LARGE SCALE PRODUCTION OF MICRO ALGAE AND EXTRACTION OF BIOOIL BY TRANS ESTERIFICATION METHOD.

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ABSTRACT

Algae are a diverse group of eukaryotic photosynthetic organisms that constitute over 40,000 species. They can be single – celled (unicellular) or multicellular such as seaweed. Microalgae has been described as nature’s very own power cells and could provide alternatives to petroleum based fuels without competing with crops. In this study, the algae species selected were Chlorella pyrenoidosa and Scenedesmus. It was initially expanded in its selective medium (Fog’s medium) and then expanded to a larger scale after putting various trials at various concentrations. It was found that chlorella well adapted to the waste water as there was increase in the absorbance and doubling time. Carbon dioxide seemed to enhance the growth of algae even more than aerators used. The algae were expanded in the Bubble tops of 25 litres capacity to get the mass production of algae for the Biodiesel production. Collected biomass The Flocculating agent used was the Aluminum Chloride that showed good separation. Decreasing the pH to 4 showed a better result and faster flocculation thus it was performed by decreasing pH to 4. Then the collected biomass was used for the Biodiesel production by mixing the catalyst mixture consisting of Sodium Hydroxide and the solvent methanol and the Trans esterification process was carried out and allowed to settle for 16 hrs. In this study, the oil was produced from the algae efficiently by Trans esterification which was environmental friendly. The amount of oil extracted was found to be 28.8 ml. By this way, Chlorella pyrenoidosa can be used as renewable energy source.

KEYWORDS: Microalgae, Chlorella pyrenoidosa, Scenedesmus, Biooil, Trans esterification.
INTRODUCTION

Biodiesel is a renewable and environmental friendly energy source that can be produced via trans esterification from various oil crops such as soy bean, sunflower, palm, and algae. In this work, the microalgae *Scenedesmus obliquus*, *S. armatus* and *S. bernadii*, isolated from natural water basins, were enriched in modified Chu 13 medium.

A study was conducted on fresh water green algae *Selenastrum* sp. to check its growth behavior and tolerance under different concentrations of sodium bicarbonate salt, carbon dioxide gas and under different levels of sodium chloride salt. The experiment aims to analyze the biomass productivity and total lipid content of the microalgae strain under various growth conditions.

MICROALGAE

Microalgae are sunlight-driven cell factories that convert carbon dioxide to potential biofuels, foods, feeds and high-value bioactives. In addition, these photosynthetic microorganisms are useful in bioremediation applications and as nitrogen fixing biofertilizers. Microalgae can generate diverse biofuels, which are mainly: bio methane produced by anaerobic digestion, biohydrogen by photobiological process, bioethanol by fermentation, liquid oil by thermal liquefaction and biodiesel. Even if industrial scale biofuels from microalgae remain at an early stage, they remain a sustainable solution as a transportation fuel (Khan et al., 2009) (Jacob-Lopes & Teixeira Franco, 2010).

*Chlorella pyrenoidosa* is a unicellular green alga that grows in fresh water. *C. pyrenoidosa* contains much more protein and chlorophyll than other plants, and is also high in vitamins, minerals, dietary fiber, and nucleic acids. The proteins contained in *C. pyrenoidosa* include all the essential amino acids necessary for human growth and maintenance of health. *C. pyrenoidosa*, other *Chlorella* species and *Chlorella* extracts have been reported to exert a variety of effects, including reducing cholesterol, 19 preventing stress-induced ulcers, 20 enhancing resistance to infection and antineoplastic activity. Furthermore, *C. pyrenoidosa* promotes the fecal excretion of orally ingested dioxins in mice. *C. pyrenoidosa* is currently widely available in many regions of the world as a nutritional supplement and health food.

BIO OIL PRODUCTION

Bio oil has a viscosity similar to petroleum diesel and can be used as an additive in formulations of diesel to increase the lubricity. Bio oil can be used in pure form (B100) or
may be blended with petroleum diesel at any concentration in most modern diesel engines. Bio oil will degrade natural rubber gaskets and hoses in vehicles (mostly found in vehicles manufactured before 1992), although these tend to wear out naturally and most likely will have already been replaced with Viton type seals and hoses which are nonreactive to biodiesel.

Bio oil higher lubricity index compared to petroleum diesel is an advantage and can contribute to longer fuel injector life. Bio oil is a better solvent than petroleum diesel and has been known to break down deposits of residue in the fuel lines of vehicles that have previously been run on petroleum diesel. Fuel filters may become clogged with particulates if a quick transition to pure biodiesel is made, as Bio oil “cleans” the engine in the process. It is, therefore, recommended to change the fuel filter within 600-800 miles after first switching to a biodiesel blend (Kay, 1991).

