



An Efficient Extraction Method for the Isolation of Newly Isolated Green Microalgae from Fresh Water Bodies

S Michael Donatus, S Vijayalakshmi, J Ranjitha*

CO₂ Research and Green Technologies Centre, VIT University, Vellore-14

Abstract: Algal culture has been isolated from pond water and tested for its growth, pigment, protein and fatty acid contents. The growth of algal biomass was assessed by the means of optical density with at one week intervals up to fifth week Then for identification of the species level, DNA was extracted and sequenced and identified *Chlorella vulgaris* and *Scenedesmus dimorphus* was stained by DAPI staining for visualization of the microalgae cells in Fluorescent microscopic observations and also lipid was determined by using BODIPY staining method. Algal oil was extraction by two effective methods were followed for the estimation of lipids level of both algal culture Bligh and Dyer Extraction and Extraction n-hexane using Soxhlet's extraction process method, After the algal lipid was extraction from both algal culture, the lipid bodies was view under two different types of Microscopy CARS Microscopy, Scanning Electron Microscopy.

Keywords: *Chlorella vulgaris*; Isolation; Growth; Pigment; Carotenoid; Proteins; fatty acid; DAPI staining method.

Introduction

The main energy consumed sources in the worldwide is derived from fossil fuels, and it is used as transportation fuels. The usage of the fossil fuels results in the emission of greenhouse gas as well as increases in the cost of petroleum products. Hence, there is a need for an alternative source for the production of energy. The significant feedstock for the production of biodiesel is the lipids derived from animal fats, vegetable oils, waste cooking oils and plant oils¹. Similarly, on the other hand, microalgae had been identified as potentially feedstocks for the production of biofuel, because of their high lipid content and rapid biomass production.

In recent years, use of microalgae is as an alternative source for biodiesel production. It has gained more interest from researchers, scientists and entrepreneurs, etc.². The biodiesel produced from algal sources which is free from smog forming, sulphur emissions, sulfuroxides, and other chemical products³. Microalgae is having an important environmental characteristic, reduces CO₂ through photosynthesis and involves bioremediation to remove the heavy metals, such as nitrogen and phosphorous in waste water⁴. *Chlorella* is one among the common algal species which has potential benefits which is used as a food and feed supplement in health and cosmetic products. The growth of the algae is depending upon various biological and chemical factors like pH, temperature, aeration, agitation, salt concentration and light illumination.

Micro algal oil consists of high triglycerides molecules which can be transformed into biodiesel⁵. Microalgae have an ability to produce energy, because of their photoautotrophic mechanism, which is used for the conversion of atmospheric carbon dioxide into biomass, fatty acids, and lipids^{6,7}. Consequently, microalgae also have certain advantages such as, a high growth rate, short growth time, high biomass production,

and low land use to compare other plant crops. Microalgae have important environmental benefits, reduces CO₂ through photosynthesis and involves bioremediation to remove the heavy metals, such as nitrogen and phosphorous in wastewater⁸. The important advantages of biodiesel as diesel fuel are liquid nature portability, ready availability, renewability, higher combustion efficiency, lower sulphur, aromatic content higher, and higher biodegradability. Main advantages of biodiesel given in the literature include domestic origin, reducing the dependency on imported petroleum, biodegradability, high flash point and inherent lubricity in the neat form⁹. Hence the present research paper describes about newly isolated micro algal species from water bodies for the biodiesel production.

Materials and Methods

Isolation of Algal strain and cultivation condition

The water sample was collected from the pond in the southern part of Tamil Nadu in Kanyakumari district. The sample was taken and serial dilution was done using Bristol medium agar plates method. The colonies were segregated, based on their morphological features using microscope. The single colony was isolated into serially diluted plates, and then, sub-cultured streak plates were done using BG 11 medium agar plates to obtain pure colonies. It was maintained in BG 11 medium in rotary shaker at 100rpm with a photoperiod of 12 hours in the presence of light/ 12 hours in dark, light intensity of 2000 lux at room temperature (28±2). The identification of the microalgae at the species level, DNA was extracted and sequenced and identified as *Chlorella vulgaris* and *Scenedesmus dimorphus* via wet fluorescent microscope.

