most appropriate for this purpose.

Chu #10 medium was the most popular of 17 media that Chu (1942) described. It is a synthetic medium designed to mimic lake water, but it lacks a chelator, vitamins, and trace metals (except for iron). It has been extensively used for a variety of algae, including green algae, diatoms, cyanobacteria (Chu 1942). Many synthetic freshwater media are derived from Chu #10, and several modified Chu #10 media have been developed.

For initial isolation purpose, half strength Chu#10 was used. Into 950 mL of dH2O, following components were dissolve. The final volume was brought to 1 liter and autoclaved.

Table 2.0 Stock Solution (Adapted from [28])

Component	*Stock Solution* ($g \cdot L^{-1}$ dH_2O)	*Quantity Used*	*Concentration in Final Medium (M)*
$Ca(NO_3)_2$	20.0	1 mL	1.22×10^{-4}
K_2HPO_4	2.5	1 mL	1.44×10^{-5}
$MgSO_4 \cdot 7H_2O$	12.5	1 mL	5.07×10^{-5}
Na_2CO_3	10.0	1 mL	9.43×10^{-5}
Na_2SiO_3	12.5	1 mL	1.02×10^{-4}
$FeCl_3$	0.4	1 mL	2.47×10^{-6}
Trace metals solution	(See following recipe)	1 mL	—
Vitamins solution	(See following recipe)	1 mL	—

Table 3.0 Trace metal solution (Adapted from [28])

Component	*1° Stock Solution ($g \cdot L^{-1}$ dH_2O)*	*Quantity Used*	*Concentration in Final Medium (M)*
H_3BO_3	2.48	1 mL	4.01×10^{-8}
$MnSO_4 \cdot H_2O$	1.47	1 mL	8.70×10^{-9}
$ZnSO_4 \cdot 7H_2O$	0.23	1 mL	8.00×10^{-10}
$CuSO_4 \cdot 5H_2O$	0.10	1 mL	4.01×10^{-10}
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.07	1 mL	5.66×10^{-11}
$Co(NO_3)_2 \cdot 6H_2O$	0.14	1 mL	4.81×10^{-10}

Table 4.0 Vitamin solution (Adapted from [28])

Component	*1° Stock Solution ($g \cdot L^{-1}$ dH_2O)*	*Quantity Used*	*Concentration in Final Medium (M)*
Thiamine · HCl (vitamin B_1)	—	50 mg	1.48×10^{-7}
Biotin (vitamin H)	2.5	1 mL	1.02×10^{-8}
Cyanocobalamin (vitamin B_{12})	2.5	1 mL	1.84×10^{-9}

After isolation, another modification of Chu#10 medium was used for small batch culture purpose. Into 950 mL of dH2O,following components were dissolve. The final volume was brought to 1 liter and autoclaved.

Table 5.0 Chu#10 solution (Adapted from [28])

Component	*Stock Solution ($g \cdot L^{-1}$ dH_2O)*	*Quantity Used*	*Concentration in Final Medium (M)*
$Ca(NO_3)_2$	40.0	1 mL	2.44×10^{-4}
K_2HPO_4	5.0	1 mL	2.87×10^{-5}
$MgSO_4 \cdot 7H_2O$	25.0	1 mL	1.01×10^{-4}
Na_2CO_3	20.0	1 mL	1.89×10^{-4}
Na_2SiO_3	25.0	1 mL	2.05×10^{-4}
$FeCl_3$	0.8	1 mL	4.93×10^{-6}

Fig.5.4. Prepared Stock Solutions for Chu#10

5.3 Batch culture

After isolating 4 unicultures, the microalgal strains were separately grown in conical flasks. Modified Chu#10 culture medium was used for this purpose. In a conical flask, 200 ml of culture medium was taken and 10 ml of isolated strain was added to it. In total 8 such batches were produced. 4 of them were kept in the growth chamber, maintaining the above mentioned temperature and light duration. Rest 4 batches were placed in a shaker machine. The RPM of the shaker was fixed at 120 RPM. After thorough observation of 16 days, it was found that the growth of the batch placed in the shaker much slower than the batch placed in the growth chamber. Possible cause of this was assumed to be limited exposure to light and rapid fluctuation in room temperature. Later on, the aforementioned batch was moved to the growth chamber.