TRANSESTERIFICATION

Trans esterification of natural glycerides with methanol to methyl esters is a technically important reaction that has been used extensively in the soap and detergent manufacturing industry worldwide for many years. Almost all biodiesel is produced in a similar chemical process using base catalyzed trans esterification as it is the most economical process, requiring only low temperatures and pressures while producing a 98% conversion yield.

The trans esterification process is the reaction of a triglyceride (fat/oil) with an alcohol to form esters and glycerol. A triglyceride has a glycerine molecule as its base with three long chain fatty acids attached. The characteristics of the fat are determined by the nature of the fatty acids attached to the glycerine. The nature of the fatty acids can, in turn, affect the characteristics of the biodiesel.

In the trans esterification mechanism, the carbonyl carbon of the starting ester (RCOOR) undergoes nucleophilic attack by the incoming alkoxide (R₂O⁻) to give a tetrahedral intermediate, which either reverts to the starting material, or proceeds to the trans esterified product (RCOOR²). The various species exist in equilibrium, and the product distribution depends on the relative energies of the reactant and product.
COLLECTION OF CHLORELLA PYRENOIDOSA, SCENESDESMUS

The algal strains were collected from NCIM, Pune and maintained properly in its particular selective medium at Genewin Biotech, Hosur.

IDENTIFICATION OF ALGAE

The collected Chlorella was grown in the medium and was identified and confirmed with its characteristics using the Microscope. According to studies available it is noticed that the bio oil extraction and lipid content is high in Chlorella pyrenoidosa.

SAMPLE COLLECTION

The lake water samples collected from Gudiyattam lake, Tamil Nadu. The samples were collected in the pre-cleaned polythene bottles with necessary precautions. The collected Lake water was analyzed for the parameters such as pH, EC, TDS, Chlorides, Hardness, COD, Turbidity as initial testing and the results were compared to ICMR standards (K. Usharani et al., 2010).

CONFIRMATION OF ORGANISM

The organism Chlorella pyrenoidosa was confirmed using Lactophenol cotton blue staining and its characteristics were observed.

The identification of molds is based on the shape, method of production, and arrangement of spores (conidial ontogeny). Lactophenol Blue Solution is a mounting medium and staining
agent used in the preparation of slides for microscopic view. Fungal elements are stained intensely blue.

- Take a sterile glass slides for microscopic view.
- Place a drop of Lactophenol Blue Solution on a slide.
- Using an inoculating needle carefully spread the fungal culture into a thin preparation.
- Place a cover slip edge on the drop and slowly lower it. Avoid trapping air bubbles under the cover slip. Wait for about 5 minutes.
- If desired, seal the edges of the cover slip with nail polish or Per mount to preserve the mount as a reference slide.
- Observe under a microscope with low power for screening in low intensity (Tripathi, A., Srivastava, S.K., 2011).

MICROSCOPIC VIEW OF CHLORELLA, SCENEDESMUS:

The Chlorella, Scenedesmus which were collected from NCIM, Pune and regularly sub cultured and maintained at Genewin Biotech was examined under the Microscope to confirm all the features of the same algae. The microscopic features observed were.

- Chlorella is Single celled, The cells of Chlorella was spherical in shape

Fig 1: Microscopic View of Chlorella.

Fig 2: Microscopic View of Scenedesmus.
Lab scale trials on the three species were conducted. It is clearly noted that the algae in its particular fertilizers showed varying levels of growth. Highest growth being noted in that of *Chlorella* in its elective media followed by *Scenedesmus*. Thus Chlorella is used for the further studies.

**MASS CULTIVATION OF ALGAE**

The lowest concentrations of FA were selected and the algae were allowed to grow in it and the Absorbance was taken for a period of week. It was found that at every concentration the algae growth increased gradually but both the algae were able to adapt itself in the lowest concentration of 0.01 when compared to the other concentrations. The Doubling time taken by algae also was less in the 0.01 concentration.

![Fig 3: Mass Cultivation Of Algae.](image)

**BIOMASS ESTIMATION:** Quantitative Analysis Using Algal Biomass: Determination Of Carbohydrate: Standard Curve For Glucose.

