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ISOLATION AND PARTIAL CHARACTERIZATION OF MICROALGAE FOR BIODIESEL PRODUCTION

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Abstract*-This study focused upon isolation and selection of local microalgae species and scaling up of the suitable species at outdoor condition for biomass production to extract biodiesel. Three different species were isolated, Scenedesmus, dark green colored Chlorella and light green colored Chlorella. Scenedesmus, dark green colored Chlorella and light green colored Chlorella were found to have specific growth rate of 0.15 day^{-1} , 0.068 day^{-1} ,and 0.062 day^{-1} respectively whereas the dry biomass production were 0.60 mg/ml. 0.33 mg/ ml, and 0.11 mg/ml respectively. Based on their growth in media, biomass production and morphological characteristic, Scenedesmus and dark green colored Chlorella were selected for further large scale test for biodiesel production. A liquid enrichment culture containing all three species showed a dry biomass productivity 3.885 mg/ml, whereas for dark green colored Chlorella alone, it was 0.58 mg/ml with air flow rate of 2.5 L/min at outdoor condition. Though the biomass productivity was not significant for dark green Chlorella but the biomass obtained in liquid enrichment culture is comparable to the other conventional photobioreator system. In addition, this wild local species showed a tolerance of large variation of temperature while using sunlight, since no controlled temperature and artificial light system were provided. From liquid enrichment culture 0.25ml/gm (dry weight) oil was obtained by following direct transesterification method.*

Keywords: Renewable Energy, Microalgae, Isolation, Biodiesel, Photobioreactor

1. INTRODUCTION

According to the BP's (British Petroleum) statistical review of world energy, at today's level of extraction, estimated proved reserves of coal would be exhausted in 113 years, the last cubic meter of natural gas in 2069, and the entirety of crude oil reserves by 2067.[1] This rapid decline in fossil fuel reserves leads to the energy crisis and aggravates global climate change.[2,3] Liquid biofuel has recently gained much attention in meeting the global demand for transport fuel [4] Biodiesel is a proven fuel and its production and use has been known for more than 50 years..[5] Among all other available biodiesel feed stocks microalgae possesses large possibilities owing to environmental concerns and the wide field of application [6]

Microalgae are a group of diverse photosynthetic organisms that can accumulate substantial amounts of lipids – up to 50% of dry cell weight in certain species while grown up as well as cleansing waste water.[5] Almost 75% algae species, contribute approximately 40% of the oxygen in the atmosphere**.[7]** It can be converted to biodiesel, bioethanol, bio-oil, biohydrogen and biomethane via thermochemical and biochemical methods.[8] Selection of algae strains for biomass conversion into biodiesel depends upon productivity and lipid content.[9].For the large scale production of microalgae biomass, cultivation carried out generally in the open-culture systems (lakes or ponds) and also in the closed-culture systems called photobioreactors (PBRs).[10] Some parameters - such as nitrogen deprivation, light intensity, salt concentration, pH, temperature etc - also effect growth as well as lipid content in some microalgae.[11] The most challenging part is to liberate the lipids from their intracellular location by using the most energy-efficient and economical way possible, avoiding the use of large amounts of solvent.[12]

Several processes exist for the extraction and conversion of algal oils to biodiesel. These include organic solvent extraction, super-critical fluid extraction, and direct transesterification. It is important to note that the search for a cost effective and efficient method using suitable solvent under optimum condition has begun since 1950s.[13]

The ultimate objective of this work was isolation of a suitable single strain and scaling it up by batch mode for biodiesel production. Effect of aeration on growth rate was also examined and the oil was extracted by following direct trasesterification method.