After completion of 30 days observation, the growth of the samples was measured. Batches of sample A and C had shown good growth. Sample D was found to form large colonies rather than forming a uniform suspension in the culture medium. Sample B's growth was not above par.

5.4 Mass cultivation

Due to unavailability of necessary technology, the 4 isolated strains could not be specifically identified. So, an attempt was made to empirically identify them. By analyzing different studies, it has been seen that, Tris-buffer solution can be a viable culture medium for this purpose. The basic colonial structures of sample A and B resembled the colonial structure of *Chlorella* sp. Tris-buffer solution is universally used for mass cultivation of *Chlorella* sp. The chemicals and nutrients used in this medium allow *Chlorella* sp. to flourish and suppresses the growth of other competing strains.

6 different batches for mass cultivation were prepared. 4 of the conical flasks contained 200 ml tris-buffer solution. 2 of them were inoculated with 20 ml of sample A and the remaining 2 were inoculated with sample C. The rest 2 conical flasks were filled with 200 ml of Chu#10 and each was inoculated with 20 ml of sample A and C respectively. All the batches were kept in the incubator and observation was made for 28 days. It has been seen that the growth of sample C in tris-buffer medium was exceptionally good and it has shown more rapid growth in tris-buffer than in Chu#10. On the other hand, sample A failed to survive in tris-buffer medium. A thick layer of foreign entity, presumably fungi, was seen to develop on the liquid surface, which was lethal for the microalgal strain. The growth of sample A in Chu#10 was not satisfactory.

Increased amount of tris-buffer was added to all conical flasks containing sample C. One of the flasks was separated and its exposure to light was increased to 14 hours a day. Necessary arrangement for periodic shaking of the flask was also made. Within 15 days, the suspension of microalgae reached its saturation point. Thick sediment of microalgae was visible at the bottom of the flask.

Fig.5.5. Mass cultivation of the microalgae samples from test-tubes

5.5 Harvesting and Lipid Extraction

After the sample reached satisfactory growth level, wet biomass was harvested from the growth medium. Gravity sedimentation was used for harvesting. The sample was kept undisturbed for 24 hours. As a result, microalgal suspension got precipitated at the bottom of the flask. Since harvesting by gravity sedimentation was satisfactory, no other process was executed.

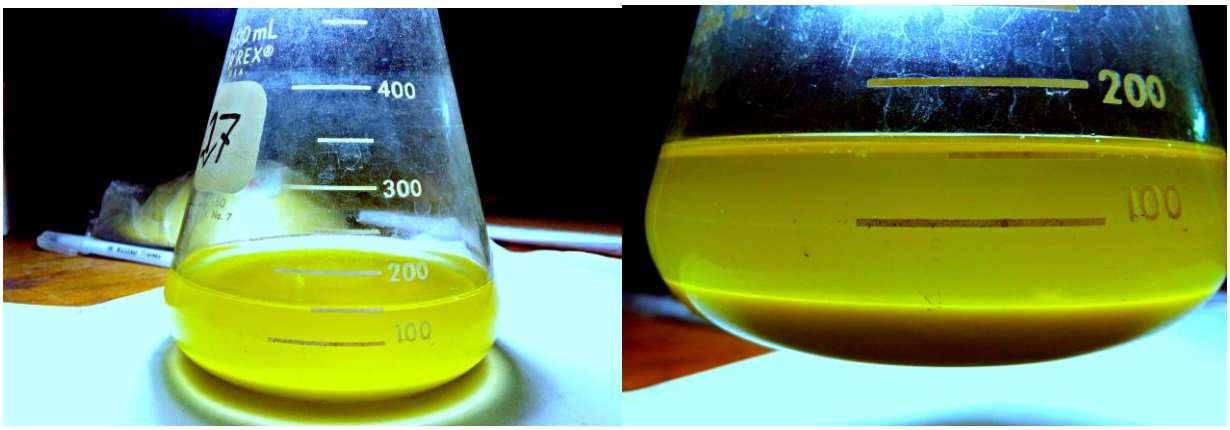

Fig.5.6. Gravity Sedimentation of the microalgae.

Growth medium was removed from the flask and wet biomass was obtained for further analysis. About 30 ml (by volume) of wet biomass was obtained from 400 ml of culture.