<table>
<thead>
<tr>
<th>Concentration (Mg)</th>
<th>Optical Density (575 Nm)</th>
<th>Carbohydrate Content (Mg/ML)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Scenedesmus</em></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>0.5</td>
<td>0.0206</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0.0511</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0.0520</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0.0948</td>
<td>0.35</td>
</tr>
<tr>
<td>2.5</td>
<td>0.1041</td>
<td></td>
</tr>
</tbody>
</table>
TOTAL CHLOROPHYLL CONTENT
Chlorophyll a (Ca) = (12.25 × OD at 663) - (2.79× OD at 645) ×10/ (1000×wt)
Chlorophyll b (Cb) = (21.50 × OD at 645) - (5.10× OD at 663) ×10/ (1000×wt)
Total Chlorophyll (C) = (7.15 × OD at 663) + (18.71× OD at 645) ×10/ (1000×wt)

<table>
<thead>
<tr>
<th>ALGAE</th>
<th>Chlorophyll a</th>
<th>Chlorophyll b</th>
<th>Chlorophyll c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella pyrenoidosa</td>
<td>1.67%</td>
<td>1.08%</td>
<td>1.15%</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>1.43%</td>
<td>1.03%</td>
<td>1.10%</td>
</tr>
</tbody>
</table>

DETERMINATION OF PROTEIN

STANDARD CURVE OF BSA (BOVINE SERUM ALBUMIN)

<table>
<thead>
<tr>
<th>Concentration (Mg/Ml)</th>
<th>Absorbance</th>
<th>Estimation Of Protein (Mg/Ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Scenedesmus</td>
</tr>
<tr>
<td>1000</td>
<td>25.8</td>
<td>980</td>
</tr>
<tr>
<td>2000</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>3000</td>
<td>72.8</td>
<td></td>
</tr>
<tr>
<td>4000</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>193</td>
<td></td>
</tr>
</tbody>
</table>
It was observed that the protein in the collected biomass was higher in *Chlorella pyrenoidosa* of 2000 mg/ml.

**TEST FOR FLAVONOIDS**

**STANDARD CURVE OF QUERCETIN**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance of std (Quercetin)</th>
<th>FLAVONOID CONTENT (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>Scenedesmus</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Chlorella pyrenoidosa</em></td>
</tr>
<tr>
<td>2</td>
<td>0.22</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.43</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.87</td>
<td></td>
</tr>
</tbody>
</table>

From the standard graph, the total flavonoids content was determined as 0.2 µg/ml in the Scenedesmus, 1µg/ml in Chlorella.
DETERMINATION OF TOTAL PHENOLIC CONTENT

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance of Std(Gallic acid)</th>
<th>PHENOLIC CONTENT (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Scenedesmus</td>
</tr>
<tr>
<td>200</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2.5</td>
<td>420</td>
</tr>
<tr>
<td>1200</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1400</td>
<td>3.7</td>
<td></td>
</tr>
</tbody>
</table>

From the standard graph, total Phenol content is found as 420 µg/ml in the *Scenedesmus* and 1000 µg/ml in the *Chlorella*.

ANTIOXIDANT ACTIVITY USING DPPH METHOD: Control=0.0893

<table>
<thead>
<tr>
<th>CONCENTRATION (µl)</th>
<th>ANTIOXIDANT ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scenedesmus</td>
</tr>
<tr>
<td>25</td>
<td>0.0902</td>
</tr>
<tr>
<td>50</td>
<td>0.1480</td>
</tr>
<tr>
<td>100</td>
<td>0.2063</td>
</tr>
<tr>
<td>200</td>
<td>0.2326</td>
</tr>
<tr>
<td>400</td>
<td>0.1854</td>
</tr>
</tbody>
</table>
The antioxidant activity was found to be more in *Chlorella pyrenoidosa* than the *Scenedesmus* which gradually increased as the concentration increased.

**DETERMINATION OF NITROGEN**

*Scenedesmus*

\[ \% N = V_1 \times N_1 \times 1.4/M \]

- \( V_1 \) - Volume in ml of standard acid
- \( N_1 \) - Normality of standard acid
- \( M \) - Mass in g of the prepared sample

Nitrogen fixation by micro-kjelDahl method

\[ V_1N_1 \times 1.4/M = (1.5)0.01 \times 1.4/0.0 = 2.1\% \]

*Chlorella pyrenoidosa*

\[ \% N = V_1 \times N_1 \times 1.4/M \]

- \( V_1 \) - Volume in ml of standard acid.
- \( N_1 \) - Normality of standard acid.
- \( M \) - Mass in g of the prepared sample.