DAPI staining for visualization of the microalgae cells

Take 1 ml of pure micro algal culture grown after 25 days at 28°C in BG-11 (non-N₂ fixer medium) & (N₂ fixer medium). The centrifuged samples (2350 g) were taken for pelletisation and the supernatant solution was discarded. The cell pellet was washed with 200 µl of McIlvaine's buffer. Fix the pellet with 200 µl of 4% formaldehyde (prepared in McIlvaine's buffer) and incubate for about 30 minutes. Wash the pellet with buffer solution. The cells permeable by treating with 200 µl of 0.3% (v/v) Triton-X-100 solution (prepared in McIlvaine's buffer) and finally washed with buffer. The cells were mixed on the slide containing 200 µl of DAPI solution and then incubated for about 30 minutes in the dark surface. The proper and gentle washing was done to minimize the background noise, due to the fluorescence of extra DAPI stain present on the slide during microscopy. Dry the slides at room temperature in the dark surface and then observe the slides under microscope. This study was used to identify the nature of the microalgae species, *chlorella vulgaris* and *scenedesmus dimorphus*, these species were used for lipid identification and bio-diesel production.

Determination of Lipid using BODIPY staining method

The BODIPY 505/501 stock solution (1 mg/ml) was prepared in DMSO. A fresh solution of BODIPY 493/503 diluted in the presence of M9 buffer at specified concentration. The vital staining was done using 2 µl of BODIPY 505/515 added to 3ml of micro algae culture.

Growth measurement of Microalgae culture

Microalgal species *chlorella vulgaris* and *scenedesmus dimorphus* were cultured using BG 11 medium and then the growth of the micro-algal cultures were measured at 680 nm using UV-Visible spectrophotometer¹⁰.

Estimation of Biomass

The total biomass quantity was estimated using Richmond and Gobbelaar (1986) standard protocol. The grown microalgal cultures were taken and filtered through vacuum suction pump. The filtered wet biomass was taken and filtered through whatman filter paper No.1 and then dried in an hot air oven at 60°C until constant weight was obtained. The biomass yield was calculated in terms of mg/ml.

Estimation of Chlorophylls

The total chlorophyll content was estimated by homogenizing a known volume of culture and centrifuged at 8000 rpm for about 10 minutes. The pellet was treated with 10 ml of 95 % methanol, shaken well and incubated at 60°C in water bath for about 30 minutes. The supernatant was centrifuged and the absorbance was measured at 652.4 and 665.2 nm using UV-Visible spectrophotometer model using 95 % methanol as a blank¹¹.

Estimation of Carbohydrates

The carbohydrate content was estimated by Dubois method¹². Algae sample was taken and to this 1 ml of 4% phenol solution and 5 ml of concentrated sulphuric acid were added. After that, the whole solution was kept in a dark room for about 30 minutes. The colour intensity was developed and the absorbance values were measured at 490 nm. The sugar content was calculated by using standard D- Glucose and the results have been expressed in terms of mg/g sugar.

Estimation of Protein

The protein content was estimated by Biurette method¹³. To the algal samples, 1ml of distilled water was added and followed by 4ml of biurette reagent, incubated for about 30 min at room temperature. The mixture was centrifuged for about 10 min at 4000 rpm. The supernatant was collected and the optical density was measured at 540 nm using UV-Visible Spectrophotometer. The protein content was calculated using BSA as standard and expressed in terms of mg/g protein.

Calculate the Dry weight of Biomass

Take a known sample of wet algal biomass in a china dish and note the weight of the sample with dish, then the sample was kept in a hot air oven for about 4 hours at 105°C to remove the moisture content. After 4 hours, cool the dish in desiccators and then the weight of the dish was noticed. The formula to calculate the percentage of dry biomass weight is,

$$\text{Dried weight of the biomass} = \frac{\text{Dired weight of the sample}}{\text{Weight of the sample}} \times 100$$

Lipid extraction from algal biomass

The algal oil extraction was carried out using two effective methods, (i) Bligh and Dyer and Soxhlet's extraction process method. The biomass of algae was harvested by centrifugation at 5,000 rpm and wet cell mass was dried at 105°C at constant weight was obtained. In soxhlet extraction process, weighed 100 grams of algal powder material and then transferred the whole powdered algal material into Soxhlet's extraction tube. About 350 ml of n-hexane was poured into a round-bottomed flask and then, the Soxhlet's apparatus was connected with LB condenser. Finally the organic solvent was heated using a heating mantle for about 48 hours at a temperature of 80°C. The algal oil was collected from extraction process using distillation process.