For extraction of biofuel, process proposed by Oh et al (2010) was followed. The total biomass of the wet biomass was raised to 100 ml by adding distilled water. For disrupting the cell wall, the biomass was kept in microwave oven at high temperature (100^0C) and 2450 MHz for 3 minutes. Lipid was extracted by mixing Chloroform-Methanol (1:1 v/v) with the sample using a slightly modified version of Bligh and Dyer's method. The mixture was shaken vigorously by hand.

Fig.5.7. Extraction of lipids from wet biomass

Chapter Six

Results and Discussion

50 ml of the sample was taken in a test tube and was centrifuged at 1300 RPM for 5 minutes. 3 distinct sub layers were formed. The topmost layer contained water, the middle layer was formed of used biomass and the bottom layer was a dense viscous layer of Chloroform-lipids mixture.

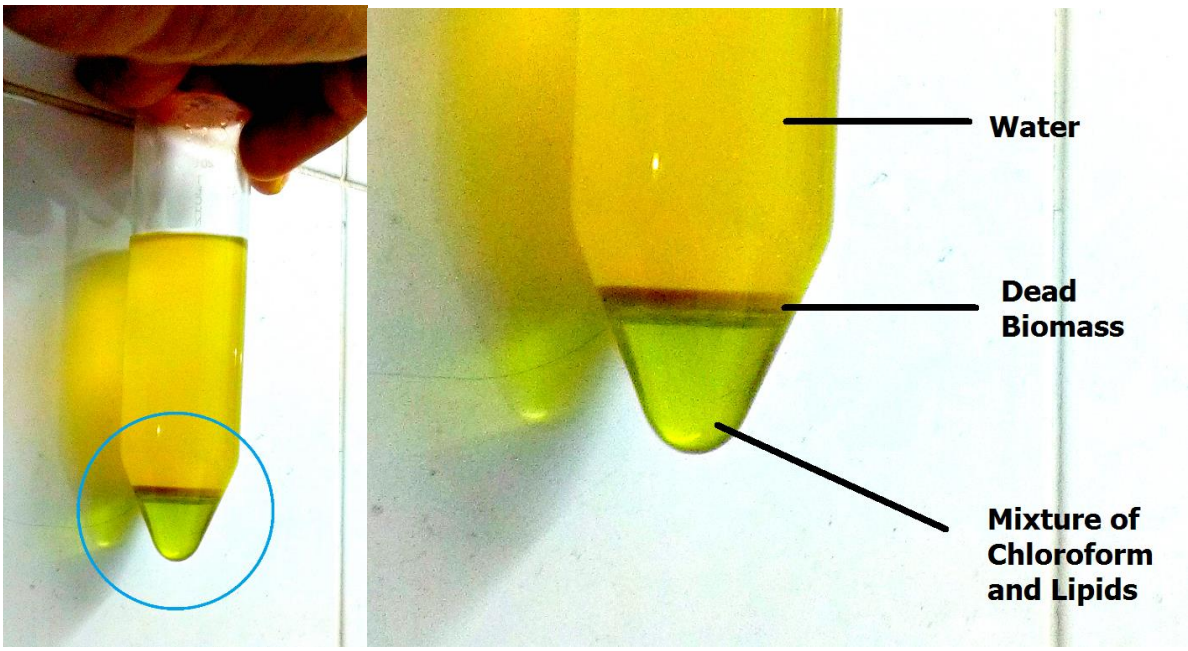

Fig.6.1. Three Distinct sub layers formed after centrifugation

The following interpretations were made during experimentation.

1. All the samples were collected from open water systems. Two of the samples were collected from paddy fields and the rest were collected from fresh water ponds. As nitrogen-rich fertilizers (Urea) are used in paddy fields, nitrogen-fixing Blue Green cyanobacteria were dominant in the collected samples. That is why, after isolation of strain from sample B and D, two different cyanobacteria were found.
2. Samples A and C has shown circular cellular pattern and rosette colonial pattern. Lipid-enriched microalgae species like *Chlorella* sp and *Botryococcus* sp shows similar type of pattern. So, both those samples were considered for further analysis.
3. Emphasis was given on finding *Cholorella* sp due to its high lipid content, so tris-phosphate buffer solution was used for mass culture of the samples. Since sample C had shown satisfactory growth in the used medium, it is interpreted that the following sample contained *Chlorella* sp. for further investigation, the sample will be sent to Department of Botany, University of Dhaka.
4. After extraction of lipid from the biomass, the colorless chloroform layer turned to bright green, which indicates the presence of organic lipid absorbed in chloroform.

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