Nitrogen fixation by micro-kjelDahl method

\[ V_1N_1 \times 1.4/M = (2.1)0.01 \times 1.4/0.01 = 2.94\% \]

The Nitrogen content was higher in *Chlorella pyrenoidosa* of 2.94% than the *Scenedesmus*. 
**Floculation**

The Floculating agent used was Aluminium Chloride for the separation of algae and the water in the bubble top for the collection of biomass.

It is found that decreasing the pH to 4 using acid seemed to help in flocculation faster followed by aluminum chloride. Trials were put and then concluded as to which method to flocculate algae in large scale production.

![Fig 4: Floculation.](image)

**A) Various Trials Used For Floculation, B) Decrease Ph To 4**

**BIOMASS COLLECTION**

The Biomass was collected after the Flocculation process and the collected biomass was used for the Biodiesel production.
The collected Biomass cake obtained from *Chlorella pyrenoidosa* after drying was observed as 42 g which was further studies.

**Fig 5 A): Collected Biomass – Algal Cake From Chlorella Pyrenoidosa.**

The collected Biomass cake obtained from *Secenedesmus* after drying was observed as 33 g. As the quantity obtained was lesser amount, it was not used for the bio oil production.

**Fig 5: B) Collected Biomass – Algal Cake From Scenedesmus.**

**Evaporation of oil from algae**

Rotary evaporators (also called "rotavaps") are used to remove solvents from reaction mixtures and can accommodate volumes as large as 3 liters. They are found in almost every organic laboratory, since they allow performing this task very quickly. A typical rotary evaporator has a water bath that can be heated in either a metal container or crystallization dish. This keeps the solvent from freezing during the evaporation process. The solvent is
removed under vacuum, is trapped by a condenser and is collected for easy reuse or disposal. The collected oil was evaporated in order to eliminate the catalyst mixture from the oil. The catalyst mixture contained Sodium hydroxide and the solvent Methanol.

Fig 6: Evaporation Of Oil.

Separation of Bio-oil

The Bio oil was separated from the algae after the Trans estertification process. The high oil yield was reported in different studies. Goveia & Oliveira, 2009 reported 29.0 and 28.7% of oil extracted from Neochlorisoleoabundans and Nannochloropsissps. Hossain et al, 2008 reported 1.8 gm and 3.0 gm of extracted oil from Spirogyra and Oedogoniumssps. Kandra et al 2010 reported 40.1% of green crude oil. Steam distillation using Clevenger apparatus proved to be most effective for algal oil extraction & petroleum ether was found to be the best solvent in our investigation. It seems that algal oil extraction from algal blooms may not be an economically viable solution for producing green sustainable energy.

Fig 7: Separation of Bio Oil.
In this study, the oil was produced from the *Chlorella pyrenoidosa* efficiently by Trans esterification which was environmental friendly, the amount of oil extracted was found to be 28.8 ml. The pH was found to be 7.62. By this way, *Chlorella pyrenoidosa* can be used as renewable energy source.

**SUMMARY AND CONCLUSION**

In this study, the algae species selected were *Chlorella pyrenoidosa* and Scenedesmus. It was initially expanded in its selective medium (Fog’s medium) and then expanded to a larger scale after putting various trials at various concentrations. Waste water was supplemented with certain fertilizers with different N:P:K ratio and growth of algae performed in order to check whether *Chlorella* was efficient enough to adapt itself to the waste water. The Absorbance was taken in UV Spectrophotometer for checking its growth at 680 nm for a period of 10 days. It was found that *chlorella* well adapted to the waste water as there was increase in the absorbance and doubling time.

The algae were expanded in the Bubble tops of 25 litres capacity to get the mass production of algae for the Biodiesel production. They were expanded in the bubble tops and then they were flocculated in order to separate the algae from the liquid. The biomass collected after mass cultivation. Collected biomass The Flocculating agent used was the Aluminum Chloride that showed good separation. Decreasing the pH to 4 showed a better result and faster flocculation thus it was performed by decreasing pH to 4. Then the collected biomass was used for the Biodiesel production by mixing the catalyst mixture consisting of Sodium Hydroxide and the solvent methanol and the Trans esterification process was carried out and allowed to settle for 16 hrs.

It was observed that the Bio oil was clearly produced after 16 hours. It was concluded that the Bio oil can be produced from the algal species efficiently and it was environmental friendly and Pollution free. It is concluded that algae species for its high availability in nature, renewable energy source and low cost could be efficiently used to produce Bio oil. In this study, the oil was produced from the algae efficiently by Trans esterification which was environmental friendly. The amount of oil extracted was found to be 28.8 ml. By this way, *Chlorella pyrenoidosa* can be used as renewable energy source.
REFERENCES