2. MATERIALS AND METHODS

2.1 Experimental Setup for Algal Growth

For studying algal growth in the outdoor condition, batch system was selected. The strains were wild and survived well in outdoor condition and displayed suitability against a moderate temperature variance (16^o C-30^oC). In this system 2L PET bottle was used as a batch reactor and properly sealed with cap stoppers to prevent any contamination. Two ports were cut into the stopper. The ports were used for air inlet, outlet and sampling. The air feed pipeline with sparger was kept immersed inside the bottom of the growth container for mixing, prevent sedimentation, and to ensure all cells of the population are equally exposed to the light and to nutrients and to improve carbondioxide exchange between the medium and air. Effect of aeration was observed under two different air flow rate. The air flowrate was 2.5 L/min and 1.25 L/min. Four batch reactor was used for each experiment containing 450 ml sterilized growth medium with 50 ml preadapted rapidly growing inoculum.

2.2 Sample Collection and Processing

Microalgal sample was collected from stagnant fresh water native pond in the Sylhet region and taken into the laboratory as soon as possible for further processing. The sample was observed under microscope to ensure that if it contains microalgae.

2.3 Liquid Enrichment

Collected sample was initially enriched in BG-11 broth to maintain the dominating species and to assure that medium is suitable for the species. Species inoculated to 1:10 ration. 1ml sample was directly inoculated into 50 ml autoclaved sterilized liquid medium into 250 ml conical flask. This ensured that the algae were not competing with native bacteria for nutrients in the medium. Conical flasks were kept at 25 ± 2^oC under cool fluorescence light with 16:8 h photoperiod with respect to blank media to visualize growth by naked eyes. The culture broth was shaken manually for three to four times a day for allowing aeration and no forced air is supplied.

2.4 Isolation and Identification

The enriched culture sample was first spread into sterilized petridish contains 15 ml BG-11 solid media to obtain a single strain. 1 ml inoculum was added on to the solidified media after 10^{-1} and 10^{-2} dilution by spreading method then it was further streak to obtain axenic culture. The plates were then kept in the culture room as inverted position at above said condition.

2.5 Inoculation into Broth tubes and vials

Single colonies were picked up by needle loop and allowed to grow in test tubes containing 3 ml liquid medium. All experiments conducted in triplicate. Test tubes were kept in the culture room for 1 week for noticeable growth. After 1 week 1 ml inoculum was transferred into 50 ml autoclaved medium with sterilized micropipette. After 7 days 5ml sterilized medium was introduced into it. After grown into the conical flask it is finally diluted with 450 ml liquid medium providing with aeration and kept into direct sunlight at outdoor condition.

2.6 Identification

Microalgal strains were identified using morphological taxonomic keys upto general level. A drop of liquid culture from test tube was placed on glass slide and observed under the compound microscope (40x). The morphological characters considered for identification were cell structure, color, spines present on the cell.

2.7 Growth Monitoring

To identify the inoculation date and specific growth rate, algal growth was measured in liquid media by using spectrophotometer at wavelength 540 nm and 680 nm after 2 days interval. The specific Growth rate was measured by the following equation:

$$\mu = \frac{\ln(OD_t) - \ln(OD_o)}{t}$$

Where OD_o and OD_t are the optical density at the start and end of the exponential phase and t is the difference of time measured in days.

The pH of the medium was adjusted around 7.1 and measured by pH meter after 2 days interval. It was calibrated using a pH 7.00 and a pH 10.00 calibrating buffer prior to the readings. For wet and dry biomass measurement, a dry and clean eppendorf tube was weighted first and recorded. Sample was taken into 1.5 ml eppendorf tube. It was then centrifuged at 5000 rpm for 5 minutes. Supernatant was discarded carefully and again measured. Wet mass was measured by the difference of this two weight. This eppendorf tube was then dried at 80^oC for 24 hour and again measured and dry mass was calculated.

2.8 Harvesting and Dewatering

Algal biomass was harvested after 14^{th} days during exponential phase prior to reach in stationary or decay phase. The algal biomass was centrifuged and washed with distilled water for removal of any metal ion. Sample was dried at 80^oC overnight in circulating airflow dryer. Dried sample was collected and grinded with mortar pestle and stored at 4^oC for further use.