Enssani¹⁴ adapted Bligh and Dyer procedure, which was used to extract lipids from the algae for further study. After the samples had been pelleted in the centrifuge tubes, they were used for extraction immediately. The algae concentrate was dried in a hot air oven at 80°C for about 40 min. About 250 g of algal biomass was weighed and then the extraction was proceed using 50 mL of chloroform, 100 mL of methanol and 40 mL of deionized water to the 250ml conical flask and the solution was sonicated using sonicator (ultra-sonication operated at a frequency of around 40 kHz for 2 minutes). This process is used to disrupt the algae cells present in the suspended mixture. The samples were then placed horizontally on a mechanical shaker for about 6-8 hours. The purpose of the shaking step was to promote the complete exposure of intracellular products into the solvents. The flasks were removed from the shaker and then additional 20 mL of chloroform and 20 mL of deionized water were added to the algal sample. After addition of solvents, the algal sample was vortex mixed for about 30 seconds to mix thoroughly with the organic solvents. The centrifuge tubes were then centrifuged at 4000 rpm for four minutes to separate the contents into layers. The green layer at the bottom, comprised primarily of chloroform, contains lipophilic material. The upper layer contains methanol and water. A thin layer of cell debris separates into the two layers and separated using separating funnel. After the algal

lipid was extracted from both the algal culture, the lipid bodies were viewed under two different types of Microscopy - CARS and SEM Microscopic technique.

Yield of oil in percentage

After the algal oil was extracted using Soxhlet's extraction process. It was then transferred into a measuring cylinder which was placed over a water bath for about 30 minutes at 70°C, to ensure complete evaporation of solvent and then the volume of the oil was measured. The percentage yield of oil was calculated using standard formula¹⁵.

$$\text{oil content(\%)} = \frac{\text{weight of the oil}}{\text{weight of the sample}} \times 100$$

Results and discussion

Identification and of microalgae for biofuels production

The water sample was collected from the pond in southern part of Tamil Nadu in Kanyakumari district. The collected water sample was done serial dilution using standard protocols. The colonies were segregated, based on their morphological features using compound microscope. The single colony was isolated from serially diluted plates, and then, sub-cultured into streak plates using BG 11 medium agar plates to obtain pure colonies. It was maintained in BG 11 medium in rotary shaker at 100rpm with a photoperiod of 12 hours light/ 12 hours dark, light intensity of 2000 lux at room temperature (28±2). DNA was extracted from algal species and then sequenced and identified as *chlorella vulgaris* and *scenedesmus dimorphus* and viewed at fluorescent microscope as shown in the figure1&2.

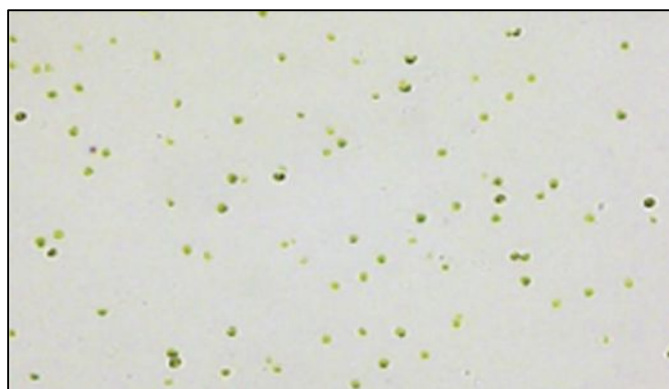


Figure 1: Microscope view of *Chlorella vulgaris*



Figure 2: Microscope view of *Scenedesmus dimorphus*

Biochemical estimation of Algae samples

The growth of *Chlorella vulgaris* and *Scenedesmus dimorphus* was determined from optical density values using UV-Visible spectrophotometer. The chlorophyll content and dry weight was measured at 5 weeks with same environmental conditions. The optical density value was increased in *Chlorella vulgaris* than *Scenedesmus dimorphus* reported by Rekha Sharma, *et al.*,¹⁶. *Chlorella vulgaris* shown that the effects of culture conditions at different temperature and light regimes on the growth. Similarly, the contents of chlorophyll-a, chlorophyll-b, total carotenoids, total protein and total free amino acids of *Chlorella vulgaris* were determined. The maximum growth was achieved for *Chlorella vulgaris* is 0.42 at 670 nm, and dry weight (30.2 mg/50 ml), and the amount of chlorophyll- a (2.16%), chlorophyll- b (0.59%) and total protein. It was found higher at the temperature 25-30°C, natural day light receiving through the north facing window of the growth room. In this present desired study two different strains BG 11 medium in natural day light receiving at room temperature (28±2). *Scenedesmus dimorphus* shows decrease in the growth rate, compared with *Chlorella vulgaris*. The dried weight and chlorophylls pigment increased activity of *Chlorella vulgaris* are in the graphs and figure 3, 4, 5.

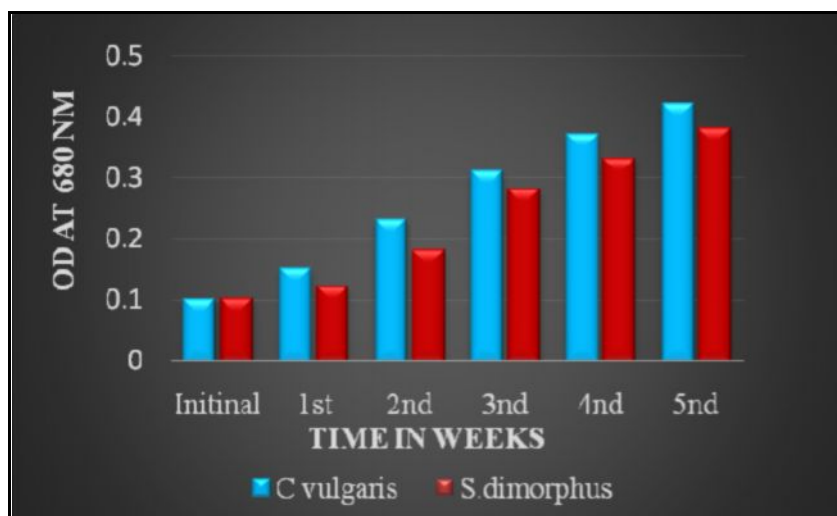


Figure 3: Effects of two different cultures in same conditions on growth (OD at 680 nm)

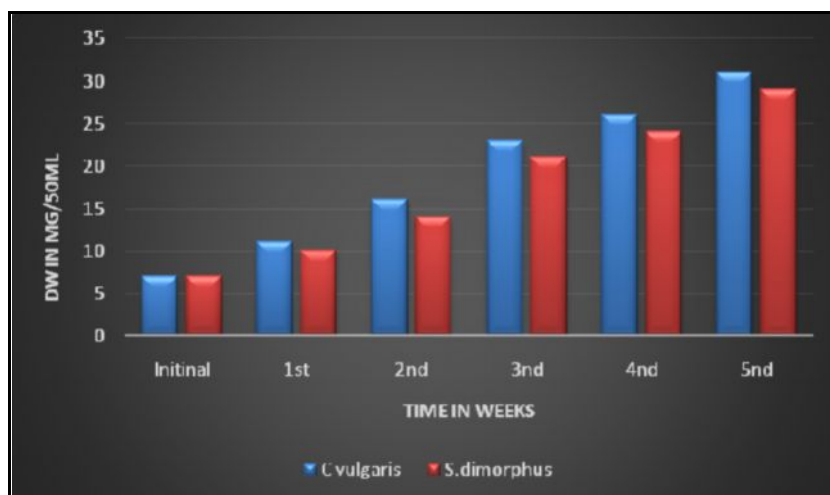


Figure 4: Effects of two different cultures in same conditions on growth (DW in mg/50ml)