2.9 Extraction Procedure

2 gm dry microalgae was grind in a mortar pestle. 6.8 ml methanol, 1.2 ml sulfuric acid, and 4.0ml chloroform was added with it. It was placed into a magnetic stirrer at 90^0C temperature for 40 minutes with thorough mixing. It was then cooled to room temperature. Further 4 ml of distilled water added and again mixed for 45 seconds. Allowed for phase separation. The content of biodiesel was too low. Water was added to the mixture for quick assay of oil and the oil floated on water.

3. RESULT AND DISCUSSION

3.1 Liquid Enrichment Culture

In primary liquid enrichment culture the sample algae

showed a good growth in the selected BG-11 medium. From microscopic view **Figure3.1** initially two different types of algae were observed. A few number of small spherical dark green color non flagellated cell were observed. Two or four cells were clustered together as well as single cell was also exist. These were similar to *chlorella.* A large number of oval shaped cells were also observed. 4-8 cells were arranged in a row. Colonies contain chloroplast and no curved spine or teeth observed. These characteristics are as similar as *scenedesmus*.

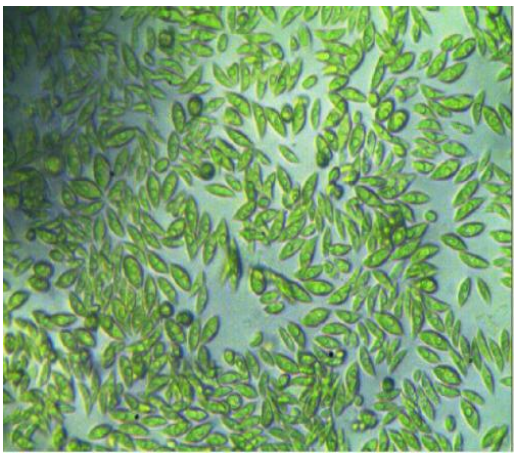


Figure 3.1: Microscopic view of liquid enrichment culture.

In incubation period, the species showed quick adaptation and good growth also.**Figure 3.2** illustrates

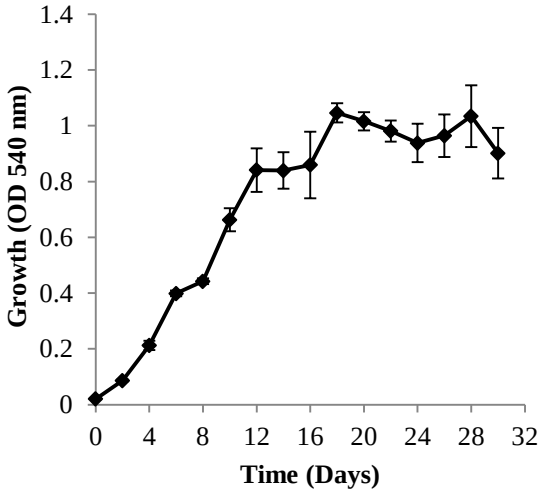


Figure 3.2: Growth observation by Optical Density 540 nm of the initial growth test in incubation period.

that, the lag phase exists for 4 days and then it exponentially increases upto day 12. After 12th day the growth of the species become limited and it gradually falls which indicates to decay phase.

3.2 Biomass Estimation

The biomass data were taken every 4 days interval. It has found that the biomass increases approximately 2 times from day 4 to day 16, and it can be considered the exponential period. At 16th day weight obtained was 15.19 mg/ml (wet) and 2.38 mg/ml (dry). The pH also increase in 9.94 in the incubation period. After the incubation period, the algae species were transferred at outdoor condition during exponential period. Scaling up was observed under two different air flow rate. From **Figure 3.3** it has found that, the maximum dry biomass obtained was 1.39 mg/ ml and 3.88 mg/ml at air flow rate 1.25 L/min and 2.5 L/min respectively.

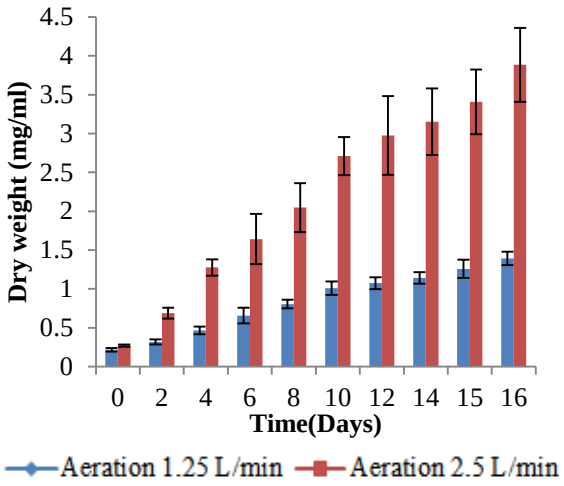


Figure 3.3: Variation in biomass accumulation (dry weight) in two different aeration condition.

Comparing these two conditions, it can be said that aeration as well as mixing has an effect upon the growth and biomass increases when aeration increase.

3.3 pH variation:

The pH of the medium at outdoor condition initially increases after adding the inoculum but decrease at day 2. The pH value of culture can decrease when CO_2 diffuses into the culture media via aeration while it increases through the photosynthetic process due to CO_2 absorption of microalgae as well as the secretion of alkaline metabolities from their cell. The change of pH for both cases shown in **Figure3.4** is similar but as the number of cells is greater in the larger aeration medium, hence the variation is greater than another one. Each experiment was done for 1 time by 4 replicates.

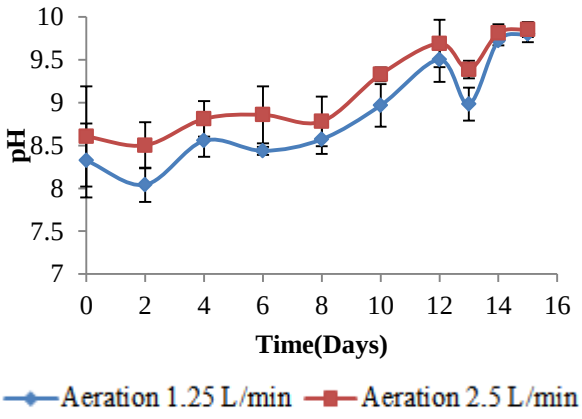


Figure 3.4: pH profile with respect to time in large scale test.

3.4 Result of Direct Transesterification

The amount of oil was nearly 0.5 ml obtained from 2 gm dry algae sample. It was insufficient for separation and further characterization. Water was added into the black paste like mass for quick assay of oil content.

3.5 Single Strain Isolation and Scale-up

The algae were isolated based on morphological characteristics. The proper colonial algae was observed and isolated into test tube after 14 days. In agar plate **Figure 3.5** three different types of colony appeared. After transfer into test tube growth was observed after 3 days and further inoculation was done after 10 days.

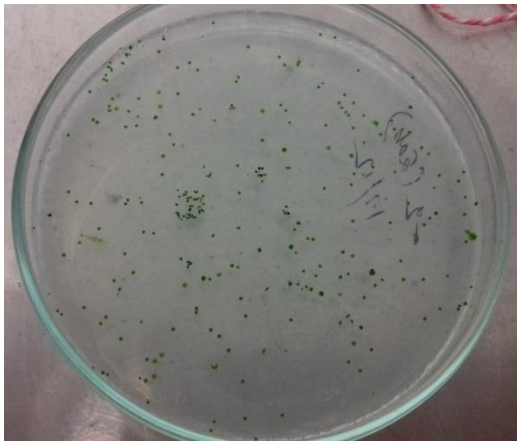
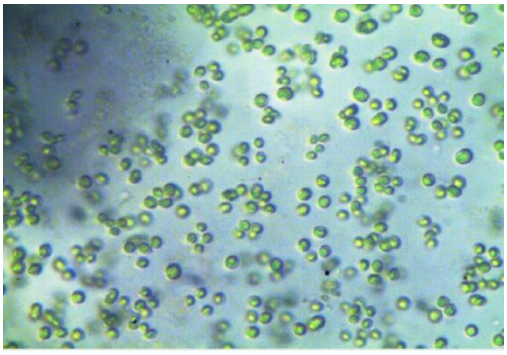