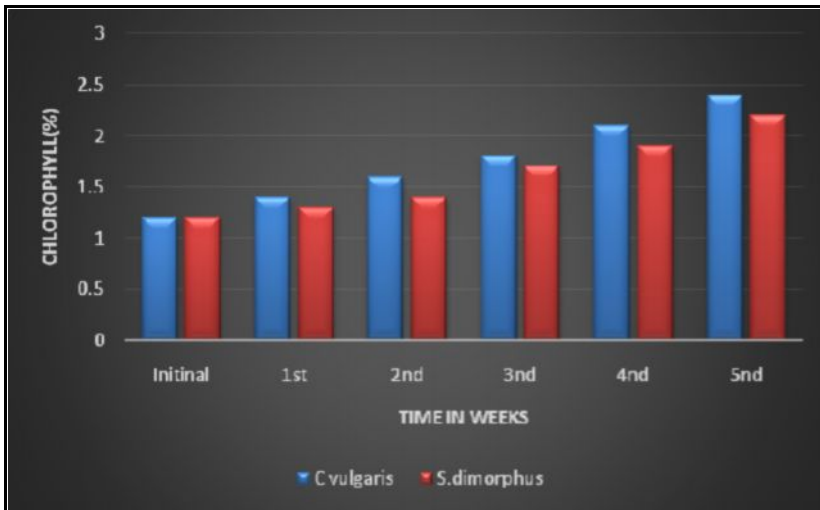


Figure 5: Effects of two different cultures in same conditions on growth (Chlorophylls %)

Estimation of Carbohydrates by Dubois method

Comparative study had been for both strains, the growth of *Chlorella vulgaris* and *Scenedesmus dimorphus* and Carbohydrates content was estimated using Dubois method. The *Chlorella vulgaris* and *Scenedesmus dimorphus* was grown in BG 11 medium up to 5 weeks. The total carbohydrate contents were calculated for *Chlorella vulgaris* $28 \pm 0.25\%$ and *Scenedesmus dimorphus* $25 \pm 0.5\%$. The total carbohydrate content was 3% higher in *Chlorella vulgaris* compared with *Scenedesmus dimorphus* as shown in the figure 6.