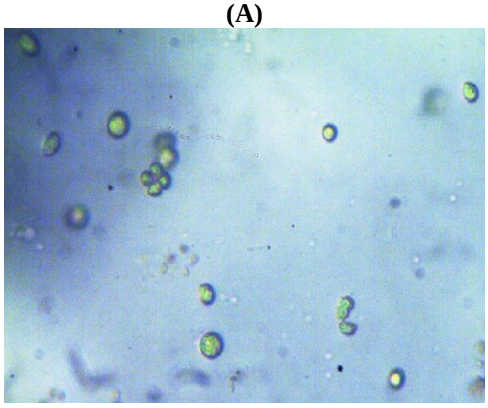
Figure 3.5: Single colony appears in BG-11 agar plate.

3.6 Microscopic Observation

From morphological characteristics, it has found that the round shaped dark and light green color colony was two different species of *Chlorella* **Figure 3.6 (A)** and **(B)**



(A)



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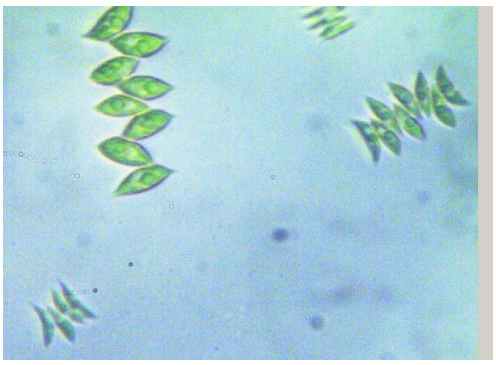

(C)

Figure 3.6: Microscopic observation at 40X (A) Dark green colored *Chlorella* species. (B) Light Green colored *Chlorella* species and (C) *Scenedesmus.*

and the other one is *Scenedesmus* Figure 3.6 (C) as mentioned before.In purity test, no contamination found in any species.

3.7 Growth Comparison of Isolated Species

For all of these isolated species growth was analyzed in incubation chamber first. From other literature review, it was expected that when isolated, *Chlorella* species will grow better in this medium. But in realistic case only *Scenedesmus* shows excellent growth in the medium as previous. Both the two species of *Chlorella* shows a long lag phase. The specific growth rate of *Scenedesmus*, dark green colored *Chlorella* and light green color *Chlorella* was 0.15 day^{-1}, 0.068 $day^{-1}$and 0.062 day^{-1} respectively. For most studies the growth rate of algae is not comparable due to different experimental condition while lipid content, composition and biomass productivity are compared. From **Figure 3.7** it is shown that, the final dry weight of biomass for

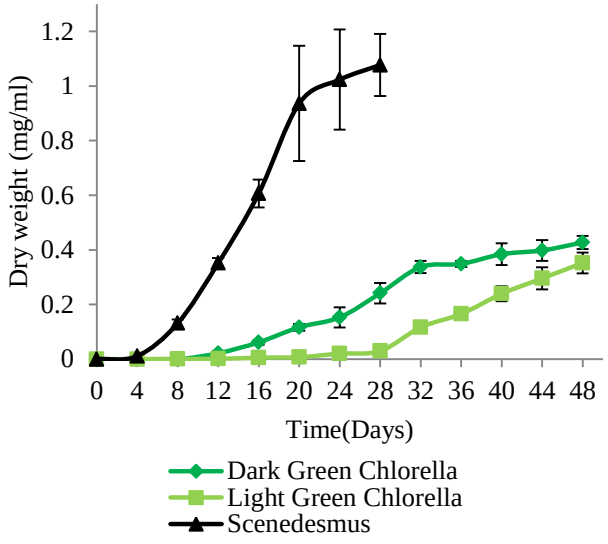

Figure 3.7: Dry weight of algae cultured in incubation chamber.

Scenedesmus is 0.60 mg/ml, for dark green colored *Chlorella* it was 0.33 mg/ml and for light green colored *chlorella* it was 0.11 mg/ml respectively at the end of the exponential period. From this comparison, it was decided that *Scenedesmus* and dark green color *Chlorella* will be scale up for larger scale biodiesel production.

3.8 Large Scale Test for single strain

Dark green color *Chlorella* and *Scenedesmus* species was given to the large scale test at three different aeration condition. These conditions are #1 without any aeration, #2 at 1.25 L/min and #3 at 2.5 L/min volumetric air flow rate.This single strain also better suit in outdoor condition and survived at a large temperature variation.
In case of condition #1 the biomass productivity increases at first two day rapidly and then it increases too slowly.No manual shaking or no agitation was done for these three reactors.

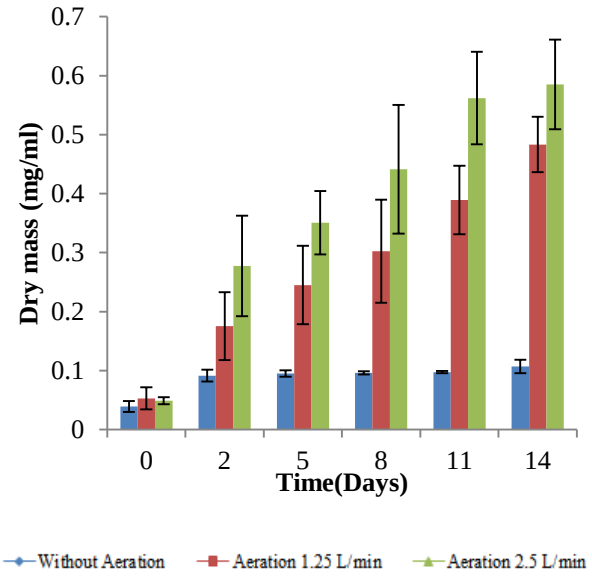

Figure 3.8: Dry weight of biomass accumulation of dark green colored *Chlorella* species of large scale test with respect to time at outdoor condition.

From **Figure 3.8** the final dry weight of biomass is 0.1 mg/ml (dry weight). One reason may be the species consumes nutrient from the medium rapidly and grow initially and then the growth limited due to the lack of nutrients. In case of condition #2 the weight of biomass is 0.48 mg/ml (dry weight). It shows that the biomass productivity increases 4.8 fold as compared to case #1. In case #3 it is seen that the final biomass obtained is 0.58 mg/ml (dry weight) . The biomass productivity increases 5.8 fold as compared to case#1 and 1.2 fold as compared to case#2 while aeration increases 2 fold for the second case. It can be concluded that, aeration has a major effect upon biodiesel productivity. In a closed photobioreactor system aeration must require due to proper mixing also.

Scenedesmus was the most suitable species in the medium. It was expected that it accumulates a measurable amount of biomass for further extraction. But the growth was affected by cloudy weather which is a big challenge for algal growth.

5. CONCLUSION

In Bangladesh perspective the idea of using algae as a fuel source is relatively new and very few researches were conducted in this field. A very few numbers of published papers were found that had reported oil production, however all of those reported species were either of foreign origin or tested in conventional photobioreactor system which is costly. The main goal of this theses was to identify and isolate a high lipid containing strain from local sources for biodiesel production rather than collecting it from other countries.

- Our experiments suggest that the isolated species have good survival capability in outdoor condition.
- Biomass productivity is considerable as compared to other conventional photobioreactor system in liquid enrichment case.
- In addition among three species, *Scenedesmus* grows very fast though the oil content per biomass unit is low.

Microalgae have the capability to remove nutrientsfrom waste water. Therefore, the isolated species can be utilized for biodiesel production and waste water

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