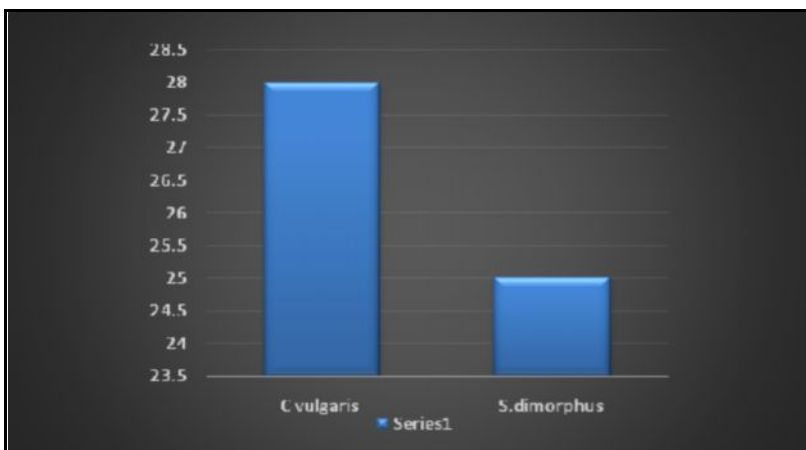


Figure 6: Estimation of Carbohydrate two different cultures in same conditions

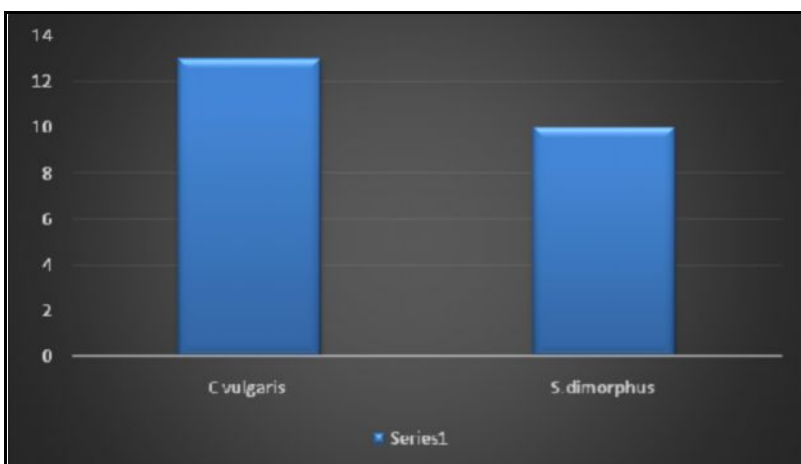


Figure 7: Estimation of Protein two different cultures in same conditions

Estimation of Protein by Burette method

To comparative study of both strains, growth of *Chlorella vulgaris* and *Scenedesmus dimorphus* the protein content was estimated by Burette method. The both strain was grown in BG 11 medium in same environmental conditions, In *Chlorella vulgaris* the protein level show that $13\pm 0.5\%$ and *Scenedesmus dimorphus* the protein content was estimated the protein level was $10\pm 0.2\%$. The protein was 3% higher in *Chlorella vulgaris* compared to *Scenedesmus dimorphus* it concluded that *Chlorella vulgaris* have rich protein content figure 9.

High-throughput measurement of lipid content using lipophilic dyes

These high throughput methodologies are based on hydrophobic (lipophilic), fluorescent dyes, such as Nile Red and BODIPY. As the dyes are absorbed by the lipids the sample fluorescence intensity increases proportionally and this principle has been used extensively in the screening for higher lipid producing cells among thousands of candidates. Furthermore, although fluorescent dyes are a powerful and potentially high-throughput approach for screening lipid-producing cells, caution has to be taken with the interpretation of fluorescence results from both BODIPY and Nile Red dyes as quantitative indicators due to the possibility of inconsistent dye-uptake between different algal cells. In addition, high throughput or high speed methods may be required to track changes in lipid content that occur across light-dark cycles. During light cycles lipids accumulate. During cell division and dark cycles these lipids are consumed by the cells for their own metabolic needs Figure 8.

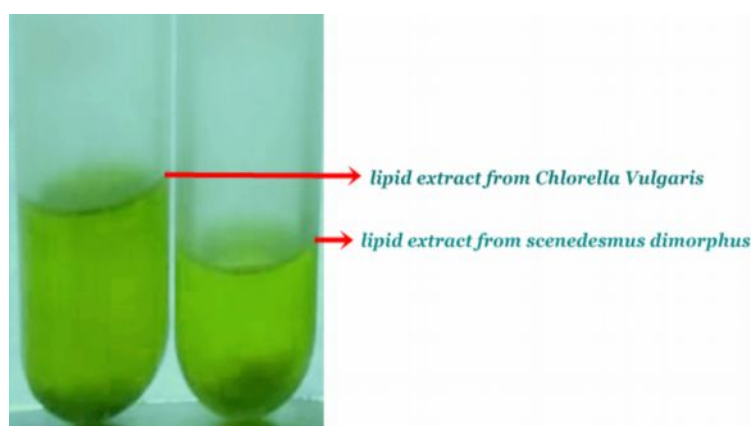


Figure 8: Measurement of lipid content using lipophilic dyes

Comparison of lipid extraction methods from two algal species

In the present study two effective methods for extraction of lipid were followed increases the yield. Lipid was major source for biodiesel production, important two methods for lipid extraction Bligh and Dyer extraction and Extraction n-hexane using Soxhlet's extraction process method was followed. In Extraction n-hexane using Soxhlet's extraction process method achieved more yield compared Bligh and Dyer extraction method Algal oil was extracted using n-hexane as an organic solvent at temperature 70°C . The maximum oil yield was achieved more than 75% in *Chlorella vulgaris*, due to the high temperature and high process duration. Extraction of Oil (Bligh and Dyer Extraction) the maximum oil yield was achieved more than 70% in *Chlorella vulgaris*. The lipid extraction from *Scenedesmus dimorphus* two methods has followed Soxhlet's extraction process and Bligh and Dyer extraction method the lipid content high in Soxhlet's extraction process compared to Bligh and Dyer extraction. In Soxhlet's extraction process the lipid extraction from *Scenedesmus dimorphus* achieved 72% and Bligh and Dyer extraction lipid content was achieved 68% higher lipid content was when using the Extraction n-hexane using Soxhlet's extraction process when compared to other method. After the algal lipid was extraction from both algal culture, the lipid bodies was view under two different types of Microscopy CARS Microscopy, Scanning Electron Microscopy Figure 9, 10, 11 and 12.

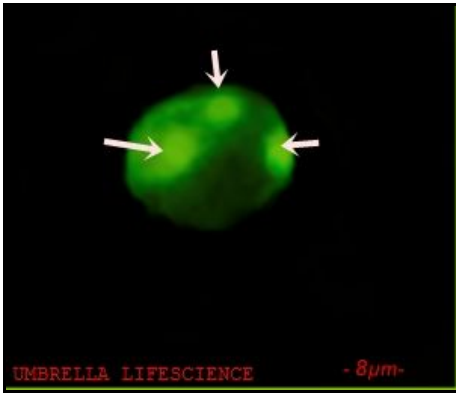


Figure 9: Lipid bodies of *Chlorella Vulgaris* Visualized by CARS Microscopy

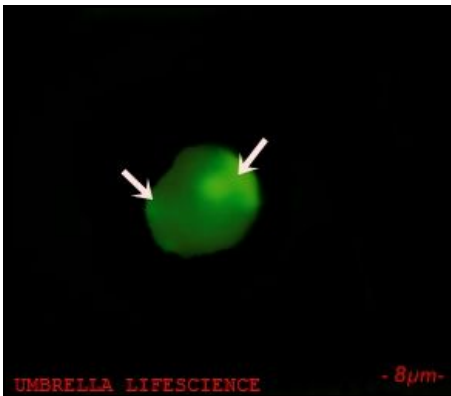


Figure 10: Lipid bodies of *Scenedesmus dimorphus* Visualized by CARS Microscopy

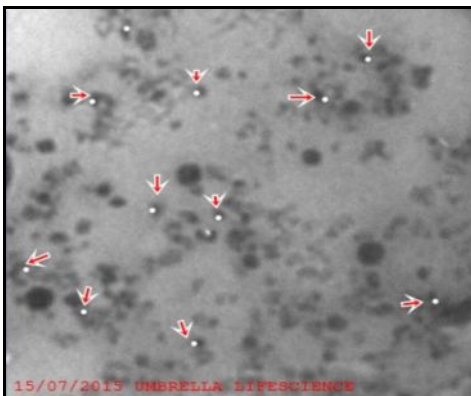


Figure 11: SEM Image for Lipid bodies of *Chlorella Vulgaris*

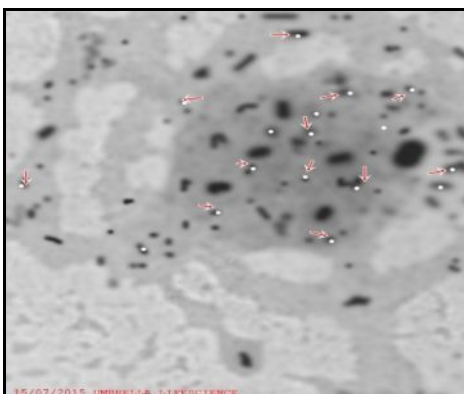


Figure 12: SEM Image for Lipid bodies of *Scenedesmus dimorphus*

Conclusion

In this study, Algal culture has been isolated from pond water and tested for its growth, pigment, protein and fatty acid contents for the selection of high lipid content species for biodiesel production, Two effective method was followed increase the yield of fatty acid contents it shows that Soxhlet's extraction process the lipid extraction from *Scenedesmus dimorphus* achieved 72% and Bligh and dyer extraction lipid content was achieved 68% higher lipid content. The maximum oil yield was achieved more than 75% in *Chlorella vulgaris*, due to the high temperature and high process duration. Extraction of Oil (Bligh and Dyer Extraction) the maximum oil yield was achieved more than 70% in *Chlorella vulgaris*

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